"PLATELET ANGIOTENSIN II BINDING IN NON-PREGNANT WOMEN AND IN NORMOTENSIVE PREGNANT AND HYPERTENSIVE PREGNANT WOMEN"

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"Pre-eclampsia, the disease of theories."

Zwiefel, 1916

"Everyone from allergist to zoologist has proposed hypotheses and suggested radical therapies based upon them, such as mastectomy, oophorectomy, renal decapsulation, trephination, alignment of the patient with the earth's magnetic field with her head pointing to the North Pole, and all sorts of medical regimens."

Chesley, 1971

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ABSTRACT

A method for measuring human platelet Angiotensin II (AII) binding was validated, and characterisation studies involving 67 non-pregnant subjects were performed. Platelets were found to possess high affinity binding sites with many of the characteristics of receptors. No correlation was found between platelet AII binding and the rise in intracellular free calcium after ex vivo AII infusion, thus formal validation of the binding sites as receptors was not achieved.

In 25 non-pregnant subjects, there was an inverse correlation between platelet AII binding and simultaneously measured plasma AII ($P<0.02$). In 10 ovulatory subjects, platelet AII binding diminished in the luteal phase of the menstrual cycle ($P<0.02$).

In a pilot cross-sectional study of platelet AII in normotensive pregnancy, incorporating 125 pregnant/postnatal patients, platelet AII binding was significantly lower in 1st trimester patients as compared to non-pregnant subjects ($P<0.001$). Platelet AII binding remained low throughout pregnancy. Higher values, approximating to the non-pregnant level, were found 6 weeks postnatally.

These findings were confirmed in a longitudinal study of 30 pregnant women, with a diminution in platelet AII binding being suggested by 5-8 weeks gestation ($P= 0.02$). Inverse correlations in pregnancy between platelet AII binding and the components of the renin-angiotensin system were found ($P<0.01$). There were also significant correlations

between platelet AII binding and the levels of serum sodium, urea and osmolality (P<0.01).

When platelet AII binding was measured in 67 patients with established hypertension in pregnancy, binding in patients with pregnancy induced hypertension (PIH) was significantly higher than in normotensive primigravidae (P<0.0001). No differences in binding were found in the puerperium.

In a prospective comparison of platelet AII binding and the AII sensitivity test in predicting the development of PIH, involving 34 subjects, platelet AII binding was a more effective discriminant than any of the parameters derived from the AII sensitivity test. There was a significant correlation between platelet AII binding and the slope of the curve relating the diastolic pressor response to infused AII (P<0.01).

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Chapter 1.

THE INTRODUCTION.

The following introduction provides a background to the studies included in this thesis. There are two reasons for considering hypertension in pregnancy in the first section. The importance of developing a suitable screening test is established from the onset, in addition, defining the various terms relating to hypertension in pregnancy facilitates their use in subsequent chapters. The renin-angiotensin system is fundamental to the thesis, and is thus considered in Section 2. Section 3 considers the renin-angiotensin system in normotensive and hypertensive pregnancy. A general review of receptor and binding site theory in general follows, (Section 4), which examines some of the concepts used in Section 5 (Angiotensin II binding sites in vascular tissue). The introduction is completed by three sections concerning platelets: morphology and behaviour (Section 6), changes in normotensive and hypertensive pregnancy (Section 7) and finally, the rationale for the use of platelets as a model for vascular smooth muscle (Section 8).

Section 1.

HYPERTENSIVE DISORDERS OF PREGNANCY.

The first definite reference to pre-eclampsia is probably Greek in origin. The pre-Hippocratic **"Coacae Praenotiones"**, XXXI, No. 523, contains the aphorism: 'In pregnancy, the onset of drowsy headaches with heaviness is bad; such cases are perhaps liable to some sorts of fits at the same time' [cited by Chesley, 1978a].

In the ensuing centuries a legion of possible aetiologies, treatments, and preventative measures have been proposed, such that Zwiefel [1916], termed pre-eclampsia 'the disease of theories'. Before this thesis proceeds to add further to the myriad of such proposals, this chapter contains a discussion of classifications, incidences, predisposing factors, mortality statistics, and the possible screening tests of the hypertensive disorders of pregnancy.

Definition and classification.

The hypertensive disorders of pregnancy, formerly called 'toxaemias of pregnancy', comprise several diseases and syndromes associated with high blood pressure. No toxin has been identified in the blood, thus the term "toxaemia" is an ambiguous misnomer. Indeed, the unknown aetiology of many of the hypertensive diseases of pregnancy has led to clinical signs such as hypertension and proteinuria, not merely diagnosing disease, but defining it. A disease cannot be defined precisely by clinical signs, and as discussed below, this has resulted in a lack of uniformity between authors.

a) Hypertension.

Carefully controlled prospective studies of blood pressure throughout pregnancy have been performed by MacGillivray, Rose and Rowe [1969] and Chesley [1978b]. These showed that the trend of blood pressure was to fall during the second trimester followed by a rise in the third trimester, with diastolic blood pressure increasing substantially more than systolic pressure as the pregnancy advanced. The main failing in both studies was an absence of blood pressure readings before pregnancy, the unproven assumption being made that blood pressure six weeks post delivery was equivalent to that prior to pregnancy.

Hypertension has been categorised using either an absolute threshold or an increment from the baseline in the first half of pregnancy. Various absolute thresholds have been set, including values as low as 125/75 mmHg. [Chesley, 1976] with some authors only using a diastolic pressure limit [e.g. Nelson, 1955]. Chesley [1976] found increased perinatal mortality rates when the blood pressure exceeded 125/75 mmHg.. However, as Redman [1987] noted, just because the group with a diastolic pressure of 75 mmHg. or more includes a relatively greater number of individuals with abnormal pregnancies does not justify labelling the whole group as abnormal; doing so confuses epidemiological and clinical concepts. A commonly used dividing line, and that adopted by the American College of Obstetricians and Gynecologists, is 140/90 mmHg. [Hughes, 1972]. This arbitrary value has the advantage of being approximately the mean diastolic blood pressure plus two standard deviations after 35 weeks gestation, [MacGillivray, Rose and Rowe, 1969]. Further justification for using 90 mmHg. as the diastolic threshold for identification of an abnormal group may be claimed by noting that the point of inflection in relation to increasing perinatal mortality is found at this level [Friedman and Neff, 1977], although Redman's criticism again applies. Tervila, Goecke and Timonen [1973] advocated the retention of the systolic value in the definition, noting that in a series of 4404 patients, systolic blood pressure provided a closer correlation with perinatal mortality, low apgar scores, and small for dates babies, than diastolic recordings.

An inherent bias in using a threshold level alone, such as a diastolic pressure of 90mmHg., as specified by Nelson [1955], is that individuals whose pressures rise from 85 mmHg are grouped with individuals who had baseline levels of 70 mmHg or less. The two groups may represent different populations; some authors categorising the former as chronic hypertension [Redman, 1987]. To counter this bias, the definition of the American College of Obstetricians and Gynecologists is based on a systolic pressure of at least 140 mmHg **or** an increment of at least 30 mmHg **or** a diastolic pressure of at least 90 mmHg **or** an increment of at least 15 mmHg [Hughes, 1972]. However, MacGillivray [1983] found a diastolic pressure increment of 15 mmHg in 72.9% of normal primigravid pregnancies. Even if the increment were to be raised to 20 mmHg., a study from this centre, found a rise of 20 mmHg in the diastolic pressure of 26% of 500 consecutive normal primigravid pregnancies [Broughton Pipkin and Symonds, unpublished data].

Whatever arbitrary threshold is chosen, there are problems in using it to divide a continuously distributed variable such as arterial pressure. This leads to patients with essential hypertension being regarded as having a discrete disease state, rather than being at the upper end of a continuous spectrum. It is incorrect for some authors to disregard such patients [e.g. Nelson, 1955], and for others to consign them to a separate category [e.g. Chesley, 1978b]. However, as some authors found an increased incidence of pre-eclampsia in such patients [Harley, 1966], there would seem to be little alternative.

A specific difficulty encountered when measuring blood pressure in pregnant women, is the necessity of using the 4th Korotkov sound for diastolic pressure. Raftery and Ward [1968] demonstrated that in healthy young women, the 5th sound correlated better with diastolic pressure recorded directly from the right brachial artery. However, in pregnancy, use of the 5th sound results in large skewing of data due to the finding of the 5th Korotkov sound at zero cuff pressure in some pregnant women [MacGillivray, Rose and Rowe, 1969].

The difficulty in diagnosing hypertension is compounded by a necessity to standardise the technique of measurement, and by random and systematic variations in blood pressure. Factors such as posture influence it, blood pressure being lowest when the patient is lying in the left lateral position; the blood pressure being measured in her right arm [Wichman, Ryden and Wichman, 1984]. The position of the arm relative to the heart also affects recordings, each centimetre of vertical height above or below the level of the heart being equivalent to a difference in pressure of 0.7 mmHg. [Murnaghan, 1987]. A detailed description of the conditions of measurement in this study is included in the methods chapter, (p. 112).

The spontaneous variability of blood pressure in hypertensive pregnant women has long been appreciated [Wiessner, 1899]. In addition, Murnaghan, Mitchell and Ruff [1980] demonstrated that in 16 normotensive patients, blood pressure varied with the circadian rhythm, values being highest during the afternoon and early evening. Under stressful conditions, Sleight, Fox, Lopez and Brooks [1978] found that a rise in blood pressure could be evoked. This latter factor may well be responsible for many of the elevated blood pressure recordings noted when patients visit their obstetricians.

Such factors can be reduced by averaging a large number of readings, and in certain definitions high values are only accepted if confirmed after a time interval, for example 24 hours [Nelson, 1955]. In clinical practice this has the disadvantage of excluding the most acute and serious cases who are delivered shortly after admission.

Finally, it must be remembered that elevated arterial resistance is a more fundamental feature of pre-eclampsia than hypertension [Ginsburg and Duncan, 1967]. The latter is only a useful screening test as the cardiac output is usually constant.

To paraphrase Redman [1987], hypertension is a difficult to measure, artificial concept, with large sampling errors, and it is not the cardiovascular abnormality of primary interest in the pathogenesis of pre-eclampsia. To this could be added the difficulty in setting the arbitrary thresholds.

b) Proteinuria.

Most authors have incorporated proteinuria in the definition of pre-eclampsia [e.g. Nelson, 1955, Hughes, 1972, Chamberlain, 1981, Davey, 1985, Rubin, 1988], although some only incorporate it in 'severe' pre-eclampsia. As discussed above, there are problems in using clinical signs to define the disease, and eclampsia has been described in the absence of proteinuria [Sibai, McCubbin, Anderson, Lipshitz and Dilts, 1981]. Nevertheless, in two large prospective studies of 13,000 patients and of 41,000 patients, Page and Christianson [1976] and Naeye and Friedman [1979] found that when significant proteinuria was present, the outcome of the pregnancy, as assessed by the perinatal mortality, was inversely proportional to the degree of proteinuria. Both studies assessed proteinuria on random urine samples, and can be criticised accordingly. Even in severe cases of pre-eclampsia, the degree of proteinuria may fluctuate widely over any 24 hour period [Gant and Worley, 1980a]. More precise quantitation using 24 hour urine collections is desirable, although may be more difficult to achieve in clinical practice; it may be necessary to deliver a patient within a few hours of admission.

c) Oedema.

Oedema was formerly regarded as one of the diagnostic signs of pre-eclampsia [Nelson, 1955]. However, in a prospective study of 83 patients, moderate oedema was found in 80% of normotensive pregnancies [Robertson, 1971]. Moreover, Robertson found a similar incidence of hypertension among women with no oedema and amongst those with early or late onset oedema. Indeed, in a series of over 4,000 patients, Tervila, Goecke and Timonen [1973] found that the presence of oedema tended to improve the fetal prognosis.

d) Hyperuricaemia.

An elevated serum urate measurement has been suggested as a specific diagnostic feature of pre-eclampsia [Rubin, 1988]. Redman, Beilin, Bonnar and Wilkinson [1976] found that in a study of 332 hypertensive pregnant patients, plasma urate levels were a better indicator of fetal prognosis than blood pressure. However, Breckenridge [1966] had previously noted serum urate to be frequently raised in essential hypertension, finding hyperuricaemia in 58% of patients under treatment for hypertension at the Hammersmith Hospital. Uric acid is filtered through the glomeruli, but is primarily excreted through the tubules and in 71 male patients with borderline and established hypertension, serum uric acid concentrations correlated inversely with renal blood flow per square metre of body surface [Messerli, Frohlich, Dreslinski, Suarez and Aristimuno, 1980]. Raised serum urate levels are probably better regarded not as a diagnostic or specific feature of pre-eclampsia, but as a sensitive indicator of impaired renal function and renal blood flow.

e) Renal biopsy findings.

A specific renal lesion has been described in pre-eclampsia. Details of the pathological changes are outside the scope of this text [see McCartney, 1964, Lindheimer, Chesley, Taylor, Spargo and Katz, 1987], but the major changes occur in the glomeruli, which become large and swollen but not hypercellular.

McCartney [1964] performed renal biopsies on 62 carefully selected primigravid patients, all known to be clinically normal in early pregnancy, who developed hypertension, proteinuria and oedema in the third trimester. Examination of the biopsies revealed that only 44 had the renal lesion of pre-eclampsia. Similar results were obtained by Fisher, Luber, Spargo, Spargo and Lindheimer [1980], from 104 primigravid hypertensive patients, with both studies finding that amongst multiparous hypertensive patients the renal lesion occurred much less commonly. Such data underscore the difficulty in making a clinical diagnosis of the hypertensive complications of pregnancy on clinical criteria alone.

There are further problems in defining and classifying hypertensive disorders of pregnancy. For instance, with regard to the time of onset of pre-eclampsia, some definitions set a cut-off time of 20 weeks [e.g. Gant and Worley, 1980a], others 24 weeks [e.g. Hughes, 1972], and others 26 weeks [e.g. Nelson, 1955]. There seems little basis for choosing any one of these above another, indeed pre-eclampsia has been described prior to 20 weeks [Sibai, Abdella and Taylor, 1982], often in conjunction with a molar pregnancy.

Nevertheless, there is a need for provisional definitions to allow different investigators to compare their experiences. Detailed below are the definitions used in this study, which are felt on balance to be the most appropriate.

Pregnancy induced hypertension (PIH).

An arterial blood pressure of 140 mmHg. or greater, and a diastolic blood pressure of 90 mmHg. or greater, occurring in a previously normotensive woman after the twentieth week of pregnancy. At least two such blood pressure recordings must be obtained, on consecutive occasions, 24 hours apart. There should be no history of chronic renal disease or essential hypertension.

Pre-eclampsia.

Pregnancy induced hypertension with the addition of significant proteinuria, significant proteinuria being the excretion of at least 0.3 gm of protein in a 24 hour urine collection, in the absence of a urinary tract infection.

Eclampsia.

The occurrence of convulsions in a patient with pre-eclampsia, in the absence of a history of epilepsy.

Essential hypertension.

The presence of a blood pressure of at least 140/90 mmHg., either before the twentieth week of pregnancy, or after three months post delivery.

Incidence.

Largely due to differences in definitions, incidences of pregnancy induced hypertension vary markedly, with estimations of up to 25% of pregnancies having been made [Chamberlain, 1981]. In data from this centre, using identical definitions to those above, Symonds [1979] found an incidence of pregnancy induced hypertension of 10.3% amongst primigravidae. The incidence was of a similar order in a study by MacGillivray et. al. [1969], who found an incidence amongst Aberdeen primigravid patients of 8.9% over a two year period, using diagnostic thresholds of at least 0.25 gm/litre proteinuria and at least 140/90 mmHg. blood pressure recordings. Hall and Campbell [1986], again studying the population in Aberdeen, found little change in incidence over the last three decades.

Predisposing factors.

In 1694 Mauriceau observed that nulliparous women were more likely to develop eclampsia than multiparous women [cited by Chesley, 1978c]. In the British Perinatal Mortality Survey the incidence of 'moderate and severe' pre-eclampsia in primigravidae was 13.5% as compared with 7.1% in multigravidae [Taylor, 1988]. Campbell, MacGillivray and Carr-Hill [1985] suggested that an abortion, either therapeutic or spontaneous, confers a protective effect, only if it occurs in the second trimester. Following a first trimester abortion, the subsequent incidence of pre-eclampsia approximated to that of the other nulliparous patients.

The association of increased maternal age and both pre-eclampsia and eclampsia is consistent [e.g. Nelson, 1955], with the morbidity being reflected in the ages of maternal deaths caused by eclampsia/pre-eclampsia in England and Wales [HMSO, 1982-84]. Over a maternal age of 35 years, a dramatic increase in maternal deaths occurs. At the other end of the age spectrum, there is a disparity of opinion. Certain studies, [e.g. Vollman, 1970] suggest an increased incidence in girls in their early teens, but this has not been found in other studies [e.g. Nelson, 1955]. These conflicting results may be explained by the difficulty in making comparisons with the early teenage group, due to the high incidence of concealed pregnancy and poor antenatal care, affecting both the observed incidence and the presentation of the disease.

Over a century ago, a familial history of the disease was suggested [Elliot, 1873]. However, it is only in the last thirty years that research has focussed on this aspect; principally due to the persistent and dedicated work of Leon Chesley. His recent analysis of data collected over a 49 year period concerned the incidences of pre-eclampsia and eclampsia in 147 sisters, 248 daughters, 74 granddaughters and 131 daughters-in-law of women who had eclampsia [Chesley and Cooper, 1986]. The observed incidences fitted closely with a single gene model with the frequency of the putative gene being 0.25. Other studies have confirmed a genetic and familial predisposition. Arngrimsson, Bjornsson, Geirsson, Bjornsson, Walker and Snaedal [1990] found a pattern of inheritance through three or four generations in 94 families from the homogenous island population of Iceland, which could fit either a single recessive gene model or a dominant model with incomplete penetrance.

It must be recognised that this conclusion relates to severe pre-eclampsia or eclampsia occurring in primigravidae. The disease is also very common in conditions associated with hyperplacentosis (multiple gestation, hydatidiform mole, diabetes, etc.) [Chesley, 1978c], and the single gene hypothesis would not explain this.

There must be other compounding factors involved in the genesis of pre-eclampsia. A detailed review of possible factors is beyond the remit of this thesis. Suffice to say, that suggestions include a fetal sex-linked factor, (MacGillivray [1983] finding an excess of male infants), low socioeconomic class [Chamberlain, 1981], maternal obesity [Hall and Campbell, 1986], maternal smoking, (a protective effect [Chamberlain, 1981]), non-caucasian ethnic origin [Taylor, 1988], and even an association with unsettled weather [Chesley, 1978c].

Mortality.

The British Births Survey 1970 studied 16,815 women who had babies in one week [Chamberlain, Phillip, Howlett and Masters, 1978]. In cases of 'severe pre-eclampsia', (this approximated to the definition of pre-eclampsia above), the perinatal mortality rate increased to 34/1000 from the normal rate of 19/1000. However, the rate was normal or even slightly lower in cases of 'mild or moderate pre-eclampsia', (this approximated to the definition of PIH above). Tervila, Goecke and Timonen [1973] found a similar pattern in 4404 cases from Helsinki and Wurzburg, reporting no effect on perinatal mortality from either hypertension alone, or hypertension and oedema. When proteinuria was also present, the perinatal mortality rose from 24/1000 to 65/1000. Should eclampsia ensue, recent figures of up to 216/1000 have been reported [Taylor, 1988].

Hypertensive disorders of pregnancy have remained the most common cause of maternal mortality in England and Wales, over recent decades [HMSO, 1982-84]. In the triennium 1982-84 there were 25 maternal deaths (13.5/million), 14 of which resulted from eclampsia.

Prevention of PIH.

The treatment of established PIH is outside the scope of this text. However, if PIH and pre-eclampsia could be prevented, eclampsia would not develop. Worley [1984] recommends a management plan of rest, regular out-patient attendance, and early hospitalisation, aimed at those patients who are at an increased risk of developing hypertension in pregnancy. Nakamura, Ito, Matsui, Yoshimura, Kawasaki and Maeyama [1986] advocated a low salt diet for such patients. Recent work has suggested prophylactic benefits of both low-dose aspirin administration [Beaufils, Uzan, Donsimoni and Colau, 1985] and fish oil administration [Secher and Olsen, 1990]. Moreover, both clinical experience and investigation [Gant and Worley, 1980b], provide evidence that a prophylactic reduction in physical activity may forestall PIH. (These concepts are discussed in greater detail in Chapter 9, p. 245.) The difficulty is in differentiating between those who are and will remain normal, and those those who appear normal but will later develop PIH. To this end, a number of possible tests have been investigated, including:

a) The angiotensin sensitivity test.

This investigation is discussed in more detail in section 3, (p. 50). Gant, Daley, Chand, Whalley and Macdonald [1973] reported that primigravid women who later developed PIH in the same pregnancy showed an increased pressor response to infused angiotensin II. Although subsequent studies found substantial false positive rates [e.g. Oney and Kaulhausen, 1982, Nakamura, Ito, Matsui, Yoshimura, Kawasaki and Maeyama, 1986], this test is regarded as the most discriminatory available. However, it is a time consuming procedure, and requires the placement of an intravenous cannula and the presence of a clinician throughout, thus rendering it unsuitable for screening the general population.

b) The roll-over test.

Gant, Chand, Worley, Whalley, Crosby and Macdonald [1974] described what later became known as the roll-over test. They reported that an increase of > 20 mmHg. in the diastolic blood pressure, when the patient was turned from the left lateral to the supine position, predicted the development of subsequent hypertension in 15 out of 16 women tested at between 28 and 32 weeks gestation. Unfortunately subsequent reports have indicated that the test is less satisfactory, with Tunbridge and Donnai [1983] finding a false negative rate of 19% and false positive rate of 80%. Dekker, Makovitz and Wallenburg [1990] found a false positive rate of 67% and they concluded that the roll-over test was of no value in the prediction of PIH.

There are several flaws in the test. Some patients have unusually low diastolic blood pressures in the left lateral position (<45mmHg.), this can create a false positive result when the value is compared with the reading in the supine position [Phelan, 1977]. Furthermore, markedly differing results from the same patient are found when the test is performed on a biweekly basis [Phelan, 1977]. Despite the fact that the test is simple to perform and requires only time and personnel rather than elaborate equipment, it has rightly found little place in routine obstetric practice.

c) Forearm venous tone.

In a cross-sectional study, Stainer, Morrison, Pickles and Cowley [1986] found that women with PIH were venoconstricted in the forearm, when compared with normotensive pregnant women. The authors suggest that measurement of venous tone may have potential as a screening test for PIH. However, the number of women studied was small, with only 11 PIH patients, (two of whom were in their second pregnancies). Moreover the degree of overlap was such that only one value of the PIH group lay outside the normal range. A prospective study by the same group demonstrated similar results, with differences in forearm venous tone occurring at least six weeks before the diagnosis of PIH [Stainer, 1989]. Patient number was again small, (33 primigravidae, of whom 7 developed PIH), and overlap was again present. Although time-consuming, and requiring both temperature control and sophisticated equipment, the technique merits further study.

d) Platelet count.

Redman, Bonnar and Beilin [1978] suggested that platelet count might be helpful in monitoring high risk pregnancies. They found that a reduction in platelet count occurred early in the development of pre-eclampsia, being detectable about seven weeks prior to delivery. However, the study group chosen were multiparous women with chronic hypertension, in whom a diagnosis of superimposed pre-eclampsia cannot be made with ease. The chosen method of diagnosing pre-eclampsia was that of an elevation in plasma urate, which as discussed above, is better regarded as an indicator of impaired renal function. In addition, the variation in counts between patients was such that no importance could be attached to a single low reading. As discussed in section 6 (p. 104), a reduced platelet count is found in only a minority of patients with pre-eclampsia, thus platelet counts cannot be regarded as a good screening test.

e) Prostacyclin synthesis.

As reviewed in section 3 (p. 52), diminished prostacyclin production may be a factor enhancing angiotensin II pressor sensitivity in patients with severe PIH. In a small study, Fitzgerald, Entman, Mulloyk and FitzGerald [1987] found elevated levels of 2,3 -dinor-6-keto-prostaglandin F_1 alpha (a stable urinary metabolite of prostacyclin) in patients who subsequently developed PIH. Moreover, in the same patients, they found no difference in the results of the angiotensin sensitivity test between those women who developed PIH and those who remained normotensive. In another small study [Schaffer, Rakhit, Martin, Hanie, Sibia, Ross, Dunton and Douglas, 1990], elevated urinary thromboxane : 2,3 -dinor-6-keto-prostaglandin $F_{1\ alpha}$ ratios were found in patients with severe PIH. A complex method was used in these studies, involving gas chromatography-mass spectrometry. Evaluation of potential as a screening test would require study of a larger population with a more easily applied method such as radioimmunoassay.

f) Doppler ultrasound.

Doppler ultrasound scanning is becoming an increasingly controversial technique, (e.g reviewed by Neilson [1987]), and detailed discussion is beyond the scope of this thesis. However, the possibility of identifying changes in perfusion in advance of clinical signs prompted Campbell, Pearce, Hackett, Cohen-Overbeek and Hernandez [1986] to perform Doppler studies of maternal arcuate arteries. They studied 126 women at 16-18 weeks gestation, and found a higher subsequent incidence of PIH in women with abnormal waveforms. The very high incidence (40%) of abnormal waveforms demonstrated in this series may be spurious, and suggests that the normal ranges obtained initially were inappropriate for the general population. In contrast, a recent controlled study of Doppler uteroplacental waveforms in untreated cases of PIH, demonstrated no difference from the normotensive controls [Hanretty, Whittle and Rubin, 1988]. Although the method of obtaining the waveform has been criticized [Pearce and MacParland, 1988], it was apparent that markedly different waveforms could be obtained from the utero-placental vessels, a finding which probably relates to the focal nature of placental pathology in PIH. The value of the technique as a screening procedure is thus as yet unproven.

At present, none of the above methods are widely used to screen the general pregnant population, in order to select those at risk of developing PIH. This thesis involves a study of human platelet angiotensin II binding, including the potential use of the technique as a marker and indeed screening test for PIH.

Section 2.

THE RENIN-ANGIOTENSIN SYSTEM.

In 1898 Tigerstedt and Bergman demonstrated that a kidney extract exerted a potent pressor response when injected into a rabbit, and they termed the active substance renin. Since this time the renin-angiotensin system has been closely associated with efforts to understand the regulation of blood pressure. This section presents a brief overview of the present state of knowledge of the renin-angiotensin system, concentrating on angiotensin II (AII). Unless stated the section considers data from human studies.

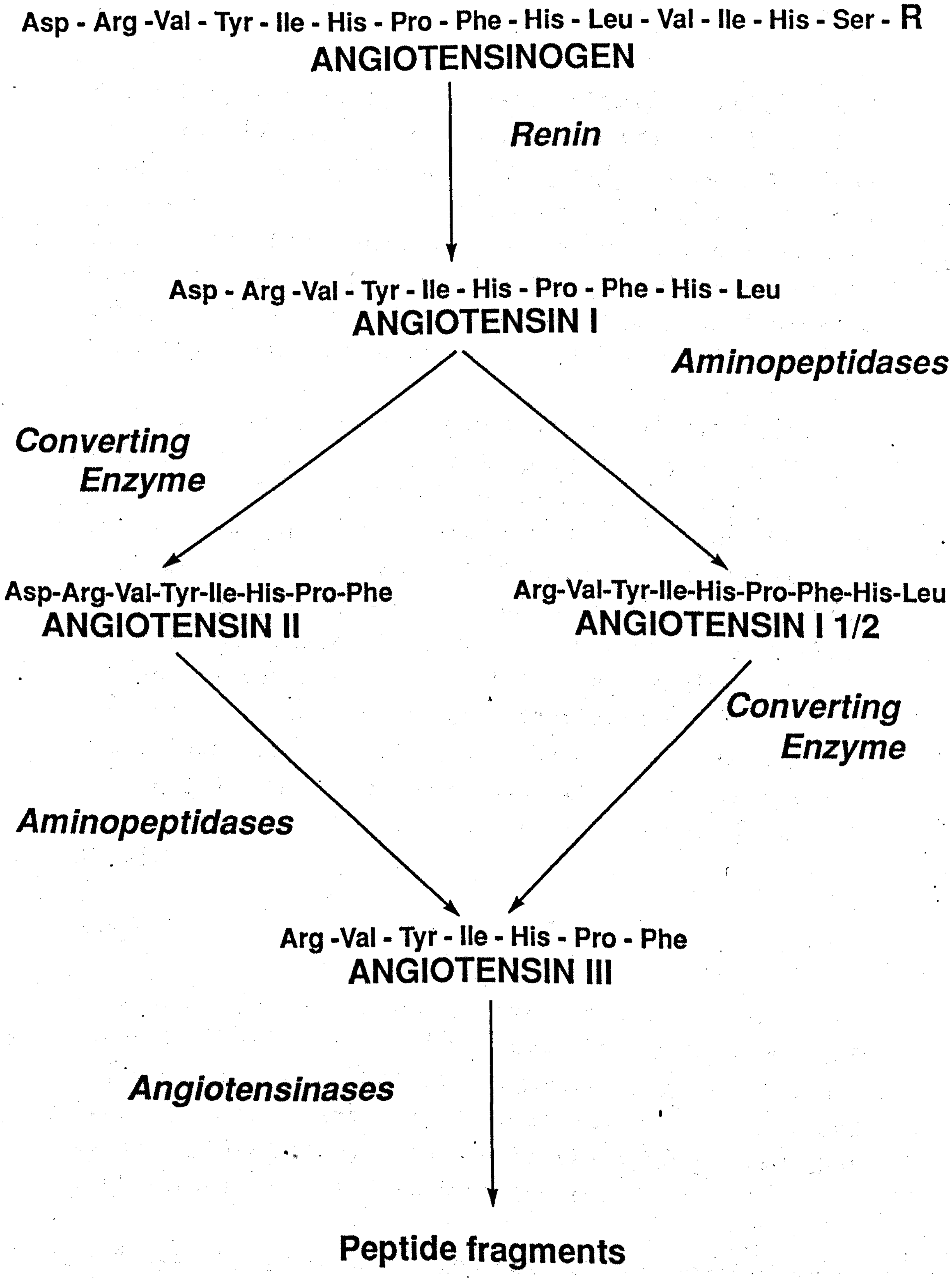
The renin-angiotensin system is summarised in Figure 1.1.

Angiotensinogen.

Angiotensinogen is the circulating $alpha_2$ globulin from which enzyme renin cleaves the decapeptide angiotensin I. Chromatographic studies by Gordon and Sachin [1977] suggested a multiplicity of forms. Subsequent characterisation studies [Campbell, Bouhnik, Coezy, Menard and Corvol, 1985] have indicated that all forms stem from a single precursor of molecular weight 46,000. The higher molecular weight forms are believed to be either aggregates or combinations with other proteins. The higher molecular weight forms increase progressively as a proportion of the total angiotensinogen throughout pregnancy [Tetlow and Broughton Pipkin, 1986].

Figure 1.1.

The renin-angiotensin system.


Asp - Arg -Val - Tyr - Ile - His - Pro - Phe - His - Leu - Val - Ile - His - Ser - R
ANGIOTENSINOGEN
Renin
Asp - Arg -Val - Tyr - Ile - His - Pro - Phe - His - Leu
ANGIOTENSIN I
Converting Enzyme
Aminopeptidases
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
ANGIOTENSIN II
Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
ANGIOTENSIN I 1/2
Aminopeptidases
Converting Enzyme
Arg -Val - Tyr - Ile - His - Pro - Phe
ANGIOTENSIN III
Angiotensinases
Peptide fragments

Fyhrquist, Rosenlof, Gronhagen-Riska, Hortling and Tikkanen [1985] noted immunological similarities between erythropoietin and angiotensinogen, and suggested that angiotensinogen might be a precursor of erythropoietin. However, these results have not been confirmed, and removal of contaminants using fast protein liquid chromatography, indicated that the substances were unrelated [Rosenlof, 1986].

A detailed review of the sources and regulation of angiotensinogen production is provided by Reid, Morris and Ganong [1978]. In summary, the majority of the angiotensinogen in the circulation is synthesised in the liver. Oestrogens increase angiotensinogen production, as does the administration of ACTH, certain corticosteroids, and AII itself. The ability of corticosteroids to increase angiotensinogen production is related to glucocorticoid rather than to mineralocorticoid activity. Thus plasma levels are elevated in patients with Cushing's syndrome but not in patients with primary aldosteronism [Krakoff, 1973].

<u>Renin.</u>

Renin is an aspartyl proteinase that cleaves the Leu-Val bond from the aspartic acid end of angiotensinogen to release angiotensinogen I (Figure 1.1). Renin exists in various forms. Morris and Lumbers [1972] studying amniotic fluid, suggested that proteinases activated an inactive form.

Renin is primarily synthesised and stored in a specialised part of the nephron, the juxtaglomerular apparatus, a complex of vascular and tubular elements located at the hilum of the glomerulus, (Figure 1.2).

The major components of the juxtaglomerular apparatus are the afferent and efferent arterioles and the macula densa, a specialised tubular area that marks the transition from the loop of Henle to the distal tubule. The afferent arteriole contains specialised cells known as the juxtaglomerular cells. These cells, which are the site of synthesis, storage, and release of renin, contain myofibrils, characteristic of vascular smooth muscle, and a well developed endoplasmic reticulum and Golgi membranes, characteristic of endocrine cells. The vascular and tubular elements of the juxtaglomerular apparatus are innervated by adrenergic nerves. More detailed description of the anatomy of the juxtaglomerular apparatus may be found elsewhere [Keeton and Campbell, 1981].

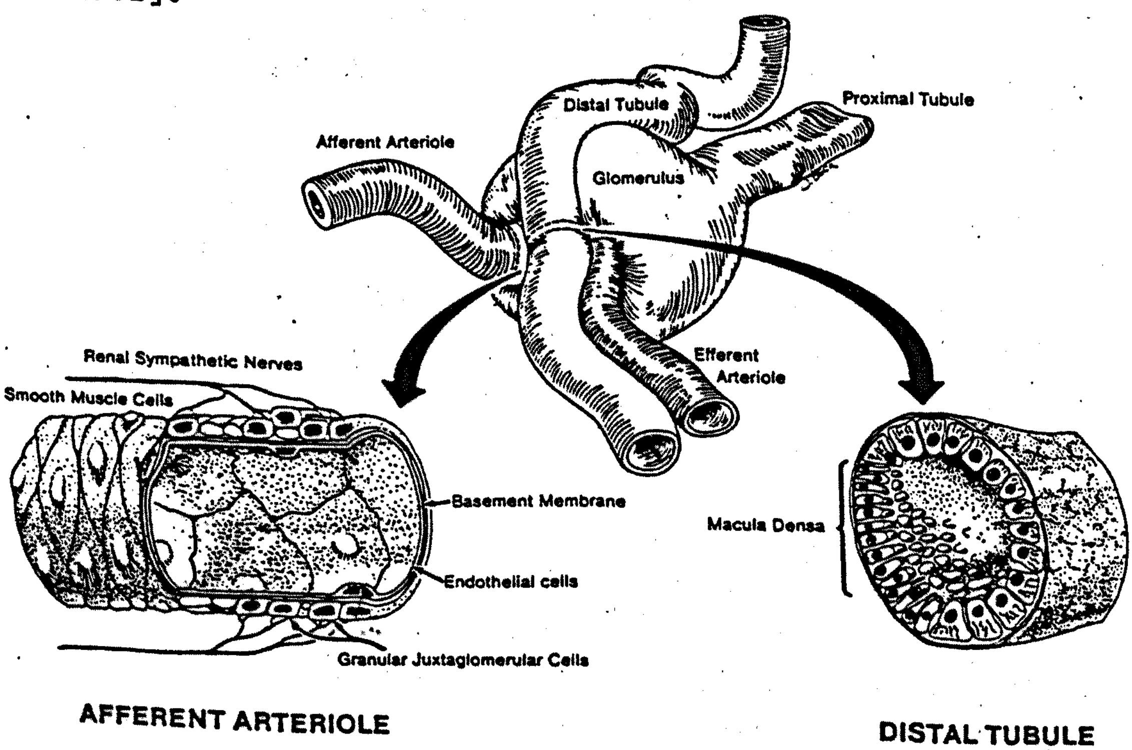


Figure 1.2. The anatomical relationship of the juxtaglomerular apparatus [Keeton and Campbell, 1981].

Renin release is controlled by interacting renal and extrarenal factors. Tobian, Tomboulian and Janecek [1959] perfused isolated rat kidneys, and noted degranulation of juxtaglomerular cells in association with increased perfusion pressure. This led Tobian to propose the 'stretch' receptor hypothesis, whereby a high renal perfusion pressure or renal (afferent arteriolar) vasodilation stretches the walls of the afferent arterioles. The juxtaglomerular cells are thus stretched, resulting in decreased renin release. Subsequent criticism of the theory has stemmed from the observation by various workers that either systemic or renal arterial renin release was usually associated with renal vasodilation. Many of these studies [e.g. Cowley and Guyton, 1972] were performed on anaesthetised animals, and are difficult to interpret in view of the reduced renal blood flow and increased renin release induced by anaesthesia <u>per se</u> [Keeton and Campbell, 1981]. However, further studies on conscious chronically cannulated dogs [e.g. Gutmann, Tagawa, Haber and Barger, 1973] confirmed the previous findings. It must be assumed that intravascular pressure rather than the radius of the afferent arteriole is the factor inducing 'stretch', i.e that a baroreceptor mechanism is present.

Evidence derived from animal studies suggests that changes in the rate of delivery of sodium [Vander and Miller, 1964] and chloride [Kotchen, Golla and Luke, 1978] to the distal tubule are detected by the macula densa, and signal reciprocal changes in renin release. The sympathetic nervous system also plays an important role in the control of renin release. In an elegant study of the innervation of the juxtaglomerular apparatus, Johnson, Davis and Witty [1971] stimulated isolated renal nerves in anaesthetised dogs, having given an intrarenal infusion of papaverine. The increased renin release noted, was thus in the absence of a functional macula densa, or any change in renal blood flow. Other factors that alter renin secretion include potassium, vasopressin, and AII, all of which inhibit secretion. The control of renin release has been extensively reviewed [Davis and Freeman, 1976, Reid, Morris and Ganong, 1978, Keeton and Campbell, 1981].

Whilst the majority of renin is renal in origin, it is known to be synthesised in a variety of extrarenal tissues, notably those of the genital tract. Symonds, Stanley and Skinner [1968] described the production of renin by <u>in vitro</u> cultures of chorion and uterine muscle. Immunohistochemical techniques have shown the presence of cells containing renin granules clustering in the interarteriolar tissue in human myometrium adjacent to the endometrium [Johnson, Johnson, Ronan and Craven, 1984]. Renin has also been identified in brain tissue from several species [e.g. Phillips, 1987], lending support to the suggestion of an intrinsic brain renin-angiotensin system, (see p. 29).

Renin is measured in the plasma either in the form of plasma renin activity (PRA) or plasma renin concentration (PRC). The former measures the rate of generation of angiotensin I (AI) under constant conditions of pH and temperature and in the presence of inhibitors of converting enzyme and angiotensinase. PRC measures the maximal AI generation obtained in the presence of excess heterologous (sheep) substrate. Under certain conditions the substrate may be the limiting factor in the generation of AI [Skinner, Lumbers and Symonds, 1969] thus PRC provides a truer measurement of enzyme concentration.

Angiotensin I (AI).

AI is of little biological activity and is rapidly converted to AII [Peach, 1977]. The enzyme responsible for this conversion is converting enzyme (ACE).

Angiotensin Converting Enzyme (ACE).

ACE is a non-specific carboxypeptidase that cleaves dipeptides from many synthetic or natural substrates, including bradykinin, as demonstrated by Yang, Erdos and Levin [1970], who found ACE to be identical to kinase II.

The primary site of functional AII production outside pregnancy is the lung, morphological and immunological studies originally performed on rabbit tissues, having indicated that ACE is predominantly localised in the luminal surface of the endothelial cells which line blood vessels [Caldwell, Seegal, Hsu, Das and Soffer, 1976]. However, using high performance liquid chromatography Miyazaki, Okunishi, Nishimura and Noboru [1984] found ACE activity in blood vessels of other tissue beds, in different species. In addition, ACE activity has been demonstrated in fetal membranes, placenta and amniotic fluid [Warren, Craven and Symonds, 1984].

Angiotensin II (AII).

The octapeptide AII is the physiologically active component of the renin-angiotensin system. The various actions of the hormone are considered in more detail below.

Cardiovascular effects.

In 1941, Hill and Andrus first presented evidence from <u>in vitro</u> experiments with animal tissues that AII possessed a direct positive inotropic myocardial effect. The vast majority of subsequent studies, performed using dog heart-lung preparations, perfused cat and rabbit hearts, isolated cat and dog papillary muscles, and isolated atria from cats and rabbits, have consistently confirmed a positive inotropic action of AII [e.g. Dempsey, McCallum and Kent and Cooper, 1971, Freer, Pappano, Peach, Bing, McLean, Vogel and Sperelakis, 1976, reviewed by Peach, 1977]. One study [Downing and Sonnenblick, 1963] concluded that there was no consistent inotropic effect when cat myocardial preparations were continuously infused with AII, yet these conclusions were based on only three such preparations.

The majority of workers have found that the administration of AII to animals with intact baroreceptor reflexes results in a bradycardia [e.g. Page and Olmstead, 1961, Brod, Hejl, Hornych, Jirka, Slechta and Burianova, 1969, reviewed by Peach, 1977]. However, Ismay, Lumbers and Stevens [1979] studying conscious sheep, found that the bradycardia evoked when blood pressure was raised by AII was less marked than when the same increase in blood pressure was produced by phenylephidrine. Moreover, Mueller, Gregory, Giannelli and Ayres [1970] found no change in heart rate when AII infusion studies were performed on 10 post heart-surgery patients. Both Ismay et. al., and Lumbers, McCloskey and Potter [1979] suggested that AII inhibited vagal discharge to the heart; the latter group recording the effect of AII on action potentials in single cardiac efferent fibres dissected from the vagus nerves of dogs.

In all species studied, the blood pressure response to intravenously injected AII begins within about ten seconds, and is characterised by a sharp rise in both systolic and diastolic pressures, the increase in diastolic pressure being the greater [Peach, 1977]. Very low doses induce a slow progressive rise in arterial pressure that may require days to develop, however, infusions that cause an immediate pressor response tend to result in tachyphlaxis over a period of several hours [e.g. Dickinson and Lawrence, 1963, Day, McCubbin and Page, 1965]. There are several components to the pressor response to AII. Direct influences of the central and sympathetic nervous systems are discussed below, however the major component results from direct vasoconstriction in several vascular beds, the splanchnic, renal, and skin beds being the most sensitive [Brod, Hejl, Hornych, Jirka, Slechta and Burianova, 1969, Reid, 1985]. Brod et. al. [1969] infused 9 subjects intravenously with AII, finding little change in forearm muscle blood flow, as compared to markedly reduced skin and renal blood flow. Paradoxical coronary vasodilation induced by AII has been reported in rabbits [Needleman, Marshall and Sobel, 1975], and has been shown to be due to stimulation of the synthesis of a prostaglandin E_2-like substance. An underlying vasoconstrictor effect was unmasked by the administration of the prostaglandin synthetase blocking agent, indomethacin.

Interaction with sympathetic nervous system.

There is good evidence that the vasoconstrictor effects of AII and sympathetic nerve activity interact. For example, Zimmerman [1962] demonstrated that sympathectomy in dogs reduced the pressor effect of AII. Conversely, AII may modify the vasoconstrictor effect of sympathetic activity. AII-induced potentiation of the contraction of sympathetic smooth muscle has been reported by many groups [reviewed by Peach, 1977].

Nicholls, Espiner, Miles, Zwiefler and Julius [1981] infused normal human subjects with AII in doses from 0.5 to 4.0 ng/Kg/min and raised plasma AII concentrations to as high as 100 pg/ml (normal range up to 35 pg/ml in man) without any significant effect on the plasma catecholamine level, suggesting no adrenal medullary effect of such physiological peptide concentrations. They also found unchanged plasma catecholamine concentrations in hypertensive patients given the converting enzyme inhibitor captopril at a dose which lowered the plasma AII levels from 22 to 6 pg/ml. Furthermore, during AII-induced hypertension, (20 ng/Kg/min for six days), in unanaesthetised dogs, there was only a transient increase in arterial adrenaline level after four hours of infusion, [Carroll, Lohmeier and Brown, 1983]. Measurement of the adrenal noradrenaline secretion rate indicated no increased release from the adrenal.

However, there are inherent flaws in the above studies. Measurement of unstimulated catecholamine levels will not have been sufficiently sensitive to detect relatively small changes in adrenergic transmitter release due to AII-induced facilitation, especially with the less specific measurement methods available in the early 1980s. In addition, baroreceptor reflexes due to blood pressure changes would tend to counteract the effect of AII on transmitter release. In contrast, concentrations of 30-1000 pg/ml AII produced consistent facilitation of adrenergic responses in isolated preparations without compensatory mechanisms [Zimmerman, Gomer and Liao, 1972, Ackerly, Blumberg and Peach, 1976]. Furthermore, in the isolated rat kidney, endogenously formed AII enhanced pressor responses and transmitter release during renal adrenergic nerve stimulation when AI was infused [Boke and Malik, 1983].

The mechanism by which AII enhances sympathetic vasoconstriction is controversial. Some investigators [e.g. Kiran and Khairallah, 1969] have concluded that the peptide directly stimulates adrenergic nerve terminals to release noradrenaline. However, in Kiran and Khairallah's work, the concentration of AII necessary to produce significant effects in the perfused rabbit aortae was high, (20 ug/ml). The majority of other studies have indicated that AII interferes with the inactivation of synaptic noradrenaline by inhibiting the neuronal uptake process for the amine [reviewed by Peach, 1977]. There is also evidence from studies of guinea pig atria adrenergic nerve endings that AII stimulates noradrenaline synthesis by enhancing hydroxylation of tyrosine [Roth, 1972].

Central actions.

The blood-brain barrier excludes polar molecules, such as AII, from most parts of the brain. However, in the rat, systemically administered radiolabelled AII was found to have a specific, high affinity uptake in the circumventricular organs, small areas of the brain that lack a blood-brain barrier [Van Houten, Schiffrin, Mann, Posner and Boucher, 1980]. A detailed discussion of whether the actions elicited by centrally administered AII can be explained solely on the basis of an interaction at the circumventricular organs, or whether a separate brain renin-angiotensin system exists, is outside the remit of this thesis.

In summary, although certain studies question the presence of AII in the brain, (for example Semple, Wacrae and Morton [1980] suggested that much of the immunoreactive "AII" found in human cerebrospinal fluid was an immunoassay artifact), the demonstration of many of the components of the renin-angiotensin system [reviewed by Phillips, 1987], and the wide variety of responses obtained, (discussed below), on injecting AII into the central nervous system has led the majority of authors to support the existence of an intrinsic brain system [e.g. Reid, 1979, Ganong, 1984].

The major central actions of AII include the control of hypovolaemia, the cyclic regulation of reproductive hormones, and possible neurotransmitter effects.

Water intake is elevated in conditions which elevate the renin-angiotensin system, for example, water deprivation, renal artery constriction, administration of isoproterenol [Severs and Summy-Long, 1975]. Fitzsimons and Simons [1969] demonstrated that AII infused intravenously caused rats to drink, the effect being greater after nephrectomy. Moreover, when AII was injected or infused into the anterior diencephalon, the minimal dose sufficient to cause water-replete rats to drink was at least 1000 times smaller than the minimal intravenous dose [Epstein, Fitzsimons and Rolls, 1969]. Dose response studies in the dog, indicate that drinking responses can be elicited by low doses of intravertebral and intracarotid AII, which produce elevations in plasma AII levels that are within the physiological range [Reid, Brooks, Rudolph and Keil, 1982]. Reid et. al. [1982] estimated that the threshold plasma AII level necessary to induce drinking in the dog was in the range 108-449 pg/ml; with plasma concentrations in the lower half of the range being commonly found in hypovolaemic states. With the caveat that the effects of the endogenous hormone are not necessarily those of exogenous infusion doses, it seems reasonable to conclude, that in animals at least, the dipsogenic effect is a physiological one.

The first evidence for a central pressor effect of AII was obtained from isolated head perfusion studies in dogs [Bickerton and Buckley, 1961]. Subsequently, dose response studies in dogs demonstrated that pressor responses could also be elicited by intravertebral or intracarotid infusion of AII in doses that produce elevations in concentration within the physiological range, (the threshold being a plasma AII level below 100pg/ml) [Reid et. al., 1982]. This dose dependant effect has been demonstrated by numerous independent groups and in several species including man [e.g. Falcon, Phillips, Hoffman and Brody, 1978, Unger, Rascher, Schuster, Pavlovitch, Schomig, Dietz and Ganten, 1981, Ganong, 1984]. Comparison of the dose response curves for administration of intravertebral and intracarotid AII in dogs, with the dose response curve for intravenous AII, suggested that centrally mediated effects constituted a significant component of the increase in blood pressure produced by intravenous AII [Reid et. al., 1982].

Some groups demonstrated relatively marked increases in plasma vasopressin with low doses of AII administered directly into the cerebral ventricles [e.g. Ramsay, Keil, Sharpe and Shinsako, 1978], whereas others failed to show any increase [Reid et. al., 1982]. Regrettably, many authors neglected to take their use of anaesthetic agents into account. Certain anaesthetic agents may increase vasopressin secretion, whilst others abolish it [Padfield and Morton, 1976]. When analysis is confined to studies on conscious animals, the variation in the dose of systemically administered AII required to stimulate vasopressin secretion may reflect difficulties in vasopressin assays [e.g. Phillips, 1987]. In human studies, Padfield and Morton [1976] found that plasma levels of vasopressin only increased at supraphysiological levels of AII, (at approximately 200pg/ml), and concluded that AII is unlikely to affect vasopressin concentrations in physiological situations. However, vasopressin is suppressed by elevations in blood pressure, and AII is a pressor agent. Thus the role of AII may be greater than predicted by results from exogenous infusions.

Phillips [1987] suggested that the central effects of AII included the control of sodium balance. AII infused into the third ventricle has been shown to cause rats to drink large quantities of 2.7% sodium chloride [Fluharty and Epstein, 1983]. Furthermore, a natriuresis has been demonstrated following intraventricular injections of AII, in rats, dogs, and goats [Brooks and Malvin, 1982, Reid, 1984]. However, the studies quoted above were on animals, with large doses of exogenous hormone elevating AII to supraphysiological levels. Coghlan, Considine, Denton, Fei, Leksell, McKinley, Muller, Tarjan, Weisinger and Bradshaw [1981] argued against a role for AII in this situation, finding that the AII antagonist saralasin did not alter the sodium intake of sodium deficient sheep.

In animal studies, AII has been shown to increase ACTH secretion when administered into the carotid arteries, or into the cerebral ventricles, [Ramsay, Keil, Sharpe and Shinsako, 1978, Reid et. al, 1982]. These studies were flawed in that increased ACTH secretion was inferred from changes in plasma corticosteroid concentration, ignoring any direct action of AII on the adrenal cortex. Nevertheless, when isolated pituitary cells were treated with AII, radioimmunoassay measurements of ACTH increased [Vale, Vaughan, Smith, Yamamoto, Rivier and Rivier, 1983]. Since ACTH modulates the aldosterone stimulatory effect of AII at the adrenal cortex, (discussed below), a delicate amplification system seems to exist.

There is evidence that AII has a functional interrelationship with the hypothalamo-pituitary sex hormone axis. In oestrogen primed, ovariectomised rats, intraventricular AII increased plasma luteinising hormone (LH) levels, whereas intravenous AII had no effect [Steele, Gallo and Ganong, 1985]. Moreover, in the presence of oestrogen and progesterone, whilst intravenous saralasin or enalapril (an ACE inhibitor) did not affect the LH surge during proestrus in rats, intravenous administration prevented the surge [Pellicer, Palumbo, DeCherney and Naftolin, 1988]. AII stimulates noradrenaline secretion, (discussed below), which is known to stimulate LH secretion, thus this could be either a direct or an indirect effect. In contrast, prolactin and growth hormone secretion by the anterior pituitary were selectively inhibited by intraventricular AII [Steele et. al., 1985]. The physiological significance of these findings was not assessed, the doses of AII being much higher than both physiological levels, and the levels used to elicit drinking [Severs and Summy-Long, 1975].

The influence of the sex hormones on AII binding is considered in a later section, (p. 83).

The final central nervous system function of AII is the stimulation and interaction with neuro-transmitters. Electrophysiological studies have shown AII to act as a neurotransmitter, being able to excite neurons at doses of 10^{-12} M and being found in synaptic terminals [Pavovcik and Phillips, 1984]. AII increases catecholamine production in the brain, with intraventricular AII increasing noradrenaline turnover in the circumventricular organs without changing the dopamine turnover [Summners and Phillips, 1983].

Peripheral AII administration has been found to increase the serotonin content of the dog hypothalamus, brainstem and pineal gland [Nahmod, Finkielman, Benarroch and Pirola, 1978]. Interactions with opiods such as enkephalins and endorphins have also been suggested [Haulica, Petrescu, Stratone, Branisteanu, Rosca, Neamtu and Slatineanu, 1982].

Aldosterone secretion.

AII in vivo is a specific stimulus to aldosterone secretion within the physiological range. The sensitivity threshold in humans varies from 0.3 to 1.0 ng AII/Kg/min in individuals consuming 100-200 mEq of sodium [Hollenberg, Chenitz, Adams and Williams, 1974]. When endogenous AII is low, a low sodium diet increased the sensitivity and magnitude of the response to AII by up to threefold [Hollenberg et. al., 1974]. The infusion rate of 0.3 ng AII/Kg/min did not produce a measurable change in the plasma AII level, suggesting that the sensitivity of the glomerular cell was greater than that of the technique used to measure AII. Potassium loading also increased the maximum output of aldosterone in response to AII infusion [Hollenberg, Williams, Burger and Hooshmand, 1975]. With prolonged infusion, a decrease in aldosterone secretion occurred, which may be a secondary effect of potassium depletion [Ames, Borkowski, Sininski and Laragh, 1965]. _In vitro_ studies using human tissue are few. Moreover, due to the profound effect of dietary sodium and potassium intake _in vivo_ and _in vitro_, human studies are difficult to interpret in view of the lack of control of these critical variables. Nevertheless, when normal human adrenal cells were used, the response was similar to that reported _in vivo_ i.e. the AII response was modified by dietary sodium intake [Williams and Braley, 1977]. Cells obtained from aldosteronomas tend to have an increased sensitivity to sodium and AII and a much greater aldosterone output, primarily because of an increased rate of conversion of corticosterone to aldosterone [Williams and Braley, 1977].

The biochemical mechanism whereby AII stimulates aldosterone synthesis is complex. Aldosterone synthesis is regulated at two steps; an early step involving the conversion of cholesterol to pregnenolone which is therefore common to many steroids, and a specific later step, the conversion of corticosterone to aldosterone [Muller, 1971]. To study these two regulated steps Campbell, Brady and Gomez-Sanchez [1986] used an inhibitor of 3 beta-hydroxy steroid dehydrogenase, cyanoketone, to block the further metabolism of pregnenolone. Using this method in isolated adrenal cells from rats, they found that AII stimulated pregnenolone synthesis in a concentration related manner; at higher concentrations it also stimulated the second step.

The control of steroid secretion by AII may differ across species. There is evidence for this in the response of fasciculata cells to AII. Studies of rat and normal human cells give no indication that AII stimulates secretion [Hollenberg et. al., 1974, Williams and Braley, 1977]. However, in bovine and in pathological human tissue, AII stimulates cortisol production [Kojima, Kojima and Rasmussan, 1986]. The implication of these findings in terms of AII stimulated aldosterone secretion are uncertain, but lead to caution in the interpretations made from animal studies.

Prostaglandins have also been proposed as regulators of adrenal steroidogenesis [e.g. Kostis, DeFelice and Pianko, 1987], and Campbell et. al. [1986] found that PGE_2 stimulated aldosterone synthesis by activating the early step. Furthermore, indomethacin, at a concentration that inhibited the synthesis of prostaglandins, reduced by 48% the stimulation of aldosterone synthesis by AII. Indomethacin, like PGE_2, acted only on the early biosynthetic step [Campbell et. al., 1986]. This data thus provides support for a role for prostaglandins in AII induced steroidogenesis.

There is a complex interrelationship between the renin-angiotensin system and prostaglandin synthesis. Various prostaglandins have been found to affect the renin-angiotensin system, with prostaglandins E_1, E_2, A_1, D_2, I_2, G_2, H_2 and arachidonic acid all being found to stimulate renin release, whereas PGF_{2alpha}

inhibits renin release [Keeton and Campbell, 1981]. In addition, direct intrarenal AII infusion has been shown in dogs, to evoke release of a PGE_2-like substance, [McGiff, Crowshaw, Terragno and Lonigro, 1970]. Indomethacin administration has been shown to block the intrarenal formation of PGE_2, and to enhance both the renal vasoconstrictor and the overall systemic pressor response to AII [Aiken and Vane, 1973, Negus, Tannen and Dunn, 1976]. Consideration of the relationship between AII and prostaglandins in pregnancy, follows in the next section, (p. 44).

Renal actions.

AII exerts a number of effects on the kidneys. Indirect actions of AII occur via three mechanisms discussed above; increased perfusion pressure due to peripheral vasoconstriction, increased sodium reabsorption and potassium secretion from stimulated aldosterone secretion, and the actions of AII-induced catecholamines.

Direct effects of AII include:

1) A potent feedback inhibition of renal renin release [Vander and Geelhoed, 1965].

2) A potent vasoconstricting effect on renal vascular smooth muscle, especially on the efferent arterioles. This results in increased renal perfusion pressure, decreased renal blood flow, and enhanced reabsorption due to a reduction in both pericapillary hydrostatic pressure and colloid pressure, [these changes are reviewed in detail by Mendelsohn, 1985a].

3) Increased tubular reabsorption of sodium (in the proximal convoluted tubule) [reviewed by Humphreys and Lin, 1988].

4) The interaction with renal prostaglandin synthesis discussed above. In addition AII infusion causes a

reduction in prostaglandin-mediated cAMP production by mesangial cells, resulting in contraction of the cells [Ichikawa and Brenner, 1984].

An intact renin-angiotensin system is crucial to the autoregulation of renal blood flow and glomerular filtration rate, (GFR). When Hall, Guyton, Jackson, Coleman, Lohmeier and Trippodo [1977] reduced renal perfusion pressure in dogs with high plasma renin concentration, autoregulation of renal blood flow and GFR was maintained. However, when converting enzyme inhibitors were infused, the GFR fell. This effect has been encountered in patients with bilateral renal artery stenosis, where the renin-angiotensin system has maintained the GFR in spite of low perfusion pressures. The addition of captopril has induced functional renal insufficiency in some of these patients [Hricik, Browning, Kapelman, Goorno, Madias and Dzau, 1983].

Angiotensin III (AIII).

The heptapeptide AIII is the first product of degradation of AII, (Figure 1.1). It is AII minus aspartic acid that is removed by aminopeptidase hydrolysis. With the exception of the adrenal cortex, where AIII seems to be more potent than AII in the conversion of cholesterol to aldosterone, AIII manifests all the activity of AII but is a less potent pressor agent, and its concentration is much lower than AII in human plasma [reviewed by Kostis et. al., 1987].

The following section moves on to a more specific consideration of the renin-angiotensin system in normal and in hypertensive pregnancies.

Section 3.

THE RENIN-ANGIOTENSIN SYSTEM IN NORMAL AND IN HYPERTENSIVE PREGNANCIES.

The renin-angiotensin system in normal pregnancy.

Two important features dominate the changes that occur in the renin-angiotensin system in pregnancy. The first is the increase in plasma concentrations of angiotensinogen, renin and angiotensin II (AII). The second is a diminution in sensitivity to infused and presumably to circulating AII.

Helmer and Judson [1967] first demonstrated the marked increase in angiotensinogen in pregnancy. They found an elevation in plasma levels of angiotensinogen in 22 women in normal pregnancy. The administration of oestrogens to 7 pregnant women caused an increase in angiotensinogen similar to that found in pregnancy, from which Helmer and Judson concluded that the changes of pregnancy were oestrogen induced. There is general agreement that the plasma concentration of angiotensinogen increases during pregnancy [reviewed by Broughton Pipkin, 1988]. Derkx, Stuenkel, Schalekamp, Visser, Huisveld and Schalekamp [1986], studying a larger group of women, confirmed that oestrogen-containing oral contraceptive pills induced similar changes. Tetlow and Broughton Pipkin [1986] examined the relative proportion of high and low molecular weight substrates in normal pregnancy using gel filtration chromatography. They found that the proportion of high molecular weight angiotensinogen was linearly correlated with gestational age, rising from approximately 5% to 15% through gestation, although no attempt was made to identify whether the high molecular weight forms were polymers of angiotensinogen, or protein bound forms of low molecular weight angiotensinogen.

Increases in plasma renin activity (PRA) and plasma renin concentration (PRC) have also been demonstrated. Brown, Davies, Doak, Lever and Robertson [1963], in a cross-sectional study of 38 patients, found a rise in PRA, with a peak at 16 weeks gestation. Although Genest, de Champlain, Veyrat, Boucher, Tremblay, Strong, Koiw and Marc-Aurele [1965] also found elevated PRA in pregnancy, the peak values they reported were much later in the pregnancy. Neither group measured angiotensinogen, and the differences may reflect either small sample size or the difficulty in using a measurement that may be limited by available angiotensinogen. The consensus from more recent studies is that PRA rises until 28-30 weeks gestation, and then diminishes towards term [e.g. Oats, Broughton Pipkin, Symonds and Craven, 1981, Karlberg, Ryden and Wichman, 1984, reviewed by Broughton Pipkin, 1988].

The data of Oats et. al. [1981], obtained from a longitudinal study of 18 primigravid patients, demonstrated a rise in PRC throughout gestation, with a level 2-3 times that seen in non-pregnant women being attained. This study also recorded serial measurements of angiotensin-converting enzyme, finding a highly significant increase after 30 weeks gestation.

Massani, Sanguinetti, Gallegos and Raimondi [1967] found that plasma AII levels in normotensive pregnant patients were increased as compared to non-pregnant women, although the numbers in their study, nine and seven respectively, were small. Cross-sectional data suggests a progressive rise to term [Gordon, Symonds, Wilmhurst and Pawsey, 1973, Weir, Brown, Fraser, Lever, Logan, McIlwaine, Morton, Robertson and Tree, 1975], with Weir et. al. finding no correlation between plasma AII and either PRC or angiotensinogen. However, in one longitudinal study of primigravidae, AII levels rose in the first trimester, reaching a maximum at mid-gestation [Oats, 1982].

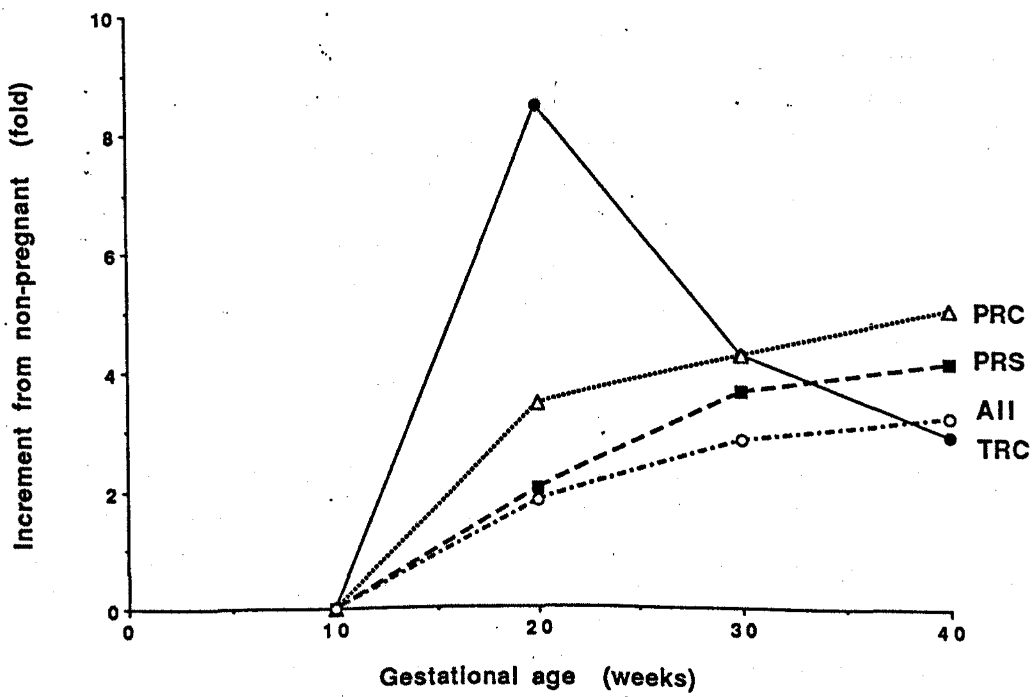


Figure 1.3 Proportional increments of the components of the Renin-angiotensin system in pregnancy. Non pregnant values form the reference point. Data: from various authors quoted above; figure: modified version from Broughton Pipkin [1988].

Most studies of angiotensinase activity in pregnancy, although small in subject number, have indicated a progressive rise throughout pregnancy [e.g. Talledo, Rhodes and Livingstone, 1967, Tapia, Johnson and Strong, 1972]. This may explain the smaller increase, in proportional terms, of plasma AII as compared to plasma renin and plasma angiotensinogen concentrations, (Figure 1.3) [Broughton Pipkin, 1988].

The increased angiotensinase activity has been suggested as a possible explanation of the diminution in pressor response to AII, (see below).

Chinn and Dusterdieck [1972] calculated that in normotensive adult women, AII was usually present in concentrations only just below those capable of affecting arterial blood pressure. As discussed above, the concentration of AII is elevated in normal pregnancy. However, the pregnant woman is protected from the potential hypertensive effects of the increased concentration.

Abdul-Karim and Assali [1961] first demonstrated that the pressor response of 12 subjects to a standard intravenous dose of angiotensin (5ug), was significantly less when tested pre- as compared to postpartum. The pressor response of 4 non-pregnant subjects was similar to that found in the postpartum studies. This diminution in pressor sensitivity appears to be specific to AII. Chesley, Talledo, Bohler and Zuspan [1965] and Lumbers [1970] confirmed the relative insensitivity to AII in pregnancy, yet found that the pressor response to noradrenaline was essentially the same in pregnant and non-pregnant women. In a semi-longitudinal study, principally of Afro-American women, Gant, Daley, Chand, Whalley and

Macdonald [1973] found that the diminution was apparent by 8 weeks gestation, and reached a maximum at 16 to 18 weeks gestation, falling slowly to term thereafter.

Kaplan and Silah [1964] devised a clinical test to separate renovascular hypertension from other forms of hypertension, based on the assumption that patients with increased endogenous levels of AII (renovascular hypertension) required greater doses of exogenous AII to achieve a given pressor response because of prior receptor occupancy. The components of the renin-angiotensin system increase at a similar gestation to that at which reduced pressor sensitivity to AII has been noted [e.g. Karlberg, Ryden and Wichman, 1984], and Talledo, Rhodes and Livingstone [1966] suggested that tachyphylaxis in response to raised AII concentration was the cause of the diminished pressor response. However, no association between threshold dose and endogenous AII was found in 22 women in the second trimester of pregnancy, in whom infusion studies were performed [Broughton Pipkin, Hunter, Turner and O'Brien, 1984]. Furthermore, Gant, Chand, Whalley and Macdonald [1974] found that suppression of basal activity of the renin-angiotensin system by acute volume expansion with normal saline had no effect on the dose of AII required to evoke a 20 mmHg diastolic blood pressure rise in pregnant subjects, despite enhancing pressor responsiveness in non-pregnant women. The rapid infusion of an equal amount of 5% saline, did, however, result in a diminution in pressor response to AII in pregnant patients, with a similar decrease in PRA. These results suggest that the principal determinant of pressor response is responsiveness of the vessels themselves, rather than alterations in activity of the renin-angiotensin system or alterations in plasma volume. There have been several suggestions of mechanisms whereby vessel responsiveness is altered.

Contraction and relaxation of vascular smooth muscle are controlled through variations in cytoplasmic calcium ion concentration. The calcium control of the actinomysin system is the final common pathway by which neurotransmitters, hormones and vasoactive drugs normally exert their short term influences on vascular tone [Johansson, 1981] (discussed further in Section 5, p. 86). Studies in isolated smooth muscle cells with calcium sensitive dyes confirm that activation is associated with increased intracellular calcium [Fay, Shlevin, Granger and Taylor, 1979]. Although little is known about intracellular ionic concentration in the pregnant human, a cross-sectional study demonstrated that in platelets, cells with many of the characteristics of smooth muscle cells, (see Section 8), intracellular free calcium rises in pregnancy [Kilby, Broughton Pipkin, Cockbill, Heptinstall and Symonds, 1990]. Such changes would be expected to cause increased rather than decreased responsiveness.

Another candidate implicated in the diminution of the pressor response is progesterone. In seven women with pregnancy induced hypertension, who had lost their refractoriness to AII, (see below), an infusion of 5 alpha-dihydroprogesterone was associated with the restoration of refractoriness to the pressor effects of AII [Everett, Worley, Macdonald and Gant, 1978]. Moreover, in five pregnant women in whom pressor sensitivity was increased by indomethacin, (see below), infusion of 5 alpha-dihydroprogesterone similarly restored refractoriness. Such data is consistent with the view that a progesterone metabolite is important in maintaining normal blood pressure in pregnancy. However, Chesley and Tepper [1967] found that progesterone administration to non-pregnant women did not blunt the AII pressor response, although the renal response to AII was diminished, whilst oestrogen administration had no effect. Curiously, whilst the dose of 300 mg / day progesterone administered by Chesley and Tepper approximates to the quantity of hormone secreted in pregnancy, the dose of 0.45 mg / day ethinyl oestradiol used, is considerably less than that found in pregnancy. One further difficulty in relating the diminished pressor response found in pregnancy to changes in steroid concentrations is the specific nature of the diminished response. Any effect of steroids, and indeed of changes in intracellular free calcium, would be expected to have a general effect on vascular responsiveness to vasoconstrictors.

The complex inter-relationship between vasodilator prostanoids and AII has been previously discussed, (Section 2, p. 35). Speroff, Haning and Ewaschuk [1976] demonstrated that in pregnant monkeys, acute elevation of blood pressure by AII was associated with a significant increase in the release of prostaglandin E from the uterus. This increase was associated with increased uterine artery blood flow. Although Terragno, Terragno, Pacholxzyk and McGiff [1974] in the pregnant dog, and Venuto, O'Dorisio, Stein and Ferris [1975] in the pregnant rabbit, found diminished uterine artery blood flow following the administration of the prostaglandin synthetase inhibitor, indomethacin, Speroff et. al. [1976] did not initially find this. However, when they substituted alternative anaesthetic agents for the cardiovascular stimulant sodium pentabarbital, their results concurred with those from other species [Speroff, Haning and Levin, 1977].

In addition to playing a role in the control of uterine blood flow, the increase in plasma prostaglandins found in pregnancy [reviewed by Ferris, 1988], may be responsible for the reduced AII sensitivity, with increased AII required in pregnancy to overcome antagonism caused by increased synthesis of vasodilator prostaglandins. Broughton Pipkin, Hunter, Turner and O'Brien [1982] examined the effect of prostaglandin E_2 on the pressor response to exogenous AII in 22 pregnant women in mid-trimester, and in 10 non-pregnant control subjects. Prostaglandin E_2 diminished the diastolic blood pressure response in both groups without altering the basal blood pressure, the effect being significant only in the pregnant group. Furthermore, indomethacin has been shown to enhance the systemic pressor response to AII in pregnancy [Everett, Worley, Macdonald and Gant, 1978].

Another possible candidate as regulator of the AII pressor response in pregnancy is atrial natriuretic peptide (ANP). ANP antagonises AII-induced arterial contractility in vitro to a greater extent than noradrenaline-induced contractility [Kleinert, Maack, Atlas, Januszewicz, Sealey and Laragh, 1984]. Moreover, the majority of studies, (all cross-sectional), suggest increased levels of ANP in pregnancy [e.g. Thomsen, Storm, Thamsborg, deNully, Bodker and Skouby, 1988, Miyamoto, Shimokawa, Sumioki, Touno and Nakano, 1988, Merkouris, Miller, Catanzarite, Rigg, Quirk and Vesely, 1990]. One dissenting study was that of Hirai, Yaniahara, Nakayama, Ishibashi and Yamaji [1988]. Hirai et. al. found that although there was a gradual increase in ANP levels as pregnancy progressed, the mean concentrations were not significantly different from those of the non-pregnant women. They noted both the wide range in pregnancy, (12.5-137 pg/ml), and the marked overlap between groups, criticising the small sample size of other studies. However, the study of Hirai et. al. was also cross-sectional, and the statistical tests used appear to ignore the skewed nature of the data.

Both Gant, Chand, Whalley and Macdonald [1974], studying the effect of acute volume expansion, and Broughton Pipkin et. al. [1982], studying the effect of prostaglandin E_2, found different results when non-pregnant as compared to pregnant subjects were used. The control of vascular responsiveness in pregnancy may well be complex, with several factors important.

One further suggestion, particularly pertinent to this thesis is that these factors mediate their effect via alterations in AII receptor concentrations. This would then be an effect specific to AII, rather than a general effect, as would be caused by changes in intracellular calcium, anatomical effects on structure, or the dilutional effect of the increased blood volume in pregnancy.

Aguilera and Catt [1981] demonstrated that in rat vascular smooth muscle tissue, conditions associated with reduced vascular sensitivity to AII, such as sodium restriction, were also associated with diminished AII receptor concentrations. Prior to this thesis, there has been no reported work on human AII receptor changes in pregnancy. However, studies of rabbit mesenteric vascular tissue, indicate reduced AII receptor concentrations in pregnancy [Brown and Venuto, 1986]. Furthermore, both increases in endogenous AII and progesterone infusions, have been found to reduce AII receptor concentrations; the former in rat vascular smooth muscle tissue [Brunner, Chang, Wallach, Sealey and Laragh, 1972], the latter in rat uterine smooth muscle tissue [Schirar, Capponi and Catt, 1980]. A more detailed discussion of AII receptors follows in Section 5.

<u>The renin-angiotensin system in hypertensive pregnancy.</u>

Section 1 contains a detailed review of many of the aspects of pregnancy induced hypertension (PIH), including detailed definitions of PIH and pre-eclampsia (PE). Discussion of the aetiology of PIH is outside the scope of this thesis, however, hyperfunction of the renin-angiotensin system has been suggested as a possible contributory cause for 20 years [Massani, Sanguinetti, Gallegos and Raimondi, 1967]. This has led to study of the components of the renin-angiotensin system in PIH.

The plasma concentration of angiotensinogen does not appear to change markedly in hypertensive pregnancies [e.g. Tapia, Johnson and Strong, 1972, reviewed by Broughton Pipkin, 1988]. However, Tewksbury and Dart [1982] reported that the proportion of high molecular weight angiotensinogen increased from 16% to 28% in pregnancies complicated by PIH. The true molecular weight of the high molecular weight angiotensinogen is unknown. Gordon and Sachin [1977] obtained an estimate using gel filtration under denaturing conditions, finding a value of 350,000 to 500,000. However the method they employed only yields valid estimates if the shape of all the proteins studied, standards and unknowns, are the same and there is no evidence that this criterion was met. Whether such a difference in the form of angiotensinogen is of functional importance has yet to be assessed.

There are conflicting reports in the literature regarding PRA and PRC in patients with PIH. Some authors suggest a relative increase relative to normotensive pregnancies [e.g. Symonds, Broughton Pipkin and Craven, 1975], some no change [e.g. Brown, Davies, Doak, Lever, Robertson and Trust, 1966, Gallery, Stokes, Gyory, Rowe and Williams, 1980], and others a decrease [e.g. Weir, Brown, Fraser, Kraszewski, Lever, McIlwaine, Morton, Robertson and Tree, 1973, Symonds and Andersen, 1974]. In many of the studies, the range of variation within the group was greater than the difference between the groups [e.g. Brown et. al., 1966]. Possible explanations of these discrepancies are legion. The lack of standardization of collection procedures may have been responsible for at least part of the conflict. Changes of posture have been shown to have a marked effect on PRA in pregnancy [Weinberger, Petersen, Herr and Wade, 1973]. Rowe, Gallery and Gyory [1979] demonstrated how commonly used collection and handling procedures could inadvertently result in cryoactivation of renin, the effect being much greater in pregnant as compared to non-pregnant women. Gallery et. al. [1980] suggested that variation in sodium intake was important. Different criteria for inclusion into the various studies were used. For example, Symonds and Andersen [1974] demonstrated the pitfalls of ignoring parity [e.g. Weir et. al., 1973], finding that changes in PRA and PRC levels were completely different in primigravid and multigravid women. Despite the difficulties in drawing any conclusions, there does seem to be some consensus that in severe, proteinuric PIH, PRA is suppressed [Symonds and Andersen, 1974, Karlberg, Ryden and Wichman, 1984].

Studies of plasma AII levels in PIH have also produced confusing data. In a small series, Massani, Sanguinetti, Gallegos and Raimondi [1967] found no significant differences in plasma AII levels in hypertensive as compared to normotensive pregnancies. The bioassay they used would not have distinguished between angiotensins I and II, and there was no matching of gestational age. On balance, it appears reasonably certain that in pre-eclampsia, AII levels are diminished, even in the face of a contracted plasma volume [Weir et. al., 1973, Pedersen, Aalkjaer, Christensen, Christensen, Danielsen, Johannesen, Kornerup, Leyssac, Mulvany and Rasmussen, 1984]. In mild late-onset PIH there may be activation of the renin-angiotensin system, significantly elevated levels of AII having been found [Symonds et. al., 1975, Symonds and Broughton Pipkin, 1978]. Interpretation of the study by Symonds et. al. [1975] is eased by their exclusion of multigravid women, and by the careful control of posture at the time of sampling which they ensured.

Such changes in angiotensinogen, renin and AII, with marked overlap between groups demonstrated in each of the above studies, are unlikely to account for the pronounced changes in AII sensitivity, (see below). This does not negate a significant role for the renin-angiotensin axis in the development or aggravation of PIH; changes may occur at a cellular level.

Alterations in pressor sensitivity to AII in women with PIH are well documented. In 1968, Talledo, Chesley and Zuspan demonstrated that pre-eclamptic patients showed increased sensitivity to infusions of AII as compared to normotensive pregnant women. Since then several prospective studies have been performed. In 1973, Gant, Daley, Chand, Whalley and Macdonald reported that primigravid women who later developed PIH in the same pregnancy, showed an increased vascular sensitivity to AII as early as the 18th to 22nd weeks of gestation. Their series contained 192 patients, mostly Afro-American teenagers, between 13 and 17 years old. 91% of the subjects who needed more than 8ng of AII/kg/min, (on at least one occasion between 28 and 32 weeks gestation), to elicit an increase in diastolic blood pressure of at least 20 mmHg, remained normotensive throughout the pregnancy. Conversely, only 9% of the women who later developed PIH had not shown an increased sensitivity to AII. There are problems in relating the results of Gant et. al. to other populations, Gant et. al. reported the incidence of PIH in the Dallas population as 37.5%, well above the incidence found in our centre of approximately 10% [Symonds, 1979]. The youth and lower socio-economic status of the Afro-American population, as well as the tendency of negroes to hypertension may have been predisposing factors in the etiology of PIH and pre-eclampsia, and thus may have influenced the data obtained. Teenagers have been suggested as suffering more frequently from hypertensive disorders in pregnancy [Vollman, 1970].

Oney and Kaulhausen [1982] also investigated the prospective value of such studies in the early diagnosis of hypertensive disorders in pregnancy. They tested an unselected European population, performing an AII infusion on 231 normotensive primigravid women between 28 and 32 weeks gestation. They confirmed the high predictive value of a negative test, (0.954), but found a high false positive rate, (approximately 50%). Similar results were obtained by Nakamura, Ito, Matsui, Yoshimura, Kawasaki and Maeyama [1986] in a smaller study of 48 Asian women of mixed parity, and by Dekker, Makovitz and Wallenburg [1990] in a study of 90 nulliparous European women. The mean effective pressor dose of AII in the three very different Afro-American, European and Asian populations differed minimally, suggesting that race is not a major factor in determining vascular reactivity to AII.

One dissenting report is that of Morris, O'Grady, Hamilton and Davidson [1978]. They examined a small group of young primigravid patients, testing them each week from the 29th to the 32nd week of pregnancy. They found a false positive rate of 93% and a false negative rate of 17%, concluding that AII infusions were an unreliable screening test. However, there were methodological differences in this study. Rather than using a sphygmomanometer to measure arterial blood pressure, Morris et. al. used a Doppler ultrasound device. For unknown reasons, they could not establish a constant baseline diastolic pressure. Therefore they calculated basal diastolic blood pressure from the average of two 30 minute periods in the left lateral recumbent position.

Sensitivity to infused AII is regarded as one of the better predictors of whether PIH will develop later in the pregnancy [e.g. Wallenburg, Dekker, Makovitz and Rotmans, 1986]. However, the test is impractical as a screening test for the general population, taking several hours to perform, and requiring both medical supervision and the placement of an intravenous cannula.

These findings are complemented by the observation that specific hyper-responsiveness to AII has been documented in isolated small arteries taken from the omentum of women with proteinuric PIH [Aalkjaer, Johannesen, Pedersen, Rasmussen and Mulvany, 1984].

The cause of such altered pressor sensitivity to PIH is unknown.. In a cross-sectional study comparing intracellular free calcium ion concentrations in women with established PIH and normotensive pregnant women, Kilby, Broughton Pipkin, Cockbill, Heptinstall and Symonds [1990] found increased levels in women with PIH. However, as discussed above, there are difficulties in relating such calcium ion changes to altered pressor sensitivity, not least the elevated levels found in pregnancy [Kilby et. al., 1990]. One attractive hypothesis is that the diminished production of vasodilator prostanoids in severe PIH is a major factor enhancing pressor sensitivity to AII. A fall in the stable metabolite of prostacyclin, 6-keto-PGF_{1alpha}, has been reported to occur early in pre-eclampsia [Pedersen, Christensen, Christensen, Johannesen, Kornerup, Kristensen, Lauritsen, Leyssac, Rasmussen and Wohlert, 1983]. Indeed, Fitzgerald, Entman, Mulloyk and FitzGerald [1987] identified an 'at risk' cohort during the first trimester, on the basis of urinary 6-keto-PG_{1alpha} excretion. Lower levels of prostacyclin synthesis has also been reported in the umbilical vessels of the babies of pre-eclamptic women [reviewed by Ferris, 1988].

Broughton Pipkin [1988] speculated that in women with PIH, altered sensitivity to AII might be due to changes in AII binding. Prior to this thesis no formal examination of AII receptor number or avidity in human pregnancy appears to has been performed, and there is no spontaneous disease nor experimentally induced condition in animals that has general acceptance as a model of pre-eclampsia.

Before discussing AII receptors at greater length, the next section considers the theory of receptors in general.

Section 4.

RECEPTOR THEORY.

One of the earliest proponents of the concept of receptors was Paul Ehrlich, a German physician, biologist and chemist. He was concerned with antibodies against bacterial toxins and snake venoms, and postulated that antitoxins were essentially free receptors which combined with the toxin, thereby preventing combination with the receptors of the cells. Ehrlich summarised his emphasis on the binding of drugs to receptors: 'If the law is true in chemistry that **corpora non agunt nisi liguida,** then for chemotherapy the principle is true that **corpora non agunt nisi fixata** (substances do not act unless bound)' [Ehrlich, 1913].

Receptor theory is crucial to this thesis, thus before AII receptors in particular are reviewed, there follows a brief discussion of conceptual models of drug-receptor interactions.

Many pharmacological and physiological observations support the hypothesis that drugs and hormones produce their effects by interacting in a specific way with some component of a living cell. This component, which is likely to be either an enzyme or a site on the cell membrane, is called a receptor.

One analogy for the action of a drug on a receptor is that of a lock, (the receptor), and key, (the drug). This image was first proposed by Emil Fischer in 1894 for the action of enzymes, where the lock is the active centre of the enzyme, and the key is the substrate. In that this is a mechanistic view of a chemical combination, the analogy is crude; nevertheless, some valid implications are conveyed. The drug 'unlocks' the response, moreover, the specificity of the 'locks' as far as the 'keys' are concerned is only relative; the lock may be turned by a different number of keys, and so a series of chemically related drugs may have the same action. In addition, the lock may be 'jammed', i.e. the response may be blocked by an appropriate substance [Fischer, 1894].

Application of the Law of Mass Action: The Occupation Theory.

Any detailed conclusions about drug-receptor interactions must rest on information derived from studies of the kinetics of drug action or of dose response curves. The kinetics of drug action, however, when measured on isolated tissues, are likely to depend on the rate of diffusion of the drug to the receptors, on the rate of reaction of the drug with the receptors, and on the rate of response of the cells to the drug-receptor reaction. These problems may arise even when responses are measured on single cells [Mackay, 1966]. Analyses of drug-receptor interactions therefore tend to be based on dose response curves measured under equilibrium conditions, so that complicated kinetic factors are eliminated. The responses obtained are then assumed to correspond to an equilibrium, or steady state, occupation of the receptors, to which the law of mass action may be applied.

The simplest assumption about the formation of a drug-receptor complex is that it can be expressed as a chemical reaction:

Drug + Receptor $\rightleftharpoons$ Drug-receptor complex

with the complex producing a biological response of magnitude E, proportional to the amount of the complex, where k_1 and k_2 represent the forward and reverse rate constants, thus:

$$D + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DR$$

and $$E = q\,[DR]$$

where q is a proportionality constant.

According to the law of mass action, the rate of forward reaction is given by $k_1[D][R]$, and the rate of the reverse reaction is given by $k_2[DR]$.

At equilibrium, the rates of association and dissociation are equal: $k_1[D][R] = k_2[DR]$

therefore:

$$\frac{k_1}{k_2} = K = \frac{[D][R]}{[DR]} \qquad (1)$$

where K is the dissociation constant, also known as the Michaelis-Menten constant. The affinity of the drug for the receptor is given by 1/K .

The total number of receptors, Rt, is the sum of the receptors involved in forming the complex, plus the free receptors, thus: $[R] = [Rt] - [DR]$

Substituting for [R] in equation (1):

$$K = \frac{[D]([Rt] - [DR])}{[DR]}$$

This may be rearranged to the form:

$$\frac{[DR]}{[Rt]} = \frac{[D]}{K + [D]} \qquad (2)$$

If Em is the maximal response of which the system is capable, obtained when all the receptors are occupied:

$$Em = q[Rt]$$

therefore:

$$\frac{E}{Em} = \frac{[DR]}{[Rt]}$$

and therefore:

$$E = \frac{Em\ [D]}{K + [D]} \qquad (3)$$

This is the familiar hyperbolic function in which E = 0 when [D] = 0, and E approaches Em when [D] becomes very large. When half maximal response is obtained:

$$\frac{E}{Em} = \frac{[D]}{K + [D]} = \frac{1}{2}$$

so that the concentration of D required for a half-maximal response is equal to K.

The derivation of equation (3) is identical to that of the classical Michaelis-Menten equation, which gives the velocity v of an enzyme reaction as a function of the substrate concentration [S], the enzyme-substrate dissociation constant K , and the maximum velocity V :

$$v = \frac{V\ [S]}{K + [S]}$$

Three critical assumptions, which underlie the derivation or usual application of equation (3) are as follows:

a) **Response is proportional to receptor occupancy.** The receptor theory of drug activity was first postulated by Clark [1926a], and simply states that the intensity of a pharmacological effect is directly proportional to the number of receptors occupied by the drug. In the case of enzyme-substrate and enzyme-inhibitor interactions, since the observed response is derived directly by the concentration of enzyme bound substrate or inhibitor, this assumption is soundly based. However, with respect to drug effects in general, the assumption may not be correct, and alternatives to the 'occupation theory' are considered below.

b) **One drug molecule combines with one receptor site.** This is the simplest reaction mechanism, from which equation (3) (and also the Michaelis-Menten treatment) is derived.

c) **A negligible fraction of the total drug is combined.** In equation (3) this enables the term [D], the uncombined drug, to be replaced by [Dt], the total drug concentration. A system in which this assumption is true, is said to be operating in zone A [Straus and Goldstein, 1943]. In experiments where binding is actually measured, as in equilibrium dialysis, uncombined drug is determined and equation (3) can be used rigorously. However, when a biological response is the criterion of effect, only the total amount of the drug added to the system is known.

If equation (3) is inverted , the equation of a straight line is obtained:

$$\frac{1}{E} = \frac{K}{Em} \times \frac{1}{[D]} + \frac{1}{Em} \qquad (4)$$

When reciprocal response magnitudes, 1/E, are plotted against reciprocal doses, 1/[D], (actually 1/[Dt], as explained above when discussing the zone A assumption), the slope is K/Em, and the intercept 1/Em. This procedure is known as the double reciprocal or Lineweaver-Burk plot [Lineweaver and Burk, 1934]. Increasing drug concentrations are to the left, so that the y-axis is at 1/[D] = 0, corresponding to infinite drug concentration, where all receptors are occupied. The y intercept gives 1/Em. Since the slope, given by equation (4), is K/Em, K can be obtained by estimating the slope: but an easier method is to extend the line downwards and to the left, as shown in Figure 1.4. The x intercept is -[1/K].

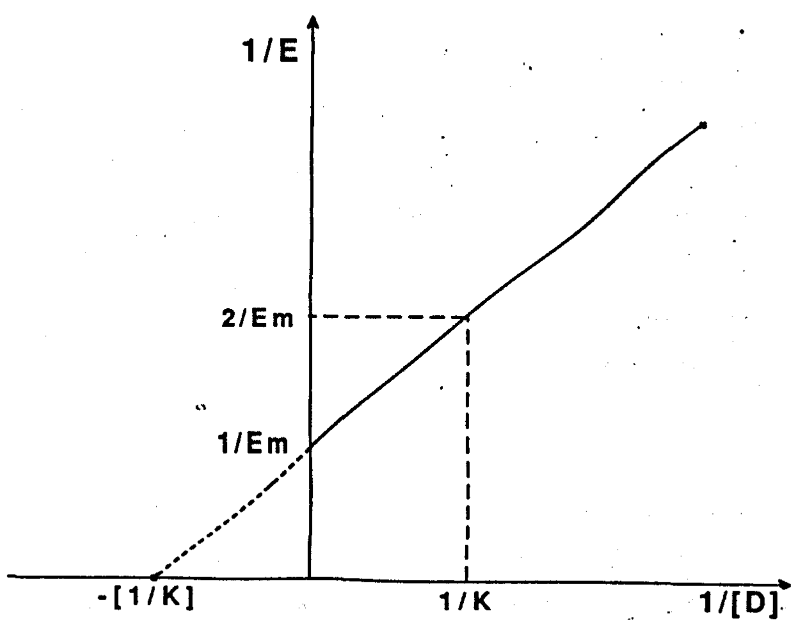


Figure 1.4 - The double reciprocal plot.

Another very useful linear transformation often used when interpreting binding data is the Scatchard plot [Scatchard, 1949]. Equation (1) states that:

$$K = \frac{[D]\,[R]}{[DR]}$$

If n is the number of binding sites per protein molecule, and P the molar concentration of protein in the system, then nP, the total concentration of binding sites, is equal to [DR] + [R], and:

$$nP[D] - [DR][D] = K[DR]$$

$$(K+[D])[DR] = nP[D]$$

If r is the number of moles of drug bound per moles of total protein, then:

$$r = \frac{[DR]}{P} = \frac{n[D]}{(K + [D])}$$

therefore: $$Kr + r[D] = n[D]$$

and therefore: $$\frac{r}{[D]} = \frac{-r}{K} + \frac{n}{K}$$

Thus plotting r/[D] against r should yield a straight line with two intercepts, n (on the x axis) and n/K(on the y axis), hence K is obtained. An example of this approach is shown in Figure 1.5. In this example, the dissociation constant of the platelet angiotensin II binding in one representative subject is shown, in an experiment examining the effect of reducing unlabelled angiotensin II on specific binding, (see p. 129).

A useful feature of this type of graph is that if there are distinct sets of binding sites with different affinities, two or more line segments may be distinguishable [Klotz and Hunston, 1971]. Figure 1.6 illustrates this type of plot for the binding of acetylcholine to a proteolipid extracted from an acetylcholine-sensitive tissue. There is one binding

Figure 1.5.

Scatchard analysis of the results from one platelet angiotensin II specific binding competition study (see Chapter 3, p. 129).

Figure 1.6.

Scatchard analysis of acetylcholine binding to a proteolipid extracted from electroplax tissue of the electric eel. From De Robertis, Lunt and La Torre [1971].

Figure 1.5.

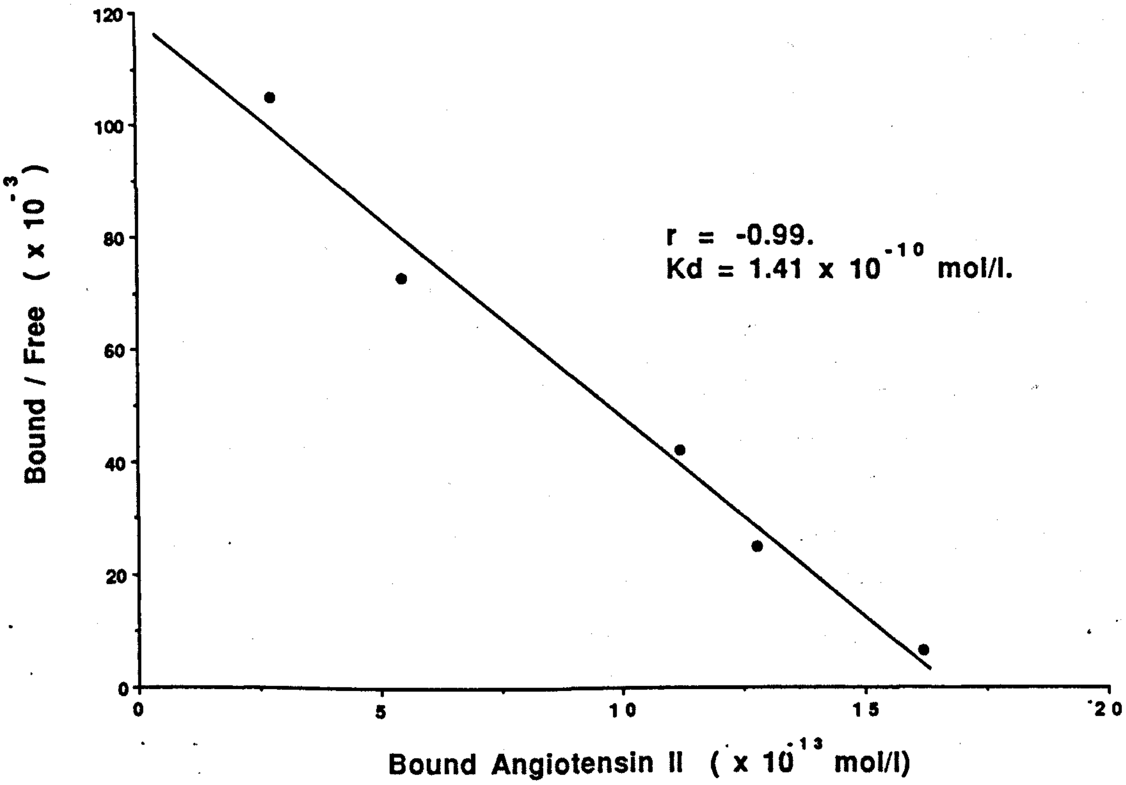
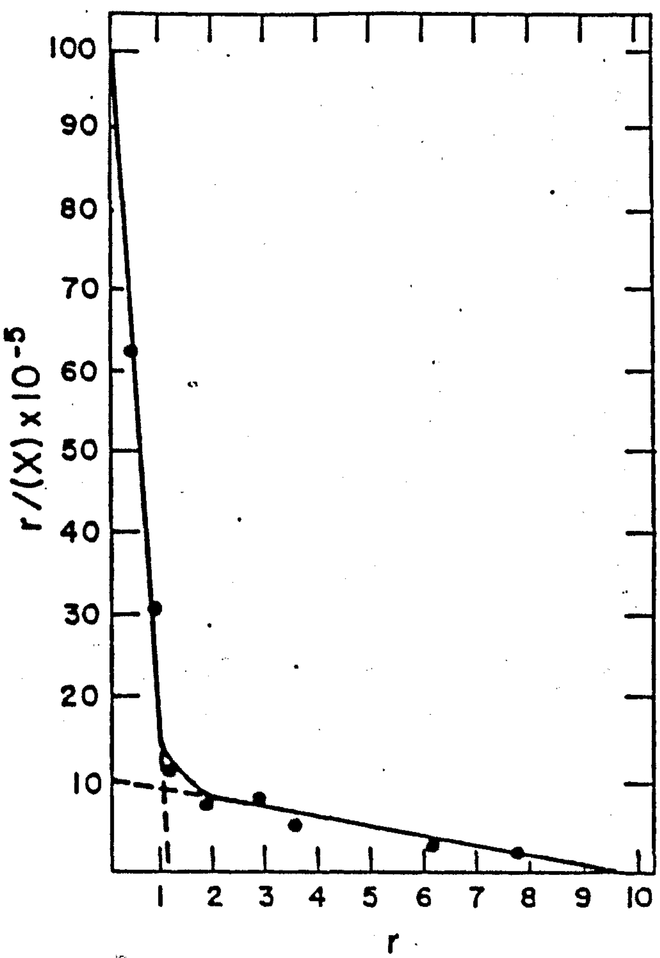


Figure 1.6.



site with high affinity and about ten other sites where the binding is much weaker [De Robertis, Lunt and La Torre, 1971].

It should be pointed out that analysis by Scatchard plots was originally developed for systems where soluble ligands interact with one or more independent binding sites on a soluble macromolecule. Such a model may not hold for particulate membrane receptors with extremely high affinity. In some cases, for example radio-labelled cholera toxin, binding can occur at concentrations well below the dissociation constant, K, of the compound [Henderson, 1973].

Alternatives to the Occupation theory.

a)Efficacy (Intrinsic Activity).

After Clark proposed the theory [1926a], loopholes were discovered. With certain series of drugs, for example alkyltrimethylammonium compounds tested on guinea pig ileum [Stephenson, 1956], a maximal response was never achieved even at extremely high doses. The biological effect did not appear to follow the law of mass action, nor did it seem to be dependent on the affinity of the drug for the receptors.

Ariens [1954] and Stephenson [1956] proposed modifications to the occupation theory in an attempt to explain such anomalous factors. They visualised the drug-receptor interaction as being a two step phenomenon:

1. Complexation of the drug with its receptor.
2. Production of the effect.

Affinity of the drug for the receptor alone is not sufficient. The compound must also have what Ariens described as intrinsic activity. The Ariens-Stephenson idea considers the ability of the drug receptor-complex to produce a biological effect. Their modification brings in the concept of agonists and antagonists, which are thought to have a strong affinity for the receptor and a tendency to form a complex. However, only the agonist gives rise to the stimulus and has intrinsic activity.

Since the biological effect of a drug at a given dose would be determined both by the extent of receptor occupancy, (determined by affinity), and by the intrinsic activity or efficacy, it follows that according to this theory equal biological responses need not imply equal degrees of receptor occupancy, and maximal responses may vary from drug to drug.

b) Spare receptors.

Much of the data on drug-receptor interactions is based on studies of smooth or striated muscle. There are many steps between a drug interacting with a receptor and the contraction of the muscle; namely changes in ion fluxes, depolarisation, intracellular release of calcium, etc. It is certainly plausible that the limiting factor may not be the total number of receptors, but rather one or more of the intervening processes. Hence a maximal response would be obtained with only a fraction of the receptors occupied. Certain anomalous effects, not readily explained by the occupation theory led to the formation of this alternative known as the spare receptor hypothesis [Stephenson, 1956, Ariens, Van Rossum and Koopman, 1960].

The 'atropine anomaly' is typical of such effects [Clark, 1926b]. Acetylcholine reduces the amplitude of contraction of the frog heart. If the heart is treated with a small dose of atropine and then retested with acetylcholine, a ten-fold increment in atropine concentration necessitates a ten-fold increase in acetylcholine dosage to achieve the same response as previously. At all atropine concentrations the same maximum response could be elicited, provided the acetylcholine concentration was high enough.

According to the occupation theory, when maximal response was obtained in the presence of atropine, the receptors should be fully occupied by acetylcholine molecules, with the atropine molecules displaced. Thus, if the tissue was removed from a high atropine and acetylcholine concentration, then replaced in a solution without atropine, and finally in solution without atropine or acetylcholine, the heart should have regained the original sensitivity to acetylcholine. In fact, the decreased sensitivity produced by atropine persists. With repeated washings, the atropine effect diminishes only very slowly, but the rate of washout is the same whether or not acetylcholine is present. The antagonism, which is apparently competitive, is at the same time almost irreversible, and not affected by the presence of the agonist.

This phenomenon is explained by the spare receptor concept. The affinity of acetylcholine for the receptors is assumed to be low, but maximal response is obtained when only a small fraction of the receptor pool is occupied. Supposing that when 0.01 of the total number of receptors, n, combine with acetylcholine to cause maximal response, 99% of the receptors are 'spare'. If atropine irreversibility blocks 90% of the receptors, leaving 0.1n available for interaction. 10% occupancy is now required to produce maximal response, thus a much higher acetylcholine concentration is necessary.

c) **Rate theory.**

Instead of attributing excitation to the occupation of receptors by drug molecules, the rate theory attributes it to the process of occupation, each association between a drug molecule and a receptor providing one quantum of excitation [Paton, 1961]. The magnitude of a response is proportional to the rate at which drug molecules associate with receptor sites.

Efficacy, or intrinsic activity, is no longer an **ad hoc** constant, but is defined by the rate constant for association of drug molecules with receptors, k_1, which may differ from drug to drug. The distinction between an agonist and an antagonist is defined by the value of k_2, the rate constant of dissociation. If the k_2 is large, then the rate of dissociation of the complex will be high, making free receptor sites available at a high rate. Such drugs are agonists. However, if the k_2 is small, the drug-receptor complex is stable, and free receptor sites become available only infrequently, with little excitation and only weak agonist action. The persistent occupancy of the sites by such a drug reduces the number of sites available to an agonist, so that the drug behaves as an antagonist. For all drugs, the potency is described by the equilibrium dissociation constant k_2/k_1 which as usual describes the affinity of the drug for the receptor.

Rate theory explains the finding that some antagonists first stimulate then block as the stimulation fades. One example is nicotine, which initially excites the autonomic ganglion cell, then blocks it, so that it no longer responds to various agonists. The stimulation is a consequence of the initial associations between drug molecules and receptors, which proceed at a high rate, as all receptor sites are vacant [Goldstein, Aronow and Kalman, 1974].

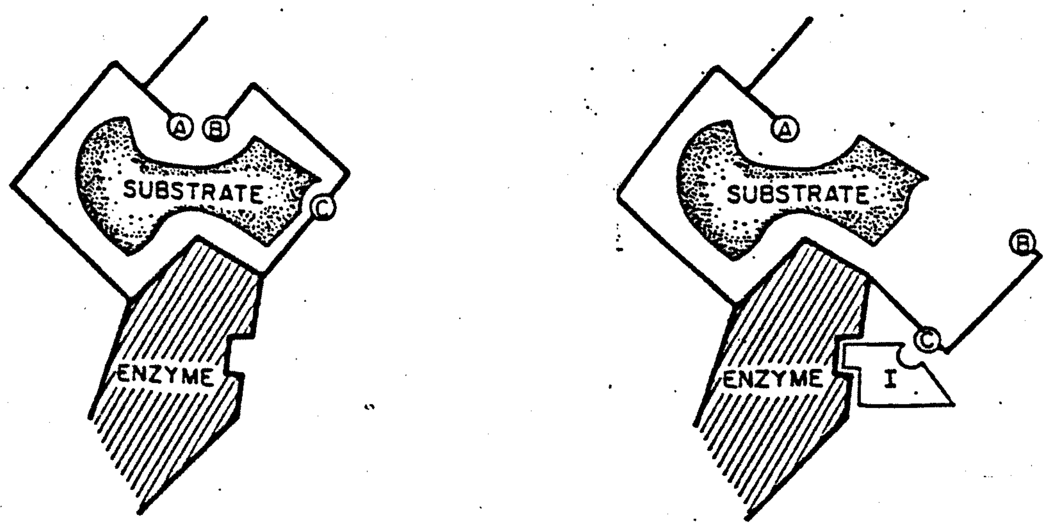
Rate theory also accounts for a phenomenon known as fade, in which an agonist causes an immediate peak response that fades off to a lower plateau at equilibrium. The theory predicts such behaviour for all agonists, since the initial rate of combination is bound to be higher than the eventual rate at steady state. However, numerous instances are known in which fade is not observed at all, with responses following those predicted by occupation theory, e.g. the action of the cholinomimetic drug norarecoline on guinea-pig atrial tissue [Bieger, Kruger-Thiemer, Lullmann and Ziegler, 1970].

d) **Allosteric effects.**

The interpretations presented above were attempts to find a consistent explanation of the observed phenomenon of partial agonism. The recognition of the general importance of allosterism in metabolic regulation has been accompanied by the realization that allosteric transitions (induced conformational changes) play a major role in the action of drugs on membrane bound receptors [Belleau, 1964, and Changeux, Thiery, Tung and Kittel, 1967]

Recent observations indicate that substrate and inhibitor may combine at different sites and yet influence each others affinity for a receptor by inducing configurational changes in the receptor. Figure 1.7 [Koshland, 1964] suggests one possibility. A, B and C represent a flexible active site, which assumes an active form under the influence of a substrate molecule. An inhibitor of a different shape combining at a different site could impede the substrate-induced change. In the model shown, the inhibition might seem competitive, if the affinity of substrate or inhibitor for their respective sites was diminished by the combination of the other with its site.

Figure 1.7. The effect of an inhibitor on a flexible active site [Koshland, 1964].

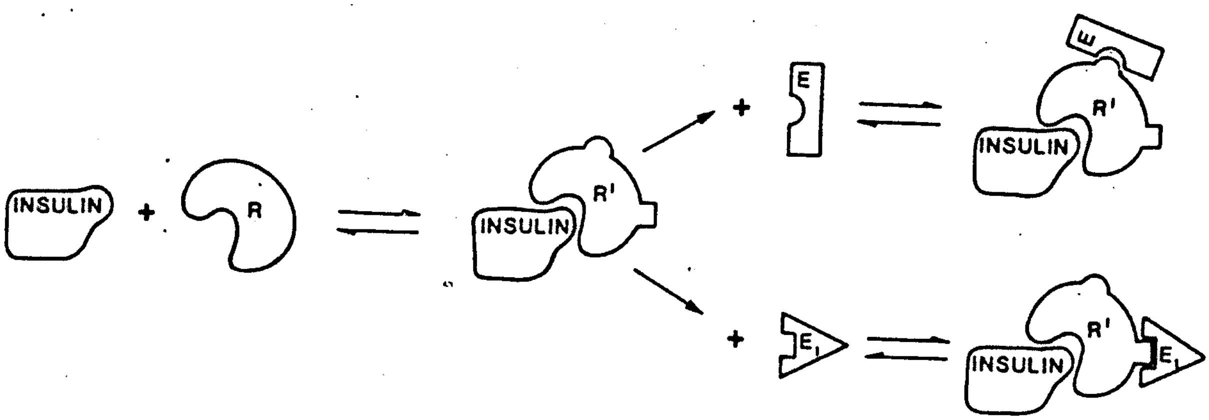


Studies with cholinergically innervated cells from the electric eel provide experimental evidence of an allosteric effect [Changeux and Podleski, 1968]. When data was obtained on the relationship between the extent of depolarisation and the concentration of cholinergic agonists, a systematic departure from the law of mass action was observed. Changeux and Podleski [1968] proposed that the acetylcholine receptor exists in two interconvertable states, a depolarized state (D) with a higher affinity for agonists, and a polarized state (P) with a higher affinity for antagonists. The membrane potential is governed by the relative proportions of the two states. An agonist selectively complexes with the (D) state, shifting the equilibrium in that direction. An antagonist selectively complexes with the (P) state preventing the shift to the (D) state. Low intrinsic activity of a partial agonist would be determined by its ability to complex with either state, but with a preferential affinity for the (D) state. Thus, differences in maximal response can be explained, for regardless of how high the concentration of such a drug was, it would hold a definite proportion of the receptors in the inactive (P) state.

e) Mobile or floating receptor model.

The two-state model outlined above, depicting a concerted conformational change of the receptor, implies a restricted association between an ionophore and the ligand recognition site. The 'mobile' or 'floating' receptor model, developed simultaneously in a number of laboratories [e.g. De Haen, 1976, Jacobs and Cuatrecasas, 1976, and Boeynaems and Dumont, 1977], proposes that a hormone-receptor complex may interact with a number of 'effector' substituents in the plane of the membrane. The above workers observed that a number of receptor-specific agonists, (e.g. catecholamines, prostaglandins, glucagon, ACTH), could independently stimulate adenylate cyclase in a cell such as the adipocyte. The manner of the response was indicative of a unique adenylate cyclase enzyme responding in a complex way to a variety of hormonal stimuli. Rather than supposing that all of the receptors cluster around the cyclase enzyme complex, (this very large complex, which would be visible at the electron microscope level, has not been observed), this model proposes that each independent receptor moiety can freely compete for the effector, (adenylate cyclase), in the plane of the membrane. Moreover, observations with fluorescent-labelled antibodies directed at cell surface constituents has demonstrated that membrane molecules can diffuse freely in the plane of the membranes [Frye and Edidin, 1970].

Figure 1.8. The mobile or floating receptor model. Using the example of insulin, the receptor (R), changes its conformation (R'). The hormone-receptor complex interacts with two separate membrane effector molecules, (E and E_1) [Hollenberg, 1985].



The model has been generalised to accommodate a variety of receptor systems, the concept of receptors as freely diffusable membrane constituents that, on binding to a ligand, can interact with other freely diffusable membrane effectors, is illustrated in Figure 1.8.

These speculative interpretations are discussed in order to emphasise the many possible ways of looking at the rather complex phenomena associated with drug actions on membrane bound receptors. All the theories discussed suffer from the same weakness. In the absence of concrete physicochemical evidence of drug receptor interactions they remain inconclusive. The simple law of mass action description including the occupation theory, adequately describe a large number of interactions in which the receptor is a soluble macromolecule. The known phenomenon of allosterism illuminates the mechanism of action of a further set of drugs. What remains is a special class of receptors that appear to be integrally bound to membranes. These sometimes yield anomalous kinetic data not readily explained by simple law of mass action theory. It seems likely that real understanding will have to await further progress in the isolation of membrane-bound receptors.

Section 5.

ANGIOTENSIN II BINDING SITES IN VASCULAR TISSUE.

Angiotensin II (AII) is known to produce effects on target tissues such as the adrenal gland, brain, kidney and myocardium, (see Section 2 - The renin-angiotensin system). Receptors for the peptide have been identified in each of these tissues, and their characteristics and function have been extensively reviewed by Aguilera, Schirar, Baukal and Catt [1980], Capponi, Aguilera, Fakunding and Catt [1981], and Mendelsohn [1985]. Discussion of the localisation and binding properties of AII receptors in these organs is beyond the scope of this thesis, and instead this section concentrates on AII binding in vascular tissue. Throughout this section, the use of the term receptor relates to cellular binding sites which demonstrate both the characteristics of a hormonal response, (see below), and a correlation between binding site occupancy and a target cell response. In tissues, such as human platelets, where binding of AII does not fulfil these criteria, (see p. 79), the term binding site is used.

The study of AII receptors in vascular tissue has been complicated by the small amount of smooth muscle in such tissue, and by the difficulty in obtaining suitable membrane preparations for the study of receptor regulation. However, AII binding has been studied in preparations from other smooth muscle tissues including the uterus and the bladder, and in human platelets. The properties of AII receptors in smooth muscle of nonvascular origin have been shown to be generally similar to those found in vascular tissue [Aguilera and Catt, 1981]. In particular, AII receptors in both the rat uterus and bladder smooth muscle demonstrate a parallel negative regulatory influence of sodium restriction to that found in the rat mesenteric artery [Capponi et. al., 1981, Aguilera and Catt, 1981]. Likewise, AII binding sites on human platelets have been found to resemble AII receptors in vascular tissue in the response to alterations in the endogenous renin-angiotensin system [Moore and Williams, 1981, Moore, Taylor and Williams, 1984, Ding, Kenyon and Semple, 1985]. AII binding studies in the above tissues are thus discussed, although the limitations of using AII binding both in platelets and in nonvascular smooth muscle as models of vascular smooth muscle AII binding must be recognised. The rationale of the use of platelet AII binding as a model of myocyte AII binding is further discussed in section 8 (p. 108).

Identification and localisation.

The earliest observations on AII binding in vascular tissues demonstrated specific binding of radiolabelled AII to rabbit aortic strips [Lin and Goodfriend, 1970] and to subcellular fractions of rabbit aorta [Devynck, Pernollet and Meyer, 1973] and guinea pig aorta [Le Morvan and Paliac, 1975]. These binding sites were of relatively low affinity, (15-50 nM), in comparison to circulating levels of AII, (10-100 pM), and may not be representative of AII receptors in resistance vessels. Moreover, contractile responses to AII in the aorta, a fibroelastic, conductive type vessel, differ from those in muscular resistance type vessels [Altura and Altura, 1970]. More recently, AII receptors have been identified in particulate fractions derived from rat mesenteric arterial arcades [Gunther, Gimbrone and Alexander, 1980a, Aguilera and Catt, 1981, McQueen, Murray and Semple, 1984], small muscular vessels representative of resistance vasculature and highly sensitive to AII [Bohr and Urchida, 1967]. These were found to be of higher affinity, (<1 nM), as discussed further below.

The first evidence of specific binding of AII to uterine tissue was provided by Lin and Goodfriend [1970] in the rat, and the presence of uterine AII receptors has subsequently been demonstrated in several species [Schirar, Capponi and Catt, 1980, Siddiqi, Koenig and Clarke, 1986]. Specific binding of AII to human platelets was first demonstrated by Moore and Williams [1981].

Autoradiographic analysis following intracardiac injection of tritiated AII in rats showed nuclear localisation in myocardial and vascular smooth muscle, suggesting a direct interaction of the hormone at a nuclear level [Robertson and Khairallah, 1971]. Recent support for the concept of nuclear receptors is provided by Re and co-workers, who suggested that AII might also interact directly with the nucleus. They demonstrated binding of AII to a rat liver nucleoprotein, following incubation of rat liver nuclei with radiolabelled AII [Re, Visard, Brown and Bryan, 1984]. The problem of how AII might reach this site in intact cells is unresolved, but one attractive possibility is that the hormone is internalised by receptor mediated endocytosis [Pastan and Willingham, 1981].

However, localisation of the receptors on the plasma membrane has been directly demonstrated biochemically. Analysis of microsomal membranes prepared from homogenates both of rabbit aorta, and of rat myometrium, by differential centrifugation and density gradient fractionation demonstrated that binding sites for tritiated AII copurify with marker enzymes of the plasma membrane, such as adenylate cyclase [Devynck et. al., 1973]. Purified microsomal membranes solubilised with deoxycholate retain their ability to bind tritiated AII [Devynck, Pernollet, Meyer, Fermandjian, Fromageot and Bumpus, 1974].

Photoaffinity labelling, in which a photosensitive ligand is covalently coupled to binding sites prior to solubilisation and fractionation, has confirmed the receptor localisation on the plasma membrane, using dog uterine tissue [Capponi and Catt, 1980]. Electron microscope autoradiography of cultured rabbit aorta smooth muscle cells also demonstrated that the initial site of interaction of AII was at the plasma membrane [Paglin, Stukenbrok, Joyce and Jamieson, 1987]. The latter technique demonstrated varying receptor density amongst cells in the population, ranging from cells that were heavily labelled to those with virtually no label. This implies that AII receptor expression may be limited to a certain progeny within the cell population or be a function of their stage within the cell cycle.

Characterisation.

The hormone - receptor interaction is the first step of a chain of cellular responses leading to the hormonal response. A hormone receptor is a particular cellular component possessing the dual function of recognition and stimulation. For the interaction of a hormone with a cellular binding site to be considered to correspond to a receptor interaction, it should fulfil the criteria of a hormonal response; exhibiting specificity, high affinity, reversibility and saturability.

Specificity of AII receptors in the rabbit aorta was demonstrated by the absence of any effect of phentolamine, methylsergide, and diphenhydramine, at doses that completely abolished the vasoconstrictor effect of noradrenaline, 5-hydroxytryptamine, and histamine, respectively [Rioux, Park and Regoli, 1973]. Rioux et. al. [1973] also demonstrated that the myotropic effect of AII was similarly unchanged in the presence of indomethacin, which significantly reduced the myotropic effect of prostaglandin E2.

The structural requirements for binding of AII have been extensively investigated. Both Gunther, Gimbrone and Alexander [1980a] and McQueen, Murray and Semple [1984] found that in rat mesenteric artery tissue, the discrimination between the affinities for angiotensin I, II, and III, and angiotensin analogues, was consistent with the pressor activity of these peptides [Bumpus, Khairallah, Arakawa, Page and Smeby, 1961, Carey, Vaughan, Peach and Ayres, 1978]. In particular, angiotensin I (AI) had virtually no effect on the binding of tracer AII.

In similar studies using uterine tissue from dogs and using human platelets, little difference was found between the affinities of AII and angiotensin III (AIII) [Gregory and Aguilera, 1981, Moore and Williams, 1981]. This seems to disagree with the affinities expected from pharmacological studies;

during AII and AIII infusions in man, the heptapeptide evokes only 15-30% of the pressor effect of AII [Carey et. al., 1978]. However, Gregory and Aguilera [1981] suggested that similar affinities should be expected, providing evidence that responses to infusion of the peptides relate to differences in degradation rates.

Binding sites of **high affinity** have been found in rat mesenteric arteries [e.g. Gunther, Gimbrone and Alexander, 1980a, McQueen, Murray and Semple, 1984]. The apparent dissociation constant Kd of 1 nM found by Gunther et. al. [1980a] suggested a lower affinity than that found by McQueen et. al. [1984] of 19-74 pM, although the observed concentrations of binding sites were similar, (54 and 80 fmol/mg of membrane protein respectively). The latter group suggested that degradation of tracer AII might explain the different dissociation constants found. McQueen et. al. [1984] encountered extensive degradation that could not be inhibited without adversely affecting binding capacity, necessitating the use of non-linear regression methods for the analysis of radioligand binding data. The lower affinity for the AII receptor reported by Gunther et. al. [1980a] may have been due to unrecognised tracer AII degradation. If the experimental results of McQueen et. al. [1984] are analysed without compensation for tracer degradation, (e.g. by Scatchard analysis), the calculated dissociation constant is in the region of 1 nM.

High affinity binding for AII has also been demonstrated in uterine tissue, (e.g. in the rat, Kd: 1 nM [Schirar, Capponi and Catt, 1980]), and in human platelets, Kd: 0.1 - 0.3 nM [e.g. Moore and Williams, 1981, Ding, Kenyon and Semple, 1984].

The concentration of AII binding sites on platelets deserves additional comment. A maximal level of approximately 10 receptor sites per platelet has consistently been found [e.g. Moore and Williams, 1981, Mann, Sis and Ritz, 1985, Ding, Kenyon and Semple, 1985a]. Assuming 1 mg of platelet membrane protein per 10 cells [Alexander, Cooper and Handin, 1978], the platelet, with 10 fmol AII bound / mg protein, has 20% of the binding site density reported in uterine smooth muscle (45 fmol/mg) and vascular smooth muscle (57 fmol/mg) [Aguilera, Hauger and Catt, 1978, Gunther, Gimbrone and Alexander, 1980b].

Scatchard analyses of data derived from steady-state binding and binding-inhibition experiments have often indicated the presence of two populations of binding sites, with differing affinities [e.g. Chevillotte, Devynck, Fyhrquist, Meyer, Rouzaire-Dubois and Worcel, 1975, McQueen, Murray and Semple, 1984, Moore and Kwok, 1987]. The significance of the low affinity binding sites is controversial. In most cases the low affinity sites have been considered physiologically insignificant, part of non-specific binding, or simply overlooked. Chevillotte et. al. [1975] and McQueen et. al. [1984] argued that it was unlikely that the low affinity binding sites were involved in any response to AII, as their low affinities would probably not permit interactions with AII at physiological concentrations, (10-100 pM). However, Moore and Kwok [1987] found that in rat uterine tissue, the value of the dissociation constant obtained by photoaffinity labelling was two orders of magnitude larger than that obtained for the high affinity membranous AII binding sites. They suggested that the low affinity binding sites represent the native 'resting' receptors, and that the high affinity

Photo-affinity labelling has been used to determine the physical characteristics of the receptor. AII receptors in the dog uterus have been shown to consist of two species with a relative molecular mass value of 126 and 64 kDa. On sodium dodecyl sulfate polyacrylamide (SDS) gel electrophoresis under reducing conditions, Capponi and Catt [1980] observed a single band of 68 kDa. AII receptors in rat liver and adrenal glomerulosa membranes have also been studied. There seems to be general agreement that the AII receptor is composed of two, (or perhaps four), subunits, each of a relative molecular mass near 68 kDa [Mendelsohn, 1985]. This is consistent with the suggestion above, that one or more disulphide bonds are essential bridges in the receptor molecule.

Whilst the techniques above are useful tools in characterising the AII receptor, they have not resulted in its purification to homogeneity. Recently it was hypothesised that there is a molecular recognition code in which peptide ligands and their receptor binding sites can be encoded by complementary nucleotide sequences [Bost, Smith and Blalock, 1985]. Elton, Dion, Bost, Oparil and Blalock [1988] demonstrated the use of this hypothesis in the purification of the AII receptor. They found that a peptide, specified by RNA complementary to the mRNA of AII, competed specifically with radiolabelled AII for AII binding sites on rat adrenal membranes. Furthermore, they used an antibody to the complementary peptide to immunoaffinity-purify a protein which specifically bound radiolabelled AII.

The complete validation of peptide hormone binding sites as receptors that mediate the biological actions of the ligand requires that a correlation be made between binding site occupancy and an appropriate target-cell response. Indirect evidence for such a correlation is obtained from binding-inhibition studies of AII and its analogues, as detailed above. In addition, a direct relationship between binding and muscle contraction was shown by Baudouin, Meyer and Worcel [1971] whilst studying the specific binding of tritiated AII to rabbit aorta.

Several studies have attempted to determine whether the low AII binding site density on platelets is sufficient to evoke some cellular response. Poplowski [1970] and Ding, MacIntyre, Kenyon and Semple [1985] have suggested that AII added to platelet suspensions enhanced the aggregation response to adrenaline, and Haller, Ludersdorf, Lenz, Distler and Philipp [1987] found that AII increased intracellular free calcium in platelets. The suggestion that the physiological action of AII on platelets may be as an enhancer of the actions of other hormones rather than as a primary effector, may explain the lower concentration of AII binding sites on platelets as compared to vascular tissue (discussed above). As yet, no attempt at correlating any cellular responses with platelet binding site concentrations has been made.

Regulation.

The sensitivity of vascular tissues to AII changes in response to certain regulatory factors, and such changes are thought to be accompanied by parallel AII receptor variations:

a) Circulating AII level.

Kaplan and Silah [1964] observed an inverse relationship between the level of endogenous circulating AII in humans and the pressor effect resulting from the injection of synthetic AII. Furthermore, when the plasma AII level in rats was lowered by nephrectomy, Brunner, Chang, Wallach, Sealey and Laragh [1972] demonstrated an enhanced pressor effect of exogenous AII.

Gunther, Gimbrone and Alexander [1980b] demonstrated that AII receptor concentrations in rat mesenteric arteries were regulated by circulating AII levels. Increased levels of AII induced by sodium restriction resulted in significantly decreased receptor concentration, whereas inhibition of AII formation by an AI converting enzyme inhibitor, SQ-14,225, had the opposite effect. Renin did not appear to play a role, as the increase in plasma renin activity (PRA) in sodium depleted rats was associated with decreased receptor concentration, whilst an even greater increase in PRA in sodium depleted rats given SQ-14,225, (due to loss of feedback inhibition of renin release by endogenous AII), was accompanied by increased receptor density. Moreover, Schiffrin, Gutkowska and Genest [1984] showed a diminution in the number of rat mesenteric artery AII receptors when the hormone was infused, the dissociation constant being unaffected.

Previous studies of the regulation of AII smooth muscle receptors had used uterine membrane fractions, and yielded conflicting results. Devynck, Pernollet, Macdonald, Mathews, Raisman and Meyer [1978] reported increased AII binding in sodium depleted rats, whilst Aguilera, Hauger and Catt [1978] found a 40% decrease. Although the reasons for this discrepancy are unclear, it illustrates the difficulty in extrapolating binding data from the uterus, a tissue which does not respond to physiological concentrations of AII, to vascular smooth muscle.

In human platelet studies, results demonstrating that significant AII binding changes inversely correlate with changes in plasma AII levels [Moore, Taylor and Williams, 1984, Ding, Kenyon and Semple, 1985] were obtained by alteration of dietary sodium.

b) Sodium balance.

The results of variations in sodium balance on the pressor effect of AII are well established, with sodium deficiency causing a decrease in pressor responsiveness to AII, in both the rat [Brunner,Chang, Wallach, Sealey and Laragh, 1972], and in vascular tissue from dogs [Oliver and Cannon, 1978]. In the latter study, altered sodium balance had little effect on the pressor responses to other vasoactive hormones such as catecholamines [Oliver and Cannon, 1978].

The selective nature of this effect suggested a specific mechanism for the changing AII sensitivity. In addition to the studies above, Aguilera and Catt [1981] found that sodium restriction and loading were accompanied by significant decreases and increases, respectively, in rat mesenteric artery AII receptors. Furthermore, during altered sodium intake, no alterations in alpha- or beta- adrenoreceptors were found.

As discussed in section 2, sodium depletion increases AII production by stimulating renin release, and the elevated levels of AII may well account for sodium induced changes in AII receptor concentration.

c) Mineralocorticoids.

Schiffrin, Gutkowska and Genest [1984] found that the decrease in rat mesenteric artery AII receptor concentration when AII was infused, did not occur at the highest infusion dose, (200ng/Kg/min). Moreover, only at this dose did they find a significant increase in plasma aldosterone. When deoxycorticosterone was infused, an increase in the number of AII receptors was noted. Increased vascular AII receptor concentrations have also been shown after infusion of aldosterone into rats _ex vivo_, and after the addition of aldosterone to cultured rat mesenteric artery cells _in vitro_ [Schiffrin, Franks and Gutkowska, 1983].

Whether these results, which have yet to be confirmed in human studies, have any physiological implications is uncertain. However, they suggest that mineralocorticoids may have an up-regulating action on vascular AII receptors.

d) Cations and guanine nucleotides.

Cations have been shown to influence the binding of AII to receptors in a wide variety of tissues, including the adrenal cortex, aorta, renal glomerulus, cerebellar cortex and liver [Wright, Alexander, Ekstein and Gimbrone, 1982]. No consistent pattern of cation effect has emerged, even when vascular tissue alone is considered. Gunther, Alexander and Gimbrone [1980a], Wright et. al. [1982], and McQueen, Murray and Semple [1984] found that the divalent cations Mn^{+2}, Mg^{+2}, and Ca^{+2} increased AII binding concentrations in rat mesenteric artery fractions, whereas the chelating agents EDTA and EGTA had an inhibiting effect. In contrast, Devynck and Meyer [1976] demonstrated that Ca^{+2} and Mg^{+2} decreased AII binding concentrations in the rabbit aorta. The significance of these differences is unclear, but they suggest that AII receptors may vary in their biochemical nature from tissue to tissue, and even from blood vessel to blood vessel. In addition to a possible alteration in the concentration of AII receptors, an Mn^{+2} induced increase in receptor affinity has been demonstrated [Wright et. al., 1982].

Guanine nucleotides have been found to inhibit AII binding [Devynck and Meyer, 1976]. Wright et. al. [1982] noted that in rat mesenteric artery tissue, the sensitivity of AII binding to inhibition by guanine nucleotides was greatly reduced when endogenous divalent cations were chelated by EDTA. They postulated the existence of receptor associated cation-sensitive sites, and suggested that a complex interplay with guanine nucleotides regulates AII binding.

e) Sex steroids.

Uterine contractility is modulated by oestrogen and progesterone, and oestrogen has been shown to increase rabbit uterine alpha-adrenergic and oxytocin receptors [Williams and Lefkowitz, 1977, Nissenson, Flouret and Hechter, 1978]. In 1980, Schirar, Capponi and Catt found that during the rat and rabbit ovarian cycles, changes in the concentrations of uterine AII receptors were directly proportional to changes in the serum levels of oestradiol. After ovariectomy, the density of AII receptors was found to decrease as a function of time elapsed from surgery, reaching 50% of the control values 8 days after castration. Furthermore, short-term infusion of oestradiol caused a progressive increase in AII receptor concentration, reaching a maximum of four- to six-fold above control values at 36 hours. Progesterone infusion for 7 days caused uterine AII receptor numbers to diminish by 93%. No effects of steroids on the affinity of AII receptors were detected.

In pregnant rabbits and pregnant women, there is a sustained rise in circulating AII levels despite a fall in systemic blood pressure [Lee, Oakes, Lam and Hobel, 1982, Karlberg, Ryden and Wichman, 1984]. During pregnancy in both species, there is an attenuated vasoconstrictor response to exogenously infused AII [Abdul-Karim and Assali, 1961, Donker, Min and Venuto, 1983].

During pregnancy in both rats and sheep, a diminution in the number of uterine AII receptors has been shown, [Schirar, Capponi and Catt, 1980, Siddiqi, Koenig and Clarke, 1986]. Schirar found that infusion of the converting-enzyme inhibitor SQ-14,225 did not modify uterine AII receptor concentration, indicating that these changes were unrelated to plasma AII levels.

Brown and Venuto [1986] demonstrated that the concentration of mesenteric artery AII receptors was lower in pregnant than nonpregnant rabbits, and suggested that the decrease in receptors was related to the associated elevation of endogenous AII. Paller [1984] had previously found no AII receptor changes during pregnancy in rats. However, Paller [1984] used nonvascular bladder smooth muscle tissue. In addition, it is particularly inappropriate to compare findings in the pregnant rats with those in other species. It has been suggested that the resistance in pregnancy to the pressor action of AII that is observed in humans [Abdul-Karim and Assali, 1961] and in rabbits [Donker, Min and Venuto, 1983], does not occur in rats [Fowler, Johnson, Kurz, Kiljoil, Love and Payne, 1981]. If this is the case, altered AII receptor concentrations in pregnant rats would be surprising.

As yet, there have been no reports regarding platelet AII binding site changes in pregnancy.

Mode of action.

The activation mechanism of AII in smooth muscle appears to be independent of cyclic nucleotide formation. Penit, Faure and Jard [1983] demonstrated that AII did not modify the cyclic AMP content of rat aortic smooth muscle cells in primary culture. This lack of response was not due to major impairment of the adenylate cyclase system, because a definite response to beta-adrenergic stimulation was found.

The action of AII is, however, calcium dependant. Reduction of extracellular calcium concentration has been shown to abolish the contractile responses of rat uterine tissue to AII [Freer, 1975]. Freer [1975] also noted that the calcium blocking agent, verapamil, prevented AII-induced uterine contractions. Another calcium channel blocker, nitrendipine, has been found to reduce AII pressor responsiveness in pregnant sheep [Lawrence and Broughton Pipkin, 1986].

Gaining insight into the biochemical mechanism by which AII mediates its effect in vascular smooth muscle has been difficult, in part because of the structural and cellular heterogeneity of blood vessels. Several workers [e.g. Penit et. al., 1983, Brock, Alexander, Ekstein, Atkinson and Gimbrone, 1985, Alexander, Brock, Gimbrone and Rittenhouse, 1985] have used cultured vascular smooth muscle cells, which express functional AII receptors, to provide a homogeneous population of intact cells in sufficient numbers for biochemical analysis.

Using the calcium-sensitive fluorescent indicator quin 2, Brock et. al. [1985] demonstrated that AII caused a rapid increase in cytosolic free calcium in cultured rat aortic smooth muscle cells, the increase being dependant on the mobilisation of intracellular calcium and the influx of extracellular calcium. In similar tissue culture, Alexander et. al. [1985] subsequently found that AII stimulated the breakdown of phosphatidylinositol bisphosphate and the generation of inositol triphosphate. The time course of the inositol triphosphate formation was comparable to that of the AII induced cytosolic free calcium increase. Inositol triphosphate has been shown to cause a rapid release of calcium, probably from the sarcoplasmic reticulum [Smith, Smith and Higgins, 1985], which triggers the contraction of smooth muscle by activating a calmodulin dependant protein kinase that phosphorylates a light chain of smooth muscle myosin [Adelstein and Eisenberg, 1980]. Anderson, Gimbrone and Alexander [1981] had previously shown that AII increased phosphorylation of the myosin light chain in cultures of smooth muscle cells derived from rat mesenteric arteries.

Although such a mechanism describes one possible mode of action of AII, it is almost certainly an oversimplification. For example, recent work by Vallega, Canena, Berk, Brock and Alexander [1988], again using cell cultures, indicated that AII regulated the activity of a vascular smooth muscle sodium ion - hydrogen ion exchange mechanism. The authors also suggested that an AII induced alkalinisation may activate a sodium ion - calcium ion exchange; a mechanism for cytosolic calcium homoeostasis.

Information regarding the mode of action and regulation of vascular smooth muscle AII receptors is constantly increasing, aided by the use of accessible tissue models. A discussion of the tissue model used in this thesis, platelets, now follows.

Section 6.

PLATELETS: MORPHOLOGY AND BEHAVIOUR.

Guilio Bizzozero was probably the first person to observe platelets in the circulating blood of intact animals. In 1882 he coined the term 'blut plattchen' and described the changes that platelets undergo when exposed to a foreign surface. These studies and subsequent work suggested that the platelet is almost solely responsible for the white thrombus that plugs a small vascular puncture [Bizzozero, 1882, cited by Wintrole, 1985].

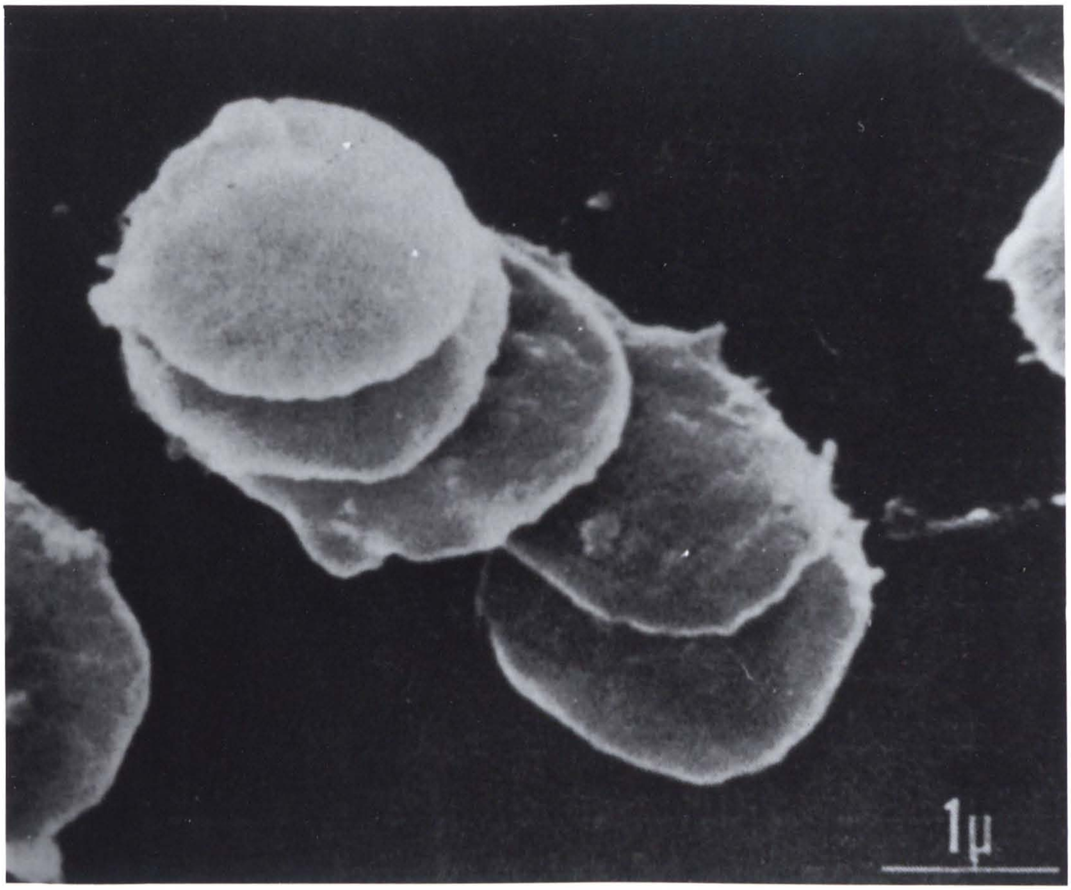
The behaviour of platelets is central to the thesis, thus there follows a discussion of platelet morphology and action.

Platelets are the smallest of the formed elements of blood, with a diameter of 2-3 microns. Normally, approximately 250,000 are present per μl (range 150,000 - 350,000) [Majerus, 1987]. Platelets are formed from megakaryocyte cytoplasm, prior to release into the circulation from the bone marrow [Majerus, 1987]. They remain viable <u>in vivo</u> for about 9 days, and microscopically they appear as flat discs (Figure 1.9). The shape of a platelet is labile; for example, exposure of platelets to the anticoagulant EDTA turns the disc shape into a 'spiny sphere' [Holmsen, 1972]. This shape change is the first response of the platelet to almost any stimulus, and has resulted in criticism of work with platelets which have been handled, washed, or stored for long periods of time [Holmsen, 1972].

Figure 1.9.

Scanning electron micrograph of human platelets drawn directly into fixative.

[Hovig, 1970]


1μ

Morphology.

Platelets can be considered as having three zones, the peripheral, the Sol-gel and the organelle zones. A detailed discussion of platelet morphology is outside the limits of this text, but is extensively discussed, amongst others by Holmsen [1972], Gerrard and White [1976], Shattil and Bennett [1980], and White, Clawson and Gerrard [1981]. The brief review below concentrates on the peripheral zone, consisting of the membranes and closely associated structures, as AII binding sites have been found to be membrane bound [Capponi and Catt, 1980] (see p. 73).

The peripheral zone is made up of three structural domains, the exterior coat, the unit membrane, and the submembrane region. It includes an intricate system of channels continuous with the plasma membrane, the open canalicular system [White et. al., 1981]. The surface area exposed to surrounding plasma is thus much greater than expected, and in this sense the platelet resembles a sponge [Adelson, Rheingold and Crosby, 1961].

The **exterior coat**, or fluffy coat, is about 50 nm thick, and runs all around the platelet. Human platelets can be stained with dyes such as lanthanum nitrate, ruthenium red, and alcian blue, all revealing a heavy glycocalyx on platelets, thicker, and more dense, than the surface coats of other blood cells [O'Brien, 1970]. When Schiff's stain is used, three characteristic bands of glycoprotein are demonstrated; I, II and III. These correspond to molecular weights of approximately 155,000, 135,000 and 100,000 respectively [Mills, 1981]. These three major glycoprotein classes can be subdivided on the basis of affinity for plant lectins, and by the relative intensity of radio-labelling [e.g. Nurden and Caen, 1978]. With a combination of these techniques, as many as 15 different glycoproteins have been identified. These glycoproteins act as receptors for stimuli, triggering platelet activation, and as discussed below, deficiencies of certain receptors may lead to disordered platelet function.

Under the electron microscope, the **unit membrane** appears as a triple layered structure with a thickness of about 8 nm, in common with all cell membranes [Hovig, 1968]. However, this three layered structure is a specific, repeatable artifact originating from fixation procedures, and any such biological membrane should be regarded as a conglomerate of lipid and protein molecules in constant movement relative to each other [Holmsen, 1972].

Lying beneath the unit membrane is the third component of the peripheral zone, the **sub-membrane region.** Electron microscopy has shown this region to contain a relatively regular system of filamentous elements [White, 1969]. Functionally, the sub-membrane filaments are thought to cooperate in maintaining platelet discoid shape, play a role in the extrusion and stabilisation of pseudopods, and interact with other elements of the platelet contractile mechanism to effect platelet cohesion and clot retraction [White et. al., 1981].

The Sol-gel zone is the matrix of the cytoplasm of the platelet. Near the surface, a microtubular system provides a cytoskeleton which maintains the discoid shape of the platelet. This system consists of a bundle of 8-24 circular profiles, each approximately

25 nm in diameter, the major protein being tubulin. Microfilaments constitute a second system of fibres in this zone. These filaments are approximately 5 nm in diameter, with actin being the major proteinaceous component [White et. al., 1981]. Pronounced changes occur in the Sol-gel zone when the platelet is activated. The randomly dispersed cytoplasmic organelles are moved into a tightly packed mass in the central region of the platelet where they are encircled by microtubules and a tight fitting web of microfilaments [Gerrard and White, 1976].

Understanding of the third zone, the organelle zone, increased with the development of electron microscopy. Mitochondria, and at least three distinct types of secretory granules have been demonstrated [Brenton-Gorius and Guichard, 1975]. Alpha granules contain coagulation factors, such as beta thromboglobulin and platelet factor 4, (both heparin neutralising factors), fibrinogen, factor V, and von Willebrand factor. Another type of secretory granule in platelets is termed the dense body. This organelle contains calcium and magnesium ions, ATP, ADP, and smaller amounts of other nucleotides plus several vasoactive hormones, particularly serotonin. A third secretory granule is the lysosome, which contains hydrolase enzymes, similar to those found in other cells.

Any consideration of platelet anatomy must include the membrane systems, the dense tubular system and the open canalicular system. The dense tubular system is thought to be analogous to the sarcoplasmic reticulum of smooth muscle cells [Gerrard and White, 1976], being the site where calcium, important for triggering contractile events, is sequestered and released

[O'Rourke, Halenda, Zavoico and Feinstein, 1985]. Also, it is the site where enzymes involved in prostaglandin synthesis are localised. The surface connected open canalicular system is made up of membranous channels providing access to the interior for plasma-borne substances, and an egress route for substances released by storage granules [White et. al., 1981].

Platelet binding sites.

In recent years, the exterior coat of the platelet has been found to contain many high affinity binding sites. The concept of a platelet receptor being an externally orientated membrane protein, accessible in the intact cell, that demonstrates specificity, high affinity and saturability, and that interacts reversibly with the agonist, forming a complex, which then initiates a measurable intracellular response, has been discussed previously in Sections 4 and 5.

The first receptor to be discussed is historically the first one identified, adenosine diphosphate (ADP). In 1972, Born found that at low concentrations, (0.1 - 0.5 µM), ADP induced human platelets to change shape. ADP also induces platelet aggregation and antagonises adenylate cyclase stimulation [Haslam, 1973].

The methods used to study the ADP receptor illustrate the difficulties of platelet receptor characterisation and isolation. The most traditional technique, that of directly measuring the binding of radiolabelled ADP to intact platelets, is not applicable, as ADP is hydrolysed by platelet membrane phosphatases [Born and Feinberg, 1975]. A second approach, measuring the binding of ADP to isolated membranes [Nachman and Ferris, 1974], is also not valid because membrane preparations expose both proteins that were externally orientated and proteins found on the cytoplasmic face.

Bennett, Colman and Colman [1978] reported the use of fluorosulfonylbenzoyl adenosine (FSBA), as a specific analogue for ADP. Washed intact platelets incubated with [^{3}H]-FSBA showed concentration-dependent covalent incorporation of radioactivity and concomitant inhibition of shape change. When platelet membranes prepared from the platelets exposed to [^{3}H]-FSBA were subjected to SDS gel electrophoresis, a single radioactive peak corresponding to a relative molecular weight (M_r) of 100 kd was found.

As discussed below, adrenaline is a powerful inducer of platelet aggregation. The effect of adrenaline on human platelets can be abolished by alpha-receptor blocking agents such as dihydroergotamine and phentolamine. Using a variety of such agents, allied to computer modelling techniques, Hoffman, De Lean, Wood, Schoken and Lefkowitz [1979] demonstrated that the receptor involved was the alpha-2, presynaptic type. Motulsky, Shattil, and Insel [1980] successfully characterised alpha-2 adrenergic receptors on human platelets, finding a dissociation constant (Kd) of 2.7 nM. This receptor has since been purified 80,000-fold to apparent homogeneity, using a five-step chromographic procedure, and is a single polypeptide, (M_r= 64 kd) [Regan, Nakata, DeMarinis, Caron and Lefkowitz, 1986]. Moreover, using a partial amino acid sequence from the purified receptor as a guide, the alpha-2 adrenergic receptor has been cloned and expressed [Kobilka, Matsui, Kobilka, Yang-Feng, Francke, Caron, Lefkowitz and Regan, 1987].

Fibrinogen binding studies have suggested that there are 45,000 fibrinogen binding sites per platelet, and using a monoclonal hybridoma antibody, immunoaffinity chromatography has isolated the binding site from a detergent-solubilised platelet membrane extract [McEver, Baenziger and Majerus, 1982]. This method has the disadvantage that it is not possible to separate intracellular and plasma membranes. Using gel filtration chromatography, Jennings and Phillips [1982] found the fibrinogen binding site to be a dimeric glycoprotein with the relative molecular weights of the subunits being 125 kd and 95 kd. The fibrinogen binding site, designated glycoprotein IIb-IIIa, has been shown to bind to purified human fibrinogen [Nachman and Leung, 1982]. The formation of the glycoprotein IIb-IIIa fibrinogen complex was calcium dependent, fibrinogen specific, saturable and inhibited by specific amino acids. The binding site has also been shown to bind platelet contractile proteins [Phillips, Jennings and Edwards, 1980]; thus suggesting a model in which IIb-IIIa serves a linking function by binding fibrinogen to its extracellular portion and binding platelet contractile proteins to its intracellular portion.

Glanzmann's thrombasthenia, a congenital platelet disorder, is characterised by mucocutaneous bleeding with absent or reduced platelet aggregation [Majerus, 1987]. Direct binding studies of monoclonal antibodies to fibrinogen binding sites, indicate an absence of binding in such patients, with 50% levels in obligate heterozygotes [McEver, Baenziger and Majerus, 1980].

However, fibrinogen binding sites should not strictly be regarded as receptors, as the interaction of fibrinogen with glycoprotein IIb-IIIa does not occur on an intact cell but requires prior stimulation of the platelet, for example by ADP [Colman, 1990].

The definition of a platelet receptor also excludes another of the well-understood platelet reactions. The autosomal recessive disease, Bernard-Soulier syndrome, has been shown to result from an absence of binding sites for von Willebrand factor (vWF) an integral factor in the blood coagulation cascade. Binding studies using purified radiolabelled vWF have confirmed the presence of platelet binding sites in normal individuals, but not in Bernard-Soulier patients [Kao, Pizzo and McKee, 1979]. Furthermore, a deficiency of glycoprotein Ib can be demonstrated in such patients using SDS electrophoresis [Nurden and Caen, 1975]. However, the interaction of vWF with vWF binding sites can occur in metabolically inert platelets, (treated with formaldehyde). The agglutination which results, does thus not require a measurable intracellular response [Colman, 1990].

High affinity binding sites, (Kd = 0.3 nM), for thrombin have been found [Harmon and Jamieson, 1985], although the receptor has not yet been isolated. Similarly, although studies using a radiolabelled thromboxane A_2 antagonist have demonstrated high affinity, saturable and reversible binding to human platelet membranes [Saussy, Mais, Burch and Halushka, 1986], isolation and characterisation of the receptor is awaited. In addition, Schafer, Cooper, O'Hara and Handin [1979] have identified high affinity binding sites for prostaglandin I_2 and D_2 in platelet particulate fractions.

Perhaps no receptor for a platelet agonist has been as controversial as that for collagen. No fewer than nine binding sites on the platelet surface have been promulgated as the platelet collagen receptor. One possibility is glycoprotein IIIb, Tandon, Kralisz and Jamieson [1989] having recently demonstrated that this protein inhibits aggregation and shape change stimulated by collagen, without inhibiting platelet aggregation stimulated by thrombin, ADP or adrenaline.

Amongst polypeptide platelet high affinity binding sites demonstrated have been those for vasopressin (Kd 1 nM) [Thibonnier and Roberts, 1985], for atrial natriuretic peptide [Schiffrin, Deslongchamps and Thibault, 1986], and as discussed previously (p. 75), for angiotensin II [Ding et. al., 1984].

<u>Platelet function.</u>

In the circulation, platelets are freely floating cells that do not adhere to each other or to vascular endothelium. However, when platelets are exposed to a non-endothelial surface, they adhere and spread to the surface. In this process, the platelet shape changes dramatically from a disc to a spiny sphere with long fine filopodia [Holmsen, 1972]. The process of adhesion requires the presence of von Willebrand factor, and other plasma factors, such as fibrinogen [George, Nurden and Phillips, 1985].

Most of the platelets that accumulate at sites of injury do not adhere directly to subendothelial structures but rather to each other. This process of platelet-platelet adherence is called aggregation. Platelet aggregation has been studied <u>in vitro</u> by measuring the aggregation of stirred platelet suspensions by monitoring changes in optical density using a modified spectrophotometer [e.g. Macfarlane and Mills, 1975]. Platelet aggregation can be triggered by several physiological agents, the most important of which are ADP and thrombin. Other potential agonists include collagen, adrenaline, vasopressin, serotonin, thromboxane A_2 and platelet activating factor [Shattil and Bennett, 1980]. ADP, serotonin and thromboxane A_2 are released from the platelet during degranulation, thus they produce positive feedback and cascade platelet activation.

Aggregation results in secretion of the contents of platelet granules, which as discussed above, stimulates further aggregation. When the granules are condensed in the centre of the cell following contraction of the microtubular circumferential band, the granular membranes fuse with the membranes of the surface-connected open canalicular system [Stenberg, Shuman, Levine and Bainton, 1984]. Secretion is thus analogous to squeezing a sponge.

The above events require calcium [Holmsen, 1972]. It is thought that the aggregating agents bind to platelet surface binding sites, and that this then triggers release of calcium from the dense tubular system. When platelets are stimulated, for example by thrombin, the intracellular free calcium levels have been shown to rise by at least tenfold, as measured with the fluorescent indicator Quin-2 [Rink, Smith and Tsien, 1982]. The role of intracellular free calcium in initiating platelet activity has been studied in a series of experiments which demonstrated that varying thresholds of intracellular free calcium elevation produced various platelet responses. Relatively low thresholds of elevation of intracellular free calcium resulted in platelet shape change and myosin light chain phosphorylation, the latter being required for platelet contraction [Simpson, Hallam and Rink, 1984]. Platelet aggregation has been shown to occur at higher thresholds of elevation [Rink and Hallam, 1984]. Even higher thresholds of elevation of intracellular free calcium result in secretory exocytosis [Rink and Hallam, 1984], liberation of arachidonic acid [Pollock, Rink and Sage, 1987] and thromboxane A_2 production [Pollock, Rink and Irvine, 1986].

The next chapter considers the changes in platelet morphology and behaviour that occur in normotensive and hypertensive pregnancy.

Section 7.

PLATELET CHANGES IN NORMOTENSIVE AND HYPERTENSIVE PREGNANCY.

Normotensive pregnancy.

Several investigators have studied the platelet count in pregnancy, with conflicting results. One of the problems in assessing the platelet studies during pregnancy relates to the definition of normal pregnancy. Moreover platelet counting techniques have differed between reports, as has study design, with instances of cross sectional data having been compared directly with results from longitudinal studies.

O'Brien [1976], Fay, Hughes and Farron [1983], and Sill and Lind [1985], all showed a small fall in platelet count towards term. In a small cross-sectional study, O'Brien [1976] found a significant progressive decrease in platelet count. The study by Fay et. al. [1983] analysed the results from over two thousand patients. However, the study design was also cross-sectional, and patient selection criteria were unclear. Furthermore, the fall in platelet number which occurred from 32 weeks gestation, was not significant when compared to non-pregnant values. Sill and Lind [1985] reported a fall in the platelet count towards term from a longitudinal study of 26 normotensive patients. However, there was considerable variation in platelet count within individuals, and the apparent fall in the group as a whole was accounted for by a considerable fall in a few individuals.

In contrast, in four longitudinal studies, where care was taken to exclude patients with pregnancy induced hypertension, no change in platelet count was found [Fenton, Saunders, and Cavill, 1977, Tygart, McRoyan, Spinnato, McRoyan and Kitay, 1986, Pekonen, Rasi, Ammala, Viinikka and Ylikorkala, 1986 and Marumoto, Kaibara and Murata, 1988], with Fenton et. al. [1977] stressing the considerable variation in the platelet counts of normal women.

It seems reasonable to conclude that any reduction in platelet count is minimal. In view of the 40% increase in plasma volume induced by normal pregnancy, platelet mass must thus increase considerably.

Mean platelet volume (MPV) and platelet distribution width (PDW) have been used as indices of bone marrow haemopoetic activity; Letsky [1985] having demonstrated that platelets become progressively smaller with age. Again, differing results have been obtained. Fay et. al. [1983] noted an increase in the MPV in the last month of pregnancy with the PDW increasing progressively. Sill and Lind [1985] confirmed the progressive rise in the PDW, but found that the MPV increased throughout the third trimester. Tygart et. al. [1986] found the MPV to be unchanged. Marumoto et. al. [1988] demonstrated a decrease in MPV from 20 to 31 weeks gestation with a marked increase from 38 to 41 weeks gestation. Although all the authors collected their samples in EDTA, none of them give venepuncture details. Holmsen [1972] amongst others, has emphasised the importance of collection and handling techniques. Any differences may have produced the discrepancies, which probably also reflect differences in the populations studied.

Both Wallenburg and Van Kessel [1978] and Rakoczi, Tallian, Bagdany and Gati [1979] have studied platelet life-span in healthy pregnant and non-pregnant women. Both studies were cross-sectional, with relatively small numbers, (between nine and nineteen), in each group. Their methods involved the use of aspirin to inhibit the platelet cyclo-oxygenase system and allow the calculation of platelet survival curves. Although the mean platelet life spans were marginally shorter in the pregnant women, the differences were not significant. Pekonen et. al. [1986] confirmed these results in a longitudinal study of 17 patients. The increased platelet mass and unaltered platelet life span suggested a pregnancy-induced increase in the total platelet turnover.

Measurement of secretory products released from platelets, into the plasma is a further means of examining platelet activity in pregnancy. In both cross sectional [Douglas, Shah, Lowe, Belch, Forbes and Prentice, 1982], and longitudinal studies [Pekonen et. al., 1986], pregnancy results in an increase in plasma beta-thromboglobulin levels. Contradictory measurements of serum thromboxane B_2, (the stable metabolite of thromboxane A_2), have been obtained. Koullapis, Nicolaides, Collins, Rodeck and Campbell [1982] and Pekonen et. al. [1986] did not find elevated levels in pregnancy, the former being a cross-sectional study involving 141 uncomplicated, normotensive pregnancies. Ylikorkala and Viinikka [1980] had previously demonstrated a rise with gestation, in a small cross-sectional study. One explanation of this difference lies in the production of thromboxane B_2 by tissues other than platelets, such as the placenta and amnion [Ylikorkala and

Makila, 1985], and again collection and handling techniques may be important.

Despite an extensive literature search, no studies of pregnancy induced changes in platelet plasma membrane binding sites have been found, apart from a study of alpha 2 adrenoreceptors in the puerperium [Metz, Stump, Cowen, Elliott, Gelder and Grahame-Smith, 1983]. The principal finding of the study was that the number of platelet alpha 2 adrenoreceptors fell after childbirth. In rabbit uterine tissue, oestrogen has been shown to increase alpha adrenergic receptors [Williams and Lefkowitz, 1977], and Metz et. al. [1983] suggested that a reduction in oestrogen and progesterone affected the number of receptors. However, they were unable to show a direct relationship between binding characteristics and serum steroid levels in either the ante-partum or the post-partum samples.

Platelet aggregation in response to various agents alters during pregnancy. Yamazaki, Motomiya, Kikutani, Sakakibara, Watanebe, Namuta and Noguchi [1979] assessed platelet aggregation, utilising platelet rich plasma and using the light-transmission method mentioned in the previous section (p. 97). They found that platelet aggregation induced by ADP and adrenaline occurred more frequently in pregnant women, as compared to non-pregnant women. One of the inherent problems of using platelet rich plasma, is that the platelet yield may not be representative of the original platelets. This problem, considered again in Chapter 2 (p. 121), may be particularly important in pregnancy [Burgess-Wilson, Morrison and Heptinstall, 1986].

Hypertensive pregnancy.

There have been many studies of the circulating platelet count in pre-eclampsia and pregnancy induced hypertension (PIH) [e.g. Howie, 1977, Trudinger, 1976, Rakoczi, Tallian, Bagdany and Gati, 1979, Gibson, Hunter, Neame and Kelton, 1982, Pekonen et. al., 1986]. The majority of these observers found a reduction in the number of circulating platelets, but comparisons between studies are made extremely difficult by the varying definitions used. For instance, whereas Rakoczi et. al. [1979] defined severe pre-eclampsia as a rise in diastolic blood pressure of 20 mmHg. with proteinuria in excess of 0.5 gm/day, Pekonen et. al. [1986] regarded the diagnostic criteria for severe pre-eclampsia as proteinuria above 5 gm/day and/or blood pressure repeatedly above 160/110 mmHg. Pekonen et. al. [1986] were one of the few groups to show no reduction in platelet count. This may be explained in part by their active management of pre-eclamptic patients, with three-quarters of pre-eclamptic patients being delivered prior to 35 weeks gestation, possibly before the platelet count diminished. Gibson et. al. [1982] found that the reduction in platelet number only occurred in about 20% of cases, and the sample size in the study of Pekonen et. al. [1986] (n = 16), may have been too small to detect any change. Moreover, Wallenburg [1987] demonstrated that platelet counts, even in patients with severe disease, show marked day to day variation.

An increased platelet consumption in PIH was suggested by Giles and Inglis [1981]. In general large platelets (macrothrombocytes) are immature platelets, and they found an increase in the mean platelet volume in a cross-sectional study of over 500 patients with PIH. Rakoczi et. al. [1979] had previously demonstrated reduced platelet survival in pre-eclamptic patients, using the non-radioisotopic method described above. Unfortunately, the value of the non-isotopic method is limited in such patients. The technique relies upon a stable platelet count throughout the test, unfortunately not often the case in pre-eclampsia. This factor, and differing populations, may account for the contrasting results of Pekonen et. al. [1986], who found that platelet survival in pre-eclampsia was unchanged.

The results of <u>in vitro</u> platelet aggregation studies are conflicting. Howie, Prentice and McNicol [1971] found decreased ADP-induced platelet aggregation in pre-eclamptic patients, unlike patients with non-proteinuric PIH and with essential hypertension, in whom platelet aggregation was unchanged. Similarly, this group demonstrated a decrease in arachidonic acid induced aggregation in the pre-eclamptic patients [Whigham, Howie, Drummond and Prentice, 1978]. Inglis, Stuart, George and Davies [1982] showed unchanged ADP-induced platelet aggregation in pre-eclamptic patients. In a study from this centre, Morrison, Crawford, Macpherson and Heptinstall [1985] found increased platelet aggregation in patients with PIH. The concentration of arachidonic acid needed to induce aggregation and release reaction was reduced, particularly in those patients who were hyperuricaemic.

Possible explanations for such discrepancies are legion. The studies are all cross-sectional. Whigham et. al. [1978] and Morrison et. al. [1985] included patients of mixed parity. Howie et. al. [1971]

failed to comment on parity. Almost a third of the patients studied by Morrison et. al. [1985] were taking labetolol, an alpha- and beta-adrenergic antagonist with known effects on platelet aggregation [Anfossi, Trovati, Lanzio, Mularoni, Massucco and Emanuelli, 1988]. Late pregnancy controls were poorly matched in the study by Howie et. al. [1971], platelet counts varying markedly between groups. However, as in previous comparisons, the most important factors in the differences shown are the problems associated with the use of platelet rich plasma, (discussed above), and the various definitions of PIH and pre-eclampsia. Patients included in the study of Howie et. al. [1971] may have had the disease more severely, as patients with blood pressures up to 190/125 mmHg, and reduced birthweights were noted. If their patients had concurrent intravascular coagulation, this could had resulted in removal of the more active platelets from the circulation.

A study by Louden, Heptinstall, Broughton Pipkin, Mitchell and Symonds [1989] also from this centre, examined whole blood platelet aggregation in 27 patients with PIH, using definitions identical to those used in this thesis. Arachidonic acid-induced platelet aggregation was found to be increased only in patients with non-proteinuric PIH, whereas adrenaline and ADP induced aggregation was increased in both the non-proteinuric PIH and the pre-eclamptic groups.

Platelet activity as assessed by plasma beta-thromboglobulin concentration, was found to be elevated in patients with PIH, by both Inglis et. al. [1982], in a well-structured study with a carefully matched control group, and by Pekonen et. al. [1986], with an increase in levels as compared to normotensive pregnancies being demonstrated.

During the second trimester, in the spiral arteries supplying the placenta, platelet aggregates can be demonstrated between the trophoblast cells that form the intimal lining of the vessels [Sheppard and Bonnar, 1974]. Howie [1977] demonstrated that in pre-eclamptic patients, platelet serotonin levels were lower than those found in non-pregnant and normal pregnant controls. The explanation suggested for this finding was that platelet emboli occur in the microcirculation in pre-eclampsia, and that the platelets release some of their serotonin. Changes of platelet behaviour may be of special importance in relation to the diminished placental blood flow that appears to be a prerequisite for the development of pre-eclampsia. In pregnancies complicated by severe growth retardation, extensive atheromatous lesions full of fibrin and lipid-laden cells are found, producing occlusion of the spiral arteries [Sheppard and Bonnar, 1976].

Ylikorkala and Makila [1985] argued that the platelet-vascular changes pathognomonic for pre-eclampsia could be explained by an imbalance between locally produced and acting prostaglandins, prostacyclin and thromboxane A_2, with the imbalance tilted towards the vasoconstrictor, platelet aggregating actions of thromboxane A_2, produced by platelets. Support for this theory is provided by Koullapis, Nicolaides, Collins, Rodeck and Campbell [1982], who found significantly higher plasma levels of thromboxane B_2, (the stable metabolite of thromboxane A_2), in 19 patients of mixed parity with PIH. Pekonen et. al. [1986] found increased thromboxane B_2 levels in patients with pre-eclampsia, although the increases were not significant.

In the studies included in this thesis, platelets have been used as a model for vascular myocytes. The next and final section of the introduction briefly considers the rationale for this model.

Section 8.

THE RATIONALE FOR THE USE OF PLATELETS AS A MODEL FOR VASCULAR MYOCYTES

In this thesis, platelet AII binding sites are used as a model for studying smooth myocyte AII binding sites. Both platelets and smooth myocytes are cells that depend upon excitation-contraction coupling mechanisms for their actions and bear many resemblances both physiologically and pharmacologically.

Both types of cell contain a contractile system composed of an actin-myosin complex [Pollard, Thomas and Niederman, 1974], although studies using electron microscopy suggest that the platelet myosin filament is relatively short with fewer cross-links which might thus form a limiting factor in the contractile force generated [Hinssen, D'Haese, Small and Sobieszek, 1978].

The presence of membranous high affinity binding sites has been demonstrated in both platelets and vascular myocytes, with intracellular changes being affected by receptor mediated signal transduction. One such example is the adrenoreceptor. Both alpha-1 and alpha-2 adrenoreceptors have been identified in vascular smooth muscle [Bylund and U'Pritchard, 1983, Van Zwieten, Van Meil and Timmermans, 1981]. As described above (p. 93), platelet alpha-2 adrenoreceptors have been fully isolated and characterised [Motulsky et. al., 1980]. Moreover, washed platelets loaded with the fluorescent indicator

Quin-2 have been demonstrated to show an elevation in intracellular free calcium when incubated with adrenaline. This effect was inhibited by the addition of yohombine, suggesting a direct involvement of the alpha-2 receptor in the modulation of intracellular free calcium [Erne, Mittelholzer, Burgisser, Fluckiger and Buhler, 1984]. A similar adrenoreceptor mediated increase in intracellular free calcium had previously been demonstrated in rabbit vascular smooth muscle tissue [Cauvin, Loutzenhiser, Hwang and Van Breeman, 1982].

The isolation of high affinity AII binding sites in both vascular smooth muscle [e.g. Gunther et. al., 1980a], and in platelets [e.g. Moore and Williams, 1981], has been discussed in Section 5 (p. 75). In both tissues, alterations in the endogenous renin-angiotensin system produce significant changes in AII binding which inversely correlate with changes in plasma AII levels [Gunther et. al., 1980b, Moore et. al., 1984].

In response to drugs, similarities between changes in platelet behaviour and changes in vascular tone have been described. Catecholamines cause both vasoconstriction and platelet aggregation [Cameron and Ardlie, 1982] whereas drugs that cause vasodilation, such as sodium nitroprusside, inhibit platelet aggregation [Levin, Weksler and Jaffe, 1982]. In a study using 26 normal volunteers, Cowley, Stainer, Cockbill and Heptinstall [1984] demonstrated a close correlation between maximal cold-induced plethysmography and the threshold concentration of arachidonate required in induce a pro-aggregatory response <u>in vitro</u>. Five subjects underwent a prostacyclin infusion. This decreased the maximal cold-induced vasoconstrictor response and increased the threshold concentration of arachidonate to induce an aggregatory response. Cowley et. al. [1984] suggested that the reactivity of both platelets and blood vessels depends up on cyclic AMP levels.

There are thus many similarities between platelets and myocytes. However, the changes which platelets undergo in normotensive and hypertensive pregnancy are complex and extensive. Such changes will inevitably provide an important caveat when any pregnancy-induced alterations in binding are considered.

Chapter 2.

METHODS.

This thesis encompasses several different studies of platelet angiotensin (AII) binding. Before considering these in turn, this chapter will discuss the methods common to all the studies, and describe some experiments performed to validate the techniques used. A detailed list of laboratory reagents and disposable equipment may be found in Appendix A.

a) **Solutions.**

Modified 199 medium: 1L of 199 medium was supplemented with 78.9 mg $MgSO_4$, 108.8 mg $CaCl_2$, 2g glucose, 1.46 g EDTA and 5 g bovine serum albumin to give a solution containing (in mmol/l): Na^+ 145; K^+ 3.8; Ca^{+2} 2.54; Mg^{+2} 1.18; SO_4^{-2} 1.18; HCO_3^- 24.9; Cl^- 128; PO_4^{-3} 1.18; glucose 11.1; EDTA 5; 0.5% (w/v) bovine serum albumin, pH 7.4.

Tris-HCl buffered saline : 0.6 g Tris and 0.5 g bovine serum albumin were added to 100 ml of normal saline to give a solution containing 50 mmol Tris-HCl/154 mmol/l NaCl, 0.5% (w/v) bovine serum albumin, pH 7.35.

These solutions were stored at 4'C.

Pthalate solution : 395 ml of dibutyl pthalate (specific gravity 1.046) was mixed with 105 ml dionyl pthalate (specific gravity 0.970) to produce 500 ml of a solution with a specific gravity of 1.030.

This solution was stored at room temperature.

b) **Patient Selection.**

Patient selection criteria for each study are discussed in relevant subsequent chapters.

However, no patient had any evidence of essential hypertension, chronic renal disease, or diabetes, and all the pregnancies were singletons. All subjects gave informed consent to inclusion in a clinical trial, including consent to venepuncture. Patients gave written consent prior to infusion studies. Ethical approval for all the studies described in the thesis was obtained from the hospital ethical committee.

c) **Blood Pressure.**

Blood pressure measurements were performed by auscultation and standard sphygmomanometry, in accordance with the proposals submitted to the International Society for the Study of Hypertension in Pregnancy [Davey and MacGillivray, 1986]. In particular, all patients were carefully positioned on their right side with a 15-30' tilt and the cuff applied to the right upper arm at the same level as the heart. The rate of cuff deflation was restricted to 10 mmHg in 5 seconds. The systolic pressure was taken at the first Korotkov sound and the diastolic pressure at the fourth Korotkov sound, (see p. 4), both being recorded to the nearest 5 mmHg. All blood pressure recordings were performed by the same observer.

d) **Venepuncture.**

Platelets may be activated at the point of venepuncture; as the vessel wall is damaged, sub-endothelial surfaces are exposed, and prostaglandins may be rapidly released. Care was thus exercised in venepuncture technique to avoid unnecessary trauma. It was not possible to quantify the artefactual activation which may have occurred as a result of exposure of the blood to a series of foreign surfaces, before mixture with anticoagulant, but the importance of standardised venepuncture techniques has been stressed [Saniabadi, Lowe, Madlick, Spowart, Shaw, Barbenel and Forbes, 1986]. A 19 gauge needle was thus employed to allow free flow of 35 ml of blood to pass from the venepuncture site in the antecubital fossa into a polypropylene syringe. A tourniquet was used to effect venous stasis, and the skin was prepared with a Medi-Swab. A traumatic venepuncture resulted in the disposal of the sample, followed by a new attempt in the opposite arm.

Blood was transferred from the syringe to the following tubes:

17.2 ml for platelet AII binding estimation into two 10 ml graduated polypropylene tubes, each containing 1 ml of the anticoagulant 3.13% (w/v) trisodium citrate dihydrate, which is isotonic with human blood, and 400 μl of acetyl salicylic acid (25 mmol/l).

10 ml for plasma AII, plasma renin substrate (PRS), and plasma renin concentration (PRC) assay into a polypropylene tube containing 250 ul o-phenanthroline (25 mmol/l) and 250 ul diaminoethanetetra-acetic acid (EDTA) (125 mmol/l).

2 ml for full blood count estimation into a glass EDTA vacutainer tube.

5 ml for serum urea and electrolytes, osmolarity, urate and creatinine estimation into a glass additive-free vacutainer tube.

The samples were gently mixed by inversion. The samples for platelet AII binding and for AII, PRS and

PRC estimation, were placed in ice and transferred to the laboratory for immediate assay.

e) Preparation of platelets.

An initial platelet count was made, (Ariane-5 platelet counter, ABX, Montpellier, France.)

Platelet preparation as described by Ding, Kenyon and Semple [1984], was initially attempted. This involved layering blood onto an iso-osmotic solution of Percoll (154 mmol/l NaCl; specific gravity 1.06). After centrifugation (400g, 5 minutes, 4'C, using an MSE Coolspin 2 centrifuge), the upper platelet-rich layer equal to the volume of added blood was aspirated with a liquipipette, washed with an equal volume of modified medium 199 and centrifuged, (1000g, 10 minutes, 4'C). This was repeated three times with the platelet-rich layer resuspended in 15 ml of modified medium 199 each time. The final aspirate was suspended in 5-6 ml of medium and the suspension filtered through 20µm nylon gauze. The platelet count in the concentrate was then counted, using the Ariane-5 counter.

However, using the above method, the recovery of platelets from whole blood (platelet yield) was consistently below 10% in 10 consecutive samples. This figure is well below the 60% achieved by Ding et. al. [1984]. The reason for this discrepancy is unclear, but may have been due to the author's inability produce a discrete layer of blood on the percoll solution.

An alternative method of platelet preparation was thus subsequently used throughout the studies included in this thesis. This method, described below, is similar to that used by Mann, Sis and Ritz [1985] when studying platelet AII binding, and was already being used in ongoing platelet studies in this centre.

After an initial platelet count, platelet rich plasma (PRP) was prepared by centrifugation, (160g, 20 minutes, 4'C). The supernatant PRP was filtered through 20 µm nylon gauze, prior to being adjusted to pH 6.4 with 0.1M citric acid; 1 u/ml apyrase being added. Apyrase was used as a 250 u/ml stock solution in 154 mmol/l NaCl, and stored at -20'C. The platelets were pelleted by centrifugation, (400g, 20 minutes, 4'C), and resuspended in modified medium 199. The platelet concentration in a small portion of the suspension was measured, (after being diluted 1:9, see p. 121), and the volume of medium adjusted to give a platelet concentration of 10^6/µl. Leucocyte and erythrocyte contamination was checked using a Minos STE counter (ABX).

Both methods involve centrifugation, which may cause the formation of aggregates of the most active sub-population of platelets, which would be discarded with the red and white blood cells. Platelet loss of between 20 and 40% has been recorded, with the suggestion that the platelets lost may have been those with the greatest metabolic activity [Corash and Gralnick, 1974].

f) Platelet AII binding studies.

The physiological concentration of AII in the plasma of many species is in the order of 10-100 pM, (see p. 27). One problem found when studying the binding of the hormone at such low concentrations, is the necessity to use a radioactive hormone of high specific activity. This is usually achieved by introducing atoms of radioactive iodine into the molecule. However, the iodination of AII results in a loss of biological activity, the potency of the iodinated AII diminishing with each iodine atom [Lin, Ellis, Weisblum and Goodfriend, 1969]. The substitution of hydrogen by tritium neither provokes major structural changes, nor perturbs the biological activity of the hormone [Morgat, Hung and Fromageot, 1970]. However, the specific activities obtained are much lower than those of the iodinated hormone. Thus either physiological concentrations of the iodinated hormone must be used, producing sub-optimal interaction with the receptor; or pharmacological concentrations of the tritiated hormone, which interacts normally with the receptor. In fact, both labelled peptides have been satisfactorily employed for quantitative binding studies on AII receptors, and there are surprisingly few areas of discrepancy between results obtained with the two tracers [Capponi, Aguilera, Fakunding and Catt, 1981]. Previous studies of AII binding on platelets have used ^{125}I-AII [Moore and Williams, 1981, Ding, Kenyon and Semple, 1984, Mann, Sis and Ritz, 1985], and the method detailed below is essentially that of Ding et. al. [1984], with only minor modifications.

^{125}I-AII (specific activity 74 TBq/mmol) was stored at -20'C in Tris-buffered saline at a concentration of 300 pmol/l. To prevent damage by freezing and thawing, the ^{125}I-AII was stored in 200 µl aliquots, this quantity being only sufficient for the assay of one sample. Unlabelled AII was stored in 20 µl aliquots at -20'C at a concentration of 1 mg/ml in saline. Each day, an aliquot was thawed at room temperature, and added to 700 µl Tris buffered saline, to give a final concentration of 25 µmol/l.

Six portions (200μl) of the platelet concentrate were each incubated with 25 μl of ^{125}I-AII (300 pmol/l) in polypropylene tubes in a Grant shaking water bath (Grant Instruments, Cambridge, England). To three of these portions, an excess of unlabelled AII (25 μl, 25 μmol/l) was added prior to the incubation. To the other three portions, 25 μl Tris-buffered saline was added. The final concentration of ^{125}I-AII was thus 30 pmol/l.

After incubation at 26'C for 90 minutes, platelets were separated from the 199 medium, by centrifuging 100 μl portions of the incubation mixture in 400 μl microcentrifuge tubes containing 200 μl of the mixture of pthalate esters. A Beckman microcentrifuge (Beckman, U.S.A.) was used, at 12,000g for 30 seconds. The microcentrifuge tubes were immediately frozen in a mixture of acetone and solid CO_2, the latter being produced from a cylinder of compressed gas using a Snow pack CO_2 pellet maker (Jencons Scientific Ltd., Leighton Buzzard, England). The frozen microcentrifuge tubes were then cut with a tube cutter (Rostfret Sollingen, West Germany), just below the oil-water interface. The lower portion of the tube, containing the platelet pellet, was then counted as the 'bound' hormone, and the upper portion of the tube, containing the supernatant, was counted as 'free' hormone, using an LKB Wallac 1275 mini-gamma counter. The non-specific binding was defined as the amount of AII bound to platelets in the presence of excess unlabelled AII. Values of platelet AII binding discussed subsequently have been corrected for non-specific binding, which ranged from 10-40% of total binding.

g) **Biochemical and haematological quantification of serum and urine samples.**

Plasma concentrations of renin (PRC), renin substrate (PRS), and AII were measured by radioimmunoassay [Poulsen and Jorgensen, 1974, Broughton Pipkin and Symonds, 1977, Craven and Symonds, 1978]. Reproducibility of these methods, as measured by replicate assays, were:

PRC	5.6% intra-assay,	10.7% inter-assay.
PRS	9.2% intra-assay,	15.3% inter-assay.
AII	4.0% intra-assay,	11.1% inter-assay.

A full blood count estimation was performed on the EDTA sample in the Haematology Department using a Coulter S Plus 4 automated blood counter (Coulter Company, U.S.A.).

The clotted blood sample for serum urea and electrolytes, urate, osmolality and creatinine estimation was sent to the Department of Clinical Chemistry. A 24 hour urine sample, consisting of all urine passed in a 24 hour period, following early morning voiding, was delivered to the same department.

Serum urea and electrolytes, urate, osmolality and creatinine estimations were performed using a Kodak 700XR Ekatachem analyser. The reproducibility of these assays is summarised in Appendix B. The corresponding urinary estimations were performed using an SMA continuous flow analyser. Urinary total protein concentration was measured by the method of salicyl-sulphonic acid turbidity.

h) **Statistical Analysis.**

Guidance was received from Mr. P. H. Riley, statistical advisor to the University of Nottingham, from the earliest stages of the project. Data was stored on the University Mainframe computer, in a form appropriate for use of the Statistical Package for the Social Sciences [SPSS-X, 1988]. Unless otherwise stated, medians and 25th and 75th centiles have been quoted as estimates of central tendency and scatter, respectively, and non-parametric statistics have been employed. (When the sample size is below 10 the range rather than the 25th and 75th centiles has been quoted.) Non-parametric statistics were used because the data for many of the parameters measured was found to be significantly skewed, and is in accord with the statistical advice received. Consistent use of non-parametric testing is appropriate for such data, because no assumption is made regarding data distribution. For paired data, the Wilcoxon matched pairs signed-ranks test has been used. For unpaired data, the Mann-Whitney U test has been used. Where other types of analysis have been performed, this will be clearly explained in the text.

In order to allow for multiple comparisons amongst groups, $P<0.01$ rather than $P<0.05$ has been taken, throughout the thesis, as the level indicating statistical significance.

The results of the studies presented in the subsequent chapters have been tabulated. In order to aid interpretation of the data, many of the results have also been presented as figures. This duplication was deliberate. Graphical interpretation of the data was reproduced by a Macintosh SE micro-computer with laser printer, and all diagrams and tables have been produced by the author.

Validation of the methods.

Unless stated otherwise, in the following validatory experiments, all the subjects were non-pregnant females, who were normotensive and had no history of renal, metabolic or cardiovascular disease. No subject was taking any medication.

Platelet preparation.

a) Platelet count.

Venous blood from six subjects, taken for platelet AII binding site assay, was repeatedly counted using the Ariene-5 platelet counter. The coefficients of variation when four platelet count estimations were performed on each sample ranged from 0.2 - 1.9%, median : 1.0%. When the same procedure was performed using the prepared platelet concentrate, the coefficients of variation ranged from 0.5 - 18.2%, median : 5.1%. However, following dilution with the Minoton solution, (see below), these values fell to a range of 0.3 - 2.1%, median : 1.2%.

Figure 2.1 demonstrates the effect of diluting the original whole blood sample with normal saline; the platelet count was found to decrease linearly in proportion to the dilution. However, this linear relationship was not found at the highest concentrations of the prepared platelet suspension, (Figure 2.2). The platelet suspension was diluted with Minoton, a proprietary isotonic salt solution, (ABX, Montpellier, France). At the high platelet concentrations, well above physiological levels, the Ariene-5 counter consistently underestimated the platelet concentration. In the subsequent studies,

Figure 2.1.

Platelet counts obtained when whole blood samples were diluted with normal saline.

These results were from three subjects, with mean +/- SEM of triplicate readings plotted.

Figure 2.2.

Platelet counts obtained when whole blood samples were diluted with minoton solution.

These results were from three subjects, with mean +/- SEM of triplicate readings plotted.

Figure 2.1

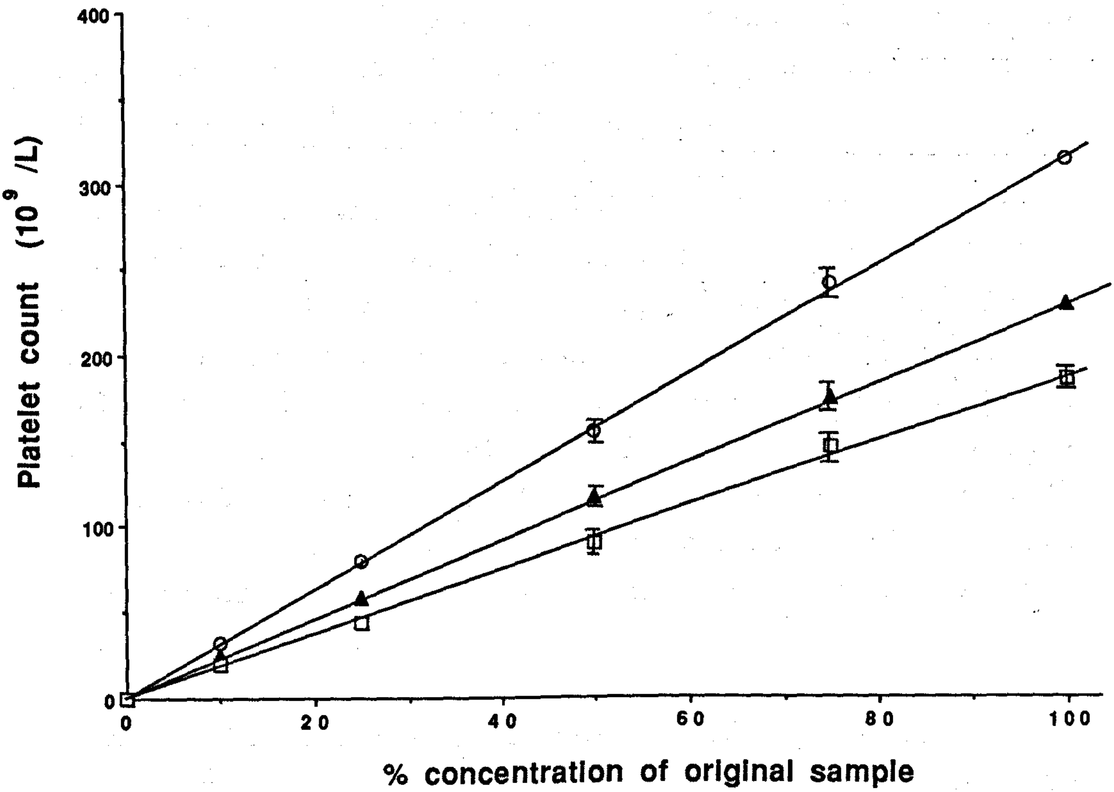
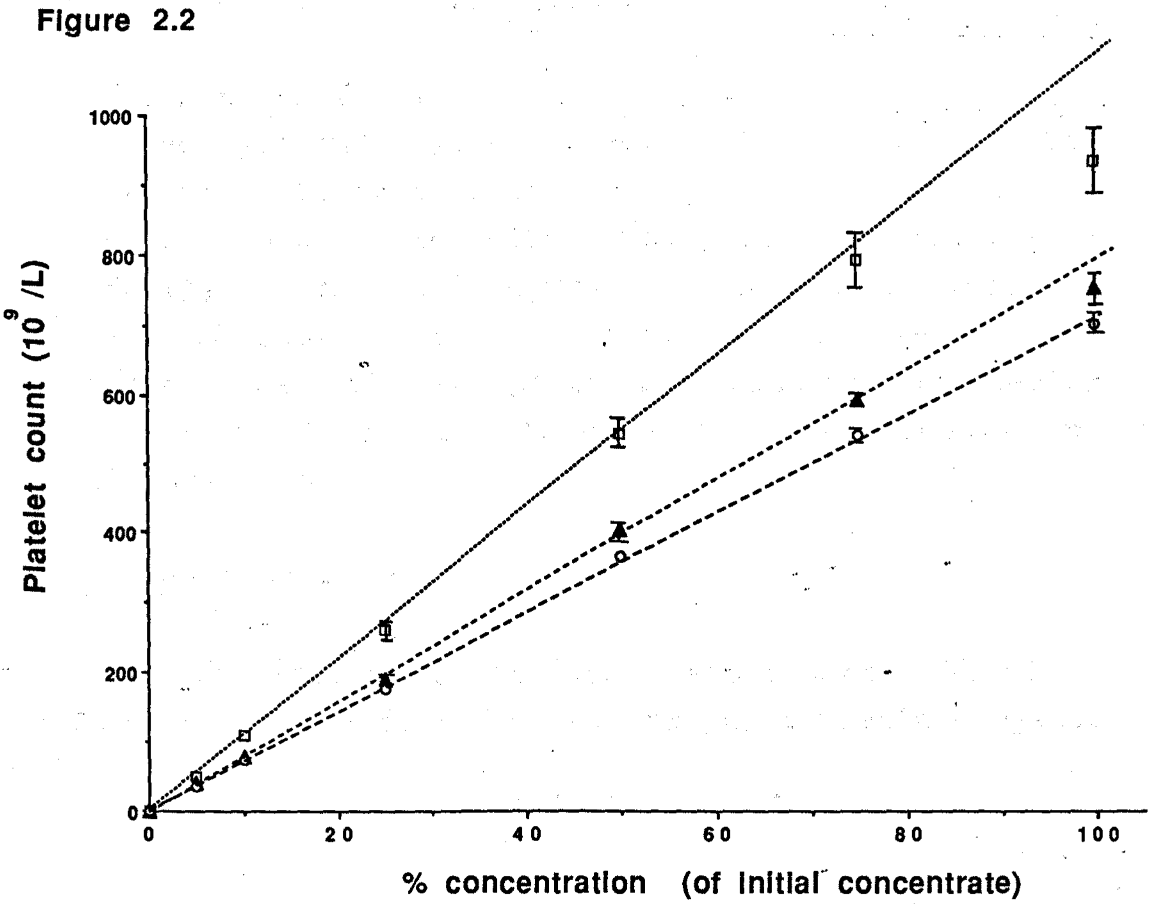


Figure 2.2



when counting the platelet suspension in order to adjust the concentration to 10^6 / μl, a 1:9 dilution with Minoton solution was used.

b) Platelet distribution curve.

As discussed above, (see p. 102 and p. 115), it has been suggested that the use of platelet rich plasma selects a particular platelet population [Corash and Gralnick, 1974]. The use of the Ariene-5 platelet counter allows comparison of the platelet volume distribution curves of the whole blood sample with that of the final platelet concentrate. Figure 2.3 illustrates a example from one representative non-pregnant subject.

When the distribution curves of the whole blood samples and the platelet concentrate were compared in six representative non-pregnant female subjects, using the non-parametric Wilcoxon matched-pairs signed-rank test, no significant difference was found in any of the subjects, ($P = 0.3 - 0.8$).

c) Purity of sample.

Leucocyte and erythrocyte contamination was checked in 20 platelet concentrates using a Minos STE counter, (ABX). Contamination with leucocytes was < 1%, (1-$5 \times 10^3/\mu l$), and no erythrocytes were detected. This low level of contamination is important in view of the finding that AII receptors are present in greater numbers on leucocytes than on platelets [Moore and Williams, 1981].

Binding Assay.

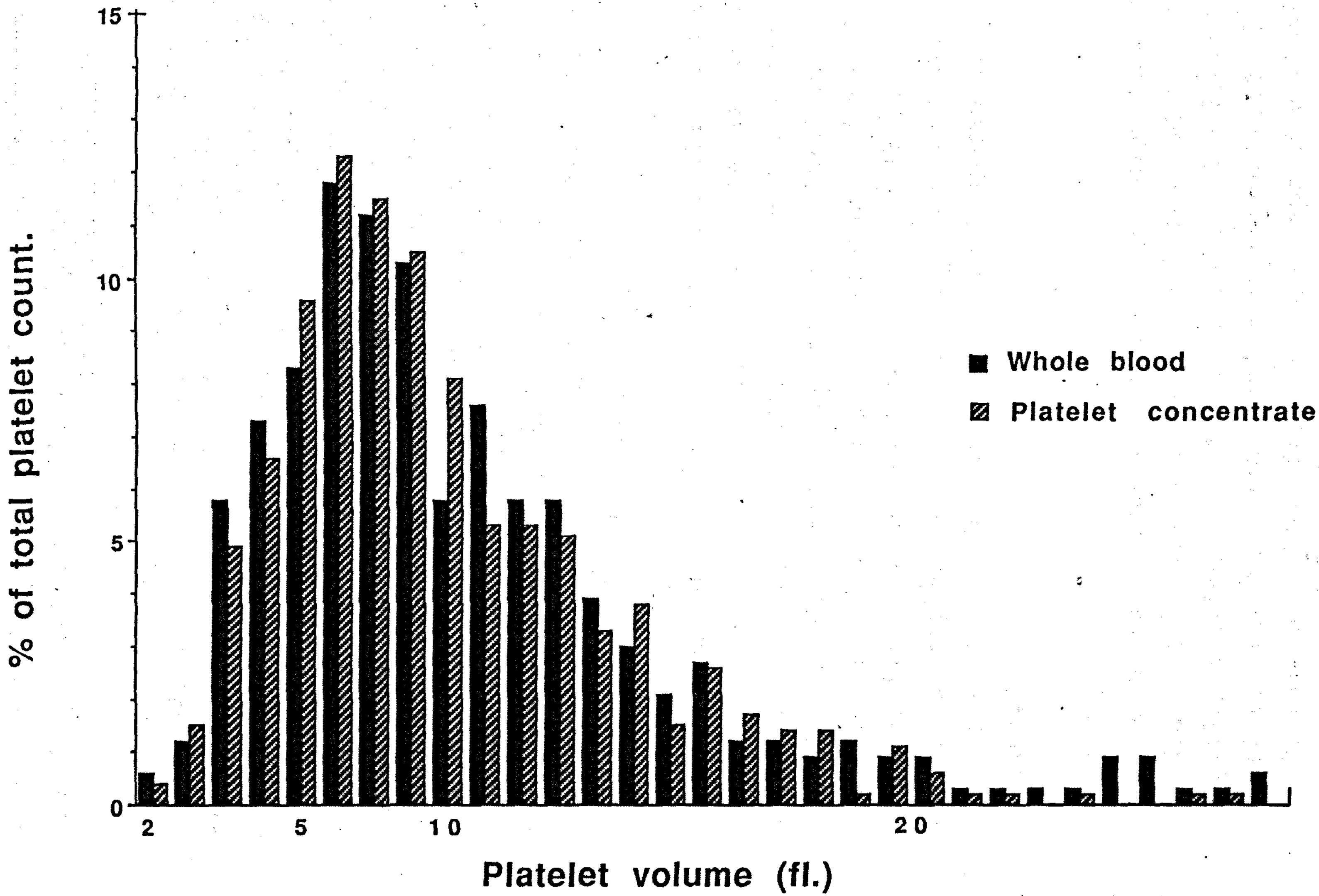
In the following validatory experiments, 52 ml of blood was taken from each non-pregnant female subject.

Figure 2.3.

Platelet Distribution curves.

This figure illustrates the platelet distribution curves obtained from whole blood and from the final platelet suspension, from one representative non-pregnant subject.

Figure 2.3



15
10
5
0
% of total platelet count.
2
5
10
20
Platelet volume (fl.)
Whole blood
Platelet concentrate

This was collected into six 10 ml polypropylene tubes, each containing 1 ml trisodium citrate, (3.13% w/v), and 400 µl acetylsalicylic acid, (2.5 mmol/l).

a) Platelet concentration.

^{125}I-AII, was incubated with platelet concentrations of 1 x 10^6 cells/µl, 7.5 x 10^5 cells/µl, 5 x 10^5 cells/µl, and 2.5 x 10^5 cells/ µl, at 26 'C for 90 minutes. There was a direct, linear relationship between cell concentration and specific binding, (Figure 2.4a). In each of four experiments the correlation coefficients relating to specific binding and platelet concentration were > 0.9, although at a concentration of 2.5 x 10^5 cells/µl, binding was consistently underestimated. Non-specific binding was also related to the platelet concentration, (Figure 2.4b).

In subsequent experiments throughout this thesis, if there were insufficient platelets to adjust the concentration to 10^6 cells/µl, the quoted value of specific binding was obtained by multiplying the concentration of specific binding found by 10^6/platelet concentration. If there were insufficient platelets to adjust to a concentration of at least 5 x 10^5 cells/µl, the experiment was abandoned.

b) pH.

In five experiments, the platelet suspension was divided into five portions, and the pH of the resulting portions adjusted to between 6.9 - 7.8. The platelets were then incubated with ^{125}I-AII at 26 'C for 90 minutes. The effect of pH on specific binding is illustrated in Figure 2.5. When the pH rose above 7.4, platelet AII binding declined, although a

Figure 2.4.

The effect of varying platelet concentration on specific and on non-specific platelet AII binding.

These results were from four subjects, with mean +/- SEM of triplicate assays plotted.

Figure 2.4a

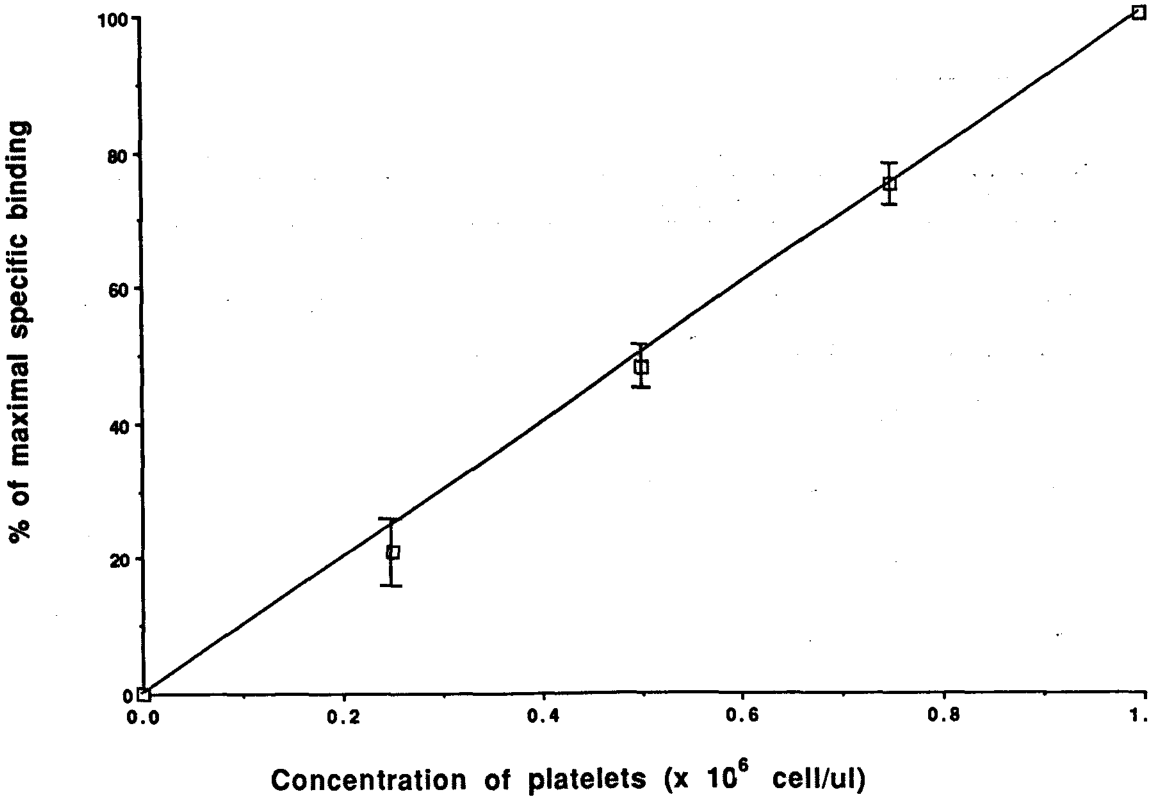


Figure 2.4b

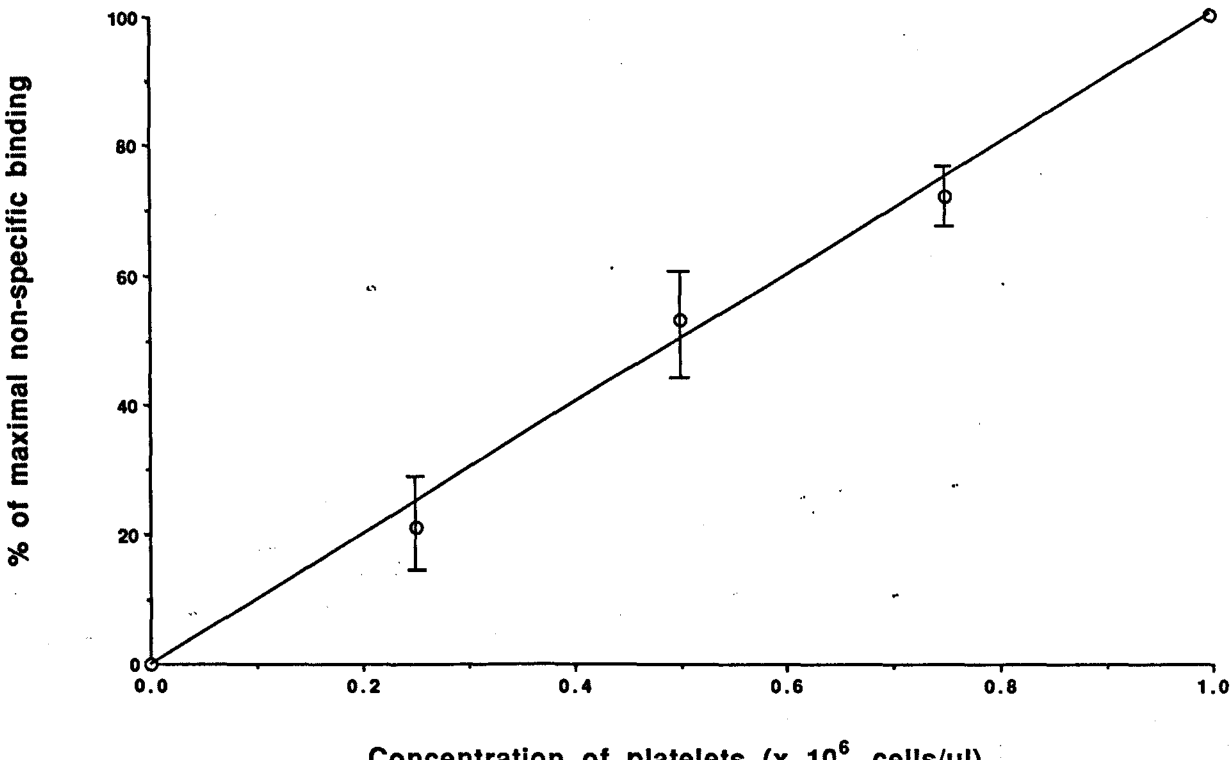
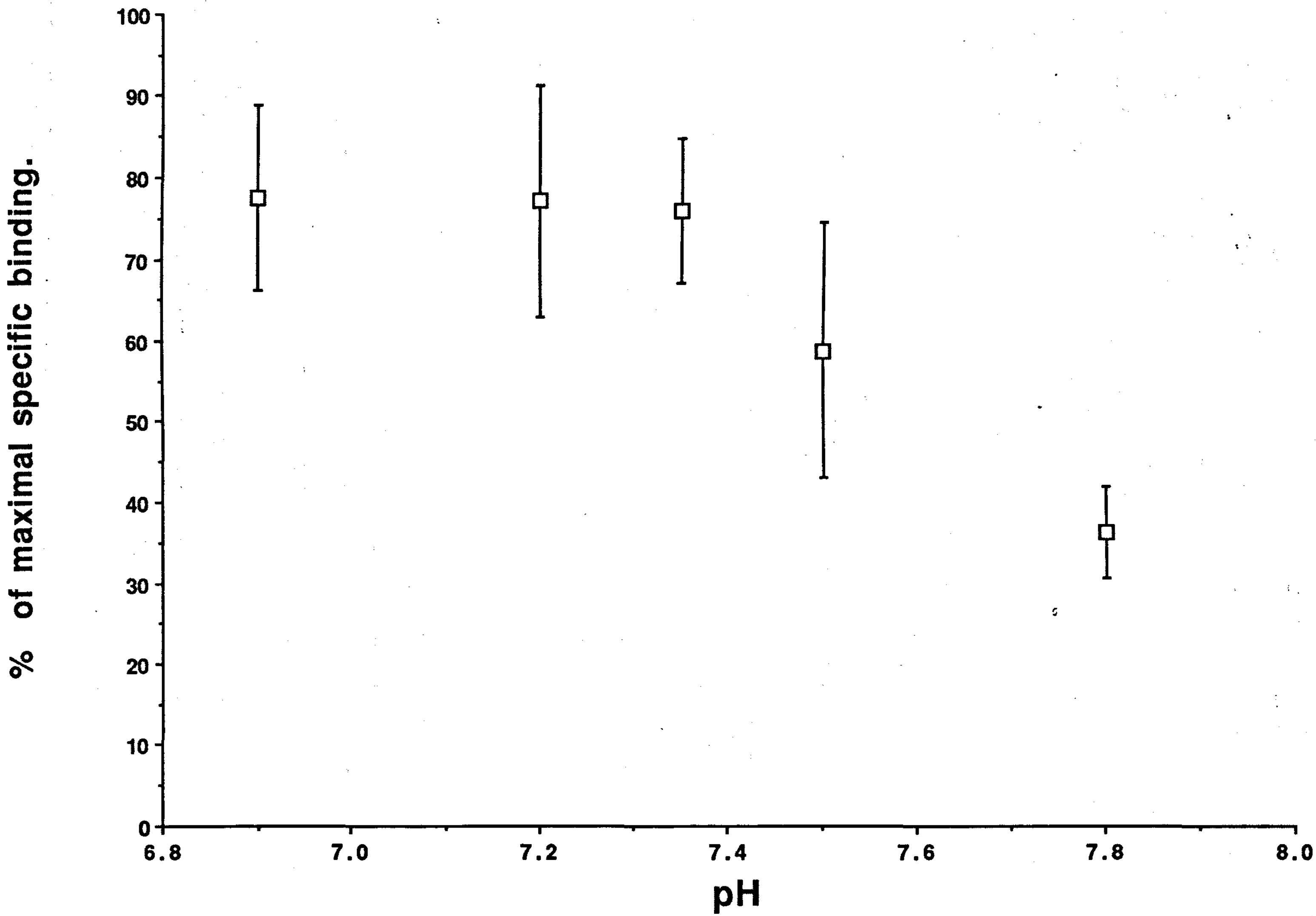


Figure 2.5.

The effect of varying pH on platelet AII binding.

These results were from five subjects, with mean +/- SEM of triplicate assays plotted. The incubation time was 90 min and the incubation temperature was 26'C.

Figure 2.5



% of maximal specific binding.
100
90
80
70
60
50
40
30
20
10
0
6.8
7.0
7.2
7.4
7.6
7.8
8.0
pH

significant difference was only attained at a pH of 7.8 ($P<0.01$, Wilcoxon matched-pairs signed-ranks test).

c) Incubation time.

In assessing the time course of binding, ^{125}I-AII (30 pmol/l) was incubated with platelets at 26 'C, the equilibrium being stopped by centrifugation at varying time intervals. Figure 2.6 demonstrates the results of five experiments. Platelet AII binding increased rapidly for 30 minutes, then more slowly between 30 and 90 minutes. There was a plateau from 90 to 150 minutes when binding remained relatively constant.

In subsequent experiments, incubation time was 90 minutes. However, on occasions, in order to facilitate the processing of more than one sample, this time period was extended to 120 minutes.

The experiments were repeated at a concentration of 15 pmol/l ^{125}I-AII, in order to derive dissociation rate constants (Kd) from kinetic data using two concentrations of ligand (see p. 130).

d) Temperature.

Incubation temperature was assessed by incubating portions of the platelet suspension in five different shaking water baths, the temperature in the water baths varying from 16 'C to 37 'C. The concentration of ^{125}I-AII was 30 pmol/l, the pH 7.4 and the incubation time 90 minutes. The results of four experiments are shown in Figure 2.7. There was significantly greater platelet AII binding ($P<0.01$) at 22 'C and 26 'C, than at 16 'C, 30 'C, or 37 'C. In subsequent experiments, the incubation temperature was 26 'C.

Figure 2.6.

The effect of varying incubation time on platelet AII binding.

These results were from four subjects, with mean +/- SEM of triplicate assays plotted. The concentration of ^{125}I-AII was 30 pmol/l. The incubation temperature was 26'C and the pH was 7.4.

Figure 2.7.

The effect of varying incubation temperature on platelet AII binding.

These results were from four subjects, with mean +/- SEM of triplicate assays plotted. The incubation time was 90 min and the pH was 7.4.

Figure 2.6

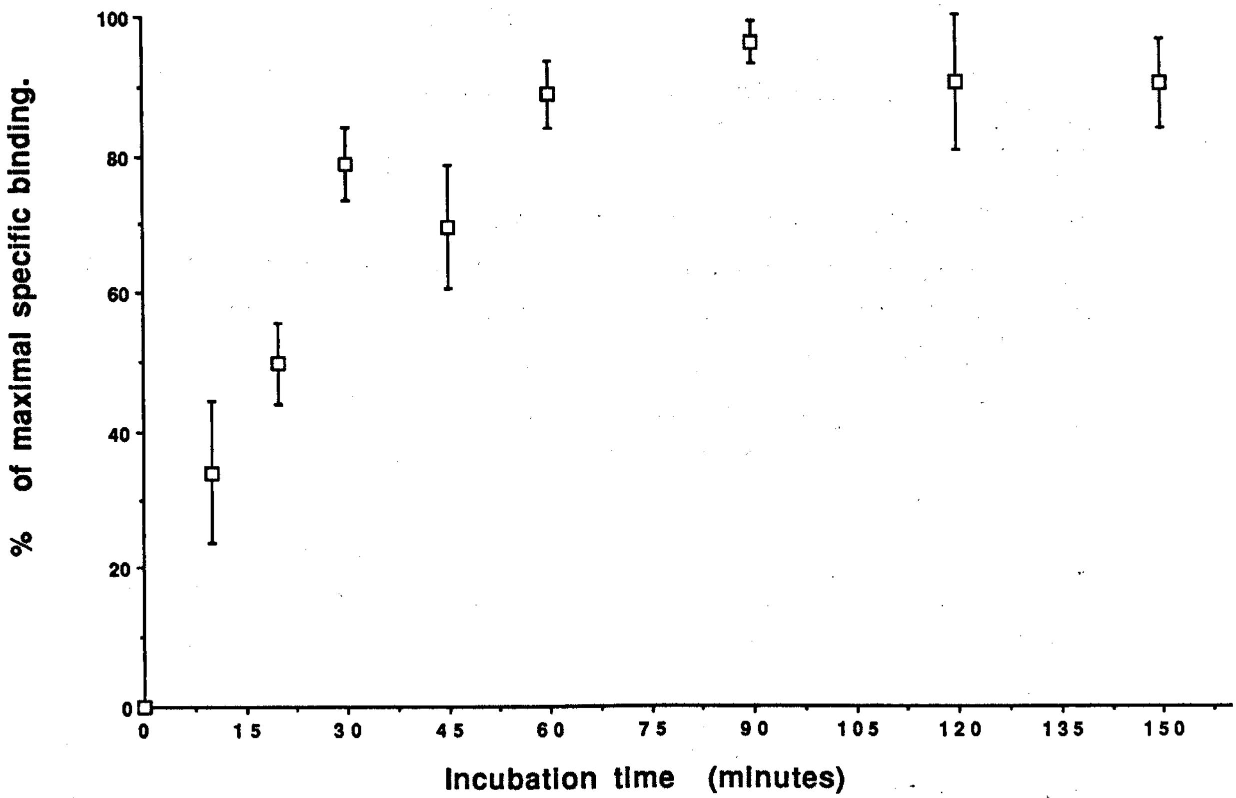
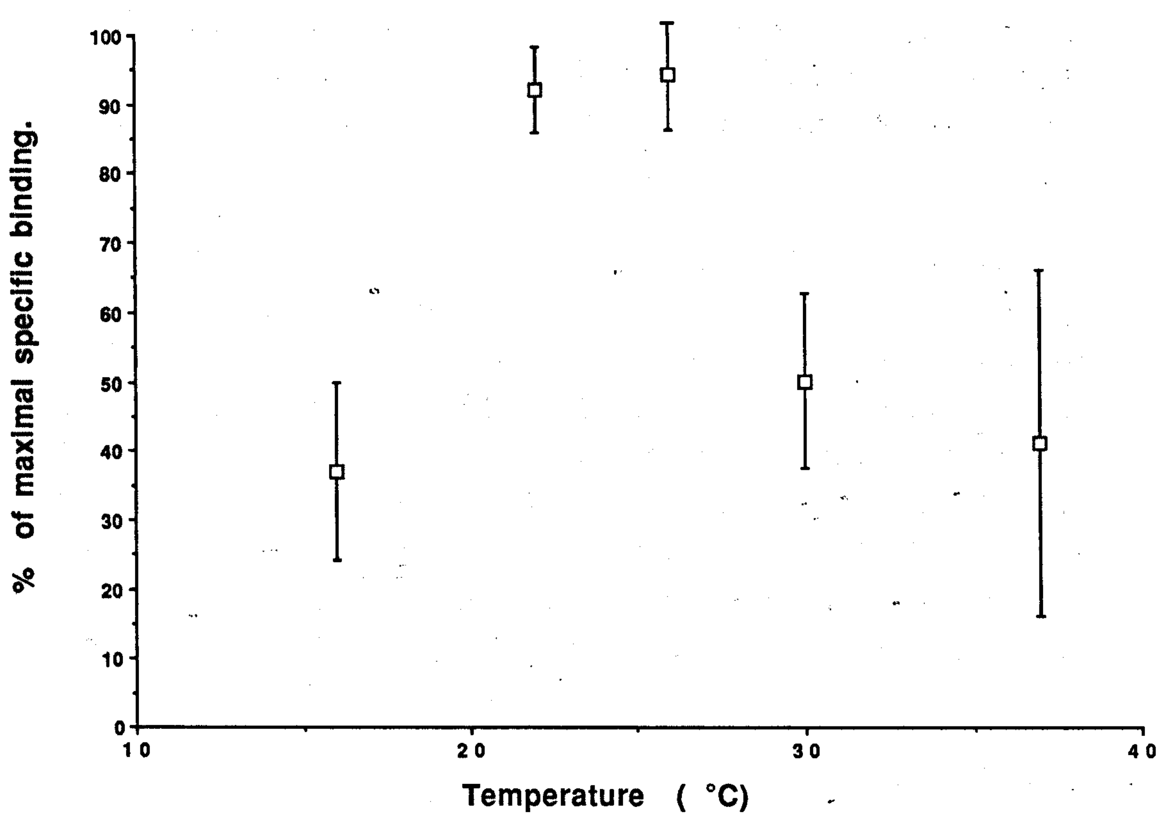


Figure 2.7



Work by both Moore and Williams [1981] and Ding, Kenyon and Semple [1984], suggests that it is difficult to separate the effects of incubation time and temperature on platelet AII binding. It is likely that at 16 'C, platelet AII binding had yet to attain maximal levels. At 37 'C both groups, [Moore and Williams, 1981, Ding et. al., 1984], found that varying incubation times resulted in a short plateau period when platelet AII binding was constant. Ding et. al. [1984] found that at 37 'C, platelet AII binding declined progressively after 30 minutes.

e) Stability.

In five experiments, following preparation of the platelet suspension, the binding assay was delayed for 60 minutes, at varying temperatures. There was a marked reduction in platelet AII binding at each temperature, with median diminutions in platelet AII binding of 82% at -20'C, 63% at 4'C, and 68% at 16'C. In subsequent experiments there was no delay between platelet preparation and the binding assay.

f) Enzyme inhibition.

Enzymatic degradation of ^{125}I-AII occurs in many tissues, including vascular smooth muscle [Gunther, Gimbrone and Alexander, 1980b]. This precludes the study of binding sites at equilibrium unless appropriate enzyme inhibitors are added. Mann, Sis and Ritz [1985], when studying platelet AII binding, found that without the addition of the enzyme inhibitor bacitracin (1mg/ml) there was a considerable loss of measurable binding. However, the incubation temperature in the experiments of Mann et. al. [1985] was 37'C. To determine whether the addition of an enzyme inhibitor was necessary at an incubation temperature of 26'C, the platelet suspension from six subjects was divided into two portions, with one half being incubated with bacitracin (1mg/ml) and the other half without bacitracin (90 min incubation at pH 7.4, 26'C).

The results of these experiments are illustrated in Figure 2.8. The median value of platelet AII binding in the samples without bacitracin added was the same as that of the samples incubated with bacitracin (3.1 fmol/10^9 cells). The addition of bacitracin did not significantly alter platelet AII binding ($P>0.3$, Wilcoxon matched-pairs signed-ranks test), thus in subsequent experiments it was not added.

g) Blood donation.

In an effort to study the effect of alterations in platelet populations on platelet AII binding, blood was taken from six subjects both immediately prior to, and one week following, the donation of approximately 500ml blood at the Nottingham Blood Transfusion Centre. The subjects were all male (in order to avoid any effect of the menstrual cycle, see p. 141) and had a median age of 28 years (range 18-42). All the subjects were normotensive, and none was taking any medication.

Prior to the blood donation, the median values of the platelet parameters were as follows: platelet count 214 x 10^9/L (range 187-321), mean platelet volume (MPV) 8.2 fl (range 7.7-8.5) and platelet distribution width (PDW) 15.7 (range 14.8-16.5). Following the blood donation, there was no significant change in the platelet count (204 x 10^9/L, 185-309, $P>0.5$, Wilcoxon matched-pairs signed-ranks test). There were, however, changes in MPV (9.1 fl, 8.2-9.9,

Figure 2.8.

The effect of the enzyme inhibitor bacitracin in platelet AII binding.

Figure 2.9.

Platelet AII binding immediately prior to, and one week following, blood donation.

Figure 2.8

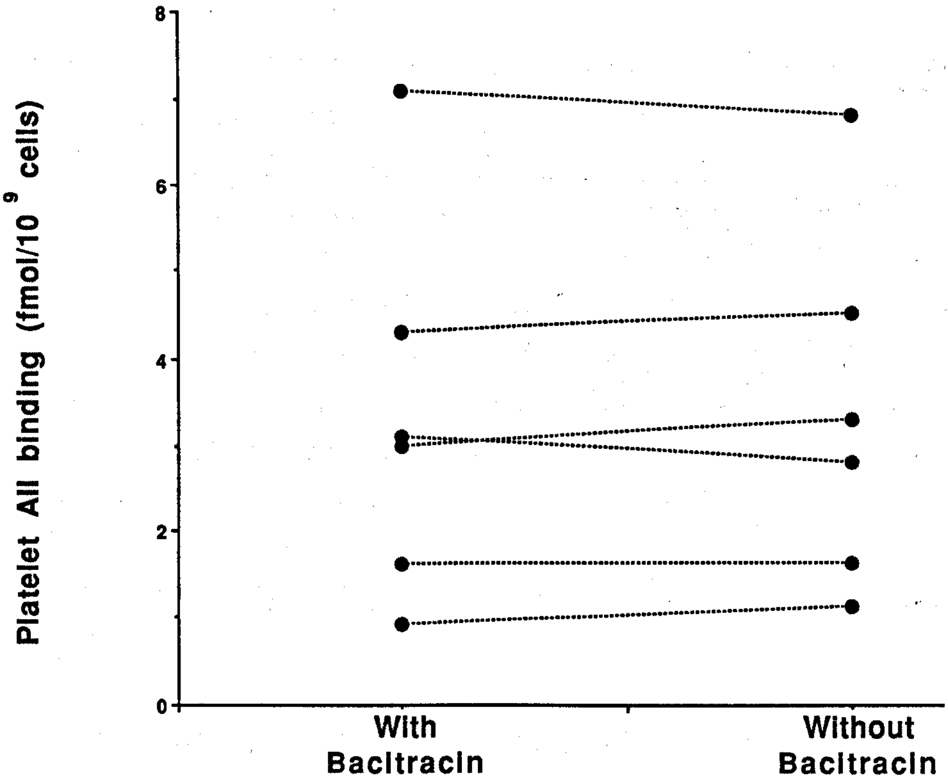
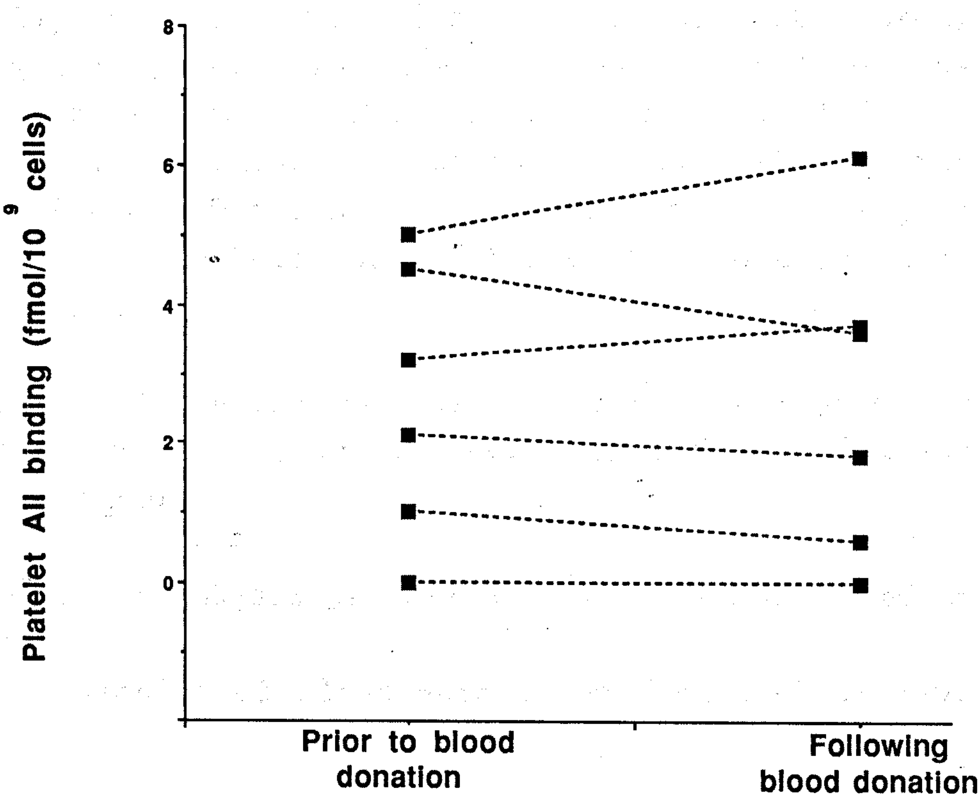


Figure 2.9



P= 0.04) and in PDW (16.9, 16.2-17.8, P= 0.03) which approached significance.

The changes in platelet AII binding are illustrated in Figure 2.9. Despite the alterations in platelet parameters, the values of platelet AII binding after the blood donation (median 2.7 fmol/10^9 cells) were not significantly different from those obtained prior to blood donation (median 2.6 fmol/10^9 cells, P>0.4, Wilcoxon matched-pairs signed-ranks test).

Quantification of inter-assay and intra-subject variation.

a) Inter-assay variation of platelet AII binding assay.

The method described above entails performing triplicate binding assays on every blood sample. Each month, the coefficient of variation found in the triplicate assays was calculated for the first 20 samples. The values ranged from 10% - 18% (median 13.5%), and were dependant on the particular study being performed. When analysis was confined to non-pregnant subjects, the lowest value of 10% was obtained. However, as would be expected, greater variation was found in samples from pregnant women, in whom lower values of platelet AII binding were demonstrated.

This inter-assay variation confirmed the need for triplicate assays, if necessary at a lower platelet concentration.

b) Intra-subject variation of platelet AII binding assay.

Six samples of blood were taken from each of five subjects, (including the author), over a fourteen day period. All the subjects were male, in order to avoid any menstrual effect, (see p. 141). The coefficient of variation ranged from 17% - 42% (median 27%). However the ranking of the subjects, (highest concentration of platelet AII binding to lowest concentration of platelet AII binding), remained constant on all bar one of the six occasions.

When this assessment of intra-subject variation was repeated after a further nine months experimentation similar results were obtained (median coefficient of variation 25%, range 11%-40%).

Chapter 3.

CHARACTERISATION OF PLATELET AII BINDING SITES.

This chapter is the first of six chapters describing studies of platelet AII binding. At the end of each chapter there is a brief discussion relating to the experiments described in that particular chapter. The thesis then concludes with a General Discussion.

As discussed in the Introduction (p. 79), for the interaction of a hormone with a cellular binding site to be considered to correspond to a receptor interaction, it should fulfil the criteria of a hormonal response; exhibiting specificity, high affinity, reversibility and saturability. The complete validation of peptide hormone binding sites as receptors which mediate the biological actions of the ligand requires that a correlation be made between receptor occupancy and an appropriate target-cell response. The purpose of the experiments detailed below was to establish whether the AII binding sites found on platelets had the characteristics of receptors.

Study sample.

Unless otherwise stated, all the subjects in the studies below were non-pregnant female members of the medical and nursing staff aged between 18 and 43 years. All subjects were normotensive and had no history of renal, metabolic or cardiovascular disease. No patient was taking any medication, including the oral contraceptive pill. 52 ml of blood was taken from each patient, the subsequent methodology being as described on page 112.

Saturation studies.

The platelet suspensions from 5 subjects were divided into seven portions, and these were incubated with varying concentrations of ^{125}I-AII. Figure 3.1 illustrates the relationship between specific AII binding and increasing concentrations of the labelled peptide, the plateau of the curve demonstrating **saturability** of the binding sites.

Scatchard analysis, (see p. 60), of specific binding results from the 5 saturation curves showed linear plots with a mean equilibrium dissociation constant (Kd) (+/- SEM) of 2.57 +/- 0.97 x 10^{-10} mol/l, thus demonstrating that the binding sites were of **high affinity.** Figure 3.2 shows a Scatchard plot from one representative saturation study.

Competition studies.

The platelet suspensions from 5 subjects were again divided into seven portions, however, for these experiments each portion was incubated with a fixed concentration of ^{125}I-AII and increasing concentrations of unlabelled AII. Figure 3.3 illustrates the results of these studies. Significant displacement of the label was noted at concentrations of unlabelled AII as low as 5 x 10^{-11} mol/l, ($P<0.01$). At a concentration of 2.5 x 10^{-8} mol/l, 97% of the specific binding had been displaced. Scatchard analysis of these results gave a Kd of 1.41 +/- 0.7 x 10^{-10} mol/l, again demonstrating **high affinity.** Figure 3.4 shows a Scatchard plot from one representative competition study.

Figure 3.1.

Saturation study.

This figure illustrates the effect of altering the concentration of radiolabelled Angiotensin II on specific binding.
These results were from five subjects, with the mean +/- SEM of triplicate readings plotted.

Figure 3.2.

Scatchard analysis of saturation study results.

The results of one representative non-pregnant subject are shown.

Figure 3.1

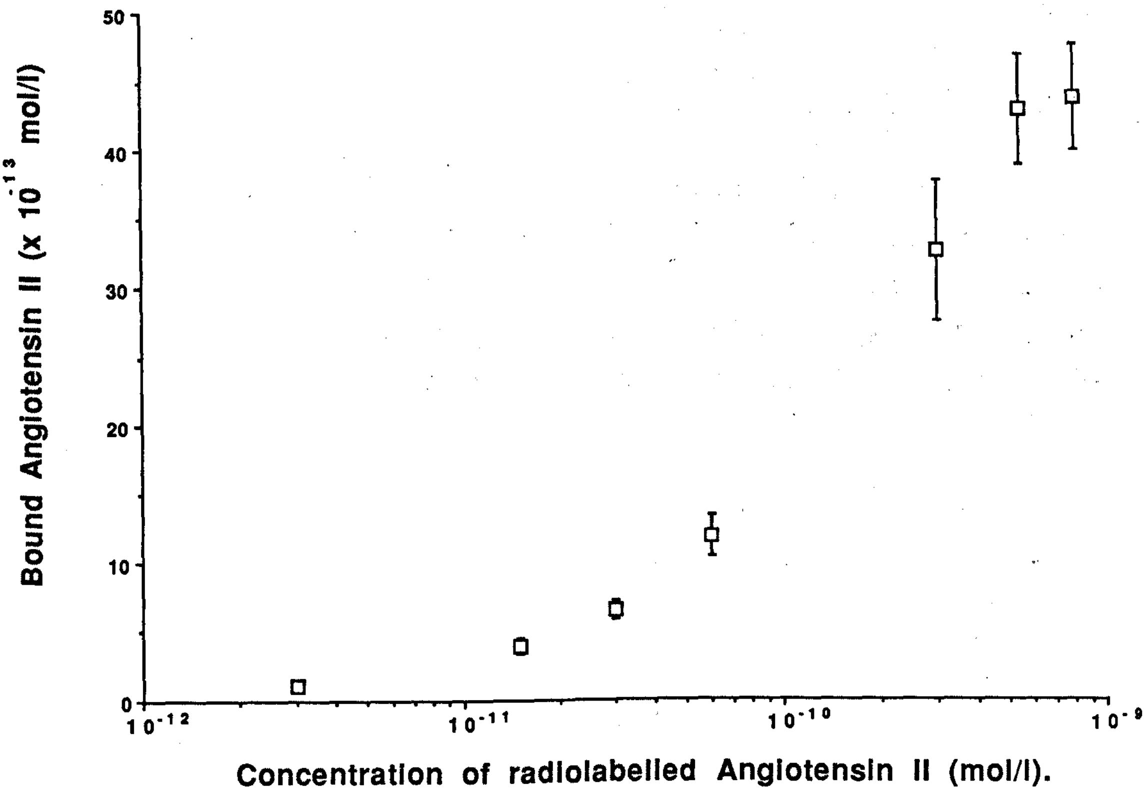


Figure 3.2

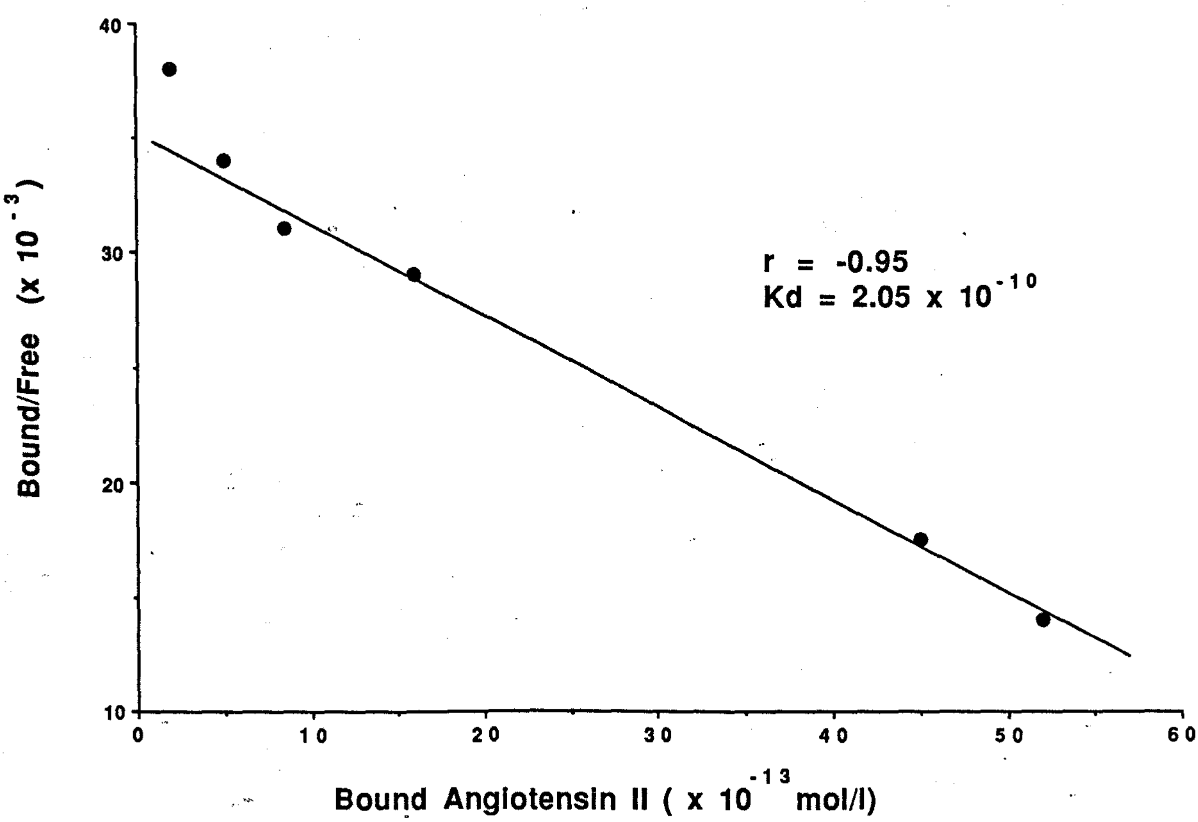


Figure 3.3.

Competition study.

This figure illustrates the effect of altering the concentration of unlabelled Angiotensin II on specific binding.
These results were from five subjects, with the mean +/- SEM of triplicate readings plotted.

Figure 3.4.

Scatchard analysis of competition study results.

The results of one representative non-pregnant subject are shown.

Figure 3.3

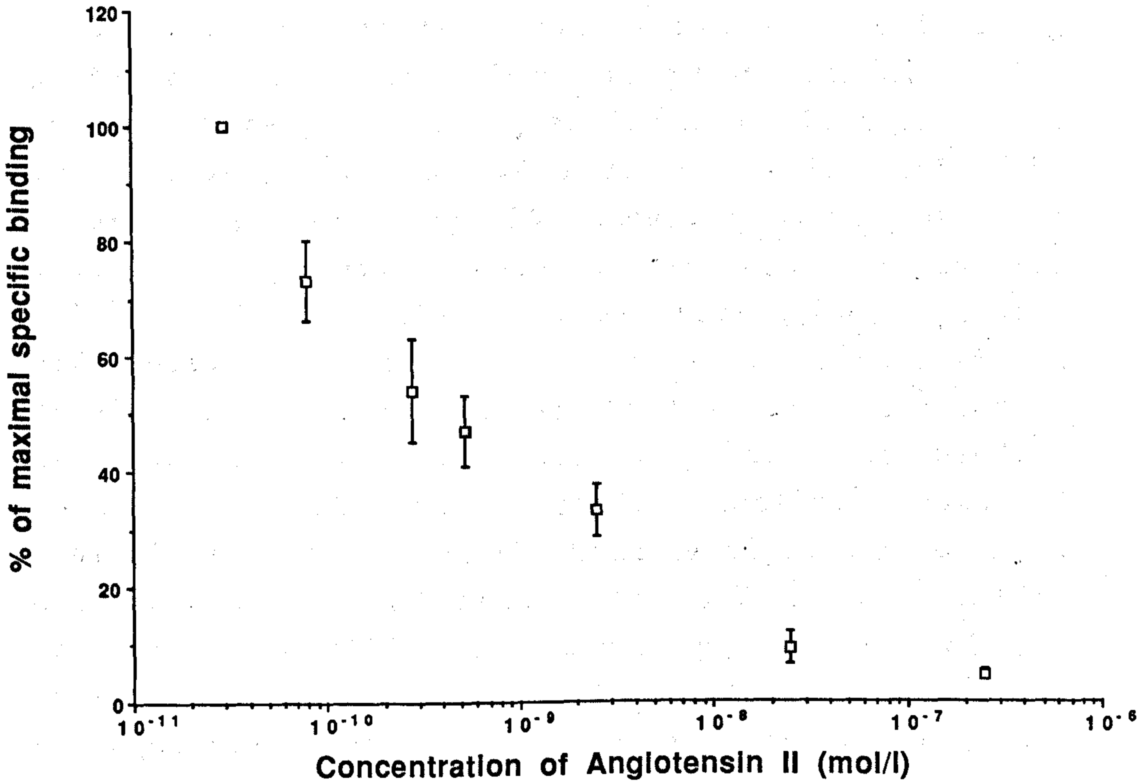


Figure 3.4

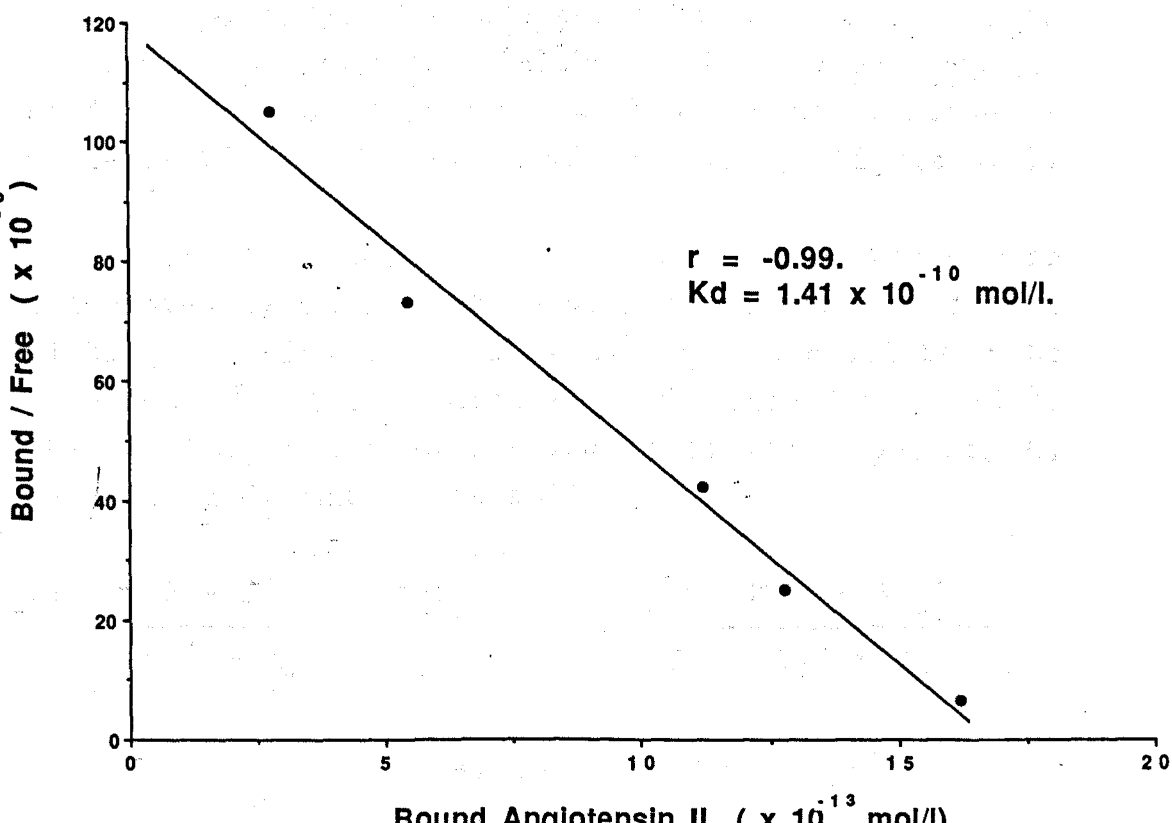


Figure 3.5.

Kinetic data obtained using two concentrations of ligand.

These results were from at least three subjects at each concentration of ^{125}I-AII, with the mean +/- SEM of triplicate readings plotted.

Figure 3.6.

AII binding expressed as the natural log of the proportion of binding at various times relative to the binding at equilibrium.

Competition studies were also attempted using platelet suspensions from pregnant women. All subjects were primiparous, taking no medication except iron preparations and were in the third trimester (median gestational age 34 weeks). However, many of these women had low or undetectable levels of platelet AII binding, making characterisation studies difficult. In order to perform successful competition studies on 5 patients, these studies were attempted in 12 subjects. The median value of platelet AII binding in these subjects was 8.0 fmol/10^9 cells. Scatchard analysis indicated that the binding sites were of high affinity, with a Kd of 2.5 +/- 0.7 x 10^{-10} mol/l, which did not differ significantly from that of the non-pregnant subjects. 3 of the 5 subjects subsequently developed pregnancy-induced hypertension.

Kinetic studies.

The effect of varying incubation time upon specific binding has been discussed in the validation of the method, (p. 123). These experiments were repeated in a further 4 subjects, (although in one subject no binding could be detected), with the concentration of ^{125}I-AII 15 pmol/l instead of 30 pmol/l, (Figure 3.5).

Forward (K_1) and reverse (K_{-1}) rate constants were calculated by a method free of assumptions made in the fitting of binding results to the appropriate rate equations [Gunther, Gimbrone and Alexander, 1980a]. This method, which is derived in full in Appendix C, calculates K_1 and K_{-1} as follows:

$$K_1 = \frac{S - S'}{D_o' - D_o} \quad \text{and} \quad K_{-1} = \frac{S'D_o - SD_o'}{D_o' - D_o}$$

Figure 3.5

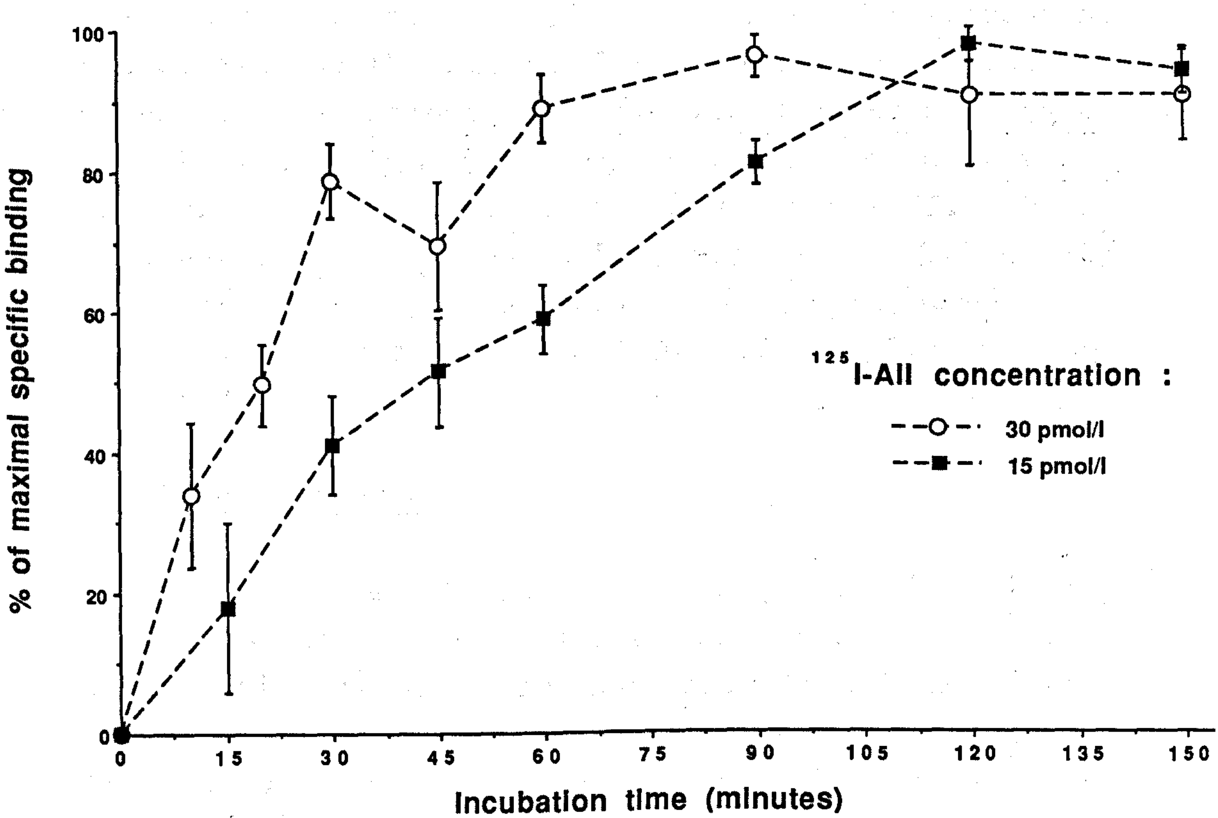


Figure 3.6

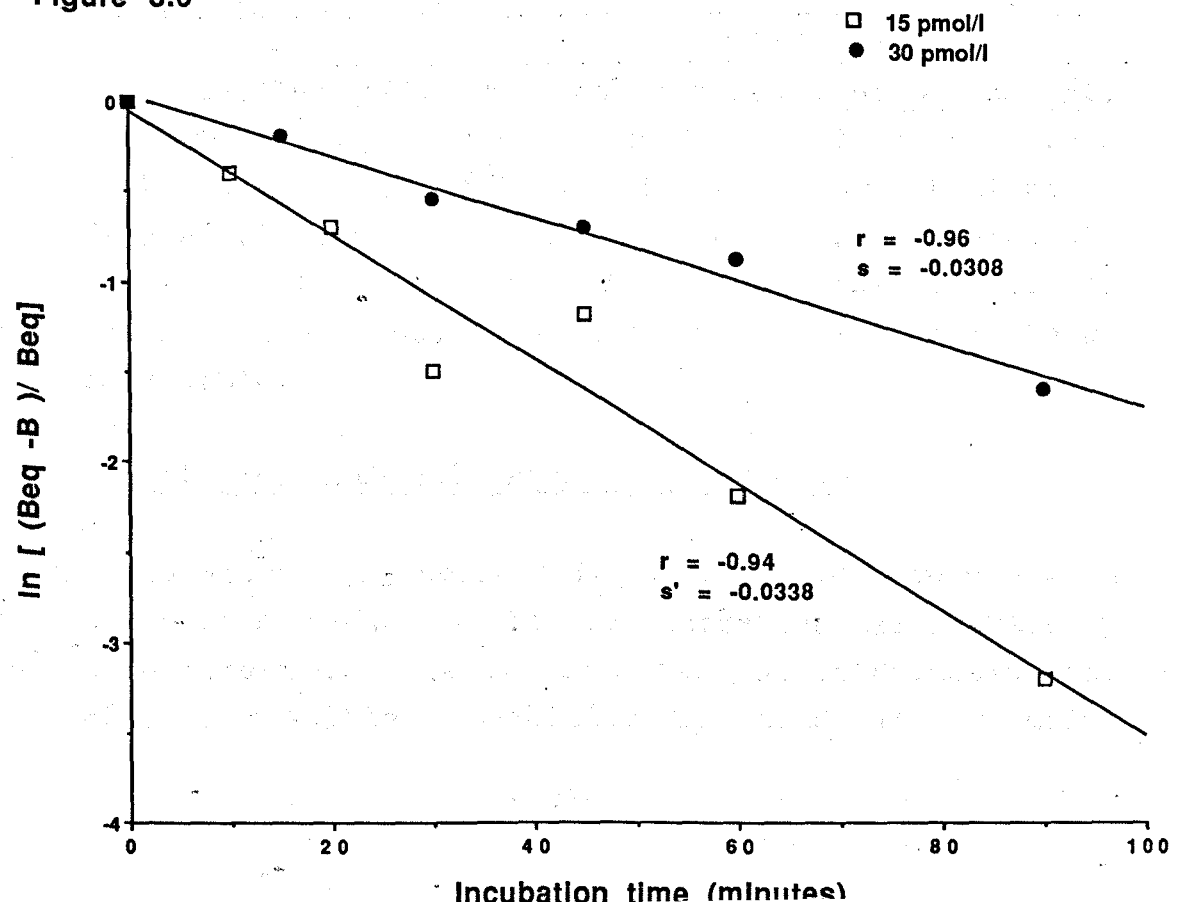


Figure 3.5.

Kinetic data obtained using two concentrations of ligand.

These results were from at least three subjects at each concentration of ^{125}I-AII, with the mean +/- SEM of triplicate readings plotted.

Figure 3.6.

AII binding expressed as the natural log of the proportion of binding at various times relative to the binding at equilibrium.

Figure 3.5

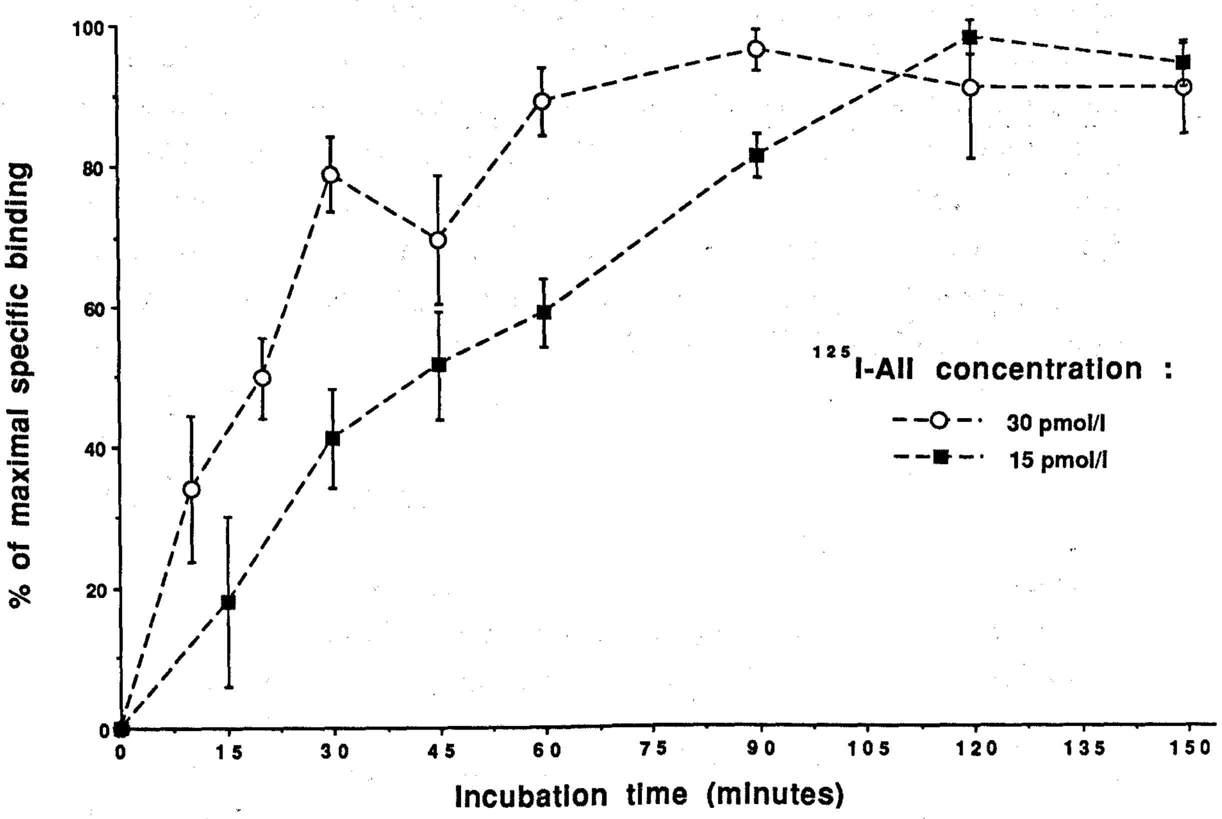
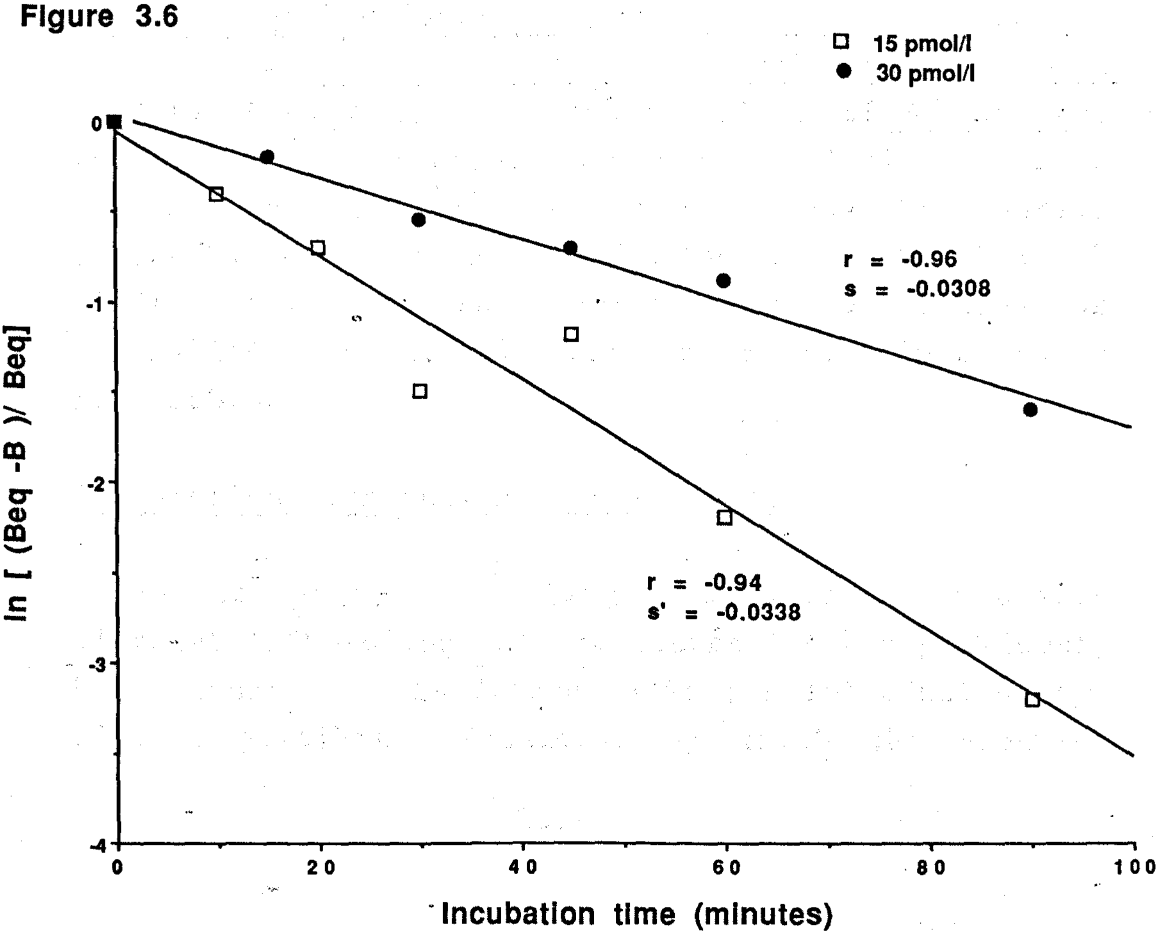


Figure 3.6



where S and S' are the slopes obtained by expressing AII binding as the natural logarithm of the proportion of binding at various times (B) relative to binding at equilibrium (Beq), (see Figure 3.6), and D_o and D_o' are the initial concentrations of ^{125}I-AII. Using this equation, K_1 was calculated to be 2.0×10^8 mol/sec/l and K_{-1} was 2.8×10^{-2} mol/sec/l. A dissociation constant Kd of 1.4×10^{-10} mol/l was thus obtained.

Specificity studies.

To determine ligand **specificity** of the binding sites, competition curves were prepared using various angiotensin analogues as the unlabelled hormone (see Figure 3.7). In this analysis, Des-Asp^1 angiotensin II (AIII) was equipotent with AII, followed by [Sar^1,Ala^8] angiotensin II, with angiotensin I (AI) found to be the least potent peptide.

Dissociation Studies.

Unlabelled AII, at a concentration of 25 µmol/l, was added to platelet suspensions which had been preincubated with ^{125}I-AII at 26 °C for 90 minutes. Figure 3.8 demonstrates the diminution in platelet AII binding following the addition of the excess of unlabelled hormone. The binding was thus found to be **reversible.**

Correlation with cell response studies.

No attempt at correlating a cellular response with platelet AII binding site concentration has previously been reported. As discussed in the Introduction (p. 85), a possible mechanism by which the vascular

Figure 3.7.

Competitive binding by analogues of AII for ^{125}I-AII binding sites.

Experiments with each peptide were performed using at least three subjects. The mean of triplicate readings has been plotted.

Figure 3.7

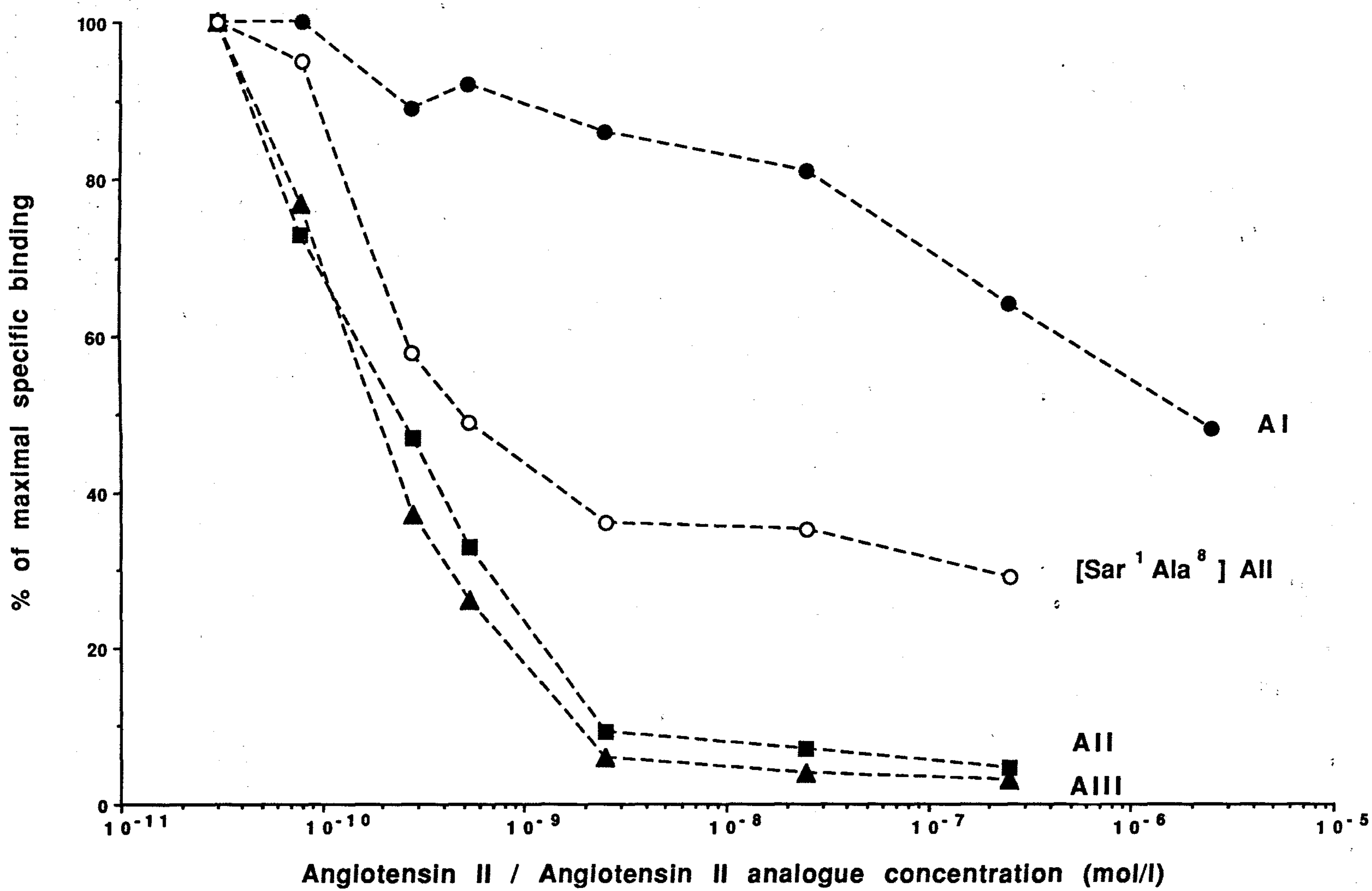
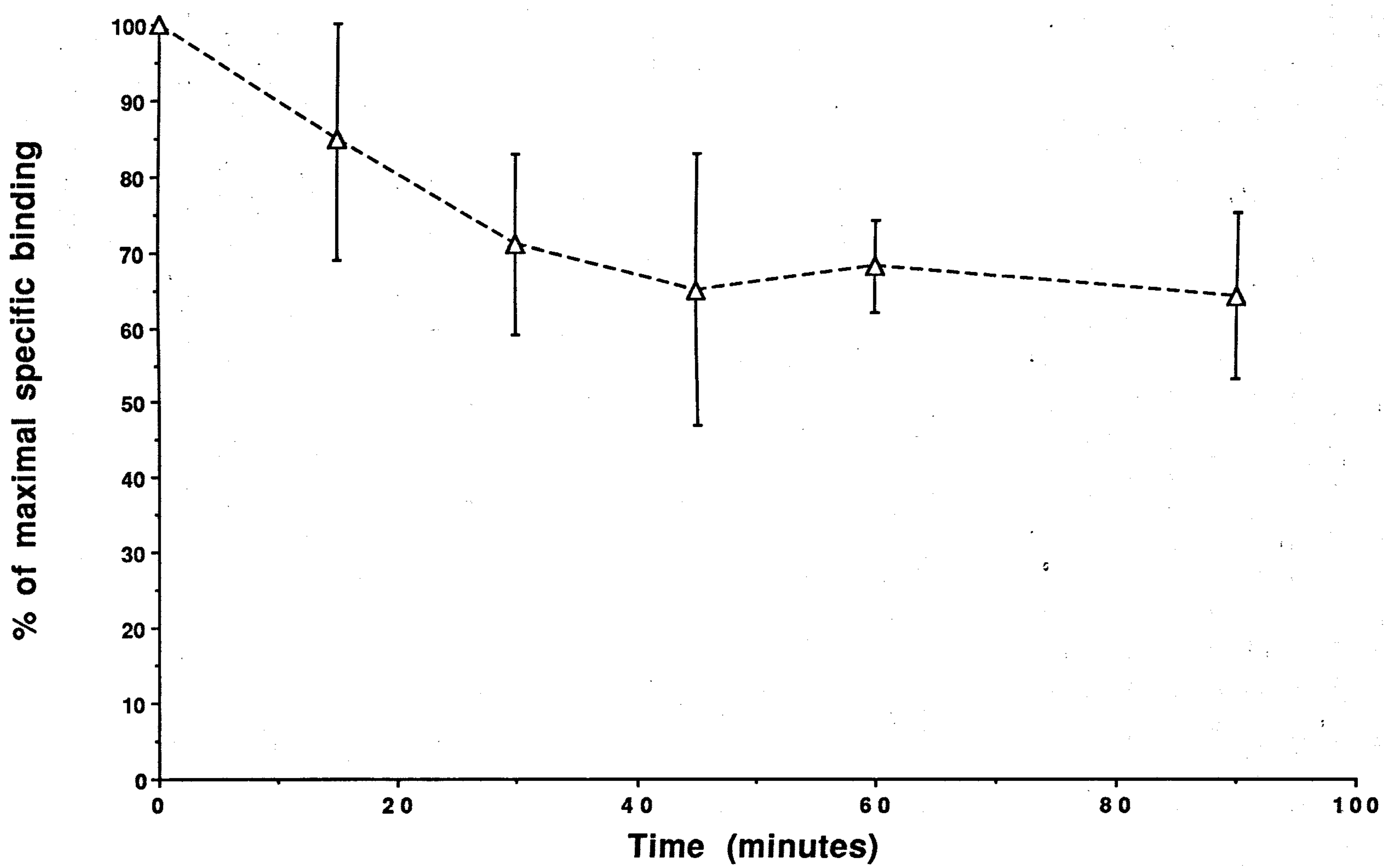

100
80
60
40
20
0
% of maximal specific binding
AI
[Sar 1 Ala 8] AII
AII
AIII
10-11
10-10
10-9
10-8
10-7
10-6
10-5
Angiotensin II / Angiotensin II analogue concentration (mol/l)

Figure 3.8.

Dissociation study.

The effect of adding excess unlabelled AII following preincubation with ^{125}I-AII is illustrated.
These results were from three subjects, with the mean +/- SEM of triplicate readings plotted.

Figure 3.8



% of maximal specific binding
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
Time (minutes)

smooth muscle cell AII binding site exerts its effect is via an increase in intracellular free calcium ($[Ca^{+2}]_i$). Moreover, platelets have homologous intracellular mechanisms for the mobilisation of $[Ca^{+2}]_i$ [O'Rourke, Halenda, Zavoico and Feinstein, 1985]. AII has been found to increase $[Ca^{+2}]_i$ in platelet suspensions from non-pregnant women [Haller, Ludersdorf, Lenz, Distler and Philipp, 1987], and pregnant subjects [Haller, Oeney, Hauck, Distler and Philipp, 1989], although the concentration of AII necessary to elicit an increase was higher than that described in experiments with vascular smooth muscle [Alexander, Brock, Gimbrone and Rittenhouse, 1985].

The relationship between platelet AII binding and the AII-induced rise in platelet $[Ca^{+2}]_i$ was therefore investigated. Experiments were performed _in vitro_, with the addition of AII to platelet suspensions, and _ex vivo_, with platelets obtained from primiparous subjects before and after they were infused with intravenous AII. The subjects were in the third trimester of pregnancy, when levels of platelet $[Ca^{+2}]_i$ have been found to be at their highest [Kilby, Broughton Pipkin, Cockbill, Heptinstall and Symonds, 1990].

The _in vitro_ and _ex vivo_ studies were performed on six subjects and on thirteen subjects respectively; their details are summarised in Table 3.1. No woman was known to be suffering from renal, metabolic or cardiovascular disease. All the subjects were studied during their first pregnancy and no patient in any group was taking any medication bar iron and vitamin supplements. All subjects were receiving _ad libitum_ sodium intake and had normal serum urea, creatinine, and electrolyte estimations.

Table 3.1.

Each study group comprised subjects in the third trimester of their first pregnancy. Medians are quoted (range).

	In vitro group (n = 6)	*Ex vivo* group (n = 13)
Maternal age (years)	25 (17-28)	25 (21-28)
Gestation (weeks)	33 (29-34)	32 (28-33)
Diastolic B.P. (mmHg)	68 (64-74)	70 (50-85)
Systolic B.P. (mmHg)	120 (115-125)	110 (100-125)
Platelet count ($10^3/\mu l$)	160 (114-225)	211 (127-333)

In vitro studies.

Platelet AII binding was measured as described in Chapter 2. Platelet $[Ca^{+2}]_i$ was measured, with the fluorescent indicator fura-2 being added to prepared platelet suspensions [Kilby, Broughton Pipkin, Cockbill, Heptinstall and Symonds, 1990].

Platelet $[Ca^{+2}]_i$ was measured immediately after the addition of AII, at concentrations ranging from 2 x 10^{-6} mol/l to 2 x 10^{-12} mol/l, to the platelet preparations of each subject.

Quantitative measurement of platelet $[Ca^{+2}]_i$ indicated a basal concentration of 111 (97-122) nM. On stimulation with AII, no change in fluorescence was elicited in any of the subjects, even at the highest doses of AII. The median value of platelet AII binding was found to be 3.1 (0-21.4) fmol/10^9 cells.

Ex vivo studies.

An initial venous blood sample was obtained from an indwelling cannula (Venflon 19 G) in an antecubital vein, prior to an AII infusion, using the protocol described by Broughton Pipkin, Hunter, Turner and O'Brien [1982]. (A brief description of this protocol is included in Chapter 8, p. 220). A second blood sample was obtained at the end of the third infusion step, at a dose of 16ng AII/kg/min. The platelet AII binding assay and the platelet $[Ca^{+2}]_i$ estimation were performed on the initial samples and the platelet $[Ca^{+2}]_i$ estimation on the second sample, as described above.

Platelet $[Ca^{+2}]_i$ in the blood samples taken prior to AII infusion was 115 (range 89-126) nM. Measurement of platelet $[Ca^{+2}]_i$ following the AII infusion indicated a statistically significant AII-induced rise in concentration ($P<0.01$, Wilcoxon matched-pairs signed-ranks test), to 138 (103-154) nM. The increase was found in 12 of the 13 subjects (Figure 3.9).

The median value of platelet AII binding, determined prior to the infusion was 2.0 (0-15.4) fmol/10^9 cells. The AII infusion caused a rise in the plasma AII concentration from 43.2 (10.9-100) pM to 130.0 (13.3-300.0) pM.

There was no significant correlation between platelet AII binding and the rise in platelet $[Ca^{+2}]_i$ ($P>0.2$, Spearman correlation coefficient), as illustrated in Figure 3.10, although the trend was positive. Furthermore, there was no correlation between the rise in platelet $[Ca^{+2}]_i$ and either the

Figure 3.9.

Platelet intracellular free calcium levels, before and immediately following AII infusion.

There was a significant rise in the levels of intracellular free calcium ($P<0.01$).

Figure 3.10.

The AII-induced rise in platelet intracellular free calcium plotted against platelet AII binding.

No significant correlation between these two parameters was found ($R = 0.36$, $P>0.2$, Spearman correlation coefficient).

Figure 3.9

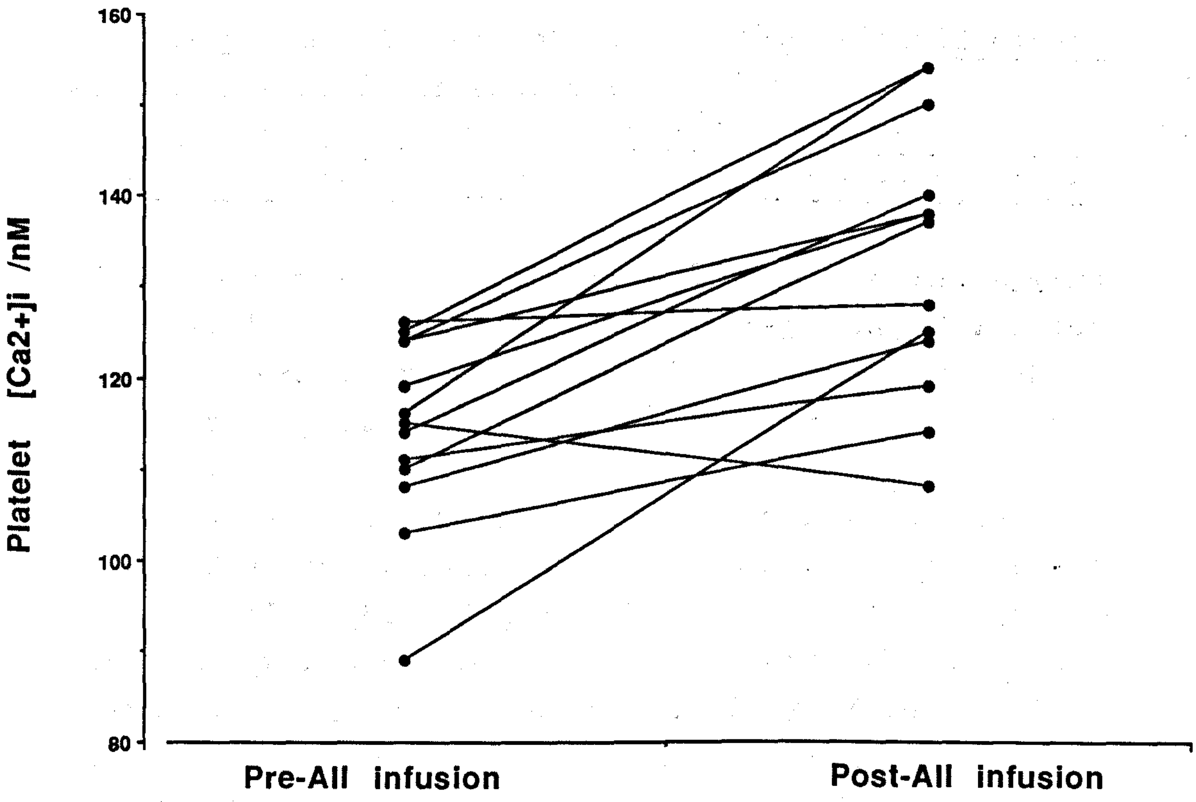
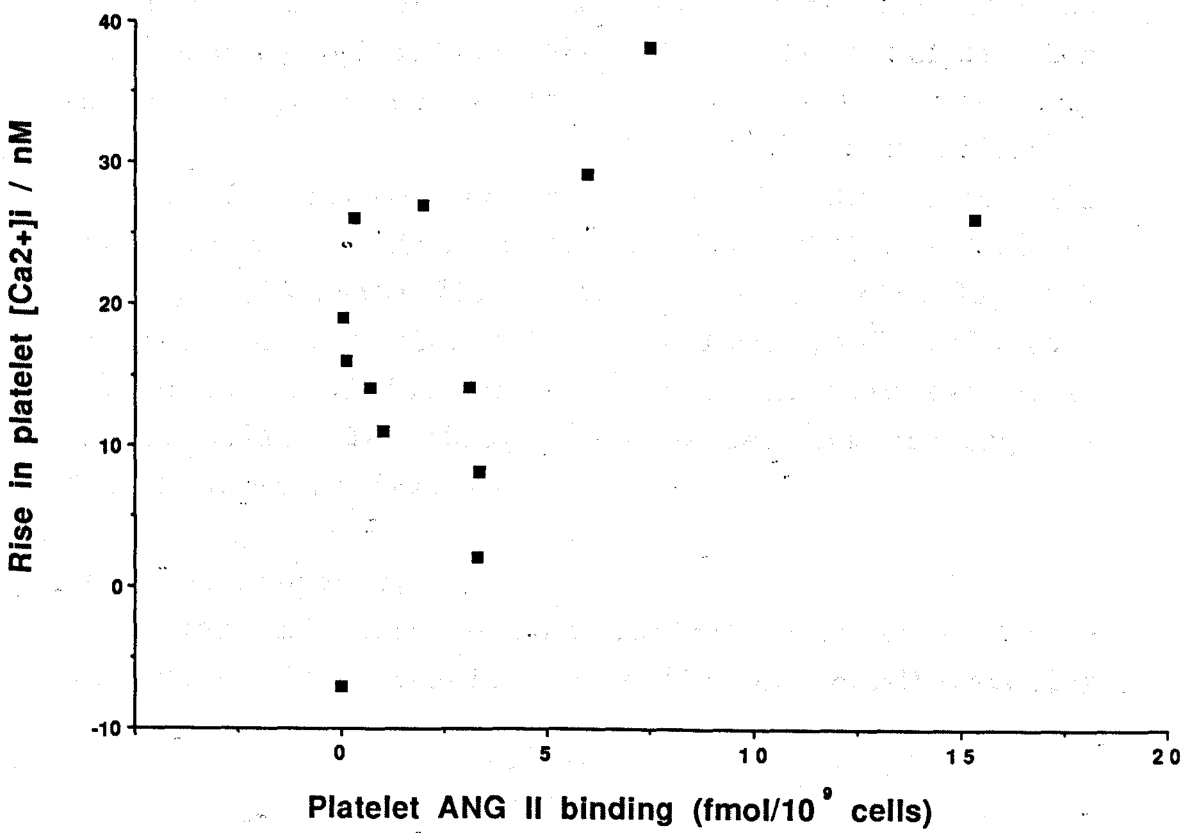


Fig. 3.10



initial plasma AII concentration or the rise in plasma AII level ($P>0.5$, in each case).

Infusion studies.

To test the effect of acute changes in the plasma AII level on platelet AII binding, AII infusion studies were performed using 9 non-pregnant and 32 pregnant subjects.

As above, an initial venous blood sample was obtained from an indwelling cannula prior to an AII infusion [Broughton Pipkin et. al., 1982]. A second blood sample was obtained at the end of the third infusion step, at a dose of 4ng AII/kg/min when the subjects were non-pregnant and 16ng AII/kg/min when the subjects were pregnant. Platelet AII binding and plasma AII levels were measured in both blood samples.

Non-pregnant subjects.

These subjects fulfilled the criteria described above (p. 128) and in addition, were all studied between days 5-9 of the menstrual cycle. Their median age was 28 years (range 18-43).

The AII infusion caused a rise in the plasma AII level from a median value of 15.4 pM (9.9-28.8) to 52.3 pM (34.5-107.7). The change in platelet AII binding from a median value of 4.9 fmol/10^9 cells to a median value of 4.0 fmol/10^9 cells is illustrated in Figure 3.11. The change in platelet AII binding was not significant ($P>0.4$).

Pregnant subjects.

None of the pregnant subjects was known to be suffering from renal, metabolic or cardiovascular

Figure 3.11.

The effect of AII infusion on platelet AII binding in non-pregnant subjects.

Figure 3.12.

The effect of AII infusion on platelet AII binding in pregnant subjects.

The median values are indicated by the horizontal bars.

Figure 3.11

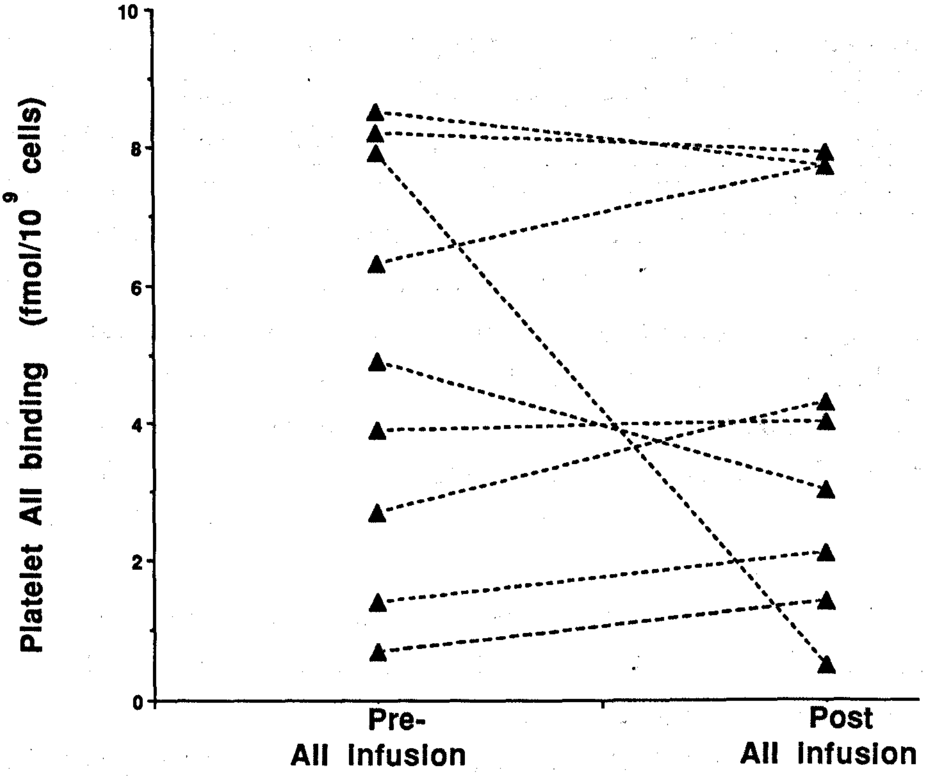
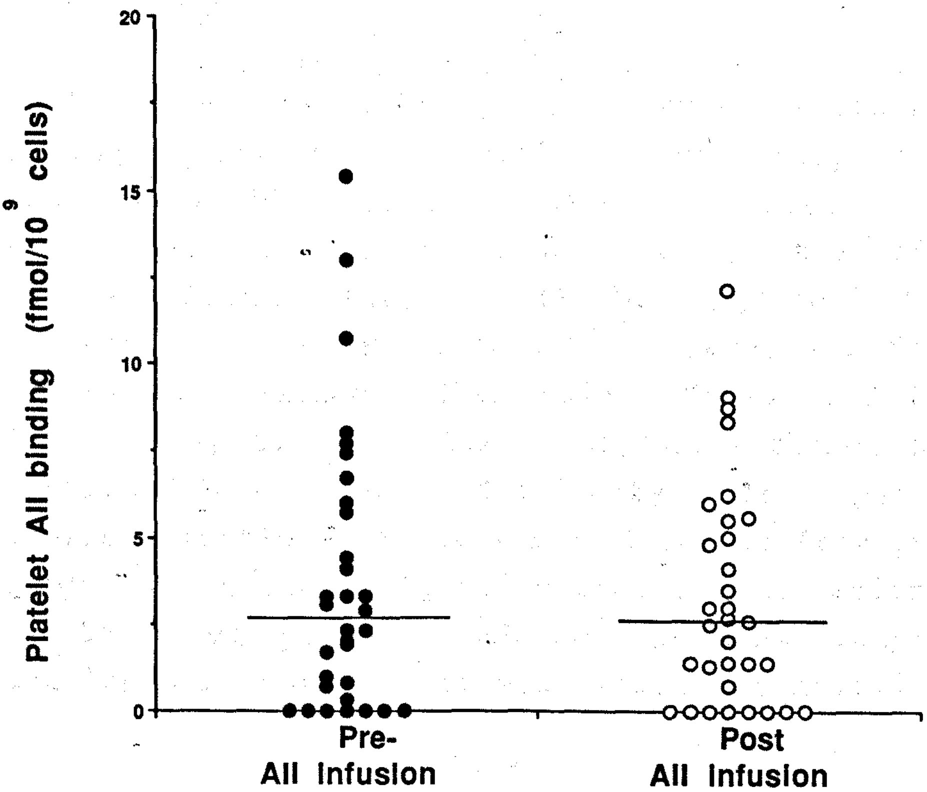


Figure 3.12



disease. All the subjects were primiparous and none were taking any medication bar iron and vitamin supplements. All subjects were receiving ad libitum sodium intake and had normal serum urea, creatinine, and electrolyte estimations. The median age of the subjects was 24 years (21-24) and the median gestational age was 32 weeks (31-32). All subjects were normotensive when the infusion studies were performed (median systolic blood pressure 117 mmHg., 114-121, median diastolic blood pressure 65 mmHg., 61-69).

The AII infusion caused a rise in the plasma AII level from a median value of 8.2 pM (3.2-60.0) to 120.1 pM (40.5-205.0). The change in platelet AII binding from a median value of 2.6 fmol/10^9 cells to a median value of 2.5 fmol/10^9 cells is illustrated in Figure 3.12. As in the non-pregnant subjects, this change in platelet AII binding was not significant ($P>0.5$).

Discussion.

These results indicate that human platelets possess a single class of high affinity, specific, saturable and reversible binding sites. Moreover, the validation experiments described in the previous chapter found platelet AII binding to be time, temperature and pH dependent. Previous findings [Moore and Williams, 1981, Ding, Kenyon and Semple, 1984, Mann, Sis and Ritz, 1985] have therefore been confirmed. The difficulty in performing characterisation studies using platelets from pregnant subjects has been alluded to above. The median value of the five pregnant subjects in whom competition studies were possible is well above the median values in the third trimester, determined in both the cross-sectional and longitudinal studies (see Chapter 5, p. 153, and Chapter 6, p. 171). That three of the five patients subsequently developed pregnancy-induced hypertension is further evidence that these women may be unrepresentative of the normotensive pregnant population.

Three different methods, performed on different subjects, indicated that the value of the dissociation constant Kd was between 1.40 - 2.57 x 10^{-10} mol/l. The fact that similar binding site affinities were found for both radiolabelled AII and for the native hormone, (i.e the ^{125}I-AII saturation curve and the competitive displacement studies), suggests that iodination of the hormone did not alter its binding to platelets, thus justifying its use as a radiolabelled tracer. In this regard, the platelets resembled rat mesenteric artery tissue, which also binds iodinated and unlabelled AII equally [Gunther, Gimbrone and Alexander, 1980a].

The values of the dissociation constant found were in close agreement with those found previously in human platelets, 1.2 - 3.0 x 10^{-10} mol/l, [Moore and Williams, 1981, Ding, Kenyon and Semple, 1984, Mann, Sis and Ritz, 1985]. The affinity of the platelet binding sites were also of a similar order to those reported in animal vascular smooth muscle [e.g. in rat mesenteric artery tissue, McQueen, Murray and Semple, 1984, discussed in the Introduction, p. 75].

There was, however, a disparity noted between these results and previous work, when the dissociation study was considered. As illustrated in Figure 3.8, the diminution in binding found following the addition of excess unlabelled AII, ceased after one hour. This contrasts with the results of Ding et. al. [1984] and Mann et. al. [1985], indeed, the latter group found that even after six hours the rate of the reduction in binding levels had not slowed. Following the 90 minute incubation with ^{125}I-AII only, clumps of platelet aggregates were visible. It was thus anticipated that the addition of the unlabelled AII would only partially displace the radiolabelled peptide. The differing findings of the above workers may reflect varying degrees of platelet aggregation, although the method of platelet preparation used by Mann et. al. [1985] was similar to that used in this thesis, with the production of platelet rich plasma as an intermediate step. The possibility that in our studies there was breakdown of the unlabelled AII cannot be discounted.

In a small sample of primiparous subjects in the third trimester of pregnancy, an AII-induced rise in platelet $[Ca^{+2}]_i$ in the <u>ex vivo</u> situation was demonstrated. However, in contrast to the work of Haller, Oeney, Hauck, Distler and Philipp [1989], no increase in platelet $[Ca^{+2}]_i$ was found, on addition of AII to platelets <u>in vitro</u>, using either physiological or pharmacological concentrations of AII. Our findings were concordant with an earlier <u>in vitro</u> study in non-pregnant subjects [Lechi, Lechi, Bonadonna, Sinigaglia, Corrandini, Polignano, Arosio, Covi and Detogni, 1987]. The methodology used in this study was similar but not identical to that used by the Haller group. Centrifugation was used to prepare the platelet suspensions and the more contemporary indicator fura-2 used to measure platelet $[Ca^{+2}]_i$, allowing a reduction in the intracellular concentration of indicator and thus reducing any buffering of the calcium signals. The response found by Haller et. al. [1987] was present only in a very narrow concentration band (10^{-7} to 10^{-6}mol/l), and it is possible that the concentration steps used in this study were too large to identify the steep peak of response.

However, the results of the _ex vivo_ data clearly demonstrate a rise in platelet $[Ca^{+2}]_i$ after infusion of AII. The increase in platelet $[Ca^{+2}]_i$ in the _ex vivo_ studies was smaller than that demonstrated by Haller et. al. [1989], and lower than the transient calcium signals measured in smooth muscle cells [Brock, Alexander, Ekstein, Atkinson and Gimbrone, 1985]. The relatively smaller effect of AII _ex vivo_ may be explained by the low number of AII binding sites on platelets as compared to smooth muscle cells [Aguilera, Hauger and Catt, 1978, Gunther, Gimbrone and Alexander 1980b] (discussed in the Introduction, p. 76).

The results demonstrated no correlation between platelet AII binding and the rise in platelet$[Ca^{+2}]_i$ after _ex vivo_ infusion of AII, although an underlying trend was apparent. Formal validation of the high affinity platelet AII binding sites as receptors has thus not been achieved.

An AII-induced enhancement of the platelet aggregation response to adrenaline has been noted [Poplawski, 1970, Ding, MacIntyre, Kenyon and Semple, 1985]. Conditions causing vasoconstriction tend to produce parallel increases in platelet aggregation [e.g. Cowley, Stainer, Cockbill and Heptinstall, 1984]. Vascular smooth muscle constriction has been shown to be related to vascular smooth muscle cell AII binding [Baudouin, Meyer and Worcel, 1971], thus an attempt to correlate AII enhancement of platelet aggregation with platelet AII binding might be fruitful.

The results of the AII infusion studies are in accord with those of Moore, Taylor and Williams [1984], who also found that short term increases in plasma AII did not alter platelet AII binding. The regulation of platelet AII binding will be discussed in subsequent chapters.

In conclusion, the characterisation studies provide strong evidence that the platelet AII binding sites are receptors. However, until a correlation between levels of binding and a biological response is demonstrated, they cannot strictly be regarded as such.

Chapter 4.

MENSTRUAL STUDY.

Changes in platelet AII binding associated with the menstrual cycle.

This chapter will describe the results from fifteen nulliparous, normotensive non-pregnant female volunteers during the follicular and luteal phases of the menstrual cycle. Changes in the renin-angiotensin system associated with the menstrual cycle are well established, with increased levels in the luteal phase of plasma renin concentration (PRC) [Brown, Davies, Lever and Robertson, 1964], plasma renin activity (PRA) [Skinner, Lumbers and Symonds, 1969] and plasma angiotensin II (AII) concentration [Sundsfjord and Aakvaag, 1970] having been found. As discussed in chapter 1 (section 5 p. 79), regulation of platelet AII binding by changes in plasma AII concentration has been suggested [Moore, Taylor and Williams, 1984, Ding, Kenyon and Semple, 1985]. It was thus decided to measure platelet AII binding in both the follicular and luteal phases of the menstrual cycle in a small sample of women.

Study sample.

Fifteen nulliparous, normotensive Caucasian women were investigated between days 5-9 of the menstrual cycle (follicular phase) and on day 21 (luteal phase). All subjects had either not been taking hormonal contraception for at least six months (40%) or had never taken this medication (60%). Subjects had no recent history of menstrual disorder, and had regular periods. All subjects were normotensive and had no history of renal, metabolic or cardiovascular disease. No subject was taking any medication. All subjects were receiving sodium intake ad libitum and had normal serum urea, creatinine and electrolyte estimations. Their ages, body weights and blood pressures are summarised in Table 4.1. In view of the small sample size, the range of values are quoted rather than the 25th and 75th centiles.

The fifteen women were divided on the basis of serum progesterone levels into those with ovulatory (n=10) and anovulatory (n=5) cycles. As discussed below, a rise in serum progesterone level from <5 nmol/l at days 5-9 to >30 nmol/l at day 21 was adjudged to indicate ovulation. The changes in serum progesterone levels are illustrated in Figure 4.2.

1) **The ovulatory subgroup.**
The ten women had a median age of 26 years. The median body weight for the follicular phase was 61.5kg. All individuals were normotensive, the median follicular phase blood pressure being 117/75 mmHg. Neither body weight (P>0.3) nor blood pressure (P>0.4) changed significantly in the luteal phase (Wilcoxon matched-pairs signed-ranks test).

2) **The anovulatory subgroup.**
The five women had a median age of 21 years. The median body weight for the follicular phase was 61.7 kg. All individuals were normotensive, the median follicular phase blood pressures being 110/60 mmHg. Again, no significant changes in either body weight (P>0.5) or blood pressure (P>0.5) were noted in the phases of the menstrual cycle.

There were no significant differences between the ovulatory and anovulatory subgroups when comparisons of age (P>0.4), body weight (P>0.5), systolic blood pressure (P>0.1) and diastolic blood pressure (P>0.1) were made (Mann-Whitney U test).

Table 4.1.

Demographic data for the sample of non-pregnant women investigated during the menstrual cycle.

At the end of the study, 10 women were considered to have ovulatory cycles and 5 were considered to have anovulatory cycles.
Median values (range) are quoted.

Table 4.2.

Hormonal data from the sample of non-pregnant women investigated during the menstrual cycle.

Median values (range) are quoted.
The lower limit of detection of the serum progesterone assay was 2.0 nmol/l. In the statistical analysis performed, values reported as <2.0 nmol/l have been deemed to be 2.0 nmol/l.

Table 4.1.

	OVULATORY		ANOVULATORY	
	Follicular	Luteal	Follicular	Luteal
Age (years)	26 (20-42)	* *	21 (20-35)	* *
Weight (Kg)	61.5 (49.9-94.2)	61.8 (50.2-94.0)	61.7 (58.5-81.7)	61.8 (59.0-81.7)
Systolic blood pressure (mmHg.)	117 (100-130)	118 (105-130)	110 (100-120)	108 (100-115)
Diastolic blood pressure (mmHg.)	75 (60-80)	75 (60-80)	60 (50-70)	70 (55-80)

Table 4.2.

	OVULATORY		ANOVULATORY	
	Follicular	Luteal	Follicular	Luteal
Progesterone (nmol/L)	<2.0 (<2.0-4.0)	56.0 (32.0-75.0)	<2.0 (<2.0-<2.0)	8.0 (<2.0-11.5)
Angiotensin II (pg/ml)	9.4 (2.9-26.9)	16.5 (3.6-42.1)	29.1 (6.7-67.5)	15.6 (12.6-66.2)
P.R.C. (ng AI/hr/ml)	1.6 (0.5-3.4)	4.9 (1.3-6.7)	1.8 (0.1-2.0)	2.1 (0.2-2.5)
P.R.S. (ug AI /ml)	1.2 (0.7-2.4)	1.1 (0.5-3.2)	1.0 (0.4-1.1)	1.0 (0.2-1.0)

40 ml. of venous blood was taken on each occasion, as previously described (chapter 2, p. 112). In addition to platelet AII binding, serum urea and electrolytes, AII, plasma renin substrate (PRS), plasma renin concentration (PRC), and progesterone estimations were performed using the methods detailed in Chapter 2.

Results.

1) Platelet AII binding.

Figure 4.1 summarises the changes in platelet AII binding.

In the ovulatory subjects, there was a diminution in binding in the luteal phase of the cycle in nine of the ten women. The fall in platelet AII binding, from a median value of 11.2 (0.7-29.5) fmol/10^9 cells at days 5-9 to a median value of 3.6 (0.0-20.4) fmol/10^9 cells at day 21, approached significance ($P<0.02$, Wilcoxon matched-pairs signed-ranks test).

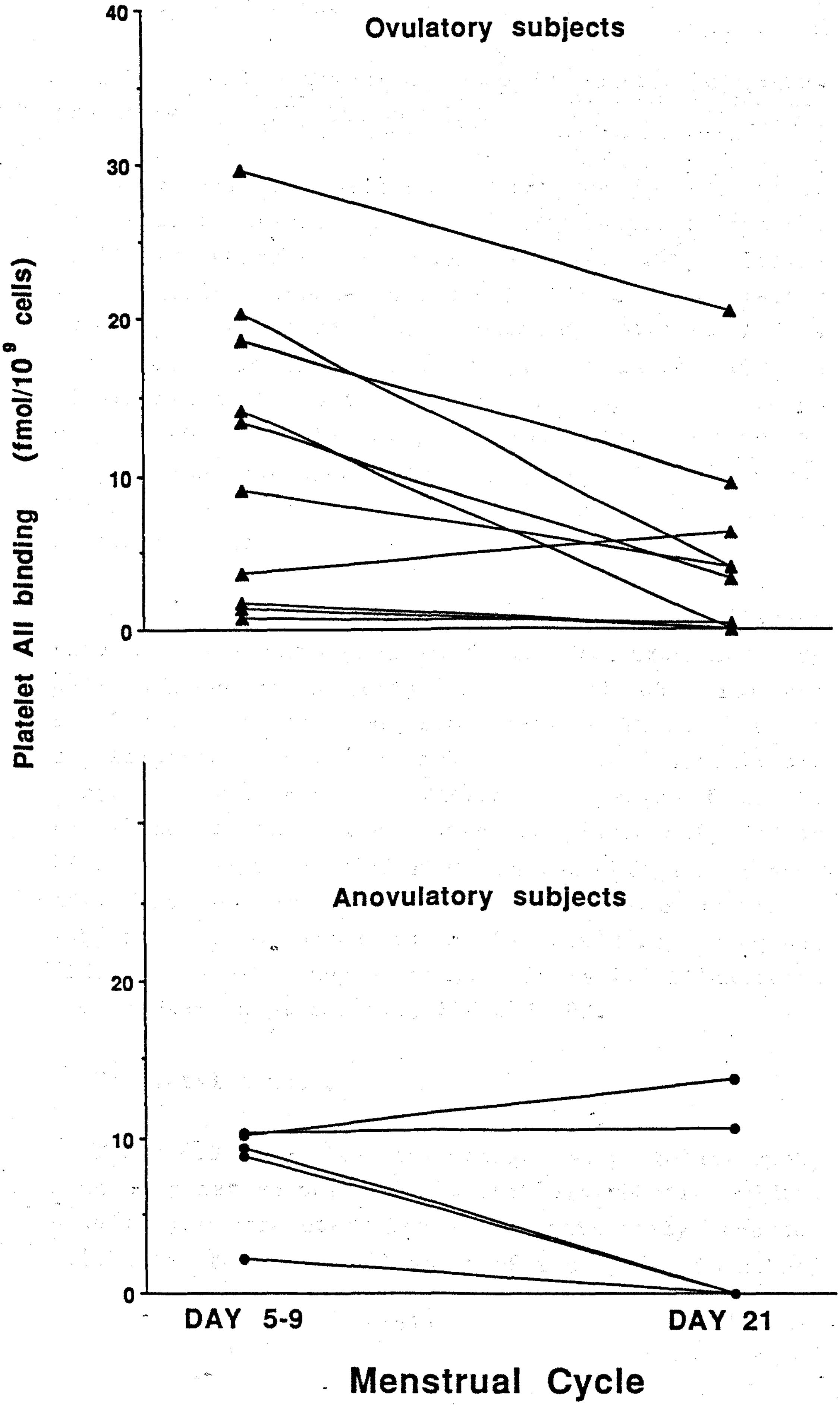
An increase in platelet AII binding was found in two of the five subjects deemed to be anovulatory. The change in binding, from a median value of 8.8 (1.9-10.6) fmol/10^9 cells at days 5-9 to a median value of 0.0 (0.0-13.7) fmol/10^9 cells, was not significant ($P>0.5$).

There were no significant differences between the ovulatory and the anovulatory subgroups when comparisons of platelet AII binding in the follicular ($P>0.5$) and luteal ($P>0.4$) phase of the menstrual cycle were made (Mann-Whitney U test).

Figure 4.1.

Changes in platelet AII binding associated with the menstrual cycle in ovulatory and in anovulatory subjects.

Figure 4.1.


Ovulatory subjects
Anovulatory subjects
Platelet AII binding (fmol/10^9 cells)
40
30
20
10
0
20
10
0
DAY 5-9
DAY 21
Menstrual Cycle

2) **Hormonal data.**

Table 4.2 summarises the changes in serum progesterone, AII, PRC and PRS.

There was a significant increase in the serum progesterone levels in the ovulatory subgroup from the follicular phase to the luteal phase ($P<0.01$, Wilcoxon matched-pairs signed-ranks test). In the anovulatory subgroup, the increase in serum progesterone levels was not significant ($P>0.1$). There was a significantly greater increase in the serum progesterone levels in the ovulatory subgroup as compared to the anovulatory subgroup ($P<0.01$, Mann-Whitney U test). Figure 4.2 illustrates the changes in serum progesterone levels.

In the ovulatory subjects, there was a significant rise in both plasma AII ($P<0.05$, Wilcoxon matched-pairs signed ranks test) and PRC ($P<0.05$) from the follicular to the luteal phase. There was no significant change in PRS. In the anovulatory subjects there were no significant changes from the follicular to the luteal phase, in plasma AII, PRC or PRS. A significant difference in the change in plasma AII, but not in PRC or PRS, was shown in these subjects as compared with the ovulatory subgroup ($P<0.05$, Mann-Whitney U test). Figure 4.3 illustrates the changes in plasma AII, PRC and PRS.

3) **Platelet data.**

Table 4.3 summarises the changes in platelet count, mean platelet volume and platelet distribution width. None of the parameters changed significantly from the follicular to the luteal phase of the cycle, in either

Figure 4.2.

Serum progesterone concentrations in the follicular and luteal phases of the menstrual cycle.

A rise in the serum progesterone concentration from <5 nmol/l at days 5-9 to >30 nmol/l at day 21 was adjudged to indicate ovulation.

Figure 4.3.

Changes in plasma AII, PRC and PRS associated with the menstrual cycle in ovulatory and anovulatory subjects..

Figure 4.2.

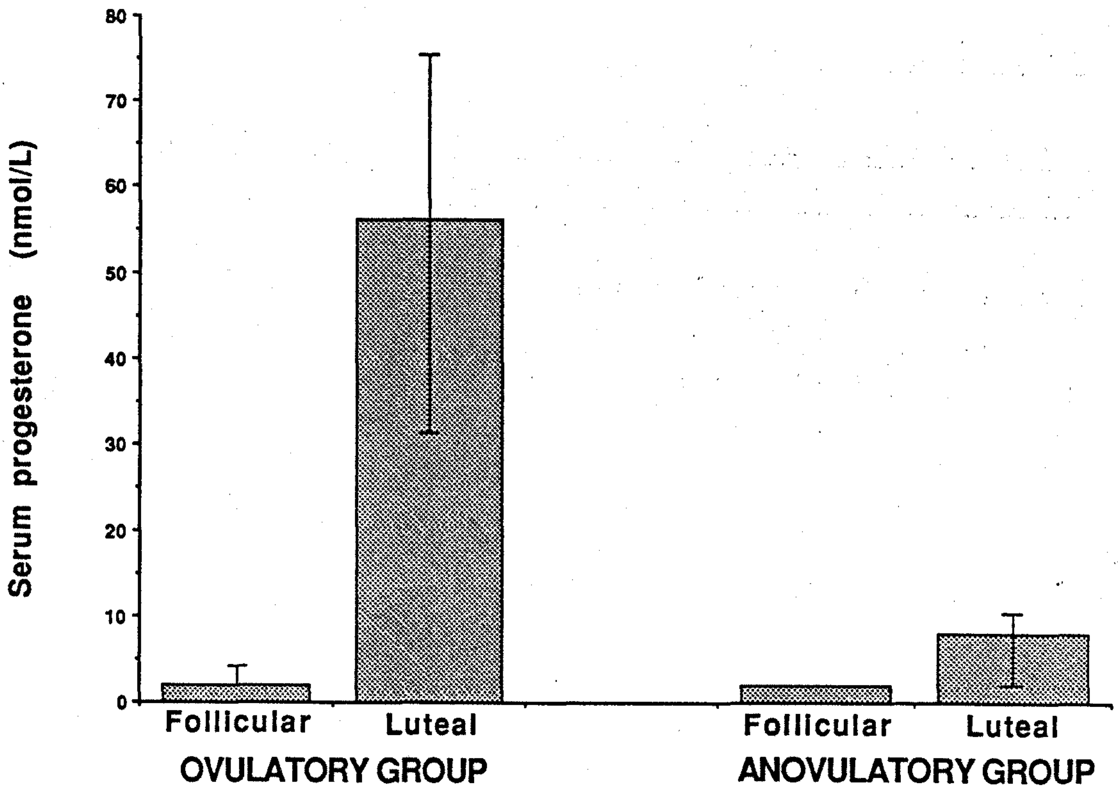


Figure 4.3.

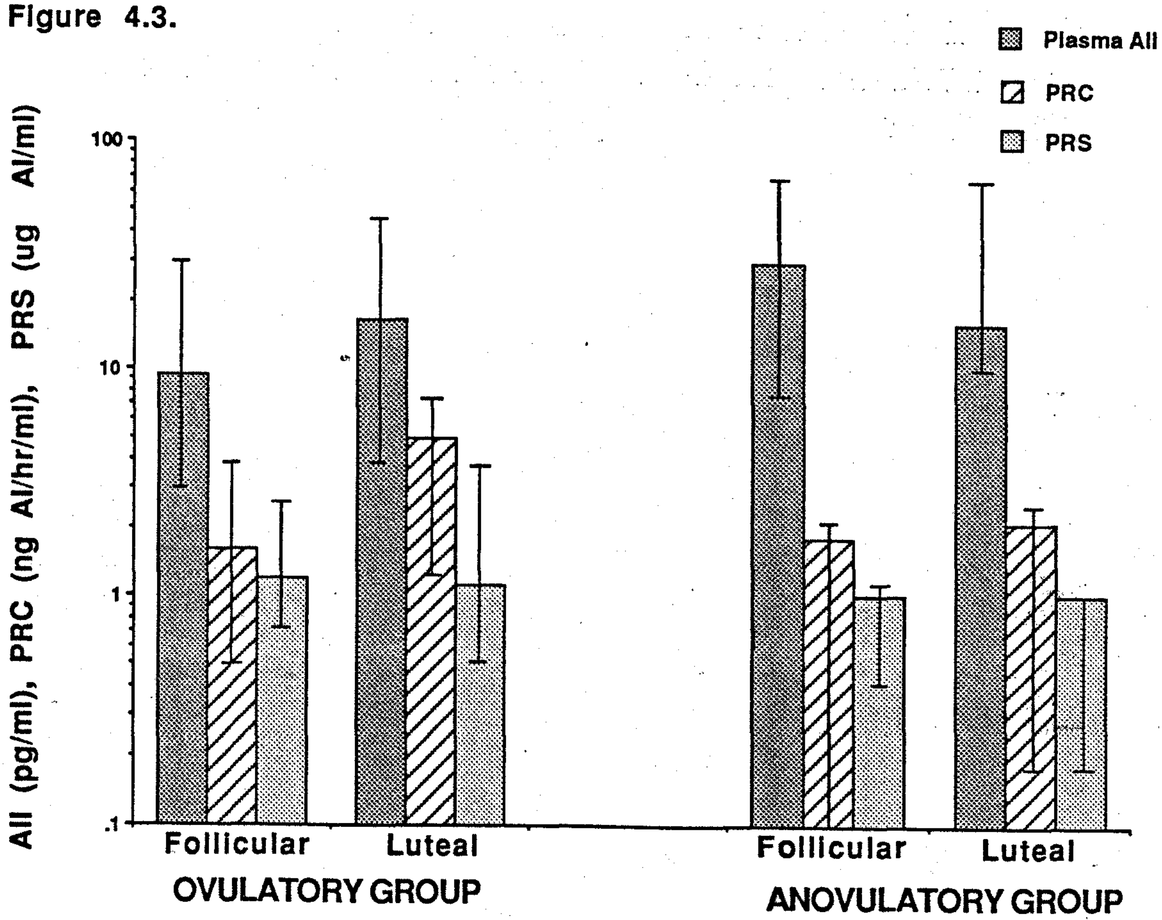


Table 4.3.

Platelet data from the sample of non-pregnant women investigated during the menstrual cycle.

Median values (range) are quoted.

Table 4.4.

Biochemical data from the sample of non-pregnant women investigated during the menstrual cycle.

Median values (range) are quoted.

Table 4.3.

	OVULATORY		ANOVULATORY	
	Follicular	Luteal	Follicular	Luteal
Platelet count (x 10^9 /L)	224 (168-346)	235 (160-344)	227 (174-252)	210 (153-221)
Mean platelet volume (fl)	7.7 (6.9-9.3)	8.1 (6.9-9.7)	7.9 (6.8-8.0)	7.6 (7.3-8.2)
Platelet distribution width	16.4 (14.4-17.9)	16.3 (14.4-17.5)	15.1 (14.7-16.2)	15.1 (14.4-16.4)

Table 4.4.

	OVULATORY		ANOVULATORY	
	Follicular	Luteal	Follicular	Luteal
Sodium (mmol/L)	140 (139-146)	139 (137-142)	140 (139-141)	141 (138-143)
Potassium (mmol/L)	4.2 (3.5-5.3)	4.1 (3.3-4.5)	4.5 (4.1-5.1)	3.9 (3.7-4.7)
Urea (mmol/L)	4.1 (2.8-5.6)	4.3 (2.9-5.9)	4.2 (2.8-5.1)	4.1 (2.5-5.0)
Creatinine (umol/L)	83 (69-98)	79 (56-98)	76 (73-87)	76 (45-91)
Urate (umol/L)	180 (167-257)	196 (167-245)	218 (198-296)	224 (207-306)
Osmolality (Osm/Kg)	285 (278-298)	280 (274-288)	281 (278-288)	281 (275-285)

the ovulatory or the anovulatory subgroup ($P>0.3$, Wilcoxon matched-pairs signed ranked test). There were no significant differences between the ovulatory and the anovulatory subgroups ($P>0.4$, Mann-Whitney U test).

4) **Biochemical data.**

Table 4.4 summarises the changes in serum sodium, potassium, urea, creatinine, urate and osmolarity. The only significant change from the follicular to the luteal phase was found in the ovulatory subgroup; a significant decrease in serum osmolarity was noted ($P<0.01$, Wilcoxon matched-pairs signed-ranks test). For all other comparisons $P>0.05$. There were no significant differences between the ovulatory and anovulatory subgroups ($P>0.1$, Mann-Whitney U test).

Discussion.

The main problem in interpreting this study lies in the small number of anovulatory patients studied. The logistics of recruitment were not easy, given the strict entry criteria and the amount of laboratory time required for each patient. Thus the possibility of incorrect acceptance of the Null Hypothesis for lack of any statistically significant difference between the groups must be recognised. However, although the sample size was small, this prospective study has demonstrated a significant diminution in platelet AII binding in the luteal phase of ovulatory menstrual cycles in nulliparous, normotensive women, with the median value of platelet AII binding decreasing by 69%.

Significant increases in both AII and PRC were found in the ovulatory subgroup, with the 75% increase in the median plasma AII concentration in the luteal phase being very similar to the 76% increase previously reported by Sundsfjord and Aakvaag [1970]. In the luteal phase of ovulatory cycles, progesterone from the corpus luteum causes a net loss in both sodium and water from the renal tubules, due to the increase in glomerular filtration rate and the natriuretic effects of progesterone [Oparil, Erlich and Lindheimer, 1975]. As discussed in the introduction (section 2), these changes are at least partially compensated for by activation of the renin-angiotensin system and its effects on renal tubular sodium handling. The observed rise in PRC in the ovulatory patients (Table 4.2) is consistent with this hypothesis. As discussed above, increases in both PRC and plasma renin activity have previously been demonstrated [Brown, Davies, Lever and Robertson, 1964, Skinner, Lumbers and Symonds, 1969]. Our data are also concordant with these physiological changes in that the serum electrolytes were unaffected by the changes in serum progesterone. The significant fall in serum osmolarity found in the luteal phase of ovulatory subjects is probably due to the effects of the progesterone surge and changes in sensory volume receptors, changes which extend into pregnancy if implantation occurs [Davison, 1980].

Increases in plasma AII concentrations consequent upon dietary sodium restriction have previously been found to cause a reduction in platelet AII binding [Moore, Taylor and Williams, 1984, Ding, Kenyon and Semple, 1985], thus the increased plasma AII concentrations in the luteal phase provide one possible explanation for the diminution in platelet AII binding.

The influence of sex steroid hormones on platelet AII binding has yet to be studied. However, as discussed in the introduction (section 5 p. 83), rat and rabbit uterine AII receptor concentrations respond in a dose-dependent manner to steroid administration [Schirar, Capponi and Catt, 1980]. Schirar et. al. [1980] found that progesterone infusion caused an increase in uterine AII receptor concentrations whereas oestradiol injection resulted in a decrease. As previously discussed (p. 71), uterine AII receptors share many characteristics with platelet AII binding sites, and steroid hormone regulation of platelet AII binding is another possibility.

The majority of studies of platelet behaviour during the different phases of the menstrual cycle suggest increased platelet reactivity in the luteal phase. Zahavi, Dreyfuss, Kalef and Soferman [1973] demonstrated an increase in the pro-aggregatory effects of ADP, and recent data from this centre has indicated that prostaglandin E_2-induced platelet rich plasma aggregation is increased in the third week of the menstrual cycle [Hughes, Broughton Pipkin, Heptinstall, personal communication]. One cause of the increased reactivity may be the elevated levels of plasma AII in the luteal phase of the cycle, Poplawski [1970] having found that AII enhanced the pro-aggregatory effects of both ADP and adrenaline, although it did not exert any direct effect itself. The alterations in platelet behaviour may themselves diminish the availability of cell surface binding sites, and thus compromise the use of platelets as a model of vascular smooth muscle cells.

In all cases a quantitative measure of ovulation was obtained. Diagnosis is in practice presumptive, based on luteal progesterone production. The critical level has been reported variously as between 9 and 30 nmol/l [reviewed by Hull, Savage, Bromham, Ismail and Morris, 1982], the wide range being at least partly due to inconsistency between cycles in 'normal' women. There is disagreement about the definition of the very term 'ovulation', which in clinical practice, and indeed most research studies, is based on indirect evidence of ovulation. The best proof of ovulation is subsequent pregnancy, thus using 21 untreated singleton conception cycles, Hull et. al. [1982] found that the critical progesterone level in a single midluteal serum sample was 28 nmol/l, this being the lower 95 % confidence limit. Their lowest observed value was 27 nmol/l. To allow for assay variation they suggested that a value of 30 nmol/l be taken as indicating ovulation. This value is in accord with that established in our own laboratory [Wood, Groom, Moore, Ratcliffe and Selby, 1985].

The importance of quantitative evidence of ovulation is underlined by the fact that all the subjects studied had normal, regular menstrual cycles, yet 5 out of 15 were anovulatory. Thus in studies of platelets in the menstrual cycle where confirmation was not obtained, (e.g. platelet aggregation in the menstrual cycle [Yamazaki, Motomiya, Kikutani, Sakakibara, Watanebe, Namuta and Noguchi, 1979]), the sample group may contain a heterogeneous group of ovulatory and anovulatory women. Unfortunately, a preliminary study of changes in the pressor response to AII during the menstrual cycle also failed to document evidence of ovulation [Broughton Pipkin and O'Brien, personal communication]. Thus although an overall diminution in pressor response was found during the luteal phase, there can be no certainty that this was related to ovulation.

In view of the above finding of reduced platelet AII binding in the luteal phase of the cycle, in all subsequent studies, non-pregnant subjects have been studied between days 5-9 of the menstrual cycle.

Chapter 5.

CROSS-SECTIONAL STUDY.

Platelet AII binding in normotensive and hypertensive pregnancy.

Prior to this study, there was no information concerning platelet angiotensin II (AII) binding in pregnancy. Although less powerful than prospective longitudinal studies, cross-sectional studies are frequently used in pregnancy to provide preliminary data. It was thus decided to perform a 'pilot' cross-sectional study, in order to assess whether a longitudinal study, following a cohort of women through pregnancy, would be justified.

Study sample.

A total of 175 women were studied, with 25 subjects in each group.

a) Normotensive patients.

25 primiparous patients in each trimester were recruited randomly from the antenatal clinic, the exception to this being 18 of the 25 subjects investigated in the first trimester who were studied prior to a termination of pregnancy (under Clause 2, Abortion Act, 1967). 25 patients were studied on the postnatal ward, 24 hours after their first pregnancy. A further 25 patients were recruited randomly from the postnatal clinic, six weeks following their first pregnancy, in 18 cases delivery having been by caesarean section. Data for comparison was obtained from 25 nulliparous volunteers at days 5-9 of the menstrual cycle. No woman was known to be suffering from renal, metabolic or cardiovascular disease. No subject was taking any medication except iron supplementation. The pregnancies of all pregnant/postnatal patients remained normotensive throughout, with no patient having any proteinuria

detectable on routine 'dipstick' testing. All subjects were receiving sodium intake ad libitum and had normal serum urea, creatinine and electrolyte estimations. The median ages, gestational ages, subject weights and both systolic and diastolic blood pressures are summarised in Table 5.1.

The median gestational ages of the trimester samples were 10.0, 18.0 and 33.0 weeks respectively. In all cases the gestational ages were confirmed by an ultrasound scan performed at approximately 18 weeks gestation. With the exception of maternal age in the first trimester group, which was significantly lower than that of any other group ($P<0.01$), there were no significant differences in maternal age ($P>0.1$), systolic blood pressure ($P>0.1$), or diastolic blood pressure ($P>0.05$), between any of the 'normotensive' groups (Mann-Whitney U test). Maternal weight increased in pregnancy, the difference from the non-pregnant control group reaching a significant level by the third trimester ($P<0.001$). Maternal weight was also significantly greater in the group studied six weeks post delivery as compared to the non-pregnant control group ($P<0.01$).

b) Hypertensive patients.

25 primiparous patients in the third trimester of pregnancies complicated by pregnancy-induced hypertension (PIH), as defined by Davey and MacGillivray [1987] (discussed further in the introduction, section 1, p. 9), were also studied. These patients were subdivided in 11 patients with non-proteinuric PIH and 14 with proteinuric PIH or pre-eclampsia, with respect to the presence or absence of 'significant proteinuria' (>0.3g/24 hour urine collection), illustrated in figure 5.1. All samples

Table 5.1.

Demographic details of the normotensive pregnant and non-pregnant patients.

Median values are shown, with the interquartile range in brackets.

Table 5.1

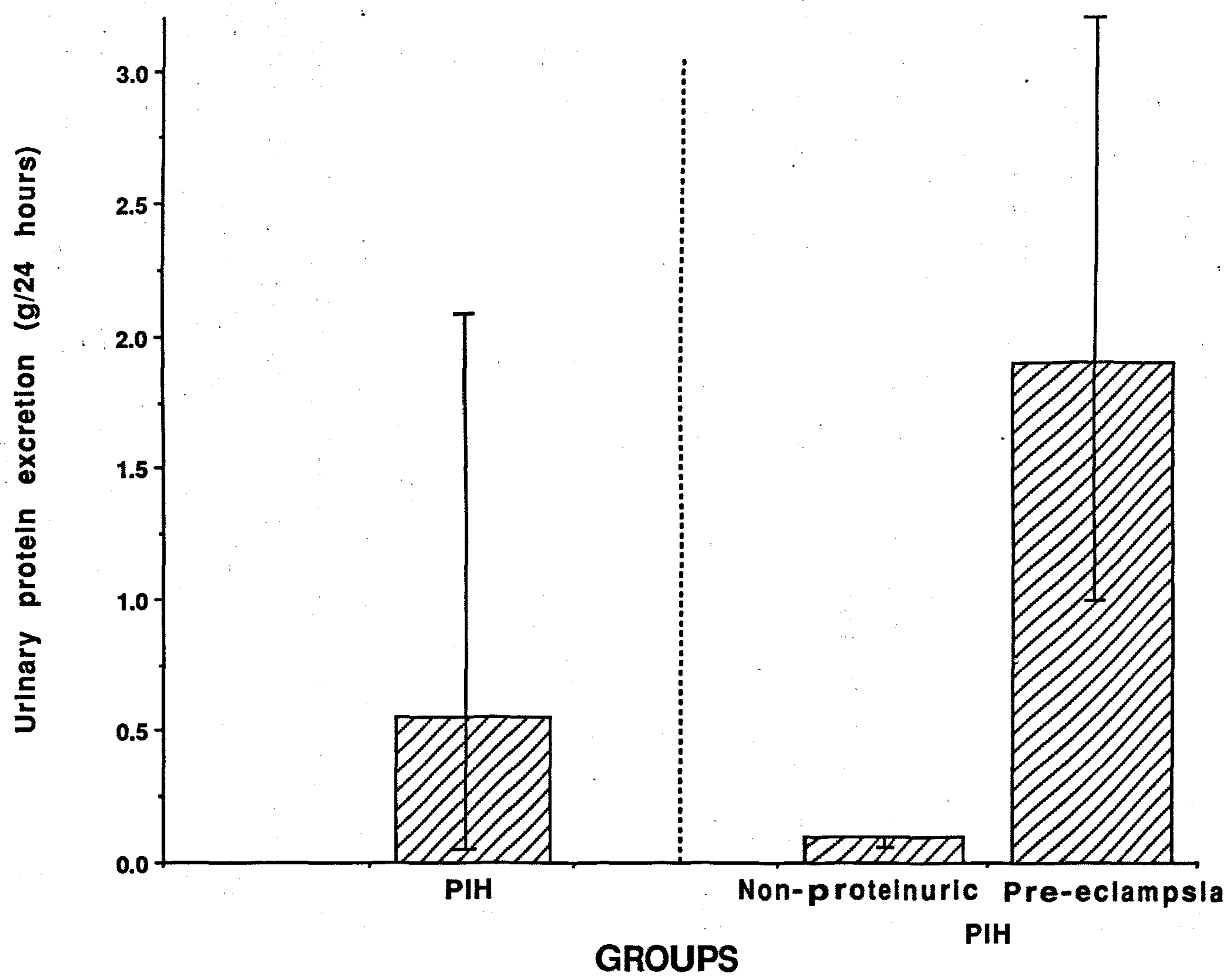
	Non-pregnant	1st trimester	2nd trimester	3rd trimester	24hrs PN	6/52 PN
Gestation (weeks)	* *	10.0 (9.5-12.0)	18.0 (16.0-21.0)	33.0 (29.5-38.0)	* *	* *
Age (years)	26 (24-29)	20 (18-23)	25 (21-28)	25 (21-28)	25 (21-28)	25 (22-27)
Weight (Kg)	59.8 (57.6-66.1)	60.6 (56.3-65.0)	67.5 (60.0-70.6)	73.0 (66.0-81.0)	68.7 (60.5-71.7)	69.2 (62.5-77.5)
Systolic blood pressure (mmHg.)	110 (104-123)	110 (110-120)	115 (110-120)	110 (110-123)	120 (110-120)	120 (110-120)
Diastolic blood pressure (mmHg.)	70 (70-75)	70 (60-70)	70 (70-70)	70 (65-75)	70 (70-80)	70 (70-80)

Figure 5.1.

Quantitative urinary protein content in the hypertensive primigravid patients.

Median values are shown, with the interquartile range marked.
The PIH group have been subdivided into non-proteinuric PIH patients (n=11) and patients with pre-eclampsia (n=14) on the basis of significant proteinuria (>0.3g / 24hours). The overall data from the PIH group and the subdivided data are shown.

Figure 5.1.


Urinary protein excretion (g/24 hours)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PIH
Non-proteinuric PIH
Pre-eclampsia
GROUPS

were taken prior to any antihypertensive medication being commenced. No woman was known to be suffering from renal, metabolic or cardiovascular disease. The subjects with PIH were followed up in the postnatal period and all patients were normotensive with no proteinuria at six weeks post delivery.

The maternal ages, gestational ages, maternal weights, systolic and diastolic blood pressures, gestational ages at delivery and infant birthweights of this group are summarised in Table 5.2, gestational age again being confirmed by ultrasound scan. Both the systolic and diastolic blood pressures of the PIH group were by definition elevated as compared to those of the normotensive third trimester patients (Figure 5.2), the differences being significant ($P<0.001$, Mann-Whitney U test). There were no significant differences in blood pressure between those patients with non-proteinuric PIH and those with pre-eclampsia ($P>0.5$). There were no significant differences between any of the groups when comparisons of gestational age ($P>0.1$), maternal age ($P>0.1$) and maternal weight ($P>0.05$) were made. Gestational age at delivery was significantly earlier in the PIH group than in the normotensive third trimester patients ($P<0.001$), no significant difference being found between the non-proteinuric PIH patients and the patients with pre-eclampsia ($P<0.1$). Differences in infant birthweight between the normotensive and the PIH third trimester groups approached significance ($P=0.012$), the difference being significant when the pre-eclamptic group were compared with both the non-proteinuric patients ($P<0.01$) and the normotensive group ($P<0.01$).

Table 5.2.

Demographic details of the normotensive and hypertensive third trimester patients.

Median values are shown, with the interquartile range in brackets.

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Table 5.2

	3rd trimester n=25	PIH n=25	Non-proteinuric PIH n=11	Pre-eclampsia n=14
Gestation (weeks)	33.0 (29-38)	36.0 (33-38)	38.0 (36-38)	35.0 (32-38)
Age (years)	25 (21-28)	24 (19-27)	23 (18-27)	24 (20-28)
Weight (Kg)	73.0 (66.0-81.0)	79.5 (71.8-86.7)	83.5 (66.8-95.8)	79.3 (72.7-86.0)
Systolic blood pressure (mmHg.)	110 (110-123)	145 (140-150)	145 (140-150)	145 (143-155)
Diastolic blood pressure (mmHg.)	70 (65-75)	95 (95-100)	97 (95-100)	95 (95-100)
Gestation at delivery (weeks)	40.0 (39.5-40.5)	38.0 (36.8-38.0)	38.0 (37.0-39.0)	37.0 (35.0-38.5)
Birthweight (Kg)	3.30 (2.99-3.53)	2.94 (2.31-3.31)	3.10 (2.54-3.34)	2.63 (2.10-3.51)

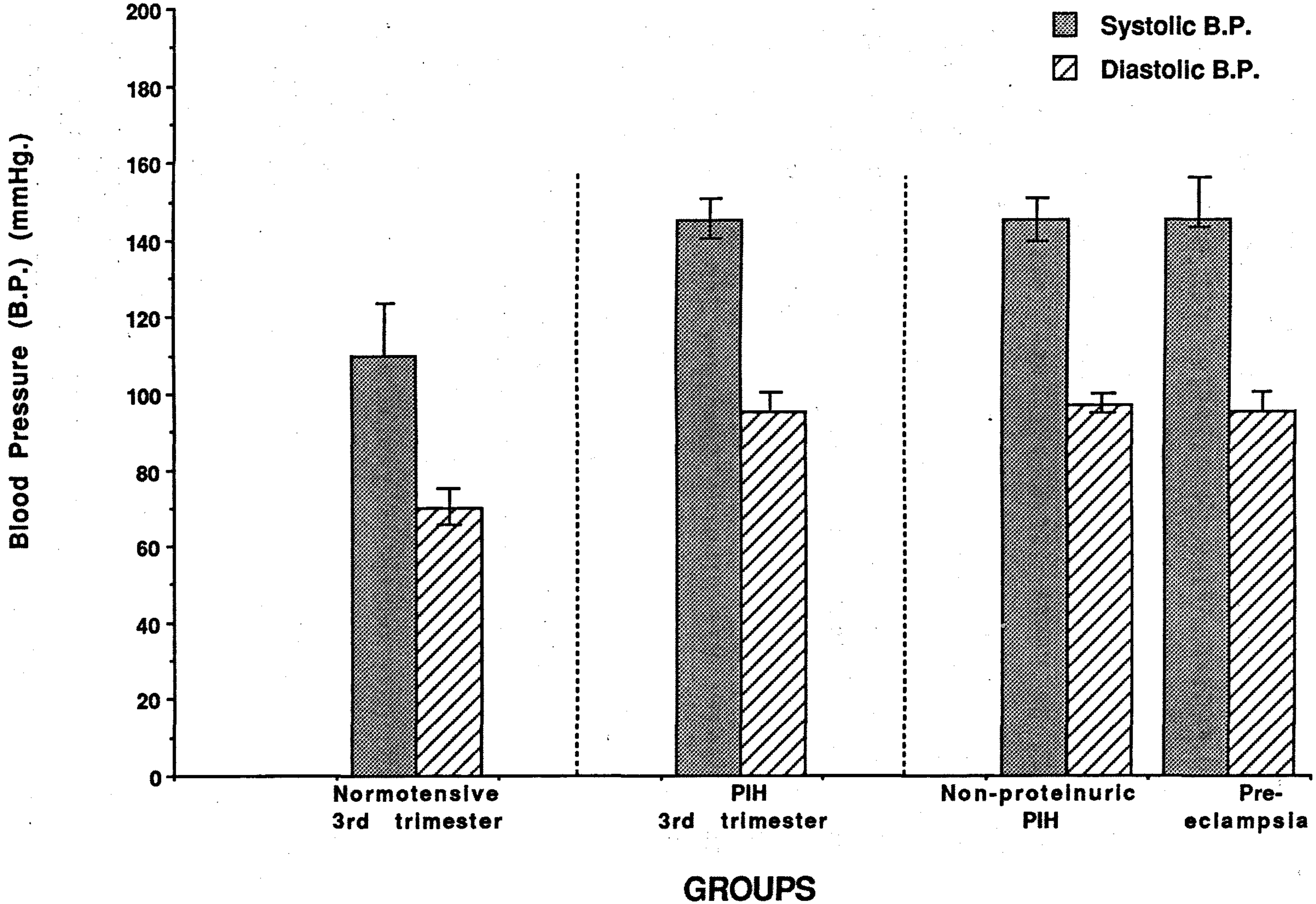
Figure 5.2.

Diastolic and systolic blood pressures of the normotensive and hypertensive third trimester pregnant patients.

Median values are shown, with the interquartile range marked.

The PIH group have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 5.2.


Systolic B.P.
Diastolic B.P.
Blood Pressure (B.P.) (mmHg.)
200
180
160
140
120
100
80
60
40
20
0
Normotensive 3rd trimester
PIH 3rd trimester
Non-proteinuric PIH
Pre-eclampsia
GROUPS

Platelet Angiotensin II (AII) binding data.

a) Normotensive patients.

The median value of platelet AII binding in the non-pregnant female volunteer group was 9.0 fmol/10^9 cells, these data not being normally distributed and with marked individual variability being found (see Figure 5.3).

Kruskal-Wallis analysis of variance indicated a significant effect of pregnancy on platelet AII binding ($P<0.0001$). There was significantly lower platelet AII binding in the first trimester group (2.7 fmol/10^9 cells, $P<0.001$, Mann-Whitney U test) as compared to the non-pregnant control group, with an apparent reduction in variability. In four women no platelet AII binding could be detected at this time, an observation not made in the non-pregnant control group. As illustrated in Figure 5.3 the median value of platelet AII binding remained low in the groups of second trimester women (3.3 fmol/10^9 cells), third trimester women (2.3 fmol/10^9 cells), and in those studied 24 hours after delivery (1.3 fmol/10^9 cells), with no significant differences between any of these groups ($P>0.1$). There was significantly higher binding in the women studied six weeks after delivery (9.0 fmol/10^9 cells, $P<0.01$) as compared to those studied 24 hours after delivery, with values similar to those in the non-pregnant control group.

b) Hypertensive patients.

The median value of platelet AII binding in the 25 PIH patients was 6.0 fmol/10^9 cells (Figure 5.4). Median platelet AII binding thus fell by 33% in the PIH patients by comparison with the non-pregnant control group, whereas in the normotensive third

Figure 5.3

Figure 5.3

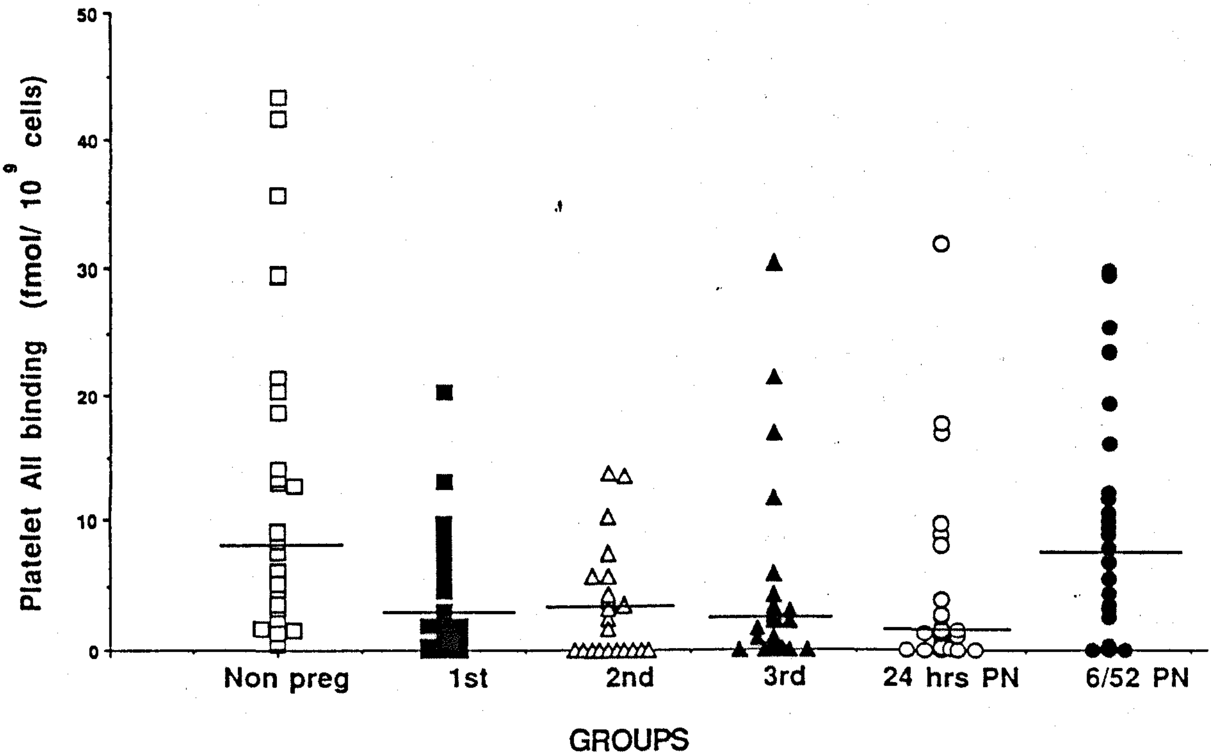
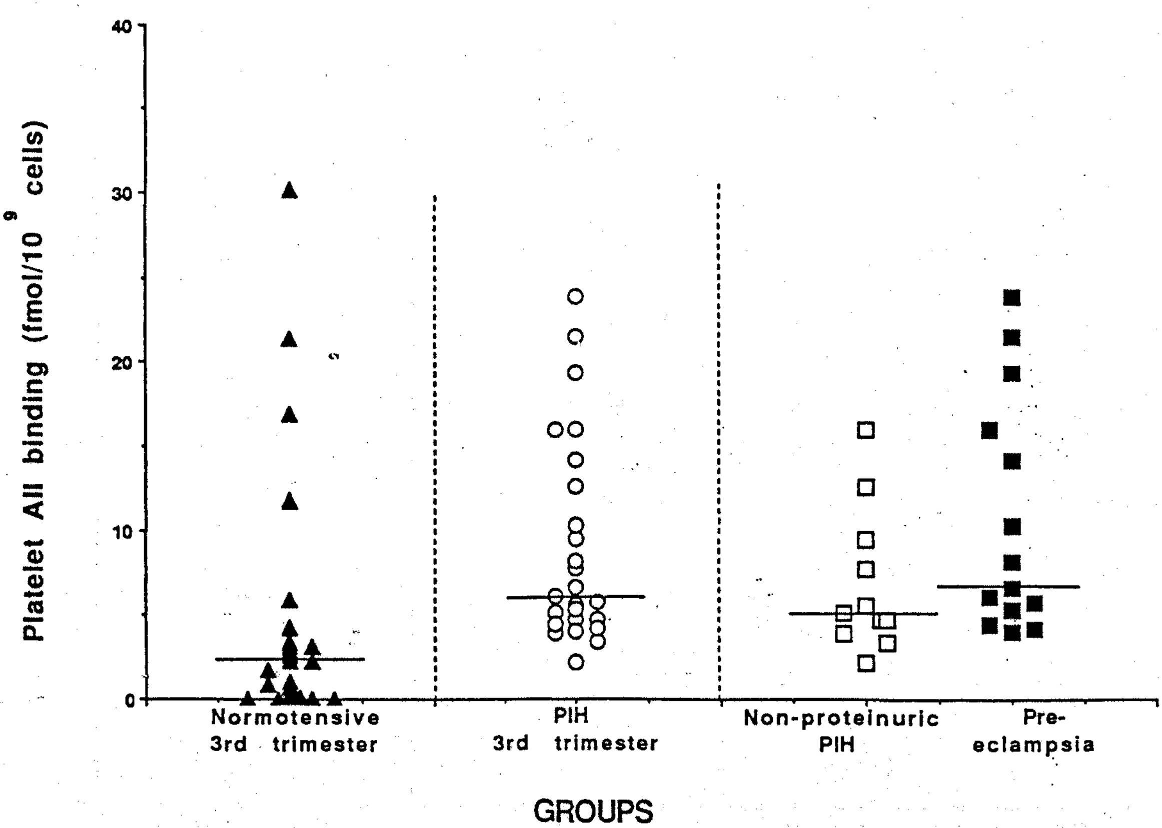


Figure 5.4.



trimester group, median platelet AII binding fell by 74%. Binding was significantly higher in the PIH group than in the normotensive third trimester patients ($P<0.001$, Mann-Whitney U test). Platelet AII binding in the 14 patients with pre-eclampsia (6.7 fmol/10^9 cells) was significantly higher than in the normotensive pregnant patients ($P<0.01$) but was not significantly higher than in the 11 patients with non-proteinuric PIH (5.0 fmol/10^9 cells, $P>0.1$). Platelet AII binding was higher in the patients with non-proteinuric PIH as compared to the normotensive pregnant group although the difference did not reach significant levels ($P>0.05$).

<u>Hormonal data</u>

a) Normotensive patients.

The median values of plasma angiotensin II (AII), plasma renin concentration (PRC) and plasma renin substrate (PRS) are summarised in Table 5.3.

Kruskal-Wallis analysis of variance indicated a significant effect of pregnancy on all three hormone estimations, with $P<0.01$ in each case.

The median plasma AII concentration was 6.7 pg/ml in the non-pregnant group (Figure 5.5). Higher AII levels were found in the first trimester group, and the difference became significant by the second trimester ($P<0.01$, Mann-Whitney U test). Levels of the peptide continued to rise with pregnancy, reaching a maximum in the third trimester (Figure 5.5). There was a reduction in AII levels in the samples taken 24 hours after delivery, with the levels at six weeks post-delivery being significantly lower than those of

Table 5.3

Hormonal data from the normotensive pregnant and non-pregnant patients.

Median values are shown, with the interquartile range in brackets.

Table 5.3

	Non-pregnant	1st trimester	2nd trimester	3rd trimester	24hrs PN	6/52 PN
Angiotensin II (pg/ml)	6.7 (3.4-12.0)	11.0 (4.5-30.0)	14.0 (10.0-21.0)	19.3 (12.7-31.6)	14.1 (5.9-31.8)	7.0 (3.5-34.1)
P.R.C. (ng AI/hr/ml)	0.9 (0.7-1.6)	2.3 (1.3-3.4)	3.2 (2.1-4.7)	2.8 (1.5-3.8)	1.9 (1.2-3.5)	1.0 (0.6-1.4)
P.R.S. (ug AI/ml)	1.2 (1.1-1.6)	1.3 (1.1-2.0)	1.9 (1.2-2.6)	2.5 (2.1-4.2)	2.0 (1.2-2.5)	1.3 (1.0-1.7)

the third trimester group ($P<0.01$) and approximating to those of the non-pregnant control group.

Increased values of PRC and PRS were also found in pregnancy (Table 5.3, Figure 5.5). The values of PRC were significantly higher in the first trimester group as compared with the non-pregnant control group ($P<0.001$, Mann-Whitney U test). There were no significant differences between the three trimesters or at 24 hours after delivery ($P>0.05$). However, the values found six weeks postnatally were significantly lower than those 24 hours after delivery ($P<0.01$). The rise in PRS became significant by the second trimester ($P<0.01$). Significant changes in PRS were found between the third trimester and 24 hours after delivery ($P<0.01$), and between 24 hours and six weeks after delivery ($P<0.01$). Values of both PRC and PRS at six weeks after delivery approximated to those of the non-pregnant control group.

b) Hypertensive patients.

The median values of AII, PRC and PRS are summarised in Table 5.4.

The values of plasma AII in the 25 PIH patients were significantly higher than those of the non-pregnant control group ($P<0.01$ Mann-Whitney U test), but did not differ significantly from the normotensive third trimester patients ($P>0.1$) (Figure 5.6). Plasma AII levels were higher in the 11 patients with non-proteinuric PIH than in the 14 patients with pre-eclampsia, although the difference was not significant ($P>0.1$).

Similar patterns were demonstrated when levels of PRC and PRS were considered (Figure 5.6). There were

Figure 5.5.

Plasma angiotensin II (AII), plasma renin substrate (PRS), and plasma renin concentration (PRC) in the normotensive pregnant and non-pregnant patients.

Median values are shown, with the interquartile range marked.
The concentrations of all three hormones were significantly elevated in pregnancy.

Figure 5.6.

Plasma angiotensin II (AII), plasma renin substrate (PRS), and plasma renin concentration (PRC) in the normotensive and hypertensive third trimester pregnant patients.

Median values are shown, with the interquartile range marked.
The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 5.5.

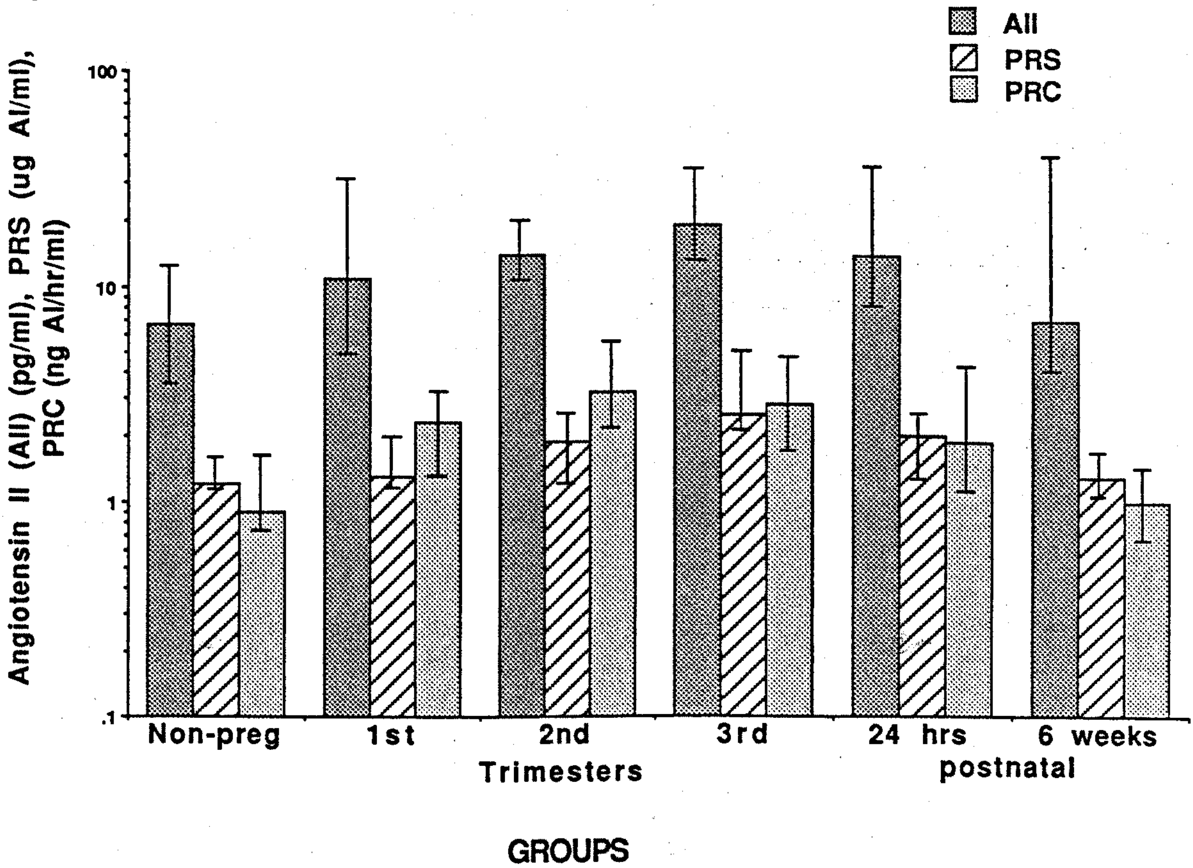


Figure 5.6.

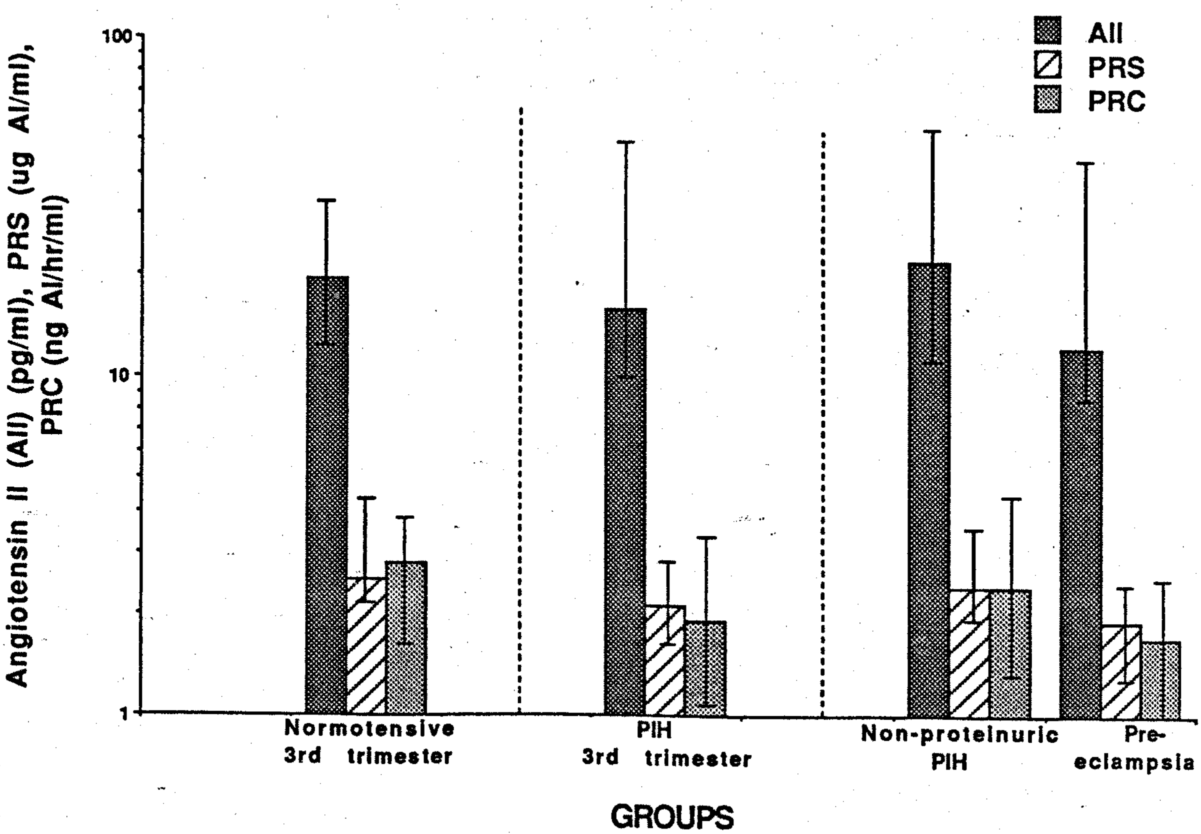


Table 5.4.

Hormonal data from the normotensive and hypertensive third trimester patients.

Median values are shown, with the interquartile range in brackets.

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Table 5.4

	3rd trimester n=25	PIH n=25	Non-proteinuric PIH n=11	Pre-eclampsia n=14
Angiotensin II (pg/ml)	19.3 (12.7-31.6)	15.6 (9.8-48.2)	21.6 (11.9-51.2)	12.1 (8.7-41.8)
P.R.C. (ng AI/hr/ml)	2.8 (1.5-3.8)	1.9 (1.1-3.2)	2.4 (1.3-4.3)	1.7 (0.9-2.5)
P.R.S. (ug AI/ml)	2.5 (2.1-4.2)	2.1 (1.6-2.8)	2.4 (1.9-3.5)	1.9 (1.3-2.4)

significantly higher levels of both hormones in the 25 PIH patients as compared to the non-pregnant control group ($P<0.01$). There were no significant differences between any of the pregnant groups, although when the values of PRS were considered, the differences between the PIH group and the normotensive third trimester group ($P=0.03$) and between those patients with pre-eclampsia and those with non-proteinuric PIH ($P=0.04$) approached significance.

<u>Correlation between platelet AII binding and hormone concentrations.</u>

In the non-pregnant control group there were inverse correlations between platelet AII binding and both plasma AII (Spearman correlation coefficient r_s = -0.48, $P=0.015$) and PRS (r_s = -0.44, $P=0.027$) which approached significance. The relationship between platelet AII binding and plasma AII is illustrated in Figure 5.7.

In none of the groups of pregnant and postnatal patients, was any significant correlation between platelet AII binding and either AII or PRS found. Spearman correlation coefficients for AII ranged from -0.31 to +0.22 and those for PRS ranged from -0.11 to +0.20. Even when platelet AII binding was considered in relation to plasma AII in the patients at six weeks <u>post partum</u>, the correlation coefficient still did not approach significant levels ($P>0.1$).

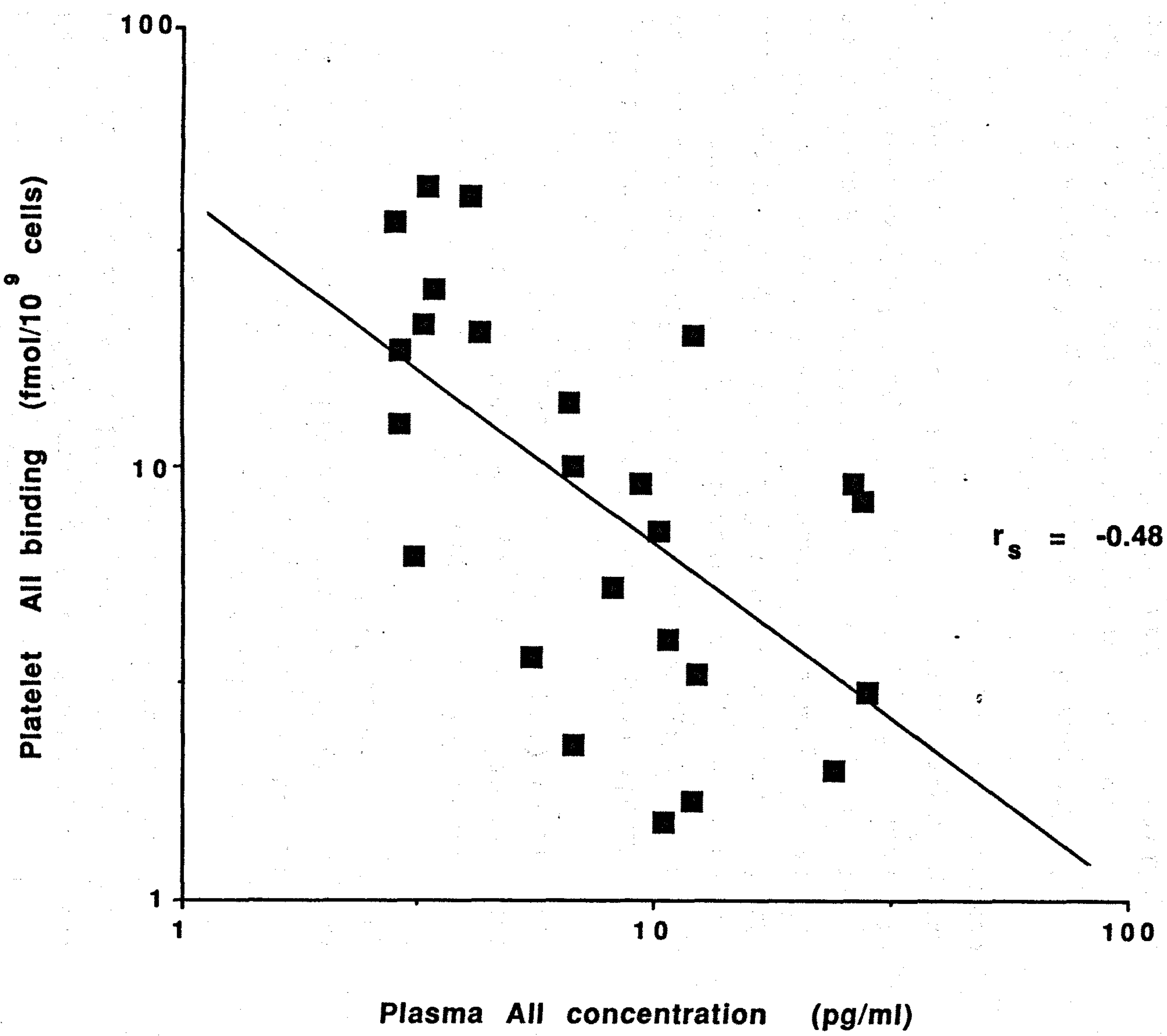
No correlation was ever demonstrated between platelet AII binding and PRC ($P>0.1$).

Figure 5.7.

Correlation between platelet AII binding and plasma AII concentration in the non-pregnant control group.

Regression characteristics: log (platelet AII binding) = 1.14 - 0.31 (plasma AII concentration).

Figure 5.7.



Platelet All binding (fmol/10^9 cells)
100
10
1
1
10
100
r_s = -0.48
Plasma All concentration (pg/ml)

Haematological data.

There were no significant differences in recovery of platelets from whole blood in any pregnant/postnatal group (47%-55%) when compared with the non-pregnant control group (56%). Contamination with leucocytes was <1% (1-5 x $10^3/\mu l$) and no erythrocytes were detected.

a) Normotensive patients.

The median values of platelet count, mean platelet volume (MPV) and platelet distribution width (PDW) are summarised in Table 5.5.

Kruskal-Wallis analysis of variables demonstrated a significant effect of pregnancy on all three parameters (P<0.001 in each case).

A diminution in platelet count occurred during pregnancy (Figure 5.8), with the fall from the values in the non-pregnant control group significant by the third trimester (P<0.01, Mann-Whitney U test). The values of platelet count remained low at 24 hours after delivery, but were significantly increased at six weeks after delivery, the platelet count of this group approximating to that of the non-pregnant control group.

MPV increased in pregnancy, the increase from the values in the non-pregnant control group became significant by the third trimester (P<0.001), and remained high at 24 hours after delivery. Although the values of MPV at six weeks after delivery fell significantly as compared to the third trimester group (P<0.01), these values remained significantly higher than those of the non-pregnant control group (P<0.01).

Table 5.5

	Non-pregnant	1st trimester	2nd trimester	3rd trimester	24hrs PN	6/52 PN
Platelet count (x 10^9/L)	220 (175-274)	198 (180-238)	183 (163-209)	170 (150-194)	167 (156-232)	243 (206-271)
Mean platelet volume (fl)	7.8 (7.3-8.2)	7.8 (7.6-8.1)	7.9 (7.5-8.6)	8.7 (7.9-9.1)	8.4 (7.5-8.7)	8.2 (7.8-8.7)
Platelet distribution width	15.5 (15.0-16.7)	16.2 (15.3-17.0)	15.7 (14.9-16.5)	17.1 (16.4-18.5)	16.4 (15.2-17.5)	16.0 (15.1-16.7)
Yield (%)	56 (48-62)	51 (48-63)	52 (51-58)	52 (49-58)	55 (50-60)	51 (47-54)

Figure 5.8.

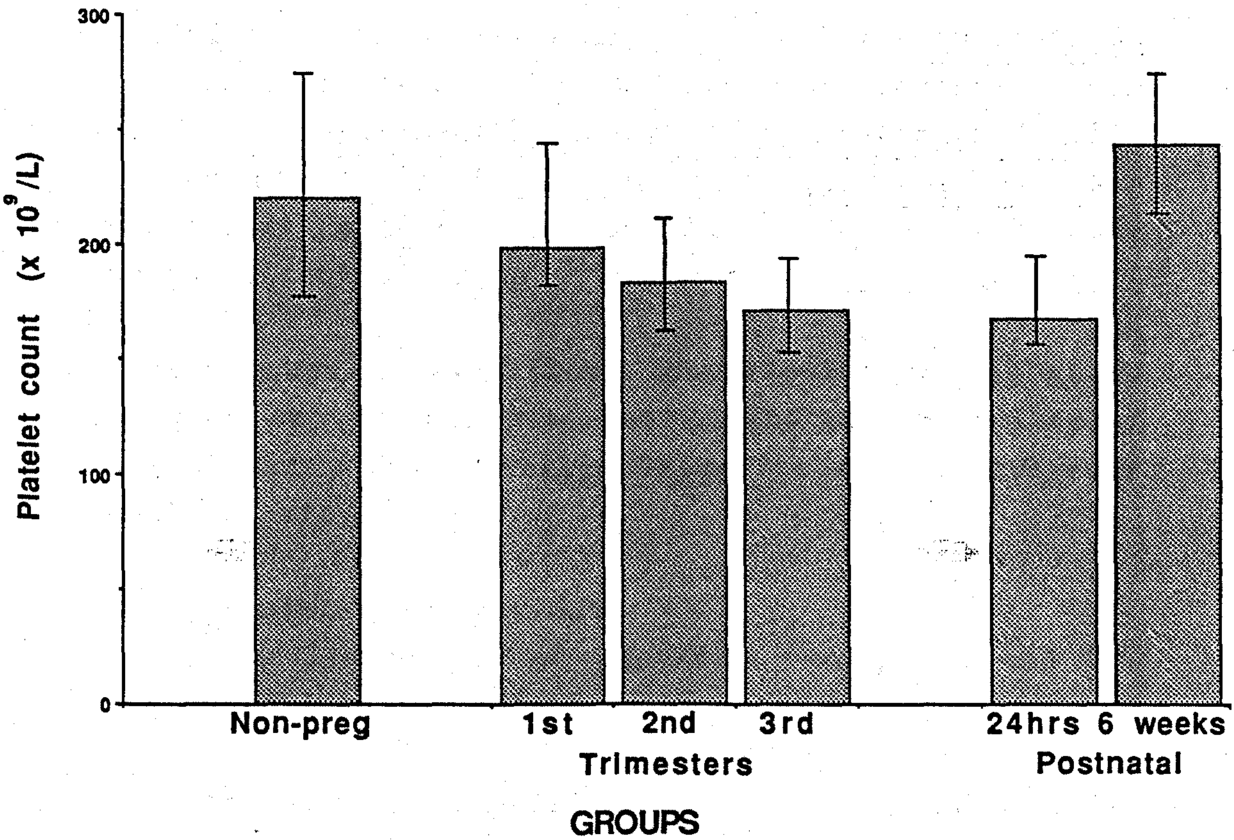
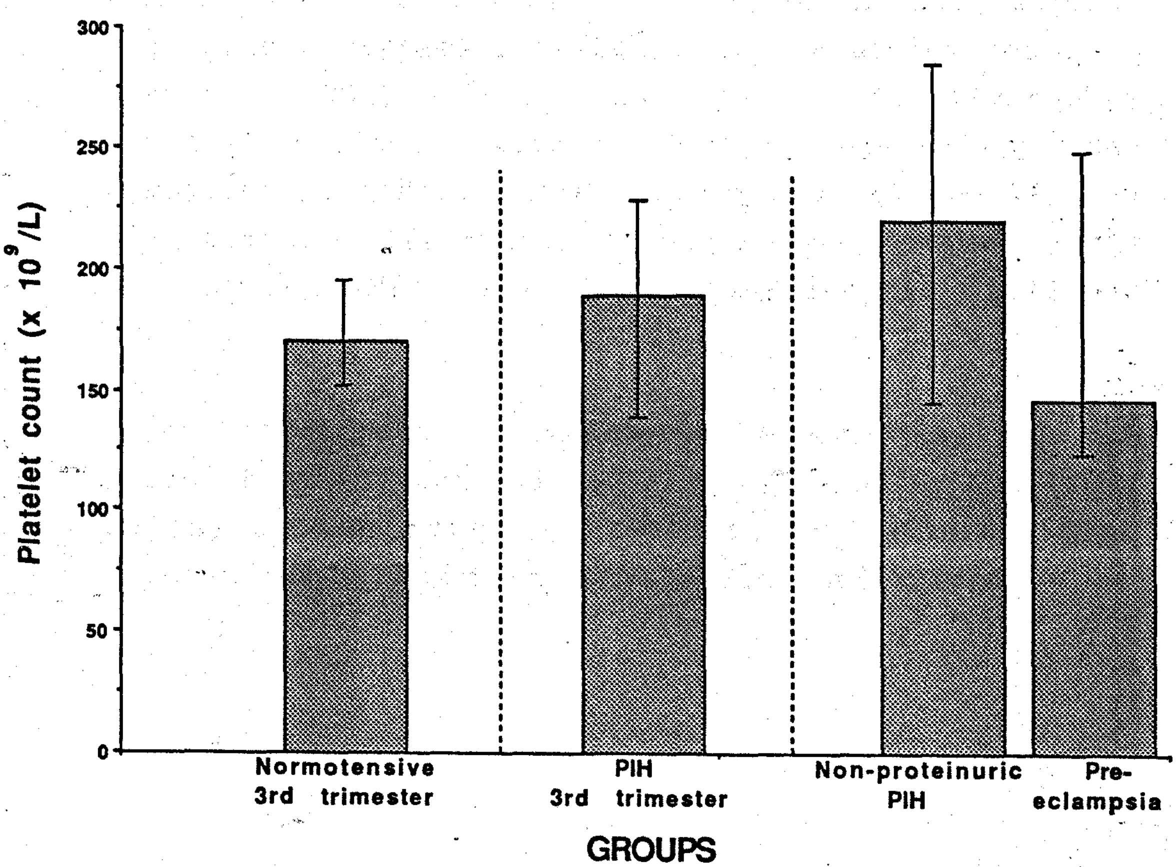


Figure 5.9.



A similar pattern was found when PDW was considered, the increase from the non-pregnant control group becoming significant by the third trimester ($P<0.001$). Values of PDW fell postpartum, with values at six weeks post delivery approximating to those of the non-pregnant control group.

There was no significant correlation in any group between any of the platelet parameters and platelet AII binding, when assessed using Spearman correlation coefficients.

b) Hypertensive patients.

The median values of platelet count, MPV and PDW are summarised in Table 5.6.

There were no significant differences in platelet count (Figure 5.9) and PDW between any of the groups, although the difference in platelet count between the patients with non-proteinuric PIH and those with pre-eclampsia approached significance ($P=0.04$, Mann-Whitney U test). One of the 14 pre-eclampsia patients was thrombocytopaenic (defined as a platelet count of less than $100 \times 10^9/L$). MPV was significantly higher in the patients with PIH as compared to the normotensive third trimester group ($P<0.001$), no significant difference being found between non-proteinuric PIH and the pre-eclampsia patients ($P>0.1$).

No significant correlation between any of the platelet parameters and platelet AII binding was found in any of the groups of hypertensive patients, when assessed using Spearman correlation coefficients.

Table 5.6.

Platelet data from the normotensive and hypertensive third trimester patients.

Median values are shown, with the interquartile range in brackets.
The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Table 5.6

	3rd trimester n=25	PIH n=25	Non-proteinuric PIH n=11	Pre-eclampsia n=14
Platelet count (x 10^9/L)	170 (150-194)	188 (138-226)	219 (145-283)	146 (123-247)
Mean platelet volume (fl)	8.7 (7.9-9.1)	10.1 (9.4-11.3)	10.0 (9.2-11.1)	10.8 (9.4-12.6)
Platelet distribution width	17.1 (16.4-18.5)	17.3 (16.9-18.1)	17.1 (16.8-17.5)	17.6 (17.0-18.3)
Yield (%)	52 (49-58)	47 (37-52)	49 (45-55)	47 (36-52)

Table 5.7.

Biochemical data from the normotensive pregnant and non-pregnant patients.

Median values are shown, with the interquartile range in brackets.

Table 5.7

	Non-pregnant	1st trimester	2nd trimester	3rd trimester	24hrs PN	6/52 PN
Sodium (mmol/L)	140 (138-141)	137 (136-138)	137 (136-138)	137 (136-139)	138 (138-139)	140 (139-142)
Potassium (mmol/L)	4.2 (4.0-4.5)	4.2 (4.0-4.2)	4.0 (3.7-4.2)	4.1 (3.9-4.3)	4.2 (4.0-4.4)	4.3 (4.1-4.5)
Urea (mmo/L)	4.1 (3.6-4.8)	3.0 (2.3-3.7)	3.0 (2.5-3.4)	3.0 (2.2-3.7)	3.4 (3.0-4.0)	5.4 (4.1-5.9)
Creatinine (umol/L)	87 (83-92)	66 (63-72)	71 (63-83)	71 (68-77)	72 (65-84)	86 (79-93)
Urate (umol/L)	202 (180-229)	159 (140-178)	193 (149-204)	212 (171-245)	244 (230-315)	280 (249-321)
Osmolality (Osm/L)	282 (277-285)	273 (270-275)	273 (270-276)	275 (274-279)	280 (276-282)	285 (283-287)

Biochemical data.

a) Normotensive patients.

The median values of serum sodium, potassium, urea, creatinine, urate and osmolality are summarised in Table 5.7.

Kruskal-Wallis analysis of variance demonstrated a significant effect of pregnancy on the values of serum sodium, urea, creatinine, urate and osmolality ($P<0.001$ in each case).

When the first trimester group were compared with the non-pregnant control group, significantly lower levels of serum sodium, urea, creatinine, urate and osmolality were found ($P<0.001$ in each case, Mann-Whitney U test). No further significant changes in any of these parameters occurred during pregnancy. Values of serum sodium, urate and osmolality at 24 hours after delivery were significantly higher than those of the third trimester group ($P<0.01$ in each case). Significant increases in serum concentrations of sodium ($P<0.01$), urea ($P<0.001$), creatinine ($P<0.001$) and osmolality ($P<0.001$) were found when the samples obtained six weeks after delivery were compared with the 24 hours after delivery group. There were significant differences between the non-pregnant control group and the women studied six weeks after delivery when the values of serum urea ($P<0.01$) and urate ($P<0.001$) were compared. Potassium concentrations were not significantly different across the sample groups studied.

There was no correlation in any group between any of these biochemical parameters and platelet AII binding when assessed using Spearman correlation coefficients.

b) Hypertensive patients.

The median values of serum sodium, potassium, urea, creatinine, urate and osmolality are summarised in Table 5.8.

There were no significant differences in the concentrations of serum sodium, potassium, urea and osmolality between any of the groups, although in comparisons of concentrations of urea, the difference between the non-proteinuric PIH patients and those with pre-eclampsia approached significance (P=0.02, Mann-Whitney U test). Significantly higher values of serum creatinine were found in the pre-eclampsia subgroup as compared to those patients with non-proteinuric PIH (P<0.01). The values of serum urate were significantly higher in the patients with PIH as compared to the normotensive third trimester group (P<0.0001), there being no significant difference between the non-proteinuric and the pre-eclamptic subgroups (Figure 5.10).

Again, no correlation was found between any of the biochemical parameters and platelet AII binding (Spearman correlation coefficients).

Discriminant analysis.

Discriminant analysis is a statistical test in which linear combinations of variables are used to distinguish between two or more categories of cases. The variables 'discriminate' between groups of cases and predict into which category or group a case falls, based on the values of the variables [SPSS-X, 1988].

Table 5.8.

Biochemical data from the normotensive and hypertensive third trimester patients.

Median values are shown, with the interquartile range in brackets.

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Table 5.8

	3rd trimester n=25	PIH n=25	Non-proteinuric PIH n=11	Pre-eclampsia n=14
Sodium (mmol/L)	137 (136-139)	137 (135-138)	137 (135-138)	138 (134-138)
Potassium (mmol/L)	4.1 (3.9-4.3)	4.1 (3.9-4.3)	4.0 (3.9-4.3)	4.1 (4.0-4.4)
Urea (mmo/L)	3.0 (2.2-3.7)	3.7 (2.8-4.3)	2.8 (2.4-4.1)	3.9 (3.5-4.6)
Creatinine (umol/L)	71 (68-77)	79 (73-89)	75 (61-80)	85 (76-104)
Urate (umol/L)	212 (171-245)	318 (260-412)	317 (239-408)	339 (277-422)
Osmolality (Osm/L)	275 (274-279)	278 (274-280)	276 (274-280)	278 (274-280)
Urinary protein (g/24hrs)	* *	0.55 (0.10-2.20)	0.10 (0.10-0.20)	1.90 (1.05-3.20)

Figure 5.10.

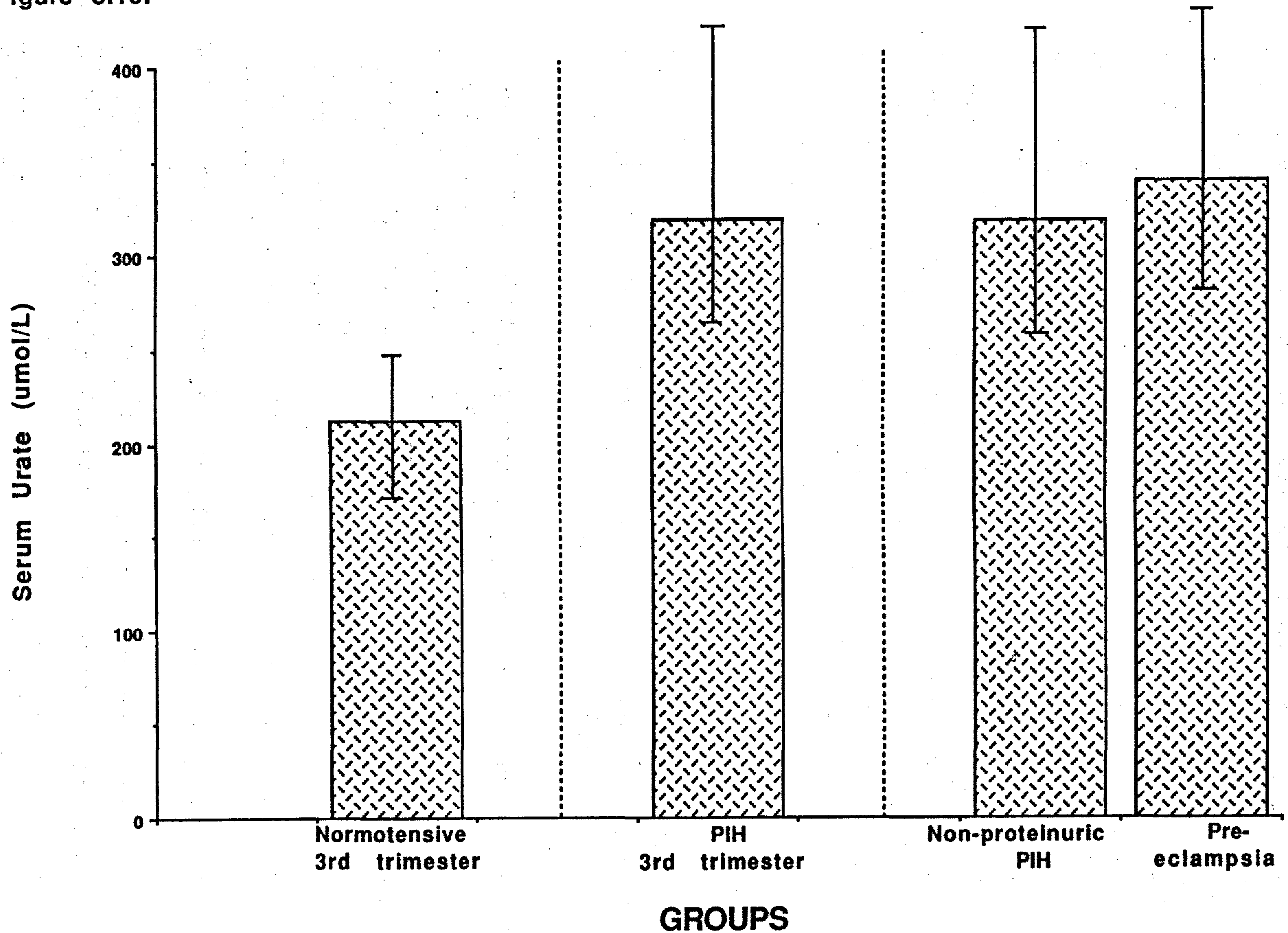
Serum urate concentrations in the normotensive and hypertensive third trimester pregnant patients.

Median values are shown, with the interquartile range marked.

Serum urate concentrations were significantly higher in the PIH patients as compared to the normotensive women ($P<0.001$).

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 5.10.



Serum Urate (umol/L)
400
300
200
100
0
Normotensive
3rd trimester
PIH
3rd trimester
Non-proteinuric
PIH
Pre-
eclampsia
GROUPS

The use of platelet AII binding to discriminate between normotensive and hypertensive third trimester patients was thus assessed using canonical discriminant analysis. There was a false positive rate of 42% and a false negative rate of 18%, 70% of patients being correctly classified. When the hormonal, platelet, and biochemical parameters measured in this study were used to discriminate between the two groups of third trimester patients, the only parameters which correctly classified more patients were serum urate and MPV (correct classification in 80% of patients in both cases). The results of such discriminant analysis were not improved when MPV and platelet AII binding were combined (74% of patients correctly classified), but when platelet AII binding and serum urate were combined there was a false positive rate of 24% and a false negative rate of 10%, 83% of patients being correctly classified.

Discussion.

The cross-sectional study thus provided evidence of a reduction in platelet AII binding in normotensive pregnancy, the change being apparent by the first trimester. It was therefore decided to perform a prospective longitudinal study (discussed in the next chapter) in order to determine whether these findings would be confirmed when a cohort of women were followed through pregnancy. Pregnancy-induced changes in platelet AII binding, and in the hormonal, platelet and biochemical parameters will also be studied in the longitudinal study. To avoid unnecessary repetition, a discussion of these changes, found in the cross-sectional study and in the longitudinal study, is included at the end of the next chapter. This discussion will only address the problems specific to the cross-sectional study.

On analysing the data from the cross-sectional study, certain deficiencies were evident. One of these was the inclusion in the first trimester group, of women attending for termination of pregnancy. At the hospital in which the women were studied, (University Hospital, Nottingham), the first antenatal clinic visit for the majority of women is at eighteen weeks gestation. It was thus logistically easier to recruit women attending for termination of pregnancy. However, the demographic differences between the first trimester patients and both the non-pregnant control group and the other pregnant/postnatal groups, which resulted from their inclusion, provide an important caveat in making other comparisons between the groups. This problem will be resolved by the longitudinal study.

The patients in the six weeks postnatal group were recruited randomly from the hospital postnatal clinic. However, the majority of six week postnatal attendances are at the General Practitioner's surgery, hospital postnatal attendances being restricted to pregnancies complicated antenatally or at delivery. This accounted for the high incidence of delivery by caesarean section in the six weeks postnatal group. It must be reiterated that none of the pregnancies were complicated by either PIH or intrauterine growth retardation, the majority of the caesarean sections being for failure to progress in the first stage of labour or for fetal distress. However, because of the large proportion of operative deliveries in the sample, these individuals may not be representative of the puerperal population as a whole. Again, the longitudinal study will address this problem.

It may be three months <u>post partum</u> before the changes consequent upon the renal alterations of normal pregnancy resolve [Davison and Lindheimer, 1984]. This was reflected in the significant differences in serum urea and serum urate found between the non-pregnant control group and the subjects studied six weeks after delivery. The time period of six weeks after delivery may not have been long enough to re-establish the inverse correlation between plasma AII concentration and platelet AII binding. In the longitudinal study patients will thus be followed until three months after delivery.

In the cross-sectional study the absence of proteinuria in the non-pregnant and the normotensive pregnant groups was assessed using urinary dipsticks, a method deemed by some [e.g. Davey, 1986] to be inaccurate. In order to obtain a quantitative measurement of urinary protein, and also to further investigate the relationship between platelet AII binding and both electrolyte balance and renal function (no correlation having been found between platelet AII binding and any of the serum biochemical parameters measured), the longitudinal study will incorporate 24 hour urine collections from every subject on each occasion platelet AII binding is measured.

In addition to a longitudinal study, with the diminution in platelet AII binding in the first trimester indicating that a study of early pregnancy would be particularly relevant, the results of the cross-sectional study suggest other lines of investigation. One of these is a larger comparison of normotensive and hypertensive third trimester patients, there being a significant difference in platelet AII binding between the two groups in the cross-sectional study, despite the small sample size. Moreover, if such a study included follow up of both groups in the puerperium it would determine whether any such differences resolve after delivery. Further study of platelet AII binding in women with hypertensive diseases of pregnancy is included in Chapter 7.

The use of canonical discriminant analysis to discriminate between normotensive primigravidae and patients with established PIH indicates that the potential use of platelet AII binding as a marker for PIH is limited, with both both MPV and serum urate estimations proving better at discriminating between the two groups of third trimester women. The potential use of the technique as a screening test for PIH may however be greater, and this possibility is discussed further in Chapters 7 and 8.

Chapter 6.

LONGITUDINAL STUDY.

Platelet AII binding in normotensive pregnancy.

Chapter 5 describes a preliminary study investigating the changes in platelet angiotensin II (AII) binding in normotensive pregnancy. Although useful information was obtained, cross-sectional studies such as this are inherently weak, as they investigate different individuals at differing time points. A formal prospective study investigating the changes in platelet AII binding in normotensive pregnancy was therefore performed.

<u>Statistical analysis.</u>

The statistical analysis used in this study differs from that used in the other studies included in the thesis, in that the nature of a longitudinal study enables more powerful statistical tests to be used.

In assessing the overall effect of pregnancy on any given parameter, the Friedman non-parametric analysis of variance has been used. This technique calculates the mean rank for each parameter over all the time points, (in this study: gestational ages), for each case [SPSS-X, 1988], instead of assessing the data as being from different individuals at different time points (as in the Kruskal-Wallis analysis of variance). When values of any parameter at any two gestations are compared, the data can be regarded as paired, thus the Wilcoxon matched-pairs signed-ranks test has been used. With the exception of the early pregnancy subgroup, the subjects in the non-pregnant control group differ from those in the other groups, thus in comparisons including this group, the Mann-Whitney U test has been used.

In determining whether there was a significant correlation between platelet AII binding and any of the parameters measured in pregnancy (for example the serum AII concentration), stepwise regression analysis has been used. This necessitated the use of parametric statistics, in accord with statistical advice received, there being no non-parametric equivalent technique. Using this method, the effect of gestation, which may exert an effect on both parameters and thus produce a falsely positive correlation between them (the Durbin-Watson effect), can be excluded [SPSS-X, 1988].

Regression analysis is a highly sophisticated technique. In an effort to ensure that no trends between platelet AII binding and any of the measured parameters were missed, preliminary analysis of the data was performed as follows. The subjects were divided into two groups on the basis of levels of platelet AII binding above or below the median for each gestation. At each gestation, the two groups were compared using the Mann-Whitney U test, in order to determine whether significant differences in any of the parameters measured existed between subjects with 'low binding' and those with 'high binding'. However, no trends were suggested that were not confirmed using regression analysis, and the results of the preliminary analysis above, will not be referred to in the text.

Study sample.

The cross-sectional study described in the previous chapter highlighted the need to study the changes in platelet AII binding in early pregnancy. At the onset of the study, thirty pre-conceptual, nulliparous, normotensive women were recruited to the study. These subjects were referred by local general practitioners and practice nurses and included women attending for advice regarding pre-conceptual health care, such as checking of rubella immune status. No subject had been taking hormonal contraception for at least six months, or had any recent history of menstrual disorder. All subjects were investigated between days 5-9 of the menstrual cycle, and were advised that after they conceived they would be studied both throughout their pregnancy and in the puerperium. This group thus formed the non-pregnant control group.

Out of the thirty patients recruited, only seven were pregnant one year into my allotted research time. It was thus decided to recruit a further 25 primiparous women, who were investigated from eleven weeks gestation and throughout their pregnancies. The first antenatal clinic visit of patients referred to the hospital used in the study (University Hospital, Nottingham) is usually at eighteen weeks gestation. A random selection was made from patients who were referred by their general practitioners at an early gestation (less than nine weeks). These patients were asked to participate in the study, and to attend for an additional antenatal clinic visit at eleven weeks gestation. Two of these subjects developed hypertension during their pregnancy (one of whom had been studied preconceptually), and were thus excluded from this longitudinal study of normotensive pregnancy (they are discussed in the next chapter). The pregnancy cohort group therefore consisted of thirty patients studied from eleven weeks gestation until twelve weeks post delivery.

None of the women included in this study were known to be suffering from renal, metabolic or cardiovascular disease. No subject was taking any medication except iron supplementation. All the pregnancies remained normotensive throughout, with no patient having significant proteinuria (>0.3g / 24 hour urine collection) at any time. All subjects were receiving sodium intake <u>ad libitum</u> and had normal serum urea, creatinine and electrolyte estimations. The gestational age of each subject was confirmed by an ultrasound scan at eighteen weeks gestation.

The median age of the non-pregnant control group was 27 years (interquartile range 25-28) and of the pregnant cohort group was 25 years (22-27). The difference in age between the two groups approached statistical significance ($P = 0.03$, Mann-Whitney U test). By chance all subjects were Caucasian, although no positive discrimination was practised. One of the thirty patients (3%) in each group was a smoker.

The median maternal weights and systemic arterial blood pressures of the two groups are shown in Table 6.1. There was no significant difference in maternal weight when the non-pregnant control group was compared with the pregnant cohort group at 11 weeks gestation ($P>0.5$, Mann-Whitney U test). Friedman analysis of variance indicated a significant effect of pregnancy ($P<0.001$), with significant increases between 18 and 28 weeks gestation ($P<0.001$) and between 28 and 36 weeks gestation ($P<0.01$) being found (Wilcoxon matched-pairs signed-ranks test). There were significant decreases in maternal weight from 36 weeks gestation to 6 weeks post delivery ($P<0.001$, Wilcoxon matched-pairs signed-ranks test) and from 6

Table 6.1.

Maternal weights and systemic blood pressures of the non-pregnant control group and the pregnant cohort group.

Median values are shown, with the interquartile range in brackets.

Table 6.1

	Non-pregnant control group	GESTATION (weeks) 11	18	28	36	6 weeks PN	12 weeks PN
Weight (Kg)	53.9 (56.8-65.4)	60.8 (55.3-67.7)	62.8 (56.3-69.6	68.8 (61.0-74.3)	71.5 (64.5-78.6)	62.3 (60.0-69.5)	58.5 (55.3-68.3)
Systolic blood pressure (mmHg.)	115 (110-120)	110 (100-110)	110 (100-120)	110 (100-120)	110 (110-120)	120 (110-120)	115 (110-120)
Diastolic blood pressure (mmHg.)	70 (70-75)	70 (60-70)	60 (60-70)	70 (60-70)	70 (61-70)	70 (60-70)	70 (60-70)

to 12 weeks post delivery ($P<0.01$). When the pregnant cohort group were weighed at either 6 or 12 weeks following delivery there were no significant differences as compared to the non-pregnant control group ($P>0.5$, Mann-Whitney U test).

There were no significant differences in systolic blood pressure between either group or between any gestation ($P>0.05$). There was a significantly lower diastolic blood pressure in the pregnant cohort group at 11 weeks gestation as compared to the non-pregnant control group ($P<0.01$). There was no significant effect of pregnancy on diastolic blood pressure ($P>0.1$) and no significant changes in diastolic blood pressure following delivery. However, the diastolic blood pressure of the pregnant cohort group at 12 weeks post partum was significantly lower than that of the non-pregnant control group ($P<0.01$).

No significant correlations were found between platelet AII binding and any of the above parameters in either the non-pregnant control group ($P>0.1$, Spearman correlation coefficient) or the pregnant cohort group ($P>0.1$, stepwise regressional analysis when pregnant, Spearman correlation coefficient following delivery).

The median birthweight in the pregnant cohort group was 3.44 Kg (interquartile range 3.04-3.75) and the median gestational age at delivery was 40 weeks (39-41). None of the birthweights were below the 3rd centile for gestational age. 20 (66%) had a spontaneous vaginal delivery, 5 (17%) were delivered using forceps, and 5 (17%) by caesarean section. Two caesarean sections were for delay in the first stage of labour, one was for a breech presentation with poor pelvimetry, and two were for fetal distress (although in each case the subsequent cord pH was normal). 16 (53%) infants were male, 14 (47%) were female, and none were admitted to the neonatal unit for any reason. 18 (60%) of the infants were breast-fed and 12 (40%) bottle fed. At six weeks following delivery only two (7%) of the women had menstruated, and at twelve weeks only 8 (27%) had menstruated.

Early pregnancy.

In addition to the seven preconceptual patients who conceived in the first year of the study, a further seven preconceptual women subsequently conceived. These women were asked to contact the hospital at as early a gestation as possible. Two of the women suffered a spontaneous abortion. Analysis of the changes in early pregnancy in the remaining twelve women is included in this chapter. One of the twelve patients developed pregnancy induced hypertension (discussed further in the next chapter, p. 198). In view of the small sample size, the ranges of values will be quoted rather than the 25th and 75th centiles. Despite the small sample size, some limited statistical analysis has been performed using the data obtained from this subgroup.

The median age of the group was 27 years (24-33). Median maternal weight increased from 58.9 Kg (47.2-75.8) when non-pregnant to 60.0 Kg (47.0-77.5) at 5-8 weeks gestation, 62.1 Kg (50.1-79.0) at 11 weeks gestation and 65.9 Kg (52.8-84.0) at 18 weeks gestation. Friedman analysis of variance indicated a significant effect of early pregnancy on maternal weight in this subgroup ($P<0.01$), the difference between 11 and 18 weeks gestation being significant ($P<0.01$, Wilcoxon matched-pairs signed-ranks test).

Median systemic blood pressure was 110/70 mmHg (110/60-125/75) when non-pregnant, 110/70 mmHg (110/60-120/70) at 5-8 weeks gestation, 110/70 mmHg (100/60-120/75) at 11 weeks gestation and 105/65 mmHg (95/60-120/70) at 18 weeks gestation. There was no significant effect of early pregnancy of either systolic or diastolic blood pressure on this subgroup ($P>0.05$).

Platelet Angiotensin II (AII) binding data.

The median value of platelet AII binding in the non-pregnant control group was 5.1 fmol/10^9 cells (interquartile range 2.5-12.8), with marked individual variability being found (Figure 6.1). There was significantly lower binding in the pregnant cohort group at 11 weeks gestation (2.1 fmol/10^9 cells, 0.3-4.1, $P<0.01$, Mann-Whitney U test), with an apparent reduction in variability. The effect of pregnancy on platelet AII binding approached significance ($P=0.02$, Friedman analysis of variance) with significantly lower binding at 18 weeks gestation (0.3 fmol/10^9 cells, 0-1.6) than at 11 weeks gestation ($P<0.01$, Wilcoxon matched-pairs signed-ranks test). There were no subsequent significant changes in pregnancy ($P>0.05$), with the median value of platelet AII binding remaining low (0.8 fmol/10^9 cells, 0.3-3.5, at 28 weeks gestation and 0.7 fmol/10^9 cells , 0-2.8, at 36 weeks gestation). There was significantly higher binding at 6 weeks following delivery (3.9 fmol/10^9 cells, 1.6-6.9, $P<0.01$) as compared to the samples taken at 36 weeks gestation, but there was no further significant change at 12 weeks after delivery (3.9 fmol/10^9 cells, 0-6.5, $P>0.1$). There were no significant differences between the values of platelet

Figure 6.1.

Platelet AII binding in the non-pregnant control group and the pregnant cohort group.

The median values of platelet AII binding are indicated by horizontal bars.

Figure 6.2.

Platelet AII binding in the early pregnancy subgroup.

The median values of platelet AII binding are indicated by horizontal bars.
Platelet AII binding fell in all the patients except one, (who subsequently developed pregnancy-induced hypertension.

Figure 6.1.

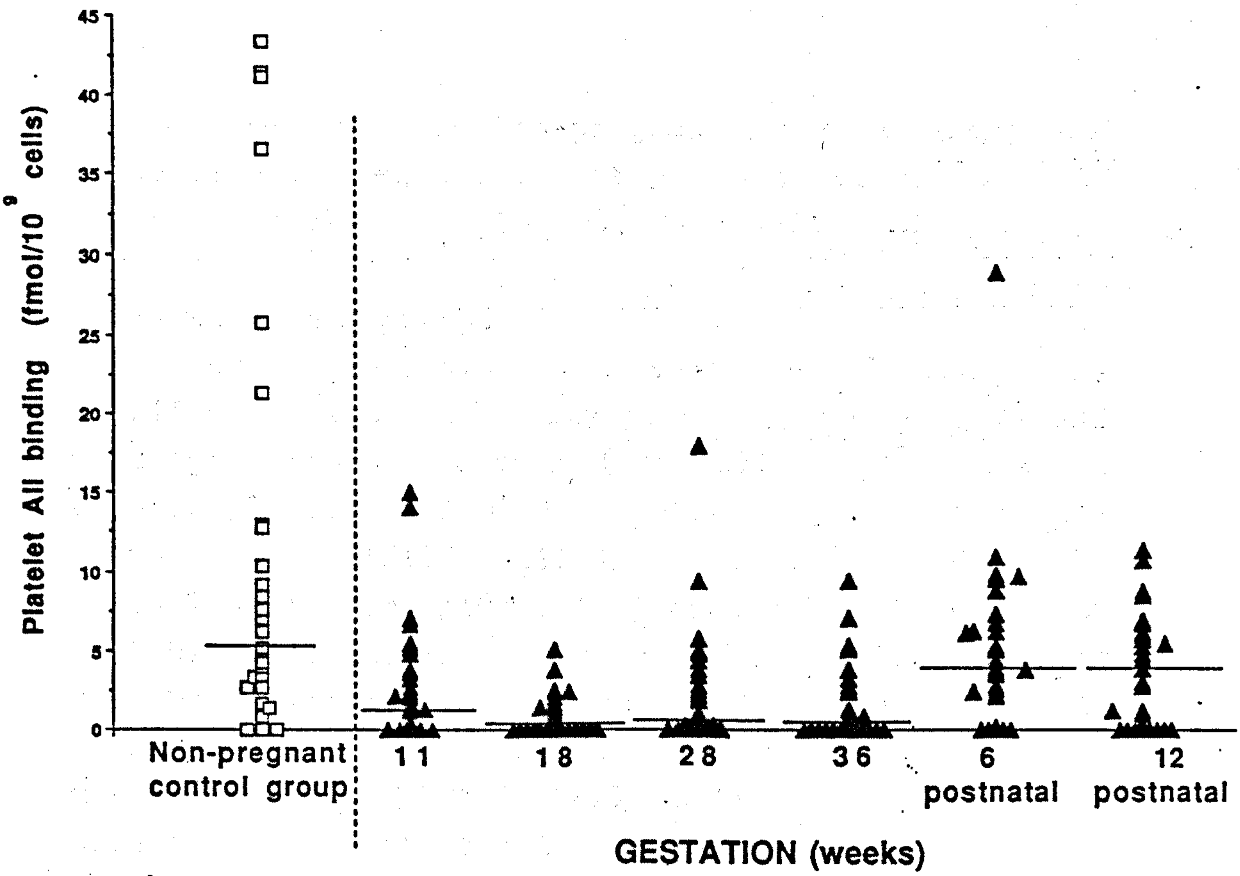
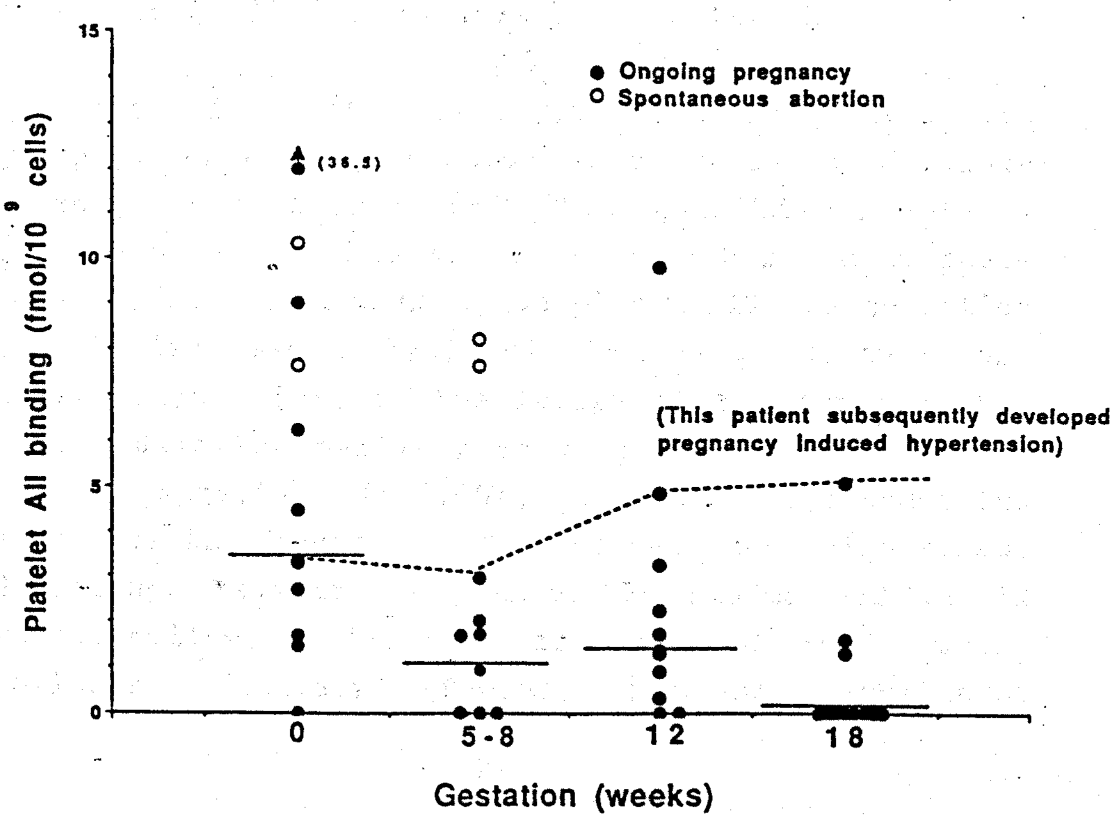


Figure 6.2.



AII binding in the non-pregnant control group and those of the pregnant cohort group at either 6 or 12 weeks after delivery (P>0.05, Mann-Whitney U test).

Early pregnancy group.

Friedman analysis of variance indicated a significant effect of early pregnancy on platelet AII binding in this subgroup (P<0.01). The difference between platelet AII binding before conception (median value 3.4 fmol/10^9 cells) to that at 5-8 weeks gestation (1.2 fmol/10^9 cells) approached significance (P=0.02, Wilcoxon matched-pairs signed-ranks test) as did the difference between platelet AII binding at 11 weeks gestation (1.5 fmol/10^9 cells) and that at 18 weeks gestation (0.0 fmol/10^9 cells, P=0.02). These changes are illustrated in Figure 6.2.

Hormonal data.

The median values of plasma angiotensin II (AII), plasma renin concentration (PRC) and plasma renin substrate (PRS) are summarised in Table 6.2.

Higher AII levels were found in the pregnant cohort group at 11 weeks gestation as compared to the non-pregnant control group (P<0.001, Mann-Whitney U test) (Figure 6.3). Within the pregnant cohort group there was no effect of pregnancy on plasma AII concentration (P>0.1, Friedman analysis of variance), but there was a significant fall in the levels of the peptide at 6 weeks following delivery as compared to those at 36 weeks gestation (P<0.001, Wilcoxon matched-pairs signed-ranks test). There was no significant difference between the values of plasma AII at 12 weeks following delivery as compared to 6 weeks following delivery (P>0.5), and no significant

Table 6.2.

Hormonal data from the non-pregnant control group and the pregnant cohort group.

Median values are shown, with the interquartile range in brackets.
The concentrations of all three hormones were significantly elevated in pregnancy.

Table 6.2

	Non-pregnant control group	GESTATION (weeks) 11	18	28	36	6 weeks PN	12 weeks PN
Angiotensin II (pg/ml)	10.7 (5.2-25.8)	29.4 (22.0-38.6)	27.8 (20.6-35.2)	33.3 (23.6-42.8)	30.9 (20.6-44.4)	12.5 (8.8-16.1)	11.7 (9.0-15.5)
P.R.C. (ng AI/hr/ml)	1.3 (0.9-1.9)	5.7 (4.5-7.5)	6.0 (4.5-8.4)	6.6 (4.8-8.7)	6.5 (4.3-9.6)	2.0 (1.1-2.8)	2.9 (1.0-3.8)
P.R.S. (ug AI/ml)	1.2 (0.9-1.6)	2.3 (1.9-2.9)	3.0 (2.6-3.4)	3.3 (2.8-4.0)	3.5 (3.2-4.0)	1.3 (0.9-1.7)	1.2 (1.0-1.9)

differences between the values of plasma AII in the non-pregnant control group and those of the pregnant cohort group at either 6 or 12 weeks <u>post partum</u> (P>0.5, Mann-Whitney U test).

Increased values of PRC and PRS were also found in pregnancy (Figure 6.3). Values of both PRC and PRS were significantly higher in the pregnant cohort group at 11 weeks gestation as compared to the non-pregnant control group (P<0.001 in each case). There was no subsequent effect of pregnancy on PRC within the pregnant cohort group (P>0.5), but there was a significant effect on PRS (P<0.0001), with significant increases between 11 and 18 weeks gestation (P<0.0001) and between 18 and 28 weeks gestation (P<0.01). The values of both PRC and PRS were significantly lower at 6 weeks following delivery as compared to 36 weeks gestation (P<0.001 in each case). There were no significant differences between the values of either PRC or PRS at 12 weeks following delivery as compared to those at 6 weeks following delivery (P>0.1), and no significant differences between the values of either PRC or PRS in the non-pregnant control group as compared to the pregnant cohort group at either 6 or 12 weeks <u>post partum</u>. (The difference in PRC between the non-pregnant control group and the pregnant cohort group at 12 weeks after delivery approached significance, P=0.011).

In twenty pregnant women, plasma oestradiol concentration was measured at 36 weeks gestation. The median concentration was 66,000 pmol/L (34,000-85,000).

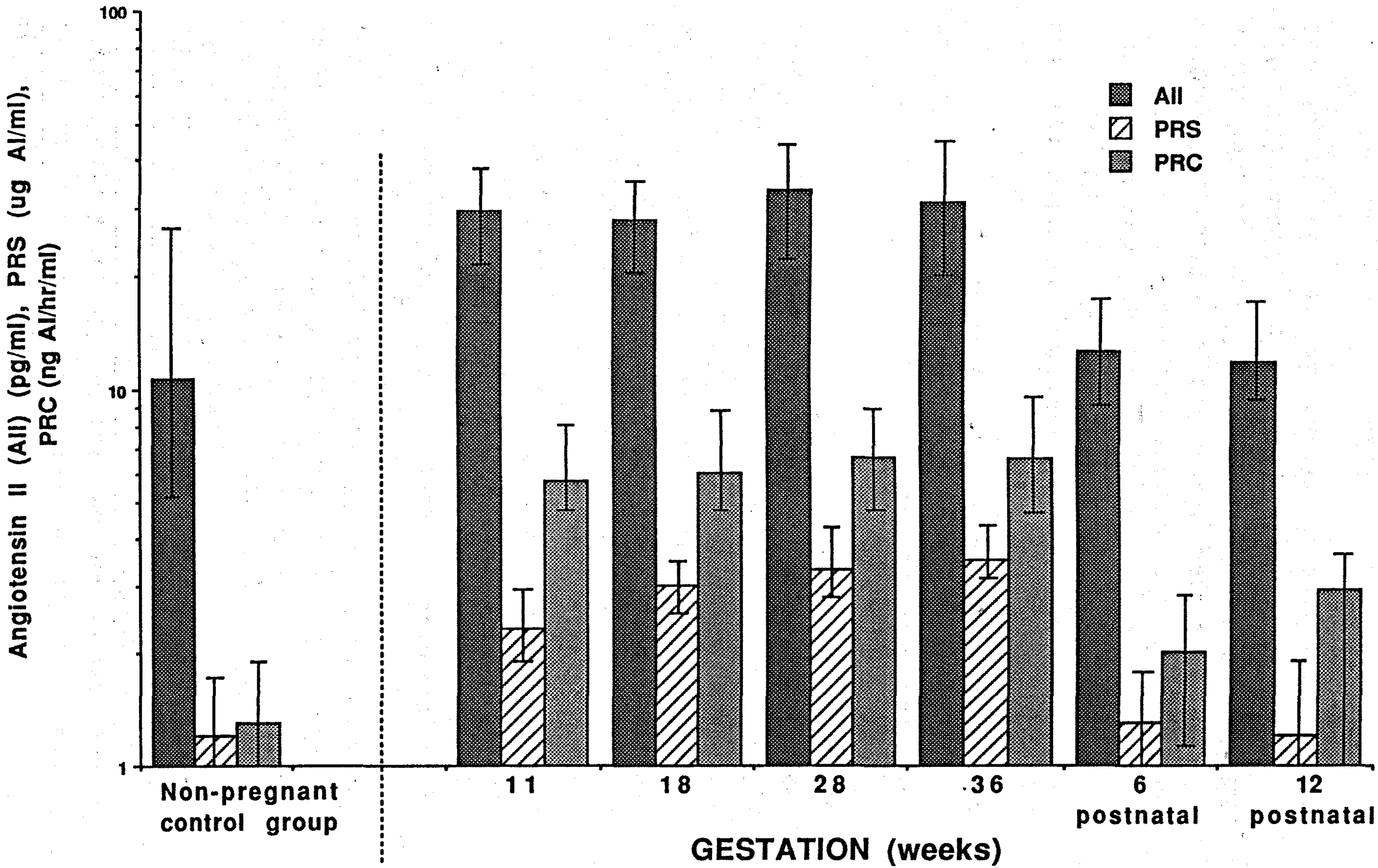
In the non-pregnant control group the inverse correlation between platelet AII binding and plasma

Figure 6.3.

Plasma angiotensin II (AII), plasma renin substrate (PRS), and plasma renin concentration (PRC) in the non-pregnant control group and the pregnant cohort group.

Median values are shown, with the interquartile range marked.

Figure 6.3.



Angiotensin II (AII) (pg/ml), PRS (ug AI/ml), PRC (ng AI/hr/ml)
100
10
1
AII
PRS
PRC
Non-pregnant control group
11
18
28
36
6 postnatal
12 postnatal
GESTATION (weeks)

AII approached statistical significance (P= 0.03, Spearman correlation coefficient r_s= 0.40). There was no correlation between platelet AII binding and either PRC or PRS (P>0.1).

When women from between 11 to 36 weeks gestation in the pregnant cohort group were considered, there were significant inverse correlations found between platelet AII binding and all of the above hormonal parameters; plasma AII (stepwise regression analysis, T= -2.61, P<0.01), PRC (T= -3.40, P<0.001), PRS (T= -2.95, P<0.01). Moreover, the correlations between both PRC and PRS with platelet AII binding were independent of any correlation between platelet AII binding and AII; PRC (T= -3.10, P<0.01), PRS (T= -2.84, P<0.01). At both 6 and 12 weeks following delivery, no correlation between platelet AII binding and plasma AII, PRC or PRS was found (P>0.1). In the subgroup of twenty pregnant women at 36 weeks gestation, there was no correlation between platelet AII binding and plasma oestradiol concentration (P>0.5, Spearman correlation coefficient) (Figure 6.4).

Early pregnancy group.

The median values of plasma AII, PRC and PRS in this subgroup are summarised in Table 6.3.

Friedman analysis of variance indicated that the effect of early pregnancy on plasma AII was not significant (P>0.05), although the difference between the values of the subgroup when non-pregnant and at 5-8 weeks gestation approached significance (P=0.014, Wilcoxon matched-pairs signed ranks test). The effect of early pregnancy on PRS was significant (P<0.01), with significant increases in the levels of PRS

Figure 6.4.

The absence of any correlation between platelet AII binding and the serum oestradiol concentration.
($P>0.5$, $r_s= -0.18$)

Figure 6.5.

Plasma angiotensin II (AII), plasma renin substrate (PRS), and plasma renin concentration (PRC) in the early pregnancy subgroup.

Median values are shown, with the range marked.

Figure 6.4.

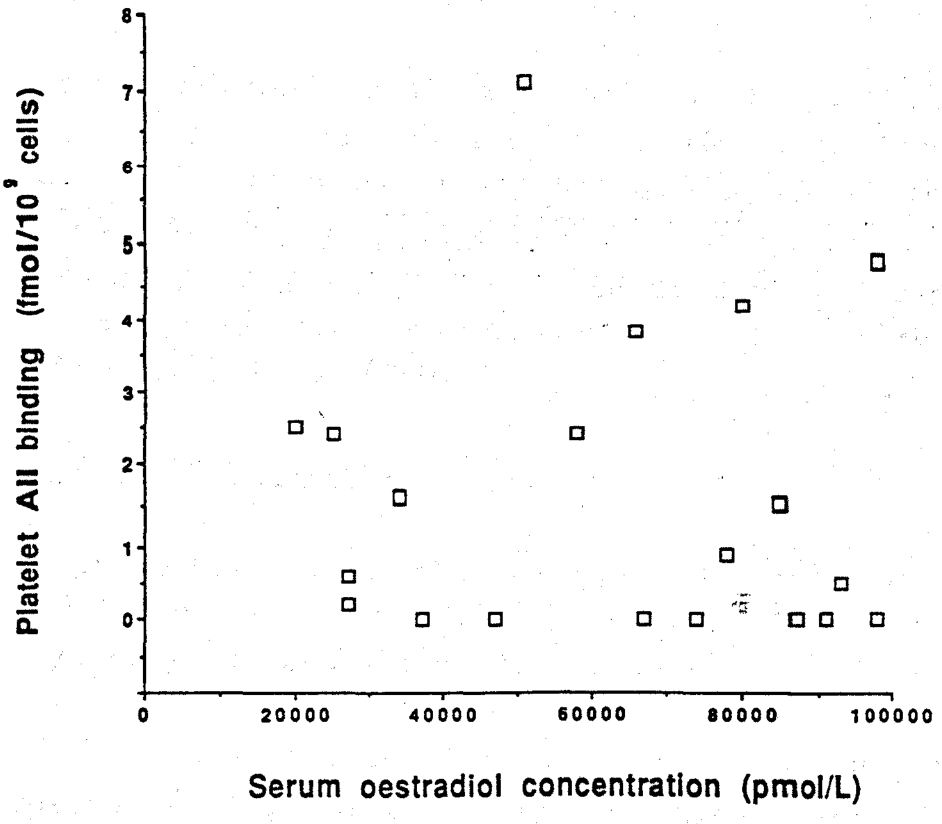
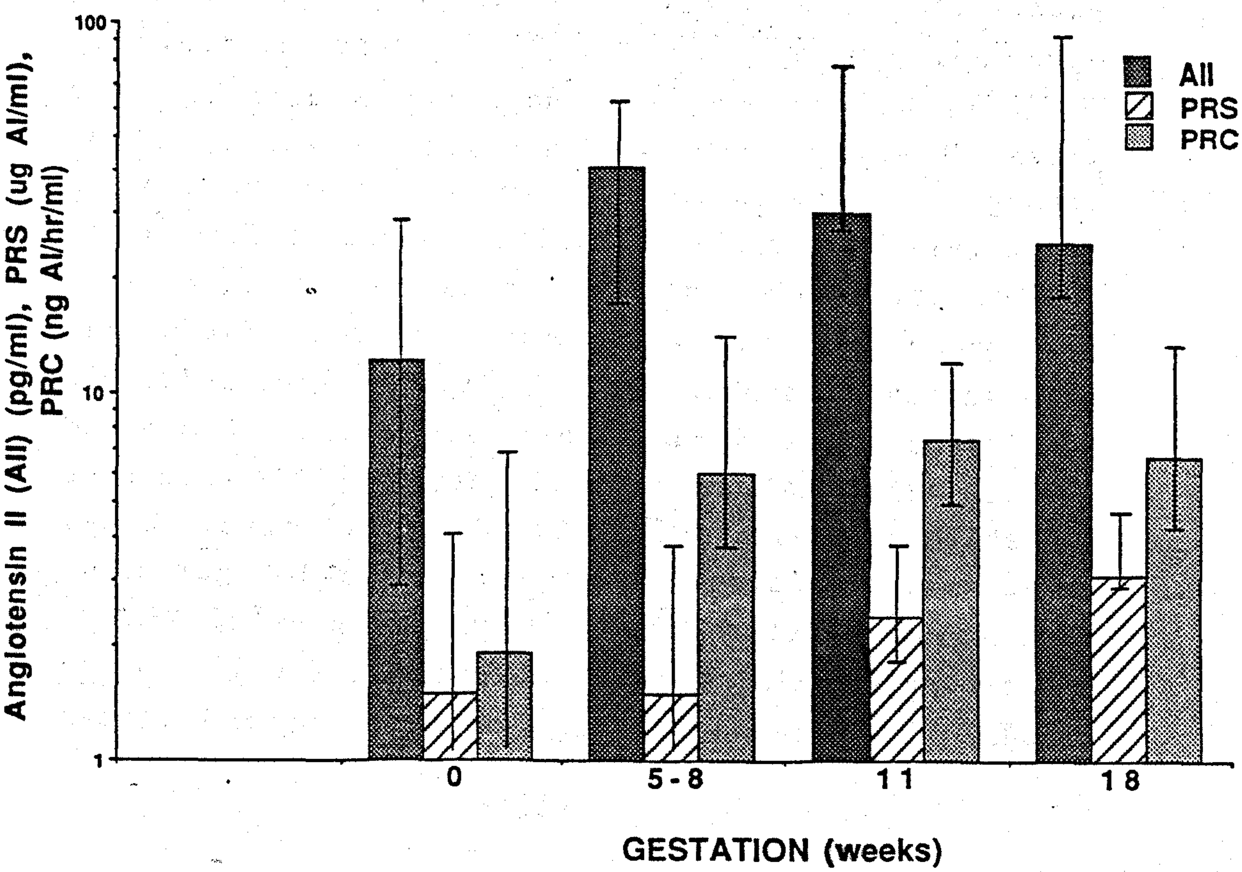


Figure 6.5.



between 5-8 weeks gestation and 11 weeks gestation and between 11 weeks gestation and 18 weeks gestation ($P<0.01$ in each case) (Figure 6.5). A significant effect of early pregnancy on PRC was also found ($P<0.01$), with the increase in levels measured when the subgroup were non-pregnant to those at 5-8 weeks being significant ($P<0.01$).

Haematological data.

Contamination of the prepared platelet suspension by leucocytes was <1% (1-5 x $10^3/\mu l$) and no erythrocytes were detected.

The median values of platelet count, mean platelet volume (MPV), platelet distribution width (PDW), recovery of platelets from whole blood (yield), haemoglobin concentration and white cell count (WCC) are summarised in Table 6.4.

There were no significant differences in the values of platelet count, MPV and PDW between the non-pregnant control group and the pregnant cohort group at 11 weeks gestation ($P>0.1$, Mann-Whitney U test). Although there was no significant effect of pregnancy on platelet count ($P>0.05$, Friedman analysis of variance), there was a significant increase in MPV ($P<0.0001$) and the increase in PDW approached significance ($P= 0.015$). The largest increases were between 28 weeks gestation and 36 weeks gestation, when both MPV ($P<0.001$, Wilcoxon matched-pairs signed-ranks test) and PDW ($P= 0.02$) were considered. Following delivery there was a significant diminution in MPV between 36 weeks gestation and 6 weeks _post partum_ ($P<0.01$, Wilcoxon matched-pairs signed-ranks test), but no other significant changes in platelet

Table 6.3.

Hormonal data from the early pregnancy subgroup.

Median values are shown, with the range in brackets.

Table 6.5.

Platelet data from the early pregnancy subgroup.

Median values are shown, with the range in brackets.

Table 6.3

	GESTATION (weeks)			
	0	5-8	11	18
Angiotensin II (pg/ml)	12.1 (2.8-26.3)	40.3 (14.8-59.2)	30.2 (24.7-71.7)	25.0 (15.8-87.8)
P.R.C. (ng AI/hr/ml)	1.9 (0.7-7.2)	6.0 (3.3-12.9)	7.4 (4.4-11.1)	6.7 (4.0-12.9)
P.R.S. (ug AI/ml)	1.5 (0.9-3.7)	1.5 (0.9-3.3)	2.4 (1.7-3.5)	3.1 (2.6-4.2)

Table 6.5

Platelet count (x 10^9/L)	207 (137-337)	218 (183-326)	216 (168-281)	199 (173-275)
Mean platelet volume (fl)	7.5 (7.2-8.9)	8.0 (7.2-9.8)	7.8 (6.0-8.6)	8.2 (6.8-9.0)
Platelet distribution width	14.6 (13.8-16.8)	15.2 (13.8-16.9)	15.3 (14.2-17.4)	15.8 (13.8-18.0)

Table 6.4.

Haematological data from the non-pregnant control group and the pregnant cohort group.

Median values are shown, with the interquartile range in brackets.

Table 6.4

	Non-pregnant control group	GESTATION (weeks) 11	18	28	36	6 weeks PN	12 weeks PN
Platelet count (x 10^9/L)	209 (182-270)	227 (198-265)	207 (191-239)	212 (195-250)	208 (174-244)	226 (187-259)	235 (211-278)
Mean platelet volume (fl)	8.1 (7.5-8.4)	7.9 (7.5-8.3)	8.0 (7.7-8.3)	8.0 (7.7-8.4)	8.5 (8.2-8.9)	8.1 (7.7-8.6)	8.0 (7.8-8.4)
Platelet distribution width	15.8 (14.7-16.7)	15.4 (14.6-16.4)	15.4 (14.7-16.0)	15.7 (14.6-16.2)	16.1 (15.4-16.9)	15.6 (14.8-16.1)	15.3 (14.9-16.4)
Yield (%)	54 (47-64)	58 (50-68)	59 (46-78)	51 (37-71)	43 (35-58)	57 (36-75)	53 (35-79)
Haemoglobin (g/dl)	13.3 (12.8-14.0)	13.1 (12.4-14.1)	12.1 (11.8-12.7)	11.8 (10.7-12.6)	11.8 (10.0-12.5)	12.6 (12.1-13.3)	12.8 (12.3-13.3)
White cell count (x 10 /L)	6.3 (5.4-7.3)	8.3 (7.1-9.0)	8.9 (7.1-9.9)	8.6 (7.8-10.4)	8.3 (7.4-10.2)	5.5 (4.8-6.9)	5.8 (4.6-6.7)

count, MPV or PDW (P>0.05). There were no significant differences between the values of platelet count, MPV and PDW in the non-pregnant control group and those of the pregnant cohort group at either 6 or 12 weeks after delivery (P>0.1, Mann-Whitney U test).

Platelet yield in the pregnant cohort group at 11 weeks gestation did not differ significantly from that of the non-pregnant control group (P>0.1). However, a significant effect of pregnancy was found (P<0.01), with significant diminutions in yield between 18 and 28 weeks gestation (P<0.01) and between 28 and 36 weeks gestation (P<0.01). The platelet yield at 6 weeks post partum was significantly greater than at 36 weeks gestation (P<0.01). There were no significant differences between the yield at 6 weeks after delivery and at 12 weeks after delivery (P>0.1), or between the yield in the non-pregnant control group and at either 6 weeks or 12 weeks after delivery (P>0.5).

At 11 weeks gestation, a significant change in WCC (P<0.001) but not in haemoglobin concentration (P>0.5) was found, as compared to the non-pregnant control group. Although there was no significant effect of pregnancy on WCC (P>0.1), there was a significant diminution in haemoglobin concentration (P<0.001), with the decreases between 11 and 18 weeks gestation (P<0.001) and between 18 and 28 weeks gestation being significant (P<0.001). Following delivery, the values of both WCC and haemoglobin concentration at 6 weeks post partum were significantly altered as compared to those at 36 weeks gestation (P<0.001 in each case). There were no significant differences between either WCC or haemoglobin concentration at 6 weeks after delivery and at 12 weeks after delivery (P>0.1 in each case), or between WCC in the non-pregnant control group and at either 6 weeks or 12 weeks gestation (P>0.1). However, the haemoglobin concentration was significantly higher in the non-pregnant control group in comparison with the pregnant cohort group at 6 weeks after delivery (P<0.01). This difference ceased to be significant when the comparison was with the pregnant cohort group at 12 weeks gestation.

There was no correlation between platelet AII binding and any of the haematological parameters at any gestation, in any group (P>0.1, stepwise regression analysis when pregnant, Spearman correlation coefficient in the non-pregnant control group and following delivery).

Early pregnancy group.

The median values of platelet count, MPV and PDW of this subgroup are summarised in Table 6.5.

There was no significant effect of early pregnancy on either platelet count (P>0.1) or MPV (P>0.1), although the increase in PDW approached significance (P= 0.04).

Biochemical data.

The median values of serum sodium, potassium, urea, creatinine, urate and osmolality are summarised in Table 6.6.

There were significantly lower levels of serum sodium (P<0.001, Mann-Whitney U test), urea (P<0.0001), urate (P<0.01), creatinine (P<0.0001) and osmolality (P<0.0001) in the pregnant cohort group at 11 weeks gestation as compared to the non-pregnant

Table 6.6.

Serum biochemical data from the non-pregnant control group and the pregnant cohort group.

Median values are shown, with the interquartile range in brackets.

Table 6.6

SERUM	Non-pregnant control group	GESTATION (weeks) 11	18	28	36	6 weeks PN	12 weeks PN
Sodium (mmol/L)	139 (138-141)	137 (136-138)	137 (134-138)	135 (133-136)	136 (133-137)	139 (138-140)	140 (139-141)
Potassium (mmol/L)	4.3 (4.0-4.6)	4.3 (4.1-4.4)	4.2 (3.9-4.4)	4.2 (4.1-4.4)	4.4 (4.1-4.6)	4.3 (4.1-4.6)	4.3 (4.2-4.7)
Urea (mmol/L)	3.9 (3.5-4.5)	3.1 (2.7-3.4)	2.7 (2.1-3.3)	2.2 (2.0-2.9)	2.1 (1.9-2.6)	4.2 (3.1-5.3)	4.1 (3.2-4.6)
Creatinine (umol/L)	87 (81-91)	61 (55-69)	53 (48-65)	52 (46-56)	57 (49-65)	75 (67-81)	70 (66-77)
Urate (umol/L)	201 (173-222)	153 (137-198)	189 (171-212)	208 (177-226)	259 (211-289)	271 (244-312)	250 (220-281)
Osmolality (Osm/L)	281 (279-284)	277 (273-279)	276 (274-279)	278 (275-280)	276 (275-279)	284 (282-287)	284 (281-288)

control group. Friedman analysis of variance demonstrated a significant effect of pregnancy on serum sodium ($P<0.01$) (with a significant decrease occurring between 18 and 28 weeks gestation, $P<0.01$, Wilcoxon matched-pairs signed-ranks test), serum urea ($P<0.001$) (a significant decrease occurring between 18 and 28 weeks gestation, $P<0.01$), serum urate ($P<0.0001$) (significant increases occurring between 11 and 18 weeks gestation and between 28 and 36 weeks gestation, $P<0.001$) and serum creatinine ($P<0.0001$) (significant decreases occurring between 11 and 18 weeks gestation, $P<0.01$, and between 28 and 36 weeks gestation, $P<0.001$). The changes in serum urate are illustrated in Figure 6.6. Following delivery, there were significant increases from 36 weeks gestation to 6 weeks <u>post partum</u> in levels of serum sodium, urea, osmolality and creatinine ($P<0.001$ in each case, Wilcoxon matched-pairs signed-ranks test). There were no further significant changes in any of the biochemical parameters from 6 weeks <u>post partum</u> to 12 weeks <u>post partum</u>, although the diminution in serum creatinine approached significance ($P= 0.03$). There were significant differences between the values of serum urate ($P<0.0001$, Mann-Whitney U test), osmolality ($P<0.01$) and creatinine ($P<0.001$) in the non-pregnant control group and those of the pregnant cohort group at 6 weeks after delivery. Significant differences in serum urate ($P<0.001$) and in serum creatinine ($P<0.001$) remained when the non-pregnant control group were compared with the cohort group at 12 weeks after delivery. There were no differences in serum potassium at any gestation ($P>0.1$).

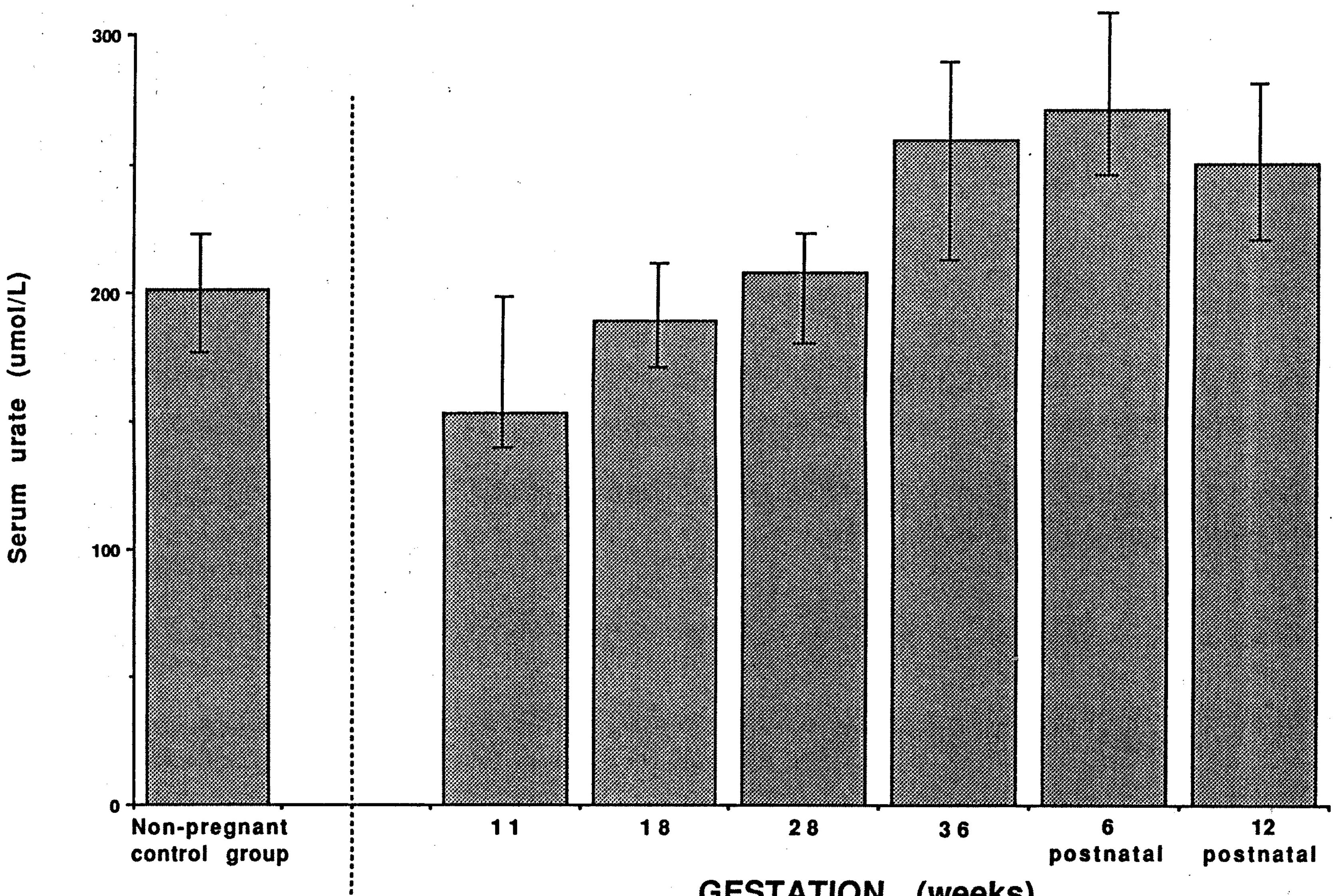
The median values of urinary sodium, potassium, urea, creatinine, urate, osmolality, protein and volume are summarised in Table 6.7.

Figure 6.6.

Serum urate concentrations in the non-pregnant control group and the pregnant cohort group.

Median values are shown, with the interquartile range marked.

Figure 6.6.



Serum urate (umol/L)
300
200
100
0
Non-pregnant control group
11
18
28
36
6 postnatal
12 postnatal
GESTATION (weeks)

Table 6.7.

Urinary biochemical data from the non-pregnant control group and the pregnant cohort group.

Median values are shown, with the interquartile range in brackets.

Table 6.7

URINARY	Non-pregnant control group	GESTATION (weeks) 11	18	28	36	6 weeks PN	12 weeks PN
Sodium (mmol/L)	88 (78-98)	103 (73-130)	108 (78-125)	100 (82-132)	100 (76-118)	120 (96-151)	121 (110-149)
Potassium (mmol/L)	49 (44-61)	57 (44-66)	54 (42-68)	49 (40-72)	45 (36-53)	61 (44-78)	56 (43-68)
Urea (mmol/L)	20 (16-23)	23 (19-29)	22 (16-29)	21 (17-25)	17 (14-21)	25 (18-31)	25 (19-27)
Creatinine (umol/L)	8.2 (6.1-9.9)	7.9 (6.7-9.3)	8.7 (5.4-10.6)	8.0 (5.3-10.5)	7.6 (6.3-9.8)	9.5 (7.8-11.0)	8.8 (7.1-9.5)
Urate (umol/L)	1.6 (1.2-2.3)	2.1 (1.8-2.6)	2.1 (1.6-2.7)	2.3 (1.8-3.2)	2.4 (1.8-3.1)	2.3 (1.7-3.0)	2.1 (1.6-2.8)
Osmolality (mmol/kg)	496 (396-530)	583 (457-719)	543 (448-684)	547 (460-680)	506 (459-628)	686 (533-802)	617 (452-696)
Protein (g/L)	0.09 (0.05-0.12)	0.10 (0.04-0.17)	0.10 (0.08-0.17)	0.09 (0.05-0.16)	0.07 (0.03-0.09)	0.05 (0-0.10)	0.03 (0-0.08)
Volume (L)	1.3 (1.1-1.8)	1.3 (1.0-1.7)	1.2 (1.0-1.7)	1.3 (0.9-1.6)	1.3 (1.0-1.5)	1.0 (0.7-1.1)	0.6 (0.4-0.8)

There were no significant changes in any of the measured urinary biochemical parameters in the pregnant cohort group at 11 weeks gestation as compared to the non-pregnant group (P>0.05). There was no significant effect of pregnancy on any of the measured urinary parameters, although the changes in urinary urea estimations approached significance (P= 0.04). Following delivery, there were significant increases in urinary sodium (P<0.01) and urinary urea (P<0.01) from 36 weeks gestation to 6 weeks *post partum*, with the changes in urinary creatinine (P= 0.02) and urinary osmolality approaching significance (P= 0.03). The only significant change between 6 weeks and 12 weeks *post partum* was a fall in urine volume (P<0.01). There were significant differences between the non-pregnant control group and the pregnant cohort group at 6 weeks after delivery when urinary sodium (P<0.001), urea (P<0.01), osmolality (P<0.01) and volume (P<0.0001) were considered. The significant differences in urinary sodium (P<0.001) and urinary volume (P<0.0001) remained when the non-pregnant control group were compared to the pregnant cohort group at 12 weeks after delivery.

In the non-pregnant control group there was no correlation found between platelet AII binding and any of the serum or urinary biochemical parameters (P>0.1, Spearman correlation coefficient). However, in pregnancy there were significant correlations between platelet AII binding and serum sodium (P<0.01, T= 2.8, stepwise regression analysis), serum urea (P<0.01, T= 2.9) and serum osmolality (P<0.01, T= 3.2). Moreover, these correlations were independent of any inverse correlation between platelet AII binding and plasma AII (serum sodium P<0.01 T= 2.8, serum urea P<0.01 T= 2.7 and serum osmolality P<0.01 T= 2.9). There were no correlations found in pregnancy between platelet AII binding and any of the other serum parameters or between platelet AII binding and any of the urinary parameters, including the urinary sodium/urinary creatinine ratio ($P<0.05$). Following delivery, there was no correlation found between platelet AII binding and any of the serum or urinary biochemical parameters at either 6 weeks or 12 weeks <u>post partum</u> ($P>0.1$).

The following derived values for renal function were calculated: ([] denotes concentration)
Urinary sodium/urinary creatinine ratio,
Glomerular filtration rate (GFR)

$$\text{GFR} = \frac{\text{urinary[creatinine] x urine volume}}{\text{plasma[creatinine] x collection time (mins)}}$$

Fractional excretion of sodium (F_eNa)

$$F_eNa = \frac{\text{urinary[sodium] x plasma[creatinine]}}{\text{urinary[creatinine] x plasma[sodium] x 10}}$$

Fractional excretion of potassium(F_eK)

$$F_eK = \frac{\text{urinary[potassium] x plasma[creatinine]}}{\text{urinary[creatinine] x plasma[potassium] x 10}}$$

Osmolar clearance (C_{osm})

$$C_{osm} = \frac{\text{urinary volume x urinary osmolality}}{\text{plasma osmolality x collection time (mins)}}$$

Water clearance (Urine flow - C_{osm})

However, there was no significant correlation found between platelet AII binding and any of the above derived values for renal function in either the non-pregnant control group or the pregnant cohort group, at any gestation (in each case P>0.05, Spearman correlation coefficient or stepwise regression analysis as appropriate).

Early pregnancy group.

The median values of serum sodium, potassium, urea, creatinine, urate and osmolality in this subgroup are summarised in Table 6.8.

There was a significant effect of early pregnancy on serum urea, creatinine and osmolality (P<0.01 in each case, Friedman analysis of variance), and the effect on serum urate approached significance (P= 0.02).

Discussion.

In the following discussion of the results of the cross-sectional and the longitudinal study, the order used in the text above will be adhered to. The discussion thus commences with a consideration of the study sample, before reviewing the changes in platelet AII binding, and then the changes in the hormonal, haematological and biochemical parameters measured.

The longitudinal study is weaker than if all the pregnant women had been investigated prior to their pregnancy and used as their own controls. However, it was not logistically possible to obtain a large enough pregnant cohort using this method. Fortunately a sufficient number of the preconceptual subjects conceived, therefore the early pregnancy subgroup did act as their own controls. One of the major flaws in the cross-sectional study, the demographic differences between the first trimester group and the non-pregnant control group, was thus corrected.

Table 6.8.

Serum biochemical data from the early pregnancy subgroup.

Median values are shown, with the range in brackets.

Table 6.8

SERUM	GESTATION (weeks) 0	5-8	11	12
Sodium (mmol/L)	139 (137-142)	137 (135-140)	137 (134-140)	137 (135-139)
Potassium (mmol/L)	4.3 (3.9-4.7)	4.2 (3.9-4.4)	4.2 (3.7-4.5)	4.2 (3.9-4.5)
Urea (mmol/L)	3.8 (3.0-4.6)	3.0 (2.0-3.4)	2.8 (2.0-3.3)	2.8 (2.2-3.1)
Creatinine (umol/L)	83 (61-92)	64 (48-74)	55 (41-69)	52 (45-58)
Urate (umol/L)	220 (164-241)	189 (171-212)	172 (148-197)	190 (152-215)
Osmolality (Osm/L)	280 (278-284)	278 (275-280)	277 (273-279)	276 (275-279)

Many of the deficiencies in the cross-sectional study have been discussed at the end of the previous chapter (p. 162). Certain more subtle deficiencies in the longitudinal study did, however, become apparent. There was a bias in the selection of the non-pregnant control group, which only included well-motivated women who attended their general practitioner or practice nurse for advice regarding pre-pregnancy health care, and who were prepared to attend the hospital and have a sample of blood sample taken. This bias may account for the difference in age between the non-pregnant control group and the pregnant cohort group which approached statistical significance. In order to concentrate on the changes in platelet AII binding in early pregnancy, the longitudinal pregnant cohort group were selected randomly from women referred at an early gestation by their general practitioners. A further source of bias is thus incorporated, as women who present late in pregnancy, and would thus be excluded from the study, tend to be younger than average, and of a lower socio-economic class [Birch, 1989].

In the discussion following the cross-sectional study, the differences between the non-pregnant control group and those women studied six weeks after delivery, particularly as regards renal function, were considered. The longitudinal study emphasises the dangers in assuming that at twelve weeks <u>post partum</u> a woman has returned to her pre-pregnant state. The haematological data agrees with both this supposition and previous studies [Louden, 1988, Kilby, 1990]. However, at twelve weeks following delivery, 22 of the pregnant cohort group had not menstruated, all of the women having had regular menstrual periods prior to their pregnancies. This finding is in accord with previous work which suggests that even in bottle-feeding mothers, menstruation does not typically occur until fourteen weeks after delivery [reviewed by Howie, 1988]. In addition there were significant differences in certain of the urinary biochemical parameters between the non-pregnant control group and the subjects studied twelve weeks after delivery.

The principal finding of the prospective longitudinal study of platelet AII binding in normotensive pregnancy was that a reduction in binding occurred, the reduction occurring early in pregnancy. Study of the early pregnancy subgroup suggests that the diminished platelet AII binding, with lower levels demonstrated at 5-8 weeks gestation, may represent a continuation of the low binding found in the luteal phase of the menstrual cycle (see chapter 4), the return to follicular levels not occurring if the woman conceives.

The results of the longitudinal study confirm those of the provisional cross-sectional study, and suggest that the fall in platelet AII binding in pregnancy parallels the diminution in pressor responsiveness to infused AII, that also occurs in pregnancy [Gant, Daley, Chand, Whalley and MacDonald, 1973]. This finding supports the use of platelets as a accessible model of vascular smooth muscle in pregnant women, in order to study AII binding site changes.

In both the cross-sectional and the longitudinal studies, there were certain subjects in whom the values of platelet AII binding were elevated, even in the third trimester of pregnancy. In the longitudinal study, individuals in whom values of platelet AII binding were well above the median at 36 weeks gestation also tended to have elevated levels at 18 and at 28 weeks gestation. The explanation for the high levels of binding in these women is unclear; in the cross-sectional study the four women with the highest values of platelet AII binding, at 36 weeks gestation all delivered male infants but this was not the case in the longitudinal study, (although it was interesting to note that 11/15 of the women with values of platelet AII binding above the median at 36 weeks gestation subsequently delivered male infants). In all of these women the blood pressure recordings were well below pathological levels throughout their pregnancies, and plasma AII concentrations did not differ markedly from the median values.

The studies discussed in this thesis were the first cross-sectional and the first longitudinal studies of platelet AII binding in pregnancy. However, Pawlak and Macdonald [1990] have recently presented preliminary data from a cross-sectional study of platelet AII binding sites in pregnancy. The numbers in the study were small (16 non-pregnant controls and 16 women in the first and third trimesters), the non-pregnant control group was of mixed parity and was tested at various stages of the menstrual cycle, and parametric statistical analysis was inappropriately used. Nevertheless, Pawlak and Macdonald demonstrated a diminution in platelet AII binding site capacity in pregnancy, with binding site capacity in the first trimester subjects being significantly lower than in the non-pregnant control group.

The cross-sectional study, the longitudinal study and the early pregnancy subgroup all demonstrated increases in plasma AII, PRC, and PRS in pregnancy. In the longitudinal study all six samples from each patient were assayed on the same day, thus minimising laboratory errors. There were, however, very few differences between the longitudinal and cross-sectional studies, with both plasma AII and PRC increasing markedly in early pregnancy, (the early pregnancy subgroup demonstrating elevated levels at 5-8 weeks gestation), and then increasing slightly towards term. These increases thus occur at a similar gestation to the diminution in platelet AII binding. In contrast, there were gradual increases in PRS throughout pregnancy. These findings are in accord with the consensus from recent studies [discussed in the Introduction, p. 40, reviewed by Broughton Pipkin, 1988].

Both the cross-sectional and the longitudinal studies, and the cross-sectional study of Pawlak and Macdonald [1990], demonstrated an inverse correlation between platelet AII binding and plasma AII in the non-pregnant control subjects which approached statistical significance. This lends support to the contention that platelet AII binding is down-regulated by plasma II in non-pregnant women [Moore, Taylor and Williams, 1984, Ding, Kenyon and Semple, 1985].

In pregnancy, the cross-sectional study described in this thesis and that described by Pawlak and Macdonald [1990] found no correlation between platelet AII binding and any of the hormonal parameters. There have been no other studies of AII binding in human pregnancy, although studies using animals suggest several other possible regulatory influences upon AII receptor concentration (these are discussed in detail in the Introduction, p. 79).

One such possibility is the serum level of oestradiol; changes in rat and rabbit uterine AII receptors in the ovarian cycle having been found to be directly proportional to serum oestradiol estimation [Schirar, Capponi and Catt, 1980]. However, there are differences in AII receptor regulation in uterine tissue as compared to vascular smooth muscle tissue. For example, in <u>in vitro</u> studies with rat tissue, sodium increases AII binding to mesenteric artery homogenates [Wright, Alexander, Ekstein and Gimbrone, 1982] but has no effect on AII binding to uterine homogenates [Bennett and Snyder, 1980]. Only twenty of the pregnant patients in the longitudinal study had serum oestradiol levels measured. Nevertheless, the absence of any correlation between platelet AII binding and serum oestradiol in these patients suggests that such a regulatory mechanism is unlikely.

Alternatively, the divalent cations, Mn^{+2}, Mg^{+2} and Ca^{+2} have been found to increase rat mesenteric artery AII receptor concentrations in <u>in vitro</u> studies [McQueen, Murray and Semple, 1984]. Plasma calcium concentrations were not measured in this study. However, in a longitudinal study of subjects drawn from the same population as those of this study, and using identical entry criteria, Kilby [1990] found that serum calcium concentrations, when corrected for albumen binding, were unchanged during normotensive pregnancy. In contrast, platelet intracellular free Ca^{+2} concentrations have been reported to rise during pregnancy, although the increase only reached statistical significance in the third trimester [Kilby, Broughton Pipkin, Cockbill, Heptinstall and Symonds, 1990]. However, when platelet intracellular free Ca^{+2} was measured in third trimester subjects, in conjunction with platelet AII binding, no correlation between the two parameters was found (Chapter 3, Characterisation of binding, p. 131).

In contrast to the cross-sectional studies, the longitudinal study did suggest inverse correlations in pregnancy between platelet AII binding and plasma AII, PRS and PRC. This was probably due to the greater statistical power of the longitudinal study being required to demonstrate the inverse correlations. As in the non-pregnant women, these hormonal parameters thus seem to be likely regulatory influences of platelet AII binding in pregnancy. Interestingly however, AII infusion studies have suggested that after the first trimester of pregnancy, pressor sensitivity to AII is also independent of endogenous plasma AII concentration [Broughton Pipkin, Hunter, Turner and O'Brien, 1984], and other regulatory influences may well affect platelet AII binding in pregnancy.

In both the longitudinal and the cross-sectional studies, there was no correlation between plasma AII and platelet AII binding in the women studied after delivery. In the cross-sectional study, a rapid fall in plasma AII, PRC and PRS, evident at 24 hours after delivery, was accompanied by a more gradual return to non-pregnant levels of platelet AII binding, confirming the dissociation between platelet AII binding and the hormonal parameters. The median values of both platelet AII binding and plasma AII at six and twelve weeks _post partum_ approximate to those of the nulliparous non-pregnant control group, although the scatter of platelet AII binding data was narrower when the subjects were studied after delivery. The dangers in regarding women at twelve weeks after delivery as having returned to the non-pregnant state has been discussed above, the reduced _post partum_ variability may be related to the delay in establishing ovulatory cycles. It seems unlikely that a different mechanism regulates platelet AII binding in parous as compared to nulliparous women, and it would be interesting to study these women at progressively longer intervals after delivery, in order to determine if and when an inverse correlation between platelet AII binding and plasma AII returns.

The conflicting reports of the changes in platelet count in pregnancy [e.g. Sill and Lind, 1985, Fenton, Saunders and Cavill, 1977] have already been discussed in the Introduction (p. 99). This thesis provides further conflicting evidence. The longitudinal study, in keeping with the majority of previous longitudinal studies, found no change in platelet count. In contrast, the cross-sectional study demonstrated a highly significant fall in platelet count with gestational age. In certain of the previous reports in which a fall in platelet count was found, the exclusion of patients with pregnancy-induced hypertension has been questioned [e.g. Fay, Hughes and Farron, 1983]. This criticism cannot be applied to the cross-sectional study described in Chapter 5, and the reason for the discrepancy in the results of the two studies is unclear. The dangers in drawing conclusions from cross-sectional studies alone are thus illustrated.

Both the cross-sectional and longitudinal studies demonstrated pregnancy-induced increases in mean platelet volume and platelet distribution width, with the majority of the increases occurring in the third trimester. These findings are in agreement with the majority of previous studies [e.g. Sill and Lind, 1985] (discussed in the Introduction, p. 100). Letsky [1985] has demonstrated that platelets become progressively smaller with age, thus this data is consistent with a pregnancy-induced increase in platelet turnover. Values of platelet AII binding are expressed per cell; the maximal increase in mean platelet volume was of the order of 6%, thus the results would be similar if expressed per unit membrane protein.

A progressive reduction in platelet yield (the proportion of the platelets in whole blood that remained after platelet preparation) was found in pregnancy, in both the cross-sectional and longitudinal studies. This may well be due to the well-documented changes in platelet reactivity [Yamazaki, Motomiya, Kikutani, Sakakibara, Watanebe, Numata and Noguchi, 1979], outlined in the introductory chapter (p. 102). The most contemporary experimental evidence is provided by a prospective study from this centre, in which both platelet aggregation (in response to arachidonic acid and adrenaline) and adrenaline-induced platelet exocytosis (as measured by radioimmunoassay of ^{14}C-serotonin) were found to increase significantly by the third trimester, in comparison to the non-pregnant state [Louden, Broughton Pipkin, Heptinstall, Fox, Mitchell and Symonds, 1990]. Increased platelet aggregation would be expected to diminish the number of platelets in the platelet-rich plasma obtained after centrifugation.

It is possible that pregnancy-induced platelet changes *per se*, may be responsible for altered platelet AII binding, which would thus not reflect AII binding to vascular smooth muscle. This possibility seems unlikely; the maximal platelet changes occur in the third trimester, whereas those of platelet AII binding occur in the first trimester, and at no gestation, in any study, was a correlation between platelet AII binding and any of the platelet parameters found. The finding of no consistent alteration in platelet AII binding following blood transfusion, when the platelet parameters were markedly altered, provides further evidence to refute such a hypothesis (Chapter 2, p. 125).

The longitudinal study also demonstrated that pregnancy induced a fall in the haemoglobin concentration and an increase in white cell count. Both of these phenomena are well described [Letsky, 1980], the former being due to haemodilution, and the latter being due to a direct effect of oestrogens.

The marked changes in serum urea and electrolytes, creatinine and urate concentrations, particularly in the first trimester of pregnancy, which were demonstrated in both the longitudinal and the cross-sectional studies, are also in accord with phenomena previously well described [Wood, 1977]. Mineralocorticoid and vasopressin secretion are increased in normotensive pregnancy. Increased plasma progesterone concentrations in pregnancy cause a hyperventilation which results in a lowering of the serum bicarbonate concentration. This is balanced by a fall in serum sodium concentration. Water retention is thus achieved despite an increase in the glomerular filtration rate and is reflected in a reduced plasma osmolality [Wood, 1977]. Although substantial renal tubular absorption of urea is thought to occur in pregnancy, the net effect is of increased urea excretion, thus leading to a fall in serum urea concentration with gestation. A complex process maintains serum urate concentration, involving both secretion and reabsorption in the proximal renal tubule and then further reabsorption in the distal tubules [Wood, 1977]. In normotensive pregnancy the serum urate concentration falls initially with gestation and then rises to reach almost non-pregnant concentrations at the end of the last trimester [Dunlop and Davison, 1977]. All these changes are reflected in the results of the studies described above.

In the longitudinal study, significant correlations were found in pregnancy, between platelet AII binding and the values of plasma sodium, urea and osmolality. It may well be that the greater numbers and statistical power of the longitudinal study is responsible for the identification of correlations not found in the cross-sectional study. Decreases in the rate of delivery of sodium to the distal tubule may be effecting reductions in platelet AII binding via alterations in the renin-angiotensin system, the altered delivery being detected at the macula densa and signalling reciprocal renin release [Vander and Miller, 1964] (outlined in the Introduction, p. 22). However, use of stepwise regression analysis suggested that these correlations acted independently of changes in plasma AII concentration. Further evidence to suggest that the plasma sodium concentration may exert a key regulatory influence on levels of platelet AII binding will be presented in Chapters 7 and 8. Consideration of possible regulatory mechanisms whereby the plasma sodium concentration exerts such an influence is included in the General Discussion at the end of the thesis (p. 239).

In an effort to study further the relationship between platelet AII binding and electrolyte balance, the longitudinal study incorporated the collection of 24 hour urine samples. The difficulties in using such collections obtained on an out-patient basis are graphically illustrated. Patients found that providing serial samples whilst pregnant was a considerable drawback to participating in the study. Even women who collected their samples with an almost missionary zeal whilst pregnant became less compliant when coping with a newborn infant. This almost certainly accounted for the volumes of several samples in the puerperium being consistent with acute renal failure (less than 400ml / day, [Warrell, 1988]), and for the highly significant differences in urine volumes obtained at six and twelve weeks post partum compared to those of the non-pregnant control group. The results of the urinary biochemical parameters must thus be treated with some scepticism, although even when the derived values for renal function were considered, no correlations were found with platelet AII binding.

Chapter 7.

Platelet AII binding in women with hypertensive diseases of pregnancy.

This chapter describes the differences in platelet angiotensin II (AII) binding of primiparous women whose pregnancies were complicated by hypertension as compared to primiparous women whose pregnancies were normotensive throughout. All definitions used in this chapter have been described in detail in the Introduction (Section 1, p. 9). This part of the thesis is essentially a cross-sectional study, although all the subjects were investigated prior to delivery and then studied prospectively after delivery, at both six and twelve weeks <u>post partum</u>. Thus when comparisons are made within groups, between samples taken before and after delivery and between six and twelve weeks <u>post partum</u>, the data has been treated as paired data.

<u>Study sample.</u>

67 third trimester primiparous patients whose pregnancies were complicated by hypertension were included in the study. 61 of these patients had pregnancy induced hypertension (PIH), as defined by Davey and MacGillivray [1987] (Introduction, p. 9). These patients were subdivided into 30 patients with non-proteinuric PIH and 31 patients with proteinuric PIH or pre-eclampsia, with respect of the presence or absence of 'significant proteinuria' (>0.3g/24 hour urine collection), as illustrated in Figure 7.1. Two of these patients were originally in the longitudinal pregnancy cohort group (described in the previous chapter). All subjects with PIH were followed up in the postnatal period and all were normotensive with no significant proteinuria at twelve weeks after delivery. In addition 6 patients whose blood pressure remained elevated at twelve weeks after delivery are

Figure 7.1.

Quantitative urinary protein content in the PIH patients in the third trimester.

Median values are shown, with the interquartile range marked.

The PIH group have been subdivided into non-proteinuric PIH patients (n=30) and patients with pre-eclampsia (n=31) on the basis of significant proteinuria (>0.3g / 24hours). The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 7.2.

Diastolic and systolic blood pressures of the PIH patients in the third trimester.

Median values are shown, with the interquartile range marked. In certain groups, the 25th or 75th centiles were equal to the median.

The PIH group have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 7.1.

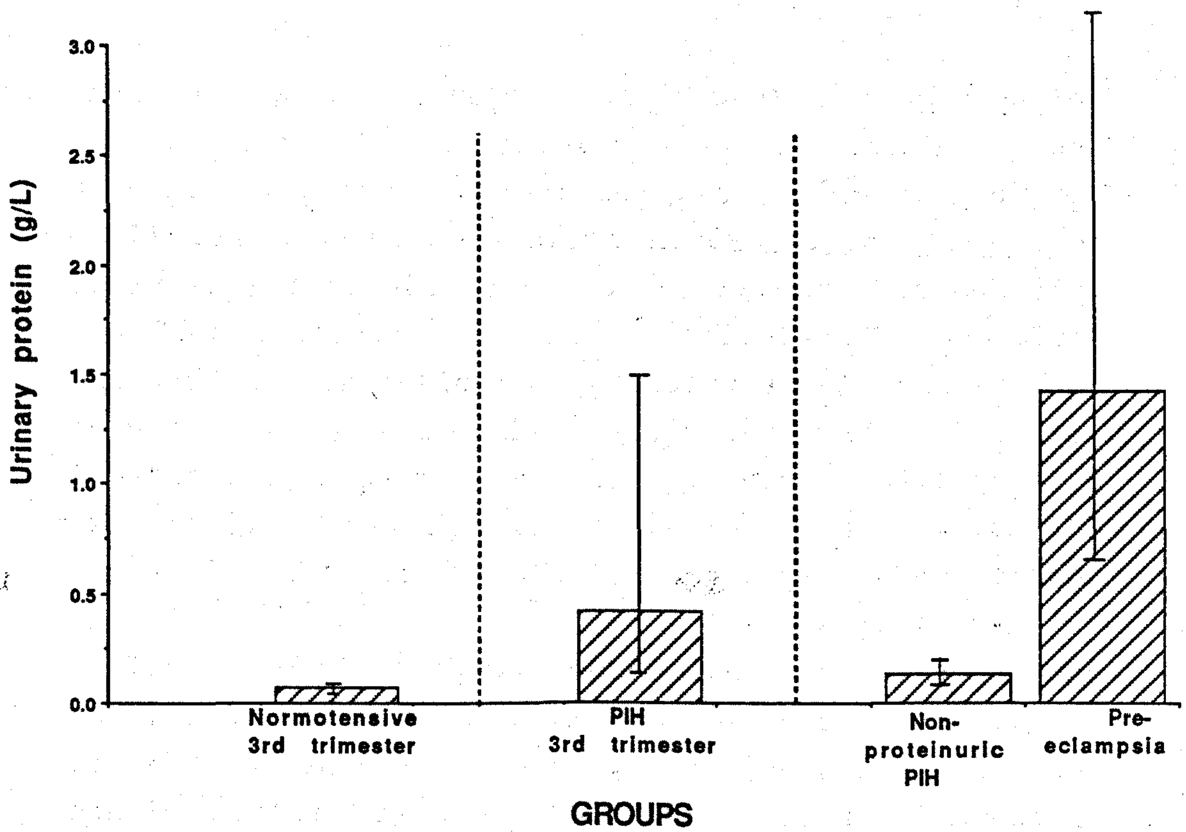
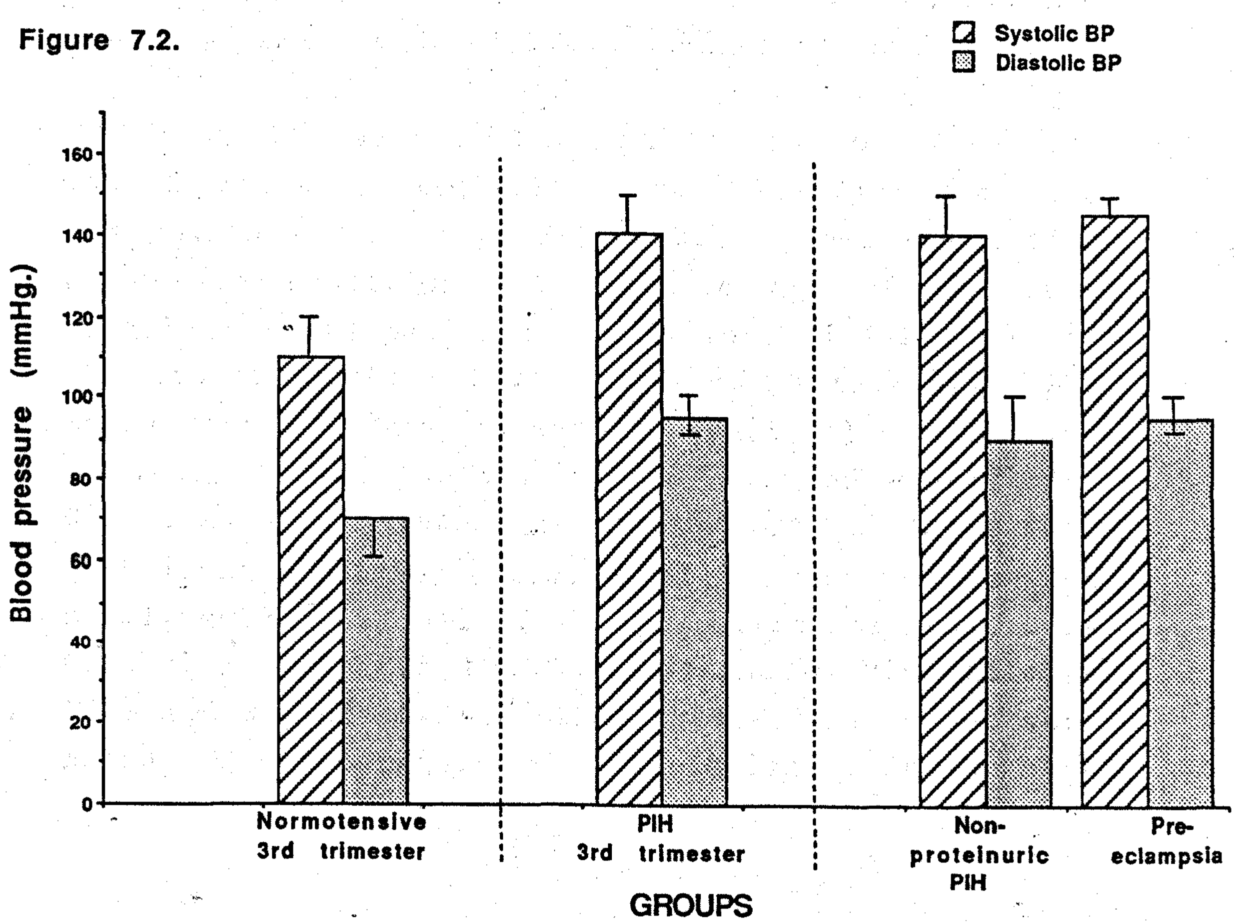


Figure 7.2.



included in the study as an essential hypertension group. None of the essential hypertension group had significant proteinuria at any time. (In view of the small size of the essential hypertension group, the range of values has been quoted, rather than the 25th and 75th centiles, and these patients have not been included in statistical comparisons.) At the time of investigation all the subjects were hospital inpatients, hypertension in pregnancy having been diagnosed. However, all samples were taken prior to any antihypertensive medication having been commenced.

These sample groups were compared in a cross-sectional manner to the 30 normotensive primigravidae of the pregnancy cohort group (described in the previous chapter) when they attended the antenatal clinic at 36 weeks gestation.

All subjects were receiving sodium intake <u>ad libitum</u> and the gestational age of each subject had been confirmed by an eighteen week ultrasound scan.

The median age of the normotensive primigravidae was 25 years (interquartile range 22-27) and of the PIH patients was 24 years (21-27). When the latter group was subdivided, the median age of the non-proteinuric PIH patients was 24 years (21-27) and of the pre-eclampsia group was 23 years (21-28). The median age of the 6 essential hypertensive women was 25 years (range 21-36). There were no significant differences in maternal age between any of the groups ($P>0.1$, Mann-Whitney U test). All of the normotensive primigravidae and all of the pre-eclampsia group were Caucasian. Two of the non-proteinuric PIH patients (7%) and one of the essential hypertensive women were Asian, the remainder being Caucasian. One of the normotensive primigravidae (3%) was a smoker, as were two of the non-proteinuric PIH patients (7%), three of the pre-eclampsia group (10%) and two of the essential hypertensive women.

There was no significant difference between the values of gestational age at the time of investigation of the PIH patients (median 36 weeks, 32-38) and those of the normotensive primigravidae (36 weeks, 35-36, P>0.1, Mann-Whitney U test). However when the PIH group was subdivided, the gestational ages of the non-proteinuric PIH patients (38 weeks, 36-39) were significantly greater than those of both the normotensive primigravidae (P<0.01), and those of the pre-eclampsia group (35 weeks, 31-37, P<0.01). The median gestational age at the time of investigation of the essential hypertensive subjects was 38 weeks (range 34-40).

The median maternal weights and systemic blood pressures of the normotensive primigravidae, of the PIH patients and of the essential hypertensive women, at initial investigation and at six and twelve weeks <u>post partum</u>, are summarised in Table 7.1. At initial investigation, the maternal weights of the PIH patients were significantly greater than those of the normotensive primigravidae (P<0.01, Mann-Whitney U test). When the former group was subdivided, the maternal weights of the non-proteinuric PIH patients were significantly greater than those of both the normotensive primigravidae (P<0.001) and the pre-eclamptic group (P<0.01). There was no significant difference in maternal weight between the normotensive primigravidae and the pre-eclampsia group (P>0.1). The median maternal weights of all the groups fell after delivery. Nevertheless, the same statistically

Table 7.1.

Maternal weights and systemic blood pressures in the third trimester and at six and twelve weeks after delivery.

Median values are shown, with the interquartile range in brackets.
The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects and the essential hypertensive women included for comparison.

Table 7.1

Third trimester groups

	Normotensive (n=30)	PIH (n=61)	Non proteinuric PIH (n=30)	Pre-eclampsia (n=31)	Essential hypertensive (n=6)
Weight (Kg)	71.5 (64.5-78.6)	79.5 (70.6-93.0)	89.0 (77.8-99.5)	76.0 (69.0-80.5)	87.5
Systolic blood pressure (mmHg)	110 (110-120)	140 (140-150)	140 (140-150)	145 (145-150)	140
Diastolic blood pressure (mmHg)	70 (61-70)	95 (90-100)	90 (90-100)	95 (92-100)	90

6 week postnatal groups

	Normotensive	PIH	Non proteinuric PIH	Pre-eclampsia	Essential hypertensive
Weight (Kg)	62.3 (60.0-69.5)	71.0 (61.2-85.9)	78.0 (70.0-91.8)	68.1 (60.6-72.4)	79.4
Systolic blood pressure (mmHg)	120 (110-120)	120 (115-130)	120 (115-125)	120 (115-130)	135
Diastolic blood pressure (mmHg)	70 (60-70)	75 (70-80)	75 (70-80)	75 (65-80)	90

12 week postnatal groups

	Normotensive	PIH	Non proteinuric PIH	Pre-eclampsia	Essential hypertensive
Weight (Kg)	58.5 (55.3-68.3)	69.5 (61.5-77.4)	76.0 (68.3-90.5)	66.1 (58.9-71.3)	76.1
Systolic blood pressure (mmHg)	115 (110-120)	120 (110-120)	120 (110-120)	119 (110-120)	140
Diastolic blood pressure (mmHg)	70 (60-70)	70 (70-75)	70 (69-78)	70 (67-72)	90

significant differences between the groups persisted at both six and twelve weeks after delivery.

By definition, at initial investigation there were significant differences in both the systolic ($P<0.0001$) and diastolic ($P<0.0001$) blood pressures of the PIH patients as compared to the normotensive primigravidae (Figure 7.2). The differences between the non-proteinuric PIH patients and the pre-eclampsia group were not significant ($P>0.1$). The systolic and diastolic blood pressures of both the non-proteinuric PIH patients and the pre-eclampsia group were significantly lower at 12 weeks after delivery as compared to those at the initial investigation ($P<0.01$ in each case, Wilcoxon matched-pairs signed-ranks test), and at twelve weeks after delivery there were no significant differences in systolic and diastolic blood pressures between any of the groups ($P>0.05$).

No significant correlations were found between platelet AII binding and any of the above parameters in any of the groups ($P>0.1$, Spearman correlation coefficient).

The birthweights in the normotensive primigravidae (median 3.44 Kg, 3.04-3.75) were significantly greater than those of the PIH patients (2.87 Kg, 2.32-3.27, $P<0.0001$, Mann-Whitney U test). When the latter group was subdivided, the difference between the birthweights of the pre-eclampsia group (2.60 Kg, 1.74-3.34) and those of the non-proteinuric PIH patients (3.08 Kg, 2.63-3.26) approached significance ($P= 0.03$). The median birthweight of the essential hypertension group was 2.64 Kg.

The gestational ages at delivery of the normotensive primigravidae (median 40 weeks, 39-41) were significantly greater than those of the PIH patients (38 weeks, 36-39, $P<0.0001$). When the latter group was subdivided, the gestational ages at delivery of the non-proteinuric PIH patients (38 weeks, 36-39) were significantly greater than those of the pre-eclampsia group (37 weeks, 34-38, $P<0.01$). The median gestational age at delivery of the essential hypertension group was 37 weeks.

5 (17%) of the normotensive primigravidae were delivered by caesarean section, as compared to 25 (41%) of the PIH patients (17 of the 25 were patients with pre-eclampsia). The ratio of male:female infants was 1.1:1 in the normotensive primigravidae and 1.9:1 in the PIH patients (1.7:1 in the non-proteinuric PIH patients and 2.1:1 in the pre-eclampsia group). 18 (60%) of the normotensive primigravidae breast-fed their infants, as compared to 26 (43%) of the PIH patients (17 (57%) of the non-proteinuric PIH patients and 9 (29%) of the pre-eclampsia group). At twelve weeks after delivery, only 8 (27%) of the normotensive primigravidae and 15 (25%) of the PIH patients had menstruated.

In addition to the above subjects, platelet AII binding was also measured in 6 subjects with 'fulminating pre-eclampsia'. This was defined as patients with pre-eclampsia, in whom the diastolic blood pressure was greater than 110 mmHg., +++ proteinuria was present on dipstick testing of a random sample, and in whom delivery was necessary before 24 hours after admission (in most cases a caesarean section was performed as soon as the blood pressure was controlled by anti-hypertensive medication). In 5 of the 6 cases, blood was taken for assay prior to antihypertensive medication being administered. In the remaining case, blood was taken only a few minutes after intravenous hydrallazine had been commenced. All 6 patients were primiparous, the median gestational age of these subjects was 31 weeks and the median platelet count was 97 x 10^9/L.

Platelet Angiotensin II (AII) binding data.

At the time of initial investigation, median platelet AII binding in the PIH patients was 4.4 fmol/10^9 cells (interquartile range 2.0-7.6) (Figure 7.3). Platelet AII binding in the PIH patients was thus significantly higher than in the normotensive primigravidae, studied at 36 weeks gestation (median value 0.7 fmol/10^9 cells, 0-2.8, $P<0.0001$, Mann-Whitney U test). When the PIH patients were subdivided, although platelet AII binding was higher in the pre-eclampsia group (5.3 fmol/10^9 cells, 3.5-16.3) than in the non-proteinuric PIH patients (3.9 fmol/10^9 cells, 1.8-6.1), the difference was not significant ($P= 0.08$). In addition, whereas several of the normotensive primigravidae had undetectable levels of platelet AII binding at 36 weeks gestation, this was not the case when any of the patients with pre-eclampsia were studied. The median value of platelet AII binding in the essential hypertension group was 0.4 fmol/10^9 cells).

The median value of platelet AII binding in the 6 patients with fulminating pre-eclampsia was 4.0 fmol/10^9 cells (range 2.9-6.1). The values of platelet AII binding in the two PIH patients who had been studied longitudinally are illustrated in Figure 7.4. The median values found in the longitudinal

Figure 7.3.

Platelet AII binding in the PIH patients in the third trimester.

The median values of platelet AII binding are indicated by horizontal bars.
Platelet AII binding was significantly higher in the PIH patients as compared to the normotensive women ($P<0.0001$).
The PIH group have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 7.4.

Platelet AII binding in the two PIH patients studied longitudinally prior to the development of PIH.

The median values of platelet AII binding found in the longitudinal study of normotensive pregnant subjects are shown for comparison.

Figure 7.3.

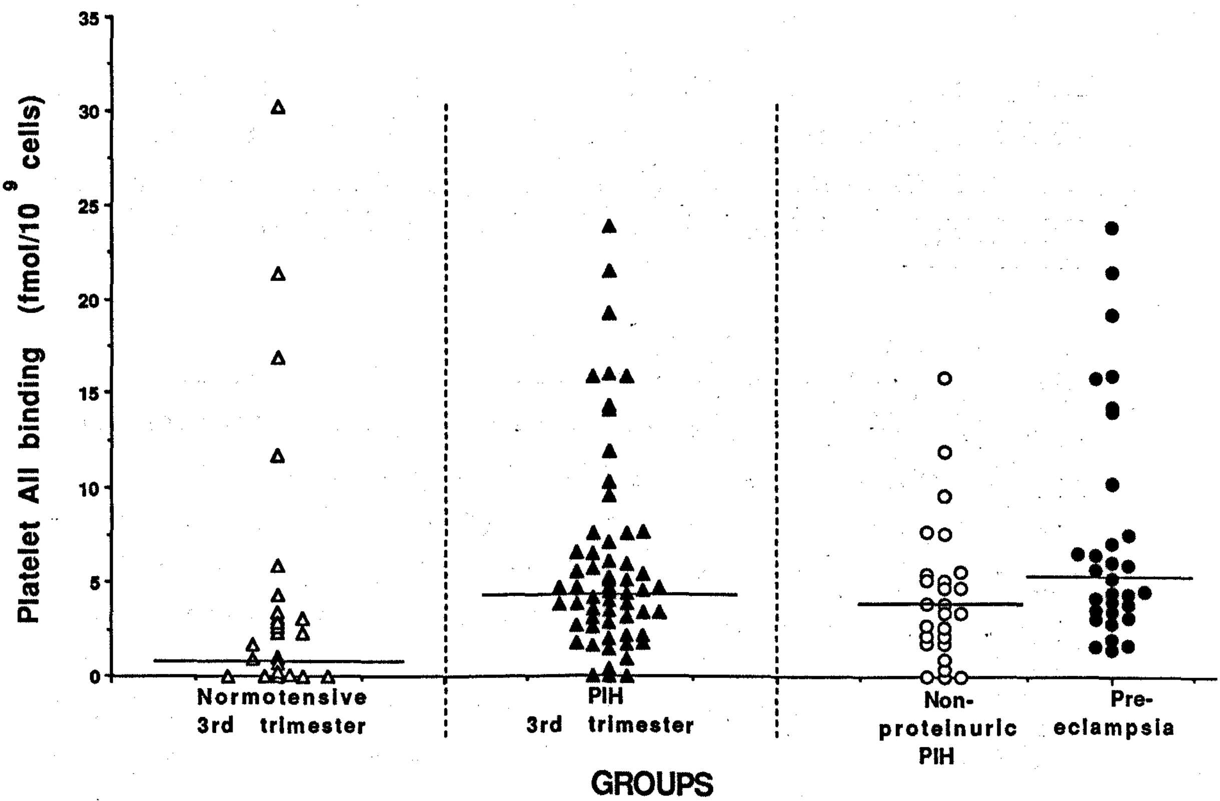
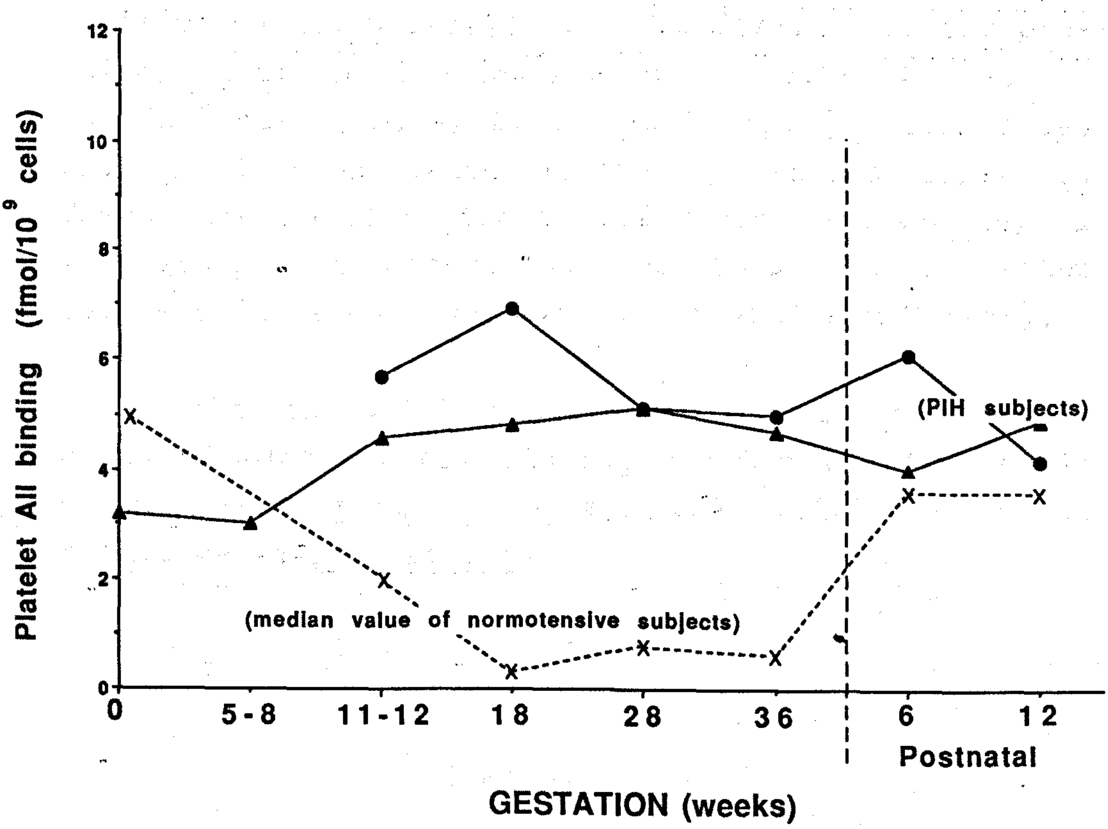


Figure 7.4.



study of normotensive pregnancy (Chapter 6) are also indicated in Figure 7.4. Neither of the PIH patients demonstrated the early-pregnancy fall in platelet AII binding that was found in the normotensive pregnant subjects.

As discussed in the previous chapter (p. 171), in the normotensive primigravidae, platelet AII binding was significantly increased at six weeks _post partum_ (3.9 fmol/10^9 cells, 1.6-6.9, $P<0.01$, Wilcoxon matched-pairs signed ranks test), with no further significant change at twelve weeks _post partum_ (3.9 fmol/10^9 cells, 0-6.5, $P>0.1$). In contrast, platelet AII binding in the PIH patients had not significantly changed at either six weeks _post partum_ (3.8 fmol/10^9 cells, 1.7-6.0, $P>0.1$) or twelve weeks _post partum_ (4.2 fmol/10^9 cells, 0.8-6.9, $P>0.5$). When the PIH patients were subdivided, there were no significant changes in platelet AII binding at either six or twelve weeks _post partum_ in the non-proteinuric PIH patients (3.7 fmol/10^9 cells, 1.3-6.1, and 5.3 fmol/10^9 cells, 0.5-8.4, respectively), or in the pre-eclampsia group (4.0 fmol/10^9 cells, 1.9-6.4, and 3.1 fmol/10^9 cells, 0.9-7.1, respectively) (in each case, $P>0.1$, Wilcoxon matched-pairs signed ranks test). There were no significant differences between any of the groups at either six or twelve weeks after delivery (in each case, $P>0.5$, Mann-Whitney U test).

In the essential hypertension group the median value of platelet AII binding was 3.9 fmol/10^9 cells at six weeks _post partum_ and 1.0 fmol/10^9 cells at twelve weeks _post partum_.

Hormonal data.

The median values of plasma angiotensin II (AII), plasma renin concentration (PRC) and plasma renin substrate (PRS) in the third trimester, and at six and twelve weeks after delivery, are summarised in Table 7.2.

In the third trimester, although the values of PRC were significantly higher in the normotensive primigravidae as compared to the PIH patients, there were no significant differences in the values of plasma AII or PRS between the two groups ($P>0.05$, Mann-Whitney U test). However, when the PIH patients were subdivided, further significant differences became apparent (Figure 7.5). The values of all three hormonal parameters were lower in the pre-eclampsia group as compared to the non-proteinuric PIH patients, with the differences approaching statistical significance in each case ($P= 0.01-0.04$). In the non-proteinuric PIH patients the values of plasma AII, PRC and PRS approximated to those of the normotensive primigravidae, with no significant differences between the two groups (although for PRC the difference approached significance, $P= 0.02$). In contrast, the values of PRC ($P<0.0001$) and PRS ($P<0.01$) were significantly lower in the pre-eclampsia group as compared to the normotensive primigravidae, with the difference between the values of plasma AII approaching significance ($P= 0.02$).

In the normotensive primigravidae, there were significant falls in the levels of all three hormones from 36 weeks gestation to six weeks post partum (in each case $P<0.001$, chapter 6, p. 172, Figure 6.3), with no further significant changes from six to twelve

Table 7.2.

Values of the hormonal parameters in the third trimester and at six and twelve weeks after delivery.

Median values are shown, with the interquartile range in brackets.

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects and the essential hypertensive women included for comparison.

Table 7.2

Third trimester groups

	Normotensive (n=30)	PIH (n=61)	Non protein-uric PIH (n=30)	Pre-eclampsia (n=31)	Essential hypertensive (n=6)
Angiotensin II (pg/ml)	30.9 (20.6-44.4)	21.7 (8.7-66.2)	32.4 (16.0-81.7)	10.7 (7.8-55.9)	25.3
P.R.C. (ng AI/hr/ml)	6.5 (4.3-9.6)	2.5 (1.3-4.8)	4.2 (2.0-9.2)	1.7 (0.9-2.5)	3.8
P.R.S. (ug AI/ml)	3.5 (3.2-4.0)	3.0 (2.6-3.6)	3.0 (2.7-3.9)	3.0 (2.3-3.3)	3.2

6 week postnatal groups

	Normotensive	PIH	Non protein-uric PIH	Pre-eclampsia	Essential hypertensive
Angiotensin II (pg/ml)	12.5 (8.8-16.1)	16.4 (10.5-22.7)	18.8 (9.9-24.1)	14.5 (9.2-23.9)	14.3
P.R.C. (ng AI/hr/ml)	2.0 (1.1-2.8)	2.1 (1.1-1.9)	2.7 (1.1-3.9)	1.9 (1.1-2.8)	1.5
P.R.S. (ug AI/ml)	1.3 (0.9-1.7)	1.2 (1.1-1.9)	1.1 (0.8-1.6)	1.2 (0.7-1.7)	1.6

12 week postnatal groups

	Normotensive	PIH	Non protein-uric PIH	Pre-eclampsia	Essential hypertensive
Angiotensin II (pg/ml)	11.7 (9.0-15.5)	16.0 (9.5-28.0)	16.6 (9.0-29.4)	14.8 (9.5-28.8)	16
P.R.C. (ng AI/hr/ml)	2.9 (1.0-3.8)	1.9 (1.4-2.8)	2.2 (1.5-3.3)	1.9 (1.3-2.7)	1.6
P.R.S. (ug AI/ml)	1.2 (1.0-1.9)	1.1 (0.8-1.8)	1.0 (0.8-1.7)	1.3 (0.8-2.8)	1.5

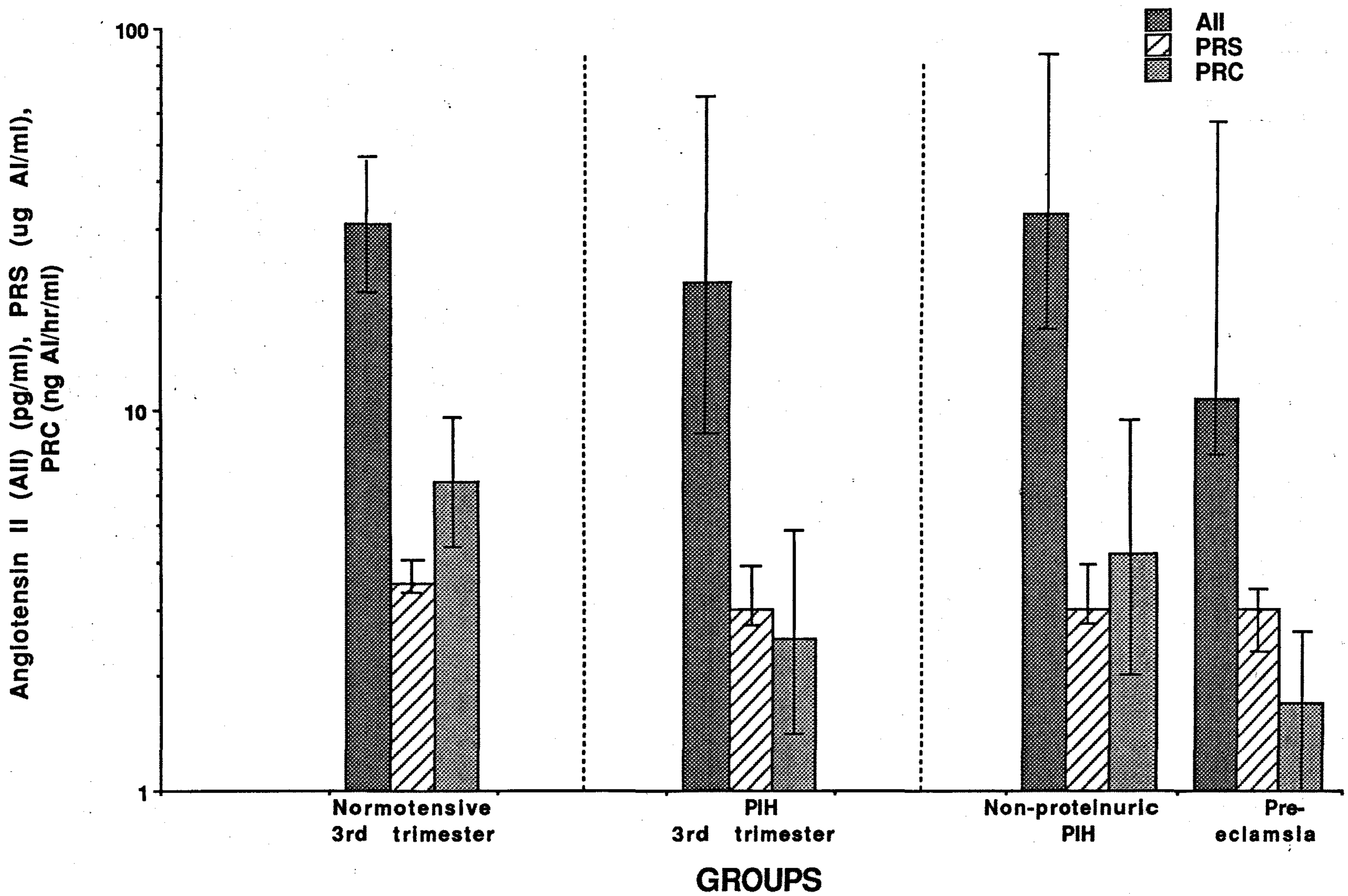
Figure 7.5.

Plasma angiotensin II (AII), plasma renin substrate (PRS), and plasma renin concentration (PRC) in the PIH patients in the third trimester.

Median values are shown, with the interquartile range marked.

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 7.5.


Angiotensin II (AII) (pg/ml), PRS (ug AI/ml),
PRC (ng AI/hr/ml)
100
10
1
AII
PRS
PRC
Normotensive
3rd trimester
PIH
3rd trimester
Non-proteinuric
PIH
Pre-
eclamsia
GROUPS

weeks _post partum_. In the PIH patients, there were no significant changes in the values of plasma AII or PRC (although the fall in the values of plasma AII from 36 weeks gestation to six weeks _post partum_ approached significance, P= 0.03, Wilcoxon matched-pairs signed ranks test), and there was a significant fall in PRS from 36 weeks gestation to six weeks _post partum_ (P<0.01). When the PIH patients were subdivided, in the non-proteinuric PIH patients, as in the normotensive primigravidae, there were significant falls in the levels of all three hormones from 36 weeks gestation to six weeks after delivery (in each case P<0.01), with no further significant changes from six to twelve weeks _post partum_. In the pre-eclampsia group, whilst the levels of PRS fell significantly from 36 weeks gestation to six weeks _post partum_ (P<0.01), the levels of plasma AII and PRC were increased after delivery, with the rise in plasma AII approaching significance (P= 0.04). There were no significant changes in the levels of plasma AII, PRC and PRS in the pre-eclampsia group from six to twelve weeks after delivery (in each case P>0.1).

At six and at twelve weeks after delivery, there were no significant differences in the values of any of the hormonal parameters between any of the groups (P>0.05).

In the normotensive primigravidae, the significant inverse correlations between platelet AII binding and the hormonal parameters have been discussed in the previous chapter (p. 187). There were no correlations found between platelet AII binding and the hormonal parameters when the PIH patients were investigated in the third trimester (P>0.1, Spearman correlation coefficient). However, there was a significant inverse correlation between platelet AII binding and plasma AII ($P<0.01$, $r_s= -0.44$) at six weeks _post partum_, and at twelve weeks _post partum_ the inverse correlation approached significance ($P= 0.04$, $r_s= -0.29$). When the PIH patients were subdivided, there was a significant inverse correlation between plasma AII and platelet AII binding in the pre-eclampsia group at six weeks _post partum_ ($P<0.01$, $r_s= -0.53$). This correlation was not present at twelve weeks _post partum_ ($P>0.1$), and was not present at either six or twelve weeks after delivery when the non-proteinuric PIH patients were considered ($P>0.1$). There were no correlations at any time between platelet AII binding and either PRC or PRS in any of the PIH groups (in each case $P>0.1$).

Haematological data.

Contamination of the platelet concentrate by leucocytes was <1% ($1-5 \times 10^3/\mu l$) and no erythrocytes were detected.

The median values of platelet count, mean platelet volume (MPV) and platelet distribution volume (PDW), in the third trimester and at both six and twelve weeks after delivery are summarised in Table 7.3.

In the third trimester, the MPV and the PDW were significantly greater in the PIH patients as compared to the normotensive primigravidae ($P<0.0001$ and $P<0.001$ respectively, Mann-Whitney U test). The difference in platelet count between the two groups approached significance ($P= 0.04$). When the PIH patients were subdivided, the difference in the platelet count between the non-proteinuric PIH

Table 7.3.

Values of the platelet parameters in the third trimester and at six and twelve weeks after delivery.

Median values are shown, with the interquartile range in brackets.

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects and the essential hypertensive women included for comparison.

Table 7.3

Third trimester groups

	Normotensive (n=30)	PIH (n=61)	Non protein-uric PIH (n=30)	Pre-eclampsia (n=31)	Essential hypertensive (n=6)
Platelet count (x 10^9/L)	208 (174-244)	190 (154-232)	221 (162-258)	173 (128-209)	213
Mean platelet volume (fl)	8.5 (8.2-8.9)	9.4 (8.6-10.1)	9.2 (7.9-10.0)	9.7 (9.1-10.2)	8.5
Platelet distribution width	16.1 (15.4-16.9)	17.1 (16.1-18.1)	16.9 (15.1-17.7)	17.3 (17.0-18.0)	16.3

6 week postnatal groups

	Normotensive	PIH	Non protein-uric PIH	Pre-eclampsia	Essential hypertensive
Platelet count (x 10^9/L)	226 (187-259)	246 (209-278)	240 (199-283)	246 (209-270)	311
Mean platelet volume (fl)	8.1 (7.7-8.6)	8.3 (7.8-9.1)	8.1 (7.8-8.9)	8.4 (7.9-9.6)	7.8
Platelet distribution width	15.6 (14.8-16.1)	15.9 (15.2-16.3)	16.0 (15.2-16.1)	15.8 (15.3-17.0)	15.4

12 week postnatal groups

	Normotensive	PIH	Non protein-uric PIH	Pre-eclampsia	Essential hypertensive
Platelet count (x 10^9/L)	235 (211-278)	238 (211-272)	246 (202-269)	223 (210-261)	297
Mean platelet volume (fl)	8.1 (7.7-8.6)	8.3 (7.9-8.8)	8.3 (7.8-8.7)	8.4 (8.0-9.1)	7.9
Platelet distribution width	15.6 (14.9-16.4)	15.8 (15.2-16.4)	15.8 (15.2-16.5)	15.8 (15.2-16.5)	15.2

patients and the pre-eclampsia group approached significance ($P= 0.02$) (Figure 7.6). Moreover, when the non-proteinuric PIH patients were compared with the normotensive primigravidae the only significantly different platelet parameter was the MPV ($P<0.001$), whereas when the pre-eclampsia group were compared with the normotensive primigravidae there were significant differences in the platelet count ($P<0.01$), the MPV ($P<0.0001$) and the PDW ($P<0.001$) (Figure 7.7).

In the normotensive primigravidae, the changes in the platelet parameters following delivery have already been discussed in the previous chapter (p. 188). In the PIH patients there was a significant rise in platelet count ($P<0.01$, Wilcoxon matched-pairs signed-ranks test) at six weeks after delivery as compared to at 36 weeks gestation, with significant falls in MPV ($P<0.001$) and PDW ($P<0.0001$). When the PIH patients were subdivided, the changes in all three parameters were significant in the pre-eclampsia group ($P<0.001$ in each case), whereas when the non-proteinuric patients were considered only the fall in MPV was significant ($P<0.001$). There were no significant changes from six to twelve weeks <u>post partum</u> in any of the PIH groups ($P>0.1$). There were no significant differences in any of the platelet parameters between any of the groups at either six or twelve weeks after delivery ($P>0.05$, Mann-Whitney U test).

In the third trimester, there was no significant difference in the proportion of platelets in whole blood that were recovered in the prepared platelet suspension (platelet yield) between the PIH patients (47%, 38-55) and the normotensive primigravidae (43%,

Figure 7.6.

Platelet count in the PIH patients in the third trimester.

Median values are shown, with the interquartile range marked.
The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 7.7.

Mean platelet volume (MPV) and platelet distribution width (PDW) in the PIH patients in the third trimester.

Median values are shown, with the interquartile range marked.
The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 7.6.

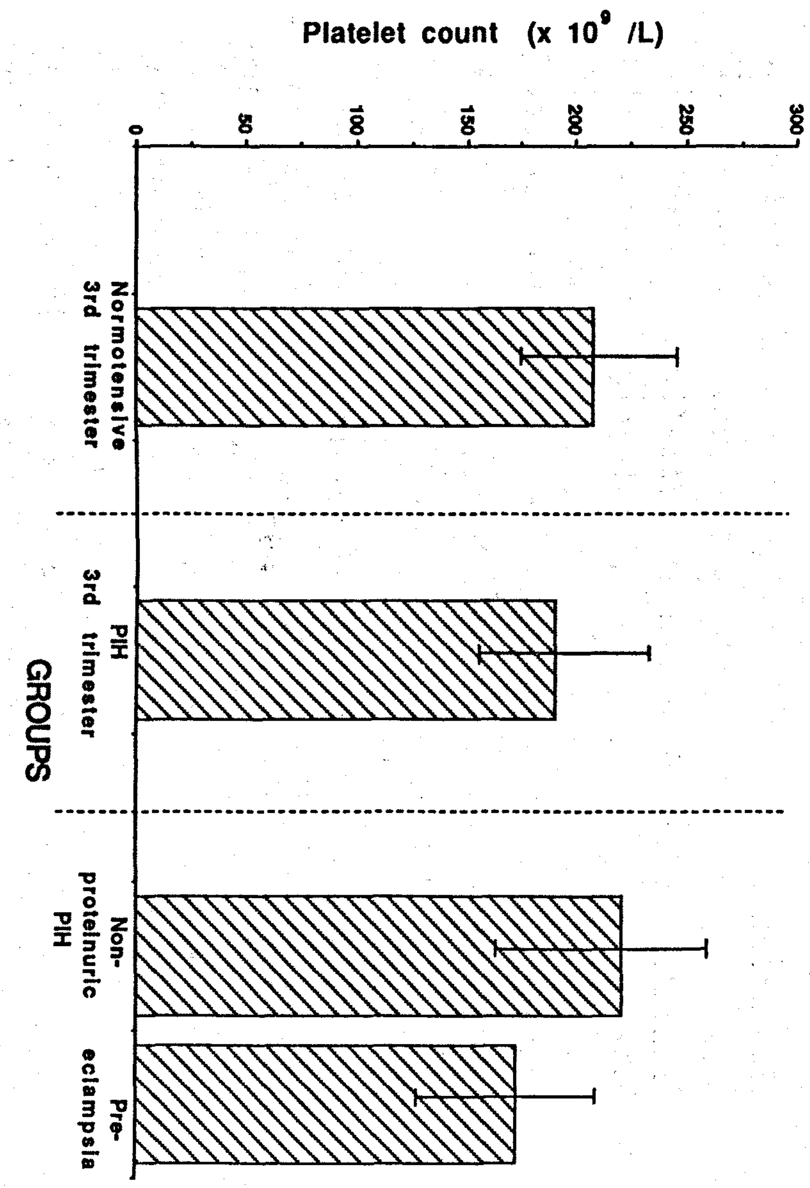
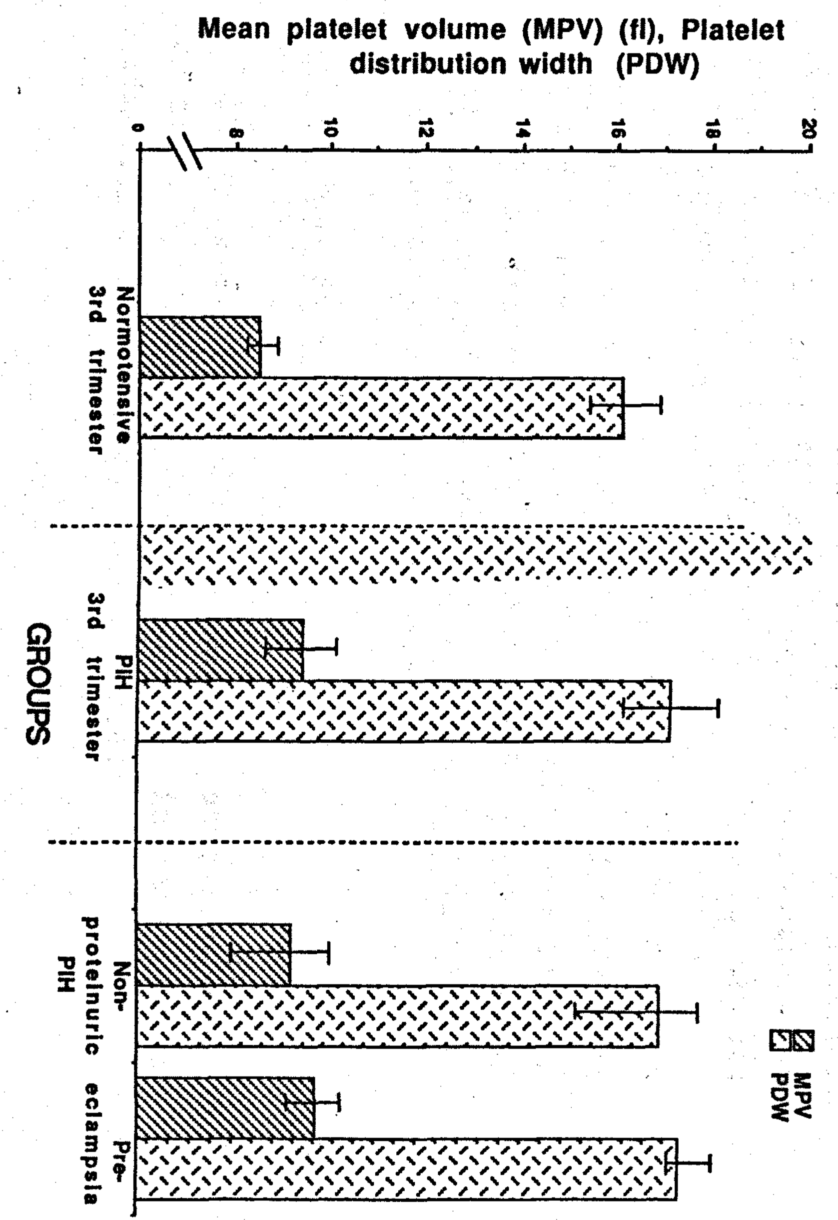


Figure 7.7.



35-58, $P>0.5$, Mann-Whitney U test). No significant differences were revealed when the PIH patients were subdivided. Similarly, there were no significant differences in platelet yield between any of the groups at either six or twelve weeks after delivery ($P>0.1$), with values in the PIH patients of 55% at six weeks post partum and 50% at twelve weeks post partum.

In the third trimester PIH patients, values of haemoglobin concentration (12.2 g/dl, 11.4-12.8) and white cell count (WCC) (9.1×10^9/L, 7.8-11.2) were higher than in the normotensive primigravidae (11.8 g/dl, 10.0-12.5, and 8.3×10^9/L, 7.4-10.2), but these differences were not significant ($P>0.1$). No significant differences were revealed when the PIH patients were subdivided. In the PIH patients following delivery there were no significant changes in haemoglobin concentration, although the WCC at six weeks post partum (6.3×10^9/L, 5.2-7.1) was significantly lower than that at 36 weeks gestation ($P<0.01$). There were no significant differences in either parameter between any of the groups at six weeks or at twelve weeks post partum.

There was no correlation between platelet AII binding and any of the haematological parameters in the PIH patients in the third trimester, at six weeks post partum or at twelve weeks post partum ($P>0.1$, Spearman correlation coefficient).

Biochemical data.

The median values of serum sodium, potassium, urea, creatinine, urate and osmolality in the third trimester are summarised in Table 7.4.

Table 7.4.

Serum biochemical data in the third trimester.

Median values are shown, with the interquartile range in brackets.

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects and the essential hypertensive women included for comparison.

Table 7.4

SERUM	Normotensive (n=30)	PIH (n=61)	Non-proteinuric (n=30)	Pre-eclampsia (n=31)	Essential hypertension (n=6)
Sodium (mmol/L)	136 (133-137)	137 (135-138)	137 (135-138)	138 (135-139)	134
Potassium (mmol/L)	4.4 (4.1-4.6)	4.2 (4.0-4.6)	4.3 (4.0-4.7)	4.2 (4.0-4.5)	4.3
Urea (mmol/L)	2.1 (1.9-2.6)	3.3 (2.6-4.1)	2.9 (2.4-3.9)	3.7 (3.0-4.4)	2.3
Creatinine (umol/L)	57 (49-65)	75 (63-83)	67 (59-78)	82 (73-90)	60
Urate (umol/L)	259 (211-289)	317 (256-398)	308 (219-408)	339 (291-398)	281
Osmolality (Osm/L)	276 (275-279)	279 (276-280)	278 (278-282)	279 (277-280)	278

In the third trimester, the values of serum urea, creatinine and urate were significantly higher in the PIH patients as compared to the normotensive primigravidae (in each case $P<0.0001$, Mann-Whitney U test), and the differences in serum sodium ($P= 0.02$) and serum osmolality ($P= 0.01$) approached significance. The differences in serum urate are illustrated in Figure 7.8. When the PIH patients were subdivided, the differences in serum sodium ($P= 0.04$), urea ($P= 0.03$) and creatinine ($P= 0.02$) approached significance. However, at both six weeks and at twelve weeks after delivery, there were no significant differences in any of the serum biochemical parameters between any of the groups ($P>0.05$).

The correlations between platelet AII binding and the serum biochemical parameters in the normotensive primigravidae have been discussed in the previous chapter (p. 191). In the third trimester PIH patients there was a significant correlation between platelet AII binding and serum sodium concentration ($P<0.01$, $r_s= 0.37$, Spearman correlation coefficient). When the PIH patients were subdivided, there was no such correlation in the pre-eclampsia group ($P>0.1$), although in the non-proteinuric PIH patients the correlation approached significance ($P= 0.03$, $r_s= 0.44$). There were no correlations found in the PIH patients, between platelet AII binding and any of the other serum biochemical parameters.

At six weeks after delivery, there were no correlations found between platelet AII binding and any of the serum biochemical parameters when the PIH group as a whole was considered. However, in the non-proteinuric PIH subgroup, the correlation between platelet AII binding and serum sodium concentration

Figure 7.8.

Serum urate concentrations in the PIH patients in the third trimester.

Median values are shown, with the interquartile range marked.
Serum urate concentrations were significantly higher in the PIH patients as compared to the normotensive women ($P<0.0001$).
The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 7.9.

Glomerular filtration rate in the PIH patients in the third trimester.

Median values are shown, with the interquartile range marked.
The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects included for comparison.

Figure 7.8.

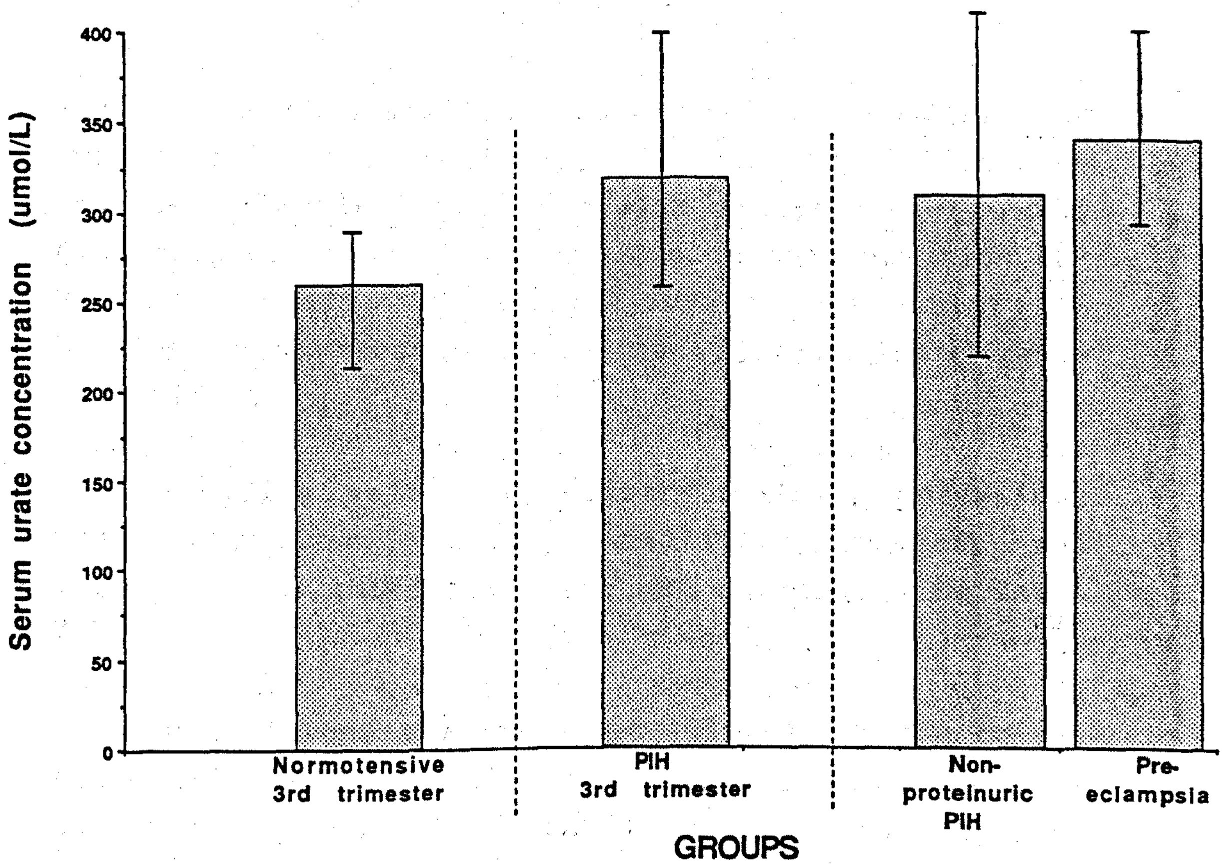
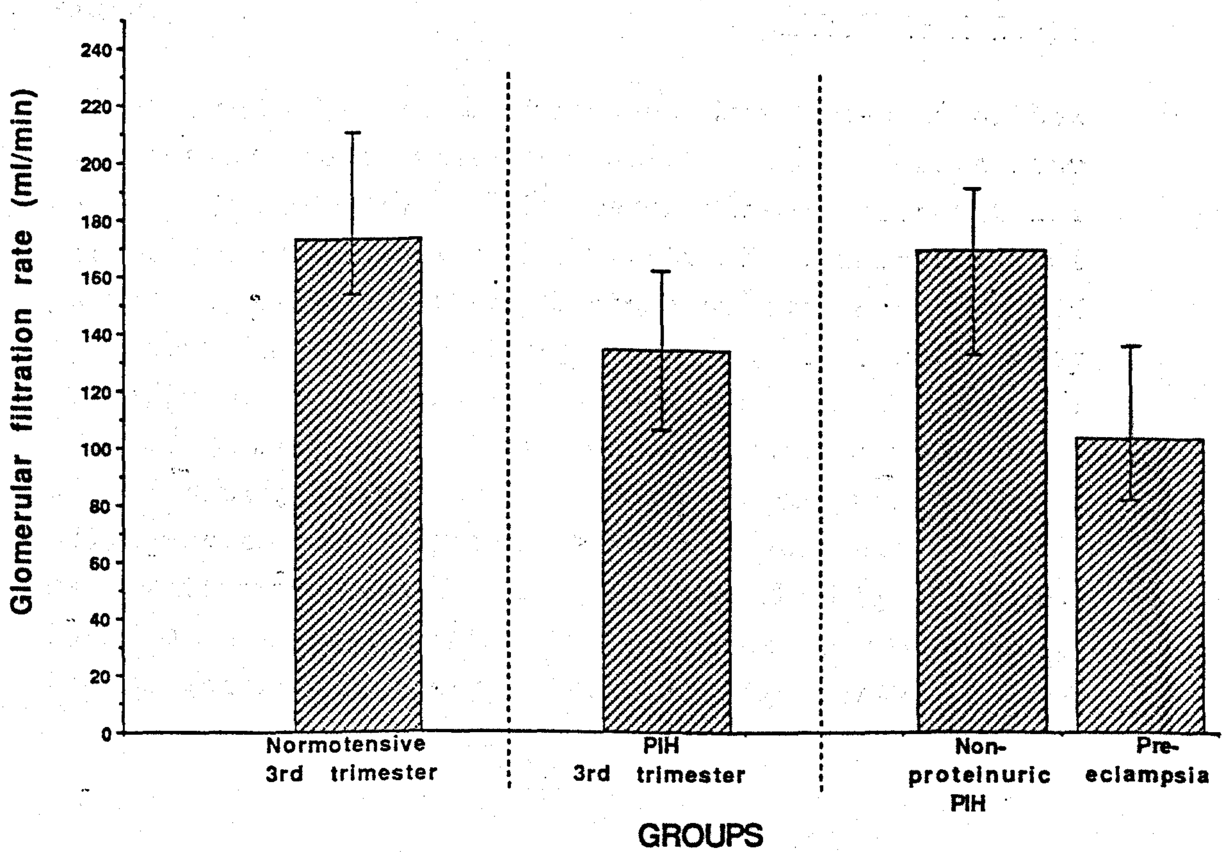


Figure 7.9.



again approached significance ($P = 0.04$, $r_s = 0.41$). At twelve weeks after delivery, the only correlation with platelet AII binding to approach significance in the PIH group was that with serum sodium concentration ($P = 0.04$, $r_s = 0.30$). No correlations with platelet AII binding were found when the PIH patients were subdivided.

The median values of urinary sodium, potassium, urea, creatinine, urate, osmolality, protein and volume in the third trimester are summarised in Table 7.5.

By definition, the values of urinary protein in the third trimester were significantly higher in the pre-eclampsia group than in both the normotensive primigravidae ($P<0.0001$, Mann-Whitney U test) and the non-proteinuric PIH patients ($P<0.0001$). These differences are illustrated in Figure 7.1. There were no other significant differences in any of the measured urinary biochemical parameters (in each case $P>0.05$).

The following derived values for renal function were calculated: urinary sodium/urinary creatinine ratio, glomerular filtration rate, fractional excretion of sodium, fractional excretion of potassium, osmolar clearance and water clearance (as outlined in Chapter 6, p. 180). There were significant differences between the groups in the derived values of glomerular filtration rate, with significantly higher values found in the normotensive primigravidae as compared to the PIH patients ($P<0.01$, Mann-Whitney U test), and significantly higher values found in non-proteinuric PIH patients as compared to the pre-eclampsia group ($P<0.001$) (Figure 7.9). There

Table 7.5.

Urinary biochemical data in the third trimester.

Median values are shown, with the interquartile range in brackets.

The PIH patients have been subdivided on the basis of significant proteinuria. The overall data from the PIH group and the subdivided data are shown, with the normotensive subjects and the essential hypertensive women included for comparison.

Table 7.5

URINARY	Normotensive (n=30)	PIH (n=61)	Non-proteinuric PIH (n=30)	Pre-eclampsia (n=31)	Essential hypertension (n=6)
Sodium (mmol/L)	100 (76-118)	98 (70-131)	91 (67-125)	110 (69-132)	92
Potassium (mmol/L)	45 (36-53)	53 (40-66)	47 (44-49)	57 (43-73)	50
Urea (mmol/L)	17 (14-21)	20 (16-26)	18 (16-27)	22 (16-26)	18
Creatinine (umol/L)	7.6 (6.3-9.8)	8.3 (6.3-10.3)	8.1 (6.1-9.7)	9.4 (6.5-12.5)	7.5
Urate (umol/L)	2.4 (1.8-3.1)	2.2 (1.8-2.9)	2.0 (1.4-2.8)	2.5 (2.0-4.1)	2.4
Osmolality (mmol/kg)	506 (459-628)	543 (417-688)	497 (394-677)	554 (435-739)	555
Protein (g/L)	0.07 (0.03-0.09)	0.42 (0.13-1.48)	0.13 (0.07-0.20)	1.43 (0.65-3.15)	0.92
Volume (L/24hrs)	1.3 (1.0-1.5)	1.2 (0.8-1.7)	1.4 (0.9-1.9)	0.9 (0.7-1.3)	1.4
Fractional excretion of sodium	0.49 (0.43-0.65)	0.59 (0.44-0.82)	0.56 (0.46-0.72)	0.69 (0.43-0.87)	0.51

were no other significant differences between the groups although the difference in the urinary sodium/urinary creatinine ratios between the pre-eclampsia group and the normotensive primigravidae approached significance ($P= 0.02$), as did the differences in fractional excretion of sodium between the normotensive primigravidae as compared to both the total PIH group and the pre-eclampsia patients ($P= 0.03$ and $P= 0.02$ respectively). The values of fractional excretion of sodium in each of the groups are given in Table 7.5.

At six and at twelve weeks post partum, the only significant difference in either the measured urinary parameters or the derived values of renal function between the groups was a significantly greater urinary volume in all the PIH groups as compared to the normotensive primigravidae ($P<0.01$).

There were no significant correlations found between any of the measured urinary parameters and platelet AII binding, in any group, at any time ($P>0.05$). The only derived value of renal function for which a correlation with platelet AII binding approached significance was the fractional excretion of sodium, when the PIH group as a whole was considered, in the third trimester ($P= 0.02$, Spearman correlation coefficient $r_s= 0.33$).

Discriminant analysis.

The use of platelet AII binding to discriminate between the normotensive primigravidae and the PIH patients, in the third trimester, was assessed using canonical discriminant analysis (discussed on p. 160).

There was a false positive rate of 42% and a false negative rate of 14%, 67% of patients being correctly classified. When the hormonal, haematological and biochemical parameters measured in this study were used to discriminate between the two groups of third trimester patients, the only parameters which correctly classified more patients were serum urate and MPV (correct classification in 76% and 75% of cases respectively). The results of such discriminant analysis were not markedly improved when platelet AII binding was combined with either serum urate (68% of patients correctly classified) or MPV (77% of patients correctly classified). However, when all three parameters were combined there was a false positive rate of 27% and a false negative rate of 1%, 81% of patients being correctly classified.

When the above parameters were used to discriminate between the normotensive primigravidae and the pre-eclampsia group, in the third trimester, similar results were obtained. With platelet AII binding as the discriminant, there was a false positive rate of 48% and a false negative rate of 14%, 68% of patients being correctly classified. Serum urate and MPV both correctly classified 80% of patients. Platelet AII binding combined with serum urate resulted in correct classification of 80% of patients and combined with MPV resulted in correct classification of 81% of patients. When all three parameters were combined, there was a false positive rate of 26% and a false negative rate of 4%, 85% of patients being correctly classified.

Discussion

The results of this study of platelet AII binding in normotensive and hypertensive primigravidae are similar to those of the small pilot study described in Chapter 5. The two studies will thus be discussed together.

This study provides further evidence to support the use of platelet AII binding as a model of vascular responsiveness to AII. Just as the fall of platelet AII binding in early pregnancy (described in the previous chapter) parallels the diminution in pressor response to infused AII [Gant, Daley, Chand, Whalley and Macdonald, 1973], in PIH the increased levels of platelet AII binding in comparison with normotensive primigravidae parallels the increased AII pressor responsiveness found in PIH [Gant et. al., 1973].

The pilot study was the first reported study of platelet AII binding in patients with PIH. Subsequently, Pawlak and Macdonald [1990] reported the preliminary results of a study of platelet AII binding sites in hypertensive pregnancy. Although the numbers in their study were small (16 in each group) and the definition of PIH used was an increase in diastolic blood pressure of 20 mmHg. or more, Pawlak and Macdonald also found that whilst platelet AII binding in women with PIH was lower than that of non-pregnant women, it was significantly greater than that of normotensive women in the third trimester of pregnancy.

Only two of the patients who developed PIH had been studied on a longitudinal basis. However, the results from these two women support the hypothesis that the elevated levels of platelet AII binding in women with PIH as compared to the normotensive primigravidae are not due to a rise in platelet AII binding towards the end of pregnancy in PIH patients, but instead, are due to the early pregnancy fall in platelet AII binding found in normotensive pregnancy, not occurring.

Although platelet AII binding was higher in the pre-eclampsia group than in the patients with non-proteinuric PIH, the difference was not significant. This is in marked contrast to the significant differences in certain of the haematological, biochemical and in particular the hormonal parameters (all discussed below). Moreover, there were highly significant differences in platelet AII binding between the normotensive primigravidae and both of the PIH subgroups. As discussed in the Introduction (Sections 1 and 3), the majority of the haematological, biochemical and hormonal differences between normotensive and hypertensive pregnant women are only apparent shortly before, or indeed after, the rise in blood pressure. If the differences in platelet AII binding are more fundamental, occurring at a much earlier gestation, this suggests a link between the aetiology of non-proteinuric PIH and pre-eclampsia. The finding that in women with fulminating pre-eclampsia, the levels of platelet AII binding were no higher than in the other PIH patients also supports this suggestion.

Only a small number of women with essential hypertension were included in the study. Nevertheless, the finding that the values of platelet AII binding in this group approximated to those of the normotensive primigravidae and were much lower than those of the PIH patients suggests that essential hypertension in pregnancy is an entirely different condition to pregnancy-induced hypertension, and provides further evidence to support the concept that platelet AII binding is central to the development of pregnancy-induced hypertension. A study of non-pregnant individuals also found that values of platelet AII binding in normotensive subjects were similar to those of individuals with essential hypertension [Ding, Kenyon and Semple, 1985].

As outlined in the Introduction (Section 3, p. 49), there is some consensus that in pre-eclampsia, activity of the renin-angiotensin system is suppressed [e.g. Symonds and Andersen, 1974], whilst in mild non-proteinuric PIH, activity is increased [e.g. Symonds, Broughton Pipkin and Craven, 1975]. The results of this study confirm that the renin-angiotensin system is suppressed in pre-eclampsia, and although no evidence of activation in non-proteinuric PIH was found, differences between the two PIH subgroups were, indeed, demonstrated.

The two studies described in this thesis, and that of Pawlak and Macdonald [1990], all failed to demonstrated any correlation between platelet AII binding and any of the hormonal parameters, in the PIH patients in the third trimester. This is in contrast to the correlations, discussed in the previous chapter, found in normotensive third trimester subjects. That in these women, regulation of platelet AII binding must be more complex than straightforward down-regulation by plasma AII, is emphasised by the non-proteinuric PIH patients. Although the median value of platelet AII binding in this group was almost six times that of the normotensive third trimester subjects, plasma AII levels in the two groups were similar.

Platelet pathophysiology in pregnancy-induced hypertension has been discussed in the Introduction (Section 7, p 103). The decreased platelet number, increased MPV and increased PDW found in pre-eclamptic patients in this study, have previously been demonstrated on numerous occasions [e.g. Giles and Inglis, 1981], the increased MPV and increased megakaryocytes found in the bone marrow [Thiagarajah, Bourgeois, Harbert and Candle, 1984] indicating increased platelet turnover. Only one of the PIH patients was thrombocytopaenic (defined as a platelet count of less than 100 x 10^9/L). This probably reflects the exclusion of the most severely affected individuals, who were delivered before the twenty four hours necessary to make the diagnosis.

It seems most unlikely that differences in any of the measured platelet parameters between the two groups were responsible for the differences in platelet AII binding. No correlation between platelet AII binding and any of the platelet parameters was found in any of the groups. In addition, values of platelet AII binding were not affected by donation of large volumes of blood, another situation in which platelet turnover is increased (Chapter 2, p. 125).

Louden, Heptinstall, Broughton Pipkin, Mitchell and Symonds [1988] demonstrated that adrenaline-induced platelet aggregation was increased in both non-proteinuric PIH patients and women with pre-eclampsia. This finding is consistent with the elevated levels of platelet AII binding in these groups, Poplawski [1970] and Ding, MacIntyre, Kenyon and Semple [1985] having demonstrated a potentiation of the adrenaline-induced platelet aggregation by AII.

A possible mechanism by which platelet AII binding is increased in PIH has been suggested by Gleeson, Ahmed, Rice-Evans and Elder [1990]. Gleeson et. al. [1990] have suggested that the platelet membrane in women with pre-eclampsia has an inherited or acquired defect, perhaps due to a change in the phospholipid content ratio and glycocalyx structure on the surface of the platelet membrane, which causes an increase in membrane viscosity. It is believed that alterations in cell membrane fluidity can affect the accessibility of cell membrane proteins which act as binding sites. Increasing the viscosity of a cell membrane decreases its capacity to solubilise integral proteins, displacing the proteins towards the aqueous phase on either side of the membrane [Borochov and Shinitzky, 1976]. This 'vertical displacement' of membrane proteins may thus allow previously concealed binding sites to be unmasked.

Platelet membrane fluidity can be modified <u>in vivo</u> and <u>in vitro</u>. Dietary n-3 polyunsaturated fatty acids, which decrease membrane viscosity, inhibit platelet aggregation [Vas Dias, Gibney and Taylor, 1982] and enrichment of platelet membranes with cholesterol <u>in vitro</u> increases membrane viscosity and sensitises platelets to aggregation by ADP and adrenaline [Shattil and Cooper, 1976]. In a small pilot study, Gleeson et. al. [1990] determined the relative membrane viscosity in platelets from six pregnant women, three of whom had PIH, and three of whom were normotensive controls. They found significantly higher platelet membrane viscosity in the PIH patients.

In this study, the pre-eclampsia group were noted to have a significantly lower median creatinine clearance (reflecting a reduced glomerular filtration rate) than either the normotensive primigravidae or the non-proteinuric PIH patients. This finding has been noted in the past, with glomerular filtration rate and effective renal plasma flow being reduced by 30% and 20% respectively in pre-eclamptic women [Lindheimer, Chesley, Taylor, Spargo and Katz, 1987], and may be due to increased intrarenal sensitivity to AII, AII having a potent vasoconstricting effect on renal vascular smooth muscle [reviewed by Mendelsohn, 1985, discussed in the Introduction, p. 36]. The elevated serum urea and uric acid concentrations, found in this study in the PIH patients, have also been well described previously, being thought to reflect a decline in renal function [Redman, Beilin and Bonnar, 1976].

In the pregnant normotensive primigravidae there was a direct correlation between platelet AII binding and and the serum sodium concentration (discussed in the previous chapter, p. 191). In the third trimester PIH patients this correlation was again found, and the possibility remains that in pregnancy, it is the serum sodium concentration rather than the plasma AII level which regulates platelet AII binding. Consideration of possible mechanisms by which such regulation may act is included in the General Discussion (Chapter 9, p. 239).

There were very few differences found between any of the groups at either six or twelve weeks post partum (although by definition, the values of systemic blood pressure were higher in the women with essential hypertension). However, the volumes of the 24 hour urinary collections were significantly lower in the normotensive primigravidae than in either of the PIH groups. Whilst there may have been poor compliance amongst the normotensive primigravidae (discussed in the previous chapter, p. 192), suffering a complication of their pregnancy presumably increased the motivation of the PIH patients.

The use of canonical discriminant analysis confirmed the finding of the pilot study, in that platelet AII binding appears to have only limited potential as a marker for PIH. Although combining the three parameters produced the highest proportion of correctly predicted outcomes, both MPV and serum urate estimations proving better at discriminating between normotensive third trimester women and those with established PIH. Interestingly, these results when platelet AII binding is used in discriminating between normotensive primigravidae and women with established PIH are very similar to those achieved when the AII infusion test (discussed in the Introduction, p. 14) was used to predict whether PIH would develop in pregnancy. Oney and Kaulhausen [1982] found a false positive rate of 55% in European women using the AII infusion test, although the false negative rate was only 5%.

In normotensive pregnancy, the diminution in platelet AII binding parallels closely the diminution in pressor sensitivity to AII [Gant, Daley, Chand, Whalley and Macdonald, 1973, discussed in the Introduction p. 14 and p. 50]. If the same is true of pregnancies complicated by PIH, and the data from the two longitudinal patients whose pregnancies were complicated by PIH suggests that this is so, then the increased platelet AII binding as compared to normotensive pregnant women should be detectable well before the clinical development of PIH. The potential use of the technique as a screening test for PIH may thus be much greater.

To investigate this possibility, it was decided to perform both the platelet AII binding estimation and the AII pressor sensitivity test in a group of normotensive, primiparous pregnant women, in order to establish whether co-linearity between the two methods existed, and to assess the success of each test in predicting the outcomes of the pregnancies. This study is described in the next chapter.

Chapter 8.

PARALLEL STUDIES OF PLATELET AII BINDING AND AII PRESSOR RESPONSIVENESS.

This chapter describes a study of the relationship between platelet angiotensin II (AII) binding and the pressor responsiveness to AII (as indicated by the AII sensitivity test, discussed in the Introduction, p. 14). This relationship was studied in a group of pregnant women, and in a smaller group of non-pregnant women. By comparing the results of the two techniques obtained from the pregnant subjects, and by following-up the outcomes of the pregnancies, the potential of the two methods as screening tests for pregnancy-induced hypertension (PIH) could also be assessed.

Study sample.

Pregnant subjects.

Primiparous pregnant women, in whom an initial diastolic blood pressure recording of 80-85 mmHg. was made at their second antenatal clinic visit (at the University Hospital, Nottingham this is at 28-32 weeks gestation), were asked if they would participate in the study. 37 patients were thus recruited. However, the arterial blood pressure of three subjects failed to stabilise prior to the AII infusion (as detailed in the protocol below), and these patients were excluded from the study. The diastolic blood pressures of all the remaining 34 pregnant subjects settled to <70 mmHg. prior to the AII infusion.

None of the 34 pregnant women were known to be suffering from renal, metabolic or cardiovascular disease. All subjects were receiving sodium intake _ad libitum_ and had normal urea, creatinine and electrolyte estimations. The gestational age of each subject was confirmed by an ultrasound scan at eighteen weeks gestation.

10 of the 34 women developed PIH subsequent to the AII sensitivity test and the platelet AII binding estimation (subsequent PIH group). Six of the subsequent PIH group developed pre-eclampsia and four developed non-proteinuric PIH, the definitions used being those described in detail in the Introduction (p. 9). The other 24 pregnancies remained normotensive throughout (normotensive group). In order to minimise bias, the results of the experiments were not available to the obstetricians responsible for the medical care of the patients, and the outcome of the pregnancies was assessed by a colleague who likewise did not have access to the experimental results.

All of the patients, apart from one Asian subject in the normotensive group, were Caucasian. None of the subsequent PIH group were taking any medication apart from iron supplementation. Of the normotensive group, two patients were using antacids, one was taking oral antibiotics and one occasionally used a salbutamol inhaler. Two (20%) of the subsequent PIH group and three (13%) of the normotensive group continued to smoke whilst pregnant. Five patients (50%) in the subsequent PIH group gave a history of essential hypertension in a first degree relative as compared to seven (29%) in the normotensive group. Four (40%) of the subjects in the subsequent PIH group stated that their mothers had suffered PIH as compared to 5 (21%) in the normotensive group.

The median maternal ages, booking visit systemic blood pressures (at the University Hospital, Nottingham, this is at eighteen weeks gestation), gestational ages at the time of the infusion, maternal weights at the time of the infusion and the systemic blood pressures immediately prior to the infusion, of both the normotensive and the subsequent PIH groups, are summarised in Table 8.1.

There were no significant differences in any of these parameters when measured at the time of the infusion ($P>0.05$, Mann-Whitney U test). However, the booking visit systolic blood pressure was significantly higher in the subsequent PIH group ($P<0.01$), and the difference in booking visit diastolic blood pressures approached significance ($P= 0.02$).

The gestational ages at delivery of the subsequent PIH group (median 37.5 weeks, 34-39) were significantly lower than those of the normotensive group (median 40 weeks, 39-41, $P<0.01$, Mann-Whitney U test). Of the normotensive group, seven women (29%) had a forceps delivery, and two women (8%) were delivered by caesarean section (both for fetal distress in the first stage of labour); the remainder had spontaneous vaginal deliveries. In contrast, in the subsequent PIH group, only three women (30%) had vaginal deliveries (forceps being used in one case), the remainder being delivered by caesarean section. The birthweights of the subsequent PIH group (median 2.86 Kg., 2.29-3.20) were significantly lower than those of the normotensive group (median 3.42 Kg., 3.19-3.54, $P<0.01$). Ten (42%) of the birthweights in the normotensive group were below the 50th centile for gestational age, as compared to seven (70%) of the birthweights in the subsequent PIH group. Eight (80%) of the infants in the subsequent PIH group were male, as were fourteen infants (58%) in the normotensive group.

Table 8.1.

Maternal ages, booking visit systemic blood pressures, gestational ages, systemic blood pressures prior to the infusion, and maternal weights at the infusion, of the pregnant subjects.

The pregnant subjects have been subdivided into those patients who remained normotensive following investigation, and those who subsequently developed PIH.

Despite the small number of subjects in the PIH group, in order to aid comparison between the two groups, the interquartile range is quoted in addition to the median.

Table 8.2.

Ages, systemic blood pressures prior to the infusion, and body weights at the infusion, of the non-pregnant subjects.

Median values are shown, with the range in brackets.

Table 8.1

	Normotensive group n=24	Subsequent PIH group n=10
Maternal age (years)	24.0 (21.5-27.0)	24.0 (19.5-31.5)
Booking visit systolic blood pressure (mmHg.)	115 (110-120)	125 (120-133)
Booking visit diastolic blood pressure (mmHg.)	70 (60-70)	77 (70-80)
Gestational age at infusion (weeks)	32.0 (31.5-32.5)	30.5 (28.0-32.0)
Systolic blood pressure prior to infusion (mmHg.)	117 (114-122)	121 (115-124)
Diastolic blood pressure prior to infusion (mmHg.)	65 (60-69)	66 (61-70)
Maternal weight at infusion (Kg)	75.0 (65.5-82.1)	78.8 (71.2-88.4)

Table 8.2

	Non-pregnant group n=24
Age (years)	27.0 (16.0-44.0)
Systolic blood pressure prior to infusion (mmHg.)	115 (109-129)
Diastolic blood pressure prior to infusion (mmHg.)	68 (58-76)
Body weight at infusion (Kg)	57.5 (52.0-73.0)

Non-pregnant subjects.

Ten nulliparous, normotensive Caucasian women were recruited into the study. The blood pressure of one subject failed to stabilise prior to the infusion. Nine subjects were thus included in the study. They were all investigated between days 5-9 of the menstrual cycle. No subject was taking any medication, including hormonal contraception. All subjects were normotensive and had no history of renal, metabolic or cardiovascular disease. All subjects were receiving sodium intake ad libitum and had normal urea, creatinine and electrolytes. Their ages, body weights and systemic blood pressures, at the time of the infusions, are summarised in Table 8.2. In view of the small sample size, the range of values are quoted rather than the 25th and 75th centiles.

Protocol.

The protocol used was that of Broughton Pipkin, Hunter, Turner and O'Brien [1982], with only minor modifications. It is described in brief below.

The subjects lay supine throughout the experiment, a pillow being placed under the pregnant subjects to support a right lateral tilt. Indwelling cannulas (Venflon, 16G on the left side and 18G on the right side) were placed in both antecubital fossae. Normal saline solution was infused continuously at approximately 30 drops/minute via the right arm. The cannula in the left arm was used solely for blood sampling. Arterial blood pressure was measured at 2 minute intervals from the left arm using a Dinamap automatic blood pressure recorder (Critikon Ltd., Ascot, U.K.).

Arterial blood pressure was allowed to stabilize for a minimum of 30 minutes after setting up, until random fluctuations in diastolic blood pressure had been within 6 mmHg. for 10 minutes. An initial blood sample, for measurement of platelet AII binding, plasma AII, plasma renin concentration (PRC), plasma renin substrate (PRS), serum urea, creatinine and electrolytes and full blood count, was taken at this point. (Details of the determination of these parameters are included in Chapter 2, p. 118).

After a further 10 minutes, provided that the blood pressure had restabilised, AII was infused intravenously via a needle connection into the indwelling cannula of the right arm, so that it was flushed in by the saline infusion. The AII was made up freshly each day from dry ampoules of 2.5 mg; the stock solution was then stored on ice. This was infused with a Vickers pump (Vickers Medical Ltd., Basingstoke, U.K.). Doses of AII given were 4, 8, 16, 32, 48 and 64 ng/kg/minute to pregnant patients and 1, 2, 4, 8 and 16 ng/kg/minute to non-pregnant subjects. Each infusion step lasted 10 minutes. At the end of the third step a further blood sample was taken for plasma AII, PRC and PRS estimation. The AII infusion was discontinued after the last planned infusion step, or earlier if a 20 mmHg. rise in diastolic blood pressure had been achieved. After a period of 30 minutes recovery and stabilisation of blood pressure, a third blood sample was obtained, again for plasma AII, PRC and PRS estimation.

Infusion parameter calculations.

The basal systolic and diastolic blood pressures were calculated using the stable blood pressures bracketing the infusion. The pressor response to AII was calculated as the mean systolic or diastolic blood pressure over the last six minutes of each infusion step. The absolute evoked change was than calculated by comparison with the baseline.

Response thresholds were calculated for both diastolic and systolic blood pressures, for each patient, as being the doses of AII required to evoke both a 5 mmHg. and a 20 mmHg. rise in blood pressure. For this purpose the linear relationship between $\log_{10}$AII infused and evoked rise in blood pressure:

$$y = a + bx$$

was used, where y = evoked response (mmHg.), a = intercept on the y axis, b = slope from the curve relating pressor response to the infused dose of AII, and x = infused dose of AII ($\log_{10}$). This method has previously been used by other workers investigating the pressor effect of AII [Chinn and Dusterdieck, 1972].

Figure 8.1 provides an illustration, using one representative subject, of the calculation of the doses of AII required to produce a 20 mmHg. rise in systolic blood pressure (TS20), and in diastolic blood pressure (TD20). Figure 8.1 also demonstrates the calculation of the slope from the curve relating pressor response (systolic SLOS, diastolic SLOD), to the infused dose of AII.

RESULTS.

<u>Hormonal data.</u>

Pregnant subjects.

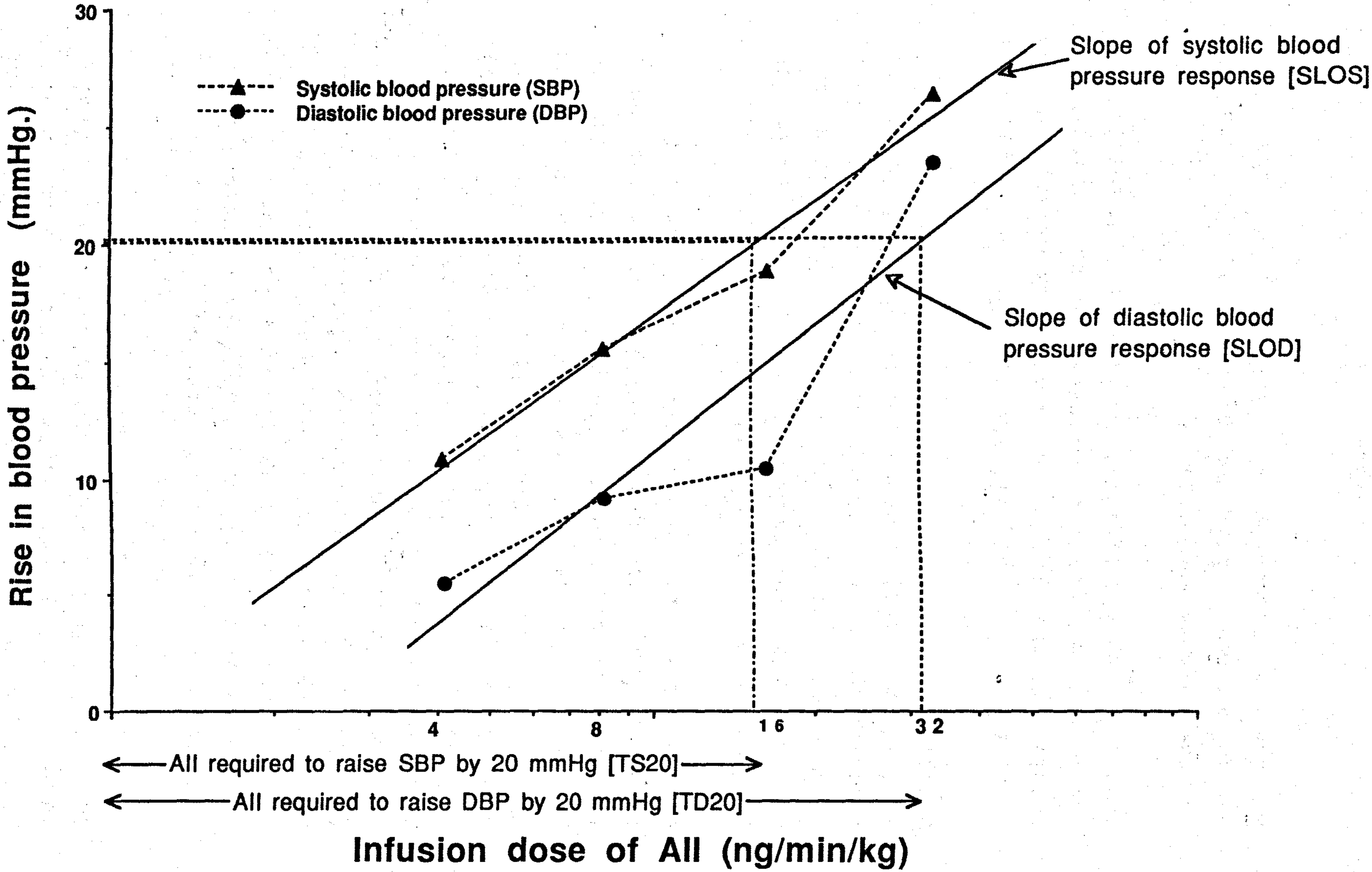
The median values of plasma angiotensin II (AII),

Figure 8.1.

Calculations derived from the AII infusion pressor dose response curves.

This figure illustrates, using one representative subject, the calculation of the doses of AII required to produce a 20 mmHg. rise in systolic blood pressure (TS20), and in diastolic blood pressure (TD20). The figure also demonstrates the calculation of the slope from the curve relating pressor response (systolic SLOS, diastolic SLOD), to the infused dose of AII.

Figure 8.1



Systolic blood pressure (SBP)
Diastolic blood pressure (DBP)
Slope of systolic blood pressure response [SLOS]
Slope of diastolic blood pressure response [SLOD]
Rise in blood pressure (mmHg.)
30
20
10
0
4
8
16
32
All required to raise SBP by 20 mmHg [TS20]
All required to raise DBP by 20 mmHg [TD20]
Infusion dose of All (ng/min/kg)

plasma renin concentration (PRC) and plasma renin substrate (PRS), immediately prior to the infusion, at the third infusion step, and following the infusion, are summarised in Table 8.3.

There was a significant rise in plasma AII levels as a result of the 16 ng AII/kg/min infusion in both the subsequent PIH group ($P<0.01$, Wilcoxon matched-pairs signed-ranks test) and the normotensive group ($P<0.001$). Plasma AII levels following the infusion were significantly lower than those at the 16 ng AII/kg/min infusion step in the normotensive group ($P<0.001$), with the difference approaching significance in the subsequent PIH group ($P= 0.015$). There were no significant differences between the plasma AII levels measured immediately prior to the infusion as compared to those found following the infusion (for each group $P>0.1$). These changes in plasma AII levels are illustrated in Figure 8.2.

There were no significant changes in the levels of PRC or PRS in either group, although in the normotensive group, the fall in PRC from that immediately prior to the infusion to that at the 16 ng AII/kg/min infusion step approached significance ($P= 0.04$).

There were no significant differences between the levels of any of the hormonal parameters in the subsequent PIH group as compared to the normotensive group, at any stage ($P>0.1$, Mann-Whitney U test).

Non-pregnant subjects.

The median values of plasma AII, PRC and PRS immediately prior to the infusion, at the 4 ng

Table 8.3.

Values of the hormonal parameters in the pregnant subjects, prior to the infusion, at the third infusion step, and following the infusion.

The pregnant subjects have been subdivided into those patients who remained normotensive following investigation, and those who subsequently developed PIH.

Despite the small number of subjects in the PIH group, in order to aid comparison between the two groups, the interquartile range is quoted in addition to the median.

Table 8.4.

Values of the hormonal parameters in the non-pregnant subjects, prior to the infusion, at the third infusion step, and following the infusion.

Median values are shown, with the range in brackets.

Table 8.3

	Normotensive group n=24		Subsequent PIH group n=10	
AII (pg/ml)				
Prior to infusion	34.1	(19.4-48.9)	36.2	(16.0-49.1)
At 16 ng AII/kg/min	107.5	(58.0-205.0)	146.1	(70.1-193.0)
Following infusion	19.5	(15.8-43.0)	14.2	(9.6-83.3)
PRC (ng AI/hr/ml)				
Prior to infusion	3.8	(3.5-6.2)	3.1	(2.2-6.9)
At 16 ng AII/kg/min	2.8	(2.4-4.4)	2.1	(1.7-3.9)
Following infusion	3.0	(2.3-4.0)	2.8	(2.4-3.3)
PRS (ug AI/ml)				
Prior to infusion	3.3	(2.8-3.7)	3.2	(2.4-3.9)
At 16 ng AII/kg/min	3.0	(2.6-3.6)	3.4	(2.8-3.9)
Following infusion	3.1	(2.6-3.5)	3.8	(3.0-4.3)

Table 8.4

	Non-pregnant subjects n=9	
AII (pg/ml)		
Prior to infusion	15.4	(9.9-28.8)
At 4 ng AII/kg/min	52.3	(34.5-106.7)
Following infusion	15.8	(10.0-35.0)
PRC (ng AI/hr/ml)		
Prior to infusion	2.8	(0.7-3.5)
At 4 ng AII/kg/min	1.5	(0.5-2.5)
Following infusion	1.5	(0.4-2.3)
PRS (ug AI/ml)		
Prior to infusion	1.0	(0.6-1.2)
At 4 ng AII/kg/min	1.1	(0.5-1.5)
Following infusion	0.8	(0.7-1.3)

Figure 8.2.

Plasma Angiotensin II (AII) levels prior to, during, and following the AII infusion, in the pregnant subjects.

Median values are shown, with the interquartile range marked.
The pregnant subjects have been subdivided into those patients who remained normotensive following investigation, and those who subsequently developed PIH.

Figure 8.3.

Plasma Angiotensin II (AII) levels prior to, during, and following the AII infusion, in the non-pregnant subjects.

Median values are shown, with the range marked.

Figure 8.2

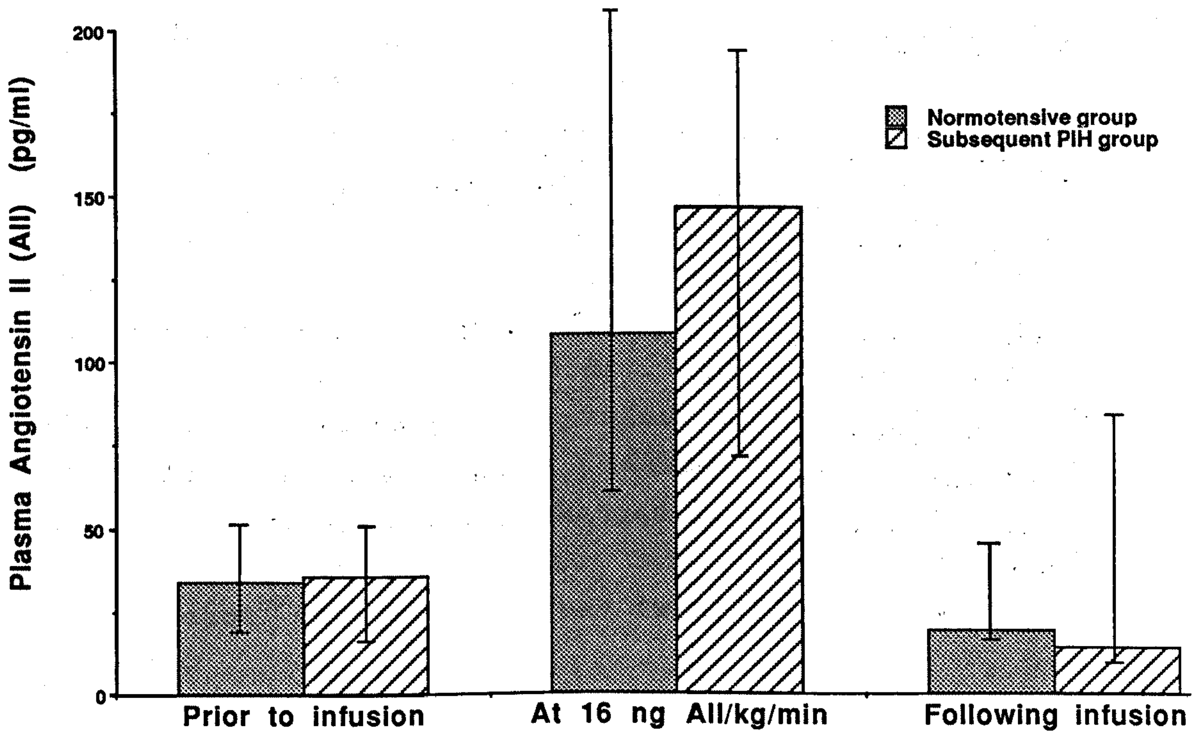
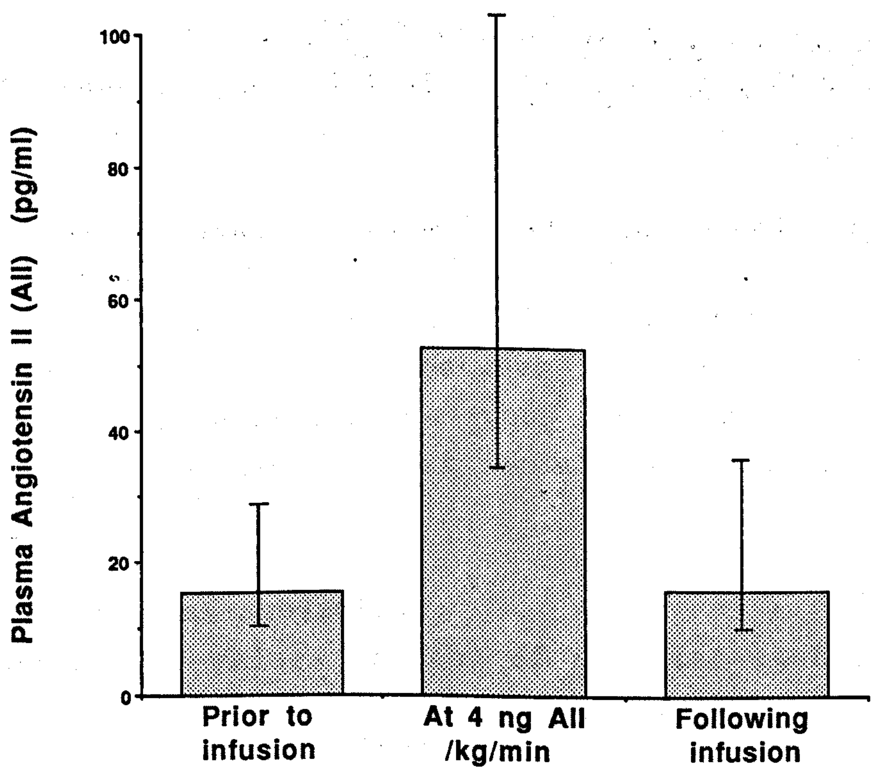


Figure 8.3



AII/kg/min infusion step and following the infusion, are summarised in Table 8.4.

The levels of plasma AII at the 4 ng AII/kg/min infusion step were significantly higher than both those measured before the infusion ($P<0.01$, Wilcoxon signed-ranks matched pairs test), and those measured following the infusion ($P<0.01$). The levels of plasma AII found following the infusion did not differ significantly from those measured prior to the infusion ($P>0.5$). These changes in plasma AII levels are illustrated in Figure 8.3. There were no significant changes in the levels of PRC or PRS ($P>0.1$).

<u>Haematological and biochemical data.</u>

Pregnant subjects.

The median values of platelet count, mean platelet volume (MPV), platelet distribution width (PDW), haemoglobin concentration (Hb) and haematocrit at the time of the infusion are summarised in Table 8.5. There were no significant differences between the subsequent PIH group and the normotensive group in any of the haematological parameters ($P>0.05$).

The median values of serum sodium, potassium, urea, creatinine, urate and osmolality at the time of the infusion are summarised in Table 8.6. There were no significant differences between the subsequent PIH group and the normotensive group in any of the serum biochemical parameters (in each case $P>0.2$, Mann-Whitney U test).

Non-pregnant subjects.

The median values (range) of platelet count, MPV,

Table 8.5.

Values of the haematological parameters in the pregnant subjects.

The pregnant subjects have been subdivided into those patients who remained normotensive following investigation, and those who subsequently developed PIH.
Despite the small number of subjects in the PIH group, in order to aid comparison between the two groups, the interquartile range is quoted in addition to the median.

Table 8.6.

Values of the biochemical parameters in the pregnant subjects.

The pregnant subjects have been subdivided into those patients who remained normotensive following investigation, and those who subsequently developed PIH.
Despite the small number of subjects in the PIH group, in order to aid comparison between the two groups, the interquartile range is quoted in addition to the median.

Table 8.5

	Normotensive group n=24	Subsequent PIH group n=10
Platelet count ($x\ 10^9$ /L)	186.0 (157-242)	189.0 (149-226)
Mean platelet volume (fl)	8.1 (7.9-8.8)	8.6 (7.7-9.3)
Platelet distribution width	16.0 (15.2-16.5)	17.4 (15.4-17.9)
Haemoglobin concentration (g/dl)	10.6 (10.1-11.3)	11.0 (10.5-11.6)
Haematocrit	0.32 (0.30-0.34)	0.33 (0.31-0.35)

Table 8.6

	Normotensive group n=24	Subsequent PIH group n=10
Sodium (mmol/L)	136 (134-138)	137 (136-137)
Potassium (mmol/L)	4.0 (3.8-4.2)	4.0 (3.8-4.1)
Urea (mmol/l)	2.3 (1.9-3.3)	2.0 (1.1-2.7)
Creatinine (umol/l)	57 (49-66)	59 (52-72)
Urate (umol/l)	206 (171-259)	197 (195-273)
Osmolality (osm/L)	277 (274-280)	278 (278-280)

PDW, Hb and haematocrit at the time of the infusion are summarised in Table 8.7. The median values (range) of serum sodium, potassium, urea, creatinine, urate and osmolality at the time of the infusion are summarised in Table 8.8.

Platelet AII binding data.

Pregnant subjects.

The median value of platelet AII binding in the normotensive group was 1.0 fmol/10^9 cells (0-3.4). Platelet AII binding was significantly higher in the subsequent PIH group (6.7 fmol/10^9 cells, 3.3-11.3, P= 0.001, Mann-Whitney U test). The differing values of platelet AII binding in the two groups are illustrated in Figure 8.4.

No significant correlations were found in either group, between platelet AII binding and any of the hormonal, haematological or biochemical parameters, although in the subsequent PIH group the inverse correlation between platelet AII binding and PRC approached significance ($P<0.02$, $r_s= -0.75$, Spearman correlation coefficient).

No significant differences were found in platelet AII binding when the pregnant subjects were divided on the basis of infant sex ($P>0.05$, Mann-Whitney U test) or on the basis of birthweight below either the 50th centile ($P>0.1$) or the 10th centile ($P>0.1$).

Non-pregnant subjects.

The median value of platelet AII binding was 4.9 fmol/10^9 cells (range 0.7-8.5). The only significant correlation between platelet AII binding and any of the hormonal, haematological or biochemical parameters

Table 8.7.

Values of the haematological parameters in the non-pregnant subjects.

Median values are shown, with the range in brackets.

Table 8.8.

Values of the biochemical parameters in the non-pregnant subjects.

Median values are shown, with the range in brackets.

Table 8.7

	Non-pregnant group n=24
Platelet count ($\times 10^9$ /L)	197.0 (156-272)
Mean platelet volume (fl)	7.8 (7.5-9.2)
Platelet distribution width	15.3 (14.0-17.0)
Haemoglobin concentration (g/dl)	11.4 (9.9-12.2)
Haematocrit	0.33 (0.29-0.35)

Table 8.8.

	Non-pregnant group n=24
Sodium (mmol/l)	139 (134-141)
Potassium (mmol/l)	4.1 (3.6-4.2)
Urea (umol/l)	4.5 (3.1-6.0)
Creatinine (umol/l)	69 (58-80)
Urate (umol/l)	250 (182-294)
Osmolality (osm/l)	282 (277-286)

Figure 8.4.

Comparison of platelet AII binding in those pregnant patients who remained normotensive and those who subsequently developed PIH.

Median values are indicated by a horizontal bar.
Platelet AII binding correctly classified 79% of patients.

Figure 8.5.

Comparison of the slope of the pressor response (SLOD) in those pregnant patients who remained normotensive and those who subsequently developed PIH.

Median values are indicated by a horizontal bar.
The slope of the diastolic pressor response correctly classified 63% of patients.

Figure 8.4

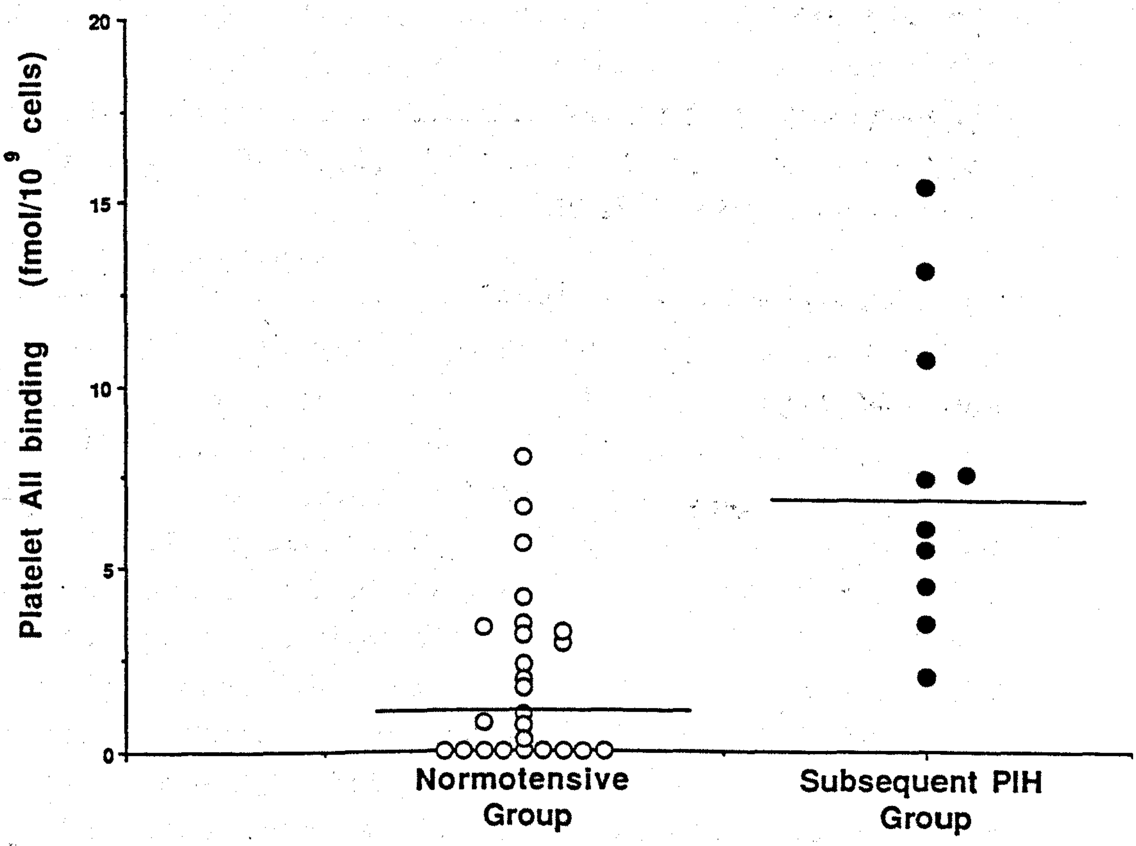
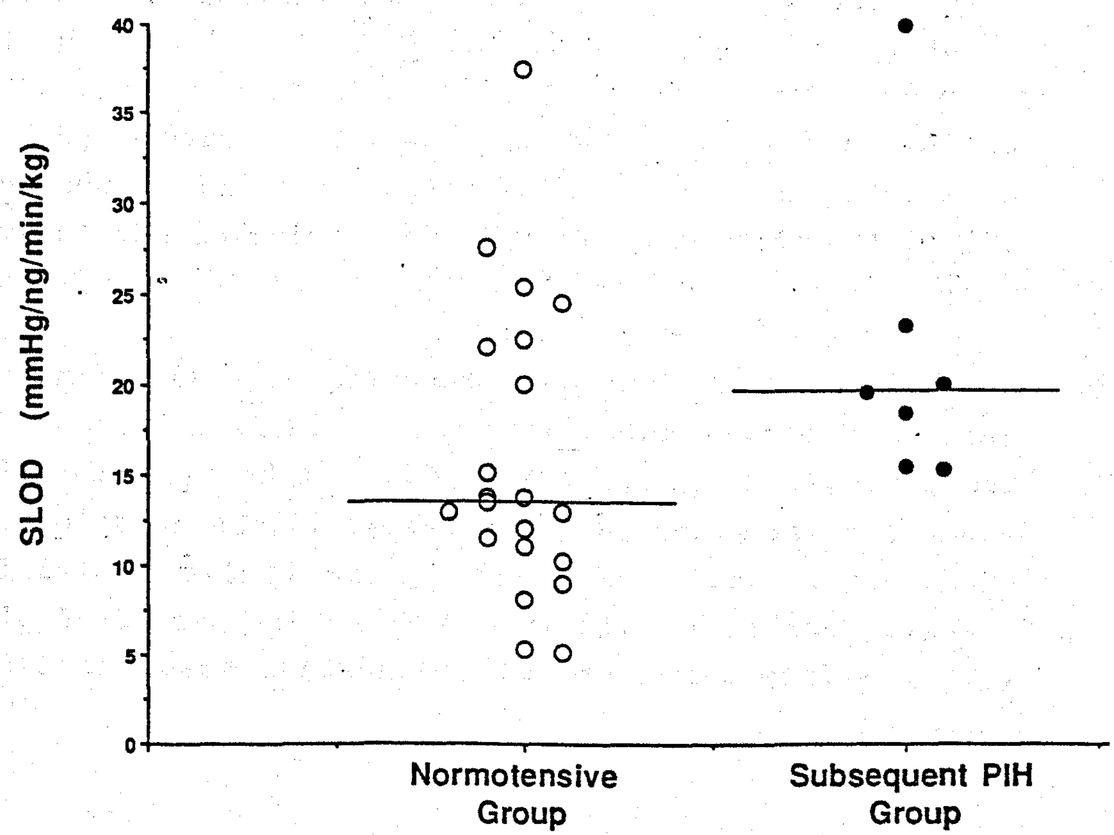


Figure 8.5



was between platelet AII binding and MPV ($P<0.01$, $r_s= 0.81$, Spearman correlation coefficient), although the correlation with serum sodium concentration approached significance ($P= 0.04$, $r_s= 0.67$).

AII infusion parameters.

Pregnant subjects.

The median values of the rises in systolic and diastolic blood pressure at 16 ng AII/kg/min, the slopes of the curves relating pressor response to infused AII (systolic SLOS, diastolic SLOD), and the doses of AII required to produce both a 5 mmHg. rise in blood pressure (systolic TS5, diastolic TD5) and a 20 mmHg. rise in blood pressure (systolic TS20, diastolic TD20), are summarised in Table 8.9, with the values of the SLOD being illustrated in Figure 8.5. A 20 mmHg. rise in systolic blood pressure was achieved in 13 (54%) of the normotensive group and 6 (60%) of the subsequent PIH group, with a 20 mmHg. rise in diastolic blood pressure being achieved in 12 (50%) of the normotensive group and 7 (70%) of the subsequent PIH group.

There were no significant differences between the subsequent PIH group and the normotensive group when any of the infusion parameters were considered ($P>0.1$, Mann-Whitney U test).

When all the pregnant patients were considered, there was a significant correlation between platelet AII binding and the SLOD ($P<0.01$, $r_s= 0.54$, Spearman correlation coefficient), with the correlation between platelet AII binding and the SLOS approaching significance ($P= 0.03$, $r_s= 0.41$). When the pregnant patients were subdivided, in the normotensive group,

Table 8.9.

Values of the infusion parameters in the pregnant subjects.

The pregnant subjects have been subdivided into those patients who remained normotensive following investigation, and those who subsequently developed PIH.

Despite the small number of subjects in the PIH group, in order to aid comparison between the two groups, the interquartile range is quoted in addition to the median.

Table 8.9

	Normotensive group n=24	Subsequent PIH group n=10
Systolic BP rise at 16 ng AII/kg/min (mmHg.)	10.7 (7.3-14.1)	12.1 (6.5-17.1)
Diastolic BP rise at 16 ng AII/kg/min (mmHg.)	13.4 (7.9-16.0)	12.7 (10.3-23.1)
SLOS	17.6 (13.2-22.5)	18.5 (16.2-27.7)
SLOD	13.5 (11.5-22.2)	18.9 (15.3-22.4)
TS5 (ng AII/kg/min)	5.8 (4.1-8.3)	6.4 (4.4-7.3)
TS20 (ng AII/kg/min)	30.8 (20.4-52.1)	29.8 (15.3-36.1)
TD5 (ng AII/kg/min)	3.5 (1.8-6.7)	4.9 (3.6-7.2)
TD20 (ng AII/kg/min)	27.3 (12.6-39.7)	28.9 (14.2-31.3)

the correlations between platelet AII binding and both the SLOD ($P= 0.03$, $r_s= 0.49$) and the SLOS ($P= 0.02$, $r_s= 0.51$) approached significance. In the subsequent PIH group there was a significant correlation between platelet AII binding and the SLOD ($P<0.01$, $r_s= 0.86$), with the correlation between platelet AII binding and the rise in systolic blood pressure at 16 ng AII/kg/min approaching significance ($P= 0.04$, $r_s= 0.70$). No other correlations between platelet AII binding and any of the infusion parameters were found ($P>0.1$).

Non-pregnant subjects.

The median values of the rises in systolic and diastolic blood pressure at 4 ng AII/kg/min, the slopes of the curves relating pressor response to infused AII (systolic SLOS, diastolic SLOD), and the doses of AII required to produce both a 5 mmHg. rise in blood pressure (systolic TS5, diastolic TD5) and a 20 mmHg. rise in blood pressure (systolic TS20, diastolic TD20), are summarised in Table 8.10. A 20 mmHg. rise in systolic blood pressure was achieved in 6 (66%) subjects with a 20 mmHg. rise in diastolic blood pressure being achieved in 7 (78%) subjects.

No correlations were found between platelet AII binding and any of the infusion parameters (in each case $P>0.2$).

Discriminant analysis.

The use of platelet AII binding as a prospective screening test, i.e. to predict the subsequent development of PIH, was assessed using canonical discriminant analysis (discussed on p.160). There was

Table 8.10.

Values of the infusion parameters in the non-pregnant subjects.

Median values are shown, with the range in brackets.

Table 8.10

	Non-pregnant subjects n=9
Systolic BP rise at 4 ng AII/kg/min (mmHg.)	9.7 (4.8-16.9)
Diastolic BP rise at 4 ng AII/kg/min (mmHg.)	12.9 (5.8-18.7)
SLOS	18.8 (14.4-40.2)
SLOD	13.2 (7.5-22.2)
TS5 (ng AII/kg/min)	1.8 (1.1-4.5)
TS20 (ng AII/kg/min)	11.2 (3.8-17.8)
TD5 (ng AII/kg/min)	0.8 (0.2-1.8)
TD20 (ng AII/kg/min)	12.5 (3.6-20.4)

a false positive rate of 40% and a false negative rate of 16%, 77% of patients being correctly classified.

When the hormonal, haematological and biochemical parameters measured at the time of the infusion were used to discriminate between those patients who would subsequently develop PIH and those who would remain normotensive, in each case, less than 60% of patients were correctly classified. In particular, serum urate and MPV correctly classified only 51% and 59% of patients correctly. The systemic blood pressure measured at the time of the infusion was also unsuccessful in discriminating between the two groups. However, when the booking visit systolic blood pressure was used to discriminate between the two groups, there was a false positive rate of 10% and a false negative rate of 50%, 62% of patients being correctly classified, and when the booking visit diastolic blood pressure was used, there was a false positive rate of 40% and a false negative rate of 21%, 74% of patients being correctly classified. The results of such discriminate analysis were improved when platelet AII binding was combined with either the booking visit systolic blood pressure (89% correct classification, 30% false positive rate, 4% false negative rate) or the booking visit diastolic blood pressure (79% correct classification, 30% false positive rate, 17% false negative rate).

None of the infusion parameters was as effective in discriminating between the two groups as platelet AII binding, the most successful being the SLOD (63% correct classification, 37% false negative and false positive rates). However, combining the TS20 with platelet AII binding produced the highest proportion of correctly classified patients of any parameter or combination of parameters (90% correct classification, 16% false positive rate, 7% false negative rate).

Discussion.

Two important conclusions can be drawn from the parallel studies of AII pressor sensitivity and platelet AII binding. The first concerns the close correlation which was found between platelet AII binding and the slopes of both the diastolic and the systolic pressor responses to AII, in the pregnant subjects. This correlation provides support for the use of platelet AII binding as a model for vascular smooth muscle sensitivity. Secondly, although a marked overlap was found when platelet AII binding was used as a predictive test for PIH, the results using platelet AII binding were better than those using any of the parameters derived from the AII sensitivity test.

The incidence of PIH amongst primigravid women in this centre (University Hospital, Nottingham), using the definition detailed in the Introduction (p. 9), has been found to be as low as 5% [Baker and Kilby, unpublished data]. In an effort to increase the proportion of patients studied, who subsequently developed PIH, women in whom a diastolic blood pressure of 80-85 mmHg. was initially recorded at their second antenatal clinic visit, were selected. The previous work of Friedman and Neff [1977], who suggested that a diastolic blood pressure greater than 75 mmHg. led to an increased incidence of PIH, was therefore confirmed; approximately one third of the patients entered into this study subsequently developed PIH. The selection of this particular population could be criticised, in that at the time of recruitment to the study, the definitions of PIH used by certain authors [e.g. Chesley, 1976] had already been fulfilled. However, it must be stressed that prior to the infusion, the blood pressures of all the subjects settled to levels well below any pathological thresholds, that two thirds of the patients subsequently had uncomplicated pregnancies and that none of the platelet counts, serum urate estimations etc. were found to be elevated. It thus seems likely that the subjects studied are representative of the primigravid pregnant population, but a much larger study would be needed to confirm this. Furthermore, the selected patient group do represent a particular clinical problem, i.e. whether to ignore an initial elevated blood pressure recording if the blood pressure subsequently settles. This study suggests that this group of patients is at a high risk of developing PIH and should be offered any suitable screening test.

When this study was commenced, the implications of the studies discussed earlier in the thesis, that differences in platelet AII binding in normotensive and hypertensive pregnant women might be apparent at a much earlier gestation, had not become fully apparent. If the fall in platelet AII binding in normotensive pregnancy does occur by eighteen weeks gestation (Chapter 6, p. 171), and if the elevated levels of platelet AII binding in PIH are due to this early gestation diminution in binding not occurring (Chapter 7, p. 210), then a study of platelet AII binding at the antenatal booking visit might be more appropriate.

The rationale for the use of platelets as a model for vascular myocytes has been outlined in the

Introduction (p. 108). The correlations found in pregnant women, between platelet AII binding and the slopes of the pressor response to AII support the use of platelet AII binding as a model of vascular smooth muscle, and suggest that the elevated platelet AII binding in PIH does reflect elevated myocyte AII binding. That the strongest correlations were found with the slopes of the curves relating pressor response to the infused dose of AII is perhaps not surprising. Considerable debate had arisen from previous studies [Nakamura, Ito, Matsui, Yoshimura, Kawasaki and Maeyama, 1986, Dekker, Makovitz and Wallenburg, 1990], as to the most appropriate threshold rise in blood pressure. Use of the slopes of the pressor responses resolves this uncertainty. Moreover, in this study, some of the subjects failed to attain the 20 mmHg. threshold rise in blood pressure.

The results obtained from the parallel studies in the non-pregnant women appear to conflict with other results presented in the thesis, and presumably were as a consequence of the very small sample size of this group. No significant correlations were found between platelet AII binding and the infusion parameters, and it would be difficult to postulate a model whereby platelet AII binding reflected vascular smooth muscle AII pressor responsiveness in pregnant women but not in non-pregnant women. In addition, the significant correlation that was found, that between platelet AII binding and mean platelet volume (MPV), was not found in any of the larger studies of pregnant or non-pregnant women presented in earlier chapters, with changes in MPV resulting from blood donation not being associated with changes in platelet AII binding (see p. 125). The entry criteria for the non-pregnant subjects in this study were exacting, and it proved more difficult to recruit subjects than had been anticipated. Nevertheless, parallel studies involving a larger number of non-pregnant subjects are necessary, even if this requires a relaxation of certain of the entry criteria.

From a pragmatic viewpoint, the most important conclusion that can be drawn from this study, is the confirmation of the potential use of platelet AII binding as a screening test for PIH. The false positive and false negative rates obtained when platelet AII binding was used as a prospective discriminant were very similar to those obtained when platelet AII binding was used to discriminate women with established PIH from normotensive primigravidae. However, the potential use of the technique as a screening test is greater than its potential use as a marker for PIH, because parameters such as MPV and serum urate which effectively discriminated women with established PIH from normotensive pregnant women, were not effective on a prospective basis.

As discussed in the Introduction (p. 52), the AII sensitivity test is widely regarded as one of the most accurate predictors of PIH [e.g. Wallenburg, Dekker, Makovitz and Rotmans, 1986]. However, a search of the literature revealed only five small studies [Morris, O'Grady, Hamilton and Davidson, 1978, Orozco, Pinsker, Hernandez and Karchmer, 1979, Oney and Kaulhausen, 1982, Nakamura, Ito, Matsui, Yoshimura, Kawasaki and Maeyama, 1986, Dekker, Makovitz and Wallenburg, 1990] which assessed the validity of the original work by Gant, Daley, Chand, Whalley and MacDonald [1973]. All of these studies found a considerable false positive rate, with, depending on the thresholds used, typical rates of 45-55%, the exception being the study of Morris et. al. [1978], in which the false positive rate was 93%. In this study, using SLOD, the parameter derived from the infusion studies which was found to be the most effective discriminant, the false positive rate was 37%. In contrast, the 37% false negative rate for the AII sensitivity test found in this study was higher than that of any of the previous studies, with all the studies except that of Morris et. al. [1978] finding a false negative rate rate below 10%.

None of the above studies were able to duplicate the very low false positive and false negative rates found in the original report [Gant et. al., 1973], which as discussed in the Introduction (p. 50), may be due to the particular Dallas population studied. Even after achieving considerably better results with the AII sensitivity test than those of this study, Oney and Kaulhausen [1982] concluded that the test could not be recommended for routine screening for PIH. In our hands, as in those of Morris et. al. [1978], the assessment of the risk of PIH using the AII sensitivity test was patently unreliable. The reason for the discrepancy between the results of this study and those of Orozco et. al. [1979], Oney and Kaulhausen [1982], Nakamura et. al. [1986] and Dekker et. al. [1990] is unclear. The population in this study was similar to that investigated by Oney and Kaulhausen [1982] and Dekker et. al. [1990]. The studies differ in that not all the above workers generated pressor dose response curves. The use of such curves would, however, be anticipated to increase the predictive accuracy of the test.

Nakamura et. al. [1986] argued that that it was imprudent to predict the outcome of pregnancy on only one performance of the AII sensitivity test, and that it was preferable to perform the test serially in the same patient for accurate evaluation. Whilst this may well be a valid criticism of our study the logistical difficulties of performing the AII sensitivity test on one occasion are considerable. Moreover, the high platelet AII binding coefficients of variation found (see p. 126) suggest that the predictive accuracy of this technique might also be improved by serial estimations.

The predictive accuracy of platelet AII binding in assessing the risk of developing PIH was at least as great as that previously derived using the AII sensitivity test (with the exception of the original report by Gant et. al. [1973]). Moreover, by including the booking visit systemic blood pressure, (information readily available), almost a 90% correct classification was achieved. Furthermore, unlike the measurement of platelet AII binding, the AII sensitivity test had the disadvantage that the failure of the blood pressures of certain patients to stabilise prior to the infusion caused the test to be abandoned in these patients. This problem was also reported by Morris et. al. [1978].

Therefore, there is an urgent need for a much larger study to assess the prospective use of platelet AII binding in determining the risk of developing PIH. Serial estimations of platelet AII binding would be preferable, as these might increase the accuracy of the evaluation, and would indicate the optimal gestation to perform the test if a single estimation were deemed to be the most cost effective. If the results of such a study were to confirm those of this study, then the more invasive, more expensive and more time-consuming AII sensitivity test would surely be redundant.

Chapter 9.

GENERAL DISCUSSION.

The characterisation studies performed using blood from non-pregnant subjects confirmed that human platelets possess a single class of angiotensin II (AII) binding sites which are of high affinity, specific, saturable and reversible. The higher variability of platelet AII binding found in the non-pregnant subjects relative to previous reports [e.g. Moore and Williams, 1981] may have resulted from all the subjects being female and at day 5-9 of the menstrual cycle, maximal variability having been demonstrated at this stage of the cycle. Although the pregnant subjects in whom characterisation studies were performed may not have been representative of the normotensive population, the preliminary results of Pawlak and Macdonald [personal communication] also indicate that platelet AII binding sites are of high affinity, with a dissociation constant similar to that of non-pregnant subjects. Although a positive trend was suggested, (p. 131, Figure 3.10), no correlation was demonstrated between platelet AII binding and the rise in intracellular free calcium after <u>ex vivo</u> AII infusion, thus validation of the high affinity affinity platelet AII binding sites as receptors was not achieved.

The cross-sectional and longitudinal studies of platelet AII binding in normotensive pregnancy demonstrated a marked reduction in binding. That this diminution in binding occurred at an early gestation was confirmed by study of the early pregnancy subgroup, with lower levels demonstrable at 5-8 weeks gestation. These levels may represent a continuation of the low binding found in the luteal phase of the menstrual cycle. These results suggest that the fall in platelet AII binding in pregnancy parallels the pregnancy-induced fall in AII pressor responsiveness [Gant, Daley, Chand, Whalley and MacDonald, 1973], and support the use of platelets as an accessible model of vascular smooth muscle in pregnant women.

Changes in binding site occupancy are unlikely to account for the changes in platelet AII binding, the AII infusions having little effect on levels of binding. Changes in platelet AII binding due to synthesis or degradation of new binding sites seem improbable as platelets possess no nuclei and minimal protein synthesis capacity. However, the suggestion that altered platelet AII binding results from the exposure or concealment of presynthesised binding sites on the cell membrane would be consistent with the data, with the 'vertical displacement' theory of Borochov and Shinitzky [1976] being one possibility (discussed in detail in Chapter 7, p. 213). The down-regulation of insulin receptors has been related to this type of binding site mechanism [Krupp and Lane, 1981].

The fall in platelet AII binding levels from those of the non-pregnant women to those in pregnancy, which were between five and ten fold, put into perspective the relatively high coefficients of variation of the technique, that were reported in the Methods chapter. Even in non-pregnant subjects, the data from this study suggests that the number of binding sites per platelet is less than ten, (similar numbers of binding sites per cell having been reported previously, e.g. Ding, Kenyon and Semple [1985]). In view of this low concentration of binding sites per platelet, the limited reproducibility of the technique, which persisted throughout the study, was perhaps not unexpected.

Once the platelet preparation and platelet binding studies had been modified and validated, one advantage of the technique used was that the laboratory methods were straightforward and easily learnt. Three medical students worked in the department at various intervals during the study (measuring platelet AII binding in neonatal platelets), and all three were able to obtain satisfactory results, with similar reproducibility to that of this study, within a short time period.

Analysis of the data obtained from the binding studies was handicapped by the necessity to use distribution-free or non-parametric statistics. Although over recent decades there has been a dramatic and expansive development of parametric statistical analysis, there have been few developments of non-parametric statistical tests [Lehmann, 1975]. Very considerable statistical advice was received regarding each of the studies included in the thesis, in order that the most appropriate non-parametric tests were selected. Nevertheless, there was a loss of statistical power as a consequence of the use of non-parametric statistical analysis; anticipated development of non-parametric tests should result in this loss being minimised in future studies.

The study of the non-pregnant subjects provided further evidence for down-regulation of platelet AII binding by plasma AII concentration. The longitudinal study of platelet AII binding in normotensive pregnancy utilised greater statistical power than that of the cross-sectional study and suggested that this mechanism may also be a factor in pregnancy. The AII infusion studies and the lack of any significant changes in platelet AII binding at 24 hours after delivery when the plasma AII levels were significantly elevated, suggest that down-regulation by plasma AII concentration does not exert a short term effect. Animal studies using various tissues have provided conflicting results. Whereas Schiffrin, Gutkowska and Genest [1984] found that short-term AII infusions reduced AII receptor concentrations in mesenteric artery fractions, Chevillotte, Rouzaire-Dubois, Devynck and Meyer [1976] found that acute pressor intravenous injection of AII did not affect the number of uterine AII receptors. Chevillotte et. al. [1976] reported that after 24 hours AII infusion, the uterine AII receptor concentration was reduced, and diminished AII binding in rat bladder particulate fractions have also been demonstrated after 2-4 days [Aguilera and Catt, 1981]. These differences serve to illustrate the dangers of drawing conclusions regarding AII binding from studies using differing tissues from differing species.

Other regulatory factors may influence platelet AII binding in normotensive pregnancy and undoubtedly influence binding in pregnancies complicated by pregnancy-induced hypertension (PIH). For example, although plasma AII concentrations were higher in women with non-proteinuric PIH as compared to normotensive primigravidae, the same was true of platelet AII binding in these patients.

One other possible regulatory factor is the serum sodium concentration, a direct correlation persistently being demonstrated in the various studies included in the thesis. Indeed, if the data from the cross-sectional, longitudinal normotensive, longitudinal hypertensive and infusion studies are pooled, then amongst subjects in the third trimester of pregnancy (n=181), the direct correlation between platelet AII binding and serum sodium concentration approaches statistical significance (P= 0.15, r_s= 0.19). In contrast, there is no significant inverse correlation between platelet AII binding and plasma AII concentration (P>0.05, r_s= 0.10).

As discussed in the Introduction (p. 81), many workers [e.g. Aguilera and Catt, 1981] have concluded that the effects of alterations in sodium balance on AII pressor sensitivity [Brunner, Chang, Wallach, Sealey and Laragh, 1972] and on vascular smooth muscle cell receptor concentrations [Aguilera and Catt, 1981], result from changes in the renin-angiotensin system. (Sodium depletion increases AII production by stimulating renin release, as outlined in the Introduction, p. 22). This concept is supported by the findings of Cowley and Lohmeir [1977], who demonstrated that short term alterations in sodium balance by haemodialysis in nephrectomised dogs did not affect vascular AII sensitivity, whereas Oliver and Cannon [1978] noted diminished sensitivity during acute sodium depletion by frusemide administration in dogs, a condition characterised by markedly increased AII levels.

However, the results of the regression analysis performed on data from the longitudinal study of normotensive pregnancy (Chapter 6), indicated that the correlation between platelet AII binding and serum sodium concentration was independent of plasma AII levels. It remains possible that there is a direct effect of the serum sodium concentration on both AII binding and vascular sensitivity. Whilst there have been several studies of the effect of dietary sodium restriction on both vascular reactivity and AII binding site concentration, as above, variations in serum sodium levels have not been observed during dietary sodium restriction, and there have been very few studies of the effect of changes in extracellular sodium concentration. In one such study, intra-arterial infusion of high concentration saline solutions via the brachial artery of male subjects resulted in alterations in extracellular sodium levels which were directly proportional to changes in vascular responsiveness to AII [Heistad, Abboud and Ballard, 1971].

Sodium and water retention in a blood vessel wall would increase wall thickness [Friedman and Friedman, 1967], and perhaps alter vascular reactivity. However, the results of in vitro experiments in which the sodium ion concentration influences the binding of agonists to a diverse variety of binding sites, including dopamine [Usdin, Creese and Snyder, 1980], opiate [Pert and Snyder, 1974], muscarinic cholinergic [Rosenberger, Yamamura and Roeske [1980] and AII receptors [Wright, Alexander, Ekstein and Gimbrone, 1982], suggests a more complex mechanism.

There is conflicting evidence from in vitro studies regarding the effect of sodium ion concentration on smooth muscle cell AII binding. In the rabbit uterus [Bennett and Snyder, 1980] and the rabbit aorta [Devynck and Meyer, 1976], alterations in sodium ion concentration have not been found to influence AII binding. However, as discussed in the Introduction (p. 71), binding sites in these tissues may not be representative of those in tissue from resistance vessels. Wright et. al. [1982] demonstrated that rat mesenteric artery AII binding was increased by increases in sodium ion concentration, whereas increases in other monovalent cations (including lithium, potassium and ammonium) resulted in reduced AII binding.

Wright et. al. [1982] suggested the existence of a membrane monovalent cation site closely related to the vascular smooth muscle AII receptor. A similar membrane site was proposed to explain the stimulatory effect of increasing sodium ion concentration on adrenal cortical AII binding [Glossman, Baukal and Catt, 1974], and is in keeping with the two-state model of receptor binding [e.g. Koshland, 1964] outlined in the Introduction (p. 66).

An alternative explanation for the regulatory influence of sodium ion concentration on AII binding involves a direct effect on the conformation of the radioligand, since interaction between AII and artificial lipid membranes have been demonstrated by Elliott and Goodfriend [1979]. These interactions were sensitive to cation concentrations (including calcium and magnesium), although a specific effect of sodium ion concentration was not investigated. However, regulation of binding by the sodium ion concentration appears to be a general phenomenon, as above, involving agonists as diverse as peptides and opiates. Thus, it seems more likely that sodium ion concentration changes act through sodium ion-sensitive membrane sites common to many hormone binding sites, rather than by a specific physical alteration in the AII molecule.

In <u>in vitro</u> studies, guanine nucleotides have been found to inhibit AII binding [Devynck and Meyer, 1976], discussed in the Introduction (p.82). Wright et. al. [1982] demonstrated that the effect of guanine nucleotides in inhibiting AII binding varied with the ambient sodium ion concentration. The guanine nucleotide-sensitive site is thus one locus where effects of alterations in sodium ion concentrations may be mediated.

Robdell [1980] has suggested that membrane guanine nucleotide-binding proteins play a pivotal role in the transduction of signals from a variety of surface receptors. The theory was originally derived from data on adenylate cyclase coupled receptors, particularly the beta-adrenergic receptors [Robdell, 1980], although it has since been extended to other guanine nucleotide sensitive receptors [Catt, Mendelsohn, Millan and Aguilera, 1984]. An agonist, but not an antagonist, induces the formation of a complex between the receptor and a membrane nucleotide regulatory protein. In the absence of guanine nucleotides, the receptor binds the agonist with high affinity. When guanine nucleotides bind to sites on the regulatory protein, simultaneous (or sequential) development of low affinity states for the agonist and coupling of the regulatory unit to the catalytic unit of adenylate cyclase lead to the activation, or inhibition of the enzyme.

Wright et. al. [1982] suggested that sodium ions facilitated the formation of a receptor-nucleotide regulatory protein complex of high affinity for AII. When guanine nucleotides bind to the complex, AII binding is inhibited. Since AII does not stimulate adenylate cyclase activity, but rather has an inhibitory effect on the enzyme [Woodcock and Johnson, 1982], AII binding sites must interact with the inhibitory form of the guanyl-nucleotide regulatory protein (Ni) that is involved in the actions of several ligands, including the $alpha_2$-agonists

[reviewed by Gilman, 1984]

In patients with PIH increased levels of platelet AII binding in comparison with normotensive primigravidae were found. The increased levels paralleled the increased AII pressor response found in patients with PIH [Gant et. al., 1973]. There would seem to be a limited application for the technique as a marker for PIH in patients with the established disease, possibly as an adjunct to the biochemical and haematological parameters more commonly measured. However, values of platelet AII binding in patients who developed fulminating pre-eclampsia were no higher than those of the other pre-eclampsia subjects. Moreover, the value of platelet AII binding in the single patient who developed eclampsia was below the median value of the PIH group.

The hypothesis, supported by the two patients from the longitudinal study who developed PIH, that platelet AII binding also parallels AII pressor sensitivity in pregnancies complicated by PIH, would suggest that differences in platelet AII binding between normotensive pregnant women and PIH patients should be apparent well before the clinical development of the disease. The potential application of the technique as a screening test for PIH was emphasised in the previous chapter; the results using platelet AII binding as a predictive test for PIH not only being better than those of the biochemical and haematological parameters measured, but also being better than those using any of the parameters derived from the AII sensitivity test.

A much larger prospective study is needed to confirm the potential use of platelet AII binding as a screening test for PIH. However, with recent studies suggesting measures which prevent the development of the disease, the need for such a practical and minimally invasive technique to determine those at risk of PIH, has never been greater.

Dietary salt restriction, as advocated by Nakamura, Ito, Matsui, Yoshimura, Kawasaki and Maeyama [1986], was suggested as a prophylactic measure as early as the 18th century [Zuckert, 1767]. Although the geographical incidence of eclampsia has been reported as varying with the dietary intake of salt [deSnoo and Remmelts, 1938], there is little evidence to support the use of salt restriction as a prophylactic measure, with Robinson [1958] reporting an increased incidence of PIH in patients advised to restrict their salt intake, as compared to patients told to increase dietary salt consumption. Similar conclusions should be drawn regarding the prophylactic administration of diuretic therapy. Finnerty and Bepko [1966] reported that 'vasoconstriction', (diagnosed on the basis of either a 10% or more rise in mean arterial blood pressure or the appearance of proteinuria), developed three times as often in untreated women as compared to patients treated prophylactically with chlorothiazide. However, not only was the study poorly controlled, but many of the patients in whom the mean arterial blood pressure rose by 10% did not have diastolic blood pressures above 70 mmHg. In contrast, Chesley [1976] reviewed six double-blind studies of diuretic drugs as preventatives of pre-eclampsia; five of the six studies indicated essentially no effect, and the one exception was based on only 74 cases. The use of both fish-oil dietary supplementation and low-dose aspirin administration offer much greater promise.

A large number of diseases have been considered to be preventable or curable by fish-oil [Gibson, 1988], but it has only recently been suggested that fish-oil supplementation might lessen the occurrence of obstetric complications such as pre-eclampsia [Romero, Lockwood, Oyarzun and Hobbins, 1988]. The possible preventative effect of fish-oil in pre-eclampsia is supported indirectly by the controlled study undertaken by the People's League of Health [1946] during 1938-39 in London. Over five thousand pregnant women were allocated alternately to treatment with a dietary supplement which included halibut liver oil, or to no treatment. The group receiving the supplement had less 'toxaemia' than the control group.

The beneficial effect of fish-oil seems to be associated mainly with its long chain n-3 fatty acids, which together with the n-6 fatty acids derived primarily from vegetables, represent the main polyunsaturated fatty acids in our diet. As discussed in the introduction (p. 52), PIH is associated with decreased prostacyclin synthesis [Ylikorkala and Makila, 1985] and increased thromboxane A_2 synthesis, which contributes to the thrombocyte aggregation and vasoconstriction seen in the disease [Walsh, 1985]. Secher and Olsen [1990] suggested that the beneficial effect of fish-oil supplementation was due to a change in haemostatic balance resulting from the increased prostacyclin production and the inhibition of thromboxane A_2 production that is found after intake of long chain n-3 fatty acids [Leaf and Weber, 1988].

However, dietary n-3 polyunsaturated fatty acids are also known to decrease platelet membrane viscosity [Vas Dias, Gibney and Taylor, 1982]. This provides an alternative explanation of the beneficial effect of fish-oil supplementation, in that supplementation may reduce the elevated platelet membrane viscosity found in pre-eclampsia [Gleeson, Ahmed, Rice-Evans and Elder, 1990]. Such an alteration in cell membrane fluidity would reduce the accessibility of cell membrane proteins which act as binding sites (discussed in detail in Chapter 7, p. 213). A reduction in AII binding would thus be anticipated and it is interesting to note that the AII pressor response is reduced after fish-oil intake [Leaf and Weber, 1988]. This is clearly an area in which further studies are required. The results of a prospective analysis of both the efficacy of administering fish-oil supplementation to women with high values of platelet AII binding in reducing the incidence of pre-eclampsia, and the assessment of the effect of fish-oil on platelet AII binding, would be particularly interesting.

The incidence of PIH has been found to be decreased in women given low-dose aspirin. In a controlled trial by Beaufils, Uzan, Donsimoni and Colau [1985], aspirin (150 mg/day) combined with dipyridamole (300 mg/day) was administered to women from the first trimester of pregnancy. The treatment regimen conferred a clear benefit, both in terms of the occurrence of PIH and intra-uterine growth retardation, and in perinatal survival. These subjects were selected on the basis of a previous obstetric history indicating that they were likely to develop PIH. A clear advantage of the subsequent study by Wallenburg, Dekker, Makovitz and Rotmans [1986] was that patients were selected to participate in their study on the basis of an increased blood pressure response to infused AII. 23 women received 60 mg aspirin daily, and the same number received matching placebo until delivery. In the placebo group PIH, pre-eclampsia, and eclampsia developed in four, seven and one cases, respectively, whereas only two women in the aspirin group developed mild PIH. Several larger studies of the effect of low-dose aspirin in pregnancy, such as the Collaborative Low-dose Aspirin Study in Pregnancy (CLASP), have been commenced. In an overview of the results of these studies thus far available, Grant [1990] reported that meta-analysis of the data indicated that there was an 80% reduction in pre-eclampsia as a consequence of low-dose aspirin administration, although no significant reduction in the perinatal mortality rate was demonstrated.

There is some concern as to the safety of aspirin ingestion in pregnancy. Some human studies [e.g. McNeil, 1973] suggest that there is an increase in congenital malformations when aspirin is given in high doses in early pregnancy. Animal data has shown that aspirin (55-90 mg/Kg) may cause constriction of the fetal ductus arteriosus [Heymann and Rudolph, 1976], and increased incidences of both ante-partum and post-partum haemorrhages have been described following aspirin ingestion in pregnancy [Collins and Turner, 1975]. Such fears may well prove to be unfounded, with large studies having found no association between aspirin ingestion and either congenital malformations [Slone, Siskind, Heineman, Manson, Kaufman and Shapiro, 1976], or persistent pulmonary hypertension [Shapiro, Siskind, Manson, Heineman, Kaufman and Slone, 1976]. Nevertheless, if platelet AII binding does prove to be an effective and practical screening test, then restricting low-dose aspirin administration to those deemed to be at high risk of developing PIH has obvious merit.

It would be interesting to determine the effect of low-dose aspirin on platelet AII binding. Spitz, Magness, Cox, Brown, Rosenfeld and Gant [1988] demonstrated that the administration of low-dose aspirin to women in the third trimester of pregnancy with increased AII pressor sensitivity caused them to become more refractory to infused AII. These women did not, however, become as refractory to infused AII as normal pregnant women. In view of this finding, a reduction in platelet AII binding as a consequence of low-dose aspirin administration might be anticipated.

The finding of the close correlations between platelet AII binding and the slopes of the diastolic and systolic pressor response to infused AII strengthen the other conclusions of the thesis, in that the correlations provide support for the use of platelet AII binding as a model of vascular smooth muscle sensitivity.

Further support for such a model has been provided by comparisons of neonatal platelet AII binding and the <u>in vitro</u> response of chorionic plate arterial strips to AII [Cook, Farquhar, Baker, Broughton Pipkin, unpublished data]. A significant inverse correlation was found between the response of the arterial strips to 10^{-7} M AII and the values of neonatal platelet AII binding. Assuming that there is a direct relationship between AII binding and the degree of contraction, as demonstrated in the rabbit aorta by Baudouin, Meyer and Worcel [1971], this suggests that the levels of platelet AII binding reflect those of vascular tissue.

There are many possible areas of further investigation of platelet AII binding. Perhaps the most important would be a large prospective study assessing the use of the technique as a screening test. The effect of fish oil administration and low dose aspirin therapy on levels of platelet AII binding would also be a valuable study. Platelet AII binding levels have not been measured in multigravid pregnant women, and it would be interesting to determine levels in women whose previous pregnancies were complicated by PIH. Other possible avenues of investigation include isolation and characterisation studies of platelet AII binding sites, particularly on platelets from normotensive pregnant and hypertensive pregnant women, and further efforts to correlate a cellular response with binding site concentrations.

"To a scientist there is no absolute truth; truth is a relative term. A hypothesis becomes more probably true the more often it stands up to experiment, and the more rigorous these experiments are." [Pickering, 1964].

Appendix A.

Materials and disposable equipment :

Acetone : obtained from BDH Chemicals Ltd., Poole, England.

Acetylsalicylic acid : obtained from Sigma Chemicals Co., St. Louis, U.S.A.

Angiotensin II : 2.5 mg ampoules of Hypertensin Ciba were obtained from Ciba-Geigy Ltd., Basle, Switzerland.

Angiotensin II analogues : Angiotensin I, [Sar^1 Ala^8] angiotensin II, [des-Asp^1] angiotensin II (angiotensin III) were obtained from Sigma Chemicals Co., St. Louis, U.S.A.

Apyrase : obtained from Sigma Chemicals Co., St. Louis, U.S.A.

Bacitracin : obtained from Sigma Chemicals Co., St. Louis, U.S.A.

Bovine serum albumin : obtained from Sigma Chemicals Co., St. Louis, U.S.A.

Calcium chloride ($CaCl_2$) : obtained from Sigma Chemicals Co., St. Louis, U.S.A.

Citric acid : obtained from BDH Chemicals Ltd., Poole, England.

CO_2 : compressed gas was obtained from BOC Ltd., Guildford, Surrey.

Earles 199 medium : obtained from Flow Laboratories, Irvine, Scotland.

EDTA (Diaminoethaneteta-acetic acid) : obtained from Fisons Scientific Apparatus Ltd., Loughborough, England.

Glucose : obtained from May and Baker Ltd., Dagenham, England.

Hydrochloric acid (HCl) : obtained from May and Baker Ltd., Dagenham, England.

^{125}I-Angiotensin II : (2000 Ci/mmol, 74 TBq/mmol) was obtained from Amersham International plc, Amersham, Bucks, England.

Liquipipettes : 5 ml plastic liquipipettes were obtained from Elkay, Eireann, Costelloe, Co. Galway, Ireland.

Magnesium sulphate ($MgSO_4$) : obtained from Fisons Scientific Apparatus Ltd., Loughborough, England.

Medi-Swab : containing 70% v/v isopropyl alcohol and 0.5% w/w chlorhexidine acetate, obtained from Smith and Nephew Medical Ltd.

Microcentrifuge tubes : 400 ul polyethylene microcentrifuge tubes were obtained from Beckman, U.S.A.

Minoton solution : isotonic salt solution obtained from ABX, Montpellier, France.

Nylon gauze : 20 um Nylon gauze was obtained from Henry Simon, Stockport, England.

Percoll : obtained from Sigma Chemicals Co., St. Louis, U.S.A.

O-Phenanthroline : obtained from Sigma Chemicals Co., St. Louis, U.S.A.

Polypropylene test tubes were round bottomed and disposable. 3 ml, and 10 ml tubes were obtained from L.I.P. Ltd., Yorkshire, England. Graduated 10 ml tubes were obtained from Sarstedt Ltd., Leicester, England. Appropriate stoppers were supplied by L.I.P. Ltd., and Sarstedt. Ltd. respectively.

Pthalate solutions : dibutyl pthalate and dionyl pthalate obtained from BDH Chemicals Ltd., Poole, England.

Tris : obtained from BDH Chemicals Ltd., Poole, England.

Trisodium citrate obtained from Hopkin and

Williams Ltd., Chadwell Heath.

Vacutainer tubes : obtained from Becton Dickinson Vacutainer Systems, Cowley, Oxford.

Appendix B.

The figures below have been provided by Mr. N. S. Brown (Top grade biochemist) of the Clinical Chemistry department, University Hospital, Nottingham. The table indicates the between-batch coefficients of variation for both serum and urine biochemistry.

Between-batch C.V. (%).

SERUM.

Sodium	1.0
Potassium	3.0 (<3mmol/l); 1.5 (>3mmol/l)
Urea	2.5 (<10mmol/l); 1.5 (>10mmol/l)
Creatinine	4.0 (<200umol/l); 1.5 (>200umol/l)
Osmolality	0.5
Urate	2.5

URINE.

Sodium	4.0
Potassium	3.0
Urea	3.0
Creatinine	3.0
Osmolality	0.5
Urate	2.5

Appendix C.

Derivation of association and dissociation constants from kinetic data using two ligand concentrations. [Gunther, 1980a].

For the reaction of a drug/hormone (D) binding reversibly with a single class of receptor sites (R) to form a receptor-bound complex B :

$$D + R \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} B$$

When the initial free drug/hormone concentration $D_o >> R_o$:

$$D_o \simeq D_t, \text{ for all times (t).}$$

Thus the rate of change of concentration of bound drug/hormone (dB/dt) is approximated by:

$$\begin{aligned} dB/dt &\simeq K_1 D_o(R_o - B) - K_{-1}B \\ &= K_1 D_o R_o - B(K_1 D_o + K_{-1}) \end{aligned}$$

The solution to the general equation: $dy/dx + p(x)y + Q(x)$ is

$$ye^{(p(x)dx} = \int Qe^{(p(x)dx} dx + Z$$

where Z is a constant.

Thus :

$$Be^{(K_1 D_o + K_{-1})t} = \frac{K_1 D_o R_o}{K_1 D_o + K_{-1}} . e^{(K_1 D_o + K_{-1})t} + Z$$

When t=0, B=0 and

$$0 = \frac{K_1 D_o R_o}{K_1 D_o + K_{-1}} + Z$$

Thus :

$$Bt = \frac{K_1 D_o R_o}{K_1 D_o + K_{-1}} \cdot [1 - e^{-(K_1 D_o + K_{-1})t}]$$

When t = infinity, B = Beq

$$= \frac{K_1 D_o R_o}{K_1 D_o + K_{-1}}$$

Thus :

$$\frac{Beq - B}{Beq} = e^{-(K_1 D_o + K_{-1})t}$$

or :

$$\ln \left[\frac{Beq - B}{Beq} \right] = -(K_1 D_o + K_{-1})t$$

When plotted graphically, the slope (S) of:

$\ln \left[\frac{Beq - B}{Beq} \right]$ against time

determines the value of $-(K_1 D_o + K_{-1})$

If two different concentrations of drug/hormone Do and D_o' are used: $-(K_1 D_o + K_{-1}) = S$

and $-(K1 D_o' + K_{-1}) = S'$

$$K1 = \frac{S - S'}{D_o' - D_o} \quad \text{and} \quad K_{-1} = \frac{S' D_o - S D_o'}{D_o' - D_o}$$

REFERENCES

Aalkjaer, C., Johannesen, P., Pedersen, E. B., Rasmussen, A. and Mulvany, M. J., [1984] Morphology and angiotensin II responsiveness of isolated resistance vessels from patients with pre-eclampsia. Scand. J. Clin. Lab. Invest. **44, Suppl. 169,** 57-60.

Abdul-Karim, R. and Assali, N. S., [1961] Pressor response to angiotonin in pregnant and nonpregnant women. Am. J. Obstet. Gynecol. **82,** 246-51.

Ackerly, J., Blumberg, A. and Peach, M. J., [1976] Angiotensin interactions with myocardial sympathetic neurons: enhanced release of dopamine-B-hydroxylase during nerve stimulation. Proc. Soc. Exp. Biol. Med. **151,** 650-53.

Adelson, E., Rheingold, J. J. and Crosby, W. H., [1961] The platelet as a sponge: a review. Blood **17,** 767-74.

Adelstein, R. S. and Eisenberg, E., [1980] Regulation and kinetics of the actin-myosin-ATP interaction. Ann. Rev. Biochem. **49,** 921-56.

Aguilera, G. and Catt, K. J., [1981] Regulation of vascular angiotensin II receptors in the rat during altered sodium intake. Circ. Res. **49,** 751-58.

Aguilera, G., Hauger, R. L. and Catt, K. J., [1978] Control of aldosterone secretion during sodium restriction: adrenal receptor regulation and increased adrenal sensitivity to angiotensin II. Proc. Natl. Acad. Sci. USA. **75,** 975-79.

Aguilera, G., Schirar, A, Baukal, A. and Catt, K. J., [1980] Angiotensin II receptors; properties and regulation in adrenal glomerulosa cells. Circ. Res. **46 (Suppl 1),** 118-27.

Aiken, J. W. and Vane, J. R., [1973] Intrarenal prostaglandin release attenuates the renal vasoconstrictor activity of angiotensin. J. Pharmacol. Exp. Ther. **184,** 678-87.

Alexander, R. W., Brock, T. A., Gimbrone, M. A. Jr. and Rittenhouse, S. E., [1985] Angiotensin increases inositol triphosphate and calcium in vascular smooth muscle. Hypertension **7,** 447-51.

Alexander, R. W., Cooper, B. and Handin, R. I., [1978] Characterisation of the human platelet alpha-adrenergic receptor: Correlation of tritiated dihydroergocryptine binding with aggregation and adenylate cyclase inhibition. J. Clin. Invest. **61**, 1136-44.

Altura, B. M. and Altura, B. T., [1970] Differential effects of substrate depletion on drug-induced contractions of rabbit aorta. Am. J. Physiol. **219**, 1698-705.

Ames, R. P., Borkowski, A. J., Sininski, A. M. and Laragh, J. H., [1965] Prolonged infusion of angiotensin II and norepinephrine and blood pressure, electrolyte balance, and aldosterone and cortisol secretion in normal man and in cirrhosis with ascites. J. Clin. Invest. **44**, 1171-86.

Anderson, J. M., Gimbrone, M. A. and Alexander, R. W., [1981] Angiotensin II stimulates phosphorylation of the myosin light chain in cultured vascular smooth muscle cells. J. Biol. Chem. **256**, 4693-96.

Anfossi, G., Trovati, M., Lanzio, M., Mularoni, E., Massucco, P. and Emanuelli, G., [1988] Effect of labetolol on human platelet function. Clin. Exp. Pharm. Physiol. **15**, 437-48.

Ariens, E. J., [1954] Affinity and intrinsic activity in theory of competitive inhibition; problems and theory. Arch. Intern. Pharmacodyn. **99**, 32-49.

Ariens, E. J., Van Rossum, J. M. and Koopman, P. C., [1960] Receptor reserve and threshold phenomena. Arch. Intern. Pharmacodyn. **127**, 459-78.

Arngrimsson, R., Bjornsson, S., Geirsson, R. T., Bjornsson, H., Walker, J. J. and Snaedal, G., [1990] Genetic and familial predisposition to eclampsia and pre-eclampsia in a defined population. Br. J. Obstet. Gynaecol. **97**, 762-769.

Baudouin, M., Meyer, P. and Worcel, M., [1971] Specific binding of tritiated angiotensin II in rabbit aorta. Biochem. Biophys. Res. Comm. **42**, 434-40.

Beaufils, M., Uzan, S., Donsimoni, R. and Colau, J. C., [1985] Prevention of pre-eclampsia by early antiplatelet therapy. Lancet **i**, 840-42.

Belleau, B., [1964] A molecular theory of drug action based on induced conformational permutations of receptors. J. Med. Chem. 7, 776-84.

Bennett, J. P. and Snyder, S. H., [1980] Regulation of receptor binding interactions of ^{125}I-angiotensin II and ^{125}I-(sarcosine^{1}-leucine^{8})angiotensin II, an angiotensin antagonist, by sodium ions. Eur. J. Pharmacol. 67, 1-10.

Bennett, J. S., Colman, R. F. and Colman, R. W., [1978] Identification of adenine nucleotide binding proteins in human platelet membranes by affinity labeling with 5'-p-fluorsulfonylbenzoyl adenosine. J. Biol. Chem. 253, 7346-54.

Bennett, J. S. and Vilaire, G., [1979] Exposure of platelet fibrinogen receptors by ADP and epinephrine. J. Clin. Invest. 64, 1393-401.

Bickerton, R. K. and Buckley, J. P., [1961] Evidence for a central mechanism in angiotensin induced hypertension. Proc. Soc. Exp. Biol. Med. 106, 834-36.

Bieger, D., Kruger-Thiemer. E., Lullmann, H. and Ziegler, A., [1970] The rate of action of cholinergic drugs in guinea-pig isolated atria. Eur. J. Pharmacol. 9, 156-62.

Birch, D., [1989] Schoolgirl pregnancies. In: Progress in obstetrics and gynaecology (ed.) J. Studd. Churchill Livingstone, Edinburgh. Vol. 7. p.75-90.

Bizzozero, J., [1882] Ueber einen neuen formstandtheil der Bluttes und dessen Rolle bie der Thrombose und der Blutgerrinnung. Virchows Arch. Pathol. Anat. 90, 261.

Boeynaems, J. M. and Dumont, J. E., [1977] The two-step model of ligand receptor interaction. Mol. Cell. Endocrinol. 7, 33-47.

Bohr, D. F. and Urchida, E., [1967] Individualities of vascular smooth muscle in response to angiotensin. Circ. Res. 20-21 (Suppl II), 135-43.

Boke, T. and Malik, K. U., [1983] Enhancement by locally generated angiotensin II of release of the adrenergic transmitter in the isolated rat kidney. J. Pharmacol. Exp. Ther. 226, 900-07.

Born, G. V. R., [1972] Human Blood Coagulation. Haemostasis and Thrombosis. Blackwell Scientific Publications, London. p.159-75.

Born, G. V. R. and Feinberg, M. J., [1975] Binding of adenosine diphosphate to intact human platelets. J. Physiol. **251**, 803-16.

Borochov, H. and Shinitzky, M., [1976] Vertical displacement of membrane proteins mediated by changes in microviscosity. Proc. Natl. Acad. Sci. **73**, 4526-30.

Bost, K. L., Smith, E. M. and Blalock, J. E., [1985] Similarity between the corticotrophin (ACTH) receptor and a peptide encoded by an RNA that is complementary to ACTH mRNA. Proc. Natl. Acad. Sci. USA **82**, 1372-75.

Breckenridge, A., [1966] Hypertension and hyperuricaemia. Lancet. **i**, 15-18.

Breton-Gorius, J., and Guichard, J, [1975] Fine structural and cytochemical identification of microperoxisomes in developing human erythrocytic cells. Am. J. Pathol. **79**, 523-36.

Brock, T.A., Alexander, R. W., Ekstein, L. S., Atkinson, W. J. and Gimbrone, M. A. Jr., [1985] Angiotensin increases cytosolic free calcium in cultured vascular smooth muscle cells. Hypertension **7**, I105-09.

Brod, J., Hejl, Z., Hornych, A., Jirka, J., Slechta, V. and Burianova, B., [1969] Comparison of haemodynamic effects on equipressor doses of intravenous angiotensin and noradrenalin in man. Clin. Sci. **36**, 161-72.

Brooks, V. L. and Malvin, R. L., [1982] Intracerebro-ventricular infusion of angiotensin II increases sodium excretion. Proc. Soc. Exp. Biol. Med. **169**, 532-37.

Broughton Pipkin, F., [1988] The renin-angiotensin system in normal and hypertensive pregnancies. In: Handbook of Hypertension, Vol. 10: Hypertension in pregnancy. (ed.) P. C. Rubin. Elsevier, Amsterdam, The Netherlands. p. 118-51.

Broughton Pipkin, F., Hunter, J. C., Turner, S. R. and O'Brien, P. M. S., [1982] Prostaglandin E2 attenuates the pressor response to angiotensin II in pregnant subjects but not in nonpregnant subjects. Am. J. Obstet. Gynecol. **142**, 168-76.

Broughton Pipkin, F., Hunter, J. C., Turner, S. R. and O'Brien, P. M. S., [1984] The effect of prostaglandin E2 upon the biochemical response to infused angiotensin II in human pregnancy. Clin. Sci. **66**, 399-406.

Broughton Pipkin, F. and Symonds, E. M., [1977] Factors affecting angiotensin II concentrations in the human infant at birth. Clin. Sci. Mol. Med. **52**, 449-56.

Brown, G. P. and Venuto, R. C., [1986] Angiotensin II receptor alterations during pregnancy in rabbits. Am. J. Physiol. **251**, E58-64.

Brown, J. J., Davies, D. L., Doak, P. B., Lever, A. F. and Robertson, J. I. S., [1963] Plasma renin in normal pregnancy. Lancet. **ii**, 900-02.

Brown, J. J., Davies, D. L., Doak, P. B., Lever, A. F. and Robertson, J. I. S. [1966] Plasma renin concentrations in the hypertensive diseases of pregnancy. J. Obstet. Gynaecol. Brit. Cwlth. **73**, 410-17.

Brown, J. J., Davies, D. L., Lever, A. F., Robertson, J. I. S. and Trust, P., [1964] Variations in plasma renin during the menstrual cycle. B. M. J. **ii**, 1114-15.

Brunner, H. R., Chang, P., Wallach, R., Sealey, J. E. and Laragh, J. H., [1972] Angiotensin II vascular receptors: Their avidity in relationship to sodium balance, the autonomic nervous system and hypertension. J. Clin. Invest. **51**, 58-67.

Bumpus, F. M., Khairallah, P. A., Arakawa, K., Page, I. M. and Smeby, R. R., [1961] The relationship of structure to pressor and oxytocic action of isoleucine angiotensin octapeptide and various analogues. Biochim. Biophys. Acta. **46**, 38-46.

Burgess-Wilson, M. E., Morrison, R. and Heptinstall, S., [1986] Spontaneous platelet aggregation in heparinised blood during pregnancy. Thromb. Res. **37**, 385-93.

Bylund, D. B. and U'Pritchard, D. C., [1983] The characterisation of alpha-1 and alpha-2 adrenergic receptors. Int. Rev. Neurobiol. **24**, 343-431.

Caldwell, P. R. B., Seegal, B. C., Hsu, K. C., Das, M. and Soffer, R. L., [1976] Angiotensin-converting enzyme: vascular endothelial localisation. Science **191**, 1050-51.

Cameron, H. A. and Ardlie, N. G., [1982] The facilitating effects of adrenaline on platelet aggregation. Prostaglandins, Leukotrienes and Medicine. **9**, 117-28.

Campbell, D. J., Bouhnik, J., Coezy, E., Menard, J. and Corvol, [1985] Characterisation of precursor and secreted forms of human angiotensinogen. J. Clin. Invest. **75**,1880-93.

Campbell, D. M., MacGillivray, I. and Carr-Hill, R., [1985] Pre-eclampsia in second pregnancy. Br. J. Obstet. Gynaecol. **92**, 131-40.

Campbell, S., Pearce, J. M. F., Hackett, G., Cohen-Overbeek, T., and Hernandez, C., [1986] Qualitative assessment of uteroplacental blood flow; early screening test for high risk pregnancies. Obstet. Gynecol. **68**, 649-653.

Campbell, W. B., Brady, M. T. and Gomez-Sanchez, C. E., [1986] Effects of angiotensin, prostaglandin E2 on the early and late steps of aldosterone biosynthesis in isolated adrenal cells. J. Steroid. Biochem. **24**, 865-70.

Capponi, A. M., Aguilera, G., Fakunding, J. L. and Catt, K. J., [1981] Angiotensin II: Receptors and mechanism of action. In; Biochemical regulation of blood pressure. Ed. Soffer, R. J., New York, J. Wiley & Sons Inc. p.205-62.

Capponi, A. M. and Catt, K. J., [1980] Solubilisation and characterisation of adrenal and uterine angiotensin receptors after photoaffinity labelling. J. Biol. Chem. **255**, 12081-86.

Carey, R. M., Vaughan, E. D. Jr., Peach, M. J., Ayres, C. R., [1978] Activity of (Des-Aspartyl)-angiotensin II and angiotensin II in man. J. Clin. Invest. **61**, 20-31.

Carroll, R. J., Lohmeier, T. E. and Brown, A. J., [1983] Renal vein norepinephrine overflow decreases during chronic angiotensin II hypertension. Fed. Proc. **43**, 473.

Catt, K. J., Mendelsohn, F. A. C., Millan, M. A. and Aguilera, G., [1984] The role of angiotensin

II receptors in vascular regulation. J. Cardiovasc. Pharmacol. 6, S575-86.

Cauvin, C., Loutzenhiser, R., Hwang, O. and Van Breeman, C., [1982] Alpha 1-adrenoreceptors induce Ca influx and intracellular Ca release in isolated rabbit aorta. Eur. J. Pharmacol. 22, 233-35.

Chamberlain, G., [1981] Raised blood pressure in pregnancy. Br. J. Hosp. Med. 26, 127-33.

Chamberlain, G., Phillip, E., Howlett, B. and Masters, K., [1978] British Births 1970, Vol. 2; Obstetric Care. Heinemann, London.

Changeux, J. P. and Podleski, T. R., [1968] On the excitability and cooperativity of the electroplax. Proc. Nat. Acad. Sci. 59, 944-50.

Changeux, J. P., Thiery, J., Tung, Y. and Kittel, C., [1967] On the cooperativity of biological membranes. Proc. Nat. Acad. Sci. 57, 335-41.

Chesley, L. C., [1976] Blood pressure, edema and proteinuria in pregnancy. Prog. Clin. Biol. Res. 7, 249-68.

Chesley, L. C., [1978a] History. In; Hypertensive disorders of pregnancy, Appleton-Century-Crofts, New York. p.17-34.

Chesley, L. C., [1978b] Orientation. In; Hypertensive disorders of pregnancy, Appleton-Century-Crofts, New York. p.1-15.

Chesley, L. C., [1978c] Epidemiology of preeclampsia-eclampsia. In; Hypertensive disorders of pregnancy, Appleton-Century-Crofts, New York. p.35-55.

Chesley, L. C. and Cooper, D. W., [1986] Genetics of hypertension in pregnancy: possible single gene control of pre-eclampsia and eclampsia in the descendants of eclamptic women. Br. J. Obstet. Gynaecol. 93, 898-908.

Chesley, L. C., Talledo, E., Bohler, C. S. and Zuspan, F. P., [1965] Vascular reactivity to angiotensin II and norepinephrine in pregnant and non-pregnant women. Am. J. Obstet. Gynecol. 91, 837-42.

Chesley, L. C. and Tepper, I. H., [1967] Effects of progesterone and estrogen on the sensitivity to angiotensin II. J. Clin. Endocrinol. Metab. 27, 576-81.

Chevillotte, E., Devynck, M-A., Fyhrquist, F., Meyer, P., Rouzaire-Dubois, B. and Worcel, M., [1975] Angiotensin-induced variations of receptors in rat uterine membranes. Clin. Sci. Mol. Med. **48**, 23S-26S.

Chevillotte, E., Rouzaire-Dubois, B., Devynck, M. A. and Meyer, P., [1976] Variations in the number of uterine angiotensin receptors following changes in plasma angiotensin levels. Contr. Nephrol. **3**, 134-141.

Chinn, R. H. and Dusterdieck, G., [1972] The response of blood pressure to infusion of angiotensin II: relation to plasma concentrations of renin and angiotensin II. Clin. Sci. **42**, 489-504.

Clark, A. J., [1926a] The reaction between acetyl choline and muscle cells. J. Physiol. **61**, 530-46.

Clark, A. J., [1926b] The antagonism of acetyl choline and atropine. J. Physiol. **61**, 547-56.

Coghlan, J. P., Considine, P. J., Denton, D. A., Fei, D. T. W., Leksell, L. G., McKinley, M. J., Muller, A. F., Tarjan, E., Weisinger, R. S. and Bradshaw, R. A., [1981] Sodium appetite in sheep induced by cerebral ventricular infusion of angiotensin: comparison with sodium deficiency. Science. **214**, 195-97.

Collins, E. and Turner, G., [1975] Maternal effects of regular salicylate ingestion in pregnancy. Lancet **ii**, 335-38.

Colman, R. W., [1990] Platelet receptors. Hematol. Oncol. Clin. North. Am. **4**, 27-42.

Colman, R. W. and Figures, W. R., [1984] Characteristics of an ADP receptor mediating platelet activation. Mol. Cell. Biochem. **59**, 101-111.

Connor, J. A., Cornwall, M. C. and Williams, G. H., [1987] Spatially resolved cytosolic calcium response to angiotensin II and potassium in rat glomerulosa cells measured by digital imaging techniques. J. Biol. Chem. **262**, 2919-27.

Corash, L. and Gralnick, H. R., [1974] High yield quantitive isolation of human platelets from whole blood. Blood **44**, 919 (abstract).

Cowley, A. J., Stainer, K., Cockbill, S. and Heptinstall, S., [1984] Correlation between platelet behaviour and cold induced vasoconstriction in man, and the effects of epoprostenol infusion. Clin. Sci. 67, 511-14.

Cowley, A. J., Heptinstall, S. and Mitchell, J. R. A., [1985] Similarity between platelet and blood-vessel reactivity. Lancet i, 154-55.

Cowley, A. W. and Guyton, [1972] Quantification of intermediate steps in the renin angiotensin-vasoconstrictor feed-back loop in the dog. Circ. Res. 30, 557-67.

Cowley, A. W. and Lohmeir, T. E., [1977] The relationship between body fluid volume, sodium ion concentration, and sensitivity to pressor effect of angiotensin II in dogs. Circ. Res. 42, 503-10.

Craven, D. J. and Symonds, E. M., [1978] Increased renin concentration in plasma and amniotic fluid during storage. Clinical Chemistry 24, 933-35.

Davey, D. A., [1985] Hypertensive disorders of pregnancy. In; Progress in obstetrics and gynaecology. Vol.5. (ed) J. Studd. Churchill Livingstone, Edinburgh. p. 89-107.

Davey, D. A., [1986] Hypertensive disorders of pregnancy. In; Dewhurst's textbook of obstetrics and gynaecology for postgraduates. (ed) C. R. Whitfield. Blackwell Scientific Publications, Oxford. p. 200-41.

Davey, D. A. and MacGillivray, I., [1986] The classification and definition of the hypertensive disorders of pregnancy. In: Hypertension in pregnancy. Proceedings of the 16th study group of the RCOG. (eds.) F. Sharp and E. M. Symonds. Perinatology Press, Ithaca, New York. p. 401-08.

Davis, J. O. and Freeman, R. H., [1976] Mechanisms regulating renin release. Physiol. Rev. 56, 1-56.

Davison, J. M., [1980] The urinary system. In: Clinical physiology in Obstetrics. F. Hytten and G. Chamberlain (eds). Blackwell, England. p. 296-327.

Davison, J. M. and Lindheimer, M. D., [1984] Pregnancy and renal disease. In; Progress in obstetrics and gynaecology. Vol.4. (ed) J. Studd. Churchill Livingstone, Edinburgh. p. 151-65.

Day, M. D., McCubbin, J. W. and Page, I. H., [1965] Limited hypertensive effect of infusion of angiotensin. Am. J. Physiol. 209, 264-68.

De Haen, C., [1976] The non-stoichiometric floating receptor model for hormone sensitive adenylate cyclase. J. Theor. Biol. 58, 383-400.

Dekker, G. A., Makovitz, J. W. and Wallenburg, H. C. S., [1990] Prediction of pregnancy-induced hypertensive disorders by angiotensin II sensitivity and supine pressor test. Br. J. Obstet. Gynaecol. 97, 817-821.

Dempsey, P. J., McCallum, Z. T., Kent, K. M. and Cooper, T., [1971] Direct myocardial effects of angiotensin II. Am J. Physiol. 220, 477-81.

Derkx, F. H. M., Stuenkel, C., Schalekamp, M. P. A., Visser, W., Huisveld, I. H. and Schalekamp, M. A. D. H., [1986] Immunoreactive renin, prorenin and enzymatically active renin in plasma during pregnancy and in women taking oral contraceptives. J. Clin. Endocrinol. Metab. 63, 1008-15.

De Robertis, E., Lunt, G. S. and La Torre, J. L., [1971] Multiple binding sites for acetylcholine in a proteolipid from electric tissue. Mol. Pharmacol. 7, 97-103.

de Snoo, K. and Remmelts, R., [1938] Die Eclampsie geographisch betrachtet. Internat. Cong. Verlosk. Gynaecol. Vol. 1. Brill, Leyden. p. 107-36.

Devynck, M-A. and Meyer, P., [1976] Angiotensin receptors in vascular tissue. Am. J. Med. 61, 758-67.

Devynck, M-A., Pernollet, M-G., Macdonald, G. J., Matthews, P. G., Raisman, R. S. and Meyer, P., [1978] Alterations of adrenal and uterine angiotensin II receptors during alteration of sodium intake and/or experimental hypertension. Clin. Sci. Mol. Med. 55, Suppl. 4 171s-74s.

Devynck, M-A., Pernollet, M-G. and Meyer, P., [1973] Angiotensin receptors in smooth muscle cell membranes. Nature. 245, 55-58.

Devynck, M-A., Pernollet, M-G., Meyer, P., Fermandjian, S., Fromageot, P. and Bumpus, F. M., [1974] Solubilisation of angiotensin II receptors in rabbit aortae membranes. Nature. 249, 67-69.

Devynck, M-A., Rouzaire-Dubios, B., Chevillotte, E. and Meyer, P., [1976] Variations in the number of uterine angiotensin receptors following changes in plasma angiotensin levels. Eur. J. Pharmacol. **40**, 27-37.

Dickinson, C. J. and Lawrence, J. R., [1963] A slowly developing pressor response to small concentrations of angiotensin, its bearing on the pathogenesis of chronic renal hypertension. Lancet **i**, 1354.

Ding, Y-A., Kenyon, C. J. and Semple, P. F., [1984] Receptors for angiotensin II on platelets from man. Clin. Sci. **66**, 725-31.

Ding, Y-A., Kenyon, C. J. and Semple, P. F., [1985] Regulation of platelet receptors for angiotensin II in man. J. Hypertens. **3**, 209-12.

Ding, Y-A., MacIntyre, D. E., Kenyon, C. J. and Semple, P. F., [1985] Potentiation of adrenaline-induced platelet aggregation by angiotensin II. Thromb. Haemostas. **54**, 717-20.

Donker, A. J. M., Min, I. and Venuto, R. C., [1983] The conscious instrumented rabbit: A model for the study of mechanisms of blood pressure regulation during pregnancy. Hypertension. **5**, 514-20.

Douglas, J.T., Shah, M., Lowe, G. D. O., Belch, J. J. F., Forbes, C. D. and Prentice, C. R. M., [1982] Plasma fibrinopeptide A and beta-thromboglobulin in pre-eclampsia and pregnancy hypertension. Thromb. Haemostas. **47**, 54-55.

Downing, S. E. and Sonnenblick, E. H., [1963] Effects of continuous infusion of angiotensin II on ventricular performance. J. Appl. Physiol. **18**, 585-92.

Dunlop, W. and Davison, J. M., [1977] The effect of normal pregnancy upon the renal handling of uric acid. Br. J. Obstet. Gynaecol. **84**, 13-21.

Elliot, G. T. Jr., [1873] Case 120: puerperal eclampsia in the eighth month: extraordinary family history: rigid cervix, douche, dilators, forceps. In: Obstetric Clinic, Appleton, New York. p. 291-93.

Elliott, M. F. and Goodfriend, T. L., [1979] Angiotensin and bradykinin interactions with phospholipids. Biochim. Biophys. Acta. **586**, 357-73.

Elliott, M. F., Siegel, F. L., Hadjokas, N. E. and Goodfriend, T. L., [1985] Angiotensin effects on calcium and steroidogenesis in adrenal glomerulosa cells. Endocrinology **116**, 1051-59.

Elton, T. S., Dion, L. D., Bost, K. L., Oparil, S. and Blalock, J. E., [1988] Purification of an angiotensin II binding protein by using antibodies to a peptide encoded by angiotensin II complementary RNA. Proc. Natl. Acad. Sci. USA **85**, 2518-22.

Ehrlich, P., [1913] Chemotherapeutics: scientific principles, methods and results. Lancet **ii**, 445-51.

Epstein, A. N., Fitzsimons, J. T. and Rolls, B. J., [1969] Drinking caused by intracranial injection of angiotensin into the rat. J. Physiol. **200**, 98-100P.

Erne, P., Mittelholzer, E., Burgisser, E., Fluckiger, R. and Buhler, F. R., [1984] Measurement of receptor induced changes in intracellular free calcium in human platelets. J. Rec. Res. **4**, 587-604.

Everett, R. B., Worley, R. J., MacDonald, P. C. and Gant, N. F., [1978] Modification of vascular responsiveness to angiotensin II in pregnant women by intravenously infused 5 alpha-dihydroprogesterone. Am. J. Obstet. Gynecol. **131**, 352-57.

Everett, R. B., Worley, R. J., MacDonald, P. C. and Gant, N. F., [1978] Effect of prostaglandin synthetase inhibitors on pressor response to angiotensin II in pregnancy. J. Clin. Endocrinol. Metab. **46**, 1007-10.

Falcon, J. C., Phillips, M. I., Hoffman, W. E. and Brody, M. J., [1978] Effects of intraventricular angiotensin II mediated by the sympathetic nervous system. Am. J. Physiol. **235**, H392-99.

Fay, F. S., Shlevin, H. H., Granger, W. C. Jr. and Taylor, S. R., [1979] Aequorin luminescence during activation of single isolated smooth muscle cells. Nature. **280**, 506-08.

Fay, R. A., Hughes, A. O. and Farron, N. T., [1983] Platelets in pregnancy; hyperdestruction in pregnancy. Obstet. Gynecol. **61**, 238-40.

Feinstein, M. B., Egan, J. J., Sha'afi, R. I. and White, J., [1983] The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP production. Biochem. Biophys. Res. Commun. 113, 598-604.

Fenton, V., Saunders, K. and Cavill, I., [1977] The platelet count in pregnancy. J. Clin. Path. 30, 68-69.

Ferris, T. F., [1988] Prostanoids in normal and hypertensive pregnancy. In: Handbook of Hypertension, Vol. 10: Hypertension in pregnancy. (ed.) P. C. Rubin. Elsevier, Amsterdam, The Netherlands. p. 102-17.

Finnerty, F. A. Jr. and Bepko, F. J. Jr., [1966] Lowering the perinatal mortality and the prematurity rate. J. A. M. A. 429-32.

Fischer, E., [1894] Einfluss der Configuration auf die Wirkung der Enzyme. Diese Berichte 27, 2985-3005.

Fischer-Ferraro, C., Nahmod, V. E., Goldstein, D. J. and Finkielman, S., [1971] Angiotensin and renin in rat and dog brain. J. Exp. Med. 133, 353-61.

Fisher, K. A., Luber, A., Spargo, B. H. Spargo and Lindheimer, M. D., [1980] A biopsy study of hypertension in pregnancy. In; Pregnancy Hypertension. (eds) J. Bonnar, I. MacGillivray and E. M. Symonds. MTP, Lancaster. p.333-38.

Fitzgerald, D. J., Entman, S. S., Mulloyk, K. and FitzGerald, G. A., [1987] Decreased prostacyclin biosynthesis preceding the clinical manifestation of pregnancy-induced hypertension. Circulation. 75, 956-63.

Fitzsimons, J. T. and Simons, B. J., [1969] The effect of drinking in the rat of intravenous infusion of angiotensin given alone or in combination with other stimuli of thirst. J. Physiol. 203, 45-47.

Fluharty, S. J. and Epstein, A. N., [1983] Sodium appetite elicited by intracerebroventricular infusion of angiotensin II in the rat: II Synergistic interaction with systemic mineralocorticoids. Behav. Neurosci. 97, 746-48.

Fowler, W. L. Jr., Johnson J. A., Kurz, K. D., Kiljoil,J., Love, S. and Payne, C. G., [1981] Role of the renin-angiotensin system in maintaining arterial pressure in conscious pregnant rats. Endocrinol. 109, 290-95.

Freer, R. J., [1975] Calcium and angiotensin tachy-phylaxis in rat uterine smooth muscle. Am. J. Physiol. 228, 1423-30.

Freer, R. J., Pappano, A. J., Peach, M. J., Bing, K. T., McLean, M. J., Vogel, S. and Sperelakis, N., [1976] Mechanism for the positive inotropic effect of angiotensin II on isolated cardiac muscle. Circ. Res. 39, 178-83.

Friedman, E. A. and Neff, R. K., [1977] Pregnancy hypertension. A systematic evaluation of clinical diagnostic criteria. PSG Publishing Co., Littleton, Massachusetts.

Friedman, S. M. and Friedman, C. L., [1967] The ionic matrix of vasoconstriction. Circ. Res. 21 **(Suppl II)**, 147-55.

Frye, L.D. and Edidin, M., [1970] The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. J. Cell. Sci. 7, 319-335.

Fyhrquist, F., Rosenlof, K., Gronhagen-Riska, C., Hortling, L. and Tikkanen, I., [1985] Evidence that renin substrate (angiotensinogen) may be a precursor of erythropoietin. Scand. J. Urol. Nephrol. Suppl. 90, 41-43.

Gallery, E. D. M., Stokes, G. S., Gyory, A. Z., Rowe, J. and Williams, J., [1980] Plasma renin activity in normal human pregnancy and in pregnancy-associated hypertension, with reference to cryoactivation. Clin. Sci. 59, 49-53.

Ganong, W. F., [1984] The brain renin-angiotensin system. Ann. Rev. Physiol. 46, 17-31.

Gant, N. F., Chand, S., Worley, R. J., Whalley, P. J., Crosby, U. D. and MacDonald, P. C., [1974] A clinical test useful for predicting the development of acute hypertension in pregnancy. Am. J. Obstet. Gynecol. 120, 1-7.

Gant, N. F., Daley, G. L., Chand, S., Whalley, P. J. and MacDonald, P. C., [1973] A study of angiotensin II pressor response throughout primigravid pregnancy. J. Clin. Invest. 52, 2682-89.

Gant, N. F. and Worley, R. J., [1980a] Classification and differential diagnosis of hypertensive disorders in pregnancy. In; Hypertension in pregnancy. Appleton-Century-Crofts, New York. p. 1-10.

Gant, N. F. and Worley, R. J., [1980b] Evaluation and management of hypertension in pregnancy. In; Hypertension in pregnancy. Appleton-Century-Crofts, New York. p. 107-165.

Genest, J., de Champlain, J., Veyrat, R., Boucher, R., Tremblay, G. Y., Strong, C. G., Koiw, E. and Marc-Aurele, J., [1965] Hypertension XIII, a monograph of the American Heart Association, Council for High Blood Pressure Research. p. 97.

George, J. N., Nurden, A. T. and Phillips, D. R., [1985] Molecular defects in interactions of platelets with the vessel wall. N. Engl. J. Med. **17**, 1084-98.

Gerrard, J. M. and White, J. G., [1976] The structure and function of platelets, with emphasis on their contractile nature. In: Pathobiology annual. (ed) Iochim, H. L., Appleton-Century Crofts, New York. Vol 6, p. 31-58.

Gibson, B., Hunter, D., Neame, P. B. and Kelton, J. G., [1982] Thrombocytopenia in pre-eclampsia and eclampsia. Semin. Thromb. Hemost. **8**, 234-47.

Gibson, R. A., [1988] The effect of diets containing fish and fish-oils on disease risk factors in humans. Aust. N.Z. J. Med. **18**, 713-22.

Giles, C. and Inglis, T. C. M., [1981] Thrombocytopenia and macrothrombocytosis in gestational hypertension. Br. J. Obstet. Gynaecol. **88**, 1115-19.

Giles, W. B., Trudinger, B. J. and Baird, P. J., [1985] Fetal umbilical artery flow velocity waveforms and placental resistance: pathological correlation. Br. J. Obstet. Gynaecol. **92**, 31-38.

Gilman, A. G., [1984] Guanine nucleotide binding regulatory proteins and dual control of adenylate cyclase. J. Clin. Invest. **73**, 1-4.

Ginsburg, J. and Duncan, S. L. B., [1967] Peripheral circulation in hypertensive pregnancy. Cardiovasc. Res. **1**, 356-61.

Gleeson, R., Ahmed, Y., Rice-Evans, C. and Elder, M. G., [1990] Platelet membrane fluidity in pregnancy hypertension. Lancet 335(i), 225-26. (letter)

Glossman, H., Baukal, A. and Catt, K. J., [1974] Cation dependent high-affinity angiotensin II binding to adrenal cortex receptors. Science 185, 281-83.

Goldstein, A., Aronow, L. and Kalman, S. M., [1974] Theory based on the rate of drug-receptor combination. In: Principles of drug action. (2nd. ed) J. Wiley & Sons, New York. p. 104-06.

Gordon, D. B. and Sachin, I. N., [1977] Chromographic separation of multiple renin substrates in women; effect of pregnancy and oral contraceptives. Proc. Soc. Exp. Biol. Med. 156, 461-64

Gordon, R. D., Symonds, E. M., Wilmhurst, E. G. and Pawsey, C. G. K., [1973] Plasm renin activity, plasma angiotensinogen and plasma and urinary electrolytes in normal and toxaemic pregnancy, including a prospective study. Clin. Sci. Mol. Med. 45, 115-27.

Grant, A., [1990] General principles of clinical trials. VIIth World Congress of Hypertension in Pregnancy. Scientific Program. p.31. (abstract).

Gregory, H. and Aguilera, G., [1981] Comparison of receptor binding metabolism and pressor effect of angiotensin II and des-asp-angiotensin II. Endocrine Society Abstracts, 63rd Annual Meeting, p. 86.

Gunther, S., Gimbrone, M. A. and Alexander, R. W., [1980a] Identification and characterisation of the high affinity vascular angiotensin II receptor in rat mesenteric artery. Circ. Res. 47, 278-86.

Gunther, S., Gimbrone, M. A. and Alexander, R. W., [1980b] Regulation by angiotensin II of its receptors in resistance blood vessels. Nature 287, 230-32.

Gütmann, F. D., Tagawa, H., Haber, E. and Barger, A. C., [1973] Renal arterial pressure, renin secretion, and blood pressure control in trained dogs. Am. J. Physiol. 224, 66-72.

Hall, J. E., Guyton, A. C., Jackson, T. E., Coleman, T. G., Lohmeier, T. E. and Trippodo, N. C., [1977] Control of the glomerular filtration rate by the renin-angiotensin system. Am. J. Physiol. 233, F366-72.

Hall, M. H. and Campbell, D. M., [1986] Geographical epidemiology of hypertension in pregnancy. In: Hypertension in pregnancy. (eds) F. Sharp, E. M. Symonds. Proc. 16th study group RCOG. Perinatology Press, Ithaca, New York. p. 33-46.

Haller, H., Ludersdorf, M., Lenz, T., Distler, A. and Philipp, T., [1987] Changes in sensitivity to angiotensin II in platelets. J. Cardiovasc. Pharmacol. **10 (Suppl. 10)**, S44-S46.

Haller, H., Oeney, T., Hauck, U., Distler, A. and Philipp, T., [1989] Increased intracellular free calcium and altered sensitivity to angiotensin II in platelets of pre-eclamptic women. Am. J. Hypertension. **2**, 238-43.

Hanretty, K. P., Whittle, M. J. and Rubin, P. C., [1988] Doppler uteroplacental waveforms in pregnancy induced hypertension: a re-appraisal. Lancet **i**, 850-52.

Harley, J. M. G., [1966] Pregnancy in the chronic hypertensive woman. Proc. R. Soc. Med. **59**, 835-38.

Harmon, J. T. and Jamieson, G. A., [1985] Thrombin binds to a high affinity -900,000 dalton site on human platelets. Biochem. **24**, 58-64.

Haslam, R. J., [1973] Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. Sem. Hematol. **6**, 333-50.

Hassid, A. and Williams, C., [1983] Vasoconstrictor-evoked prostaglandin synthesis in vascular smooth muscle. Am. J. Physiol. **245**, C278-82.

Haulica, I., Petrescu, G., Stratone, A., Branisteanu, D., Rosca, V., Neamtu, C. and Slatineanu, S., [1982] Possible functions of brain renin. In: (ed.) Ganten, D., Printz, M., Phillips, M.I. and Scholkens, B.A., Heidelberg: Springer-Verlag. p335-52.

Heistad, D. D., Abboud, F. M. and Ballard, D. R., [1971] Relationship between plasma sodium concentration and vascular reactivity in man. J. Clin. Invest. **50**, 2022-32.

Helmer, O. M. and Judson, W. E., [1967] Influence of high renin substrate levels on the renin-angiotensin system in pregnancy. Am. J. Obstet. Gynecol. 99, 9-17.

Henderson, P. J. F., [1973] Steady state enzyme kinetics with high affinity substrates or inhibitors. Biochem. J. 135, 101-07.

Heymann, M. A. and Rudolph, A. M., [1976] Effect of acetylsalicylic acid in the ductus arteriosus and circulation in foetal lambs in utero. Circ. Res. 38, 418-22.

Hill, W. H. P. and Andrus, E. C., [1941] The cardiac factor in the 'pressor' effects of renin and angiotensin. J. Exptl. Med. 74, 91-103.

Hinssen, H., D'Haese, J., Small, J. V. and Sobieszek, A., [1978] Mode of filament assembly of myosins from muscle and nonmuscle cells. J. Ultrastruct. Res. 64, 282-302.

Hirai, N., Yaniahara, T., Nakayama, T., Ishibashi, M., Yamaji, T., [1988] Plasma levels of atrial natriuretic peptide during normal pregnancy and in pregnancy complicated by hypertension. Am. J. Obstet. Gynecol. 159, 27-31.

HMSO [1982-1984] Reports on confidential enquires into maternal death in England and Wales, 1952-1954 to 1982-84.

Hoffman, B. B., De Lean, A., Wood, C. L., Schoken, D. D. and Lefkowitz, R. J., [1979] Alpha-adrenergic receptor subtypes: quantitative assessment by ligand binding. Life Sci. 24, 1739-46.

Hollenberg, M. D., [1985] Receptor models and the action of neurotransmitters and hormones; some new perspectives. In: Neurotransmitter receptor binding, (eds. H. I. Yamamura, S. J. Enna and M. J. Kuhar). Raven Press, New York. p. 2-38.

Hollenberg, N. K., Chenitz. W. R., Adams, D. F. and Williams, G. H., [1974] Reciprocal influence of salt intake on adrenal glomerulosa and renal vascular responses to angiotensin II in normal man. J. Clin. Invest. 54, 34-42.

Hollenberg, N. K., Williams, G. H., Burger, B. and Hooshmand, I., [1975] Potassium's influence on the renal vasculature and the adrenal gland, and their responsiveness to angiotensin II in normal man. Clin. Sci. Mol. Med. 49, 527-34.

Holmsen, H., [1972] The platelet: its membrane, physiology, and biochemistry. Clin. Haematol. **1**, 235-66.

Holmsen, H., [1985] Platelet metabolism and activation. Semin. Haematol. **22**, 219-40.

Hovig, T., [1968] The ultrastructure of blood platelets in normal and abnormal states. Ser. Haematol. **1**, 3-64.

Hovig, T., [1970] Influence of various compounds and surfaces of blood platelets and platelet aggregates. A scanning electron microscopic study. Ser. Haematol. **3**, 47-67.

Howie, P. W., [1988] The puerperium and its complications. In: Dewhurst's textbook of obstetrics and gynaecology for postgraduates. (ed) C. R. Whitfield. Blackwell Scientific Publications, Oxford. 4th edition. p. 463-81.

Howie, P. W., [1977] The haemostatic mechanisms in pre-eclampsia. Clin. Obstet. Gynaecol. **4**, 595-611.

Howie, P. W., Prentice, C. R. and McNicol, G. P., [1971] Coagulation, fibrinolysis and platelet function in pre-eclampsia, essential hypertension and placental insufficiency. J. Obstet. Gynaecol. Br. Commonw. **78**, 992-1003.

Hricik, D., Browning, P., Kapelman R., Goorno, W., Madias, N. and Dzau, U., [1983] Captopril-induced functional renal insufficiency in patients with bilateral renal artery stenosis or renal artery stenosis in a solitary kidney. N. Engl. J. Med. **308**, 373-76.

Hughes, E. C., [1972] Obstetric-Gynecologic terminology. Davis, Philadelphia. p.422-23.

Hull, M. G., Savage, P. E., Bromham, D. R., Ismail, A. A. and Morris, A. F., [1982] The value of a single serum progesterone measurement in the midluteal phase as a criterion of a potentially fertile cycle (ovulation) derived from treated and untreated conception cycles. Fertil. Steril. **37**, 355-60.

Humphreys, M. H. and Lin, S. Y., [1988] Peptide hormones and the regulation of sodium excretion. Hypertension **11**, 397-410.

Ichikawa, I. and Brenner, B. M., [1984] Glomerular actions of angiotensin II. Am. J. Med. 76, 43-49.

Inglis, T. C. M., Stuart, J., George, A. J. and Davies, A. J., [1982] Haemostatic and rheological changes in normal pregnancy and pre-eclampsia. Br. J. Haematol. 50, 461-65.

Ismay, M. J. A., Lumbers, E. R. and Stevens, A. D., [1979] The action of angiotensin II on the baroreflex response of the conscious ewe and the conscious fetus. J. Physiol. 288, 467-47.

Jacobs, S. and Cuatrecasas, P., [1976] The mobile receptor hypothesis and "cooperativity" of hormone binding. Application to insulin. Biochim. Biophys. Acta. 433, 482-95.

Jennings, L. K. and Phillips, D. R., [1982] Purification of glycoproteins IIb and IIIa from human platelet plasma membranes and characterisation of a calcium dependent glycoprotein IIa-IIIa complex. J. Biol. Chem. 257, 10458-66.

Johansson, B., [1981] Vascular smooth muscle reactivity. Ann. Rev. Physiol. 43, 359-70.

Johnson, J., Johnson, I. R., Ronan, J. E. and Craven, D. J., [1984] The site of renin in the human uterus. Histopathology 8, 273-78.

Johnson, J. A., Davis, J. O. and Witty, R. T., [1971] Effects of catecholamines and renal nerve stimulation on renin release in the non-filtering kidney. Circ. Res. 29, 646-53.

Kao, K-J., Pizzo, S. V. and McKee, P.A., [1979] Demonstration and characterisation of specific binding sites for factor VIII/vWF on human platelets. J. Clin. Invest. 63, 656-64.

Kaplan, N. M. and Silah, J. G., [1964] The effect of angiotensin II on the blood pressure in humans with hypertensive disease. J. Clin. Invest. 43, 659-69.

Karlberg, B. E., Ryden, G. and Wichman, K., [1984] Changes in the renin-angiotensin-aldosterone and kallikrein-kinin systems during normal and hypertensive pregnancy. Acta. Obstet. Gynaecol. Scand. Suppl. 118, 17-24.

Keeton, T. K. and Campbell, W. B., [1981] The pharmacological alteration of renin release. Pharmacol. Rev. **31**, 81-227.

Kilby, M. D., [1990] Platelet intracellular free calcium in normotensive and hypertensive pregnancy. DM Thesis, University of Nottingham.

Kilby, M. D., Broughton Pipkin, F., Cockbill, S., Heptinstall, S. and Symonds, E. M., [1990] A cross-sectional study of basal platelet intracellular free calcium concentration in normotensive and hypertensive primigravid pregnancies. Clin. Sci. **78**, 75-80.

Kiran, B. K. and Khairallah, P. A., [1969] Angiotensin and norepinephrine efflux. Eur. J. Pharmacol. **6**, 102-08.

Kleinert, H. D., Maack, T., Atlas, S. A., Januszewicz, A., Sealey, J. E., Laragh, J. H., [1984] Atrial natriuretic factor inhibits angiotensin-norepinephrine-, and potassium-induced vascular contractility. Hypertension, **6, Suppl. 1,** 143-47.

Klotz, I. M. and Hunston, D. L., [1971] Properties of graphical representations of multiple classes of binding sites. Biochem. **10**, 3065-69.

Kobilka, B. K., Matsui, H., Kobilka, T. S., Yang-Feng, T. L., Francke, U., Caron, M. G., Lefkowitz, R. J. and Regan, J. W., [1987] Cloning, sequencing and expressing of the gene coding for the human platelet alpha-2 adrenergic receptor. Science **28**, 650-656.

Kojima, I., Kojima, K. and Rasmussan, H., [1985] Characteristics of angiotensin II-, K-, and ACTH-induced calcium influx in adrenal glomerulosa cells; Evidence that angiotensin II, K , and ACTH may open a common calcium channel. J. Biol. Chem. **260**, 9171-76.

Kojima, I., Kojima, K. and Rasmussan, H., [1985] Intracellular calcium and adenosine 3',5'- cyclic monophosphate as mediator of potassium-induced aldosterone secretion. Biochem. J. **228**, 69-76.

Koshland, D. E., [1964] Conformational changes at the active site during enzyme action. Fed. Proc. **23**, 719-26.

Kostis, J. B., DeFelice, E. A. and Pianko, L. J., [1987] The renin-angiotensin system. In: Angiotensin converting enzyme inhibitors. (eds.) J. B.

Kostis, E. A. DeFelice. Alan R. Liss Inc., New York. p. 1-18.

Kotchen, T., Golla, L. and Luke, R., [1978] Contribution of chloride to the inhibition of plasma renin by sodium chloride in the rat. Kidney Int. **13**, 201-08.

Koullapis, E. N., Nicolaides, K. H., Collins, W. P., Rodeck, C. H. and Campbell, S., [1982] Plasma prostanoids in pregnancy-induced hypertension. Br. J. Obstet. Gynaecol. **89**, 617-21.

Krakoff, L. R., [1973] Measurement of plasma renin substrate by radioimmunoassay of angiotensin I: Concentration in syndromes associated with steroid excess. J. Clin. Endocrinol. Metab. **37**, 110-17.

Krupp, M. and Lane, M. D., [1981] On the mechanism of ligand-induced down-regulation of insulin receptor level in the liver cell. J. Biol. Chem. **256**, 1689-94.

Lawrence, M. R. and Broughton Pipkin, F., [1986] Effects of nitrendipine on cardiovascular parameters in conscious pregnant sheep. Scientific program, International Society for the Study of Hypertension in Pregnancy, 5th World Congress, Abstract 77.

Leaf, A. and Weber, P. C., [1988] Cardiovascular effects of n-3 fatty acids. N. Engl. J. Med. **318**, 549-57.

Leary, W. P. P. and Ledingham, J. G., [1970] Inactivation of angiotensin analogues by perfused rat liver and kidney. Nature **227**, 178-79.

Lee, M. I., Oakes, G. K., Lam, R. and Hobel, C. J., [1982] The rabbit; a suitable model for investigation of vascular responsiveness during pregnancy. Clin. Exp. Hyper. **B1**, 429-39.

Lechi, A., Lechi, C., Bonadonna, G., Sinigaglia, D., Corrandini, P., Polignano, R., Arosio, E., Covi, G. and Detogni, P., [1989] Increased basal and thrombin-induced free calcium concentrations in platelets of essential hypertensive patients. Hypertension **9**, 230-35.

Lehmann, E. L., [1975] Nonparametrics: Statistical methods based on ranks. Holden-Day, San Francisco.

Le Morvan, P. and Paliac, D., [1975] Characterisation of the angiotensin receptor in the guinea-pig aorta. J. Pharmacol. Exp. Ther. **195**, 167-75.

Letsky, E. A., [1980] The haematological system. In: Clinical physiology in Obstetrics. (eds) Hytten, F., and Chamberlain, G. Blackwell Publications, U. K.

Letsky, E. A., [1985] Haemostasis and tests of the integrity of haemostatic systems in health. In: Coagulation problems during pregnancy. Churchill Livingstone, Edinburgh. p. 1-28.

Levin, R. I., Weksler, B. B. and Jaffe, E. A., [1982] The interaction of sodium nitroprusside with human endothelial cells and platelets, nitroprusside and prostacyclin synergistically inhibit platelet behaviour. Circulation. **66**, 1299-307.

Lin, S-Y., Ellis, H., Weisblum, B. and Goodfriend, T. L., [1969] Preparation and properties of iodinated angiotensins. Biochem. Pharmacol. **19**, 651-62.

Lin, S-Y. and Goodfriend, T. L., [1970] Angiotensin receptors. Am. J. Physiol. **218**, 1319-28.

Lindheimer, M. D., Chesley, L. C., Taylor, J. R., Spargo, B. H. and Katz, A. I., [1987] Renal function and morphology in the hypertensive disorders of pregnancy. In; Hypertension in pregnancy, (eds) Sharp, F., Symonds, E. M., Proc. 16th study group RCOG, Perinatology Press, Ithaca, New York. p. 73-99.

Lineweaver, H. and Burk, D., [1934] The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**, 658-66.

Louden, K. A., [1988] Platelet reactivity and thromboxane production in pregnancy and pre-eclampsia: the effect of low dose aspirin. DM Thesis, University of Nottingham.

Louden, K. A., Heptinstall, S., Broughton Pipkin, F., Mitchell, J. R. A. and Symonds, E. M., [1988] Whole blood platelet behaviour in non pregnant and pregnant women with PIH, and in neonates. Presented at 6th international congress of the International Society for the Study of Hypertension in Pregnancy, Montreal, 1988. Clin. and Exper. Hyper. in Pregnancy **B8**, (ii). (abstract)

Louden, K. A., Broughton Pipkin, F.,Heptinstall, S., Fox, S. C., Mitchell, J. R. A. and Symonds, E. M., [1990] A longitudinal study of platelet behaviour and thromboxane production in whole blood in normal pregnancy and the puerperium. Br. J. Obstet. Gynaecol. **97**, 1108-14.

Lumbers, E. R., [1970] Peripheral vascular reactivity to angiotensin II and noradrenaline in pregnant and non-pregnant women. Aust. J. Exp. Biol. Med. Sci. **48**, 493-500.

Lumbers, E. R., McCloskey, D. I. and Potter, E. K., [1979] Inhibition by angiotensin II of baroreceptor-evoked activity in cardiac vagal efferent nerves in the dog. J. Physiol. **294**, 69-80.

Macfarlane, D. E. and Mills, D. C. B., [1975] The effects of ATP on platelets: evidence against the central role of released ATP in primary aggregation. Blood. **46**, 309-20.

MacGillivray, I., [1983] Pre-eclampsia: The hypertensive disorders of pregnancy. University Press, Cambridge.

MacGillivray, I., Rose, G. A. and Rowe, B., [1969] Blood pressure survey in pregnancy. Clin. Sci. **37**, 395-407.

Mackay, D., [1966] The mathematics of drug-receptor interactions. J. Pharm. Pharmacol. **18**, 201-22.

Majerus, P. W., [1987] Platelets. In: The molecular basis of blood diseases. (eds) Stamatoyannopoulos, G., Nienhuis, A. W., Leder, P. and Majerus, P. W., W. B. Saunders Co., Philadelphia. p.689-721.

Mann, J. F. E., Sis, J. and Ritz, E., [1985] I-Angiotensin II binding to human blood cells. J. Hypertens. **3**, 131-37.

Marumoto, Y., Kaibara, M. and Murata, T., [1988] Hemorheological studies on platelet counts and size in normal pregnancy and pregnancies with pre-eclampsia and intrauterine growth retardation. Nippon Sanka Fujinka Gakkai Zasshi. **41**, 1380-86.

Massani, Z. M., Sanguinetti, R., Gallegos, R. and Raimondi, D., [1967] Angiotensin blood levels in normal and toxemic pregnancies. Am. J. Obstet. Gynecol. **99**, 313-17.

McCartney, C. P., [1964] Pathological anatomy of acute hypertension in pregnancy. Circulation. 30 (suppl II), 37-42.

McEver, R.P., Baenziger, N. L. and Majerus, P. W., [1980] Isolation and quantitation of the platelet membrane glycoprotein defect in thrombasthenia using a monoclonal hybridoma antibody. J. Clin. Invest. 66, 1311-18.

McEver, R. P., Baenziger, J. U. and Majerus, P. W., [1982] Isolation and structural characterisation of the polypeptide subunits of membrane glycoprotein IIb-IIIa from human platelets. Blood. 59, 80-85.

McGiff, J. C., Crowshaw, K., Terragno, N. A. and Lonigro, A. J., [1970] Release of a prostaglandin-like substance into renal venous blood in response to angiotensin II. Circ. Res. 26/27, Suppl 1, 121-130.

McNeil, J., [1973] The possible teratogenic effect of salicylate on the developing fetus: brief summaries of eight suggested cases. Clinical Paediatrics. 12, 347-50.

McQueen, J., Murray, G. D. and Semple, P. F., [1984] Identification of the angiotensin II receptor in the rat mesenteric artery. Biochem. J. 223, 659-71.

McQueen, J. and Semple, P. F., [1987] Cation-dependent and cation-independent stages in regulation of the vascular angiotensin receptor after alteration of sodium balance in the rat. Endocrinol. 121, 1256-64.

Mendelsohn, F. A. O., [1985a] The actions of angiotensin II. In; The Renin-Angiotensin system, (ed) J. I. S. Robertson, Merck and Co., Inc., Singapore. p.3.14-3.26.

Mendelsohn, F. A. O., [1985b] Localisation and properties of angiotensin receptors. J. Hypertens. 3, 307-16.

Merkouris, R. W., Miller, F. C., Catanzarite, V., Rigg, L. A., Quirk, G. and Vesely, D. L., [1990] Increase in the plasma levels of the N-terminal and the C-terminal portions of the prohormone of atrial natriuretic factor during normal pregnancy. Am. J. Obstet. Gynecol. 162, 859-64.

Messerli, F. H., Frohlich, E. D., Dreslinski, G. R., Suarez, D. H. and Aristimuno, G. G., [1980] Serum uric acid in essential hypertension; an indicator of renal vascular involvement. Ann. Int. Med. **93**, 817-21.

Metz, A., Stump, K., Cowen, P. J., Elliott, J. M., Gelder, M. G. and Grahame-Smith, D. G., [1983] Changes in platelet alpha 2-adrenoreceptor binding postpartum: possible relation to maternity blues. Lancet **i**, 495-98.

Mills, D. C. B., [1981] The basic biochemistry of the platelet. In: Haemostasis and thrombosis. (Eds) Bloom, A. L. and Thomas, D. P., Churchill Livingstone, Edinburgh. p.50-60.

Miyamoto, S., Shimokawa, H., Sumioki, H., Touno, A. and Nakano, H., [1988] Circadian rhythm of plasma atrial natriuretic peptide, aldosterone, and blood pressure during the third trimester in normal and preeclamptic pregnancies. Am. J. Obstet. Gynecol. **158**, 393-99.

Miyazaki, M., Okunishi, H., Nishimura, K. and Noboru, T., [1984] Vascular angiotensin-converting enzyme activity in man and other species. Clin. Sci. **66**, 39-45.

Moore, G.J. and Kwok, Y. C., [1987] Angiotensin receptors in resting smooth muscle are the low affinity sites observed in binding studies. Life Sci. **41**, 505-11.

Moore, T. J., Taylor, T. and Williams, G. H., [1984] Human platelet angiotensin II receptors: Regulation by the circulating angiotensin level. J. Clin. Endocrinol. Metab. **58**, 778-82.

Moore, T. J. and Williams, G. H., [1981] Angiotensin II receptors on human platelets. Circ. Res. **51**, 314-20.

Morgat, J. L., Hung, L. T. and Fromageot, P., [1970] Preparation of highly labelled H-angiotensin II. Biochim. Biophys. Acta. **207**, 374-76.

Morris, J. A., O'Grady, J. P., Hamilton C. and Davidson, E. C., [1978] Vascular reactivity to angiotensin II infusion during gestation. Am. J. Obstet. Gynecol. **130**, 379-84.

Morris, B. J. and Lumbers, E. R., [1972] Activation of renin in human amniotic fluid by proteolytic enzymes. Biochim. Biophys. Acta. **289**, 385-91.

Morrison, R., Crawford, J., Macpherson, M. and Heptinstall, S., [1985] Platelet behaviour in normal pregnancy, pregnancy complicated by essential hypertension and pregnancy induced hypertension. Thromb. Haemostas. 545, 607-11.

Motulsky, H. J., Shattil, S. J. and Insel, P. A., [1980] Characterisation of alpha 2-adrenoreceptors on human platelets using tritiated yohombine. Biochem. Biophys. Res. Comm. 97, 1562-70.

Mueller, H. S., Gregory, J. J., Giannelli, Jr., S. and Ayres, S. M., [1970] Systemic hemodynamic and myocardial metabolic effects of isoproterenol and angiotensin after open-heart surgery. Circ. 42, 491-500.

Muller, J., [1971] Regulation of aldosterone biosynthesis. Monographs in Endocrinology 5, 4-29.

Murnaghan, G. A., [1987] Methods of measuring blood pressure. In; Hypertension in pregnancy, (eds) Sharp, F., Symonds, E. M., Proc. 16th study group RCOG, Perinatology Press, Ithaca, New York. p. 19-28.

Murnaghan, G. A., Mitchell, R. H. and Ruff, S., [1980] Circadian variation of blood pressure in pregnancy. In; Pregnancy Hypertension. (eds) J. Bonnar, I. MacGillivray and E. M. Symonds. MTP, Lancaster. p. 107-12.

Nachman, R. L. and Ferris, B. J., [1974] The binding of adenosine diphosphate by isolated membranes from human platelets. J. Biol. Chem. 249, 704-10.

Nachman, R. L. and Leung, L. L. K., [1982] Complex formation of platelet membrane glycoproteins IIb-IIIa with fibrinogen. J. Clin. Invest. 69, 263-69.

Naeye, R. L. and Friedman, E. A., [1979] Causes of perinatal death associated with gestational hypertension and proteinuria. Am. J. Obstet. Gynecol. 133, 8-10.

Nahmod, V. E., Finkielman, S., Benarroch, E. E., Pirola, C. J., [1978] Angiotensin regulates release and synthesis of serotonin in the brain. Science. 202, 1091-93.

Nakamura, T., Ito, M., Matsui, K., Yoshimura, T., Kawasaki, N. and Maeyama, M., [1986] Significance of angiotensin sensitivity test for prediction of pregnancy-induced hypertension. Obstet. Gynecol. 67, 388-94.

Needleman, P. G., Marshall, G. R. and Sobel, B. E., [1975] Hormone interactions in the isolated rabbit heart: synthesis and coronary vasomotor effects of prostaglandins, angiotensin and bradykinin. Circ. Res. 37, 802-08.

Negus, P., Tannen, R. L. and Dunn, M. J., [1976] Indomethacin potentiates the vasoconstrictor actions of angiotensin II in normal man. Prostaglandins 12, 175-80.

Neilson, J. P., [1987] Doppler ultrasound. Br. J. Obstet. Gynaecol. 94, 929-34.

Nelson, T. R., [1955] A clinical study of pre-eclampsia. J. Obstet. Gynaecol. Br. Emp. 49, 48-57.

Nicholls, M. G., Espiner, E. A., Miles, K. D., Zwiefler, A. J. and Julius, S., [1981] Evidence against an interaction of angiotensin II with the sympathetic nervous system in man. Clin. Endocrinol. 15, 423-30.

Nissenson, R., Flouret, G. and Hechter, O., [1978] Opposing effects of estradiol and progesterone on oxytocin receptors in rabbit uterus. Proc. Natl. Acad. Sci. USA. 75, 2044-48.

Nurden, A. T. and Caen, J .P., [1975] Specific roles for platelet surface glycoproteins in platelet function. Nature, 255, 720-22.

Nurden, A. T. and Caen, J. P., [1978] Membrane glycoproteins and human platelet function. Br. J. Haem. 38, 155-60.

Oats, J. N., [1982] A prospective study of angiotensin converting-enzyme activity in primigravid pregnancy. M. D. Thesis, University of Melbourne.

Oats, J. N., Broughton Pipkin, F., Symonds, E. M. and Craven, D. J., [1981] A prospective study of angiotensin converting enzyme in normotensive primigravidae and their infants. Br. J. Obstet. Gynaecol. 88, 1204-10.

O'Brien, J. R., [1970] Some effects of mucopolysaccharide stains on platelet aggregation. J. Clin. Path. 23, 784-88.

O'Brien, J. R., [1976] Platelet counts in normal pregnancy. J. Clin. Path. 29, 174.

Oliver, J. A. and Cannon, P. J., [1978] The effect of altered sodium balance upon renal vascular reactivity to angiotensin II and norepinephrine in the dog. J. Clin. Invest. 61, 610-23.

Oney, T. and Kaulhausen, H., [1982] The value of the angiotensin sensitivity test in the early diagnosis of hypertensive disorders in pregnancy. Am. J. Obstet. Gynecol. 142, 17-20.

Oparil, S., Ehrlich, E. N. and Lindheimer, M. D., [1975] Effects of progesterone on renal sodium handling in man. Clin. Sci. Mol. Med. 49, 139-47.

O'Rourke, F. A., Halenda, S. P., Zavoico, G. B. and Feinstein, M. B., [1985] Inositol 1,4,5-Trisphosphate releases calcium from a calcium transporting membrane vesicle fraction derived from human platelets. J. Biol. Chem. 260, 956-62.

Orozco, J. Z., Pinsker, V. S., Hernandez, E. M. and Karchmer, S., [1979] Valor de la prueba de la angiotensina II y del 'roll over test' como metodos predictivos de la enfermedad hipertensiva aguda del embarazo (preeclampsia y eclampsia). Ginec. Obstet. Mex. 46, 235-44.

Padfield, P. L. and Morton, J. J., [1977] Effects of angiotensin II on arginine-vasopressin in physiological and pathological situations in man. J. Endocrinol. 74, 251-59.

Page, E. W. and Christianson, R., [1976] Influence of blood pressure changes with and without proteinuria upon outcome of pregnancy. Am. J. Obstet. Gynecol. 126, 821-33.

Page, I. H. and Olmstead, F., [1961] Hemodynamic effects of angiotensin, norepinephrine, and bradykinin continuously measured in unanaesthetised dogs. Am. J. Physiol. 201, 92-96.

Paglin, S., Stukenbrok, H., Joyce, N. C. and Jamieson, J. D., [1987] Interaction of angiotensin II with functional smooth muscle cells in culture. Am. J. Physiol. 253, C872-82.

Paller, M. S., [1984] Mechanism of decreased pressor responsiveness to ANG II, NE, and vasopressin in pregnant rats. Am. J. Physiol. 247, H100-08.

Pastan, I. H. and Willingham, M. C., [1981] Journey to the center of the cell: role of the receptosome. Science **214**, 504-09.

Paton, W. D. M., [1961] A theory of drug action based on the rate of drug-receptor interaction. Proc. Roy. Soc. **B154**, 21-69.

Pavovcik, R. A. and Phillips, M. I., [1984] Saralasin increases activity of hippocampal neurons inhibited by angiotensin II. Brain Res. **323**, 345-48.

Pawlak, M. A. and Macdonald, G. J., [1990] Possible regulatory disorder of Angiotensin II receptors in pregnancy-induced hypertension. VIIth World Congress of Hypertension in Pregnancy, Perugia, Italy, 1990. Scientific program. p 247.

Peach, M. J., [1972] In: Chemistry and Biology of Peptides, (ed) J. Meienhofer, Ann Arbor Sci. Publ., Ann Arbor, Mich., USA. p. 471-93.

Peach, M. J., [1977] Renin-angiotensin system: Biochemistry and mechanisms of action. Physiol. Rev. **57**, 313-70.

Peach, M. J., Cline, W. H. and Watts, D. T., [1966] Release of adrenal catecholamines by angiotensin II. Circ. Res. **19**, 571-75.

Pearce, J. M. and MacParland, P., [1988] Lancet (letter) **i**, 1287.

Pedersen, E. B., Aalkjaer, C., Christensen, N. J., Christensen, P., Danielsen, H., Johannesen, P., Kornerup, H. J., Leyssac, P. P., Mulvany, M. and Rasmussen, A. B., [1984] Renin, angiotensin II, aldosterone, catecholamines, prostaglandins and vasopressin; the importance of pressor and depressor factors for hypertension in pregnancy. Scand. J. Clin. Lab. Invest. **44, Suppl. 169**, 48-56.

Pedersen, E. B., Christensen, N. J., Christensen, P., Johannesen, P., Kornerup, H. J., Kristensen, S., Lauritsen, J. G., Leyssac, P. P., Rasmussen, A. and Wohlert, M., [1983] Pre-eclampsia, a state of prostaglandin deficiency? Hypertension **5**, 105-11.

Pekonen, F., Rasi, V., Ammala, M., Viinikka, L. and Ylikorkala, O., [1986] Platelet function and coagulation in normal and preeclamptic pregnancy. Thromb. Res. **43**, 553-60.

Pellicer, A., Palumbo, A., DeCherney, A. H. and Naftolin, F., [1988] Blockage of ovulation by an angiotensin antagonist. Science. **240**, 1660-61.

Penit, J., Faure, M. and Jard, S., [1983] Vasopressin and angiotensin II receptors in rat aortic smooth muscle cells in culture. Am. J. Physiol. **244**, E72-82.

People's League of Health, [1946] The nutrition of expectant and nursing mothers in relation to maternal and infant mortality and morbidity. J. Obstet. Gynaecol. Br. Emp. **53**, 498-509.

Pert, C. B. and Snyder, S. H., [1974] Opiate receptor binding of agonists and antagonists affected differentially by sodium. Mol. Pharmacol. **10**, 868-879.

Phelan, J. P., [1977] Enhanced prediction of pregnancy-induced hypertension by combining supine pressor test with mean arterial pressure of middle trimester. Am. J. Obstet. Gynecol. **129**, 397-400.

Phillips, D. R., Jennings, L. K. and Edwards, H. H., [1980] Identification of membrane proteins mediating the identification of human platelets. J. Cell Biol. **86**, 77-86.

Phillips, M. I., [1987] The functions of angiotensin in the central nervous system. Ann. Rev. Physiol. **49**, 413-35.

Pickering, G., [1964] Physicians and scientists. B. M. J. **2**, 1615-19.

Pollard, T. D., Thomas, S. M. and Niederman, R., [1974] Human platelet myosin. I. Purification by a rapid method applicable to other nonmuscle cells. Anal. Biochem. **60**, 258-66.

Pollock, W. K., Rink, T. J. and Irvine, R. F., [1986] Liberation of [^{3}H] arachidonic acid and changes of $[Ca^{+2}]_i$ in Fura-2 loaded platelets stimulated by ionomycin and collagen. J. Biochem. **235**, 869-77.

Pollock, W. K., Rink, T. J. and Sage, S. O., [1987] ^{45}Ca extrusion from fura-2 loaded platelets: no evidence for Na^+:Ca^{+2} exchange. J. Physiol. **389**, 142.

Poplawski, A., [1970] The effect of angiotensin II on the platelet aggregation induced by adenosine-diphosphate, epinephrine and thrombin. Experientia 26, 86.

Poulsen, K. and Jorgensen, J., [1974] An easy radioimmunological microassay of renin activity, concentration and substrate in human and animal plasma and tissues based on angiotensin I trapping by antibody. J. Clin. Endocrinol. Metab. 39, 816-25.

Printz, M. P., Ganten, D., Unger, T. and Phillips, M. I., [1982] Minireview; The brain renin angiotensin system. In; The brain renin angiotensin system. (ed.) Ganten, D., Printz, M., Phillips, M. I. and Scholkens, B. A., Heidelberg: Springer-Verlag. p5-52.

Raftery, E. B. and Ward, A. P., [1968] The indirect method of recording blood pressure. Cardiovasc. Res. 2, 210-68.

Rakoczi, I., Tallian, F., Bagdany, S. and Gati, I., [1979] Platelet life-span in normal pregnancy and pre-eclampsia as determined by a non-radioisotope technique. Thromb. Res. 15, 553-56.

Ramsay, D. J., Keil, L. C., Sharpe, M. C. and Shinsako, J., [1978] Angiotensin infusion increases vasopressin, ACTH, and 11-hydroxycorticosteroid secretion. Am. J. Physiol. 234, R66-71.

Re, R. N., Visard, D. L., Brown, J. and Bryan, S. E., [1984] Angiotensin II receptors in chromatin fragments generated by micrococcal nuclease. Biochem. Biophys. Res. Commun. 119, 220-27.

Redman, C. W. G., [1987] The definition of pre-eclampsia. In; Hypertension in pregnancy, (eds) Sharp, F., Symonds, E. M., Proc. 16th study group RCOG, Perinatology Press, Ithaca, New York. p.3-13.

Redman, C. W. G., Beilin, L. J., Bonnar, J., Wilkinson, R. H., [1976] Plasma urate measurement in predicting fetal death in hypertensive pregnancy. Lancet. i, 1370-73.

Redman, C. W. G., Bonnar, J. and Beilin, L. J., [1976] Renal function in pre-eclampsia. J. Clin. Pathol. 29, 91.

Redman, C. W. G., Bonnar, J. and Beilin, L. J., [1978] Early platelet consumption in pre-eclampsia. B. M. J. 1, 467-69.

Regan, J. W., Nakata, H., DeMarinis, R. M., Caron, M. G. and Lefkowitz, R. J., [1986] Purification and characterisation of the human platelet alpha-2 adrenergic receptor defect. J. Biol. Chem. **261**, 3894-900.

Reid , I. A., [1979] The brain renin-angiotensin system: a critical analysis. Fed. Proc. **38**, 2255-59.

Reid, I. A., [1984] Actions of angiotensin II on the brain: mechanisms and physiologic role. Am. J. Physiol. **246**, F533-43.

Reid, I. A., [1985] The renin-angiotensin system and body function. Arch. Intern. Med. **145**, 1475-79.

Reid, I. A., Brooks, V. L., Rudolph, C. D. and Keil, L. C., [1982] Analysis of the actions of angiotensin on the central nervous system of conscious dogs. Am. J. Physiol. **243**, R82-91.

Reid, I. A., Morris, B. J. and Ganong, W. F., [1978] The renin-angiotensin system. Ann Rev. Physiol. **40**, 377-410.

Rink, T. J. and Hallam, T. J., [1984] What turns platelets on? Trends in Biochem. Sci. **9**, 215-19.

Rink, T. J., Smith S. W. and Tsien, R. Y., [1982] Cytoplasmic free calcium ion in human platelets: calcium ion thresholds and calcium independent activation for shape change and secretion. FEBS Lett. **148**, 21-26.

Rioux, F., Park, W. K. and Regoli, D., [1973] Application of drug receptor theories to angiotensin. Can. J. Physiol. Pharmacol. **51**, 665-72.

Robdell, M., [1980] The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature **284**, 17-22.

Robertson, A. L. and Khairallah, P. A., [1971] Angiotensin II: Rapid localisation in nuclei of smooth and cardiac muscle. Science **172**, 1138-39.

Robertson, E. G., [1971] The natural history of oedema during pregnancy. J. Obstet. Gynaecol. Br. Commonw. **78**, 520-29.

Robinson, M., [1958] Salt in pregnancy. Lancet. i, 178-181.

Romero, R., Lockwood, C., Oyarzun, E. and Hobbins, J. C., [1988] Toxaemia: New concepts in an old disease. Sem. Perinatol. 12, 302-23.

Rosenberger, L. B., Yamamura, H. I. and Roeske, W. R., [1980] Cardiac muscarinic cholinergic receptor binding is regulated by Na^+ and guanyl nucleotides. J. Biol. Chem. 246, 1872-76.

Rosenlof, K., [1986] Immunological comparison of renin substrate with erythropoietin. Scand J. Clin. Lab. Invest. 46, 497-504.

Roth, R. H., [1972] Action of angiotensin II on adrenergic enhancement of norepinephrine biosynthesis. Fed. Proc. 31, 1358-64.

Rowe, J., Gallery, E. D. M. and Gyory, A. Z., [1979] Cryoactivation of renin in plasma from pregnant and non-pregnant subjects and its control. Clin. Chem. 25, 1972-74.

Rubin, P. C., [1988] Hypertension in pregnancy: clinical features. In; Handbook of hypertension, Vol. 10. (ed) P. C. Rubin. Elsevier, Amsterdam. p. 10-15.

Saniabadi, A. R., Lowe, G. D. O., Madlick, R., Spowart, K., Shaw, B., Barbenel, J. C. and Forbes, C. D., [1986] A critical investigation into the existence of circulating platelet aggregates. Thromb. Haemostas. 56, 45-49.

Saussy, D. L. Jr., Mais, D. E., Burch, R. M. and Halushka, P. V., [1986] Identification of a putative thromboxane A2/prostaglandin H2 receptor in human platelet membranes. J. Biol. Chem. 261, 3025-29.

Scatchard, G., [1949] The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660-72.

Schafer, A. I., Cooper, B., O'Hara, D. and Handin R. I., [1979] Identification of platelet receptors for prostaglandin I2 and D2. J. Biol. Chem. 254, 2914-17.

Schaffer, A. V., Rakhit, A., Martin, L., Hanie, L., Sibai, B., Ross, M. G., Dunton, A. W. and Douglas, F. L., [1990] The thromboxane/prostacyclin ratio in pre-eclampsia: a potential biochemical marker (abstract). VIIth World Congress of Hypertension in Pregnancy, Scientific program, p. 252.

Schiffrin, E. L., Deslongchamps, M. and Thibault, G., [1986] Platelet binding sites for atrial natriuretic factor in humans. Characterisation and effects of sodium intake. Hypertension. **8(suppl II)**, II6-II10.

Schiffrin, E. L., Franks, D. J. and Gutkowska J., [1983] Aldosterone up-regulates vascular angiotensin II receptors in vivo and in cultured vascular smooth muscle cells in vitro. (abstract) Clin. Res. **31**, 688A.

Schiffrin, E. L., Gutkowska, J. and Genest, J., [1984] Effect of angiotensin II and deoxycorticosterone infusion on vascular angiotensin II receptors in rats. Am. J. Physiol. **246**, H608-14.

Schirar, A., Capponi, A. M. and Catt, K. J., [1980] Regulation of uterine angiotensin II receptors by oestrogen and progesterone. Endocrinol. **106**, 5-12.

Secher, N. and Olsen, S., [1990] Fish-oil and pre-eclampsia. (commentary) Br. J. Obstet. Gynaecol. **97**, 1077-79.

Semple, P. F., Wacrae, W. A. and Morton, J. J., [1980] Angiotensin II in human cerebrospinal fluid may be an immunoassay artifact. Clin. Sci. **59**, 61s-64s.

Severs, W. B. and Daniels-Severs, A. E., [1973] Effects of angiotensin on the central nervous system. Pharmacol. Rev. **25**, 415-49.

Severs, W. B. and Summy-Long, J., [1975] Minireview: The role of angiotensin in thirst. Life Sci. **17**, 1516-26.

Shapiro, S., Siskind, V., Manson, R. R., Heineman, O. P., Kaufman, D. W. and Slone, D., [1976] Perinatal mortality and birthweight in relation to aspirin later in pregnancy. Lancet **i**, 1375-76.

Shattil, S. J. and Bennett, J. S., [1980] Platelets and their membranes in hemostasis: Physiology and pathophysiology. Ann. Int. Med. **94**, 108-18.

Shattil, S. J. and Cooper, R. A., [1976] Membrane microviscosity and human platelet function. Biochemistry 15, 4832-37.

Sheppard, B. L. and Bonnar, J., [1974] The ultrastructure of the arterial supply of the human placenta in early and late pregnancy. J. Obstet. Gynaecol. Br. Commonw. 81, 497-511.

Sheppard, B. L. and Bonnar, J., [1976] The ultrastructure of the human placenta in pregnancy complicated by fetal growth retardation. J. Obstet. Gynaecol. Br. Commonw. 83, 948-59.

Sibai, B. M., Abdella, T. N. and Taylor, H. A., [1982] Eclampsia in the first half of pregnancy. Report of three cases and review of the literature. J. Reprod. Med. 27, 706-08.

Sibai, B. M., McCubbin, J. H., Anderson, G. D., Lipshitz, J. and Dilts, P. V., [1981] Eclampsia: Observations from 67 recent cases. Obstet. Gynecol. 58, 609-13.

Siddiqi, T. A., Koenig, B. B. and Clarke, K. E., [1986] Pregnancy causes a decrease in the number and affinity of myometrial angiotensin II receptors. Obstet. Gynecol. 68, 820-24.

Sill, P. R. and Lind, T., [1985] Platelet values during normal pregnancy. Br. J. Obstet. Gynaecol. 92, 480-83.

Simpson, A., Hallam, T. J. Rink, T. J., [1984] TMB-8 inhibits secretion evoked by phorbal ester at basal cytoplasmic free calcium in Quin-2 loaded platelets much more effectively than it inhibits thrombin-induced calcium mobilisation. F.E.B.S. 176, 139-43.

Skinner, S. L., Lumbers, E. R. and Symonds, E. M., [1969] Alteration by oral contraception of normal menstrual changes in plasma renin activity, concentration and substrate. Clin. Sci. 36, 67-76.

Sleight, P., Fox, P., Lopez, R. and Brooks, D. E., [1978] The effect of mental arithmetic on blood pressure variability and baroreflex sensitivity in man. Clin. Sci. Mol. Med. 55, 381-82.

Slone, D., Siskind, V., Heineman, O. P., Manson, R. R., Kaufman, D. W. and Shapiro, S., [1976] Aspirin and congenital malformations. Lancet i, 1373-75.

Smith, J. B., Smith, L. and Higgins, B. L., [1985] Temperature and nucleotide dependence of calcium release by myo-inositol 1,4,5,-triphosphate in cultured vascular smooth muscle cells. J. Biol. Chem. 260, 14413-16.

Speroff, L., Haning, R. V. Jr. and Ewaschuk, E. J., [1976] Uterine artery blood flow studies in the pregnant monkey. In: Hypertension in pregnancy. (eds.) M. D. Lindheimer, A. I. Katz, F. P. Zuspan. John Wiley and Sons, New York. p. 315-27.

Speroff, L., Haning, R. V. and Levin, R. M., [1977] The effect of angiotensin II and indomethacin on uterine artery blood flow in pregnant monkeys. Obstet. Gynecol. 55, 611-14.

Spitz, B., Magness, R. R., Cox, S. M., Brown, C. E. L., Rosenfeld, C. R. and Gant, N. F., [1988] Low-dose aspirin. 1. Effect on angiotensin II pressor responses and blood prostaglandin concentrations in pregnant women sensitive to angiotensin II. Am. J. Obstet. Gynecol. 159, 1035-43.

SPSS-X [1988] Statistical package for social sciences. Users guide. 3rd edition. McGraw-Hill Book Co., New York.

Stainer, K., [1989] The peripheral venous system in health and disease. PhD Thesis. University of Nottingham.

Stainer, K., Morrison, R., Pickles, C. J. and Cowley, A. J., [1986] Abnormalities of peripheral venous tone in women with pregnancy-induced hypertension. Clin. Sci. 70, 155-57.

Steele, M. K., Gallo, R. V. and Ganong, W. F., [1985] Stimulatory or inhibitory effects of angiotensin II upon LH secretion in ovariectomised rats: a function of gonadal steroids. Neuroendocrinol. 40, 210-16.

Steele, M. K., McCann, S. M. and Negro-Vilar, A., [1982] Modulation by dopamine and estradiol of angiotensin II on anterior pituitary hormone release. Endocrinol. 111, 722-29.

Stenberg, P. A., Shuman, M. A., Levine, S. P. and Bainton, D. F., [1984] Redistribution of alpha granules and their contents in thrombin stimulated platelets. J. Cell. Biol. 98, 748-60.

Stephenson, R. P., [1956] A modification of receptor theory. Br. J. Pharmacol. **11**, 379-93.

Straus, O. H. and Goldstein, A., [1943] Zone behaviour of enzymes. J. Gen. Physiol. **26**, 559-85.

Sumners, C. and Phillips, M. I., [1983] Central injection of angiotensin II alters catecholamine activity in rat brain. Am. J. Physiol. **244**, R257-63.

Sundsfjord, J. A. and Aakvaag, A., [1970] Plasma angiotensin II and aldosterone excretion during the menstrual cycle. Acta Endocrinol. **64**, 452-58.

Symonds, E. M., [1979] Hypertension in pregnancy. In: Recent advances in obstetrics and gynaecology 13. (eds) J. Stallworthy, G. Bourne. Churchill Livingstone, Edinburgh.

Symonds, E. M. and Andersen, G. J., [1974] The effect of bed rest on plasma renin in hypertensive disease of pregnancy. J. Obstet. Gynaecol. Brit. Cwlth. **81**, 676-81.

Symonds, E. M. and Broughton Pipkin, F., [1978] Pregnancy hypertension, parity and the renin-angiotensin system. Am. J. Obstet. Gynecol. **132**, 473-79.

Symonds, E. M., Broughton Pipkin, F. and Craven, D. J., [1975] Changes in the renin-angiotensin system in primigravidae with hypertensive disease of pregnancy. Br. J. Obstet. Gynaecol. **82**, 643-50.

Symonds, E. M., Stanley, M. A. and Skinner, S. L., [1968] Production of renin by in vitro cultures of human chorion and uterine muscle. Nature **217**, 1152-53.

Talledo, O. E., Chesley, L. C. and Zuspan, F. P., [1968] Renin-angiotensin system in normal and toxemic pregnancies. III. Differential sensitivity to angiotensin II and norepinephrine in toxemia of pregnancy. Am. J. Obstet. Gynecol. **100**, 218-21.

Talledo, O. E., Rhodes, K. and Livingstone, E., [1966] Renin-angiotensin system in normal and toxemic pregnancies. I. Angiotensin infusion test. Am. J. Obstet. Gynecol. **96**, 141-43.

Talledo, O. E., Rhodes, K. and Livingstone, E., [1967] Renin-angiotensin system in normal and toxaemic pregnancies. II. Inactivation of angiotensin in normal pregnancy. Am. J. Obstet. Gynecol. **97**, 571-72.

Tandon, N. N., Kralisz, V. and Jamieson, G. A., [1989] Identification of glycoprotein IV (CD36) as a primary receptor for platelet collagen adhesion. J. Biol. Chem. **264**, 7576-83.

Tapia, H. R., Johnson, C. E. and Strong, C. G., [1972] Renin-angiotensin system in normal and in hypertensive disease of pregnancy. Lancet **ii**, 847-50.

Taylor, D. J., [1988] The epidemiology of hypertension during pregnancy. In; Handbook of hypertension, Vol. 10. (ed) P. C. Rubin. Elsevier, Amsterdam. p. 223-40.

Terragno, N. A., Terragno, D. A., Pacholxzyk, D. and McGiff, J. C., [1974] Prostaglandins and the regulation of uterine blood flow in pregnancy. Nature. **249**, 57-58.

Tervila, L., Goecke, C. and Timonen, S., [1973] Estimation of gestosis of pregnancy (EPH-gestosis). Acta. Obstet. Gynec. Scand. **52**, 235-43.

Tewksbury, D. A. and Dart, R. A., [1982] High molecular weight angiotensinogen levels in hypertensive pregnant women. Hypertension **4**, 729-34

Tetlow, H. J. and Broughton Pipkin, F., [1986] Changing renin substrates in human pregnancy. J. Endocrinol. **109**, 257-62.

Thiagarajah, S., Bourgeois, F. J., Harbert, G. M. and Candle, M. R., [1981] Thrombocytopaenia in pre-eclampsia: associated abnormalities and management principles. Am. J. Obstet. Gynecol. **150**, 1-7.

Thibonnier, M. and Roberts, J. M., [1985] Characterisation of human platelet vasopressin receptors. J. Clin. Invest. **76**, 1857-64.

Thomsen, J. K., Storm, T. L., Thamsborg, G., deNully, M., Bodker, B. and Skouby, S., [1988] Increased concentration of circulating atrial natriuretic peptide during normal pregnancy. Eur. J. Obstet. Gynecol. Reprod. Biol. **27**, 197-201.

Tigerstedt, R. and Bergman, P. G., [1898] Niere and Kreiselauf. Skand Arch Physiol. **8**, 223-71.

Tobian, L., Tomboulian, A. and Janecek, J., [1959] The effect of high perfusion pressure on the granulation of juxtaglomerular cells in an isolated kidney. J. Clin. Invest. 38, 605-10.

Trippodo, N. C., McCaa, R. E. and Guyton, A. C., [1976] Effect of prolonged angiotensin II infusion on thirst. Am. J. Physiol. 230, 1063-66.

Trudinger, B. J., [1976] Platelets and intrauterine growth retardation in pre-eclampsia. Br. J. Obstet. Gynaecol. 83, 284-86.

Tunbridge, R. D. G. and Donnai, P., [1983] Pregnancy-associated hypertension, a comparison of its prediction by roll-over test and plasma noradrenaline measurement in 100 primigravidae. Br. J. Obstet. Gynaecol. 90, 1027-32.

Tygart, S. G., McRoyan, D. K., Spinnato, J. A., McRoyan, C. J. and Kitay, D. Z., [1986] Longitudinal study of platelet indices during normal pregnancy. Am. J. Obstet. Gynecol. 154, 883-87.

Unger, T., Rascher, W., Schuster, C., Pavlovitch, R., Schomig, A., Dietz, R. and Ganten, D., [1981] Central blood pressure effects of substance P and angiotensin II: role of the sympathetic nervous system and vasopressin. Eur. J. Pharmacol. 71, 33-42.

Usdin, T. B., Creese, I. and Snyder, S. H., [1980] Regulation by cations of [^{3}H] spiroperidol binding associated with dopamine receptors of rat brain. J. Neurochem. 34, 669-76.

Vale, W., Vaughan, J., Smith, M., Yamamoto, G., Rivier, J. and Rivier, C., [1983] Effects of synthetic ovine corticotrophin releasing factor, glucocorticoids, catecholamines, neurohypophysial peptides and other substances on cultured corticotrophic cells. Endocrinol. 113, 1121-31.

Vallega, G. A., Canena, M. L., Berk, B. C., Brock, T. A. and Alexander, R. W., [1988] Vascular smooth muscle sodium ion - hydrogen ion exchanger, kinetics and its activation by angiotensin II. Am J. Physiol. 254, C751-58.

Van Dias, F. W., Gibney, M. J. and Taylor, T. G., [1982] The effect of polyunsaturated fatty acids of the n-3 and the n-6 series on platelet aggregation and platelet and aortic fatty acid composition in rabbits. Atherosclerosis. 43, 245-57.

Vander, A. J. and Geelhoed, G. W., [1965] Inhibition of renin secretion by angiotensin II. Proc. Soc. Exp. Biol. Med. **120**, 399-403.

Vander, A. and Miller, R., [1964] Control of renin secretion in the dog. Am. J. Physiol. **207**, 537-45.

Van Houten, M., Mangiapane, M. L., Reid, I. A. and Ganong, W. F., [1983] (Sar,Ala)Angiotensin II in the cerebrospinal fluid blocks the binding of blood borne angiotensin II to the circumventricular organs. Neurosci. **10**, 1421-26.

Van Houten, M., Schiffrin, E. L., Mann, J. F. E., Posner, B. I. and Boucher, R., [1980] Radiographic localisation of specific binding sites for blood borne angiotensin II in the rat brain. Brain Res. **186**, 480-85.

Van Zwieten, P. A., Van Meil, J. C. and Timmermans, P. B., [1981] Vascular aspects of calcium antagonists. Pharmacol. Weekbl. **6**, 237-47.

Venuto, R. C., O'Dorisio, T., Stein, J. H. and Ferris, T. F., [1975] Uterine prostaglandin E secretion and uterine blood flow in the pregnant rabbit. J. Clin. Invest. **55**, 193-97.

Vollman, R. F., [1970] Rates of toxaemia by age and parity. In : 'Die Spatgestose (EPH Gestose)'. (ed) E. T. Rippman. Schabe, Basel.

Wallenburg, H. C. S., [1987] Changes in the coagulation system and platelets in pregnancy-induced hypertension and pre-eclampsia. In: Hypertension in pregnancy, Proceedings of the 16th study group of the RCOG. (eds) Sharp, F. and Symonds, E. M. Perinatology Press, Ithaca, New York. p. 232-38.

Wallenburg, H. C. S., Dekker, G. A., Makovitz, J. W. and Rotmans, P., [1986] Low dose aspirin prevents pregnancy-induced hypertension and pre-eclampsia in angiotensin sensitive primigravidae. Lancet **i**, 1-3.

Wallenburg, H. C. S. and Van Kessel, P. V., [1978] Platelet lifespan in normal pregnancy as determined by a nonradioisotopic technique. Br. J. Obstet. Gynaecol. **85**, 33-36.

Walsh, S. W., [1985] Pre-eclampsia: An imbalance in placental prostacyclin and thromboxane production. Am. J. Obstet. Gynecol. **152**, 335-40.

Warrell, D. M., [1988] Urinary tract disorders in pregnancy. In: Dewhurst's textbook of obstetrics and gynaecology for postgraduates. (ed) Whitfield, C. R. Blackwell Scientific Publications, Oxford. p. 277-83.

Warren, A. Y., Craven, D. J. and Symonds, E. M., [1984] Angiotensin converting enzyme in human fetal membranes, placenta, amniotic fluid and cord venous serum. Clin. and Exper. Hyper. in Pregnancy. B3, 51-60.

Weinberger, M. H., Petersen, L. P., Herr, M. J. and Wade, M. B., [1973] The effect of supine and lateral recumbency on plasma renin activity during pregnancy. J. Clin. Endocrinol. Metab. 36, 991-94.

Weir, R. J., Brown, J. J., Fraser, R., Lever, A. F., McIlwaine, G. M., Morton, J. J., Robertson, J. I. S. and Tree, M., [1975] Relationship between plasma renin, renin substrate, angiotensin II, aldosterone and electrolytes in normal pregnancy. J. Clin. Endocrinol. Metab. 40, 108-115.

Weir, R. J., Brown, J. J., Fraser, R., Kraszewski, A., Lever, A. F., McIlwaine, G. M., Morton, J. J., Robertson, J. I. S. and Tree, M., [1973] Plasma renin, renin substrate, angiotensin II and aldosterone in hypertensive disease of pregnancy. Lancet i, 291-94.

Whigham, K. A. E., Howie, P. W., Drummond, A. H. and Prentice, C. R. M., [1978]. Abnormal platelet function in pre-eclampsia. Br. J. Obstet. Gynaecol. 85, 28-32.

White, J. G., [1969] The submembrane filaments of blood platelets. Am. J. Path. 56, 267-77.

White, J. G., Clawson, C. C. and Gerrard, J. M., [1981] Platelet ultrastructure. In: Haemostasis and thrombosis. (Eds) Bloom, A. L. and Thomas, D. P., Churchill Livingstone, Edinburgh. p. 22-49.

Wichman, K., Ryden, G. and Wichman, [1984] The influence of different positions and Korotkov sounds on the blood pressure measurements in pregnancy. Acta. Obstet. Gynecol. Scand. Suppl. 118, 25-28.

Wiessner, [1899] Uber Blutdruckmessungen wahrend der Menstruation und Schwangerschaft. Centralbl. Gynaekol. 23, 1335. (abs)

Williams, G. H. and Braley, L. M., [1977] Effects of dietary sodium and potassium intake and acute stimulation on aldosterone output by isolated human adrenal cells. J. Clin. Endocrinol. Metab. **45**, 55-64.

Williams, L. T. and Lefkowitz, R. J., [1977] Regulation of rabbit myometrial alpha-adrenergic receptors by estrogen and progesterone. J. Clin. Invest. **60**, 815-18.

Wintrole, M., [1985] Discovery of the platelet. In: Haematology, the blossoming of a science. Lea and Febiger, Philadelphia. p. 28-31.

Wood, S. M., [1977] Assessment of renal function in hypertensive pregnancies. Clin. Obstet. Gynaecol. **4**, 747-58.

Wood, P., Groom, G., Moore, A., Ratcliffe, W. and Selby, C., [1985] Progesterone assays: guidelines for the provision of a clinical biochemistry service. Ann. Clin. Biochem. **22**, 1-24.

Woodcock, E. A. and Johnson, C. I., [1982] Inhibition of adenylate cyclase by angiotensin II in rat renal cortex. Endocrinol. **111**, 1687-91.

Worley, R. J., [1984] Eclampsia. Progress in Obstetrics and Gynaecology, Vol.4. (ed) J. Studd. Churchill Livingstone, Edinburgh. p. 183-96.

Wright, G. B., Alexander, R. W., Ekstein, L.S. and Gimbrone, M. A., [1982] Sodium, divalent cations, and guanine nucleotides regulate the affinity of the rat mesenteric artery angiotensin II receptor. Circ. Res. **50**, 462-69.

Yamazaki, H., Motomiya, T., Kikutani, N., Sakakibara, C., Watanebe, S., Numata, M. and Noguchi, K., [1979] Platelet aggregation during menstrual cycle and pregnancy. Thromb. Res. **14**, 333-40.

Yang, H. Y. T., Erdos, E. G. and Levin, Y., [1970] A dipeptidyl carboxylase that converts angiotensin I and inactivates bradykinin. Biochim. Biophys. Acta. **214**, 374-76.

Ylikorkala, O. and Makila, U. M., [1985] Prostacyclin and thromboxane in gynecology and obstetrics. Am. J. Obstet. Gynecol. **152**, 318-29.

Ylikorkala, O. and Viinikka, L., [1980] Thromboxane A2 in pregnancy and puerperium. B. M. J. **281**, 1601-02.

Zahavi, J., Dreyfuss, F., Kalef, M. and Soferman, N., [1973] ADP-induced platelet aggregation in healthy women with and without a combined and sequential contraceptive pill. Am. J. Obstet. Gynecol. **117**,107-11.

Zhuo, J., Harris, P. J. and Skinner, S. L., [1986] Modulation of proximal tubular reabsorption by angiotensin II. Clin. Exp. Pharmacol. Physiol. **13**, 277-81.

Zimmerman, B. G., [1962] Effect of acute sympathectomy on responses to angiotensin and norepinephrine. Circ. Res. **11**, 780-87.

Zimmerman, B. G., Gomer, S. K. and Liao, J. C., [1972] Action of angiotensin on vascular adrenergic nerve endings: facilitation of norepinephrine release. Fed. Proc. **31**, 1344-50.

Zuckert, J. F., [1767] Diat der Schwangern und Sechswochnerinnen. Mylius, Berlin. p. 40-72.

Zwiefel, P., [1916] Eklampsie. In; Handbuch der Geburtshilfe, Vol II, (ed) Doderlein, A. Wiesbaden, Bergmann. p.672-723.