

FACTORS AFFECTING MAILLARD INDUCED GELATION OF PROTEIN- SUGAR SYSTEMS

by

Azhar Mat Easa

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Department of Applied Biochemistry and Food Science
Faculty of Agricultural and Food Sciences
University of Nottingham
Sutton Bonington Campus
Loughborough
Leicestershire
LE12 5RD

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Abstract

Gelation due to the Maillard reaction took place when solutions containing a low level of bovine serum albumin were heated in the presence of carbonyl compounds. The Maillard reaction caused a change in colour, a decrease in the pH and induced gelation. These changes were dependent on the type and concentration of sugars or protein and on the heating conditions used. Reducing sugar and Maillard reaction products (e.g. glyoxal) affected these changes, yet their order of reactivity for browning and gelation were not necessarily the same. Loss of available lysine and arginine plus changes in the thio amino acids showed that these were implicated in the reaction.

The gelation kinetics (gelation time and development of storage modulus) were followed in real time using a Bohlin CS Rheometer at a temperature of 90 °C. These studies showed that the gels did not form at a specific pH, the pH being lower for samples where the more reactive carbonyl compounds were used. Measurement of the charge on the protein after the Maillard reaction showed an increased negative charge, hence causing a lowering in the protein's isoelectric point. This had the effect of changing the critical protein concentration necessary for gelation at any pH above the isoelectric point. This reasoning would also explain the low syneresis seen in the Maillard gels. Hydrodynamic studies on dilute solution showed that the protein molecules heated in the presence of xylose associated in an orderly manner despite having a low pH. The aggregates could be described as "stiff and rod like". The linkages holding the aggregates together were mostly attributed to additional non-disulfide linkages resulting from the Maillard reaction. Similar types of crosslinks were formed in the Maillard gels heated at 90 °C and were thought to have enhanced the gel strength. Extrusion of soya grits with reducing sugars did not form a retort-stable product. However, microwave heating of the extruded product was successful in producing a product that survived a typical canning process. This suggests that formation of crosslinks may be controlled to produce novel food products.

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INTRODUCTION

One of the most important contributions that proteins make to the development of structure and texture of foods is through the formation of gels. Egg albumen for instance is widely used as a gelling agent in many bakery products. Since this property is a useful attribute of proteins, any information on enhancement of the gelling behaviour is undoubtedly beneficial. Recent work within the Food Science section, University of Nottingham has shown that gelation behaviour can be altered if a protein solution is heated in the presence of a reducing sugar. This could be expected to be of commercial and general interest as the mechanism involves a natural chemical reaction that is known to occur in heat-processed foods and allows protein gelation to occur at low concentrations. The reaction is also known in biological systems. For example, the development of protein crosslinks in collagen and lens crystallins, has been postulated to occur as a result of the Maillard reaction.

The work described in this thesis is a continuation of the studies on "Maillard gels". These are gels formed by heating a protein in the presence of a reducing sugar. They are typified by homogeneous brown gels with little syneresis. Practical work to elucidate the kinetics and possible mechanisms for the formation of bovine serum albumin products formed in the presence of substances that encourage the Maillard reaction is described in this thesis. In the final results chapter some work carried out on processing a commercial sample of soya grits is explained.

As anticipated from previous work, important factors for Maillard gelation are; charge, new covalent crosslink formation and pH of the system. Why these factors are relevant to protein gelation and their connection with the Maillard reaction is explained, with reference to current literature, in the opening chapters of this thesis.

There is much published work for protein gelation and on the Maillard reaction, therefore only areas which would seem to be directly pertinent to the systems studied are discussed.

The initial chapter gives some of the terminology and important factors for gel formation. The next issue addressed is whether the Maillard reaction could alter any of these factors. Therefore Chapter 2 describes some details of the Maillard reaction which may be relevant to the systems used in this thesis. Chapter 3 reviews previous work on protein gelation which may be due to the Maillard reaction. The important steps and mechanisms proposed before the start of this work are given.

To achieve some of the aims of the project, a range of experimental techniques were necessary. For some work the methods had to be developed to achieve the values required. Methods and some of the relevant theoretical details are given in Chapter 4. These methods apply to the work described in the next five chapters. It is in these chapters that the experimental work carried out on bovine serum albumin is presented and explained.

Chapter 10 gives the reasons for carrying out an extrusion study on soya protein. The methods and results are given in the same chapter.

Finally, a general discussion of the findings from all the experimental work described in this thesis is given in Chapter 11.

CHAPTER 1

PROTEIN GEL FORMATION

Protein gels can be formed in several ways but perhaps the most common method occurs by heating solutions of the protein. It is gels formed in this way which are discussed in this thesis.

1.0 *Definition of terms*

The terms *gelation*, *coagulation*, *association*, *aggregation* and *precipitation* are often used to describe changes to protein upon heating. It is important to understand and differentiate between these terms. Upon heating, protein molecules may undergo full or partial denaturation. The dissociated molecules may then *associate* and hence form a *coagulate*, *precipitate*, or *gel* depending upon the conditions of heating.

Protein *association* reactions generally refer to changes occurring at the molecular or subunit level while *aggregation* reactions generally involve the formation of higher molecular weight complexes from *association* reactions. *Gelation* may be defined as a protein *aggregation* phenomenon in which polymer-polymer and polymer-solvent interactions occur so that the attractive and repulsive forces are balanced such that a well-ordered tertiary network is formed. Such a network is capable of immobilizing a substantial amount of water (Schmidh, 1981). Thus *gelation* can sometimes be differentiated from *coagulation*, which involves a more random *aggregation* where polymer-polymer interactions are favoured over polymer-solvent reactions. At extreme conditions (i.e. rapid heating or change in pH) protein molecules may not have time to interact sufficiently and this results in less hydrated *aggregates* which *precipitate*.

1.1 *Classification of gels*

Gels may be classified into two types, the polymer network and the aggregated dispersion (Dickinson and Stainsby, 1982).

a *Polymer network*

Polymer networks are formed by gelatin and polysaccharides such as agarose and carrageenan. The network is formed by aggregation of disordered chains with regions of local order. These gels are characterised by their low polymer concentrations, fine texture and transparency. They may be formed by a variety of methods including pH adjustment, ion addition and heating or cooling. Gelatin is perhaps the most useful and versatile protein gelling agent in this category.

b *Aggregated dispersion*

Aggregated dispersions are usually formed following heating and denaturation of globular proteins. These gels are characterised by their higher polymer concentration (5-10 %), which is an order of magnitude greater than that of the polymer network gels. Since globular proteins are used in the work discussed in this thesis, it is appropriate to focus on gelation due to aggregated dispersion.

1.2 Formation of globular protein gels

Formation of protein gel structures can occur under conditions which disrupt the native protein structure provided that the concentration, thermodynamic and environmental conditions are optimal for the formation of the tertiary matrix. For thermal gelation, heat is applied to disrupt the native protein and expose a variety of functional groups available for interactions. It is generally accepted that gelation involves a two-step mechanism (Ferry, 1948) involving unfolding or dissociation followed by aggregation to form a network of gel matrix (Equation 1.1; Fig. 1.1).



where P_n is the native protein and P_d is the heat denatured protein.

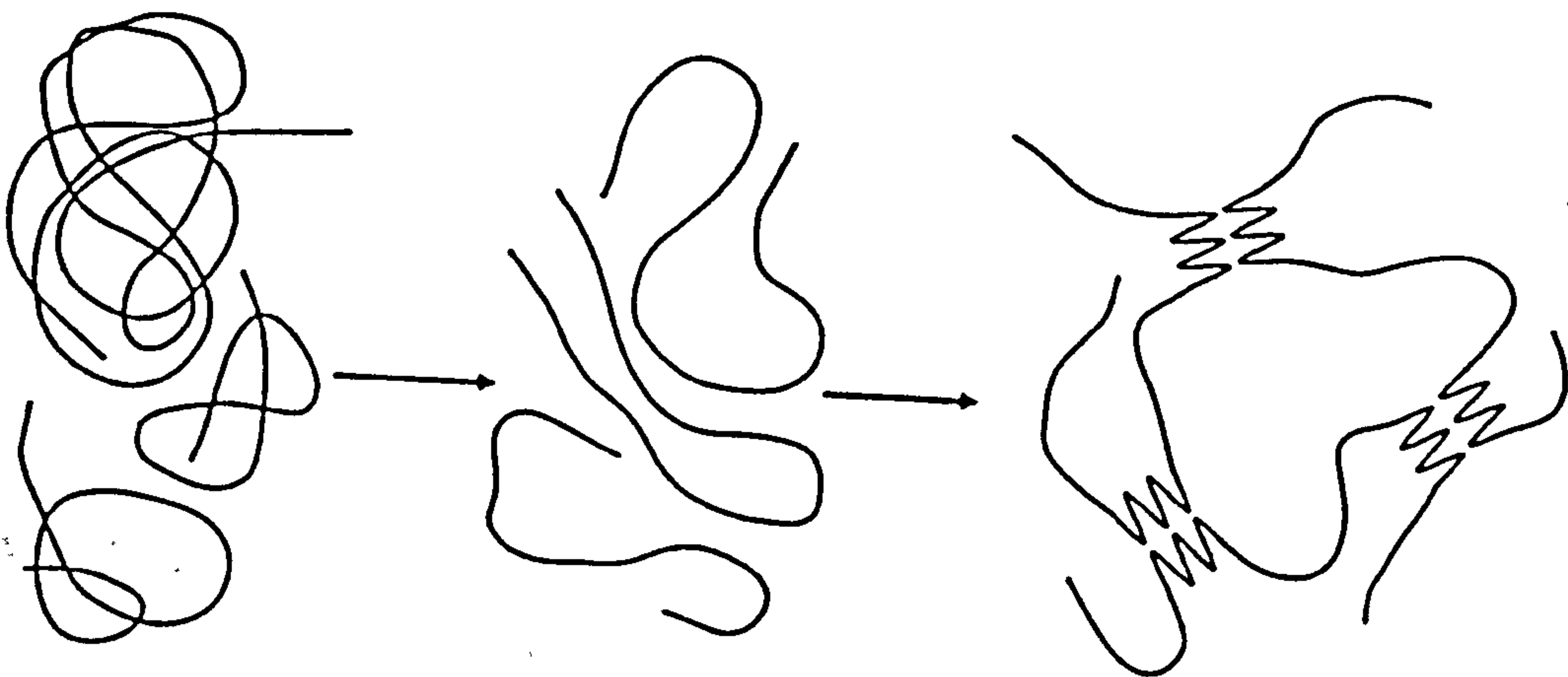


Fig. 1.1 Schematic diagram of gelation of a globular protein (Oakenfull, 1987)

Denaturation causes a change in the three-dimensional structure of the native protein, but without involving the rupture of peptide bonds. Aggregation of the denatured molecules will cause complexes of higher molecular weight. For the formation of a highly ordered gel it is necessary that the aggregation step proceeds at a rate slower than the unfolding step. The resulting gels are usually clearer, firmer and hold more water (less syneresis). In contrast, if aggregation occurs too rapidly, or simultaneously with denaturation, a coagulum may be formed which results in gels having higher opacity, lower elasticity and generally more syneresis. Coagulum formation often occurs if the pH of the system is close to the isoelectric point of the protein.

1.3 Factors affecting the gelation of globular proteins

The quality of the gels produced by heating a protein solution may depend on features that are associated with the protein, the solvent and the method used to produce the gel and, of course, a combination of these factors. For the gels discussed in this thesis some of the relevant factors may include the following;

1.3.1 Protein characteristics - crosslinking and charge

(a) Crosslinkings

Crosslinking is important for the formation of protein gels. This may occur via specific bonding at specific sites on the protein molecules or by nonspecific bonding occurring along the protein strands. The nature and degree of crosslinking would vary with the type of protein and the gelation environment.

Covalent

Covalent bonding is often regarded as a major influence in protein crosslinking. The most significant bond is normally considered to be the disulfide bond, with an energy

of formation of around 200 kJ/mol. The number of disulfide bonds varies between proteins; bovine serum albumin has 17, lysozyme has 4, soya bean 11S is thought to have 21 whilst soya bean 7S has none (Damodaran, 1988). Disulfide bridges in proteins enhance their tertiary structure and confer molecular stability. The crosslinking between molecules can occur via the sulfhydryl-disulfide interchange reactions (Fig. 1.2);

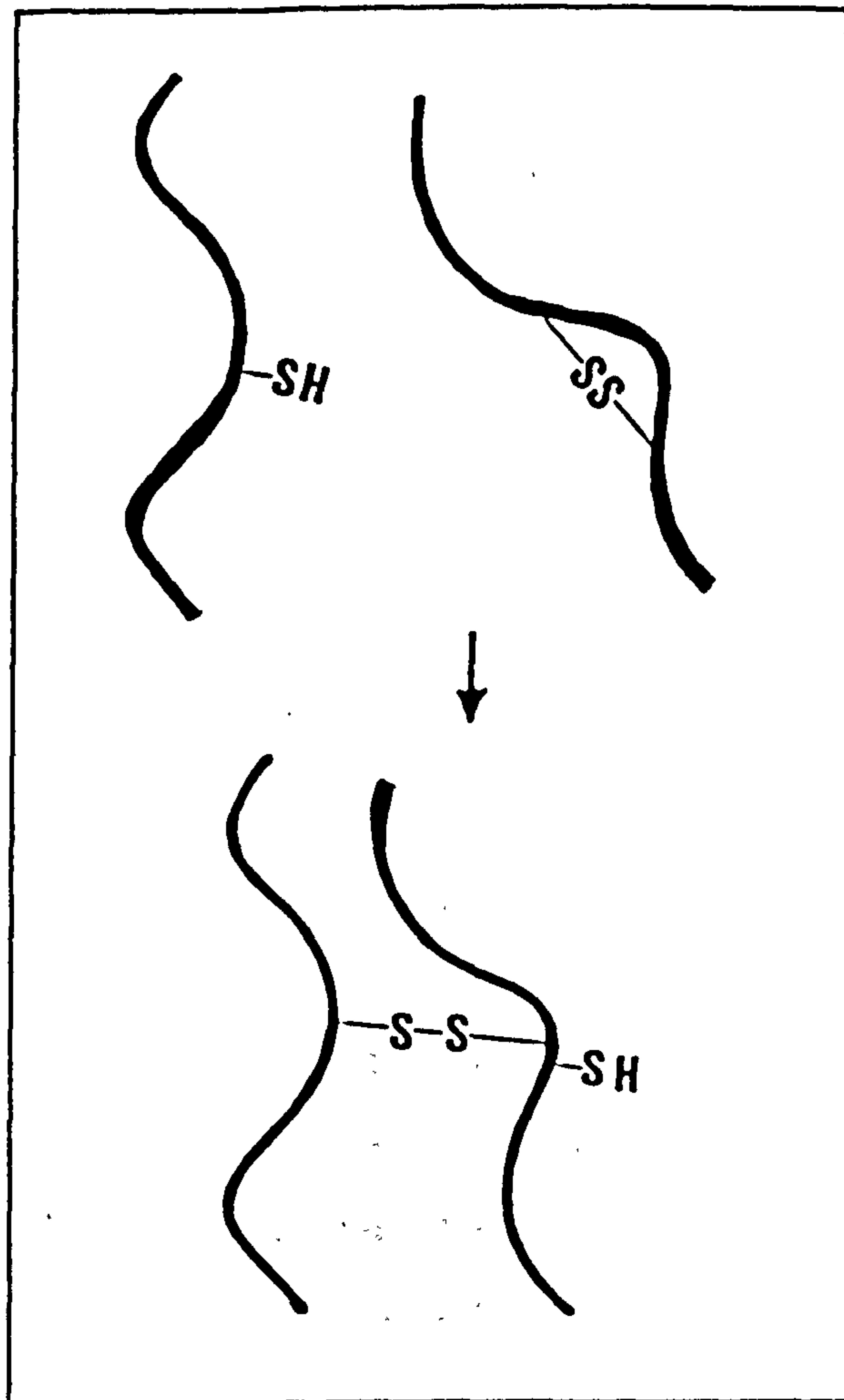


Fig. 1.2 The sulfhydryl-disulfide interchange reactions

The free sulfhydryl group reacts with a disulfide group in a neighbouring molecule to form an intermolecular disulfide bond while generating a new sulfhydryl group.

Gelation may be achieved if a sufficient amount of intermolecular disulfide crosslinks are formed.

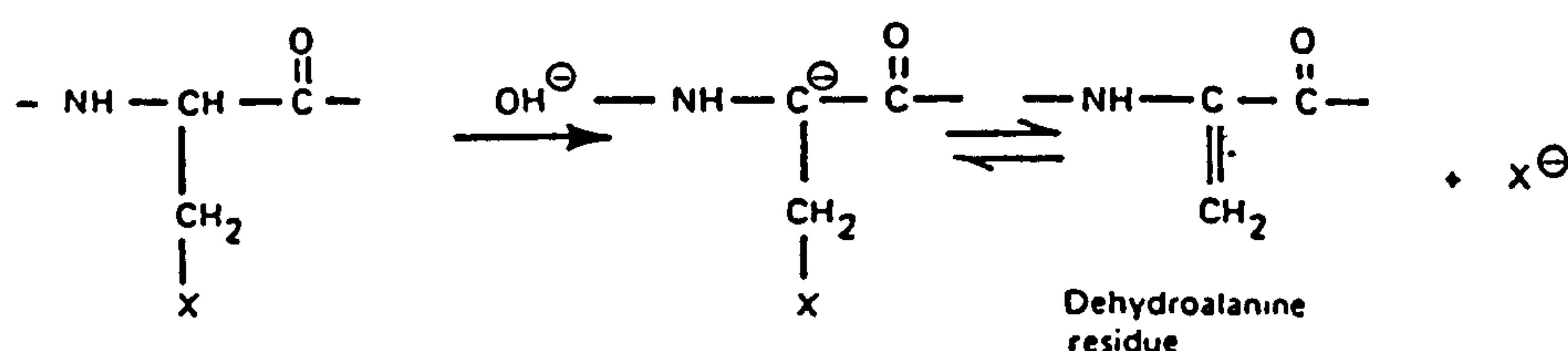
The importance of disulfide bridges for gelation of proteins has been shown by several authors. Mori *et al* (1986) reported that disulfide, hydrophobic, and hydrogen bonds were all essential for the thermal gelation of soybean 11S globulin. Disulfide links have been shown to contribute to the gel formation of bovine serum albumin solution on the alkaline side of its isoelectric point (Yasuda *et al* 1986).

As the formation of disulfide bonds is important for gel formation, agents that stop their formation would be expected to affect the gelation properties. This does appear to be substantiated. Mercaptoethanol is a disulfide bond breaking agent and its addition to preformed gels of soya protein caused them to become soluble (Mori *et al* 1986; Cabodevila *et al* 1994). Addition of mercaptoethanol to soya protein stopped it from gelling on heating (Mori *et al* 1986). Addition of N-ethylmaleimide (a sulfhydryl blocking agent) caused a decrease in the gel strength of whey protein concentrate (Schmidt and Morris, 1984) and in the hardness of bovine serum albumin (pH 8) gels (Matsudomi *et al* 1991). Howell and Lawrie (1985) showed that the addition of cysteine to whole plasma, bovine serum albumin or egg albumen resulted in a decrease in the gel strength.

However, changes in the disulfide bridging potential is not the only consideration in prediction of gelation. When the availability of sulfhydryl groups of bovine serum albumin was increased by thiolation (Murphy and Howell, 1990), the disulfide-sulfhydryl interchange reactions were not promoted and gelation was inhibited. This was thought to be due to the detrimental effect of increased overall negative charge on the molecule and decrease α -helix conformation and hydrophobicity.

In certain circumstances, covalent bonds other than disulfides can also link protein molecules. These bonds may be generated during heating a protein at elevated temperatures (Whitaker, 1986; Finley, 1989). The new crosslinks being formed in the presence of heat can take place within proteins, between proteins and between proteins and other components. The bonds generated include lysinoalanine and lanthionine type crosslinks and isopeptide bonds. The former type of crosslink is normally formed when food proteins are heated at elevated temperatures at alkaline pH. The initial step

in the reaction is the formation of a dehydroprotein e.g dehydroalanine, by a β -elimination reaction (Fig. 1.3).



where X = SH or OPO₃H₂.

Fig. 1.3 β -elimination reaction in proteins

The products of the reaction are very reactive with various nucleophilic groups, including ϵ -amino, sulfhydryl, imidazole, indole and guanidino groups of amino acid residues of proteins (Feeney and Whitaker, 1988). The reactions cause intramolecular and intermolecular crosslinkages in proteins, which give them properties quite different from those of the original proteins.

Isopeptide type crosslinks can also link protein molecules. It has been claimed that during high temperature processing of proteinaceous materials, such as occurs in extrusion, the glutamic acid or glutamine residues of the protein undergo browning reactions involving the evolution of water and the formation of covalent linkages (Ledward and Mitchell, 1988). The free carboxyl group of glutamic (or aspartic) acid or amide group of glutamine (or asparagine) may crosslink directly with lysine to form glutamyl (or aspartyl) lysine isopeptides with the liberation of ammonia (Bjarnson and

Carpenter, 1970). A recent study by Yoong *et al* (1994) indicated a liberation of water and loss of available lysine and glutamine residues occurred during a dry heat processing (160 °C for 30 min) of crude gluten. In the presence of reducing sugars, the loss of these amino acids increased by amounts dependent on the type of sugar and its concentration. These changes were thought to be indicative of isopeptide formation. Another example of processing where isopeptide formation may be important is extrusion. It has been suggested that isopeptide linkages help to create the initial structure of extrudates. On cooling of the product, they are further stabilised by the disulfide bonds and hydrophobic interactions (Yoong *et al* 1994; Ledward and Mitchell, 1988).

Another type of crosslink that can be formed between protein molecules is one involving formaldehyde with amino groups, primary amides or guanidyl groups (Fraenkel-Conrat and Olcott, 1948). These crosslinks may occur by the formation of covalent methylene bridges, with resulting increase in molecular weight and polymers that are not soluble in hydrogen bond breaking solvents (Fraenkel-Conrat and Meecham, 1949).

In cases where a protein is heated in the presence of reducing sugars, other forms of crosslinks in addition to the isopeptide link, have been postulated. These types of bonds are thought to be derived from the Maillard reaction between amino side chains occurring in the protein (for example, the ϵ -amino group on lysine) and carbonyl compounds of the reducing sugar. This reaction causes some changes to the protein behaviour and this will be discussed in some detail in Chapter 2.

Non-covalent

Non-covalent bonds are also involved in maintaining protein structure. These include hydrogen bonds, hydrophobic associations and electrostatic interactions. Most of these bonds are relatively weak with the energies of 0.4-80 kJ/mol.

A hydrogen bond is normally formed when a hydrogen atom is shared between an acid (proton donor) group and a base (proton acceptor). Hydrophobic interactions can be formed between non-polar residues as they associate to minimise hydration. The energy of formation is approximately 0.6 kJ/mole per water molecule displaced (Banaszak *et al* 1981). A good example of the importance of hydrophobic interactions was shown by Murphy and Howell (1991). Their study showed that the covalent attachment of the long chain valine to increase hydrophobicity of bovine serum albumin improved its gelling properties.

Electrostatic interactions may involve associations of charged amino acids. Protein molecules are zwitterions which carry a net positive or a net negative charge depending on their pI and the pH of the environment. Electrostatic interactions are repulsive for like charges and attractive for opposite charges. As this form of interactions is considered an important factor for protein gelation, this will be discussed in more detail in Section 1.3.2.

(b) Charge

The size and shape of the aggregates formed on heating are strongly influenced by the net charge on the molecules. Barbu and Joly (1953) proposed that two types of aggregates could be formed depending on how much charge the protein carried. These were linear aggregates formed when repulsion was large and 'random' aggregates when repulsion was small. For example, at pH values near the isoelectric point, denatured proteins aggregate randomly by, for example, hydrophobic interactions. At pH values far from the isoelectric point, electrostatic repulsive forces hinder the formation of random aggregates, and more ordered linear polymers are formed. Barbu and Joly (1953) proposed a kinetic scheme to explain the cause of linear aggregation i.e. reduction in the activation energy barrier near the ends of a growing chain. If the energy barrier against random aggregation is sufficient, the molecules can arrange themselves into strings and a fine stranded network structure is obtained.

Models for these aggregates have been presented by Tombs (1974), as random aggregation and the 'string of beads' structure. The 'string of beads' model refers to the appearance of the gels under the microscope. In this model, gels form as a result of association of molecules to produce small spherical aggregates which link to form linear strands. This is followed by the interaction of the strands to give the gel mesh.

The random aggregation model is consistent with the coagulation or clumping which occurs under conditions favouring a more random association of molecules since this would lead to a smaller effective chain length. For example, the adjustment of pH to a value near the isoelectric point of the protein would generate more bonding sites through decreased electrostatic repulsion and this would result in less oriented, more random interactions. The random nature of aggregation is thought to be the main factor which determines the relatively high protein concentration required in these types of gels (Tombs, 1974).

Random aggregates have been observed in heat-set gels of β -lactoglobulin (Hermansson, 1988) and acid-induced gels of casein (Fox and Mulvihill, 1990), while 'string of beads' structures have been observed in heat-set gels of serum albumin (Barbu and Joly, 1953; Murata *et al*, 1993). Models for the two gelling processes are depicted in Fig. 1.4.

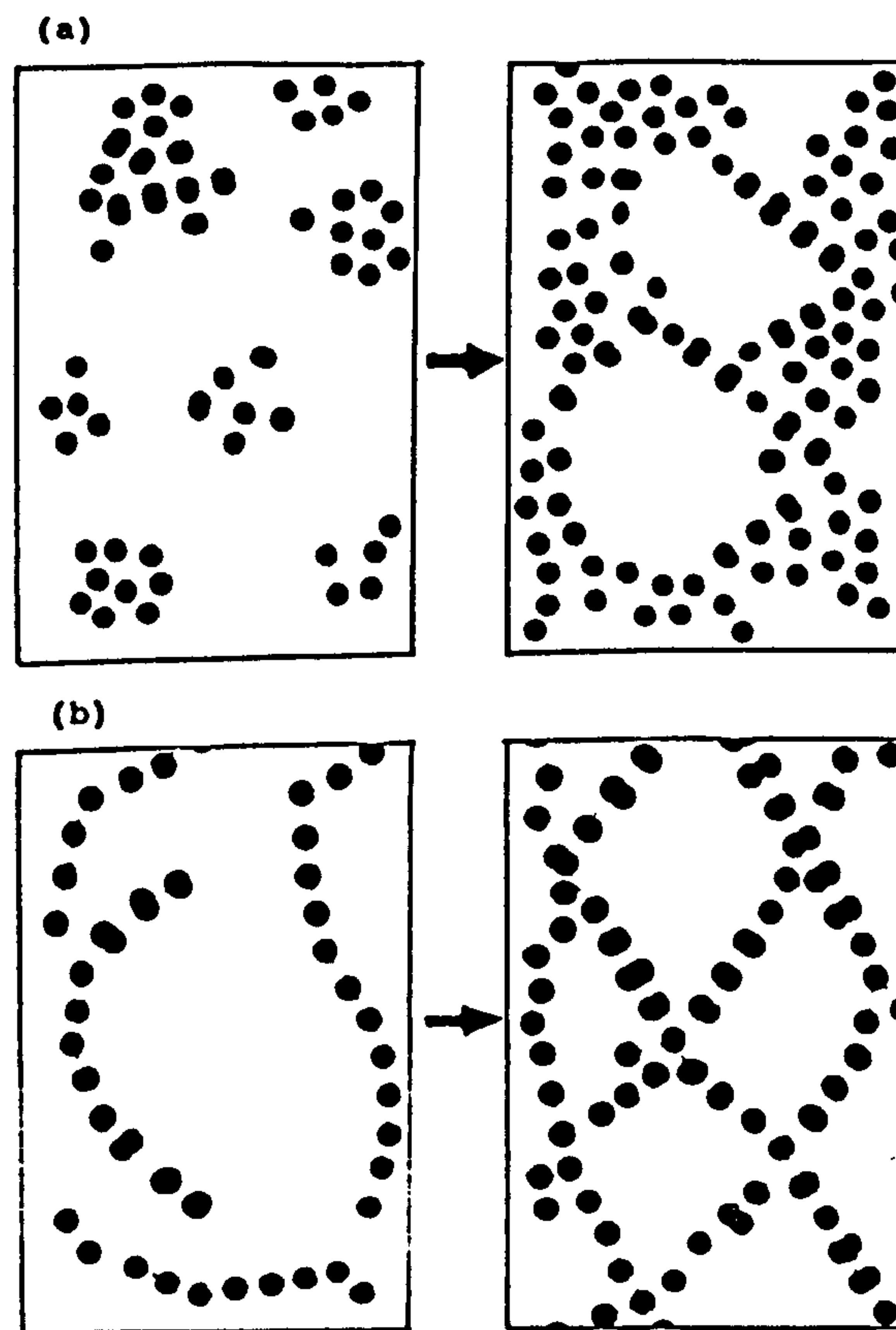


Fig. 1.4 Two types of gel networks formed by the aggregation of globular proteins: (a) random aggregation of molecules; (b) aggregation of 'string of beads' polymers (Doi, 1993).

1.3.2 Solvent characteristics - charge effects

(a) *pH*

In gel formation not only will the protein-protein interactions be important, but also the manner in which the protein interacts with the solvent. The charged amino acids may play an important role in stabilising the globular protein by their interaction with the surrounding water (Kinsella, 1982). It has been estimated by Bull and Breese

(1968) that aspartic and glutamic acid residues can bind 6-7 molecules of water per charged residues. This would be expected to have a major influence on network formation.

Movement of pH towards the isoelectric point of the protein will reduce the number of charged groups. As the charged groups become less in number, it could be expected that there will be an enhanced protein-protein interaction. This has been shown by Poole (1986) who studied gelation of mixed protein systems. He showed that bovine serum albumin did not form a gel at a concentration of 3 % at neutral pH. However, at pH 5 which is close to its isoelectric point (4.9), a coarse gel was formed.

At higher protein concentrations, the pH of heated protein dispersions have also been shown to exert a profound effect on the quality and appearance of the gels subsequently formed. Matsudomi *et al* (1991) demonstrated that bovine serum albumin gels (10 %) possessed maximum hardness at pH 6.5, and as the pH was increased above this value, the gel strength decreased. At pH values below 6.0, the gel hardness decreased markedly and white, opaque sponge-like coagula were obtained. The difference in gel strength at different pH values may reflect the importance of electrostatic attractive and repulsive forces between unfolded molecules for gel network structure.

The effect of pH on the appearance of bovine serum albumin gels (6 %) has been studied by Murata *et al* (1993). The gels formed were transparent at pH 4.0 and 6.5, translucent at pH 4.5 and 6.0 and turbid at the two pH values (pH 5.0 and 5.5) nearest the isoelectric point of bovine serum albumin. Langton and Hermansson (1992) differentiated between gels that form under conditions where the electrostatic repulsion between molecules is moderately high ($\text{pH} < 4$ or > 6) and gels which form under conditions closer to the isoelectric point ($\text{pH} 4 - 6$) by calling the former fine stranded gels and the latter aggregated gels.

In cases where there is an increase in charge on the protein, it should be expected that this can have an impact on the attractive-repulsive balance associated with network

formation. Therefore, modifications of charged amino acid residues can alter the properties of the proteins. Succinylation and acetylation are two of the most popular methods for such modification (Kinsella and Shetty, 1979). The techniques involve substituting succinyl and acetyl groups onto nucleophilic side chains of proteins. Most of this occurs on the ϵ -NH₃⁺ group of lysine therefore converting the cationic ϵ -amino groups to anionic or neutral residues and hence increasing the net negative charge on the protein. An example of such modification was shown by Murphy and Howell (1991) in which the methods of succinylation and thiolation were employed to enhance the net negative charge on bovine serum albumin.

Gels can be formed from mixtures of proteins. Difference in charge between protein molecules can also cause synergistic gelling effects. Murphy and Howell (1991) used mixtures of thiolated proteins and native or lysyl-bovine serum albumin to form gels. The interactions between the proteins increased as the difference between the protein charge increased. However the gelling properties of proteins which had been succinylated were inferior when gelled alone or when mixed with other proteins. The use of a basic protein such as clupeine (pI 12), to enhance gelation of bovine serum albumin is another example of the importance of electrostatic interaction for gelation (Poole, 1986; Poole and Fry, 1987). In this case, gelation occurred over the pH range of 4 to 9. At pH range 6-9, bovine serum albumin and clupeine are oppositely charged and the charge difference is great enough to cause association between molecules. At pH 5, a lower concentration of bovine serum albumin was required for the gelation of mixed bovine serum albumin-clupeine gels. When examined by electron microscopy, the structure of the gels appeared to resemble bovine serum albumin gels which were made at a similar pH.

As proteins will coagulate at pHs near their isoelectric point, this could be utilised for gel formation. Producing a solution of protein, followed by acidification will result in a gel. A good example for this is the gelation of soybean 11S protein by addition of glucono- δ -lactone (Kohyama and Nishinari, 1992; Kohyama *et al* 1992). Gluconic acid is produced by the hydrolysis of glucono- δ -lactone. This generates protons, lowers the pH and initiates coagulation of the protein. Gelation of this type does not

depend so much on temperature and hence the activation energy for gelation was quite low i.e. 15 kJ/mol (Kohyama *et al* 1992). However, the gelation kinetics of this system are not similar to the heat induced gelation of bovine serum albumin. The most probable reason for this is that the rate of pH fall could be expected to depend on temperature, glucono- δ -lactone concentration and the buffering capacity of the system.

(b) Ionic strength and specific ion effects

Ionic strength has a profound influence on gel formation. This effect can be shown by the addition of salts such as NaCl, KCl and CaCl_2 . Salts exert a charge shielding effect, and suppression of ionic repulsion at higher salt concentration, thus enhancing protein-protein interaction and formation of gels. An example of the application of specific ionic effects is the use of calcium chloride (CaCl_2) for gelation of bovine serum albumin. It is thought that gels are formed by calcium bridging between negatively charged groups of unfolded protein molecules. However, at too high a concentration e.g. 5 mM CaCl_2 , excessive crosslinkings between molecules may lead to compaction and collapse of protein matrices with syneresis and formation of a coagulum (Mulvihill and Kinsella, 1988).

Ionic strength can also influence the formation of aggregates. This has been demonstrated by Murata *et al* (1993). They studied the effect of different NaCl concentrations on heat treated bovine serum albumin solutions by an electron microscopy technique. The results indicated that the heat-denatured bovine serum albumin molecules connected to each other to form linear polymers at low NaCl concentrations (10 mM). With increasing salt concentration, the length of the linear polymers increased and interactions between linear polymers also increased. However at salt concentration > 200 mM the formation of ordered polymers was interrupted and massive aggregates were formed.

1.3.3 Method of preparation

(a) Heating

Heating may cause at least a partial denaturation and unfolding of the protein molecules. During heating various functional groups i.e. side chains of amino acids, are exposed and are capable of undergoing reactions with other proteins (protein-protein interaction) and/or polysaccharides, lipids and food additives.

The unfolding temperature varies with the type of protein. In the case of bovine serum albumin, the protein of interest to this thesis, the value is approximately 57 °C (Richardson and Ross-Murphy, 1981b). Morales *et al* (1976) showed that denaturation of bovine serum albumin began at 60 °C and increased up to near 90 % at 70 °C. Richardson and Ross-Murphy (1981b) distinguished the processes of unfolding and aggregation; above the unfolding temperature of 57 °C, unfolding was fast and spontaneous and aggregation was a slower process whilst below this temperature the unfolding was rate determining. At higher temperatures, the gels formed were transparent whilst below or at 57 °C the gels formed were slightly opalescent.

Alterations in heating conditions can change the microscopic and macroscopic structural attributes of gels. Tombs (1970) showed by electron microscopy, differences in the structures of aggregated bovine serum albumin heated at 70 °C (30 min) and 100 °C (8 seconds). The formation of bovine serum albumin gels at 70 °C required formation of protein strands. At this temperature, only a slight change in conformation occurred. It was supposed that only some of the SH groups became accessible to intermolecular crosslinking and the protein aggregated in an orderly manner. Heating at 100 °C for 8 seconds produced coagulates which appeared as clump-like aggregates, a combination of chains and spheres. It was thought that at such a high temperature a more complete unfolding of the polypeptide chain occurred, the number of interaction centres increased and aggregation was of a random manner.

The methods of heating can also influence the appearance of the gels formed. A "two-step heating method" has been introduced by Kitabatake *et al* (1987). This method enables preparation of transparent gels at high salt concentrations (normally turbid by "one-step heating") if a protein solution is heated in advance under salt free conditions.

(b) Concentration

The aggregates formed on heating protein molecules can become so large that they are no longer soluble and consequently precipitate. If the protein concentration is sufficiently high (i.e. exceeding a certain critical value), a gel is normally formed. This concentration is termed critical concentration for gelation (denoted as C_0), below which no gel network is formed.

The C_0 for gelling globular proteins is normally quite high (5-10 %). This is one of the major disadvantages of globular proteins that are used as a gelling agent in food applications. The value of C_0 is often determined rheologically (more details are given in Chapter 4) and in the case of bovine serum albumin a value of 6.8 % at pH 6.5 has been reported by Richardson and Ross-Murphy (1981b). C_0 is highly dependent on pH, and is at a minimum at the protein isoelectric point. For example, the gelation threshold concentration for ovalbumin at its isoelectric point is ~ 1 % compared with ~ 8 % at pH 7.0 (Bikbov *et al* 1985). Similar differences for β -lactoglobulin gels have been reported by Stading and Hermansson (1990).

As gel formation is dependent on the formation of a continuous matrix, the concentration of the proteins and the rate of formation of the crosslinks between the proteins are obviously important factors in the kinetics of gel formation. Heating provides the activation energy for chemical reactions that lead to the processes required for gelation. The heating time at a specific temperature, required to form a protein gel structure is generally considered to decrease with increased protein concentration. This relationship has been shown by Richardson and Ross-Murphy

(1981a) for the thermal gelation of bovine serum albumin and Nakamura *et al* (1984) for the thermal gelation of soybean globulin 11S. A similar tendency was also observed for an enzymically induced gelation, specifically casein micelle clotting by rennet (Tokita *et al* 1982).

1.4 *Gelling systems in this thesis*

In this thesis, the protein most used in the study of the so called 'Maillard gelation' was bovine serum albumin. The physical and biological properties of bovine serum albumin have been discussed in a very comprehensive review by Peter (1985). The ability of bovine serum albumin to form gels has been reported previously (Richardson and Ross-Murphy, 1981a and b; Tombs 1974; Yasuda *et al* 1986) and the gelation of bovine serum albumin was addressed in depth by Clark and Lee-Tuffnel (1986). Some important information on bovine serum albumin is summarised in Table 1.1.

Table 1.1 Summary of bovine serum albumin properties.

Molecular weight	65 000	Tanford (1961)
Denaturation temperature	57 °C	Richardson and Ross-Murphy (1981b)
Critical concentration, C ₀ for gelation (% , pH 6.5)	6.8 %	Richardson and Ross-Murphy (1981b)
Net charge at pH 7.0	- 18.0	Peter (1985)
Isoelectric point	pH 5.0	Peter (1985)
Disulfide (per molecule)	17	Peter (1985)
Lysine (per molecule)	59	Peter (1985)
Sulfhydryl (per molecule)	1	Peter (1985)
Intrinsic viscosity (globular) (ml/g)	3.7	Tanford (1961)
Intrinsic viscosity (denatured) (ml/g)	52.2	Tanford <i>et al</i> (1967)

CHAPTER 2

CHANGES TO PROTEIN AS A RESULT OF THE MAILLARD REACTION

The Maillard reaction has been implicated in;

- (a) Formation of coloured compounds
- (b) Change in pH
- (c) Change in the charge on proteins
- (d) Indicators of increased crosslinking between proteins

As these features are seen in the gel systems studied within this thesis it is reasonable to consider that the Maillard reaction is implicated in gel formation.

The chemistry, products and changes to protein due to the amino-carbonyl reaction are therefore reviewed in this Chapter. However, no attempt has been made to review all the literature available on the Maillard reaction. The general topic is covered briefly with only areas of specific interest to this thesis given in more depth.

2.0 *Study of the Maillard reaction*

The Maillard reaction is important in foods containing reducing sugars and protein. Typically, studies have focused on the effect of the reaction on the quality of foodstuffs following heat processing and storage. Colour and flavour development are frequently being studied in detail. The studies are often associated with products of low water activity. Studies to elucidate the mechanisms and the chemical components are normally carried out on a model system consisting of a single amino acid and a reducing sugar. However, it is known that even in these simple systems many compounds can be generated via the reaction (Apriyantono and Ames, 1993). Similarly it is expected that many of these compounds can also be generated when a protein, such as bovine serum albumin is reacted with a reducing sugar. It might be expected that the compounds formed in the Maillard reaction may then interact with the protein and hence changing the subsequent behaviour of the protein.

2.1 *Chemistry and products of the Maillard reaction*

The Maillard reaction is a series of chemical reactions that occur between amino and carbonyl compounds resulting in complex changes in biological and food systems. The reaction may take place slowly at low temperatures e.g. 30 °C, but proceeds more rapidly at higher temperatures.

The Maillard reaction scheme by Hodge (1953) is still the most accepted pathways of chemical reactions that lead to formation of brown polymers (Fig. 2.1). More recent reviews on the reaction are given by Mauron (1981), Danchy (1986) and O'Brien and Morrissey (1989). A typical way of describing the Maillard reaction is to divide it into three stages, an initial reversible stage, intermediate pH dependent stage and the final polymerisation stage.

The initial step is the combination of reducing sugar to protein-amino groups i.e. amino-carbonyl condensation. The trivalent nitrogen atom of the amine, which has

an unshared electron pair, acts as a nucleophile towards the carbonyl group (Fig. 2.2).

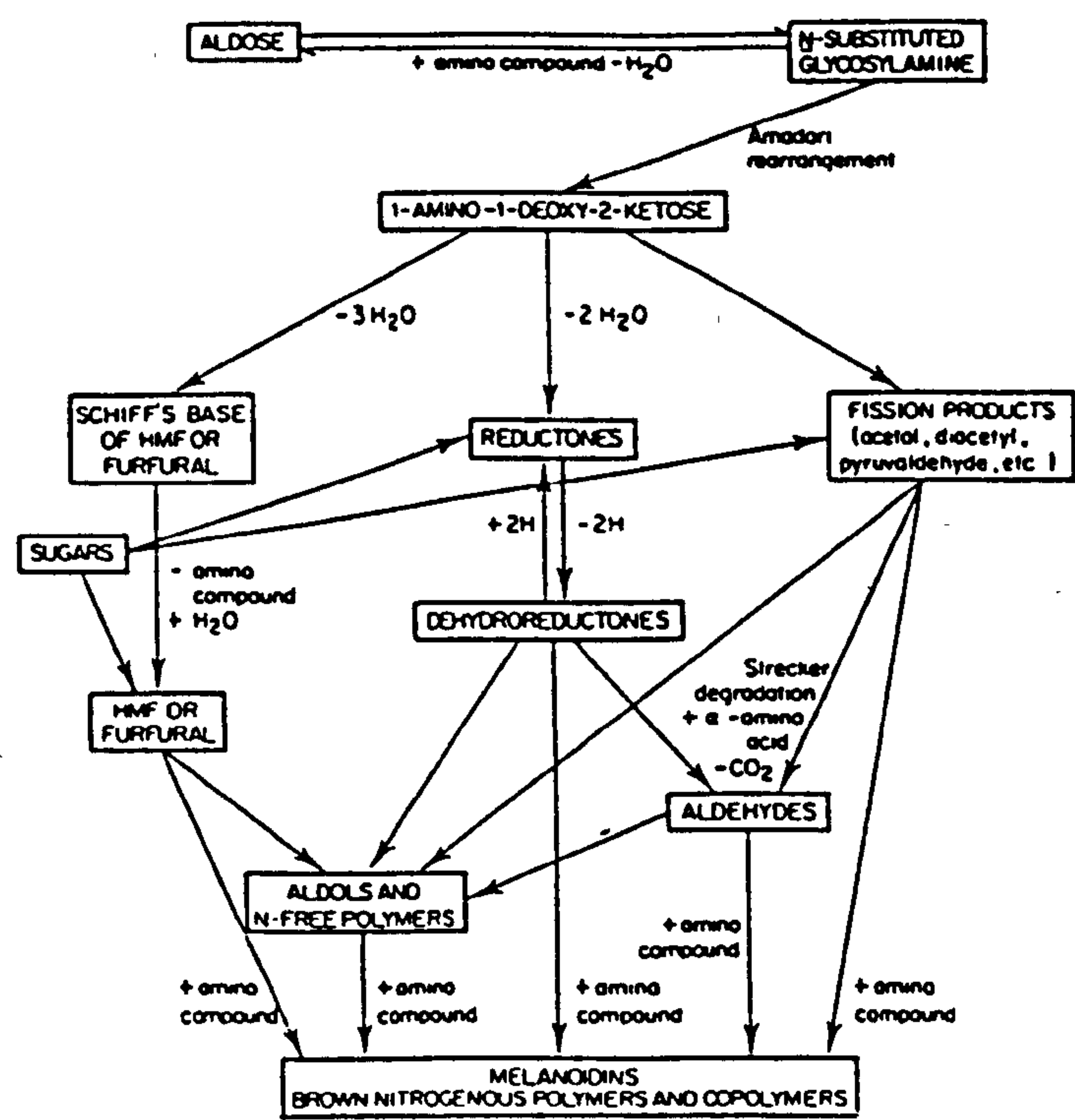


Fig. 2.1 The Maillard reaction scheme adapted from Hodge (1953).

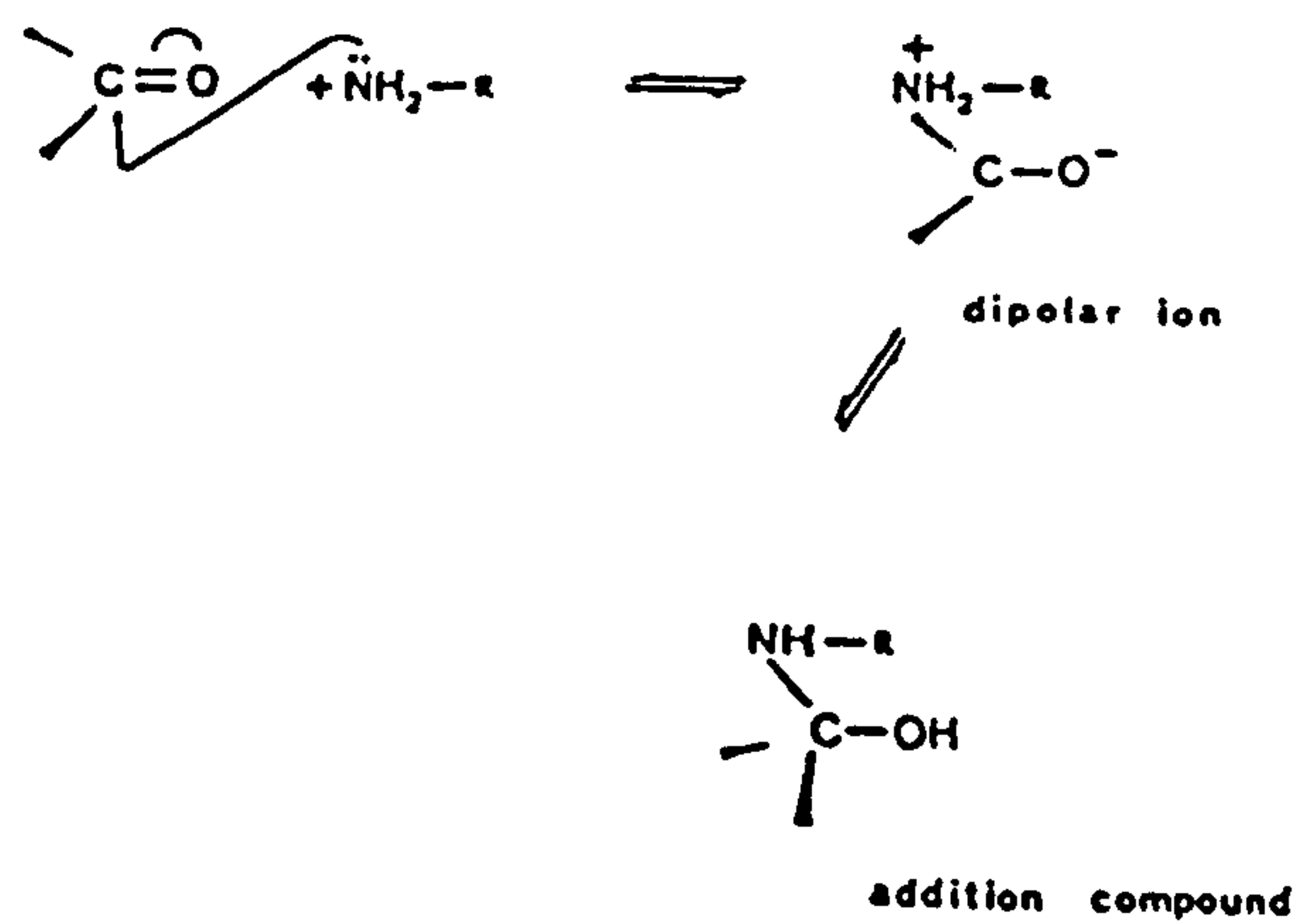


Fig. 2.2 Initial steps of the amino-carbonyl condensation

Several factors may influence the kinetics of the reaction. The rate of condensation of the carbonyl group with the free amine depends on the pK of the protonated amino group and the type and concentration of the sugar. A base (B) can catalyze such reactions by removing a proton (H) from the nucleophile (Nu), thus converting a weak nucleophile into a stronger one.



The addition compound then forms a Schiff's base, followed by cyclisation to form the corresponding N-substituted glycosylamine. The rate of Schiff's base formation has been shown to depend on the proportion of the sugar which exists in the carbonyl form (rather than in the ring form) in aqueous solution (Bunn and Higgins, 1981).

There is a subsequent rearrangement of the N-substituted glycosylamine to form a deoxyketose or deoxyaldose compound, depending on the type of sugars included in the system. This is the first irreversible step in the Maillard reaction and is termed the Amadori rearrangement (Fig. 2.3).

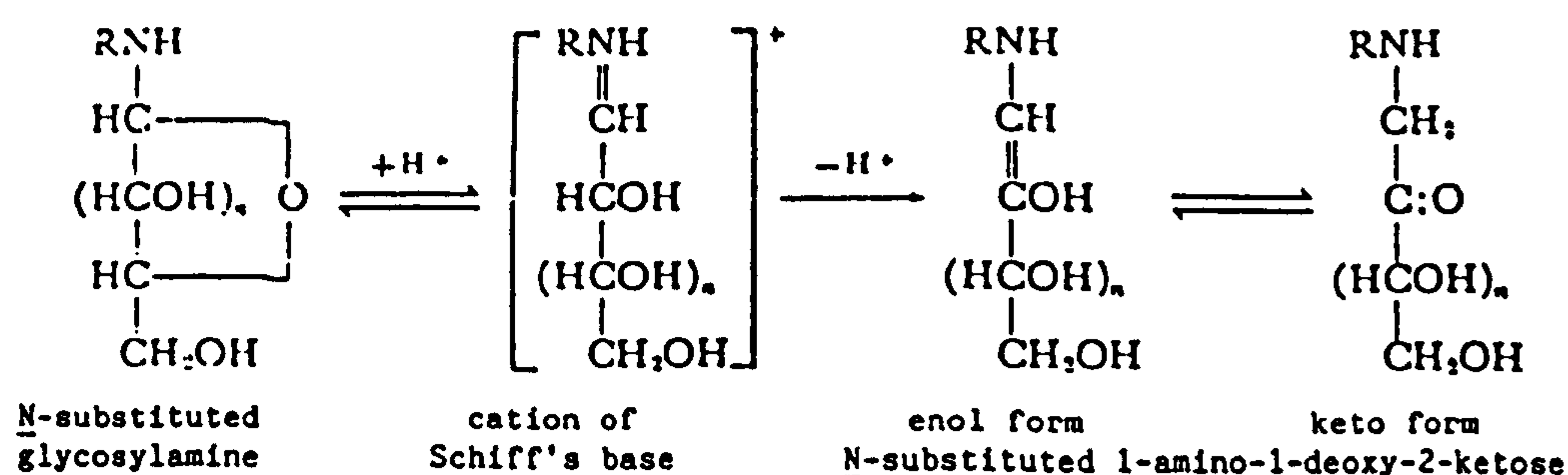


Fig. 2.3 The Amadori rearrangement

During the intermediate stage, the Amadori compounds may undergo either dehydration or fission reactions. In general, different compounds are produced depending on the pathways of the breakdown of the Amadori compounds.

Dehydration of Amadori compounds are dependent on the pH of the reaction (Fig. 2.4). α -Dicarbonyls and C-methyl reductones (e.g. glyoxal, 3-deoxyglucosone, diacetyl, hydroxydiacetyl and pyruvaldehyde) are known to be generated if the pH is high (above pH 5) and the reaction follows 2,3 enolisation (2,3 E) pathway after loss of 2 molecules of water. On the other hand at low pH (at pH 5 or lower), the reaction would follow 1,2 enolisation (1,2 E) pathway with the subsequent production of α -hydroxycarbonyl and furfural compounds (e.g. 5-hydroxymethylfurfural and glyceraldehyde) after loss of 3 molecules of water. An example of a study on the effect of pH on the Maillard reaction products has been demonstrated by Apriyantono and Ames (1993) who studied the volatile reaction products generated during the reaction of xylose and lysine. They concluded that heating without pH control (final pH 2.6) increased the total yield of volatile reaction products, due mainly to an increased yield of 2-furfural. When pH was maintained at 5.0, there was an increase in the total yield and numbers of nitrogen containing reaction products with pyrazines being the second most abundant class of volatiles in the system.

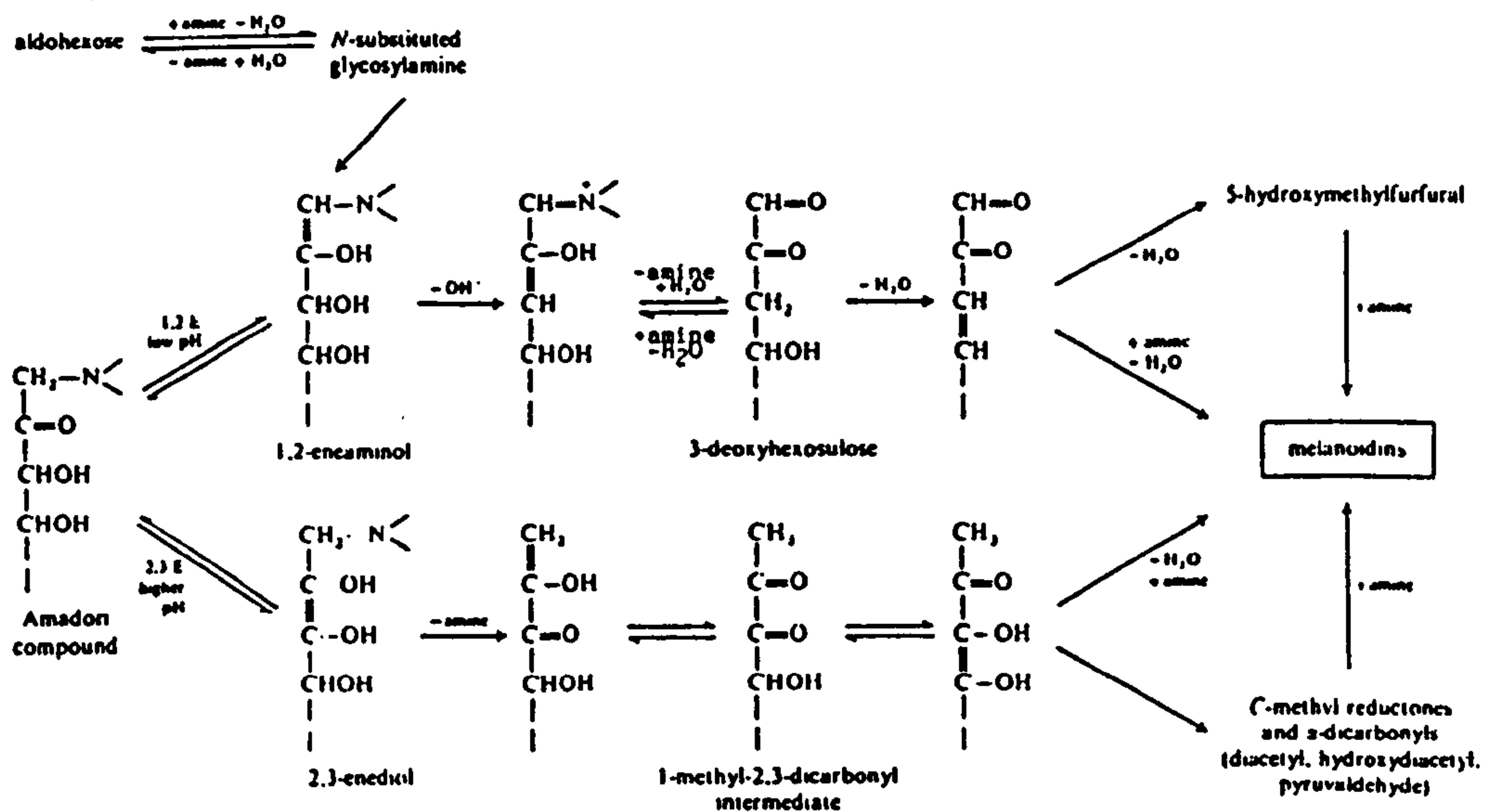


Fig. 2.4 1,2- and 2,3- enolisation and some subsequent reactions.

The Amadori compounds may also undergo a fission reaction which occurs mainly by retro- or de-aldolisation. Fission products include diacetyl and pyruvaldehyde. Formation of free radicals before the Amadori rearrangement has been reported (Namiki, 1988). There appears to be a direct pathway from the glycosylamine to reactive fission products without involving the Amadori compound pathways. This has been demonstrated to involve fragmentation of the Schiff base product at the first stage of the Maillard reaction to give a very reactive low molecular weight enaminol product followed by the formation of a pyrazinium free-radical product as well as browning products.

The final stages of Maillard browning are complex and poorly understood and involve conversion of low molecular weight precursors, such as furfural, fission products and reductones into the high molecular weight melanoidin pigments. While melanoidins are the ultimate products of the reaction, many compounds of low molecular weight are formed which are very important in flavour and off-flavour production.

Other than pH and reactants, the formation of Maillard reaction products is also dependent on temperature. It has been shown that the activation energy for the formation of 5-acetyl-2,3-dihydro-1H-pyrrol-izine (5-ADP) in a proline/glucose system was 180 kJ/mol (Stahl and Parliment, 1994). Other compounds detected from the reaction (maltoxazine and 2-acetyl tetrahydropyridine) had lower activation energy indicating that their formations occurred under milder conditions. Benzing-Purdie *et al* (1985) concluded that increased temperature may lead to an increase in aromatic character in both high and low molecular products. They showed that Melanoidins formed at high temperatures had fewer unsaturated carbons bonds and different aliphatic carbons compared to the coloured compounds formed at room temperature (22 °C).

2.2 Browning

Browning is a well-known result of the Maillard reaction. Most studies on browning have concentrated on the effect of the colour production on quality of processed foods. Browning can be an advantage when a dark brown colour is desirable, for instance during frying or roasting of meat. However it is not desirable during processing and storage of milk.

The extent of browning can be measured by measuring the absorbance of the reducing sugar/protein system at 420 nm (Kato *et al* 1988) or estimating the L-values on a Hunter L*a*b colour scale (Hill *et al* 1992). These will allow the study of sugar reactivity on the rate of browning. Hashiba (1981) estimated that the reducing power and extent of browning with ribose was about twice that of xylose and 7 times that of glucose. The extent of browning with glycine was reported as about 30 times that of glucose. The reactivity of these compounds to browning is related to their structures. The structures of xylose, ribose and glucose are shown in Fig. 2.5.

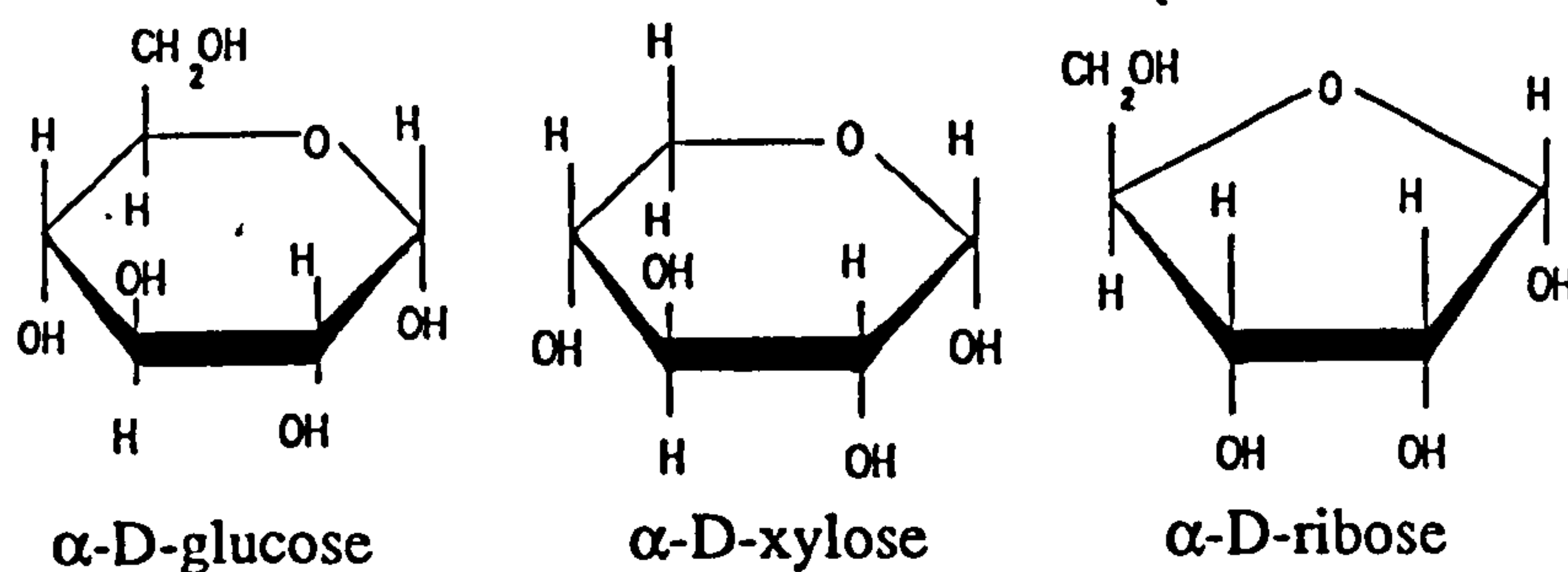


Fig. 2.5 Structures of reducing sugars used in the thesis

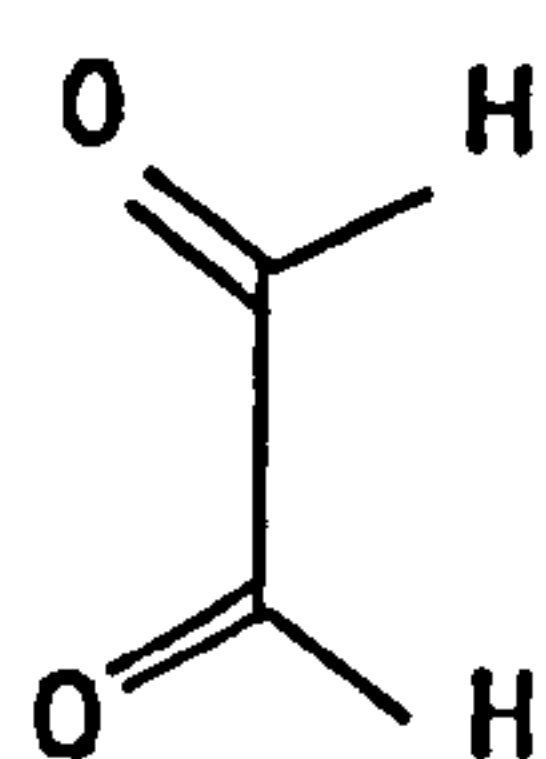
Browning reactions occur much more rapidly if a protein or amino acid is reacted with fragmentation products of the sugars (Hayashi and Namiki, 1986). These reactive carbonyl compounds include glycoaldehyde, methylglyoxal, glyceraldehyde and glyoxal. The relative values for glycoaldehyde and glyceraldehyde were about 2000 times and that of methylglyoxal was about 650 times that of glucose. 3-deoxyglucosone and glucosone had higher abilities to brown than glucosylamine and its Amadori products. However, the browning values for glycoaldehyde and glyceraldehyde were far higher than those of these intermediates. This may suggest that even if a small amount of sugar fragmentation occurs, browning by sugar fragmentation will make a major contribution to browning in the early stage of the Maillard reaction.

The intensity of browning produced by heating sugars with proteins is also dependent on the types of amino acid used or contained in the protein. Based on the browning reaction with glucose, fructose, ribose and lactose at 121 °C for 10 min, common amino acids have been classified into three groups (Ashoor and Zent, 1984). Lysine, glycine, tryptophan and tyrosine were included in the highest browning group, whilst histidine, threonine, aspartic acid, arginine, glutamic acid and cysteine were included in the low browning amino acids. The remaining amino acids were included in the intermediate browning group.

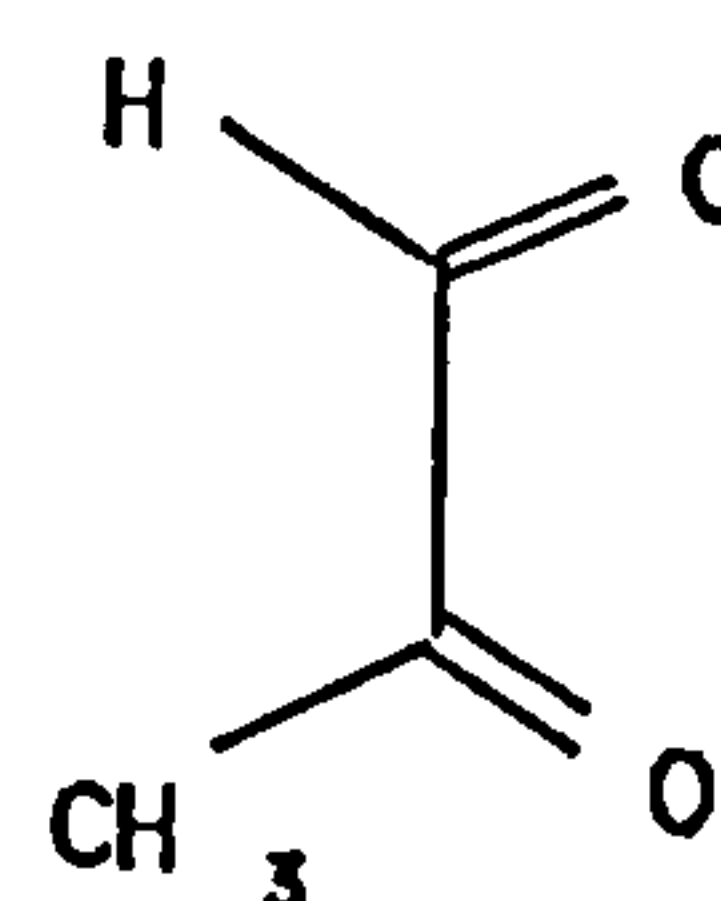
It is well known that sucrose, a non-reducing sugar does not participate in the Maillard reaction. However, at elevated temperatures sucrose can be broken down, presumably by the splitting of the glycosidic bonds to yield glucose and fructose which may

undergo Maillard reaction (Hurrel and Carpenter, 1977b). The process of the hydrolysis of sucrose is favoured at low pH values and relatively high moisture levels.

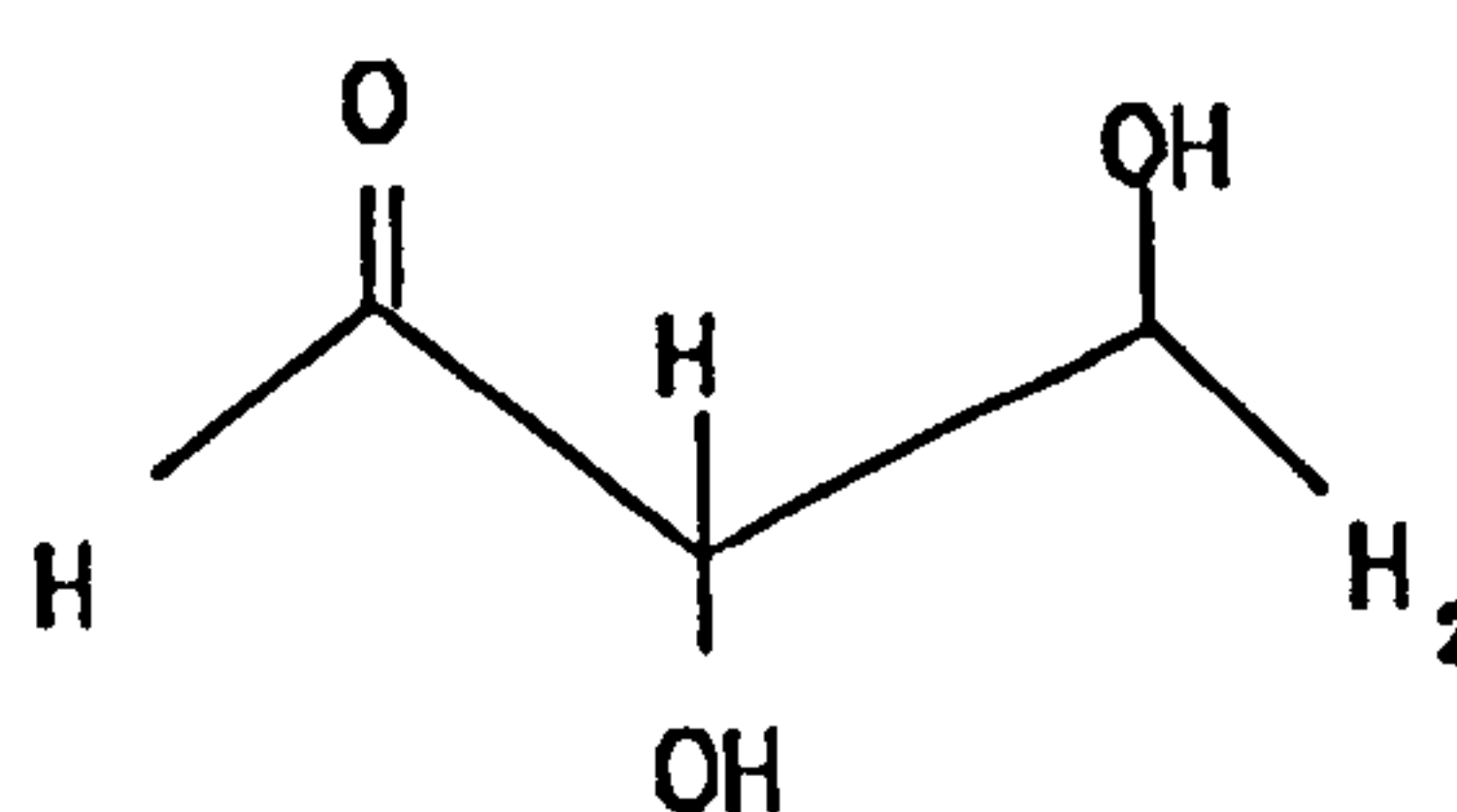
In this thesis, compounds known to be produced from the Maillard reaction were also used to induce the Maillard gelation. These were glyceraldehyde and glyoxal and the relative reactivity based on browning measurement was ; glyoxal < methyl glyoxal < glyceraldehyde < glycoaldehyde (Hayashi and Namiki, 1986). The structures of glyoxal, methyl glyoxal and glyceraldehyde are shown in Fig. 2.6.



Glyoxal



Methyl glyoxal



Glyceraldehyde

Fig. 2.6 The structures of Maillard products

2.3 *Decrease in pH*

The Maillard reaction can cause a substantial decrease in the pH of the system. It is thought that this is due to the formation of organic acids, Maillard reaction products and loss of basic amino groups. The loss of basicity of the system may result in a decrease in the buffering potential against acid formation and hence produce a lower final pH. This pH fall depends on the time and temperature of heating and reactivity of reducing sugar included in the system (Hill *et al* 1992).

It has been shown that during the reaction of histidine and glucose at 100 °C in 0.1 M phosphate buffer, a drop in pH from 7.0 to pH 5.0 occurred within 5 h of reaction (Lingnert and Eriksson, 1980). The pH then levelled out at approximately pH 4.5. Even with a stronger buffer, a considerable drop in pH occurred. When xylose and lysine were heated (121 °C for 10 min), the pH decreased from the initial pH of 4.9 to 2.6 (Apriyantono and Ames, 1993).

Typically, the initial pH of the system used in this thesis (bovine serum albumin + xylose solution) was around 6.5 to 7.0. Attempts to control the pH during the Maillard gel formation proved to be difficult, unless very high ionic strength buffers were employed. The addition of buffers or high concentration of alkaline materials, although control the pH, would change the gelation properties of protein, as explained in Chapter 1. The pH fall was therefore monitored in the systems discussed in this study, but not controlled.

2.4 *Alteration to charge and amino acid contents*

The Maillard reaction can provoke a decrease in amino acid availability in food proteins (Mauron, 1981) and the mechanisms for this decrease could be attributed to either or both of the following two factors;

1. Blocking of amino side chains, for example lysine
2. Involvement in the crosslinking reactions

Lysine is well known to be susceptible to the browning reaction due to the ϵ -amino group on the side chain. As well as reducing sugars, lysine may also react with active intermediate compounds formed during the advanced Maillard reaction such as the carbonyls and aldehydes. Loss of positive charged amino group will effect the total charge on the protein and its isoelectric point.

The loss of nutritionally available lysine as a result of the Maillard reaction has been reported (Okitani *et al* 1984; Yen *et al* 1989; Armstrong, 1994). An early report by Mohammad *et al* (1949) indicated a decrease in isoelectric point and the number of basic groups in bovine serum albumin when reacted with glucose. Lea and Hannan (1950) investigated the reaction of a casein-glucose mixture and found a slight increase in the number of acidic groups as compared to the original casein. There was a variation in the rate of reaction of different aldoses and aldose derivatives with the casein. The rate of loss of free amino nitrogen was highest with xylose and arabinose, much lower with glucose and galacturonic acid and lower still with lactose, maltose and glucuronic acid. Polyacrylamide gel electrophoresis studies on protein stored in the presence of sugars indicated that the protein stored with sugar had an electrophoretic mobility greater than that of the native protein. This indicated that the positive charge of protein was decreased presumably by the blocking of amino group with sugars (Kato *et al* 1986).

The decrease in net positive charge that occurs when the amino side groups interact with carbonyl group is relevant to the system used in this thesis. In Chapter 1 (page 20) it was noted that bovine serum albumin will have a net negative charge of - 18 at a neutral pH. Loss of about 20 % of the lysine residues as reported by Yaylayan *et al* (1992) would therefore increase its overall charge. The enhanced net negative charge on bovine serum albumin molecules could lead to gelation and/or alteration in the quality of the gels.

As destruction of lysine impairs nutritional quality, one may question the suitability of the Maillard reaction in the production of food gels. However, in many cases it is the functionality which make the product of commercial importance, rather than its nutritional quality (Hill *et al* 1992). Recognition of loss of nutritional value however, should not be ignored.

In addition to lysine, the loss of some other amino acids have also been reported. Mohammad *et al* (1949), Okitani *et al* (1984) and Kato *et al* (1986) reported a loss in lysine as well as arginine, tryptophan, histidine and methionine residues when glucose was incubated with lysozyme or bovine serum albumin. In real food such as cod muscle, losses of cystine, lysine and arginine have been reported when the food was heated with glucose (Miller *et al* 1965). Lysine, arginine and histidine were lost in 'doce de leite', a Latin American dessert produced by heating at 90 °C for 150 min (Pavlovic *et al* 1994).

In many cases the availability of all amino acids may have been reduced due to crosslinkages being formed between the protein chains via the breakdown products of the Maillard reaction (Hurrel and Carpenter, 1977b). This topic is discussed in the next section.

2.5 Polymerisation of proteins

It is recognised that the interaction of reducing sugars with proteins via the Maillard reaction may induce changes that are important in the subsequent functional properties of the protein. Authors when discussing the Maillard reaction of proteins use the term polymerisation to describe the binding of a carbonyl group to a protein and also for the subsequent crosslinking of the proteins. Within this thesis, *glycation* will be used to denote sugar or other carbonyl compounds covalently attaching to protein while *crosslinking* will denote protein-protein crosslink formation.

Polymerisation is reported to be responsible for the reduced rate of protein digestion

(Hurrell and Carpenter, 1977a) and to lead to the toughening of foods during thermal processing or storage (Sullivan, 1981). The binding of sugar with protein often decreases its solubility (Kato *et al* 1990) and can impair food proteins that are dried in the presence of sugars e.g egg whites. The addition of a group to an active enzyme can cause a marked loss in enzyme activity.

2.5.1 *Binding of sugars to protein*

Within the protein structure there are few available free amino groups to interact with sugars. However, covalent interaction with the N-terminal amino group and to free amino side chains of amino acid residues may occur. This is likely to occur in a time/temperature/concentration dependent manner (Yaylayan *et al* 1992). An early report by Lea and Hannan (1950) indicated that, as storage times and reaction increased, greater amounts of carbohydrate combined with lysine and other reactive side chains resulting in insolubility and colour development.

It was thought that the binding of sugar to the free amino groups of proteins was dependent on the form of the sugars involved. For instance, the proportion of sugar existing in the carbonyl form is higher for galactose than for glucose or mannose (Hayward and Angyal, 1977), therefore it was expected that the initial binding of the sugar to the free amino group would be faster for galactose than for glucose and mannose. However, in studies on ovalbumin-aldohehexose systems, it has been shown that there was no significant difference in the amino group decrease in ovalbumin stored with glucose, mannose or galactose (Kato *et al* 1986). It appears that in these low moisture system that the differences in reactivity for binding to the amino groups cannot be explained by the ratio of carbonyl:hemiactal, but are more likely to depend on the rate of formation of Amadori rearrangement products, and the degradation products of the reaction.

2.5.2 Crosslinking

There is substantial evidence that the Maillard reaction is implicated in the formation of additional crosslinks that cause proteins to be covalently linked to other protein. Loss of solubility and change in mobility by SDS-PAGE have been used as indicators of the additional crosslinking. Ludwick (1974), Watanabe *et al* (1980) and Kato *et al* (1990) showed the presence of high molecular weight compounds formed after incubation of proteins with reducing sugars.

Although crosslinking does occur, what is less well established is how the mechanism proceeds and the chemical nature of the crosslink or crosslinks. Another less well understood area is the importance of the state of the model system being investigated. Typically powders are mixed together and held at temperatures between 50-80 °C at relative humidities of 55-75 %. The relevance of these results to those occurring in solutions or at temperatures greater than 100 °C, the conditions of most relevance to this thesis, is questionable.

"Pentosidine"

Perhaps one of the easiest crosslinks to envisage is that of the "pentosidine". The proposed mechanism for the formation of pentosidine is given in Fig. 2.7. Sell and Monnier (1989) proposed this imidazo [4,5-b] pyridium molecule comprising a lysine and an arginine residue crosslinked by a pentose (ribose or xylose).

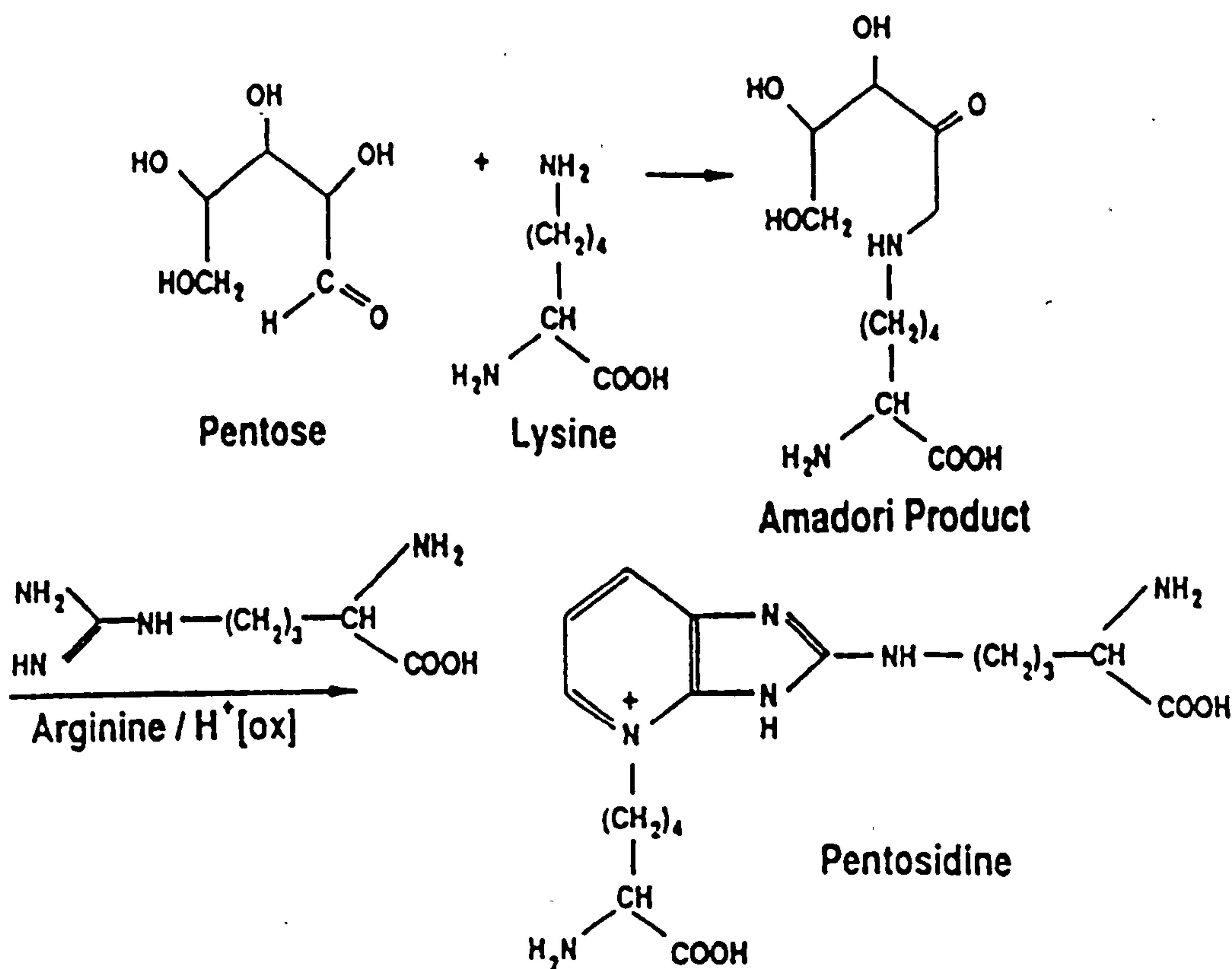


Fig. 2.7 Proposed mechanism for the formation of pentosidine (Sell and Monnier, 1989).

Very small amounts of pentosidine could be quantified in various food samples (Henle, 1996). However, these amounts would indicate that this type of crosslink would only play a minor part in crosslinking of food proteins.

Disulfide bonds

The covalent bonds that link proteins are normally disulfide bridges. Addition of 'Maillard crosslinks' has been shown to occur when proteins have been pretreated with SDS + β -mercaptoethanol, and therefore cysteines may not be a prerequisite for the new crosslink.

However, disulfide bridges have been implicated in the polymerisation of protein. Andrew (1975), Ludwick (1979) and Feeney and Whitaker (1988) reported that there

was a destruction of the disulfide bonds during the course of the Maillard induced crosslinking. Cabodevila *et al* (1994) also suggested that soya proteins containing disulfide linkages may crosslink by the Maillard reaction while the 7S soya protein, with no disulfide bridges may not.

2.5.3 Degradation products

There is an accumulating set of evidence that the Maillard crosslink is not formed directly from interactions of the sugar with the amino group, but from the subsequent products. Japanese workers stored lysozyme-glucose systems at 50 °C and 75 % relative humidity for 30 days. They noted losses of lysine, arginine and tryptophan residues. There was an increase in molecular weight of the protein, but there was some lysozyme at the monomer weight. This material was extracted and incubated for a further 10 days, in the absence of glucose. This resulted in polymerisation, with additional impairment of the lysine and arginine residues, indicating that the sugar was only required for the first steps of the reaction. Therefore it can be postulated that the polymerisation of protein via the Maillard reaction occurs mostly due to the Maillard reaction products, rather than the binding of sugars to the proteins.

The roles of lysine and arginine in protein polymerisation have been demonstrated by Cho *et al* (1984) and Okitani *et al* (1984). Acetylation of the protein which resulted in blocking the free amino groups of lysine, prevented both browning and protein crosslinking. This indicated that a reaction with the lysine is an essential first step. This was confirmed by a study on acetylated lysozyme, which was incubated with glucose in the presence and absence of free lysine (Cho *et al* 1986). These studies again indicated that the lysine in the protein was essential for the polymerisation. The work suggested that arginine and, also possibly tryptophan, residues could subsequently be attacked by compounds formed from the glucose/lysine adducts.

When the arginine residues of lysozyme were masked with cyclo-hexane-1,2-dione, browning and protein polymerisation occurred in a similar manner to that in the

protein-glucose system (Okitani *et al* 1984). This would suggest that the dione was released from the arginine residues and reacted in place of glucose, resulting in the development of colour and protein polymerisation.

The Japanese workers considered that their studies indicated that a certain substance generated through a reaction between glucose and amino groups of proteins had polymerised the protein via covalent crosslinks. Therefore, the effect of various carbonyl compounds known to be generated in amino-carbonyl reactions involving glucose were examined by Cho *et al* (1986). Their results indicated that 2,3-butanedione, glyoxal, methylglyoxal, dihydroxyacetone and glyceraldehyde polymerised both lysozyme and acetylated lysozyme, and impaired their arginine residues. However, a year later Kato *et al* (1987) concluded that 3-deoxyglucosone (3-DG), which is the major dicarbonyl intermediate, was the major crosslinker responsible for the glucose-induced polymerisation of protein. This component was shown to bring about similar polymerisation behaviour to glucose in protein-sugar systems based on lysozyme, ovalbumin, bovine serum albumin and insulin, but the rate of protein polymerisation was greater with 3-deoxyglucosone (Igaki *et al* 1990).

Feeney and Whitaker (1988) also noted that fragmentation of sugars could give rise to α -Dicarbonyl compounds. They considered that these bifunctional groups may react with two molecules of protein. It is possible that the bifunctional carbonyl compounds attach themselves to lysine, arginine or tryptophan residues via one of their functional groups. The proteins may then polymerise by binding of the second functional site with the remaining lysine and/or arginine residues of the protein molecules.

Shin *et al* (1988) suggested that the ϵ -amino groups of lysine act as the generators of 3-deoxyglucosone in protein-glucose mixtures and that the arginine residues subsequently attack these protein bound 3-deoxyglucosone moieties.

Thiols or sulfhydryl groups may also be possible sites of interactions. Thiols may react with aldehydes and ketones to produce thioacetals and thioketals. This is

because the degradation of Amadori compounds may lead to formation of deoxy osones e.g. 3-deoxyglucosone, and their dehydration products.

Fragmentation reactions presumably occur at all stages of the Maillard reaction to produce reactive low molecular weight aldehydes such as glyoxal (2 carbon). Strecker degradation of amino acids can lead to the formation of further aldehydes e.g. hydroxymethylfurfural. Reactive dicarbonyl compounds are capable of reacting rapidly with amino compounds to form a variety of flavour compounds and brown melanoidins (Henle, 1996).

2.5.4 Importance of protein glycation and crosslinking

The phenomenon of protein polymerisation is not unique to food. Nonenzymatic glycation has been shown to occur *in vivo* with various proteins and to affect protein function in several ways (Monnier and Cerami, 1983). This indicates that the reactions will occur at low temperatures (37 °C) if the storage times are sufficiently long. Glucose-induced polymerisation of protein has been hypothesized to be responsible for the ageing of tissues or accelerated ageing in diabetes (Monnier *et al* 1990). This can be achieved after the formation of crosslinking molecules resulting from the Maillard reaction.

Although much effort has been made to elucidate the structure of the Maillard crosslinks, their exact nature is not yet known. This is mostly due to the complex nature of the Maillard reaction and the structure of proteins. However, it is almost certain that the Maillard reaction products allow the crosslinks to form between protein molecules. It is therefore possible to use the Maillard reaction and/or its products to promote the gelation of proteins, and to form novel food products.

CHAPTER 3

PREVIOUS WORK ON THE MAILLARD GELATION OF PROTEINS

This chapter reviews some of the previous work on the use of the Maillard reaction to enhance gelation of proteins. All of the work was carried out within the Food Science section, University of Nottingham.

Most of these works have been confined to the study of gels produced by autoclaving solutions of protein with or without reducing sugar in a laboratory autoclave at 120°C for 30-60 min. Hill *et al* (1992) showed that a range of globular proteins (egg white, bovine serum albumin and soya) gelled at low concentrations when retorted in the presence of reducing sugars. The gels formed had a 'cooked' smell similar to that produced from the Maillard reaction in thermally processed foods and they were brown in colour. The intensity of browning and strength of the gels, as estimated by measuring the breaking force, increased with reducing sugar reactivity (Table 3.1). These were indicators that gel formation could be due to the Maillard reaction and the gels were termed 'Maillard gels'.

However, during the reaction of protein with reducing sugar, there is a decrease in pH, from around 6.5 to ~ 5.0 which may be due to the production of organic acids and loss of protons from the ϵ -amino groups. As the pH approaches the isoelectric point, the repulsion between protein molecules is reduced, and protein-protein interactions are promoted. This will encourage gel formation (Chapter 2). Hence, the Maillard-induced decrease in pH was considered as possibly the primary reason for gelation in the Maillard gels. However comparison between the Maillard gels and gels formed during a continuous fall of pH provided by the presence of glucono- δ -lactone (GDL) indicated that the former had a higher rupture force than the GDL gels, even at the

same final pH (Hill *et al* 1992). This suggested that the pH was not the sole factor in promoting gelation.

Table 3.1 A comparison of the colour and gel strength of samples prepared from 2 % bovine serum albumin and 2 % sugars and retorted for 60 min at 121 °C (Mitchell and Hill, 1995)

Sugar	Gel strength (N)	*Colour (L)
No sugar	No gel	ND
Sucrose	No gel	ND
Lactose	1.27	77.60
Fructose	1.50	69.12
Mannose	1.96	64.53
Sorbose	2.20	62.60
Xylose	2.91	43.16

ND, Not determined.
* Values of L, 100 = white, 0 = black.

Another possible reason for the enhanced gel properties could be the increase in net negative charge due to modification of lysine residues by the Maillard reaction. These ideas will be retested in this thesis by studying the rate of gelation and pH fall and estimating the isoelectric point of the proteins.

Some evidence for the importance of the charge effect was shown by Cabodevila *et al* (1994). Gels produced by heating soya solutions either with xylose or GDL were compared and the results indicated that the Maillard gels had a lower level of

syneresis compared to that of the GDL gels at the same pH. The most probable explanation for the higher water holding capacity of the Maillard gels is the loss of protons from the ϵ -amino groups during the Maillard reaction, resulting in a higher net charge on the protein molecules. This supports the suggestion that charge can be one of the important forces in the gelation of globular proteins.

Beside these changes, occurrence of new covalent bonds during the course of the Maillard reaction at elevated temperatures has been reported (Armstrong, 1994; Cabodevila *et al* 1994). This conclusion was derived from the solubility data of conventional, GDL induced and Maillard gels in SDS (sodium dodecyl sulphate) + β -mercaptoethanol solvents. Conventional and GDL gels were completely soluble in these solvents but this was not the case for the Maillard gels. This suggested the presence of other bonds in addition to the disulfides and non-covalent bonds in the Maillard gels.

The nature of the additional bonds that reinforce the gel is yet to be elucidated. The solubility data from Cabodevila *et al* (1994) indicated that despite the different solubility in the mixture of SDS + mercaptoethanol shown by GDL and Maillard gels, their solubility in SDS was very similar (about 30 % of the total protein). This could suggest that the additional crosslinks occurred in the region of the network containing disulfide bonds. Further supporting data from a large deformation study of Maillard and conventional gels (Armstrong *et al* 1994) suggested that the additional covalent crosslinks in the Maillard gels reinforced the strong regions already containing disulfide linkages. The measured stress relaxation was thought to occur in weaker, noncovalently linked regions of the gels, whereas the stronger regions determined the apparent modulus at larger deformations and longer times. Therefore, as expected, the rupture strength of the Maillard gels obtained was much higher than the GDL gels.

The interaction of reducing sugar and protein is of academic and commercial interests. A knowledge of the nature of the crosslinks and the mechanism for Maillard gelation could be important in real foods. Understanding the mechanism could lead to modification of production methods for foods, thereby enhancing quality. A demonstration of such an application is given in Chapter 10.

Aims

Since previous works have been confined to the study of autoclaved gels, it was considered desirable to carry out a study of gelation at lower temperatures in real time. Therefore, the aims of this thesis were:

1. To develop a method to follow the gelation processes in real time, using a single sample.
2. To study the rate of gelation and decrease in pH of the Maillard systems. To use this to test the hypothesis that the decrease in pH is not the sole cause of gelation.
3. To show that alteration in net charge can occur due to the Maillard reaction, by estimating the isoelectric point (I_p) of the protein after heating in the presence of a reducing sugar.
4. To study the size and shape of the associated molecules formed in dilute solutions during the Maillard reaction.
5. To investigate the nature of the additional bonds generated via the Maillard reaction.
6. To elucidate the possible mechanisms that lead to the formation of these crosslinks.
7. To investigate if the principles for Maillard gelation can be used to form novel food products.

CHAPTER 4

MATERIALS AND METHODS

This chapter deals with the Materials and Methods used to study the factors affecting the Maillard gelation. For completeness some theory is given about the methods as well as specific information of the methodology employed in this study. The chapter is divided into three sections:

Section 1 describes the Materials and the rationale of choosing the reactants to induce the Maillard gelation.

Section 2 describes the development of the techniques to measure the rheological properties of the gels as they form and to estimate the time required for the systems to gel.

Section 3 describes other general methods that were used to further investigate factors for gelation such as pH, charge and crosslinking.

The Methods and Materials used for the production of a novel food product are given in Chapter 10.

Section 1

4.1 Materials

The major components used to produce the products studied were:

- (a) A protein
- (b) A carbonyl compound

Their choice depended on their availability at a known purity, reactivity, relevance to real food products and costs.

Proteins

Two proteins were studied; bovine serum albumin and soya grits. Bovine serum albumin can be obtained in a purified form (96-99 %). Earlier experimental work has been carried out using this protein and hence comparisons with the current studies were possible. Many features of bovine serum albumin are summarised on page 20, were considered to be important in gel formation and for their interaction with the sugar.

Some of the studies undertaken required large amounts of protein and the cost and relevance of using bovine serum albumin makes its use less satisfactory than using soya grits. Soya grits are readily available and there is much detailed knowledge about the protein structures (Wolf, 1977). However, it must be recognised that batch to batch variation can be considerable and other components as well as the storage proteins may be present.

Carbonyl compounds

A range of sugars have been used in this study. The choice of sugar or Maillard reaction product mostly depended on their rate of reaction. As most of the studies were carried out at 90 °C, the time required for many experiments was hours. To keep the duration of the experiments reasonable the studies often employed the more reactive compounds.

The ranking order of reactivity in the Maillard reaction for the components used (Hayashi and Namiki, 1986) was ; glucose < mannose < xylose < ribose < glyoxal < glyceraldehyde.

Other chemicals

A wide range of chemicals were used for this study. All were supplied by Sigma Chemical Company or Fisons Chemical Company and were of reagent grade unless stated otherwise in the methods. The materials used are summarised in Table 4.1.

Table 4.1 Summary of proteins, carbonyl compounds and other chemicals used in the thesis.

(a) Proteins	Supplier	Code	Purity
1. Bovine serum albumin	Sigma	A-3350 (Fraction V)	96-99 %
2. Myoglobin (horse heart)	Sigma	M 1882	95-100 %

cont/Table 4.1

(b) Carbonyl compounds	Supplier	Code
1. D(-) Ribose	Sigma	R-7500
2. D(-) Glucose	Fisons	G/0450
3. D(+) Xylose	Sigma	X-1500
4. D(+) Mannose	Sigma	M-4625
5. Glyoxal (Ethanedial)	Sigma	G-5754
6. DL-Glyceraldehyde	Sigma	G-5001
(c) Solvents and reagents	Supplier	Code
1. Sodium dodecyl sulphate (SDS)	Fisons	S/5202
2. β -Mercaptoethanol (β -ME)	Sigma	M-6250
3. Ellman's reagent (DTNB)	Sigma	D-8130
4. Folin's reagent	Sigma	F-9252

Section 2

4.2 *Monitoring the gelation processes in real time*

A main objective of this thesis was to study the kinetics of the Maillard gelation. Therefore it was necessary to assess the time or point at which gelation begins.

To do this it was necessary to consider what is meant by gelation and to define a point where it starts. Stading and Hermansson (1990) defined the gel point as the point when the network spans the sample from one side to the other. In many assessments this point is correlated to the time when the sample can be tipped through 180 ° without the sample moving. However this method is difficult to perform on sufficient samples to get very accurate timings. Other methods rely on a rheological definition of gelation and this has been used for this study. As the development of an assay to measure the onset of gelation is central to this thesis, it is dealt with in detail. However, as the methodology depends on the rheological assessment, the terminology and theory involved also have been reviewed.

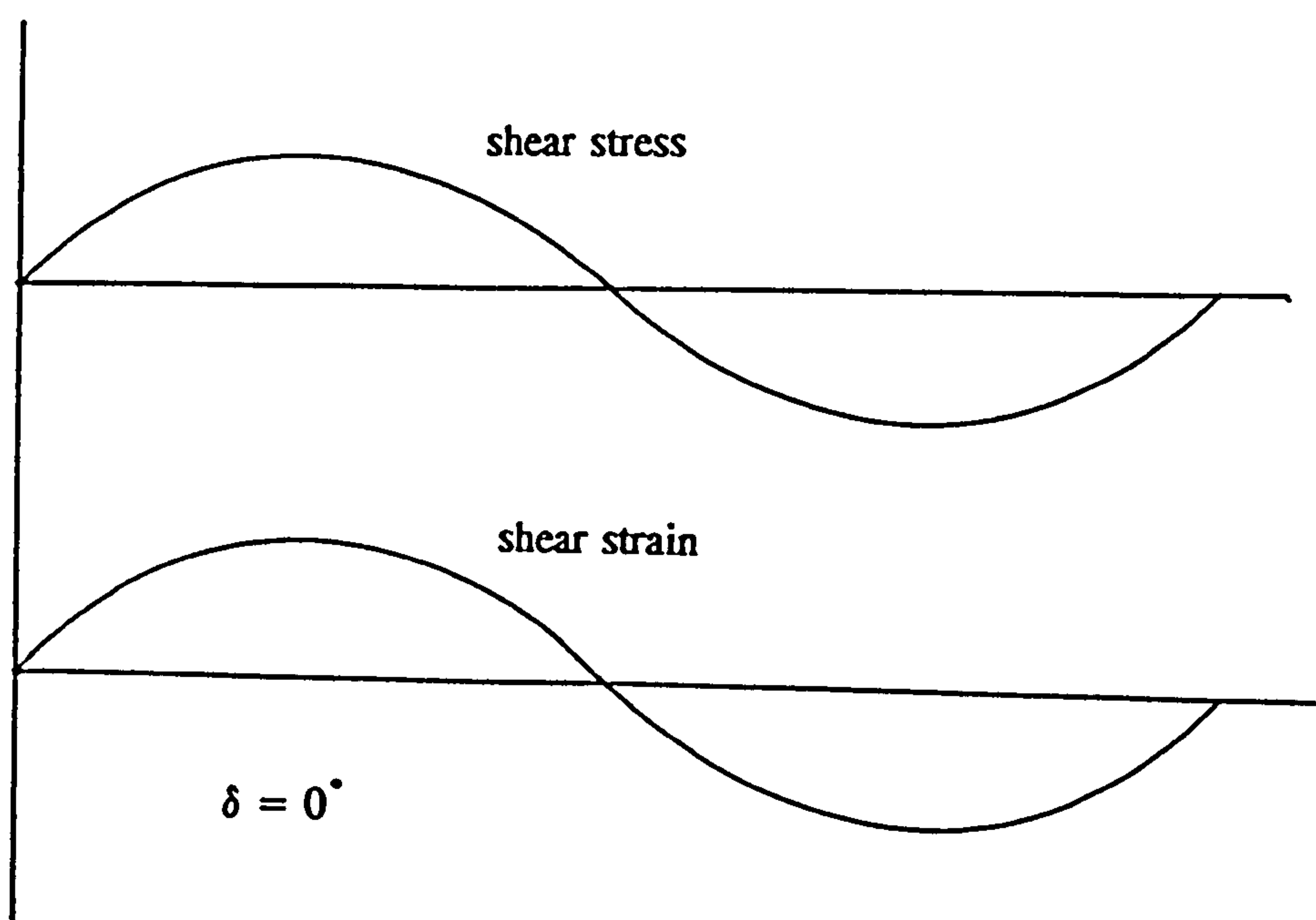
(a) *Viscoelasticity*

Many materials are said to exhibit viscoelastic behaviour. A gel is such a material. A viscoelasticity is exhibited when the rheological behaviour combines liquid and solid like characteristics. These characteristics depend on behaviour when a strain is applied. For elastic solids, stress is always directly proportional to strain and independent of the rate of strain in accordance with Hooke's law. For classical viscous liquids, the stress is always directly proportional to the rate of strain but independent of the strain magnitude in accordance with Newton's law.

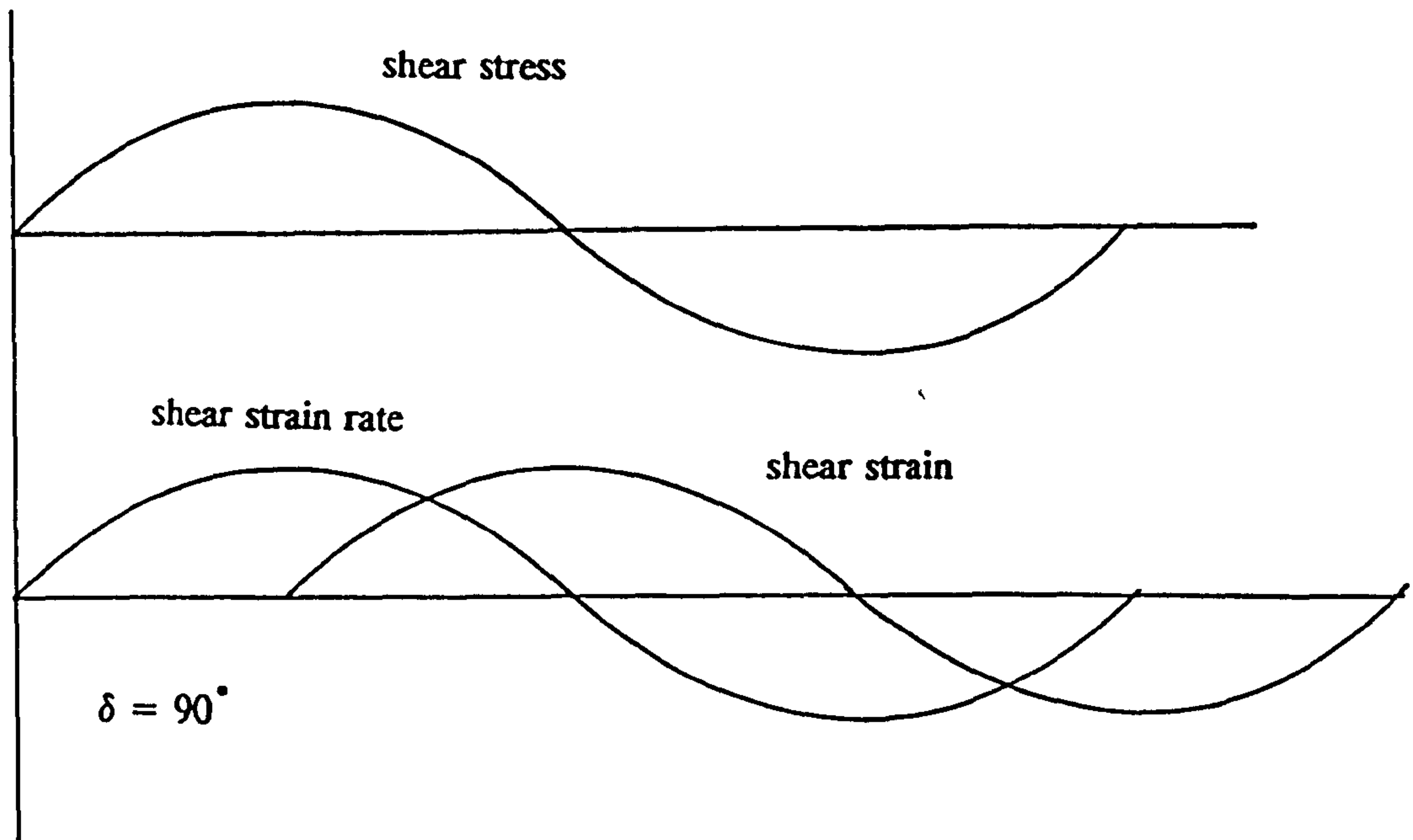
A measure of viscoelasticity would therefore indicate if a gel was formed. Viscoelasticity can be characterised by static or dynamic experiments (Mitchell, 1980). However in this section only the principles of dynamic experimentation are reviewed in some detail since the experiment development was of this type.

(b) Dynamic estimation of viscoelasticity

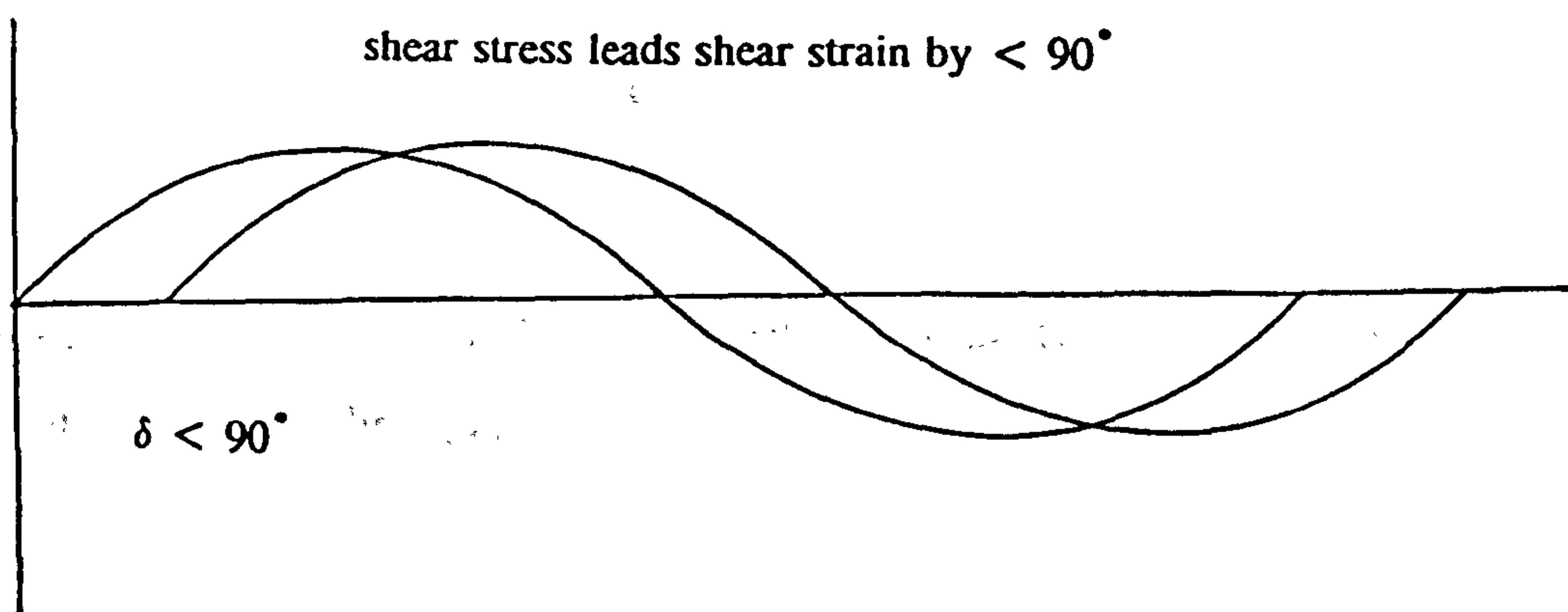
For the estimation of a sinusoidally oscillating stress, a strain is applied to the material. If we consider one complete cycle of the sine wave as 360° then differences of phase between the stress and strain waves i.e. phase angle or δ , can be estimated. For an ideal elastic solid, the strain will be at maximum when the stress is a maximum and zero when the stress is zero. Therefore, the strain response will be totally in phase with the applied stress and the resultant phase angle is 0° .



For an ideal liquid it is the strain rate which follows the stress. Therefore, when the strain is at maximum, the rate of change of strain will be zero, likewise when the strain is zero, the rate of change of strain will be a maximum. The resultant strain will therefore be totally out of phase to the applied stress i.e. phase angle = 90° .



For materials which contain both components e.g. a gel, the measured phase angle will be somewhere between 0° and 90° .



In the oscillation test the stress and strain are constantly changing but any number of 'instantaneous' values can be considered to obtain a value of 'viscoelastic G'. This

is referred to as the material's complex modulus G^* and is obtained from the ratio of the stress amplitude to the strain amplitude. Two moduli could be obtained from the rheological measurements; elastic component G' (storage modulus), which is a measure of the energy stored elastically per cycle, and G'' (loss modulus), which is a measure of the energy dissipated as heat per cycle (i.e viscous component).

Therefore, by measuring the ratio of stress to the strain (G^*) as well as the phase difference between the two (δ), G' and G'' can be defined in terms of sine and cosine functions. G' is determined from the component of the stress which is in phase with the strain and is given by the equation;

$$G' = \frac{\tau_o}{\gamma_o} \cos \delta \quad (4.1)$$

and G'' is determined from the component which is 90° out of phase and is given by the equation;

$$G'' = \frac{\tau_o}{\gamma_o} \sin \delta \quad (4.2)$$

where τ_o is the stress amplitude, γ_o is the strain amplitude. $\tan \delta$, the mechanical loss tangent is given by the equation;

$$\tan \delta = \frac{G''(\omega)}{G'(\omega)} \quad (4.3)$$

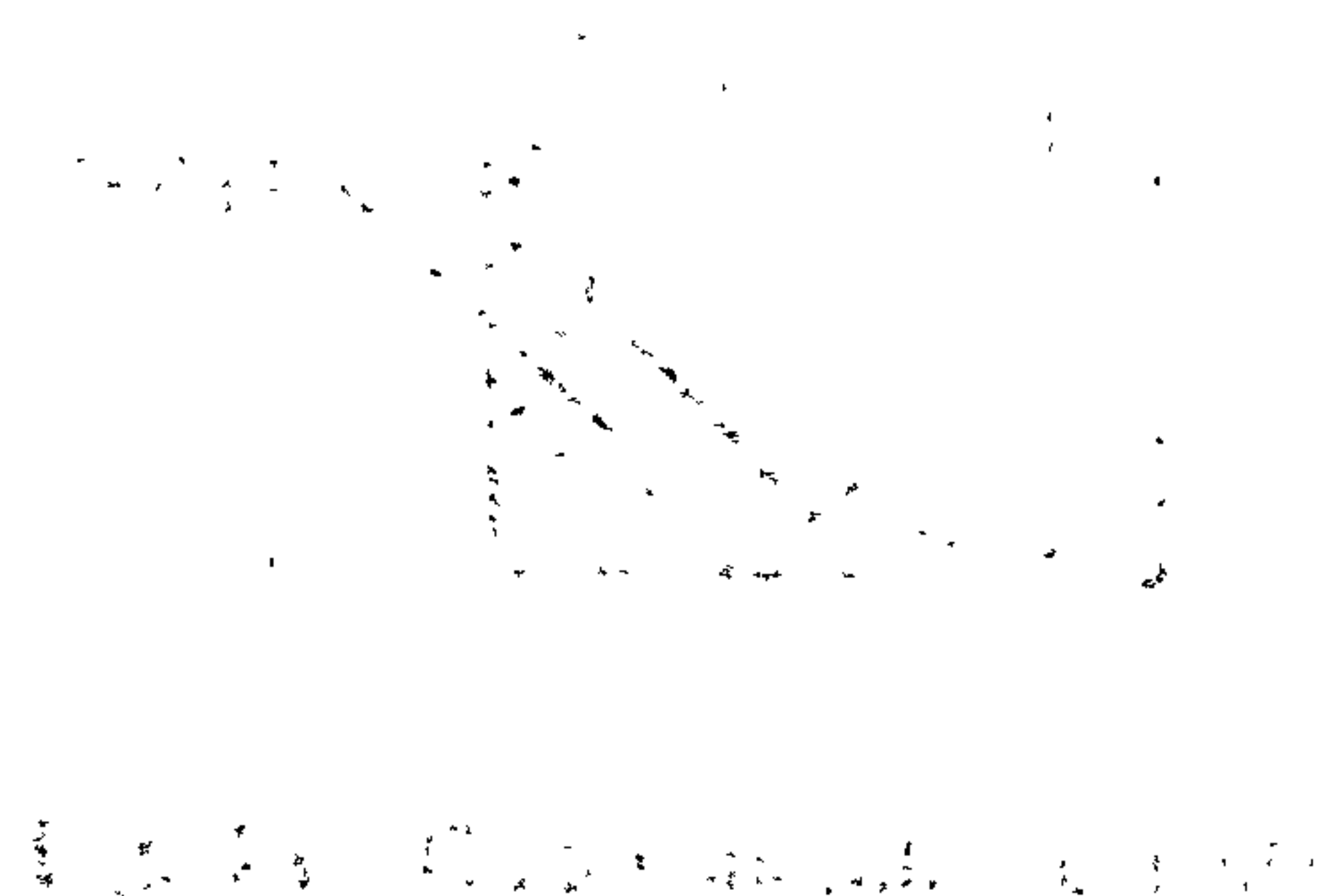
Since G is essentially stress/strain, G' and G'' have units of Pascal (N/m^2).

(c) *Definition of gelation time*

If the rheological parameters are followed with time, the network formation can be followed by means of the storage modulus G' . There are three ways in which the gel point is defined rheologically:

- (1) The point where the storage modulus starts growing, or when G' is greater than the noise level.
- (2) The point at which G' and G'' cross-over, i.e when $G'=G''$. This can also be expressed as the point where the phase angle δ equals 45° ($\tan \delta = 1$).
- (3) The point where G'' has a maximum.

In this thesis initially, the gelation time has been defined as the time when the storage modulus G' equals the loss modulus G'' (definition 2). However, it was not possible to obtain reliable measurements of these parameters prior to gelation. Therefore the gelation time was obtained by extrapolating the approximately linear increase of G' versus time relation after gelation to zero G' (Fig. 4.3). This definition is similar to (1).



(d) *Detailed methodology for the estimation of gelation time*

(1) $90\text{ }^{\circ}\text{C}$

A Bohlin CS Rheometer (Bohlin Rheologi AB, Sweden) fitted with a concentric cylinder (C 25) geometry was used to follow the evolution of viscoelastic parameters on heating (Fig. 4.1). Each run required 13 ml of sample and was carried out in duplicate. A thin film of parafin oil was applied on top of the sample to prevent drying out at the surface. Measurements were made as a function of time at a constant temperature of $90\text{ }^{\circ}\text{C}$. The solution was added to the preheated rheometer. The circulating water heating system was used to raise the temperature to $90\text{ }^{\circ}\text{C}$ (this took between 12 and 19 min) and to hold it constant. In all measurements, a target strain of 0.01 and a frequency of 1 Hz were used. Preliminary experiments showed that for the gels this strain was within the linear region. A summary of the parameters is given in table 4.2.

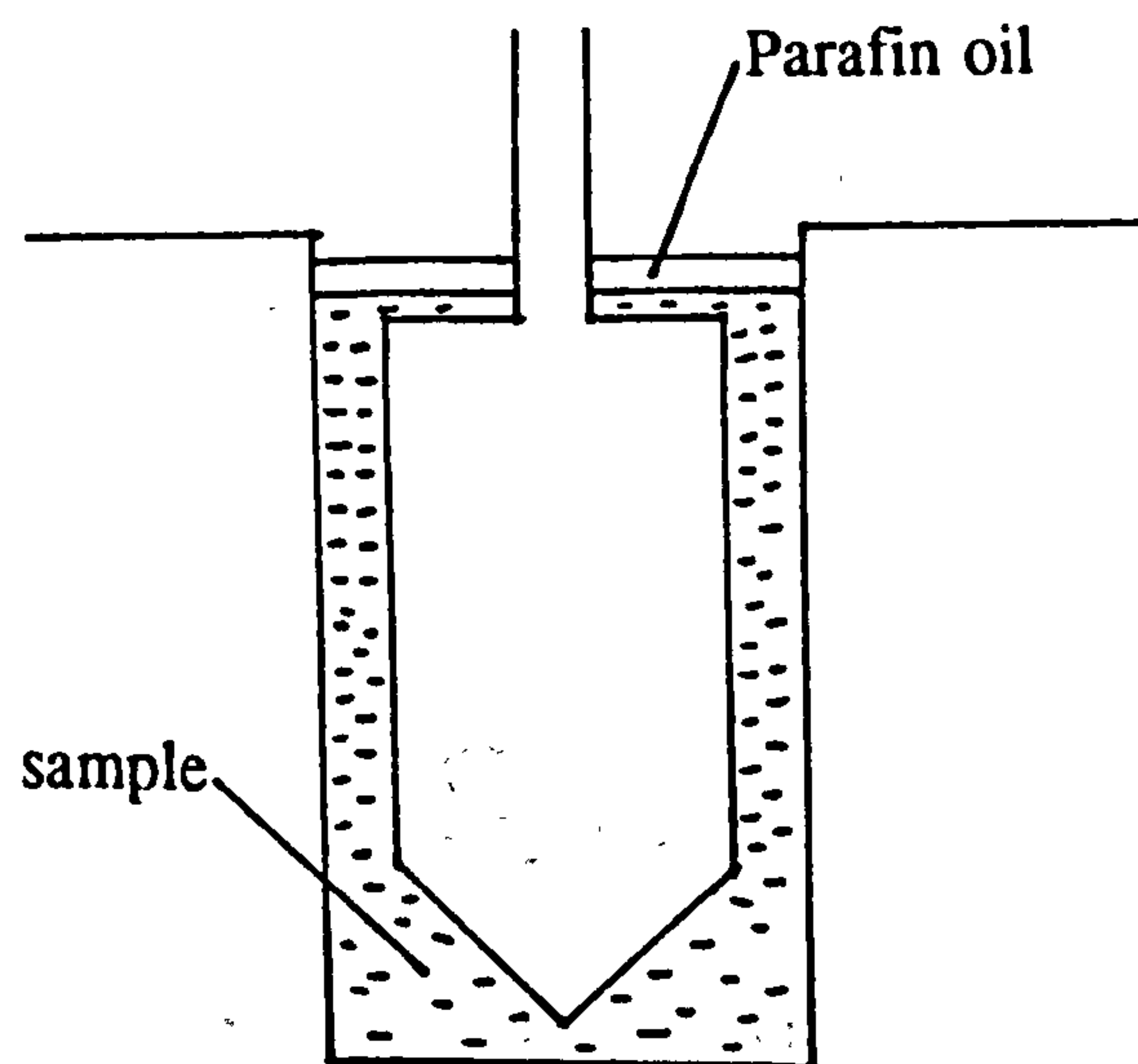


Fig. 4.1 C 25 concentric cylinder system

(2) *Elevated temperatures*

It was not possible to perform rheological measurement at temperatures above 95 °C using the C 25 geometry and water bath as the heating system. Therefore a high pressure cell (HPC) was fitted to a Bohlin CS Rheometer and an oil bath was used as the heating element (Fig. 4.2). This enabled measurements of rheological data for less reactive systems (e.g. glucose) as temperatures in excess of 120 °C can be used and heating can be prolonged without affecting the performance of the rheometer. A thermocouple and a pressure gauge were fitted to the cell to monitor the temperature generated by the oil bath. The parameters used were essentially the same as that using the water bath, but each measurement required about 3-4 ml of samples.

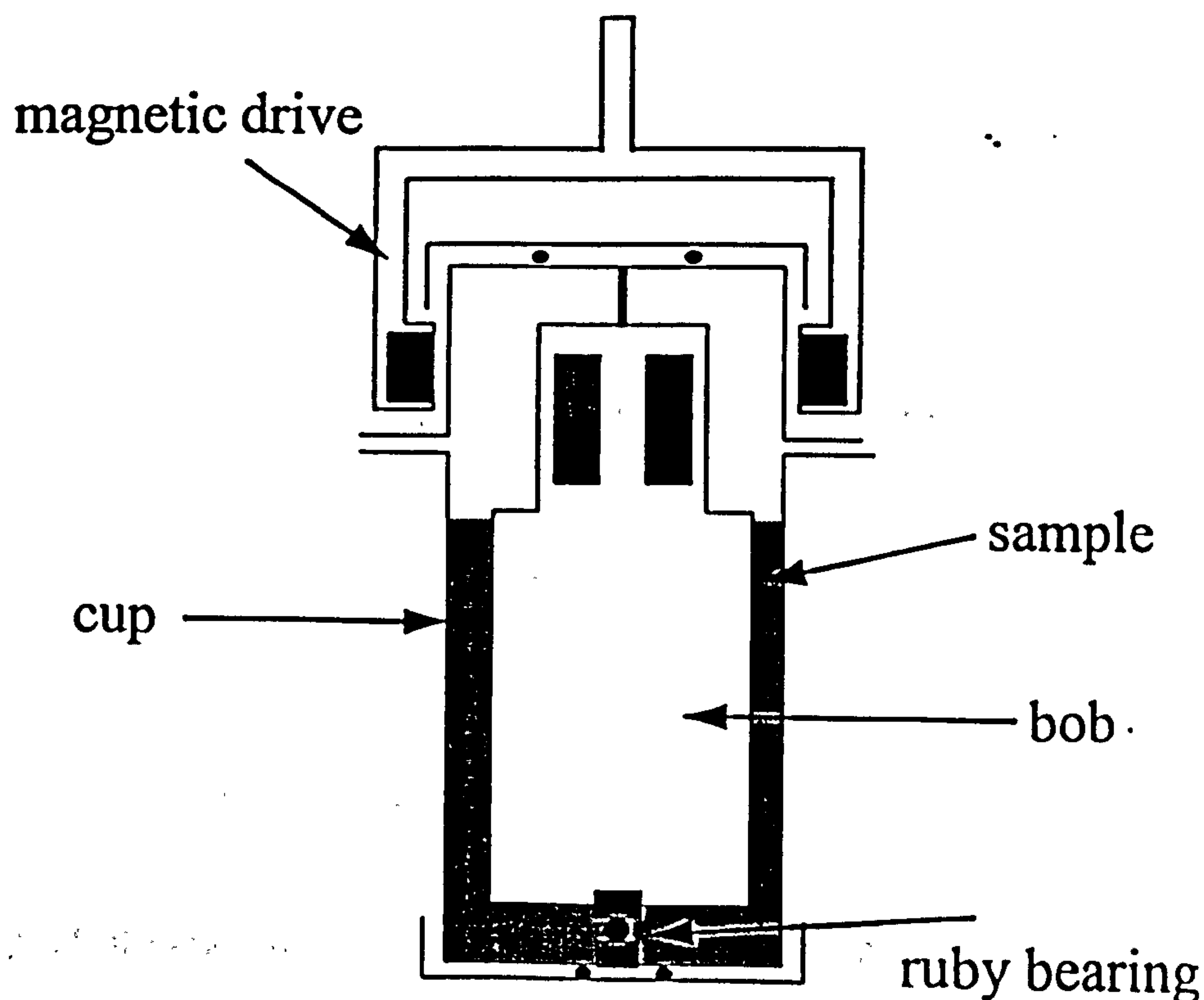


Fig. 4.2 High pressure cell

(e) *Typical features of the results from the dynamic experiment*

Dynamic experiments have been used to monitor many gelation processes. Recent examples include the use of the techniques to monitor the formation of milk gels by acidification (Gastaldi *et al* 1996) and whey protein isolates gels (Labropoulos and Hsu, 1996). Fig. 4.3 illustrates the typical results obtained using the Bohlin CS Rheometer for the Maillard system.

Table 4.2 Parameters for the rheological measurement using the Bohlin CS Rheometer

Geometry :	C 25
Target strain :	0.01
Frequency :	1 Hz
Temperature :	90 ± 2 °C

Typically, the essential features of the kinetics of gel modulus development can be divided into three stages:

1. A lag time before the gel point
2. A period of rapid growth of the storage modulus after the gel point
3. A period of levelling off during which the components of the moduli and $\tan \delta$ either become constant or change slowly.

It can be seen in Fig. 4.3 that the values of the moduli were very small during the initial stage of heating, with G'' greater than G' , as expected for a liquid. Also the values of $\tan \delta$ were essentially high (between 6-8) which implies that the phase angle

is close to 90° . This is another verification that the system was still in a liquid state. As heating proceeds further, eventually a crossover of the moduli occurred. This is the point where $G' = G''$, or $\tan \delta = 1$. The time at which this event took place was taken as the gelation time. In many cases however, the crossover was difficult to assess, therefore the gelation time was measured by a simple extrapolation of the rapidly rising storage modulus back to the time axis.

After the gel point, as the network of the gels developed further, both G' and G'' rise. At this stage the ratio of G'/G'' increased and this may indicate an increase in elastic character of the system. The rate at which G' values change with time or dG'/dt , can sometimes be taken as the rate of structure development (Sarkar, 1995). Therefore the higher dG'/dt , the faster the rate of gel structure development.

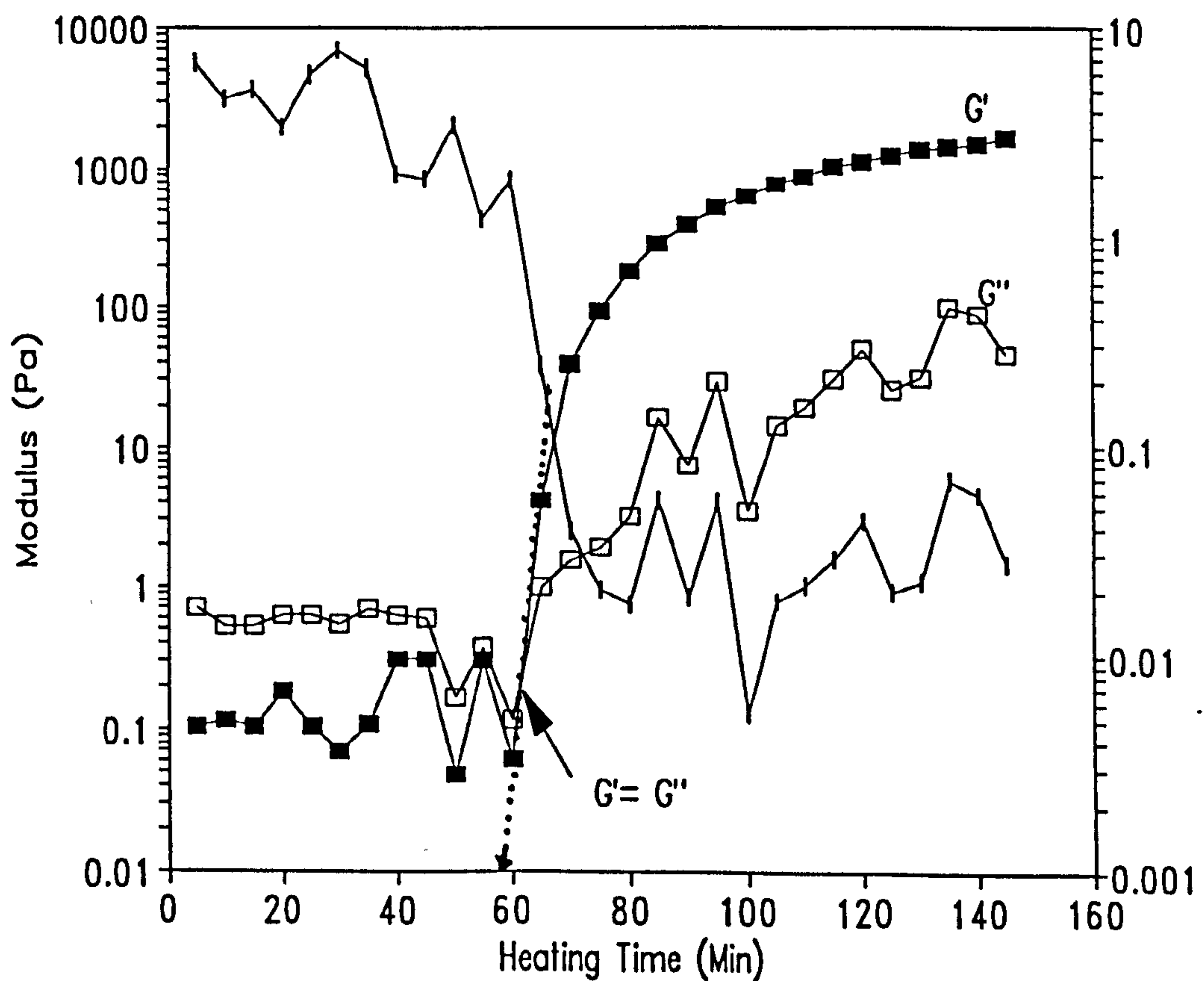


Fig. 4.3 Typical plot of storage modulus G' (■), loss modulus G'' (□) and $\tan \delta$ (|) as a function of heating time during gelation of 4 % bovine serum albumin + 3 % ribose solution at 90 °C. The dotted line illustrates another method for the estimation of gelation time.

Tan δ is the ratio of G'' to G' . The value of tan δ varies from zero for a perfectly elastic solid to infinity for an ideal liquid. The values of tan δ decreased to values between 0.005 to 0.07. This indicates that the gel was more liquid at the initial stage of gelation, and finally a more solid gel was formed. Many systems which have tan δ values of around 0.1 belong to the so called 'weak gels' or colloidal dispersions (Clark and Ross-Murphy, 1987). Tan δ values for typical biopolymer gels are about 0.01 (Ross-Murphy, 1984). It may be possible to consider the Maillard gels produced at this temperature to be slightly more liquid than these biopolymer gels.

In many cases a plateau or maximum of the moduli can be developed. However, this was not observed during the gelation of the Maillard systems. The primary reason for this is the limited heating time employed to produce the gels. The temperature and the time of heating were limited to 90 °C and 145 min respectively, for practical reasons and because these conditions were sufficient for the measurement of gelation times.

(f) Samples preparation for rheological assessment

For most of the rheological measurements, samples consisted of a mixture of a carbonyl compounds with bovine serum albumin, although occasionally other proteins were used. The summaries of the protocols used for the sample preparations are given in Fig. 4.4.

(1)

1, 2, or 3 % bovine serum albumin or 3 % myoglobin solutions



with ribose, xylose, glucose, mannose, glyoxal or glyceraldehyde



rheological assessment

(2)

3 % xylose or ribose solutions



with a range (0.5-14 %) of concentration for bovine serum albumin



rheological assessment

Fig. 4.4 Summary of sample types used for the rheological assessment; (1) gelation kinetics at low protein level, (2) gelation kinetics at high protein levels.

Section 3

4.3 *Methods of assessment*

The methods utilized in the present study for the assessment of the Maillard reaction in the promotion of protein crosslinking are given in this section. These techniques were employed on mixtures of protein-carbonyl which had been heated for various times and at different temperatures. Some techniques were carried out on the product formed by heating at conditions of such moderation that gels did not occur, while others were carried out on gelled material.

The previous section (section 4.2) reviewed the method for monitoring gel formation as it occurred in the rheometer. Other rheological methods and other assessments were made. The methods were carried out on samples of various physical states (gels, gel particles, solutions). The methods and sample types are summarised in Table 4.3.

Table 4.3 Summary of methods and sample preparation conditions

Methods of analysis	Section	Heating conditions applied to samples	Sample form
Breakstrength	4.3.3	121 °C/60 min	cylindrical gels
Stress relaxation	4.3.4	90 °C/30-240 min	cylindrical gels
pH	4.3.5	90 °C/15-75 min	solutions and gels
Isoelectric point	4.3.6	121 °C/30 min	gel particles
Intrinsic viscosity	4.3.7 (b)	95 °C/10-80 min	solutions
Diffusion coefficient	4.3.7 (c)	95 °C/10-80 min	solutions
Sedimentation velocity	4.3.7 (d)	95 °C/10-80 min	solutions
Gel solubility	4.3.8	90 °C/80-240 min	gel particles
Disulfide and sulfhydryl	4.3.9	95 °C/15-90 min	solutions
Amino acid	4.4.0	95 °C/75 min	solutions

4.3.1 *Preparation of gels and gel particles*

Gels were normally produced by heating solutions of protein with carbonyl compounds. Cylindrical gels were obtained by heating the solutions in dialysis tubing (14 mm diameter) either in a water bath or a laboratory autoclave. The typical protocol used to produce the gels and gel particles is shown in Fig. 4.5.

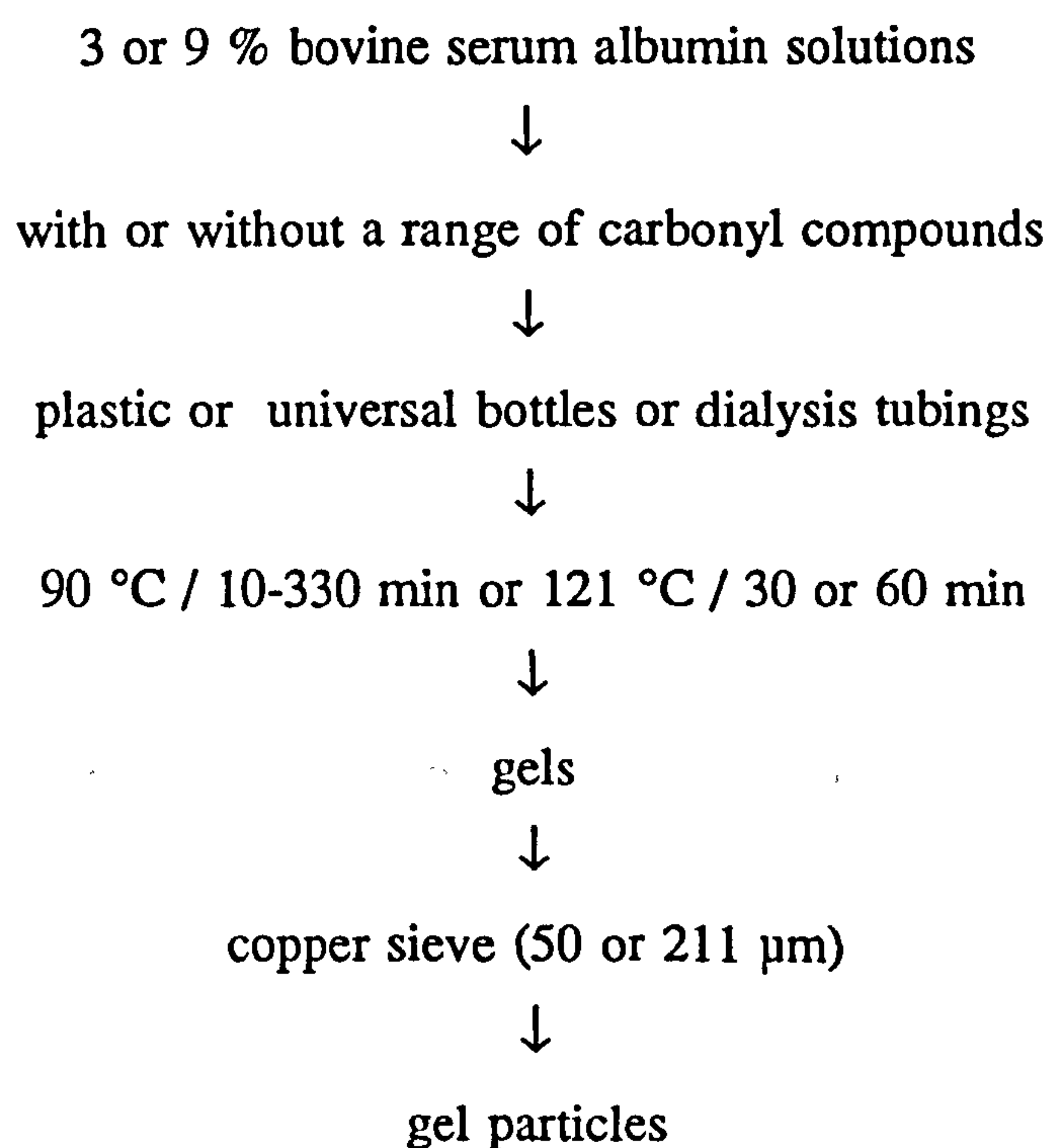


Fig. 4.5 Schematic protocol for the preparation of gels and gel particles.

4.3.2 *Preparation of solutions*

The typical protocol used to produce the solutions for the study is shown in Fig. 4.6.

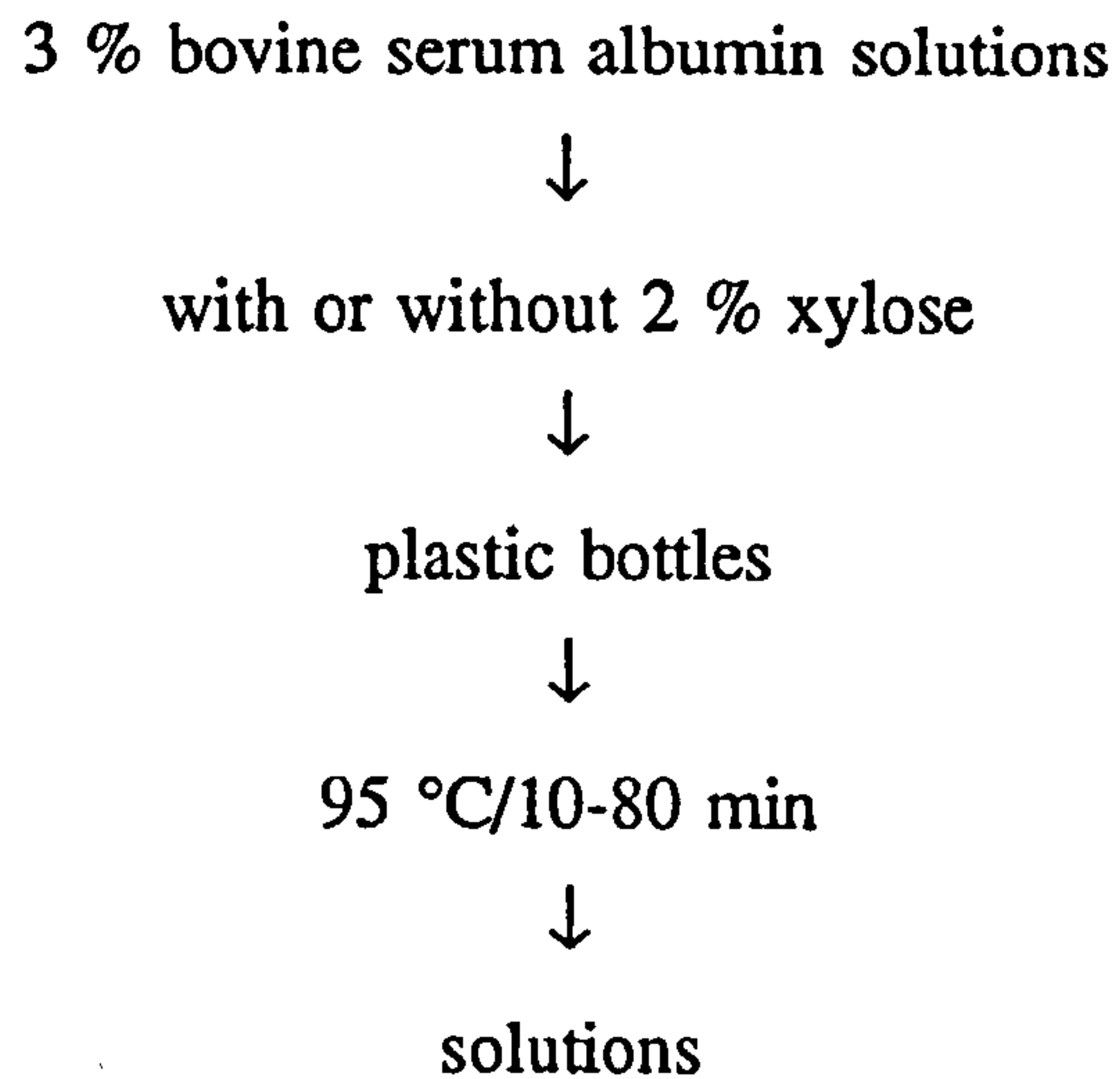


Fig. 4.6 Schematic protocol for the preparation of solutions for analysis.

4.3.3 Breakstrength

Gel samples (2.0 x 1.5 cm) obtained using dialysis tubings were compressed using a texture analyser TAXT2 (Stable Micro System) until fracture occurred, as identified by a peak in the force time plot. This was taken as the gel break strength. The plunger speed was 2 mm/s and measurements were performed in an air-conditioned room (22 ± 2 °C).

4.3.4 Stress relaxation experiment

Stress relaxation is a static experiment which can be used to characterise the viscoelastic behaviour of gels. The methods utilised was based on that of Armstrong *et al* (1994), but with some modifications. Cylindrical gels were produced at a temperature of 90 °C for range of times between 30 and 240 min. In order to reduce the loss of water during the heating process, the dialysis tubing was covered with a double coat of cling film. Cylindrical samples of 2 cm in height were cut using a parallel razor blade device. The average diameter was 1.5 cm.

After storage for 18 h at 4 °C, the cylindrical gel samples (2.0 x 1.5 cm) were compressed using a TA.XT-2 Texture Analyser (Stable Micro Systems) and the force measured. The plunger speed was 2 mm/s and measurements were performed in an air-conditioned room (22 ± 2 °C). Using the software available, the stress relaxation response from at least two tests were averaged.

The stress relaxation experiments were performed at a constant strain level (25% deformation, i.e. the gels were compressed by 5 mm). The gel was initially compressed at a rate of 2 mm/s and then the decay in force was followed for 240 s. The relaxation curves obtained were then normalised and linearized using the method previously applied to foods and gels by Peleg (1979). The equation employed was :

$$\frac{F_o t}{F_o - F(t)} = K1 + K2t \quad (4.4)$$

where F_o and $F(t)$ are the initial and momentary force and $K1$ (s) and $K2$ (dimensionless) are constants.

4.3.5 pH

(a) Measurement of pH during heating

Solutions of the Maillard mixtures (bovine serum albumin/reducing sugar) and bovine serum albumin alone were placed in 15 ml plastic bottles, sealed and heated in a water bath at a temperature of 90 °C. After 3 min to equilibrate the samples, heating was carried out for times between 15 and 75 min. The samples were then cooled to room temperature and the pH measured. Plots of pH against time were prepared.

Preliminary experiments showed that the pHs obtained were not significantly dependent on the cooling rate. Cooling the tubes under running water gave a resultant pH value at room temperature which was within 0.1 of a pH unit of the value obtained when the samples were allowed to cool at ambient temperature.

(b) Measurement of pH at the gel point

After the gelation time had been predicted from the rheological studies, pH at the gel point was estimated by using the time/pH curves created in (a) above. The pH values and gel times were also checked by heating the Maillard mixtures for their gelation times as estimated from the rheological study in a water bath at 90 °C. Visual assessment indicated that the gelation times measured by the rheometer compared favourably with heating the solutions in the water bath.

4.3.6 Estimation of charge

Determination of Isoelectric point

The gels for this study were all prepared by heating 10 ml solutions in universal bottles in a laboratory autoclave at 121 °C for 30 min. Gels were homogenised by pressing them through a copper sieve (linear size aperture 50 µm). A known amount of distilled water was added to assist in sieving and to dilute the gels to a protein concentration of 0.3 %. The pH of the gel particles was adjusted by dropwise addition of 0.1 M NaOH or 0.1 M HCL.

Titration was performed using a Muetek PCD-Titrator and a Particle Charge Detector PCD-02 (Carisprwoke Instrument, Surrey, England). The polyelectrolytes used were sodium polyethylenesulphate (anionic) and poly-diallyl-dimethyl-ammonium-chloride (cationic) at a concentration of 0.001 M. Each titration required 10 ml of samples and was carried out in duplicate. The specific charge density (µeq/g) was calculated automatically using the equation:

$$q = \frac{v c (1000)}{w} \quad (4.5)$$

where q is specific charge density (µeq/g), v is the volume of electrolyte (ml), c is the polyelectrolyte concentration (mol/L), w is solid content of sample (g) and 1000 is the conversion factor. The isoelectric point was defined as the pH at which the specific charge density was zero and this was estimated by extrapolating the apparent charge-pH relationship to zero charge.

4.3.7 Association of bovine serum albumin and xylose in dilute solution

(a) Sample preparations

Solutions of 3% (w/v) bovine serum albumin and 3% bovine serum albumin + 2% xylose were prepared by dispersing the components in deionised distilled water with stirring. Aliquots (10 ml) of these solutions were placed in 25 ml glass bottles, sealed and incubated in a water bath at 95 °C for a range of times: between 10 and 80 min (at longer times the bovine serum albumin/xylose samples began to gel). Samples were allowed to cool and then, after pH measurements had been recorded, used in the original or diluted form to carry out subsequent studies.

Bovine serum albumin solutions (3% (w/v)) were also prepared using deionised distilled water that had been passed through a 0.2 µm Millipore filter. The pH of this solution was then lowered using 1M HCl to a value of 6.30 and heated at 95 °C as previously described. For diffusion coefficient measurement it is necessary to remove particulate matter. Samples are therefore normally filtered through a 0.45 µm filter. However it was found that the bovine serum albumin samples prepared for this study formed aggregates that were removed by filtering stage. It was therefore decided to filter the water through a small aperture filter (0.2 µm) before preparation of the samples and not to filter the final samples.

Samples of the low pH bovine serum albumin and bovine serum albumin/xylose solutions were also prepared for analysis by dynamic light scattering after heating at 95 °C for 20 min. Solvents used to dilute these samples to the required 3 mg/ml for the experiment included 1% sodium dodecyl sulphate (SDS), 1% SDS + 1% β-mercaptoethanol (β-ME) and phosphate chloride buffer (0.1M) at pH 6.8. These were all carefully prepared using filtered deionised distilled water.

(b) *Intrinsic viscosity*

A Schott-Geräte automatic capillary viscometer was used for the determination of intrinsic viscosities. Aliquots of each sample were injected into an Ostwald-type viscometer (2 ml capacity) which was suspended in a water bath held at a constant temperature of 26.35 ± 0.06 °C. The temperature of the water was recorded by a platinum resistance thermometer accurate to ± 0.005 °C. The sample was allowed to equilibrate for 5 min and then four measurements of flow time were electronically recorded by photoreceptors capable of detecting the passage of the solution meniscus. The original solutions were serially diluted (using 1 and 0.1 M phosphate chloride buffer) to 27 mg/ml, 19.3 mg/ml, 10.7 mg/ml and 6.4 mg/ml and the flow times of these recorded.

From the ratio of the flow times for the solution and solvent, the kinematic viscosities were obtained and from the extrapolation of the reduced specific viscosities to zero solute concentration (Fig. 4.7), the kinematic intrinsic viscosities $[\eta']$ (ml/g) were determined.

To obtain the dynamic intrinsic viscosity $[\eta]$ from η' , the correction factor of Tanford (1955) was used.

$$[\eta] = \frac{1 - \bar{v}\rho_o}{\rho_o} + [\eta'] \quad (4.6)$$

where \bar{v} is the partial specific volume (ml/g) and ρ_o is the density of the solvent (g/ml).

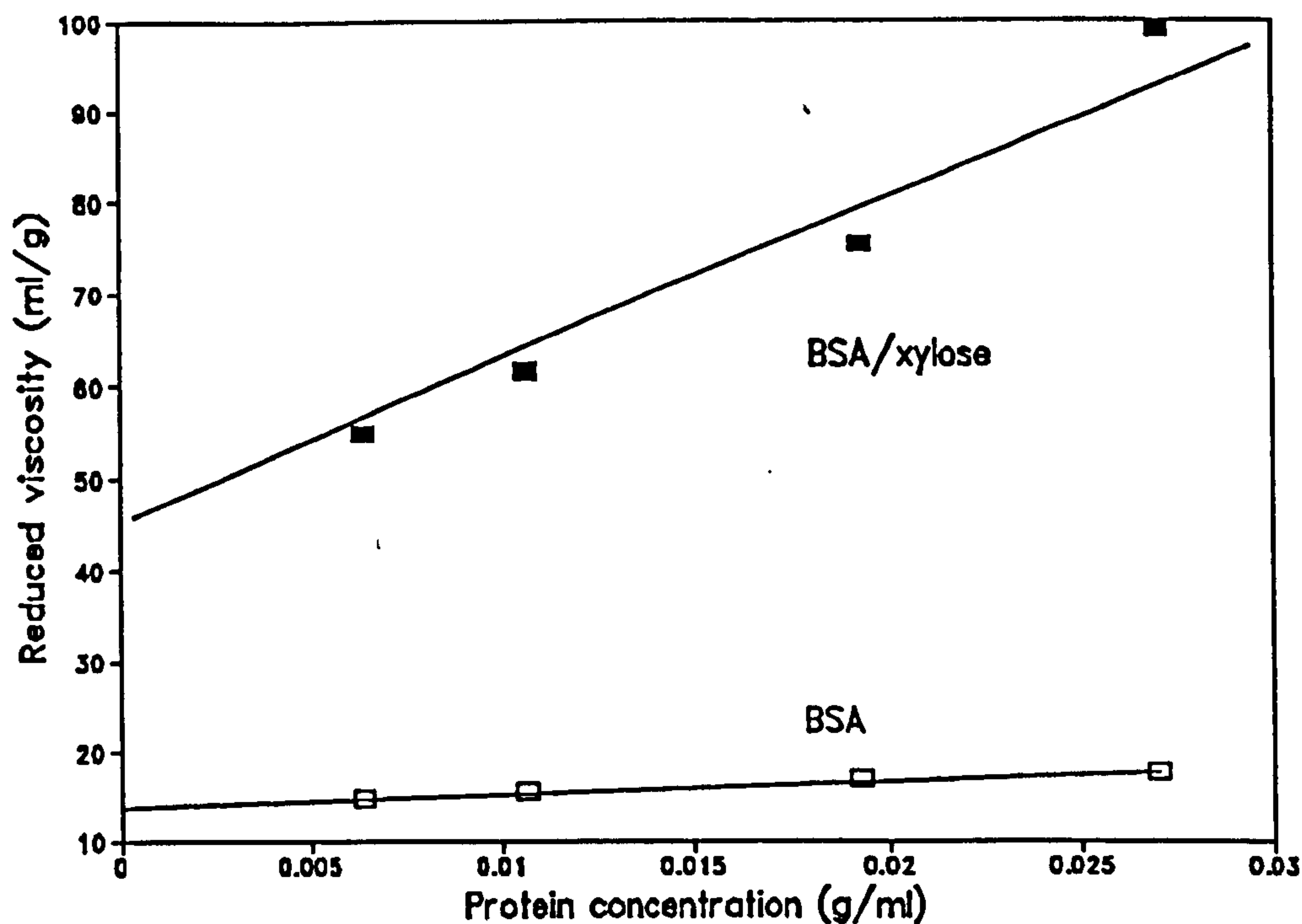


Fig. 4.7 Determination of kinematic intrinsic viscosity of bovine serum albumin solution heated alone (\square) or with xylose (\blacksquare)

(c) *Diffusion Coefficient measurement: Dynamic light scattering*

theory

The net rate at which a molecule moves through a liquid is expressed in terms of the diffusion coefficient, D , which is large if the molecule is very mobile. The net distance travelled in a time t from the starting point is measured by the root mean square distance, d , with

$$d^2 = 2Dt \quad (4.7)$$

Thus, the net distance increases only as the square root of the time.

The net effect of diffusion is very slow in liquids, each step takes only a very short time. If it is supposed that each step is of length λ and takes a time τ , then the Einstein-Smoluchowski equation states that;

$$D = \frac{\lambda^2}{\tau} \quad (4.8)$$

Therefore, the longer the step and the shorter the time it takes for the steps to occur, the greater the diffusion coefficient.

methods

A Malvern Instruments (Malvern, UK) System 4700C dynamic light scattering photometer was used to determine the translational diffusion coefficient for the bovine serum albumin and bovine serum albumin/xylose samples. This system consisted of a 40 mW He-Ne laser, a water bath maintained at 25.00 ± 0.05 °C, a filter and pump system for cleaning the water bath, a variable angle photomultiplier and a 64-channel correlator. To process the data and obtain diffusion coefficients an I.P.C. personal computer using Malvern Automeasure software was employed.

To clarify the solutions and remove dust, the samples were passed through a 0.45 μm Millipore filter. A sterile hypodermic needle was used to place the samples into the light scattering cells. The cells were kept dust-free according to a procedure similar to that described by Sanders and Connell (1980).

In order to obtain a mean (z-average) diffusion coefficient, values were calculated from 5 measurements with an experimental time of 120-1000s and a sample time of 2-15 μs . The scattered light was measured at an angle 90° to that of incident light.

The diffusion coefficient values obtained were converted to standard conditions (temperature 20 °C; viscosity of water as a solvent at 20 °C) using the following

equation (Van Holde, 1985);

$$D_{20,w} = \frac{293.1}{T} \frac{\eta_{T,b}}{\eta_{20,w}} D_{T,b} \quad (4.9)$$

where T is the absolute temperature, $\eta_{T,b}$ is the solvent viscosity and $\eta_{20,w}$ is the viscosity of water at 20 °C.

For the values obtained for the samples diluted with solvents other than water ratios of $\eta_{T,b}/\eta_{20,w}$ had to be determined. This was carried out using an Ostwald viscometer to obtain a ratio of flow times at the given temperatures. These were 0.8904 for SDS, 0.8800 for SDS/ β -ME and 0.9988 for the buffer.

(d) Sedimentation Velocity measurements

Sedimentation velocity experiments were performed using an XLA (Beckman, USA) analytical ultracentrifuge equipped with scanning absorption optics. Experiments were carried out at 20 °C and at rotor speeds between 15-40,000 rev/min. Absorption measurements were carried out at 280nm. Consecutive scans (10) were used to determine each sedimentation coefficient $s_{T,b}$. These values were converted to standard conditions (temperature 20 °C; viscosity of water as a solvent at 20 °C) using the following equation (Van Holde, 1985);

$$s_{20,w} = \frac{(1-\bar{v}\rho_o)_{20,w}}{(1-\bar{v}\rho_o)_{T,b}} \frac{\eta_{T,b}}{\eta_{20,w}} s_{T,b} \quad (4.10)$$

where \bar{v} is the partial specific volume (ml/g) and ρ_o is the solvent density (g/ml).

(e) *Analysis*

molar mass

By combining the (z-average) diffusion coefficient from dynamic light scattering with the (weight average) sedimentation coefficient from the sedimentation velocity in the analytical ultracentrifugation, an apparent weight average (Pusey, 1974) molar mass M_w could be estimated from the Svedberg Equation (Tanford, 1961);

$$M_w = \frac{s_{20,w} RT}{D_{20,w}(1 - \bar{v}\rho_o)} \quad (4.11)$$

where R = gas constant ($\text{JK}^{-1}\text{mol}^{-1}$) and T = absolute temperature (K). It should be stressed that these are apparent molar masses, since the $s_{20,w}$ and $D_{20,w}$ values are not "infinite dilution" values.

Gross Conformation

By "double-log" plotting the ranges of values of intrinsic viscosity, $D_{20,w}$ and $s_{20,w}$ over the heating period on a log scale against the calculated M_w the slopes of the lines can be taken as a function of conformation of the complex using the Mark-Houwink-Kuhn-Sakarada (MHKS) equations (Harding *et al* 1991);

Intrinsic viscosity

$$[\eta] = K'M^a \quad (4.12)$$

Sedimentation Coefficient

$$s = K''M^b \quad (4.13)$$

Diffusion Coefficient

$$D = K'''M^{-\epsilon} \quad (4.14)$$

The coefficients a , b and ϵ depend on the conformation (Harding *et al*, 1991). Once the gradients have been obtained from the double log plots of intrinsic viscosity, Sedimentation Coefficient and Diffusion Coefficient against the molar mass, the coefficient a , b and ϵ can be positioned on the "Haug's Triangle" (Fig. 4.8) to give an indication of conformation of the associated molecules.

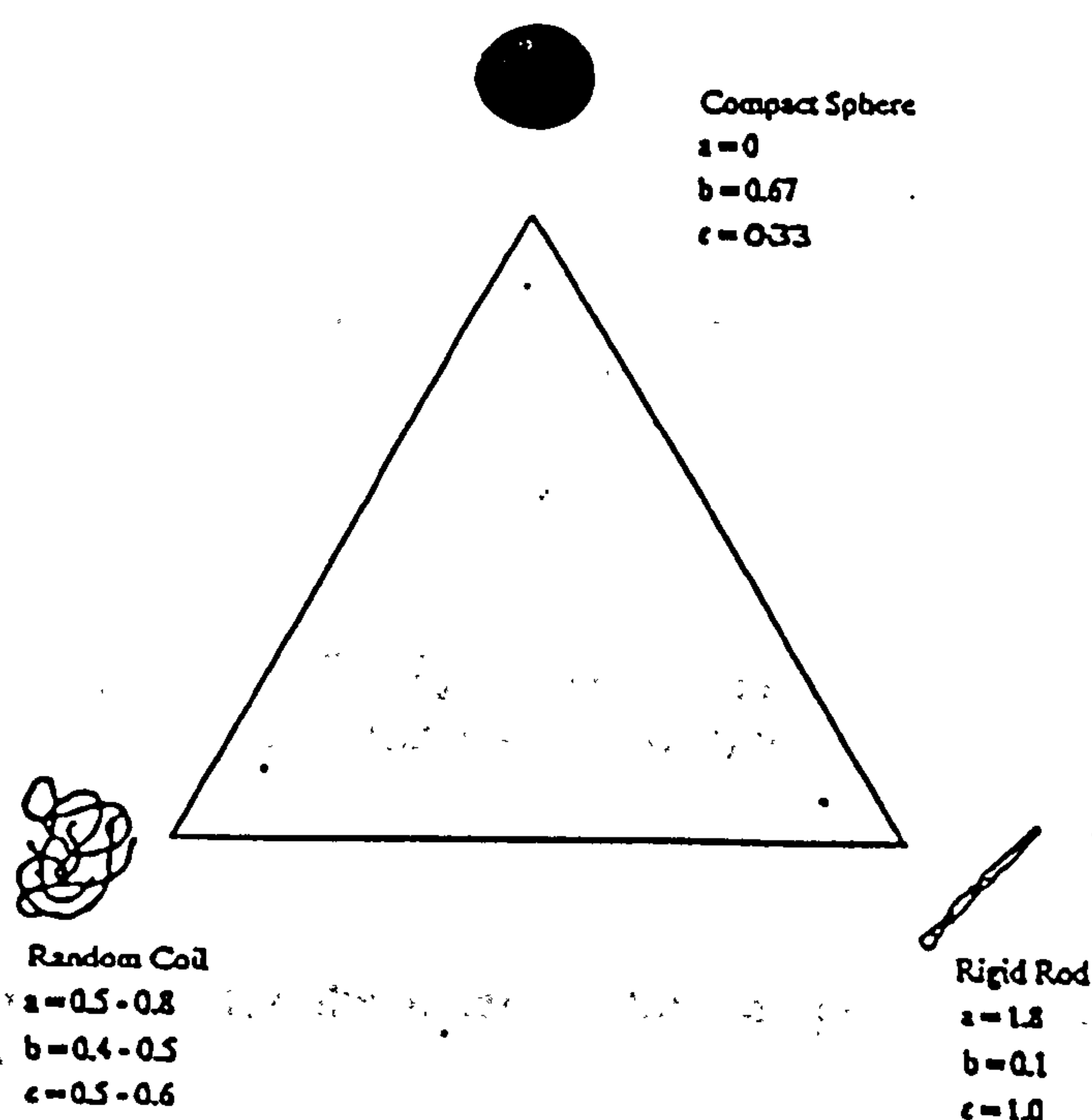


Fig. 4.8 The "Haug's Triangle"

4.3.8 Investigation of additional covalent bonds at 90 °C

Gel solubility

Gels were homogenised by pressing through a copper sieve (linear size of aperture 211 μm). Samples (0.5 g) of the homogenised gel were extracted in 10 ml of a mixture of 1 % sodium dodecyl sulphate (SDS) and 1 % β -mercaptoethanol (β -ME). The extraction was performed by shaking the sample of gel in the presence of solvent for about 14 h (overnight). Any undissolved material was removed by centrifugation (2000 g for 10 min). The concentration of the protein in the filtrate was determined by a modified Lowry method (Mohammed, 1995). The standard curve for the methods using 0.05 % bovine serum albumin is shown in Fig. 4.9.

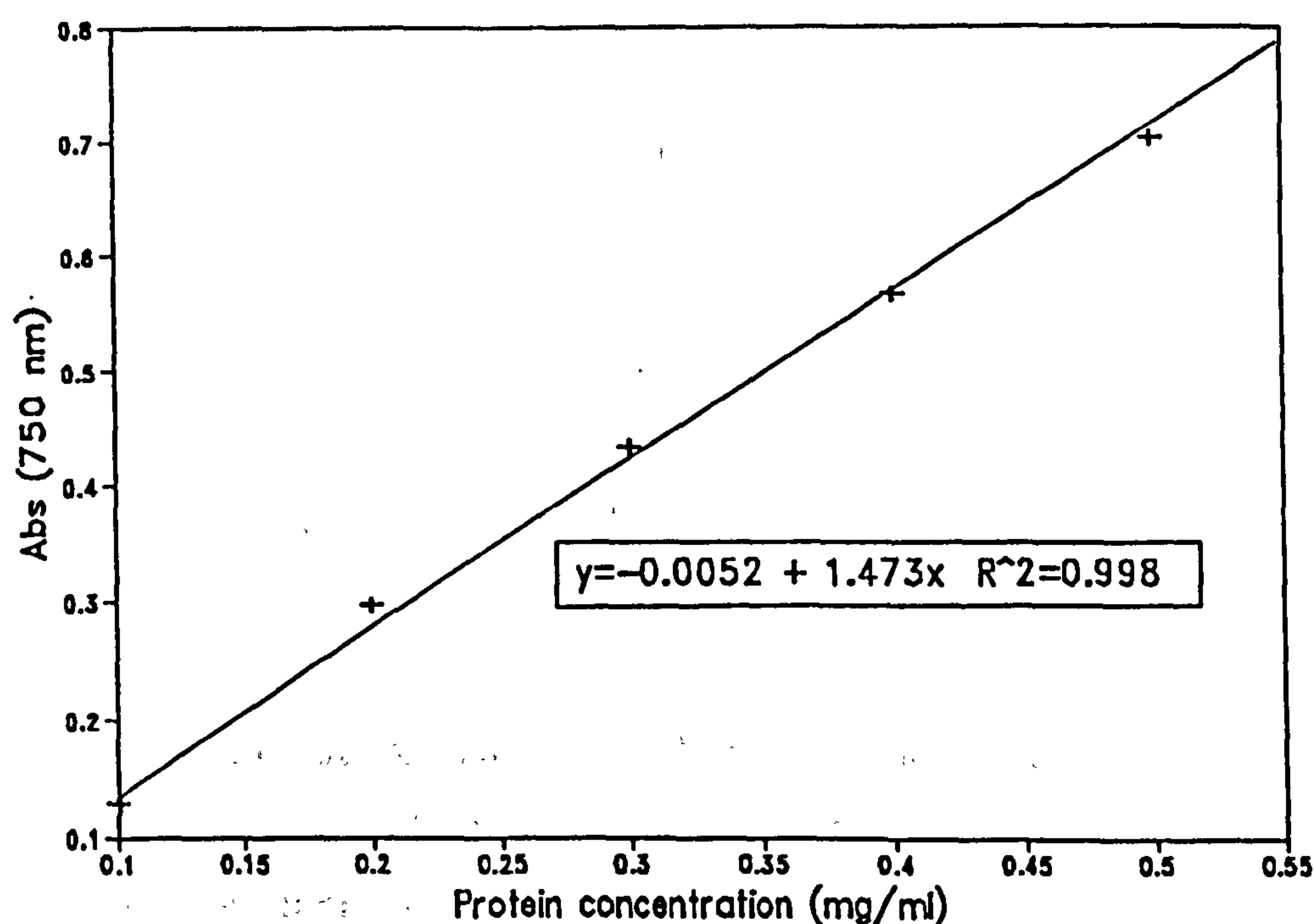


Fig. 4.9 Standard curve for the determination of protein concentration using a modified Lowry method

4.3.9 Estimation of disulfide (SS) and sulfhydryl (SH) groups

(a) Rheological measurements

To see if protein containing no disulfide can gel in the presence of reducing sugar, the rheological changes during heating of 3 % myoglobin with a range of ribose concentrations were monitored in real time using the Bohlin CS rheometer. The methods used are similar to those described in section 2(d). The rate of change of storage modulus was taken as the initial slope of the curve of G' vs time (Sarkar, 1995). This was considered as a measure of the rate of gel structure development.

(b) Reagent and sample preparations

Reagent grade chemicals were used to prepare the following: Tris-glycine buffer (10.4 g Tris, 6.9 g glycine and 1.2 g EDTA per litre, pH 8.8 denoted as Tris-gly), Ellman's reagent (5,5'-dithiobis-2 nitrobenzoic acid) in Tris-gly (4 mg/ml), 12 % trichloroacetic acid (TCA) and 8, 10 and 12 M urea in Tris-gly.

All samples were prepared in deionised distilled water. Samples for SH and SS analysis were prepared by heating 3 % bovine serum albumin solutions with or without 2 % xylose for a range of times between 0 and 75 min at 95 °C. Data from Chapter 8 showed that the additional covalent bonds were already formed within this period even though all samples were still in solution.

To show that SH groups can react with glyoxal and glyceraldehyde, native bovine serum albumin was modified by reducing the SS groups using β -mercaptoethanol. This method is similar to the reduction of disulfides prior to the reaction with the Ellman's reagent. After modification and dilution with 8M urea, the bovine serum albumin concentration was 0.072 %. This was either heated alone or with glyoxal (0.025 - 0.1 %), glyceraldehyde (0.1 %) and ribose (0.5 %)

(c) *Determination of SH and SS groups*

This was carried out according to the methods of Beveridge *et al* (1974) with some modifications. For SH, 0.2 ml of samples was diluted with 10 ml of 8 M urea in Tris-gly. To 3 ml of this, 0.02 ml of Ellman's reagent was added for colour development. For SS, 0.2 ml of samples, 1 ml of 10 M urea in Tris-gly and 0.02 ml of β -mercaptoethanol were incubated at 25°C for 1 h. After an additional 1 h incubation with 7 ml of 12 % TCA, the samples were centrifuged at 2000 g for 10 min. The precipitate was twice resuspended in 5 ml of 12 % TCA and centrifuged to remove β -mercaptoethanol. The precipitate was dissolved in 25 ml of 12 M urea in Tris-gly. The colour was then developed by adding 0.02 ml of Ellman's reagent to 3 ml of the dissolved precipitate. Within 10 min of mixing, the absorbance was measured at 412 nm on a Ultrospec 4050 spectrophotometer.

(d) *Calculation*

$$\mu M \text{ SH/g} = \frac{73.53 A.D}{C} \quad (4.15)$$

where A = Absorbance at 412 nm, C = the protein concentration (mg/ml), D = the dilution factor, 73.53 is derived from $10^6/(1.36 \times 10^4)$; 1.36×10^4 is the molar absorptivity (Ellman's, 1959) and 10^6 is for conversions from the molar basis to $\mu M/ml$ basis and from mg solids to g solids.

4.4 *Amino acid analysis*

Amino acid analysis was performed to follow up the results obtained in the previous section (4.3.9).

(a) Extraction and hydrolysis methods

Samples used in this section were 3 % bovine serum albumin heated alone or with 2 % xylose at 95 °C for 75 min. Performic acid was prepared by mixing 90 ml of formic acid, 10 ml of hydrogen peroxide and 500 mg of phenol. The flask was left to stand for 1 h at 30 °C and then cooled to 0 °C for 30 min. Performic acid (25 ml) was added to 3 ml of sample and this was left in the cold room for 16 h. Sodium metabisulphite (4.2 g) was added into the mixture of sample-performic acid to remove excess acid. The sample was then hydrolysed under reflux for 22 h after an addition of 75 ml of 6 M hydrochloric acid. The mixtures were evaporated under vacuum and the dried solids were dissolved in buffer and kept in a cold room prior to analysis.

(b) Analysis

A Pharmacia BIOCHROM 20 Amino Acid Analyser (Cation exchange chromatography) was used to analyse the amino acid contents of the samples. The instrument sensitivity was 10 nmoles (optimum) and a lower limit detection was 1 nmole. Complete separation of amino acids required 2.5 h using sodium citrate buffers (4 buffer programmes) and ninhydrin detection for colour development. NLEU was used as the internal standard. Quantitation of amino acid concentration was carried out automatically using a computerised integrator package.

CHAPTER 5

FORMATION OF MAILLARD GELS

The gels made for this project were developed in an aqueous medium and the formation of the gels was initiated by raising the temperature either to 90 °C or 121 °C. The high temperature employed led to a change in conformational states of the protein and hence to the processes of association (Chapter 2). Due to the addition of carbonyl compounds to the protein solution, other changes also take place when the solution is heated. The most important changes are thought to be due to the Maillard reaction, as evidenced by the change in the colour of the solution from light yellow to brown. It is these chemical changes and their effect on network formation that have been mostly studied and reported in this thesis.

Previous studies on Maillard gelation (Hill *et al* 1992) have used a system where samples were heated in an autoclave and their gel strength measured by estimating breakstrength using rupture techniques. Fracture phenomena using these techniques can be problematic. It is necessary to repeat the breaking force measurement, using more than one sample. Moreover, the measurement of breaking force cannot follow the gelation processes in real time. It was the intention of this study to measure the gelation time and G' (storage modulus) using the Bohlin CS Rheometer. This technique allowed measurement of rheological properties using a single sample and gelation can be followed in real time thus enabling the study of the gelation kinetics.

5.1 *Visual assessment of Maillard gels*

Fig. 5.1a shows the appearance of the products formed by heating 1 % bovine serum albumin and 3 % ribose (A) and 3 % bovine serum albumin solution alone (B) at an elevated temperature of 121 °C for 30 min. It is clear that bovine serum albumin solution did not gel at this concentration, despite the severe heating conditions employed. In the presence of ribose however, a solid gel matrix was formed. This gel was firm to the touch and had a smooth interior. The change in colour of the sample from light yellow to dark brown provides a clear indication of the occurrence of the Maillard reaction. More evidence for this reaction can also be shown by the pH values of the final products. The brown gel had a pH of 3.90, while the clear solution had a pH of 7.00.

Fig 5.1b shows the formation of 1 % bovine serum albumin and 3 % ribose gels at 90 °C for a range of heating times between 50 to 130 min. The extent of browning is higher in the sample heated at an elevated temperature (121 °C). However, all samples containing ribose are brown in colour. The gels formed at 90 °C were more transparent than those formed at the higher temperature. The intensity of browning in the gels formed at 90 °C increased slightly with heating time. After heating to 90 °C and cooling of the samples, a marble was inserted into each bottle to give an estimate of the extent of gelation. It can be seen that gelation had occurred between 80 to 100 min of heating. At 90 min the gel was strong enough to hold a marble. It is also clear that the gels formed were not due to protein precipitation.

The gels left after heat treatment (90 °C) at ambient temperature continued to develop more colour and the strength of the gels increased. Solutions (50 and 70 min) may form gels after long holding periods (e.g a week) at ambient temperature.

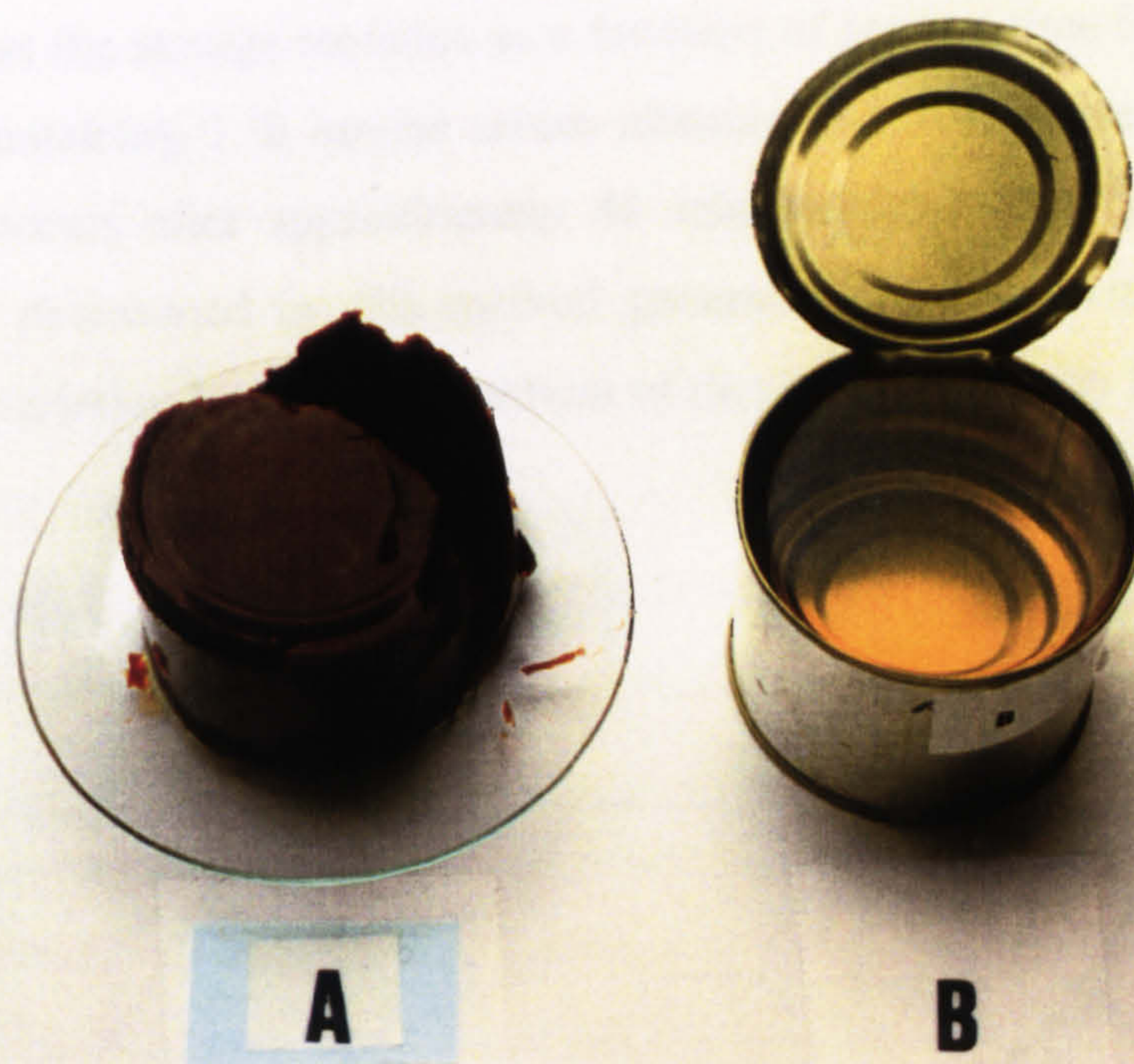
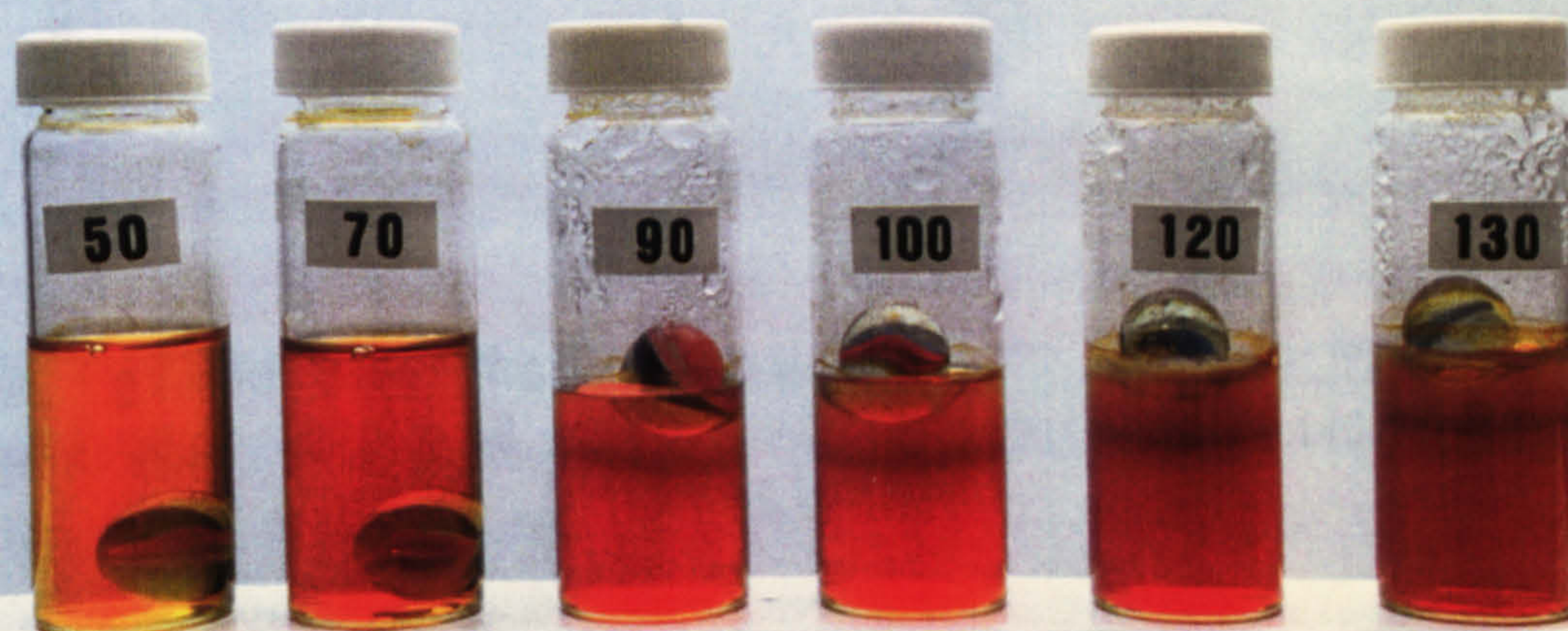
a**b**

Fig. 5.1 (a) Appearance of products formed by heating 1 % bovine serum albumin with 3 % ribose (A) and 1 % bovine serum albumin solution (B) at 121 °C for 30 min. (b) Formation of 1 % bovine serum albumin + 3 % ribose gels at 90 °C. The numerical labels indicate heating time (min).

5.2 The rheological assessment of the gelation processes

Fig. 5.2 displays the storage modulus as a function of heating time for duplicate runs on solutions containing 1 % bovine serum albumin and 3 % ribose. It can be seen that gelation occurs after approximately 80 min heating. The gelation times for duplicate runs determined by this method generally agreed to within 3 min. The coefficient of variation for the measurement of rheological property (G') was between 4 - 8 %.

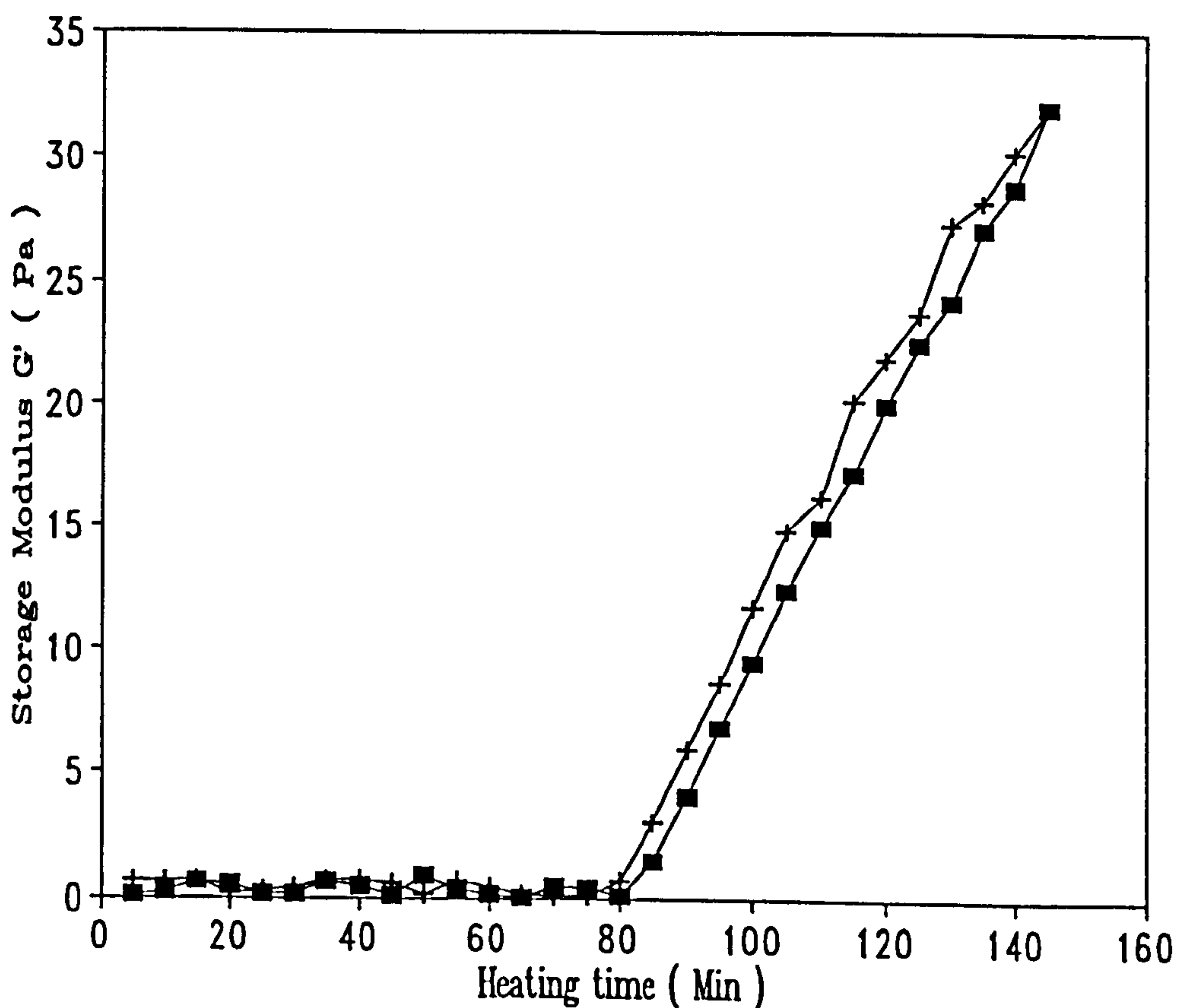


Fig. 5.2 Storage modulus as a function of heating time at 90 °C for 1 % bovine serum albumin + 3 % ribose measured at 1 Hz. The two sets of data points refer to measurements carried out on different days with different solutions

The structure formed during heating is indicated by a rapid increase in G' after the gelation time. As the crosslinkings proceed further, the elastic component of the gel increased. The maximum value of G' was not obtained as the rheometer was only run for 145 min for practical reasons.

It is clear from Fig. 5.2 that the techniques give good reproducibility even at the low bovine serum albumin concentration of 1%. Aqueous solutions (1% or 3 %) of bovine serum albumin heated in the absence of sugars or in the presence of sucrose, a non-reducing sugar did not show this gelation behaviour (Fig. 5.1a).

Maillard gelation was also shown to occur if bovine serum albumin solutions were heated with the reactive Maillard reaction products. Fig. 5.3 shows the differences in gelation time and G' of four Maillard gelling systems. Gelation time was dependent on the reactivity of sugars or the Maillard compounds used. The most reactive, glyoxal, was used at 0.25 % while glyceraldehyde was used at 1 %. This compares to a 3 % concentration used for ribose and xylose. Even though in terms of browning glyceraldehyde is considered to be the most reactive among these compounds (Hayashi and Namiki, 1986), its gelation time was not the shortest. This would seem to suggest that the kinetics of protein gelation via the Maillard reaction and the development of colour may not necessarily be connected. They may occur independently via the Maillard reaction. A similar suggestion has been put forward for protein polymerisation and the browning which developed after heating ovalbumin with aldohexoses (Kato *et al* 1986). This was supported by a study by Kato *et al* (1988) in which it was shown that if free glucose was removed from the ovalbumin-glucose system at an early stage of the reaction, colour development was suppressed, whereas protein polymerisation still took place.

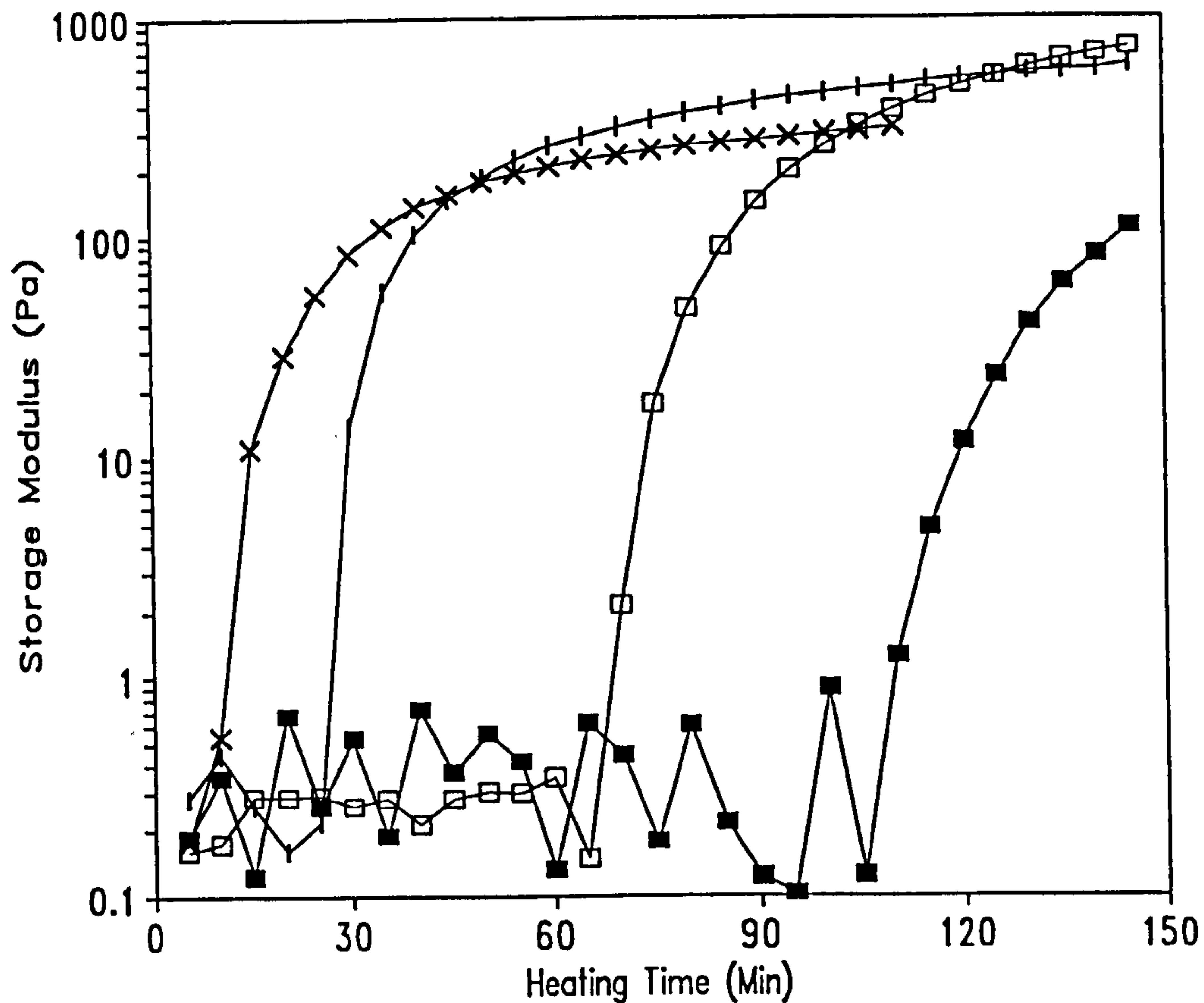


Fig. 5.3 Gelation of 3 % bovine serum albumin solutions heated in the presence of 0.25 % glyoxal (X), 1 % glyceraldehyde (|), 3 % ribose (□) and 3 % xylose (■) at 90 °C.

5.3 Gelation at elevated temperatures

Gelation of low levels of bovine serum albumin alone or bovine serum albumin with less reactive reducing sugar e.g. glucose, could not be followed using the C 25 geometry (90 °C) as it took too long for gelation to occur (> 4 h). However, this could be overcome by using a high pressure cell (HPC) fitted to the rheometer, and an oil bath as the heating element. Using these techniques, elevated temperatures in excess of 100 °C can be used to monitor the gelation processes of proteins in the presence less reactive reducing sugars. Such an application is shown in Fig. 5.4, in which solutions of 3 % bovine serum albumin + 3 % glucose were heated at three different temperatures of 95 °C, 100 °C and 105 °C.

It can be seen that gelation can be monitored in systems where a less reactive sugar (glucose) is incorporated, provided that the temperature is sufficiently high. No gelation occurred at 95 °C within the heating period of 0-190 min. At 105 °C the gelation time was approximately 105 min. A decrease in temperature by 5 °C increased the gelation time from 105 to 120 min.

It is hoped that more kinetic study at these elevated temperatures will be carried out in the future so that more information about changes in gelation properties at typical processing temperatures are obtained.

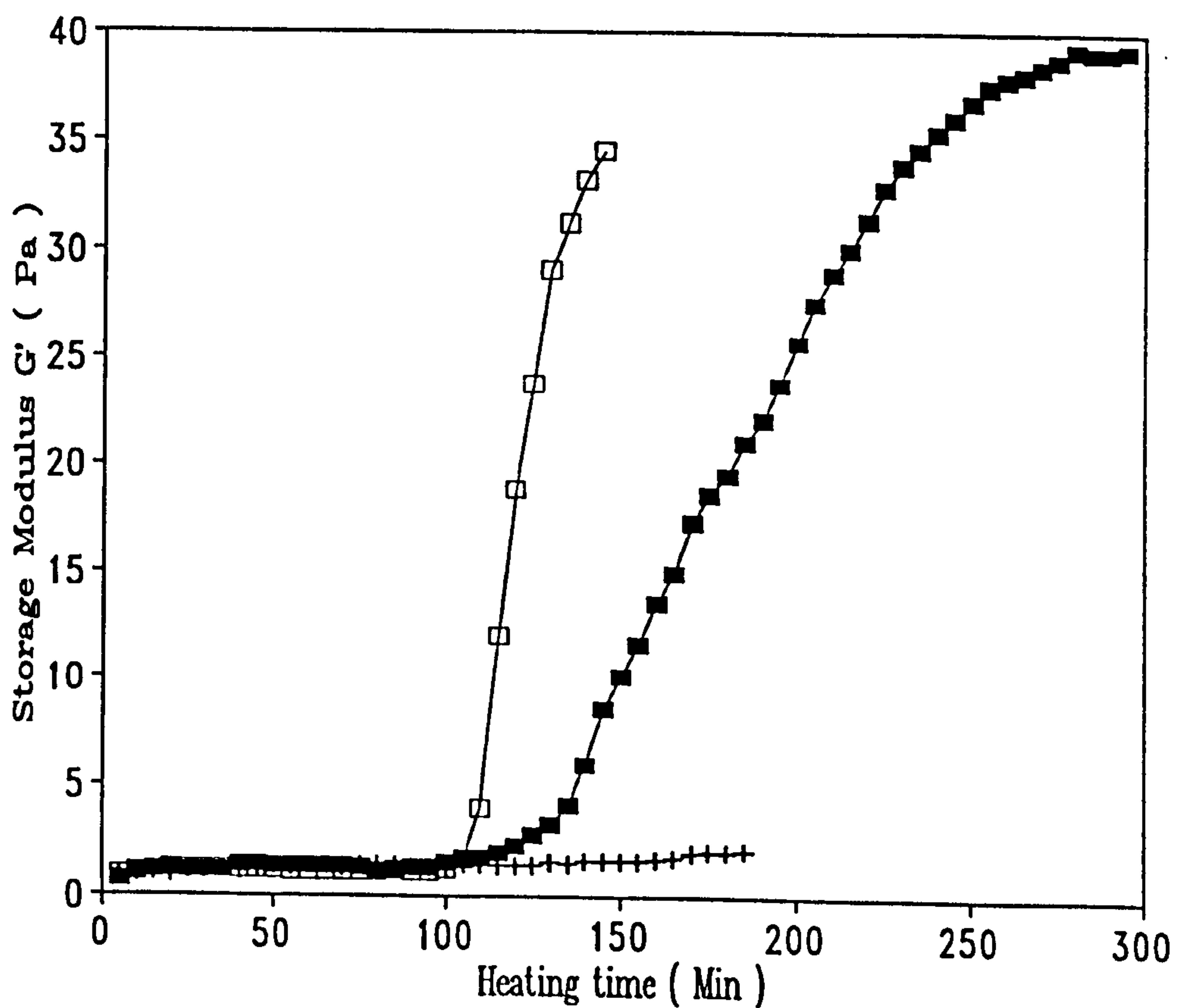


Fig. 5.4 Gelation of 3 % bovine serum albumin + 3 % glucose solutions at 3 different temperatures, 95 °C (+), 100 °C (■) and 105 °C (□).

Conclusion

Gelation of bovine serum albumin and reducing sugar solution can be followed in real time, using oscillatory rheometry. The methods show good reproducibility and can be used to establish a gelation time and the development of the storage modulus with heating conditions. This method was developed for this project and has been used in the work described in the following chapters.

Using these techniques it has been shown that Maillard gelation can also take place if a protein solution is heated in the presence of compounds that are known to be generated via the Maillard reaction. Glyoxal and glyceraldehyde were more reactive, in terms of causing gelation after shorter times and requiring lower concentration of the carbonyl compounds, than the hexoses, glucose and xylose or a pentose, ribose. There is some indication that the ranked reactivity order for carbonyl compounds is not the same for induction of gelation compared to colour development.

It is possible to follow the gelation processes in real time at temperatures above 100 °C, using the new development of pressure cell attachments to controlled shear rheometers.

KINETICS OF MAILLARD GELATION

Of concern at the start of the project was the characteristic low pH of the Maillard gels. The pH fell during the heating procedure used for gel formation. Action to stop or reduce the change in pH introduced other factors (e.g. high salt concentration) that were known to alter the gelation properties of protein (Chapter 2). Gel quality is known to be dependent on the final pH of the gel and the pH throughout the denaturation and aggregation processes undergone by the protein forming the final gel network. It was possible that the differences in protein systems heated with and without carbonyl compounds was solely due to the variation in hydrogen ion concentration throughout the heating cycle.

A hypothesis was put forward that *"gelation occurred when the pH of the system reached a specific level, termed the 'critical pH'. How this critical pH was reached was not important to the system, and the Maillard reaction was only relevant to the gelation processes in that it induced a pH fall by organic acid production"*.

To test the importance of pH and gel formation, it was necessary to have a method that gave accurate information on when the gel network was formed; the gel point. The pH of the matrix could then be measured at this time and the relationship between the two investigated.

To alter the rate of pH fall and to change the time required to gel the protein, the following were varied;

- (1) protein concentration
- (2) carbonyl compound concentration
- (3) type of carbonyl compound

Information on the gelation rate was used to ascertain information on the kinetics of gelation reaction, in low and high protein systems, as well as for testing the proposed hypothesis. The method developed for the estimation of the gel point is given in Chapter 4.

6.1 Gelation kinetics at low protein levels

Fig. 6.1 displays the gelation time as a function of reducing sugar concentration. The more reactive reducing sugar ribose gives shorter gelation times than xylose. The gelation time decreases with increasing reducing sugar and protein concentration.

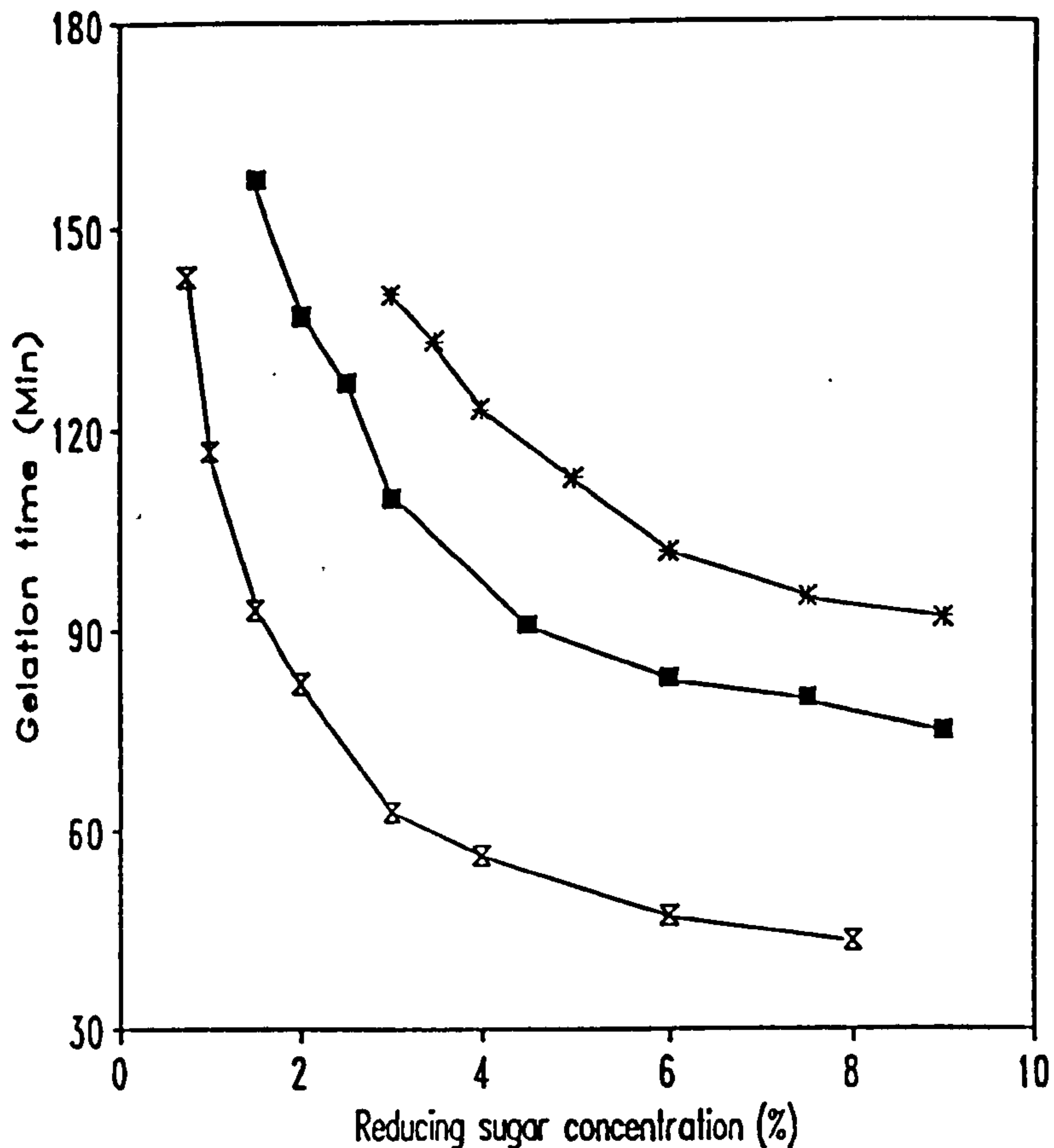


Fig. 6.1 Gelation time of 3 % bovine serum albumin + xylose (■), + ribose (X) and 2 % bovine serum albumin + xylose (*) at 90 °C

In contrast to some studies when protein gels are formed via acidification using glucono- δ -lactone (Kohyama *et al*, 1992) alteration of bovine serum albumin concentration from 3% to 2% in the presence of xylose does increase the gelation time. It is known that in order to achieve a gel the molecules have to be linked to form a continuous network. Additional protein could be expected to reduce gelation time as there would be a higher concentration of possible crosslinker, e.g. additional hydrophobic interactions. The small molecular weight sugars would not be expected

to take a primary role in the gel network. Their action was considered to be either in the pH reduction (and therefore change in the electrostatic interactions between the protein), or by their role in the Maillard reaction to form a crosslinker between the proteins and hence markedly changing the molecular weight of the protein.

It is known that the Maillard reaction produces organic acids and therefore causes a reduction in pH. An additional reason for the pH fall could be the loss of protons from lysine amino groups involved in the Maillard reaction. Fig. 6.2 and 6.3 display the pH changes as a function of time for 3% bovine serum albumin as a function of ribose and xylose concentrations.

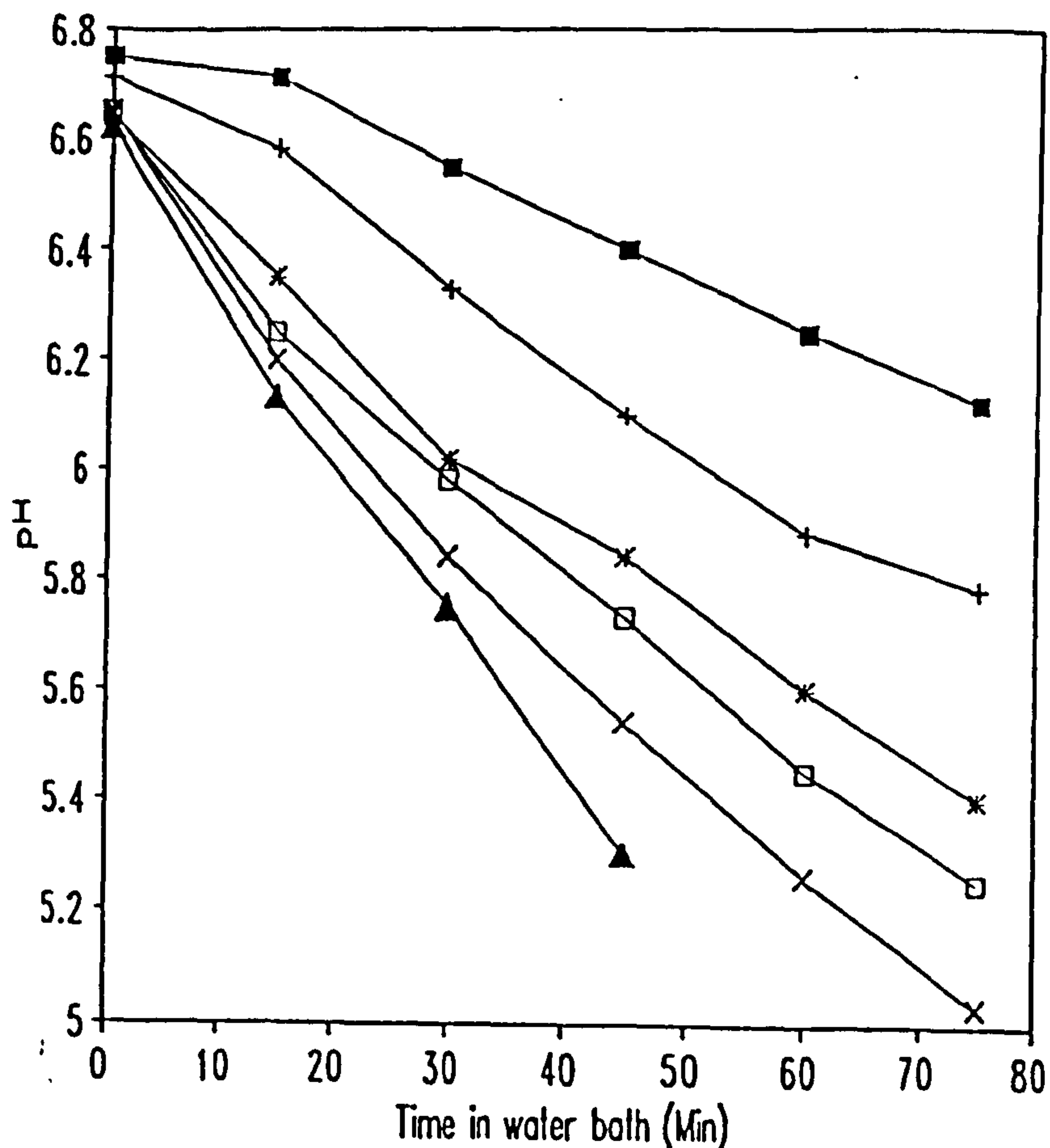


Fig. 6.2 pH of 3 % bovine serum albumin solutions after heating for different time at 90 °C in the presence of 1 % (■), 2 % (+), 3 % (*), 4 % (□), 5 % (X) and 6 % (▲) ribose

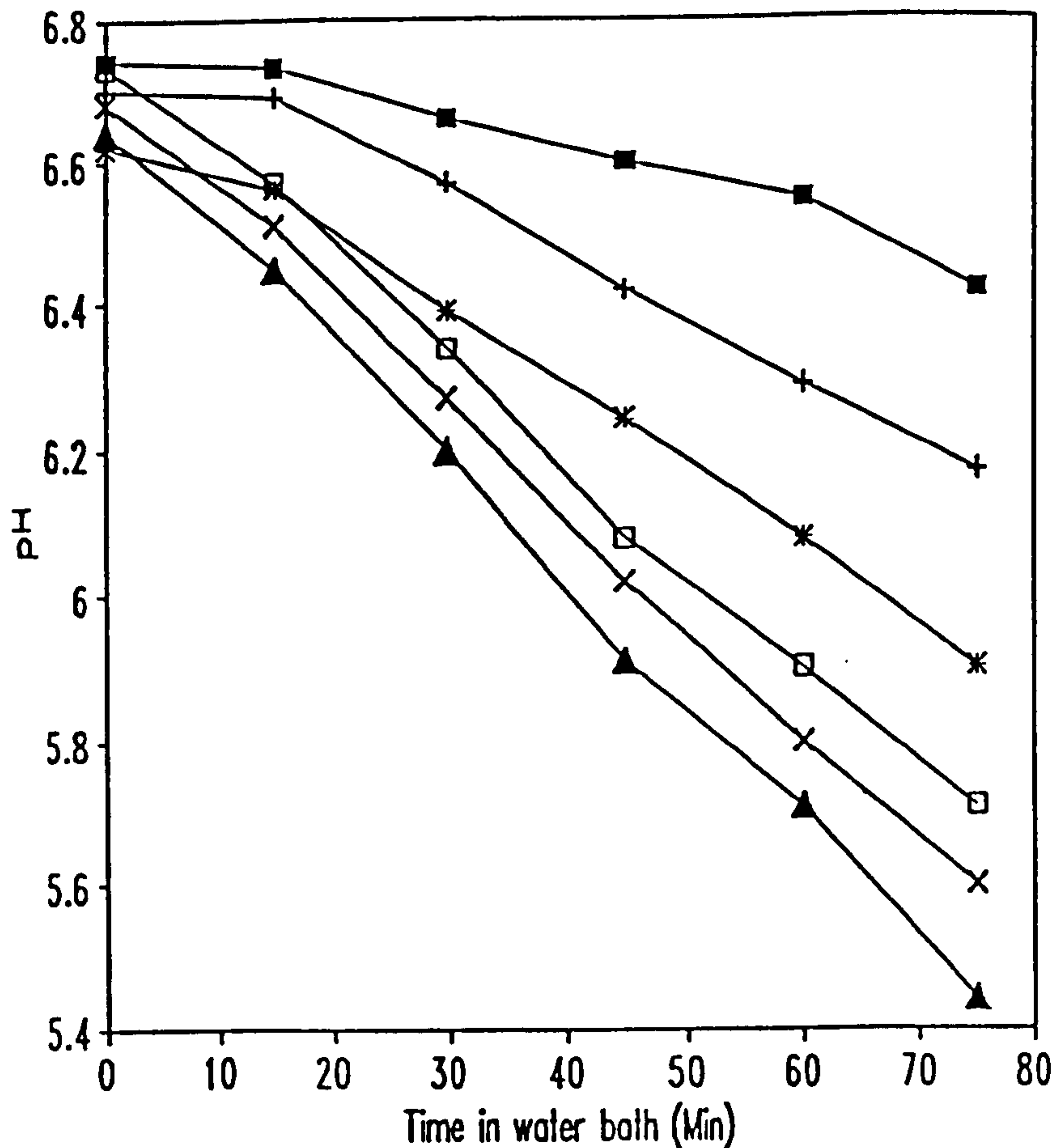


Fig. 6.3 pH of 3 % bovine serum albumin solutions after heating for different time at 90 °C in the presence of 1 % (■), 2 % (+), 3 % (*), 4 % (□), 5 % (X) and 6 % (▲) xylose

Before heating, the initial pHs of the solutions were in the range 6.65-6.75 which were independent of the reactivity and concentration of reducing sugars. The rate of decrease in pH on heating was greater for the sugar of higher reactivity (ribose) and increased with reducing sugar concentration.

It was considered possible that this pH fall affected the gelation of the protein and that a continuous network formed when the reaction mixture was reduced to a critical pH value. As the exact time for gelation could be determined from the rheological data, the pH of the system could be measured at that specific time point. This was carried

out for each sample and the results plotted against sugar concentration in Fig. 6.4. If the hypothesis of a critical pH was to hold, no variation in pH at the gel point would occur whatever the sugar concentration or type. Clearly the results in Fig. 6.4 demonstrated that the hypothesis was incorrect and needed to be modified or changed to explain why the Maillard reaction enhanced the gelation properties of the protein.

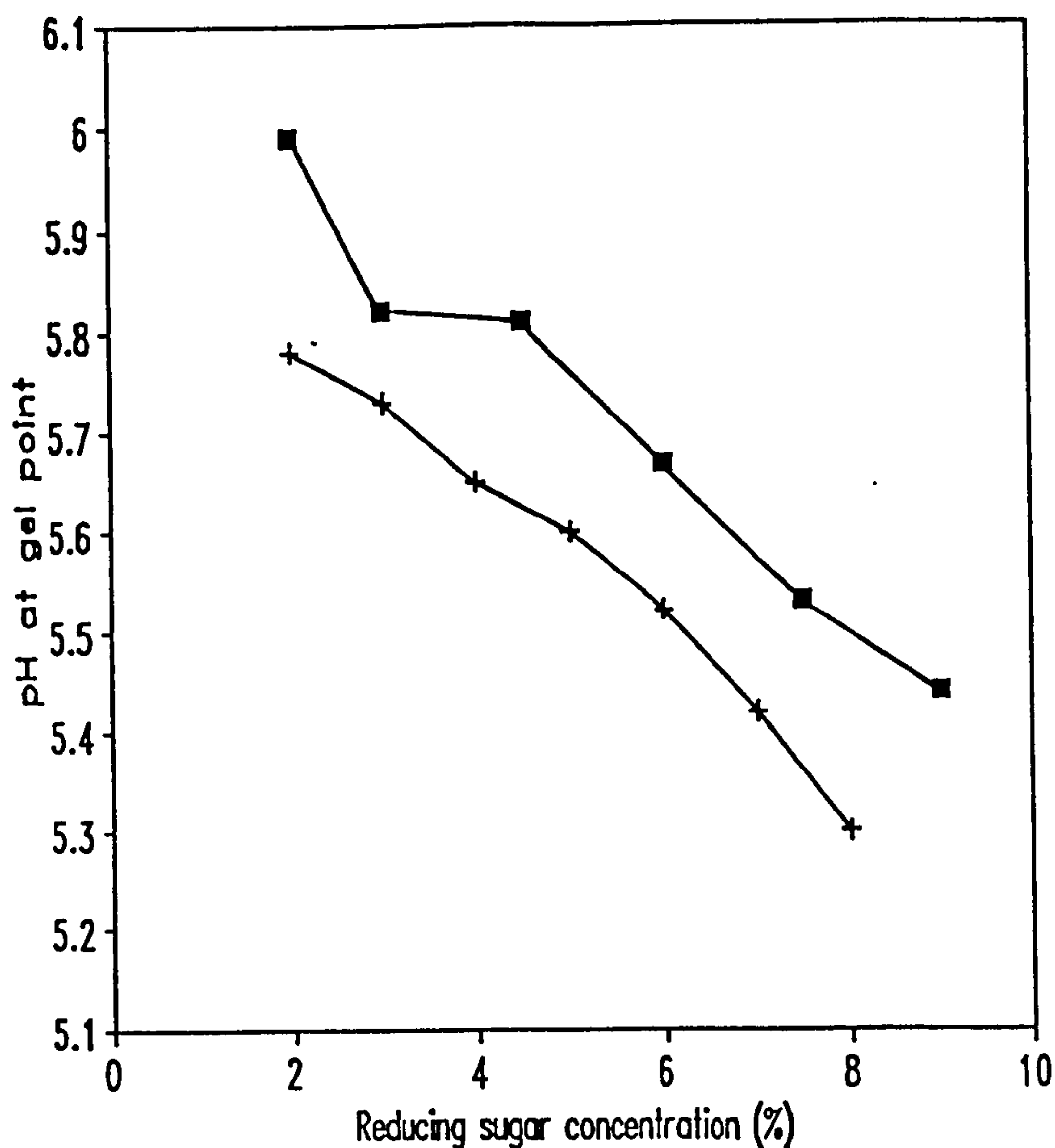


Fig. 6.4 pH at the gel point as a function of reducing sugar concentration for 3 % bovine serum albumin + xylose (■) and + ribose (+)

From the work reported in this section, three important factors for gelation could be explored and used to revise the hypothesis. The first involved the relevance of charge on the protein and its relationship to pH. The second reviewed the concept that for gelation the macromolecular concentration has to be sufficient to form a network that can span the system. The third was that the duration and order of the multiple reaction occur was important.

(a) *Charge on the protein and pH*

For a protein to gel it is necessary for the protein molecules to associate. At the isoelectric point (pI) of the protein electrostatic repulsion will not occur hence the molecules will associate. The concentration of protein required for association is minimum at the pI and increases with the increasing absolute net charge on the molecules. The gelation behaviour of bovine serum albumin at different pH values has been widely investigated (Poole *et al* 1986; Poole and Fry, 1987; Matsudomi *et al* 1991). At neutral pH values bovine serum albumin concentrations in excess of 7% are required to give gels. As the pH is lowered and electrostatic repulsion is reduced, coarse, opaque gels can be formed at much lower concentrations (Clark and Lee Tuffnell, 1986; Matsudomi *et al* 1991). For bovine serum albumin at pHs close to the protein isoelectric point, precipitation rather than gelation normally occurs. In their review, Ziegler and Foegeding (1990) summarise the normal pH range for bovine serum albumin gelation as 5.8 to 8.0 and 3.5 to 4.5, with slight variations with ionic strength. Fig. 6.4 indicates that the pH of the Maillard gels formed in this study were in the range of 5.3 to 6, which is above the expected isoelectric point of bovine serum albumin (pH 5.0), but are low for bovine serum albumin gel formation without precipitation. It is also clear that the pH at the gel point decreases with increasing reducing sugar concentration and sugar reactivity. The low pH could explain the gelation at low concentration of the protein, but not the clarity and homogenous characteristics of the gels (see Fig. 5.1).

It has been reported that heating bovine serum albumin at 37 °C for five weeks with glucose, a far less reactive reducing sugar than ribose or xylose, had resulted in the glycation of about 20 amino groups on the protein (Yaylayan, 1992). Bovine serum albumin contains 59 lysine residues. Modification of a proportion of these by the Maillard reaction so that the ϵ -amino group cannot assume a positive charge, will have a significant effect on the net charge on the protein. At pH 7.0 bovine serum albumin has been calculated to have a net charge of - 18 e.u. (Peter, 1985) which will decrease to zero as the pH moves to the protein's isoelectric point.

Loss of positive groups (i.e glycation of ϵ -amino groups) will increase the net negative charge and therefore the pI of the protein will be shifted downwards.

The change in pI would consequently mean a change in the critical protein concentration at any specific pH. This idea is illustrated in Fig. 6.5. If high reactivity relates to large numbers of amino groups losing their charge, the pI will be lower. Moving from a neutral pH, a lower pH would be necessary for the bovine serum albumin/ribose system to gel at the same concentration as a bovine serum albumin/xylose system. It follows that the critical pH for gelation above the isoelectric point would decrease with increasing extent of the Maillard reaction, if one of the consequences of the reaction is for the protein to assume a higher net charge.

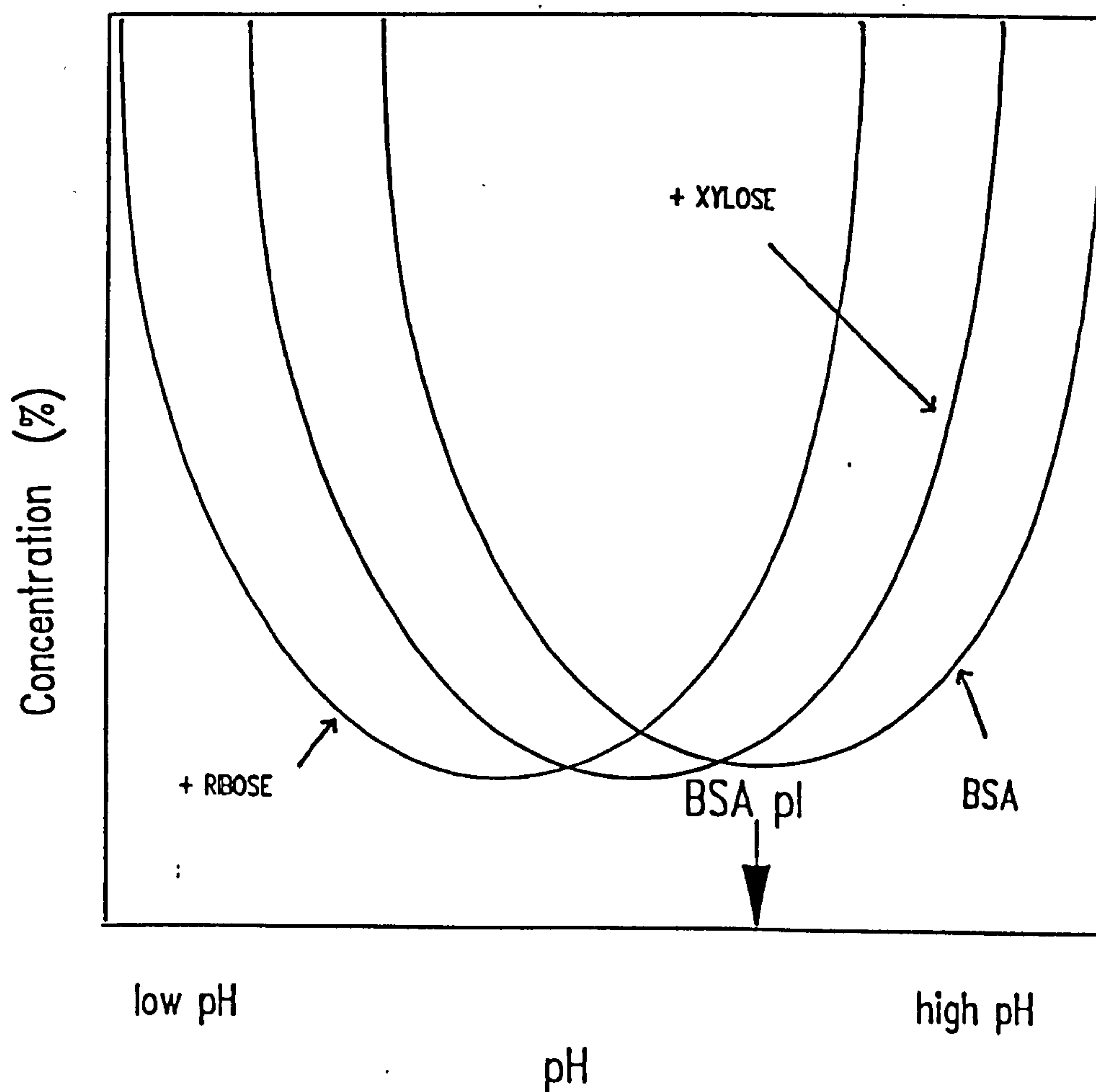


Fig. 6.5 The relationship between C_0 and pH of a protein after modification via the Maillard reaction

(b) *Critical concentration of gelling agents*

The concept of creating a network at a critical concentration has been introduced in the previous section. A method to predict the minimal concentration of the large macromolecules involved in gelling is well known (Richardson and Ross-Murphy, 1981a). It was considered interesting to use this type of methodology to see if the prediction of a minimal concentration of reducing sugar that would evoke gelation of the 3 % protein solution could be made. Therefore extrapolation a log-log plot of reactant against G' to predict a C_0 (minimal concentration) of reducing sugar was carried out. Fig. 6.6 shows the plots obtained for sugar concentration against the G' value at 145 min. The xylose data is shown at protein concentration of 2 and 3 %. The minimum xylose concentration required for gelation of 3 % protein (4.5×10^{-4} M) is about 1.7 % (0.113 M) whereas only 0.7 % ribose (0.047 M) is needed. The xylose level was about 2.4 times higher than ribose. This may suggest that ribose was 2.4 times more reactive than xylose in causing gelation of the protein. It would appear that the equilibrium value of G' obtained at high reducing sugar concentrations is higher for the more reactive reducing sugar ribose compared with xylose. This could probably be the result of additional covalent crosslinks between protein molecules with the more reactive reducing sugar. These additional crosslinks would compensate for the decrease in modulus which would normally be expected as a result of increased charge on the protein. Thus a combination of charge effects and additional crosslinking mechanisms provide an explanation for the high modulus gels of high water holding ability which can be formed at pHs close to the original isoelectric point of the protein.

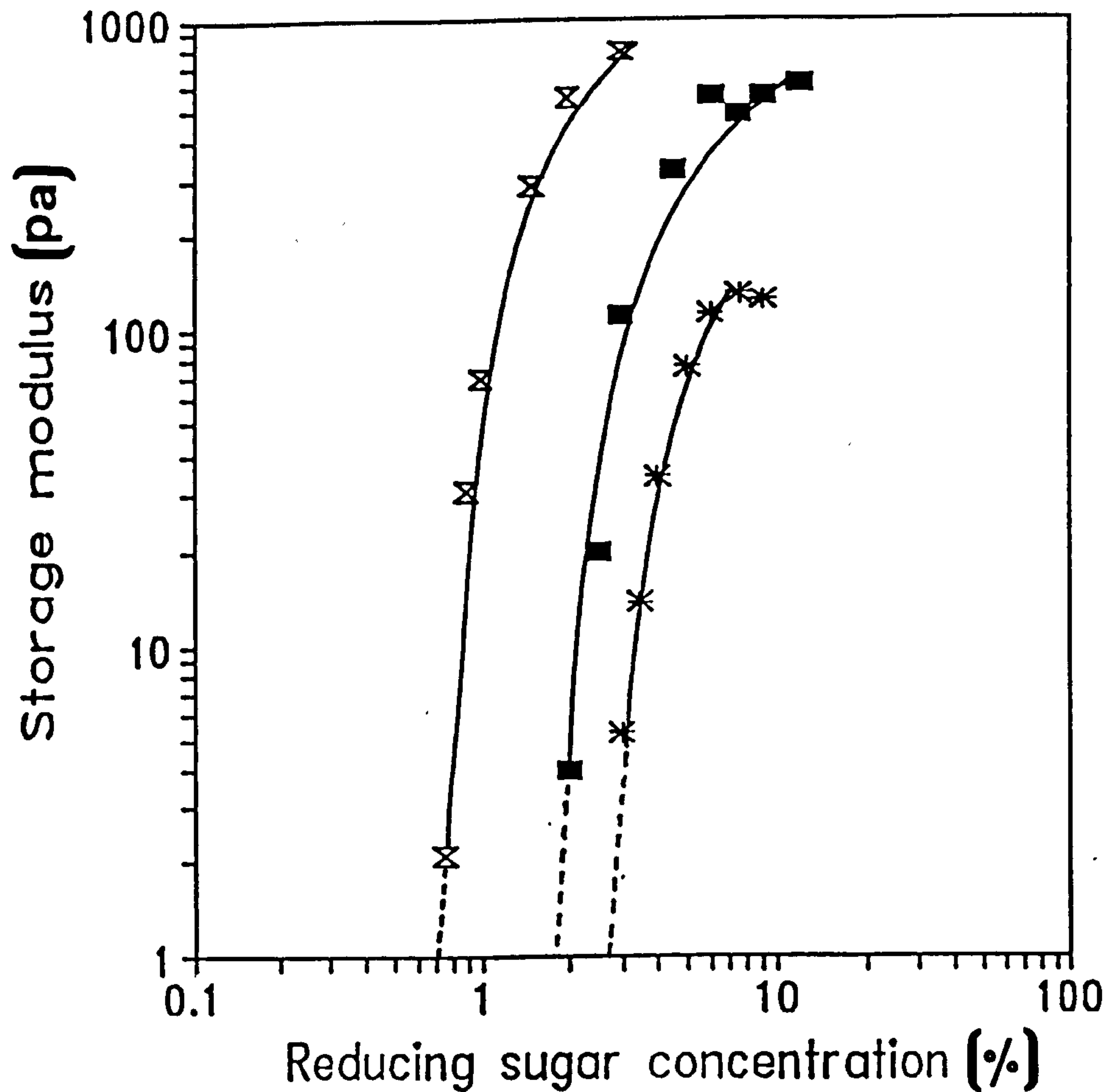


Fig. 6.6 Log-log plot of storage modulus obtained after heating for 145 min at 90 °C against reducing sugar concentration for 3 % bovine serum albumin + ribose (X), + xylose (■) and 2 % bovine serum albumin + xylose (*)

(c) *Rate of change during the gelation processes*

The speed of the pH fall, glycation and molecular interactions cannot be ignored in this work. By trying to determine the reaction order for the Maillard gelation processes some indication of the relative importance of the possible factors in the gel formation may be apparent.

Oakenfull and Scott (1986) suggested that the "reaction order" of a gelation reaction can be determined from the log-log plot of gelation rate and concentration. For this study the logarithmic plots of the two measures of the Maillard reaction rate (the

reciprocal of gelation time and the rate of increase in the hydrogen ion concentration as obtained from the pH) against the reducing sugar concentration are shown in Fig. 6.7. These plots are approximately linear and the slopes are 1.4 and 1.2 as obtained from the hydrogen ion results for the xylose and ribose systems respectively, and 0.5 from the gelation time for both gelling systems. Even though the validity of this approach is questionable, the dependence of these parameters on sugar concentration may provide some information on gelation mechanism. The weaker dependence of the gelation rate as compared to the rate of pH fall on reducing sugar concentration may suggest that the decrease in pH was not the sole cause of gelation. This supports the results obtained in Fig. 6.4.

At this stage, it would appear that pH fall, change in charge and change in the protein may all be relevant to the Maillard gelation. A reason for the low protein concentration at which these gels can be formed could be that the hydrodynamic volume achieved by the unfolded protein molecule at concentrations initially below the threshold concentration for gelation may be higher than is achieved where the unfolding process is immediately followed by aggregation. The unfolding process may be slow and hence the slower reaction rates for the gelation, although a pseudo first order reaction is seen.

Where gel formation is the result of a slow pH change following denaturation aggregation will be a slower process than would be the case when protein is heated directly at its isoelectric point. This would explain why it is possible to form gels at the isoelectric pH of bovine serum albumin using glucono- δ -lactone (Armstrong *et al* 1994). The possibility of additional covalent linkages being formed should also not be ignored.

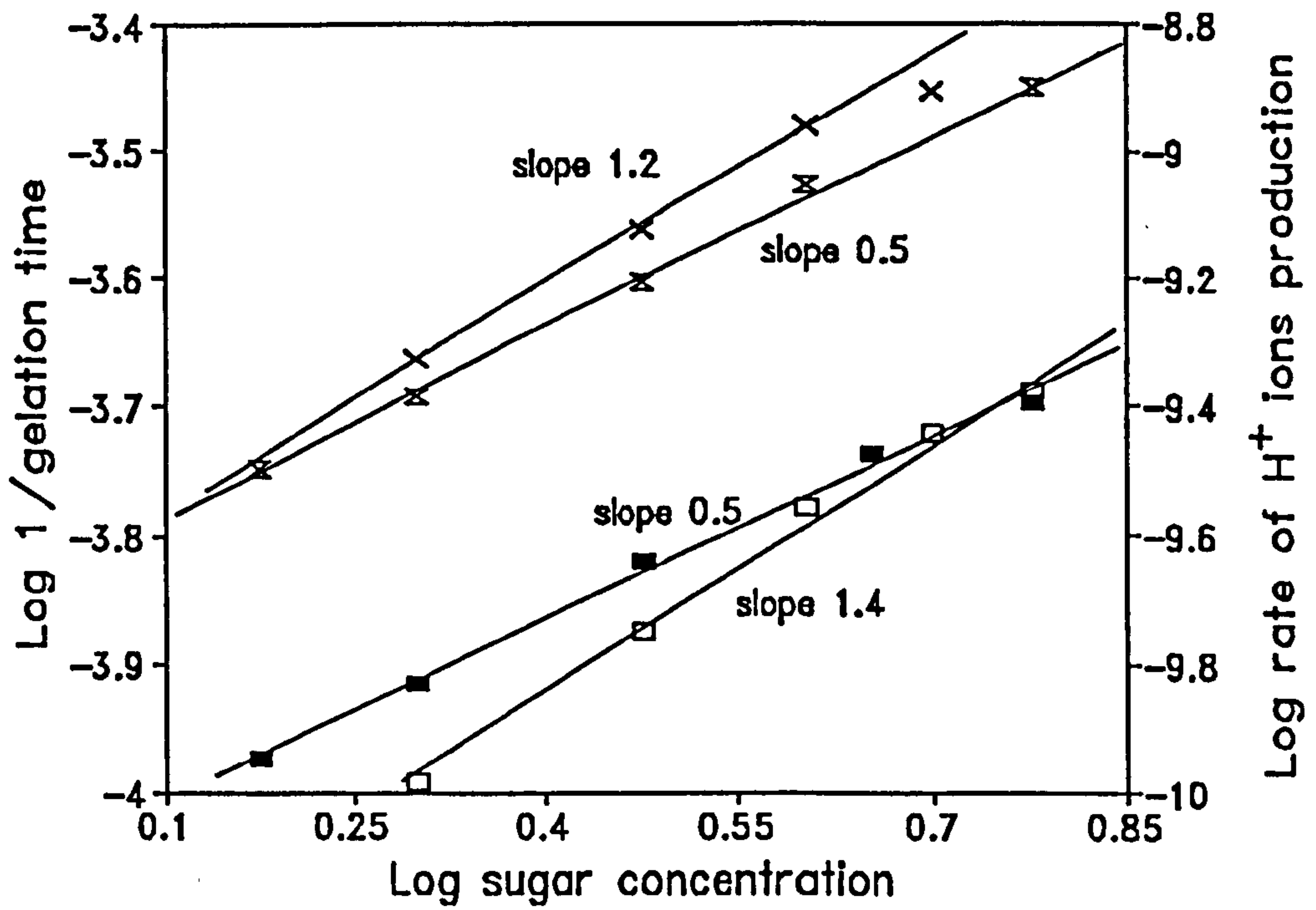


Fig. 6.7 The log-log plot of 1/gelation time and rate of hydrogen ions production against reducing sugar concentration respectively for 3 % bovine serum albumin + ribose (X and X) and + xylose (■ and □)

Conclusions

The time required for gelation is dependent on the concentration and reactivity of the sugar. It is these factors which also govern the pH fall of the system. However, although there was correlation between pH fall and the time required for gelation, gelation did not occur at a specific pH value. It must be considered therefore that as well as reaching a particular pH value other factors are required to form the gel. It is suggested that these other factors are change in the charge on the protein and time required for macromolecular interactions.

The methods used to predict minimum concentrations for gelling macromolecules, seem to be applicable for estimating the amount of reducing sugar concentration required to gel bovine serum albumin. Using this technique it was predicted that gels could be formed with 3 % bovine serum albumin and 1.7 % xylose and the sugar concentration could be reduced to 0.7 % if ribose was used. If less bovine serum albumin (2 %) was used then a higher amount of xylose was required (2.8 %).

The methods used for predicting kinetic order used for reversible gels that form by junction zones have been applied to the Maillard gels.

6.2 Gelation kinetics at high protein levels

In food applications globular proteins are normally used at concentration in excess of 5 % (Poole and Fry, 1987). It is expected that changes due to the chemical dimensions brought about by the Maillard reaction would also change the gelling properties of bovine serum albumin at concentrations where the protein is capable of gelling by itself. Bovine serum albumin with and without reducing sugar were studied at a range of concentrations to investigate the gelation characteristics.

The critical concentration C_0 for gelation of bovine serum albumin at pH 6.5 has been reported as 6.8 % (Richardson and Ross-Murphy, 1981a). Therefore, as expected gelation occurred when a 9 % solution of bovine serum albumin (pH 6.6) was heated, its final G' value obtained was about 20 Pa (Fig. 6.8).

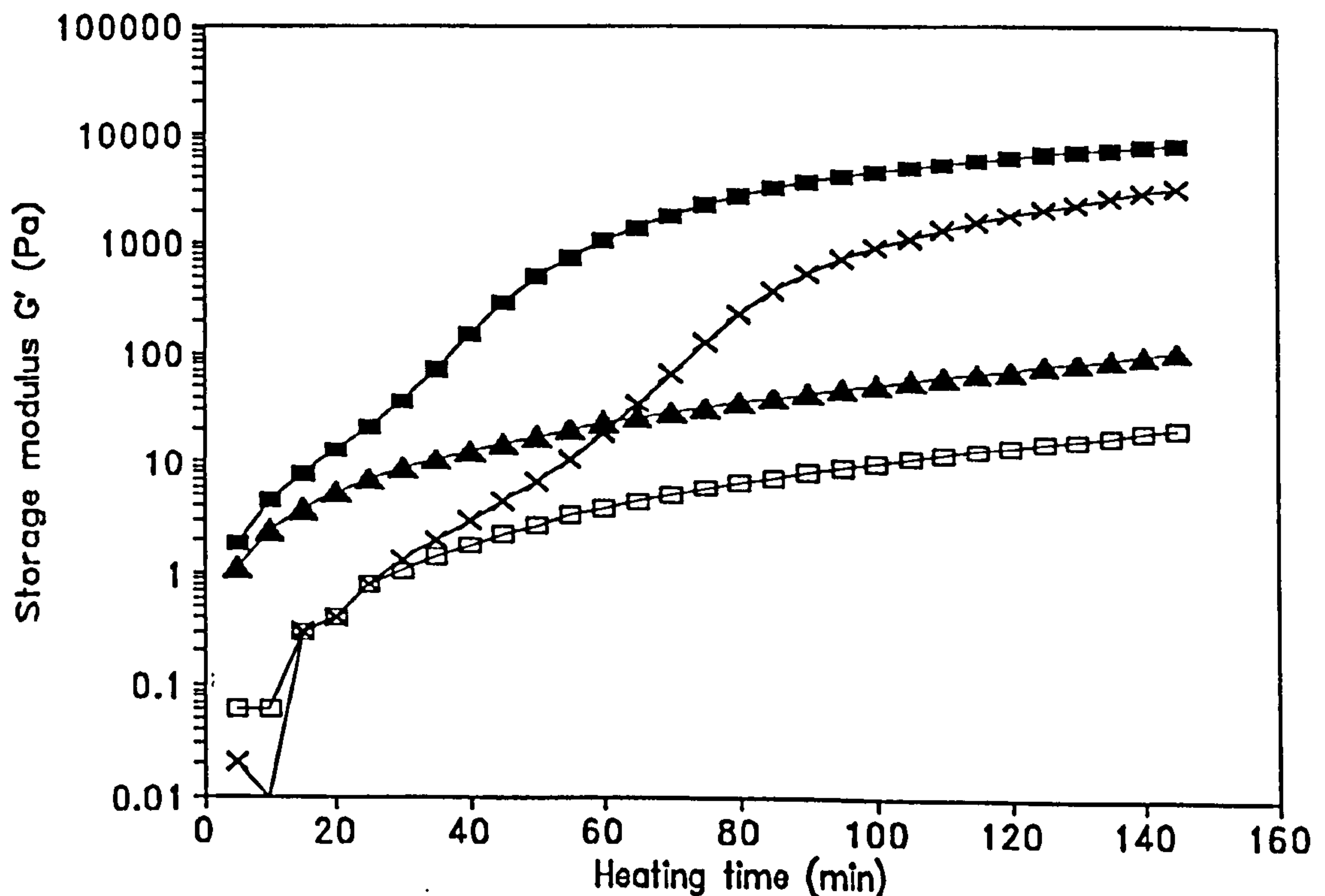


Fig. 6.8 The evolution of storage modulus as a function of time for 9 % bovine serum albumin heated alone (\square), with 3 % mannose (\triangle), with 3 % xylose (\times) and with 3 % ribose (\blacksquare)

However when 9 % bovine serum albumin solutions were heated with 3% reducing sugars, the final G' values obtained were about 8×10^3 , 3×10^3 and 1×10^2 Pa for ribose, xylose and mannose respectively. In comparison to bovine serum albumin heated alone, the gels formed by heating the protein with reducing sugars had lower pHs. For instance, the initial pH of bovine serum albumin/xylose was 6.61. After heating for 15 min, there was a slight rise. The pH decreased to 6.55 after 45 min, and at 75 min the pH was 6.20. Attempts to buffer this decrease were not successful as the ionic strength of the buffer interfered with gelation. From the values of G' , it appears that the gel strength of the Maillard systems follows the series for the reactivity of reducing sugar as reported by Hill *et al* (1992). It should be noted however that the G' values used in this thesis were not the limiting values as it was not possible to heat the systems for a period longer than 145 min or until curing of the gels occurred.

Information on the gel structure has been obtained from rheological measurements by studying the concentration dependence of the storage modulus (Stading *et al* 1993). This can be done by using a kinetic model based on Flory-Stockmayer kinetics (Flory, 1953). The value of critical concentration C_0 for bovine serum albumin has also been predicted from the log-log plot of G' against polymer concentration (Richardson and Ross-Murphy, 1981a). The double-logarithmic plots of G' against bovine serum albumin concentration is shown in Fig. 6.9, and the estimated values of the minimum protein concentration for gelation and slopes of the lines are displayed in Table 6.1.

Richardson and Ross-Murphy (1981a) suggested that G' should be zero for concentration less than C_0 and for high concentrations, G' should increase as the bovine serum albumin concentration increased. For concentration just greater than C_0 , the concentration dependence of G' should be more pronounced. This relationship is reasonable because the increase in protein concentration (i.e polymer) should increase the crosslink density at a rate proportional to its concentration.

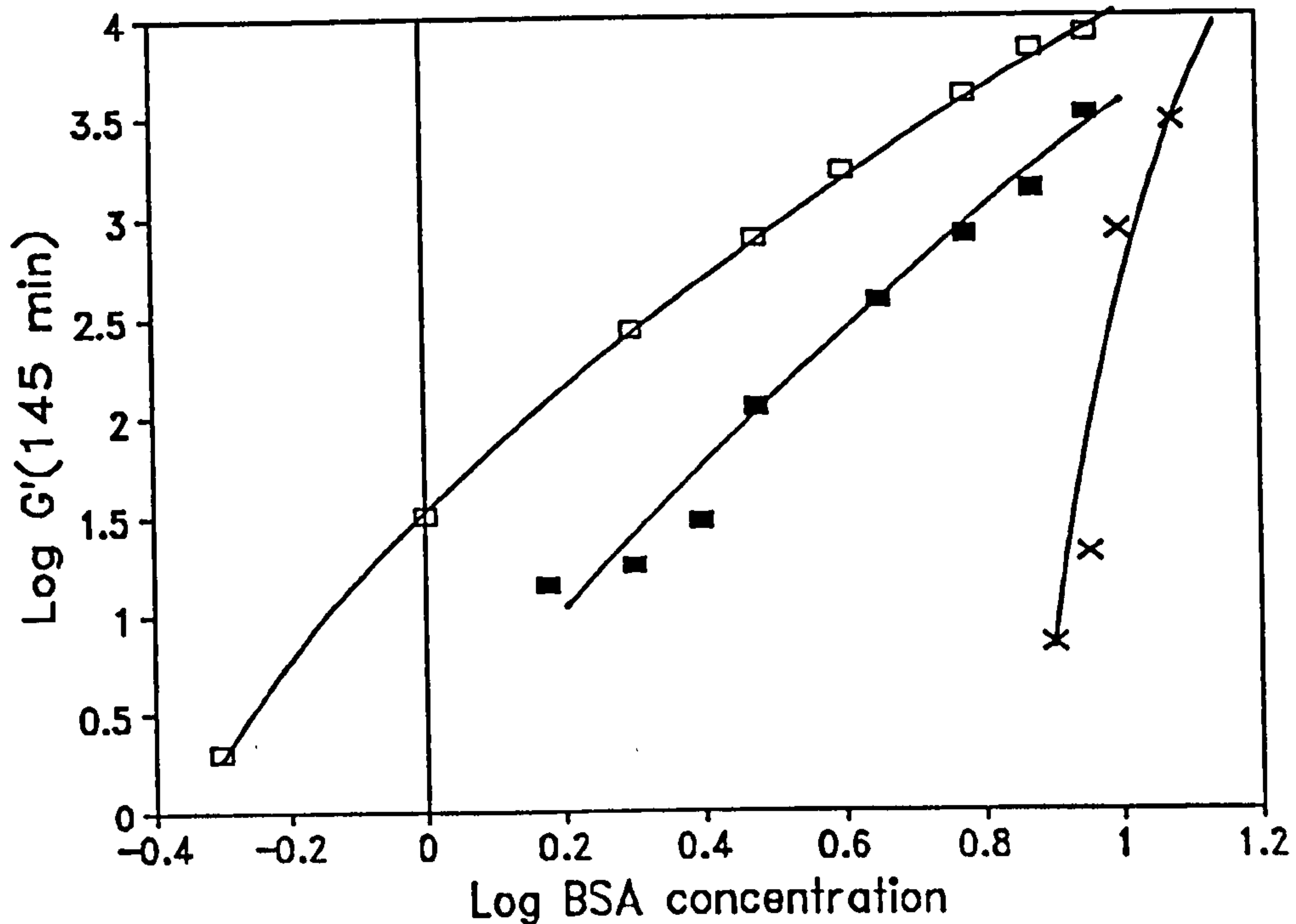


Fig. 6.9 Log-log plot of storage modulus (145 min) as a function of bovine serum albumin concentration for bovine serum albumin heated alone (X), with 3 % xylose (■) and with 3 % ribose (□)

The slopes obtained from the Maillard systems are close to the value proposed by Clark (1992) for typical biopolymer gels (Table 6.1). The rather high slope of bovine serum albumin heated alone may indicate that the concentrations used (7.5 - 12%) were too close to the critical gel concentration to give a true linear relationship of $\log G'$ versus \log polymer concentration. The value of minimum concentration for bovine serum albumin was slightly higher than previously reported at similar pH (7.0 %) and this is likely to be due to the values of G' used, not being the maximum or plateau value.

It is clear that the minimum concentration required for gelation can be reduced by incorporation of reducing sugars into solutions of bovine serum albumin (Table 6.1). The lowest minimum protein concentration could be achieved by heating bovine serum albumin with the most reactive sugar ribose, followed by xylose. Hill *et al* (1992) showed that the minimum gelling concentration for a range of plant proteins could be reduced substantially by heating them with xylose. For bovine serum albumin heated with 3 % xylose, the minimum protein concentration required was between 2 - 2.5 %.

The effect of the decrease in pH may have contributed substantially to this decrease, since it is known that the value of C_0 is very dependent on pH of the solvent. However, subsequent changes in charge and crosslink are also expected to contribute to this observation. These aspects will be studied in more detail in chapter 7-9.

As the maximum value of storage modulus could not be obtained, the rate of gel development was studied by investigating the dependence of rate of change of storage modulus on bovine serum albumin concentration. The log-log plots of dG'/dt against concentration are plotted in Fig. 6.10.

Table 6.1 Slopes of the log-log plots of bovine serum albumin concentration and G' (145 min) and corresponding minimum concentration for gelation.

Gelling systems	slope and concentration range	minimum concentration of protein (%)
Bovine serum albumin	13 (7.5 - 12.0 %)	7.0
+ 3 % xylose	3.2 (1.5 - 8.0 %)	1.2
+ 3 % ribose	2.9 (1.0 - 8.0 %)	0.5

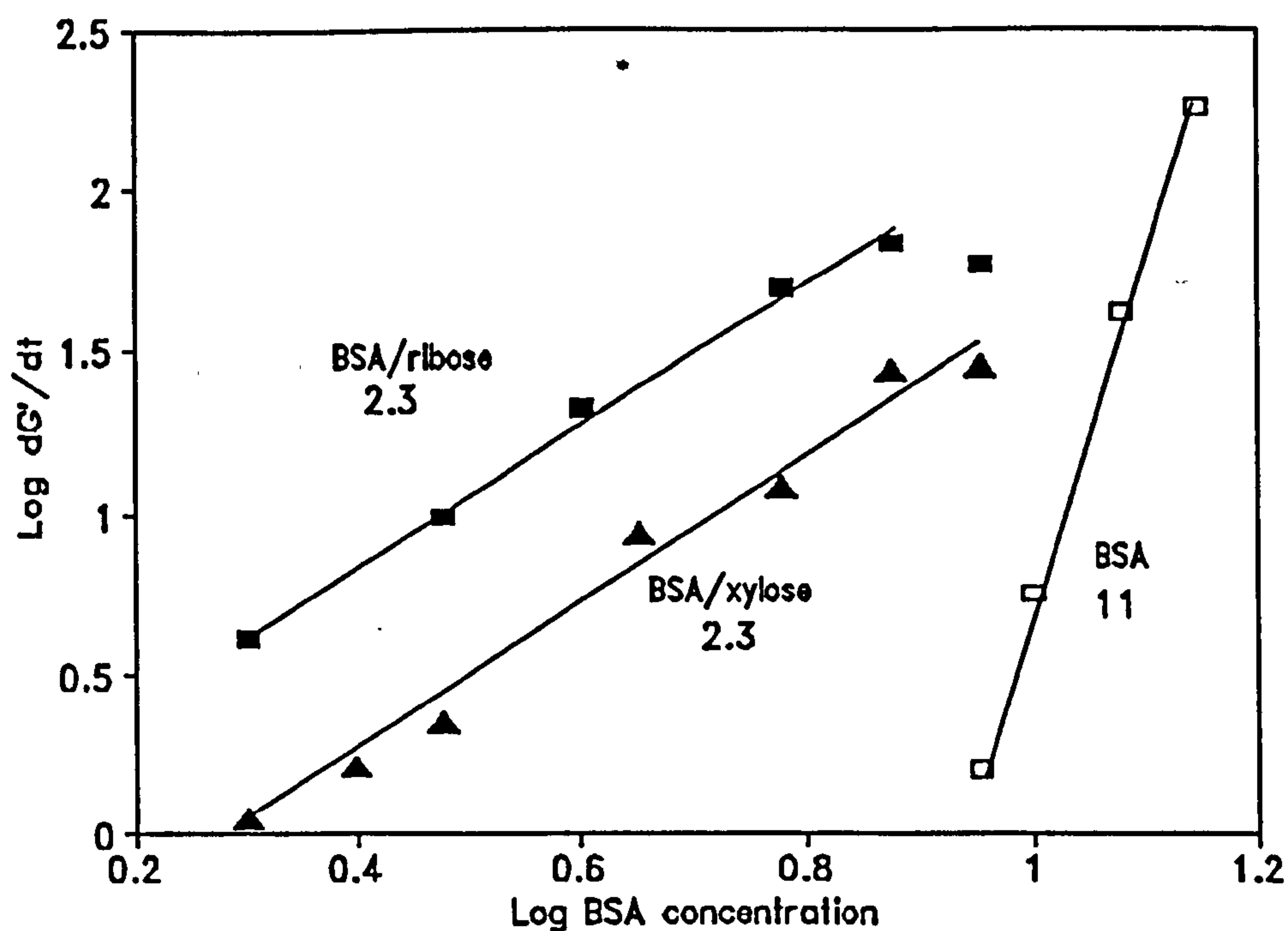


Fig. 6.10 Log-log plot of dG'/dt against bovine serum albumin concentration for bovine serum albumin heated alone (\square), with 3 % xylose (\blacktriangle) and with 3 % ribose (\blacksquare)

The rate of structure development increased with concentration. The more reactive sugar ribose showed a higher rate of dG'/dt at a given time. However, the slopes obtained for both bovine serum albumin/ribose and bovine serum albumin/xylose were essentially the same (i.e. about 2.3). For bovine serum albumin alone a high dependence of dG'/dt on concentration is marked, with a slope of 11 being reached over the protein concentration range of 7.5-14 %. The log value dG'/dt at 7.5 % bovine serum albumin was too small to be included in the log-log plot of dG'/dt versus concentration. The value of the slope for the protein in the absence of sugar is lower than that obtained by Ross-Murphy (1981); a slope of 22 at low concentration and 6 at high concentration range. Different experimental conditions could be the major attribute for these differences.

In reversible gel forming systems low power dependance of the systems may imply a small number of chains in junction zones (Oakenfull and Scott, 1986). In bovine serum albumin/reducing sugar systems it may represent low crosslinking density. However interpretation of these complex systems should also be based on the change in the apparent critical concentration (C_0) of protein with pH. As protein concentration is increased, its buffering capacity is enhanced, and the decrease in pH will be smaller at higher protein levels. Also the number of sites on the protein that can be glycated will increase. This may change the rate at which the gel strength increases.

Incorporation of reducing sugars into bovine serum albumin had also affected its gelation rate (Fig. 6.11). As expected, increasing bovine serum albumin concentration had the effect of lowering the gelation time. This was reasonable because the amount of crosslinks was increased (e.g. hydrophobic interaction) as protein concentration increased. Gelation time was longer in the less reactive system and this would reflect kinetic effects between sugar reactivity on gelation rate.

When bovine serum albumin concentrations were in excess of 7.5 % there was a marked change in the gelation times and discontinuity in the relationship between gel time and protein concentration. The reason for this may be attributed to the ability of the protein to gel at these concentrations and pHs without the addition of reducing sugar. It would appear that the normal gelation mechanism is faster than that of the Maillard system. It is probable that once the normal gels are formed the mechanism involved for the Maillard system might modify the gel (seen by the increase in G'), but not radically alter it.

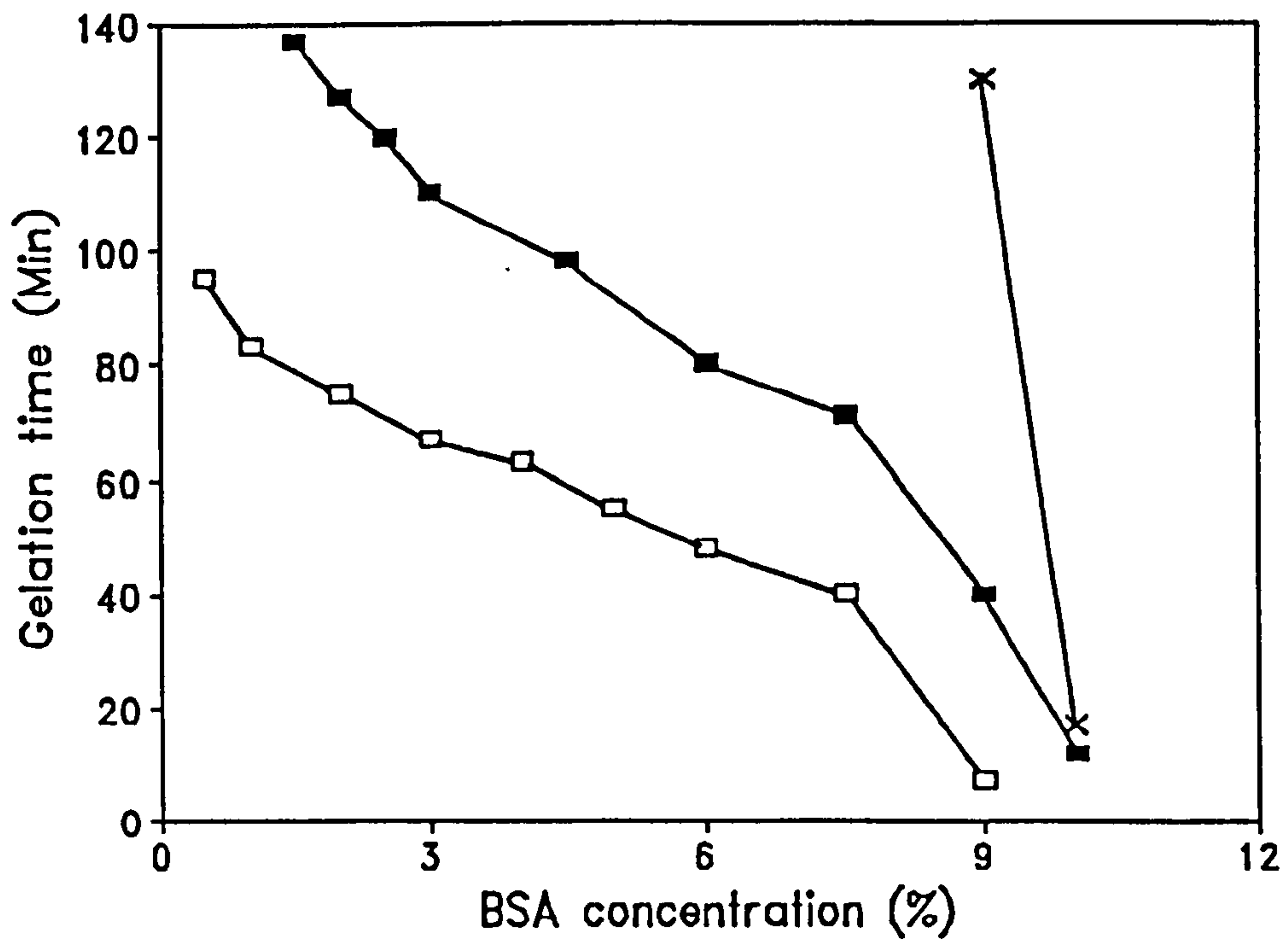


Fig. 6.11 Gelation time as a function of bovine serum albumin for bovine serum albumin heated alone (X), with 3 % xylose (■) and with 3 % ribose (□)

Gelation time, bovine serum albumin concentration and C_o can be related by an equation proposed by Ross-Murphy (1991);

$$gelation\ time = \frac{k}{([C_p/C_o]^{n-1})^p} \quad (6.1)$$

where k is a proportionality constant, C_o is critical bovine serum albumin concentration, n is the number of cross-linking loci which form a junction zone, C_p is the bovine serum albumin concentration and $p > 0$ is the critical exponent. This equation described fairly well the gelation kinetics of bovine serum albumin, even

though the concept of junction zones has to be changed to protein-protein interaction sites.

The results for the bovine serum albumin/reducing sugar systems are in agreement with this equation since an increase in polymer concentration shortens the gelation time (Fig 6.11). This is in contrast to the gelation of soybean 11S protein by glucono- δ -lactone (GDL) which showed an opposite relationship between gelation time and protein concentration (Kohyama and Nishinari, 1992 and Kohyama *et al* 1992). In the GDL/soya system, gelation processes were due mostly to acidic coagulation. Gluconic acid produced by the hydrolysis of glucono- δ -lactone generates protons to lower the pH to initiate coagulation of soybean 11S protein. Therefore, increasing protein concentration inhibits the tendency of the system to go to a lower pH and hence slows down the gelation processes.

In protein/reducing sugar system an increase in protein would have increased the concentration of free amino groups and this could have been expected to increase the amount of Maillard by products. This would decrease pH, yet increased protein would help buffer against this. When reducing sugar level was constant the pH at the gel point increased with bovine serum albumin concentration (Table 6.2), and the gelation time became shorter. The total number of lysine groups would be greater with increasing protein levels and therefore the number requiring glycation to cause a change on the protein would increase. It is possible therefore that the net charge on the protein when present at high concentrations may be lower than if the protein concentration is low. The higher protein density would enhance interactions between the protein molecules, either by hydrophobic interactions or through additional covalent linkages. It would seem that it is the greater proximity of the macromolecules that relates to the lowering in gelation time.

The increase in the pH at the gel point was attributed partly by the buffering mechanism of the protein as concentration increased. This may suggest that acidic coagulation due to protein molecules approaching their isoelectric point may not be the sole cause for the enhanced gelation at high protein concentration.

Table 6.2 pH at the gel point of bovine serum albumin/3 % xylose gelling systems heated at 90 °C.

Bovine serum albumin (%)	pH at gel point
1.25	5.60
2.0	5.70
3.0	5.82
4.5	5.95
6.0	6.05
7.5	6.48

6.3 Activation energy for gelation

Having studied the importance of protein and sugar concentration on the formation of gels, the other factor relevant to gelation time is temperature. Knowing the gelation time over a range of temperatures will allow activation energies for the process to be calculated.

1 % glyceraldehyde/3 % bovine serum albumin, and 10 % bovine serum albumin gelling systems were chosen to do this experiment because of their reactivity and ability to gel at this temperature range. At low temperatures, it took a long time for gelation to occur in the glyceraldehyde system, therefore the lowest temperature used was 75 °C and for practical reasons the highest temperature used was 92.5 °C. The plot of the gelation time against temperature is shown in Fig. 6.12.

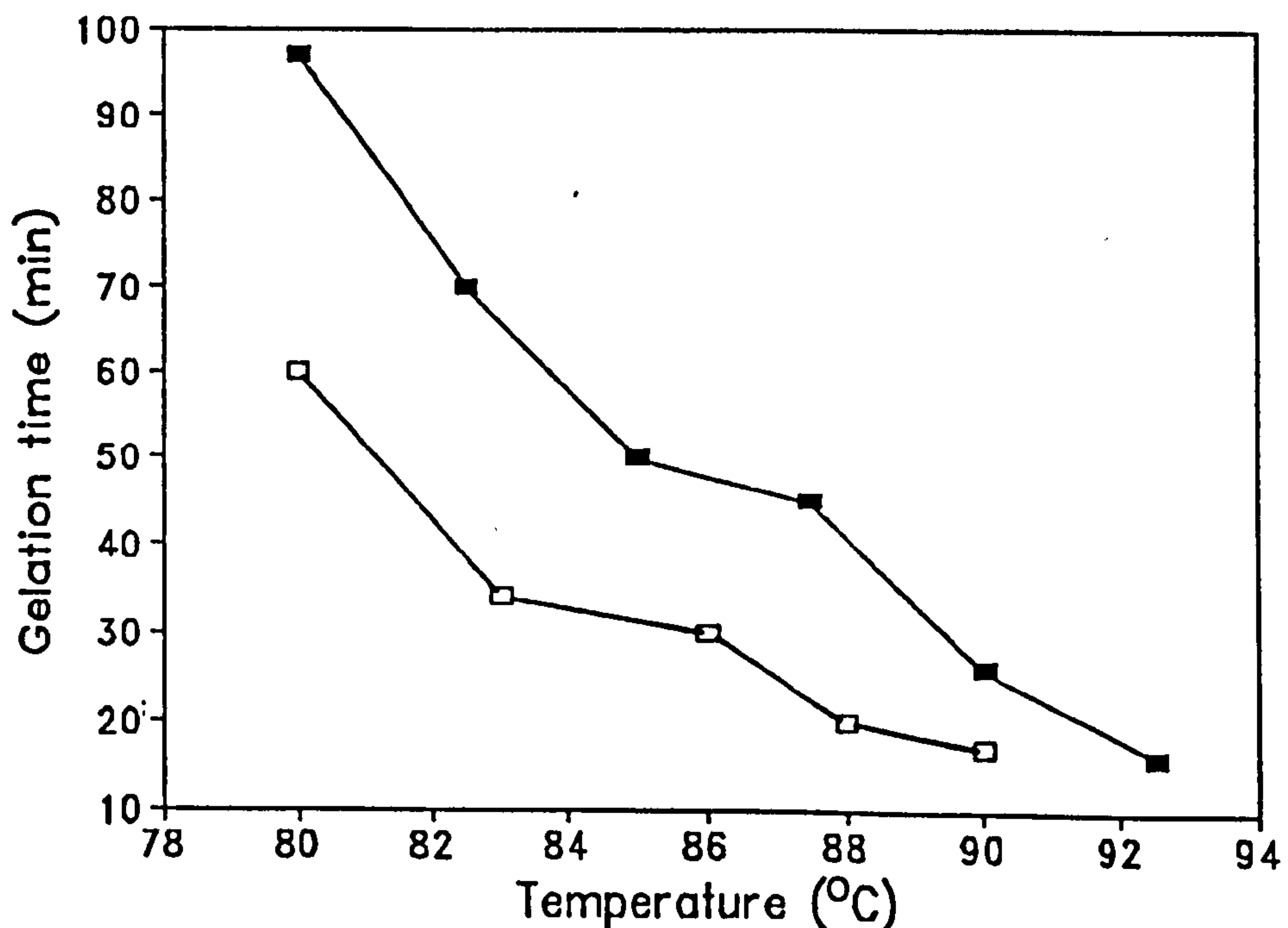


Fig. 6.12 The plots of gelation time as a function of temperature for 1 % glyceraldehyde/3 % bovine serum albumin (■) and 10 % bovine serum albumin (□)

As expected for most of the globular protein gelling systems, the effect of increasing temperature was a decrease in gelation time. The gelation times at various temperatures were logarithmically plotted against the reciprocal of the absolute temperature (Fig. 6.13). The gelation times lie on a straight line except for one measured at 75 °C. This was thought to be attributed to the slow gelation rate at 75 °C thereby making it difficult to measure the rheological properties. The data point at 75 °C was therefore excluded. The activation energy for the gelation can be estimated from the slope of this Arrhenius plot (Richardson and Ross-Murphy, 1981b).

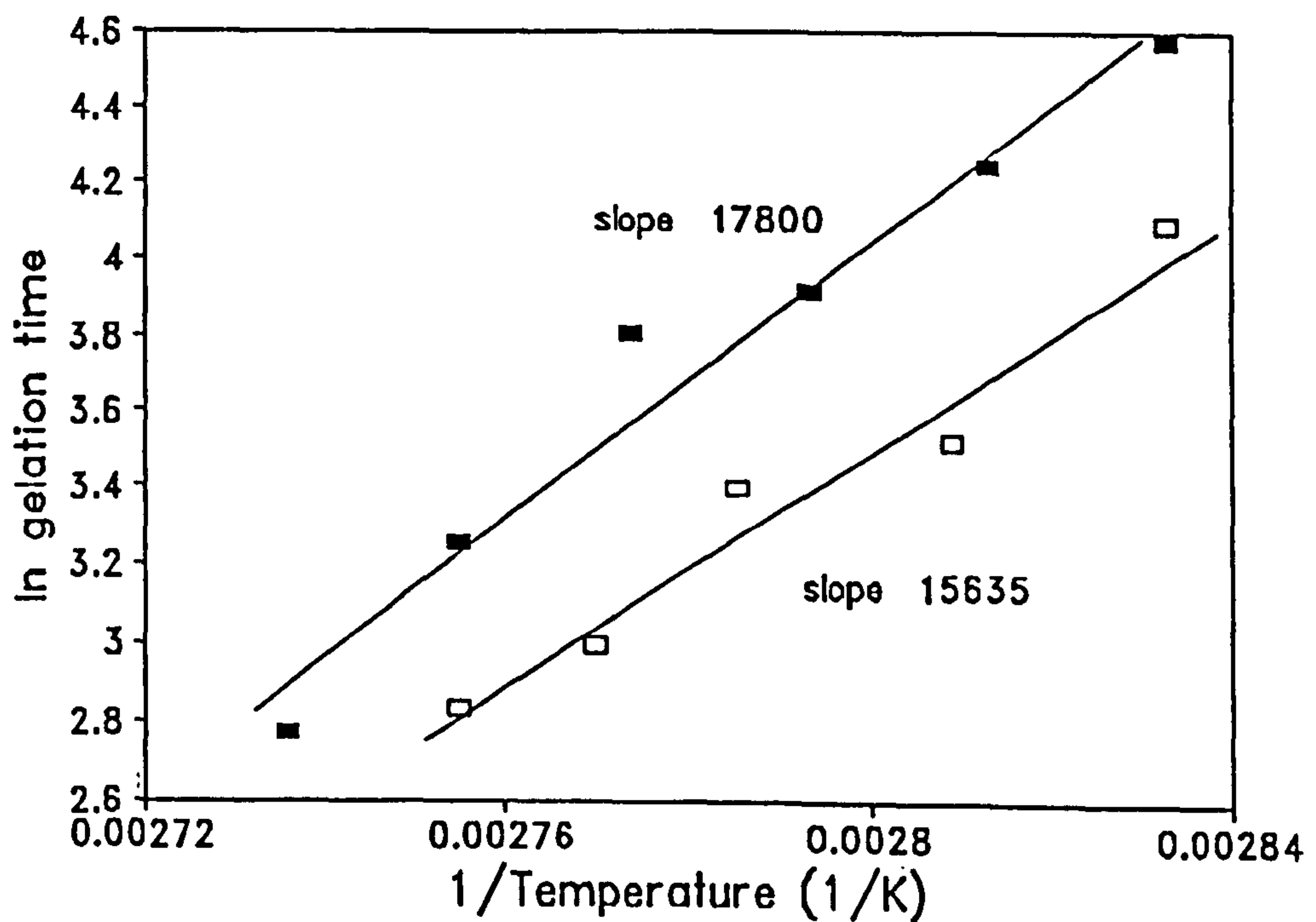


Fig. 6.13 The Arrhenius plots for 1 % glyceraldehyde/ 3 % bovine serum albumin (■) and 10 % bovine serum albumin (□)

The slope obtained for the Maillard system was 17.8×10^3 and the activation energy was calculated as 148 kJ/mol. The activation energy for the gelation of 10 % bovine serum albumin was calculated as 130 kJ/mol. Therefore, the gelation of 3 % bovine serum albumin via the Maillard reaction using 1 % glyceraldehyde was slightly more temperature dependent than that of 10 % bovine serum albumin heated alone. However, the values of the activation energy for both system were much higher than that reported for 11S soya protein gelation by acidification using glucono- δ -lactone; 15 kJ/mol (Kohyama *et al* 1992). The activation energy for gelation of soya via acidification was small because gelation was not greatly dependent on temperature. The Maillard and bovine serum albumin gelation processes required higher temperatures. It may be suggested that for the Maillard systems a high temperature was necessary for the chemical interactions to occur, which led to gelation. The activation energy for Maillard browning of bovine serum albumin with glucose was estimated to be around 120 kJ/mol i.e. 30.3 kcal/mol (Mohammad *et al* 1949). These high activation energies are often associated with covalent linkage formation rather than cooperative noncovalent association.

If it is assumed that one slope of the Arrhenius plot represents one process (Richardson and Ross-Murphy, 1981b), the Maillard gelation should therefore consist of a single process between 80 and 92.5°C. In this temperature range, higher temperatures increased gelation rate but would not effect the gelation mechanism.

From the values of activation energy obtained for the Maillard gelation, it is possible to derive an Arrhenius equation relating the gelation time, activation energy and temperature of heating;

$$gel\ time\ (min) = \frac{1}{(7.4 \times 10^{19}) e^{-(14.8 \times 10^4)/RT}} \quad (6.2)$$

where R is gas constant, T is the temperature in Kelvin and 7.4×10^{19} is the pre exponential factor and 14.8×10^4 is the activation energy for gelation (J/mol). The gelation time-temperature data can also be used to calculate the accelerating factor Q_{10} , given by the following equation;

$$Q_{10} = \frac{\text{Rate at } T + 10}{\text{Rate at } T} \quad (6.3)$$

where T is the temperature in $^{\circ}\text{C}$, and the rate can either be taken as gelation time or the reciprocal of gelation time. The calculated Q_{10} values were 4.7 and 4.5 for Maillard and bovine serum albumin gelling systems respectively. This means that the gelation rate increased 4.7 and 4.5 times for each 10°C rise in temperature for the Maillard and bovine serum albumin gelling systems respectively.

It would be useful if these estimations could be obtained for the gelation of typical food proteins in the presence of common reducing sugars such as glucose or lactose. In Chapter 5 it has been indicated that the study of gelation kinetics using less reactive sugars are possible at higher temperatures using high pressure cells. The information on the effect of carbonyl compounds on the activation energy for gelation may also be useful. By understanding the time/temperature relationship for gel formation, useful predictions on food quality might be achievable. For example, it might be possible to predict the gelation time of processed milk which is then stored.

Conclusion

The Maillard reaction can change the gelation kinetics of bovine serum albumin at concentrations where the protein can gel by itself. After a prolonged heating in the presence of reducing sugar, it is proposed that crosslinking resulting from the Maillard reaction may add interactions and impart higher G' values to the gels. Even though an enhanced protein-protein interaction due to the decrease in pH may have contributed to the enhanced gelling behaviours of the Maillard systems, other factors such as charge and covalent bondings may also be important. The kinetics of bovine serum albumin gelation in the presence of reducing sugar is not similar to that of soya 11S protein in the presence of glucono- δ -lactone via acidification processes.

ALTERATION OF CHARGE DUE TO THE MAILLARD REACTION

The protein gels formed at a pH close to the isoelectric point are normally less homogeneous than those formed at a pH far from the isoelectric point (Van Kleef, 1986).

The homogeneous texture of the Maillard gels and their low syneresis levels are indicative of a gel with a large number of charged groups. The pH values of the Maillard gels are close to the isoelectric point of bovine serum albumin and therefore low net charge might be expected. However, it is postulated that the chemical interaction of the reducing sugar with bovine serum albumin alters the net charge on the protein. Therefore the aim of the work reported in this chapter was to measure the charge occurring at a range of pH values for bovine serum albumin after heating in the presence and absence of reducing sugars.

Before the details of the experimental work are given, the reasons for the development of this hypothesis is given, using information from the literature and values from the Maillard gels produced for this work.

7.1 *Reported relationship between modification of charge and gel quality*

The alteration of net charge on amino acid side chains usually affect profound changes in the properties of the proteins. The most obvious change is in the isoelectric point of the protein, but the changes in charge can also affect the conformation of the proteins and thus its overall functional activity (Feeney, 1977).

One way of altering the charge on the protein molecules is by the technique of succinylation. The proteins modified using these techniques were shown to have inferior gelling properties either when gelled alone or when mixed with other proteins (Murphy and Howell, 1991). It was concluded that the availability of amino groups is an important contribution to the mechanism of gelling, since the blocking of such groups retarded gelation. Ma and Holme (1982) demonstrated that the isoelectric point of egg albumen was reduced from 4.65, to lower values with increasing level of succinylation, indicating an increase in net negative charge. As a result of this, the coagulation of protein was retarded. At a high level of modification, a clear solution was formed. Nakamura *et al* (1978) indicated that formation of coagulum can be retarded by either increasing the net negative charge or positive charge on the molecules.

7.2 *Maillard reaction and alteration of charge*

The Maillard reaction has also been studied as a way of altering the charge properties of proteins. With respect to this, the change in the physical properties of ovalbumin due to the Maillard reaction has been reported by Kato *et al* (1981). Ovalbumin-glucose complexes which were formed in the early stage of the Maillard reaction were shown to possess a marked resistance towards destruction of conformation and coagulation. The glycation of the amino acid side chains was thought to be the major factor. Watanabe *et al* (1980) reported that, during a prolonged storage of ovalbumin-glucose complex, the changes in charge balance were the major factor which promoted the unfolding of ovalbumin.

A schematic diagram of the alteration of net charge via the Maillard reaction is depicted in Fig. 7.1. As a result of this reaction, the glycated protein will have fewer free amino groups, and hence the number of positive charge group. At pH 7.0 bovine serum albumin is reported to have a net charge of -18 e.u. (Peter, 1985), loss of the positive charge will increase this number.

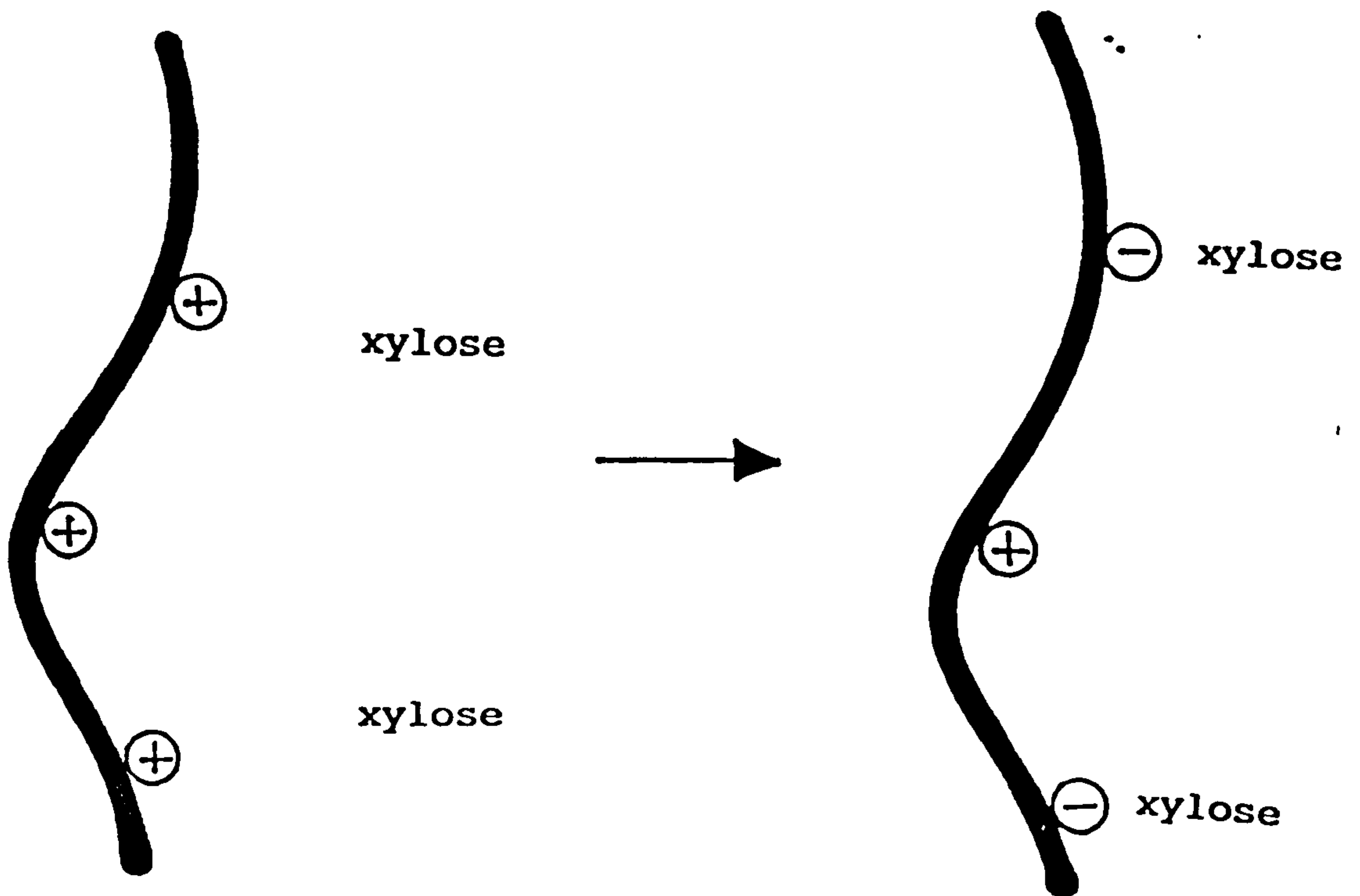


Fig. 7.1 Alteration in net charge on protein molecule via the Maillard reaction

7.3 Syneresis values of the Maillard gels

The amount of water expelled from gel structures can provide some indication of the extent of water interaction with the matrix. If the protein-protein interactions are too strong, the protein network will draw together and water will be expelled from the "pores" as they become smaller (Mangino, 1992). The levels of syneresis after heating solutions of protein with reducing sugars, Maillard reaction products and glucono- δ -lactone are displayed in Table 7.1.

Table 7.1 The percentage of syneresis of 3 % bovine serum albumin solutions after heating (121 °C for 60 min) with different compounds.

samples	% syneresis	Final pH	Breakforce (g)
0.25 % glyoxal	5.2	3.2	450
3 % ribose	13.1	3.9	334
3 % xylose	17.8	4.3	288
0.1 % glucono- δ -lactone	28.0	5.0	ND

ND, not determined

The results in Table 7.1 indicated that the gels formed by heating the protein with glyoxal had the lowest level of syneresis but the highest value for breakforce, followed by those formed with ribose and xylose. A Low level of syneresis in the more reactive systems is indicative of a higher water holding ability. The pH of the samples span the normally reported pI of bovine serum albumin (pH 5.0) (Peter, 1985), yet the high water holding capacity of the samples would seem to indicate that the proteins are carrying a charge. From this it is possible to infer that the pI of the protein had been shifted. The more reactive carbonyl compounds would seem to produce systems with the highest charge.

It is interesting to note that glyoxal was only used at 0.25 %, as compared to 3 % concentration for the sugars, yet still caused the largest change in pH and apparently the charge. The results also show that the glycation of the amino acid side chains could also take place via the carbonyl products of the Maillard reaction .

In many cases the decrease in pH was more dramatic in the samples containing a more reactive carbonyl compound (see also Chapter 6). Efforts to mimick a similar

decrease in pH of the Maillard systems at this conditions using glucono- δ -lactone were less than successful due to the buffering effect of the protein. However, Armstrong (1994) showed that 0.1 % glucono- δ -lactone can produce a decrease in pH equivalent to 2 % xylose when heated with 3 % bovine serum albumin solutions at 121 °C for 30 min. She also indicated that at a similar final pH, the level of syneresis was lower in the samples containing xylose. Similar measurements of syneresis have also been performed on soya protein isolate gels (Cabodevila *et al* 1994). For the glucono- δ -lactone gels, the syneresis percentage was high and decreased with increasing soya concentration, however for the Maillard gels, the level of syneresis was low and not dependent on soya concentration.

Typically a heat set protein gel formed near the isoelectric pH (e.g. for bovine serum albumin, pH 5.0) tends to exude water more readily than a gel formed at a pH far from the isoelectric point (e.g. pH 10.0). Thus, the water holding ability of soya protein isolate gels decreased as the isoelectric pH was approached (Van Kleef, 1986). In contrast to the gels containing carbonyl compounds, the level of syneresis decreased despite a decrease in pH upon heating. The glycation of the amino acid side chains and hence increased net charge on the molecules has been suggested to be a major attribute to the low syneresis gels. However under these heating conditions, additional covalent crosslinks resulting from the Maillard reaction may also be important. These crosslinks enable the network to entrain more water, presumably via physical means. The different values of breaking force and solubility in the solvents that disrupt conventional bonds provided some evidence of the presence of such crosslinks (Hill *et al* 1992; Cabodevila *et al* 1994). Investigations of the crosslinks in the gels are described in Chapter 9.

7.4 *Estimation of charge on the systems of interest*

Syneresis levels would indicate a change in charge on the proteins. To confirm these findings the actual charge was estimated on the protein. The gels used in this study were all prepared by autoclaving at 121 °C for 30 min. The temperature of 121 °C was chosen as being typical of that used in sterilisation processing, and the time was extended to 30 min to exaggerate possible changes for ease of analysis.

The charge associated with the gel particles or solutions was estimated using the Muetek Titrator system as described in section 4.3.6. The instrument consisted of a piston which oscillated to create a strong liquid flow in the cell. This resulted in the separation of counter-ions from the sample. The moving counter-ions induced a potential. After rectification and amplification, the result was shown as a streaming potential on the display. The amount of charge in the sample was estimated by performing a titration using an oppositely charged polyelectrolyte. The sample charge was neutralised by the ions in the polyelectrolyte. The endpoint of the titration was identified when the streaming potential reached 0 mV.

The plots of charge density against pH of the three gelling systems are shown in Fig. 7.2. It can be seen that the charge measured on gel particles or solutions varied with pH. The net charge on the protein changed from positive to negative as the pH increased from 3.5 to 6.5. The data for bovine serum albumin was obtained using two different concentrations of protein; 3 and 10 %. However the estimated isoelectric point obtained was very similar i.e. pH 4.8 - 4.9. At 3 %, no gelation occurred but at 10 % concentration a white opaque gel was formed. The isoelectric point of bovine serum albumin obtained from this study (4.9) compares favourably with that reported in the literature (Peter, 1985; Armstrong, 1994). The estimated isoelectric point was shifted from 4.9 to 4.4 for bovine serum albumin heated with xylose and to 4.1 for bovine serum albumin heated with ribose. This shift of the isoelectric point to a more acidic pH with the increasing reactivity of reducing sugar suggests a decreased net positive charge on the modified bovine serum albumin. Loss of the positively charged side groups from lysine or arginine could account for this change. A similar

suggestion has been put forward by Kato *et al* (1986). The ϵ -amino group interaction with the sugar, via the Maillard reaction, would account for loss of the positive charge and creation of a neutral or negative group (Fig. 7.1).

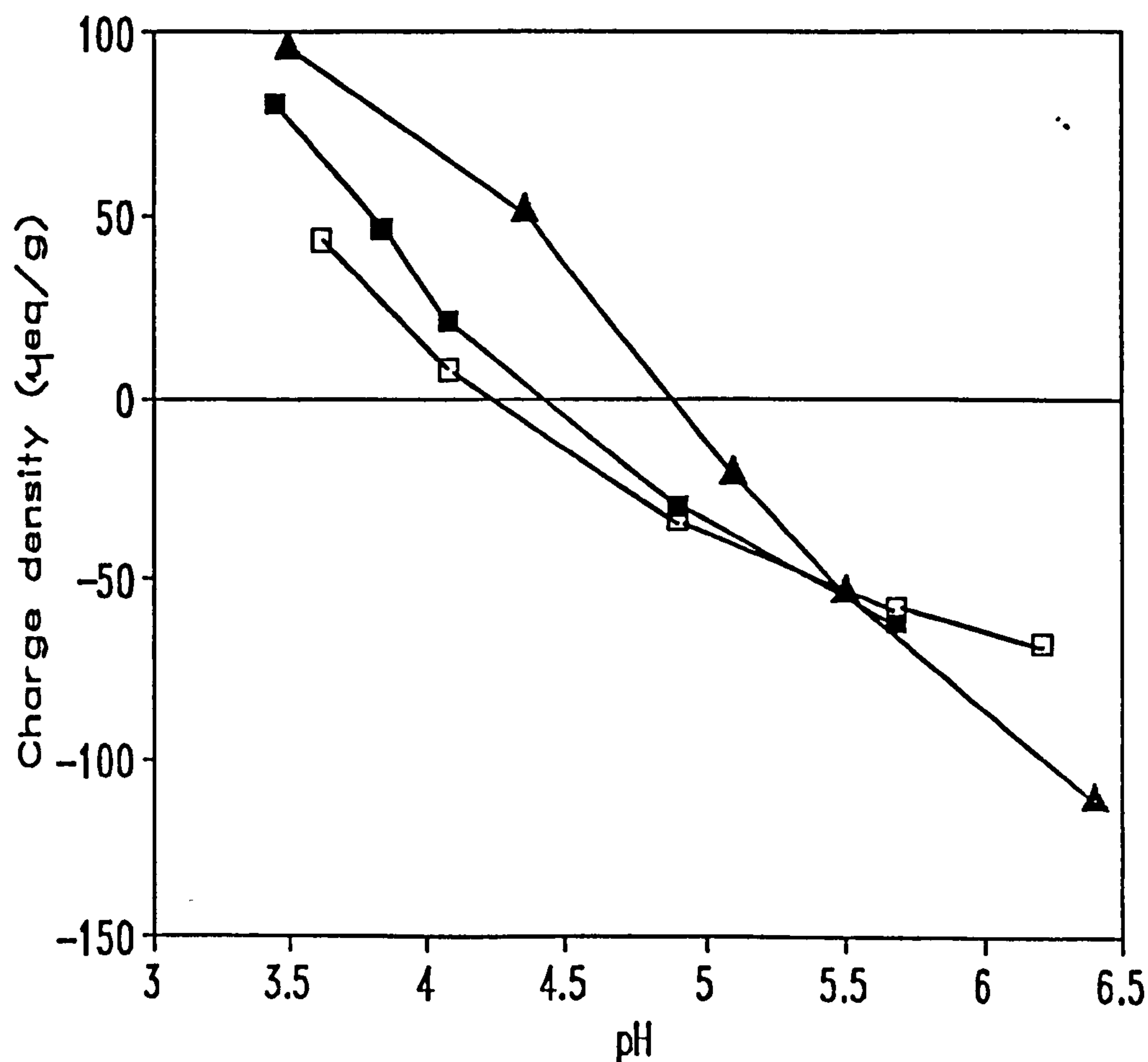


Fig. 7.2 The plots of charge density as a function of pH for the gels or solutions formed using bovine serum albumin alone (▲), with 3 % ribose (□) and with 3 % xylose (■).

At pH 4.5 the equivalent charge per mole of bovine serum albumin heated alone was + 2.3, but it decreased to - 1.0 if the bovine serum albumin is heated with xylose, or - 1.5 if the reducing sugar is ribose. If the change in charge was due solely to altering the charge on the lysine from positive to neutral, about 6 % and 6.5 % of all lysine

would be altered in the protein/xylose and protein/ribose systems respectively. If however the lysine charge changed from positive to negative, the number of lysine altered in both systems could be reduced to only around 3 %. Other amino acids with positively charge side chains at pH 4.5 could have also been changed by the Maillard reaction. It cannot be ruled out that acid groups might also be reactive hence causing a comparative increase in charge.

7.5 Estimation of lysine residues

More evidence for alteration of the amino acid side chains is provided by the decrease in total assayable lysine after the Maillard reaction, using an amino acid analysis (Table 7.2). Even though the samples were heated less severely (90 °C, for 75 min), the decrease in lysine can be taken as an evidence for glycation of the protein. Similar findings have also been shown by Armstrong (1994).

Table 7.2 Total available lysine residues in the solutions of 3 % bovine serum albumin either heated alone or with 3 % xylose at 95 °C for 75 min (estimated by an amino acid analysis).

sample	number of lysine residues /protein molecule*
protein alone	49
protein + 3 % xylose	41

* Average of duplicate samples

In the bovine serum albumin solution which had been heated alone the number of lysine residues was measured as 49 per molecule of bovine serum albumin. This compares with the known value for bovine serum albumin of 59. The variation might be due to poor experimental methods or that heat treatment alone caused the loss of

lysine available to the assay procedure. This might be chemical loss, i.e. reaction of the side chains with sugar closely associated with impure protein.

Heat treatment of the bovine serum albumin with xylose does reduce the number of lysine residues to 41 per molecule. This is 16 % reduction over the bovine serum albumin samples or a 31 % reduction over true number of lysine in bovine serum albumin. Yaylayan *et al* (1992) reported glycation of about 30 % lysine (18 mole of glucose per mole of bovine serum albumin) when incubating solutions of bovine serum albumin (0.1 mg/ml) with D-glucose (0.22 M) in 0.1 M phosphate buffer (pH 7.2) at 37 °C for 35 days.

The estimation of the change in charge of lysine as calculated in section 7.4 indicated that only 6 % of the lysine might be changed even though the heating conditions were more severe. It has also been noted that the change in charge could occur in several ways.

conclusion

Alteration in net charge presumably due to glycation of amino acid side chains resulted in a decrease in the isoelectric point of the protein. The increase in net charge on the molecules can affect the quality of the gels. Some work in the literature also indicated that the effect of the enhanced net charge was to retard formation of coagulum and gels.

The relative timing of the chemical interaction i.e. glycation and the corresponding change in charge, and crosslinking, may well be important. The chemical modification may occur much earlier than gel formation. This can affect the association and aggregation behaviours of the systems, which may be important for gel formation. How the carbonyl compounds modify protein-protein interactions before gelation is investigated in the following chapter.

CHAPTER 8

MAILLARD INDUCED COMPLEXES OF BOVINE SERUM ALBUMIN - A DILUTE SOLUTION STUDY

Within this thesis it has been shown that the Maillard reaction can cause a substantial decrease in the pH of solutions containing protein and carbonyl compound (Chapter 6). However, the reaction can also influence the charge on the molecules via the process of glycation (Chapter 7). These two factors can influence the quality of a protein gels formed by using the Maillard reaction. The changes in pH and charge are particularly important during the early stage of gelation when the molecules denature and associate.

The objective of this chapter was to investigate the conformation of Maillard associates and to detect the earlier stages of the linking processes between protein molecules. To do this the bovine serum albumin/xylose mixtures were heated under less severe conditions than those known to produce a macromolecular network that spanned the system, i.e. conditions were used so that the protein mixture was still a solution rather a gel. The techniques employed in this section include sedimentation velocity, dynamic light scattering and intrinsic viscosity.

8.1 Decrease in pH

As shown in the earlier chapters the pH of the system was important for the prediction of gelation quality. Therefore the pH values for the systems used for this chapter are shown in Fig. 8.1. Heating 3 % bovine serum albumin at 95 °C changed the pH of the solution.

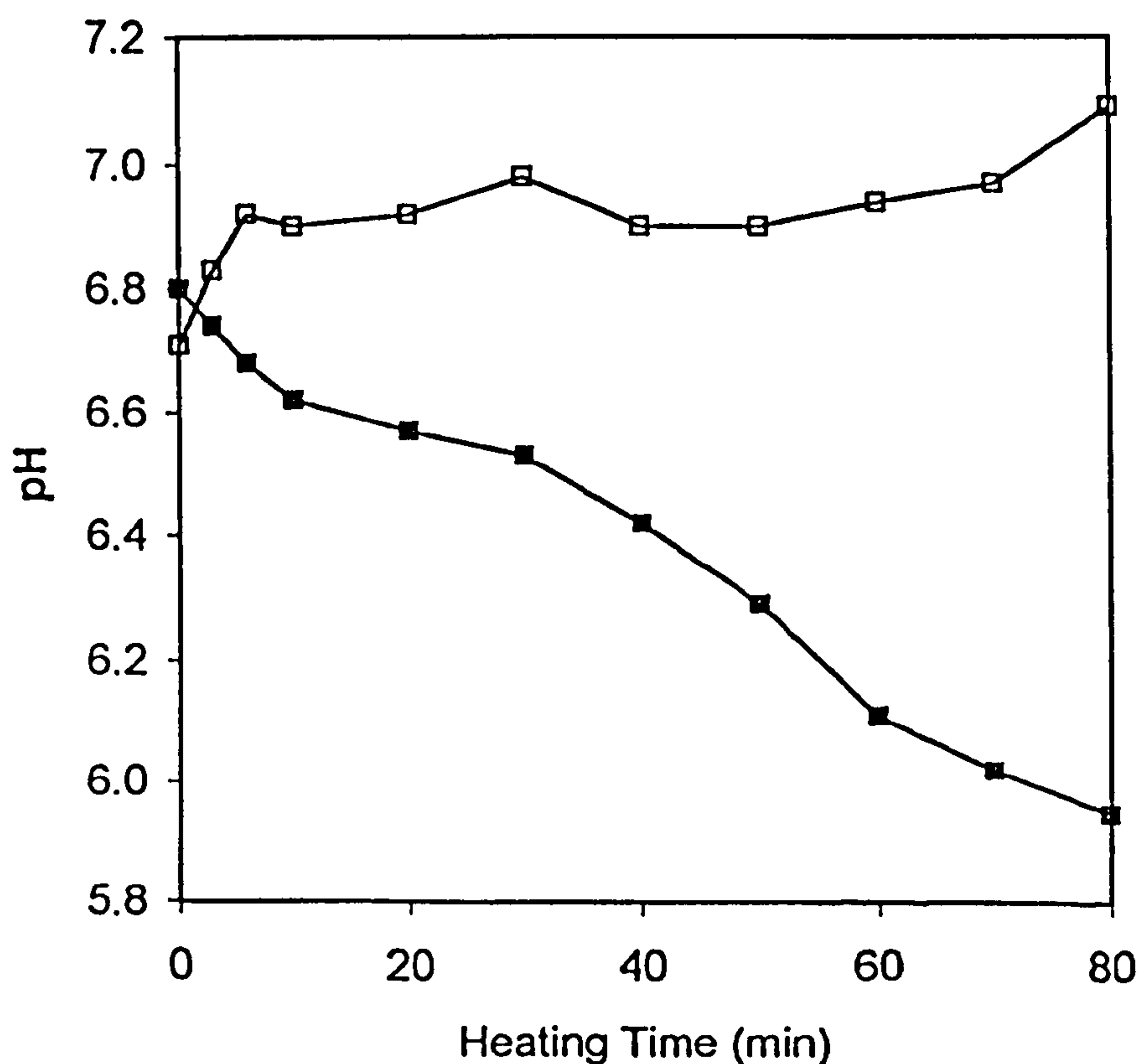


Fig. 8.1 Variation of the pH as a function of heating time at 95 °C for 3 % bovine serum albumin (□) and 3 % bovine serum albumin + 2 % xylose (■)

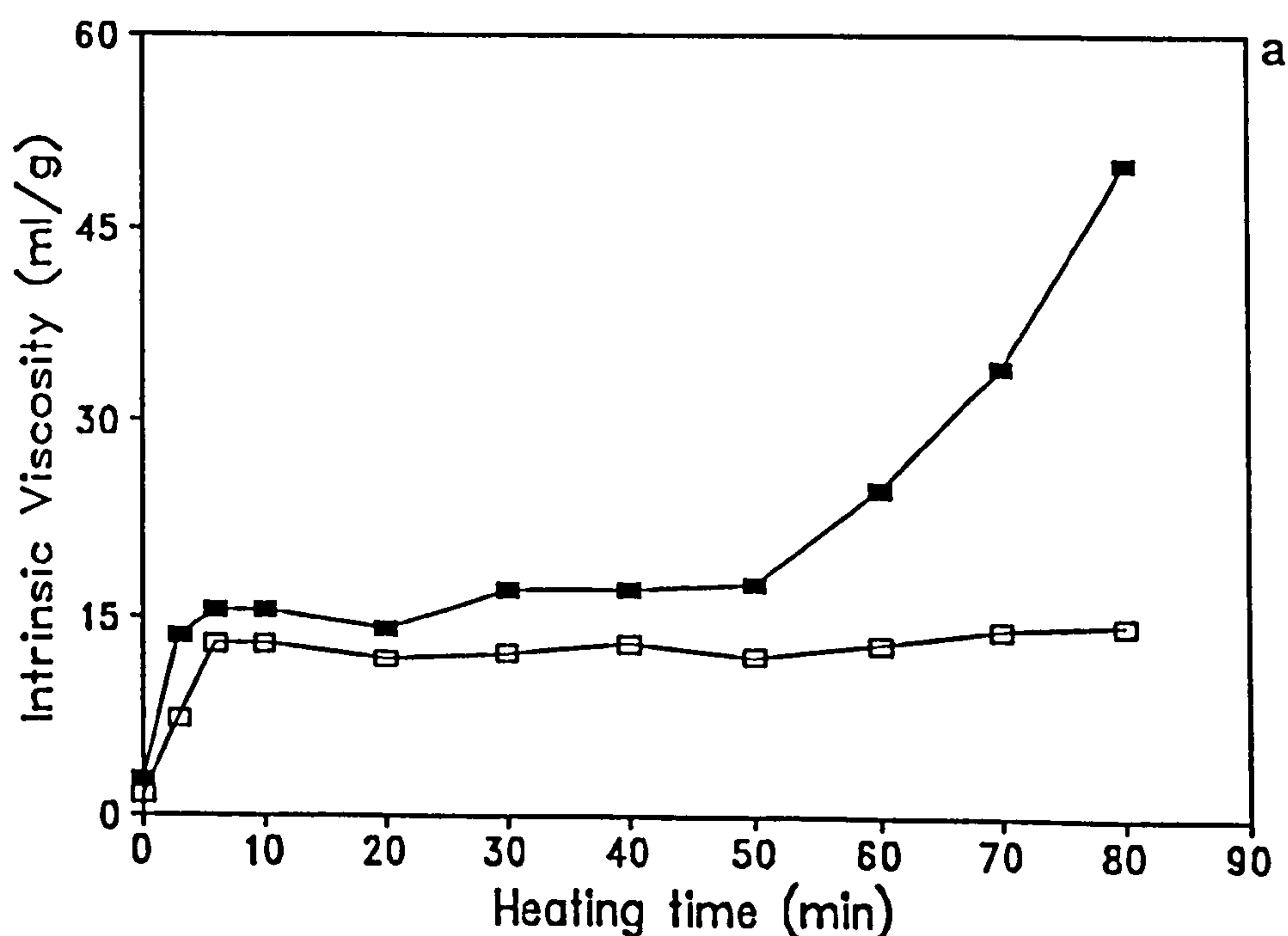
In the absence of the reducing sugar there was an increase in the pH within the first 10 min and then values remained constant at approximately pH 7.0 for the next 70 min of heating. However, in the presence of xylose a pH fall occurred and the pH changed from 6.8 to a pH of less than 6. The fall occurred at a gradual and continual rate and was still decreasing when after 90 min of heating, gelation of the protein solution began. This pH fall may be a consequence of the alteration of the protein e.g. removal of the basic ϵ -amino group of lysine and hence liberation of protons, and

because of the formation of acidic Maillard reaction products. When heating was carried out at 121 °C the fall was more severe with a pH of 5.0 being reached after 10 min (Hill *et al* 1992).

8.2 Changes in hydrodynamic parameters

Samples after heating were used to assess the shape and size of the molecular aggregates formed. Three methods were used: light scattering (to obtain diffusion coefficient), analytical ultracentrifugation (to obtain sedimentation coefficient) and capillary viscometry (to obtain intrinsic viscosity). Details of these assessments are given in Chapter 4, section 4.3.7.

The changes in the intrinsic viscosity, diffusion and sedimentation coefficients following heating of the protein are shown in Fig. 8.2.



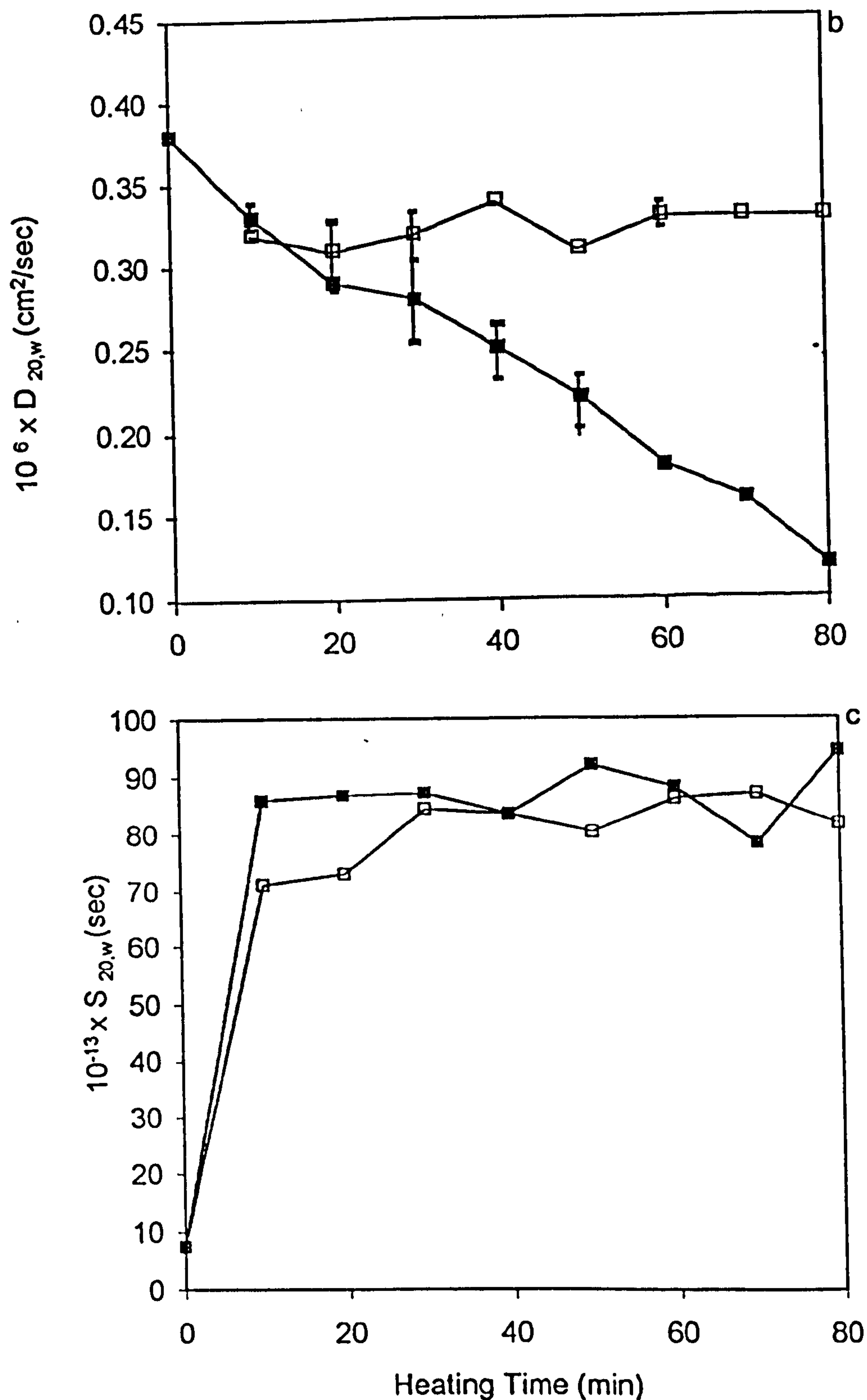


Fig. 8.2 Variation of (a) intrinsic viscosity (b) Diffusion coefficient and (c) Sedimentation coefficient for 3 % bovine serum albumin (□) and 3 % bovine serum albumin + 2 % xylose (■) as a function of heating time at 95 °C. Error bars indicate standard deviation of 3 determinations

For bovine serum albumin heated alone there was an initial rapid increase in the intrinsic viscosity and sedimentation coefficient and decrease in the diffusion coefficient during the first 10 min of heating. The values then remained approximately constant. This can be explained by the processes of denaturation and subsequent aggregation where the native protein molecules unfold on heating exposing hydrophobic regions which then promote association.

When bovine serum albumin was heated in the presence of xylose after 10 min the three parameters were essentially the same as observed for bovine serum albumin alone. However, as heating proceeds there was an increase in the values of intrinsic viscosity with heating time (Fig. 8.2a). The increase in intrinsic viscosity for the solution heated with xylose indicated an increase in the hydrodynamic volume of the protein. There was also a continuous and approximately linear decrease in the diffusion coefficient (Fig. 8.2b). Little difference was observed between the sedimentation coefficient values for the different systems (Fig. 8.2c).

The apparent molar masses obtained by combining the sedimentation and diffusion coefficients are shown in Fig. 8.3. For bovine serum albumin alone the molar mass changed from a value of 125 kg/mol (denoting a dimer of the native protein) to a value of 2000 kg/mol which indicates aggregates of approximately 30 bovine serum albumin molecules. In contrast the calculated molar mass of bovine serum albumin when heated in the presence of xylose continued to increase indefinitely throughout the heating period. From 20 to 45 minutes of heating the increase in molar mass was small, but at longer times the rate of increase became more rapid eventually resulting in gelation. This occurred when the pH decreased to 5.95.

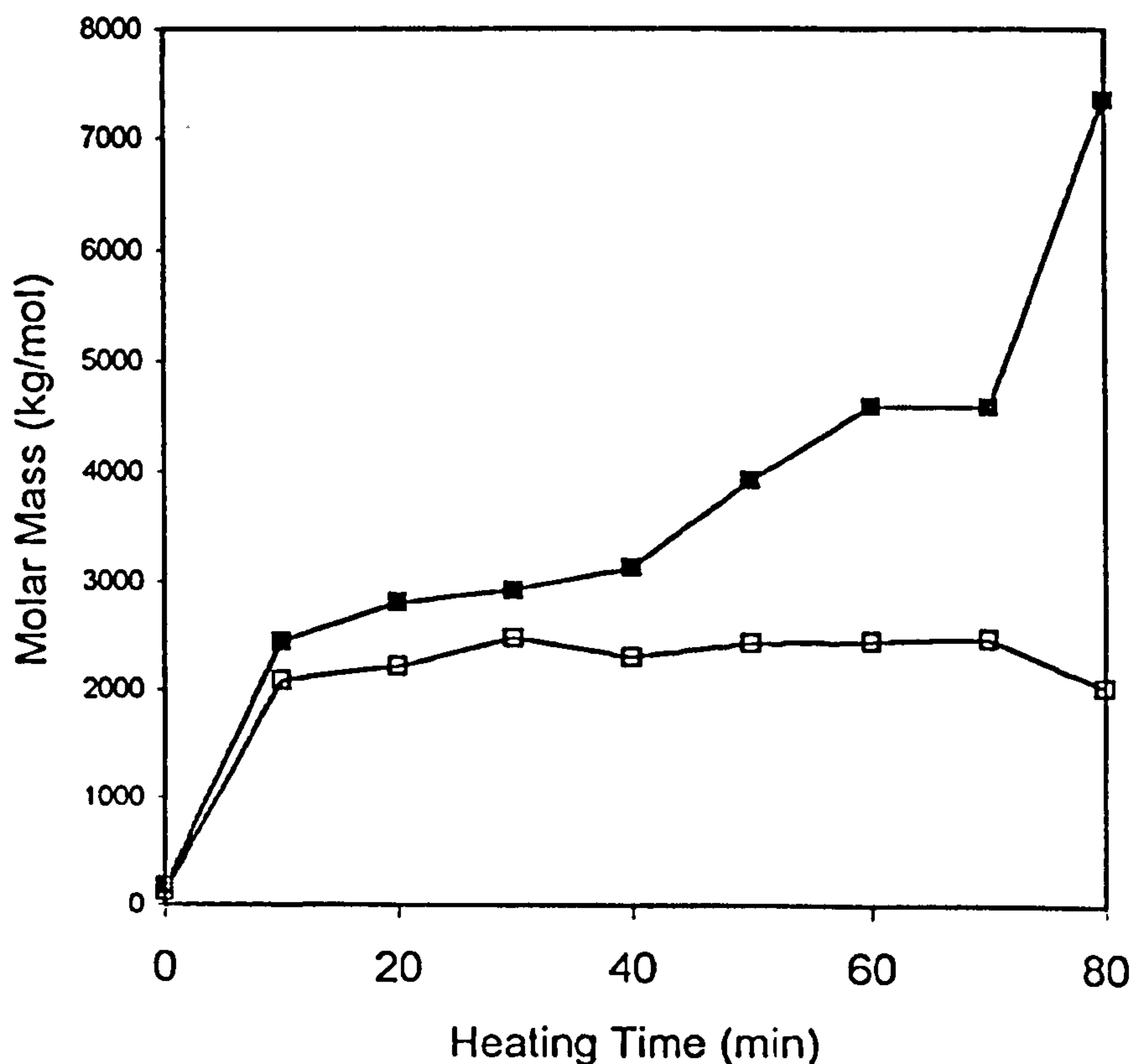


Fig. 8.3 Variation in Molar mass as calculated from sedimentation and diffusion coefficients of 3 % bovine serum albumin (\square) and 3 % bovine serum albumin + 2 % xylose (\blacksquare) as a function of heating time at 95 °C.

An obvious question was whether this increase in molar mass observed for the bovine serum albumin/xylose system was due to decrease in pH. To investigate this the pH of a bovine serum albumin solution was lowered to 6.3, using 0.1 M HCl and heated at 95 °C. Fig. 8.4 displays the pH and diffusion coefficient as a function of heating time.

It can be seen that under these conditions the pH returns rapidly to near neutral. However, the decrease in the diffusion coefficient on heating was much greater than found for the xylose system even after this had obtained a pH as low as 5.9. Despite the small diffusion coefficient no gelation was observed for the pH 6.3 system. The diffusion coefficient should normally reflect the size and the extent of solvent associated with a molecule or aggregate of molecules. The very small diffusion coefficients obtained for the heated bovine serum albumin originally at a low starting pH would indicate a highly aggregated system and/or a system which is highly expanded trapping a great deal of solvent.

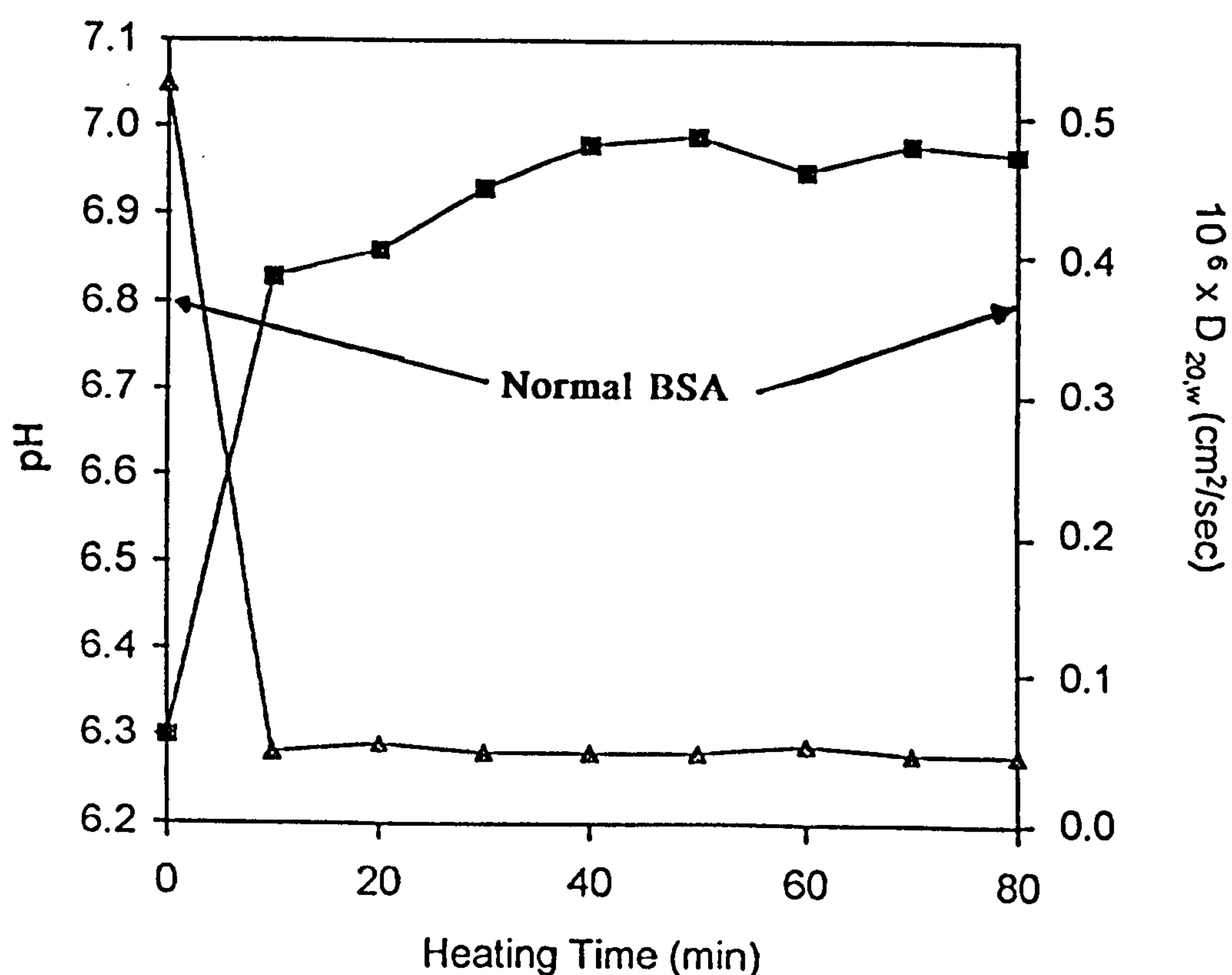


Fig. 8.4 Variation of Diffusion coefficient (▲) and pH (■) of low pH of bovine serum albumin with heating time at 95 °C. The final pH and diffusion coefficient for bovine serum albumin without altering the pH (normal) are noted on the figure.

So far in this study the linking of the protein in conditions of the changing degree of denaturation, pH of the environment and charge density could be due to variations in the electrostatic or hydrophobic interactions. However the possibility of the production of additional covalent linkages was not ignored. To obtain information about the bonds maintaining the association products the heated protein was diluted in 1% SDS (to disrupt any hydrogen bonding and electrostatic interactions) or 1% SDS + 1% β -ME (to disrupt non-covalent linkages and disulfide bonds) prior to the measurement of the diffusion coefficient (see Table 8.1). In the case of the xylose system the results obtained were almost identical to those found when the diffusion coefficient was measured in native buffer. In contrast the protein which had aggregated by heating initially at a pH 6.3 gave diffusion coefficients in SDS and SDS + β -ME which were much higher than those found in the buffer. These results suggest that the aggregates formed in the xylose system involve non-disulfide covalent linkages resulting from the Maillard reaction.

TABLE 8.1 - Diffusion coefficient values for 3% bovine serum albumin (initial pH 6.3) and 3% bovine serum albumin/2% xylose after heating at 95°C. The bovine serum albumin/xylose system was heated for 80 min and had resultant pH of 5.95. The lowered pH bovine serum albumin was heated for 20 min and the pH at this time was 6.8. Samples were mixed in buffer (pH 6.8) plus sodium dodecyl sulphate (SDS) or SDS with beta mercaptoethanol (β -ME). Control experiments indicated that SDS did not interfere with the diffusion estimates.

System	(10 ⁶) x Diffusion coefficient (cm ² /sec)		
	Buffer (pH 6.8)	SDS	SDS/ β -ME
BSA (pH 6.3)	0.026 \pm 0.001	0.05 \pm 0.001	0.17 \pm 0.002
BSA/xylose	0.12 \pm 0.002	0.12 \pm 0.003	0.13 \pm 0.009

8.3 Molecular shape

It was possible to monitor the increasing size of the aggregates formed on heating bovine serum albumin/xylose until the system gelled. Some information about the shape of these aggregates can be obtained from the double logarithmic plots of either intrinsic viscosity, sedimentation coefficient or diffusion coefficient against molar mass (Harding *et al* 1991). These plots are shown in Fig. 8.5, and the values obtained from the gradient of the slopes are :

$$a=1.20$$

$$b=0.08$$

$$\epsilon=0.92$$

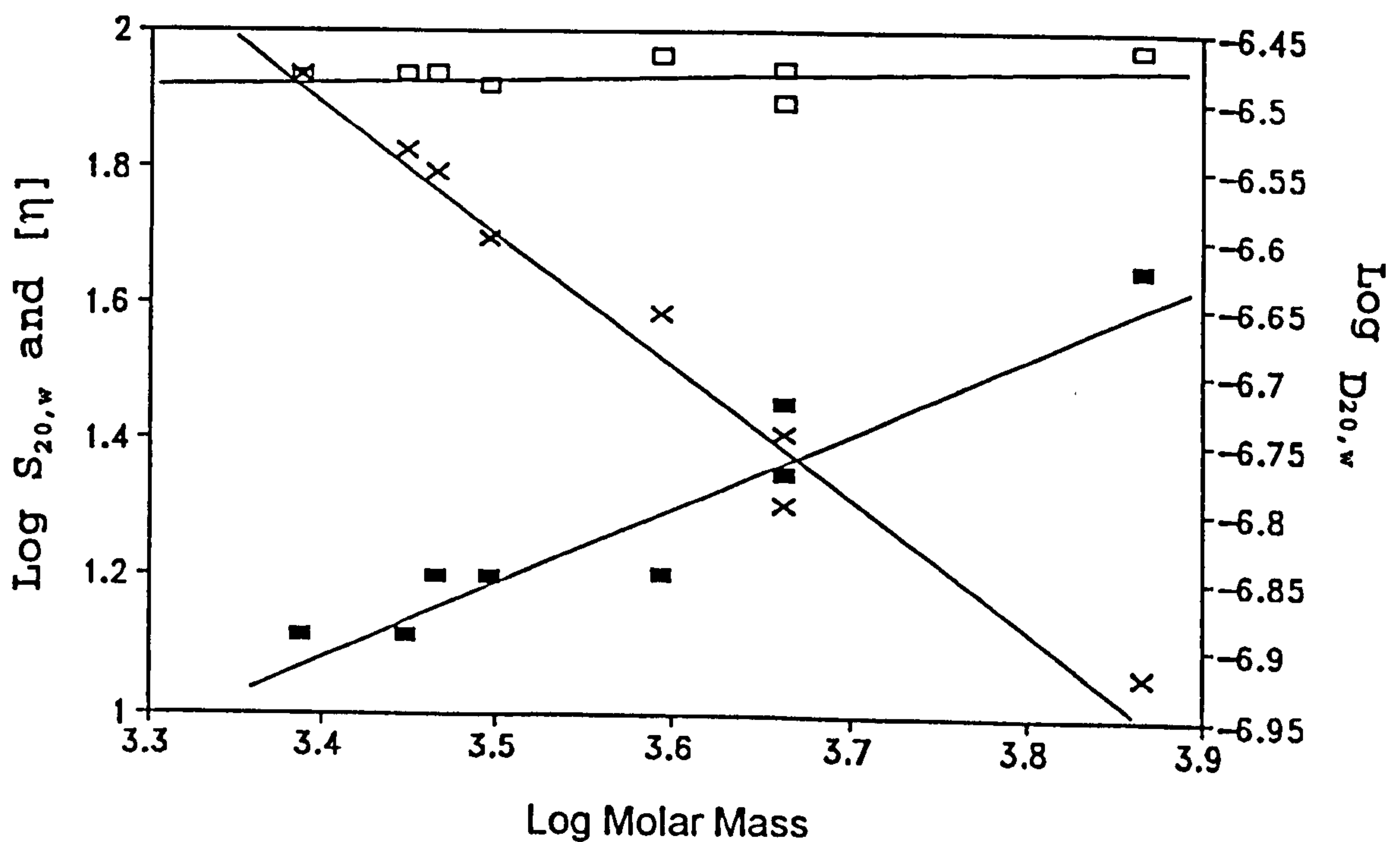


Fig. 8.5 Double logarithmic plots of sedimentation coefficient (□), translational diffusion coefficient (X) and intrinsic viscosity (■) versus molar mass for a 3 % bovine serum albumin + 2 % xylose mixture.

The values for ϵ and b are close to the expected values for a rod ($\epsilon=0.85$ and $b=0.15$), but the value of a lies between a rod ($a=1.8$) or a random coil conformation ($a=0.5-0.8$).

Based on the values of ϵ and b , it may be suggested that the aggregates became larger by protein molecules associating in a linear (i.e end-to-end) fashion. This is consistent with the so called "string of beads" model originally proposed to explain the structure of protein gels (Tombs, 1970). It could be imagined that this organised aggregation would lead to fine stranded gels. A distinction can be made between fine stranded gels that form under conditions where electrostatic repulsion between molecules is moderately high and aggregated gels that form under conditions closer to the protein isoelectric point and/ or high ionic strength (Langton and Hermansson, 1992). Van Kleef (1986) reported that an extensive (almost complete) unfolding took place when an ovalbumin solution was heated at a high pH, prior to network formation. However, at low pH, only some unfolding took place, followed almost immediately by an aggregation reaction.

It was previously suggested that Maillard gels are fine stranded in nature despite having a low pH (Armstrong *et al* 1994). The primary reason for this may be the enhanced net negative charge as a result of the loss of amino side chains from the protein (Yaylayan *et al* 1992). This high charge would also explain why the diffusion coefficient of the bovine serum albumin/xylose system is higher than that found for the system heated initially at pH 6.3 in the absence of the reducing sugar. Since it seems probable that the xylose system will have a higher net negative charge when the pH of the system is approximately 6 and thus non-covalent aggregation has not occurred. However, in the system lacking the reducing sugar aggregates are formed which the diffusion data indicates can be broken up by the inclusion of disrupting solvents.

8.4 Model for the Maillard aggregates

The molar mass calculated for the Maillard systems indicates that 50 to 60 molecules are joined in the aggregated system. These molar masses come from a combination of the diffusion and sedimentation data. Both these assessments used to predict molecular shape suggest that the aggregated form is "rod" like. The conformation estimated for the intrinsic viscosity is not so clearly rod like, but has some random coil characteristics. It is of interest to try and use the information to picture the aggregated molecules.

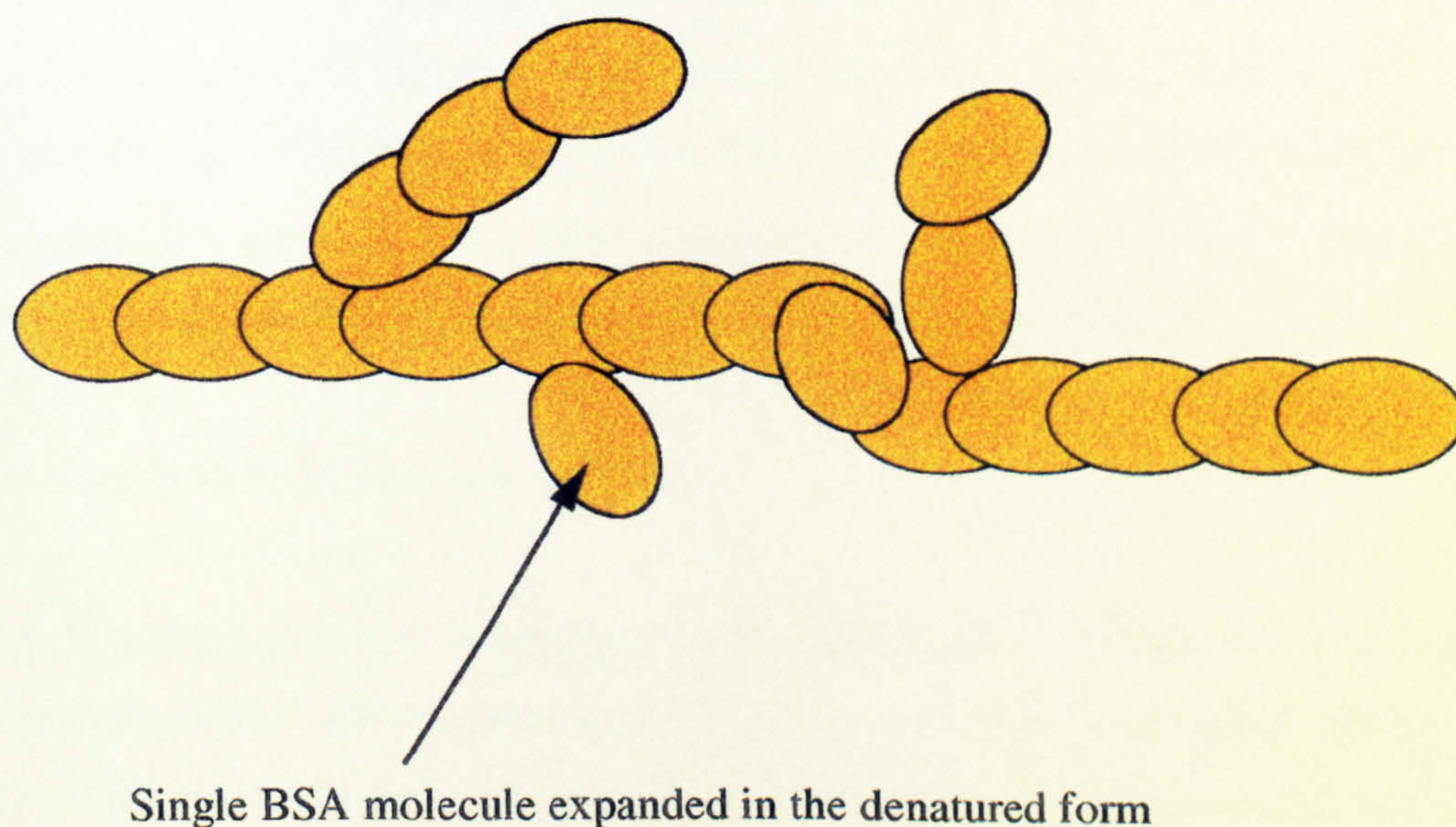


Fig. 8.6 Proposed model for bovine serum albumin/xylose aggregates based on diffusion and sedimentation coefficient plus intrinsic viscosity data

Fig. 8.6 gives a proposed model for the bovine serum albumin/xylose aggregates. It is proposed that the aggregates resemble a long stiff conformation, perhaps with some side groupings. About 50 to 60 molecules of bovine serum albumin would seem to associate before sufficient crosslinking occurs between the aggregates to form a gel network.

Conclusion

When xylose is heated with protein, there was a continuous change in the hydrodynamic properties with time. The increase in molar mass has been associated with the occurrence of additional non-disulfide linkages resulting from the Maillard reaction. Based on the gross conformation, it was suggested that the Maillard protein aggregates associated mostly in a linear fashion: i.e. the association is of an "end to end" nature, even at a pH value of less than 6. Modification of the amino acid side chains via the Maillard reaction is thought to contribute to this association.

OCCURRENCE AND NATURE OF MAILLARD GEL CROSSLINKS

In the last three chapters there is suggestive evidence that change in pH and alteration of net charge encourage the formation of gels in the presence of reducing sugar. However, as well as the change in pH and net charge, additional crosslinkings resulting from the Maillard reaction are likely to be of significant importance to the Maillard gelation. Even though the occurrence of the additional covalent bonds in the Maillard gels formed at elevated temperatures has been reported (Cabodevila *et al* 1994; Armstrong, 1994; Hill *et al* 1992), the nature of the crosslinks has not been investigated. It is possible that a similar types of bonds were formed at a temperature below 100 °C and within this chapter this possibility is investigated.

This chapter is divided into two sections;

Section 1 investigates the occurrence of the additional crosslinks by studying the solubility of protein and Maillard gels in solvents known to disrupt hydrogen and disulfide bonds.

Section 2 investigates the nature of some of the additional crosslinks by estimating the availability of important groups on the protein after reacting the protein with a reducing sugar.

In all cases the gels or solutions used were prepared at temperatures below 100 °C.

Section 1

Occurrence of additional crosslinks

9.1 *Gel solubility*

(a) *Low level of protein*

Previous rheological assessments indicated that gelation occurred after 110 min, 83 min and 13 min for 3 % solutions of bovine serum albumin when heated at 90 °C in the presence of 3 % xylose, 6 % xylose and 0.25 % glyoxal respectively. Therefore gels studied in this section were prepared by heating in excess of these times. Gels were then aged overnight at 4 °C and then passed through a sieve to produce gel particles.

The gel particles were shaken overnight in a mixed solvent of 1 % SDS + 1 % β -ME. These solvents were used since it is known that SDS is capable of disrupting hydrogen bondings and electrostatic interactions, whilst β -mercaptoethanol is capable of disrupting disulfide bonds. Therefore it could be expected that the gels would be completely soluble in these solvents if the conventional bonds were the main features of network formation. The appearance of the gel particles in the mixed solvents is shown in Fig. 9.1. The gel particles used in this figure were obtained by heating 3 % bovine serum albumin solution with 6 % xylose for a range of time, between 2-5 h. It can be seen that the gel particles were more soluble at shorter heating time (a homogeneous solution) and were less soluble at longer heating time (particles settling out of solution).

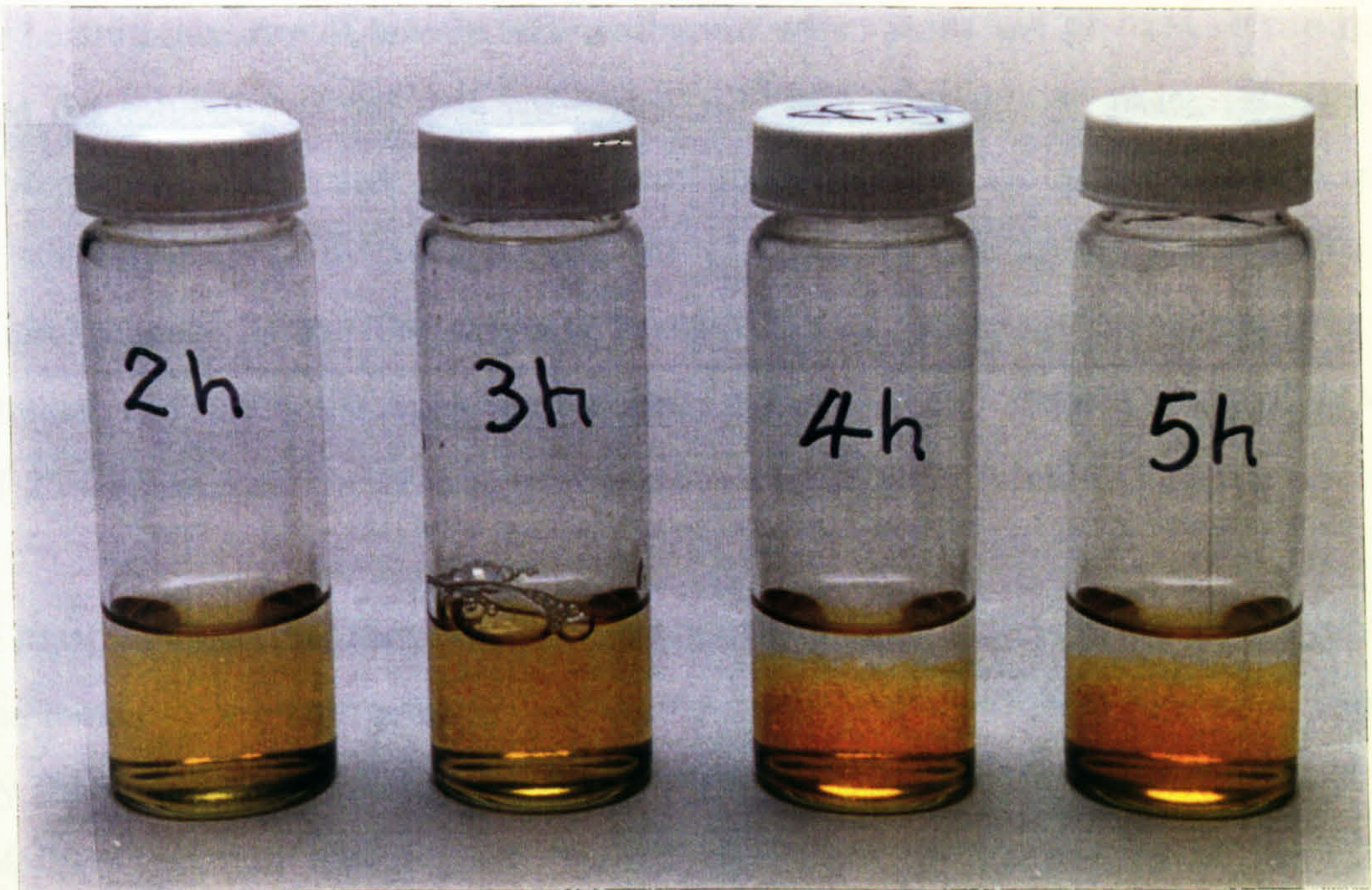


Fig. 9.1 Appearance of Maillard gel particles in 1 % SDS + 1 % β -ME. The gels were formed by heating solutions containing 3 % bovine serum albumin + 6 % xylose for a range of time of; 2 - 5 h.

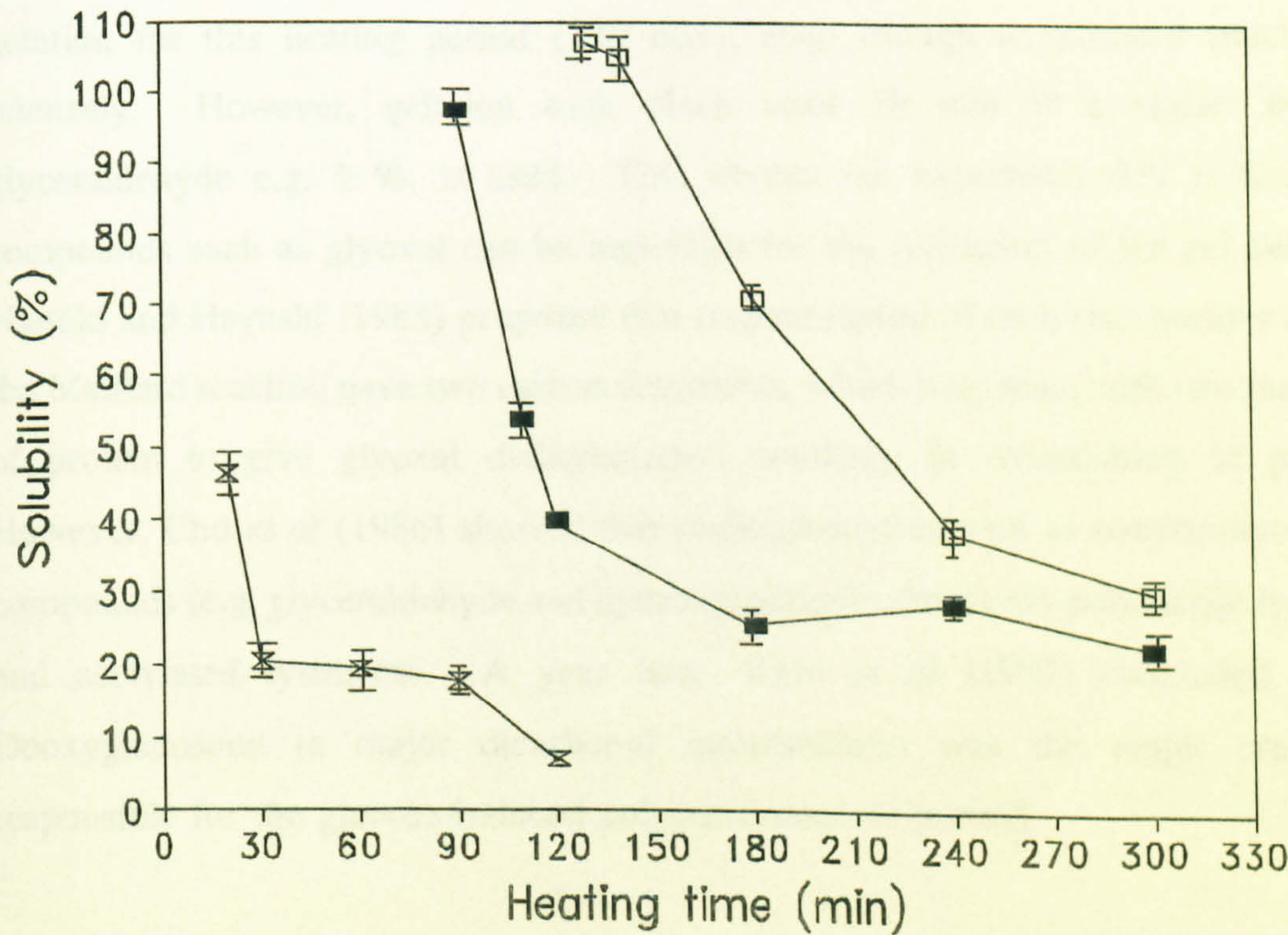


Fig. 9.2 The solubility of Maillard gels in 1 % SDS + 1 % β -ME as a function of heating time at 90 °C. The gels were formed by heating 3 % bovine serum albumin with 0.25 % glyoxal (X), 3 % xylose (□) and 6 % xylose (■). Error bars indicate standard deviation of 3 determinations.

Fig. 9.2 displays the relationship between heating time and solubility of gels formed by heating mixtures of bovine serum albumin with xylose and glyoxal. It can be seen that the solubility of the gels decreased 5-20 min after the gel point. Even though glyoxal was only used at such a low concentration, the gels formed were not completely soluble in the solvents. At 120 min of heating the gel solubility was approximately 10 %. The decrease in solubility of xylose system seems to be more gradual, and finally the solubility remained approximately constant at solubility values of 25 - 35 %.

It must be noted that the combined action of SDS and β -ME should have disrupted all the bonds in ordinary gels. Therefore the fraction of bonds that cannot be destroyed by the combination of solvents are likely to have appeared as a consequence of the Maillard reaction taking place between the carbonyl compounds of the reactants (xylose and glyoxal) and the amino groups of the protein. Note that glyoxal gelling system showed the fastest onset of gelling and decrease in solubility, even though its concentration used was only 0.25%. A similar amount of glyceraldehyde did not show gelation for this heating period (120 min), even though it browned much more intensely. However, gelation took place after 28 min if a higher level of glyceraldehyde e.g. 1 %, is used. This invites the hypothesis that α -dicarbonyl compounds such as glyoxal can be important for the formation of the gel networks. Namiki and Hayashi (1983) proposed that fragmentation of monosaccharides early in the Maillard reaction gave two carbon fragments, which may react with two molecules of protein to give glyoxal dialkylamines, resulting in crosslinking of proteins. However, Cho *et al* (1986) showed that α -dicarbonyl as well as α -hydroxycarbonyl compounds (e.g. glyceraldehyde and hydroxymethylfurfural) can polymerise lysozyme and acetylated lysozyme. A year later, Kato *et al* (1987) concluded that 3-Deoxyglucosone (a major dicarbonyl intermediate) was the major crosslinker responsible for the glucose-induced polymerisation of protein.

The loss of solubility with heating time can sometimes be taken as an indication of an increase in molecular weight. This has been demonstrated in Chapter 8 using intrinsic viscosity, light scattering and ultracentrifugation techniques on a similar

system (3 % bovine serum albumin + 2 % xylose) in dilute solution. It is possible therefore that the Maillard reaction produces a form of crosslinker which links the protein molecules together. Another indication that the Maillard gels have additional crosslinks comes from the dependence of storage modulus G' on reducing sugar concentration at fixed protein concentrations (2 and 3 %) as shown in Chapter 6. At a given time, it would appear that more crosslinks were formed in the system containing the more reactive carbonyl compounds. This idea is supported by the values of $K1$ and $K2$ from stress relaxation data (Fig. 9.3). The parameters of $K1$ and $K2$ have been used to characterise the viscoelastic properties of conventional and Maillard protein gels (Armstrong *et al*, 1994). Results from this thesis indicated that the less reactive 3 % bovine serum albumin/3 % xylose showed more elastic properties compared to the 3 % bovine serum albumin/3 % ribose gels, as evidenced by the higher values of $K1$ and $K2$.

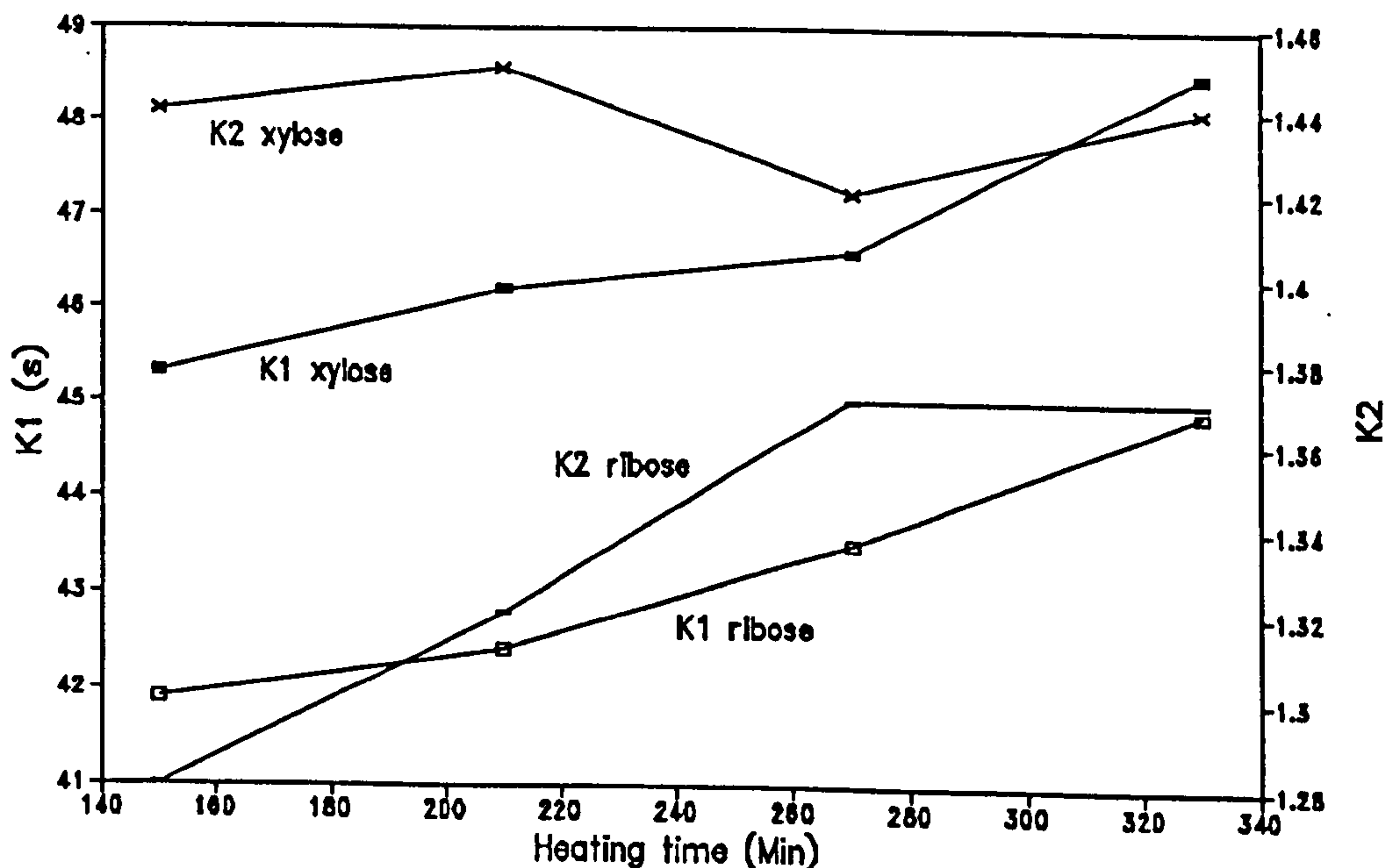


Fig. 9.3 $K1$ and $K2$ of Maillard gels at 25 % deformation as a function of heating time at 90 °C, for 3 % bovine serum albumin + 3 % xylose ($K1$, ■ and $K2$, X) and 3 % bovine serum albumin + 3 % ribose ($K1$, □ and $K2$, -)

A gel containing a smaller number of crosslinks for a given polymer concentration is more elastic than a gel with a higher crosslink density (Mitchell, 1976). The increase in parameter K1 with heating time may indicate an increase in covalent bonding.

Glyoxal and glyceraldehyde are two of the most reactive Maillard reaction products. These compounds are thought to be generated during the reaction between reducing sugar (e.g. xylose) and amino groups of protein (Namiki and Hayashi, 1983). Since the protein required low concentrations of glyoxal, short times to gel and the gels formed were not completely soluble in the mixture of solvents it is likely that this compound may be involved in the crosslinking reactions.

It is interesting to speculate that it is the bifunctional nature of the dicarbonyl compounds, such as glyoxal, that enables it to crosslink protein. This may be achieved through attachment of one of the carbonyl groups to lysine, arginine and/or other functional sites of the protein. Crosslinks may be achieved by binding of the second carbonyl group with the remaining sites on the protein. Even though this may seem plausible, it is difficult to obtain a conclusive evidence to support this idea.

(b) *High level of protein*

At pH 7.0, bovine serum albumin solution may form a gel by itself at a concentration in excess of 7 %. However at this concentration the gels formed were not strong, and it took a long time for gelation to commence. Therefore in this experiment 9 % bovine serum albumin solutions were heated for a range of times and the solubility of the gels formed measured (Fig. 9.4).

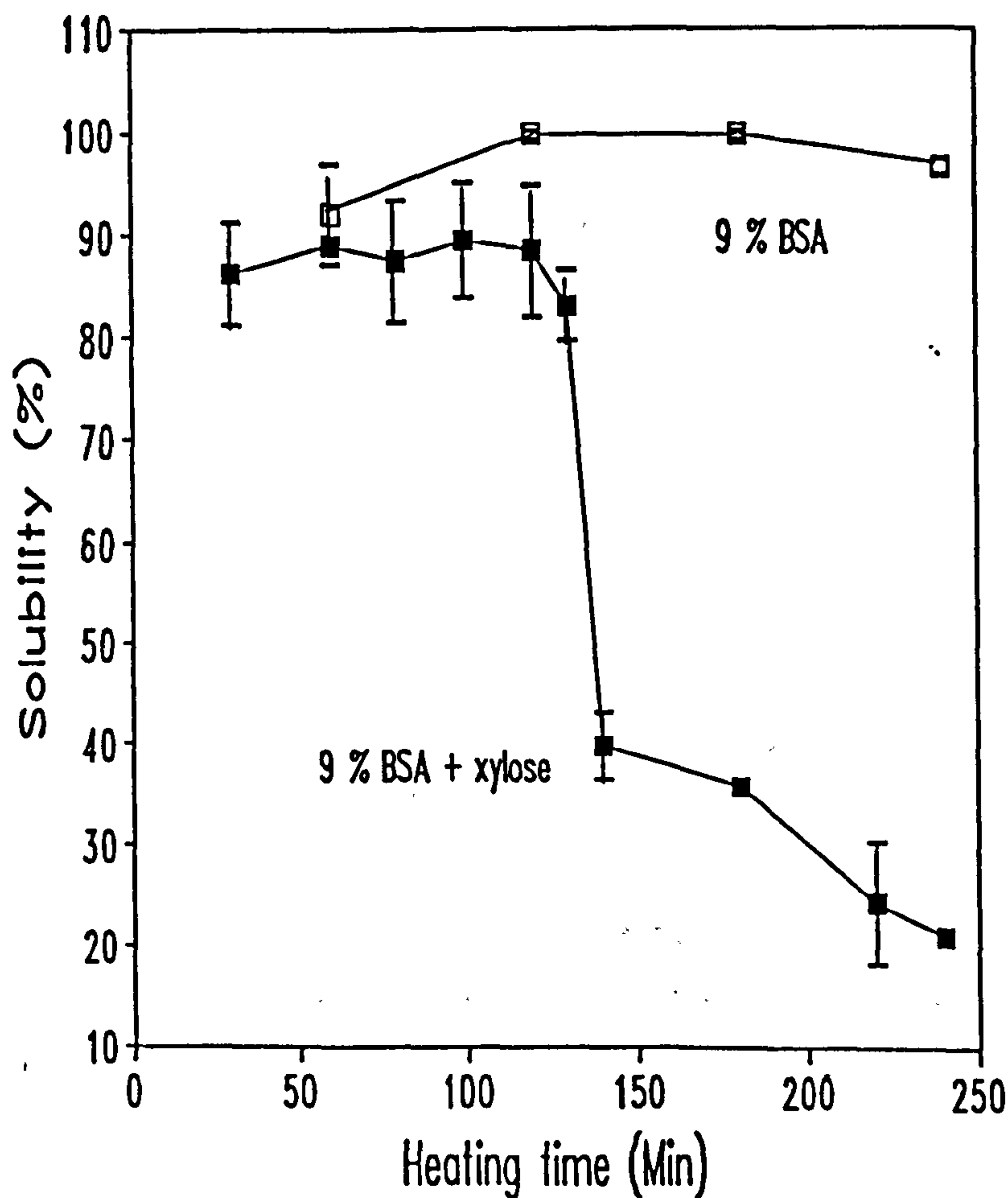


Fig. 9.4 The solubility of 9 % bovine serum albumin gels alone (\square) or with 3 % xylose (\blacksquare), in 1 % SDS + 1 % β -ME, as a function of time at 90 °C. The error bars indicate standard deviation of 3 determinations.

The bovine serum albumin gels formed in this way were almost completely soluble in the disrupting solvent for all the heating times used. This is a clear indication that only conventional bonds e.g. disulfide and noncovalent bonds, were the main features

of these gels. However, the gels produced by heating 9 % solutions of the protein with 3 % xylose showed a decrease in solubility to less than 40 % after about 90 min of gelling. Even though the gels formed with xylose had higher G' values (Chapter 6) compared to normal bovine serum albumin gels at the same concentration, the solubility of the Maillard gels only decreased after about 90 min of gelling. Two features of Maillard gelation may explain the difference in G' and solubility values observed for the 9 % bovine serum albumin gels with and without xylose. Early in the Maillard reaction the pH falls. This would give rise to higher levels of hydrophobic interactions and hence higher G' values for the Maillard gel. This gel would still maintain its solubility in SDS + β -ME. At longer heating times (around 90 min) a chemical crosslink could occur causing a decrease in solubility. The crosslink would reinforce the gel and it could be expected that G' would increase.

It should not be ruled out that the presence of sugars alters the disulfide linkages normally supporting the gel network. Ludwick (1979) reported the formation of additional disulfide bridges when β -lactoglobulin was heated with lactose at 95 °C. Ludwick (1979) also concluded that the presence of disulfide bridges helped to protect lysine from interacting with the lactose. Results reported in Fig. 9.4 show that the decrease in solubility of Maillard gels occurred later than would be anticipated from the gelation time and for solubility data for low protein Maillard gels. It is therefore suggested that disulfide linkages prevent or delay the formation of the Maillard crosslink.

Results from this section would suggest that the additional bonds that appear as a result of the Maillard reaction can also be generated after heating the protein/sugar system at a temperature less than the typical retort processing temperature (121 °C). However the nature and mechanism of formation of these covalent bondings are not yet understood. It is probable that some reactive Maillard products are involved in the reactions. However the sites of such interactions on the protein molecules have not yet been ascertained. It is the intention of the following section to investigate this.

section 2

Nature of Maillard crosslinks

9.2 *Rheological assessment*

Sulfhydryl (SH) and disulfide (SS) groups have been implicated as important functional groups in the gelation phenomenon of several globular proteins. Intermolecular disulfide crosslinking may be formed through the oxidation of SH groups or through the disulfide-sulfhydryl interchange reactions. It was also thought that disulfide bridges could become involved in the formation of additional non-disulfide crosslinks as a result of the Maillard reaction (Bishop, 1992) and impart some rigidity to the Maillard gels. To elucidate the involvement of disulfides in the gelation reactions, the gelling behaviour of two different proteins were compared. The proteins used were bovine serum albumin and myoglobin.

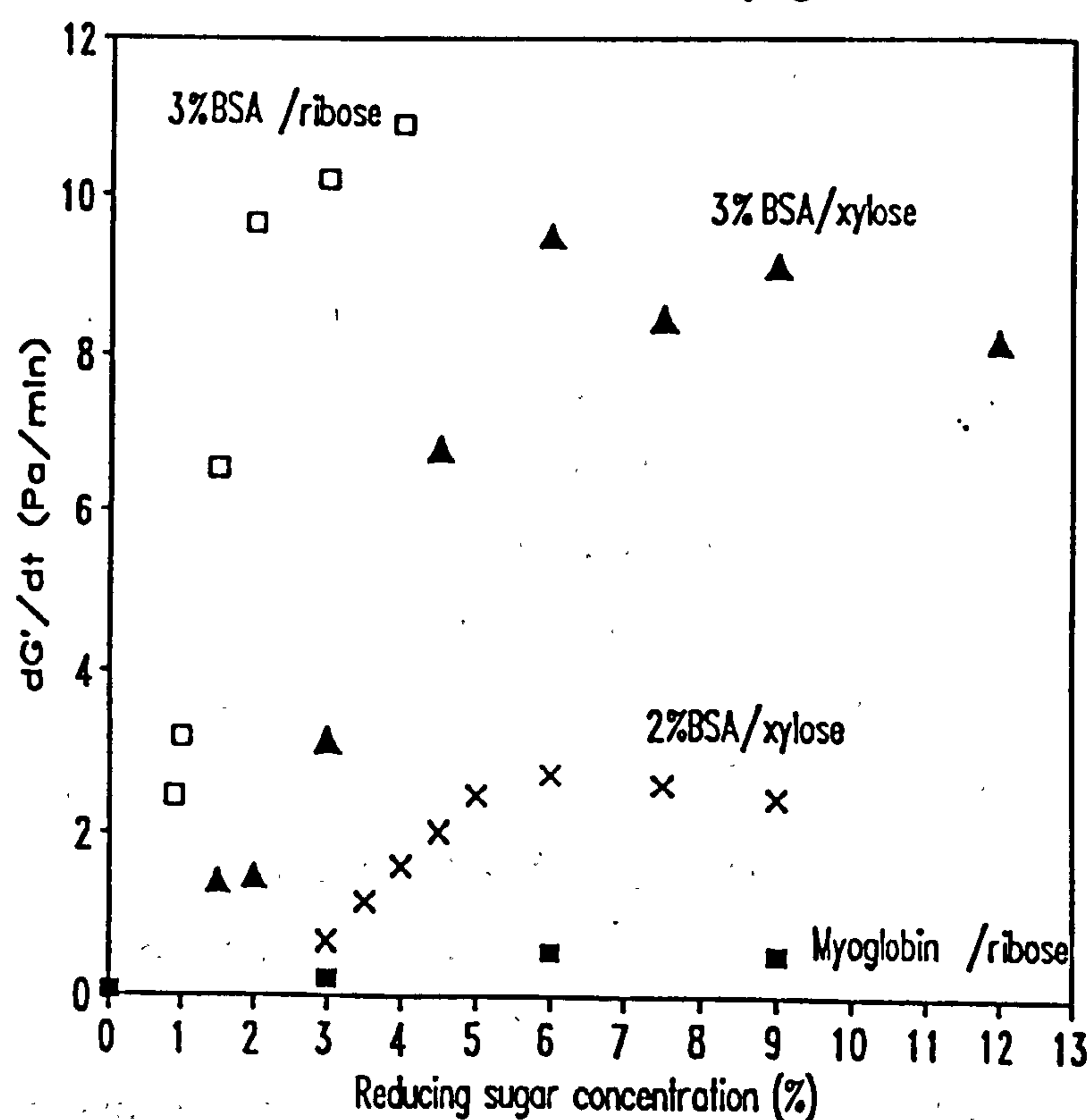


Fig. 9.5 dG'/dt of 3 % bovine serum albumin/ribose (□), 3 % bovine serum albumin/xylose (▲), 2 % bovine serum albumin/xylose (X) and 3 % myoglobin/ribose (■) as a function of reducing sugar concentration.

Both systems showed an approximately similar decrease in pH upon heating. The major difference between the two systems was that the pH for bovine serum albumin/ribose was decreasing towards the isoelectric point of the protein (pH 5.0), while on the other hand the pH of myoglobin/ribose systems was decreasing away from its isoelectric point (pH 6.9). In both cases, the decrease in pH indicated the occurrence of the Maillard reaction. Differences were seen in the gelation behaviour when the proteins were heated with reducing sugars. Data presented as the rate of change of storage modulus with time (dG'/dt), as a function of reducing sugar concentration (Fig. 9.5) indicates these differences. Unlike bovine serum albumin, there was no indication that ribose can affect the gelation of myoglobin (i.e. no gel point), even though it was heated with up to 9 % ribose for over 2 h. Visual assessments indicated that flocculates rather than gels were formed after heating of the myoglobin/ribose systems.

As well as the difference in pI. of the two protein an additional reason for the difference in the gelling behaviours of the proteins could be due to the variation in the structure and amino acid content of the two proteins. The major difference between the two systems is that myoglobin has no disulfides, whilst bovine serum albumin has 17. A similar suggestion has been put forward to explain differences in gelation between 7S soya which lacks disulfides and 11S soya which can form disulfide bonds (Cabodevila *et al* 1994). These present results could suggest that disulfides of bovine serum albumin may play a part in the formation of low concentration bovine serum albumin gels via the Maillard type crosslinks. One possible mechanism for this could involve lysine-xylose complexes interfering with the disulfides and linking molecules of protein together, resulting in an increase in molecular weight. If this is the case the total available disulfides and lysine would be reduced after the Maillard reaction between bovine serum albumin and xylose.

Despite low solubility values of the Maillard gels in the solvents that disrupt disulfide bridges, it would still be possible that such bonds were formed and imparted some gel strength. It can be imagined that additional nondisulfides crosslinks formed via the Maillard reaction "hid" the disulfides from being attacked by the disrupting solvents.

It is possible that the new covalent linkages formed from the Maillard reaction might locate protein chains in such a way as to encourage the formation of disulfide linkages by neighbouring chains.

9.3 Estimation of sulfhydryl groups

The classic method using Ellman's reagent was employed to estimate the availability of SH and SS groups of bovine serum albumin. The methods are given in detail in Chapter 4 (section 4.3.9). This method was rapid and fairly simple to perform. It is based on the reaction of DTNB (Ellman's reagent) with protein SH groups at neutral and alkaline pH to yield thionitrophenylated protein and a yellow thionitrophenylate anion (Kalab, 1970). Therefore, the stronger the intensity of colour produced from the reaction, the higher the amount of SH groups present in the solution. Estimation of disulfides was performed after reduction with mercaptoethanol in 10 M urea. The reducing agent was then removed by acid precipitation and centrifugation. It was found that the precipitates of bovine serum albumin + xylose dissolved less readily as compared to bovine serum albumin heated alone. This could be overcome by using higher molar strength (10-12 M) of urea.

The SH contents of bovine serum albumin/xylose and bovine serum albumin alone are shown in Table 9.1. Unheated samples exhibited SH values of 1 moles/mol. This is very similar to that proposed by Peter (1985), but consistently higher than those reported by Fernandez-Diez *et al* (1964), Hoshi and Yamauchi (1983) and Howell and Taylor (1991). For the protein heated alone there was a decrease in SH content for the first 15 min of heating. The value increased slightly at 45 min to 0.85 moles/mol, and at 75 min the SH value was 0.98 moles/mol. The initial decrease in SH content could be attributed to the oxidation of the SH groups by air. Petrucelli and Anon (1995) also reported a decrease in SH values after heat treatment (100 °C for 90 min) at pH 7, 8 and 9. They suggested that the thermal treatment favoured both the reactions of protein aggregation and oxidation of SH groups. A similar decrease in SH contents of ovalbumin upon heating, has also been shown by Legowo *et al* (1996).

As heating proceeded unfolding continued, and it is possible that more molecules became exposed and available for reacting with Ellman’s reagent.

Table 9.1 SH content (moles/mol) of bovine serum albumin heated alone or with xylose at different heating time at 95 °C.

	SH groups (moles/mole)*	
Heating time (min)	Bovine serum albumin	Bovine serum albumin + xylose
0	1.04 ± 0.04	1.00 ± 0.01
15	0.80 ± 0.02	0.85 ± 0.01
45	0.85 ± 0.06	0.73 ± 0.07
75	0.98 ± 0.03	0.51 ± 0.07

* Values expressed as a mean ± standard deviation of at least 3 determinations. Calculation was based on the assumption that the molecular weight of bovine serum albumin was 66000 g/mole.

For bovine serum albumin containing xylose, little difference in the SH content was observed during the first 15 min of heating. At 45 min, 73% of SH groups were detectable and at 75 min only 50% of the SH groups reacted with Ellman’s reagent. The decrease in the SH content could indicate either the formation of additional SS bonds through the oxidation of SH groups or side reactions with the products of the Maillard reaction. In Chapter 8, the protein solution containing xylose showed an increase in intrinsic viscosity with heating time. An increase in gel firmness has been related to a decrease in SH groups of soy protein isolate gels (Shimada and Cheftel, 1988). However, this cannot be considered conclusive evidence for the critical

involvement of SH groups in the formation of Maillard type crosslinks since it is known that the protein molecules had adopted different conformation if heated with xylose at these conditions (Chapter 8). As a result of this, it is possible that only a portion of the SH groups on the protein could become available for the reaction with the Ellman's reagent.

To show that some reactive Maillard reaction products (e.g glyoxal and glyceraldehyde) can react with SH groups, bovine serum albumin was modified in such a way that the initial number of SH groups per molecule was high (greater than 1 mole/mole) and more available for the reaction with the Maillard products. This was achieved by breaking the disulfide bridges using β -mercaptoethanol. The total of measureable SH groups increased to a range of 29.5 - 30.5 moles/mole of bovine serum albumin. This compares to the theoretical values of 35 reported in the literature (Peter, 1985). The relationship between SH content and heating time is shown in Fig. 9.6. The pH of the solutions after dissolving the precipitates in 12 M urea was between 7.5 - 7.8. Above the pH of 7.5, the SH groups became highly reactive (McKenzie, 1971).

After heating at 90 °C the number of SH groups decreased with time in the samples containing glyoxal and glyceraldehyde, the value however remained relatively constant in the samples heated alone or with ribose. Preliminary works also showed that glyoxal but not ribose can react readily with cysteine and resulted in a dark brown solution.

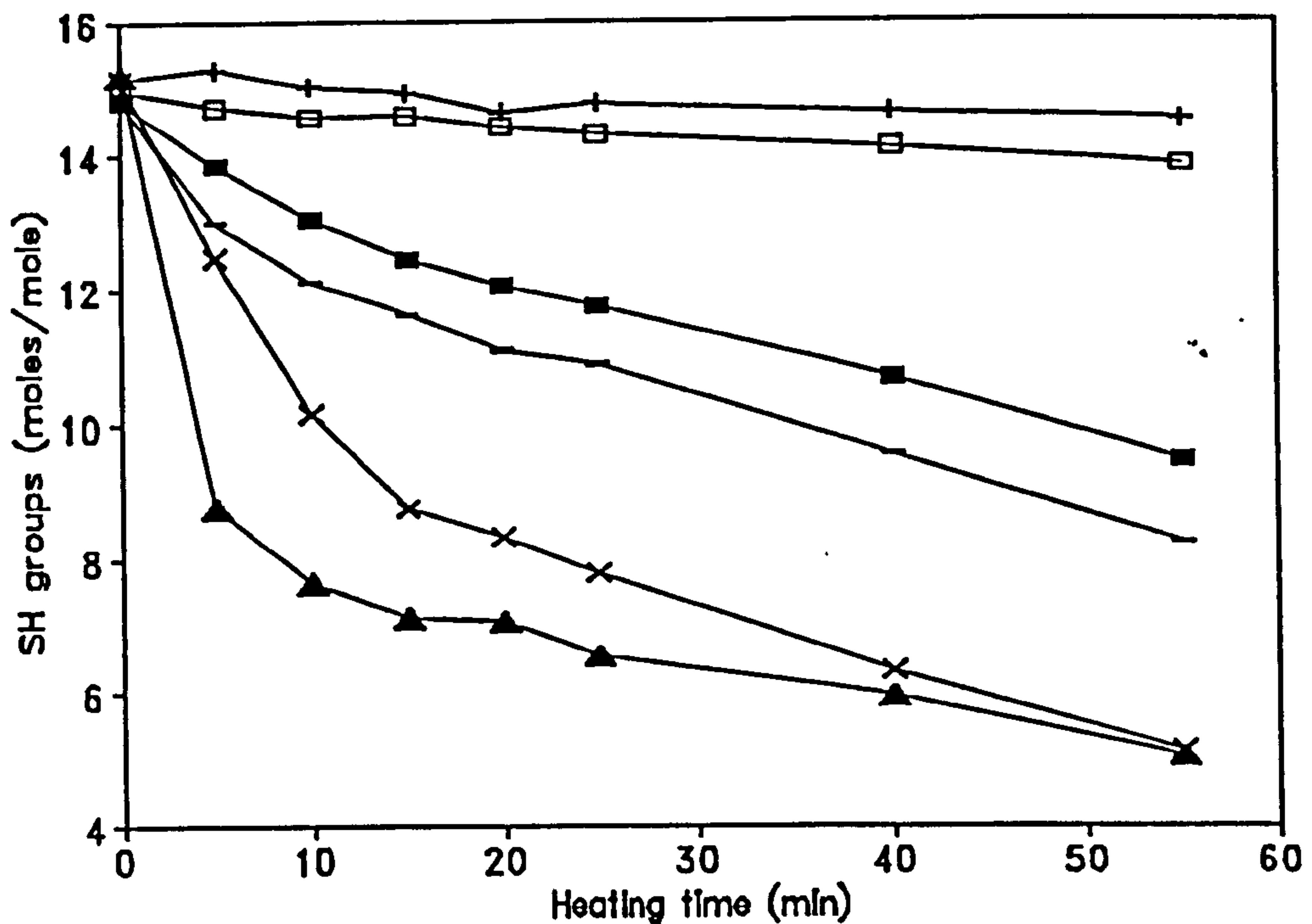


Fig. 9.6 Relationship between heating time (90 °C) and SH contents of modified bovine serum albumin heated alone (□) or with 0.5 % ribose (+), 0.1 % glyceraldehyde (-), 0.025 % glyoxal (■), 0.05 % glyoxal (X) and 0.1 % glyoxal (▲).

These results seem to suggest that some of Maillard products were capable of reacting with the SH groups of protein. However these measurements were performed at a high pH and at conditions where the SH groups were made more available for interactions. In the Maillard systems the pHs of the solutions were normally low (< pH 6). It has been suggested that at low pH, the SH groups were relatively inert and did not contribute to the maintenance of whey protein gel structure (Mangino *et al* 1987). It therefore remains unclear whether the SH groups are involved in the Maillard crosslinking reactions that enhance the gel formation being discussed in this thesis.

9.4 Estimation of disulfide content

The relationship between disulfide content and heating time of bovine serum albumin heated alone or with xylose are presented in Fig. 9.7.

Native bovine serum albumin exhibited SS values of 15.9 moles/mol which were slightly lower than those reported in the literature, i.e. 17 disulfides (Peter, 1985). This shows that the methods have a reasonable indication and that incubation in 10 M urea and β -mercaptoethanol was quite sufficient to reduce the SS bonds and that washing steps with 12 % TCA (trichloroacetic acid) were sufficient to remove β -mercaptoethanol.

Upon heating bovine serum albumin the number of SS in the protein decreased to about 11 moles/mol, and remained at that value for the rest of the heating period. This could be explained by the increased molecular weight seen on heating the protein (Chapter 8). The increased size of the molecular aggregate could make it more difficult for the disrupting solvent to reach and cleave the SS groups. A decrease in the content of cystine due to heating of cod muscle has been reported (Miller *et al* 1965).

Unheated bovine serum albumin/xylose contained approximately 14 moles/mol SS which were slightly lower than that of the unheated protein. This number decreased on heating; from 11.9 moles/mol at 15 min to 8 moles/mol at 45 min and at 75 min the SS content in the bovine serum albumin/xylose was 4.9 moles/mol. The percentage of SS groups remaining at 75 min was 45 % of the total SS groups present in bovine serum albumin heated alone. Miller *et al* (1965) showed a loss of 33 % of cystine of cod muscle after heating with glucose at 85 °C for 27 h. This data may suggest that the products of the Maillard reaction may have interfered with the remaining 55% of the SS groups and formed non-disulfide covalent bonds that could not be disrupted by the combination of urea and β -mercaptoethanol.

Further evidence for this is displayed in Fig. 9.8 which shows a relationship between xylose concentration and SS contents of bovine serum albumin heated at 95°C for 15 and 30 min.

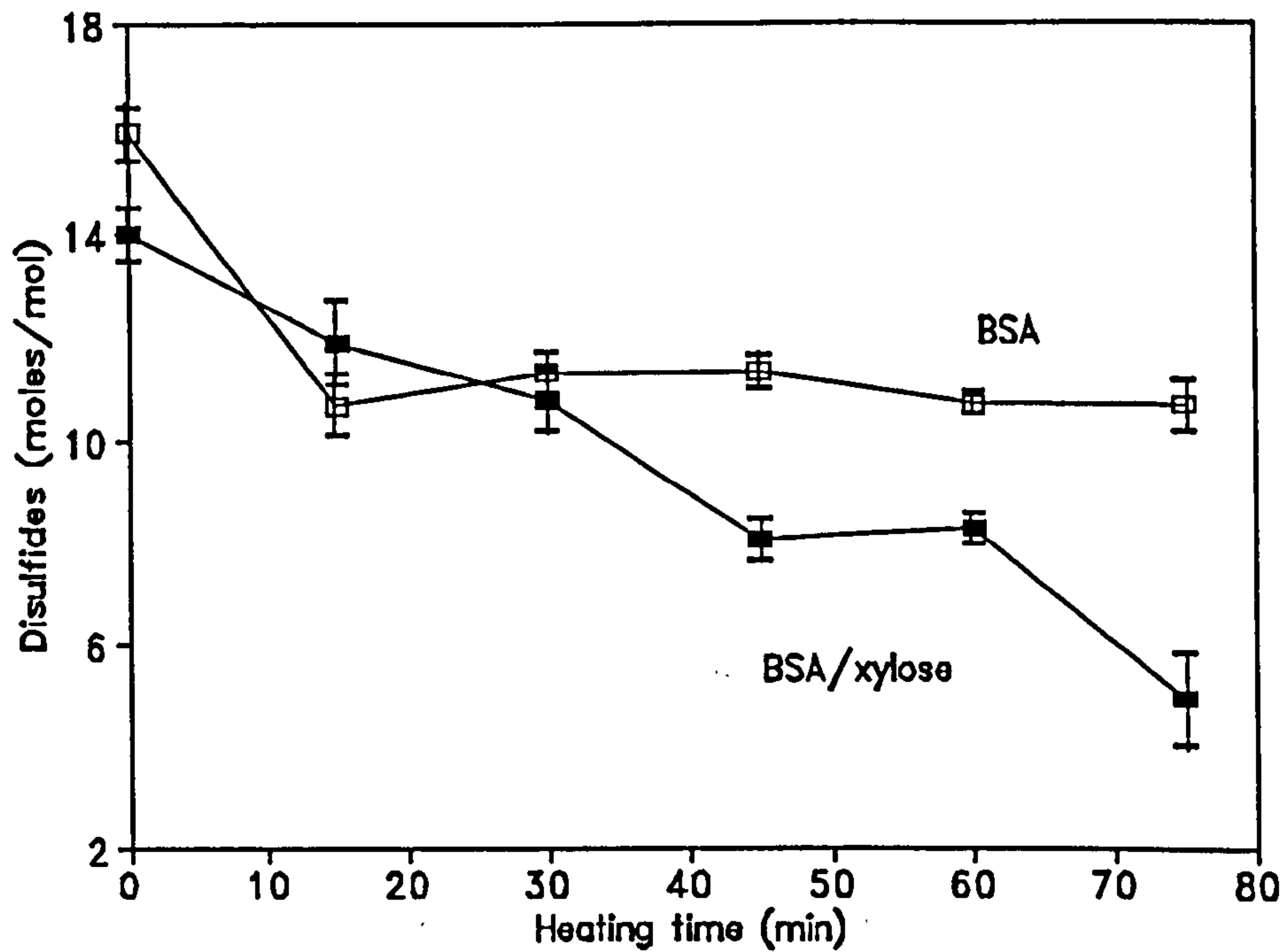


Fig. 9.7 Disulfide content of bovine serum albumin heated alone (\square) or with xylose (\blacksquare) as a function of heating time at 95 °C. The error bars indicate standard deviation of 3 determinations.

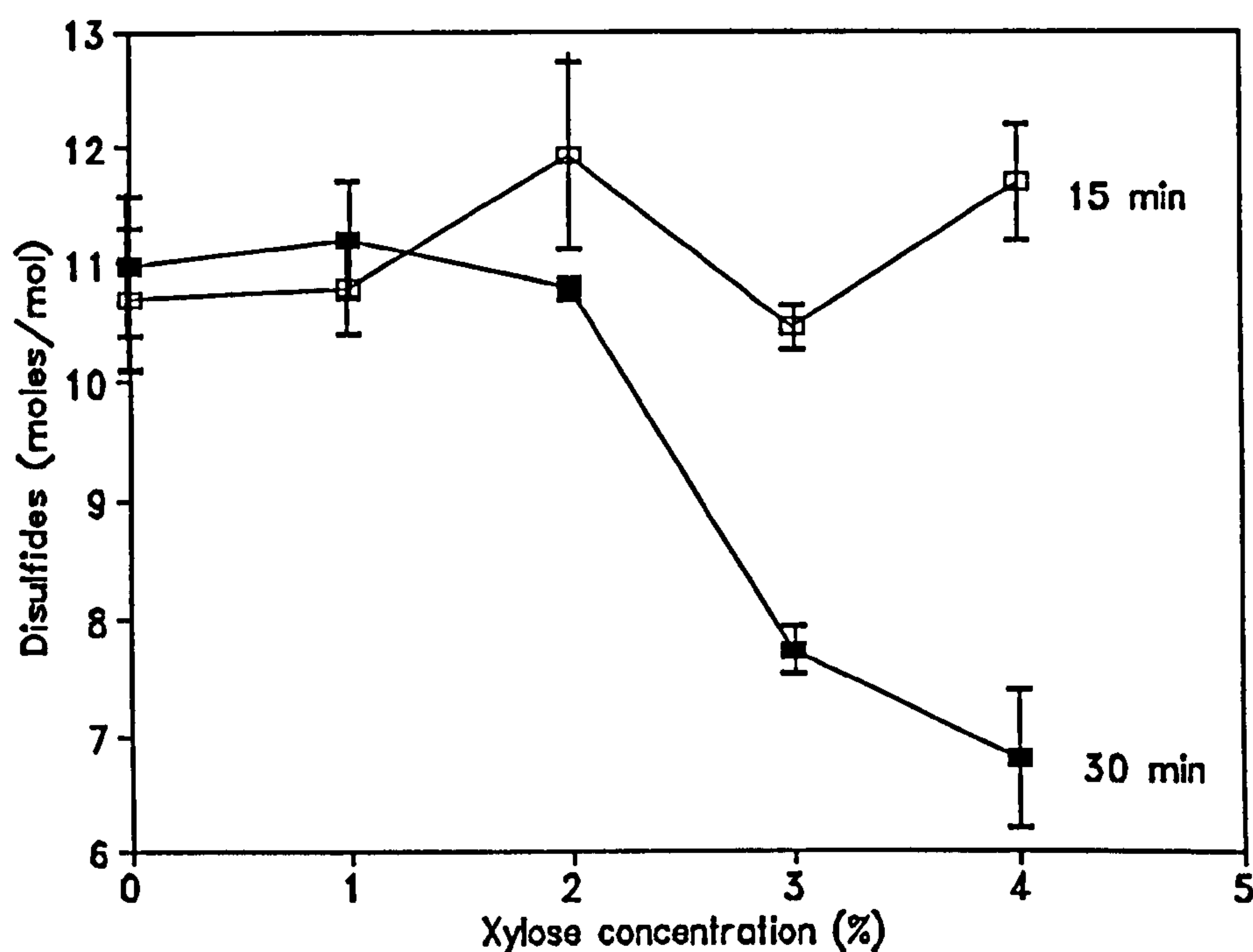


Fig. 9.8 Disulfide content of bovine serum albumin solutions heated with xylose, as a function of xylose concentration after heating at 95 °C for 15 (□) and 30 (■) min. The error bars indicate standard deviation of 3 determinations.

At 15 min the disulfide content was essentially the same for all xylose concentrations used. However at 30 min the number of disulfides decreased with increasing xylose concentration. The disappearance of the disulfides from bovine serum albumin heated with xylose is likely to have occurred as a result of the Maillard reaction as it was dependent on heating time and xylose concentration. The destruction of disulfides bridging in the course of the Maillard reaction has also been reported elsewhere (Andrew, 1975; Feeney and Whitaker, 1988). However similar results could have been obtained if the change in conformation of bovine serum albumin/xylose complex 'hid' some of the disulfides against mercaptoethanol, resulting in only a portion of disulfides being reduced and assayable. The development of more suitable methods for the determination of SH groups (and eventually of SS bonds), in insoluble proteins are required in the future.

9.5 Amino acid analysis

The profiles of amino acid analysis for bovine serum albumin either heated alone or with xylose in dilute solutions are compared in this section. Most of the values of amino acids obtained from this experiment were lower than those reported in the literature. However, the main aim of this section was to see whether differences in the contents of important functional groups were detectable, and whether the Maillard reaction could alter any of the amino acids in the protein.

Table 9.2 displayed the results from the amino acid analysis. The most noticeable difference is the decrease in the lysine content (i.e. about 16 % loss) of the protein heated with xylose. This is not unexpected because many works have indicated that due to the reaction of the carbonyl compounds with the ϵ -amino groups of lysine (glycation) a decrease in the available lysine is observed (Mohammad *et al* 1949; Armstrong, 1994). There is also some indication of a decrease in arginine residues. The loss of these basic groups of the protein could also be one of the factors that resulted in a decrease in the isoelectric point of the protein (see Chapter 7).

The content of cystine were essentially the same for bovine serum albumin solutions either heated alone or with xylose suggesting that disulfides were not involved in the crosslink formation. SH groups in the protein solutions were not detectable. However, it is worth noting that the effect of acid hydrolysis prior to analysis could have broken the additional crosslinks and rendered the SS and SH groups to oxidation.

One possible reason for the decrease in lysine and arginine could be due to their involvement in the formation of the additional crosslinks. This idea has been suggested by Shin *et al* (1988). The ϵ -amino groups of lysine may act as the generators of 3-Deoxyglucosone by reacting with the reducing sugars. The arginine residues subsequently attack the protein bound 3-Deoxyglucosone moieties, resulting in protein crosslinking. However, this idea needs further investigation by e.g. estimating the quantity of 3-Deoxyglucosone produced from the Maillard reaction.

Table 9.2 Amino acid composition per molecule of bovine serum albumin

<u>Amino acids</u>	<u>Literature*</u>	<u>BSA alone</u>	<u>BSA + xylose</u>
Gly	16	14	14
Ala	46	37	37
Val	36	27	29
Leu	61	48	49
Ileu	14	11	11
Phe	27	22	21
Tyr	19	15	14
Ser	28	21	22
Thr	34	25	25
CySH	1	0.42	Nm
CySSCy**	34	23	24
Met	4	Nm	4
Trp	2	Nm	Nm
Arg	18	19	17
Lys	59	48	41
His	17	16	16
Asp	41	43	44
Glu	59	74	75
Pro	28	38	38

* From Peter (1985), ** determined as cysteic acid
Nm Not measured

9.6 *Additional disulfide bonds*

The SS and SH contents of the protein solution heated without xylose remained at an approximately constant value throughout heating. This may indicate that no additional SS bridges (inter or intramolecular) were formed, either through the oxidation of SH groups or the SS-SH interchange reactions.

The decrease in the pH, which approached the isoelectric point and the glycation processes during heating of a Maillard mixture would seem to bring molecules of protein together. It is possible for additional SS bonds to be formed in this system due to the close proximity of the protein chains. Ludwick (1979) has reported a formation of additional disulfide bridges when β -lactoglobulin was heated with lactose in the wet state at high temperatures. The results from this chapter however did not indicate the occurrence of additional SS bonds in the Maillard system, since the estimated number of SS groups was decreasing with heating time, despite the measured decrease in SH groups. The amino acid analysis also indicated very little difference between the disulfide content of bovine serum albumin heated alone or with xylose.

Conclusion

The results from this section indicated that the gels formed at 90 °C via the Maillard reaction were not completely soluble in the mixture of solvents that disrupt hydrogen bonds, electrostatic interactions, non-covalent forces and disulfide bonds. This is an indication of the presence of additional covalent bondings in these gels. Reactive Maillard products are considered to be the possible compounds that crosslinked the protein. These new bonds may well reinforce the gel strength.

The reduced availability of SH and SS groups could indicate their involvement with the crosslinking reactions as a result of the Maillard reaction. The proximity of the protein chains held together by the Maillard induced covalent linkages could encourage disulfide bridging. Or perhaps the presence of disulfides could enable the correct spacing for the Maillard crosslink. The thiol groups can take part in interaction with reducing sugars and the possibility that this provides an initiation of the crosslink. Cysteine and cystine groups are not the only amino acids of importance. It is likely that other amino acids may also play decisive roles in the formation of the polymers.

It is now clear that the Maillard reaction is capable of forming additional crosslinks when the reaction is initiated at 90 and 95 °C. It would be useful if such a phenomenon could be applied and understood in real food systems. One area where this has potential is in the field of extrusion cooking. Therefore, the effect of the addition of xylose, glucose and sucrose on the extrusion behaviours of soya grits is investigated in the following chapter.

CHAPTER 10

**THE EFFECT OF INCLUSION OF REDUCING SUGARS ON THE
QUALITY OF SOYA GRIT EXTRUDATES**

The extrusion of soya protein has been widely investigated (Sheard *et al* 1986; Mitchell and Areas, 1992; Ledward and Mitchell, 1988). One of the most important factors that determine the extrusion behaviour of proteins is the extent to which a continuous "molten" phase is formed within the extruder. This phase has been associated with material that is soluble in solvents that disrupt non-covalent or disulfide bonds. Mitchell and Areas (1992) have predicted that formation of covalent crosslink that would not breakdown within the extruder, would reduce the formation of a homogeneous melt within the barrel. This would be expected to reduce the stability of the extrudate matrix and possibly stop the texturisation of the product as it leaves the extruder barrel.

The Maillard reaction has been suggested to have a negative effect on the extrusion behaviour of proteins, even in the absence of added carbohydrate (Ledward and Mitchell, 1988). Within this thesis it has been postulated that the Maillard reaction between reducing sugars and bovine serum albumin produced stable 'covalent bonds'. While the work of Cabodevila *et al* (1994) and Sousa *et al* (1994) demonstrated nondisulfide covalent linkages in soya and lupin when heated in the presence of reducing sugar. It is postulated that these added covalent linkages would alter the extrusion behaviour of the proteins.

There are very few studies of the effect of such reactions on protein during extrusion. One study by Racicot *et al* (1981) demonstrated the effect of the inclusion of lactose on the extrusion behaviour of corn meal. Adding sugars increased protein insolubility, and solubility decreased at higher moisture contents, indicating the possibility of Maillard type crosslinking reactions. It is therefore possible that the Maillard reaction will take place during extrusion, or after extrusion if the extrudates containing reducing sugars are further heated.

Within this chapter work to investigate the extrusion of soya grits in the presence of compounds that are known to undergo the Maillard reaction is reported. Some possible ways of using the Maillard reaction to increase the retort stability of soya extrudates are also explained.

Materials and methods

Materials

Soya grits with a protein dispersibility index of 70 were obtained from Food Maker - Protein Technologies Int. (Foodmaker Ltd., Corby, Northants). The quoted protein and moisture contents (supplier data) were 49.1 and 9.9 % respectively. D(+) xylose, D(+) glucose and other chemicals were obtained from Sigma Chemical Company, Poole, UK.

Sample preparation

Soya grits were dry mixed with either 5 or 10 weight percent of xylose, glucose or sucrose for 12 min in a planetary mixer. The preparations were allowed to stand for 24 h before extrusion.

Extrusion

A Clextral BC21 twin screw extruder was employed. The Screw configuration is given in Table 10.1. Normal screw speed was 300 rpm. The die end temperature was varied from 85 °C to 190 °C. Water (1.1 kg/h) was injected into the barrel of the extruder close to the feed end. The material was extruded through a single circular die of diameter 3 mm. The product was observed, collected and sealed in polyethylene bags. The moisture content was assessed by drying the product for 6 h in a vacuum oven at 70 °C.

Table 10.1 The screw configuration in different section of the extruder (from the hopper to the die).

Screw Section	1	2	3	4	5	6	7*	8
Length (mm)	100	50	50	50	50	50	25	25
Pitch (mm)	33	25	25	16.7	16.7	16.7	16.7	16.7
Temperature zone	1	2		3		4		

* A reverse pitch with three 3x4 mm slots

Microwave heating

Some of the textured products were further processed to induce the Maillard reaction. To do this, extrudates (20 g) were uniformly distributed on a plate and heated in a microwave oven (De Longhi MW 329, output 750 w) at a full power for 3 min.

Protein solubility

Protein solubility was assessed according to the methods given in section 4.3.8. A mixed solvent containing 1 % sodium dodecyl sulphate (SDS) plus 1 % β -mercaptoethanol (ME) was used. The extrudate was ground to a fine powder and 0.5 g of sample was mixed with 10 ml of the solvent and shaken overnight. The samples were then centrifuged for 10 min at 2000 g and filtered through Whatman no 4 paper. The supernatant (0.5 ml) was diluted to 13 ml with water and the protein solubility determined using the Lowry method (Lowry *et al* 1951). The results are expressed as a % of the total protein present in the sample, as estimated by the supplier.

Retort stability and texture assessment

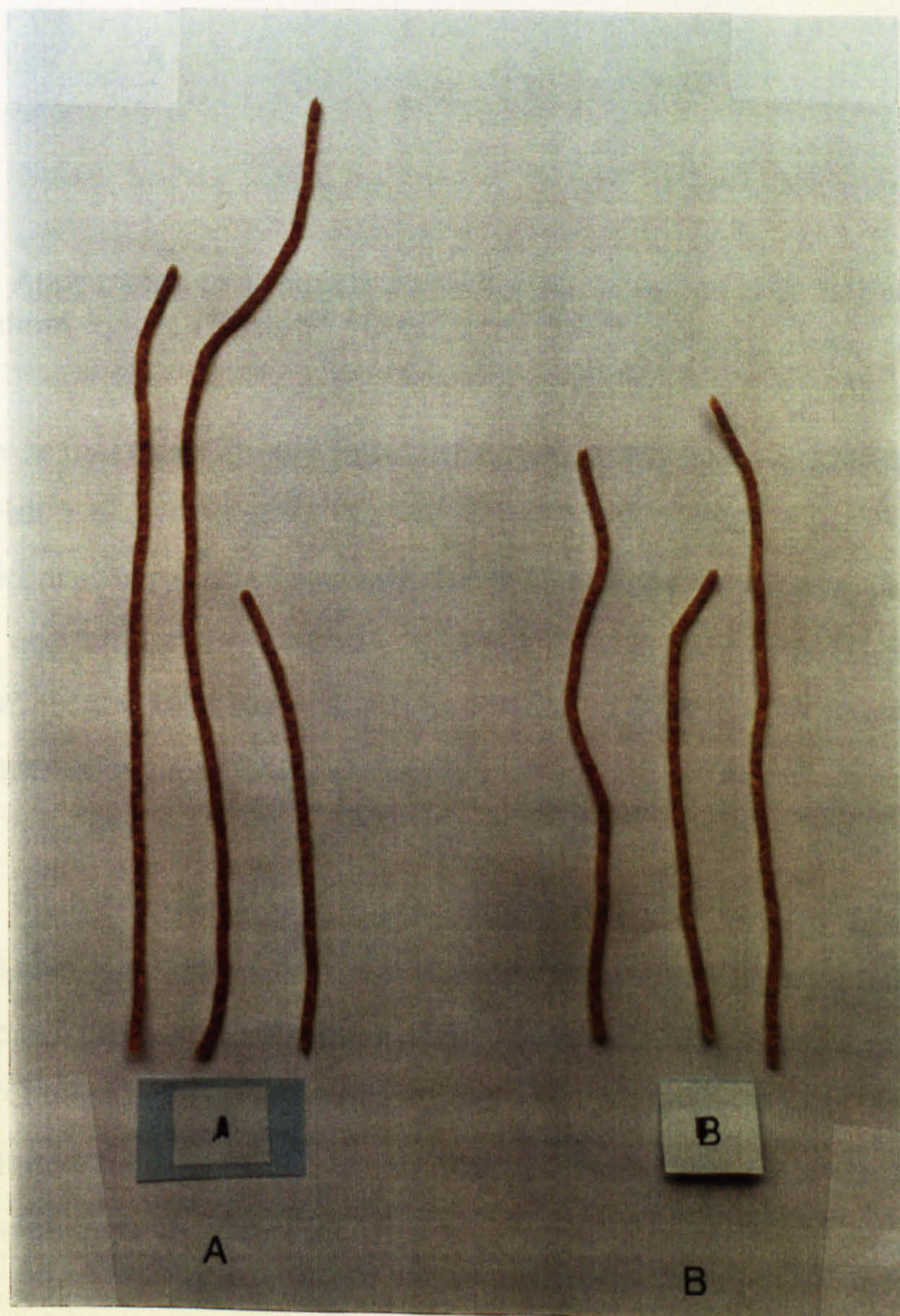
To assess the retort stability of the extrudates, the methods of Frazier *et al* (1984) were used with some modifications. Extrudates (50 g) were canned and retorted with 175 g of water at 121 °C for 60 min. The cans were cooled rapidly to ambient temperature and allowed to stand for 24 h before opening and draining excess water. The remaining sample was visually assessed, and then placed in an Ottawa Texture Measuring System (OTMS) cell with a 30 cm² base area fitted with a nine wire grid. An Instron 1140 with a plunger speed of 200 mm/min was used to determine the maximum force during compression (compression force) and extrusion of samples through the grid. Each measurement was carried out in duplicate.

Results

10.1 *Extruded products*

Soya grit was extruded with or without sugars at a range of die end temperatures. Visual assessments for soya extrudate formed alone or with 5 % sucrose indicated that more stable and continuous extrudates were formed as the die end temperature was increased. The appearance of the extrudates formed using either soya with xylose (A) or soya alone (B) at a die end temperature of 85 and 190 °C are shown in Fig. 10.1.

1



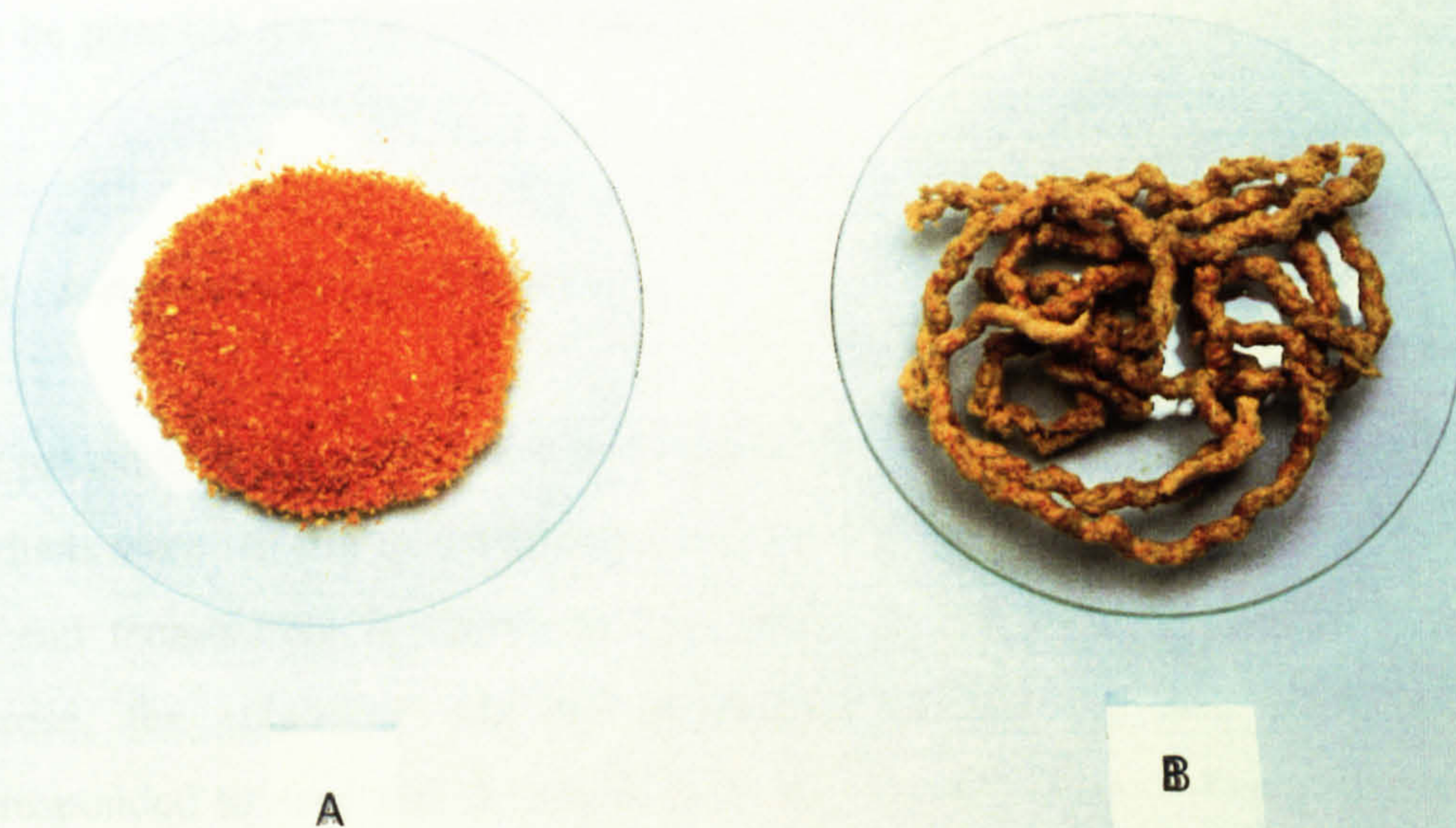


Fig. 10.1 Appearance of products formed after soya grit was extruded with xylose (A), or without xylose (B) at 85 °C (1), and 190 °C (2)

It can be seen that there is very little difference between the soya extrudates textured alone or with 5 % xylose at 85 °C. Despite the use of such a high concentration of xylose, there was very little browning due to the Maillard reaction. The combination of low die end temperature and short resident time in the extruder may explain these observation. Fig. 10.1 also depicts the products at a die end temperature of 190 °C. At this temperature the products containing xylose (A) are markedly different from the extrudates formed using soya grits alone (B). The former are much darker in colour and exited the barrel as a fine powder rather than a well textured product. Increased colour was also observed as the die end temperature was increased from 85 to 190 °C. The failure to form a continuous extrudate occurred at a die end temperature of greater than 110 °C when xylose was incorporated at 5 % concentration. If glucose (10 %) was substituted for the xylose then failure to form a textured product occurred at higher temperatures (> 130 °C).

The measured back pressure fell to zero in product not forming continuous extrudates. The colour formation and the loss of extrudate integrity could be related to the Maillard reaction. If additional crosslinks occur early in the barrel of the extruder it may be possible that the protein does not unfold to form the homogeneous melt.

10.2 *Measurement of solubility*

The presence of the postulated crosslinks can be assessed by evaluating if the extruded products were soluble in disrupting solvents. The relationship between solubility and die end temperature is shown in Fig. 10.2. For soya alone and soya containing sucrose, the solubility was not dependent on the die end temperatures, and corresponded to near 100 % solubility in the mixed solvent. This is not unexpected since it has been shown that soya isolate extrudates are held together by a combination of hydrophobic, electrostatic and disulfide bonds (Mitchell and Areas, 1992).

At the lowest die end temperature (80 °C), there was no loss in solubility for the samples containing reducing sugars. Yet at temperatures greater than 80 °C the sample containing xylose showed a reduction in solubility. This reduction was only around 20 % up to die end temperatures of 150 °C, but then there was a marked decline in solubility. Glucose incorporation did not seem to reduce solubility over the range 90 - 150 °C but products formed at temperatures in excess of this showed a large reduction in soluble products.

The steep decline in solubility (occurring at 150 °C) did not correspond to the loss of stable extrudate. The solubility of the glucose system was higher than that of the xylose at the point when extrudate formation was no longer possible. The loss of solubility at the point where the dramatic effect on extrudate stability occurred was really quite small, in the region of a loss of 5-15 %. The residence time in the barrel of the extruder was 30 to 60 s. Within this time period the samples of protein, sugar and water are intimately mixed and physical as well as chemical modifications happen. Measurements of solubility made on the extruded product show the full history of

these effects. It is possible that the critical stage of loss of solubility and formation of crosslinking occurred early in processing. The changes would seem to be small and not detectable within the greater variations seen during full passage of the product in the extruder.

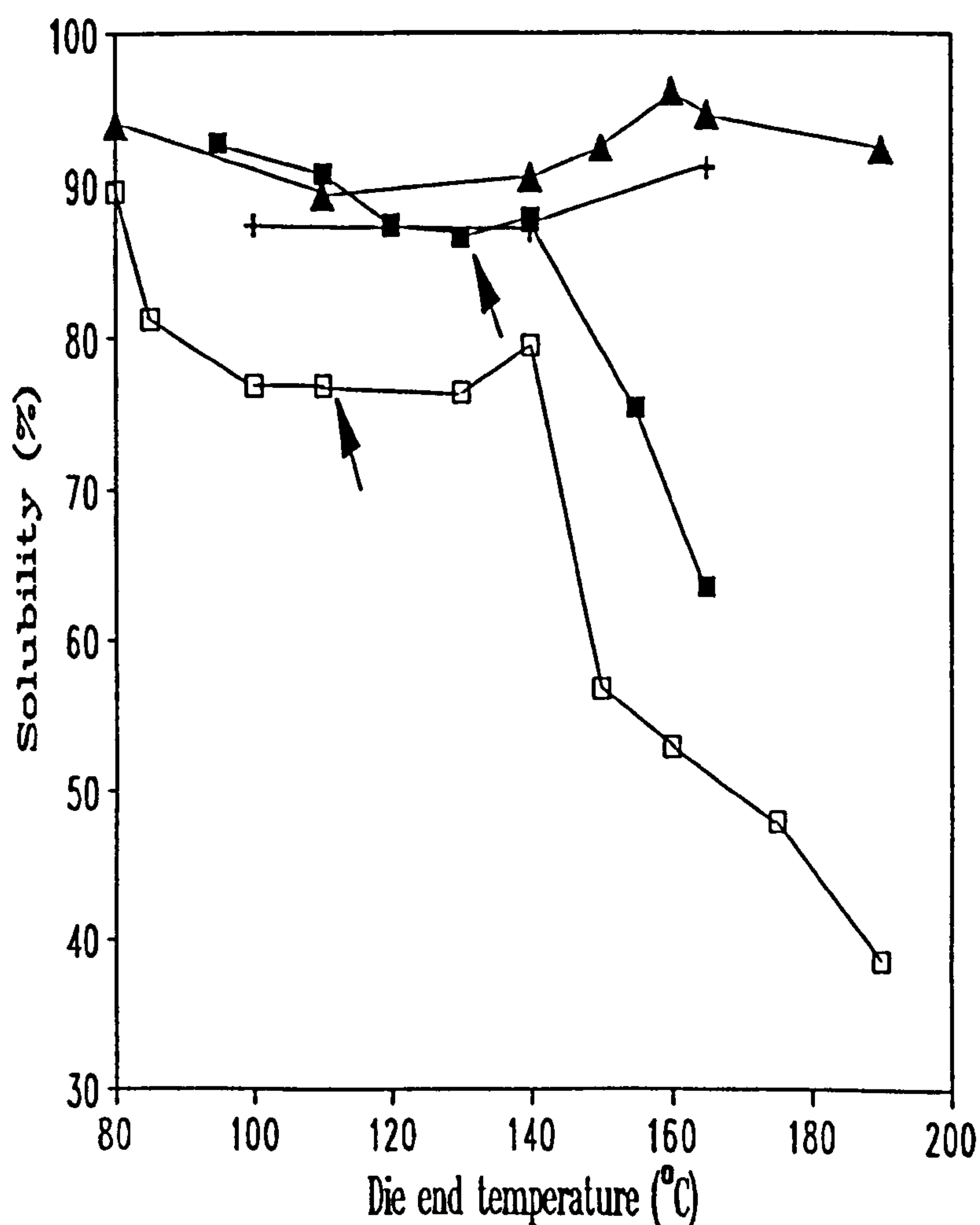


Fig. 10.2 Solubility of soya extrudates without sugar (▲), with 5 % sucrose (+), 10 % glucose (■) or 5 % xylose (□) as a function of die end temperature. The arrows in the figure indicate the point at which the failure to form stable products occurred

10.3 Retort stability

Several food products are formed using material that has been texturised within the extruder and subsequently canned and heat processed. A requirement of such products is the integrity of the products post retorting (Frazier *et al* 1984). The samples produced for this study by extrusion were therefore investigated using such techniques.

The retort stability of the extrudates were assessed by retorting in water for 60 min at 121 °C. Samples without reducing sugar were assessed for a die end temperature range of 90 - 165 °C. Due to instability of extrudates, the data for products containing xylose and glucose were limited to 3 (85 - 110 °C) and 4 (95 - 130 °C) die end temperatures respectively. Post retorting, the samples were drained of excess water and visually assessed. The strength of the samples was measured using the Ottawa Texture Measuring System (OTMS) and the data for compression force as a function of die end temperature is shown in Fig. 10.3. The compression force for all four systems was effectively similar for the range of die end temperatures of 85-130 °C. This is an indication that reducing sugars did not affect the retort stability of soya extrudates. After the die end temperature of 130 °C, the compression force for the non-reducing sugar soya controls increased as the die end temperature was increased.

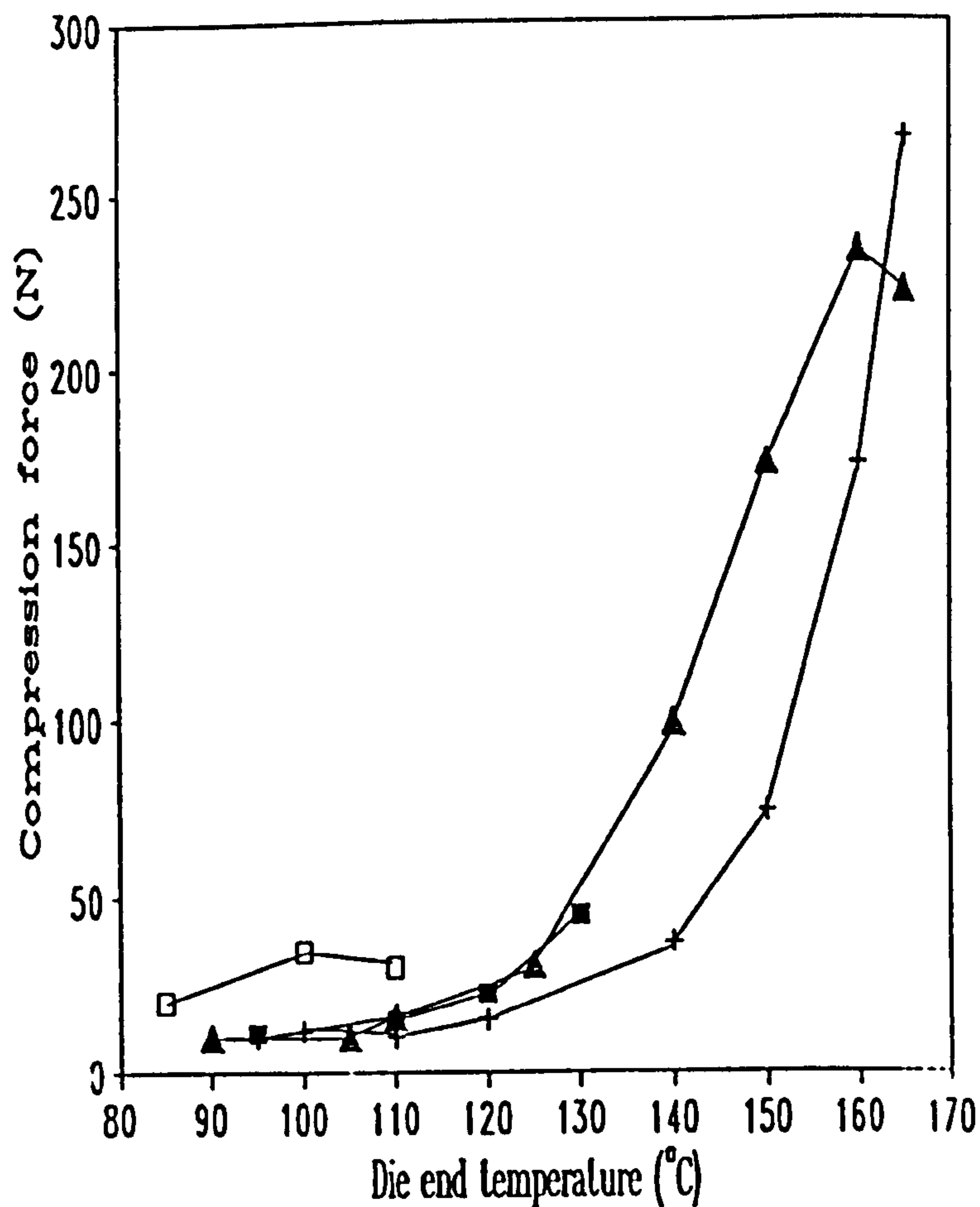


Fig. 10.3 Compression force as a function of die end temperature for soya extrudates formed without sugar (▲), with 5 % sucrose (+), 10 % glucose (■) and 5 % xylose (□)

10.4 *Post heating of the extrudates*

The evidence so far would indicate that incorporation of reducing sugar can render the sample unsuitable for extrusion. However, high die end temperature does seem to evoke a positive retort stability to the products formed without reducing sugar. It is possible that additional crosslinking, although not great enough to change solubility did occur at the higher die end temperatures.

It was considered that low crosslinking in the extruder was beneficial, but a high linking density in the product before retorting was also desirable. It had been noted that at low die end temperatures, the extrudates were not heavily coloured even though xylose or glucose were present. To see if the components would still interact in these low moisture systems, they were heated in a microwave for 3 min at full power.

The appearance of the products is shown in Fig. 10.4, the upper row shows the microwaved extrudates and the lower row the microwave extrudates after retorting. A considerable difference in the appearance and performance existed between the two types of extrudates, with and without reducing sugar, even though the processing conditions were similar for both samples. No browning was observed in the samples containing soya grits alone, but in the samples containing xylose browning occurred as a result of the microwave heating. Browning occurred even after heating at full power for only 1 min (results not shown). The change in the colour of the products from light yellow to dark brown and a slight drop in pH would indicate that the Maillard reaction had taken place.

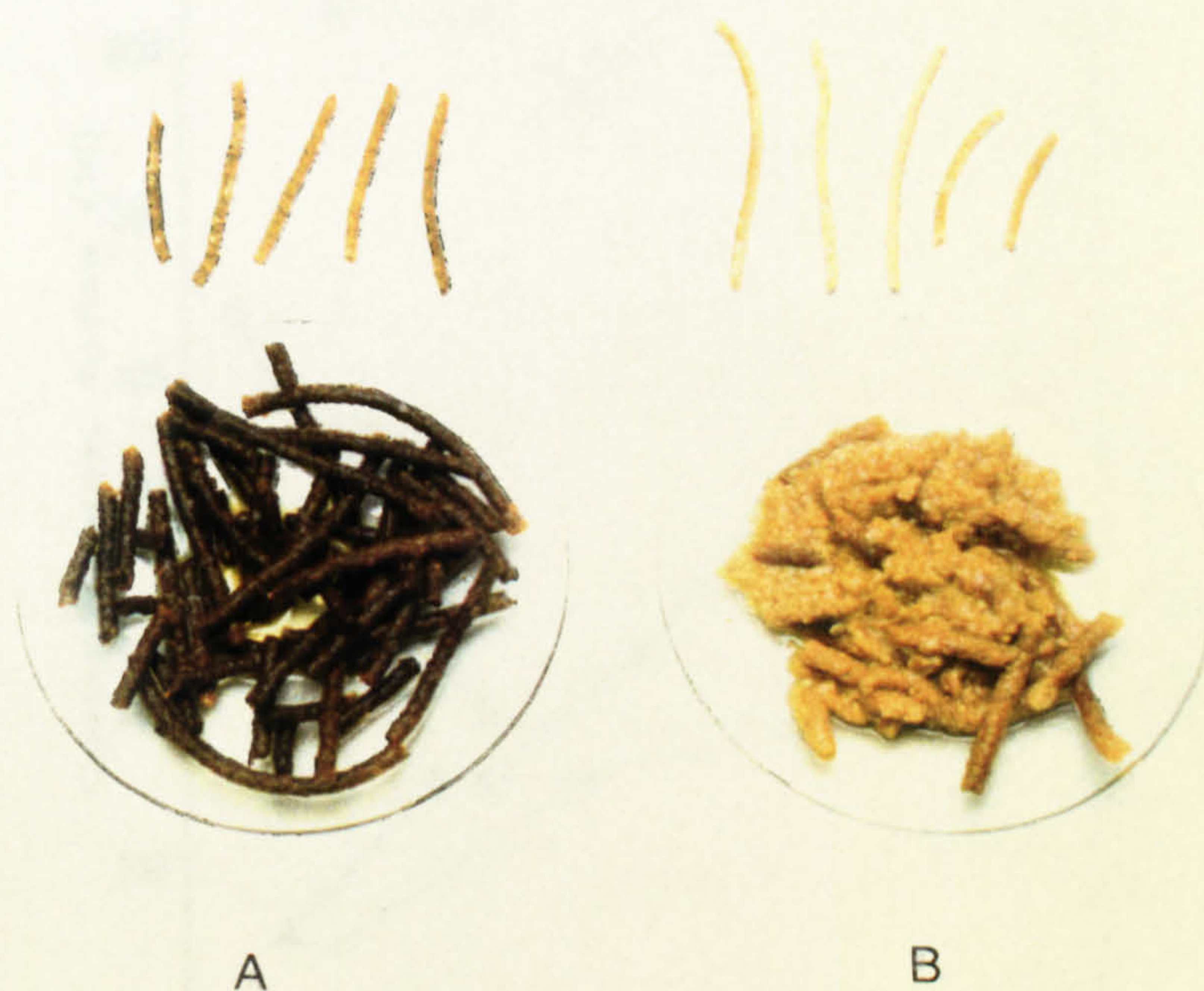


Fig. 10.4 Appearance of microwaved extrudates before (upper row) and after (lower row) retorting for soya grit containing xylose (A) and without sugar (B)

Upon retorting, the products containing soya alone crumbled almost completely, producing very fragile chunks. However, products containing 5 % xylose survived retorting and retained their texture throughout 60 min of heating at 121 °C.

Measurement of the retorted product strength (Fig. 10.5) shows that the control samples which had been microwaved showed a similar behaviour to the non-microwaved extrudates, but the values of compression force were slightly elevated. This is not unexpected since heating in the microwave did seem to decrease the solubility.

A more dramatic increase in the compression force is shown by the microwaved extrudates containing reducing sugars. The compression force for the microwaved products containing glucose was higher than that containing xylose. This difference may be related to the reactivity of the sugars, and therefore their behaviour during extrusion.

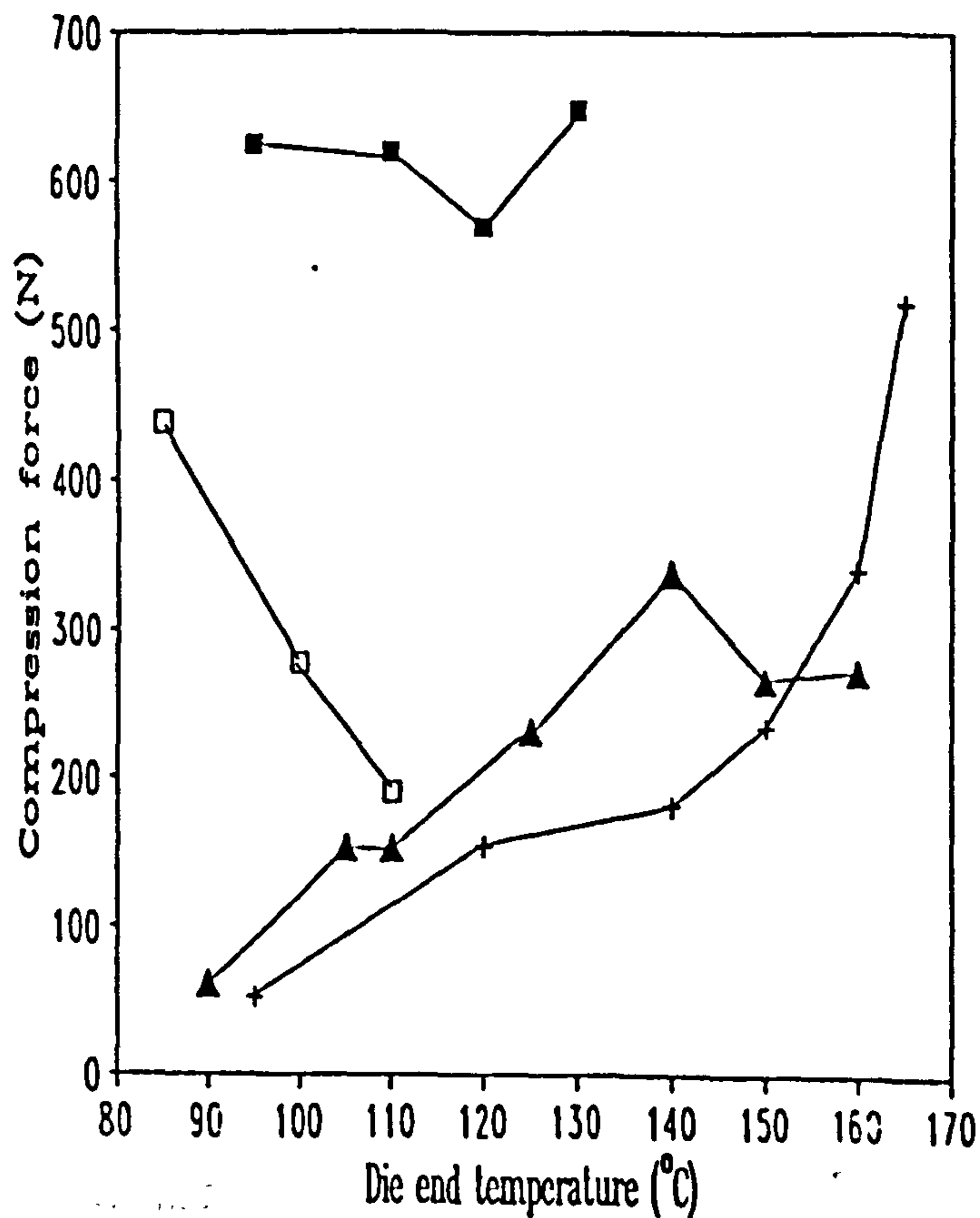


Fig. 10.5 Compression force as a function of die end temperature for microwaved soya extrudates formed without sugar (▲), with 5 % sucrose (+), 10 % glucose (■) and 5 % xylose (□)

10.5 Solubility of microwaved samples

To establish the type of bonds holding the microwaved products together, their solubility in 1 % SDS + 1% β -ME were determined and the results are displayed in Fig. 10.6.

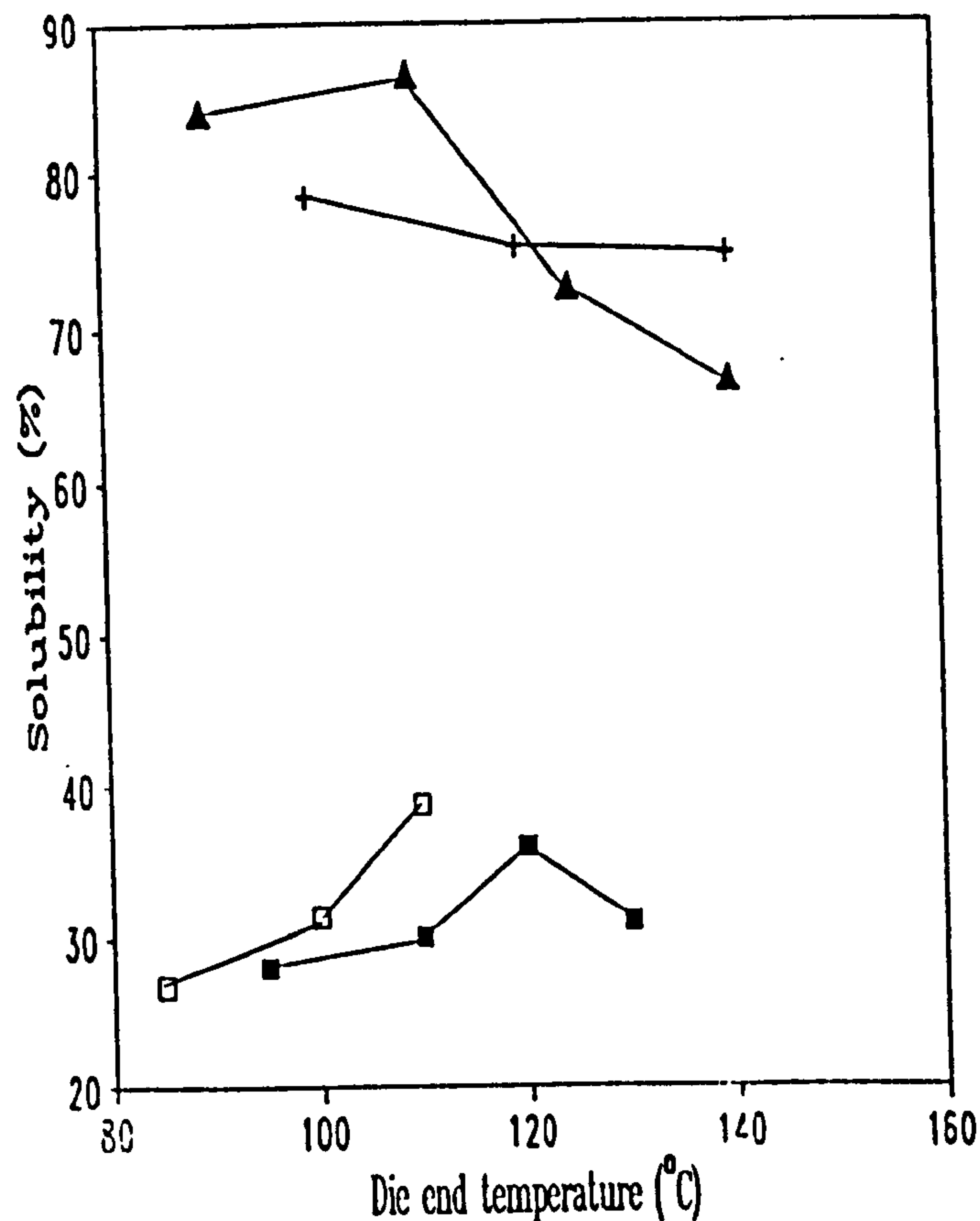


Fig. 10.6 Solubility of microwaved soya extrudates without sugar (▲), with 5 % sucrose (+), 10 % glucose (■) or 5 % xylose (□) as a function of die end temperature.

The microwaved soya and soya containing sucrose extrudates showed high values of solubility. This is an indication that conventional bonds (disulfides and non-covalents) were the major forces in these products. The extrudates containing reducing sugar gave solubility values in marked contrast to the control samples, solubility values were not greater than 40 %. Unlike the control where higher die end temperature seemed to reduce solubility, for the samples containing reducing sugar the solubility values were higher at higher temperatures. For the reducing sugar samples it is possible that some sugars had already reacted with amino acid side chains during

extrusion and were therefore not available for further interaction (during microwave heating).

10.6 *Changes to the protein during processing*

The work indicated that the processing induced crosslinks between the protein. However, the number and the time at which the linking took place appeared to be critical to the production of good textured products. The data from the studies is summarised in Table 10.2. From the work it is possible to predict the conditions that should allow stable extruded products to be formed.

It may be predicted from Table 10.2 that if crosslinking occurs early in the extruder barrel an unhomogeneous melt will result, and hence no textured product could be formed. Microwave heating may induce further crosslinks, at sites not already associated during extrusion. If many early crosslinks were formed, further heat treatment would be less effective in promoting formation of more crosslinks. Natural reducing sugar or breakdown products of sucrose (glucose and fructose) within the soya grits may give rise to some crosslinks at the highest die end temperature. The latter may stabilise the product.

The summary in Table 10.2 also indicates that intimate mixing of the sample within the extruder is beneficial in the later production of crosslinked products. However, if the crosslinks were formed too early then the ability to form a textured product is decreased. It seems likely that the best products occur at a low link density in the extruder, with further linking being potentiated through additional heating of the extruded product. To investigate this further extrusion was carried out using a range of xylose concentration at one die end temperature (section 10.7).

Table 10.2 Summary of quality of extrudates.

Soya grit	Die end temperature	Form of product	Further processing	Solubility	Retort stability
+ xylose* or glucose	High	Brown powder	NA	Low	NA
+ xylose or glucose	Low	Light brown extrudate	No	Moderate to high	Low
+ xylose or glucose	Low	Light brown extrudate	Yes	Low	Good
+ sucrose* or alone	High	Extrudate	No	High	Moderate
+ sucrose or alone	Low	Extrudate	No	High	Low
+ sucrose or alone	High or low	Extrudate	Yes	Moderate	Good

NA not applicable

* xylose and sucrose were used at 5 % concentration, glucose was used at 10 % concentration

10.7 Effect of xylose concentration on compression force and solubility

Die end temperature was important in producing textured products. At 5 % xylose and 100 °C, good quality products were produced, but compared to glucose some increase in solubility was observed for the further processed samples suggesting that the Maillard reaction was greater than optimum during the extrusion process for the xylose (5 %, 100 °C) sample. It is suggested that this was due to excessive early crosslinking or too great a loss of xylose. It was therefore decided to use lower levels of xylose in this section.

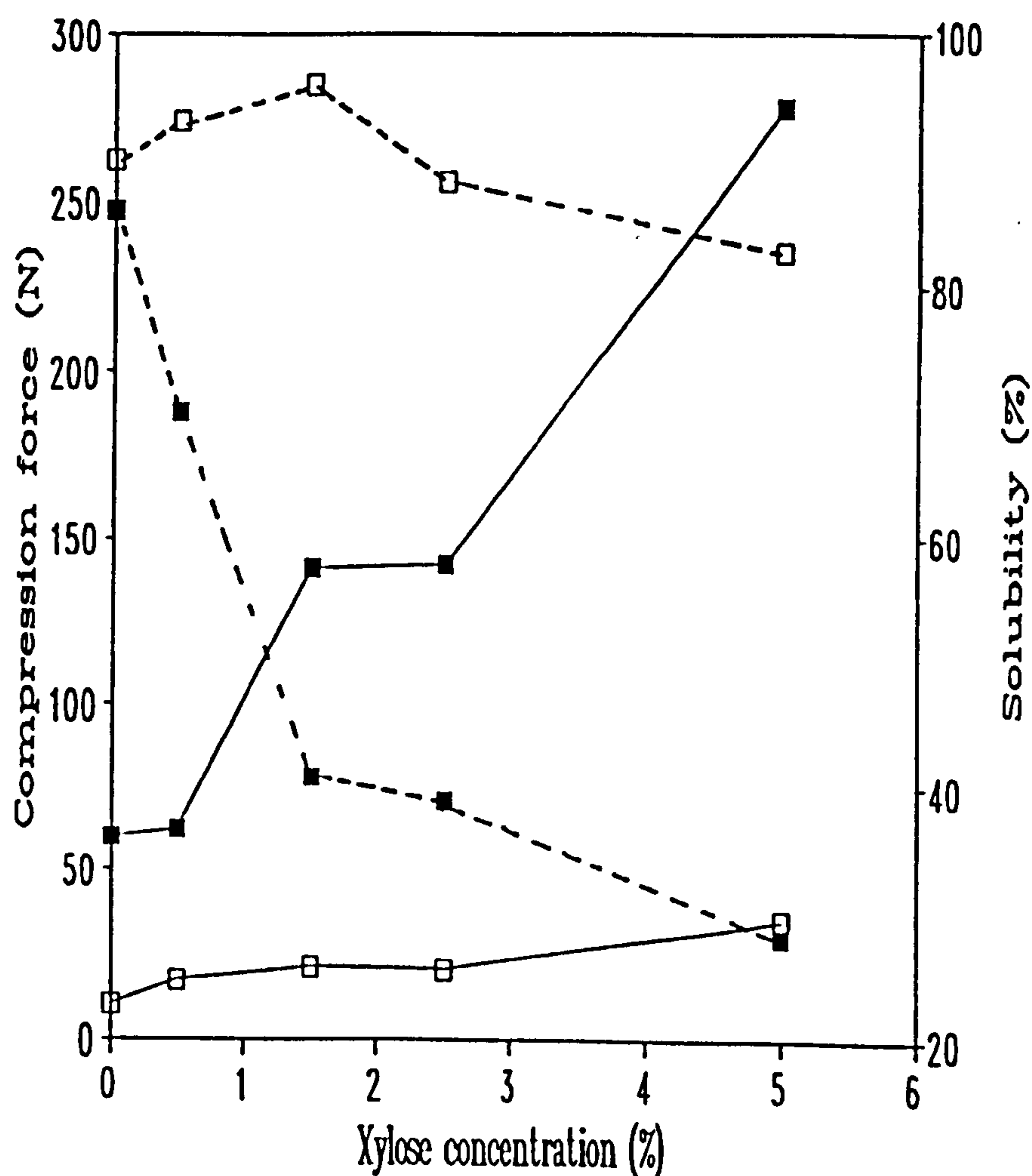


Fig. 10.7 Compression force (—) and solubility (---) of soya extrudates containing xylose before (□) and after (■) microwave heating as a function of xylose concentration. The extrudates were formed at a die end temperature of 100 °C

The compression force and solubility as a function of xylose concentration is presented in Fig. 10.7. Before microwave heating, the compression force was not dependent on the concentration of xylose. The solubility values were high ($> 80\%$). However after microwaving, the compression force became dependent on xylose concentration. The solubility of the extrudates in the mixture of solvents also decreased. These results indicate that the increase in compression force with xylose concentration is related very closely to the decrease in solubility. It is reasonable to postulate that the crosslinks resulting from the Maillard reaction were the major forces involved in enhancing the retort stability of soya extrudates containing reducing sugars. Again the results show if the levels of crosslinking can be kept low during extrusion, the induction of Maillard linkages later in the process would seem to enhance product stability.

conclusion

Additional crosslinks due to the Maillard reaction can be induced in the extruded products containing reducing sugar, after these extrudates were formed at low die end temperatures. These crosslinks are useful where retort stability of products e.g. canned foods, are required. However, the occurrence of excessive crosslinking in the barrel during extrusion at high temperatures can pose some problems to the production of a textured product. This might also explain the narrow optimum temperature conditions quoted for some products. If these products were capable of forming non-sulfide covalent bonds early in the extrusion process the homogeneous melt may never occur.

CHAPTER 11

DISCUSSION

The main aim of this project was to study the mechanism of the Maillard interaction and to consider its application in food processing technology. To do this it is necessary to consider the following questions;

1. What methodology can be used to follow gelation in real time?
2. How did the Maillard gels form?
3. Why did the gels have different properties?
4. Can the Maillard interaction techniques be used commercially?

These questions are discussed in this chapter in light of the work achieved for this project.

To study gelation kinetics, it was necessary to develop a methodology which follows gel formation in real time, without disturbing the gel network. This was achieved using a Bohlin CS Rheometer. The parameters generated by the instrument (storage modulus G' and loss modulus G'') were used to estimate the point of gelation, thus enabled the study of gelation kinetics. A temperature of 90 °C was considered as the optimum temperature as below this temperature gelation rate would be too slow for some systems, and higher temperatures are not possible using the normal geometries. In some cases, a high pressure cell was fitted to the rheometer to follow gelation at temperatures in excess of 90 °C thus allowing rheological parameters of less reactive systems to be measured. This instrumentation may be exploited in the future.

11.1 *Factors effecting the Maillard gelation*

Using the techniques of gelation kinetics it was shown that the type and concentration of sugars and proteins were the key factors to the speed and quality of gelation. Different carbonyl compounds produced gels at different rates when heated with a similar amount of protein. All these compounds may affect the rate of Maillard reaction and other changes associated with it e.g. the decrease in pH and change in charge. Even though the changes induced by the Maillard reaction may be the key factor for the different gelation rate, the extent of gelation may not be necessarily connected to the degree of browning.

It is known that some Maillard products are capable of crosslinking the proteins. If these crosslinkers were available in the solution, either added to the protein or formed via the Maillard reaction, crosslinking between protein chains may occur. More water molecules would be trapped or immobilised via physical means as a result of these crosslinks. It was shown that the crosslinks can form even after the heating of the solution has stopped.

The nature of the protein used was another important factor for Maillard gelation. The protein provided binding sites for the carbonyl compounds which initiated the reaction, and also sites for crosslinking. However a general view that any protein can form gel via the Maillard reaction is certainly unacceptable. There should be specific sites or functional groups that are involved in the crosslinking reactions. It was therefore hypothesised in Chapter 9 that such functional groups may be disulfides and sulfhydryls. It was considered that these might be important spacially and/or chemically. However the use of myoglobin to test the involvement of disulfide bridges in the Maillard crosslinks was questionable, since the pH of the systems was decreasing away from the protein's isoelectric point. This condition was not similar to the normal Maillard systems using bovine serum albumin. Proteins containing little or negligible amount of disulfide e.g. 2S or 5S soya isolate may be used in the future.

Heating and processing conditions also governed the Maillard gel formation. This

may be related to the different extent of the Maillard reaction and changes to the protein at different temperatures. Therefore the gels formed at 121 °C were more brown in colour and opaque in appearance, as compared to those formed at 90 °C. The Maillard reaction was also shown to occur in the barrel during extrusion at high temperatures, and in a microwave during a post extrusion heating. The former was considered detrimental since the occurrence of such a reaction hindered formation of stable extrudates. In fact only brown powders were generated as a result of this interaction. At lower temperatures, it was possible to form stable products by extruding soya grits with xylose or glucose. The improvement in the retort stability of the Maillard extrudates proved that the enhancement of protein functionality by using the Maillard reaction may not necessarily be restricted to thermal gelation.

11.2 *Mechanism of Maillard gelation*

The most obvious change during the Maillard reaction other than browning, was a decrease in pH. The electrostatic interactions between protein chains would increase as the pH of a protein solution was shifted towards its isoelectric point. Therefore, the decrease in pH due to the Maillard reaction was initially considered as the most important mechanism for the Maillard gelation. However, a comparison between the Maillard gels and glucono- δ -lactone gels (Armstrong, 1994) indicated quite a number of differences between the two types of gels. Therefore some other factors should also be considered. At temperatures below 100 °C, the pH of the Maillard systems decreased more slowly as compared to the rapid drop in pH of the glucono- δ -lactone gels. It is possible that this could result in a less aggregated structures of the Maillard gels. Chapter 6 investigated this idea using a combination of rheological techniques and pH measurements. The idea that a similar level of protein would need a similar 'critical' pH for gelation, was tested. Chapter 6 showed that the pH at the gel point was dependent on the type and concentration of sugars. The pH was certainly an important factor, but was not the sole cause of gelation.

The glycation of amino acid side chains via the Maillard reaction caused an increase in net negative charge on the molecules, and therefore the pI of the protein will be shifted downwards. The resultant change in pI would alter the critical concentration required for gelation. Therefore bovine serum albumin (3 %) gel required lower pHs for the critical concentration to occur if a more reactive reducing sugar was used to gel the protein, as compared to a less reactive sugar.

The change in charge was also considered as a reason in the formation of homogeneous gels, with low levels of syneresis, even though the pH of solutions was close to the isoelectric point normally associated with bovine serum albumin. The increase in net charge may cause an increase level of protein-water interactions, which in turn reduced protein-protein interactions. These effects can influence the ways in which the molecules associate and hence the quality of the gels formed. Chapter 8 indicated that the protein aggregates associated mostly in a linear fashion, i.e. "end to end". This could lead to formation of fine stranded gels.

The decreased protein-protein interaction due to glycation resulted in a more uniform distribution of the protein chains and formation of a homogeneous gel. If the decrease in pH alone is considered as the sole cause of gelation, it would be expected that the gels formed to be less homogeneous with a high level of syneresis. It could be imagined that at a pH close to the isoelectric point, the protein-protein interactions were so strong that it could create different levels of protein in certain regions within the system.

As the pH was decreasing towards the isoelectric point, the distance between protein chains was reduced, allowing for crosslinkages between adjacent chains to form. Chapter 5 showed that a heated solution containing the Maillard mixture may form gel if the solution was left to stand at ambient temperature. Covalent and noncovalent bonds were formed and enabled the network to immobilise water molecules. However, it was the Maillard crosslinks which enhanced the strength of the Maillard gels and stabilised the Maillard extrudates during retorting. This conclusion was derived after the Maillard gels and extrudates were not completely soluble in the

solvents that are known to destroy conventional bonds. The attempts to study the nature of these bonds however, were not successful due to the difficulty in dissolving the insoluble products. However it can be postulated that the additional crosslinks occurred within the regions of the protein that contained disulfide bridges, since these crosslinks 'hid' the disulfides against β -ME.

In food science it is the application that matters. Even though the knowledge of the nature of the bonds can be very important, its unavailability will not slow down the use of the techniques in food processing.

11.3 *Possible uses of the Maillard chemical interaction in food processing technology*

Results shown in this thesis suggest that the Maillard chemical interactions may be applied in several other food products. Four possible applications are suggested below;

a. *Pet foods*

Pet foods are normally processed by heating a mixture of ingredients in cans. The interactions of these and association of protein components will result in the formation of textured gel-like foods. Incorporation of reducing sugar into this system may enhance the functionality of the protein components. A lower level of protein may be needed to perform the same function.

b *Simulated meat products for canning*

One way of producing an inexpensive simulated meat products is by blending a high amylose material with an animal or vegetable protein. The high amylose material can act as a binder in the textured products. The problem with this is that amylose is not

stable under normal retort conditions. Amylose, either natural or modified tends to leach out from the textured pieces and retrograde in solution to form a gel or thick layer on the outside of the textured pieces. As a result, the retorted canned products will have an unappetizing appearance and mouthfeel. It may be possible to use the Maillard reaction to replace the use of amylose as a binding agent in these products. The crosslinks formed via the Maillard reaction may stabilise the textured products when they are subjected to retorting (Chapter 10), and may give good simulated meat products with good appearance and mouthfeel. This area may create a research interest.

c Binders for textured soya protein

Textured proteins either in the form of particles, extrudates or granules can be bound together to form a shaped meat analog product. Typically, 7S soya bean protein isolate at concentrations in excess of 50 % is used as a binder for these products. After coating the products with the binder, the products are shaped by heating them in a mould. Heating causes the protein to set or gel, thus holding the textured proteins together. The Maillard reaction may be incorporated in this system by adding a reducing sugar into the soya bean protein isolate. This effect may result in an increase in gel strength and the binding ability of the protein. Alternatively, to save cost, the amount of binding protein can be reduced to lower levels. Gelation due to the Maillard reaction may take place at low levels of proteins. This area may also create a research interest.

d Tofu gels

Packed tofu gels made by coagulating soymilk with GDL often lose 30-40 % of their weight through retort cooking (Nonaka *et al* 1996). The loss consists mainly of water and some water soluble materials. This loss is not desirable as it may affect the appearance and texture of the gels. It may be possible to incorporate a reducing sugar

into the soymilk to initiate glycation of amino acid side chains during retorting. This would be expected to increase the net charge on the protein, thus reducing the amount of water loss. However, the reaction must be controlled so that no excessive polymerisation or crosslinkings take place as these may result in the undesirable tough texture of tofu.

11.4 *Conclusion and further work*

The prospect of the use of the Maillard reaction in food processing technology is promising. The reaction is natural and occurs quite readily in the presence of heat. Some Maillard products have been detected in many daily foods. Exploitation of the Maillard reaction, especially using it to crosslink proteins could well improve the texture of many heated foods. An understanding that it can occur will allow processing of foods to optimum of texture and nutritional quality.

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