

SOYA / ALGINATE INTERACTIONS IN
EXTRUSION COOKING

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Condensation reactions in extrusion.

LEDWARD, D. A., GATHURA, M. K. G. & OATES, C. G.

Role of humectants in chemical stability of
intermediate moisture meats.

Abstract

Previous work has shown that following the inclusion of alginate high in mannuronic acid there is a reduction in the viscosity of soya melt during extrusion, this work attempts to identify the mechanism for this effect.

The effect of water content and hydrocolloid inclusion on the denaturation behaviour and water binding properties of soya protein has been investigated, mainly by the use of differential scanning calorimetry. Of a range of parameters investigated, transition onset temperature and enthalpy, change in specific heat function and cooperativity of the transition all show a dependence on initial moisture content. These parameters are all changed if soya is heated in the presence of a high mannuronic acid alginate. With the exception of changes in enthalpy these effects are not seen to the same extent with other added hydrocolloids. These changes have been attributed to the production of water during heating. Measurements of freezable water and sorption isotherms suggest that alginate addition increases the water binding ability of soya isolate after denaturation.

When heated at high temperatures only soya and gluten (out of 7 proteins tested) produced measurable quantities of additional water. In the presence of 2% Manucol DM the formation of water was markedly increased in the soya and gluten samples but not in the other proteins. Production of increased amounts of water after alginate addition was demonstrated to be dependent on the quantity of mannuronic

acid in the alginate. The dependence of this effect on water activity suggests that it is the result of browning or condensation reactions. Glutamic acid was implicated with the specific reaction between soya and alginate, though in heated soya both loss of lysine and serine were found.

Differential rates of degradation were found between a high mannuronic acid alginate and a high guluronic acid alginate after heat treatment. The alginate high in mannuronic acid was shown to depolymerise to a far greater extent. Various estimates for the molecular weight of the heat treated samples were obtained and no correlation could be found between increased reducing capability of the sample and extent of depolymerisation. Furthermore the production of volatiles that may be expected from sugar ring degradation were not found.

Increased brown colour formation and water production and concomittant decreased glutamic acid content were found in soya samples extruded in the presence of 1% Manucol DM. It was concluded that the chemical reactivity of glutamic acid with alginates rich in mannuronic acid explains the well established effect of this type of polysaccharide in extrusion processing.

INTRODUCTION

Introduction

In the last 15 years there has been a great deal of interest in the development of textured products from protein rich sources, especially soya, by extrusion processing. However studies aimed at understanding the changes undergone during processing have tended to be empirical using either response surface methods to show the effect of process variables on the properties of the product, or involving the use of physiochemical techniques to determine changes in the protein structure, such as denaturation, disaggregation, alignment and reaggregation of the fractions. The extruder has been defined as being simultaneously a pump, a forming device, a mixer, a heat exchanger and a chemical reactor (Clark, 1978). It is the last function which has to date been poorly studied.

It is well established that the addition of alginates, particularly those rich in mannuronic acid residues will lead to a decrease in torque, product temperature and expansion during soya extrusion (Smith, et al., 1982, Boison, et al., 1983, Berrington, et al., 1984 and Imeson, et al., 1985). These effects can be explained by a reduction in viscosity of the soya melt on alginate addition. The objective of this work was to establish a mechanism for this viscosity reduction. To achieve this protein - water relationships were investigated in heated soya systems in the presence and absence of added

polysaccharide.

It is thought that protein denaturation is a key step in the extrusion process and it is well known that the denaturation characteristics of proteins are dependent on the moisture content of the system, the complexity of this dependence is a result of the complexity of protein structure (Fujita & Noda, 1981 and Luesher, et al., 1974). The dependence will become even more complex when other than simple, one subunit proteins are investigated (Donovan & Ross, 1973). In the first part of this study, differential scanning calorimetry (DSC) is used to follow the events taking place during the denaturation of the 7 S globulin of soya at low water contents, with and without hydrocolloid.

It is known that proteins undergo several types of degradative and condensation reactions at elevated temperatures, the reactions being most rapid at low moisture contents (Ledward, 1978). These reactions are accelerated in the presence of reducing components. Since these reactions may well be of importance in the extrusion of soya and soya plus hydrocolloid systems methods were devised to look at them in some detail and evaluate their relevance to extrusion processing.

LITERATURE REVIEW

2.1 Macromolecular Structure

2.1.1 Proteins

2.1.1.1 Protein Structure .

Amino acids have both an amino and carboxyl group on the carbon atom so, depending upon the pH of the solution the molecule may carry either a positive or a negative charge. The R group or side chain of amino acids have been classified depending upon charge, structure or solubility. Amino acids condense together with the concomitant formation of a peptide bond between the α -carboxyl group of an amino acid and the amino group of another. Many such amino acids are linked together in this way to form a polypeptide chain. This amino acid sequence identifies a given protein and determines all the chemical and biological properties of the protein. It is known as the primary structure.

The polypeptide chain is composed of a range of units all with different charge and hydrophilic or hydrophobic properties. Consequently interactions will take place between different regions of the chain and also between the solvent and the polypeptide. It is these interactions which cause the chain to undergo regular folding into stable structures known as the secondary structure.

Further folding to give three dimensional structures occurs, known as the tertiary structure. Most proteins are composed of more than one peptide chain (sub - unit) which

are aggregated together into the quaternary structure.

2.1.1.2 Bonding

Many interactions are responsible for the formation of the secondary or higher structures, these include:

1) Electrostatic

Electrostatic forces occur between any two charged species. If the charges are of opposite sign then the interaction becomes attractive. The interactions are effected by the polarisability of the solvent molecules which will diminish the electrostatic interaction between ions.

Electrostatic interactions are unique in that they are significant over relatively large distances. Uncharged, polar molecules may interact electrostatically due to the distribution of electrons in the molecule. This results in an excess of negative charge at one part of the molecule and a corresponding positive charge at some other point.

2) Hydrogen bonds

Hydrogen bonds occur between polar molecules. A hydrogen atom is shared between a potential acid (proton donor) and a base (proton acceptor). The hydrogen bond may be described as an intermediate in the transfer of electrons from an acid, AH to a base, B (Fig 2.2).

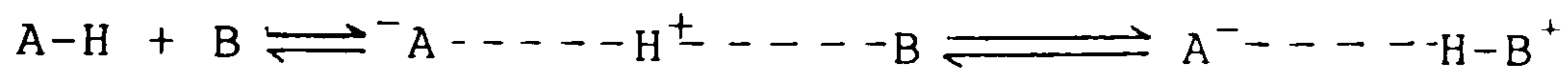


Figure 2.2 Hydrogen bond formation.

The geometric arrangement of A, H and B is very important, as for optimal hydrogen bond stability the arrangement of the atoms should be linear.

Normally only the oxygen and nitrogen atoms of proteins participate in forming significant hydrogen bonds but residues of -OH and -NH can act as good hydrogen bond donors. The polypeptide backbone has an excellent hydrogen bond donor, $\begin{array}{c} H^+ \\ | \\ -N- \end{array}$, and an excellent acceptor, $\begin{array}{c} O^- \\ || \\ -C- \end{array}$, in each

residue. Hydrogen bonds are transitory in nature but because of the vast number of these bonds they can impart a strong stabilizing influence on the protein structure.

3) Van der Waals Forces

Van der Waals forces are derived from three main sources;

a) Dipole - dipole interactions: Polar molecules have an unbalanced charge distribution and thus a permanent dipole. When two molecules are close together their dipoles interact, if of the opposite charge, and so a force exists between them.

b) Dipole - induced dipole interaction: The presence of a polar molecule in the vicinity of another molecule (polar or non - polar) will polarise the second molecule. The

induced dipole can then interact with the permanent dipole of the first molecule, thus the two molecules will become attracted together.

c) Induced dipole - induced dipole: The electron cloud surrounding a non polar molecule will be permanently fluctuating, thus an instantaneous dipole moment is created which is changing rapidly in magnitude and direction. A dipole created by one molecule will induce the opposite dipole in a neighbouring molecule and thus be attracted. As the dipole in the first molecule changes direction the induced dipole will also change direction, thus the attraction will remain.

These forces have been shown to dominate at the interfaces of subunits (Lawham & Cater, 1971).

4) Hydrophobic Interaction

Hydrophobic groups when in contact with water are subjected to a " thrust " by the surrounding water structure with its lowered entropy. As a result of this and of the tendency of the water to reduce its interface with hydrophobic groups the potential for the latter to associate with one another, aided by Van der Waals forces is increased.

Water molecules form a cavity around the non-polar amino acid groups (Franks, 1977). The energy required to order these molecules can easily be supplied as a result of the interaction of polar groups with water due to hydrogen

bond and electrostatic interactions (Franks, 1977).As the non polar groups will gain only minor Van der Waals with the solvent. To compensate the solvent will rearrange to reform the maximum number of hydrogen bonds possible. Thus highly ordered ' cage ' like structures are formed, known as clathrates (Franks,1977).

Hydrophobic interactions may well also be responsible for subunit association (Cantor & Shimmel, 1980).

5) Disulphide Linkages and other non peptide covalent bonds

Non peptide covalent bonds between different amino acid residues play an important part in ' higher ' protein structure as they serve to crosslink peptide chains or to join different segments of the same chain together.

The most common crosslink is the disulphide bond which is formed between two half cystine residues (Friedmann, 1973). This crosslink is very stable.

Orthophosphate groups will also form crosslinks in protein chains via diester links with the hydroxyl group of the appropriate amino acid ie. two seryl residues.

Most other crosslinks found in proteins are not natural but are readily formed during processing, examples being lysinoalanine (Finley, 1983) or isopeptide crosslinks such as that between glutamic acid and lysine (Pisano, et al., 1968 and Otterburn, 1983). These will be discussed in detail later.

2.1.1.3 Protein Hydration

Careri et al ., (1979) give the following description of the events occurring during protein rehydration:

a) Dry protein has a conformation similar to that of protein in solution. It makes few contacts with its neighbours.

b) Water interacts initially, with ionizable groups. This leads to the formation of strongly bound water which is equivalent to about 0.25 g water / g protein.

c) At a concentration of around 0.1 g / g clusters develop centered on polar and charged protein surface atoms.

d) Between 0.2 and 0.3 g / g, hydration of the bonding sites is complete.

e) Condensation of water molecules over the weakest interacting portions of the surface, the non polar region, leads to monolayer coverage at around 0.4 g/ g.

2.1.1.4 Bound Water

A portion of the total water present in a protein is " bound ". This is often described in terms of non - freezable water, values given in the literature range from 0.2 to 0.4 g/g (Kuntz, 1971).

Work with IR and NMR indicates that the water remaining unfrozen possesses substantial mobility and does not show the orderly structure expected of ice like aggregates

(Kuntz, 1971). Work by Kuntz (1971) and Leader & Watt (1974) would suggest that unfreezable water represents an indication of the water molecules around each polar group, as they were able to determine the hydration contribution of different amino acid residues to unfrozen water. Bull & Breese (1970) obtained a correlation between the sum of ionic residues plus hydroxyl groups in proteins and the number of water molecules sorbed.

Ruegg et al., (1975 & 1974), Haly & Snaith (1969) and Hansen (1976) propose the presence of four types of water in protein systems:

- 1) Non freezable.
- 2) Freezable with both heat and temperature of fusion different from ordinary water.
- 3) Freezable water with heat of fusion lower than bulk water.
- 4) Water indistinguishable from bulk water.

The difference in the properties of the various types of water is the result of different degrees of interaction with the protein. This results in hydrogen bonded defects in the ice lattice (Luscher et al., 1979).

The water described by 2 & 3 is probably associated with the protein surface by repulsive hydrophobic interactions and by hydrogen bonds with some polar groups of the protein or the primary hydration water.

Work with RNase - S and lysozyme (Finney, et al., 1980)

has shown these two proteins to have 586 to 627 and 511 to 600 hydrogen bonding sites, respectively. In the folded state of both these proteins about 300 (48 to 52 %) of the polar sites form internal hydrogen bonds, while the remainder interact with the solvent. Consequently all hydrogen bonding sites are occupied. In lysozyme the remaining polar groups provide hydrogen bonding for approximately 260 solvent molecules, (Finney, et al., 1980) this amounts to 0.32 g water / g protein and is the same as the amount of non freezable water associated with the protein.

2.1.2 Polysaccharide Structure

Polysaccharides are polymers of monosaccharides linked by glycosidic bonds. The sequence of these units forms the basis of the primary structure. Covalent bonds occur from C1 through an oxygen to any of the other carbons, though the preferred carbon is C4 thereby forming a 1, 4 - glycosidic bond. The structure may be a straight chain or highly branched, it may be composed entirely from the same units (homopolysaccharide) or from a range of different units (heteropolysaccharide).

The different polysaccharide units may take up a variety of conformations due to twisting of the carbon ring. As a result of steric restraints of fixed bond lengths and angles there are only a limited number of unrestrained pyranose ring shapes. The chair configuration is

energetically preferred as in the alternate boat shapes several energetically unfavourable interactions are evident.

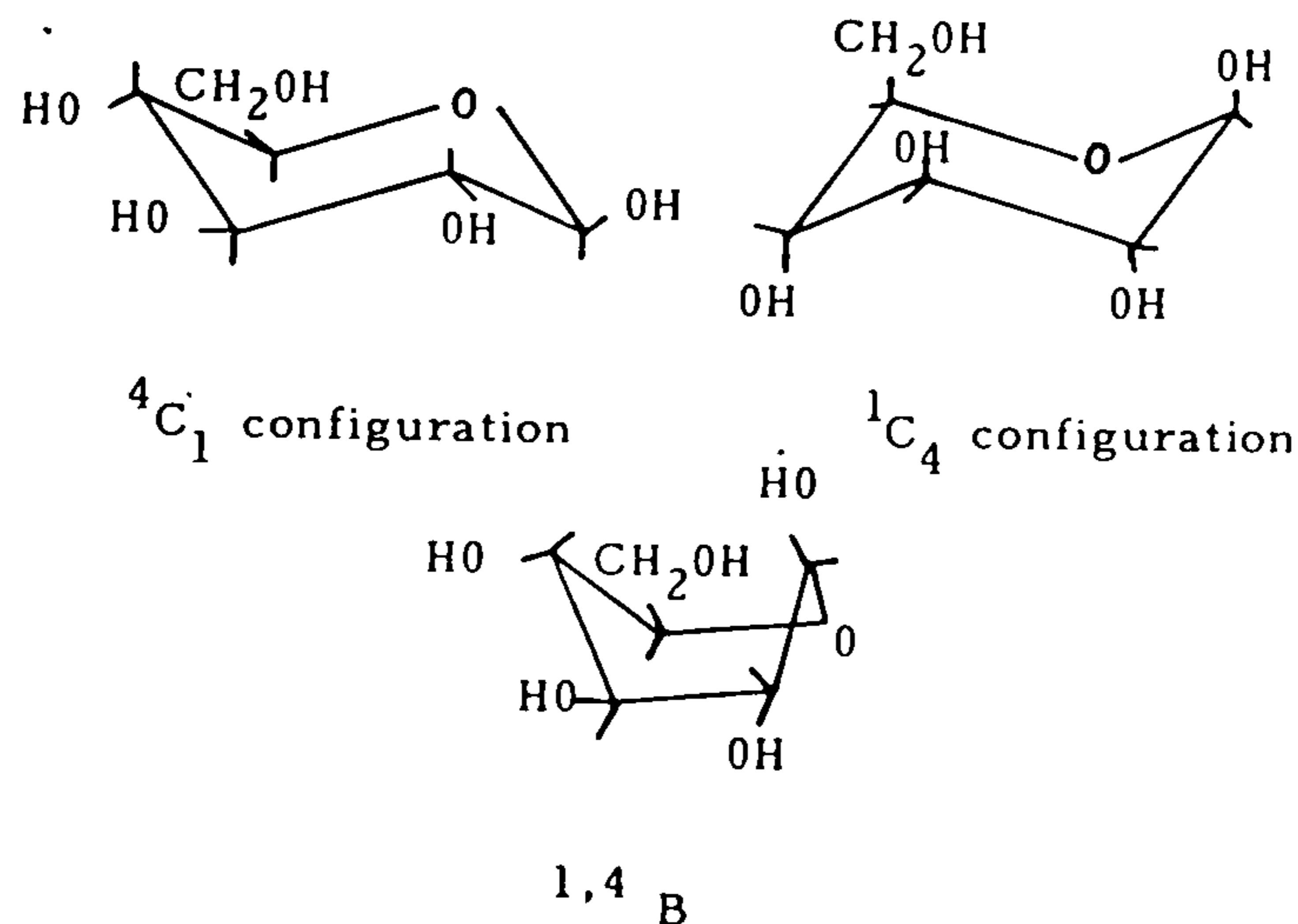


Fig 2.3 The different configurations adopted by monosaccharide units.

Two forms of chair configuration exist. The choice as to which shape will be adopted will result from a minimisation of steric repulsions between the axial substituents, which for hexoses is governed by the need for an equatorial orientation at C6 ie D - sugars will always adopt the 4C_1 and L - sugars the 1C_4 configuration (Morris, 1979).

The conformation of the polysaccharide is the result of the orientation of the component sugars around the glycosidic bond. Rotation around this bond is restricted by axial linkages and bulky equatorial substituents (eg OH) on a position adjacent to the inter residue C - O bond (Rees, 1969).

The polysaccharide may further fold into higher ordered

structures by favourable non covalent interactions and a need for efficient packing of the individual units (Morris, et al., 1977). The main ' families ' of polysaccharide structure are based around the regular helix shape and depend on the number of monomers per turn (n) and their unit length, which ultimately is a function of orientation (Rees, 1977). Two main structures may be defined :-

a) Ribbon: This structure occurs when n is between 2 and 4 and the length of the unit is the actual length of the sugar residue. In this structure each unit is almost parallel to the helix axis.

b) Hollow helix: For this structure n can be up to 10 and the actual length can be down to unity the resulting shape is like a flexible wire spring.

2.2 Chemistry of Reactive Groups

2.2.1 Carbonyl Group

The chemistry of the carbonyl group has been reviewed by Feeney, et al. (1975).

The carbonyl group, $C=O$, is the common structural feature of a large number of functional groups. The electronic structure of the carbonyl group is that of an sp^2 hybridised carbon atom and an oxygen atom in which s and p orbitals are unmixed. These two atoms form a bond by

overlap of the sp^2 orbital of carbon with a p orbital of oxygen. A second bond is formed from the overlap of p orbitals of carbon and oxygen. This leaves two sp^2 orbitals on the carbon and an s and p orbital on the oxygen (the two lone pairs).

As oxygen has a greater electronegativity, both C—O bonds are polarised, with the carbon atom bearing a partial positive charge. This +ve charge causes an inductive displacement of electrons along the bonds joining the carbon atom to adjacent groups.

The important reaction of carbonyl groups usually involves additions to the carbon - oxygen double bond, these reactions may be of two types, both having a common initial pathway (Fig 2.4).

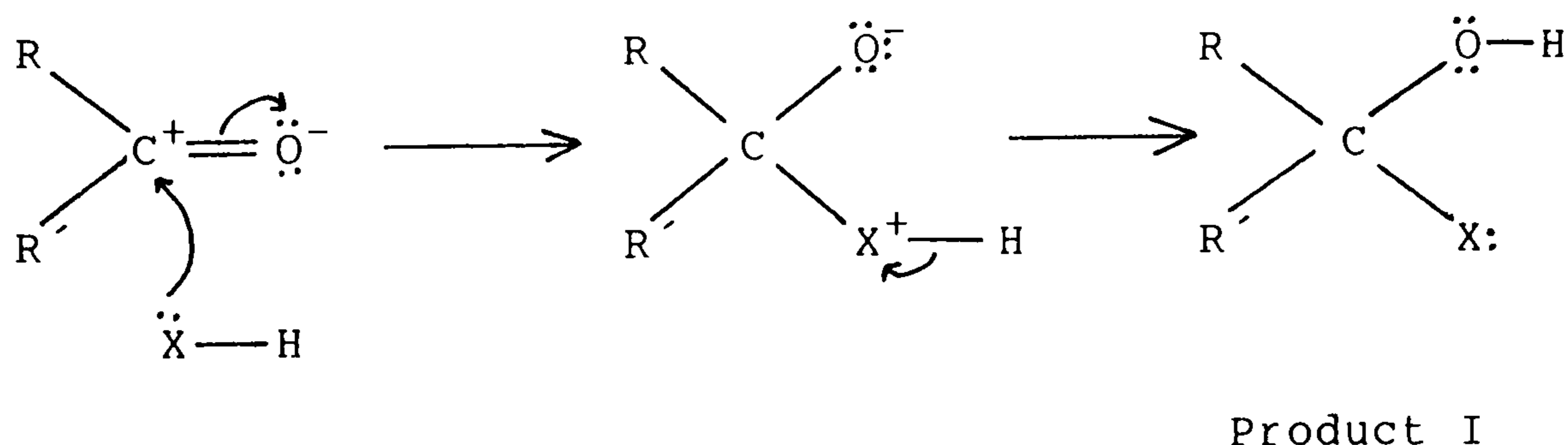


Figure 2.4 Initial stages in carbonyl addition.

The carbon atom has a partial positive charge due to polarisation of the carbonyl group, the nucleophilic group X (of reagent H—X) attacks electrons of the oxygen atom. The unstable intermediate, which carries a double charge,

will rapidly form the product I by loss of a proton from X and acquisition of an hydrogen ion by the oxygen. Alternatively the reaction may proceed via the ionisation of $\text{H}-\text{X}$ to H^+ and X^- with initial nucleophilic attack by X^- followed by protonation of the oxygen.

The second part of the reaction will depend upon the reaction conditions. In acidic conditions product I may lose a molecule of water following protonation of the oxygen atom of the hydroxyl, (Reaction type A).

Reaction Type A:

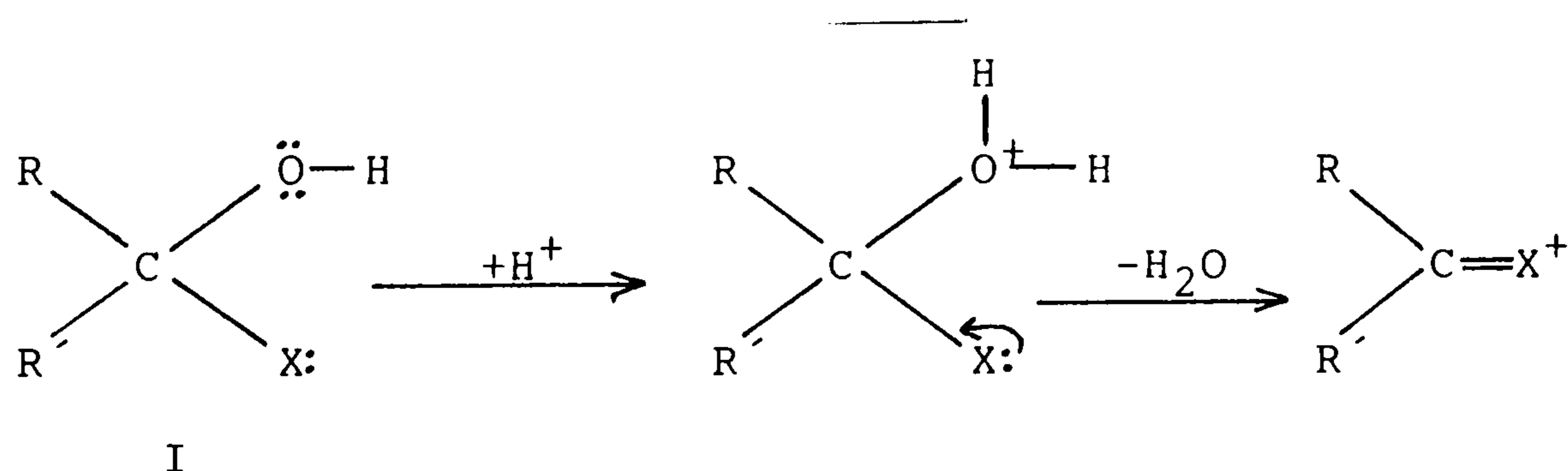


Figure 2.5 Type A addition reaction.

Under basic conditions rapid substitution of the hydroxy group of the product I by another group, Z, may occur (Fig 2.6).

Reaction Type B:

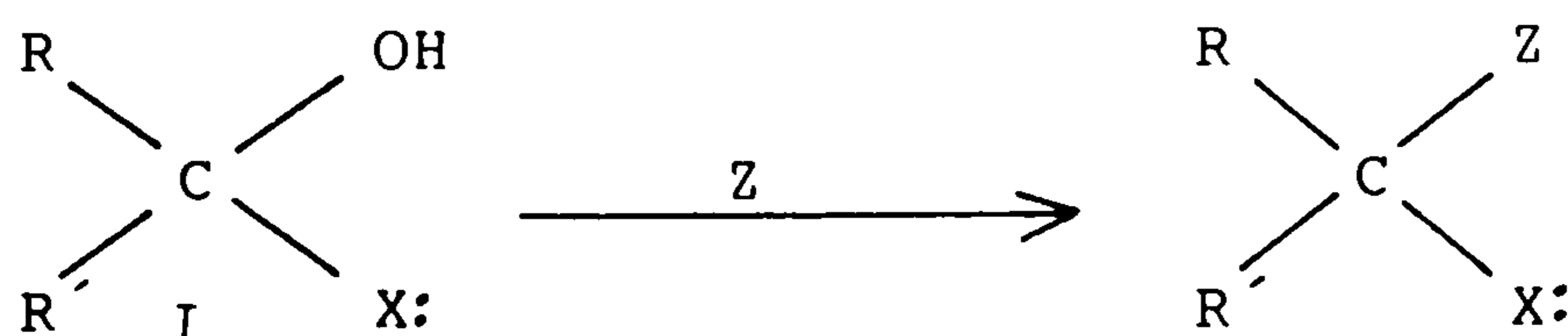


Figure 2.6 Type B addition reaction.

The reaction rate is very dependent upon the nucleophilic strength of the amine, the product formed is known as a Schiff's base. The carbonyl amine reaction is generally pH dependent with a maximal rate at a pH of about 4.5 (Feeney, et al., 1975).

At a neutral pH the rate limiting step is the loss of water from product I, at a low pH protonation of the amine becomes the rate limiting step.

2.2.4 Nonenzymic (Maillard) Browning

This is a series of reactions first discovered by Maillard (1912) leading to the formation of coloured products as a result of heat treatment of a reducing sugar with an amino acid. Reviews on the chemistry of this reaction are by Hodge (1953), Wedzicha (1984) and Reynolds (1963, 1965). The initial stage (Fig 2.8) of the reaction is a type of addition reaction between the carbonyl of the sugar and a free amine of the amino acid. The product of this reaction is known as an aldosylamine. This rapidly undergoes a facile and irreversible rearrangement, the Amadori rearrangement (Hodge, 1953) (Fig 2.9) to produce the 1-amino-1-deoxy-2-ketose ketosamine, this reaction is catalysed by weak acid and the carbonyl group.

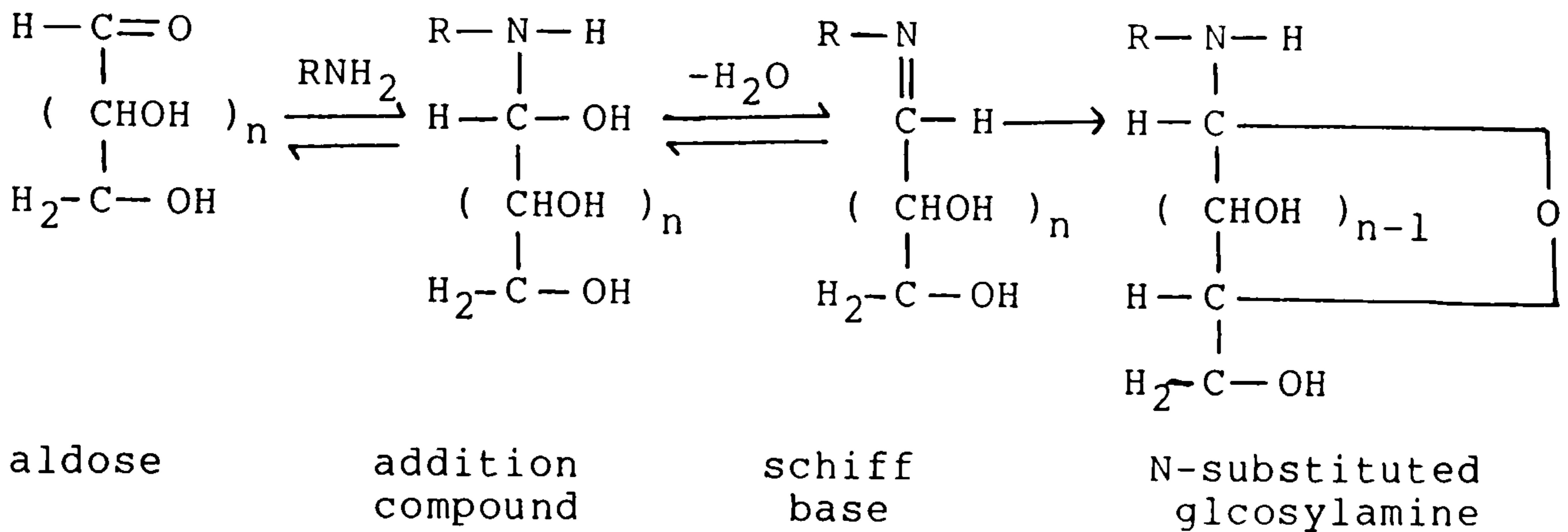


Figure 2.8 Early stages of non enzymic browning (Hodge, 1953).

If the sugar was a ketose then the product will be an aldosome via the Heyns rearrangement Fig 2.9 (Reynolds, 1965).

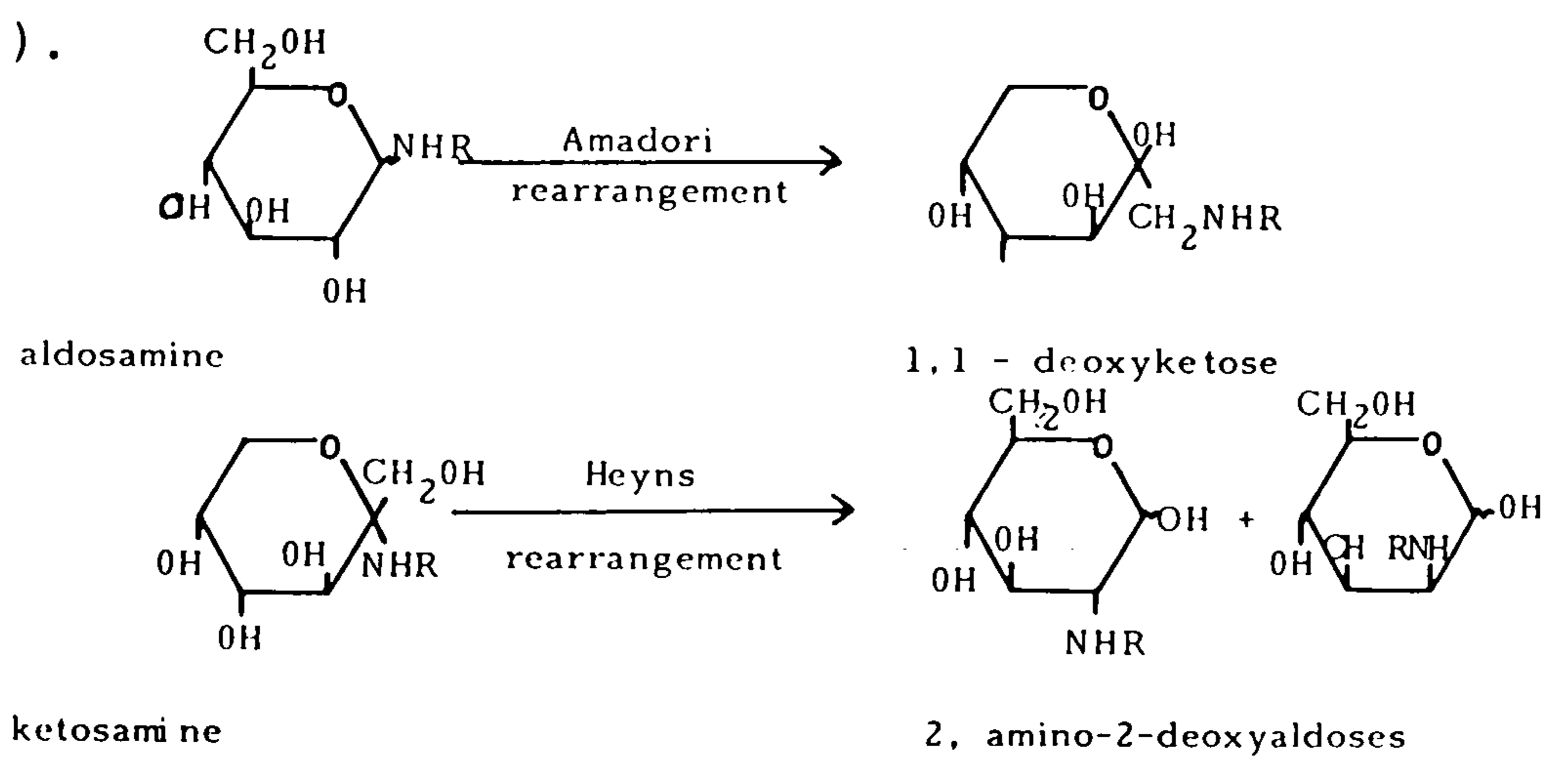


Figure 2.9 Amadori and Heyns rearrangements.

The ketosamine may undergo a further type A addition reaction with a second molecule of aldose due to the free secondary amine group . A further Amadori rearrangement will produce a diketosamine, this product is unstable and will revert back to the more stable monoketosamine. In many food systems the reaction will stop at this point, no browning will be evident but loss of nutritive value may have taken

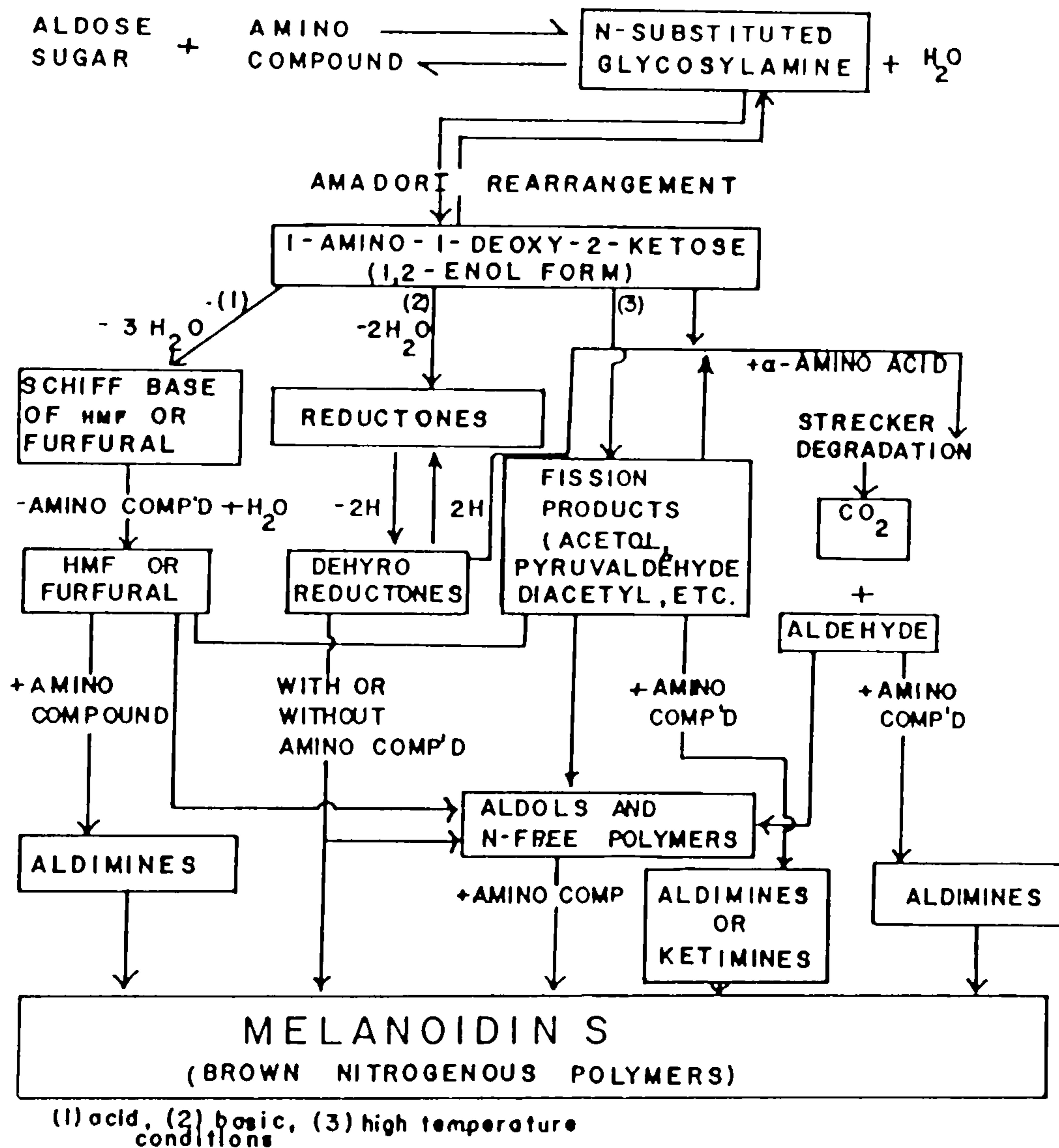
place due to loss of available lysine (Mauron, 1970 and Hurrel & carpenter, 1974).

These reactions are far from simple and other reactive species may well be formed during these initial stages (Hayashi & Namiki, 1980). Also free radicals have been demonstrated to be present (Hayashi & Namiki, 1981) which may react with amine and thus enter the pathway.

There is increasing evidence that low molecular weight carbonyl compounds are involved in the early stages of the reaction resulting in crosslinking and the formation of a variety of products (Acharya & Manning, 1980 and Acharya & Manning, 1981).

Figure 2.10.

Pathways in non-enzymic browning
(from Hodge, 1953)



Intermediate reactions involve the removal of the amino groups from the sugar moiety followed by dehydration, cyclization, fragmentation or amine condensation. Three major pathways are thought to exist (Fig 2.10):

1) Acidic conditions: Dehydration and cyclization occur resulting in the production of hydroxymethyl furfural (hexoses) or furfural (pentoses).

2) Basic conditions: Rearrangement of the 1, 2-enol glycosyl to the 2,3-enol followed by dehydration and oxidation to produce a range of reductones and dehydroreductones. The dehydroreductones can combine with amino acids to produce CO₂, aldehydes and amino keto derivatives. This path is known as the Strecker degradation (Fig 2.11).

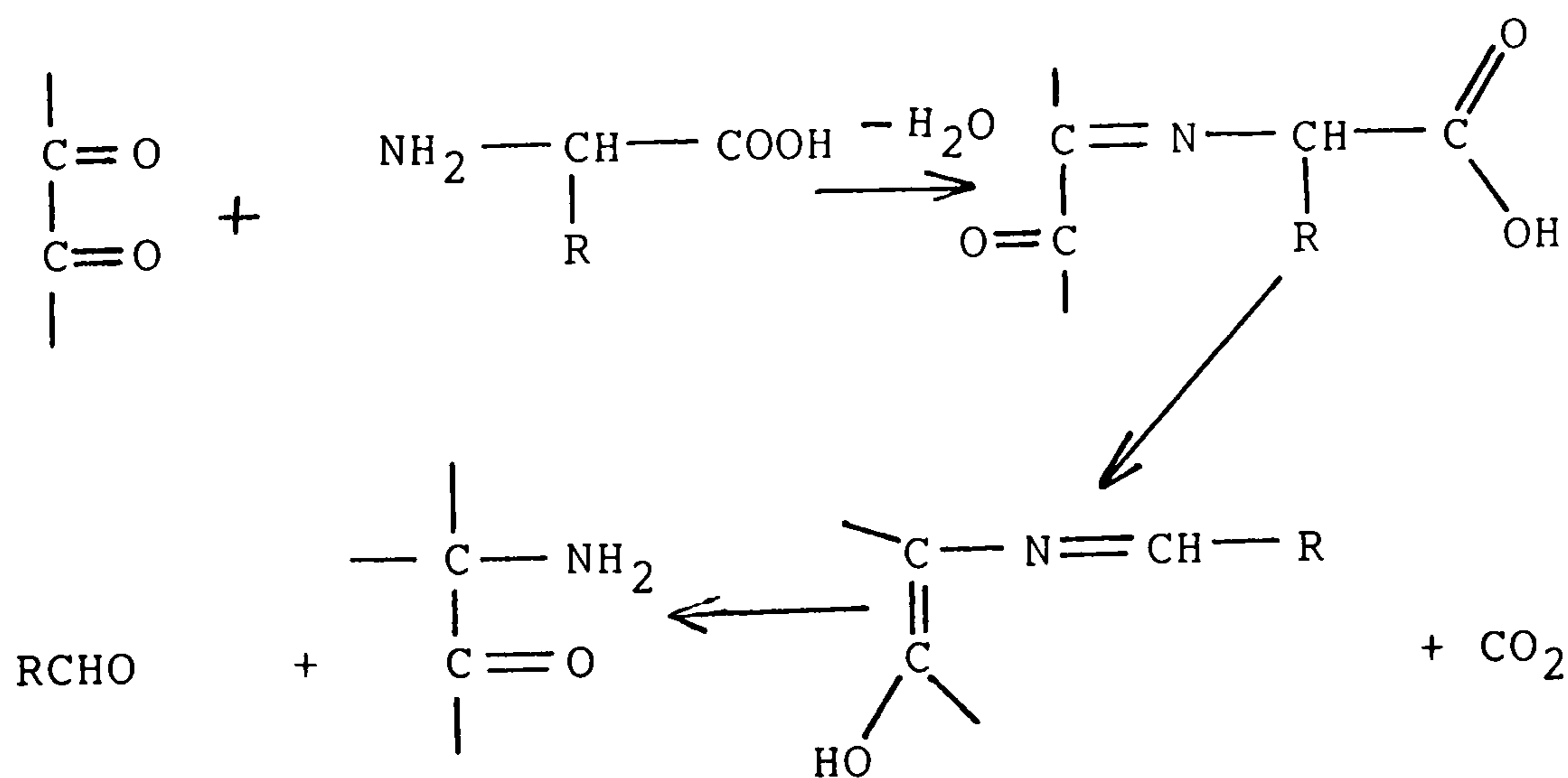


Figure 2.11 Strecker degradation (Mauron, 1981).

3) High Temperature: Fragmentation of the Amadori product producing three or four carbon aldehydes, alcohols, acids or ketones can occur at high temperature (Sullivan, et al., 1974).

The work in this study will be based on the effect of heat and so the third route, only will be considered in detail. Ketosamines and diketosamines lose their amines and amino acids forming 3 - deoxyhexosulose and 3,4 dideoxy hexosulos-3-ene. such compounds are also formed by sugar dehydration (Anet, 1964) (Fig 2.12).

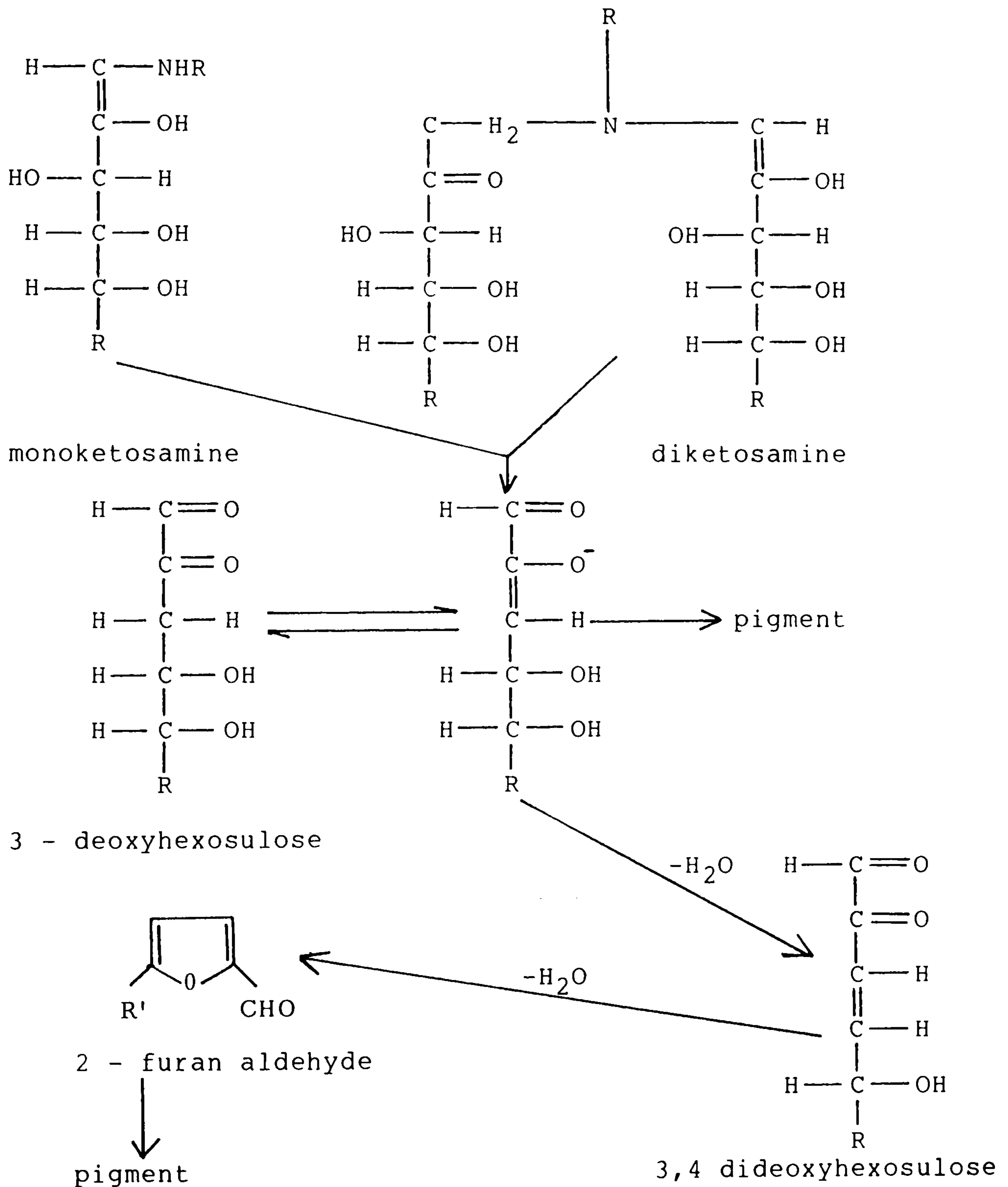


Figure 2.12 Amine assisted sugar dehydration (Anet, 1964).

Non enzymic browning has been investigated in many food systems: cane juice (Binkley, 1969), orange juice (Wolfrom, et al., 1974), soya sauce (Hashiba, 1976),

soya (Jokinen & Reineccuss, 1976) and tomato juice (Miki, 1974). It is the consensus that in food systems the principal amino acid to be lost is lysine, due to the reactivity of its ϵ -amino group (Lea & Hannan, 1949). In mild heat treatment little browning is evident (Rosenberg & Rohdenburg, 1951), but on more intense conditions the Maillard reaction is pushed past the deoxyketosyl stage and other amino acids are affected, particularly arginine and to a lesser extent tryptophan, cystine and histidine.

In foods Maillard intermediates have been implicated as flavour and aroma intermediates (Mills, *et al.*, 1969 and an excellent review by Fors, 1983).

2.2.5 Elimination Reactions

These reactions involve the removal from a molecule of two atoms or groups without there being any replacement by other atoms or groups.

In most reactions loss of atoms or groups from adjacent carbon atoms occurs, resulting in the formation of a multiple bond, a 1,2- (or β -elimination).

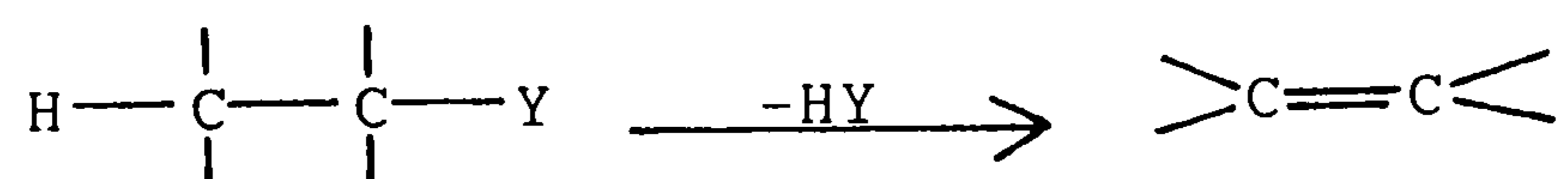


Figure 2.13 β - elimination.

Elimination may occur from the same atom (1,1) or from atoms further apart ie. 1, 5- and 1, 6-. The most common

elimination is the 1, 2-elimination also known as β -elimination, these reactions are usually associated with the loss of a proton from the α -carbon atom.

The reaction may be base catalysed or acid catalysed, three different mechanisms may exist:

A) This is a one step process with the formation of an intermediate by nucleophilic attack of a base with subsequent loss of the leaving group Y^-

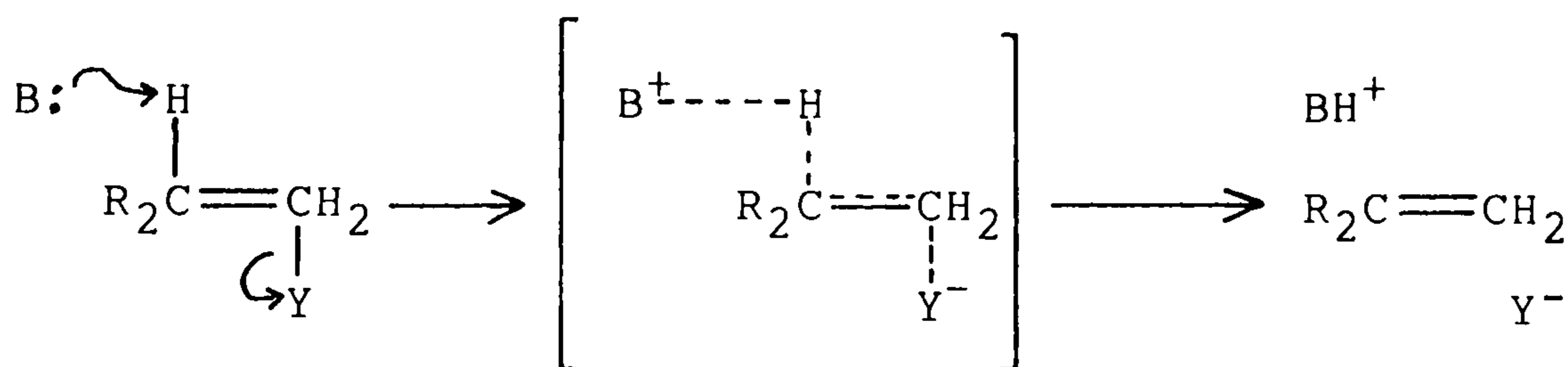


Figure 2.14 . . . β elimination reaction.

This is the most favoured mechanism.

B) The other two mechanism involve the separate cleavage of the H - C and C - Y bonds, either bond may break first.

2.2.6 Condensation Reactions

Sugar dehydration reactions begin by elimination of the elements of water which are bound to carbon atoms adjacent to the carbonyl group of the open chain. The reaction is mainly by β -elimination, whereby the hydroxyl group adjacent to the carbonyl group is eliminated, probably following protonation (Fig 2.15)

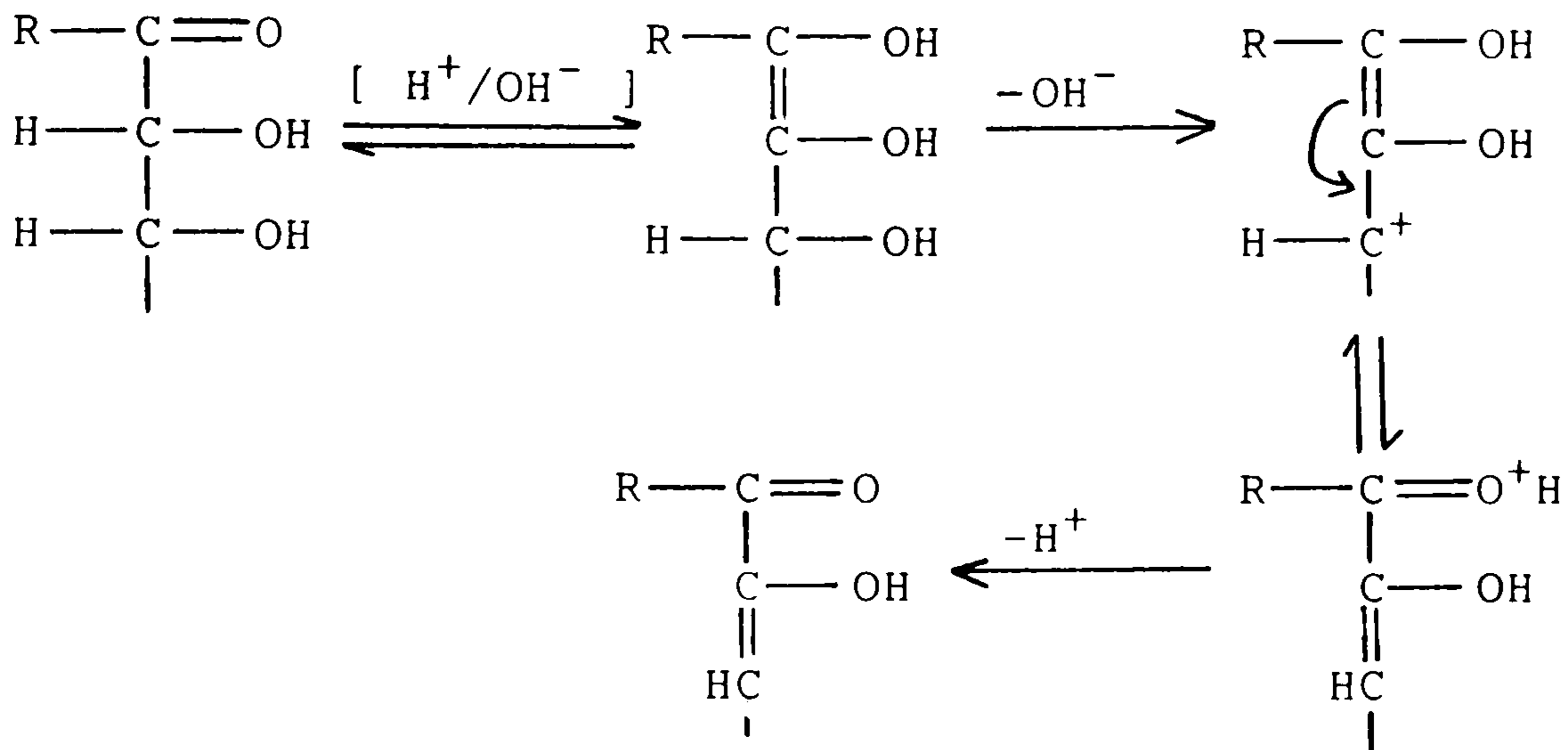


Figure 2.15 Sugar condensation reactions.

2.2.7 Acid Catalysed Reaction

These reactions are similar to the amine assisted dehydration mechanism (Feather & Harris, 1973). An aldose will lose three molecules of water to form 2-furaldehyde (Fig 2.16).

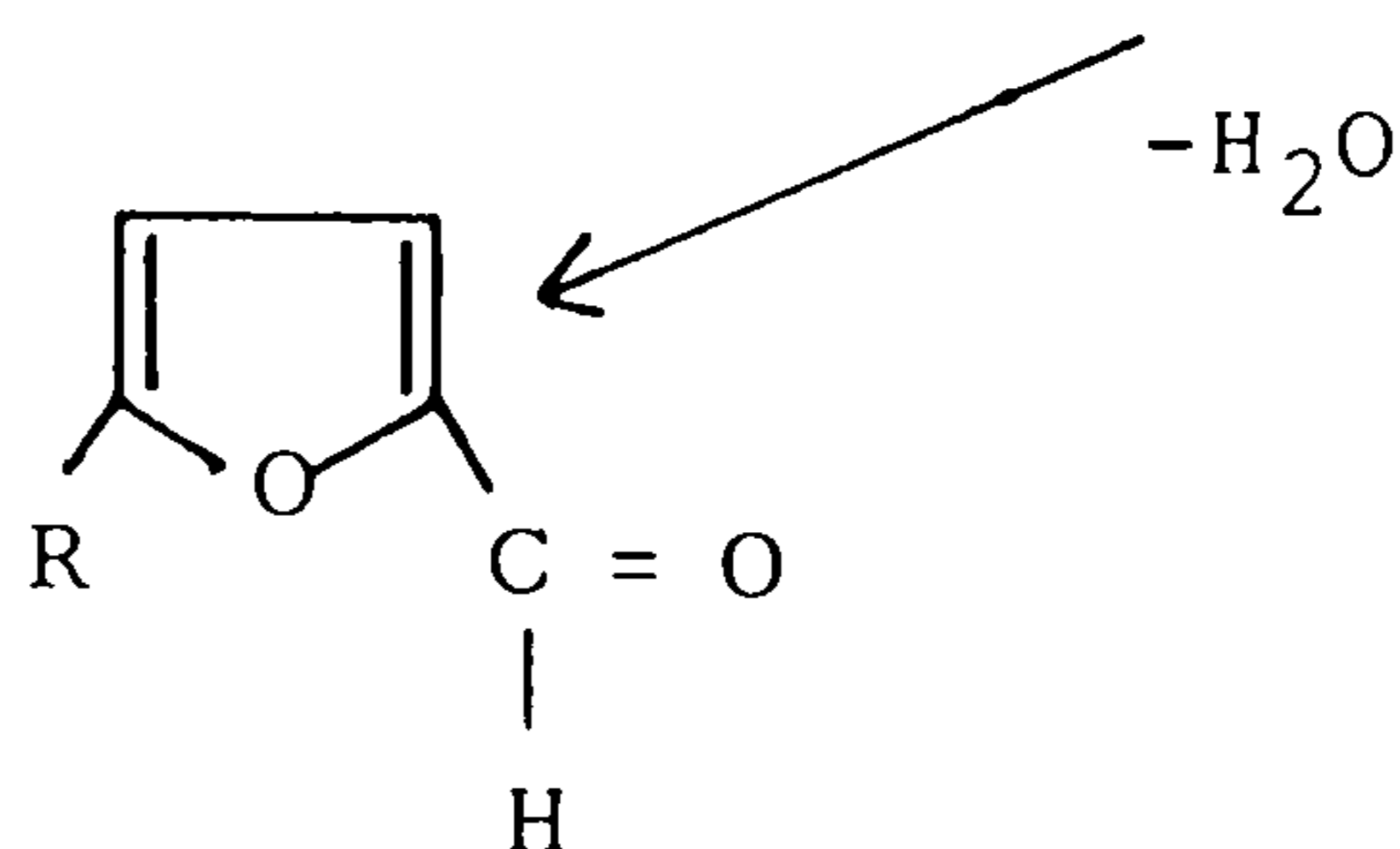
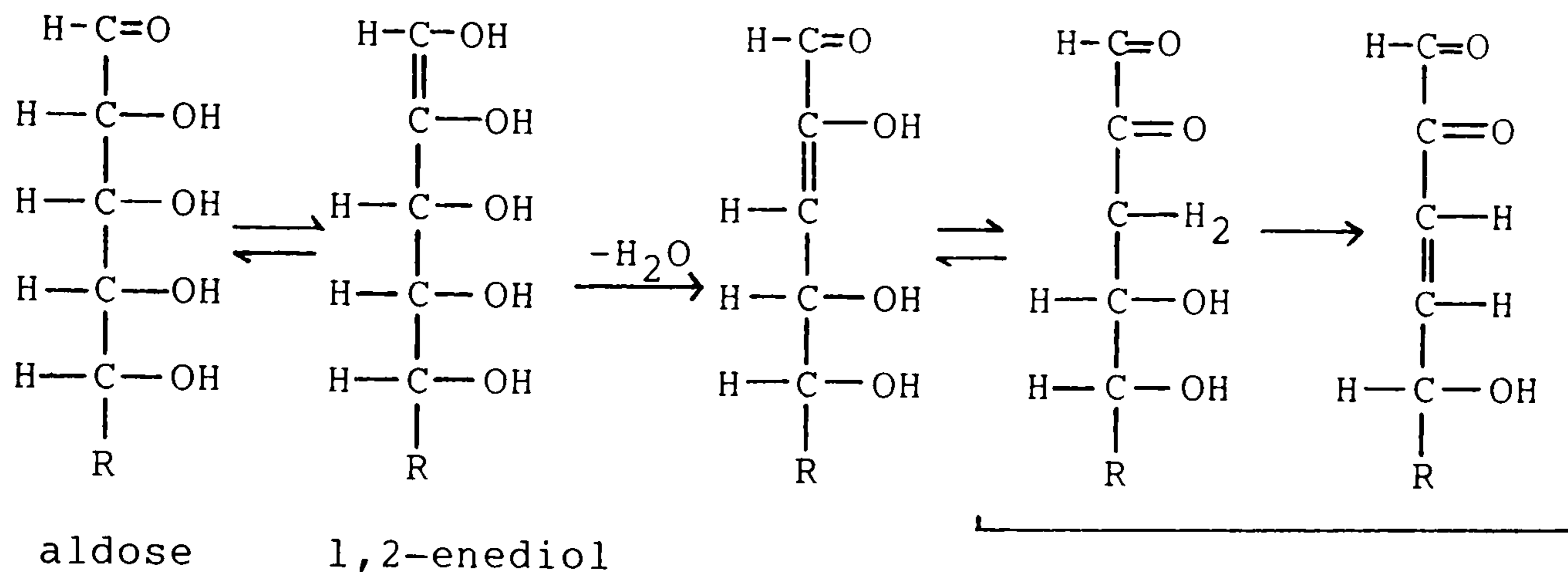


Figure 2.16 Acid mediated sugar dehydration (Feather & Harris, 1973)

The initial step in the mechanism is an acid-base catalysis interconversion of the aldose to an enol. This takes place via the Lobry de Bruyn Alberda van Ekenstein transformation which involves enolization of an aldehyde or keto sugar. The enediol can rearrange in three ways: regeneration of the original sugar, the ketose form may be generated or in very small amounts the epimer at C2 will be formed (Sowden & Schaffer, 1952) followed by two subsequent dehydrations to form 2-furaldehyde (Anet, 1960, 1962). Pentoses may follow a similar path (Bonner & Roth, 1959) as does the dehydration+decarboxylation of hexuronic acids (Anderson & Garbutt, 1963 and Stutz & Devel, 1958).

The principal products from the action of bases on hexoses are a series of 6-carbon deoxy acids and lactic acid (Sowden, 1957).

Upon further acid / base reaction the enolization may progress down the carbon chain as a new carbonyl may form at C2 leading to an increase in lability of the hydroxyl at C4. The mechanism for formation of the enol will depend upon the conditions. In acid media it is due to a dehydration reaction, a true enolization reaction can occur in acid media involving protonation of the carbonyl group and subsequent extraction of the weakly acidic hydrogen atom by base anions (Fig 2.17).

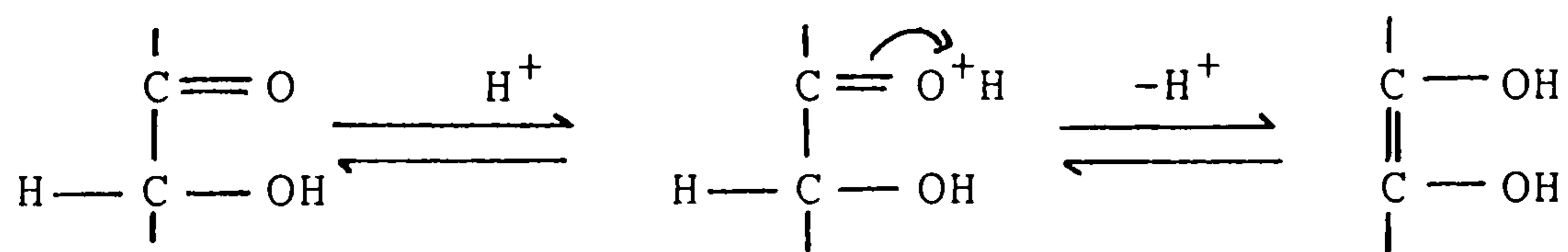


Figure 2.17 Enolization.

This reaction is slow and will be dominated by dehydration reactions. In alkali conditions the reaction will be dominated by β -elimination.

2.2.8 Sugar Mutarotations

The first and simplest structural changes in sugars which leads to their decomposition are changes in the enomeric form of the pyranose and furanose rings. The changes between the two anomeric forms for each ring type is known as mutarotation. These reaction can be catalysed by

both hydrogen and hydroxyl ions simultaneously. The reaction involves the simultaneous transfer of a proton from the acid catalyst to the sugar whilst a proton is transferred from the sugar to the base catalyst resulting in an aldehydo-sugar (Fig 2.18).

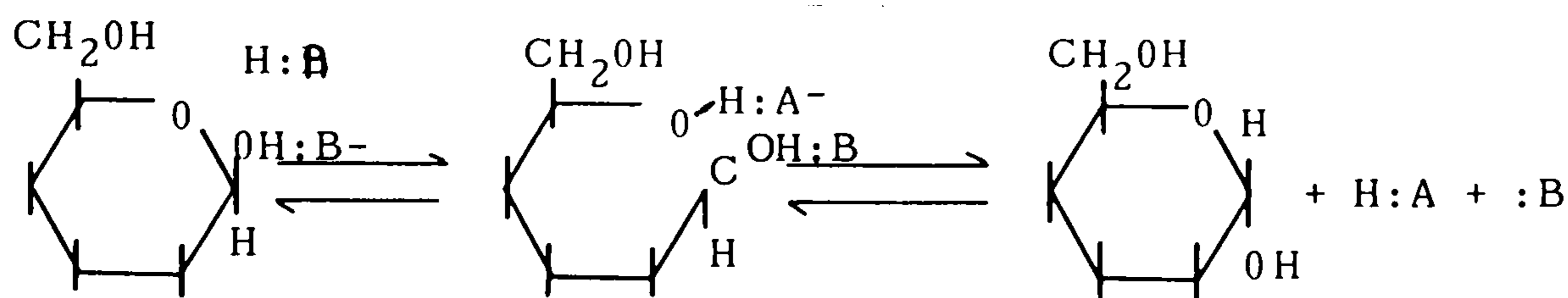


Figure 2.18 Sugar mutarotation.

Water usually acts as a complete catalyst due to its amphoteric dissociation into ions : $\text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{OH}^-$

Mutarotations can only occur at C1 due to the O—C—O bonding allowing the relative ease of formation of the intermediate aldehyde as a result of the delocalising effect of the oxygen atoms on the carbon moiety (Rees, 1977).

2.2.9 Hydrolysis of the glycosidic bond

Normally hydrolysis is with hot dilute acid. The H^+ ions probably act on the glycosidic oxygen causing it to become protonated. The reaction may then proceed via a carbonium ion. This involves a transitory change in ring conformation and so energy must be supplied, often as heat. Changes in ring conformation occur as the orbitals of the carbonium ion

and the ring oxygen align in an endeavor to spread the charge and thus stabilise the structure. Sugars carrying electron attracting groups such as -COOH or -NH_3^+ stabilise the glycosidic bond as they can make the +ve charge more unstable.

2.3 Macromolecular chemistry.

2.3.1 Thermodynamic Properties of proteins.

Changes in the state of a protein are often accompanied by a change in the energy level. These changes are reflected as either the absorption (endothermic) or liberation (exothermic) of heat.

Such changes in heat due to a change in state are associated with the term enthalpy. Enthalpy, ΔH , is the quantity of heat absorbed by a closed isothermal system, when at constant pressure it undergoes a change of state.

When proteins denature, both intra and intermolecular bonds are disrupted, often cooperatively. The protein is thought to change its conformation from a highly ordered state to one that is less ordered.

The heat changes associated with a protein transition in dilute solution are thought to be composed essentially of three components:

- 1) A negative effect, exothermic, resulting from the interaction of hydrophobic groups as a result of the protein

unwinding. Heat is evolved as water molecules are ordered into clathrate structures around the hydrophobic groups (Brandts & Hunts, 1967).

2) A positive effect which is due to heat evolution when inter and intramolecular hydrogen bonds are broken and reformed with water molecules (Privalov, 1979).

3) Van der Waals interaction are thought by some to contribute to the ΔH of the transition, Frinkelstein & Shakhanovich (1982) argues such interactions lead to positive heat changes and form the major contributor to ΔH . Privalov (1982) argues that large heat changes are not possible during the disruption of Van der Waals bonds.

Heat changes in a system of a complex protein may also be influenced by other factors, the most important being aggregation of the protein or protein fragments, a process that has been shown to be exothermic. The forces maintaining the highly complex quaternary structure exhibited by soya will affect the observed ΔH value. The influence of such effects is very difficult to assess as little is known about the stability of the complex quaternary structure of, for example, soya protein.

There has also been shown a dependence on temperature for the enthalpy of denaturation upto a temperature of 383 K (Privalov, 1974). Temperature has little effect upon the energy of hydrogen bond disruption but will greatly influence the interaction of water around the hydrophobic

groups as with rising temperature the water molecules will exhibit a decreasing ability to form bonds with themselves or other protein molecules.

The thermal stability of a range of proteins have proved to have much in common (Privalov, 1974). With increasing thermostability the enthalpy of denaturation increases and the transition becomes more cooperative (Privalov, 1982).

It has also been shown that prior to denaturation a gradual change of state of the protein molecules takes place with increasing temperature. These changes are not cooperative (Privalov, 1974) and have been assigned to changes in the microenvironment of the molecule ie a redistribution of groups, particularly an increase in the number of hydrophobic groups in contact with water due to structure expansion (Privalov, 1982).

2.3.2 Alkali treatment

Alkali treatment of proteins is often used in the preparation of protein concentrates and isolates. Unfortunately alkali treatment may lead to the formation of new amino acids which are nutritionally unavailable. The amino acids most affected by these reactions are cystine, lysine, arginine and serine. A number of reactions may take place during alkali treatment, for example:

1) Hydrolysis: Essentially two main types of hydrolysis can take place the first

β -elimination is the most dominant reaction of proteins during processing in alkali (Whitaker & feeney, 1983). A number of amino acids are implicated in this reaction including cystine, serine and threonine (Friedman, 1979). Involvement of these amino acids in β -elimination results in their destruction. A generalized reaction mechanism can be given as:

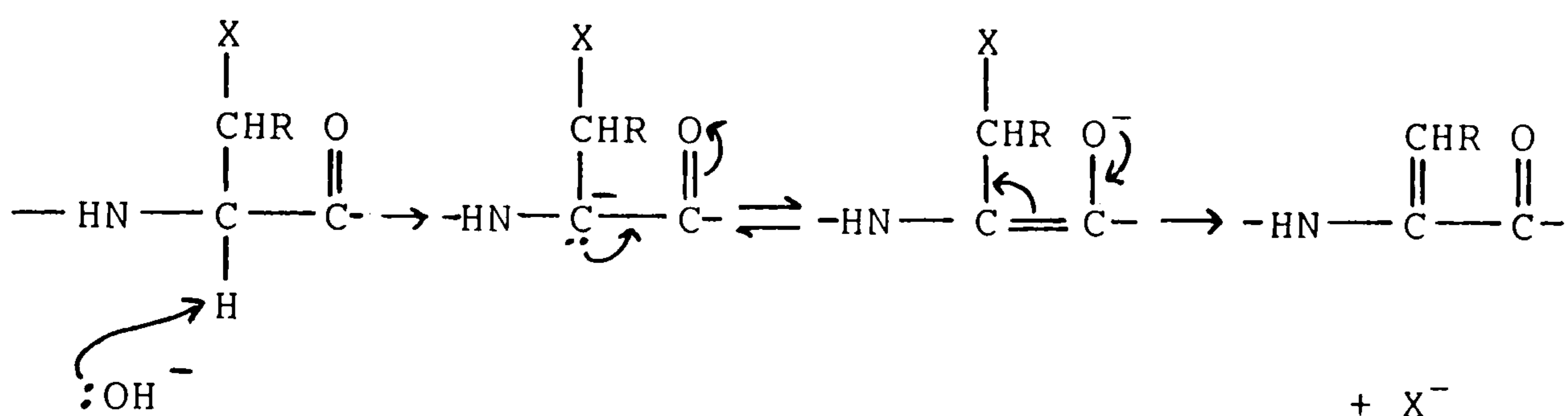


Figure 2.20 β -elimination reaction in proteins.

The unsaturated products formed during β -elimination are prime targets for nucleophilic attack and subsequent addition reactions resulting in cross links (Hasegawa & Iwata, 1982).

3) Addition Reactions

The alkali mediated scission of disulphide bonds and subsequent β -elimination of unsaturated products can lead to further reaction with a number of the nucleophilic groups on proteins and other compounds leading to the formation of new interchain crosslinks (Nashef, 1977). The main products of serine, cystine or threonine are dehydroalanine and methyldehydroalanine (Fig 2.21).

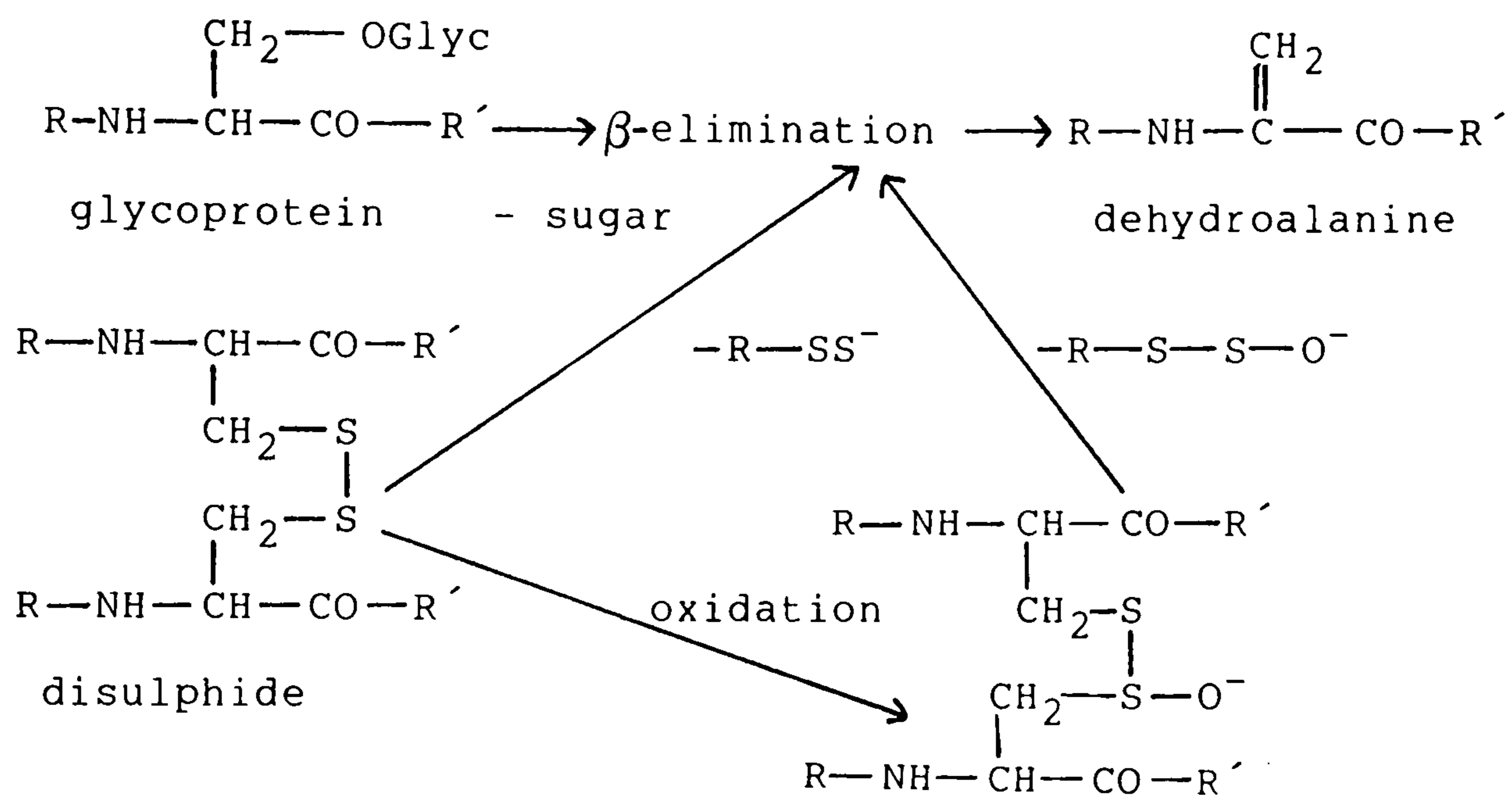


Figure 2.21 Dehydroalanine formation (Finley, 1983).

The most reactive nucleophile found on a protein is the amino group of lysine, reactions of this group with dehydroalanine lead to the formation of a crosslinked amino acid, lysinoalanine (LAL). Reaction of cysteine with dehydroalanine may also take place which results in the formation of lanthionine (LAT).

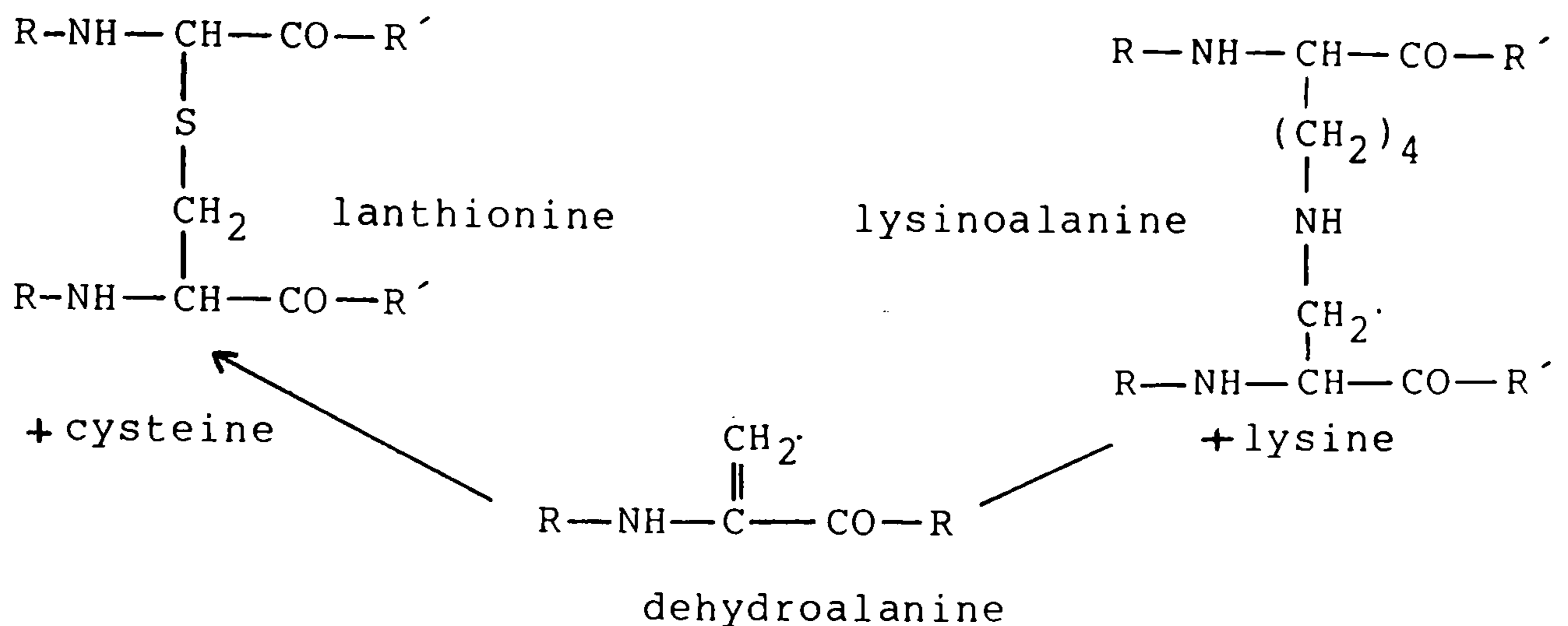


Figure 2.22 Lanthionine and lysinoalanine formation (Finley, 1983).

The formation of these crosslinks is rapid, for instance in 0.1 M NaOH DeRham et al (1977) suggest that the majority of easily formed LAL is present within 1 hour. Increasing the pH increases the rate of LAL formation (Hayashi & Kameda, 1980). The formation of LAL is competitively inhibited by the presence of carbohydrate which will involve the ϵ -amino groups of lysine in browning reactions (Friedman, 1982).

The work of Bohak (1964) and Patchornik & Sokolovsky (1964) suggests that LAL formation is purely an intramolecular event within a single polypeptide chain, but later work by Nashef et al . (1977) and Sen et al. (1977) does not agree.

LAL and LAT have been found in many alkali treated proteins (Bohak, 1964) but casein, soya protein and egg albumen have been found to be particularly susceptible to LAL formation (Dworschak, 1980; Walsh et al., 1979 and

Friedman, 1982).

2.3.3 Heat treatment of proteins

As well as the denaturant effects described earlier proteins may undergo specific chemical changes following heat treatment. The type and severity of these changes depends upon the intensity of exposure.

In a pure protein the following changes are to be expected:

TEMP / C	Change / Degradation
70 - 80	Disulphide bond splitting
80 - 100	Losses in disulphides
100 - 150	Lysine decomposition Serine and threonine loss Isopeptide formation Lysinoalanine formation
200 - 250	Pyrolysis of all amino acids
> 300	Formation of carcinogenic pyrolysis products

Table 2.1 Effects of heat treatment on protein.

This can only be a general scheme as the presence of other constituents, such as carbohydrate or lipid, will

drastically affect the course of the reactions.

The type of heat treatment is also very important, moist heat has been shown to lead to more complex effects than dry heat (Neucer & Cherry, 1982).

In this study temperatures greater than 473 K (200⁰C) were rarely used. Thus reactions occurring above this temperature will not be considered. A review of non enzymatic browning has been given elsewhere (section 2.2.4) and so this section will be limited to those changes that can be expected to occur in " pure " proteins.

1) Amino Acid Racemization

Protein quality is affected by amino acid racemization during heating (Hayase et al., 1973, 1975).

The mechanism is thought to involve the removal of the proton from the amino acid to produce a carbanion. The proton can be added back to either side of this optically inactive intermediate. Hurrel (1984) has measured amino acid racemization in different proteins after intense heating and found that only aspartic acid, glutamic acid, alanine and lysine underwent racemization to any significant extent. This reaction will occur also during alkaline treatment.

2) Protein Crosslinks

The formation of LAL and LAT will take place as a result of heat treatment by a mechanism similar to that given above (Cross, 1977), the severity of the heating

process will increase the amounts of LAL formed (Lorient, 1979). Following heating the most common crosslink is that due to isopeptide formation (Mauron, 1972 and Ford, 1973). Many crosslinking mechanisms exist but the most likely is the formation of isopeptide bonds by reaction of the ϵ -amino group of lysine with either the carboxyl group of aspartic / glutamic acids (Mecham & Olcott, 1947) or with the amide groups of glutamine and asparagine (Bjarnason & Carpenter, 1970) leading to the formation of glutamyl - lysine or aspartyl - lysine isopeptides. Such bonds have been found in heated foods (Hurrel et al., 1976).

Hurrel et al., (1976) found that isopeptides recovered from a range of proteins accounted for 0 to 50 % of those lysine units made inaccessible to FDNB. Asquith & Otterburn (1977) suggest, possibly a third of bound lysine may be tied up in these linkages.

Mauron (1972) suggests that glutamic acid and aspartic acid may form imide links with asparagine/ glutamine.

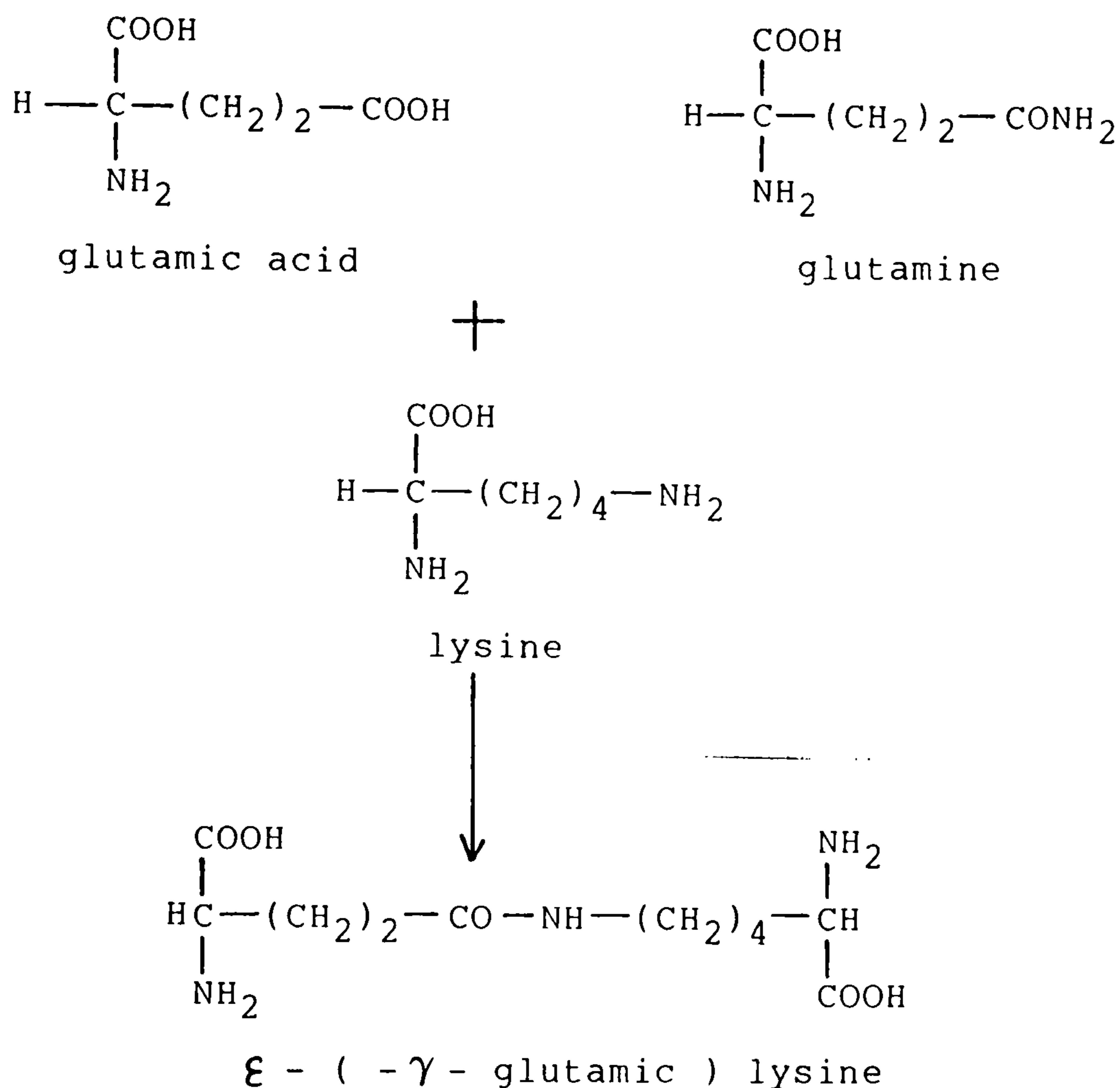


Figure 2.23 Isopeptide bond formation.

Thioester linkages with the thiol group of cysteine or ester links with the hydroxyl groups of threonine and serine can take place.

The ease of formation of the isopeptides in any specific protein depends upon the concentration of the reactive groups.

3) Cystine Loss

Decreases in cystine content of up to 60 % have been reported following heat treatment (Bjarnason & Carpenter, 1970). It

is known that at temperatures above 373 K in carbohydrate free systems H_2S is evolved as a result of the breakdown of the sulphhydryl bond.

The sulphur of cystine is thought to be liberated through an elimination reaction to yield dehydroalanine.

2.4 Soya

2.4.1 Proteins

FRACTION	% TOTAL PROTEIN	COMPONENT	MWT × 1,000
2S (conglycinin)	22	trypsin inhibitors cytochrome C	8 - 21.5 12
7S (β - and γ - conglycinin)	37	heamagglutinin lipoxxygenase β - amylase 7S globulin	110 102 61 180 - 210
11S (glycinin)	31	11S globulin	350
15S	11	-	600

Table 2.2 Major proteins found in soya (Wolf, 1970).

The major proteins are listed in table 2.2. Nomenclature varies in the literature the two main types are: The ' S ' nomenclature based on sedimentation coefficients (molecular weight) (Hill & Breidenbach, 1974 and Wolf, 1970) and the con / glycinin system based on immunological reactions (Koshiyam, 1972). Four antigenically different components have been isolated (Catsimpoolas et al ., 1969). The S nomenclature will be used in this study.

Both the 7 S and the 11S fractions have complex quaternary structures which can undergo association - dissociation reactions. They have a low content of α -helix (Hermansson, 1978) and are primarily composed of antiparallel pleated sheet structure and disordered regions (Fukushima, 1968). A 15 S globulin can often accompany purified 11S in minor amounts. Over 1,000 polypeptides have been isolated from soya (Hu & Essen, 1982) but most of these polypeptides are of minor importance in soya structure, which is essentially composed of only 50 to 60 major polypeptide fragments.

Soya protein is thought to be stabilised by the interactions between the different sub-units. Work has mainly concentrated on the interaction between the 7S and the 11S globulins, and it has been confirmed that stabilisation results from such interaction (German, et al., 1982,). A loss of solubility on freeze drying soya isolate has been shown to be the result of the formation of crosslinks between the 7S and 11 S globulins (Nash & Wolf, 1980). Utsumi (1985) observed that on heating soya both the 7S and 11 S globulins dissociated and subsequently interacted with each other to form stable macrocomplexes.

2.4.1.1 11S Globulin

The 11S globulin is composed of 12 subunits of which 6 are acidic in nature and 6 are basic. This results in a complex with a molecular weight between 302,000 and 375,000

(Badley, etal., 1975 and Utsumi, et al., 1981). The acidic and basic units are linked together in specific combinations of alternating acidic / basic units via disulphide bonds (Kitamura, et al., 1976) and hydrophobic bonds to form two packed hexagons placed one over the other held together by hydrogen bonds, the result is a hollow oblate cylinder (Badley, et al., 1975). Further studies suggest that a more complex structure exists (Iyenger & Ravestan, 1981 and Moreira, et al., 1979). Within these structures 14 % of the total ionizable groups are buried (Catsimpoolas, et al., 1971a).

The 11S globulin is low in methionine but high in lysine. The proportions of hydrophobic residues (ala, val, ile, leu and phe) and hydrophilic residues (lys, ala, arg, asp and glu) are 23.5 and 46.7 % respectively (Takagi, et al., 1979). The primary structure is arranged as a mixture of α -helix, β -sheet and random coil. There is some disagreement in the literature in regard to the proportions of each (Table 2.3).

Helix	Sheet	Random coil	
5.2	34.8	60.0	Koshiyam (1972)
20.0	17.0	63.0	Isino & Kudo (1980)
6.0	40.0	55.0	Yamauchi <u>et al.</u> (1979)

Table 2.3 Percentage secondary structures found in 11S globulin.

The acidic units have a higher content of glutamic acid, proline and half cystine while the basic units have higher contents of the hydrophobic amino acids. The acidic amino acids in the basic subunits are primarily in the amide form (Peng, 1984). In total 55 % of the total acidic amino acids in the 11S are in this form (Catsimpoilas, et al., 1971b).

Within the 11 S globulin there are 48 moles of half cystine per mole of protein (Catsimpoilas, et al., 1969). The number of free sulphhydryl groups increases from 2 to 3 on heating (Saio, et al., 1971).

2.4.1.2 7S Globulin

50% of the 7S globulin fraction is the 7 S protein which is equivalent to 18 % of the total protein (Wolf & Sly, 1967).

The 7S globulin is a glycoprotein (Koshiyama, 1969) and contains the carbohydrate as one unit attached to the aspartic acid residues at the N - terminal end of the molecule (Yamauchi, 1975). The carbohydrate content of the 7S globulin is composed of 38 mannose and 12 glucosamine residues per molecule which is equivalent to 5 % of the total globulin weight.

This globulin has molecular weight between 150,000 and 175,000 and is composed of 3 subunits (α , α' and β) which interact to produce 6 isomeric forms (B₁ to B₆), each with different properties. β - subunits have a high hydrophobic

amino acid content which is essentially responsible for the folding pattern of this globulin. Disulphide bonds do not seem to be involved in binding these subunits (Koshiyam, 1971) and in general cystine residues are at low concentrations (2 per mole of 7S globulin).

2.4.2 Carbohydrate Content of Soya

In addition to the 7S globulin haemagglutinin is also a glycoprotein (Lis, et al., 1966). This contains 3 to 5 glucosamine and 25 mannose units per molecule.

Soya flour has a carbohydrate content of 38 %, composed of oligosaccharides and insoluble polysaccharides in approximately equal proportions (Morita, 1965 a, b and Aspinal, et al., 1967 a, b, c). The composition is given in Table 2.4.

	%
Soluble carbohydrate	17
hexose	trace
sucrose	5.7
raffinose	4.1
stachyose	4.5
verbascose	trace
Insoluble carbohydrate	21
hemicellulose	
cellulose	
lignin	
pectin	
others	

Table 2.4 Carbohydrate composition of soya flour (Sheard, 1985).

2.5 Alginates

Alginate is the principle skeletal material of all brown algae and can be upto 40 % of the total dry weight of the material (Steiner & McNeeley, 1954). In the native state they are present as a mixed salt of all the cations found in the sea (Baardseth, 1969), mainly Ca^{2+} , Na^+ and Mg^{2+} . The genera of the phaeophyceae most used are;

Macroystis, Ecklonia, Laminaria and Ascophylum. Native alginate exists as an insoluble gel crosslinked by Ca^{2+} and Sr^{2+} ions, these ions must be exchanged for an alkali metal or ammonium ion to convert the material into a more soluble form. Usually this is performed at neutral pH with EDTA or ammonium oxalate following treatment with 0.1 or 0.2 M mineral acid (Haug, 1964).

Alginates are composed of D - mannuronic acid (M) and L - guluronic acid (G) in various proportions

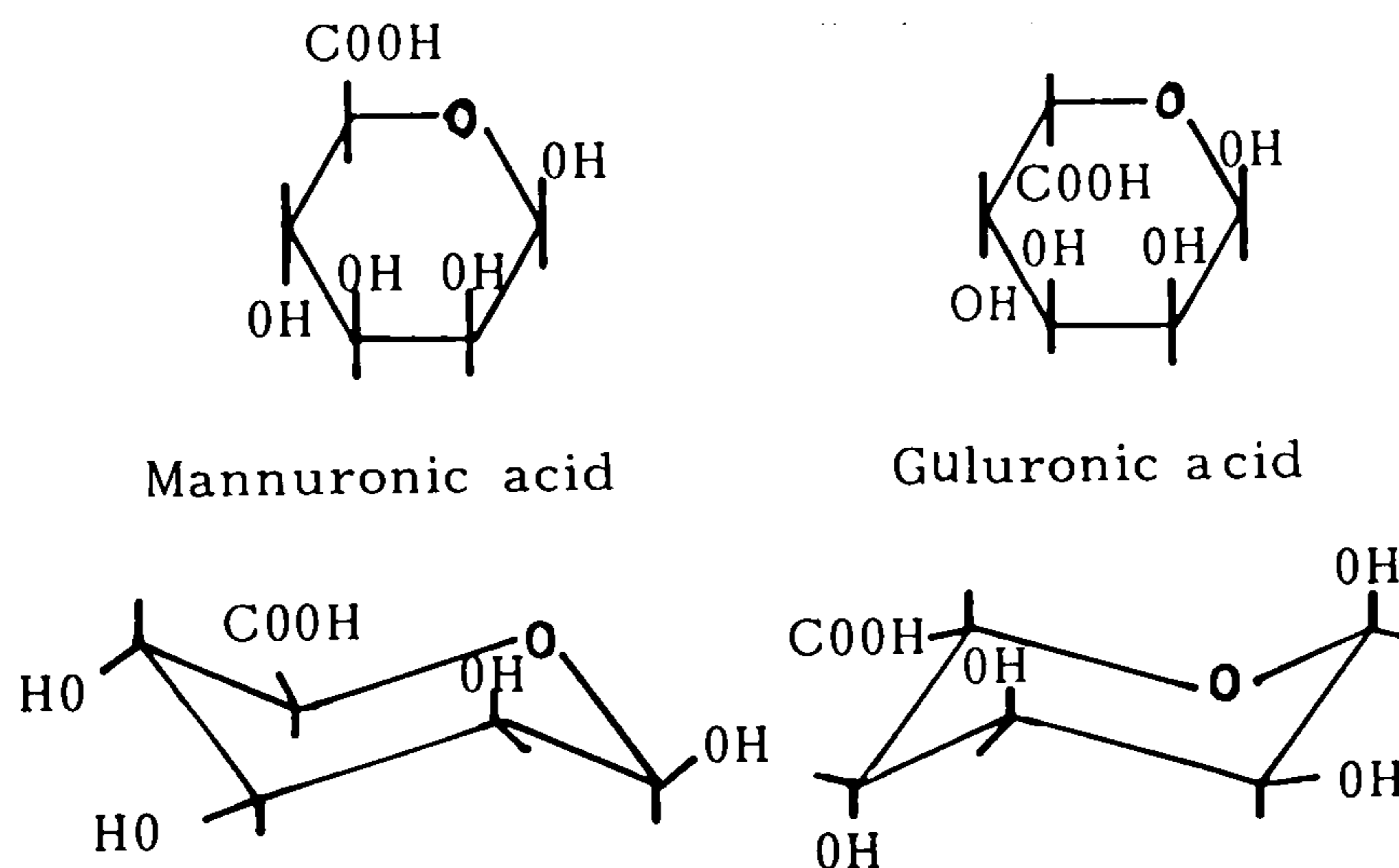
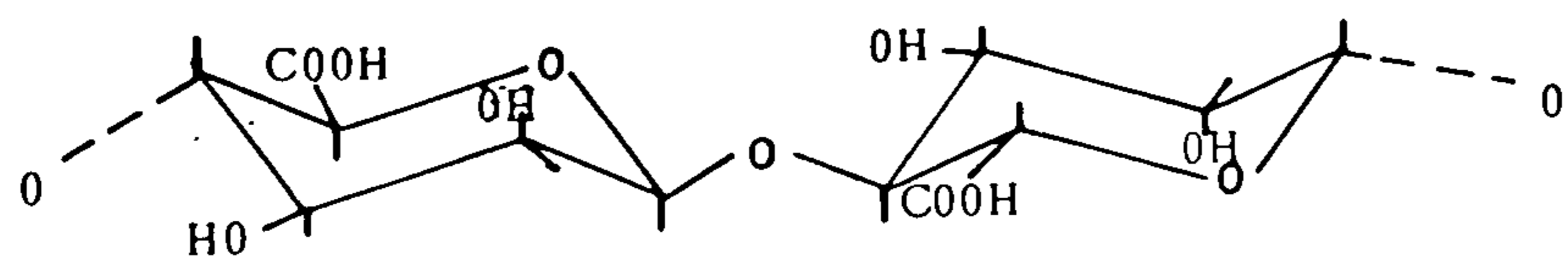


Figure 2.24 D mannuronic acid and L guluronic acid.

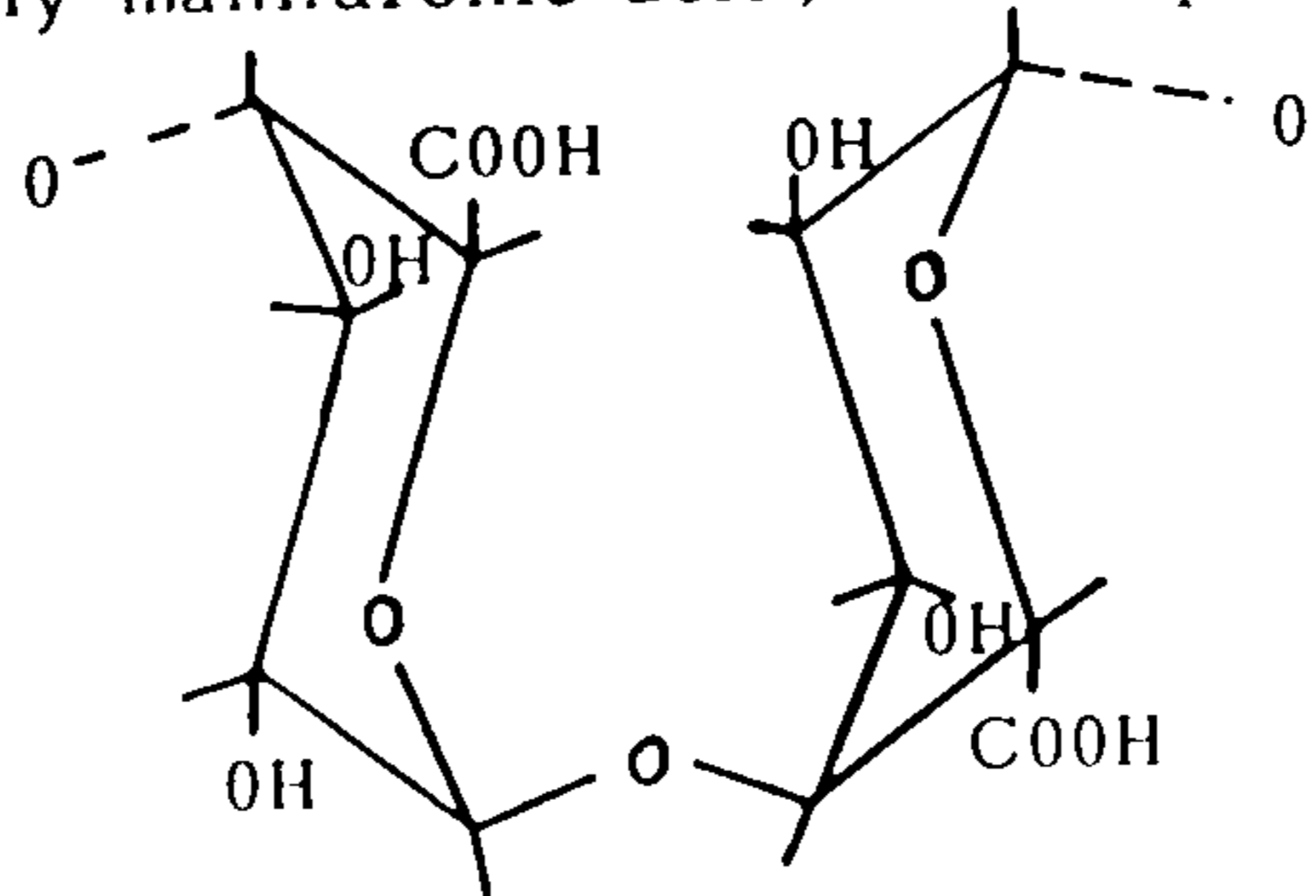
The amounts of the different units is dependent upon many criteria such as source (Haug & Larson, 1962) or part of plant used (Haug, et al., 1974). Seasonal variations have been found whereby there is a high mannuronate content during rapid growth.

Following mild acid hydrolysis three types of polymer segment have been found (Haug, et al., 1966, 1967a & 1967b). The monomers are assumed to be arranged in blocks of repeating mannuronate, repeating guluronate and regions

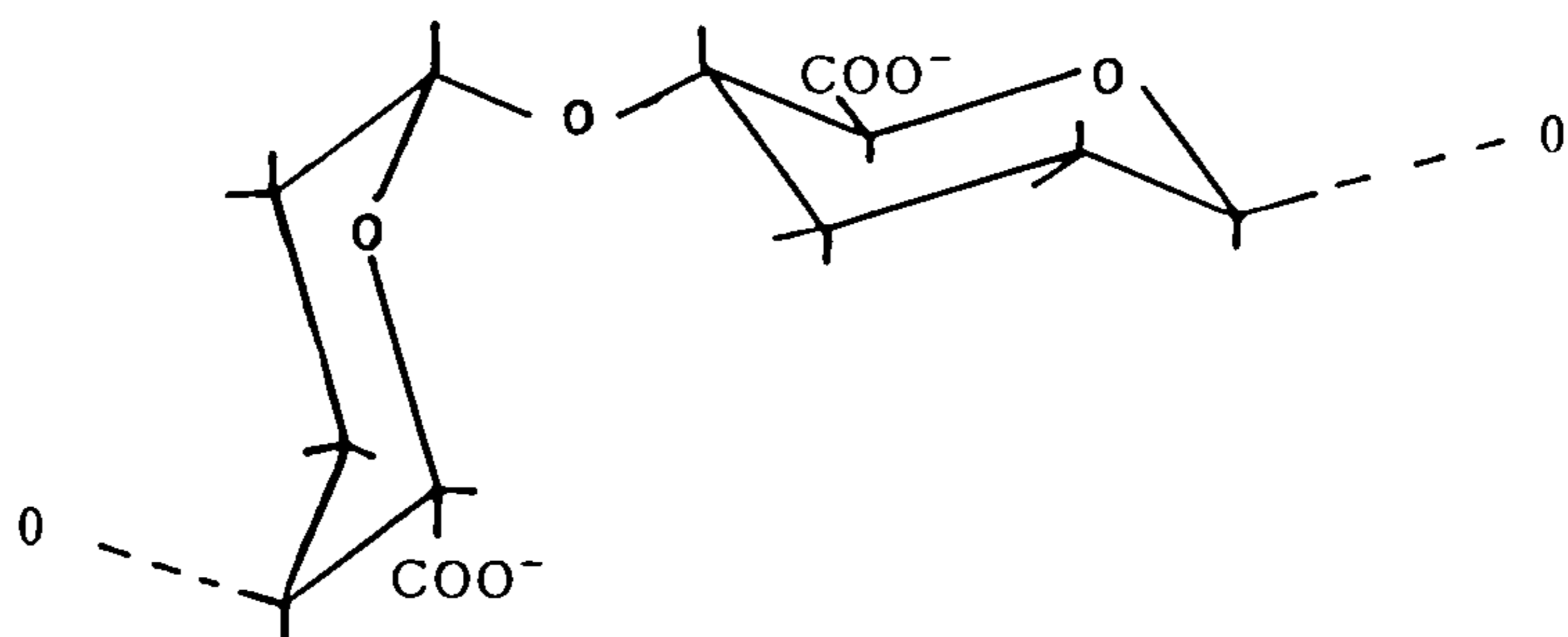
where the two alternate.



Poly mannuronic acid, 1-4 diequatorial



Poly guluronic acid, 1-4 diaxial linked



1,4 linked guluronic-mannuronic acid

Figure 2.25 Block sequences found in alginate.

Each block has been found to have a degree of polymerisation of about 24 units (Boyd & Turvey, 1978). The alternating regions form the weakest point in the polysaccharide chain. Boyd and Turvey (1978) and Grasdalen, et al. (1981) both conclude that the block sequences present in the MG blocks are not strictly alternating M-G but that both -M-M-G- and -G-G-M- sequences are also present.

The ratio of the different sugars have been studied by a variety of techniques including anion exchange liquid chromatography (Gacesa, et al., 1983), high performance liquid chromatography (Annison, et al., 1983), NMR

(Penman & Sanderson, 1972 and Grasdalen, et al., 1979), ^{13}C - NMR (Boyd & Turvey, 1978) and circular dichromism (Morris, et al., 1980a and Craige, et al., 1984). Extreme values for M:G range from 0.4 for alginate from *Laminaria hyperborea* to 2.4 for alginate from *L. digitata*. Similar ranges have been found in a single plant, for instance in *Ascophyllum nodosum* the ratio is 2.6 in the tips of the fronds and 0.9 in the holdfast (Haug, et al., 1974). Table 2.5 lists the relative proportions of the different block sequences found in three common sources of alginate.

Source	Poly-M	Poly-G	Alternating
<i>Macrocystis pyrifera</i>	40.6	17.7	41.7
<i>Ascophyllum nodosum</i>	38.6	20.7	41.0
<i>Laminaria hyperborea</i>	12.7	60.5	26.8

Table 2.5 Proportions of the different block types from alginates derived from different sources. (Penman & Sanderson, 1972).

It is expected that D- mannuronic acid adopts the $^4\text{C}_1$ conformation which will result in the maximum number of bulky ring constituents in the equatorial position. ' X ' - ray studies of fibres show that a molecular chain of poly M is a flat extended ribbon like molecule. The structure is stabilised by the formation of an intra molecular hydrogen bond between $-\text{O}_2\text{H}$ of one unit and the ring oxygen of another. The chains themselves are bonded into sheets via

hydrogen bonds (Smidsrod, 1971; Fig 2.26).

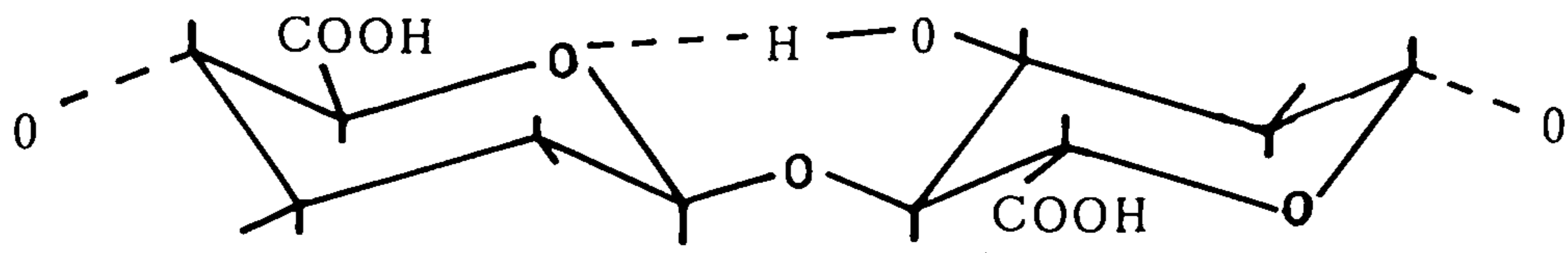


Figure 2.26 Hydrogen bonding between mannuronic acid units.

Poly G is quite different in that it is a buckled ribbon like molecule, which is the result of the L-guluronic acid being in the 1C_4 conformation. In this conformation less bulky equatorial groups are evident thus allowing tighter packing. The rod like structure is stabilised by hydrogen bonds between $-O_2H$ and O6 in adjacent units, The interchain bonds are more complex than in the case of Poly M and are thought to involve binding with water molecules (Atkins, 1971)(Fig 2.27).

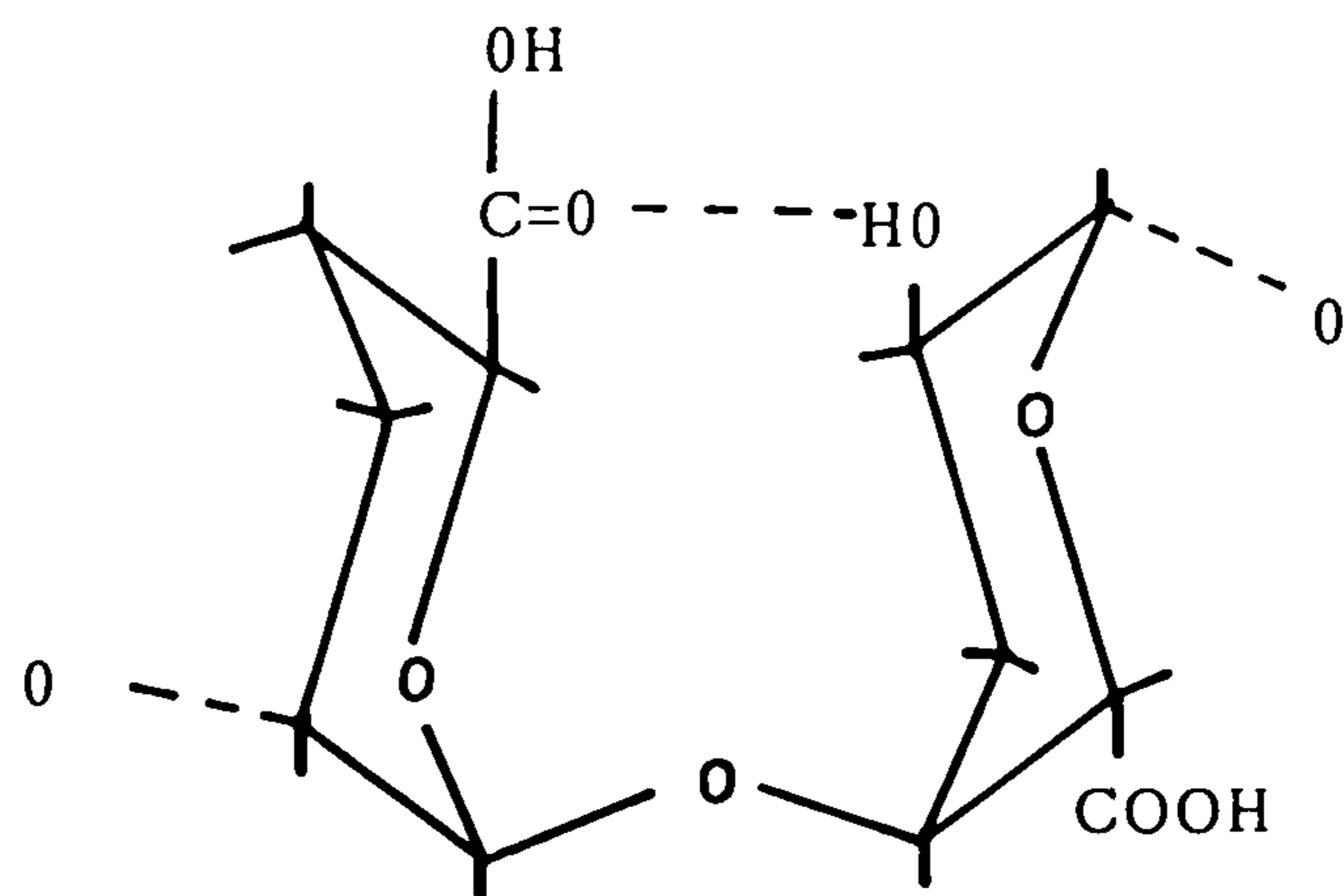


Figure 2.27 Bonding between guluronic acid units.

The differences in conformation lead to a range of different properties of the two alginate types. For example

gelation where the polyguluronate blocks are involved in calcium binding. Hence gel strength increases with the proportion of guluronate blocks. The stiffness of the alginate regions decreases in the following order:

poly G > poly M > alternating units (Whittington, 1971a, b and Bailey, et al., 1977).

In alkali conditions alginates undergo β -elimination (Preiss & Ashwell, 1962) by the following reaction:

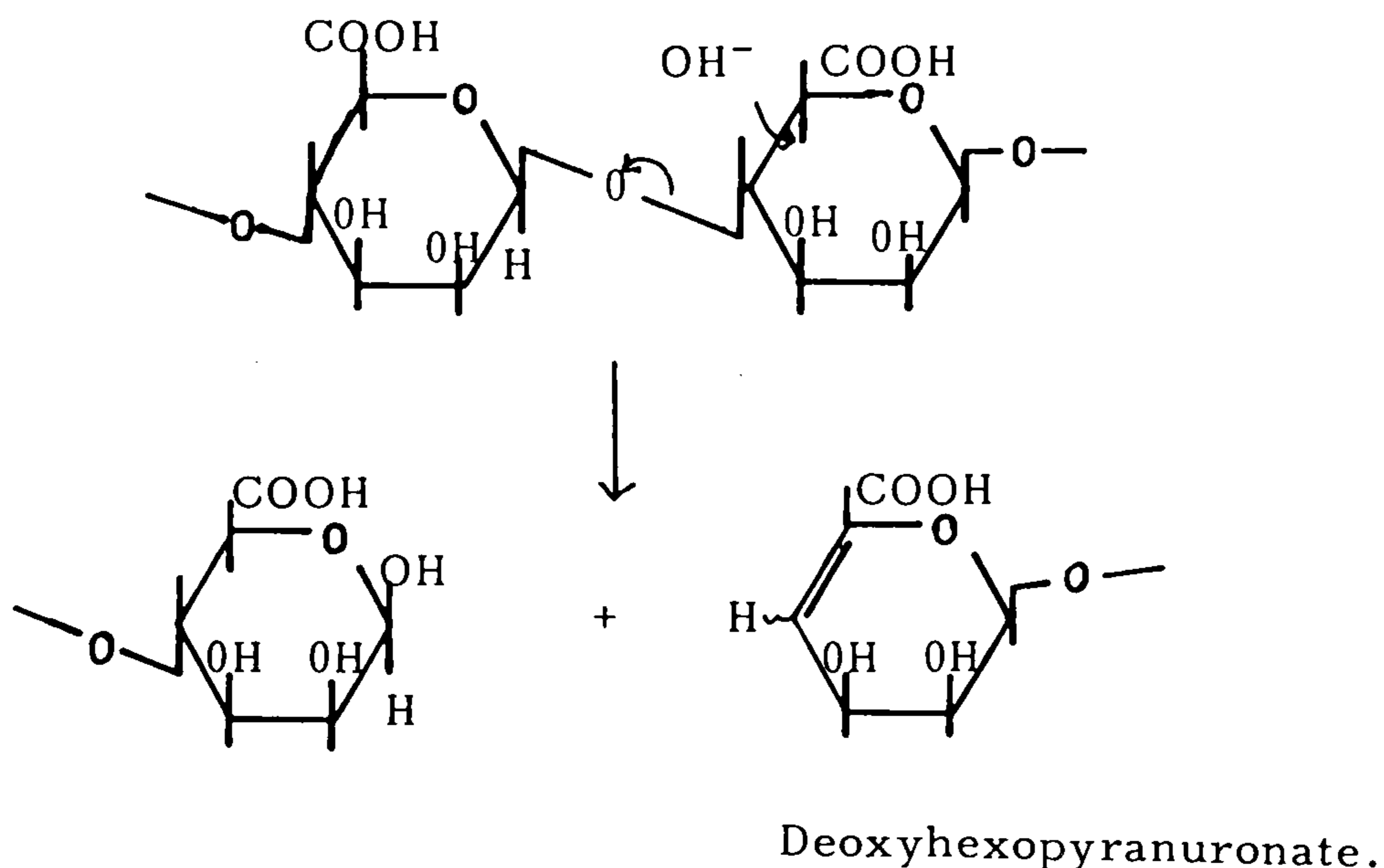


Figure 2.28 β -elimination of alginic acid.

Haug, et al. (1963) has found that β -elimination of alginates is pH dependent the rate increasing with increase in pH above 10. Niemela & Sjostrom (1985) found that a range of mono and di - carboxylic acids could be formed as the result of β -elimination, the proportion of each depending

on the incubation temperature and alkali concentration. At pH values below 5 degradation of the alginate chain takes place via proton mediated degradation.

2.6 Extrusion

2.6.1 The extruder

Many types of extruder exist, operating over a wide range of conditions (Harper, 1981). In this study work was restricted to a high temperature short time (HTST) extruder. These possess high shear and operate at high temperatures with short residence times. An extrusion cooker is simply a pump which can simultaneously transport, mix, stretch, shape and impose shear on a material under increased pressure and temperature.

2.6.1.2 Characteristics of Food Extruders

Referring to Fig 2.29 the extruder can be envisaged as consisting of several discrete sections (Linko, et al., 1981)

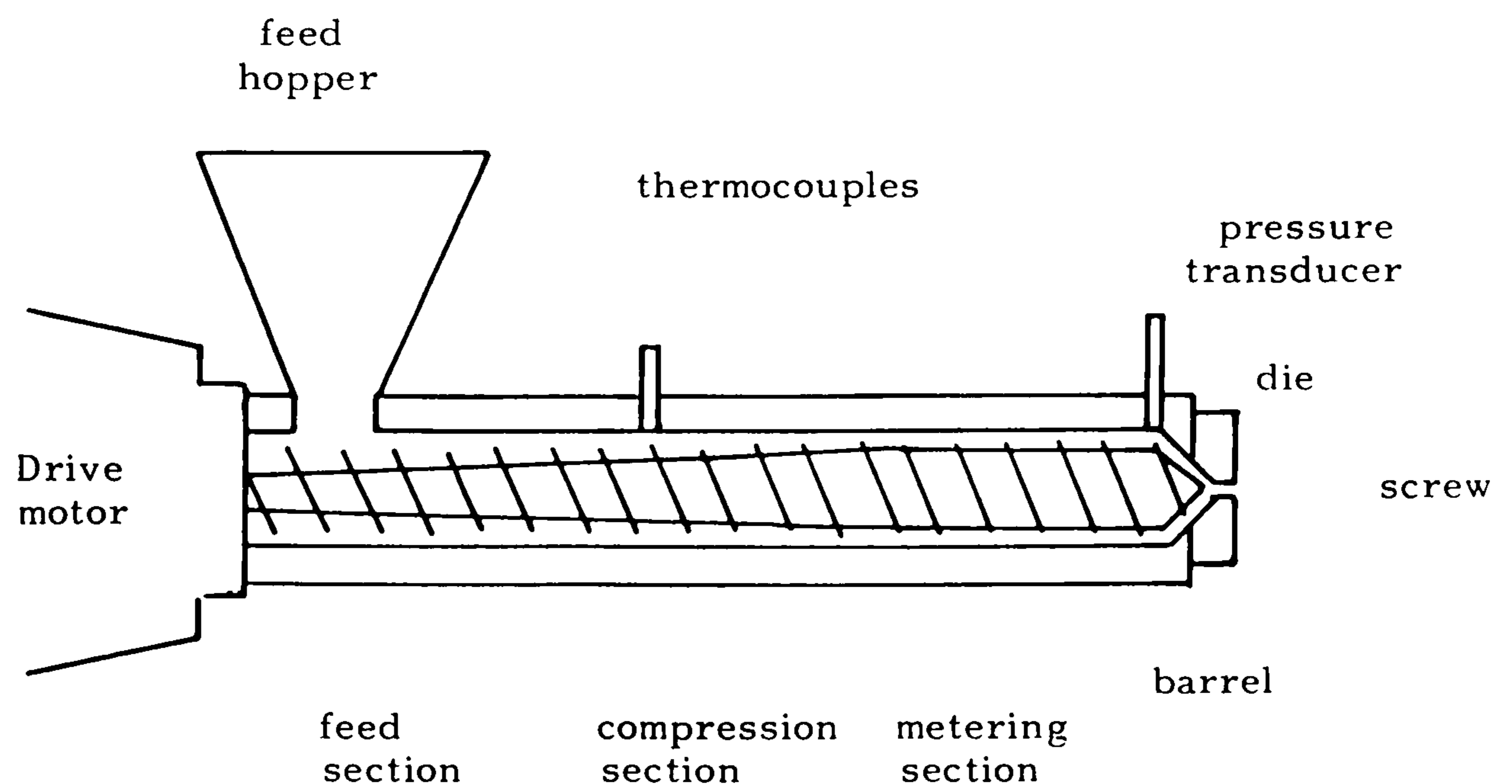


Figure 2.29 Schematic representation of a single screw food extruder.

a) Screw: The design of the screw will influence the operation and product type of the extruder. The screw is normally long in relation to its diameter ($L/D > 10$), this allows greater operating flexibility and finer control of the process variables. The screw can be divided into three sections:

1) Feed section: This section is characterised by deep flights which easily accept feed material, the purpose is to feed material down the screw. During the feed process the food material is continuously worked allowing air and voids to be expelled thus leading to complete filling of the screw flights.

2) Compression or transition section: This section has decreasing flight heights or internal restrictions which lead to compression. The compression increases the rate and mechanical energy input to the food, resulting in temperature rises that convert granular / particulate material to a plasticized dough.

3) Metering section: This section has shallow flights or flights with decreasing pitch. Internal mixing is increased and a maximum dissipation of mechanical energy ensues which increases the temperature. The temperature rise reaches a maximum just prior to the product emerging from the die.

b) The Barrel: Extruder barrels can be heated either electrically or by steam. The inner surface of the barrel is normally grooved to reduce slippage and thus increase pumping capability. This surface is hardened so as to avoid wear which results from the inevitable contact of the screw with the barrel wall (Winter, 1980).

c) The Die: Extrusion dies are small openings which shape the food material as it flows out of the extruder. There are many shapes of dies, the simplest, which is used in this study, is a hole, though it is common for annular openings and slits to be used.

2.6.2 Modelling the Extrusion Process

Many of the basic concepts that form the mathematical models for food extruders are ' borrowed ' from plastic extrusion systems. Plastics are relatively homogenous and so allow simple characterisation both physically and chemically. Unfortunately food systems are considerably more complex both in the heterogenous mix of feed material and complex ' cooking ' reactions that occur and thus such idealised modelling must be viewed with caution.

Modelling theory assumes the following conditions:

- 1) Flow is laminar, steady and fully developed.
- 2) The screw is considered to be stationary, whilst the barrel rotates.
- 3) The fluid is incompressible.

- 4) Gravity and inertia forces are negligible
- 5) There is no slip at the walls
- 6) The material is Newtonian.

Obviously not all of these conditions are acceptable for food extrusion.

2.6.2.1 Flow

The net flow (Q_N) is determined by :

$$Q_N = Q_D - Q_P$$

The drag flow (Q_D) is proportional to screw speed (N) and the pressure flow (Q_P) is proportional to the pressure gradient (P/L). In most food applications $Q_D \gg Q_P$ and hence the volumetric flow rate will increase linearly with N . Thus Harmann & Harper (1974) and Bruin, et al. (1978) have shown that the flow rate increases linearly with screw speed for both soya and corn grit systems.

Visually the components of the net flow rate are shown by the following velocity profiles, Fig 2.30.

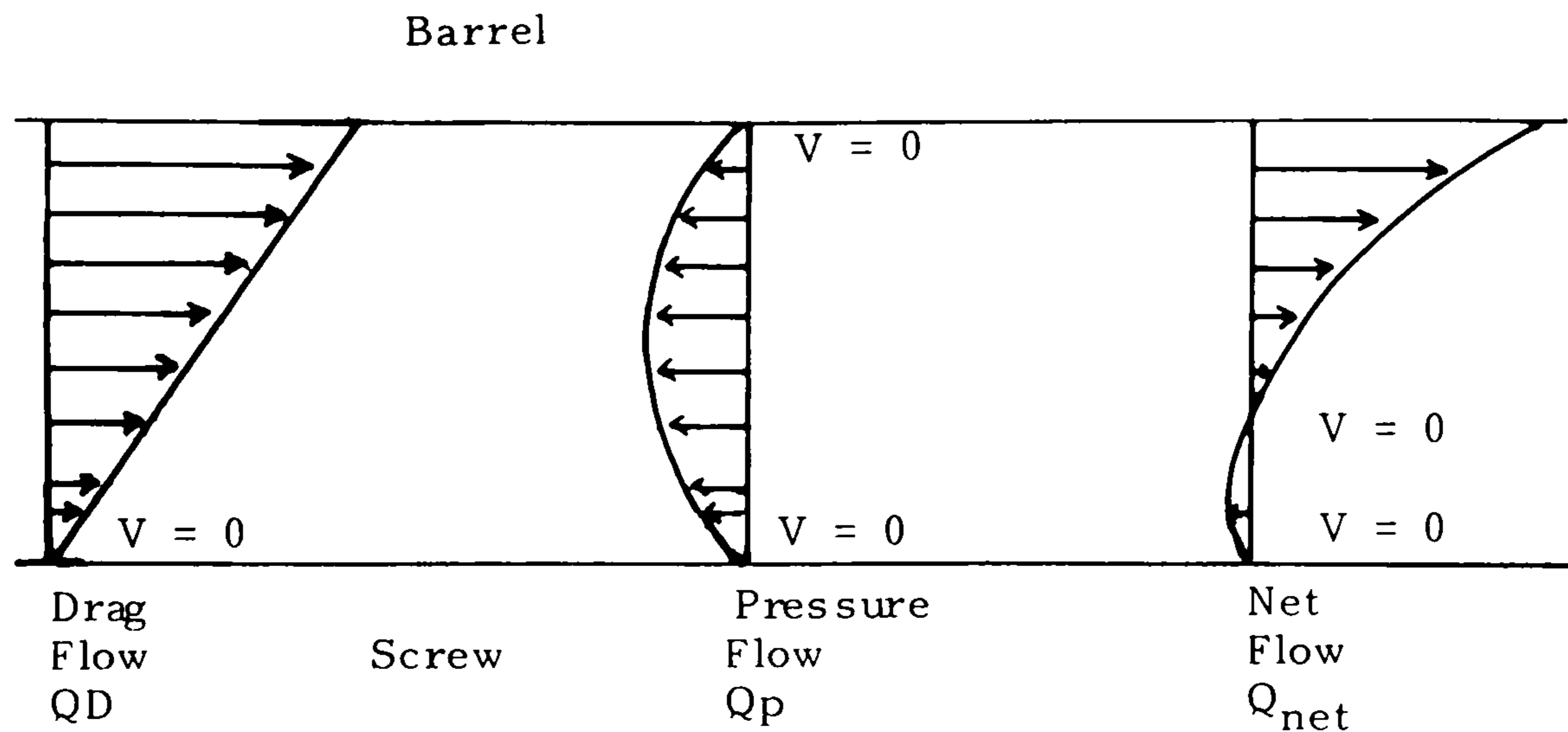


Figure 2.30 Flow patterns in the extruder.

The drag flow varies linearly from zero velocity at the screw root to a maximum at the barrel wall. The back flow shows a typical parabolic flow profile, with maximum flow mid distance between the screw and the barrel. The net flow when a large pressure flow rate is considered, shows a negative region near the screw, and a positive flow region near the barrel surface.

As the flow in the extruder is composed of two components, the second component to be considered is that across the channel ie at right angles to the flow down the barrel. This flow profile (Fig 2.31) is resolved similarly to the flow rate down the extruder.

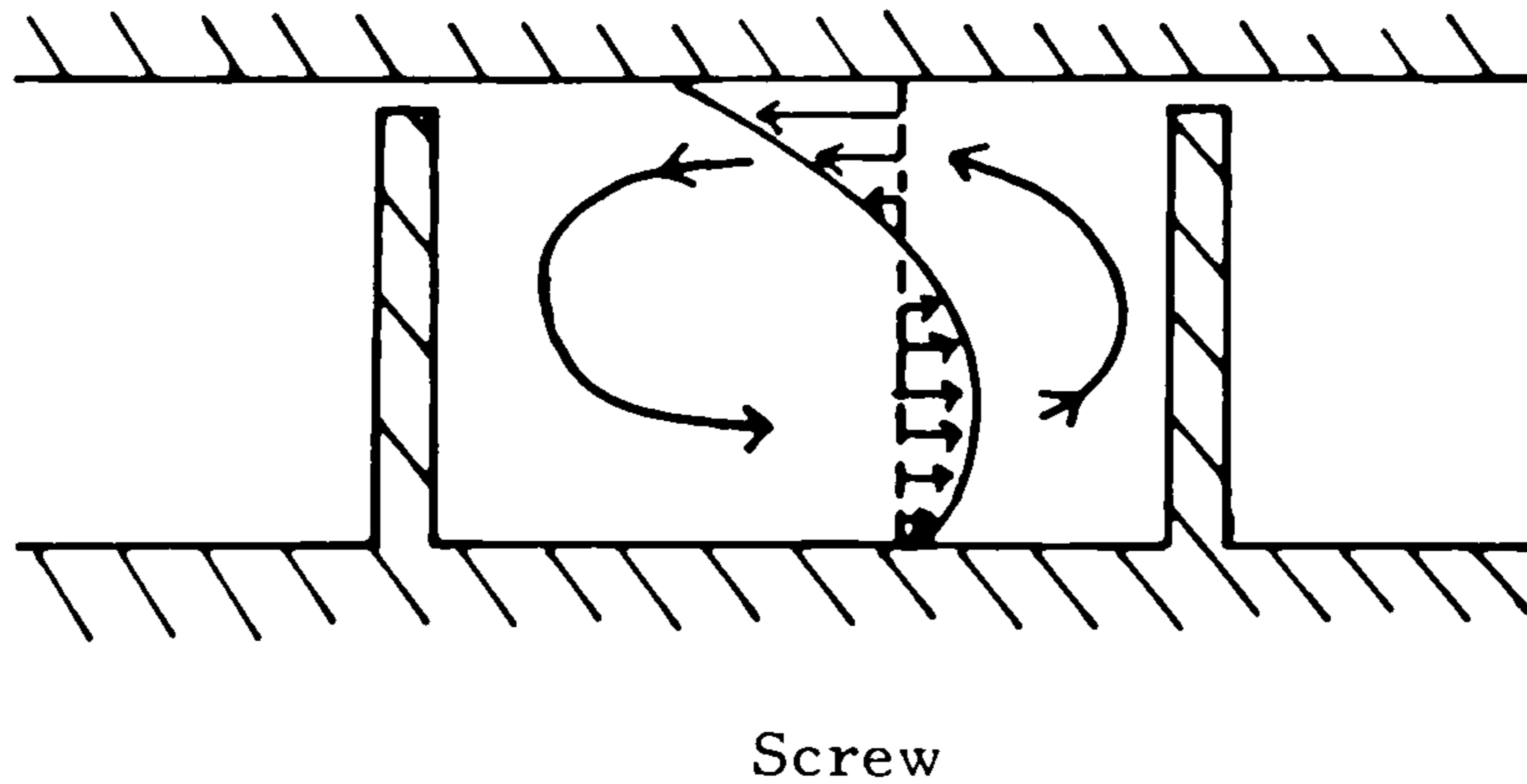


Figure 2.31 Cross channel flow in the barrel.

As the walls of the channel are impervious, the net flow rate across the channel must be zero. This means that the cross channel velocity component creates a circular flow across the channel (Fig 2.31). When the two flows are superimposed the effect is helical (McKelvey, 1962) which allows for efficient mixing.

Due to a gap between the screw flight and barrel wall leakage flow away from the die must inevitably occur. The leakage flow component is often negligible in relation to the other factors, though it does allow for better mixing. The grooves in the barrel create still further leakage flows.

The cross channel component is a function of the location of a particle of dough relative to the root of the screw. The down channel component is a function of the dough rheology and pressure distribution in the channel (Holay & Harper, 1982).

2.6.3 Biochemical Aspects

The restructuring of protein molecules in extrusion can be regarded as the result of an initial unfolding, followed by alignment in the flow and crosslinking between adjacent molecules. As a result of the pressure drop either side of the die, the superheated water 'flashes' off when exposed to atmospheric pressure. This results in a porous expanded structure.

Upon denaturation it is thought that interaction between the exposed protein chains can take place (Rhee, et al., 1981). Hager (1984) and Jeunink & Cheftel (1979) have both suggested that disulphide and non covalent interactions occur extensively during extrusion, whilst Burgess & Stanley (1976) and later Simonsky & Stanley (1982) suggested that inter molecular peptide bonds were responsible for the texture of the soya extrudate. Results obtained by Sheard (1985) do not lend support to the conclusion that isopeptide bonds are present to any great extent in the extrudate. It would thus seem that the major forces are hydrophobic interactions, hydrogen bonds, ionic interactions and covalent intermolecular disulphide bridges, Hager (1984). The intermolecular forces thought to be responsible for texture (Jenkins, 1970 and Cumming, et al., 1973) are similar to those implicated in the formation of spun fibres (Kelly & Pressey, 1965).

Shear forces are thought to be necessary to align the protein molecules thereby allowing the reactive sites on

adjacent molecules to crosslink the chains (Holay & Harper, 1982). Over shear will result in loss of texture as bonds will be broken . Moisture content of the dough will alter its rheology (Clark, 1978) and therefore change the shear environment within the screw channel. Nierla, et al (1980) propose that the feed moisture is the dominant factor in fundamental transformations. Moisture will also increase the mobility of the reactive components thereby increasing the potential for reactive sites to come close together.

Mayer (1984) lists the relative importance of the various bond types, he shows that the predominant bonds in extrudates are covalent disulphide bonds but does stress the existance of possibly more than one type of bond whose presence are dependent on temperature. At low (< 423 K) temperature thermoplastic extrusion forms structural proteins primarily by intermolecular disulphide bridges accompanied by changes in noncovalent bonding. At higher temperatures (> 453 K) extrusion may lead to protein polymerization through formation of inter molecular peptide bonds. (Burgess & Stanley, 1976) . Melius (1975) has reported that thermal polymerisation by peptide bond formation requires a temperature of at least 453 K. Pham & Del Rosario (1984) suggest that possibly the polymerization of amino acids rich in di—amino and di—carboxyl groups plays a role in the texturization of protein by the extrusion process.

Formation of protein crosslinks to form unusual amino

acids, particularly lysinoalanine and lanthionine can occur in heated proteins (Sternberg et al., 1975). Jeunink & Cheftel (1979) suggest that these reactions are not particularly prevalent in soybean extrudates, but that isopeptide crosslinks may form, particularly ϵ -(γ - glutamyl) - lysine or ϵ -(β - aspartyl) - lysine.

Areas (1986) stresses the importance of electrostatic and hydrophobic interactions to the texturization process as he found the presence of phospholipid modified the final product quite markedly.

GENERAL EXPERIMENTAL MATERIALS

AND METHODS

3.0 General Materials and Methods

3.1 Materials

3.1.1 Soya isolate

Soya isolate was prepared from a 70 PDI flour as outlined by Wolf (1970).

Approximately 2 kg of soya flour (McCauley Edwards, Ltd., code 200 / 70) was mixed with 18 litres of distilled water and the pH of the suspension adjusted to 8 with 10 % sodium hydroxide. All insoluble residue was removed by centrifugation at 2000 g for 20 minutes, using a Mistral 64 MSE centrifuge. The supernatant was adjusted to pH 4.5 with 6M HCl and the precipitate removed by centrifugation. The sediment was resuspended in distilled water to give a final dispersion of about 6 % and neutralised with 6M HCl. Samples were freeze dried, ground in a hammer mill to 1 mm and stored at 275 K in sealed containers.

3.1.2 Alginates

Alginates used in this study were supplied by Kelco International Ltd. All were blended samples principally derived from *Ascophyllum nodosum* and *Laminaria hyperborea*, except for an unblended sample obtained from *Durvillea potatorum* and Keltone which is a blended alginate but principally derived from *Macrocystis pyrifera*.

The various proportions of mannuronic and guluronic

acid (M : G ratio) are shown in table 3.1.

ALGINATE	M : G RATIO
Manucol DM	1.90 ^a
Manugel GMB	0.70 ^a
Keltone	1.56 ^a
Durvillea potatorum	1.56 ^a
Sample A	1.70 ^b
Sample D	0.70 ^b
Sample G	1.30 ^b
Sample E	2.00 ^b
Sample F	2.10 ^b

Table 3.1 Proportions of mannuronic acid : guluronic acid found in the alginates used in this study.

^a Private communication, Kelco International Ltd.

^b Smith (1984).

3.2 Moisture Determination

3.2.1 Karl Fischer

3.2.1.1 Theory

Karl Fischer analysis is a chemical means of estimating water which depends upon the oxidation of SO₂ by iodine in the presence of water. This method is usually carried out in the presence of pyridine and methanol. Normally the Karl

Fischer solution contains pyridine, methanol, sulphur dioxide and iodine, in this study a 'new' solution was used, the makers (Fisons, Ltd.) claim the titrant is a pre-adjusted solution of iodine and methanol containing no pyridine. The precise composition was not available from the manufacturers. Free sulphur dioxide is not formed as water is extracted into a solution, buffered at the optimum pH (5.7 ± 0.2) for the formation of monomethylsulphite.

3.2.1.2 Procedure

The BTL design of Karl Fischer apparatus (Baird and Tatlock [London] Ltd.) was used.

The burettes were filled with :

- 1) Aqua - Fi titrant (Fisons Ltd.)
- 2) Standard water in methanol (5 mg H₂O in 1 ml).

Prior to analysis the sample (approximately 0.3 g) was dissolved in 10 ml formamide (AR grade, Fisons, Ltd.) by stirring in a closed vessel for 30 minutes. After this time 10 ml Aqua - Fi solvent (Fisons, Ltd.) was added and the solution stirred for a further 5 minutes.

With the stirrer on, titrant was added and the end point determined using a dead stop end point procedure with a platinum electrode. Karl Fischer titrant was standardised by means of a solution of water in methanol (Fisons, Ltd.). Appropriate solution blanks were analysed and the amount of water was calculated from:

$$\% \text{ water (wsb) } = \frac{\text{Average titre} \times E}{10 \times \text{sample weight}}$$

where E is the water equivalent of Aqua Fi titrant.

3.2.2 Oven Drying

A vacuum oven at 343 K, 100 mm Hg was used for the determination of moisture contents, drying was for 24 hours. All water contents are calculated on a wet weight basis (change in weight / initial weight).

3.3 Differential Scanning Calorimetry

3.3.1 Theory

Differential scanning calorimetry (DSC) is a technique for recording change in physical and chemical properties of materials as a function of temperature. Changes in a sample are associated with a thermal event, either the absorption or evolution of heat. If the heat flow to the sample and an inert material is measured during controlled heating, then any thermal event taking place in the sample will lead to a differential heat flow, this may be recorded as a peak on a plot of heat flow against temperature.

The heat capacity of most substances varies with temperature. A change in heat capacity is represented by a shift in base line, such effects do make subsequent measurements of peak areas and widths difficult.

Protein transitions are usually cooperative in nature

(Biliaderis, 1983) and so heat changes associated with the rupture of the many bonds will be summative and produce a measurable response. The broader the transition, the less co-operative the process. The area under the peak is directly related to the enthalpic change and its direction indicates whether the thermal event is endothermic or exothermic.

The temperature of the transition can be taken from several points. T_{max} , the temperature at maximum heat flow, is the easiest point to determine but has no physical meaning, being dependent on sample geometry among other things. This point is a useful marker for identifying the transition and is the only parameter that can be easily recorded when overlapping transitions take place. A more accurate estimate of the transition temperature is the onset temperature (T_{onset}), this point is difficult to determine, as the point at which base line deviation occurs is not always clear. An extrapolated value is often used (T_m) this is taken as a tangent from the slope of the transition to the baseline (Fig 3.1).

3.3.2 Procedure

A Perkin Elmer DSC II was calibrated using a sample of 99.999% indium with a melting point of 429.60 K and latent heat of fusion of 6.80 cal / g.

Previously hydrated samples were equilibrated for 12 hours, accurately weighed 5 to 10 mg portions were

hermetically sealed into aluminium DSC pans. Pan sealing was achieved using a Perkin Elmer sample pan crimper press. Samples were heated at 5 K / min against an empty sealed pan used as a reference. Samples which were analysed for non freezable water were scanned at 10 K / min and thermal lag was compensated for by scanning a pan containing distilled water at the same rate. Data were collected on a BBC microcomputer (model B) which was interfaced with the DSC.

Pans were reweighed after heating to ensure no " leakage " had occurred. Heated and unheated pans were punctured and placed in a vacuum oven for 48 hours at 343 K. After cooling in a desiccator the moisture content (wsb) was determined.

3.3.3 Interpretation

A typical thermogram is shown in Fig 3.1

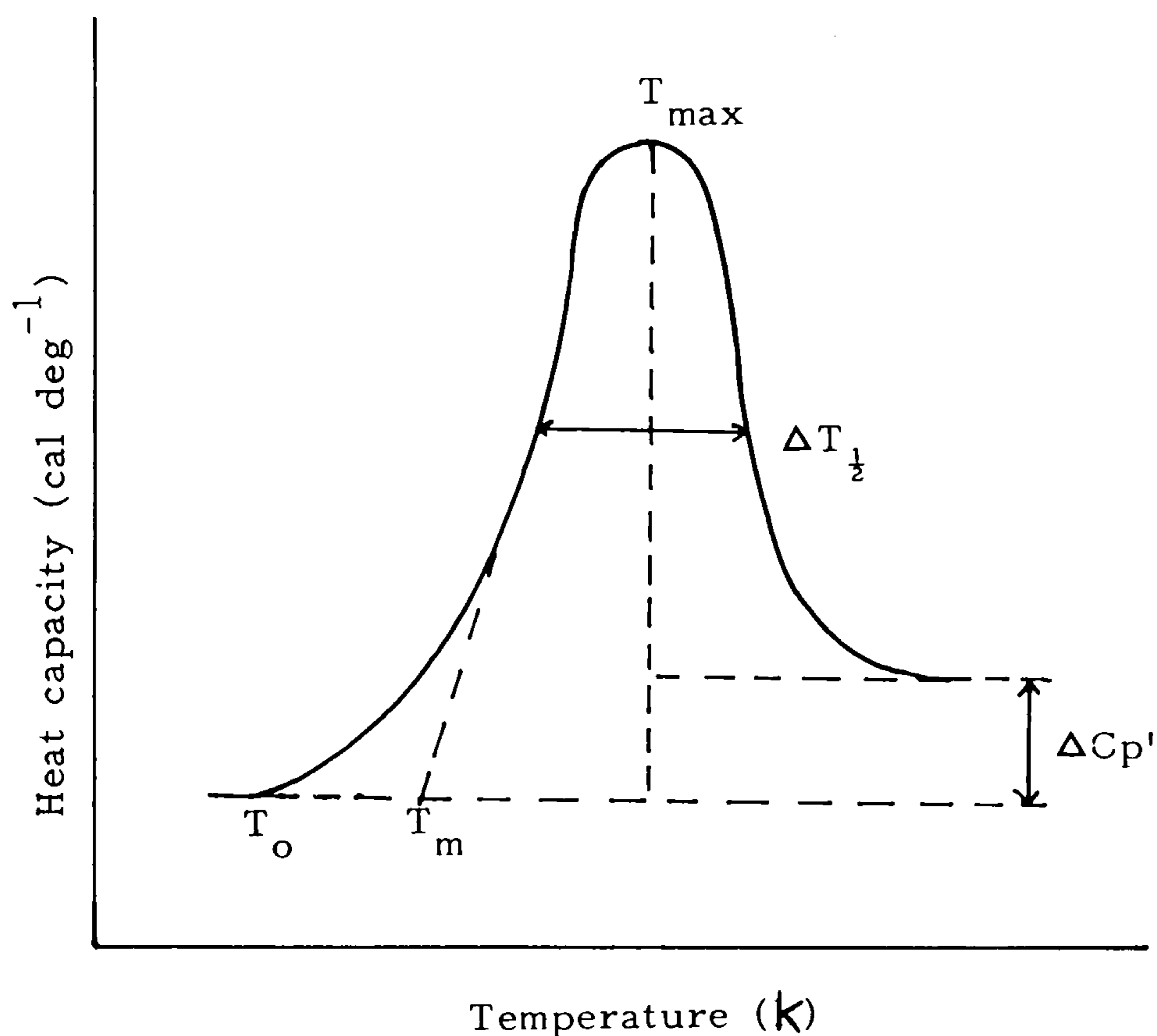


Figure 3.1 A DSC transition with associated parameters.

The transition temperature T_{onset} was taken as the point of deviation from the base line. The area under the curve was determined by the computer though base line construction, particularly for the 11 S fraction was difficult. The enthalpy (ΔH_D) was subsequently determined by measurement of this area and comparison with the value found for indium, this was done by the computer.

The change in heat capacity ΔC_p was determined from

the difference between the base line at the start and end of the transition, both base lines were extrapolated to the point of maximum heat change (Fig 3.1) and a measure of the difference in the heat flow was taken, which was assumed to be proportional to heat capacity. Exact changes in specific heat (heat capacity) are not reported as a sapphire standard was not available for calibration. Thus in the results the value is called $\Delta C_p'$ and not ΔC_p as is usual and was determined from:

$$\Delta C_p' = \frac{\text{difference in baselines}}{\text{heating rate} \times \text{sample weight}} \quad \text{cal K}^{-1} \text{g}^{-1}$$

The cooperativity of the transition was taken as the peak width at half peak height ($T_{1/2}$). Difficulties in the determination of this parameter were again the result of possible base line errors (Fig 3.1).

Van't Hoff enthalpy (ΔH_{VH}) assumes a two state process and is derived as :

$$\Delta H_{VH} = \frac{4RT_m^2}{T}$$

where R is the gas constant.

Comparison of ΔH_{VH} with the calorimetric determined ΔH_D will give an indication of how close the transition approximates to being two state.

3.4 Gas chromatography

3.4.1 Theory

Gas chromatography is a separation technique in which compounds of a mixture are carried by a mobile gas phase, at different rates, through a column of a stationary phase, usually a non-volatile liquid (silicone oils, phthalates, etc.). The solid is coated onto a packing material or the walls of the column.

Species dissolve in the stationary phase at rates governed by their vapour pressure and the temperature of the column. However, if the sample molecule contains a polarising or polarisable group, and the stationary phase is polar, then the sample molecules will be retained longer. The retention time of a molecule is the sum of time spent in the liquid phase plus time spent in the gas phase. With the type of capillary column used in this study the stationary phase is coated on the inner walls of the column. These columns have a small internal surface area, therefore only a limited availability of stationary phase, hence sample passing through the column must be restricted, by some form of inlet splitting. In this work a 'Grob' splitless injector, was used in which all the sample is initially loaded onto the column but residual solvent is removed from the column by splitting.

The type of detector used was a mass selective detector (Hewlett Packard). This type of detector was

chosen as it enabled the mass spectra of the samples to be determined and complex data manipulation was possible with the computer software supplied with the apparatus.

Samples passing through a gas chromatography column must be volatile. Sugars are relatively non-volatile as a consequence of their strongly hydrogen bonded character. A range of sugar derivatives may be made volatile by blocking the hydroxyl groups. Common derivatives are acetates, methyl ethers and trimethylsilyl ethers. Prior to derivatisation it is advantageous to break the asymmetric anomeric centre of the molecule, thereby allowing only the open chain configuration to be formed. The number of peaks recorded is thus reduced (Birch, 1973). One such technique is the formation of the corresponding alditol by the addition of an alcohol group to the anomeric carbon, (C1). Unfortunately alditols are less well resolved than the parent sugar, a problem overcome by the use of capillary columns. Also some aldoses give the same alditol as ketoses as:

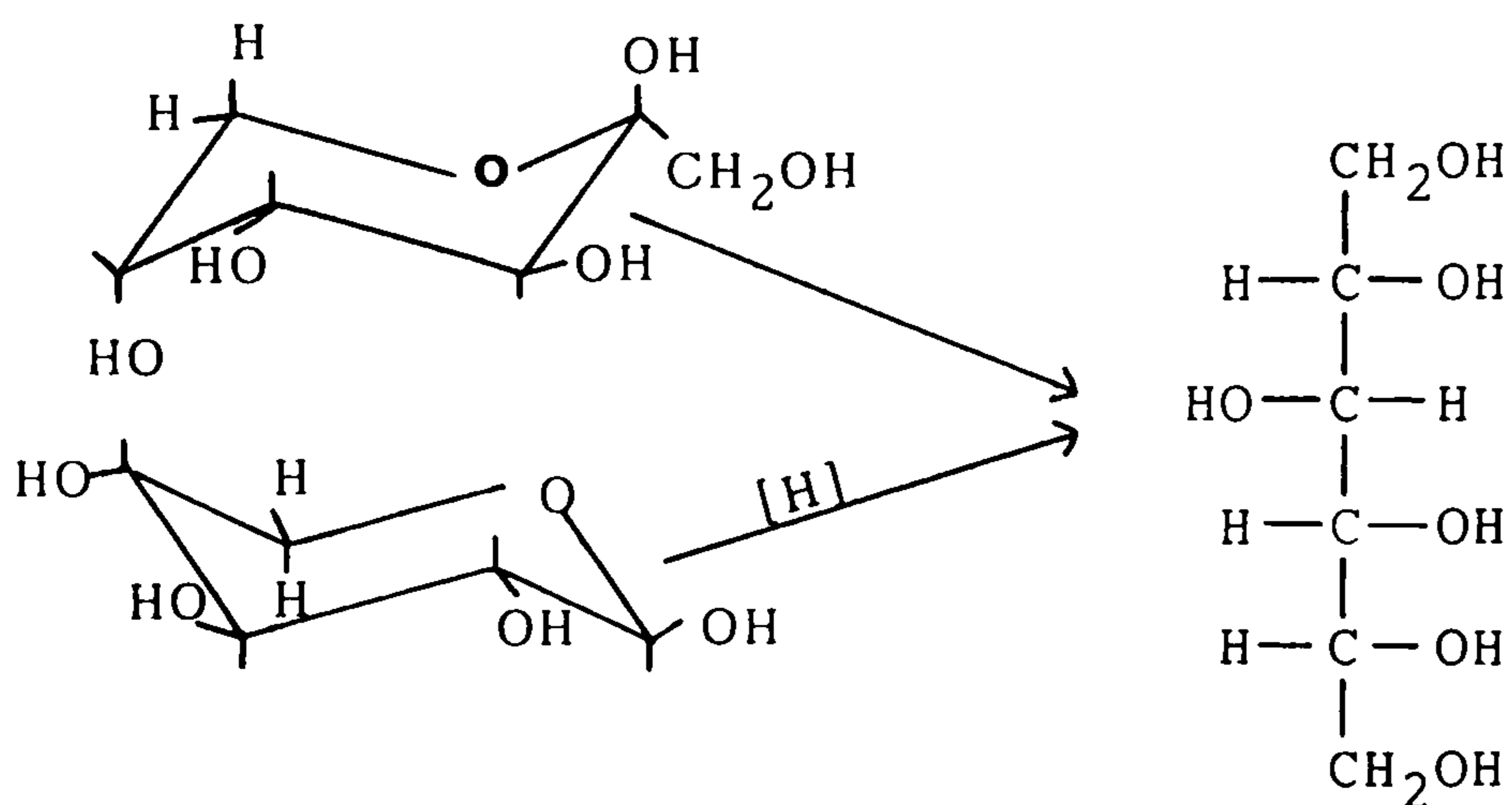


Figure 3.2 Production of alditols (Birch, 1973)

The derivatisation to acetates was chosen as it has

been used for the determination of monosaccharides produced on hydrolysis of polysaccharides (Dutton, 1973, Englyst, et al., 1982). For the production of the alditol acetates experimental details are given in section 5.2. The procedure involves reduction with sodium borohydride and catalytic conversion to acetate by 1 - methylimidazole (Blakeney, et al., 1983). All hydroxyls are equally acetylated producing the penta acetate.

Quantitative analysis was achieved by reference to an internal standard. Peak areas were assumed to be relative to the concentration of the sugar. The internal standard used was 1, 2, 3, 4, 5, 6 - hexahydroxy-cyclohexane, myo - inisotol. This compound gave a characteristic mass spectrum and had a retention time of about 36.1 minutes.

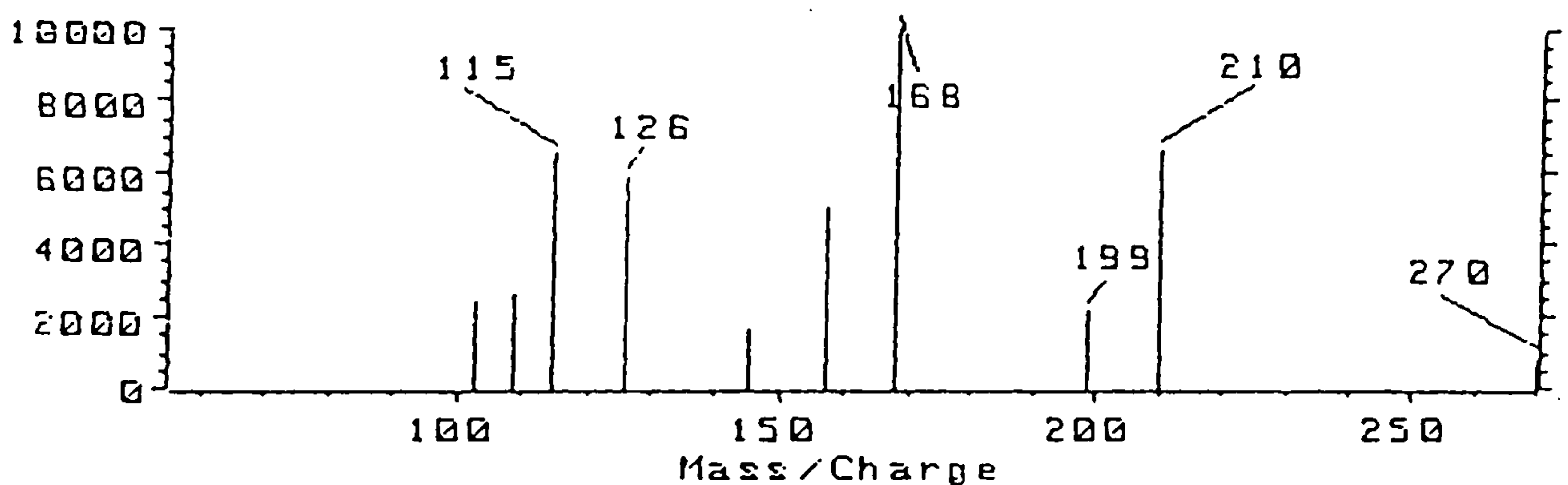


Figure 3.3 Mass spectra of myo inisotol

3.4.2 Procedure

2.5 μ l of sample was injected into a Perkin Elmer Open Tubular (capillary) Column Injector (0332 - 4013) connected to a glass capillary column, in a Perkin Elmer

Sigma chromatograph. The split valve was closed during injection but after 30 seconds the valve was opened to clear residual solvent from the injector. On opening the split valve the oven temperature programme was started. Sample was carried through the column by nitrogen (Air products, Ltd.) at 18 psi (equivalent to a flow rate through the column of about 1 ml / sec). Sampling by the mass spectral detector (Hewlett Packard 5970 series) was started after 4 minutes. Fragments were sampled with a molecular weight of between 50 and 550 at a rate of 1 scan per second. Data analysis was performed using the Hewlett Packard Chem station (9,000 series).

3.4.3 Interpretation

Sugar identity can be made by reference to the retention time of the sugar relative to the known time of a standard. All retention times quoted in this study are retention times relative to myo - inisitol. Unfortunately the mass spectra of sugars cannot differentiate between stereoisomers and so the mass spectra are limited to identifying the fact that the compound is indeed a sugar.

Alditol acetates exhibit the simplest fragmentation pattern of all the common derivatives. Acetates are characterised by an intense peak at $m/e = 43$ which corresponds to the acetyl ion ($\text{CH}_3\text{—C—O}^+$). Fragmentation patterns seen are :

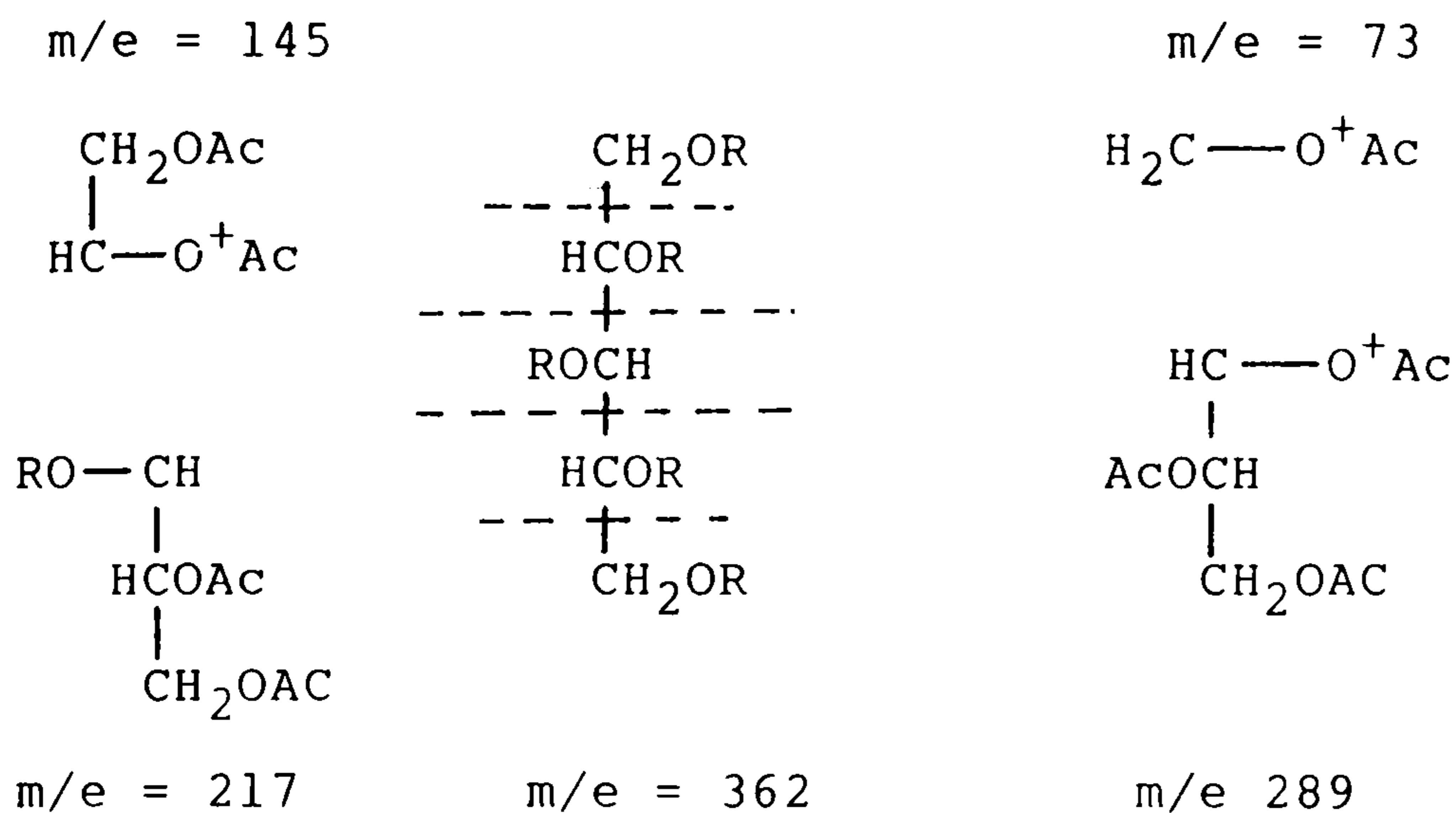
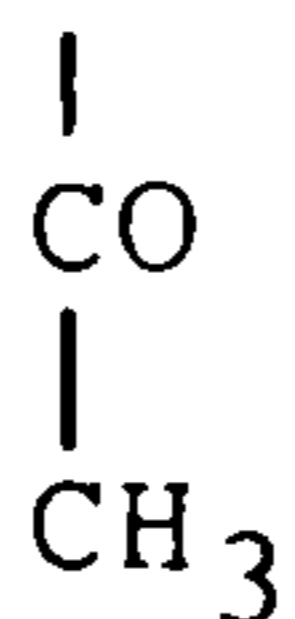
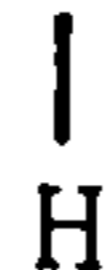


Figure 3.4 Alditol Acetate derivative Fragmentation Pattern.

Polyacetates exhibit intense peaks at m/e = 43 (-AC), 59 (-OAc), 73 (-CH₂OAc), 42 (CH₂-C=O), 145 (triacetoxonium, CH₃-CO-O⁺-CO-CH₃)



and 103 (diacetoxonium, CH₃-CO-O⁺-CO-CH₃)



molecular ions are rare for alditol acetates.

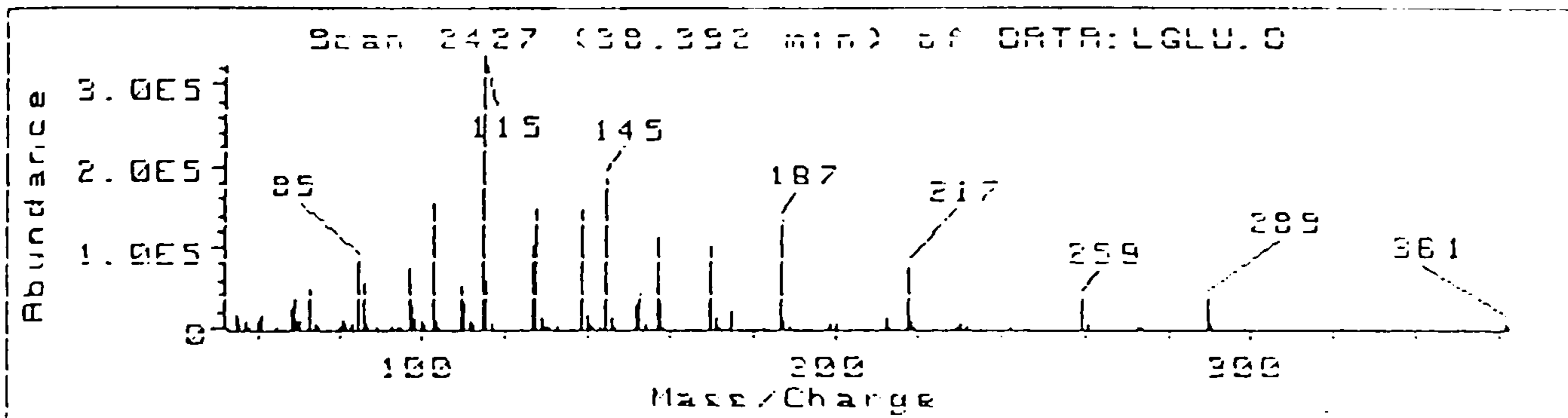


Figure 3.5 Mass Spectra For Glucose.

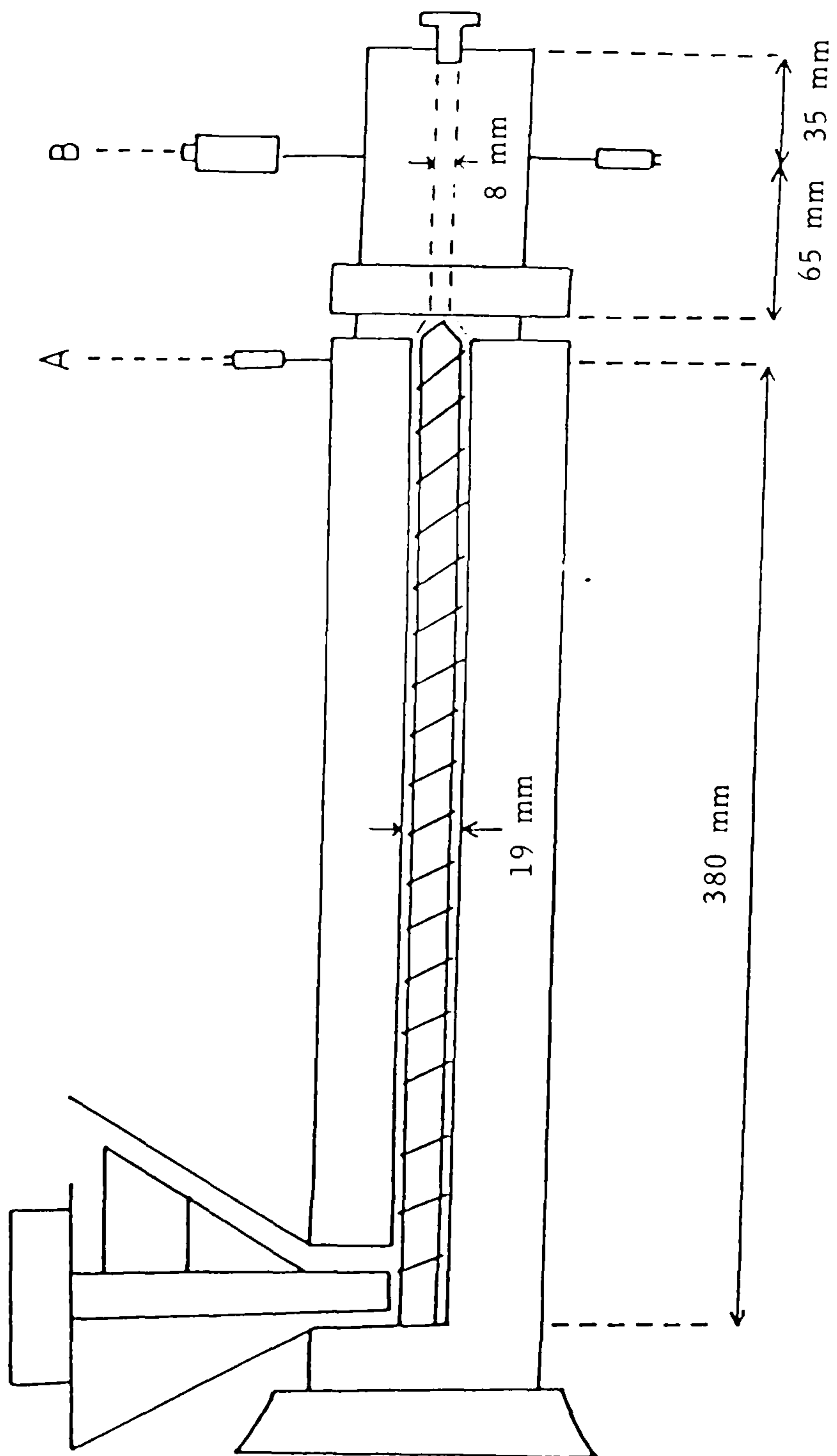
3.5 Extrusion

3.5.1 The Extruder

A Brabender laboratory extruder, (Fig 3.6) model DN (internal diameter 19.1 mm), driven by a Brabender DO - Corder drive (model E) with a speed range 0 to 250 rpm was used in this study. The screw speed used throughout was 250 rpm (Smith, 1984).

Figure 3.6.

Schematic diagram of the Brabender extruder.



A feed hopper was attached to the extruder, the base of the hopper was water cooled. To aid feeding of material an auger was used in the feed hopper, the speed of which was set at 125 rpm.

Single flight screws of various compression ratios were used.

The extruder could be heated in three independent sections, the feed section, second section of the barrel and the die section. To maintain a steady temperature the die and barrel were also air cooled.

3.5.2 Extrusion Measurements

3.5.2.1 Temperature, pressure and torque

Torque was measured continuously using a DCE - 330 corder connected to a sensor in the control cabinet.

Pressure measurements were made using a Dynisco pressure transducer PT - 480 - 20M, which had a maximum measurable pressure of 20,000 psi. Temperature was measured with iron - constantan thermocouples. These sensors were mounted in such a manner that their surfaces were flush with the surface of the barrel and die. The placements are shown in Fig 3.6. A BBC microcomputer (Model B) was interfaced with the extruder (Berrington, 1985) which was used to measure the signals from the pressure transducer and the temperature probes. Prior to extrusion the pressure transducer and torque signals were calibrated.

3.5.2.2 Flow Rate

When steady conditions had been attained samples were collected over one minute and placed in polythene bags. These samples were weighed and their moisture determined by drying in vacuo at 343 K for 24 hours. Flow rate was calculated from:

$$\text{flow rate (s)} = \frac{(100 + \text{MC})}{100} * \text{DW g min}^{-1}$$

where: MC = original moisture content

DW = Dry weight of samples

assuming the density of the grits to be 1.4 g ml^{-1}

$$\text{the volumetric flow rate} = \frac{\text{s}}{1.4 * 60} \text{ ml sec}^{-1}$$

3.5.2.3 Expansion Ratio

At least five measurements of product diameter were made on each sample with a micrometer. The expansion ratio was then given as the ratio of the diameter of the extrudate to the diameter of the die.

3.5.3 Feed Moisture

Moisture content of the feed material was determined by vacuum oven drying at 343 K for 24 hours. A kilogram of material (soya grit or soya + alginate) was placed in the bowl of a Kenwood chef food mixer and sufficient distilled water was added to give the required feed moisture content (dsb). The feed was mixed to give an even distribution of

water and samples were sealed in polythene bags and equilibrated at 275 to 276 K for 16 hours. Prior to extrusion feed material was allowed to equilibrate to room temperature.

The amount of water added was determined as:

$$x = (y/100 * [100 - z]) - z$$

where: y = required moisture content on a dry solids basis

z = original moisture content (wsb)

3.5.4 Extruder Operation

Prior to assembly the extruder barrel was cleaned. Once assembled the heaters were set to the required temperature and cooling air and water applied. The system was equilibrated for 1 hour. The feed hopper and DO corder drive were switched on and the screw speed adjusted to 250 rpm before adding the feed. When steady state conditions were attained, usually after about 15 minutes, samples and data were collected.

Before stopping the extruder, the feed hopper was left to run dry, then switched off. The extruder drive motor was stopped when the extrudate ceased to emerge from the die. Finally the extruder was stripped and allowed to cool prior to cleaning.

DENATURATION STUDIES

4.1 Denaturation Studies of Soya Proteins and the Role of Moisture

4.1.1 Introduction

Relatively little information is available about the physical and chemical changes that occur when proteins are heated at the low moisture contents found in extrusion cooking, although it is established that the denaturation characteristics of proteins are dependent on moisture content. The temperature of denaturation invariably increases with decreasing water content whereas the enthalpy of the transition (ΔH_D) generally decreases (Finch & Ledward; 1972, Hagerdal and Martens; 1976; Ruegg et al., 1975; Fujita and Noda, 1981 and Sheard et al., 1986). Tropocollagen however is unusual as it exhibits an increase in ΔH_D as the water content decreases from 60 to 28% wet sample basis (w.s.b.), although a further reduction in water content causes a rapid decrease (Luesher et al., 1974). The 7S and 11S globulins of soya exhibit the expected increase in stability with decreasing water content (Sheard et al., 1986) but the dependence of ΔH_D on moisture content is not known.

The dependence of some thermodynamic parameters for soya denaturation on the moisture content was investigated using DSC .

4.1.2 Materials and Methods

Soya isolate was prepared as outlined in section 3.1 from a 70 PDI flour.

A range of moisture contents between 5 and 85 % were used. Hydration was achieved using a variety of methods:

a) For samples of moisture contents between about 30 and 85% water, soya isolate was mixed with appropriate amounts of distilled water and allowed to equilibrate at room temperature for 24 hours.

b) For samples of moisture contents between about 5 and 30 % soya was equilibrated over silica gel or solutions of known water activity (Rockland, 1950) at 298 K to constant weight.

4.1.2.2 Differential Scanning Calorimetry

Aliquots of approximately 5 mg of soya isolate dispersions, doughs or powders were subjected to calorimetric analysis using a Perkin Elmer DSC 2. The heating rate was 5 K / min. The moisture contents of the samples were determined following denaturation by puncturing the lids of the sample pans and drying in vacuo at 343 K for 48 hours. All sample pans were checked for weight loss during DSC analysis by weighing before and after the determination of the thermogram.

Enthalpies and specific heat function $\Delta C_p'$ were

calculated for soya isolate on the assumption that the 7S globulin comprises a sixth of the protein content, which was determined as 92 %, and that this protein component was in the native form.

4.1.3 Results

Two endothermic peaks were seen on the thermograms of the soya isolate (Fig 4.1) corresponding to denaturation of the 7S and 11S globulins.

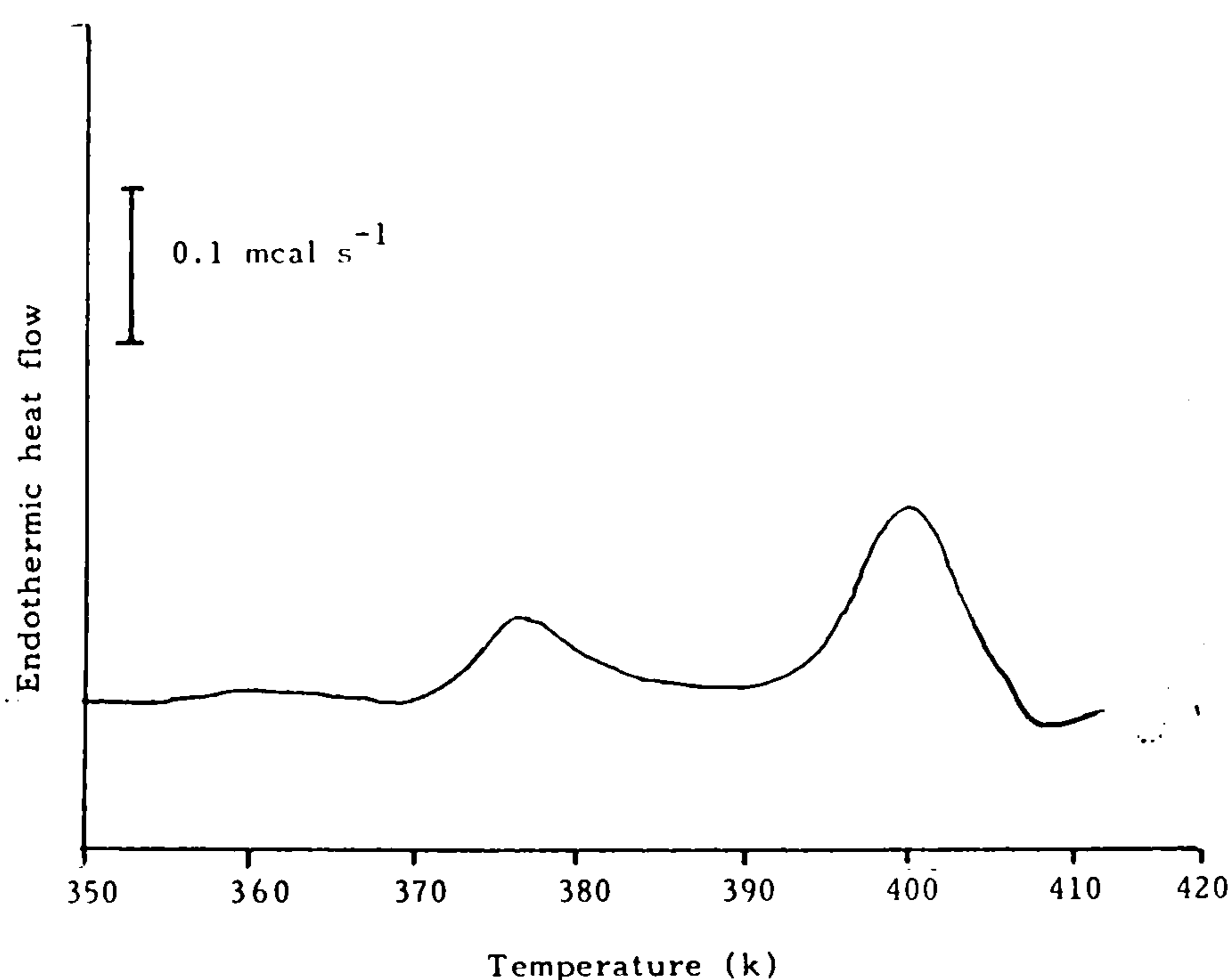


Figure 4.1 Thermogram of 14.3 mg of soya isolate, containing 40% water.

The endothermic peaks of both the 7S and 11S globulins were characterized by a change in the specific heat function following denaturation (Hermansson, 1978). With most samples a large exotherm was seen following denaturation of

the 11S globulin, often occurring at the same time as the change in specific heat function. The presence of this exotherm coupled with the change in specific heat function (shift in base line) made accurate determination of many of the parameters difficult, especially with regard to those associated with the 11S globulin. For this reason most measurements were restricted to the 7S globulin.

With decreasing moisture content there was a corresponding increase in the onset temperature of the transition (T_0), the rate of increase was far greater at moisture contents below 40 % water. Both globulins exhibited a similar dependence of T_0 on moisture content (Fig 4.2).

The enthalpy of the transition for the 7S globulin was dependent on moisture content (Fig 4.3). The profile may be split into three distinct regions:

- 1) Upto about 30 % water the enthalpy associated with the transition increases with increasing water content.

- 2) Between 30 and 55 % water there is a rapid decrease in the measured enthalpy.

- 3) At moisture contents greater than 55 % there is no dependence on moisture content of the measured enthalpy.

The maximum enthalpy (5.4 cal/g for the 7S) occurred at a moisture content of about 30 %, the minimum value (1.44 cal/g for 7S) was at 55 % water.

As we would expect the transition from the native to the denatured state was less co-operative at low moisture contents (Fig 4.4). Fig 4.4 shows the dependence of $T_{1/2}$ on

water content, the wide scatter of points reflects the difficulty of determining an accurate baseline from which accurate measurement could be made. Measurement of the $T_{1/2}$ values for the 11S globulin were not determined for reasons stated at the beginning of this section.

Moisture contents up to 30 % are associated with a steady decrease in $T_{1/2}$ values. At moisture contents from 30 to 50 % the $T_{1/2}$ values are relatively constant but drop suddenly at about 50 % water. At moisture contents above about 50 % water the $T_{1/2}$ values decrease steadily.

Calculations of " ΔH_{VH} " were attempted using the $T_{1/2}$ values but due to the imprecise estimate for the molecular weight of the 7S globulin the result was thought to be of very little value.

The change in specific heat function ($\Delta C_p'$) following denaturation was also dependent upon the moisture content (Fig 4.5). Again base line determination was difficult and so a corresponding scatter of points is shown in the profiles. There is a steady increase in $\Delta C_p'$ upto a value of $0.44 \text{ cal K}^{-1} \text{ g}^{-1}$ for the 7S globulin at a moisture content of 60 %. At higher moisture contents the $\Delta C_p'$ values decrease.

Figure 4.2.

Dependence of the onset temperature (T_0) for Soya 7S and 11S globulins on moisture content.

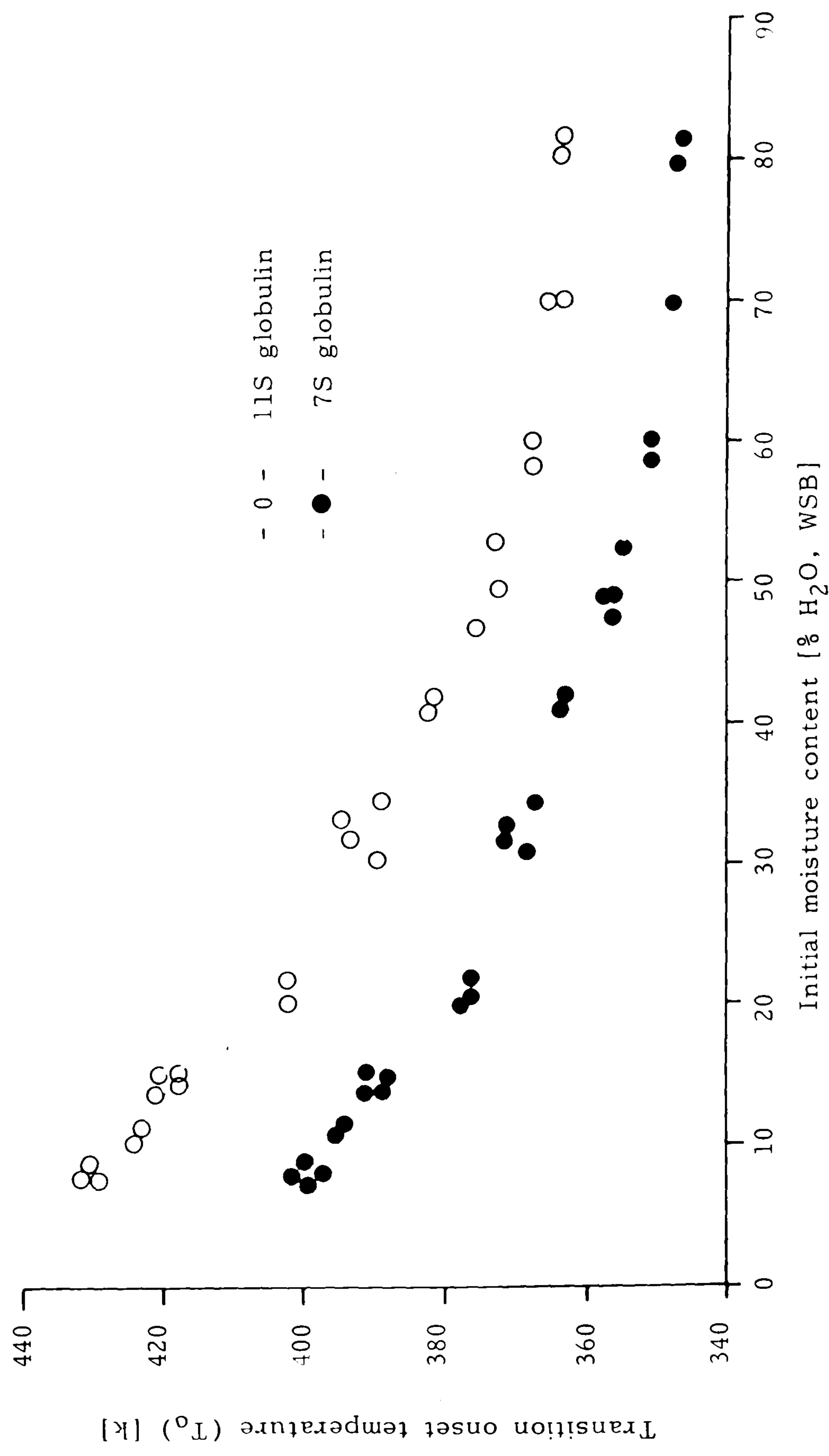


Figure 4.3.

Dependence of enthalpy for the Soya 7S globulin on moisture content.

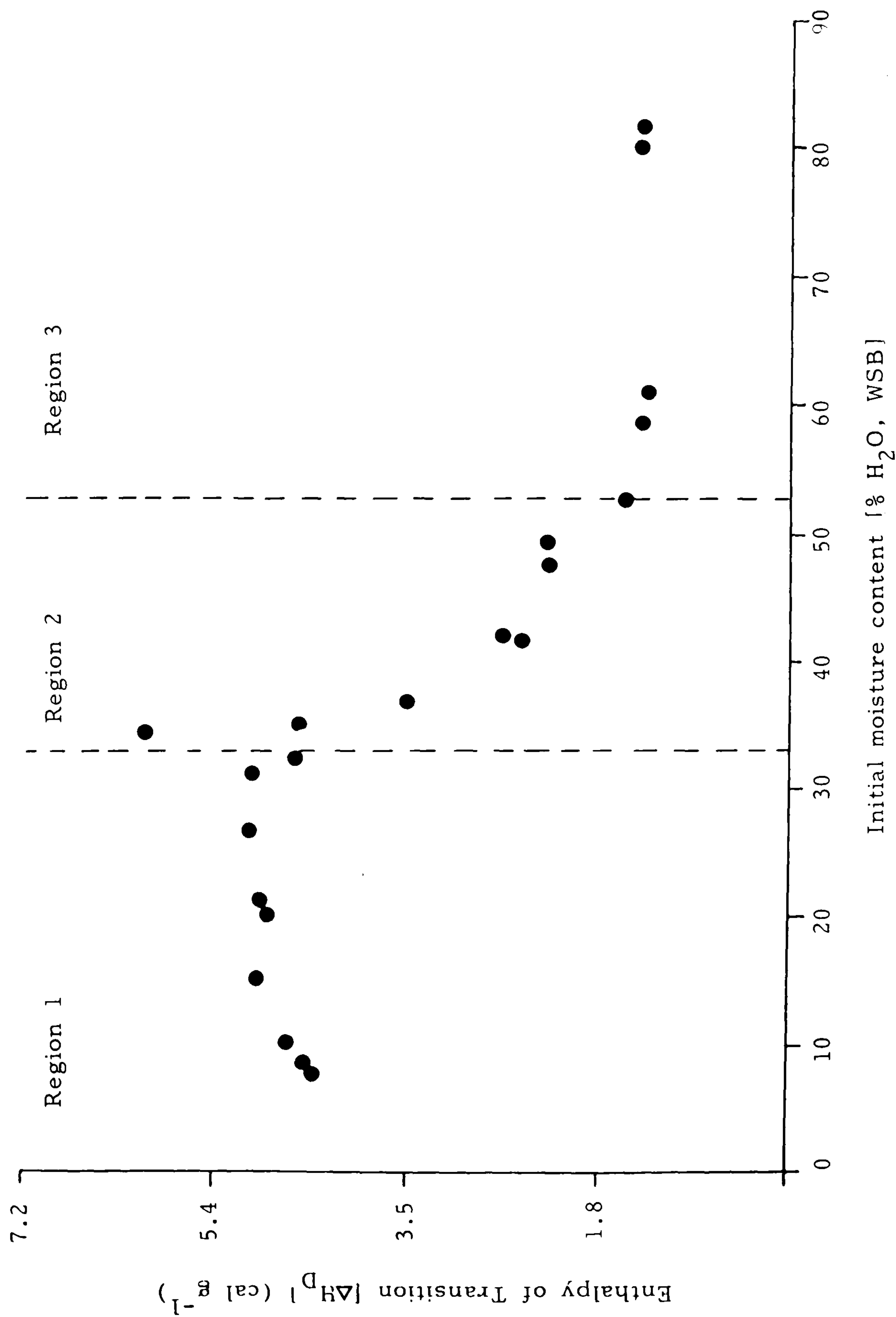


Figure 4.4.

Dependance of $T_{\frac{1}{2}}$ values for Soya 7S globulin on moisture content.

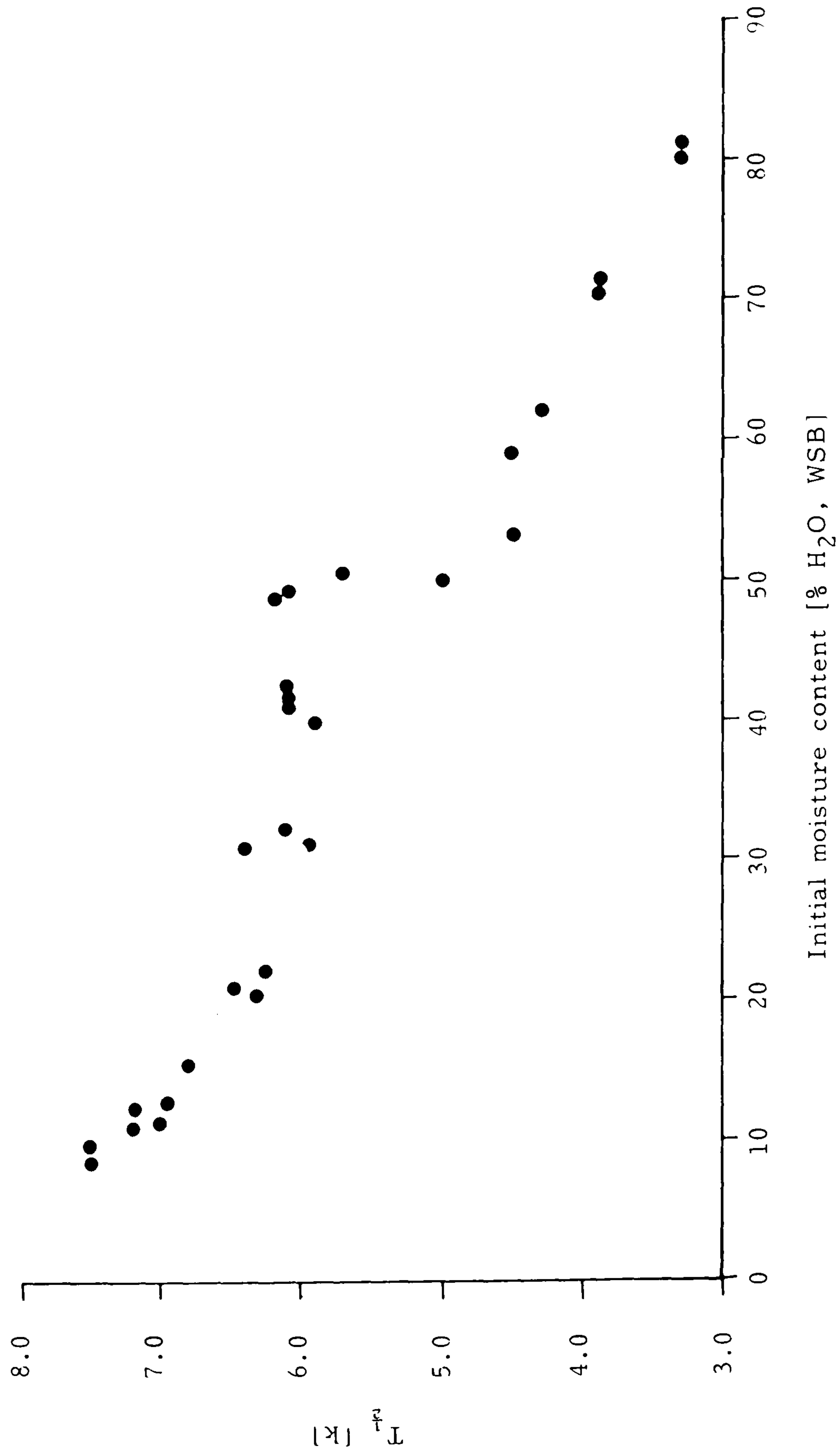
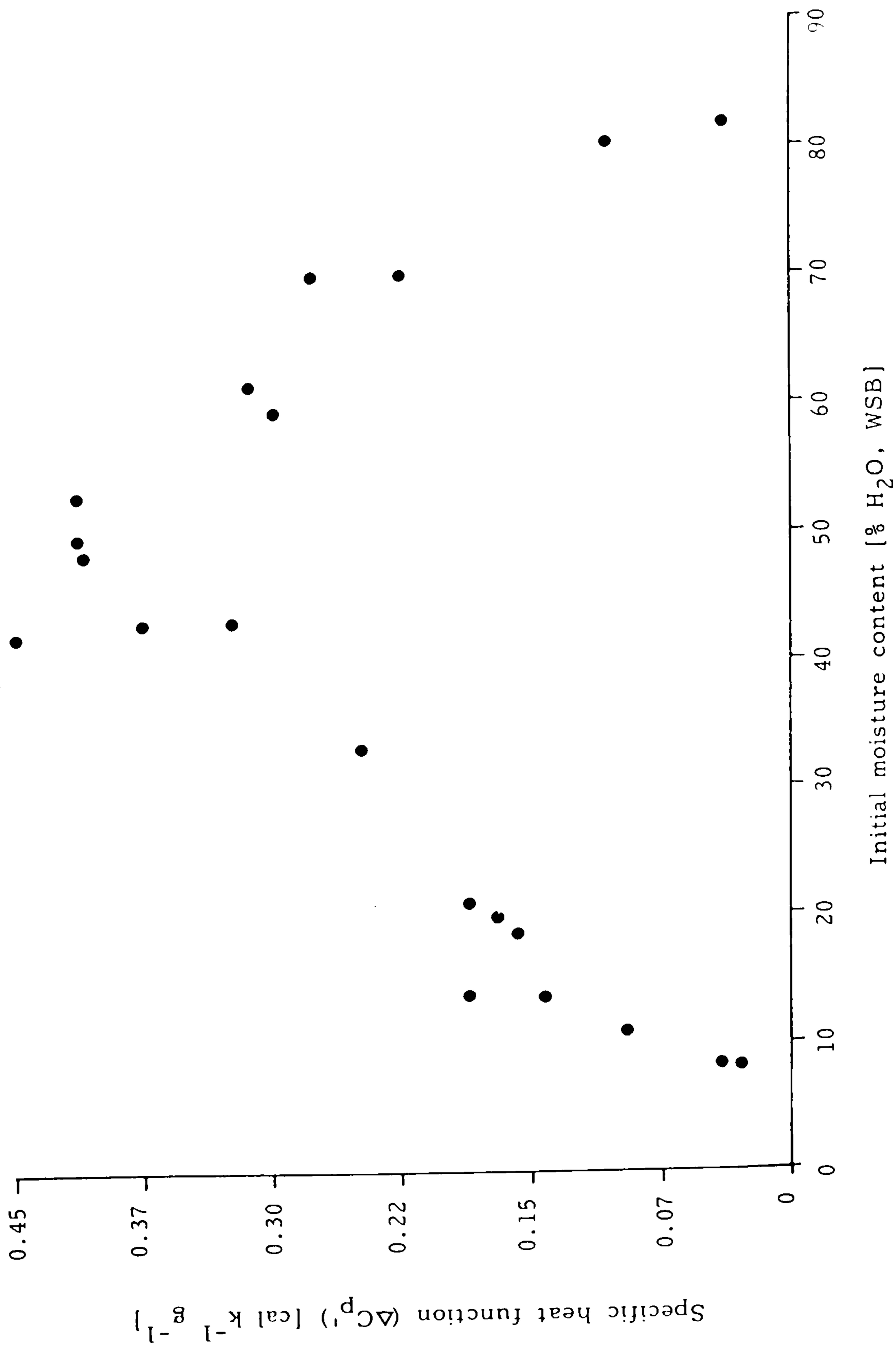


Figure 4.5.

Effect of moisture on the specific heat function after denaturation (ΔC_p)
for Soya 7S globulin.



4.1.4 Discussion

The enthalpy of the transition as a function of water content has been determined for several proteins. For chymotrypsinogen-A (Fujita and Noda, 1981), β -lactoglobulin (Ruegg et al., 1975) and myoglobin (Hagerdal and Martens, 1976) the enthalpy of the transition increases with increasing water content up to approximately 50 % . At higher water contents there is little or no change in the measured enthalpy. However Luesher et al. (1974) found a very similar profile for the enthalpy of the denaturation of tropocollagen, as a function of water content, to that exhibited by the soya 7S globulin. The differences in the enthalpy profiles may well represent different degrees of complexity between proteins as soya consists of several subunits and tropocollagen is a rigid rod not a simple globular protein.

The transition temperatures of soya proteins have been reported at a range of moisture contents (Hermansson, 1978; Wright and Boultier, 1980; Arntfield and Murray, 1981 and Sheard et al., 1986) and the values are in good agreement with those reported here. These authors unfortunately did not measure the enthalpy of the transition.

The energy changes occurring during the denaturation process have been discussed elsewhere (section 2.3.1).

For the 7S globulin the dependence of the enthalpy of

the transition on moisture content can be divided into three areas (Fig 4.3). The decrease in enthalpy associated with a decrease in water content in region 1 can be attributed to incomplete unfolding of the protein molecule as the structure becomes stabilized by inter and intra molecular bonds. These bonds may well form in the voids created by the removal of water from the molecule (Bull and Breese, 1968). Evidence for such an effect has been shown by Careri et al.(1979). The increased thermostability and limited unfolding possible will lead to increased transition temperature and decreased enthalpy. At low water contents, hydrogen bonds that are disrupted will not be able to reform with water molecules and consequently will reform with suitable vacant sites on another protein molecule (Fujita and Noda, 1981). This increase in protein hydrogen bond formation could well be expected to result in thermostability, coupled with the protein aggregation there will be an increase in the exothermic component of the measured endotherm (Donovan and Ross, 1973).

The decrease in enthalpy will be closely associated with decreased values of $\Delta C_p'$ due to decreased side chain mobility as water is removed from the system.

At low moisture contents there is less cooperativity of the denaturation process (greater $T_{1/2}$). Privalov (1982) envisages a protein as consisting of two or more cooperative regions, these regions are interspersed by a few amino acids in such a way that they are non cooperative (Wyman, 1981).

The non cooperative regions are thought to be relatively unstable. It is thus conceivable that when water is withdrawn from a protein a proportion of these regions become stabilised and decrease the number of protein fragments that will take place in the transition and additionally decrease the degree of cooperativity.

The drop in enthalpy with increasing water content in region 2 (30 - 53 %) is most probably associated with increased hydrophobic interactions (which rupture exothermally), as more water becomes available for interaction and the protein is more extensively unwound. Less protein - protein aggregation will be taking place as increased numbers of water binding sites become available, but this increasing effect upon the enthalpy will not be as great as the component due to increased hydrophobic interactions.

At water contents of 53 - 55 % the enthalpy is minimal (Fig 4.3) $\Delta C_p'$ is maximal (Fig 4.5) and the process becomes highly cooperative (small $T_{1/2}$). As will be explained later (section 4.3), it is thought that at this moisture content tertiary water becomes the dominant hydration factor. The large increase in cooperativity is thought to result from an increased number of hydrophobic interactions (Privalov, 1982). The high $\Delta C_p'$ value is thought to be due to interaction of non - polar groups which previously had been buried (Kauzman, 1959).

At moisture contents greater than about 55 % ΔH_D and T_0 are independent of water content, which is indicative of

complete chain unfolding with all associated interactions being complete. The change in the cooperativity at this water content is thought to signify a change in the denaturation mechanism. The unexpected effect (Fig 4.5) on the specific heat function at moisture contents above 55% could well be the result of environmental artifacts such as increases in water vapour pressure due to pressure increases.

It is not expected that any of the other observed changes are artifacts resulting from increases in pressure in the DSC pans. Gill and Glogovsky (1965) have shown that 680 atm. of pressure will lower the temperature of denaturation of ribonuclease by only about 2 K. Thus the comparably low pressures generated will be expected to have only a negligible effect upon the transition temperature. But the pressure changes occurring within the pans will mean that the specific heats discussed are not true ΔC_p values. Even if no pressure change took place the $\Delta C_p'$ function is only a relative value since no calibration was performed using a known standard (Finch & Ledward, 1972).

Interaction between subunit particles have been shown not to be taking place under these conditions (German, et al., 1982) and so it is unlikely that stabilising / unstabilising effects between globulin particles contribute to the observed effects.

4.2 Denaturation Studies of Soya Protein in the Presence of Hydrocolloid and the Role of Moisture

4.2.1 Introduction

The addition of small quantities of alginate is known to affect the behaviour of the soya melt in the extruder (Berrington et al ., 1984). The behaviour of the melt is believed to be dependent upon the degree of denaturation of the soya protein and the water content and type (Sheard, 1985). It was thus decided that the addition of small amounts of alginate to soya should be investigated with respect to changes in the denaturation processes. In this section DSC is used to monitor the temperature, enthalpy, specific heat change and degree of cooperativity of denaturation of the 7 S globulin in soya isolate after the addition of alginate. As with the soya alone these parameters were determined at different water contents.

4.2.2 Materials and methods

Soya isolate was prepared from a 70 PDI flour (section 3.1).

Alginate samples as described in section 3.1.2 were donated by Kelco International Ltd.

Xanthan gum (lot G1253), gum arabic (lot G9752), guar gum (lot G4129) and a partially methylated pectin (lot P9135) were obtained from Sigma Chemical Co. and a sample of carboxy methyl cellulose, medium viscosity (CMC) was

obtained from BDH Ltd.

4.2.2.2 Differential Scanning Calorimetry

Samples of soya isolate containing varying amounts of hydrocolloid (1 to 7 %) were hydrated to a moisture content of either:

a) 50%, by the addition of water, followed by 12 hours equilibration.

b) 10 % by equilibrating over a saturated salt solution of a_w 0.57 (NaBr).

Soya isolate containing Manuacol DM (2%) was hydrated to moisture contents from 5 to 60 % (section 4.1).

Approximately 5 mg of the hydrated sample was sealed into pans (section 3.3.2), and scanned at a rate of 5 K/min. Moisture contents of the samples were determined following denaturation (section 3.3.2).

4.2.3 Results

4.2.3.1 Effect of Alginate Concentration

The temperature of transition (T_0), enthalpy of transition (ΔH_D), $T_{1/2}$, and change in specific heat capacity ($\Delta C_p'$) following denaturation for 7S globulin of soya isolate hydrated to a moisture content of 50% water and containing various concentrations of Manuacol DM (2 to 7 %) are shown in Table 4.1.

MANUCOL DM %	T _{ONSET} K	ΔH _D cal g ⁻¹	ΔC _p ' cal K ⁻¹ g ⁻¹	T _{1/2} K
1	356.5 ± 0.05	2.40 ± 0.18	0.14 ± 0.01	4.4 ± 0.01
2	360.4 ± 0.04	1.98 ± 0.02	-0.25 ± 0.08	5.7 ± 0.11
5	360.0 ± 0.04	1.62 ± 0.37	-0.20 ± 0.01	5.7 ± 0.38
7	360.1 ± 0.05	2.02 ± 0.06	-0.16 ± 0.06	5.5 ± 0.01
Soya alone	355.9 ± 1.20	2.22 ± 0.03	0.41 ± 0.08	4.8 ± 0.32

Table 4.1 Effect of Manucol DM concentration on the denaturation characteristics of the 7S globulin of soya isolate, initial moisture content 50 %. Values are means ± standard error of 12 determinations.

The transition temperature is little affected by concentrations of Manucol DM below 2% but at higher concentrations it is elevated by about 4 K, for the 7S globulin (Table 4.1).

Values for ΔH_D, T_{1/2} and ΔC_p' changed following the addition of 2 % Manucol DM but no differences were evident when 1 % was added to the mix (Table 4.1). Further addition of the alginate led to no further changes. The values of ΔC_p' and ΔH_D are reduced compared to soya alone whilst the T_{1/2} values are increased. Interestingly ΔC_p' values are negative following the inclusion of 2% alginate.

4.2.3.2 Effect of Hydrocolloid Type

Results for all the parameters measured are shown in Table 4.2. for samples of soya isolate mixed with 2% hydrocolloid. All samples were hydrated to 50 % water.

HYDROCOLLOID	T_{ONSET} K	ΔH_D cal g ⁻¹	$\Delta C'_P$ cal K ⁻¹ g ⁻¹	$T_{1/2}$ K
Soya alone	355.9±1.20	2.22±0.03	0.41±0.02	4.8±0.11
Manuacol DM	360.4±0.04	1.98±0.02	-0.22±0.01	5.7±0.08
Manugel GMB	356.2±0.10	2.02±0.06	0.04±0.01	4.5±0.24
Pectin	356.7±0.10	1.96±0.07	0.34±0.07	4.6±0.30
Xanthan gum	356.2±0.40	1.98±0.10	0.30±0.07	4.5±0.23
Guar gum	355.3±0.40	2.02±0.02	0.35±0.07	4.4±0.25
Gum arabic	356.1±0.20	1.98±0.05	0.46±0.01	4.1±0.07
Keltone	357.6±0.02	1.84±0.08	-0.17±0.04	4.7±0.25

Table 4.2 Effect of the addition of 2% of various hydrocolloids on the denaturation characteristics of the 7S globulin of soya isolate hydrated to 50 ± 1 % water. Values are means ± standard error of 5 determinations.

Samples hydrated to 10 % water showed similar trends in denaturation characteristics to those hydrated to 50 ± 1 %, (Table 4.3).

HYDROCOLLOID	T_{ONSET} K	ΔH_D cal g ⁻¹	$\Delta C_p'$ cal K ⁻¹ g ⁻¹	$T_{1/2}$ K
Soya alone	396.2 \pm 1.20	4.62 \pm 0.42	0.16 \pm 0.01	7.3 \pm 0.02
Manucol DM	404.1 \pm 1.05	3.72 \pm 0.12	-0.17 \pm 0.04	8.5 \pm 0.25
Xanthan	394.4 \pm 0.80	3.12 \pm 0.72	0.21 \pm 0.01	6.8 \pm 0.09
Guar gum	394.2 \pm 0.66	3.18 \pm 0.06	0.19 \pm 0.01	7.4 \pm 0.11
Gum arabic	394.9 \pm 0.33	2.60 \pm 0.12	0.17 \pm 0.02	6.7 \pm 0.07
CMC	393.5 \pm 0.10	2.76 \pm 0.12	0.15 \pm 0.02	5.9 \pm 0.04

Table 4.3 Effect of the addition of 2% of various hydrocolloids on the denaturation characteristics of the 7S globulin of soya isolate hydrated to 10 ± 0.5 % water. Values are means \pm standard error of 5 determinations.

The transition temperature is affected only by Manucol DM and Keltone for samples hydrated to 10 ± 0.5 % and 50 ± 1.0 % moisture. With the exception of the alginates there was no significant change in $\Delta C_p'$ and $T_{1/2}$ following addition of various hydrocolloids. The alginates all made $\Delta C_p'$ very small or negative. All alginates investigated produced this effect but the increase in the $T_{1/2}$ values was specific to Manucol DM.

The changes described above were observed at both 10 &

50 % moisture but the effects on ΔH_D differed at the two moisture contents. At 50 % moisture ΔH_D was little effected by hydrocolloid addition (Table 4.2) all the hydrocolloids decreased ΔH_D by a similar amount at the lower moisture content (10%) except Manuacol DM which led to only a very small decrease. These effects were thus studied in more detail.

4.2.3.3 The Role of Moisture Content on the Denaturation of Soya Isolate With the Inclusion of 2% Manuacol DM.

The effect of water content on the temperature and enthalpy of the transition of the soya 7S and 11S globulins following the inclusion of 2% Manuacol DM are shown in Figs 4.6 and 4.7. The profiles for the temperature of the transition are similar to those found for soya alone (Fig 4.2). At any given moisture content determined after heat treatment, T_0 is elevated by about 2 to 4 K.

The enthalpy of the transition at moisture contents above approximately 40 % water are similar to those for soya alone but at water contents below this value, whilst the shape of the profile is similar there is a decrease in value of ΔH_D of about 0.9 cal g^{-1} for the soya 7S (Fig 4.7).

Figure 4.6.

Dependence of the onset temperature (T_0) for Soya 7S and 11S globulins on moisture content in the presence of 2% Manucol DM.

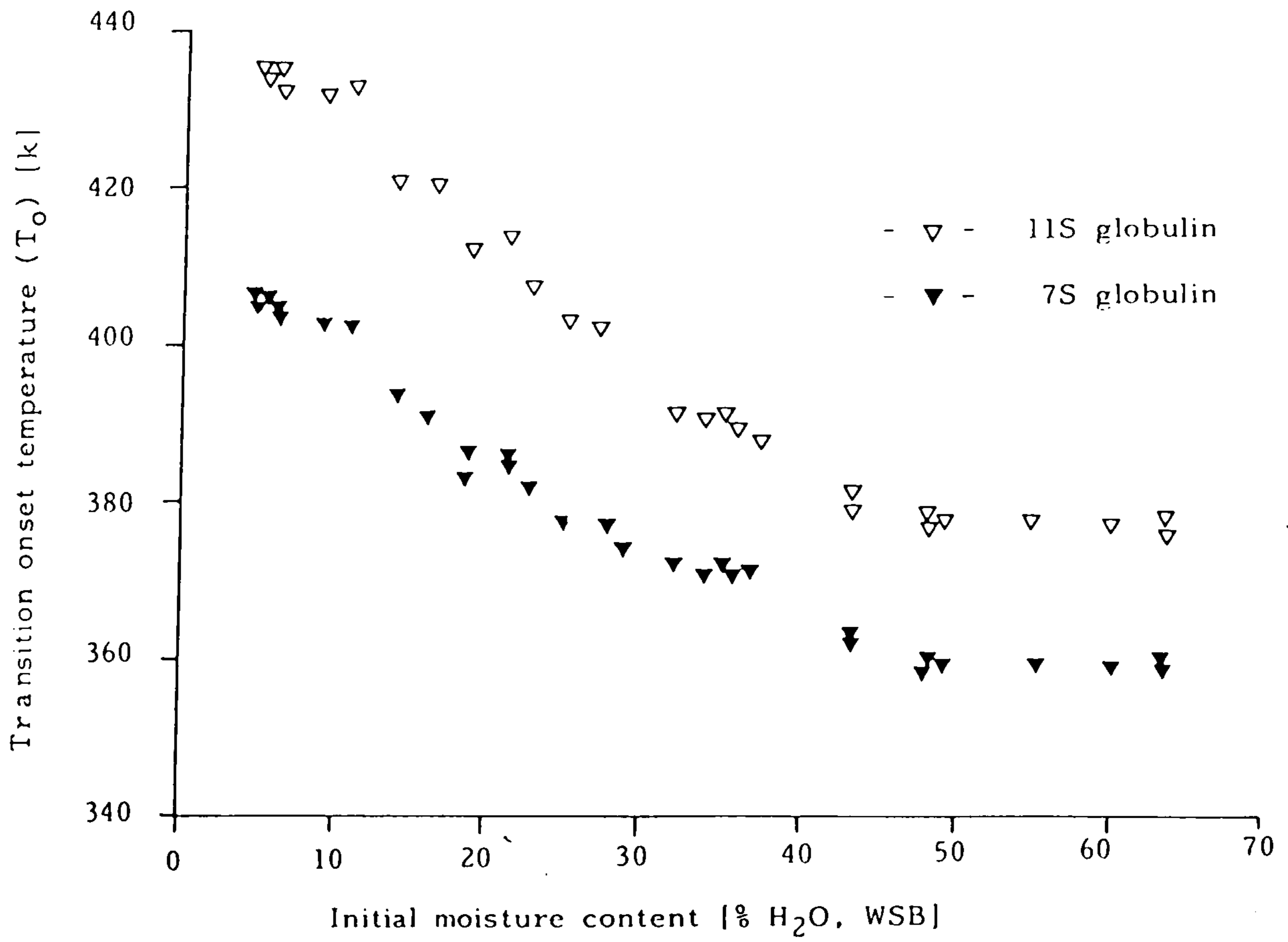
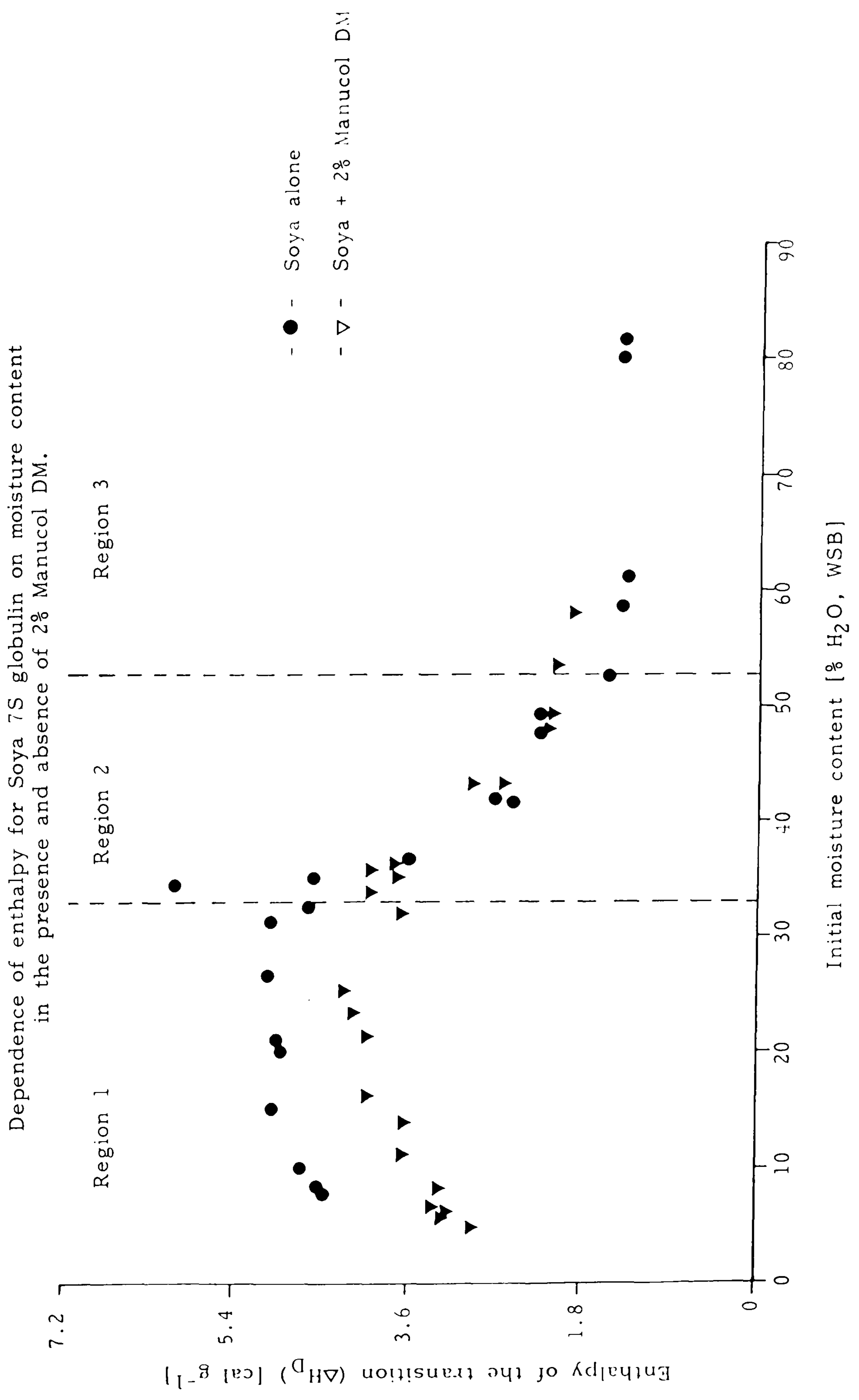


Figure 4.7.



Values of $T_{1/2}$ for the 7S globulin as a function of water content are shown in Fig 4.8. These values show a similar dependence on water content to those found for soya alone, at moisture contents below 50 %. At moisture contents above this value $T_{1/2}$ values for samples containing Manuacol DM do not exhibit the large decrease shown by samples of soya alone (Fig 4.8).

The most dramatic difference between the two systems are the values for $\Delta C_p'$. The two profiles are similar but the samples containing Manuacol DM are virtually all negative (Fig 4.9).

Figure 4.8.

Dependence of T_1 values for Soya 7S globulin on moisture content in the presence of 2% Manucol DM.

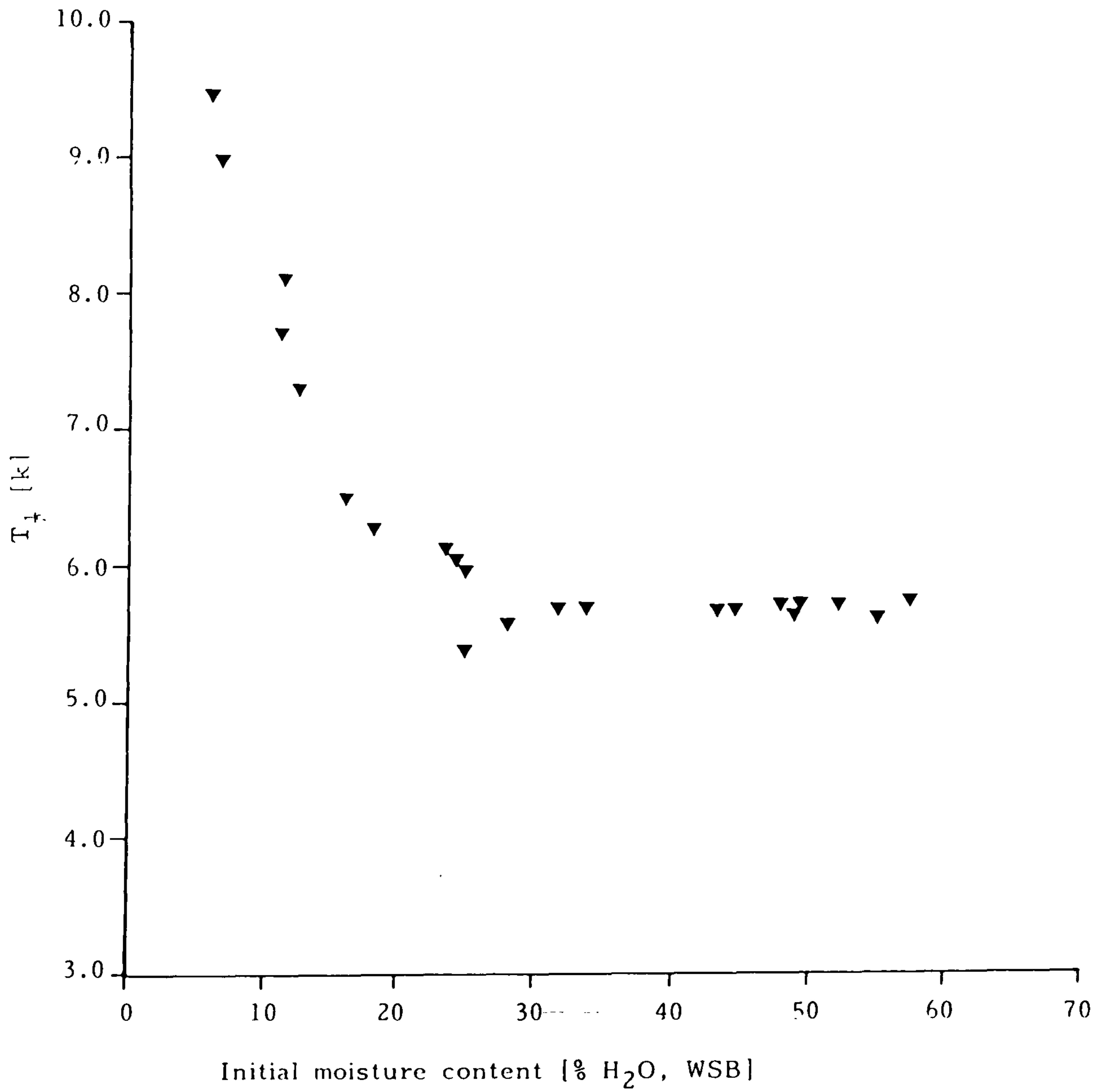
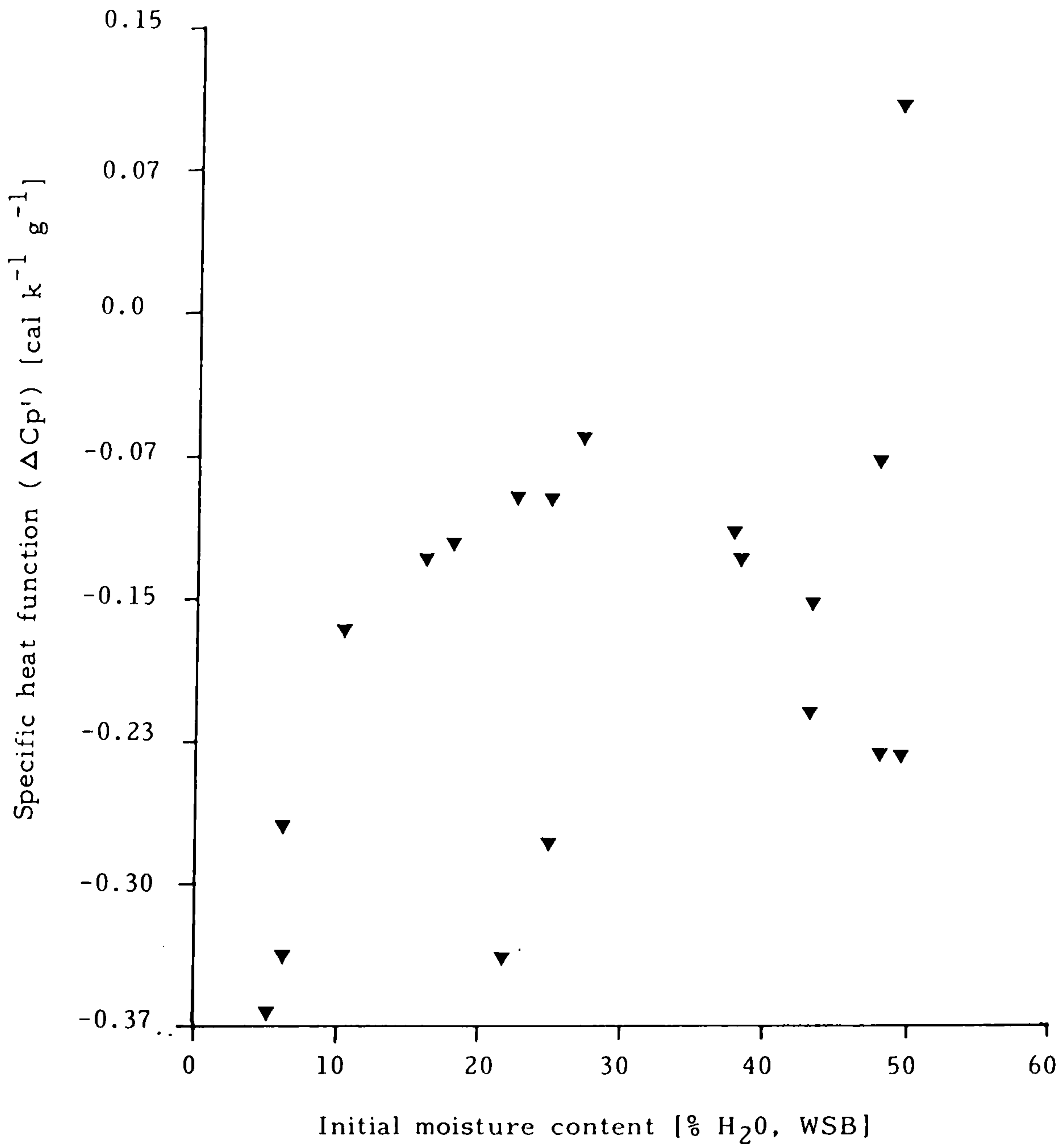


Figure 4.9.

Dependence of the specific heat function ($\Delta C_p'$) on moisture content for the Soya 7S globulin, denatured in the presence of 2% Manucol DM.



4.2.4 Discussion

To investigate the effect of hydrocolloid type and concentration a moisture content of 50 % water was chosen, it was thought that this moisture content represented a point where moisture dependence effects could be regarded as being minimal.

Values for the moisture contents did not vary by more than ± 1.0 %.

As effects upon ΔH_D occurred below 40 % moisture a lower moisture content was also studied (10 %).

If electrostatic protein - polysaccharide complexes are present, this would explain the decrease in ΔH_D at the lower moisture contents as such complex formation may be expected to result in a decrease in unfolding due to protein stabilisation. However stabilisation of the protein structure would be assumed to be associated with increased temperature tolerance. Manucol DM does lead to a small level of temperature tolerance, but the other hydrocolloids investigated had no such effect. Further evidence that complex formation is not occurring can be obtained from Donovan and Ross's (1973) work with avidin - biotin complexes. These workers proposed that during complex dissociation there should be an increase in ΔH_D .

The large drop in $\Delta C_p'$ can at best be explained in terms of reduced side group mobility following denaturation. However this effect is very specific to alginates

containing a high proportion of mannuronic acid residues and this specificity would tend to rule out electrostatic interactions as other hydrocolloids would be expected to behave in a similar fashion. The most likely explanation for these effects must thus lie with specific chemical interactions between the hydrocolloid and the protein.

The effects described fall broadly into two categories, those that are non specific and those which are highly dependent upon the presence of a large proportion of mannuronic acid groups. The alginate specific effects are very difficult to discuss in physical terms of for example charge shielding or complex formation. It seems probable that the answer must be derived from chemical events that are taking place.

4.3 A study of bound water associated with soya protein globulins in systems of soya and soya plus hydrocolloid.

4.3.1 Introduction

Protein hydration has been discussed previously (section 2.1.1.2). Water association with a protein affects not only the denaturation process but also to some extent the degree and type of protein - protein interaction. Such interactions may well play vital roles in the texturization process (Burgess and Stanley, 1976 and Rhee et al., 1981).

Within the moisture content range of 30 to 50 % it is

expected most of the water associated with the protein will be present as primary water (Muffet and Snyder, 1980). Thus at the levels of moisture used for the feed material in extrusion most will be present as primary water. It seems possible that small changes in protein structure may have a marked effect upon water binding. For this reason the hydration of protein, pre and post denaturation, in the presence and absence of alginate, was studied.

4.3.2 Materials and Methods

Soya isolate was prepared from a 70 PDI flour (section 3.1).

Alginate samples described in section 3.1.2 were donated by Kelco International Ltd. UK.

4.3.2.2 Differential Scanning Calorimetry

Samples of soya and soya plus 2% alginate were prepared for DSC analysis (section 3.3.2). Samples were cooled in the DSC as rapidly as possible to 233 K, heated at 10 K/min to 310 K and then at 5 K/min until all the thermal transitions were complete (423 to 443 K). Samples were then cooled as rapidly as possible to 233 K and the ice melting transitions reexamined. The DSC was calibrated against distilled water to evaluate the ice water transition.

Moisture contents were made by puncturing the pans and heating at 343 K for 48 hours in vacuo.

The weight of freezable water in the sample was determined assuming a heat of fusion for water of 79.6 cal/g.

4.3.2.3 Sorption Isotherms

Soya isolate, premoistened to about 35 % water was denatured by retorting at 394 K for 30 minutes in sealed cans. Calorimetric analysis indicated that denaturation was complete.

Native soya isolate, the denatured material, and native and denatured soya isolate containing 2 % Manucol DM were dried by heating in vacuo at 343 K for 48 hours. Samples (approximately 1g) of the dried material were equilibrated at 298 K over saturated salt solutions of known a_w (Rockland, 1950). Equilibration was complete within 48 hours at $a_w < 0.90$ and within 9 days at $a_w > 0.90$. The moisture content of the equilibrated samples was determined by drying in vacuo at 343 K.

4.3.3 Results

4.3.3.1 Differential Scanning calorimetry

Typical thermograms for the fusion of water associated with native and denatured soya isolate over the temperature range 243 to 310 K are shown in Fig 4.10. Below a water content of 30 % no peaks corresponding to water melting were

observed. Native samples with moisture contents between 30 and 50 % water were characterized by two peaks , a sharp peak centered around 273.5 K and a broad peak centered about 4 to 10 K lower, this peak started to deviate from the baseline at about 255 K. With increasing moisture content, to about 55 %, both peaks increased in size , though at different rates. The peak at the lower temperature increased, at a much greater rate and the temperature of maximum heat absorption for this peak was also seen to shift from about 263.5 to 269 K with increasing water content.

At water contents greater than about 55 % the peak at 273.5 increased at a far greater rate than that at the lower temperature, which eventually became only a shoulder on the sharper peak.

Interestingly Fig 4.11 indicates that an increase in moisture content leads to a corresponding increase in the concentration of unfreezable (primary) water. This increase is approximately linear from 35 to 56 % moisture. The amount of primary water associated with the soya protein was the same with or without alginate.

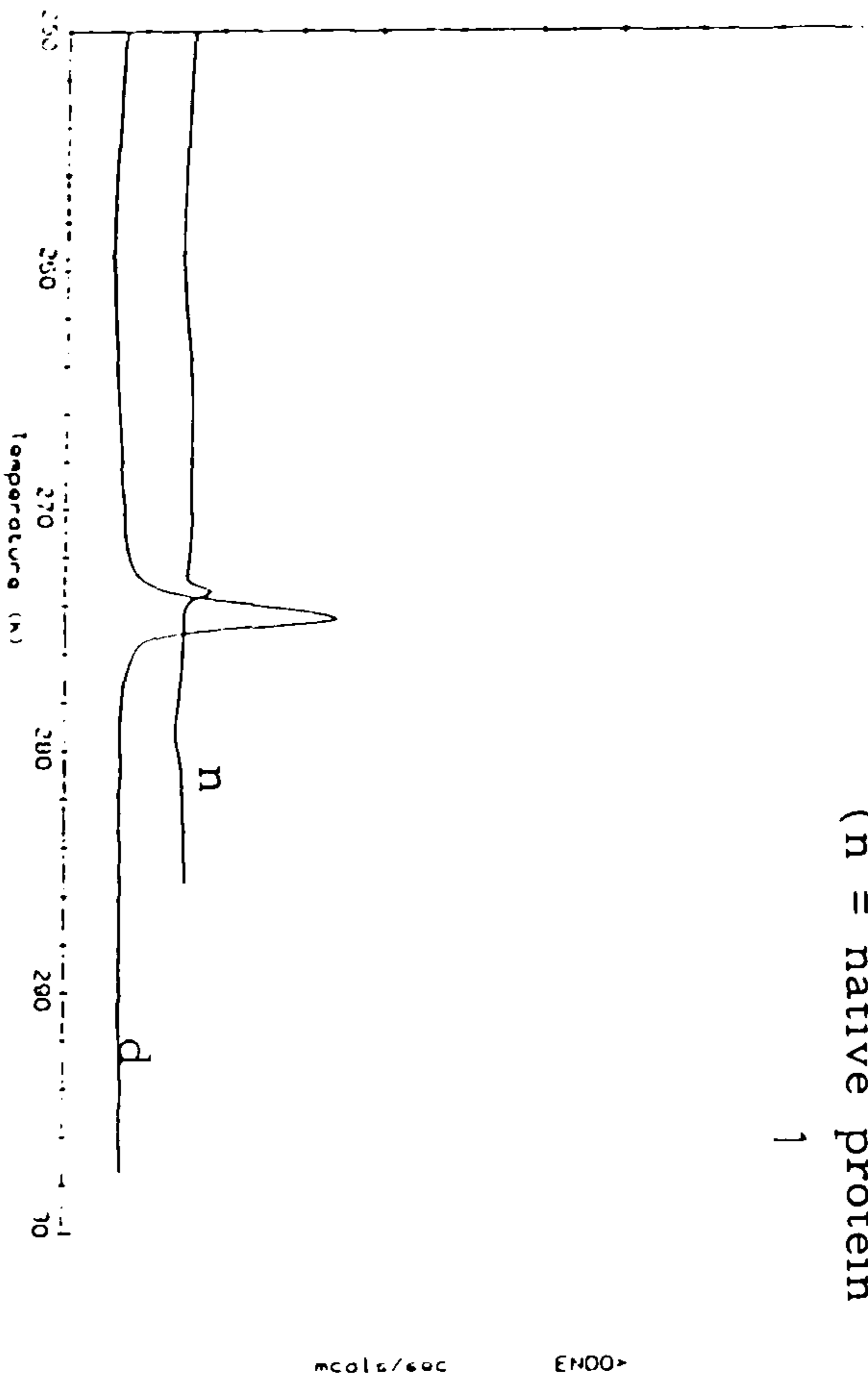
The difference between the thermograms obtained before and after protein denaturation were more pronounced at water contents below 60 % water. There was both a significant decrease in the low melting peak (266.5 K) and a significant increase in the peak at 273.5 K, suggesting that secondary water had been released from the protein following denaturation. Table 4.4 would suggest that following

denaturation there is a small increase in the quantity of unfreezable (primary) water of about 5 mg/g.

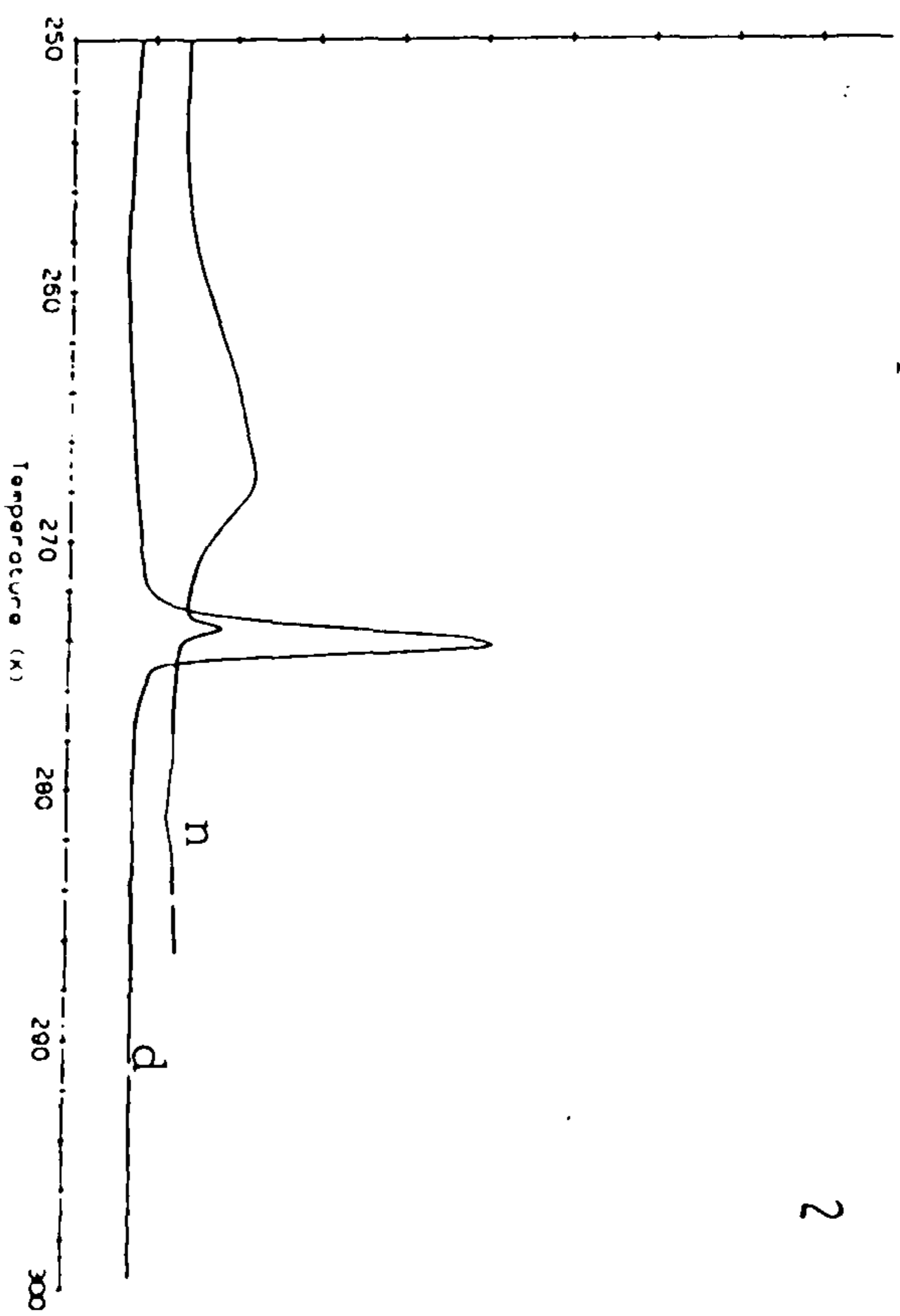
Figure 4.10.

Typical thermograms of the water associated with soya protein at initial water contents of
(1) 38%, (2) 43%, (3) 55% and (4) 65%;
sample weights are 7.13, 7.54, 9.66 and 11.48 mg, respectively
(n = native protein d = denatured protein)

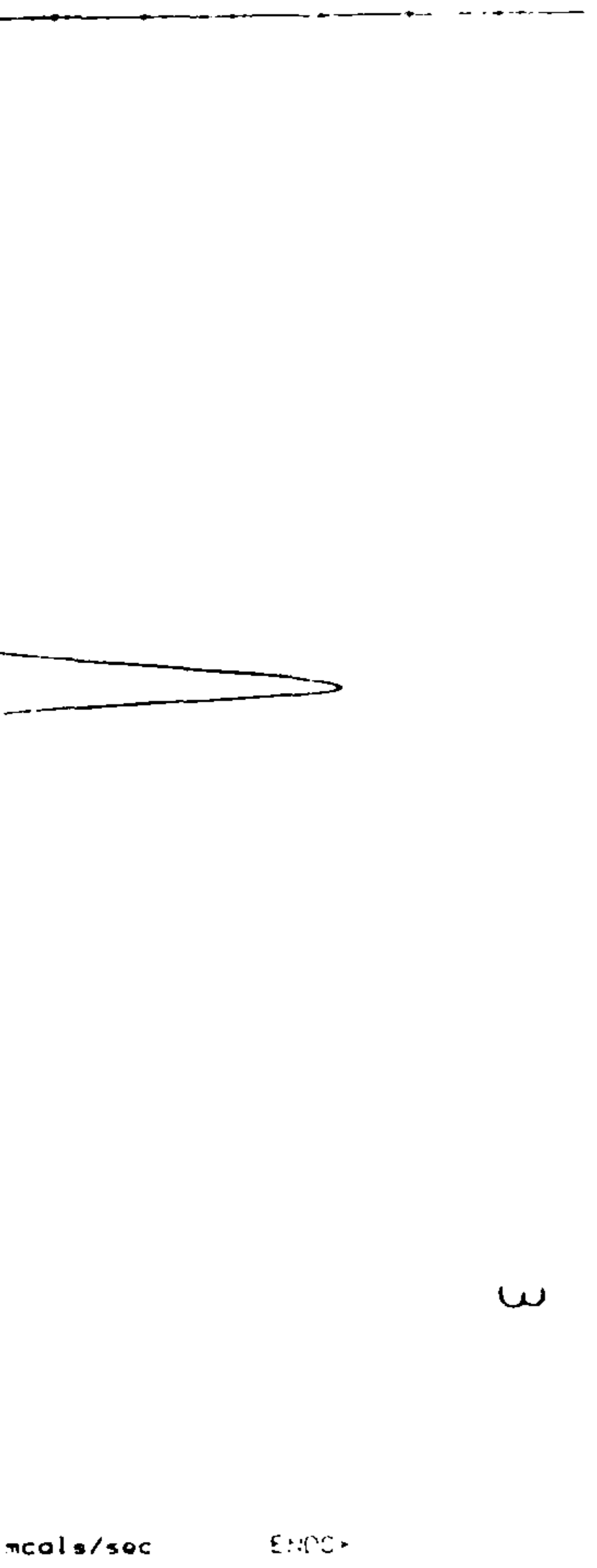
1



2



3



4

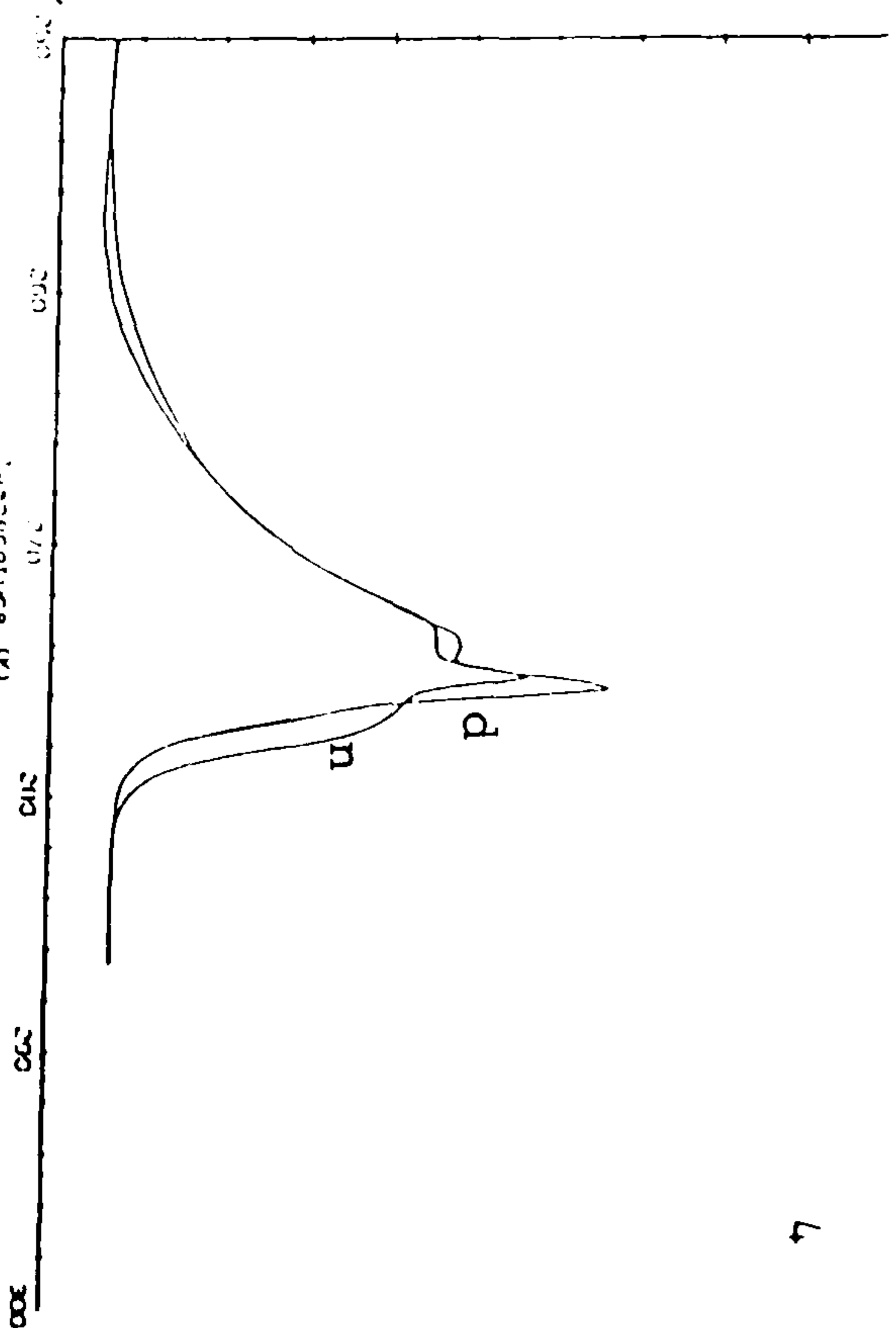
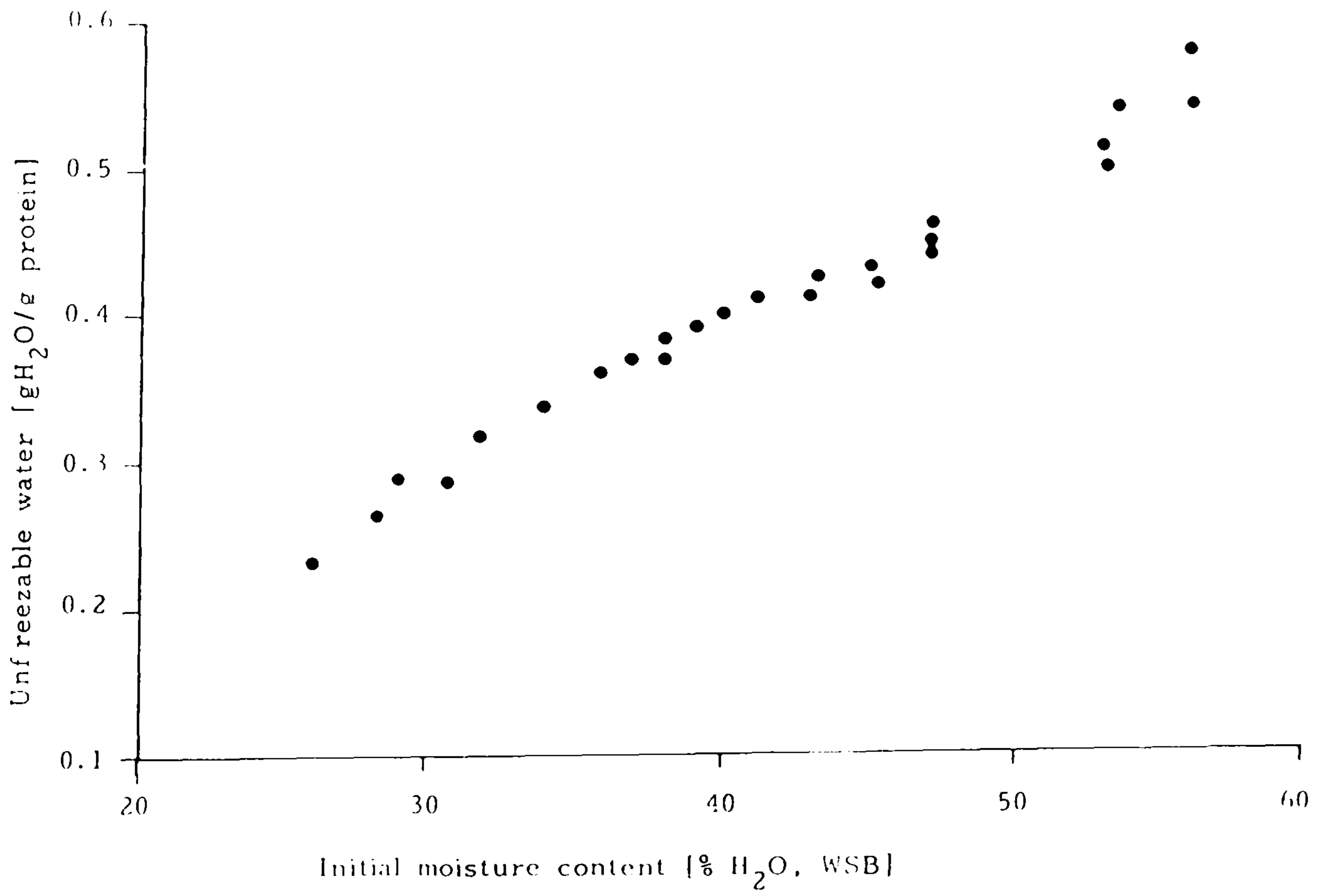


Figure 4.11.

Amount of unfreezable water associated with soya isolate
as a function of initial water content.



FEED MOISTURE %	UNFREEZABLE WATER g water / g protein		DIFFERENCE g water/g
	NATIVE	DENATURED	
36.3	0.352	0.360	0.008
38.2	0.373	0.379	0.006
40.8	0.401	0.406	0.004
43.0	0.408	0.411	0.003
45.0	0.426	0.427	0.001
46.0	0.448	0.451	0.003

Table 4.4 Effect of denaturation on the amount of measured unfreezable water associated with soya isolate.

Following the addition of 2 % Manucol DM no difference could be seen in the thermograms for the native samples compared with those obtained for soya alone. Following denaturation differences between the two systems were apparent (Fig 4.12). The decrease in the peak at around 263.5 K was slightly less pronounced than in the soya only system, at all moisture contents.

Figure 4.12.

Typical thermograms of the water associated with soya protein plus 2% Manucol DM at initial water contents of
(1) 42% (2) 45% (3) 51% and (4) 54%
Sample weights 5.71, 7.94, 9.26 and 10.72 mg respectively
(n = native protein d = denatured protein)

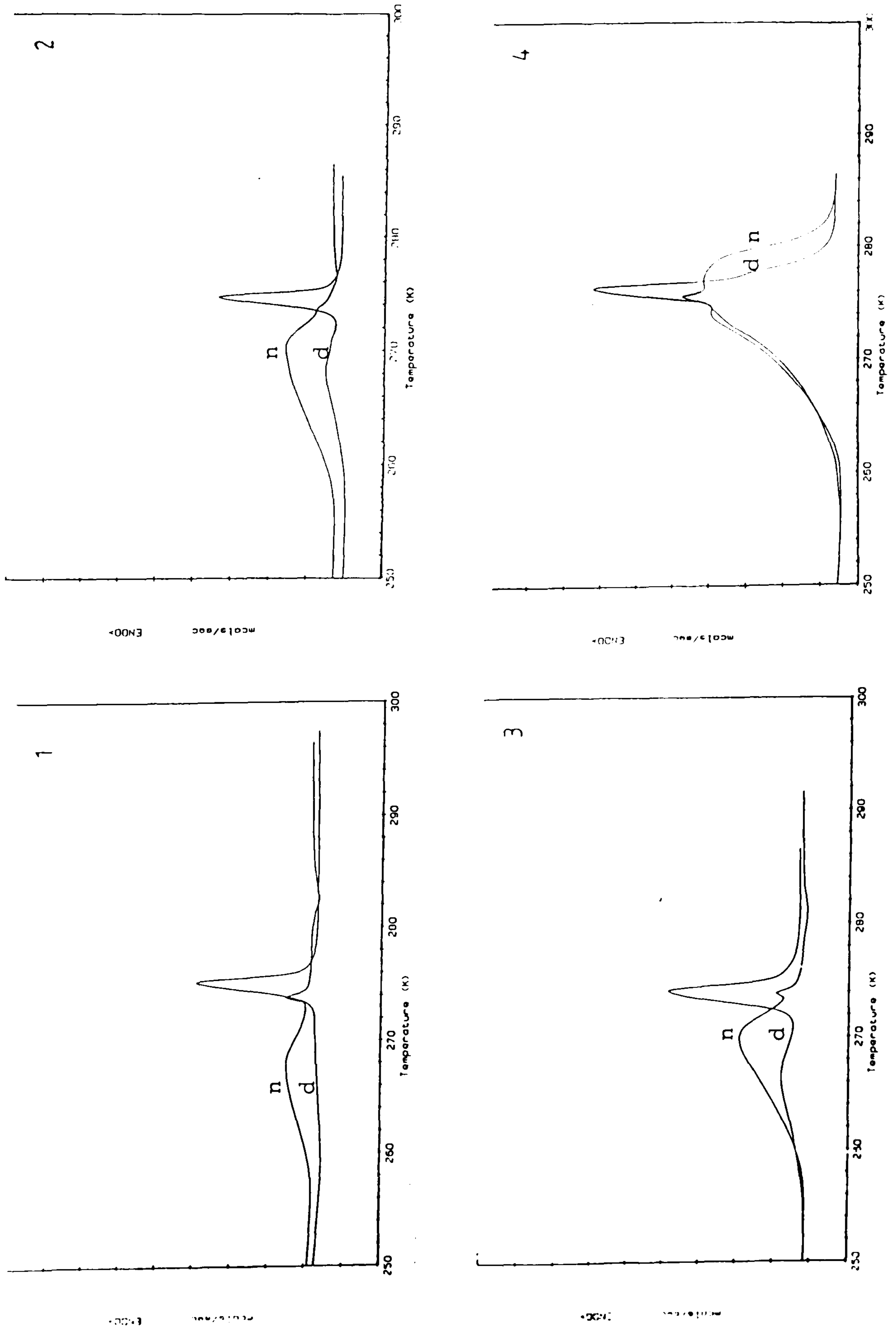
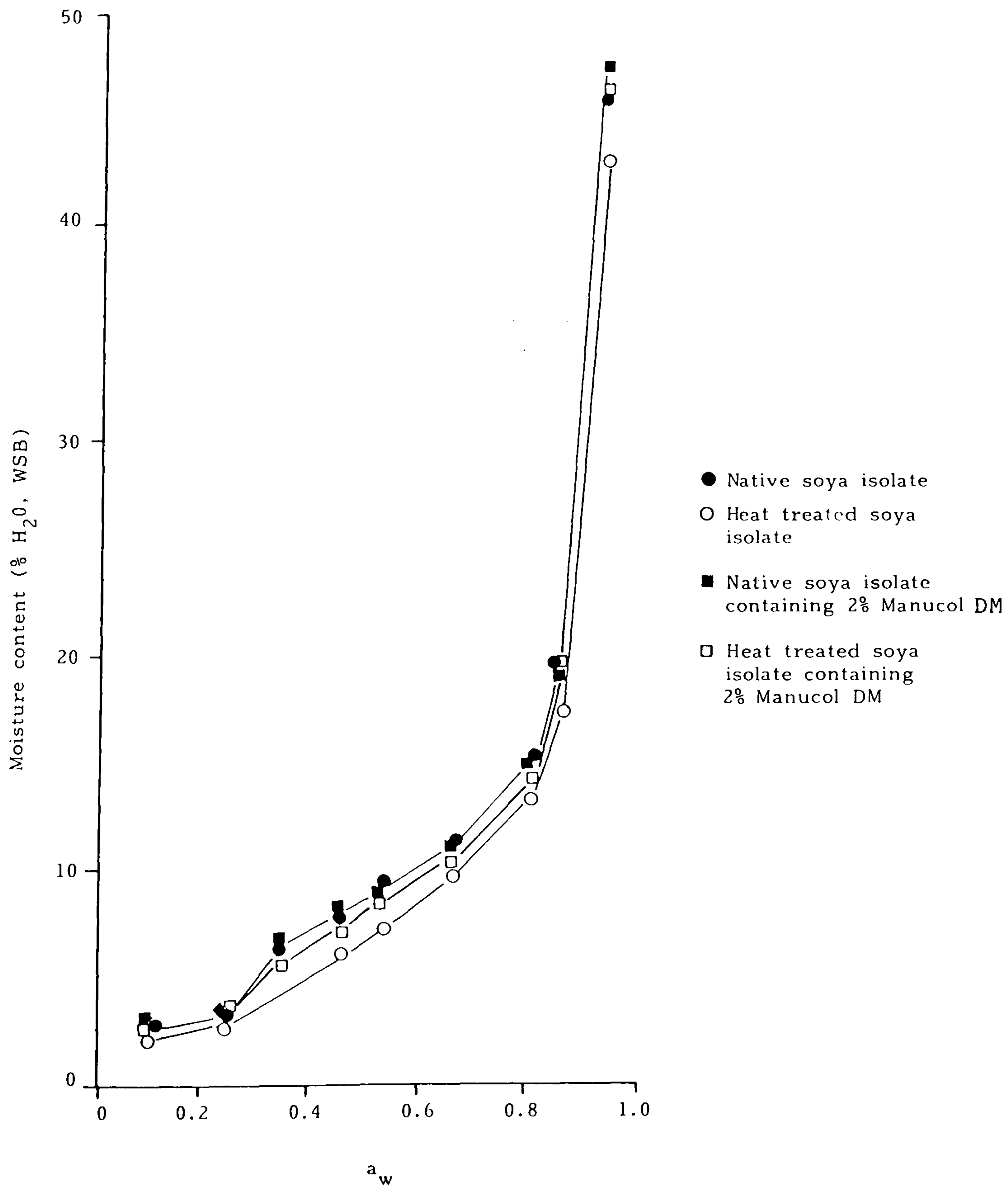


Figure 4.13.

Sorption isotherms of soya isolate.



4.3.3.2 Sorption Isotherm

With the exception of the denatured isolate all isotherms were, within, experimental error, identical at 298 K. At all water activities the denatured soya exhibited reduced water binding (Fig 4.13).

4.3.4 Discussion

Throughout this work the apparent heat of fusion of water has been assumed to be equivalent to that of pure water at 273 K, 79.6 cal/g. This value may well not be correct for some of the water associated with the protein. The true value may well lie between 56 and 79.5 cal/g (Ruegg et al., 1975).

The thermograms suggest that the water associated with soya is present in three states:

- a) Non freezable (primary) water
- b) Secondary water
- c) Bulk (tertiary) water

The threshold for freezable water (approximately. 31 %) does not differ from that observed for other proteins, albumins (Bull and Breese, 1968), collagen (Haly and Snaith, 1971), keratin (Haly and Snaith, 1969), β - lactoglobulin and casein (Ruegg et al ., 1974). The value of about 30 % was shown earlier to be associated with the significant differences in the enthalpy of denaturation of these systems suggesting these changes are primarily the

result of differences in the hydration states of the protein. The second point of major significance occurred at a moisture content of about 55 % when all the secondary hydration sites were assumed to be filled and any further water was present only as bulk water.

Muffet and Snyder (1980) determined two major regions in the hydration of soya protein, the first termed the critical value, occurred at 50% water and was the point at which free water peaks first seen. The second , occurred at a moisture content of 32% and was the point at which the major proportion of added water remained unfrozen. These findings are in very good agreement with those reported here.

The transition points shown earlier (section 4.1) for the enthalpy, $T_{1/2}$ and the $\Delta C_p'$ profiles all occurred at around 50 % water so it may be concluded that these changes were also brought about by the change in available water.

The surface area of the soya protein available to water for binding presumably increases with increasing moisture suggesting that at low moisture the protein packs more tightly than at high water contents (Richards, 1977). It is most likely that the increase in surface area is due to a relaxation of the protein complex, rather than any significant unfolding (Privalov, 1982).

Upon denaturation there is a small but consistent increase (approximately 5 mg/g) in the amount of unfreezable water. This may well be indicative of an

increase in the number of ionic groups which become exposed to the liquid medium. According to Kuntz and Kauzman (1974) ionic groups may bind upto about 6 molecules of water compared to the binding of only 1 such molecule by each of the less polar atoms (O or N).

Protein unfolding would be expected to be associated with an increase in the amount of water associated with the protein, but for soya it was shown that following denaturation there was less water bound to the protein. The water lost was that loosely held by the protein by long range charges (secondary water) and thus we may conclude that following denaturation charge redistributions occur that are unfavourable to attracting water molecules, possibly due to the emergence of previously buried hydrophobic groups. In addition if the denatured soya aggregates then less sites will be available for water binding.

The addition of Manucol DM is seen to restore some of the water binding capacity of the protein (Fig 4.12 and 4.13) after heating. It is possible that this effect is due to reasons other than by ionic interactions or perturbation of the primary water as both the uptake of water into the primary layers and amounts of unfreezable water do not seem to be affected. As will be discussed (chapter 5) it is thought that specific chemical reactions may be taking place between the protein and alginate, it may well be that as an outcome of these reactions the stability of the

secondary water is in some way increased.

A STUDY OF WATER PRODUCTION

IN HEATED PROTEIN

5.1 Evidence for the Production of Water

5.1.1 Introduction

Proteins in the presence of reducing compounds may, on heating, produce water as a result of a series of dehydration reactions that are some of the early steps involved non -enzymic browning. The reducing component may well be present as a carbonyl on an extraneous sugar or derived from a protein itself. The presence of such reactions will ultimately lead to the formation of new protein structures as a result of crosslinks. It was thought that the formation of such crosslinks or the concomitant formation of water may be important in the texturization process involved in extrusion. It was evident from the results presented in the previous chapter that following the heating of soya isolate, particularly in the presence of alginate, changes in the aqueous environment occurred.

The lowering of the soya melt viscosity following the addition of a high mannuronate alginate may well be due to such reactions, these hydrocolloids leading to a further increase in apparent water formation. Thus the production of water by these systems was investigated.

The presence of water is likely to be very important in controlling the operation of the extrusion / texturization process. It may well be involved in acting as a reactive medium for promoting the reactions responsible for texturization or the effect may be physical and act by

modifying the viscosity of the soya melt itself.

5.1.2 Materials and Methods

Soya isolate was prepared from a 70 PDI flour as outlined in section 3.1.

A range of alginates were donated by Kelco International Ltd.

Xanthan gum (lot G1253), gum arabic (lot G9752), guar gum (lot G4129) and pectin, partially methoxylated (lot P9135) were obtained from the Sigma Chemical Co. and carboxy methyl cellulose (CMC), medium viscosity, from BDH Ltd.

Methanol (A.R. grade), pyridine (A.R grade) and 2,4 dinitrophenylhydrazine, Aqua Fi solvent, Aqua Fi titrant and formaldehyde were obtained from Fisons, Ltd.

Pyridine stabiliser was prepared by mixing pyridine and water in a 80 : 20 v/v ratio.

2,4 dinitrophenylhydrazine (2,4 DNPH) solution was prepared by adding 50 mg of 2,4 DNPH to 25 ml of methanol and 2 ml of concentrated HCl. The solution was diluted to 50 ml using distilled water.

The potassium hydroxide solution used was a 33 % solution made up in methanol.

5.1.2.2 Differential Scanning Calorimetry

Approximately 5 mg of hydrated soya isolate or soya isolate plus 2 % hydrocolloid were prepared for DSC analysis

(section 3.3.2).

Samples were hydrated by equilibrating over saturated salt solutions, (Rockland , 1950).

The lids of some of the sample pans were punctured to allow the determination of the apparent moisture content of the samples to be made by drying in vacuo at 343 K for 48 hours. The remaining samples were subjected to calorimetric analysis at a scan rate of 5 K per minute. The samples were heated until the IIS fraction was seen to denature (temperature range 363 to 433 K). The samples were reweighed to ensure no weight loss had occurred and the pans punctured to determine the apparent moisture content by drying in vacuo at 343 k for 48 hours.

5.1.2.3 Determination of Carbonyl Content

Protein samples (approximately 0.3 g) were heated in closed glass vials at 458 K for 35 minutes. The glass vials were broken under 50 ml of methanol. A 2 ml aliquot of this sample was mixed with 2 ml of 2,4-DNPH reagent in a stoppered cylinder. After 30 minutes 10 ml pyridine stabilizer was added, followed by 2 ml potassium hydroxide solution and the solution mixed well. Absorbance against the appropriate blank was read at 480 nm, using 1 cm cells, within 10 minute of adding the potassium hydroxide solution.

5.1.2.4 Karl Fischer Analysis

Samples of soya isolate and soya isolate plus hydrocolloid, (about 0.3g) were sealed into glass

ampoules, a number of these ampoules were heated at 458 K for 35 minutes.

Both heated and unheated ampoules were subjected to Karl Fischer analysis. The glass ampoules were broken under 10 ml formamide and after 30 minutes of mixing 10 ml of Aqua Fi solvent was added mixing was continued for a further 5 minutes. The samples were titrated against Aqua Fi titrant as described in section 3.2.1.2.

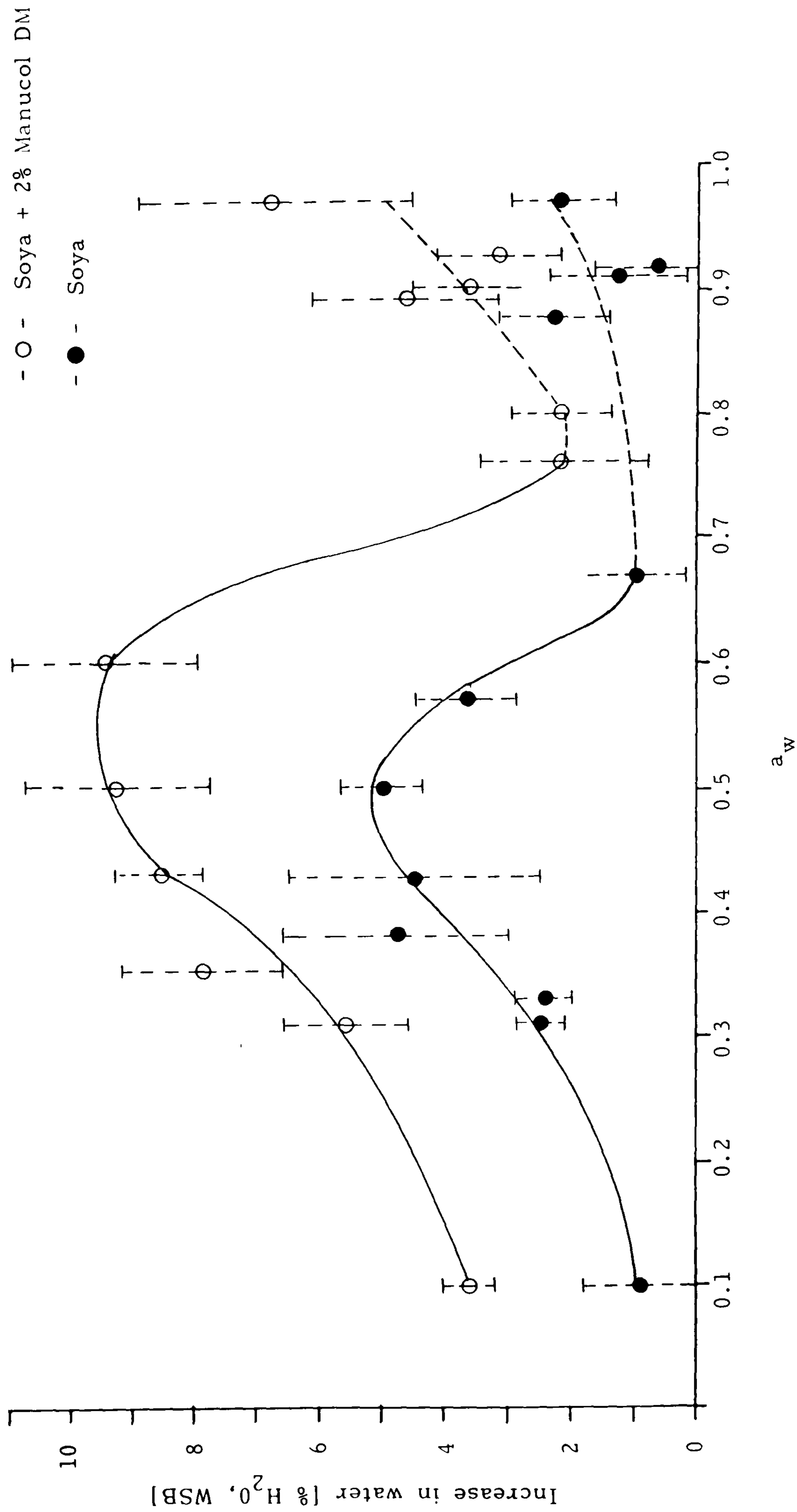
5.1.3 Results

5.1.3.1 Effect of a_w on the Increase in Apparent Water

The apparent water content of the heated protein was found to be dependent upon the initial water activity, a_w (Fig 5.1). The maximum amount of apparent water produced (approximately 5.5 %) occurred at an initial a_w of about 0.55. At a_w 's less than 0.1 and greater than 0.7 only about 1 % extra water was formed.

Figure 5.1

Dependence of apparent water contents for soya isolate on a_w ,
in the presence and absence of 2% Manucol DM.



Following the addition of 2 % Manucol DM the quantities of water produced throughout the range of a_w 's examined followed a similar profile to that of soya alone, except the a_w of maximum formation appeared to be about 0.05 of an a_w unit higher. The increase in apparent water was a_w dependent and varied between 3 and 10 % (Fig 5.1).

The standard errors for each data point are large which reflects the difficulty experienced in accurately determining a moisture content on only 5 mg of sample and possibly highlighting the inefficient drying process.

5.1.3.2 Carbonyl Determination

The concentration of carbonyls in the heated soya and soya plus alginate mixes were not significantly different ($p > 0.05$). The concentrations were 0.93 ± 0.04 and 1.0 ± 0.11 absorbance values per gram for the soya and soya plus alginate mixes respectively. The values are the means of 12 determinations with the associated standard error.

The values obtained represent a composite figure as the degree of colour formation is dependent upon the molecular weights of the carbonyl compounds. The values reported are as measures of absorbance / g; it was decided not to convert to a reducing equivalents since this would only be truly representative of the calibrant used.

5.1.3.3 Hydrocolloid Specificity

The production of increased amounts of water following

the heating of soya is dependent upon the presence of small amounts of Manucol DM (Table 5.1), all the other hydrocolloids examined showed a reduction in the amount of apparent water , as judged by drying in vacu^o at 343 K, compared to the soya isolate alone. This suggests that the hydrocolloids other than Manucol DM actually bind water produced on heating so that it will not be determined (liberated) by vacuum drying.

HYDROCOLLOID	MOISTURE CONTENT (%)
Soya alone	9.3 ± 0.43
Manucol DM	15.0 ± 1.1
Gum arabic	6.7 ± 0.6
Guar gum	6.2 ± 0.5
Xanthan	6.1 ± 0.5
CMC	7.4 ± 1.3

Table 5.1 Moisture contents (wsb) after heating to the temperature of about 433 K, for 35 minutes, of soya isolate (initial moisture content 5.5 ± 0.1 %) on its own or in the presence of 2 % hydrocolloid, as determined by vacuum oven drying. Values are means \pm standard error on 12 determinations

Values obtained for apparent water measured by Karl

Fischer analysis were somewhat higher than those recorded by vacuum oven drying (Table 5.2). Though it can still be seen following the addition of hydrocolloid only Manucol DM leads to any significant increase in the apparent water. Alginate samples from *Durvillea potatorum* and Keltone both brought about significant increases in the amount of water formed (Table 5.2), though both these compounds did not bring about increases as great as those found in the presence of Manucol DM .

There was also an apparent dependence on the ratio of mannuronic to guluronic acids in the alginate and its ability to create water in the presence of soya. Broadly speaking the greater the mannuronic acid content the more water is produced (Fig 5.2).

The block ratios were determined by Smith, (1984). Unfortunately there is no information as to how these different blocks are arranged in the molecule.

The values plotted in Fig 5.2 show a wide variability, which may reflect on the limitations of the technique (Karl Fischer) to discriminate between small differences , It may also be the outcome of inhomogenous mixing of the samples and possible interference by the alginate or their thermal degradation products.

HYDROCOLLOID	MOISTURE %
Soya isolate	12.2 ± 0.5
Manuacol DM	20.4 ± 1.3 ^a
Manugel GMB	12.2 ± 0.24 ^c
D.potatorum	15.6 ± 0.5 ^b
Keltone	15.8 ± 0.6 ^b
CMC	12.2 ± 2.5 ^c
Pectin	12.9 ± 1.04 ^c

Table 5.2 Pool percentage moisture content determinations of soya isolate (initial moisture content $8.5 \pm 0.1\%$) and soya plus 2% hydrocolloid, heated at 458 K for 35 minutes as determined by Karl Fischer analysis.

Values are mean standard error on 20 determinations.

Probability of no significance from the control (heated soya isolate) determined by Dunnetts test.

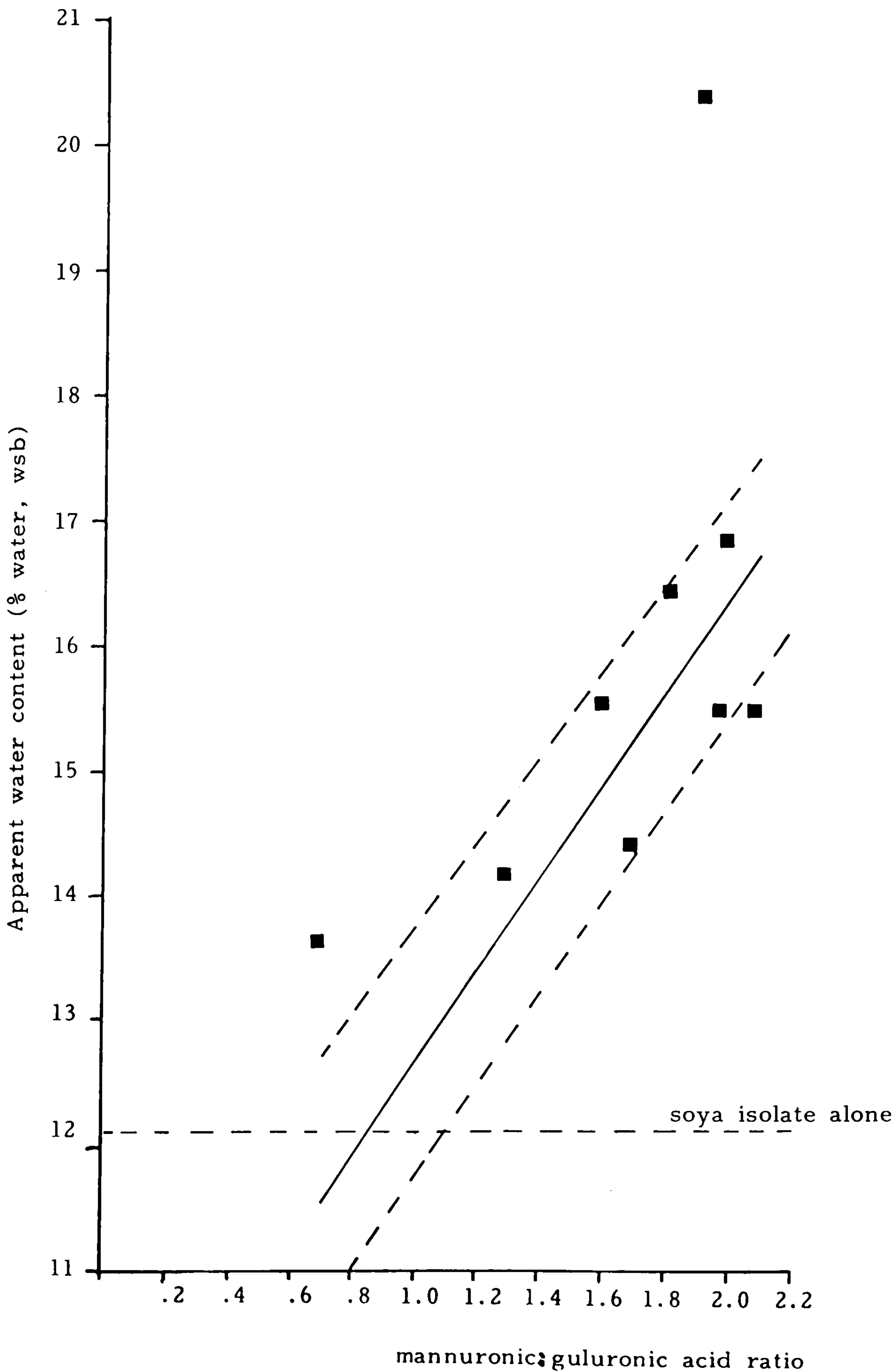
a $p < 0.001$

b $p < 0.05$

c No significant difference

Figure 5.2.

The dependence on mannuronic : guluronic acid ratio of added alginate (2%) to the water content of soya isolate after heating (458 k, 35 minutes).
Regression line of means with associated 95% confidence limites (broken line).
Population regression is linear ($P > 0.10$) (Zar, 1974)



5.1.4 Discussion

Apparent water production occurs in heated samples of soya isolate; this is interesting since these samples contain little carbohydrate (less than 0.3%, Sheard, 1985) and thus the browning between a sugar carbonyl and the amino group of an amino acid is not likely to be of importance. Soya does though contain large amounts of glutamic acid, the carbonyls of which may react with free amino groups, such as those found in lysine. Such a reaction may be limited by the accessibility of the glutamic acid residues. The result of such a reaction will form a glutamic and lysine crosslink with the concomitant production of water. The possibility of such reactions will be explored more completely in section 5.2. An involvement of the sugar moiety of the 7S glycoprotein must not be overlooked. The carbohydrate component of this glycoprotein, 12 glucosamine and 39 mannose residues per mole of 7S globulin ($\approx 5\%$) (Wolf, 1972) may well provide a significant contribution to possible browning / condensation reactions.

The condensation /browning type reactions postulated above are known to be moisture dependent, with maximum activities occurring at low moisture levels (Loncin, etal., 1968 and Eichner, 1975). Reaction maxima have been recorded for a variety of proteins and in all cases the maxima lies

in the a_w range 0.6 to 0.7 (Loncin et al, 1968 and Lea & Hannan, 1949).

The influence of water on the rate of these reactions is complex. At high water activities there is a general diluting effect of the reactants and an inhibition of the condensation reactions due to the law of mass action (Eichner, 1975). At low water contents mobility of the reactants is restricted. According to Duckworth (1962) the diffusion of solutes can only occur at water contents above the amount for minimum unbound water which for soya is around 30 % water (a_w approximately 0.9), (Hansen, 1976).

The reactions occurring within the extruder will be driven by both the heat and pressure generated, by heating the samples in sealed pans it was hoped that, to some degree, the effects of pressure could be brought to bear on the system. It must not be forgotten that the pressures generated within the pans will not be as high as those encountered in the extruder. The pressure within the system will affect the final outcome of any chemical reaction, the main effects in soya will be relocation of equilibria and phase change inhibition of potential volatiles.

The addition of alginate led to further quantities of water being produced following heating of the soya, again the reaction was moisture dependent. It is assumed that the presence of the alginate is in some way contributing reactive groups to specific browning / condensation type reaction .

During heating of proteins it is conceivable that a range of volatile carbonyl products will be formed which may interfere with the determination of the apparent moisture contents determined by Karl Fischer and oven drying. Even if this is so there is no significant difference in the concentration of these compounds in the heated systems (section 5.1.3.2). Thus the differences observed are presumably due to differences in water content.

Of a range of hydrocolloids tested only Manucol DM, alginate derived from *D.potatorum* and Keltone when heated in the presence of soya led to the production of significantly greater amounts of water than found on heating the protein on its own. Two methods were used, the first involved drying in a vacua and the second the total solubilisation of the sample prior to Karl Fischer analysis. It is apparent that although similar effects are seen between the two methods in terms of water production, in the different systems, oven drying invariably gives a lower 'water' content. The differences reported may result from two sources, firstly differences between the heating regimes. The oven dried material was originally heated in small quantities with good thermal contact, to temperatures around 430 K, this heating process lasted about 20 minutes with only a very short holding time at the highest temperature. On the other hand the samples that were analysed by Karl Fischer were heated in comparatively larger amounts with poorer thermal contact but for longer periods

of time, 35 minutes at the maximum temperature. These differences could account for some of the differences found in Tables 5.1 and 5.2.

In addition oven drying has associated problems due to the transport of soluble components to the food surface, whereby subsequent evaporation of the water may cause case hardening (Joslyn, 1970) which limits the loss of water. Drying is also affected by shape and size of sample, method of heat transfer and the way air passes over the sample (Akanbi, 1985 and Oxley & Pixton, 1961). It is apparent that the drying of the sample in DSC pans is not very efficient . Invariably much of the primary water will remain after the mild heating treatment, according to Nelson & Hulett (1920) moisture remains in biological samples even at temperatures as high as 638 K. Problems have also been found with regard to removal of water from structural food matrices and capillaries. On the other hand Karl Fischer analysis requires the total destruction of the material as it dissolves in the solvent, thus this should measure all water present, including primary water. The value will be elevated as a result of reaction with other reducing groups present in the soya (Oxley et al., 1960), However as discussed above these groups are present at similar concentrations in the heated samples.

There is a strong specificity for alginates containing a high proportion of mannuronic acid residues in the formation of significant amounts of water in the heated soya

systems. The level of water production increases as the content of such units increases. Interestingly the alginates derived from *D. potatorum* and Keltone which have similar block ratios to Manucol DM are not as reactive. It is possible that some other factor such as the number of junction zones present, which are high in Manucol DM, may be a factor in such reactions.

According to Miki (1974) and Seaver & Kertesz (1946) uronic acids are more reactive than aldoses in browning / condensation reactions. It may well be that polysaccharides breakdown to some degree upon heat treatment to give a range of sugar derivatives and smaller polysaccharide fragments. This will have the overall effect of increasing the number of potential reducing groups. From the data available it is very difficult to predict whether the differences in reactivity between the various hydrocolloids are a result of the breakdown products or due to differential rates of breakdown. The thermal breakdown of alginate is explored in more detail in chapter 6.

5.2 PROTEIN CHARACTERISATION

5.2.1 Introduction

Very little work has been performed involving analysis of the chemical composition or reactions occurring during the extrusion or texturization processes. Previously it was found (section 5.1) that soya protein apparently produced water on heating. The amount of such water was increased in the presence of alginate. Alginate is known to be active in altering the conditions of the extrusion melt. It was decided to investigate the specificity of the reaction with alginate with regard to protein type.

It is known that on heating proteins, changes in their amino acid composition take place (Cheftel, 1986). These changes are thought to be important, in that the quaternary protein structures will be modified to some extent due to the loss of amino acid side chains. These interactions can occur as a result of reaction with other components present, for example carbohydrates. The formation of a wide range of new inter / intra protein crosslinks may occur. The formation of these crosslinks and changes in the amino acid composition are thought to be important in the texturization process and with the possible interaction with alginate. It was for this reason that analysis of such reactions was undertaken.

5.2.2.1 Materials and Methods

A range of commercial soya isolates were obtained: Purina 860 and Purina 610 from McCauley Edwards Ltd.; Ardex R from British Arkady Ltd. and a laboratory prepared soya isolate was prepared from a 70 PDI flour (section 3.1).

Soya flour was obtained from McCauley Edwards, Ltd. and soya grits (Pretona Pale) from RHM Ingredient Supplies.

Glycine (analar grade) was obtained from BDH Chemicals Ltd., lysine monochloride (lot L5626) and sodium glutamate (lot G1626) from Sigma Chemicals Co. Aspartic acid obtained as the free base was purchased from Hopkin & Williams Ltd.

The proteins used in this study were:- pancreatin (lot P1750), ovalbumen (lot A5253) and haemoglobin (lot H3760) from Sigma Chemicals Co.; whole blood albumen (WBA) and casein (UFI US extra grade) from Fibrisol Services Ltd., gluten from Tenstar Products Ltd. and gelatin (a commercial brand of high bloom gel strength) was donated by Parr's, Nottingham.

2,3,6 - trinitrobenzene sulphate (TNBS) (lot P3402), nicotinamide adenine dinucleotide (lot N6754), L- glutamic dehydrogenase (type 4 lot G4008), α - ketoglutarate (lot 410-2), glutamic oxaloacetic transaminase (200 units/ mg, lot C 2751) 2,4-dinitrophenyl hydrazine (lot D2630), aniline hydrochloride (lot A8524) and nor-leucine (lot N6752) were all obtained from Sigma Chemicals Co.

2,4-dinitrophenyl hydrochloride solution was prepared

by dissolving 100 mg DNPH in 20 ml concentrated HCl and adding 80 ml distilled water.

Aniline citrate solution was a mixture of equal amounts of aniline hydrochloride solution (8g in 20 ml water) and citric acid solution (1:1 w/v).

Alcoholic potassium hydroxide was a mixture of 1M KOH and ethanol at a volume ratio of 2.5 : 1.

5.2.2.2 Physiochemical Analysis

The ionic strength of 10% dispersions of the proteins was measured with a FSA Laboratory Supplies conductivity meter, PTI - 58 referenced against standard potassium chloride solutions.

The pH of the 10% dispersions was measured using a Uniprobe ' 320 ' pH meter.

The degree of native structure for all of the proteins was determined by DSC analysis with the exception of gluten which according to Hosney and Zeleznak (1986) and Eliasson and Hegg (1980) can not be determined by DSC analysis. Casein exists as a random coil and consequently has no " native " structure. For all analysis 35 % water doughs of each of the proteins was used, (section 3.3.2). The scan rate used was 5 K / min with a sample size of about 5 mg.

5.2.2.3 Differential Scanning Calorimetry

Samples of protein and protein plus 2 % Manucol DM were prepared for DSC analysis as described in section 3.3.2. Samples were hydrated by equilibrating over the relevant saturated salt solutions (section 4.1.2). Moisture contents were determined by drying in vacuo at 343 K as described previously (section 3.3.2) before and after heating to temperatures in the range 360 to 433 K.

5.2.2.4 Karl Fischer Analysis

Approximately 0.3g of protein or amino acid with and without 2 % alginate were sealed in glass ampoules (approximately 2 cm³) and heated at 443 K for 35 minutes. Ampoules were broken under 10 ml formamide and stirred for 30 minutes after which Karl Fischer analysis was performed as previously described (section 3.2.1.1), using Aqua Fi solvent and titrant.

5.2.2.5 Amino Acid Determination

The amino acid content of native and denatured samples of soya isolate, denatured samples of soya isolate plus 2 % Manucol DM or Manugel GMB were determined using a LKB 400 amino acid analyser (LKB Instruments Ltd.).

Samples (approximately 0.1g) were refluxed in 75 ml 6M HCl under nitrogen for 22 hours. Subsequent dilution to 100 ml with distilled water was followed by evaporating to dryness at 310 K and a second washing , the final residue

was dissolved in 2 ml lithium citrate buffer. An internal standard of 1.0 μ mole Nor - leucine was employed.

Due to the susceptibility of cystine and methionine to hydrolysis, further samples were additionally hydrolysed to oxidise these amino acids to cysteic acid and methionine sulphone respectively (Moore, 1963). 100 mg of sample was equilibrated overnight at 273 K with 10 ml of performic acid, 48 % hydrobromic acid was added immediately prior to evaporation to dryness at 310 K. The resulting residue was treated as before except hydrolysis was with 30 ml of 6M HCl and was not carried out under nitrogen.

Operating conditions used :-

Column	250 * 4.5 mm stainless steel
Buffers	1] pH 2.8 2] pH 3.0 3] pH 3.02 4] pH 3.45 5] pH 3.33
Column pressure	45 bar (at start of run)
Column temperature	308 to 333 K
Regeneration	0.3 M LiOH
Equilibration	
buffer	pH 2.8
Column flow	20 ml / hour
Ninhydrin flow	20 ml / hour

The colour was read at 570 and 440 nm, the 570 nm

channel was integrated by a Spectra Physics SP4270 auto analyser.

5.2.2.6 Lysine Determination

Tubes containing approximately 10 mg of finely ground sample and 1.0 ml 4% NaHCO_3 , pH 8.5 were incubated, with shaking, in a water bath at 310 K for 10 minutes. After this time 1.0 ml of 1% TNBS solution was added and incubation continued for a further 2 hours. After incubation 5.0 ml of concentrated HCl was added. The tubes after sealing were autoclaved at 394 K / 15 psi for 1.5 hours. After cooling the volume was made up to 10 ml with distilled water and the contents extracted twice with 10 ml A.R. grade diethyl ether, any residual ether was removed by standing the tubes in hot water. After suitable dilution with distilled water the absorbance was measured at 346 nm, against the appropriate blank. The concentration of lysine was determined by comparison with known amounts of lysine.

5.2.2.7 Glutamic and aspartic acid determinations

Prior to analysis of either of these amino acids the samples were hydrolysed. Approximately 0.01 g of ground sample together with 7.5 ml 6 M HCl were sealed in high pressure glass vials. Samples were incubated in a convection oven at 383 K for 16 hours. Samples were then resuspended in pH 7.8 phosphate buffer.

a) glutamic acid (Balis, 1971)

reaction mixtures were made as follows:-

0.70 ml Hydroxylamine, 2M pH 8.0

0.10 ml Unknown solution

0.15 ml 1% NAD solution

0.05 ml Glutamic dehydrogenase (1%)

The reaction was started with the addition of the glutamic dehydrogenase prior to the commencement of the readings. All absorbances were taken at 340 nm against a blank containing 0.10 ml distilled water. Readings were taken until completion of the reaction, around 20 minutes.

The amount of glutamic acid was calculated on the basis of differences in increase between sample and blank and compared against known amounts of glutamic acid. Corrections were made for sample size and dilution.

b) Aspartic acid (Balis, 1971)

Reaction mixtures were made as follows:-

0.5 ml, Na - α - ketoglutarate, 0.1 M

0.5 ml, Unknown

0.1 ml, GOT (100 units / ml)

Tubes were incubated at 310 K for 30 minutes.

To the reaction mixture was added 0.22 ml of 50 % trichloroacetic acid, tubes were shaken and 0.1 ml aniline citrate was added followed by incubation at 310 K for 10

minutes. 0.1 ml of dinitrophenylhydrazide hydrochloride was added followed by a further incubation for 5 minutes at 310 K. 2 ml toluene was added and the tubes shaken, when the layers had separated 1 ml of the DNP layer was added to 5 ml alcoholic KOH. All solutions were mixed and the absorbance at 540 nm read after 5 minutes against the appropriate blank.

Aspartic acid levels were determined against a calibration graph constructed using pyruvate.

5.2.2.8 Solution Studies on Heated Alginate Samples

Ampoules (approximately 2ml) containing dry alginates (0.1g) heated at 428 K were broken under 10 ml of 0.1 M of the amino acid solution. Following incubation in a boiling water bath for 1 hour the increase in absorbance at 420 nm was taken as an index of browning potential.

5.2.3 Results

5.2.3.1 Physiochemical analysis

The ionic strength, pH and whether native structure or not of the proteins are shown in Table 5.3 and Fig 5.3.

PROTEIN	pH	IONIC STRENGTH KCl / M	STATE
Soya isolate	6.68	0.046	native
Purina 860	6.28	0.010	native 11S
Purina 610	6.54	0.020	denatured
Ardex	4.72	0.005	native
Ovalbumen	6.38	0.026	native
W.B.A.	8.24	0.114	native
Gelatine	5.45	-	denatured
Haemoglobin	7.40	0.020	native
Casein	6.85	0.002	-
Gluten	5.61	0.004	-

Table 5.3 Physiochemical properties of the proteins. All solutions were 10 % in deionised water at 299 K, structural state was determined by DSC analysis.

All the proteins used in this study contained little salt and exhibited a range of pH values when dissolved in water (4.72 to 8.24). Of the soya isolates , Ardex R had a significantly lower pH and lower salt content than the others.

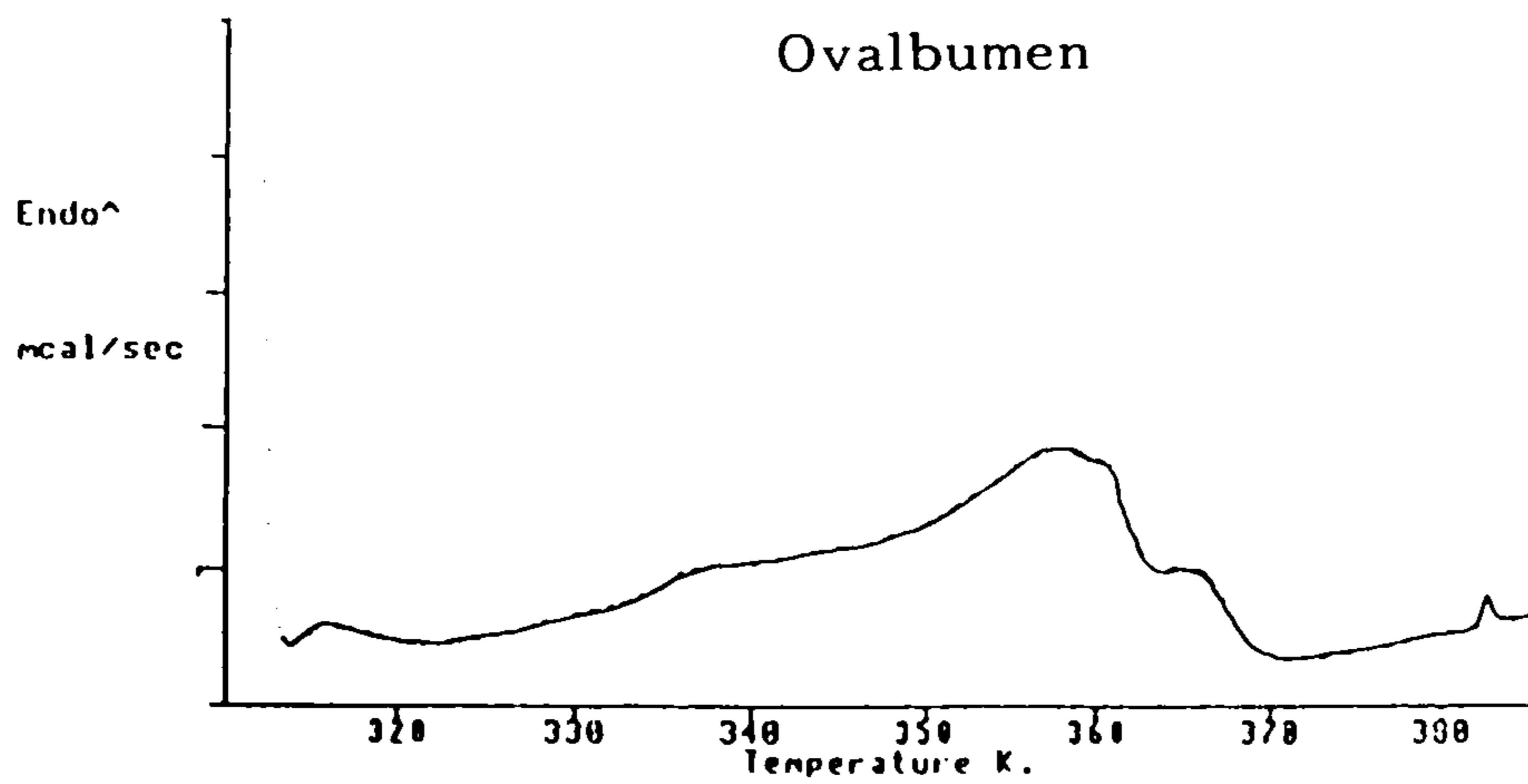
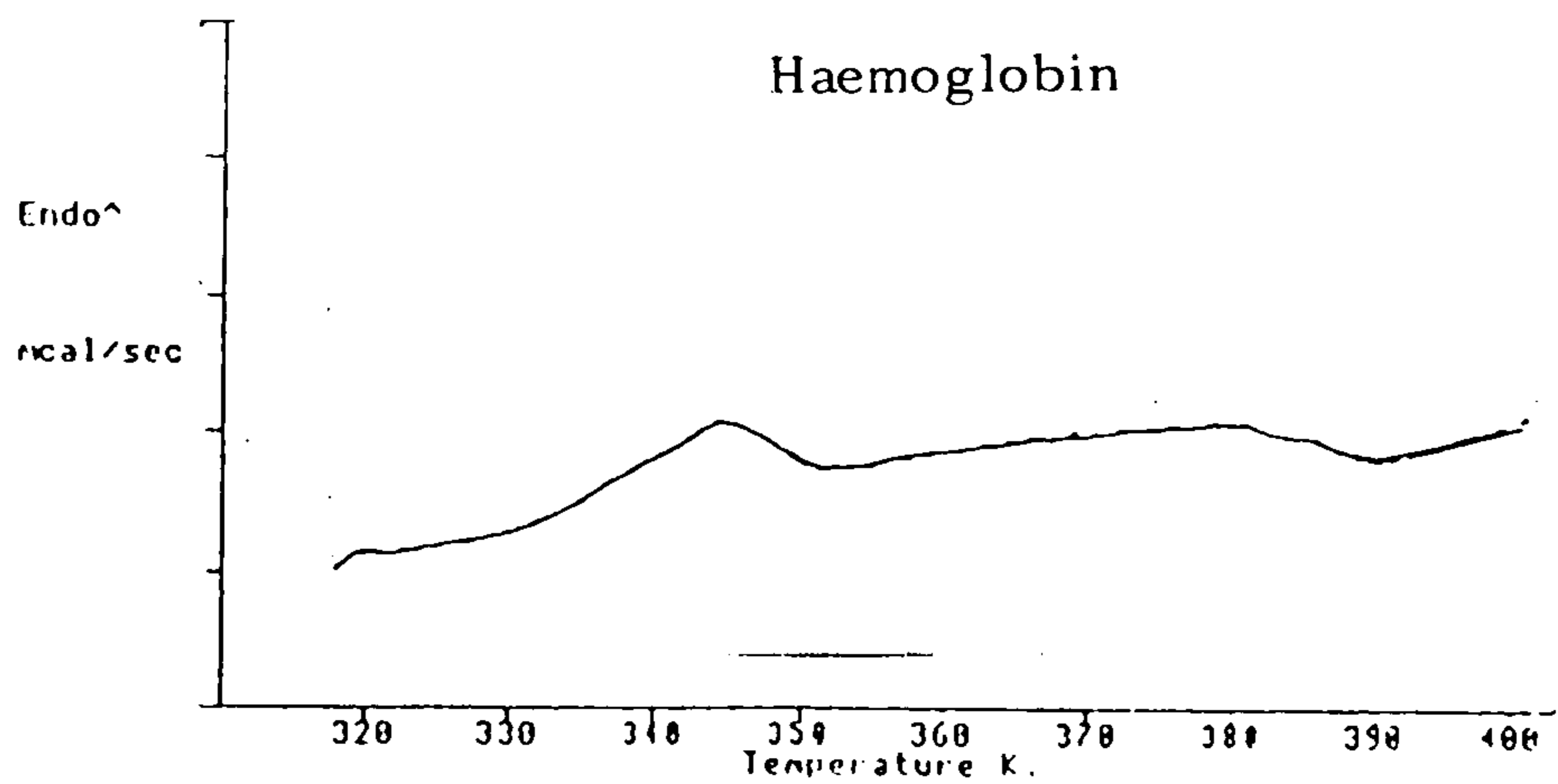
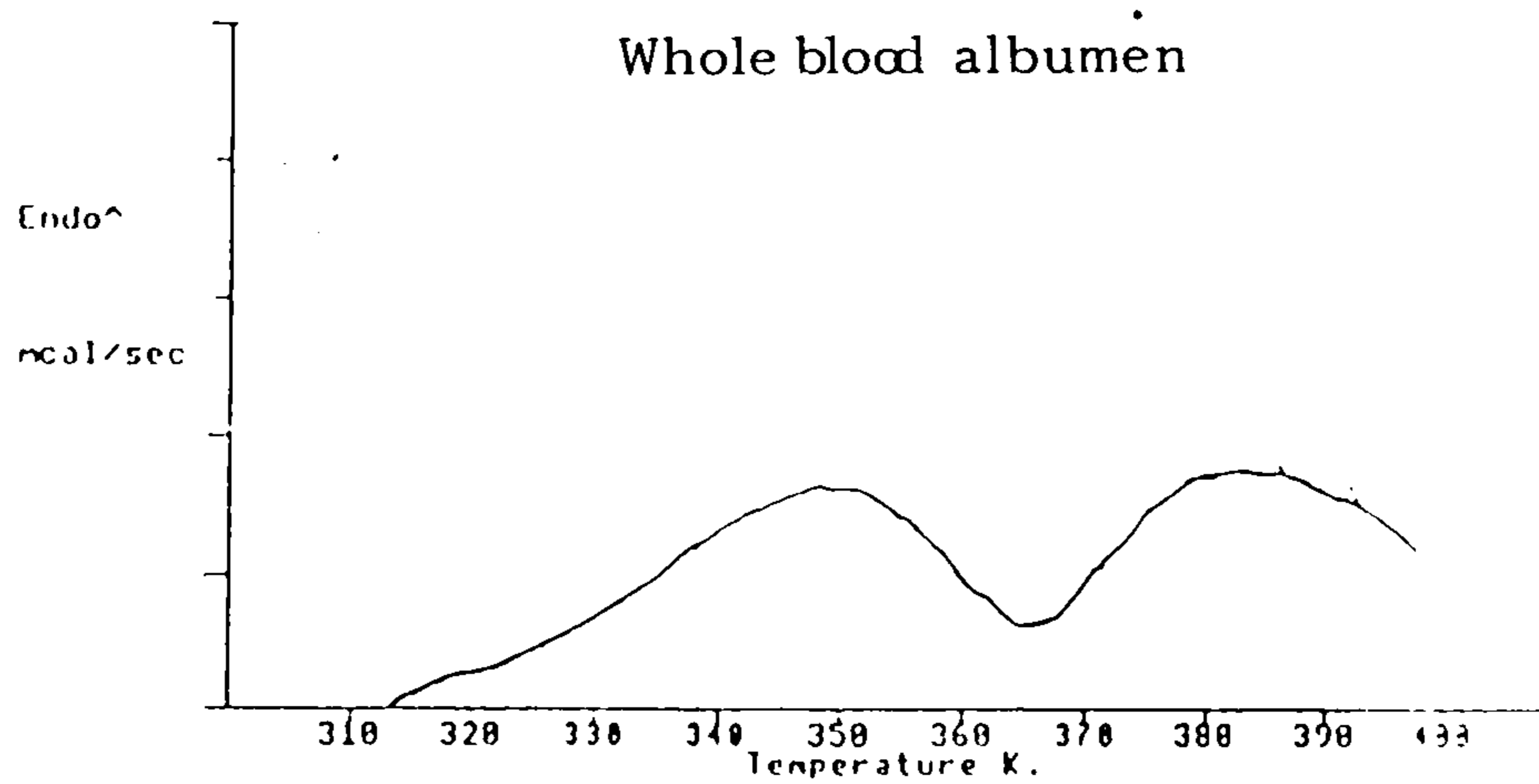
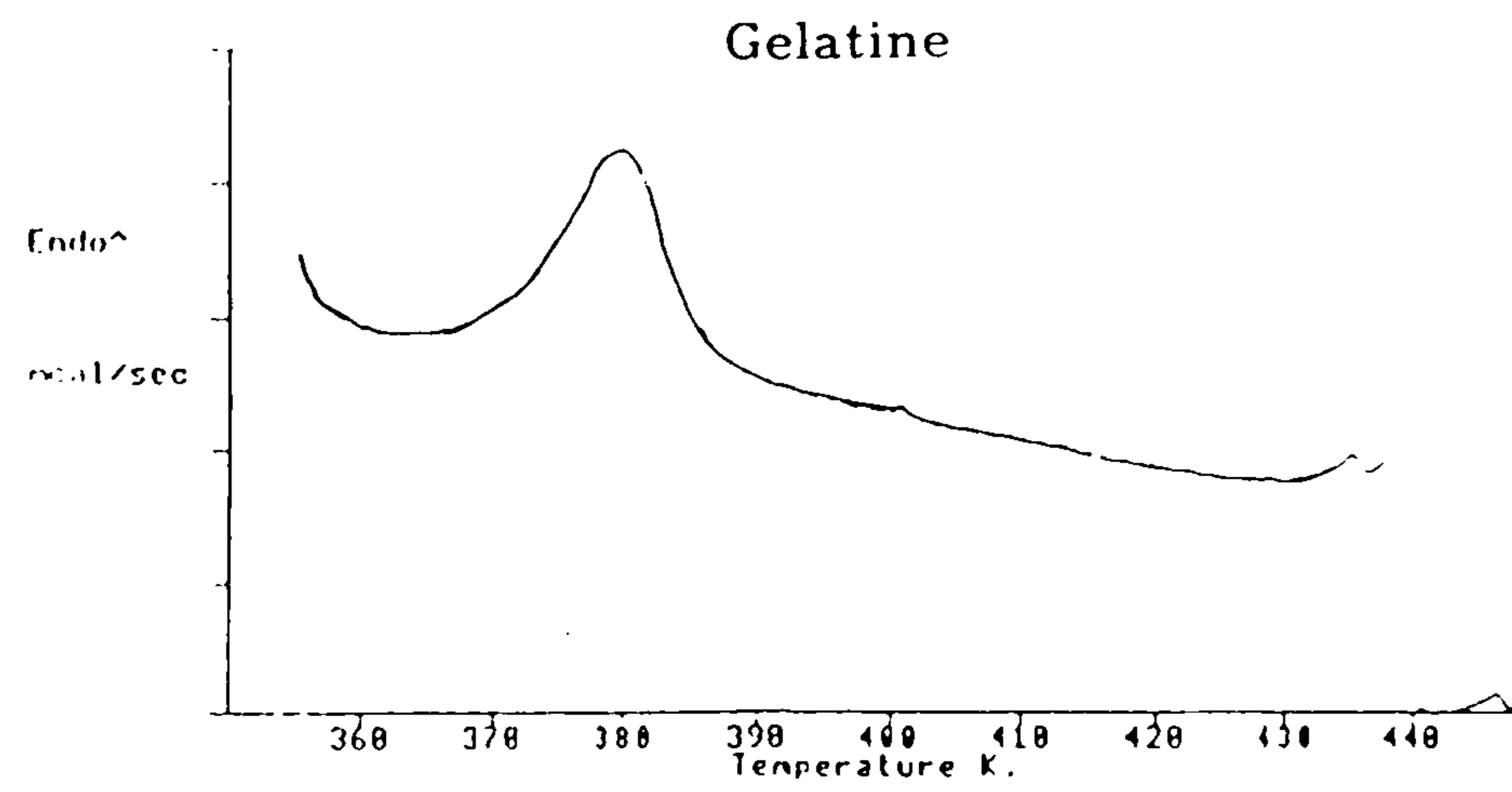


Figure 5.3.

Typical thermograms of a range of proteins, hydrated to 35% water (WSB)

Soya isolates were chosen because they were of similar pH and salt contents yet differed structurally. The laboratory prepared isolate was native, purina 860 had the 11S fraction in the native conformation but the 7S fraction was denatured whilst purina 610 was completely denatured.

5.2.3.2 Protein specificity and water creation

The amount of water generated by a range of proteins on heating is given in Table 5.4. Significances were tested by a two way analysis of variance employing a multiple comparisons test to locate the differences. For each sample values for pre and post heat treatment are shown. The moisture contents of the unheated samples containing 2 % Manucol DM were, not unexpectedly, found to be the same as for the unheated protein alone.

PROTEIN	EFFECT OF HEAT			SIG
	NO HEAT	PROTEIN	+ MANUCOL DM	
Haemoglobin	12.80±0.90(4)	13.10±1.25(4)	13.80±1.10(4)	NS
Gelatin	12.20±0.70(4)	11.70±0.26(6)	11.60±0.28(6)	NS
Casein	10.00±0.30(4)	10.00±0.17(6)	10.30±0.08(5)	NS
W.B.A.	9.80±0.23(4)	11.40±0.43(6)	10.82±0.54(5)	NS
Ovalbumen	11.40±0.31(4)	11.70±0.50(5)	11.31±0.34(5)	NS
Pancreatin	19.20±0.42(4)	19.70±0.19(5)	20.11±0.14(5)	NS
Ardex R	10.90±0.14(4)	10.70±0.47(5)	14.50±0.43(6)	*
Purina 610	8.10±0.35(4)	8.30±0.53(5)	10.20±0.45(6)	**
Purina 860	8.05±0.27(4)	8.15±0.26(4)	9.70±0.45(6)	*
Soya grit	8.70±0.10(4)	11.40±0.40(4)	13.34±0.22(5)	**
Soya flour	8.80±0.21(5)	13.70±2.20(5)	15.40±1.40(4)	**
Soya isolate	8.50±0.30(20)	12.20±0.24(20)	20.40±1.40(20)	***
Gluten	7.62±0.31(13)	9.47±0.22(13)	10.95±0.45(14)	***

Table 5.4 Apparent water content before and after heating at 458 K for 35 minutes. All values are mean ± S.E.. The sample size is shown in parentheses. SIG is the result of a 2 - way analysis of variance (Zar, 1974).

NS Not significant
 * 0.05 > p > 0.005
 ** 0.005 > p > 0.001
 *** p < 0.001

It was shown following heat treatment that samples of gluten and soya, with the exception of the commercial isolates, exhibited a significant ($p < 0.005$) increase in apparent water content. This increase was greater when 2 % Manuacol DM was included in the mix. Following the addition of this alginate to the commercial isolates a significant ($p < 0.05$) increase in apparent water content was seen for Purina 610 and 860 and Ardex R.

Of the remaining proteins tested, none showed any increase in water resulting from heat treatment either with or without Manuacol DM (Table 5.4).

A second analysis was performed with proteins at a range of a_w 's around that known to produce maximum browning (0.43 to 0.57; section 5.1). When determined by vacuum oven drying the proteins had lower water contents following heating (Table 5.5).

PROTEIN	NO HEAT	PROTEIN	+ MANUCOL DM
Water activity 0.43			
Gluten	7.20 ± 0.32	4.26 ± 0.71	6.15 ± 0.43
W.B.A	10.20 ± 0.41	6.70 ± 0.51	8.10 ± 1.75
Casein	8.40 ± 0.76	8.80 ± 1.50	7.10 ± 0.06
Gelatin	10.30 ± 0.66	10.30 ± 0.74	10.10 ± 0.63
Water activity 0.50			
Gluten	8.80 ± 0.35	5.71 ± 1.00	7.60 ± 0.49
W.B.A.	12.10 ± 0.89	11.00 ± 0.78	10.70 ± 0.40
Casein	10.30 ± 0.45	7.20 ± 0.66	7.90 ± 0.45
Gelatin	12.52 ± 0.61	11.80 ± 0.58	11.30 ± 0.54
Water activity 0.57			
Gluten	9.20 ± 0.29	3.00 ± 1.00	5.40 ± 0.56
W.B.A.	12.90 ± 0.88	11.50 ± 1.05	9.90 ± 1.20
Casein	10.20 ± 0.90	7.60 ± 0.60	8.10 ± 0.65
Gelatin	12.00 ± 0.40	11.60 ± 0.14	11.00 ± 0.75

Table 5.5 Effect of heating proteins with a range of water activities to around 430 K on the amount of apparent water measured by vacuum oven drying. Values are means ± S.E.

With the exception of gluten inclusion of Manucol DM did not produce any further water on heating. Gluten produced significantly more water on inclusion of the alginate, at the three a_w 's examined (Table 5.5).

5.2.3.3 Amino Acid Analysis

The amounts of the different amino acids found in soya isolate is shown in table 5.6.

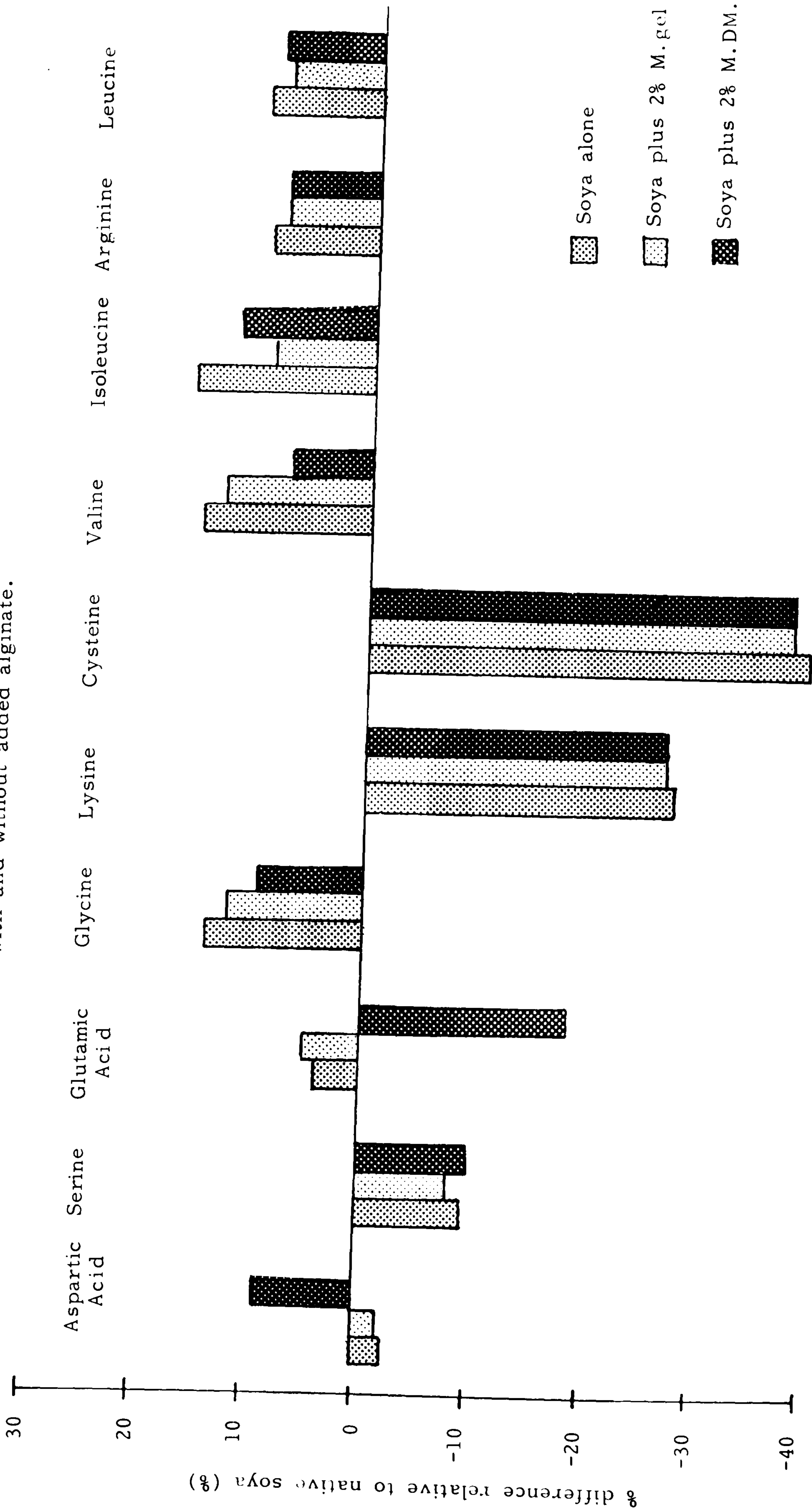
AMINO ACID	AMINO ACID CONCENTRATION (g / 100 g protein)
Alanine	4.1
Arginine	7.5
Aspartic acid	11.9
Glutamic acid	21.5
Glycine	4.2
Histidine	2.6
Isoleucine	4.9
Leucine	8.1
Lysine	6.3
Methionine	1.3
Phenylalanine	5.4
Proline	5.5
Serine	5.2
Threonine	3.7
Tryptophan	4.0
Valine	4.5

Table 5.6 Amino acid content (g / 100 g protein) of soya isolate.

After the heat treatment of the laboratory prepared soya isolate, changes in the relative concentration of certain amino acids were observed (Fig 5.4); serine, cysteine and lysine levels appeared to decrease while valine, isoleucine, alanine, leucine and glycine all appeared to increase. However the most dramatic differences were in the levels of glutamic acid between the different heated protein mixes. The heated samples containing 2 % Manuacol DM exhibited a large decrease in glutamic acid.

Figure 5.4

Changes in amino acid constituents in heat treat soya isolate with and without added alginates.



content (15 %) while the other heated protein mixes showed slight increases in this amino acid. Samples of soya in the presence of Manucol DM exhibited an increase in the level of aspartic acid which was not evident in the other heated samples.

5.2.3.4 Lysine

Values for the amounts of available lysine found in the samples are shown in Table 5.7.

NO HEAT		HEAT TREATMENT	
SOYA ALONE	SOYA ALONE	SOYA + MANUCOL	SOYA + MANUGEL
6.44 ± 0.16	4.40 ± 0.10	4.20 ± 0.08	4.48 ± 0.14
(29)	(29)	(29)	(23)

Table 5.7 Content of available lysine g / 100 g protein. Values are means ± standard error, sample size in parenthesis.

A one way analysis of variance performed on the samples of heated proteins shows there to be no significant differences ($p < 0.05$) between these heated samples.

The effect of heating is to reduce the level of available lysine in the sample by approximately 32 %, from 6.4 to 4.4 g lysine / 100 g protein.

5.2.3.5 Glutamic and Aspartic Acid Analysis

Following the heating of samples of soya isolate containing 2 % Manucol DM there was a significant loss ($p < 0.05$) of glutamic acid, no such loss was observed in heated samples of soya isolate or soya heated with 2 % Manugel GMB, (Table 5.8).

AMINO ACID	UNHEATED		HEAT TREATED	
	SOYA	SOYA	SOYA + MANUCOL DM	SOYA + MANUGEL GMB
Glutamic acid	18.8 ± 0.4	19.5 ± 0.2	7.9 ± 0.4	19.7 ± 0.2
Aspartic acid	9.1 ± 0.4	11.0 ± 0.7	14.6 ± 1.6	11.6 ± 0.8

Table 5.8 Glutamic and Aspartic acid contents (g / 100 g protein) of soya isolate before and after heat treatment at 458 K. Values are means ± standard error on 15 determinations.

The levels of aspartic acid increase when soya is heated in the presence of Manucol DM. This amino acid is increased, but to a lesser degree, in samples of heated soya and soya plus Manugel GMB (Table 5.8).

On a mole per mole basis the decrease in glutamic acid is nearly double the increase in aspartic acid, 0.074 and 0.040 M / 100 g sample respectively.

5.2.3.6 Effect of Heat on Individual Amino Acids

Heat treatment of glutamic acid in the presence of Manucol DM led to a significant ($p < 0.05$) increase in the apparent water content (Table 5.9). No such increase was seen when this amino acid was heated alone or in the presence of Manugel GMB.

AMINO ACID	INITIAL WATER (%)	INCREASE IN PERCENTAGE WATER CONTENT ON HEATING		
		ALONE	+ MANUCOL DM	+ MANUGEL GMB
Glutamic acid	8.5	0.7 (-0.2 to 1.4)	3.2 (2.0 to 5.5)	1.0 (-2.0 to 1.7)
Aspartic acid	3.5	0.0 (-1.8 to 1.1)	0.0 (-2.0 to 1.5)	0.0 (-2.3 to 1.3)
Lysine	5.5	0.7 (-1.7 to 1.4)	0.7 (-1.3 to 2.3)	0.7 (-1.0 to 2.1)
Glycine	3.0	0.0 (-0.2 to 1.0)	0.0 (-1.6 to 1.0)	0.0 (-1.8 to 1.0)

Table 5.9 Apparent increase in water content (% water, wsb) of selected amino acids in the presence or absence of alginate after heating to 440 K for 35 minutes. All values are the means of 10 determinations. Ranges are shown in parenthesis.

Aspartic acid and glycine did not produce significant

amounts of water following heating, but lysine appeared to produce small amounts of apparent water in all three systems (Table 5.9).

5.2.3.7 Solution Studies

Browning was initiated by the heated Manuacol DM samples in all of the amino acid solutions investigated, greatest browning was generated in the glycine solution. When heated Manugel GMB was added to the solutions there was significantly less browning in all solutions than seen with Manuacol DM. Significantly the solution of glutamate did not brown in the presence of heated Manugel GMB (Table 5.10).

ALGINATE	GLYCINE	LYSINE	GLUTAMATE	GLUTAMATE + LYSINE
Manuacol DM	28.0 ± 9.4	19.5 ± 2.4	8.0 ± 0.3	10.0 ± 3.1
Manugel GMB	5.5 ± 1.1	3.0 ± 1.1	0.0 ± 0.3	5.0 ± 2.0

Table 5.10 Effect of heat degraded alginate on browning in some 2M amino acid solutions, measured at 420nm after incubation at 373 K. Values are means of absorbance per gram ± standard error of 15 determinations.

5.2.4 Discussion

When most proteins hydrated to a_w 's in the range 0.43 to 0.57 are heated, the apparent moisture content, as determined by vacuum oven drying, is reduced. This suggests that in some way the water in the proteins has become entrapped, most probably as the result of the formation of

an aggregate or gel network. The removal of water from food systems that is entrapped in capillary networks is notoriously difficult. Interestingly gluten did produce more water following the inclusion of Manucol DM prior to heating, at all a_w 's examined.

Following Karl Fischer analysis, in which the water is liberated from the network by solubilisation in formamide, no loss of water was apparent in any protein mix. However some samples led to the apparent formation of water on heating. These were gluten, laboratory prepared soya isolate, soya flour and soya grits. The three forms of soya produce similar amounts of water ($\approx 30\%$ more) following heating which is substantially more than the apparent increase seen with gluten (approximately 19%). If the formation of this water is the result of browning / condensation type reactions it might be expected that the soya flours and grits would produce more water than the laboratory prepared soya isolate, as they possess greater carbohydrate contents.

Interestingly the commercial soya isolates did not increase their apparent moisture contents after heating, indicating that in some way during processing they have undergone steric / chemical changes. The exact nature of these reactions are not known.

All soya preparations, including the commercial isolates and gluten produced further quantities of apparent water after heating in the presence of Manucol DM. This

phenomenon was not observed with any of the remaining proteins. It would seem that the presence of the alginate initiated reactions in addition to those occurring in the samples heated alone. This contention is supported by the fact that the commercial isolates only produce water in the presence of the alginate.

It is apparent that proteins that are relatively easily extruded, soya and gluten, are able to form water on heating. Examination of the degree of denaturation and a range of other characteristics failed to suggest any relationship between these characteristics and water formation. For example, the commercial isolates examined had different degrees of native structure but no correlation could be found between the level of denaturation and the production of water. Possible relations between the degree of denaturation and intensity of reaction may have been expected, as the lack of reactivity of the commercial isolates could possibly be explained by supposing that steric effects limit the reaction.

Amino acid analysis of the different heated and unheated soya preparations produced results that were generally in good agreement with those published in the literature. Heating of pure proteins will lead to losses of nitrogen digestibility, but when amino acid analysis is performed on acid hydrolysed material only small losses are evident, these are usually in lysine. Acid or alkaline hydrolysis can lead to breakage of sugar amine bonds and

other linkages.

The amino acid most susceptible to loss as a result of thermal processing is lysine as this is the major source of free amino groups (Hannan and Lea, 1952). Overheating of soyabean protein causes some destruction of lysine, arginine and cystine (Rio Iriarte and Barnes, 1966). Badenhop and Hackler (1971) roasted soyabeans at 343, 353 and 358 K at water contents above 43 % . They found tryptophan to be the most heat labile, its content decreasing by some 35 % . Decreases were also seen in the lysine, cystine and histidine contents, 17, 15 and 6 % respectively; a slight drop of 4 % in serine was noted. Glutamic acid, proline, alanine and isoleucine all increased following roasting. These results are in good agreement with those obtained in this study with the exception that there was no change in the level of tryptophan ,histidine or proline. But increases in glycine, valine and leucine were found. It must be noted that the study by Badenhop and Hackler (1971) involved the use of soyabeans which had not been defatted, thereby the potential formation of lipid - protein interactions existed. Cheftel (1986) has found a loss in serine following extrusion of soya which he states is the result of non - enzymic browning. It is the loss of the serine and lysine that may be responsible for the production of water in the protein alone system.

No distinction from amino acid analysis can be made between aspartic or glutamic acids and their amides as the

amide group will be hydrolysed during hydrolysis. According to Catsimpoolas, et al., 1971b) the glutamyl and aspartyl residues in the acidic subunits of the soya protein will be present as the acid form whilst in the basic units they will be present as the amide.

Numerous workers have ascertained that losses of lysine can exist in carbohydrate free systems, Bohak (1964); Sternberg etal. (1975) and Carpenter & Booth (1973). Exact mechanisms are difficult to postulate due to the multiplicity of functional groups which may lead to further reactions. Initial reaction steps may occur due to interaction of the ϵ -amino group of lysine with either the carboxyl group of aspartic / glutamic acid with the subsequent formation of water (Meham & Olcott, 1947, Lorient, 1977) or with the amide groups of asparagine / glutamine with the formation of ammonia (Bjarnason & Carpenter, 1970). The extent of either reaction will depend upon the number or accessibility of the acid or amide groups. According to Bjarnason & Carpenter, (1970) the reaction between carboxyl groups and amino groups will be thermodynamically less feasible, but the temperatures used in this study are higher than they used. The production of substantial amounts of water would tend to point to possible intervention with the acid groups. The resulting ϵ - (γ - glutamyl) - lysinyl crosslink which may be formed between inter or intra peptides may undergo further reaction in such a manner that the lysine residue is consumed, as both amino

acids can be recovered following acid hydrolysis, or lysine also takes part in other reactions as lysine losses are still significant. For example lysine losses may occur as a result of the formation of novel cross - linking amino acids, for instance lysinoalanine (LAL) and lanthionine (LAT). Following the extrusion of field bean and soyabeans Jeunink & Cheftel (1979) could find no evidence on the formation of LAL or LAT. But in the fieldbean there was evidence of the presence of ϵ -(γ - glutamyl) - lysine. In contrast a further study (Cheftel, 1986) reported that ϵ -(γ - glutamyl) lysine could not be detected in samples of commercially extruded TVP. No indication is given as to the method of analysis or the conditions under which extrusion was performed. The presence and detection of small amounts of these crosslinks is likely to be highly dependent upon these conditions and will be very difficult to detect within the many interactions present in textured protein.

Following heat treatment cysteine is lost in quite large proportions (approximately 50 % in this study). Carbonyls formed during degradation of cysteine may bind some of the amino groups of lysine, but this value will probably be quite small due to the low cysteine content in soya.

The losses of serine may either be due to crosslinking with alanine to form lysinoalanine (Sternberg et al, 1975) or serine may be lost as a result of β - elimination of water.

The most common method for measuring the available lysine is to 'tag' the α -amino group with 1-fluoro-2,4-dinitrobenzene (FDNB) (Carpenter, 1960, Booth, 1971).

Later methods are based on the work of Kakade & Liener (1969) which uses as the tag 2,3,6-trinitrobenzene sulfonate (TNBS), the method used was modified by Ousterhout & Wood (1970). This method was used as it was quicker and cheaper to perform.

The TNBS method and autoanalysis both gave values for lysine content in good agreement, yielding a loss of around 32% after heat treating soya, a value which does not depend on the presence of alginate.

The loss of glutamic acid and increase in aspartic acid following the inclusion of 2% Manuacol DM was substantiated following biochemical assay. Discrepancies are evident between the two methods. When the values for glutamic acid in heated soya plus Manuacol DM are examined, auto-analysis showed a difference of 15% and following biochemical analysis differences of 58% were found. Similarly with aspartic acid, samples containing Manuacol DM are 9.7 or 33% higher in this residue, depending upon the method used for their determination. The biochemical method gives the higher value.

Glutamic acid - Manuacol DM crosslinks may be more stable to hydrolysis during sample preparation for the biochemical method than the auto analysis.

Using data obtained by enzyme assay the decrease in glutamic acid is nearly double the increase in aspartic acid on a mole to mole basis. It is interesting that a similar ratio exists following autoanalysis (2.9 and 2.75 respectively). It is very tempting to suggest that the formation of aspartic acid is brought about by the loss of one carbon atom from glutamic acid as a result of interaction with alginate.

The biochemical method for glutamic acid analysis is based on the enzymic breakdown by glutamic dehydrogenase to α -ketoglutarate with concomitant formation of reduced NADPH, which can be measured by determining its absorbance at 340 nm (Fig 5.5).

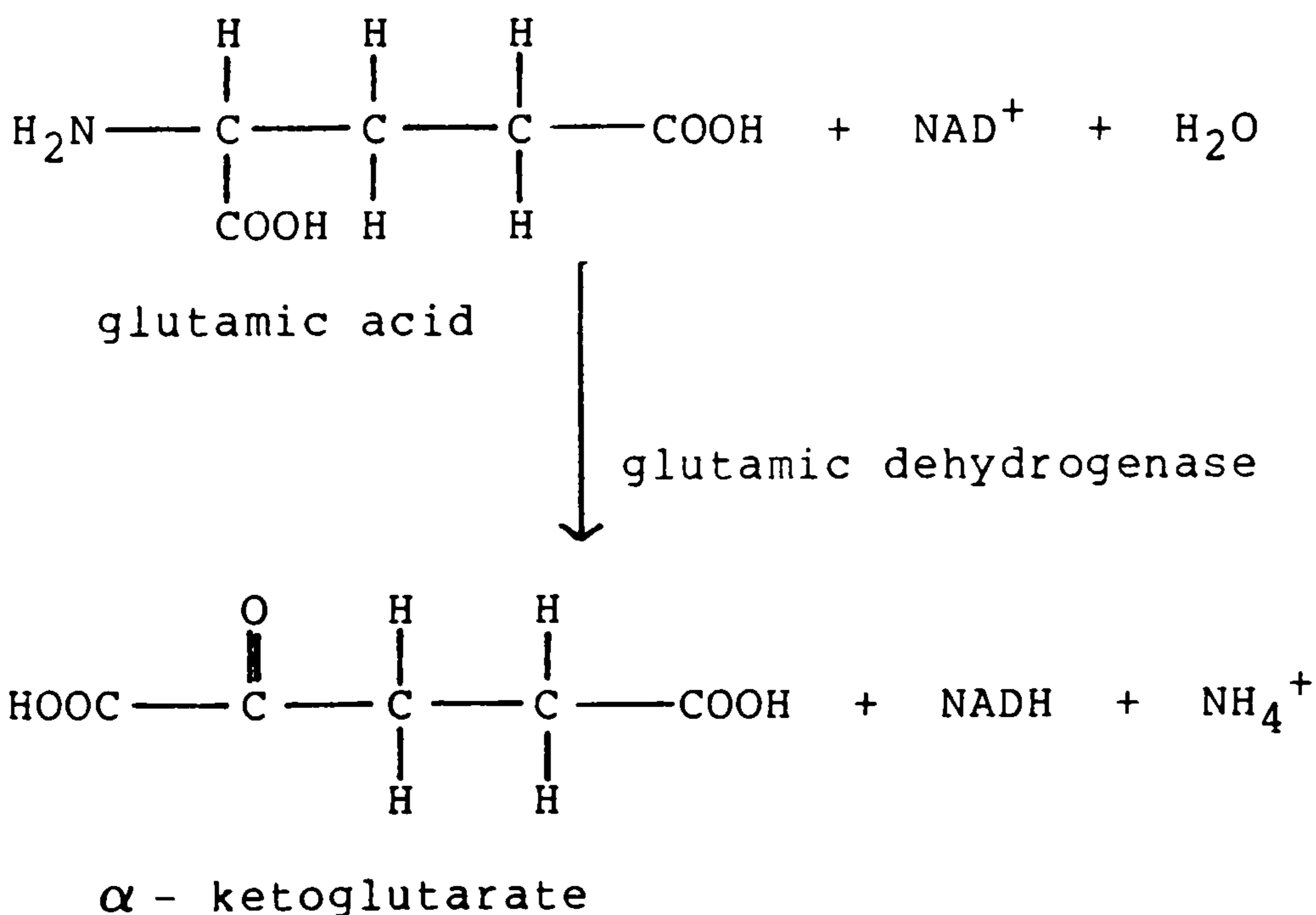


Figure 5.5 Glutamic acid analysis.

Aspartic acid is measured by first converting it to

steric effects may possibly prevent similar reactions with the carboxyl group of aspartic acid.

Further evidence for glutamic acid involvement can be derived from the solution studies using heated alginate samples. Only those solutions of glutamic acid that had Manucol DM present were able to undergo browning.

Rosenthal (1978) and Miki (1974) have implicated glutamic acid in browning systems. In these studies a range of heat regimes were employed. Miki (1974) has demonstrated a browning reaction in tomato juice that is dependent upon the presence of fructose, galacturonic acid and glutamic acid, whereas the browning system described by Seaver and Kertesz (1947) between uronic acids and amino acids is dependent upon the presence of both a carboxylic acid group and a free carbonyl group. Conversely, over a range of heating conditions Nafisi & Markakis (1983) showed that aspartic and glutamic acids will inhibit the formation of brown pigments; unfortunately this report is not very detailed and exact comparisons cannot be made.

To summarise this chapter has shown that following the heating of proteins some proteins, soya and gluten, will undergo specific reactions that lead to the formation of increased amounts of apparent water. A further specific reaction with the alginates, especially those high in mannuronic acid has been demonstrated. This reaction leads to the formation of further amounts of apparent water, an involvement of the glutamic acid residues is implicated.

HEAT DEGRADATION OF ALGINATE

6.1 Effect of Heat on Alginate

6.1.1 Introduction

Previous work has shown that the reaction between soya and alginate, leading to the formation of water is specific to alginates rich in mannuronic acid residues. It was thus decided to investigate the effect of heat on alginates of different mannuronic to guluronic acid contents. Initially the breakdown of the alginate (depolymerization) and its subsequent reaction with amino acid solutions was studied. As browning / condensation reactions, which were assumed to be taking place, require reducing groups it was also decided to evaluate the reducing powers of the heated alginates and thus obtain an indication of the number of reducing end groups.

Heat degradation of sugars will lead to the production of products which may enter into browning type reactions and thus GC-MS was used to enable the breakdown products of the polysaccharides to be identified and gel permeation chromatography was used to look at the degrees of depolymerisation. It was hoped to determine whether the different reactivities were the result of differences in the availability of the reducing groups or to different levels of depolymerisation.

6.1.2 Materials and Methods

Alginate samples were donated by Kelco International

Ltd. and are described in section 3.1.2.

Sephacryl S400 (lot S9759), sodium borohydride (lot S9125), dimethyl sulphoxide (lot D 5879), methylimidazol (lot M8878), acetic anhydride (lot A6404), dichloromethane (lot 17-3), myo-inositol (lot 15125), lysine (lot L5626), sodium glutamate (lot G1626), neocuproine hydrochloride (lot N1626) and all standard sugars were obtained from Sigma Chemicals Co.Ltd.

Ammonia (880), glacial acetic acid, urea, sulphuric acid, glycine and cupric sulphate pentahydrate were all obtained from Fisons, Ltd., all were analar grade.

Aspartic acid as the free base was obtained from Hopkin & Williams, Ltd.

Sodium borohydride solution was made by dissolving 2.00g sodium borohydride in 100 ml anhydrous dimethyl sulphoxide at 373 K.

Solution A consisted of 40.0 g anhydrous sodium carbonate, 16.0 g glycine and 450 mg cupric sulphate pentahydrate dissolved in 600 ml distilled water and made up to 1 litre with distilled water.

Solution B consisted 0.15 g of neocuproine hydrochloride dissolved in 100 ml distilled water. This solution was stored in the dark.

6.1.2.2 Sample Preperation

Samples of alginate (approximately 0.5 g) were sealed in glass ampoules (approximately 2 ml) and heated for 35

minutes at temperatures between 413 and 453 K.

6.1.2.3 Colour Formation

Samples of alginate (approximately. 0.3 g) were sealed in glass ampoules (approximately 2 ml) and heated in a convection oven at temperatures in the range 413 to 463 K for 35 minutes. Samples were dissolved in 0.1M amino acid solutions as described in section 5.2.2.8 and the absorbance at 420 nm measured after 60 minutes at 373 K.

6.1.2.4 Reducing Power

Samples of alginate were heated in sealed glass ampoules. The glass ampoules were broken under 5 ml distilled water. After dissolution of the alginate a further dilution of 1 in 6 was made.

200 μ l of the diluted sample was added to 400 μ l of solution A and 400 μ l of solution B and the contents mixed. Solutions were heated at 373 K for exactly 12 minutes, cooled rapidly in ice and 1.0 ml of distilled water added. The absorbance of the mixed solutions was measured at 450 nm using a Pye Unicam SP600 Series 2 spectrophotometer.

The number of reducing end groups was calculated assuming the moles of reduced copper were stoichiometric with reacting end groups, the number of end groups was thus found from:

$$\text{Number of end groups} = \text{moles end group} \times N_A$$

where N_A is Avogadro's number = 6.022×10^{23}

The number average molecular weight (\bar{M}_N) was calculated from:

$$\bar{M}_N = \frac{1}{\text{moles of reduced glucose / g sample}}$$

assuming that 1 mole of alginate has the reducing equivalent of 1 mole glucose.

6.1.2.5 Gel Permeation Chromatography

Approximately 7 mg of sample was dissolved in 10 ml mixed phosphate buffer (pH 6.5, ionic strength 0.3). Following deaeration a 5 ml aliquot was applied to a LKB 2137 column (1.6 cm internal diameter * 75 cm gel bed height) packed with Sephacryl S400 (bead size [wet] 40 - 150 μm). A mixed phosphate and NaCl buffer (pH 6.5, ionic strength 0.3) was used at a flow rate of approximately 10 ml / hr. 2 ml fractions were collected by a Atto mini collector.

Sugar contents of each fraction were determined by mixing 1 ml of sample with 1 ml 5 % phenol and 5 ml

concentrated sulphuric acid. After cooling the absorbance was determined at 485 nm using a Pye Unicam SP600 series 2 spectrophotometer. Sugar concentrations were determined from a calibration graph for glucose.

Molecular weight distribution was plotted using the calibration graph (Fig 6.18) determined by Ball, et al. (1988).

Weight average (\bar{M}_W) and number average (\bar{M}_N) molecular weights were determined after assessing the sugar concentration for each fraction and the molecular weight of the fragments in that fraction (assumed to be constant and taken at the volume eluted at the end of collection of that fraction). Weight average molecular weight was thus determined from: Where c = concentration.

$$\bar{M}_W = \frac{\sum (mwt \times c)}{\sum c}$$

The number average molecular weight was determined from:

$$M_N = \frac{\sum c}{\sum (c / mwt)}$$

and the polydispersity index (PI) was calculated from:

$$PI = \frac{\bar{M}_W}{\bar{M}_N}$$

6.1.2.6 GC-MS

Heated and unheated alginate samples (0.06 g) were hydrolysed in 15 ml of 3 % nitric acid containing 0.05 %

w/v urea at 373 K for 16 hours. After heating all solutions were neutralised with 15 M ammonia and evaporated to dryness at a temperature below 333 K. Samples were subsequently redissolved in 2.0 ml 1 M ammonia and 0.05 ml of 200 mg / ml myo - inositol was added as an internal standard.

Derivatization to form alditol acetates was by the method of Blakeney, et al. (1983). 0.1 ml of the sample was mixed with 1 ml sodium borohydride solution and incubated for 90 minutes at 313 K (for some of the samples this reduction step was repeated). After reduction excess sodium borohydride was removed by the addition of 0.1 ml of 18M ammonia. To the solutions 0.2 ml of 1 - methylimidazole was added followed by 2 ml acetic anhydride, after mixing they were left at room temperature for 10 minutes and 5 ml of distilled water added. When cool 1 ml of dichloromethane was added. The tubes were mixed with a vortex mixer and after the phases had separated the lower one was removed and stored in a 1 ml septum cap vial at 253 K.

The alditol acetates were separated on a 25 M BP20 capillary column:

Bonded phase	vitreous silica
Polarity	polar
Film thickness	0.25 um
Internal diameter	0.22 mm
Outer diameter	0.33 mm

The column was fitted to a Perkin Elmer Sigma 3B

chromatograph equipped with a Hewlett Packard 5970 series Mass Selective Detector. 1.5 μ l of sample were injected via a SGE splitless injector system and data collected and analysed on a Hewlett Packard Chem. Station. The oven temperatures were;

Initial Temp.	453 K for 4 mins.
Ramp rate	2 K / min.
Maximum Temp.	493 K for 20 mins.

6.1.3 Results

6.1.3.1 Colour Formation

Following treatment at very high temperatures (>430 K) excessive charring of the alginates occurred, which prevented any reliable measure of browning to be made when placed in the amino acid solutions.

Manucol DM (MDM) induced browning in all the amino acid solutions although glutamate did not react unless the alginate had been heated to 413 K (Table 6.1). The degree of browning increased with increasing temperature. Manugel GMB (M GEL) only induced browning after heating to temperatures higher than those needed with Manucol DM (Table 6.1). None of the heated Manugel GMB samples induced browning in the glutamate solution.

TEMP K	SAMPLE	Amino Acid			
		Glycine	Lysine	Glutamic acid	Aspartic acid
		pH 5.2	pH 5.4	pH 6.5	pH 6.3
373	M.DM	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
	M.GEL	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
393	M.DM	2.2 ± 0.2	2.0 ± 0.1	0.0 ± 0.1	2.9 ± 0.3
	M.GEL	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
413	M.DM	2.4 ± 0.1	2.5 ± 0.5	0.4 ± 0.1	4.5 ± 0.5
	M.GEL	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 1.0
423	M.DM	3.9 ± 0.8	4.4 ± 1.1	3.2 ± 1.3	3.3 ± 0.9
	M.GEL	0.0 ± 0.5	2.4 ± 1.0	0.9 ± 0.2	3.0 ± 0.5
428	M.DM	28.0 ± 9.4	19.5 ± 2.4	8.0 ± 0.3	10.0 ± 3.1
	M.GEL	5.5 ± 1.1	3.0 ± 1.1	0.0 ± 1.0	5.0 ± 2.0

Table 6.1 Increase in absorbance at 420 nm / g of alginate, preheated to the temperatures shown and held in 0.1 M amino acid solution at 373 K for 1 hour. All values are the means ± standard error of 15 determinations.

6.1.3.2 Reducing Power

The reducing power of the heated alginate samples were compared against standard glucose solutions. Native samples of Manugel GMB had significantly ($p < 0.05$) greater reducing power than Manucol DM (24% higher). Heat

treatment of the samples at temperatures greater than 443 K, led to Manuacol DM becoming the more powerful reducing agent ($p < 0.05$). At temperatures between 433 and 453 K the reducing power of both the alginates rose dramatically (approximately 300%).

Assuming that only the terminal reducing end groups can take part in this reaction, the number of such groups may be determined by calculating the number of molecules in the solution. For both the alginates used, the calculated number of end groups after heat treatment increased, the values were much greater for Manuacol DM (Fig 6.6). A value for \bar{M}_N was calculated assuming each reducing end group represented one molecule (Table 6.3).

GLUCOSE EQUIVALENCE		
TEMPERATURE / K	MANUCOL DM	MANUGEL GMB
Native	5.30 ± 0.16	7.00 ± 0.26
413	6.21 ± 0.32	6.83 ± 0.12
433	9.99 ± 0.32	7.14 ± 0.35
453	40.00 ± 1.22	20.66 ± 1.00

Table 6.2 Effect of heat treatment on the reducing power of alginates, before and after heat treatment. Values are means (glucose equivalence [μ moles glucose / g sample]) ± S.E of 12 values.

Figure 6.1.

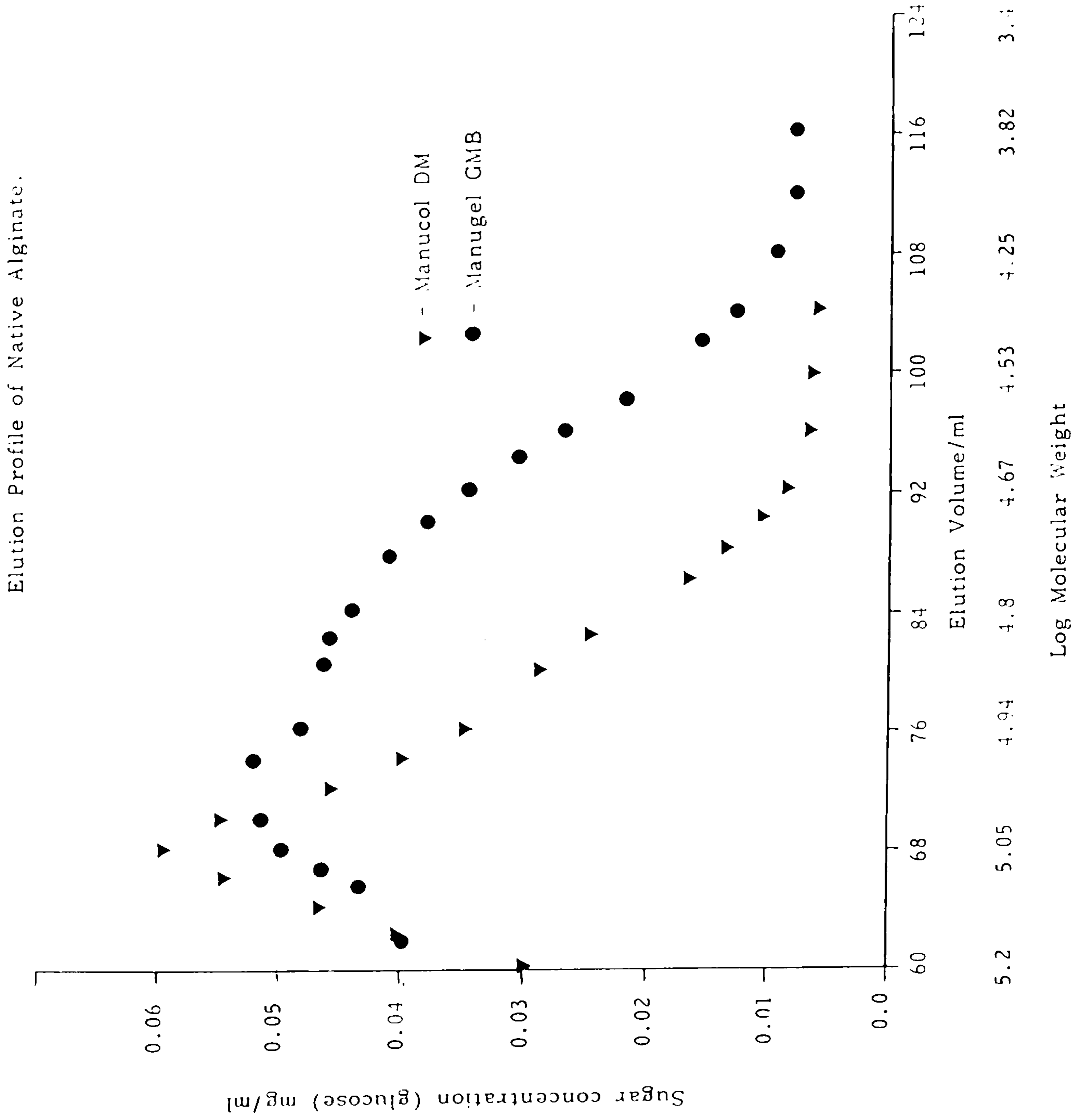


Figure 6.2.

Elution Profile of Alginate Heat Degraded for 35 minutes at +13 k.

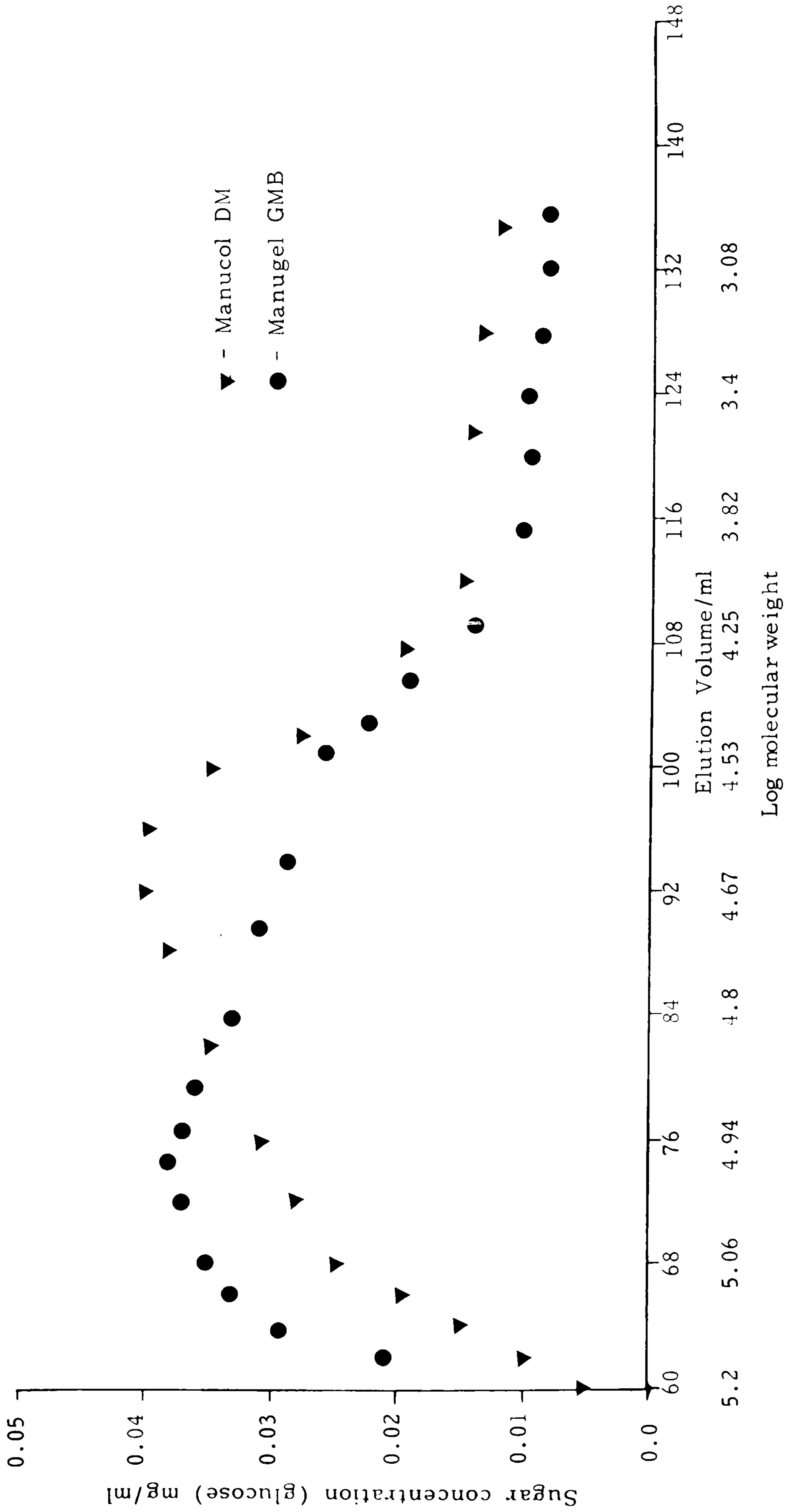


Figure 6.3.

Elution Profile of Alginate Heat Degraded for 35 minutes at 433 k.

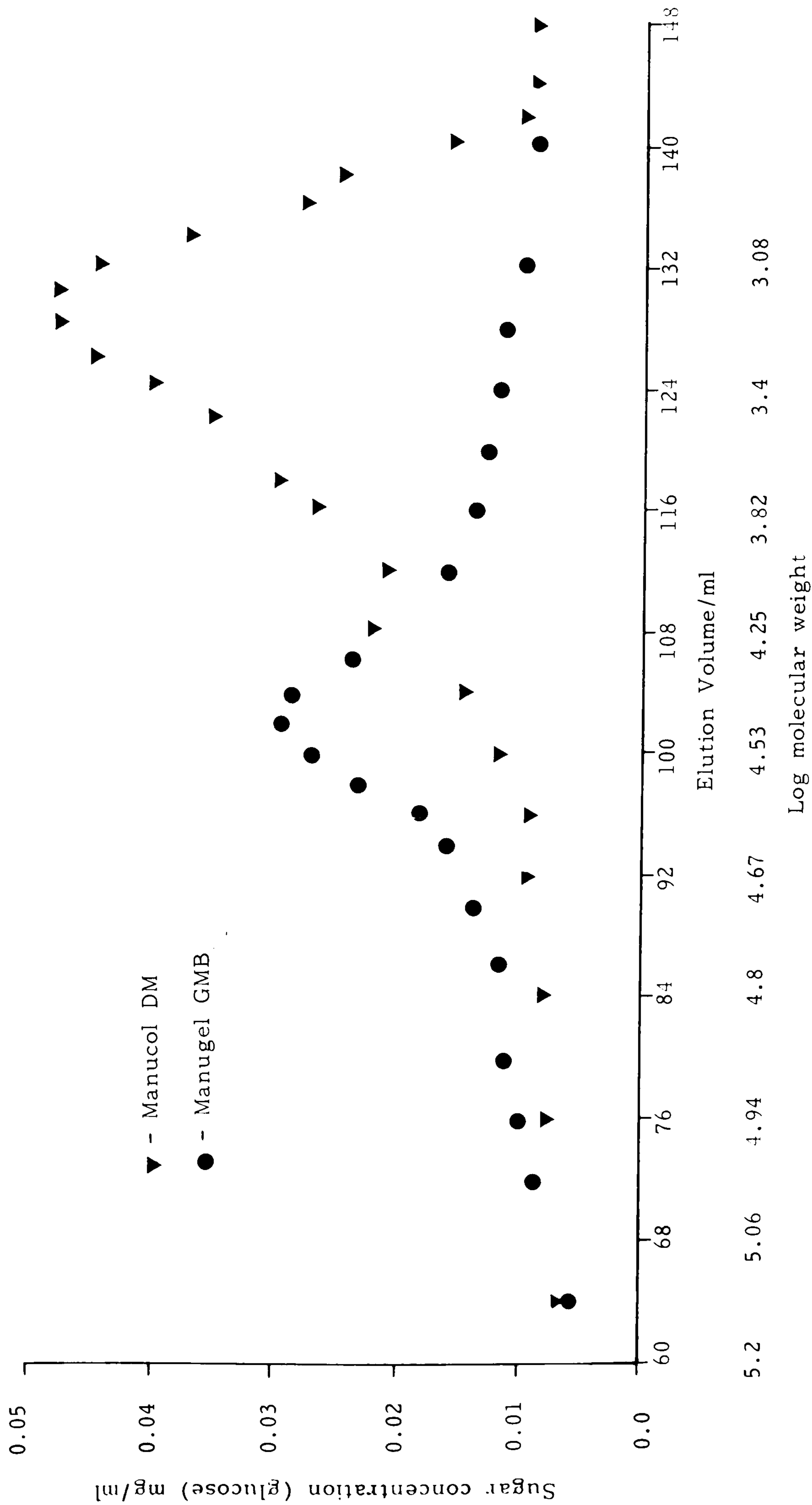


Figure 6.4.

Elution Profile of Alginate Heat Degraded for 35 minutes at 453 k.

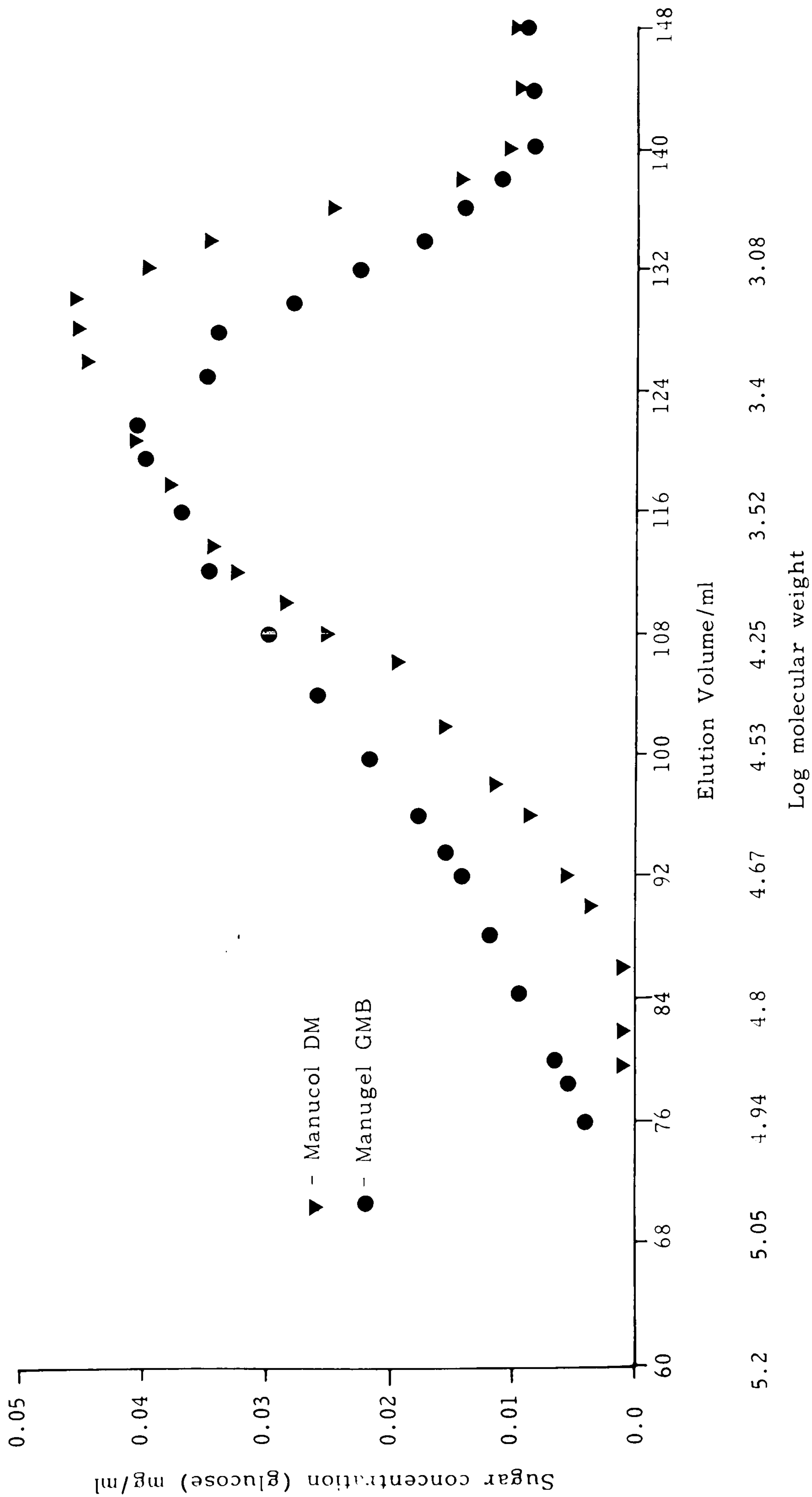
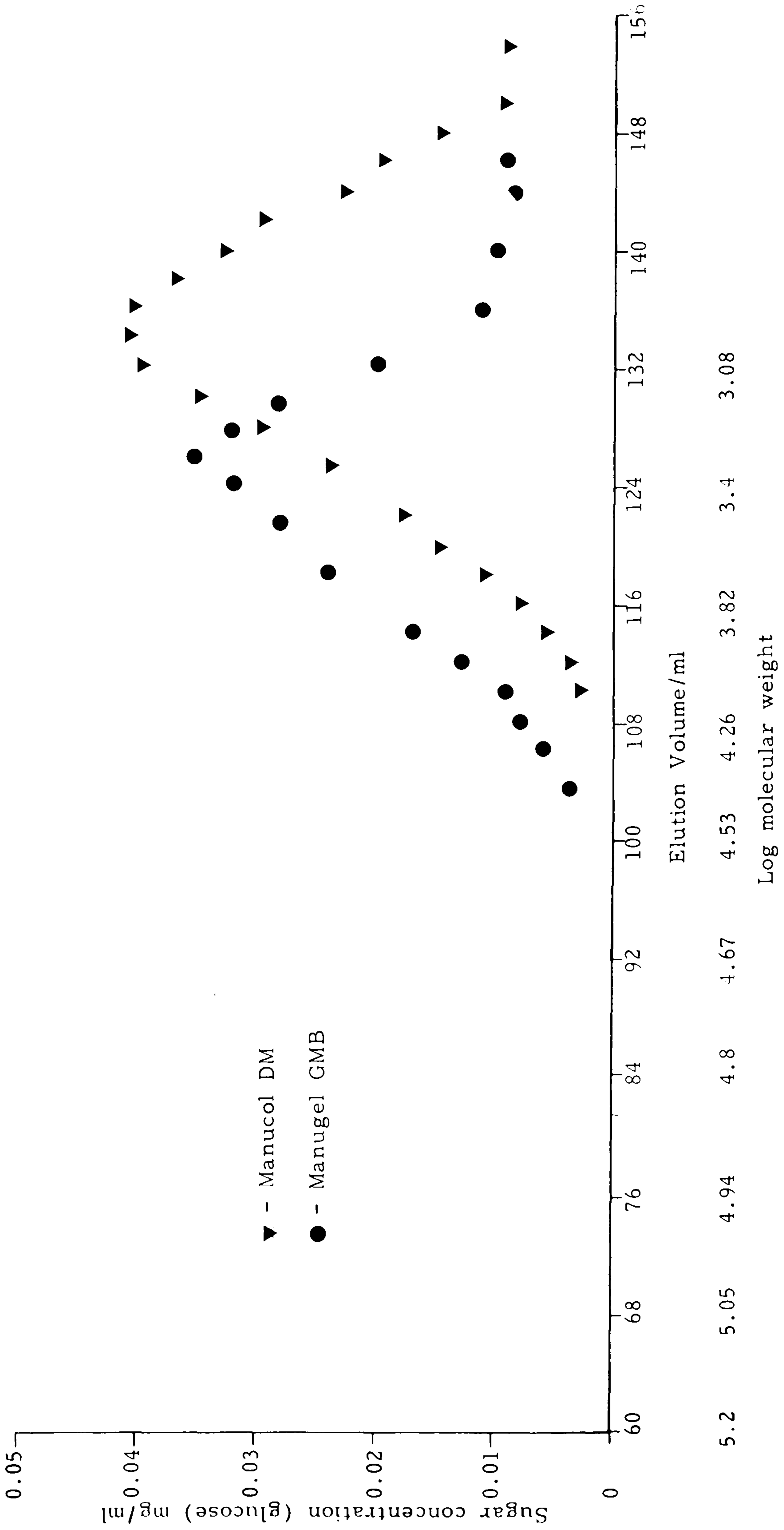


Figure 6.5.

Elution Profile of Alginate Heat Degraded for 35 minutes at 478 k.



6.1.3.3 Gel Permeation Chromatography

Molecular weight distributions (MWD) of native and heat degraded alginates are shown in Fig 6.1 to 6.5. The weight average molecular weight (\bar{M}_W), number average molecular weight (\bar{M}_N) are given in table 6.3. It is apparent that with increasing heat treatment the molecular weight decreases, though Manucol DM does so at a far greater rate. Generally samples of Manugel GMB are more polydisperse compared to Manucol DM. On initial heating both alginates exhibit increased polydispersity but subsequent heating leads to a decrease in polydispersity (Table 6.3) for samples of Manucol DM.

HEAT TREATMENT	SAMPLE	\bar{M}_N^*	\bar{M}_W	\bar{M}_N	PI
Native	MDM	189,000	109,400	102,400	1.06
	MGEL	143,000	90,400	70,000	1.29
413	MDM	161,000	52,000	17,000	3.08
	MGEL	146,000	65,600	28,000	2.34
433	MDM	100,000	3,500	2,150	1.60
	MGEL	140,000	31,000	10,900	2.80
453	MDM	25,000	3,400	1,900	1.80
	MGEL	48,000	11,400	4,750	2.40

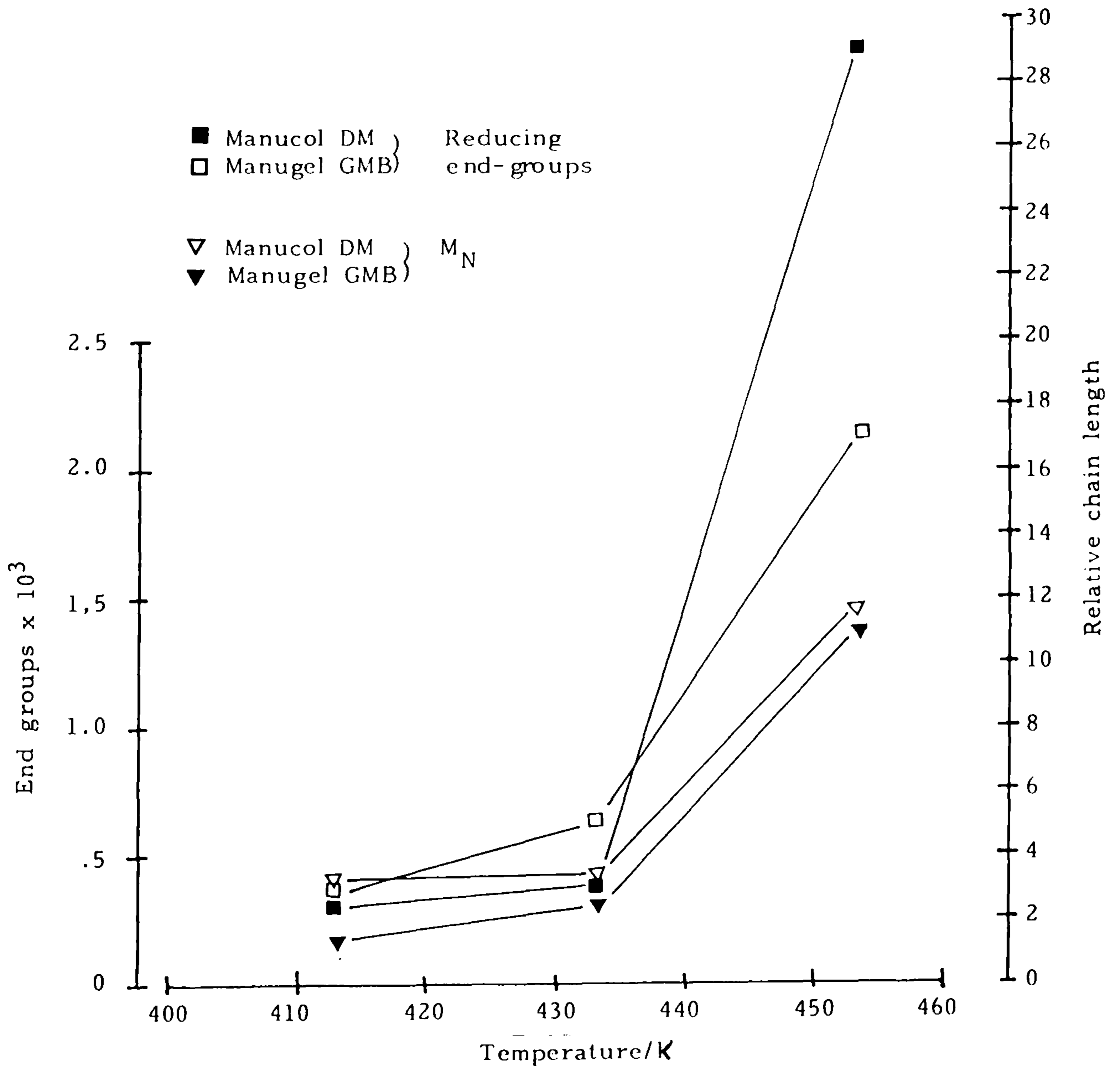
Table 6.3 Weight average, number average molecular weights and polydispersity index for samples of native and heat treated (35 minutes) Manucol DM and Manugel GMB.

* Determined by end group analysis.

Assuming that a decrease in \bar{M}_N reflects a proportionate number of polysaccharide scissions the relative increase in the number of fragments compared to the native material can be calculated (Fig 6.6). It can be seen that with increasing number of chain scissions calculated from \bar{M}_N (MWD) the expected increase in the number of end groups is far greater than when determined by end group analysis.

Figure 6.6.

Effect of heat treatment on the number of reducing end groups and relative chain numbers as determined from \bar{M}_N for Manucol DM and Manugel GMB.



6.1.3.4 GC / MS

Prior to sample analysis the extent of acid hydrolysis was estimated by gel permeation chromatography (Fig 6.7)

Typical chromatograms obtained from hydrolysed alginates are shown in Fig 6.8 and 6.9 . Of the peaks four had mass spectra typical of sugars (section 3.4.3). Further analysis against the retention times of standard sugars confirmed identification of three of the compounds (Table 6.4), rhamnitol, mannitol and glucitol. Comparison of the fourth peak (T_R 0.66) with a range of standards (Appendix I) and the retention times of sugars quoted in the literature (Blakeney, et al., 1983) would suggest that it is one of the *aldopentose* sugars.

Other unidentified components were found in varying and non reproducible amounts.

Figure 6.7.

Elution profile of acid hydrolysed Manuocol DM.

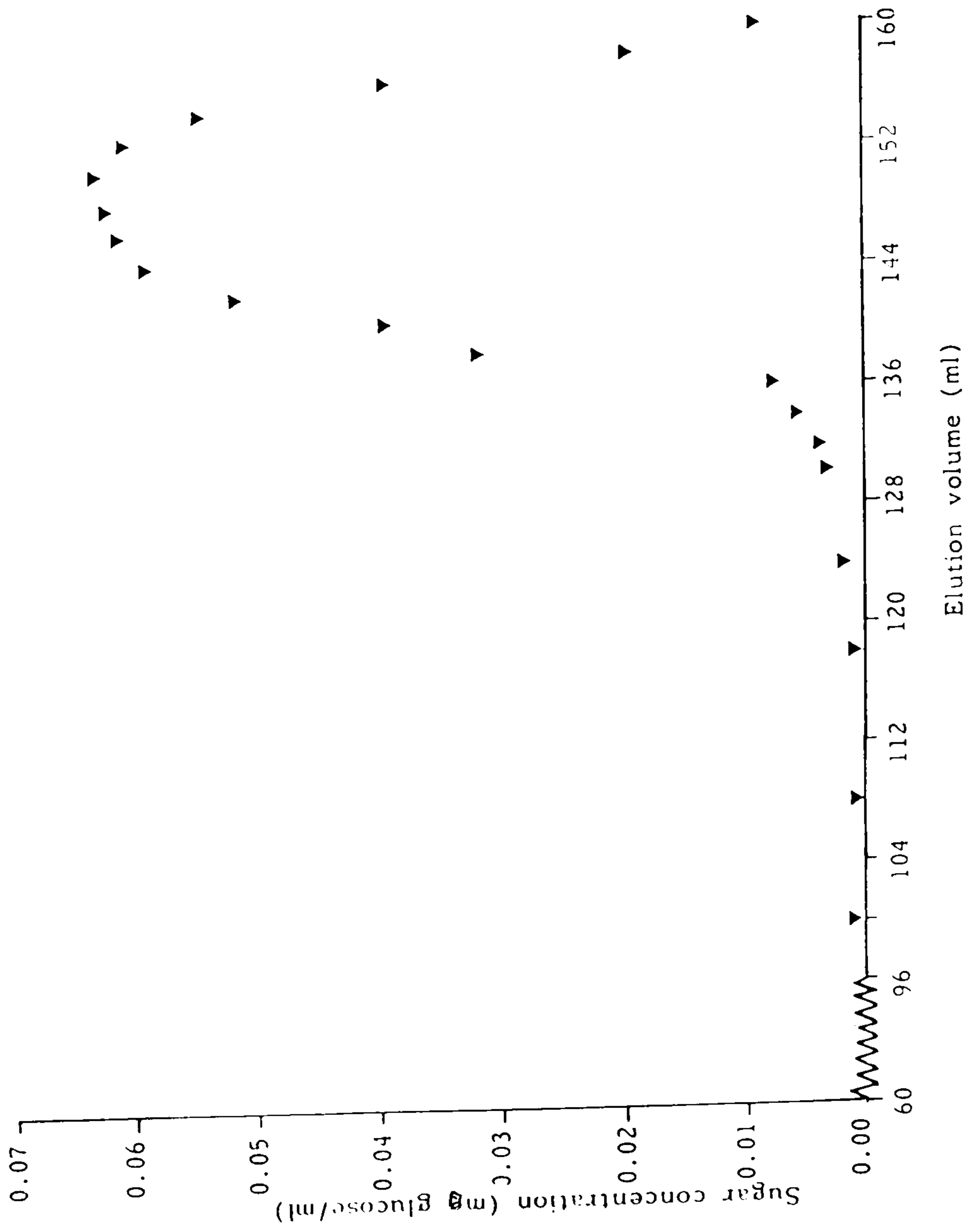


Figure 6.3.

Total ion chromatograph of heat treated (+53 k) Manuocol DM.

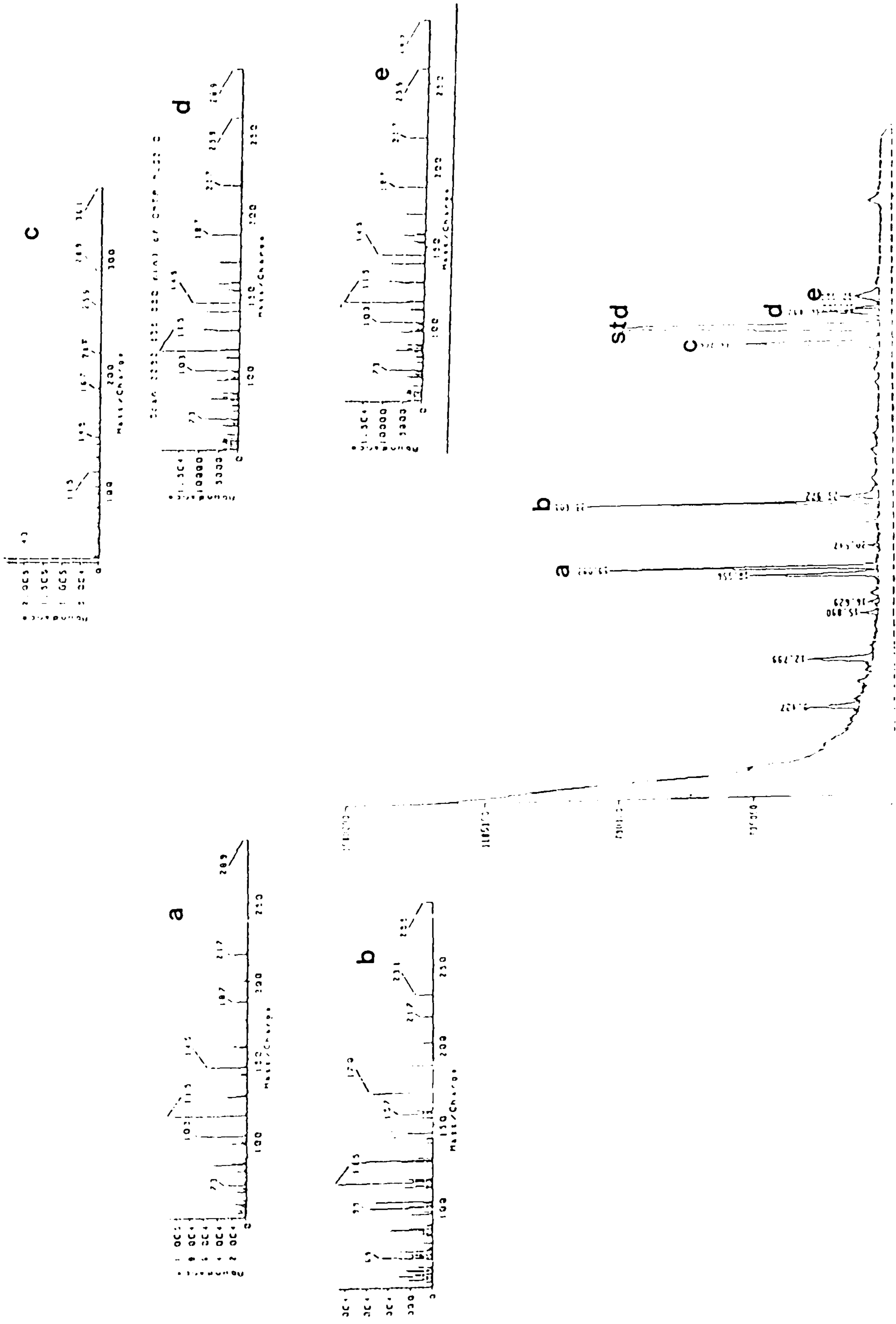
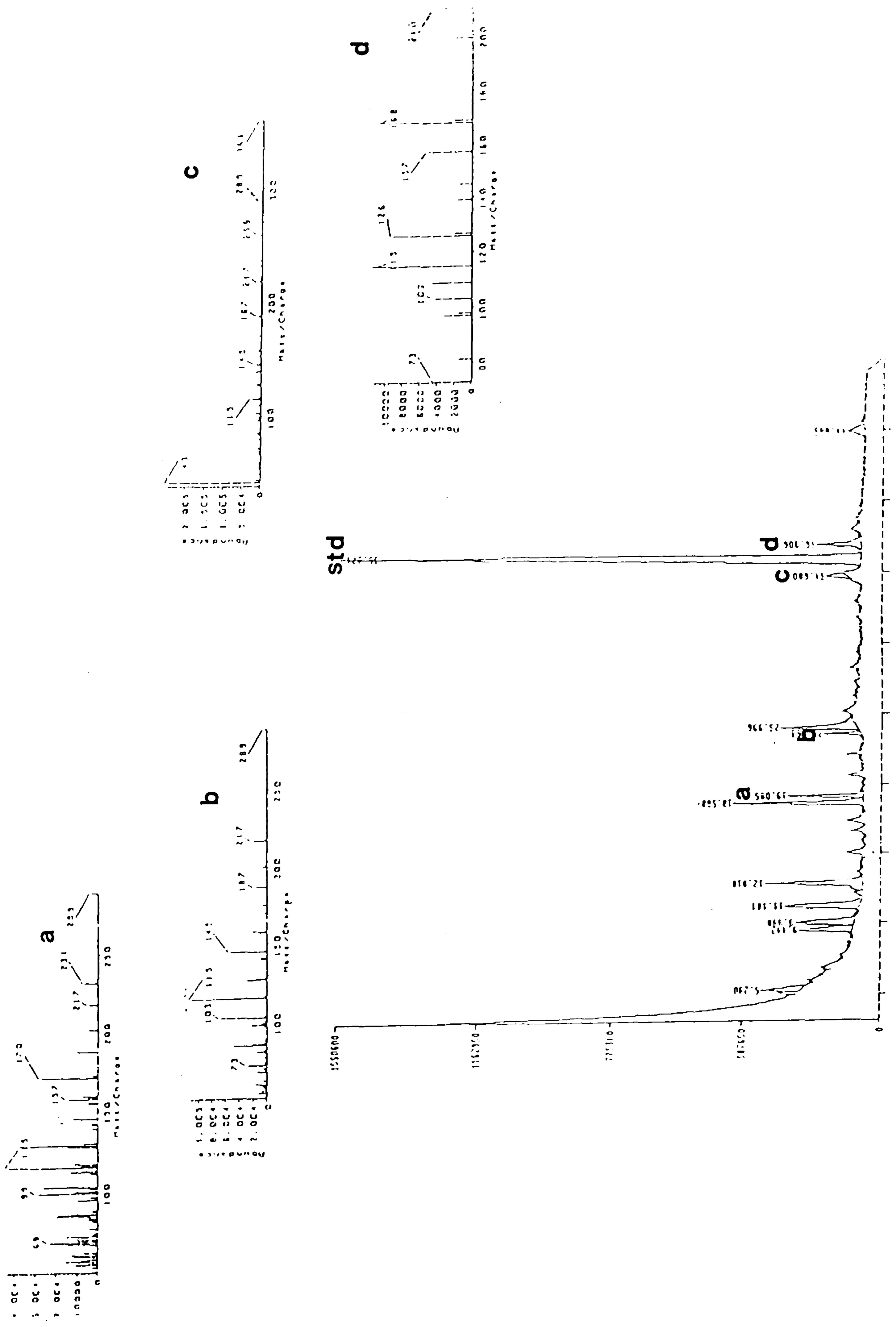


Figure 6.9.

Total ion chromatograph of heat treated (453 k) Manugel GMB



T _R	IDENTITY	HEAT TREATMENT / K			
		413		453	
		M.DM	M.GEL	M.DM	M.GEL
0.53	Rhamnitol	24.0±2.00	11.0±1.00	32.0±0.70	10.5±2.00
0.66	Unknown	22.0±1.36	5.0±0.38	13.0±1.83	3.8±0.51
0.97	Mannitol	20.0±1.30	9.2±1.00	23.0±4.21	5.0±0.09
1.02	Glucitol	7.8±1.80	2.4±0.70	NP	NP
1.10	Unknown	2.0±0.21	2.8±0.45	2.0±0.09	NP

Table 6.4 Products formed as the result of heat treating alginate. Values are means (mg /g alginate) ± standard error of 30 determinations. Compounds that were not present are designated as NP.

Manucol DM on the whole led to the formation of far greater amounts of ' novel ' new sugars following heating as compared to Manugel GMB.

Samples of unheated derivatised alginates produced chromatograms similar to that of the solvent blank (Fig 6.10).

When samples of alditol acetates of uronic acid are repeatedly reduced increasing proportions of the uronic acid, present as lactones, will be released as the free aldose (Lehrfeld, 1981). The samples that were double reduced showed profiles with increased amounts of glucitol

and mannitol (Fig 6.11). Unfortunately the level of background interference was also much increased, so much so that the 3 times reduction was dominated by background interference. Repeated reduction also led to increasing loss of sample.

Figure 6.10.

Total ion chromatograph of native Manuocol DM sample preparation involving 2 reduction steps.

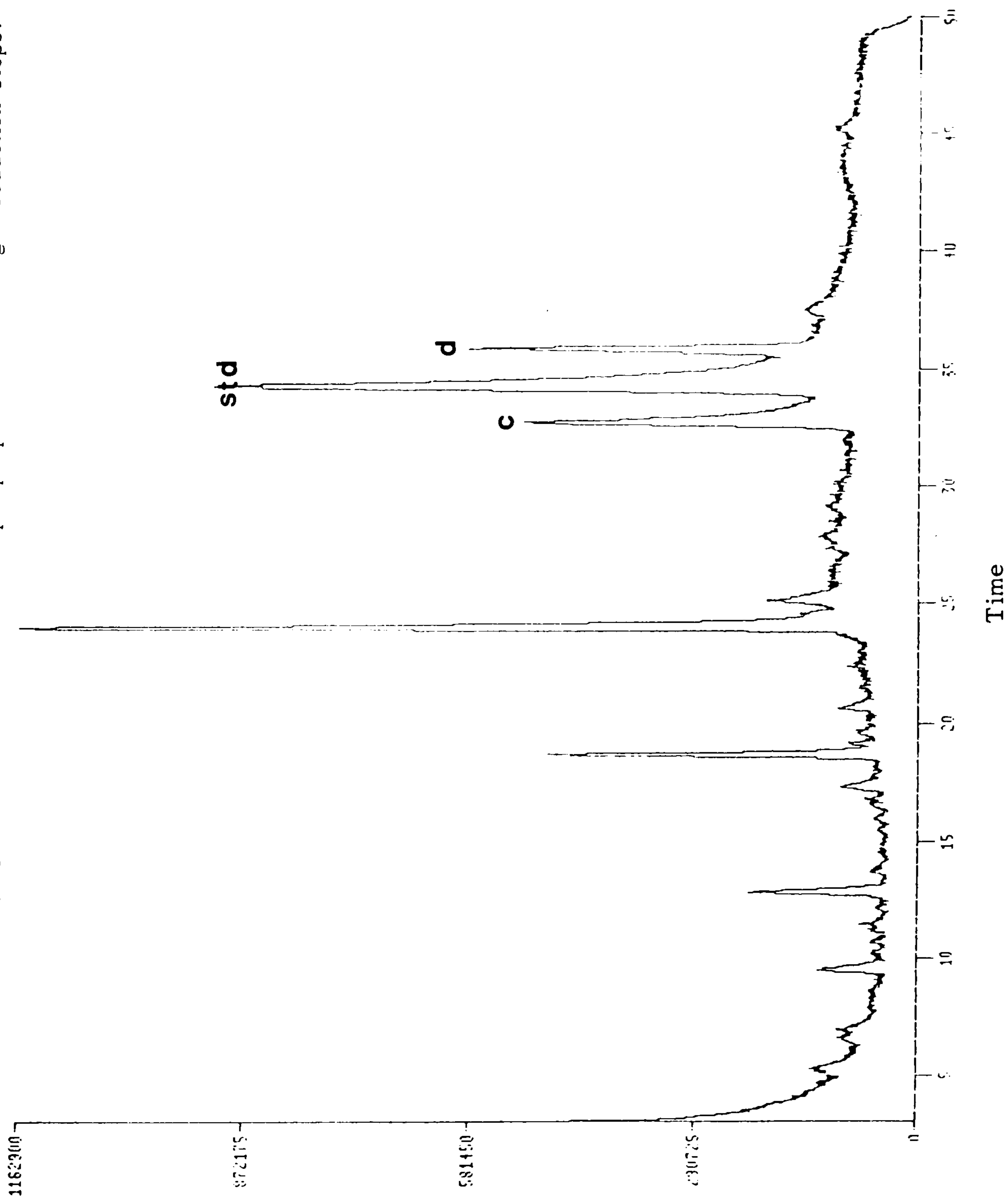
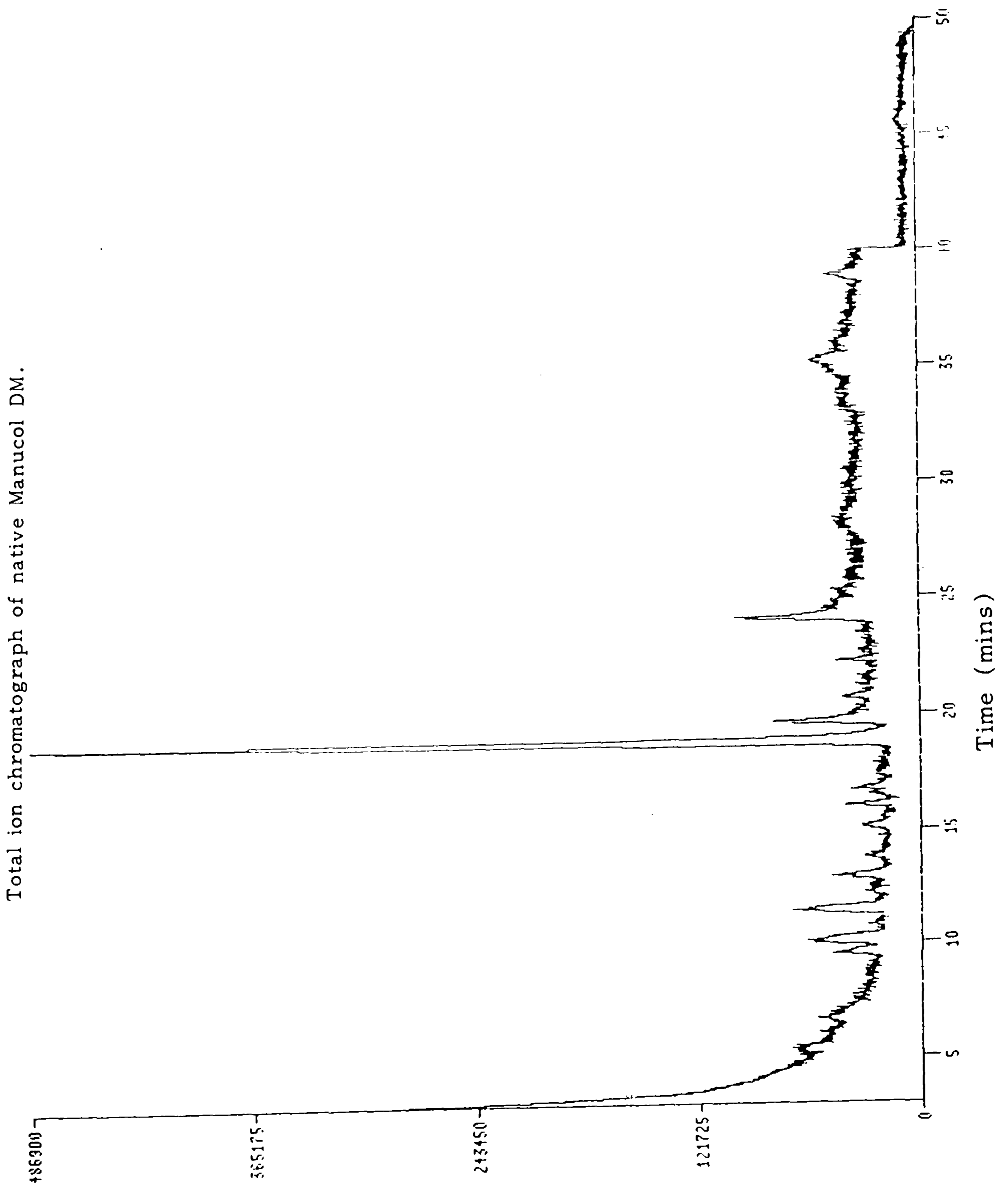


Figure 6.11.



6.1.4 Discussion

The simple system of measuring colour development gave some indication of the action of heat on the alginates. Extrapolation from these results as to how alginate may react with soya at the low moisture contents used during extrusion is difficult for two reasons. The first is that the reaction mechanisms may differ between the systems at high and low moisture. Secondly, the temperature at which the solutions were incubated was lower than that used to degrade the dry powders. In these simple model systems we are looking at the interaction between the primary amine of the amino acids and the carbonyl group on the sugar moiety. If the amino acid contains a secondary amino group, for instance the ϵ -amino group in lysine, interaction will also take place with this group (figure 6.12).

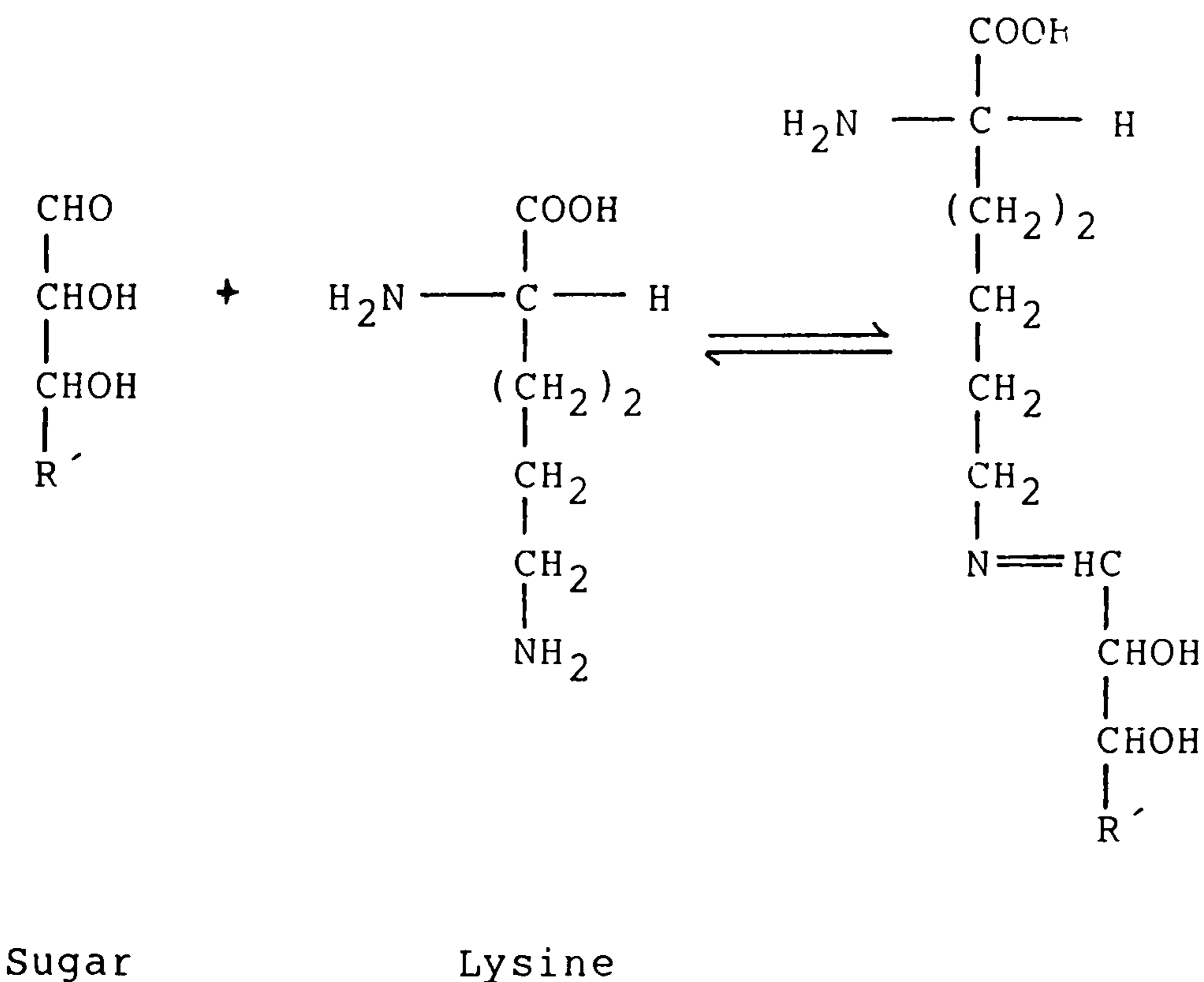
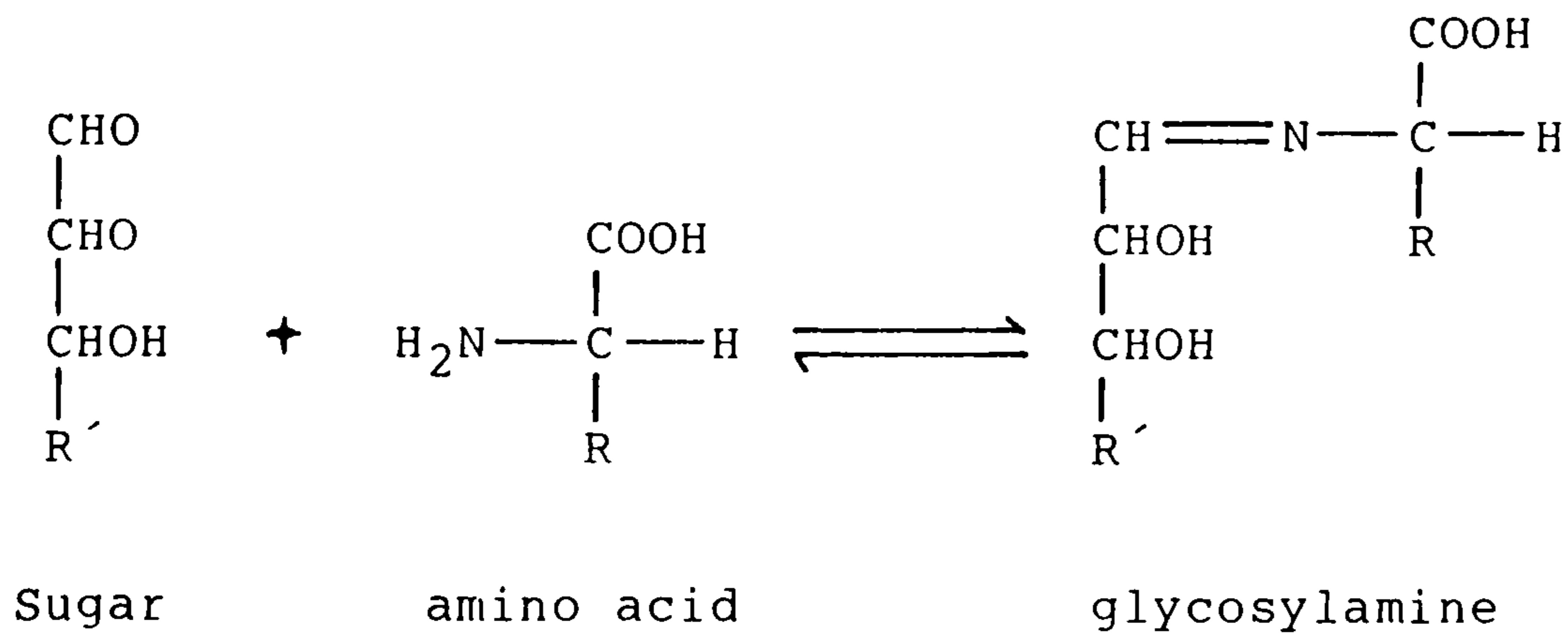


Figure 6.12 Amine - carbonyl interaction.

This accounts for the high reactivity of the lysine solutions. The reaction described here is presumably different to that discussed earlier (chapter 5), which was believed to involve a very specific reaction between the alginate and the the carboxyl group of glutamic acid.

The results do however show an increase in browning potential of the alginates following heating, especially Manucol DM. There is progressively more reactive species formed with increasing temperature. Interestingly solutions of glutamic acid did not brown with any of the heat treated Manugel GMB samples. This may be because the reactive group on the alginate is not accessible to the glutamate.

As stated in section 3.4.1 polysaccharides must be reduced to smaller fragments for gas chromatographic analysis, to achieve this the polysaccharide is hydrolysed. This may cause errors since conditions have to be sufficiently severe to degrade the polysaccharide but not so severe that dehydration or decarboxylation takes place. For this reason nitric acid with urea was chosen as the hydrolysing agent. The urea prevents protonation of the carboxyl group, which is known to stabilise these polysaccharides, thus allowing hydrolysis to be performed at lower acid concentrations. Alkaline hydrolysis is known to lead to extensive breakdown due to β -elimination so this cannot be used (Priess & Ashwell, 1962 and Haug, et al., 1963).

Gel chromatography of the hydrolysed sample confirmed that a high level of degradation had been achieved.

The lactones and uronic acids may be progressively reduced with sodium borohydride (Lehrfeld, 1981) to yield the corresponding aldoses. The multiple reductions would suggest the presence of either the lactones or uronic acids

of mannitol or glucitol. Glucitol may be derived from either glucose or gulose, this would be consistent with the known structure of the alginates (Section 2.5).

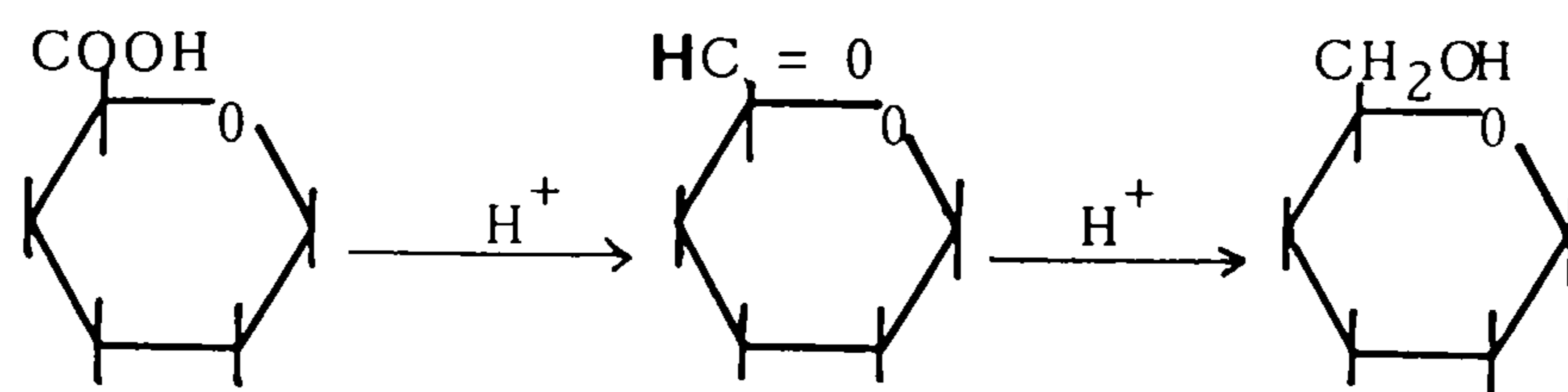


Figure 6.13 Reduction of uronic acids to the corresponding aldose.

Alditol acetates were used because of their ease of creation and such derivatives exhibit the simplest patterns of fragmentation (Blakeney, 1983). As the alditol acetate derivatives of uronic acids are not volatile the method is sensitive in detecting the minor products formed during heating, since the chromatogram will not be dominated by the major constituent peaks.

It must be stressed that the products detected after GC - MS of the heat degraded alginate are not necessarily those created during heating. The reasons for this lie in the fact that prior to GC - MS analysis, samples were hydrolysed with acid, reduced and derivatised. As a consequence classes of sugars will be 'lost', for instance anhydro sugars, these sugars will be hydrolysed to their parent aldose as the

result of acid hydrolysis (Carney & Stanck, 1977). The technique was originally employed to look for the presence of pyrolytic products, which were not found.

It can be seen that the main products of alginate heated to 453 K are sugars. It is not evident from the results obtained that any degradation of the carbon ring had occurred. This would be explained as studies on a range of polysaccharides have established the temperatures at which the degradative processes take place. For ring breakage temperatures in excess of 513 K are required; anhydro sugars and cellulose 513 K - 633 K (Glassmer & Pierce, 1965), aldoses 573 — 773 K (Heynes & Klier, 1968), 1,6 - anhydro - α -D - glucose, 423 - 623 K and starch 593 K (Greenwood, 1967), differential thermal analysis of Keltone suggests that no major structural changes occur until a temperature of about 523 K is reached (Kelco test literature) an observation substantiated in this work. At lower temperatures some depolymerisation and concurrent formation of anhydro sugars is likely to occur (Wolfrom & Thompson, 1958 ; Shafizadeh, et al., 1972 and Shafizadeh & Fu, 1973).

The mechanisms whereby degradation of the polysaccharide chain occurs involves cleavage of the glycosidic bond. Two possible mechanisms are:

a) Homolytic cleavage: this involves free radical formation, which is not favoured at the low temperatures used in this study (Shafizadeh, 1968 and Shafizadeh, et

al., 1971) (Fig 6.14)

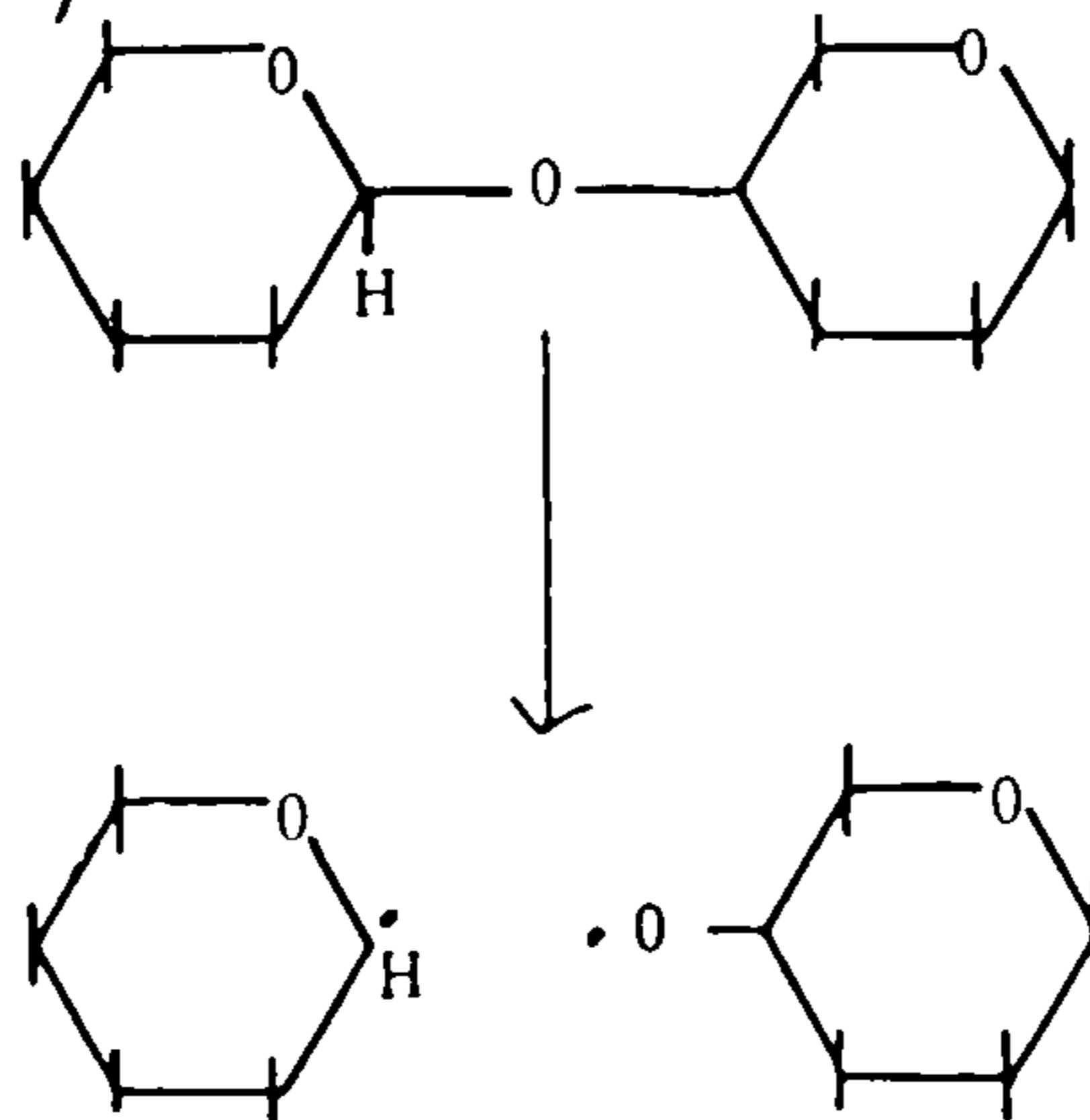


Figure 6.14 Homolytic cleavage of the glycosidic bond

b) ~~Heterolytic~~ Heterolytic cleavage; this may be brought about by several reactions, for example transglycosylation (Fig 6.15) involves attack on the glycosidic bond by either a sugar molecule or water (Houminer & Patai, 1969 and Shafizadeh, et al., 1971).

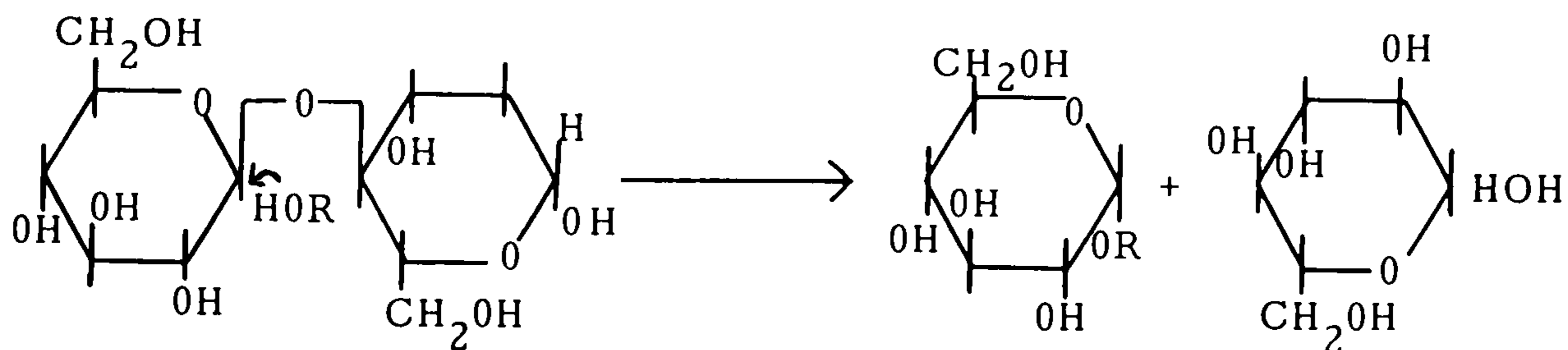


Figure 6.15 Heterolytic cleavage of a glycosidic bond

A second possible mechanism involves anhydro sugar formation (Fig 6.16) and this is possibly the most favoured mechanism (Shafizadeh, 1968 and Greenwood, 1967).

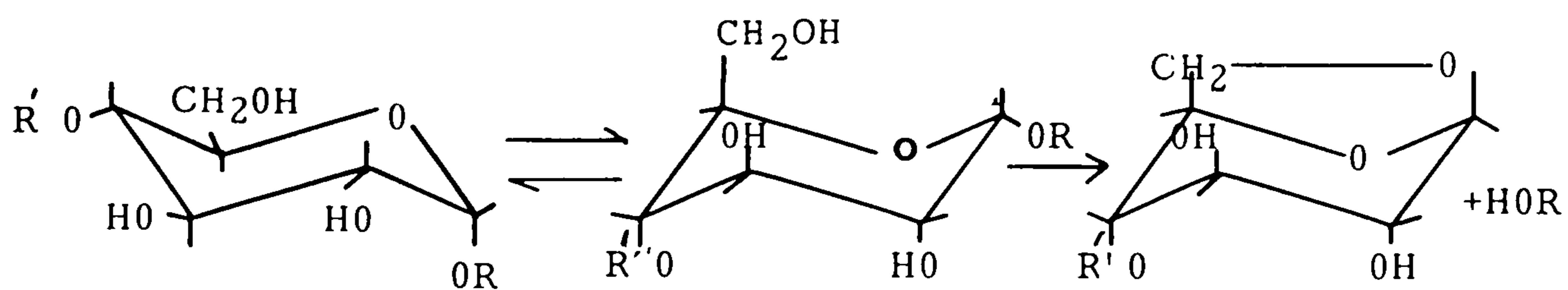


Figure 6.16 Anhydro sugar formation.

This mechanism involves nucleophilic attack by a hydroxyl on Cl. Involvement of Cl is essential at temperatures below 493 K (Liskowitz & Carrol, 1967). This carbon is the most susceptible to nucleophilic attack as it is bonded to two oxygens which will pull electrons away from it. Attacking groups may be any of the hydroxyls, though those in axial positions are most favoured (Liskowitz & Carrol, 1967). The most susceptible is that on C6 and thus it is this group that is most often involved in the production of anhydrosugars (Houminer, 1973). The formation of anhydro sugars other than 1, 6 will undergo rearrangements to the more stable 1, 6 form (Shafizadeh & Fu, 1973). The ease with which the group can be eliminated is of importance in these reactions, thus any steric hinderance of this leaving group will severely limit the degree of depolymerisation.

Uronic acids will have a negatively charged oxygen on C6, though a small amount of the oxygen will be bound to hydrogen ions. The O^- will be a stronger nucleophile than the hydroxyl and so will react with the Cl centre more vigorously. In the 4C_1

form of mannuronic acid these potential reacting groups are in close proximity (section 2.5) and thus should readily react. The product formed from such a reaction is the 1,6 anhydro sugar which due to presence of the second oxygen on C6 is also a lactone, glucopyranurono - 6,1 - lactone. None of the possible configurations of guluronic acid will allow the two centres to come close enough together to react. Thus steric constraints will prevent direct nucleophilic attack by O^- on the C1 centre.

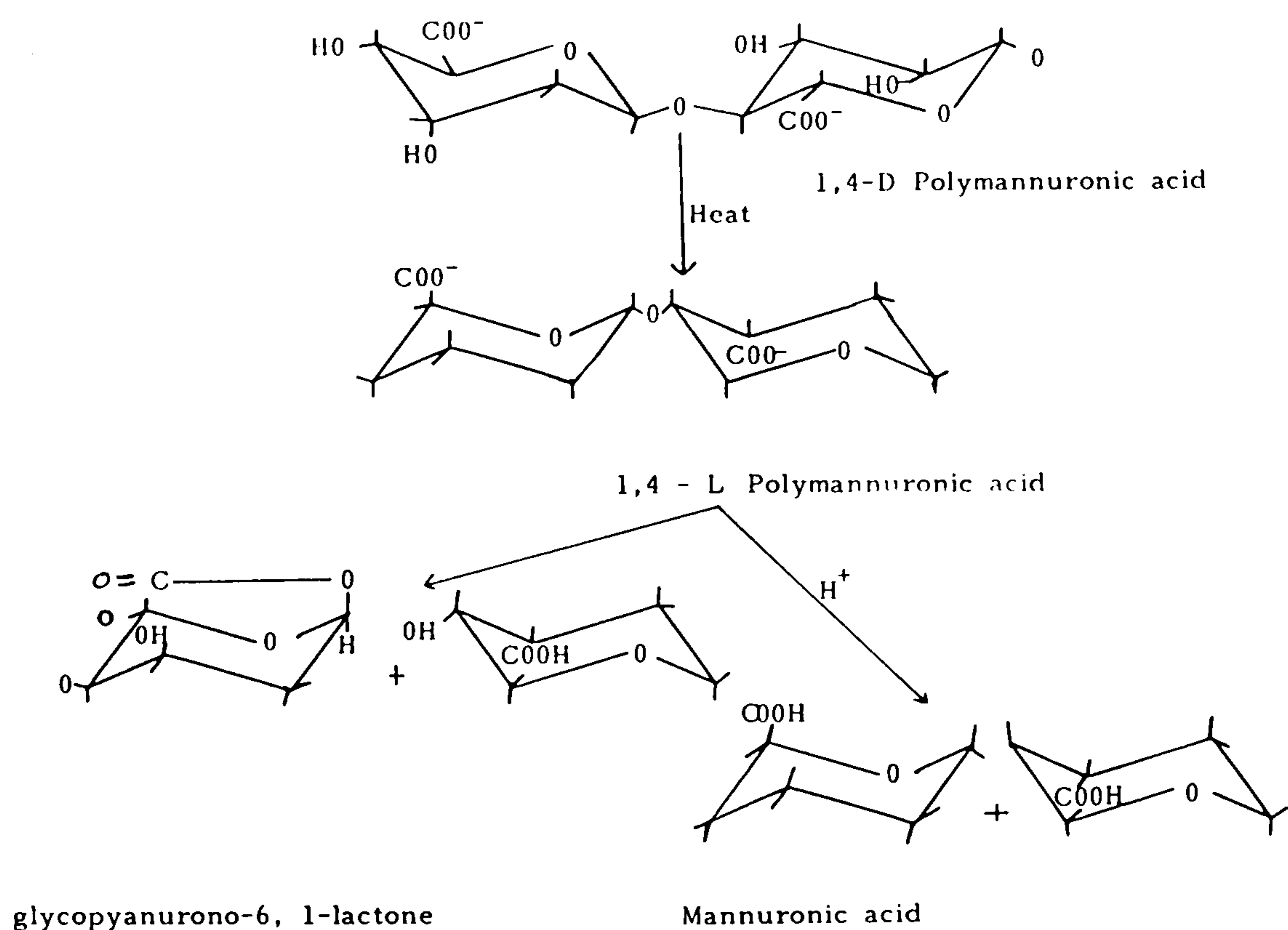


Figure 6.17 Depolymerisation of the glycosidic bond in alginate.

At temperatures greater than 473 K decarboxylation of

the alginate probably takes place (Feather & Harris, 1966) with the evolution of 1 mole carbon dioxide per mole uronic acid.

The MWD obtained for the alginate samples exhibit the phenomena of polydispersity, this " spread " of molecular weights is typical of most polysaccharides; pectin (Deckers, et al., 1986), carrageenans (Lecacheux, 1986), carboxymethyl cellulose (Hamacher, 1985), guar gum (Vijayendran & Bone, 1984) and xanthan (Lambert et al, 1982).

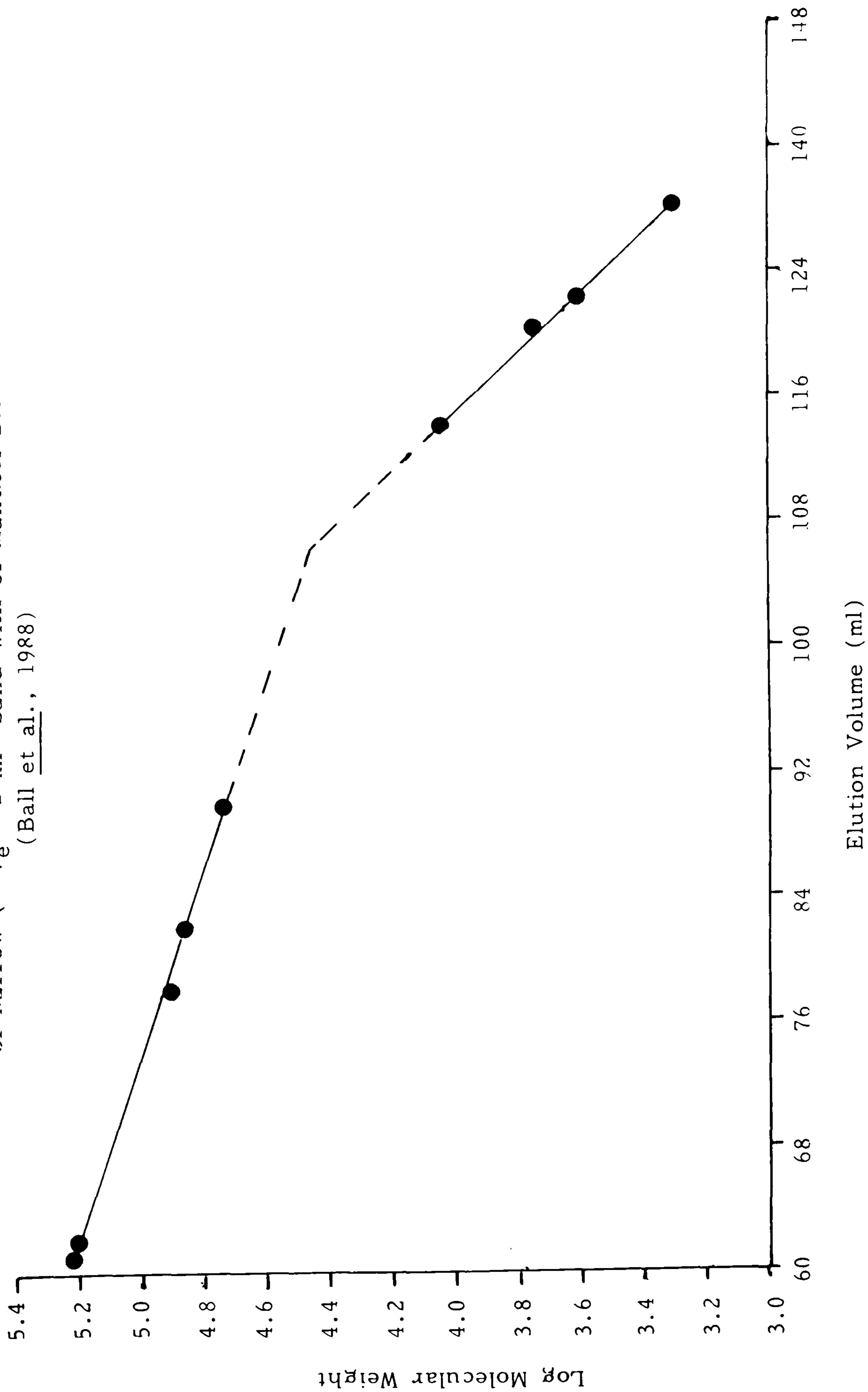
Examination of the MWD's should be undertaken with care, especially the low molecular weight portion. The column was calibrated by short column low speed sedimentation equilibrium in an analytical ultracentrifuge (Ball, et al., 1988), such a technique is not sensitive toward the low molecular weight fragments.

It was assumed that there were only negligible differences in hydrodynamic volumes between the alginate samples, at a given molecular weight. Whittington (1977) has measured the radii of gyration of homopolymers of mannuronic and guluronic acids in solutions of high ionic strength. The radii he quotes are 1,170 and 1700 Å for poly M and poly G respectively. Consequentaly it is unlikely that differences in the radii of gyration between Manucol DM and Manugel GMB will be great.

The second assumption is based upon the fact that no

Figure 6.18.

Calibration curve, using low speed sedimentation equilibrium on isolated fractions of narrow ($V_e \approx 2$ ml) band with of Manucol DM
(Ball et al., 1988)



changes in the native hydrodynamic volume of the two materials occurs after heating. This is an assumption that may not be strictly correct. Examination of the calibration graph (Fig 6.18) shows two linear plots. Material used in the construction of this curve were derived from two sources, native and heat degraded Manuacol DM. Considering the fact that the relationship between retention time and molecular weight for each sample is linear. It is tempting to speculate that the passage, through the column of the degraded material is in some way increased. Such an effect may well be due to changes in the hydrodynamic volume.

The weight average molecular weight (\bar{M}_W) calculated from the MWD data for native Manuacol DM is lower than estimates obtained by other methods; 180,000, end group analysis; 130,000, ultracentrifuge (Ball, 1988); 147,000, viscosity studies (McDowell, 1977); 203,000, sedimentation diffusion coefficients using the Svedberg equation and 198,000 by laser light scattering (Wedlock, et al., 1986). The disparity with the particularly high values of Wedlock, et al (1986) are discussed by Ball (1988), though the values calculated from end group analysis support these higher values.

A measure of the polydispersity of the MWD is given by the polydispersity index (PI). This value will be equal to unity for a monodisperse system. The PI's of heat degraded alginates are given in table 6.3. Native Manuacol DM exhibited a PI close to 1, clearly this value is not correct

as this sample is far from monodisperse. Possibly the value obtained for \bar{M}_w is low, due to the large proportion of sample which elutes very quickly. Heat degraded Manugel GMB, for all treatments, has a greater polydispersity as compared with Manucol DM and both alginates after heating have increased polydispersity. Manucol DM initially exhibits a very high polydispersity at the lower heat treatment but this value decreases with an increase in temperature. It is assumed that cleavage of the polypeptide chain occurs at defined regions, for instance -M-M- or -M-G- bonds. The ranges of different fragment sizes will depend upon the positioning of these bonds. Differences may exist in the thermal lability of the two bond types, at the lower temperature the most heat labile bond will break first whilst greater heat treatment will cause the breakage of both bond types. In the high guluronic acid alginate the presence of mannuronic acid groups will be low and so only a few randomly dispersed segments will be available for chain scission. The high mannuronic acid alginate will contain a large number of mannuronic acid groups along the chain. Thus at higher temperatures the probability of creating segments of equal size is increased as the breakage points are less randomly dispersed. At lower temperature specific breakage of the least heat labile bonds will occur which will be more randomly distributed along the chain.

The extent of chain degradation of the alginates would suggest that following alginate degradation little

repolymerisation and chain branching occurs.

The value for \bar{M}_N is a measure of average chain length in a polymer, thus assuming that the chain degrades proportionally the number of end groups may be calculated (Fig 6.6) relative to the native material. The two alginates used in this study apparently had far more end groups after heating, calculated from \bar{M}_N than estimated by chemical analysis. Both estimates though indicate more end groups are formed from Manuacol DM. As discussed earlier the mechanism of depolymerisation by many polysaccharides involves the formation of a terminal anhydro sugar, these sugars do not reduce Fehlings solution (Carney & Stanck, 1977). It is thus conceivable that the chief mechanism for chain scission in alginates is via the formation of these sugars.

This section has shown that following heat treatment of alginate, sugars or sugar derivatives are formed. No major ring breakdown is apparent and an alginate high in mannuronic acid produces higher concentrations of these compounds. In addition depolymerisation is far greater in the mannuronic acid rich alginates.

EXTRUSION

7.1 Extrusion

7.1 Introduction

The addition of certain polysaccharides to soya has been shown to markedly affect extrusion behaviour (Smith, et al., 1982; Berrington; et al., 1984 and Imeson, et al., 1985).

Previous sections have shown that, in model systems, heat treatment of soya isolate, flours and grits at temperatures similar to those achieved in extrusion processing leads to the formation of measurable amounts of water (chapter 5). This work also shows that in the presence of alginate rich in mannuronic acid residues, increased amounts of water are formed. Amino acid analysis suggested glutamic acid residues reacted specifically with alginates to produce water. In many cases a model system may not give the true reactivity in a real system (Wedzicha, 1984). Thus work described in this section was carried out to see if similar changes occurred during extrusion.

7.1.2 Materials and Methods

7.1.2.1 Soya Grits

Soya grits (Protena pale grit) with a PDI of 25, were obtained from RHM Ingredient Supplies Ltd., who supply the following specification: protein (N * 6.25, dry basis) 50 %, fat 1%, ash 6 %, fibre 3 %, carbohydrate 39.5 %,

moisture 6.5 %.

7.1.2.2 General

Sodium alginate (Manucol DM) was donated by Kelco International Ltd. (section 3.1.2).

Glutamic acid analysis kits were obtained from Boehringer Mannheim (cat No. 139092).

Acrylamide, methylenebisacrylamide, SDS, tris buffer, glycine, ammonium persulphate, 2 - mercaptoethanol and bromophenol blue were obtained from Fisons Ltd. All of the compounds were of at least analytical grade.

Coomassie blue, ammonium persulphate (ampers) (lot A9164) and N, N, N', N'- tetramethyl-ethylenediamine (temed) (lot T8133) was obtained from Sigma Chemical Co.

7.1.2.3 Extrusion

The extruder described in section 3.5 was used, fitted with a 4:1 compression screw and a 3 * 25 mm die. The temperatures for the feed, compression and die zones were: 423, 473 and 423 K respectively. These values were determined by Smith (1984) as being optimal for producing an expanded soya product. The screw speed was 250 rpm and auger feed rate 125 rpm.

7.1.2.4 Feed Material

A range of moistures were used, 24 to 50 % (DSB), the hydration was performed as described in section 3.5.3.

To some of the samples, prior to hydration, 10 g of alginate was added to 990 g of soya. The moisture content of the alginate was similar to that of the soya.

7.1.2.5 Glutamic Acid Analysis

Dried extrudate was powdered (< 1mm) in a Glen Creston Ultra Centrifugal Mill, samples (\approx 0.01g) were hydrolysed with approximately 15 ml 6M HCl at 383 K for 18 hours. After this time samples were evaporated to dryness using a rotary evaporator and resuspended in 2 ml of pH 7.8 phosphate buffer. Prior to analysis a further 1:10 dilution was necessary. Analysis was performed using a Boehringer L-glutamic acid analysis kit and the change in absorbance at 492nm was measured using a Pye Unicam Series 2 spectrophotometer. Drift due to residual reducing compounds was compensated for by extrapolation of the rate of change of absorbance with time. The concentration of glutamic acid was found by reference to standard solutions.

7.1.2.6 Colour Formation

Approximately 0.25g of finally ground material (>1 mm) was homogenised for 1 minute using a Ultra-turrex homogeniser with 10 % acetic acid. After centrifugation, in a bench centrifuge at 3,000 rpm for 10 minutes the absorbance of the supernatant was determined at 420 nm using a Pye Unicam SP600 Series 2 spectrophotometer.

7.1.2.7 SDS Electrophoresis

Electrophoresis was performed using a modified Pollard method (White, 1985). Samples of ground extrudate were dissolved by gentle heating, in sample buffer (Table 7.1) to a final concentration of about 2 mg/ml.

Component	Volume (ml)
10g SDS in 100ml	20.00
30.3g Tris buffer + 144g glycine in 1l	2.00
glycerol	20.00
Bromophenol blue	10.00 mg

Table 7.1 Sample buffer. Final volume made upto 100 ml with distilled water.

The gel was prepared as shown in Table 7.2

Component	Volume (ml)
30g Acrylamide + 0.8g Bis in 100ml	31.750
10g SDS in 100ml	12.500
30.3g Tris buffer + 144g glycine in 1l	0.500
Temed	0.025
Ampers	0.375

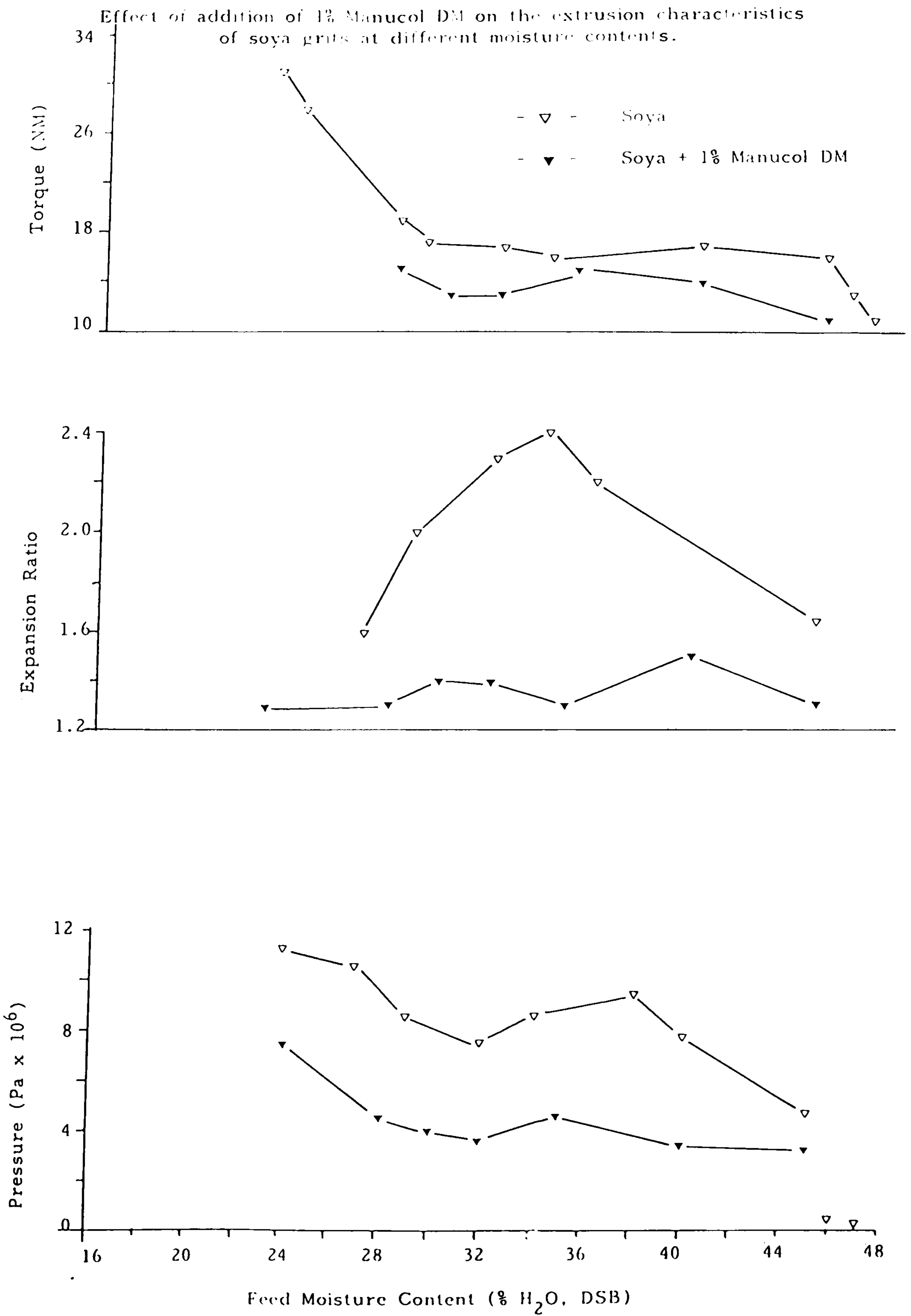
Table 7.2 Gel preparation. All solutions were in distilled water.

138g of urea was added to the solution prior to the addition of "ampers". The gel was prepared in a Biorad protein dual slab gel electrophoresis system. A comb, forming 15 slots, each 0.4 * 0.3 cm, was placed in the middle of the gel by means of spacers to give the sample wells. After 30 minutes polymerization of the gel was complete and the spacers removed. Each slot was loaded with approximately 20 µg protein in sample buffer (20 µl solution) and run at 50 mA (100V) at 293 K until completion (approximately 20 hours), with a circulating buffer of 250 ml tris - glycine buffer and 25 ml (10 %) SDS made upto 2.5 l with distilled water. Several slots were loaded with a series of marker proteins to act as molecular weight standards. The gels were fixed for 4 hours in 10 % trichloroacetic acid, stained for 12 hours in 0.2 % coomassie brilliant blue in 50 % methanol; 12 % acetic acid and then destained in several changes of 50 % propan-2-ol; 10 % acetic acid and finally fixed in 10 % acetic acid.

7.1.3 Results

The three parameters measured; pressure at the die, torque and expansion ratio, all showed a dependence on feed moisture content. With increasing feed moisture content, from 24 to 50 %, there was a corresponding decrease of 62 and 75 % in the torque and pressure respectively. (Fig 7.1). Expansion ratio showed a more complex dependence on

Figure 7.1.



moisture content with an increase with increasing water to 34 % moisture. However further increases in moisture content led to decreases in expansion ratio (Fig 7.1).

Browning was also very dependent on moisture content (Fig 7.3). The degree of browning is dependent on two main parameters, water content (Eichner, 1975) and the time and extent of heating, which in extrusion is a function of the flow rate and the temperature of the extruder jackets.

Figure 7.2 shows that the flow rate is dependent on feed moisture content. Upto 38 % water there is an increase in flow rate to a maximum of about 1.8 ml/sec. At higher moistures the rate declines with increasing water content. Thus the dependence of the browning reaction on water content is not unexpected. After the addition of 1 % Manuacol DM there was little change in the flow rate dependence on water content (Fig 7.2), except that the degree of browning increased in the presence of Manuacol DM, at all water contents, and showed a far greater dependence on moisture than the soya alone. Maximal browning occurred at a water content of about 33 % (Fig 7.3).

The glutamic acid contents of the samples of extrudate containing alginate were significantly lower ($p < 0.05$) than in samples extruded in the absence of alginate; 0.14 and 0.18 g glutamic acid / g protein respectively (Table 7.3).

Figure 7.2.

The effect of the addition of 1% Manucol DM on the flow rate for soya extrusion at different feed moisture contents.

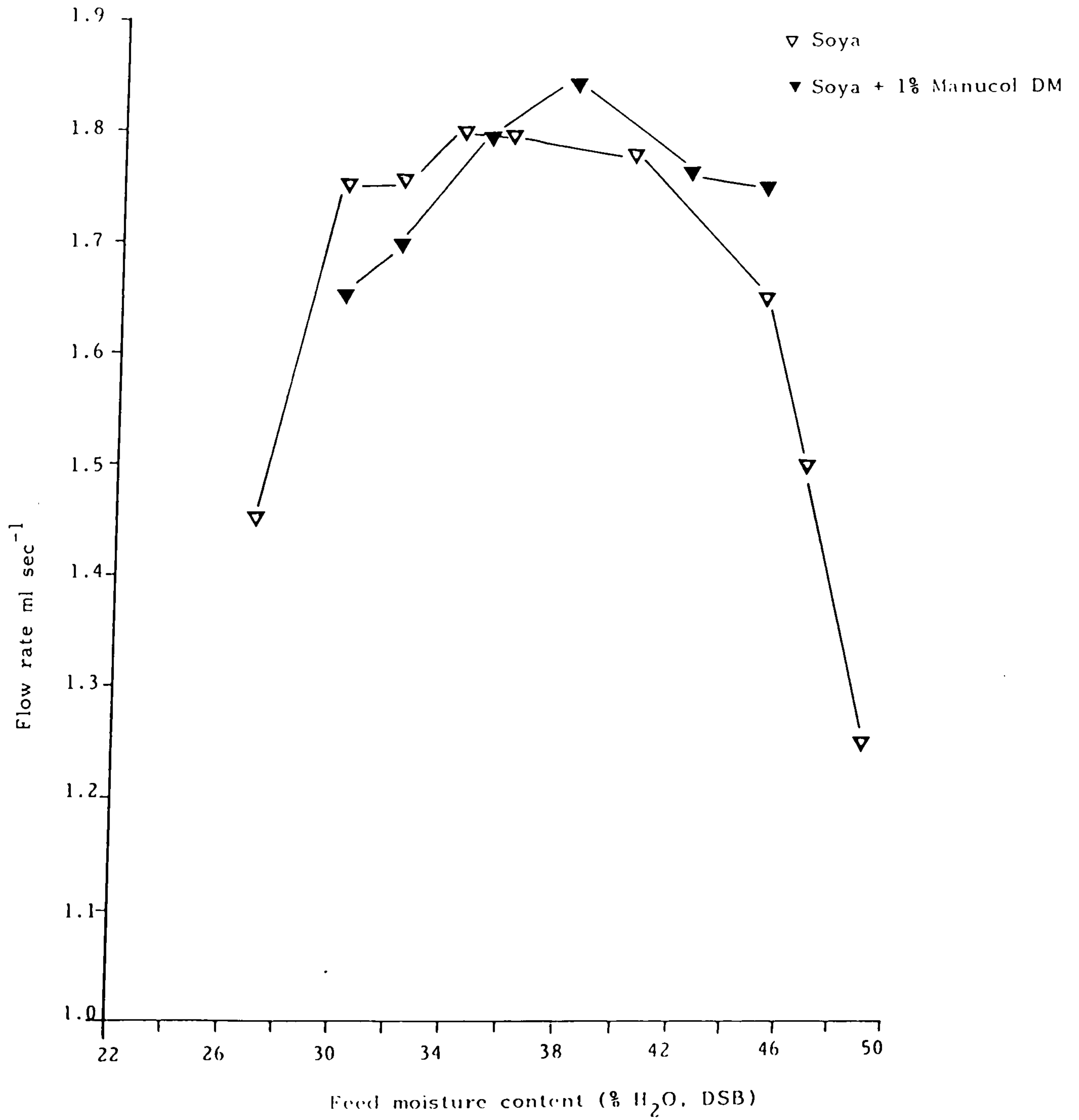
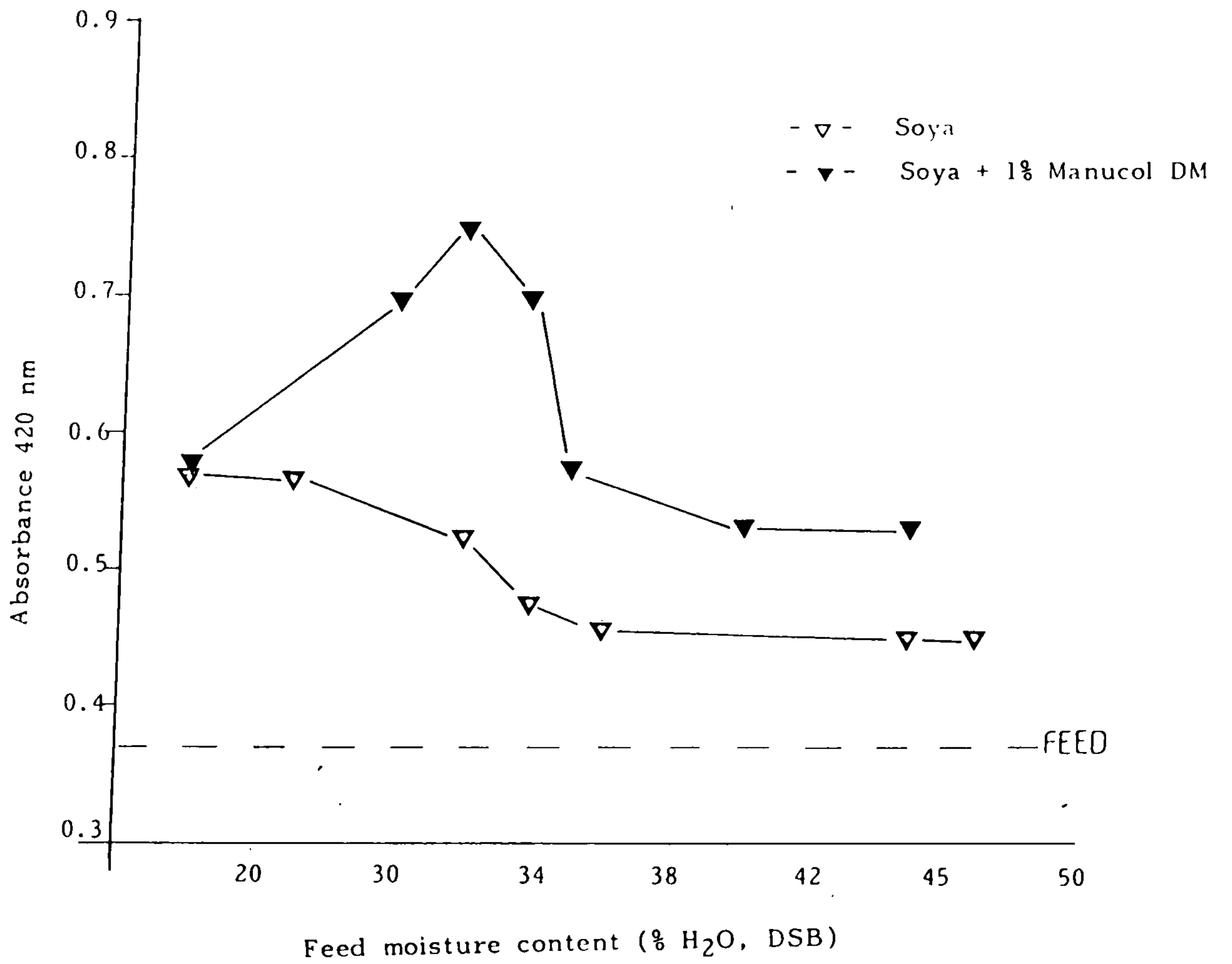


Figure 7.3.

Absorbance at 420 nm of acetic acid extracts from textured soya and soya plus 1% Manucol DM.



SAMPLE	GLUTAMIC ACID CONTENT (g/g)
Soya alone	0.180 ± 0.004
Soya + manucol DM	0.140 ± 0.007
Feed	0.180 ± 0.005

Table 7.3 Glutamic acid contents (g glutamic acid / g Protein) of samples of extruded soya, soya plus alginate and feed material. Values are means ± standard error of 20 determinations.

The glutamic acid content of the feed was the same as that of the extrudate of soya alone. Surprisingly, in the view of the dependence of browning on water content, water content did not significantly effect the loss of glutamic acid. Thus the results in Table 7.3 are pooled values for the different feed moisture contents.

The measured moisture content of the extrudates increased with increasing feed moisture (Fig 7.4) and extrudates containing 1 % Manucol DM had far higher (6 to 8 %) water contents than the corresponding alginate -free extrudates, at all feed moistures (Fig 7.4).

Fig 7.5 shows the electrophoretic patterns for a range of feed and extruded soya samples. There is no detectable difference between the samples of feed and extruded soya, with or without alginate.

Figure 7.4.

Effect of 1% Manucol DM on the apparent water content of soya extrudates as a function of feed moisture.

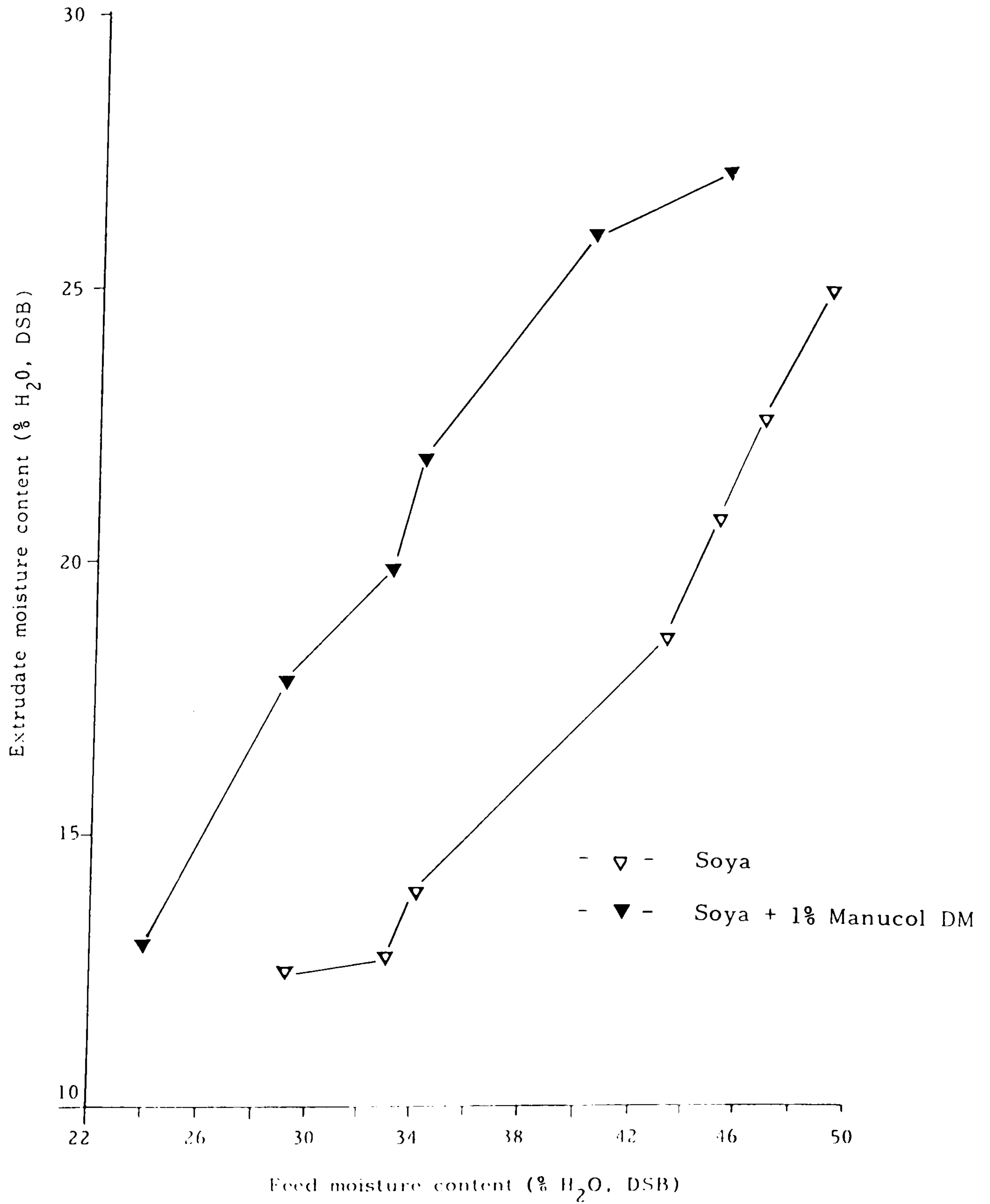
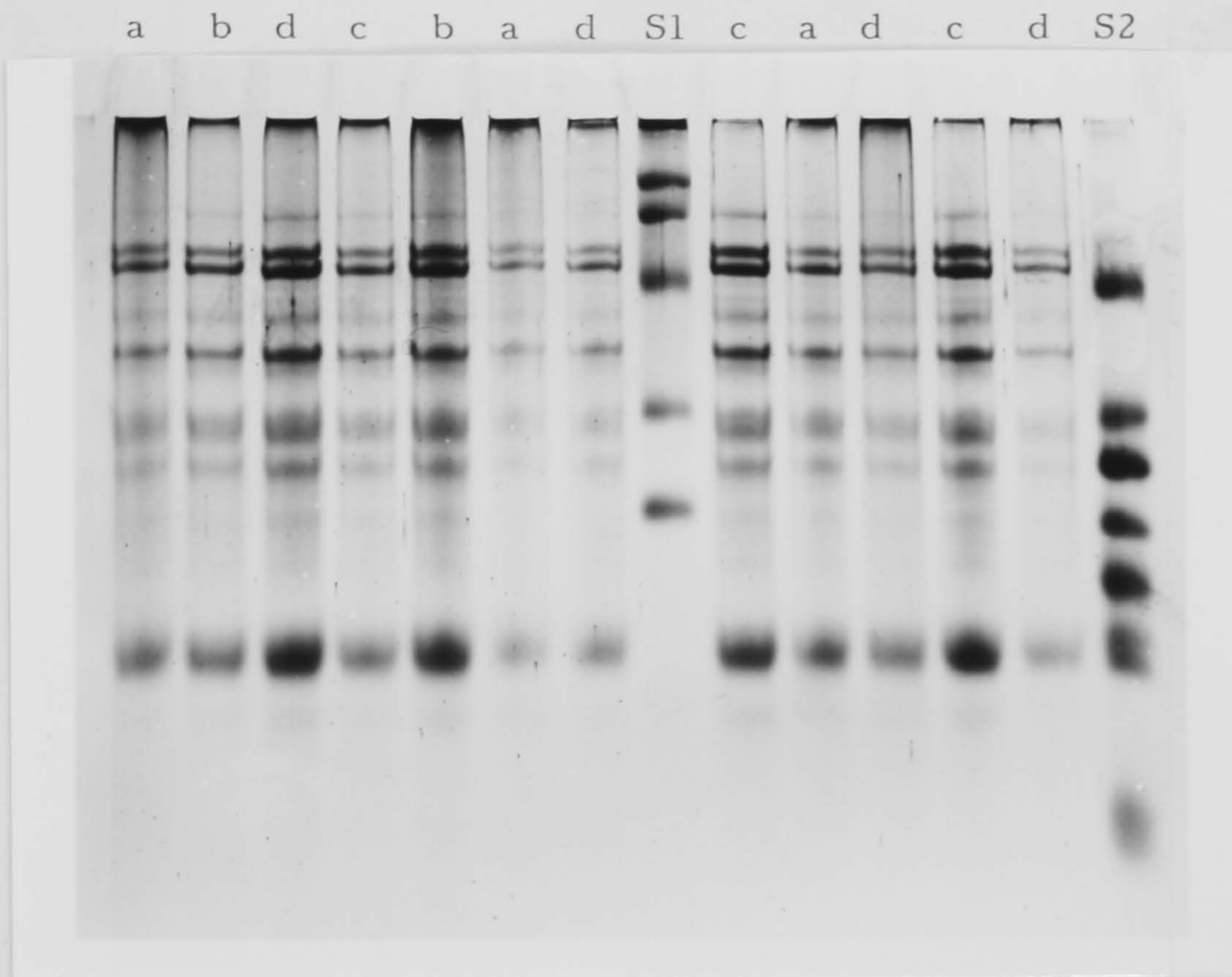


Figure 7.5.

Electrophoretogram of native soya (a) and soya + 1% Manucol DM (b) and samples of extruded soya (c) and soya + 1% Manucol DM (d), compared against sigma standards SDS 6H (S1) and SDS7 (S2).



7.1.4 Discussion

It is evident from this and previous work (Smith, et al., 1982; Berrington, et al., 1984 and Imeson, et al., 1985) that the addition of alginate to soya lowers the viscosity of the extruder dough, as shown by a reduction in torque and pressure at constant flow.

In this study the values of all parameters measured, at all moisture contents, are lower than obtained by previous workers. This may reflect on the heavy wear of the screw used in this study (Berrington, 1985).

Conditions in the extruder are ideal for browning reactions, lysine losses up to 80 % following or during extrusion cooking of soya have been found by Beufrond, et al. (1978) and Thompson, et al. (1976). Non soluble disaccharide sugar fragments found in soya grits will be broken down in the shear fields of the extruder to their composite units or anhydro sugars. It is the reaction of these and the soluble sugars with the protein that may be the cause of the browning seen when soya is extruded. Water will be formed at the rate of 3 moles water per mole sugar via simple Maillard type reactions. Lysine, arginine, histidine, aspartic acid and serine residues have all been implicated in browning reactions occurring during the extrusion of soya. Condensation reactions have been shown to occur in heated soya isolate (chapter 5). These reactions, occurring in an environment deficient in carbohydrate, may

be the result of interactions between lysine and the glyco portion of the 7S protein or protein -protein interactions involving lysine and / or the carboxyl and / or amide groups of the acidic side chains of aspartic and glutamic acid.

Earlier work with model systems (section 5.1.3) suggests that the presence of high mannuronate alginate leads to still further water being produced in heated soya mixes. Reactions probably involving glutamic or glutamide residues have been implicated. In this chapter the involvement of these residues has been demonstrated in a ' real ' system. These reactions lead to an increase in browning which is dependent upon feed moisture. Unfortunately the feed moisture contents cannot be related to a_w as the activity of the water in the extruder is not known. Extrudates containing alginate were also found to contain more water. Previous work by Berrington, et al. (1984) showed that following alginate inclusion a lowering of product temperature occurs, prior to its exit from the die. The lower product temperature would result in less "flash - off" of water (Harper, 1981). Using the data of Berrington, et al. (1984) the reduction in product temperature was found to be equivalent to only 1 % less water being " flashed - off " (Appendix II), thus the increased water must result from chemical reactions and not be related to less evaporative loss on expansion.

Production of water on heating was shown to be generated in proteins that extrude relatively easily

(section 5.2), the interaction of these proteins with alginate results in more water being formed. Smith (1984) suggests a minimum water content is necessary to fully hydrate the protein molecules and thus minimise the resistance in the extruder. With increasing moisture content, the viscosity decreases until no further hydration is possible. The excess water present at high moisture contents is thus thought to act as a lubricant in the extruder reducing both the torque, product temperature and pressure (Berrington, 1985).

Models of flow behaviour and energy balance in an extruder (Harper, 1981) show that a drop in torque, product temperature and pressure at an approximately constant output is due to a decrease in the viscosity of the melt. A rheological model study by Berrington (1985) describes a reduction of some 35 % in viscosity following alginate addition at a constant shear rate of 500 s^{-1} , feed moisture 30 % DSB and a temperature of $120 \text{ }^{\circ}\text{C}$ (393 K). The viscosity lowering effect decreased with increasing temperature, shear rate and moisture content, all of which reduced the viscosity on their own.

The production of water, if it occurs just prior to denaturation may aid the course of the denaturation and rearrangement reactions (chapter 4). The presence of quantities of free water could also act as a reaction medium.

The condensation reactions occurring in browning result

in the concomitant formation of covalent bonds. In soya it is believed (section 5.2) that such reactions will occur between the amino acid residues glutamic acid (or amide) and alginate or lysine. Such reactions may also take place between lysine or serine and the glyco residue of the 7 S globulin. The covalent bonds formed may well be stable in the extruder (temperatures > 453 K). Thus the formation of such heat stable linkages may be important in the texturisation process. Under these conditions the bonds usually responsible for maintaining structure in textured protein (hydrogen bonds, hydrophobic interactions and disulphide bonds) will not form. Thus covalent bonds formed during these reactions may well " hold " the protein strands together as they exit from the die.

Burgess and Stanley (1976) and Simonsky and Stanley (1982) have presented evidence for the formation of such bonds although Sheard (1985) does not believe such interactions occur. This conclusion was reached as he found that all extrudates were highly soluble in solutions of sodium dodecyl sulphate (SDS) plus mercaptoethanol(ME). However examination of his data reveals that extrudates lost some solubility (upto 10 %) in SDS / ME on extrusion. This suggests that, in fact, a small amount of non disulphide covalent links are present in the textured material. The addition of alginate may increase the formation of such crosslinks and so aid texturisation, this is supported by work of Boison, et al. (1983). Evidence from the

electrophoresis work quoted here at first examination does not support these ideas. However the method involved solubilising the extrudate SDS and mercaptoethanol buffer and applying the suspension to the gel, a small amount of insoluble material, the portion expected to contain the isopeptides, would thus not migrate.

The work with the extruder confirms that specific reactions between alginate and soya protein, that result in the production of water, increased browning and glutamic acid loss, take place during extrusion.

GENERAL DISCUSSION

AND CONCLUSIONS

8.0 General Discussion and Conclusions

It is evident from this and previous work (Smith,et al., 1982, Berrington, et al. 1984 and Imeson, et al., 1985) that the addition of alginate to a soya feed lowers the viscosity of the extrusion dough. To understand these reactions in more detail the action of alginate on soya was studied in both physiochemical terms and in relation to specific chemical reactions.

8.1 Physiochemical changes during soya protein denaturation

It was found that the physiochemical changes taking place during soya denaturation were similar to those observed with globular proteins in dilute solution. A range of parameters associated with the denaturation were dependent on moisture content. The complex dependence on water content could be related to the degree and type of hydration of the soya.

Inclusion of hydrocolloid modified the denaturation characteristics of the 7S soya globulin. The action of hydrocolloids was thought to be due to:

a) Complex formation; All the hydrocolloids tested lowered the enthalpy of denaturation at low moisture. This non specific effect was attributed to complex formation between the hydrocolloid and the protein.

b) Chemical reaction; The addition of alginates high in mannuronic acid residues modified some of the parameters in

a very specific manner. Thus the temperature of the transition decreased. This effect was small and may be the result of water production during heating, since the moisture contents of the samples were determined after heating. The most striking effects of alginate inclusion were a decrease in the cooperativity of the denaturation process and a decrease in specific heat following the transition. The latter parameter is often given as an indication of the mobility of the molecule, especially the non polar side groups. These effects again may well be related to the production of water prior to or during denaturation. The degrees of hydration of the samples in this study are sufficiently low to ensure only small amounts of free water are available. Thus the creation of further amounts of water in such a system will be expected to have profound effects on the reactivity of the molecule. For example the unwinding of the protein will involve an increase in accessibility and mobility of previously buried hydrophobic groups, which will find the more aqueous environment less favourable for mobility.

8.2 Chemical effects

A specific reaction between soya or gluten and alginate rich in mannuronic acid, was found to take place leading to the formation of water, loss of glutamic acid and increased browning. The extent of the reaction was dependent on the mannuronic : guluronic acid ratio of the alginate. It is not

clear whether it is the mannuronic acid groups themselves that cause this effect or the weaker glycosidic links between the units in the alternating region since these regions are in greater abundance in alginates high in mannuronic acid residues. The thermal breakdown of the alginates high in mannuronic acid was found to result in an increased number of small molecular weight fractions and an increased number of reducing end groups. No extensive carbon ring degradation was evident. It is not clear as to whether it was the reactivity of the monosaccharide units formed during thermal treatment or the increased number of free end groups on the depolymerised polysaccharide that was important in giving the high reactivity of the heated high mannuronic acid alginates. Both may well be important.

Glutamate residues were involved in the reaction with alginate though it is not known if the residues involved in the reaction were in the acid or amide form. The quantities of glutamic acid and glutamide are similar in the 11S globulin (Catsimpoolas, et al., 1971b). The reason for the involvement of these residues is not clear. It may be that all the freely available lysine has been consumed in other reactions, or it may be that both glutamic acid and glutamide have high hydration values which will make these residues susceptible towards reaction in a water 'starved' environment. The amide form is perhaps the most likely reactant since the amino group should readily react with carbonyls generated on heating. It is unfortunate that no

glutamide was available for study in the model systems (section 5.2). It is interesting that gluten, another protein that exhibits reactivity with alginate is rich in glutamide (Booth, 1987).

Following extrusion of a soya alginate mix browning or condensation reactions were found to occur as indicated by an increase in the brown colour and water content of the extrudates in the presence of alginate. Loss of glutamate residues also occurred in these samples.

In the absence of alginate soya and gluten still produced water on heating. This was assumed to be the result of interaction between lysine and / or serine and the sugar component of the 7S glycoprotein or with the glutamic acid / amide residues, though in the heated protein alone no evidence of glutamic acid consumption could be found. This reaction would lead to the formation of stable crosslinks. These reactions may be important in the texturization process in " holding " together the open polypeptide strands during extrusion and passage out of the die. They may well be vital for subsequent orientation of the polypeptide fibres (Fig 8.1). Increased occurrence of these condensation reactions after alginate addition may well lead to improved textural properties, as shown by Boison, et al. (1983).

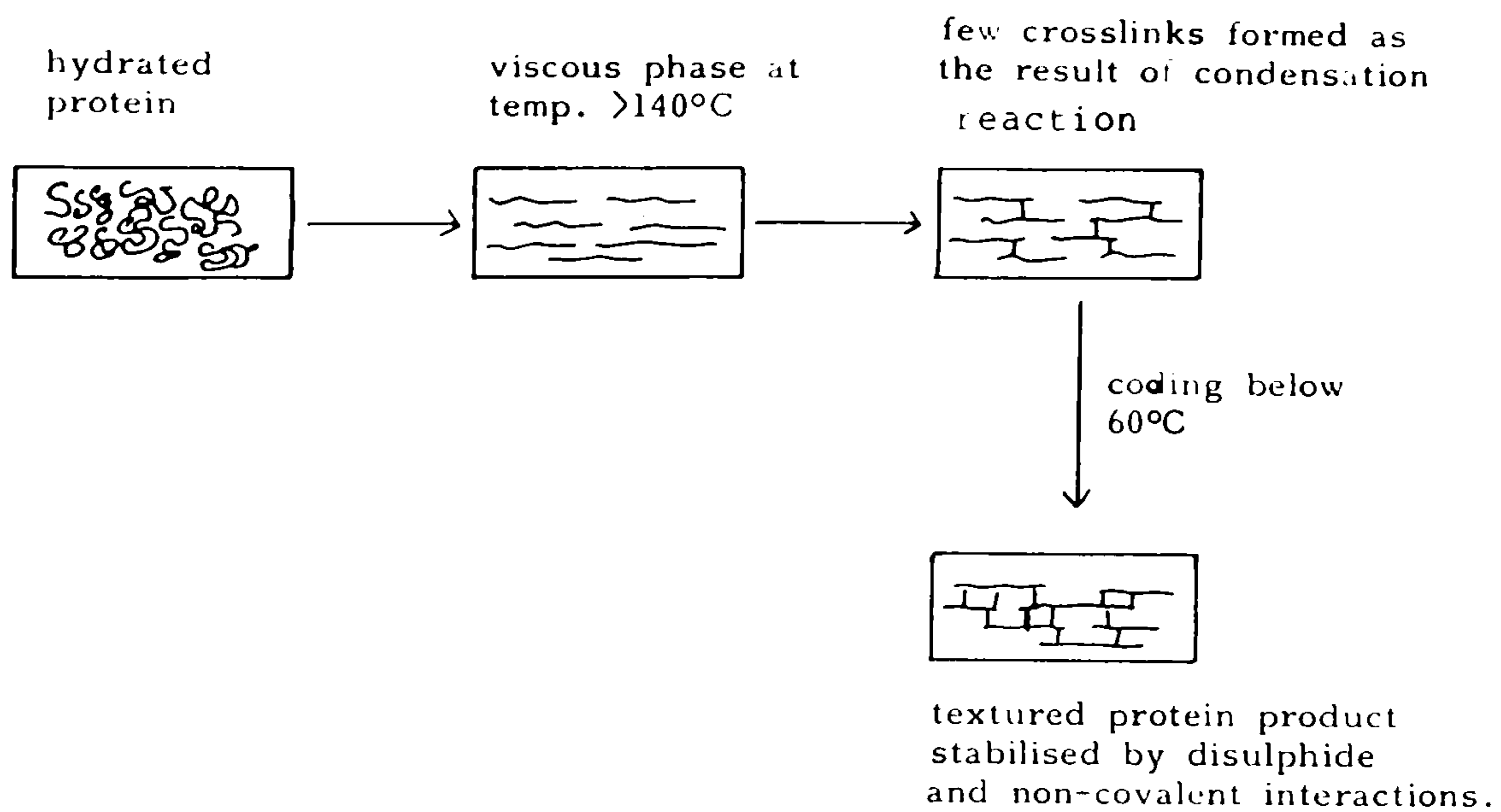


Figure 8.1 Formation of stable crosslinks during extrusion.

In conclusion the effect of reduced melt viscosity following alginate inclusion can at best be attributed to the production of water at some point along the extruder barrel. The presence or creation of this water may well aid texturisation.

The creation of this apparent water on heating soya protein may well be derived from protein - protein interactions (Table 8.1) or amine carbonyl reactions with the glyco component of the 7S fraction.

The source of the " extra " water, created when soya and alginate are heated is difficult to predict. Generally alginates are more heat labile than other hydrocolloids and this study suggests that those alginates high in mannuronic acid are the least heat stable. The predicted number of end groups, assuming all were reactive would not account for the large quantities of water which are formed. Even " total " destruction of Manucol DM to its component atoms of C, H and O would produce in the soya alginate mixtures only 1 to 2 %

Table 8.1.

Some possible protein-protein and protein-sugar reactions involving the amino acids known to be 'lost' in this study. Reaction with those groups involved in the peptide bond are not documented.
'P' - protein 'R' - sugar.

REACTIONS	MECHANISM	COMMENTS
1) Esterification		
a) γ - β -Carboxyl group	$P - COOH + \begin{array}{c} COOH \\ \\ OH - C - H \\ \\ (CH_2O)_4 \end{array} \rightarrow P - \begin{array}{c} COOH \\ \\ COOCH \\ \\ (CH_2O)_4 \end{array} + H_2O$	γ/β carboxyl of glu/asp with hydroxyl of sugar
b) Uronic carboxyl	$P-OH + \begin{array}{c} COOH \\ \\ (CH_2O)_6 \end{array} \rightarrow P - \begin{array}{c} OOC \\ \\ (CH_2O)_6 \end{array} + H_2O$	Serine hydroxyl with carboxyl of uronic acid.
c) Protein-protein	$P-OH + P-COOH \rightarrow P - OOC - P + H_2O$	γ/β carboxyl of glu/asp with hydroxyl of serine.
2) Reaction with aldehydes	$P - NH_2 + R - \begin{array}{c} C - H \\ \\ O \end{array} \rightarrow P - N = CH - R + H_2O$	ϵ -amino of lysine with aldehyde of sugar.
3) Reaction of carboxyl group.		
a) amino-carboxyl	$R(p') - COOH + P - NH_2 \rightarrow R - CO - NH - P + H_2O$	γ/β carboxyl of glu/asp or carboxyl of seric acid with ϵ -amino of lysine or γ/β amide of glu/asp.
b) carboxyl-carboxyl	$P - COOH + R(p') - COOH \rightarrow P - CO - P + CO_2 + H_2O$	γ/β carboxyl of glu/asp and carboxyl uronic acid.
4. Amino-amide	$R - NH_2 + R' - \begin{array}{c} O \\ \\ C - NH_2 \end{array} \rightarrow P - NH - \begin{array}{c} O \\ \\ C - P' \end{array} + NH_3$	ϵ -amino of lysine with γ or β amide of glu/asp.
5) Reaction with dehydroalanine	See section	Reaction of lysine with β elimination product of cystine or serine.

' new ' material of which only a small proportion would be water. It is thus apparent that the role of alginate is most likely ' catalytic ' in nature, possibly, a few protein - alginate crosslinks hold protein chains in such an orientation which allows protein - protein interactions to take place more readily (Table 8.1).

Again it must be stressed that many of the protein - protein crosslinks are not resistant to acid hydrolysis, therefore the techniques used in this study will break the crosslinks and regenerate the amino acids. Further study must involve analysis of heated protein mixes with non - destructive or separation techniques thereby allowing the protein - protein crosslinks to be examined intact.

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APPENDICES

APPENDIX I Retention times for a range of standard sugars.

RELATIVE RETENTION TIME	SUGAR
0.44	2 Deoxy - ribitol
0.53	Rhamnitol
0.57	6 Deoxy - glucitol
0.60	Ribitol
0.63	Arabitol
0.67	Xylitol
0.70	2 - Furoic acid
0.77	2 Deoxy - galactotol
0.78	2 Deoxy - glucitol
0.97	Mannitol
1.02	Glucitol
1.08	Galacitol

APPENDIX II Calculation to estimate the loss of water resulting from " flash-off " .

An energy balance can be written around the discharge die to estimate the loss of moisture in food dough caused by the flashing off of steam as the pressure is reduced to ambient. (Harper, 1981). This is represented by:

$$Q_p c_p (T_1 - T_2) = Q_p (M_1 - M_2) \Delta H_{fg}$$

where Q_p = mass flow

M = moisture fraction wet basis

ΔH_{fg} = latent heat of vaporisation at ambient pressure

c_p = specific heat of food

subscript 1 = before product emerges from die

subscript 2 = after product emerges from die

If it is assumed that the loss of moisture is small compared to the total mass volume then the mass flow of the material will be the same at either side of the die, therefore:

$$M_2 = \frac{M_1 \Delta H_{fg} - c_p (T_1 - T_2)}{\Delta H_{fg}}$$

Taking values from Berrington (1985):

Feed moisture 38% (dsb) = 41% (wsb)

Product temperature prior to exit from the die:

a) Soya alone = 413 K

b) Soya + 1% alginate = 402 K

and using values from Harper (1981):

$$c_p = 2.7 \text{ KJ Kg}^{-1} \text{ K}^{-1}$$

$$\Delta H_{fg} = 2675.8 \text{ KJ Kg}^{-1}$$

$$M_2 = 343 \text{ K}$$

The calculated moisture losses for the two feed materials will be 28 and 29 % (wsb) for soya and soya + 1% alginate respectively.