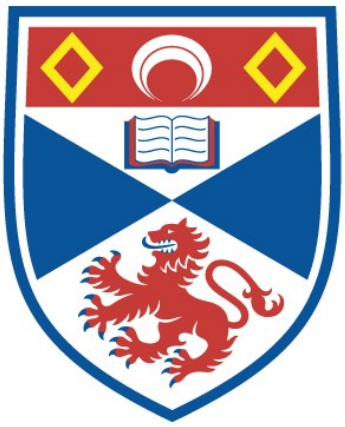
THE DISPERSIVE ACTION OF CONCENTRATED SALTS ON COLLAGEN

John Kerr Candlish

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1963

THE DISPERSIVE ACTION OF CONCENTRATED SALTS ON COLLAGEN

being a thesis presented by

JOHN KERR CANDLISH, B.Sc.

to the University of St. Andrews in application for the degree of Doctor of Philosophy.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Th 5127

D E C L A R A T I O N

I hereby declare that the following thesis is based on investigations carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research, the results of which are presented here, was carried out in the Department of Physiology and Biochemistry, St. Salvator's College, St. Andrews, under the direction of Dr. G. R. Tristram.

C E R T I F I C A T E

I hereby certify that JOHN KERR CANDLISH has spent nine terms based on research work under my direction, and that he has fulfilled the conditions of Ordinance No.16 (St. Andrews), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD.

I matriculated at the University of St. Andrews in October, 1956, passed the examination for the degree of Bachelor of Science in June, 1959, obtained Second Class Honours in Biochemistry, and graduated in June, 1960. Thereafter I was admitted as a research student to the Department of Physiology and Biochemistry, St. Salvator's College, University of St. Andrews, holding a research grant from the Scottish Hospital Endowments Research Trust until February 1963, when I was appointed Assistant in the above Department.

I have been engaged in the present research throughout this time, the results of which are now submitted as a thesis for the degree of Doctor of Philosophy.

ACKNOWLEDGMENTS.

I am first of all indebted to Dr. G. R. Tristram for his encouragement and guidance throughout this work.

I wish to offer sincere thanks also to The Scottish Hospital Endowments Research Trust for their financial support.

Throughout this work I have been aided by many valuable discussions with Drs. F. S. Steven, J. Worrall, and D. Thirkell.

I am grateful to Mr. I. Kinghorn and Mr. E.A. Carstairs for photography, and to Mrs. McAlister for typing.

C O N T E N T S

B/

CONTENTS (CONTD.)

CONTENTS (CONTD.)

4./

CONTENTS (CONTD.)

Note on nomenclature.

In this thesis the term "dispersion" has been used in its most general sense of dividing a colloid more finely (Chambers's Dictionary) without necessarily implying a concommitant denaturation. The terms "lyotropic effect" and "lyotropic agent" have been avoided since they do not appear to be used in any precise sense in the literature.

Where "collagen" is used without a definition of source or solubility, it indicates either the acid soluble calf skin collagen used in this work, or collagen in general, the alternative to be inferred from the context. Discussions of the definition of collagen itself have been provided by Gustavson (1956) and Harrington and von Hippel, (1961).

SECTION I - INTRODUCTION

1. General: arrangement of thesis : properties of calf skin collagen.

Certain concentrated salts have a dispersive or solubilising effect on collagen, this effect forming the basis for the series of experiments hereafter described and discussed. The study fell into three parts each of which is defined and discussed with reference to the relevant literature in the following sections (I, 2 - 4). First of all the dispersive effect was studied, not only as a phenomenon of interest in itself, but also to explore any application of it to the two main projects of this laboratory, namely, studies of the action of enzymes on collagen and the fractionation of its subunits. Secondly, the rate of dispersion of collagen fibres in concentrated salts was used as an index of their stability when reconstitution took place in the presence of other physiological substances. Thirdly, an attempt was made to prepare the non-protein nitrogen of collagen by manipulations in concentrated salt solutions, as a contribution to one of the general activities of the laboratory.

Interrelationships between these topics are discussed where appropriate, especially in Section IV.

The main source of collagen for this work was calf skin. This has been much used for collagen research due to its cheapness and ready availability. A summary of the important properties of calf skin collagen as reported in recent literature is now given.

1) Extraction methods.

Calf skin yields collagens soluble in dilute organic acids (Nageotte, 1927), dilute neutral salt (Jackson and Fessler, 1955), and insoluble collagen (extractable as gelatin by hot water). Gross, Highberger and Schmitt (1955) and Gross (1958) showed that in fact collagen could be extracted from connective tissues over wide continuous ranges of ionic strength and pH. Jackson and Bentley (1960) rationalised the extraction procedures to some extent when they showed that progressively "harsher" solvents (low ionic strength salt → high ionic strength salt → dilute acid → hot water) yielded collagen preparations whose constituent molecules were progressively more aged, i.e. had been in the tissue (in this case guinea-pig skin and granuloma) for progressively longer times. These authors concluded that tissue collagen is a "continuous spectrum" of aggregates, continuously variable in resistance to depolymerisation.

2.) Molecular size and shape.

The data are given in Table 1.

Table 1. Molecular weight and size (calf-skin collagen).

Extract	Method	M.W.	Length and Diameter	Reference
Citrate buffer, pH 3.4	Sedimentation	352,000	-	Doty and Nishihara, (1958)
	Light scattering.	360,000	3100 Å	
	Hydrodynamic	-	3500 Å	
Acetic acid, pH 3.5	Electron microscopy	-	3000 x 15 Å	Rice (1960)
	Hydrodynamic	-	3000 Å	
Acid and neutral salt	Electron microscopy	340,000	2600 x 15-20 Å	Gross, (1958)
Acetic acid	Sedimentation - diffusion	470,000	-	Hannig and Engel (1961)
	Light scattering	350,000	-	
	Sedimentation - viscosity	400,000	-	

3.) Amino acid composition.

The data in Table 2 are the values of each amino acid as residues per 1000 residues.

Purified soluble collagens have a small carbohydrate

content, generally less than 2% (Harrington and von Hippel, 1961). An analysis of the sugar content of calf skin collagen by Gross and Kirk (1958) yielded 1% hexose, 0.2% hexosamine and 0.1% uronic acid.

Table 2. Amino acid composition of calf skin collagen.

Amino acid	1	2	3
alanine	112	107.8	111.7
glycine	320	327.3	325.4
valine	20	22.6	22.4
leucine	25	26.6	24.6
isoleucine	11	12.2	10.3
proline	138	131.8	134.6
phenylalanine	13	14.7	13.3
tyrosine	2.6	3.9	3.0
serine	36	29.2	37.2
threonine	18	18.8	17.0
methionine	4.5	4.5	6.4
arginine	50	54.5	50.7
histidine	5	6.5	5.0
lysine	27	28.8	26.7
aspartic acid	45	47.6	45.1
glutamic acid	72	75.8	71.3
hydroxyproline	94	82.3	86.0
hydroxylysine	7.4	5.1	7.3

1. Piez and Gross (1960), extract as gelatin.
2. Grassmann et al. (1960), for citric acid extract.
3. Piez et al. (1960), for acetic acid extract.

4.) Amino acid sequence and chain configuration.

It has been established that there are discrete regions of high polar amino acid content (bands) and low polar amino acid content (interbands) along the length of the collagen molecule, (Grassmann, 1956; Hodge and Schmitt, 1960). The interbands contain sequences rich in proline and hydroxyproline which are digestible with collagenase (Nordwig et al., 1961). Suggestions for the structure of collagen based on X-ray diffraction studies apply only to the "crystalline" or apolar regions.

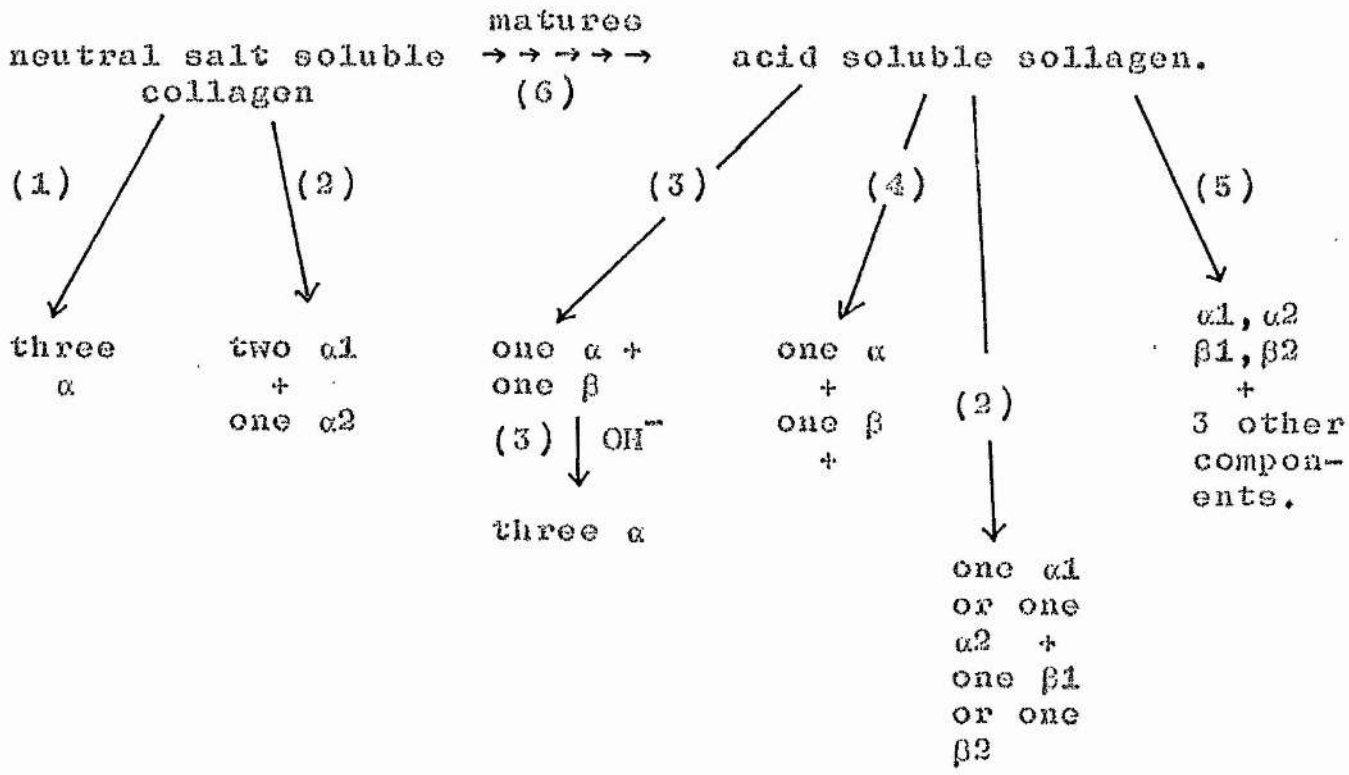
Suggestions for the polypeptide configuration of typical fibrous collagen have been based on (a) the likely repeating amino acid sequence and (b) the high angle X-ray diagram, (Ramachandran and Kartha, 1955) together with (c) the proposed configurations of poly-L-proline (Cowan et al., 1955) and poly-L-glycine (Rich and Crick, 1955). On these bases all agreed that collagen had a triple chain, coiled-coil structure, stabilised by one or two hydrogen bonds per repeating unit. The exact details of this structure have yet to be decided. Reviews of this aspect of collagen chemistry have been provided recently by Astbury (1960), Rich and Crick (1961), and Harrington and von Hippel, (1961).

The X-ray workers cited all assumed that glycine occurs at every third position in the polypeptide chains, since it represents one third of the total residues. The Rich and Crick (1955) structure and the Cowan et al. (1955) structure, which are essentially the same both accommodate the common sequence of -gly-pro-hypro- (Kroner et al., 1955; Schrohenlohor et al., 1959). The absence of hydroxyproline residues in peptide sequences of over twenty amino acids was demonstrated by Manahan and Mandl (1961), demonstrating once again that the collagen sequence is heterogeneous.

5). Subunits.

Calf skin collagen has been much used in studies of the subunits to be obtained from collagen on denaturation of the native molecule. Results are summarised in Figure 1. Subunits have been named α, β and γ by workers in order of increasing molecular weight.

Figure 1 - Subunits of skin collagens.



1. Orekhovich et al., (1960) for rat skin.
2. Piez et al.,(1961) for rat skin.
3. Doty and Nishihara (1958) for calf skin.
4. Grassmann et al., (1961) for calf skin.
5. Schleyer (1962) for calf skin.
6. Harkness, (1961).

From this body of work it appears that the smallest components ($\alpha 1$ and $\alpha 2$) correspond in molecular weight to about one third of the collagen molecule, and three of these make up a molecule of the neutral salt soluble material. In more mature collagen these link through an alkali-labile bonding system to give β components, one of which together

with one α component represents the subunit composition of the acid soluble collagen. In this there is also found a component (γ) which has a molecular weight close to that of the tropocollagen unit itself. These concepts are discussed in more detail in relation to results obtained in the present work. (Sections IV, 1 and IV, 2).

2. Influence of concentrated salts on collagen.

It is well known that electrolytes such as ammonium sulphate and sodium chloride will precipitate or salt-out a given protein should their concentrations reach critical values related to the nature of the protein. However, certain salts can have an opposite effect, namely, the power to disperse or solubilise proteins. With regard to collagen, this difference was early noticed in the leather industry, since when ammonium chloride was used as a deliming agent before tanning, a softer, looser type of leather resulted than when ammonium sulphate was used. (This is quoted by Gustavson, 1956). Gustavson (1926) and Thomas and Kelly, (1927) investigated the powers of salts to solubilise hide and hide powder respectively. They found (see Table 3) that certain

halides, along with KCNS, had a marked dispersive power; sulphates and thiosulphates, however, were less effective in this respect than pure water.

Carpenter and Lovelace (1935) conducted a series of experiments on the effect of salts on the optical rotation of gelatin. (Table 3). It was found that the salts studied caused a drop in the laevorotation of gelatin. The extent of this effect followed the Hofmeister series for anions, the influence of the cations being much less marked, and they concluded that the salts caused an association of protein molecules.

Kuntsel (1937) found that the shrinkage temperature of collagen in 8M KCNS was lowered to 2-3°C. Gustavson (1956) reported an earlier finding that $NaClO_4$ above 1M also caused a drastic lowering of the shrinkage temperature. It was the most destructive salt of the many tested by him.

In recent years the dispersive effect of concentrated neutral salts has often been used as a tool during enzymic or structural studies on collagen. Thus Boedtker and Doty (1956) used 2M KCNS as a solvent for parent gelatin to prevent re-aggregation of its subunits, since this salt was known to inhibit the gelation of ordinary gelatin (Katz and Wienhoven, 1933). Parent gelatins produced by 2M KCNS and by heating had identical viscosities and light scattering molecular weights after dialysis and cooling

respectively. Sanathanam (1959) claimed that nickel nitrate produced in mature tendon an X-ray pattern resembling that of the developing tissue, whereas $CaCl_2$ produced a pattern similar to that of elastin. In each case the original pattern could be recovered by washing away the salt.

Courts (1958) tested 78 chemical compounds at neutrality for their power to liquefy an aqueous gelatin mass, this giving an index of their hydrogen bond breaking potency. The order of activity of salts is shown in Table 3. Courts in the same study extracted alkali-pretreated ossein with LiBr and KCNS. The physical properties of such extracted gelatins "correspondod closely" with those obtained by conventional (heating) methods. Courts noted that the denaturation of the pretreated collagen by salts was irreversible since removal of the salt by dialysis led to a gelatin gel only and not to precipitated collagen. However Courts quotes a communication by Saunders (1956) that the action of KCNS on gelatin is completely reversible.

Blout and Fasman (1958) found that KCNS lowered the optical rotation and viscosity of poly-L-proline II (the structure of which was used by Cowan et al., 1955, as the basis of their collagen structure). This effect

contrasted with that of urea, and these authors therefore concluded that simple explanations cannot therefore be expected to account for the actions of various hydrogen bond breakers on a complex system such as collagen.

Bradbury et al. (1958) reported a lowering of the optical rotation of rat skin extracts treated with concentrated KCNS, KI, and KBr. Harrington (1958) showed that gelatins have a lowered rotation in 12M LiBr, and attributed this to cis-trans isomerism at proline-proline peptide bonds. Venkataraman (1960) found that $CaCl_2$ lowered the rotation of ichthyocol and that the change was reversible on removal of the salt.

von Hippel and Wong (1962) studied the effect of salts on the collagen fold by allowing gelatin solutions to cool in the presence of salts and following changes in specific rotation. They found that they could grade the effect of the salts (inhibition of helix formation) by the use of two constants, one a kinetic measure of the effect of salt on the nucleation of the collagen fold, the other an equilibrium measurement of their effect on the stability of that fold. (See Table 3 for grading of salts).

Some investigators have used $CaCl_2$ solutions as solvents for collagen during enzyme studies. Gallop et al.

(1957) stated that ichthyocol was attacked by collagenase in the presence of 0.5M $CaCl_2$, but not by trypsin. The fall in specific viscosity due to trypsin (about 15% in 2 hours) they attributed to the hydrolysis of gelatin produced by denaturation at the working temperature of 20°. These authors noted that KCNS, $MgCl_2$ $CaCl_2$, and $Mg_2S_2O_3$ solubilised the protein at 0.3 to 1M. (See Table 3). KCNS was considered to have an effect distinct from the others due to its well known denaturing power; this however is erroneous since many of the salts have been shown to lower the rotation and viscosity of collagen, the thiocyanate ion at the extreme of the Hofmeister series being merely the most effective (cf. Table 3). Grant and Allburn (1960) found that in the presence of 0.3M $CaCl_2$ trypsin chymotrypsin and elastase solubilised rat-tail tendon. Hodge et al. (1960) and Kuhn et al. (1961) studied the effect of trypsin on collagen dissolved in tris buffer and 0.5M $CaCl_2$. Soejima and Shimura (1961) noted that the breakdown of gelatin by papain was promoted by Ca^{++} and other bivalent cations. Giffee et al. (1962) found that an enzyme or enzymes from three strains of the Pseudomonas genus plus 2M $CaCl_2$ caused complete solubilisation of calf skin corium, neither being

completely effective on its own. The enzyme collagenase is activated by small amounts of Ca^{++} ions. (Mandl, 1961).

No effect similar to the salt dispersion of collagen as demonstrated in the work described above has been discovered as a normal process *in vivo*, although suggestions and studies with this in mind have been made. Courts (1961) suggested that in the absence of a known enzyme system to disperse collagen in, for example, the involuting uterus, some form of catalysed hydrogen bond breaking mechanism may be involved. Grant and Allburn (1960) suggested that it is possible that calcium concentrations can reach levels in tissues sufficient to enhance the solubility of collagen.

Delaunay et al. (1956) suggested that the abnormality present in many collagen diseases is a derangement of the mucopolysaccharide-collagen bond brought about by the presence of certain electrolytes in non-physiological concentration or by organic (bacterial) substances not normally present. Nometscheck and Ganslor (1961) injected $CaCl_2$ subcutaneously in rats and found that swelling occurred in the collagen with concentrations over 0.5M. The fibres then resembled elastin.

The literature concerning the salt dispersion of collagen is diffuse, much of the information being given

Table 3 - The influence of neutral salts on collagen and gelatin.

Parameter	Decreasing order of activity.	Salt Concentration	Reference
Loss of substance from hide powder treated 14 days.	Cations:- $Ca^{++} > Sr^{++} > Ba^{++} > Mg^{++} > Na^{+} > K^{+}$ Anions:- $CNS^{-} > I^{-} > Br^{-} > Cl^{-} > SO_4^{=} > S_2O_3^{=}$	1M	Gustavson (1926)
Solubilisation of hide powder.	$NaBr > CaCl_2 > NaI > MgCl_2 > LiCl > NaCl > H_2O > Na_2SO_4 > Na_2S_2O_3$	4M	Thomas and Kelly (1927)
Drop in the specific rotation of gelatin.	Cations:- $Li^{+} > Na^{+} > Cs^{+} > Rb^{+} > K^{+}$ Anions:- $CNS^{-} > I^{-} > ClO_3^{-} > Br^{-} > Cl^{-} > CH_3COO^{-}$	soluble range	Carpenter and Lovelace, (1936)
Solubilisation of ichthyocol.	KCNS $MgCl_2$ > NH_4Cl > $MgSO_4$ > NH_4SO_4 > NaCl $CaCl_2$ $Na_2S_2O_3$ KCl	1M	Gallop et al. (1957)
Solubilisation of gelatin.	LiBr, NaI, KI, KCNS > $ZnCl_2$, $FeCl_3$, $NaClO_4$ > $CaCl_2$ > NH_4NO_3, KBr, NaBr	2M	Courts (1958)
Inhibition of helix formation in cooling gelatin.	$BaCl_2 > KCNS > CaCl_2 > MgCl_2 > KNO_3 > KBr > LiCl > CsCl_2 > NaCl > KCl > RbCl > NH_4Cl > CH_3.COOK > (NH_4)_2SO_4$.	0-1M	von Hippel and Wong (1962)
Extraction of collagen from skin.	citrate buffer, pH 3.6 > 10% $CaCl_2$ > ½sat. $Cu(OH)_2$ > 0.1N NaCl.	-	Deasy (1960)

incidentally in papers concerning other aspects of collagen chemistry. The most comprehensive original papers are probably those by Carpenter and Lovelace (1935) and by von Hippel and Wong (1962) but these deal rather with gelatin. Gustavson (1956) has provided a review of the earlier work only.

When the studies reported in this thesis were initiated, there had been no attempts to explore the details of salt dispersion of collagen over a wide concentration range using one or two salts and studying their dispersing actions in detail as examples. It was hoped that this type of study would shed some light on the subject of the salt dispersion and be a contribution to protein chemistry in general.

Mechanism of salt dispersion of collagen and other proteins.

For a review of the earlier ideas on the salt dispersion of collagen we are indebted to the monograph by Gustavson (1956). Earlier workers had used the term "hydrolysis" as appropriate to the phenomenon. (Thomas and Kelly, 1927; Wilson, 1928). Gustavson (1926a) had pointed out that there could be no question of the breakage of primary or covalent bonds; the drastic

effect of some salts on collagen must rather be due to breakage of cross links between protein units. These cross links are now thought to be (in the main) hydrogen bonds. Gustavson (1926b) noted that the action of dispersing salts on hide powder did not affect the electrovalent groups of the material, and so proposed (1956) that salt links were not affected by the treatment. Weir and Carter (1950) came to the same conclusion with respect to the lowering of the shrinkage temperature of collagen by salts.

Gustavson (1956) pointed out that the concentrations of neutral salts necessary for disruption of collagen are those at which the salts are largely unionised, and so the Hofmeister ion series could have no significance. Gustavson visualised salt molecules with coordinating potency competing with the protein subunits for their mutually binding secondary valence forces (hydrogen bonds) and so wedging the chains apart. In support he cited work by Pfeiffer and Wurgler (1927), who had isolated a series of coordination compounds between molecules of salt and amino acids whereby the solubility of the amino acids was enhanced.

The Hofmeister series has been discussed by some workers in relation to their findings for the dispersion

of collagen and gelatin by salts and ignored by others. Carpenter and Lovelace (1935) found that the series was closely followed for the effect of salts on the specific rotation of gelatin (Table 3). Gustavson (1956) while discounting the series finds that it is followed for the solubilisation of hide powder by salts. (Table 3).

The Hofmeister series has its origin in the intensity of the electrostatic field round ions, the small ions having a more intense field than the large ions. A more intense field leads to a greater hydration, and differences in hydration are the immediate cause of the series in aqueous systems (Bull, 1951). Moreover anions and cations tend to orientate water in different directions. The Li^+ ion for example tends to hydrate a colloid since it carries water with the hydrogens sticking outward, these being able to form hydrogen bonds with the colloid, while the SO_4^{2-} ion binds water whereby such interactions cannot occur and thus sulphates tend to have a precipitating effect. If salt dispersion of collagen occurs when almost all the salt is unionised, as Gustavson pointed out, then apparently the ions carry through the properties described above to the salt molecules. Since the cationic Hofmeister series shows a much smaller diversity of effect from member to member

than the anionic, it is probable that the onus of the dispersing effect for collagen will be on the anion portion of the salt molecule; this has been shown to be the case by Gustavson (1926b) and Carpenter and Lovelace (1935).

The central problem to be considered here is how salt can disrupt the collagen structure. We can imagine two possible methods for the breakage of bonds - (a) by competitive binding to the protein and (b), by an alteration in the solvent properties through the presence of salt. Alternative (a) is of course the theory of Gustavson referred to above. Alexander (1951) in this connection suggested that the lithium ion, when water is scarce, fills its hydration shell with such functional groups as OH, CO and NH on the protein.

In the context of alternative (b), Kauzmann (1956b) suggested that lithium salts may act as protein denaturants by ordering water molecules, thereby causing the hydrogen atoms of the water of hydration to become more effective formers of hydrogen bonds than ordinary water. Bigelow and Geschwind (1961), investigating the effect of neutral salts on ribonuclease, tried to relate the degree of denaturation to the activity of water in the denaturing salt solutions. They concluded that there could be no

simple relationship between the two.

Recently von Hippel and Wong (1962) have concluded that the effect of dispersing salts on the collagen fold is due, not to a binding mechanism, but to specific effects on the solvent associated with the protein chains. Consider the equation:-

$$\frac{[SG]}{[S][G]} = K_{eq}$$

where [S] = concentration (activity) of free salt.

[G] = concentration of free binding sites. (The one in maximal possible concentration will be the peptide bond).

[SG] = concentration of filled binding sites.

K_{eq} = equilibrium constant of the system.

Assuming that there might be a process of salt binding, von Hippel and Wong have discussed the implications of all possible values of G and K_{eq}. There are four possible combinations:-

1. K_{eq} large and G large, i.e. the ratio G/S is large and so most of the added salt must be bound. This is unreasonable when one considers that the maximum concentration of peptide bonds in the system was 0.01M, whereas complete suppression of helix formation could only be achieved in 0.7M salt.

2. K_{eq} is large and G is small, i.e. the equilibrium constant favours binding and there are few effective binding sites per chain, which after blocking result in very large segments of the chain being "inactivated". If this were the case, at low concentrations of protein trace amounts of salt would be expected to suppress helix formation completely. Experimentally this is untrue.

3. K_{eq} small and G large, i.e. the ratio SG/S is large. However many salts were found experimentally to cause suppression of helix formation at moderate concentrations, when S = G. This is impossible if K_{eq} is small and G large.

4. K_{eq} small and G small. This implies that sufficient salt can not be added to fill all the sites. Experimentally, however, complete suppression of helix formation is often achieved. With the assumptions of 4, successive concentrations of salt as a function of the stability of the helix should follow a simple adsorption isotherm; experimentally, there is a linear relationship between the two.

In summary, von Hippel and Wong consider that direct binding of ions to gelatin is incompatible with at least one of the following experimental facts:-

(a) The effect is independent of concentration of protein, (b), stability is a linear function of molarity of added salt, and (c), complete suppression of helix formation can be achieved with a variety of salts at moderate concentrations.

A theoretical refutation of the conclusions of von Hippel and Wong has recently been published by Bello (1963). Bello agrees that, referring to points 1 and 2 above, a large value of K_{eq} is incompatible with direct salt binding as a cause of helix suppression, but disagrees with the larger part of von Hippel and Wong's reasoning and especially with points 3 and 4 above. With regard to point 3, Bello states that one ion may possibly "inactivate" four or five (or more) peptide bonds as far as helix formation is concerned. This means that G can be larger than S with complete helix suppression achieved, and thus SG/S can be large and K_{eq} small to fit the equation. As for point 4, this is criticised on several counts, Firstly, it is not necessarily true that the plot of total molarity of salt against fraction of helix suppressed is linear. Deviation is very marked if, for example, values of K = 1 and G = 0.01 are taken. Secondly, the adsorption

isotherm concept may not be applicable to the binding of ions by proteins, since the nature of the protein is altered by partial denaturation. Thirdly, partial denaturation by whatever mechanism may (in Bello's words) "destabilise" the protein toward additional denaturation leading to a proportionately greater effect when additional salt is added.

Bello does not claim that ion binding is in fact operative during the suppression of helix formation by salts, but that the evidence available at present is not sufficient to enable a decision to be made on the mechanism.

It was hoped that experiments planned for the present work might shed some light on these problems.

3. Redispersion of thermally reconstituted collagen.

During investigations of the salt dispersion of collagen, the varying effects of salts were demonstrated by allowing collagen fibres to form at 37° in the presence of a dispersing salt (KI) and in the presence of a precipitating salt (NaCl). (Section III,2 (i)). Consequent to this some studies were made of the effect of different salts on fibres already formed (II, 4, (ii)) and of the effect of various physiological substances in the fibre-forming protein solution on the resistance of the fibres to redispersion.

The thermal reconstitution of collagen is one of the most striking manifestations of its power to reconstitute itself from solution into well defined morphological forms. Gross, Highberger and Schmitt (1955) and Jackson and Fessler (1955) extracted collagen from skin with neutral salt solutions (dilute NaCl and Na_2HPO_4) and observed that these extracts quickly gelled on warming to body temperature. Electron micrographs showed that fibres were produced having the native 640Å longitudinal periodicity.

Nageotte (1927) had first shown that collagen could be reconstituted from solution into fibres, in this case by dialysis of an acid extract. Precipitation of

collagen with dilute NaCl (up to 0.2M) also yields fibres of the native periodicity, more concentrated salt gives axial periods of 220Å, while by 0.5M salt, all the fibres are non-striated. (Gross, 1956). Other distinct fibre forms are the fibrous long spacing (FLS) having an axial period of about 2400Å, (Highberger et al, 1950) and the segment long spacing (SLS) which is in the form of short segments of varying width having lengths averaging 2400Å. (Schmitt et al., 1953). These different forms are considered to result from different alignments of the tropocollagen monomer, inferred to be about 2600Å long and 15-20Å wide. Kahn et al. (1961) have prepared fibres with "flared" regions along their axes. The following discussion deals exclusively with work on native type fibres (640Å longitudinal periodicity).

The spontaneous formation by neutral collagen solutions of fibres having the native structure is of great interest since it has been suggested that this is similar to the process of fibrillation at the fibroblast surface. (Gross, Highberger, and Schmitt, 1955; Jackson and Fessler, 1955). The balance of evidence at present is that collagen is converted from the soluble form into fibres outside the fibroblast (Porter and Pappas, 1959; Gould, 1960), so that collagen molecules shed into

the extracellular space are imagined to polymerise spontaneously. The neutral salt soluble collagen fraction (warmed collagen solutions will only yield fibres in the near-neutral range) is the most recently synthesised of the extracts; it is thought to represent collagen in the first day of its extracellular existence (Harkness, 1961) and some of it may be associated with the intracellular microsome fraction (Green and Lowther, 1959). The process of collagen fibre formation in vivo is irreversible in the sense that in all except specialised tissues or situations, (for example the post-partum uterus), the fibres are metabolically stable and never reabsorbed. (Harkness, 1961). It is striking that the thermal reconstitution of collagen from neutral solutions is also irreversible if the fibres are not recooled within 24 hours (Gross, 1958a). After that time almost none of the collagen will redissolve.

There have been several theories to account for the progressive cross linking between collagen molecules in vivo. Gustavson (1957) has proposed that the process is a form of self tanning. Reactive groups are imagined to be split off from the amino acid residues, the hydration of the structure being lowered, and thus its stability increased. Gustavson states that some amino acids may

take on a quinoid structure, creating potential cross linking facilities. Gross, (1958b) proposed that adjacent collagen molecules, through a process of random thermal movement, gradually assume favourable steric positions for cross linking.

Gross (1956) has discussed the idea that collagen fibre formation is a simple example of the spontaneous association or crystallisation of large molecular weight building blocks to give structures and organelles such as cell membranes, mitotic spindles and connective tissue components; the units have an intrinsic specificity and ability to associate in the form of well ordered structures.

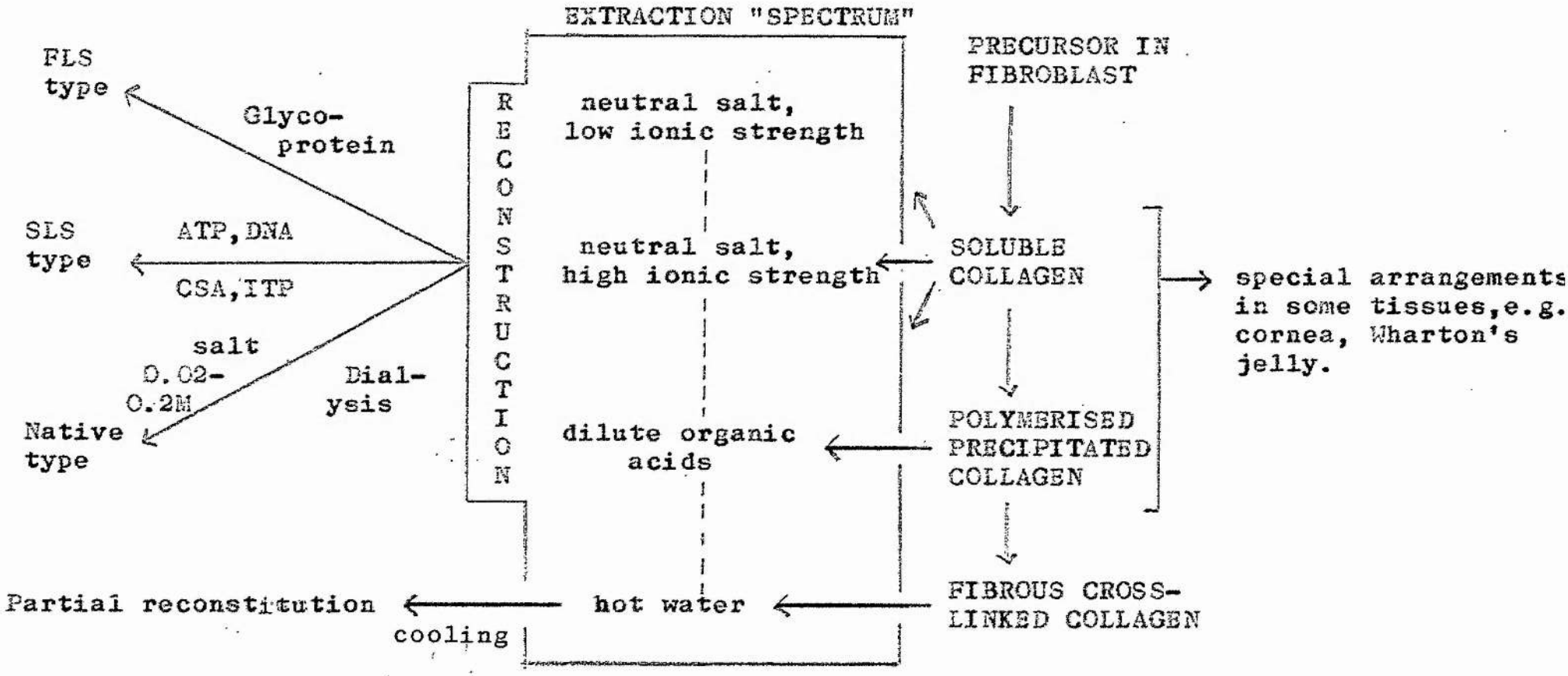
Although it is striking that neutral solutions of collagen polymerise when warmed to 37°, a temperature at which in acid solution it is well denatured, (Doty and Nishihara, 1958), the process will also take place at room temperature, though at a much slower rate. The pH range over which fibres can be made to precipitate is 4-9. Nearly all the collagen in the solution becomes insoluble. (Gross, Highberger, and Schmitt, 1955).

The details of the protein-protein interactions necessary for fibre formation are becoming known. Thus Hodge and Schmitt (1958) gained evidence from ultrasonic disintegration of collagen that randomised "end-tails"

were essential for fibre formation. Hodge and Schmitt (1960) using differential electron opaque stains, demonstrated that polar amino acid residues occur in five discrete regions along the length of the tropocollagen monomer; in the fibre these regions are juxtaposed such that each tropocollagen monomer overlaps the next by a quarter of its length. Kuhn et al., (1960) support this concept. Bensusan et al. (1962) presented evidence that the formation of ionic bonds between the arginyl residues and the acidic residues in the five loci constitute the mechanism of quarter staggering; the ϵ-amino(lysl) groups are seen as strengthening coulombic attractions in general rather than being specific sites for interaction. These authors are in disagreement with Martin et al., (1961) who concluded that histidine has to be non-protonated for normal fibre formation to occur.

Fessler (1960b) found that neutral salt soluble collagen was heterogeneous with respect to fibre formation. It contained three components of which A formed fibres reversibly, B did not yield fibres at all, and C formed fibres irreversibly. Wood (1960a) has interpreted the kinetics of the formation of native-type fibres as implying (a) a nucleation step, i.e. an aggregation into the smallest particles capable of

Figure 2 - The extraction and reconstitution of collagen.



existing as a separate phase, and (b), a growth phase, involving the reaction of soluble collagen with the surfaces of enlarging fibres. Wood and Keech (1960), allowing fibres to form in phosphate buffer at 25° found that the rate of formation was greater at pH 6 and pH 8 than at pH 7 - 7.5. They noted that the process was susceptible to even mild mechanical agitation, and that it was probable that different rates of precipitation might be obtained by the use of different ionic strengths and buffers.

Gross and Kirk (1958) studied the effect of a large variety of substances on the rate of thermal reconstitution of collagen. Their results suggested that low molecular weight substances might markedly influence the rate of collagen formation in vivo. Wood (1962) gained evidence that neutral salt soluble collagen is heterogeneous with respect to the ability of the constituent collagen particles to aggregate to form fibrils, and that this is due to the subunit composition of the molecules. (Fessler's (1960b) fractions A and C gave different proportions of α and β components on thermal denaturation). Acid soluble collagen was found to be less heterogeneous than neutral salt soluble collagen.

Further details of the foregoing literature will be cited where relevant in Section IV. A summary of modern

ideas on the maturation and extraction of collagen is presented in Figure 2.

In view of the intimate association of the polysaccharide components of connective tissue with collagen fibres, many attempts have been made to ascertain whether these are concerned with stabilising or otherwise influencing the properties of the fibres. Thus Gross et al., (1952) found that chondroitin sulphate and glycoprotein could be used to precipitate collagen from solution, but that the effect was non specific. Bazin and Delaunay (1958) showed that polysaccharides prevented the swelling of rat tail tendon collagen. Jackson (1953) concluded from the decrease in stability of tendon after hyaluronidase treatment that chondroitin sulphate was a cementing substance for collagen fibres. Gross and Kirk (1958) tested several polysaccharides for power to speed or retard the rate of thermal reconstitution of collagen, with negative results. Fessler (1960a) gained evidence to suggest that in some tissues a collagen-hyaluronic acid complex is responsible for resistance to mechanical compression. Nemeth-Csoka (1960) found that rat tail tendon fibres, reconstituted in the presence of chondroitin sulphate-A, were more stable than controls. Wood, (1960b) suggested that mucopolysaccharides might be concerned with regulating the growth or nucleation of fibres, or

determining fibril width or size. Keech (1961) found that chondroitin sulphate increased the rate of precipitation of calf dermis collagen, yielding fibres resembling fresh dermis. Heparin prevented gelation - Ohlwiler et al. (1961) found that scar formation was inhibited by heparin - and gave abnormal fibres. Hyaluronic acid gave results similar to controls. Kuhn (1962) regards carbohydrates as contributing to the ageing (lack of solubility) of collagen fibres. Houck (1962) concluded that glycoprotein was not directly involved in the formation of insoluble collagen. Partington and Wood (1963) determined the load-extension curves for rat tail tendon fibres, in conjunction with trypsin and hyaluronidase treatment, and concluded that by their criteria non-collagenous proteins were important for stability rather than chondroitin sulphates A or C or hyaluronic acid.

It would appear that chondroitin sulphate at least interacts with collagen fibres, and was one of the substances which would have to be tested for power to prevent the redispersion of thermally reconstituted fibres during the present work. Other physiological substances which were obvious choices were non-collagenous protein, other polysaccharides, amino acids, salts, cortisone and ascorbic acid.

4. The association of non-protein nitrogen with collagen.

During studies of the salt dispersion of thermally reconstituted collagen it was found that several amino acids, if present in collagen solution during reconstitution, tended to increase the resistance of the resultant fibres to dispersion by salt. This was of much interest in view of the fact that Steven and Tristram (1962a), had separated from collagen and gelatin a mixture of physically bound amino acids and small peptides. These could be removed from the protein by dialysis of acetone precipitation at acid or alkaline, but not at neutral, pH. The material amounted to approximately 2% of the total nitrogen of the collagen. The predominant amino acid was serine. Others present were glycine, glutamic acid, valine, leucine, phenylalanine, and proline. There were only trace amounts of hydroxyproline. Since the physiological function, if any, of this non-protein nitrogen was not immediately obvious, it was of much interest to study any stabilising action by amino acids on collagen fibres.

As well as studying the resistance to dispersion of fibres formed in the presence of non-protein nitrogen, this material was prepared from collagen and studied independently. Three main methods of preparation were used. These were (a) electrodialysis at pH 3.5, (b),

elution from cellulose phosphate columns at pH 3.5 and 5.5, and (c) precipitation by acetone of neutral salt (KBr) extracts of skin. This latter was an attempt to prepare the material without using extremes of pH at any stage to obviate doubts about the possible rupture of weak acid- or alkali-labile bonds.

SECTION II - EXPERIMENTAL.

1. Preparation of collagens.

(i) Acid soluble calf-skin collagen.

Fresh skin from young calves was dehaired and defatted with a scalpel, minced finely, then homogenised in a Waring Blendor with 0.2M Na_2HPO_4, pH 7.6. The phosphate was decanted after two days and fresh phosphate added. This treatment should remove haemoglobin and a large part of the non-collagenous proteins (albumins and globulins) in the skin. After treatment with phosphate for several days, the mince was collected by centrifugation, and extracted three times with 0.1M acetic acid, pH 3.5. The extract was filtered through cotton wool to remove particles of skin and hair. This appears to remove in addition a sticky mucoid-like substance. Collagen was precipitated by dropwise addition of 30% NaCl to a final concentration of 7% (both w/v). The collagen was reprecipitated three times by the same method and stored in this form in the deep freeze. Before use it was dissolved in 0.1M acetic acid and desalted by prolonged dialysis against distilled water.

(ii) Calf bone collagen.

Long bones from young calves were substantially freed from muscle, tendon, etc., and broken into small

pieces with an axe. Fragments with soft tissue still adherant were discarded. The bone was then further disintegrated in the impulse render, after which it was successively extracted with (a), 0.9% (w/v) NaCl to remove non-collagenous proteins, (b), light petroleum for defatting, and (c), sufficient daily changes of E.D.T.A. (15% w/v, pH 7 - 7.5) to produce maximum demineralisation as judged by ash content. The resultant soft fibrous material was finally dried with acetone and stored at 7°C.

(iii) Calcium chloride extract of calf-skin.

Skin was cleaned and extracted with phosphate as before (II,2,(i)) and extracted with 3M $CaCl_2$, pH 7.0. The collagen was reprecipitated three times from this solvent by dialysis against running tap water and subsequently desalted by prolonged dialysis against distilled water. The end product, an opaque gel, was stored in the deep freeze.

2. Preparation of non-protein nitrogen from collagen.

(i) Electrodialysis.

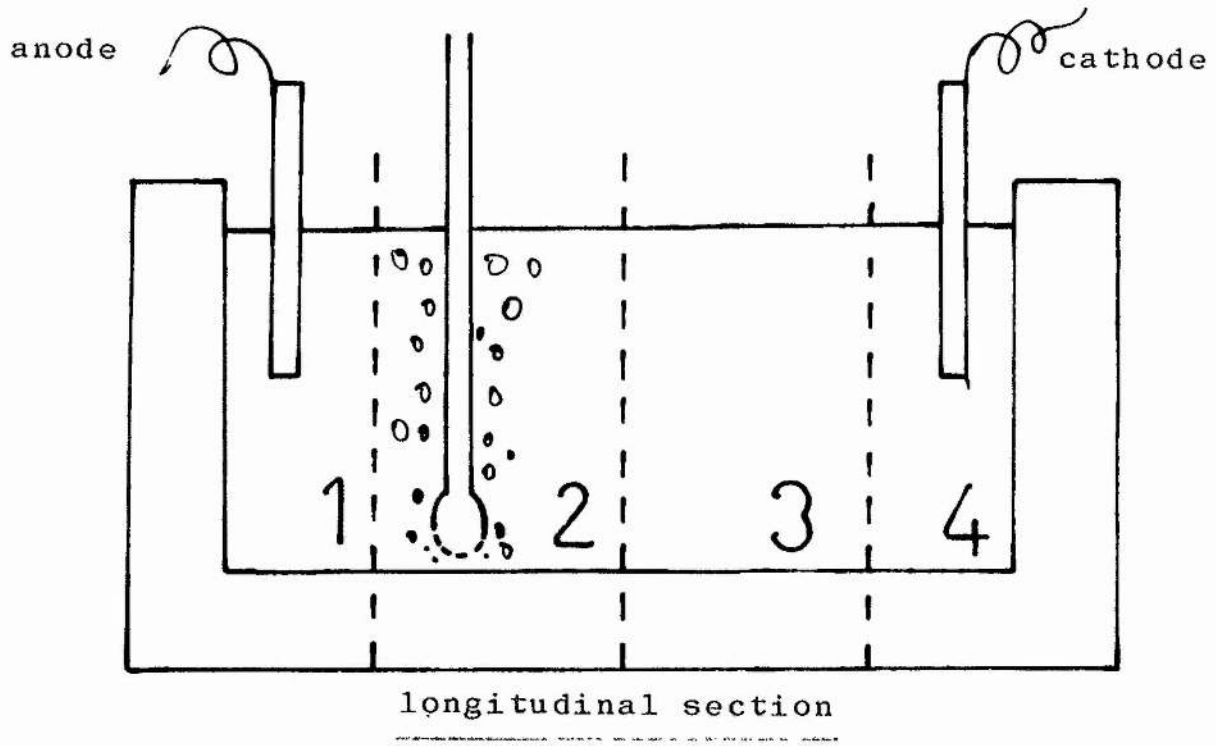
In this work electrodialysis was employed preparatively in an attempt to remove any amino acids or small peptides (non-protein nitrogen) in association with the collagen (see Section I,4). Collagen was held

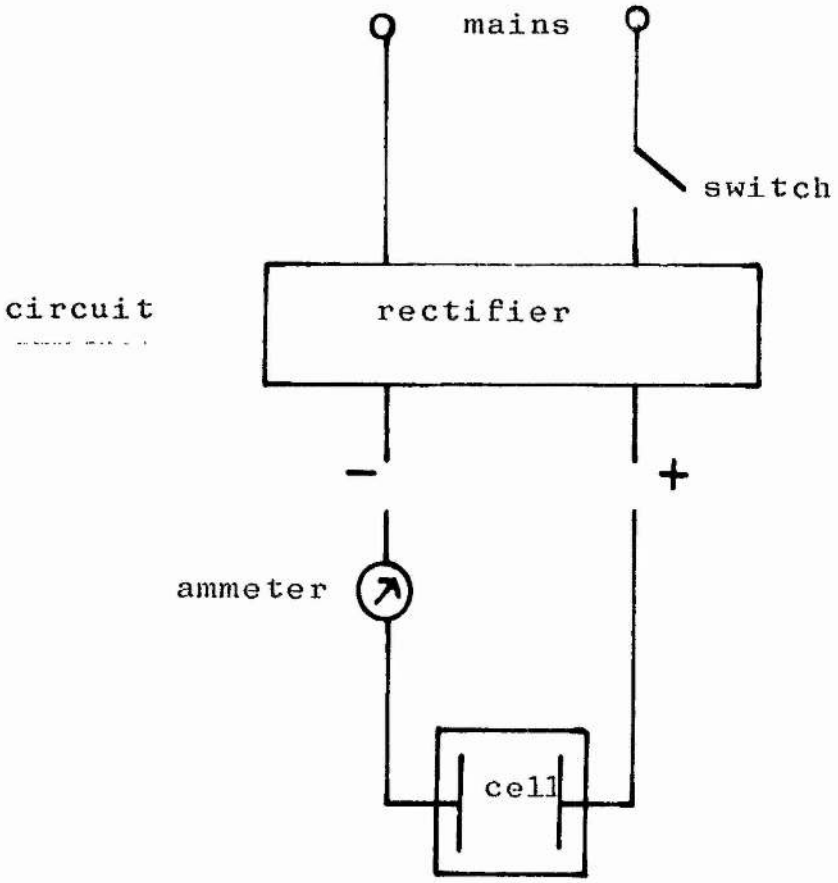
between two electrodes in acid solution so that the non-protein nitrogen, being predominantly in the cationic form, would migrate to the cathode compartment and be collected there.

The arrangement is shown in Figure 3. The units which in juxtaposition formed cells were constructed of 1.5 cm. thick perspex. With three dividing membranes four compartments were produced with individual capacities of 100 ml. The units were clamped together by means of steel frames screwed tight with wing nuts.

Compartment 1 contained the carbon anode bathed in 0.01N H_2SO_4. This was separated from compartment 2 by an anion exchange membrane. (Permaplex A20, supplied by B.D.H.). Compartment 2 contained 75 ml. collagen (27.7 mg.) in 0.1M acetic acid, pH 3.5. The collagen solution was stirred with a stream of nitrogen, and covered with octanol to prevent frothing. Compartment 3, designed to collect the non-protein nitrogen, was filled with distilled water, and was separated from compartments 2 and 4 with cationic exchange membranes. (C20, supplied by B.D.H.). Compartment 4 held the carbon cathode, bathed in 0.01N NaOH. Each compartment was cooled by means of circulating tap-water, the temperature being 18° or below throughout the operation.

Figure 3 - Electrodialysis assembly.





The current from the rectifier being switched on, and set to give a potential drop of 4 volts across the system, any low molecular weight material (ionised) will migrate to the electrodes. Positively charged material travelling to the cathode will be accepted by the cation exchanger, passed to compartment 3, accepted by the second cation exchanger, but the alkali in compartment 4 will tend to reverse the charge of the migrating material (amino acids and peptides) and transmission to this compartment will not take place.

An experiment was performed with this result in mind, the current being passed for ten days. After this time compartment 3 was emptied, the solution therefrom being desalted on Zeocarb 225 (Section II,12). After concentration by vacuum distillation the resultant material was subjected to paper chromatography (Section II, 14).

(ii) Elution of collagen from cellulose phosphate columns.

Tristram (1961) and Kessler et al. (1960), studying the column fractionation of collagen at denaturing temperatures, noted small peaks in the effluent outwith the main collagenous fractions. Since it seemed likely that this material might constitute or contribute to the non-protein nitrogen of collagen described by Steven and

Tristram (1962a), an attempt was made to prepare it by the following method.

Cellulose phosphate (P10, Whatman & Co.) was treated twice with N NaOH (containing also NaCl at 1M to prevent excessive swelling) on a steam bath to remove ammonia, and subsequently washed to neutrality with 1N NaCl. The material was then equilibrated with 0.2M acetate buffer, pH 3.3, and packed in a column of 3.5 cm. x 12 cm.

Collagen in the same solvent (200 ml. containing 24 mgm.) was then applied to the column. It was presumed that physically bound non-protein nitrogen associated with the collagen, if present, might be bound to the ion-exchanger to a different extent than the collagen itself. At this pH exchange takes place between the phosphate groups and protonated basic amino acids and amino acid residues. Two additional factors might be expected to come into play:-

1. Differential hydrogen bonding. It would be expected that in general a large protein molecule would offer more facilities for hydrogen bonds with the cellulose hydroxyls than small peptides or amino acids. Non-protein nitrogen would thus be expected to pass through the column at a faster rate than the collagen.

2. Sieving effects. The soluble collagen molecules (tropocollagen) being highly assymetric and bulky would be

expected to be trapped or retarded to some extent by the intermeshing cellulose chains. Twisting and rotating in random thermal movement they will undergo many collisions (this of course increasing the chances of binding by the ion-exchanger) in contrast to low molecular weight material which will tend to pass through the mesh at a greater rate.

It was hoped that the sum of these factors would yield a separation of non-protein nitrogen from collagen.

Experimentally, the collagen solution was applied to the column and 10 ml. cuts of the effluent taken, the draining times of these being measured in an Ostwald viscometer at 20°. It was found that typically the draining times were the same for the first 15 - 20 cuts, when the viscosity suddenly rose. The first tube with a high viscosity and the one preceding it were rejected and the remainder pooled, desalted (Section II,13), concentrated by vacuum distillation, and examined by paper chromatography (Section II, 14).

(iii) Other methods.

Extracts of skin with neutral salt ($CaCl_2$, Section II, 1,(ii)) were examined for the presence of non-protein nitrogen. There were two main reasons for this. Firstly, it was desired to find if this extract was in

any respect different from the acetic acid extract of skin. Secondly, it was felt necessary to establish whether the non-protein nitrogen could be prepared from collagen without using extremes of pH. If this could be accomplished then objections that the material is an artefact produced by the hydrolysis of weak covalent bonds could be met. The procedures listed below were adopted. In each case the end product was desalted (Section II, 13), concentrated, and examined by paper chromatography (Section II, 14).

1. Dispersion in 0.1M acetic acid and precipitation with 8 vols. acetone.
2. Elution from cellulose phosphate columns with 0.2M acetate buffer, pH 5.5, after dispersion in the same solvent. (cf. Section (ii) above).
3. Dispersion in 3M KBr (pH 7.0) and precipitation therefrom with 8 vols. acetone.
4. The first extract of skin with $CaCl_2$ was dialysed against further quantities of the same salt, this latter being subsequently desalted and treated like the other preparations.

The following procedures were adopted on bone collagen (Section II, 1,(iii)) -

1. Stirring with a mixture of one part of 0.1M acetic

acid to 5 parts acetone. This medium was decanted off after several days.

2. Extraction with neutral 3M KBr, the extracted protein being precipitated with 10 vols. acetone.

In the case of the acetone precipitations, the volume of acetone used was that required to give maximal precipitation of solids (protein + salt).

3. Preparation of dehydro-L-ascorbic acid.

The procedure was that described by Patterson, (1950). 1.08 g. ascorbic acid was dissolved in 10 ml. water and shaken for 15 min. with an equal volume of ether containing 0.66 g. freshly sublimed quinone. The ether layer was removed and the water layer subsequently washed with five 10 ml. portions of ether. Traces of ether were finally removed by suction. The solution resulting from this was used immediately and assumed to contain 100 mg. dehydro-L-ascorbic acid per ml.

4. Production of fibres by warming neutral collagen solutions.

(i) Effect of salts on precipitation time.

Acid soluble collagen in the form of the desalted gel was suspended with an equal volume of 0.2M acetate

buffer, pH 5.5, and homogenised in a Waring blendor for 2-3 minutes. Residual pieces of collagen gel were filtered off on a mat of cotton wool. 4 ml. aliquots of the filtrate (2.2 mgm. protein) were placed in test tubes, up to 2 ml. KI or NaCl (1M) was added to give the required concentration, plus water to make up 6 ml., or 2 ml. water was added for the controls. Thereafter the tubes were placed in a water bath at 37°. The time at which fibres started to appear (visualised by rotating the tubes gently in a strong beam of light) was noted with a stop-watch.

(ii) Effect of physiological substances in the fibre-forming medium on the subsequent resistance of the fibres to dispersion.

A collagen solution in buffer at pH 5.5, prepared as above was the starting material. Some experiments were performed at pH 5.5, but generally, immediately before the start of an incubation at 37°, quantities of this solution were adjusted to pH 7.4 with 0.2N NaOH. 4 ml. aliquots of this were pipetted into test tubes, up to 2 ml. of the desired concentration of substance under study was added, water to make up 2 ml./water alone for the control tubes. The solutions were then immediately placed in a water bath at 37°. In some experiments fibres were allowed to form by warming for

30 minutes, but in the main 15 hours was allowed to ensure complete equilibration. At the end of the incubation time the test tubes were shaken, which resulted in syneresis and the appearance of macroscopically visible protein threads. Thereafter 2 ml. urea or KI was added, and the time taken for complete dispersion of the fibres in each tube noted. The concentration of dispersing agent was chosen such that the dispersion time was neither too long or short to measure.

In experiments involving ascorbic acid, papain digestion (Section II, 10) and ultrasonic treatment (Section II, 12) were used as indices of resistance of fibres to dispersion. In these experiments 10 ml. of the collagen solution at pH 7.4 plus 1 ml. ascorbic acid solution or 1 ml. water were warmed to 37° for 15 hours, shaken, and subjected to papain or ultrasonication for specific lengths of time. Thereafter the residual fibres were filtered off on mats of cotton wool and the filtrate estimated for collagen nitrogen. Tubes without ascorbic acid acted as controls. Corrections were made for the amount of collagen still in solution at zero time, and the nitrogen of the added papain.

5. Estimation of nitrogen.

Samples containing over 0.5 mg. nitrogen were measured by the standard microkjeldahl technique. Samples containing less than this amount were estimated as ammonium chloride by Nesslerisation. In this procedure the nitrogen containing sample was digested for 12 hours with 2 ml., A.R. H_2SO_4, made alkaline and distilled in the microkjeldahl apparatus into 5 ml. 0.1M HCl in a 25 ml. standard flask. The volume was made up and two 10 ml. aliquots taken. 0.5 ml. Nessler's reagent (B.D.H.) was added to each. The extinction was then read immediately at 480 mμ; delay in reading had to be minimised due to the tendency of the reaction mixture to become turbid. Blanks were routinely done with the original solvent, which was not always of A.R. grade. The Nessler's reagent was standardised from time to time with A.R. NH_4Cl.

6. Estimation of protein.

Routinely, collagen solutions were estimated by applying the conversion factor of 17.98% nitrogen (Table 8) to the results of nitrogen estimation. (Section 5 above).

The effluent from columns was estimated by the technique of Lowry et al. (1951). In this, the Folin-Ciocalteau reagent (a phosphotungstic phosphomolybdic acid) reacts

with the alkaline copper-protein complex to give a blue complex with an absorption peak at 750 mμ. Required reagents are:-

A. 2% $NaCO_3$ in 0.1N NaOH.

B. 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartrate.

C. 50 ml. A + 1 ml. B (must be made up daily).

D. Folin-Ciocalteau reagent (B.D.H.) diluted two-fold with distilled water.

0.5 ml. effluent (withdrawn from every second tube) is placed in a test tube and 5 ml. reagent C added. After leaving 10 minutes, 0.5 ml. reagent D is added. After thirty minutes, extinction is measured at 750 mμ. A standard graph was not constructed since it is stated by the authors of the method that absorption is not proportiona to protein concentration. The method however gives a reliable guide to the course of a fractionation.

7. Hydroxyproline estimation.

The Leach (1960) modification of the Neumann and Logan (1950) method for hydroxyproline measurement was used. This is essentially, (a), the oxidation of hydroxyproline with hydrogen peroxide in the presence of alkaline copper sulphate, (b), destruction of excess peroxide with heat, and (c), the reaction of the oxidation

product with para-dimethylaminobenzaldehyde to produce a red complex. In Leach's modification, stages (a) and (b) are combined; the oxidation is carried out at 40° and is complete before destruction of the excess peroxide.

Reagents are:-

1. 0.05M $CuSO_4$ (A.R.)
2. 2.5N (approx.) NaOH (A.R.)
3. 6% (approx.) H_2O_2 (A.R.)
4. 3N (approx.) H_2SO_4 (conc.)
5. 5% para-dimethylaminobenzaldehyde (A.R.) in n-propanol (freshly distilled 95.8 - 98° - fraction).
6. Standard hydroxyproline 0.05 gm. made up to 1000 ml., with the inclusion of approximately 20 ml. concentrated HCl as a bacteriostatic agent. This yields a solution containing 100 µg. per ml.

About 0.1 g. freeze- or oven-dried protein was weighed accurately and hydrolysed by boiling for 15 hours with 50 ml. freshly distilled 5.7N HCl. Thereafter the hydrolysate was made up to 1000 ml.

Procedure:- for one estimation, thirteen test tubes are placed in a rack, to each of which is added 1 ml. of the following:-

1. water.

2-4. hydroxyproline, 5 µg. per ml.

4-6. hydroxyproline, 10 µg. per ml.

7-10. hydroxyproline, 15 µg. per ml.

11-13. test solution.

Thereafter is added 1 ml. copper sulphate solution, and 1 ml. alkali. After thorough mixing the rack is placed in a water bath at 40°. 1 ml. peroxide is added after the mixtures have reached the bath volume (5 minutes). The reaction is allowed to proceed for 10 minutes after thorough mixing, when the tubes are cooled in cold water. 4 ml. sulphuric acid and 2 ml. para-dimethylaminobenzaldehyde solution is added, with mixing after each pipetting operation. The rack is placed in a water bath at 70° for 16 minutes. The resultant red solutions are allowed to cool and the absorption measured at 555 mµ.

Leach (1960) recommends that the line in the standard graph be drawn from point to point rather than straight in the usual manner, in order to allow for negative deviations from Beer's law.

8. Optical rotation studies.

(i) Information available from optical rotation studies.

Recent reviews devoted to the optical rotatory characteristics of proteins have been those of Schellman and Schellman (1958), Todd (1960), and Urnes and Doty (1961). Optical rotation depends upon the symmetry properties of the perturbing forces on molecular electrons and is thus a function of the orientation of groups about the bonds in a molecule. However the complexity of interactions in an optically active molecule overtaxes present theories of molecular structure so that it is generally impossible to set up a theoretical correspondenc between rotation and configuration (Schellman and Schellman, 1958). Regarding proteins specifically, Todd (1960) has stressed that optical rotatory properties cannot as yet be interpreted absolutely and unambiguously in terms of polypeptide structure and therefore great care should be taken in drawing conclusions.

However specific rotation measurements, especially if compared with other measurements of physical change such as viscosity, are capable of yielding much information about alterations in the conformation of protein molecules. If absolute conclusions cannot be drawn from optical rotation measurements, it remains

one of the few physical parameters available which is specially sensitive to spatial configuration.

The factors contributing to the optical rotation of a native protein are:-

1. The asymmetries of the α-carbon and other asymmetric carbons of the constituent amino acids.
2. The specific main chain configuration of the polypeptide superimposed on or otherwise altering or distorting the optical rotation from that of the sum of the amino acids.
3. Environmental conditions, i.e. protein-protein, salt-protein or solvent-protein interactions which tend to produce or relieve strains on the architecture of the protein without altering configuration as such. (Todd, 1960). The sum of these factors determines the specific rotation of the protein.

Schellman and Schellman (1958) have discussed the utility of the quantity "specific rotation", which they state to have no fundamental significance since it is a measure of rotation per unit mass, i.e.

$$[\alpha] = \frac{\text{observed angle of rotation}}{\text{length of tube in dm. x concentration in g. per ml}}$$

whereas the electrons, whose motion results in the rotation of plane polarised light, make a negligible

contribution to mass. They suggest that a more useful quantity is the "mean residue rotation" defined as

$$[R_m] = [\alpha] \cdot \frac{\text{mean residue weight}}{100}$$

For complex protein molecules containing very long chains and many amino acids $[R_m]$ is approximately equal to the average residue rotation, [R], of an interior residue since in such a case the range of vicinal action of residues at the ends of the chains is negligible.

The most common quantity employed in the literature is still however specific rotation, usually measured at the D-line of sodium.

The limitations on the interpretation of data on specific rotation referred to above were kept in mind throughout the present work.

The specific rotation of collagen and gelatin.

Literature values for $[\alpha]_D^{20}$ of skin collagens are given in Table 4 and of gelatins in Table 5. Using the data of Cohen (1955a), Schellman and Schellman calculated $[R_m]$ of collagen as -326° and of hot gelatin as -138°.

Native globular proteins generally exhibit a specific rotation of about -30° to -60° (Cohen, 1955b). To take examples of fibrous proteins, silk fibroin yields

Table 4 - The specific rotation of skin collagens.

$-[\alpha]_D^{\text{Room temp.}}$	Source and solvent.	Reference
350°	Ox hide, 0.2M KCl.	Harrington, (1958).
415°	Calf skin, citric acid soluble	Doty and Nishihara, (1958)
380°	Calf skin, acetic acid.	Rice, (1960)
377°	Guinea pig, neutral salt.	Fessler, (1961)
416°	Rat skin, citric acid.	Burge and Hynes, (1959)
415°	Human neonatal skin, citric acid.	Bakerman, (1961)
408°	Calf skin, acetic acid.	Hannig and Engel, (1961)

Table 5 - Specific rotation of gelatin (equilibrium values)

Source	$-[\alpha]_D$ value for collagen.	$-[\alpha]_D$ hot value for gelatin	$[\alpha]_D$ cold value for gelatin	Reference
Calf skin, acetic acid soluble	380°	120° after 30 min. at 50-90° in acetic acid.	280° after 15 hrs. at 15°.	Rice,(1960)
Calf skin, Citric acid soluble	1000° (405mμ)	200° after 30 min. at 38° in citric acid.	810° after 3 hrs. at 4°.	Engel, (1962)
Ichthyo-col.	350°	110° after 30 min. at 40°.	290° at 2°.	Cohen, (1955b)

-53^{0} (Harrington and Schellman, 1957) whereas feather keratin yields -450^{0} (Dweltz and Mahedavan, 1961). In the unfolded state almost all proteins give specific rotations in the range -80^{0} to -120^{0}.

According to Cohen (1955b) the main chain configuration of a globular protein affects the contribution to specific rotation of the individual L-amino acids (the sum of which would otherwise give a value of -80^{0} to -120^{0} as in the randomised protein) in either of two ways:-

1. The main chain configuration is such as to oppose or reduce the contributions of the amino acids weakly.
2. There are large cancelling effects from enantiomorphic chain configurations.

The abnormally high laevorotation of collagen is then seen by Cohen as probably resulting from the presence of a uniquely large proportion of imino residues producing helices of one sense of twist only. Justification for this statement has been provided by studies on the synthetic polypeptide poly-L-proline II, the structure of which was used as a model for a proposal of the configuration of collagen by Cowan, McGavin and North (1955). (This structure is a left-handed helix). Harrington and von Hippel (1961) have pointed out that, since the specific rotation of poly-L-proline II is -540^{0} and the residue rotation is -290^{0}, the "configurational contribution" is

290°. The corresponding figures for collagen are -400° minus 125° equals 270°, and so it would appear that the two configurations must be similar. Urnes and Doty (1961) have further pointed out that since single gelatin chains appear to be able to form poly-L-proline type helices of high laevorotation, then this quantity must be characteristic of these types of helices rather than the more gradual right-handed three-chain helix of native collagen (Rich and Crick, 1955).

(ii) Experimental procedure.

Two types of polarimeter were used in this work, the Hilger standard polarimeter and the automatic polarimeter manufactured by Bendix Ericcson Ltd. With the former instrument the angle of rotation was read directly by matching the triple field. The automatic polarimeter records on a chart a reading in millivolts proportional to the angle through which the plane of light is rotated. A variable resistance in parallel increases the sensitivity of the millivolt reading. The tube normally used was of 0.2 dm. length. The protein solution was introduced into the tube with an automatic pipetting syringe, which was used to transfer the protein solution from a beaker through the tube and into a waste container, or, if necessary, back into the beaker to form a closed system. The apparatus

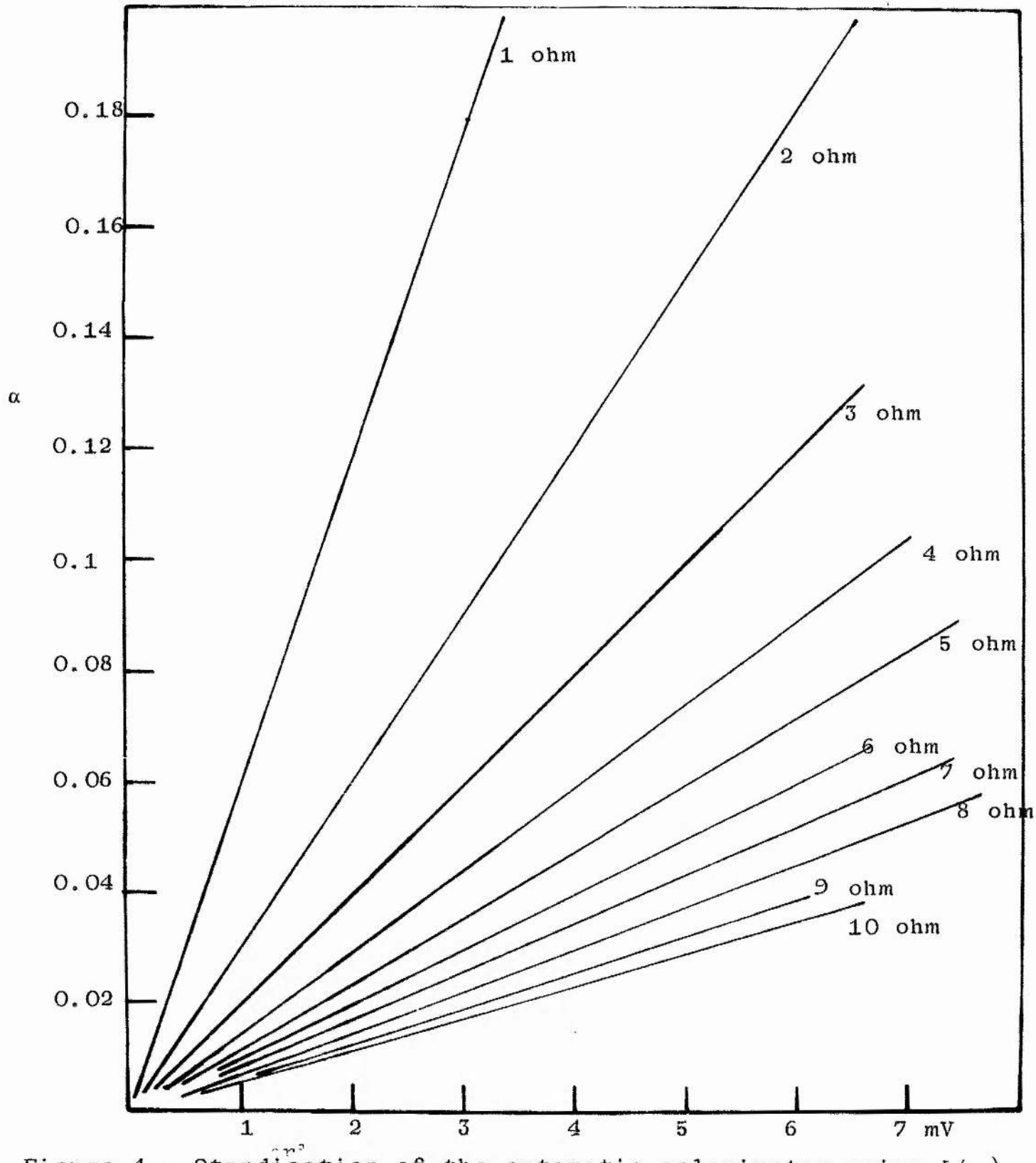


Figure 4 - Standisation of the automatic polarimeter using L(-) cysteine. Ordinate - angle of rotation. Abscissa - deflection in mV.

was calibrated using standard solutions of L-(-) cysteine, which has a specific rotation of -214° in 1 N HCl. (Figure 4)

Measurements of the specific rotation of heated collagen were made with a jacketed one decimeter tube, the water being circulated from an external bath.

For a specific rotation of about 350°, the probable error for any one reading (both methods of measurement) was calculated to be about ±30° if the protein concentration was in the region of 0.1%. The correction for refractive index of the solvent (Harrington and Sela, 1958) falls well within this error. Preparations whose specific rotations were to be compared were made up with the same protein concentration.

The method for making up collagen solutions in salt was as follows,-

Collagen in the form of the desalted gel was suspended with an equal volume of twice the concentration of salt finally required. The mixture was then homogenised and subsequently shaken or stirred for about two days to ensure maximum solubilisation. Thereafter residual collagen was filtered off on a mat of cotton wool. If the concentration of the salt was critical, this solution was then dialysed against a quantity of accurately prepared salt of the correct molarity. Otherwise it could be used at that point after adjustment of the protein concentration if necessary. The

protein content of the solutions was calculated by nitrogen estimation.

9. Viscosity.

(i) Information obtainable from viscosity studies.

The viscosity of a protein solution is related to the asymmetry of the dissolved molecules. Asymmetric molecules in a solvent, turning and twisting in Brownian motion, give the effect of occupying more volume than they do. This interrupts the streamlines of flow and requires additional expenditure of work to maintain a given velocity of flow. The work is manifested as an increase in viscosity (Bull, 1951). Collagen in solution can yield the highest viscosity of any body constituent. Also, since viscometry is quick and convenient it has been much used in research on collagen.

Specific, reduced, and intrinsic viscosities were all employed in the present work, depending on the type of information required. Strictly speaking, these measurements are not of viscosity, since they do not have the dimensions of viscosity, $Mt^{-1}L^{-1}$ (Yang, 1961); however, they are generally used in the literature.

An Ostwald capillary viscometer was used throughout the present work. Assuming that the viscometer is constructed in such a way as to render the kinetic correction

negligible and that the density of the protein solution under investigation is close to that of the calibration liquid (distilled water) rendering a correction for density unnecessary, then the viscosity of the solution is proportional to its draining time in the viscometer, and relative viscosity.

$$\eta_r = \frac{t}{t_o}$$

where t is the draining time of the solution and t_o is the draining time of water at the same temperature. Specific viscosity,

$$\eta_{sp} = \frac{t - t_o}{t_o}$$

The concentration of the protein is introduced into the viscosity measurement by the term specific reduced viscosity, η_{sp}/C, where the concentration C is expressed in arbitrary units. This last quantity is not however independent of concentration since the more concentrated the solution the more will protein-protein interactions tend to contribute to the viscosity. Kraemer (1938) introduced the term intrinsic viscosity, η_i, which corrects for this by taking η_{sp} / C when C approaches infinite dilution.

$$\eta_i = \lim_{C \to 0} \frac{\eta_{sp}}{C}$$

The intrinsic viscosity is thus found by plotting specific reduced viscosity against concentration, and extrapolating

to zero concentration. Its dimensions are dl. $g.^{-1}$ when the concentration is expressed in g. per 100 ml. Intrinsic viscosity is obviously the best quantity to use when comparing the viscosity characteristics of different proteins or different preparations of the same protein. For the changes taking place in a given solution as a function of some other quantity it usually is sufficient to calculate specific or specific reduced viscosity. Intrinsic viscosity can theoretically be used to calculate the axial ratio and molecular weight of a protein, assumptions about the hydration of the protein must however be made and such calculations have not been attempted here.

Tables 6 and 7 show some literature values for the intrinsic viscosity of collagen and gelatin respectively.

Table 6 - Values of intrinsic viscosity for skin collagens.

Value $dl.g^{-1}$	Temperature.	Source and solvent.	Reference
16.5	Not stated	Rat skin, citric acid.	Orekhovich and Shpikiter(1957)
13.5	20°	Calf skin, acetic acid.	Doty and Nishihara (1958)
15.0	20°	Calf skin, acetic acid.	Rice, (1960)
14.5	4°	Calf skin, acetic acid.	Gross and Kirk, (1958)
13.5	20°	Calf skin, citric acid.	Engel, (1962)

Table 7 - Values of intrinsic viscosity for calf skin gelatin.

(Values for η_i of the original collagens can be seen in Table 6).

Solvent	Hot value $dl.g^{-1}$	Cold value $dl.g^{-1}$	Reference
Acetic acid	0.3 at 40°	6-8 after 1000 hrs. at 5°	Rice,(1960)
Citric acid	1 at 38°	1-3 after 100 hrs. at 4°	Engel,(1962)

(ii) Experimental procedure.

The viscometer used throughout most of this work was of the Oswald type with a flow time for water of 52 seconds at 20°. 10 ml. of the required solution was introduced into one of the chambers of the viscometer through a length of polythene tubing attached to a 10 ml. syringe; this avoided the formation of bubbles in the apparatus.

For the preparation of solutions of collagen in KI, $CaCl_2$ or buffer for viscometry, some collagen in the form of the desalted gel was suspended with an equal volume of the solvent of twice the required final concentration, homogenised, and filtered. Since the final solutions were invariably dilute (0.05 - 0.1%) any increase in total volume caused by unfolding of the protein after contact with salt was well

within the experimental error. When the concentration of solvent was really critical, e.g. in the measurements of initial drop in viscosity on heating as a function of KI concentration, the filtered protein solution was dialysed against solvent of the exact final concentration.

The viscosities of KI and $CaCl_2$ solutions *per se* were corrected for by plotting the draining times of the pure salt solutions as a function of concentration and subtracting the appropriate values from the draining times of salt-protein solutions. Temperature was controlled at 20°C. with a water bath.

The protein content of the solutions was calculated from nitrogen estimation.

10. Estimation of proteolytic activity.

Enzyme studies were carried out during this work for the following purposes:-

(a) to determine whether proteinases would degrade collagen in a medium of concentrated salt.

(b) to provide a relative measure of the resistance to degradation of diversely prepared collagen fibres.

For the first purpose a variety of enzymes was used, namely, trypsin, collagenase, papain, hyaluronidase, pronase, and fungal protease. Of these, trypsin was the crystalline

commercial preparation and the rest were commercial powders. The drop in the viscosity of a collagen solution in salt was used as an index of their activity, with an enzyme:substrate ratio of 1:10. A weighed amount of the solid enzyme was added to 20 ml. collagen solution of known concentration (approximately 0.1%) such that this ratio was obtained, and the specific reduced viscosity of this system compared with that of the original. The equilibrium condition was allowed to occur before the final reading. The viscometric technique is treated in detail in Section II, 9, (ii).

For (b) above, the amount of nitrogen released from insoluble collagen fibres after papain treatment was measured. Crude papain is a mixture of enzymes (Smith and Kimmel, 1957). Papain itself has a very wide specificity (Smith and Kimmel, 1957) and so relative resistance to degradation by this enzyme system should be a good index of the relative stability of collagen fibres. 1 ml. papain solution (5% in 0.01M cysteine) was added to fibres prepared as in Section II,4(ii). After set times the residual fibres were filtered off on mats of cotton wool and the filtrate estimated for nitrogen (Section II, 5). Correction was made for the contribution of the papain and cysteine to the nitrogen value and the amount of nitrogen brought into solution calculated as a percentage of the original total nitrogen.

11. Solubility studies.

The measurement of the solubility of a protein which becomes more soluble on denaturation presents something of a problem since at normal temperatures it will be subject to a slow thermal denaturation and the solubility will be partially a function of time. This can be obviated to some extent by allowing the solubilisation to take place in the cold room, but it is obviously essential to allow exactly the same lengths of time for treatments which are to be compared. The major difficulty is then to prepare the starting material, i.e. insoluble collagen, in such a way that the particles have the same total surface area in each aliquot treated. The first method attempted (to find the relative solubility of collagen in salts as a function of pH) was to weigh out equal portions of freeze-dried collagen and shake these with the appropriate solution for 48 hours. Consistent results could not be obtained, almost certainly due to different surface areas of the sponge-like particles. In an attempt to obviate this, 10 ml. aliquots of an acetic acid solution of collagen of known concentration (0.25%) were run from a burette into segments of Visking tubing and the protein precipitated by dialysis against distilled water for 7 days. The sacs were then placed in 1 litre of the appropriate solution (salt + buffer or buffer alone as control

and left for ten days in the cold room (7°C) with occasional manual agitation. At the end of this time, the sacs were washed out with a small volume of distilled water, centrifuged, and the nitrogen content of the precipitate and supernatant measured. This method gave reasonably consistent results but is still not entirely satisfactory since (a) it is difficult to ensure that all the sacs are the same shape on dialysis, which is necessary if the same surface area of precipitate is to be obtained, and (b) it is difficult to ensure that all the material in the sacs has been removed at the end. Moreover the technique is extremely cumbersome.

Later, a technique similar to that described by Gallop et al. (1957) was employed. Collagen in the form of the desalted gel was homogenised with a little distilled water to try to achieve a uniform suspension of small collagen particles. 2 ml. aliquots of this were pipetted into centrifuge tubes, and 2 ml. of the required salt solution added. After shaking for 6 hours, the residual suspension was centrifuged down, washed with two 2 ml. portions of the same solvent, and analysed for nitrogen. Subtraction of this value from the total initial nitrogen present yielded the percentage solubilised. This gave consistent results and is now the method of choice, although it is open to the

same doubts about the achievement of identical surface areas in aliquots of suspension. Even prolonged homogenising left a small proportion of large particles which tended to block the pipette.

To measure semi-quantitatively the power of a variety of salts to solubilise collagen, times taken by them to disperse portions of thermally reconstituted fibres (Section II, 4, (ii)) were noted. 2 ml. portions of collagen in solution/0.2M acetate buffer (pH 7.4) containing 0.8 mg. protein were warmed to 37° for 15 hours. After shaking the tubes to produce visible collagen threads, 0.5 ml. aliquots of various salts of 3M concentration were added, and, with mechanical shaking, the time taken for the threads to completely disappear was noted.

12. Use of ultrasonic disintegration.

In this work ultrasonic disintegration was used to provide an index of the resistance to dispersion of collagen fibres prepared by heat precipitation (see Section II, 4,(ii)) The apparatus used was the M.S.E. ultrasonic disintegrator which has an output of 60 watts at 20 kc./sec.

Experimentally, 10 ml. aliquots of a solution of collagen in 0.2M acetate buffer, pH 7.4, (containing 2.5 mg. protein) plus 1 ml. ascorbic acid solution or 1 ml. water

as control were placed in test tubes and warmed at 37° for 15 hours. Thereafter the tubes were shaken to produce syneresis and macroscopically visible fibres. These were then subjected to ultrasonic treatment for various lengths of time. The residual fibres were then strained off on mats of cotton wool, and the nitrogen content of the filtrate measured. The initial total nitrogen being known, this provided a measure of the protein dispersed from fibres formed with and without ascorbic acid.

The tubes were not jacketed with ice since the experiment was a test of resistance to dispersion in general.

13. Desalting of amino acid solutions.

The cationic exchange resin, Zeocarb 225, was used to desalt amino acid solutions prior to paper chromatography. 2 x 5 cm. columns of the resin (in the hydrogen form) were prepared, and neutralised or weakly acid amino acid solutions allowed to pass slowly through them. Anions and neutral molecules pass through the resin but amino acids and cations are bound. Amino acids were then eluted with concentrated (2N) ammonia. Inorganic cations and arginine are retained by the resin

but amino acids are displaced by the ammonia and can be collected in the effluent. The ammonia was distilled off *in vacuo* before chromatography of the amino acids.

14. Paper chromatography of amino acids.

The system of Redfield (1953) was used exclusively. It is advantageous to have a two-dimensional system for amino acids since a mixture of anything up to 20 of them may be found and these are difficult to separate in one dimension.

Paper - Whatman no. 20. 20 cm. x 20 cm.

Solvent 1 - methanol / water / pyridine
80 / 20 / 4

The papers were left in this solvent overnight, then dried for 30 minutes at room temperature.

Solvent 2 -

tertiary butanol/methyl ethyl ketone/water/diethylamine
40 / 40 / 20 / 4

The papers were left in the second solvent overnight, dried in the air for 30 minutes, and then autoclaved for one hour to remove diethylamine which would otherwise interfere with the ninhydrin stain.

Stain - 0.2% ninhydrin in acetone was used to stain the amino acids. Small peptides are also stained.

A single one-dimensional chromatogram was prepared using n-butanol/glacial acetic acid/water (70:12:24) as the developing solvent.

15. Fractionation of collagen subunits on Sephadex G-50 gel.

Lathe and Ruthven (1956) studied the behaviour of substances passing through starch columns and found that a molecular sieving effect was obtained, produced by the extent of trapping of different molecules of different sizes in the starch. This method of separation has been greatly developed using instead cross-linked dextran ("Sephadex") as the sieving material (Porath and Flodin, 1959; Porath, 1959; Porath, 1960).

Sephadex consists of granules of the polysaccharide dextran, cross-linked to give a three-dimensional network or mesh of polysaccharide chains. The material is water insoluble but strongly hydrophilic, and the polar content is small, being due to the hydroxyls of the glucose units. The dextran chains can be given various degrees of cross-linkage, and thus various degrees of permeability to large molecules. When the substance is placed in water, it swells enormously, and can be packed into a column in the form of a gel. The process of gel filtration consists

of the separation of a substance, for example, a salt, which will enter the gel granules, from a substance, for example, a protein, which will be excluded and thus pass through the column quickly; alternatively, substances entering the granules at different rates can be separated, or large molecules which are excluded from the granules, but travel down the column at different rates due to trapping and frictional effects exerted by the network of interlacing polysaccharide chains. It is most probable that any separation of collagen subunits on Sephadex G-50 will be in the last category since its granules are stated by the manufacturers to exclude dextran molecules above the molecular weight 8,000 - 10,000. If the production of subunits by heat denaturation can be taken as a guide, it is evident that the smallest component (α, of molecular weight 115,000) would be excluded, as would the others (β, 215,000, and γ, 290,000) (Grassman et al., 1961). It is obvious that an attempted separation of collagen subunits based on molecular weight differences has some chance of success, since $\alpha : \beta : \gamma = 1 : 2 : 3$ approximately

A column of dimensions 2 cm. x 21 cm. was found suitable for the present work. Gravity only was used for packing. It did not seem to be necessary to buffer

the collagen-concentrated salt solutions. The course of the fractionations was traced by means of the Folin-Lowry protein assay (Section II, 6). 2 ml. cuts were taken with an automatic fraction collector.

SECTION III - RESULTS.

1. Characteristics of collagens studied.

Ash, nitrogen, hydroxyproline, specific rotation and intrinsic viscosity values are given in Table 8 for the three collagen preparations used in the present work.

Table 8 - Characteristics of collagens studied.

Preparation	Ash %	Nitrogen (ash-free basis).%	Hypro (ash-free basis) %	$[\alpha]_D^{20}$ c = 0.05%	η_i dl.g^{-1}
calf skin acetic acid soluble	0.46	17.98	13.94	-370°	17.5
calf skin 3M $CaCl_2$ soluble	1.33	17.9	13.98	-270°	11.5
calf bone (insoluble)	1.46	17.2	12.78	insoluble	

Since the ash value of the calcium chloride extracted material is so much higher than that of the acid fraction, it is probable that contact with the concentrated salt solutions results in the firm binding of some of the salt. It is also evident that, since the rotation and viscosity values are low, treatment with salt of this nature causes

some unfolding of the protein. An alternative conclusion might be that the calcium chloride extracts from skin a different protein which possesses these physical characteristics, but this can definitely be discounted, not only on the grounds that the hydroxyproline content is almost identical to that of the acid soluble fraction, but also because the treatment of the acid soluble fraction with concentrated salt results in the same type of physical change (e.g. see Table 10 and Figure 5).

For similar preparations of collagen Bowes, Elliot and Moss (1953) obtained 13.62% hydroxyproline (citric acid soluble fraction of ox-skin) and Gross and Kirk (1958) 12.9% (acetic acid soluble fraction of calf skin). The value for the specific rotation of acid soluble calf skin collagen found in the present work is within the literature range but rather lower than the average (cf. Table 4) while the value for intrinsic viscosity is slightly higher than that usually obtained for skin collagens (cf. Table 6).

The comparatively low values for nitrogen and hydroxyproline of insoluble bone collagen presumably indicate an incomplete removal of insoluble impurities during the preparative procedure.

2. The dispersion of collagen by concentrated salt solutions.

(i) Equilibrium optical rotation studies.

Figure 5 shows the values of laevorotation of approximately 0.1% collagen solutions as a function of concentration of KI and $CaCl_2$ in the solvent. The value at zero concentration has been taken as that in 0.2M acetate buffer, pH 5.5. It can be seen that below 0.5M there is no change in rotation, but that at higher concentrations laevorotation drops until at about 3M it is minimal (-90°) for both salts. The drop as a function of concentration is however faster for KI than for $CaCl_2$.

Figure 5 can be compared with Figure 6, where the laevorotation of a solution of collagen in 0.2M acetate buffer, pH 5.5, is plotted as a function of temperature. The native value in this case begins to fall at about 30° but at 35° the collagen precipitates to give opaque fibres (a phenomenon first described for collagen solutions by Gross et al., 1955, and Jackson and Feesler, 1955). The system liquefies again in the region of 50° to give a solution which rapidly approaches the limiting value, (-135° at 55°C). On recooling, a specific rotation of -180° was attained at the starting temperature of 20°.

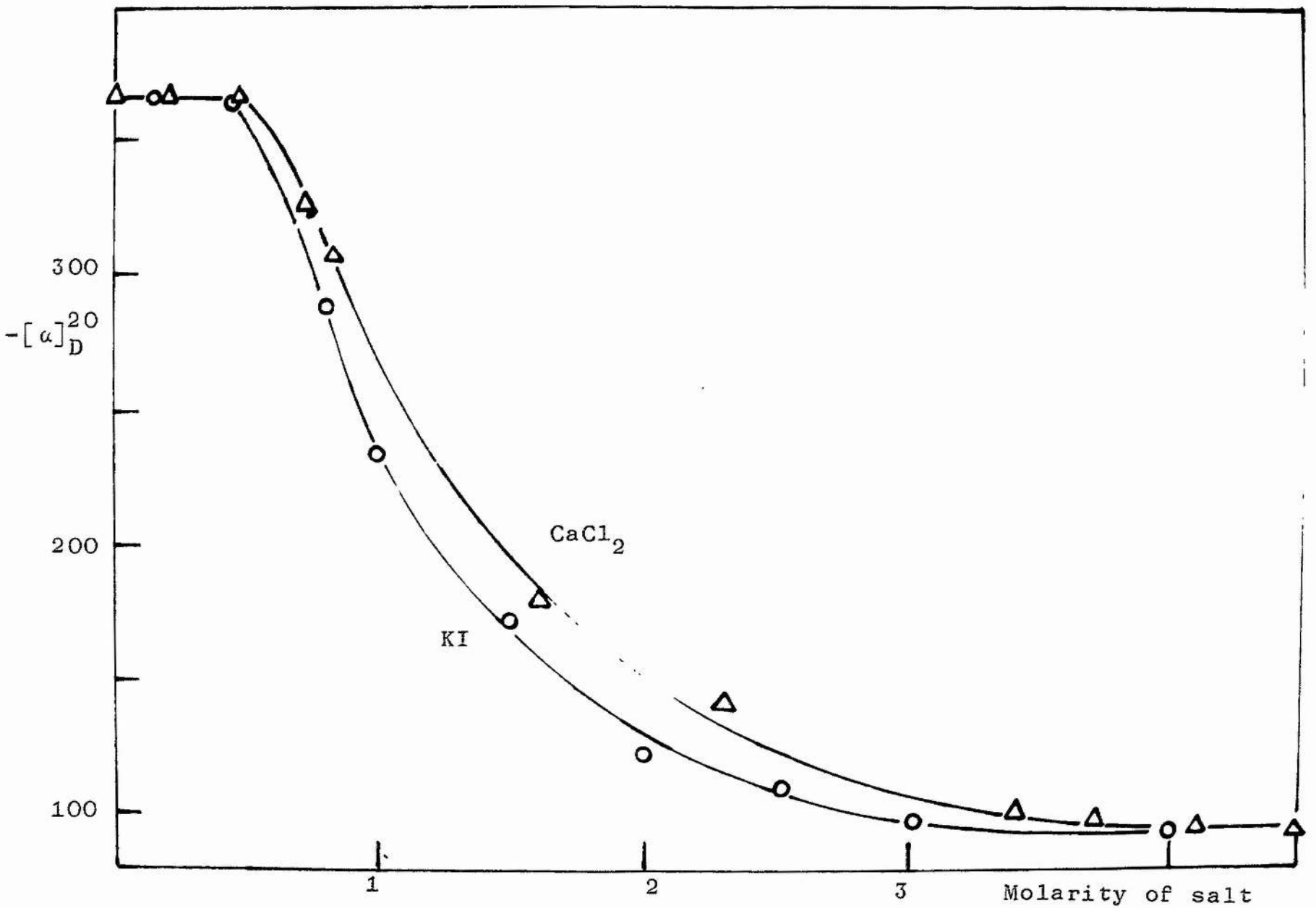


Figure 5 - Specific rotation of collagen in KI and $CaCl_2$ solutions as a function of salt concentration. Protein solutions made 0.1% approximately.

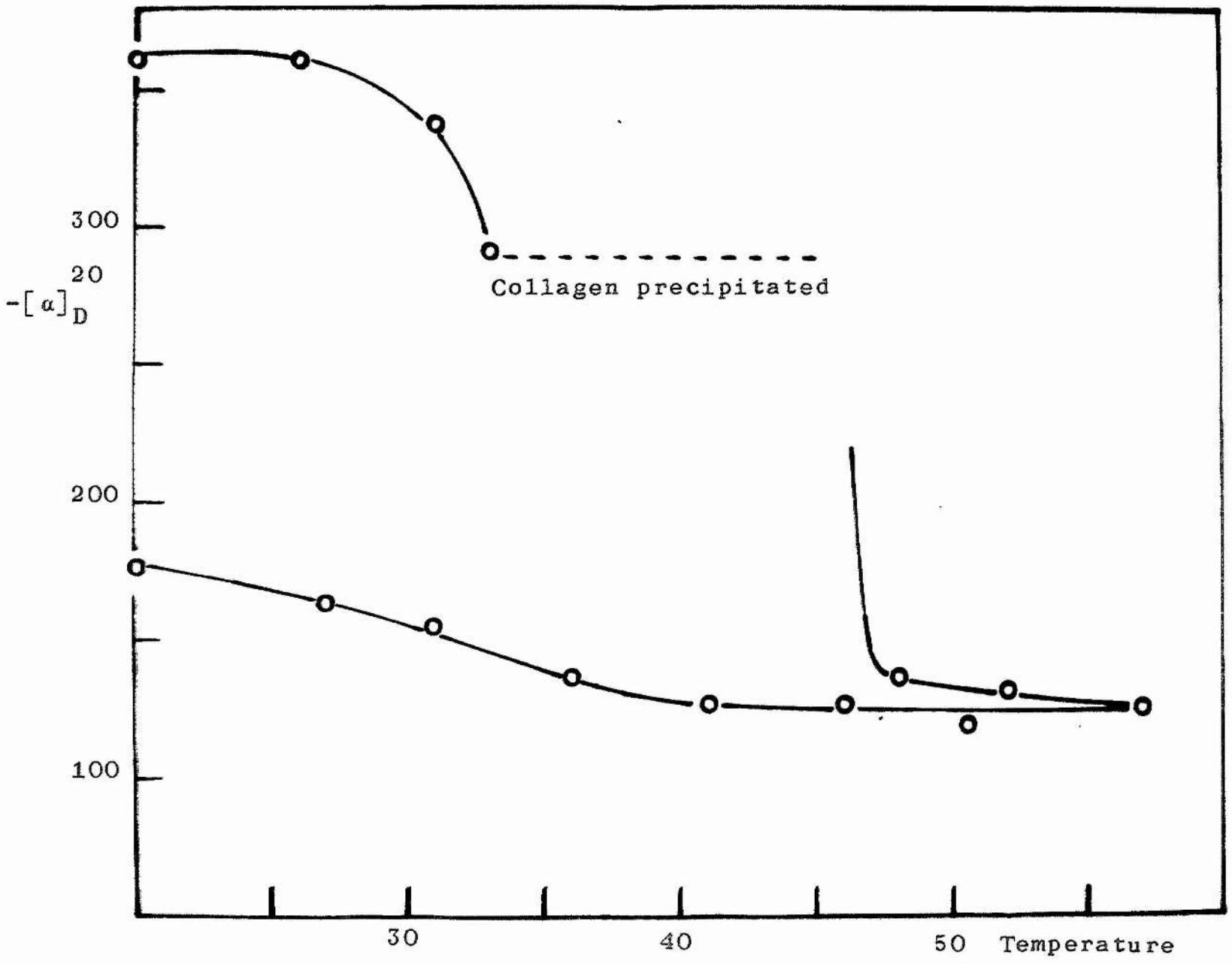


Figure 6 - Specific rotation of collagen as a function of temperature. Upper curve - heating. Lower curve - cooling. Collagen solution - 0.05% in 0.2M acetate buffer, pH 5.5.

The limiting rotation in salt thus appears to be about 40 degrees lower than the limiting rotation in hot buffer. It cannot be inferred from this that the extent of denaturation is greater in salt than in hot buffer; as discussed in Section II 8(i), this difference may be but a reflection of the protein-protein or solvent-protein interactions taking place in two slightly different systems. It is noteworthy that Doty and Nishihara (1958) also found that the limiting laevo-rotation of heated acid-soluble calf skin collagen was 135°.

The importance of a constant protein concentration for comparative optical rotation studies can be appreciated from Figure 7; several solutions of collagen in 1.5M KI were prepared and it was found that the specific rotation tended to vary quite widely with protein concentration.

(ii) Equilibrium viscosity studies.

In Figure 8 the reduced specific viscosity of collagen as a function of KI and $CaCl_2$ concentration in the solvent is presented. There is, as with specific rotation, a threshold at about 0.5M for both salts below which the viscosity is equivalent to the native value (in 0.2M acetate buffer, pH 5.5.). Thereafter the KI

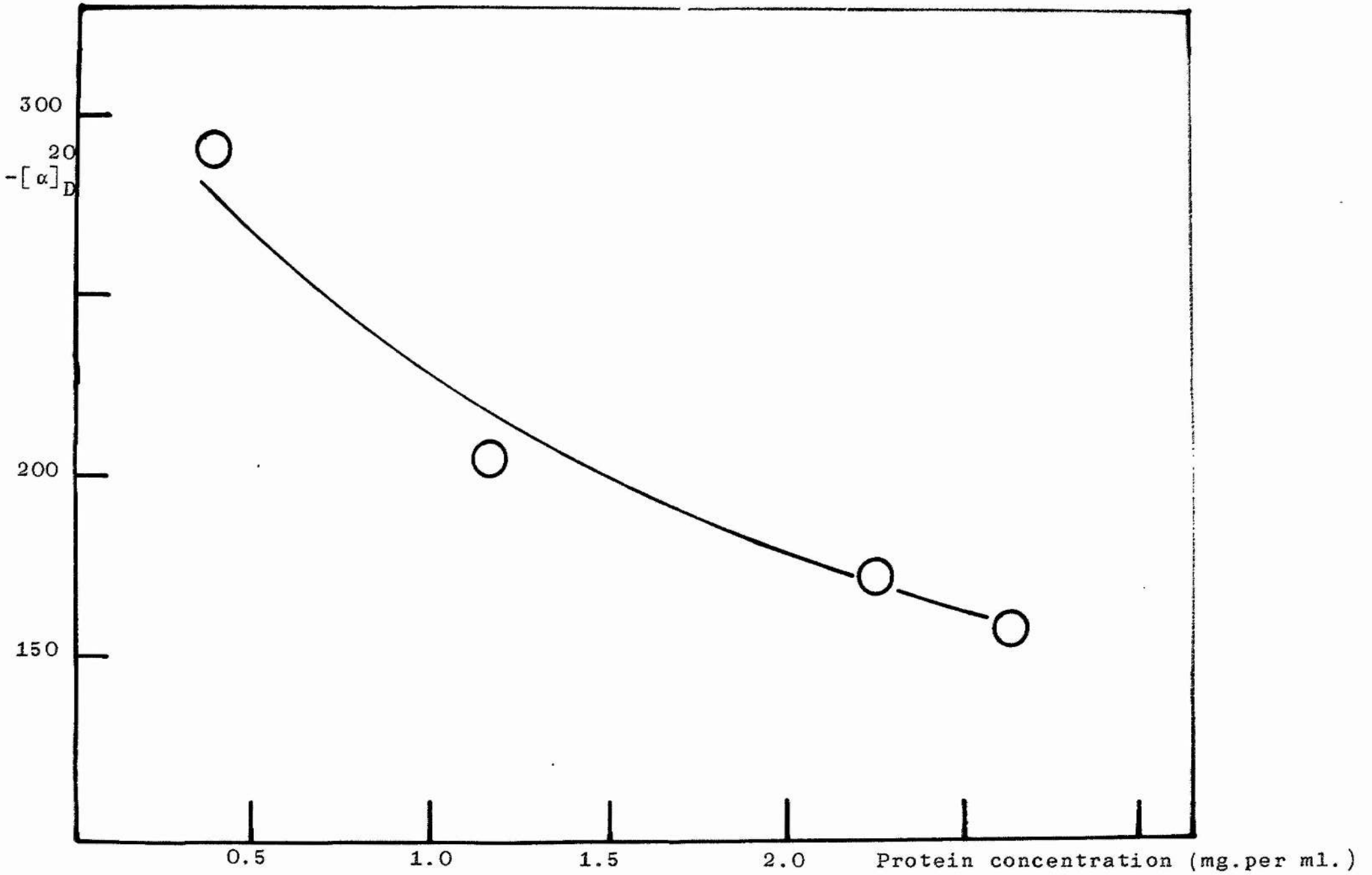


Figure 7 - The specific rotation of collagen dispersed in 1.5M KI as a function of protein concentration.

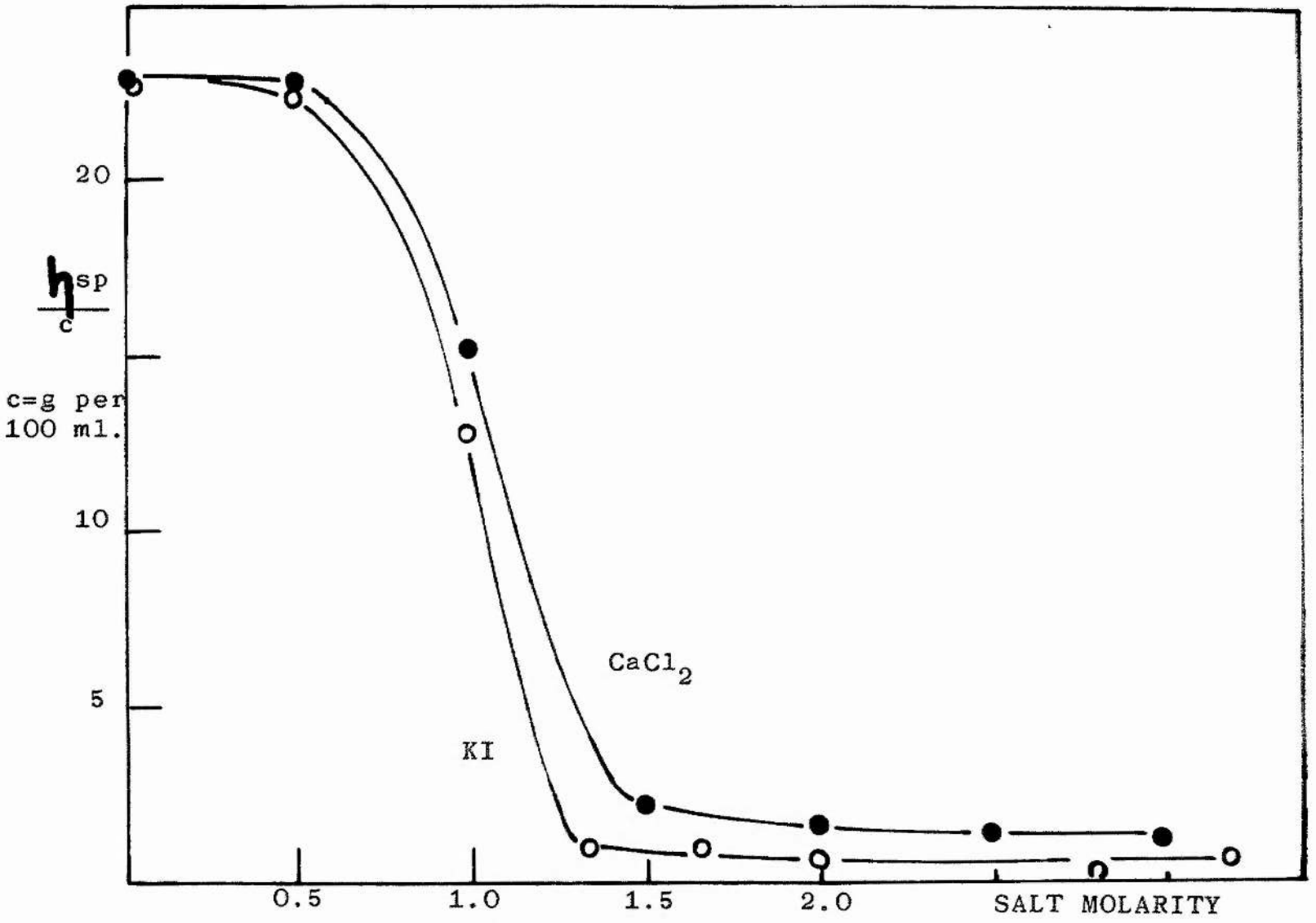


Figure 8 - Specific reduced viscosity of collagen in KI and $CaCl_2$ solutions as a function of salt concentration. Collagen - 0.1% approx.

solutions drop rapidly in viscosity with increasing salt concentration, and the $CaCl_2$ solutions slightly more slowly, but both reaching low limiting values at about 1.5M salt.

Figure 8 can be compared with Figure 9 which shows the reduced specific viscosity changes with heating of 0.2M acetate buffer solutions of collagen, pH 4.0 and 5.5. The draining time for each temperature was taken at the equilibrium for that temperature. At pH 4.0 the viscosity falls rapidly on heating and reaches a limiting value of about 2 dl./g. at 38°; on recooling there is only a slight recovery. At pH 5.5 thermal reconstitution takes place at 36°, and the mixture liquefies again at about 48°. (In Figure 6 it can be seen that in the specific rotation studies thermal reconstitution took place at 35°; this is because in this case readings were taken 30 minutes after adjustment of temperature rather than at complete equilibrium. Complete equilibrium in some cases appeared to take days and this was much easier to follow by draining times than by the more lengthy and difficult optical rotation determinations).

On recooling at pH 5.5, there was a much greater recovery of viscosity and it appears from the shape of

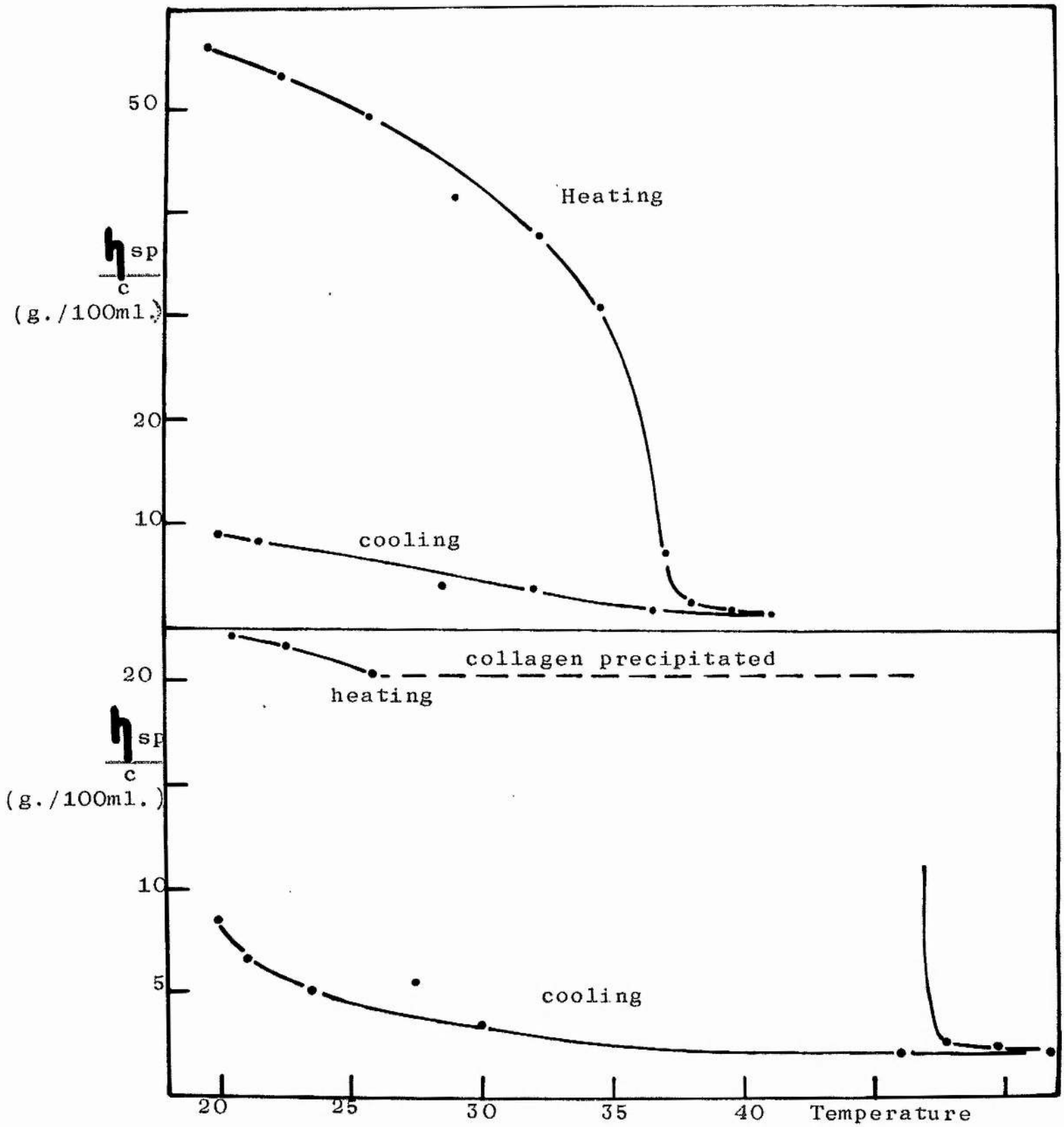


Figure 9 - Viscosity of collagen as a function of temperature.
Upper block - collagen (0.1%) in 0.2M acetate buffer, pH 4.0
Lower block - collagen (0.05%) in 0.2M acetate buffer, pH5.5

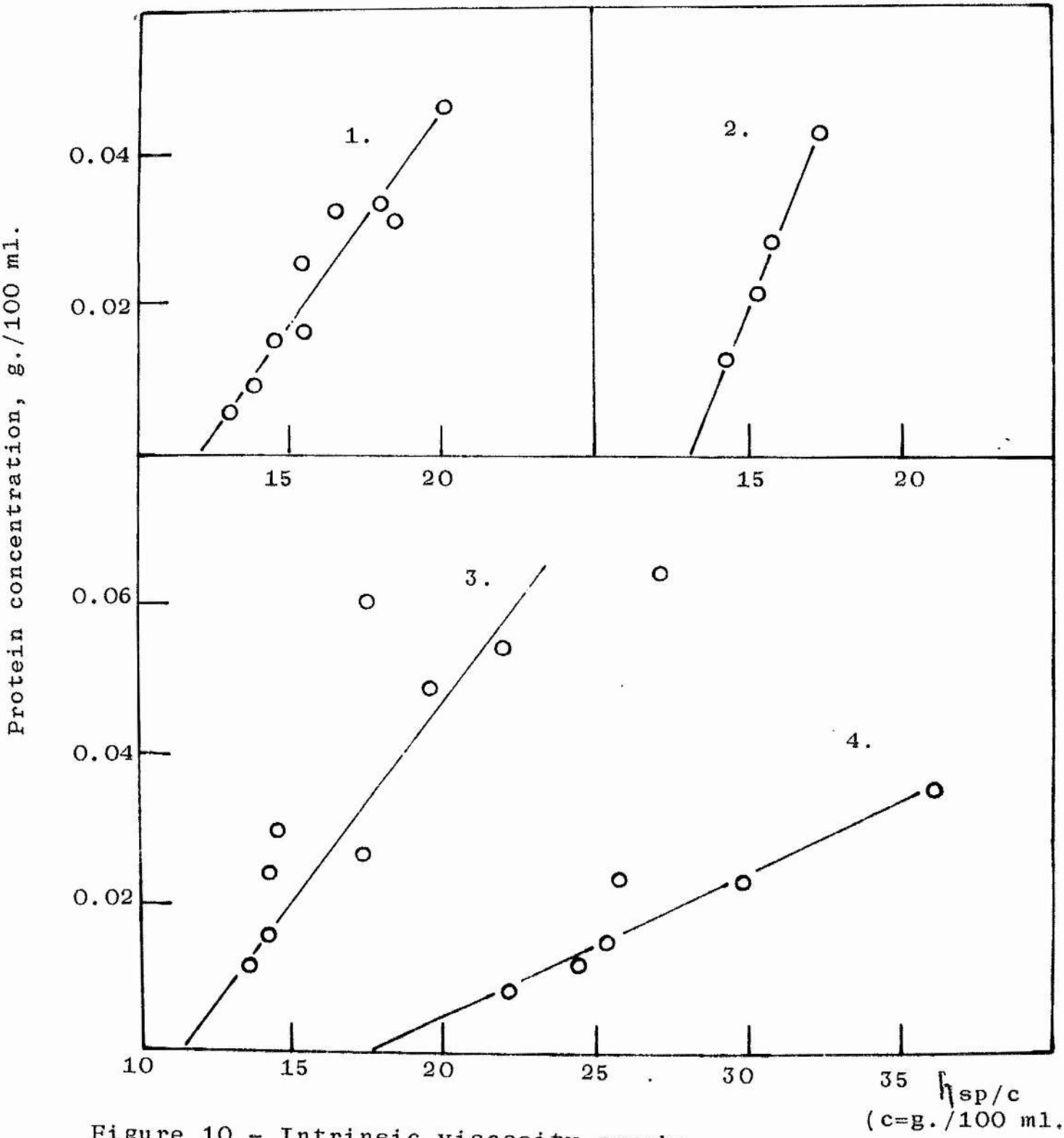
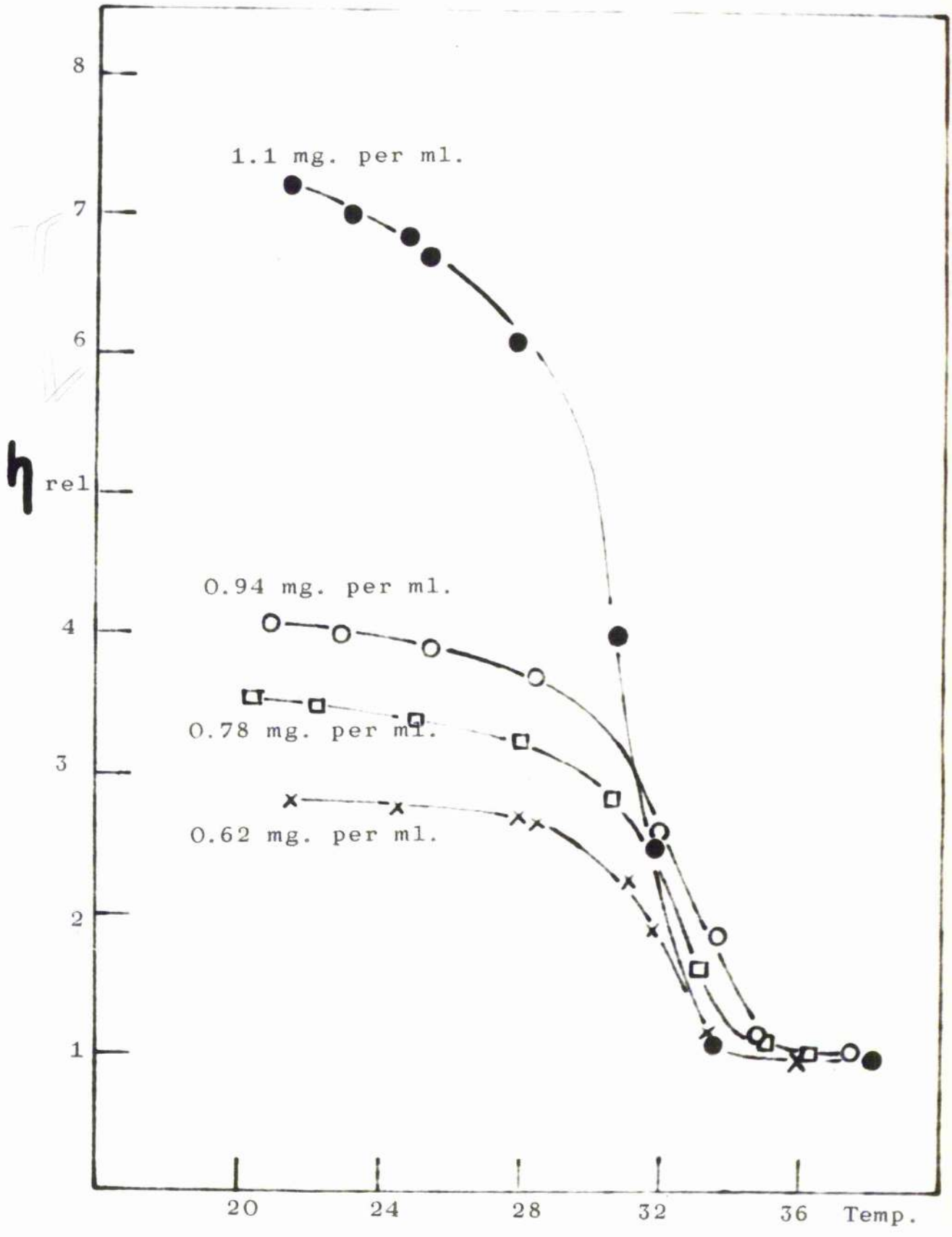


Figure 10 - Intrinsic viscosity graphs.
1.- Collagen denatured by 3M KI, after dialysis. 2.-Collagen denatured by 8M urea, after dialysis. 3.- Collagen precipitated three times from 3M $CaCl_2$. 4.- Native collagen. All dissolved in 0.1M acetic acid, pH 3.5.

the cooling curve that the solution is capable of much higher viscosities at temperatures lower than the defined starting temperature.

The effect of KI on the structure of collagen was explored in another way by measuring the viscosity of collagen in KI solutions of various strengths as a function of temperature. First of all, the viscosity-temperature curves for collagen solutions in 0.5M KI were drawn. (Figure II) There was a sharp drop in viscosity at about 30^0 instead of at 35^0 as for native collagen (see Figure 9). The quantity which was taken for comparison was the temperature at which the first inflexion of the curve takes place. This will hereafter be referred to as the temperature of initial drop in viscosity. This quantity is independent of protein concentration for 0.5M KI and was assumed to be independent of protein concentration for any given salt molarity. Determination of the temperature of initial drop in viscosity for other salt solutions were thus made with arbitrary protein concentrations. In Figure 12 are shown the values of the temperature of initial drop in viscosity for various KI concentrations. The value for zero KI concentration is taken as the temperature of initial drop in viscosity

Figure 11 - Relative viscosity of collagen in 0.5M KI as a function of temperature: the effect of protein concentration.



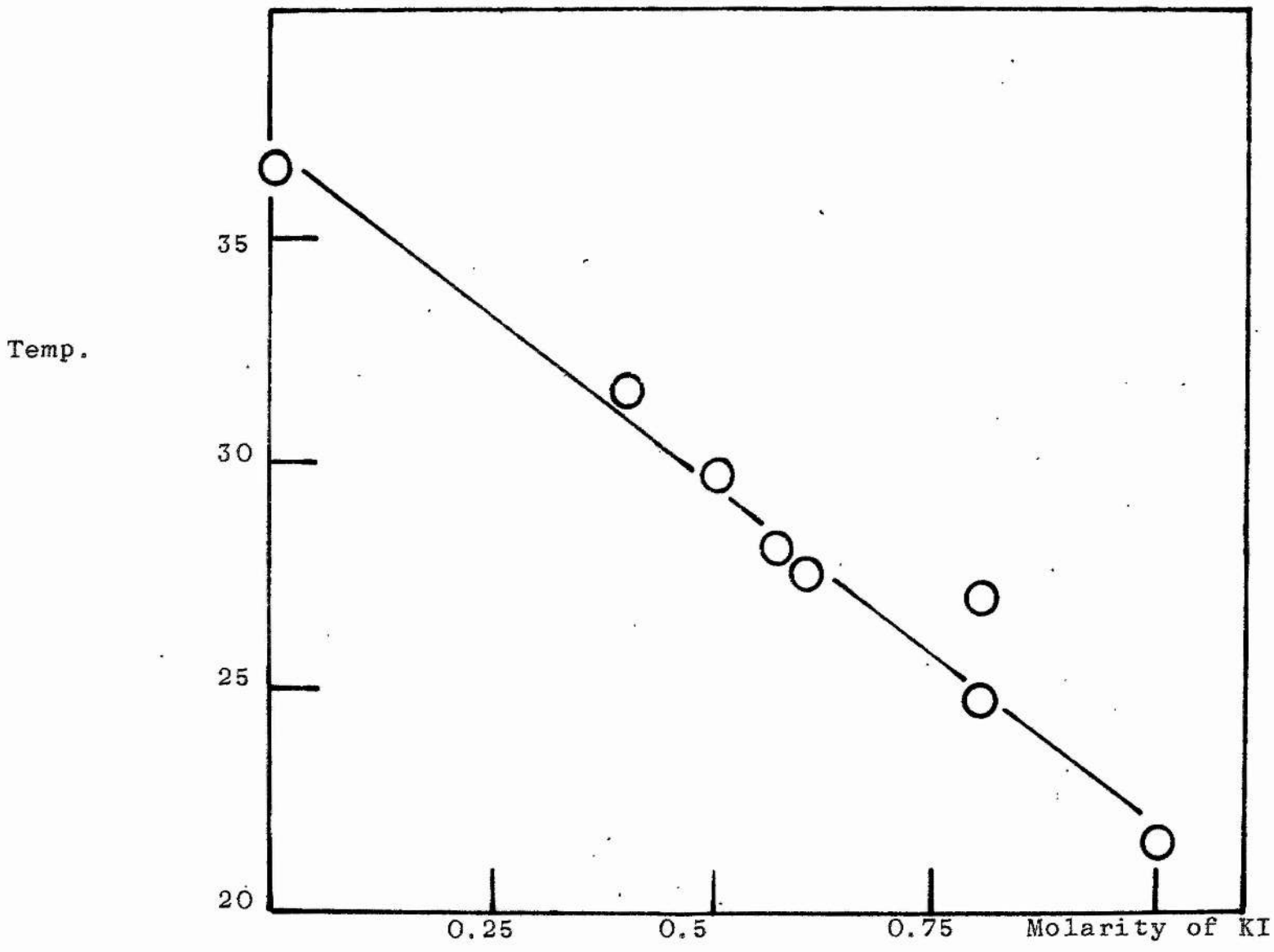


Figure 12 - Temperature of initial viscosity drop of collagen in KI solutions as a function of KI concentration.

for a collagen solution in 0.2M acetate buffer, pH 4.0 (Figure 9). A linear relationship is obtained. Below 0.4M readings could not be taken in neutral solution due to the tendency towards thermal reconstitution at these lower salt concentrations.

In Figure 10 are shown the intrinsic viscosity graphs for 0.1M acetic acid solutions of native, KI treated, $CaCl_2$ treated and urea treated collagen. The values obtained are referred to in other parts of the text. (Section IV, 1).

(iii) Kinetic viscosity and optical rotation studies.

To study the rate of salt denaturation of collagen (i.e. dispersion in salt above 0.5M), the first method attempted was to bring the desalted collagen gel into contact with a specific concentration of KI, and then, taking this time as zero, quickly homogenise the mixture in a Waring blendor, filter it through cotton wool, and transfer 10 ml. of the filtrate to a viscometer. Although this was done as rapidly as possible and the first reading was taken within about five minutes of the original contact no subsequent change could be detected in draining time after the first reading. This was attempted for final concentrations of 0.75M and 3M KI.

To try to speed up the first reading to some extent;

a solution of collagen in 0.35M KI was prepared. From Figures 5 and 8 it is apparent that no denaturation occurs at this concentration, and thus KI of higher (denaturing) concentration can be added, mixed quickly with the collagen solution, and introduced into the viscometer. It was found with this method that the first reading could be obtained within three minutes of the mixing. This was tried using final KI concentrations of 1.18M and 0.72M, but in neither case could any subsequent drop in draining time be detected after the first reading. An attempt using 0.2M acetate buffer, pH 5.5 as starting solvent yielded similar results. It must be concluded that the viscosity drop of collagen caused by KI treatment is accomplished in a very short time i.e. under five minutes. A temperature of 20° was used for these experiments.

In a parallel study, the automatic polarimeter was used to try to find the rate of change of laevorotation of collagen after contact with concentrated KI. Here, 25 ml. of a solution of collagen in 0.2M acetate buffer, pH 5.5, were flushed through the cell of the polarimeter and a beaker continuously in a closed system until the trace was steady giving $[\alpha] = -381°$. 10 ml. of buffer was then added to the system, and mixed with it as rapidly

as possible. The time for final equilibrium due to dilution with the buffer could be read from the chart after final equilibrium conditions were obtained and used as a control. In one experiment where the initial concentration of collagen was 0.3 mg. per ml. addition of buffer as above gave a drop in rotation which was complete in about 140 secs. When however 10 ml. of 4M KI were added (to give for the whole solution 1.6M KI) the final equilibration took 210 secs., reaching a lower value on the chart than the control. It appears from this that the total denaturation time (as indicated by rotation) was about 3½ minutes, but that the rate for two-thirds of this time could not be followed quantitively since it takes 140 secs. for the solutions to be mixed and for the apparatus to adjust for the dilution. This is then hardly a practical method of measuring rate of salt denaturation; it would appear that other methods will have to be tried for this, perhaps involving the use of low temperatures to slow down the reaction.

(iv) Effect of protein concentration.

Equilibrium studies of the effect of KI and $CaCl_2$ on collagen showed that this effect is independent of protein concentration in the limited sense that above

0.5M salt a decrease in viscosity and specific rotation was always qualitatively observed. However both specific viscosity and specific rotation are dependent on protein concentration (Figures 7 and 10) due to protein-protein interactions. An indication that the cohesion of collagen is independent of protein concentration in KI can nevertheless be obtained from the fact that the temperature of initial drop of viscosity in KI is independent thereof. (Figure 11).

(v) Solubility studies.

In Figure 13 the solubility of collagen in dilute solutions of calcium salts over the neutral pH range is shown. There is a solubilising effect at lower concentrations than the 0.5M threshold for denaturation (Figures 5 and 8). (The technique using collagen in dialysis sacs as described in Section II,11, was employed for this).

The effect of extremes of pH on the solubility of collagen in 1.5M KI and $CaCl_2$ is shown in Table 9 (the technique with collagen suspensions being used). The maximum solubilising effect of the salts appears to be exerted around pH 6, although the effectiveness rises from pH 8 to pH 13. The solubilising effect is depressed

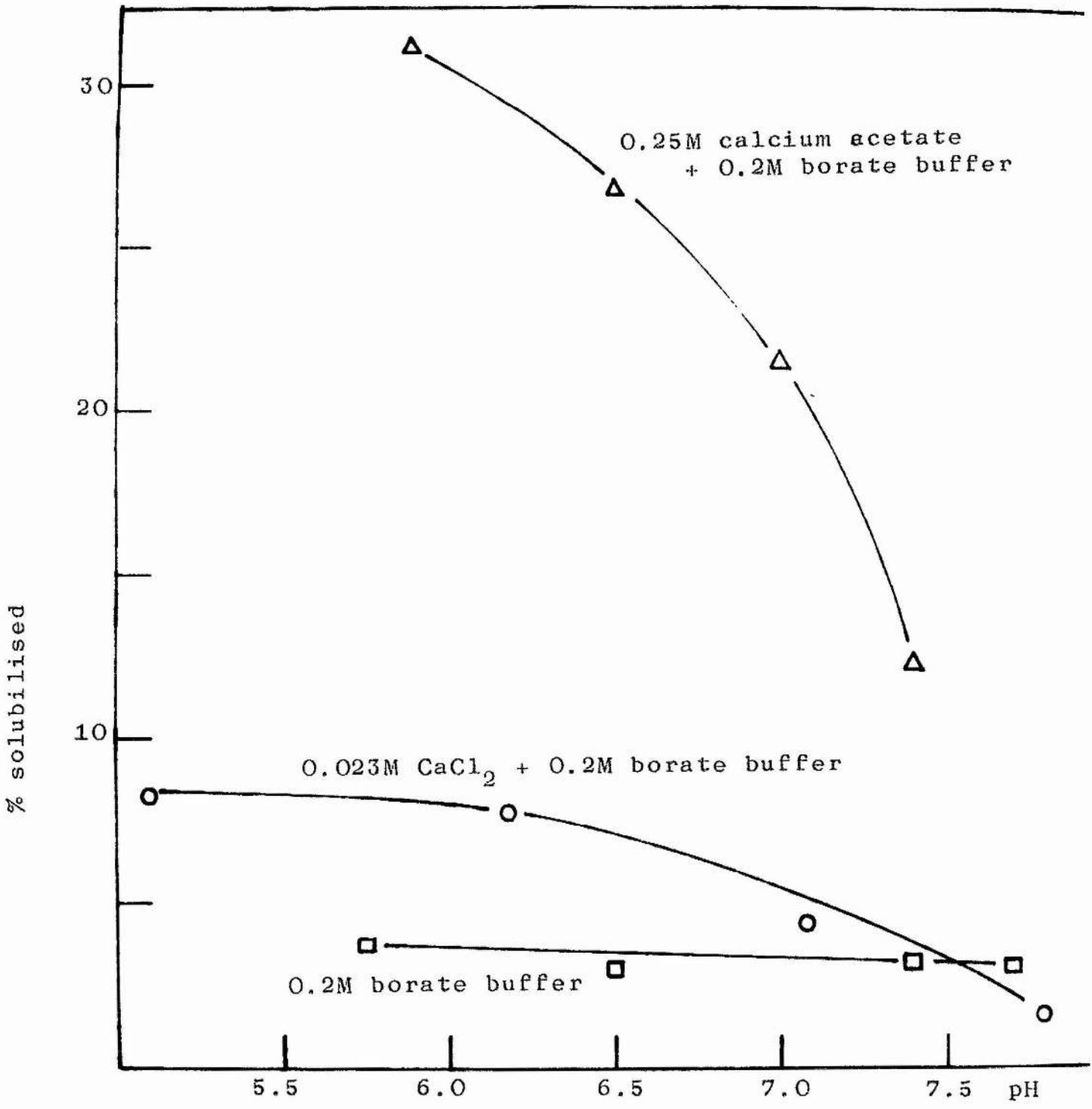


Figure 13 - Solubility of collagen in dilute calcium salts as a function of pH

Table 9 - Solubility of collagen in salt media at extremes of pH.

Salt solution *	Initial pH of salt solution	pH of collagen-salt mixture after shaking for 6 hours.	% collagen solubilised after 6 hours.
1.5M $CaCl_2$ in HCl solution	0.9	2.6	16
	1.5	5.6	60
	1.95	6.0	78
	5.0	6.35	68
1.5M $CaCl_2$ in acetic acid solution	0.5	1.65	66
	0.7	1.7	57
1.5M KI + 2M NaCl in HCl solution	0.7	1.8	20
1.5M $CaCl_2$ in NaOH solution	8.9	8.3	20
	13.1	13.0	39

* To obtain these solutions aliquots of 3M KI or $CaCl_2$ solutions were titrated with concentrated HCl or 2N NaOH to give a suitable pH; after adjustment with water to give 1.5M salt the pH was rechecked.

in strong acid, unless acetic acid is present which has a marked dispersing power. The dispersing action of weak acids on collagen has been treated theoretically by Gustavson (1956) who considers that the hydrogen bonding power of unionised molecules of the acids is responsible for wedging the protein chains apart. NaCl was added to aliquots in an attempt to suppress ionisation of $CaCl_2$ but this does not appear to affect the result (see Table 9).

For the effect of various salts at 0.6M on the solubility of aliquots of thermally reconstituted fibres, the following series, decreasing in order of effectiveness, was obtained:-

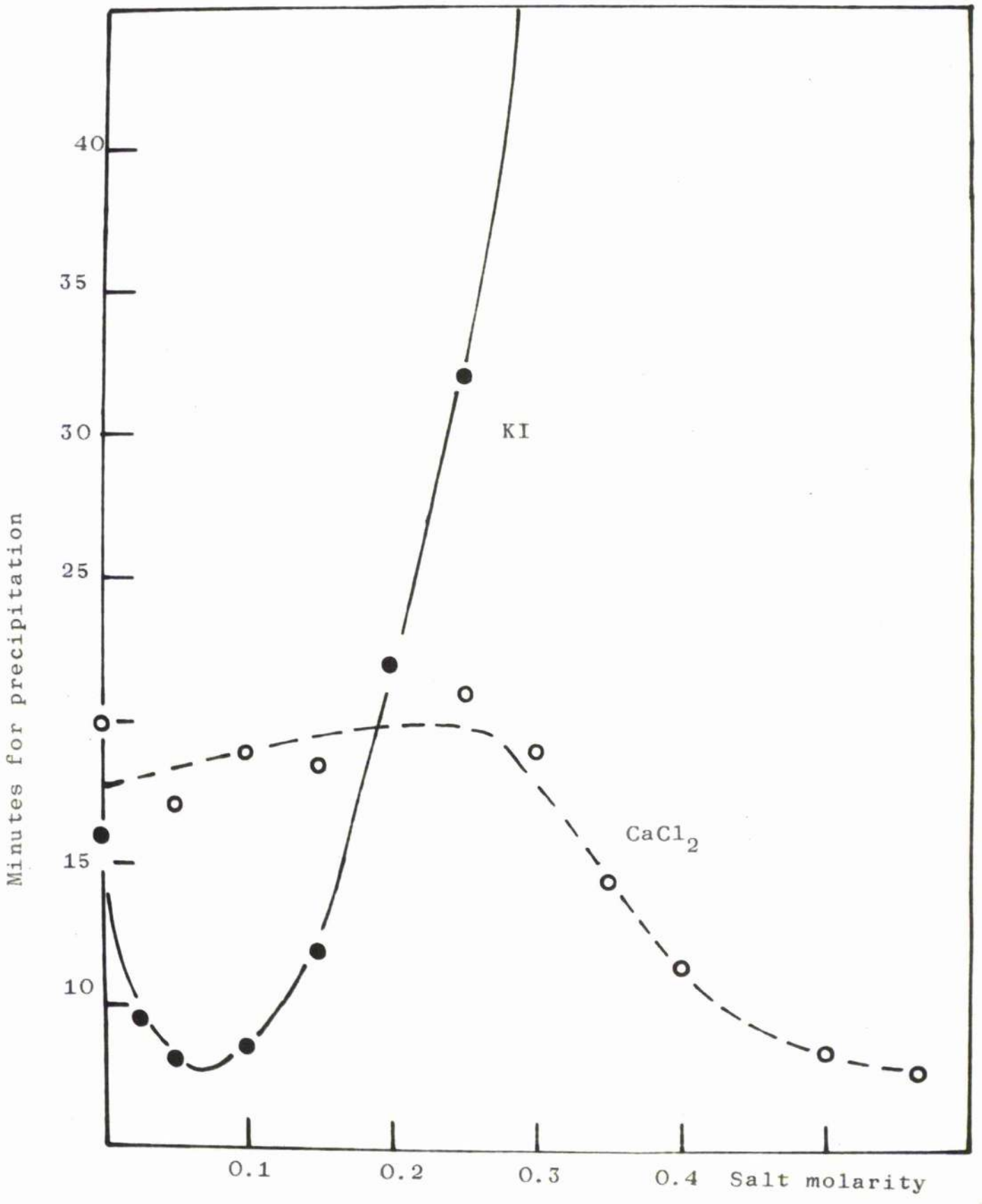
$$\text{Na salicylate} > \text{KCNS} > \text{KI} > \text{NaI} > CaCl_2 > \text{LiBr} > \begin{matrix} \text{NaBr} \\ NaNO_3 \end{matrix} > \begin{matrix} NH_4Cl \\ \text{NaF} \\ \text{KCl} \end{matrix}$$

(vi) Fibre formation.

In Figure 14 the diverse effects of NaCl and KI on the thermal reconstitution of collagen can be seen. With 0.2M acetate buffer, pH 5.5, alone the time taken for precipitation was 18 $\pm$ 2 minutes. The effect of the salts is concentration dependent. Finally at 0.4M salt, KI inhibits fibre formation completely, whereas NaCl gives times below those for zero concentration, the salts

Figure 14 - Time required for heat precipitation of collagen in the presence of KI and $CaCl_2$.

Temperature of incubation - 37°



Collagen - 0.5 mg. aliquots in 0.2M acetate buffer, pH 5.5.

thus giving salting-in and salting-out effects respectively under these conditions. At 0.4M KI is approaching its denaturing range (Figures 5 and 8). Using exactly the same conditions as for Figure 14, it was found that if KI and NaCl were in the same solution they had a cancelling effect on each other, i.e. when KI was 0.35M and NaCl was 0.26M the solution yielded fibres at about the same time as it did in the buffer alone.

(vii) Reversibility.

Table 10 shows the recovery of specific rotation and intrinsic viscosity of collagen treated with KI, $CaCl_2$, urea, or heat, after dialysis or cooling as appropriate.

Table 10 - Values of specific rotation and intrinsic viscosity of denatured collagen after dialysis or cooling.

Treatment (details in text)	$[-\alpha]_D^{20}$ c=0.05%	% native specific rotation regained	η_i $dl.g^{-1}$	% native intrinsic viscosity regained.
3M KI	311°	84	12	68
8M urea	313°	85	13.2	75
Precipitation from 3M $CaCl_2$ 3 times.	270°	75	11.5	66
Heating*	180°	49	8.5 c=0.02%	36.2

* These data are obtained from Figures 6 and 9. (Explanation in text).

The exact conditions were as follows:- (a) for KI and urea,

a homogenised suspension of desalted collagen gel was mixed with three times its volume of 4M KI or 11M urea, re-homogenised, filtered, and the filtrate stored at room temperature for 24 hours before dialysis against tap water and distilled water. These treatments were designed to ensure complete denaturation of the protein. The filtrates were left for 24 hours to ensure complete equilibration. The limiting concentrations of 3M KI and 8M urea were inferred from Figures 5 and 8, and the results of Steven and Tristram (1962b) respectively. Dialysis was performed without disturbing the sacs to allow realignment of molecules to take place if possible. (b) The 3M $CaCl_2$ treated material was in fact the collagen extracted directly from skin with the salt, (Section II, 1,(iii)). It was examined to ascertain the effect of repeated contact with the salt, i.e. precipitation three times from it by dialysis. (c) For heat denaturation, a treatment paralleling as closely as possible that by KI and urea was first attempted, i.e. some of the neutral desalted collagen gel was heated at 50° for 24 hours, then recooled by bringing the temperature down to 20° slowly over a period of 48 hours. This treatment however resulted in a very poor grade gelatin indeed, with a viscosity and specific rotation hardly above the values typical of hot

gelatin (Figures 6 and 9). For the table therefore the final value of the recooled protein in acetate buffer, pH 5.5, (Figure 9), was taken.

It can be seen from the table that KI and urea denaturation are approximately equally reversible, these being more reversible than heat denaturation in this case. Repeated contact with $CaCl_2$ gives a protein which on removal of the salt by dialysis shows a greater degree of unfolding than that prepared after short contact with KI. None of these preparations would form fibres if heated to 37° in acetate buffer at pH 5.5, which was a characteristic of the native collagen preparation.

(viii) Fractionation of collagen in concentrated salt.

Three peaks were obtained on eluting collagen from Sephadex G-50 with 3M KI (Figure 15) after denaturation by the same concentration of salt. (This is the concentration of salt which apparently promotes complete breakdown of the helical structure, see Figures 5 and 8). Thereafter a fractionation was attempted in which the salt concentration was stepped up by 0.5M intervals during the elution (Figure 16). For this, some collagen was dissolved in 0.2M acetate buffer, pH 5.5. 2 ml. of this solution containing 3 mg. collagen was applied to the column and elution was carried out with (a) 20 ml.

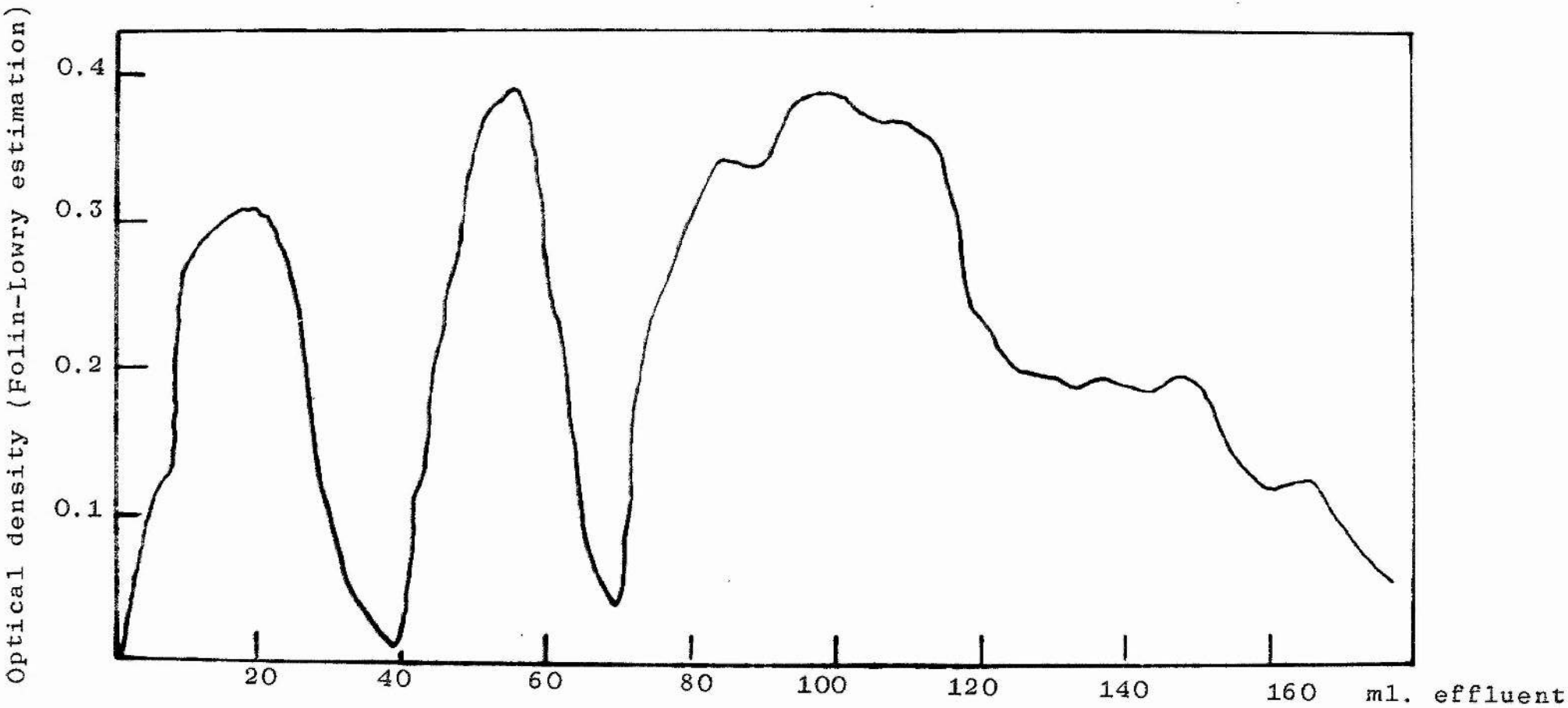


Figure 15 - Fractionation of collagen in 3M KI using Sephadex G-50.

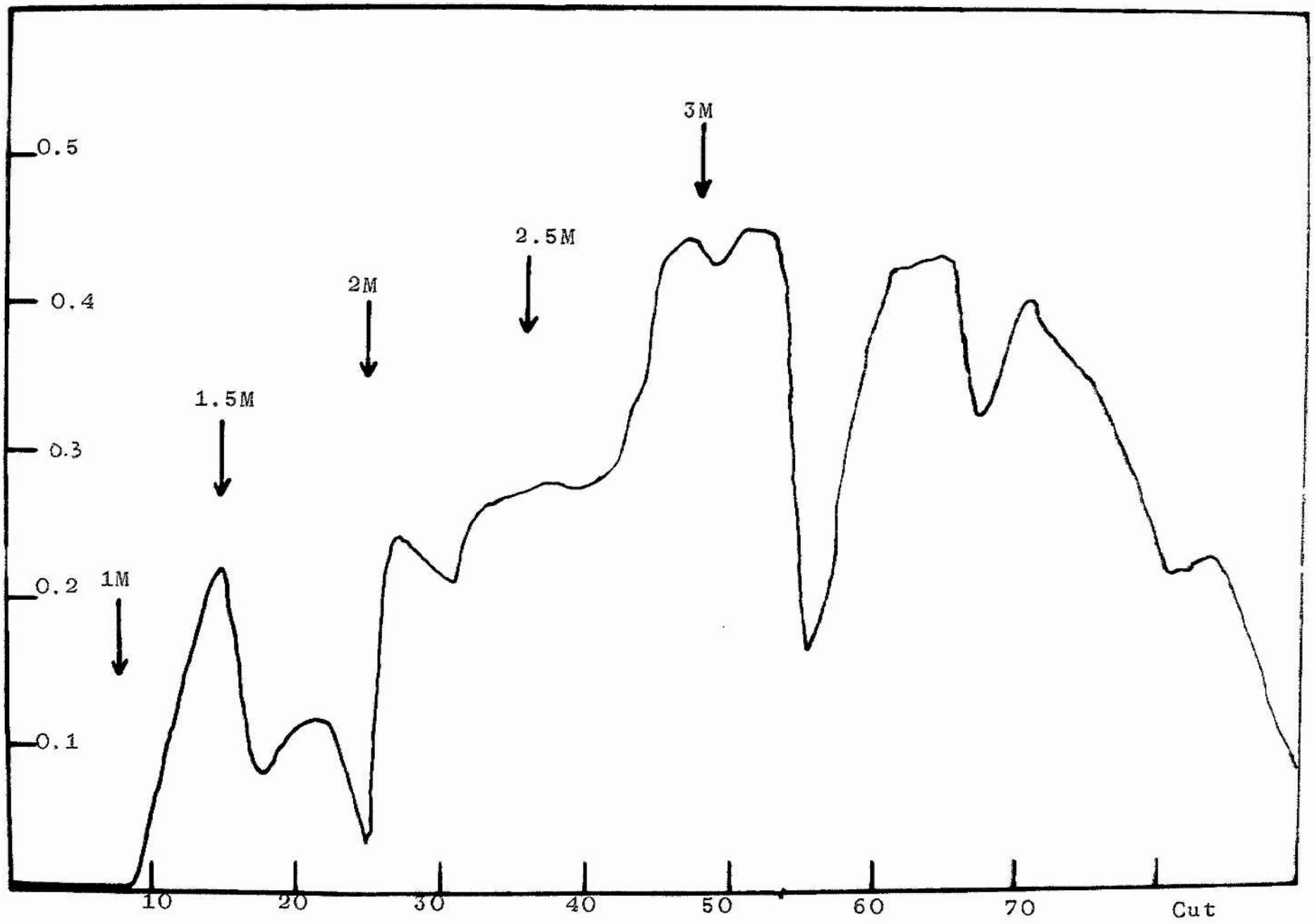


Figure 16 - Elution of collagen from Sephadex G-50 with increasing concentrations of KI.

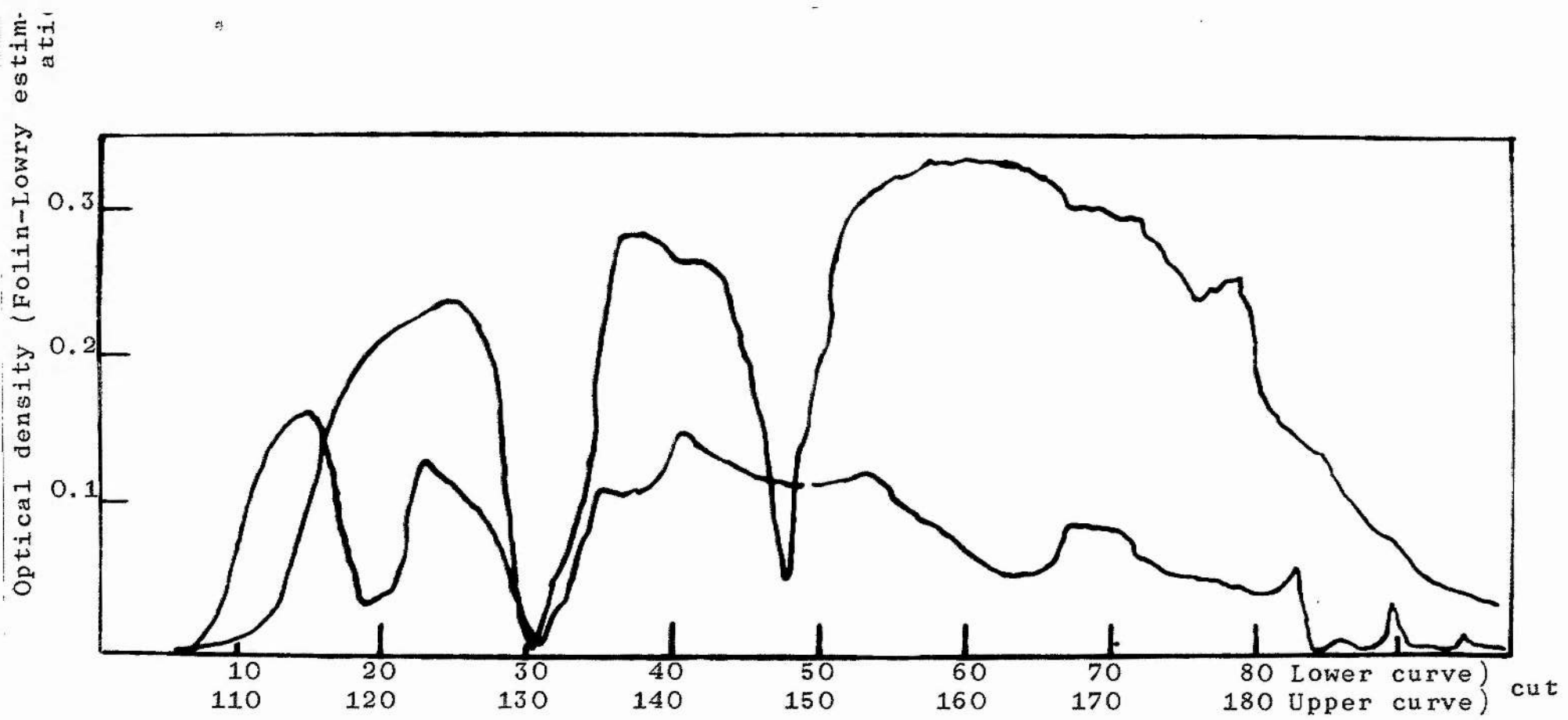


Figure 17 - Fractionation of collagen. Elution from Sephadex G-50 with 1M KI (lower curve) and subsequently with 2M KI (upper curve).

buffer, (b) 20 ml. 0.5M KI, (c) 20 ml. of 1, 1.5, 2, 2.5, and 3M KI in that order and finally (d) 3M KI to the completion of the run. A complex multi-peak elution pattern was obtained, but no protein appeared to be eluted by buffer and 0.5M KI. In the following experiment, 5.5 mg. collagen in 3 ml. acetate buffer, pH 5.5, was applied to a column of the usual dimensions (21 cm. x 2 cm.) and elution commenced with the solvent buffer. No protein could apparently be eluted from the Sephadex with buffer only. The attempted elution with buffer was discontinued and 1M KI flushed through the column (Figure 17) when three fractions were obtained. When no more protein could be eluted from the column with this molarity of salt it was flushed through the gel for several hours before stepping up the concentration of KI to 2M when again three peaks were eluted.

In experiments where elution was done with 3M KI it was found that after exhaustive washing with this concentration of salt an average of 11% of the applied collagen could apparently not be eluted. (This was estimated by extruding the gel, washing it with 0.1M acetic acid, and finding the amount of nitrogen in the washings).

Table 11 - Effect of enzymes on the viscosity of collagen in 1M KI with E : S = 1 : 10.

Enzyme	Percentage drop in specific reduced viscosity (c = 0.11 g. per 100 ml.)
Trypsin	18
Collagenase	100
Papain	9
Hyaluronidase	0
Pronase	30
Fungal protease	13

(ix) Enzyme studies.

Table 11 presents the percentage drop in specific reduced viscosity of collagen in 1M KI after treatment with various enzymes. Similar values to some of those have been reported in the literature, pertaining however to the drop in viscosity of native collagen on enzyme digestion. Thus Hodge and Schmitt (1960) found that trypsin was capable of lowering the relative viscosity of calf skin collagen by about 17%, whereas Gallop et al., (1957) found that for ichthyocol a drop in specific viscosity of 8% could be obtained with trypsin and 100%

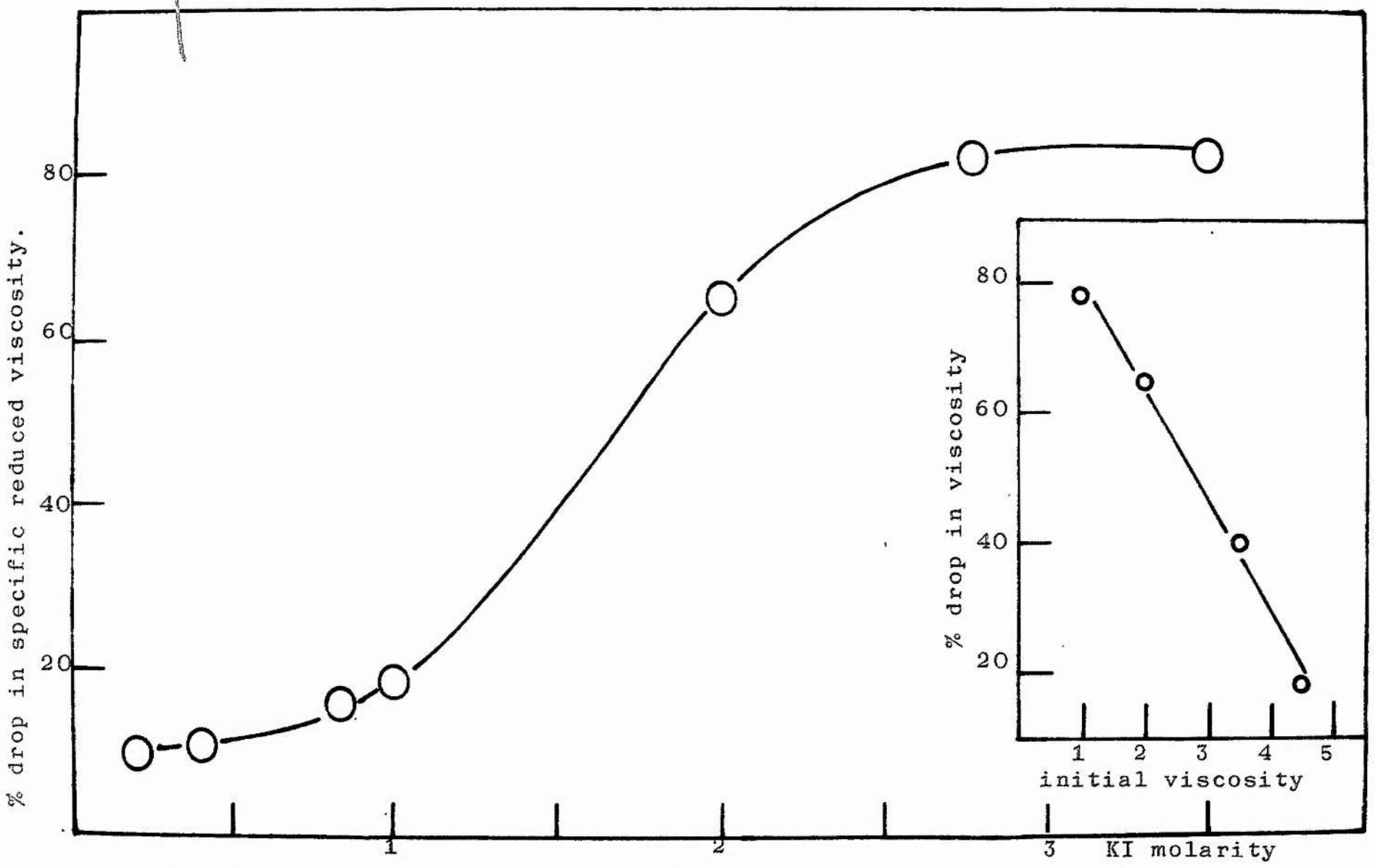


Figure 18 - Drop in specific viscosity of collagen in KI solutions, after the addition of trypsin, as a function of KI concentration. E:S = 1:10.
Inset - drop in specific reduced viscosity of collagen in KI solution after addition of trypsin (E:S = 1:10) as a function of the initial specific reduced viscosity of the collagen.

with collagenase. Tyson (1963) gives the drop in relative viscosity of calf skin collagen on treatment with pronase as about 30%. The drop in viscosity of collagen in 0.2 - 3.5M KI after addition of 10% of its weight of trypsin is shown in Figure 18. Appropriate controls were done in this experiment for the viscosity of KI solutions, which decrease in viscosity with increasing concentration. Figure 18 (inset) shows this percentage drop after addition of trypsin as a function of the initial viscosity of the collagen in the KI solution. A linear relationship is obtained when the initial specific reduced viscosity is 1 - 5, with corresponding salt molarities of 1 - 3M.

(x) Non-protein nitrogen.

It was found that collagen extracted from skin with $CaCl_2$ and collagen after denaturation with salt contained small amounts of the non-protein nitrogen described in other collagens by Steven and Tristram (1962). This was prepared and examined, and is discussed in the general context of the studies on non-protein nitrogen (Sections I,4 and IV,4).

(xi) The treatment of skin with 3M $CaCl_2$.

After no more collagen can be removed from skin with 0.1M acetic acid, 3M $CaCl_2$ extracts further quantities of protein. This however, as is quite clear from the present work, is not a different collagen but a gelatin releaseed by the breakage of hydrogen bonds in "insoluble" collagen.

Solutions in 3M $CaCl_2$ have some interesting properties. With concentrated (3%) solutions, protein fibres can be obtained simply by squirting these solutions through an orifice into tap water; salt presumably diffuses rapidly into the water and causes precipitation of the protein as a thread. The $CaCl_2$ is bacteriostatic and solutions of collagen in it can be stored indefinitely at room temperature without any apparent deterioration.

Table 12 - Effect of various physiological substances on the dispersion by KI solutions of thermally reconstituted collagen.

Substance	Concentration range in fibre-forming solutions.	Resistance as compared to controls.
Bovine serum albumin(Armour & Co.)	8%-25% of collagen present	None
Chondroitin sulphate (Light & Co.)	8%-20% of collagen present	None
BSA + CSA (1:1, w/w)	25% of collagen present	None
Urea	0.05M	Decreased
L-Arginine	0.05M	Decreased
L-Aspartic acid	0.05M	Increased
Extract of calf skin, 0.2M Na_2HPO_4 (protein after dialysis)	5%-15% of collagen present	None
L-ascorbic acid	0.1-1.5mg. per 100 ml.	Increased
D-glucose	0.1-1.5mg.per 100ml.	None
D-glucosamine	0.1-1.5mg. per 100 ml.	None
NaF	0.05 - 0.2M	Increased
Na_2SO_4	0.05 - 0.2M	Increased
Sodium citrate	0.05 - 0.2M	Increased
Na_2HPO_4	0.05 - 0.2M	Increased
LiBr	0.05 - 0.2M	Decreased
NaCl	0.05 - 0.2M	None

3. Resistance to dispersion of thermally reconstituted collagen.

(i) General results.

Within the range of protein concentration used (0.1 - 0.4 mg./ml.) the fraction of collagen precipitated by warming to 37° for 15 hours was 70-80%. A test at pH 5.0 under the same conditions, with initial protein concentration 1.1 mg. per ml., revealed that approximately the same percentage was rendered insoluble. The thermal reconstitution of collagen seems to be a very delicate process; even mild mechanical agitation during incubation inhibited gelling, and care was taken to avoid this. Since the phenomenon will occur at temperatures in excess of 40° it appears that some degree of unfolding is compatible with fibre formation, but extensively denatured collagen will not reconstitute on warming (Section III,2,vii).

In these experiments a balance had to be found between the protein concentration and the concentration of dispersing agent (KI or urea) such that in a given experiment the time for complete dispersion of the fibres produced was measurable i.e. if the time was too short obviously accurate comparisons could not be made and if it was too long (> 60 min.) it was impossible to tell at which precise time all the fibres had disappeared.

Qualitative results regarding the influence of a variety of substances, when in the fibre-forming solutions, on the subsequent resistance to dispersion of the fibres are set out in Tables 12, 13, 14, and 15. Conditions were designed specifically to allow stabilising effects to be manifested, but marked ease of dispersion, as compared to the controls with buffer only, was often noticeable, and has been noted where appropriate. Table 12 deals with the influence of substances on dispersion by 1-2M KI. Since ascorbic acid appeared to inhibit dispersion this was tested again along with some substances having similar structural features (Table 13). Some of the substances in Table 12 were tested again, along with some new ones, using 2M urea, (Table 15). Amino acids in particular are dealt with in Table 14.

All the succeeding data will refer to reconstitutions which took place at 37° and at pH 7.4.

Table 13 - Effect of some organic acids on the resistance to dispersion by KI solutions of thermally reconstituted collagen. Concentration of acids in the fibre forming solution - 0.2-1 x 10^{-4}M.

Substance	Resistance to dispersion as compared to control.
L-ascorbic acid	Increased
D-isoascorbic acid	Increased
dehydro-L-ascorbic acid	None
dihydroxymaleic acid	Increased
oxalic acid	None
fumaric acid	None

Table 14 - Effect of amino acids on the resistance to dispersion by urea of thermally reconstituted collagen. Concentration of the amino acids in the fibre forming solution - 10^{-5} - 10^{-2}M.

Amino acid	Resistance to dispersion as compared to control.
aspartic acid	Increased up to 10^{-2}M
lysine	"
arginine	"
hydroxyproline	"
tyrosine	"
serine	"
glycine	Increased above, decreased below 10x10^{-4}M
histidine	Increased above, decreased below 9x10^{-4}M

Table 15 - Resistance to dispersion by urea of collagen thermally reconstituted in the presence of various physiological substances.

Substance	Concentration range in fibre forming solution	Resistance to dispersion as compared to control.
histamine	10^{-4} - 10^{-2}M	Decreased
glutathione	10^{-4} - 10^{-2}M	Increased below 10^{-3}M
heparin 119 units/mg.	2%-20% of the collagen in the solution	Above 10% inhibits fibre formation.
hyaluronic acid (B.D.H.)	"	None
C.S.A. (Light & Co.)	"	None
cortisone acetate	0.1 - 1 mg. per 100ml.	None
deoxycorticone acetate	0.1 - 1 mg. per 100ml.	None
ascorbic acid	0.05 - 10 x 10^{-5}M	Increased

(ii) Effect of salts.

After the qualitative observations for sodium salts (Table 12) the relative potencies for delaying dispersion were determined using the same techniques and conditions. Figures 19 and 20 show the times for complete dispersion of the fibre aliquots with the salts as a function of their concentrations, using 0.75M and 1M KI as dispersing agents respectively. The order of effectiveness of the anions is:-

$$SO_4^{=} > HPO_4^{=} > F^{-} > Citrate^{\equiv}$$

If the tangents of the straight (upward) parts of the curves of Figure 20 are calculated, and taking the value for citrate as unity, then the ratio of effectiveness as above is:-

12 : 9 : 3 : 1

(iii) Effect of amino acids.

An attempt was made to study representatives of all the chemical types of amino acids and the results are summarised in Table 15 and Figure 21. The tripeptide glutathione was included. All these had a rather similar effect on the resistance to dispersion of collagen fibres i.e. a small effect (as compared, for example, to ascorbic acid, Figure 24) which is manifested at quite low

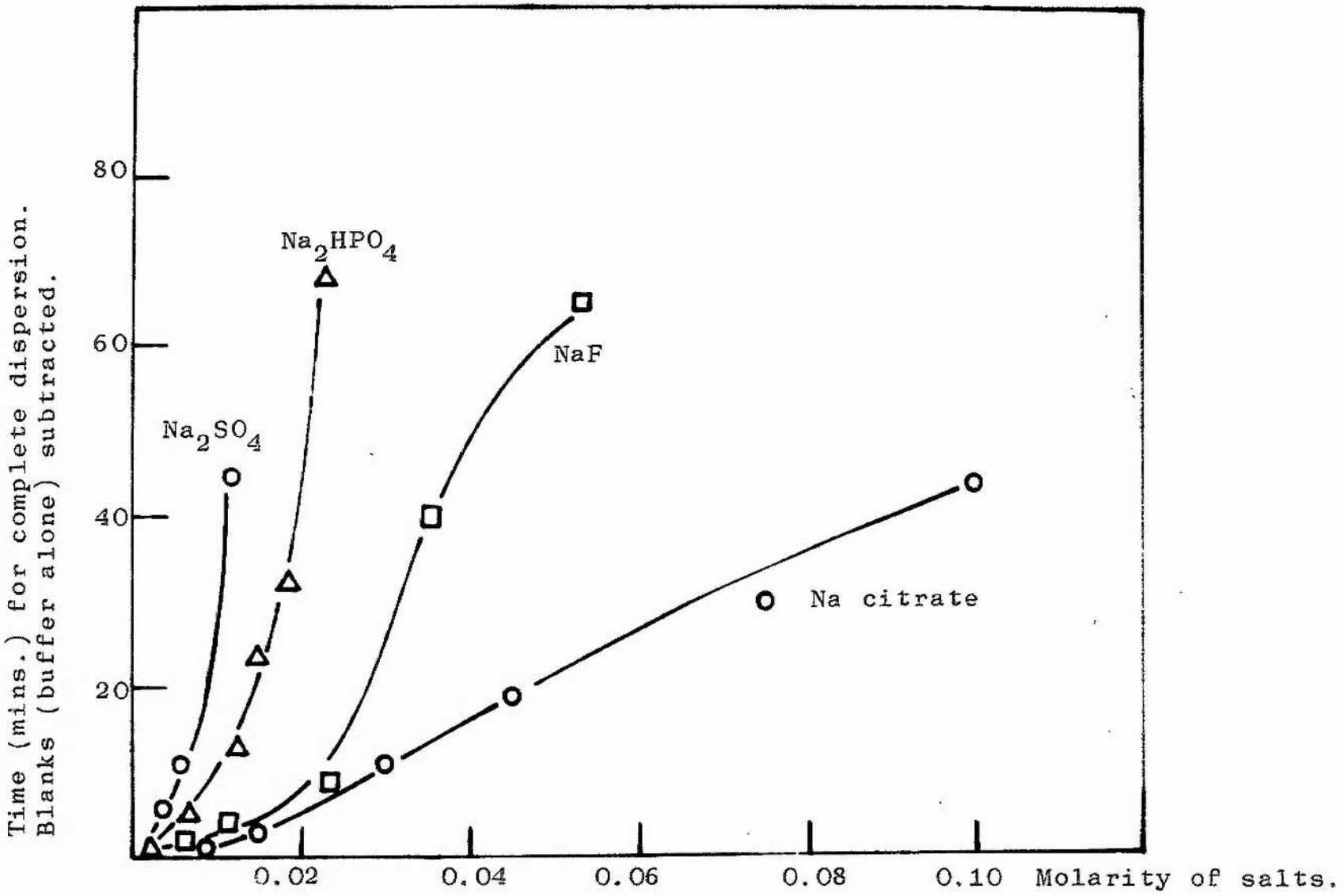


Figure 19 - The redispersion of thermally reconstituted collagen fibres by 0.75M KI: the effect of salts in the fibre-forming solution. Collagen - 0.4 mg. aliquots in 0.2M acetate buffer, pH 7.4.

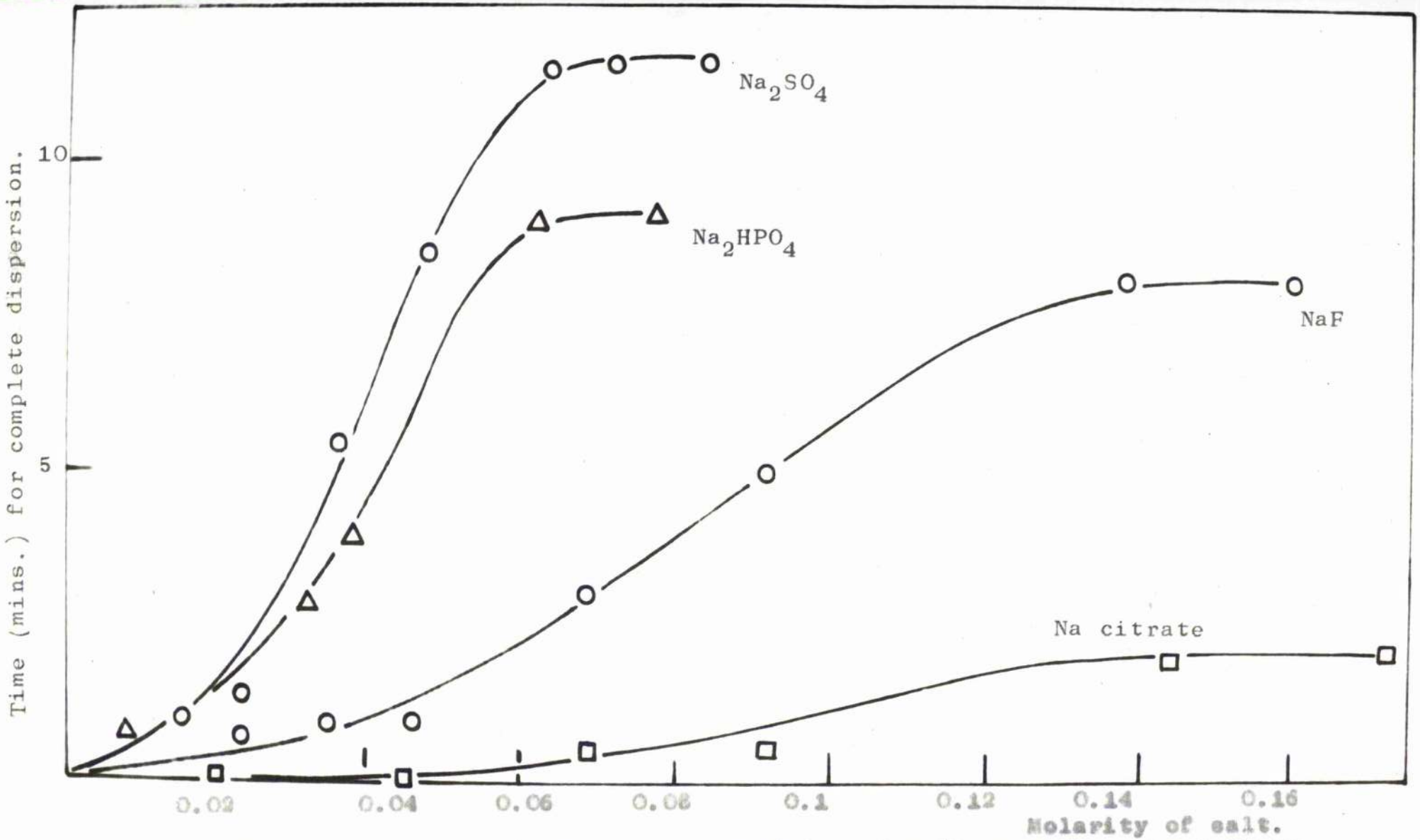


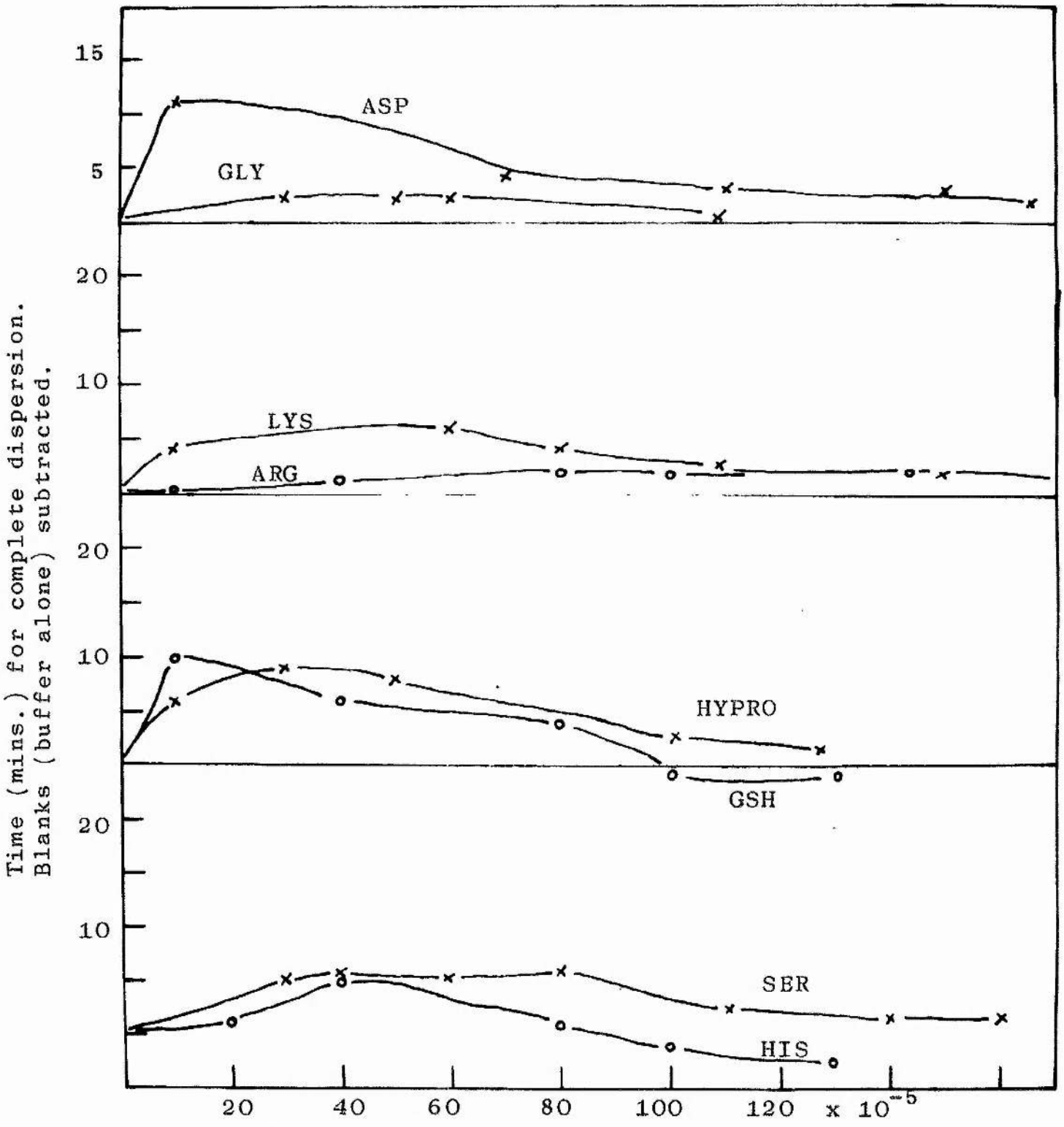
Figure 20 - The redispersion of thermally reconstituted collagen fibres by 1.0M KI: the effect of salts in the fibre-forming solution. Collagen - 0.4mg. aliquots in 0.2M acetate buffer, pH 7.4. Blanks (buffer alone) have been subtracted.

concentrations (10^{-4}M) and which dwindles or is reversed by about 10^{-5}M. The fibre dispersing effect was accentuated at still higher concentrations, e.g. 0.05M, for two amino acids tested, arginine and hydroxyproline.

It was in fact found difficult to obtain precisely repeating results in these experiments, and representative runs have been shown in Figure 21; the fluctuation, from experiment to experiment, of the exact times for dispersion in the presence of a specific amino acid is presumably caused by the fact that the very small increase in resistance compared to controls makes observational errors proportionately very large.

Since Steven and Tristram (1962a) found that collagen contained small amounts of free amino acids a solution was made up containing these in the approximate proportions described by these authors, and tested as hitherto described for influence on the rate of dispersion of fibres by 2.5M urea. The collagen sample used here was one which had been dialysed for a week against 0.1M acetic acid, pH 3.5, a treatment which according to Steven and Tristram should remove 1% of the total nitrogen in the system (50% of the non-protein nitrogen). The result is shown in Figure 22. It can be seen from the graph that the resistance to dispersion is low, and that

Figure 21 - Redispersion of thermally reconstituted collagen by 2.25M urea: the effect of amino acids in the fibre-forming solution.



Collagen - 0.65 mg. aliquots in 0.2M acetate buffer, pH 7.4.

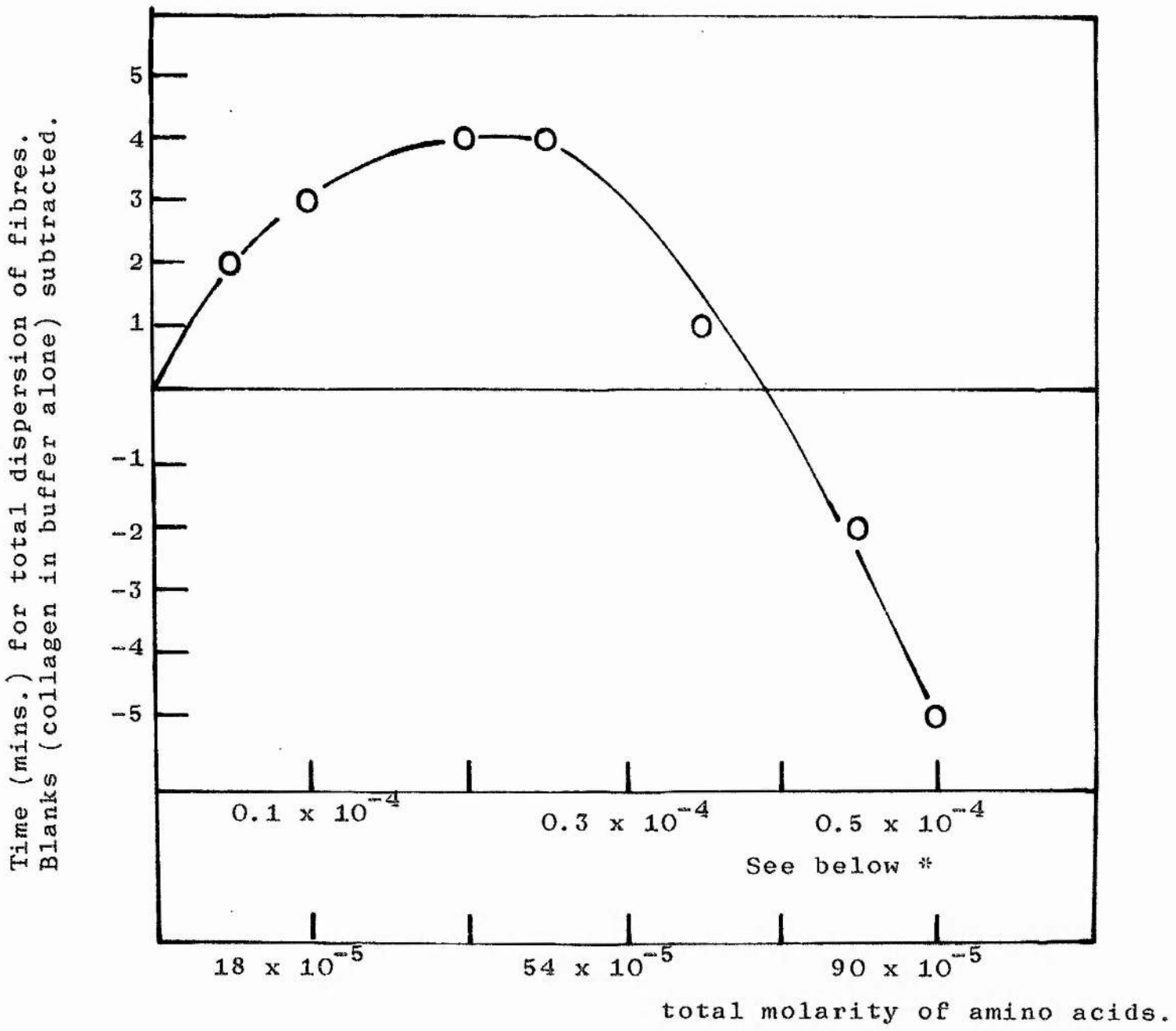


Figure 22 - Effect of an amino acid mixture on resistance to dispersion by 2M urea of thermally reconstituted collagen. (1.7 mg. aliquots in 0.2M acetate buffer, pH 7.4).

* Molarity of valine, phenylalanine, proline and glutamic acid.
Double those values for molarities of glycine, serine, aspartic acid, threonine, alanine, tyrosine and leucine.

a marked dispersing effect compared to the control sets in at about 7 x 10^{-4}M for total amino acid concentration. This value is sufficiently close to the concentrations where the effect dwindles or is reversed in Figure 21 to allow the conclusion that the amino acids have an additive effect, i.e. none of the amino acids in this mixture exerts an effect different to that of the others except in degree.

(iv) Effect of ascorbic acid.

In Figure 23 are shown the times for complete dispersion of collagen fibres by KI solutions as a function of ascorbic acid concentration (up to 10^{-4}M) in the fibre-forming solution. At concentrations higher than this range different effects are obtained: at 10^{-3}M ascorbic acid gelling is markedly inhibited, although on shaking fibres can still be produced which show a resistance to denaturation above that of controls, while at 10^{-2}M a yellow liquid is obtained on incubating the system at 37°, due obviously to chemical degradation of the collagen. In this latter connection it is interesting to note that Zaides (1962) treated cow corium with ascorbic acid and interpreted the changes occurring as signifying

a disruption of collagen structure. At 0.1M ascorbic acid degradation was said to be marked, but to exist also at 0.01 and 0.001M. Since ascorbic acid has such a marked effect at low concentrations it is the most efficient substance of all those examined for preventing the redispersion of collagen fibres. The data in Figures 19 and 23 were obtained using the same conditions and the same stock collagen solution, so that a measure of the relative potency of ascorbic acid and the most effective anion, sulphate, can be obtained by comparing the concentrations at which these have equivalent effects. For dispersion in 0.75M KI the concentrations of sulphate and ascorbic acid to which the two graphs are asymptotic (i.e. when complete dispersion by KI just fails to be achieved after a very long period) are in the ratio of 350 to 1.

The effect of the pH of the fibre forming solution on the subsequent dispersion of the fibres is shown in Figure 24. This gives the times for dispersion of fibres formed in the presence of ascorbic acid and in the presence of buffer only as a function of the pH. It can be seen that there is relatively a larger rise in stability with pH of the fibres formed in the presence of 4×10^{-5}M ascorbic acid.

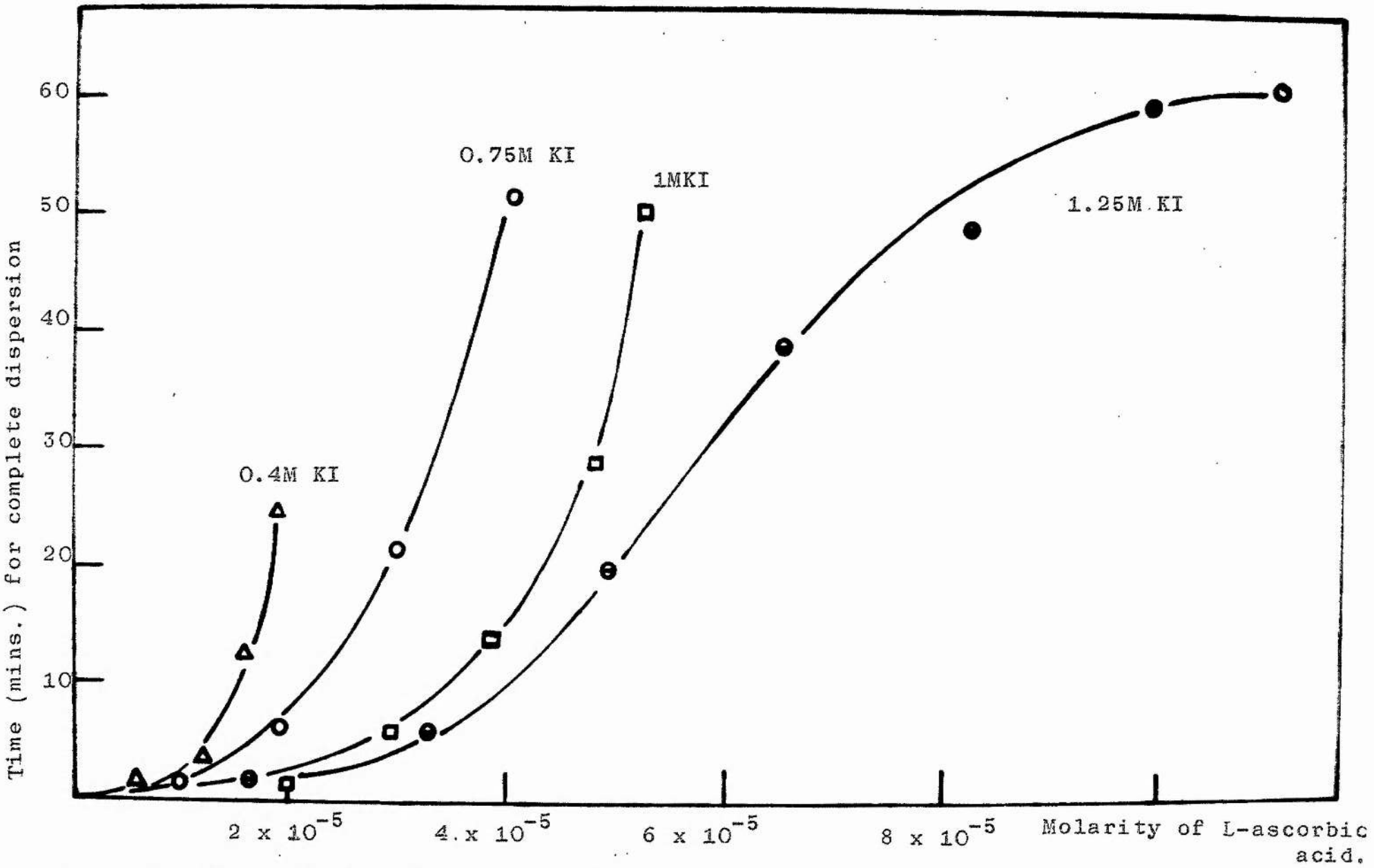
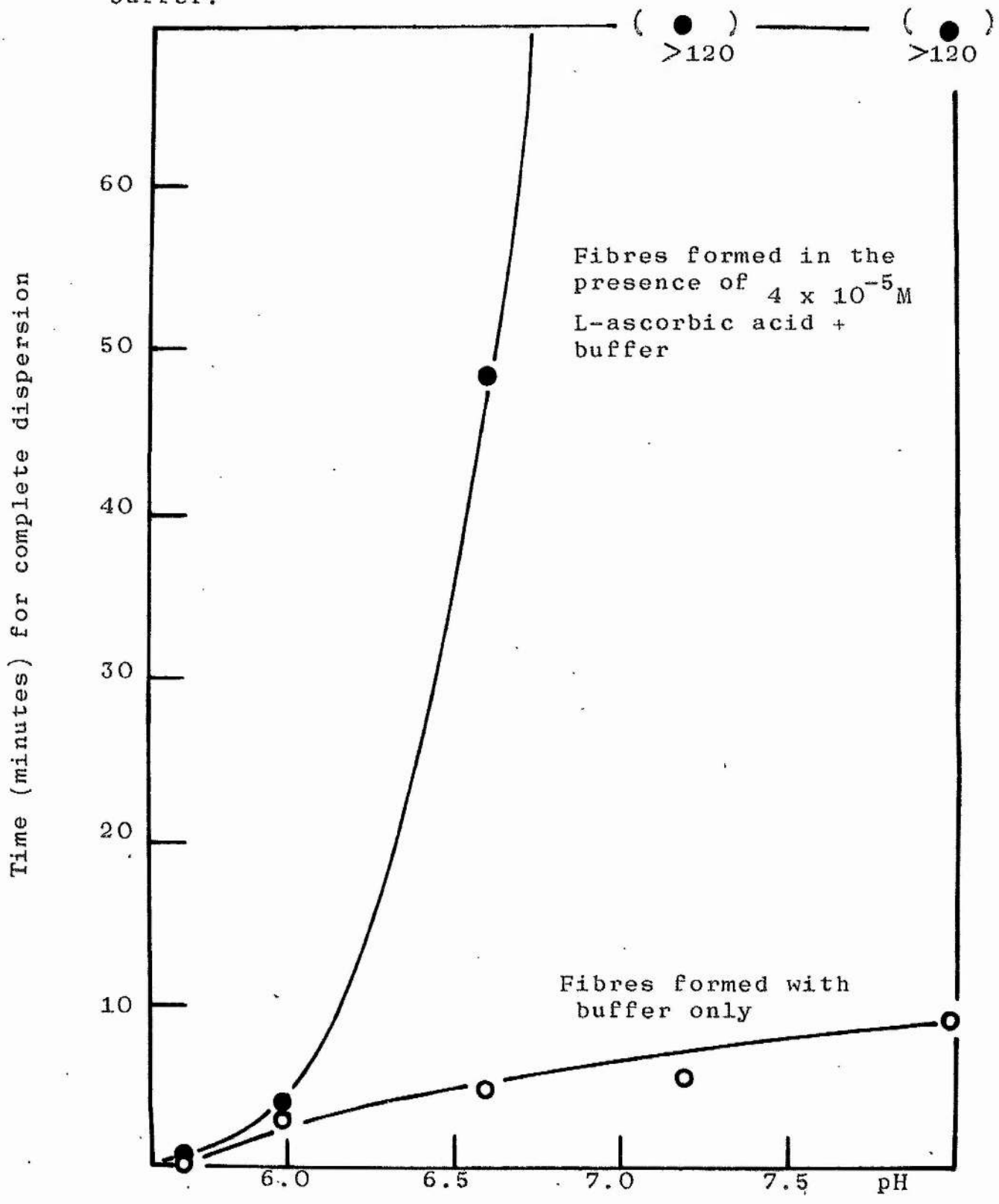


Figure 23 - The redispersion of collagen fibres by KI: the effect of ascorbic acid in the fibre-forming solution. Collagen - 0.5 mg. aliquots in 0.2M acetate buffer, pH 7.4. Blank values (buffer alone) have been subtracted.

Figure 24 - Time for complete dispersion of collagen fibres (formed with ascorbic acid and with buffer only) as a function of pH. Dispersing agent - 2.25M urea. Collagen-0.76 mg. aliquots in 0.2M acetate buffer.



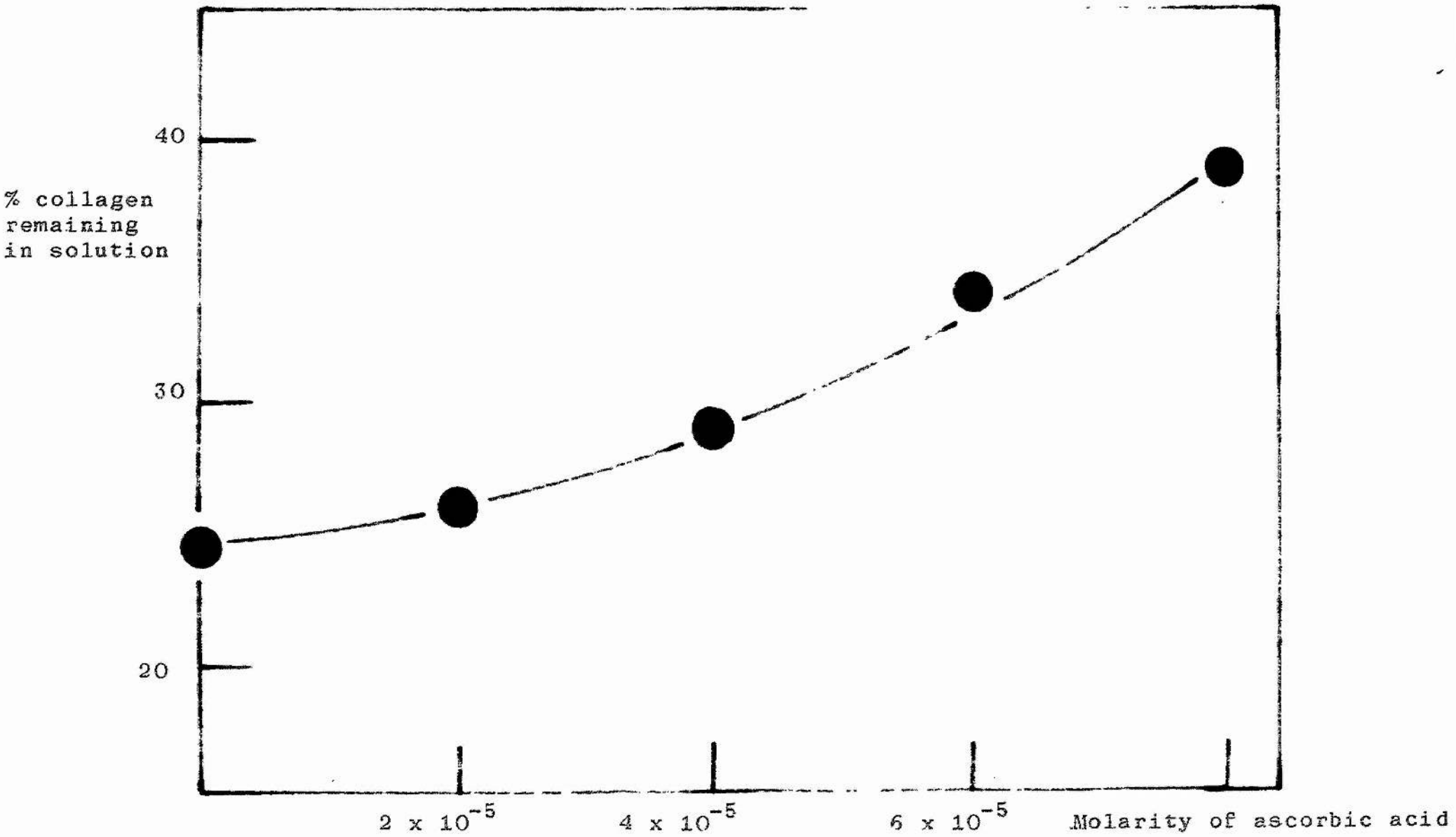


Figure 25 - Percentage of collagen (0.02% in acetate buffer) precipitable by warming to 37° in the presence of L-ascorbic acid.

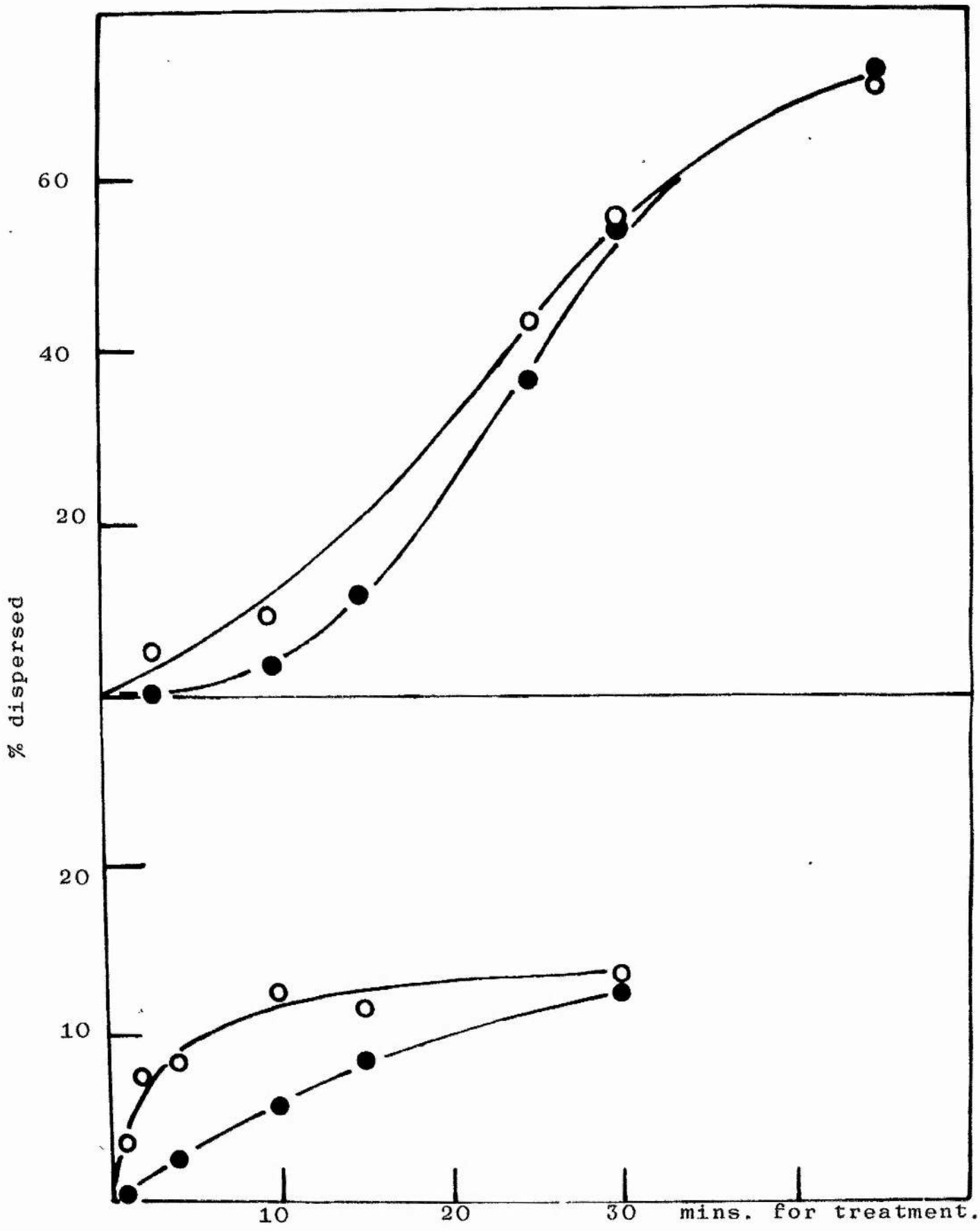
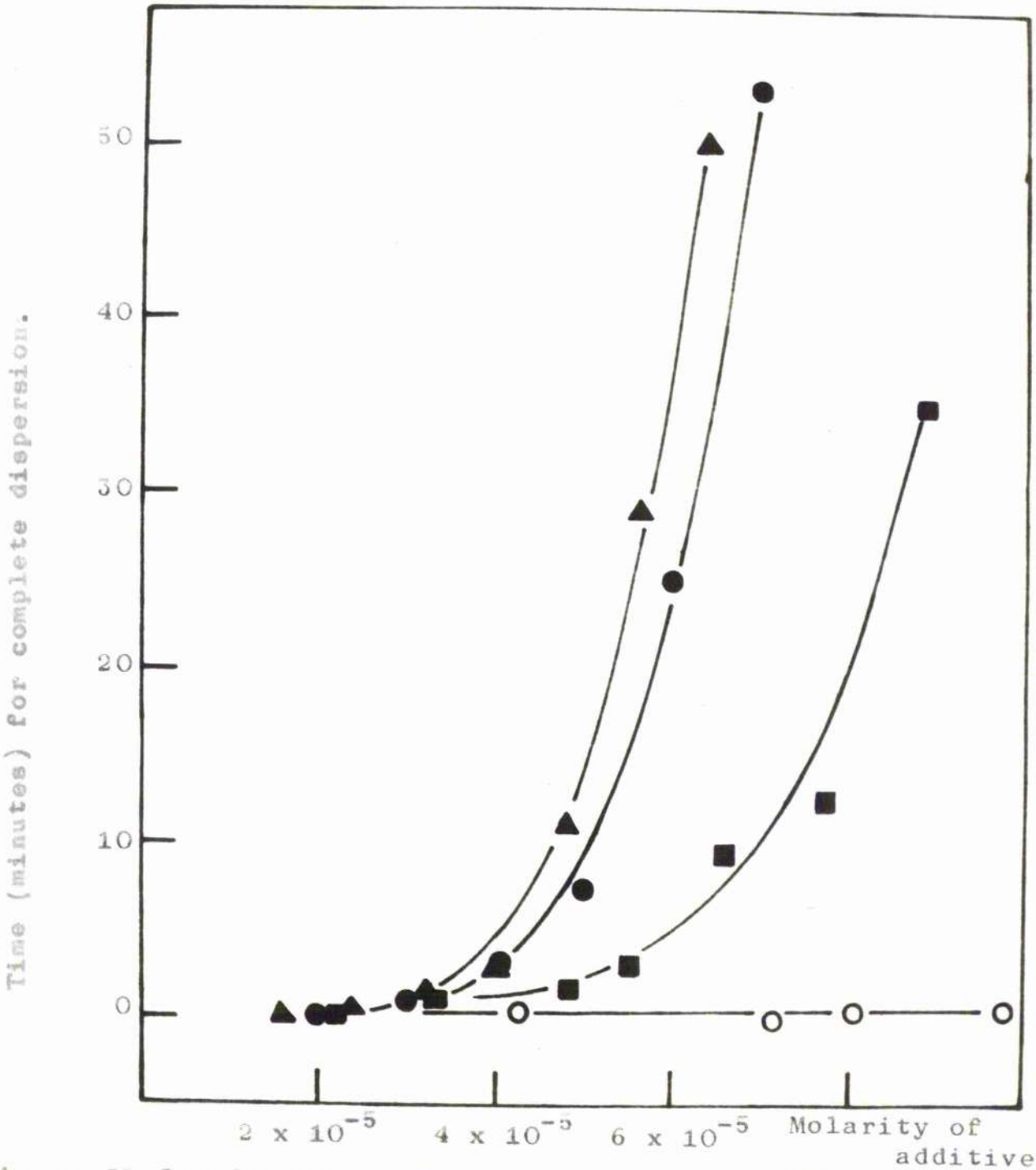


Figure 26 - Fraction of thermally reconsituted collagen redispersed after ultrasonic treatment (upper block) and papain treatment (lower block). O, aliquots in buffer alone. ●, aliquots with 4×10^{-5}M L-ascorbic acid. Collagen - 2.5 mgm. aliquots in 0.2M acetate buffer, pH 7.4, warmed to 37°.

Figure 27 - Time for complete dispersion of collagen fibres as a function of the concentration of various additives.



See Figure 32 for formulae. ▲, L-ascorbic acid. ● D-isoascorbic acid. ■ dihydroxymaleic acid. ○, dehydro-L-ascorbic acid. Dispersing agent - 1.25M KI. Blanks (collagen in buffer alone) have been subtracted. Collagen - 0.6 mg. aliquots in 0.2M acetate buffer, pH 7.4.

The dispersing effects of papain and ultrasonic treatment instead of KI were used as an index of the effect of ascorbic acid in the experiments summarised in Figure 26, which shows the percentage of collagen fibre aliquots solubilised by these treatments, the effect of 4×10^{-5}M ascorbic acid and of buffer alone being compared. It can be seen that ascorbic acid delays the dispersion of the fibres but that the final percentage solubilised in both cases is about the same. It was found that ascorbic acid decreased the percentage of collagen precipitable by warming at 37° for 15 hours (Figure 25) this decrease being about 4% at the most frequently employed concentration of ascorbic acid, 4×10^{-5}M.

Some substances with chemical similarities to L-ascorbic acid were tested in the same way as it (i.e. as in Figure 23) for influence on the dispersion of fibres by KI. In Figure 27 it can be seen that D-isoascorbic acid was approximately as effective as L-ascorbic acid, dihydroxymaleic acid was rather less effective than these, and dehydro-L-ascorbic acid was completely ineffective. Oxalic and fumaric acids were also tested in the same concentration range (up to 10^{-4}M) and had no effect distinct from the controls.

A qualitative test revealed that at room temperature (i.e. fibre formation being allowed to take place at 20° for three days at pH 7.4) ascorbic acid similarly seemed to make reconstituted fibres resistant to dispersion.

4. Non-protein nitrogen associated with collagen.

(i) Results using acid conditions.

It was found that non-protein nitrogen could be prepared by submitting acid soluble calf skin collagen both to electrodialysis and to elution from an ion-exchange material at pH 3.5. These have been named Preparations 1 and 2 respectively. Yields are seen in Table 16 and the amino acid patterns of the chromatogramed materials in Figure 28.

The predominant amino acids were gly, ala, val, ser, and leuc, but preparation 1 contained lysine, in distinction to preparation 2, and 1 also appeared to contain peptides (Spots near the origin of the chromatogram). A very much smaller yield of non-protein nitrogen was obtained in preparation 2, and it would seem to be of little practical use if any non-protein nitrogen were required for further experiments.

TABLE 16

Preparation from collagen of non-protein nitrogen.

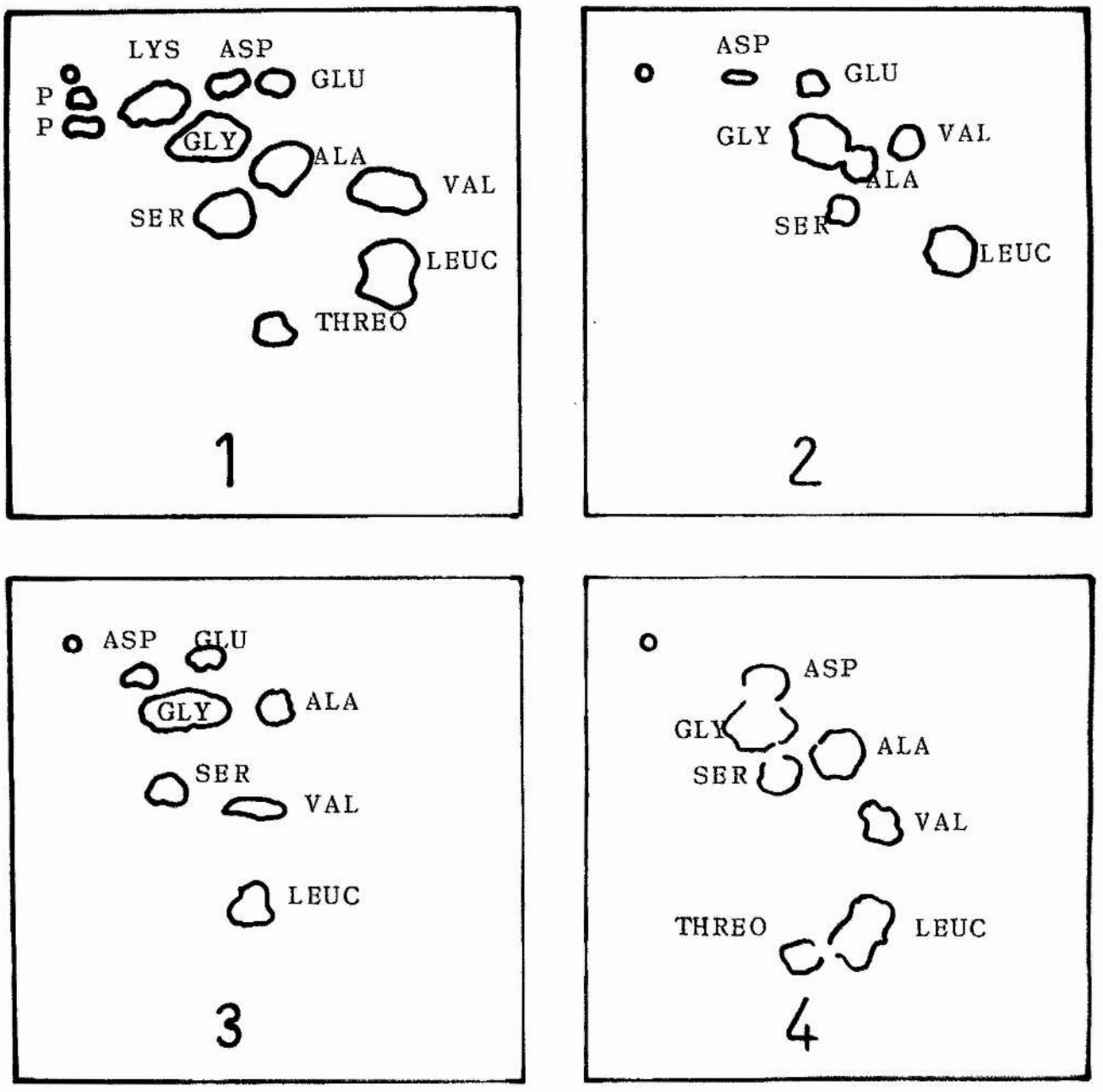
Method of prep.of collagen	pH of prep. of coll.	Method of prep.of N.P.N.
1. Acetic acid extract of calf skin, 3 x repptd.	3.5	Electrodialysis.
2. do.	3.5	Elution from ion-exchange material (cellulose phosphate)
3. Calf long-bone, EDTA (15%) demineralised	7 - 7.5	Stirring with acetic acid/acetone, 1:5 (v/v)
4. do.	7 - 7.5	Dispersion in 3M KBr pptn. with 10 vols. acetone.
5. Collagen extracted from calf skin with 3M $CaCl_2$.	7.0	Dialysis against 3M $CaCl_2$.
6. do.	7.0	Dispersion in 0.1M HAc, pptn. with 8 vols. acetone.
7. do.	7.0	Elution from cellulose phosphate with 0.2M acetate buffer, pH 5.5, 0.1M w.r.t. $CaCl_2$.
8. do.	7.0	Dispersion in 3MKBr, pptn. with 13 vols. acetone.

	pH of prep.of N.P.N.	Yield of N.P.N. as % original N.	Products identified
	3.5	1.3	Gly,Ala,Ser,Threo, Val,(Leuc,Ileu),Asp, Glu,Lys;2 peptides.
	3.5	0.2	Gly,Ala,Val,Asp,Glu, Ser,(Leu,Ileu).
	5.5	Trace	Gly,Val,Ala,Asp,Glu, Ser,(Leuc.Ileuc).
	7.0	1.32	Asp,Gly,Ser,Ala,Val, Threo(Leuc.Ileuc.).
	7.0	Trace	Asp,Gly,Ser,Ala,Val. (Leu.Ileuc.) + one peptide.
	3.5	0.24	Asp,Glu,Gly,Ser,Ala, Val,Threo.(Ileuc.Leuc) + one peptide.
	5.5	0.13	Asp,Gly,Ala,Ser,Threo, Val. (Leuc.Ileuc.) + one peptide.
	7.0	0.54	Gly,Val,Ala,Asp,Glu, Ser,(Leuc.Ileuc.).

(ii) Results using neutral or near-neutral conditions.

In Section II, 2, (iii) a list was given of procedures it was intended to carry out to ascertain whether non-protein nitrogen could be obtained from collagen using pHs near neutrality. The results of these are summarised in Table 16 (Preparations 3 - 8) and the chromatograms obtained are shown in Figures 28 and 29. It can be seen that it is indeed possible to prepare the material under neutral conditions. The strong hydrogen bond breaker 3M $CaCl_2$ itself does not remove the non-protein nitrogen (Preparation 5) but the addition of acetone or the presence of an ion-exchanger appears to result in the removal of the material.

The amino acid composition of all these preparations was very much the same, gly, ala, ser, leuc, and val. predominating. Again the preparation using cellulose phosphate (7) gave a very low yield.



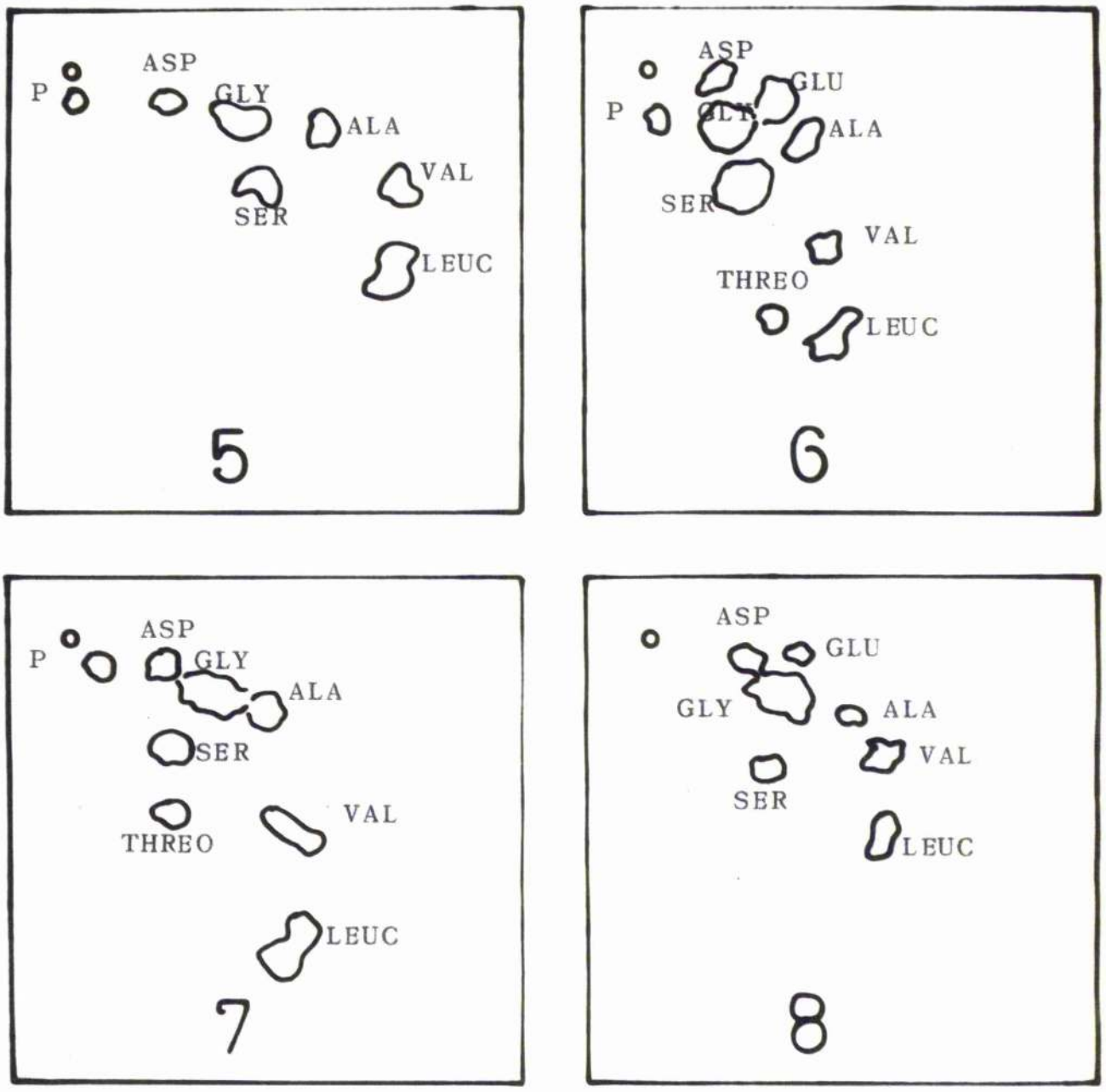
O = origin

P = peptide

O → 1st dimension (see Section II,13)

↓ 2nd dimension

Figure 28 - Chromatograms of non-protein nitrogen fractions. Numbers refer to preparations in Table 16.



O = origin

P = peptide

O → 1st dimension (See Section II, 1J)

↓ 2nd dimension

Figure 29 - Chromatograms of non-protein nitrogen fractions. Numbers refer to preparations in Table 16.

A. Hydrolysed collagen

B. Hydrolysed preparation 4.

C. Hydrolysed preparation 2.

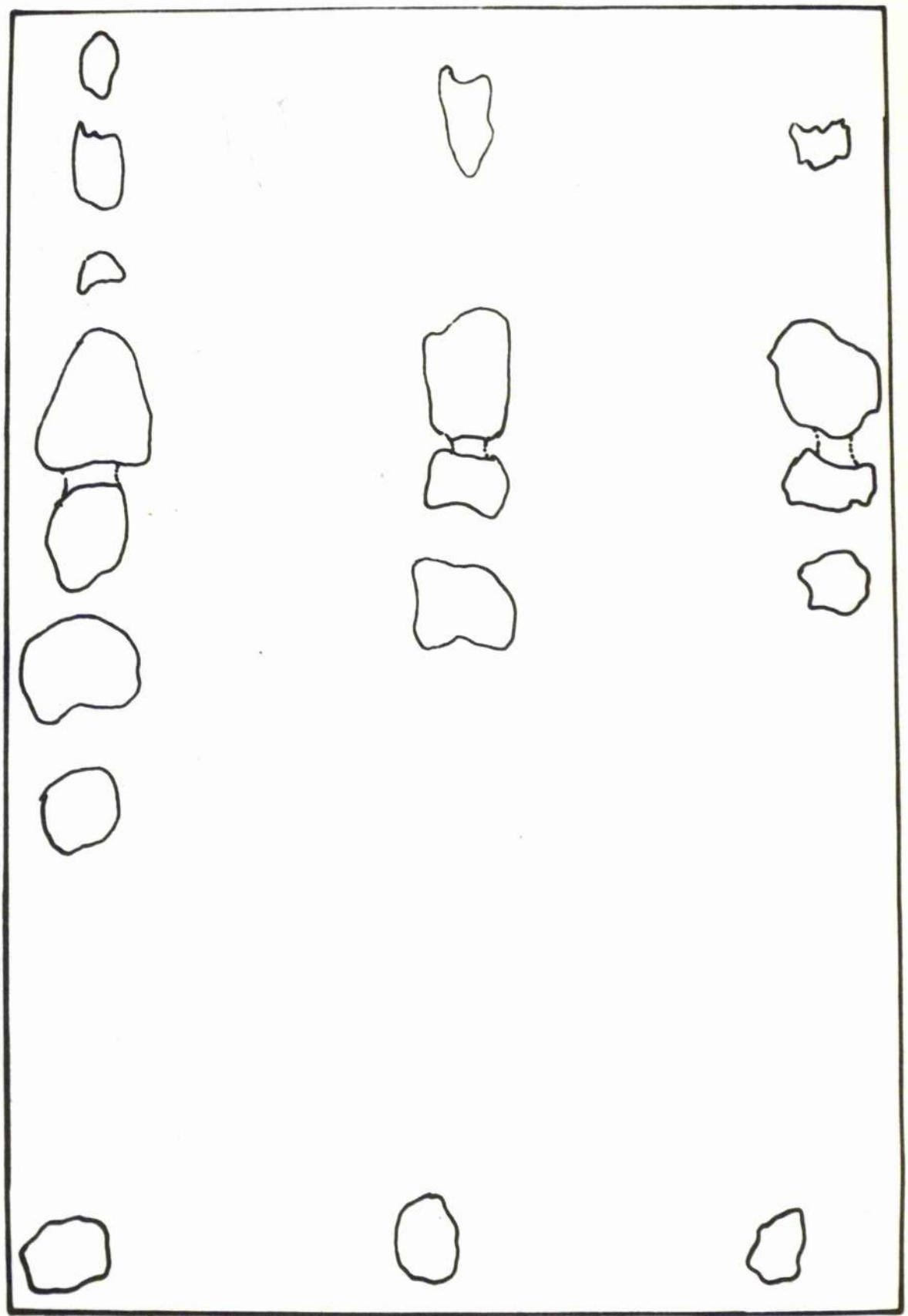


Figure 30 - Chromatographic comparison of hydrolysed collagen with two hydrolysed non-protein nitrogen preparations (see Table 16). Solvent system - butanol/acetic acid/water. (see Section II,13).

SECTION IV - DISCUSSION.

1. Nature and mechanism of the dispersion of collagen by KI and $CaCl_2$.

When precipitated collagen is brought into contact with KI or $CaCl_2$, the resultant dispersion falls into three phases with regard to the nature of the dispersed protein, depending on the concentration of the salt. In phase 1 (salt concentration less than 0.5M) there is a solubilising effect over the neutral pH range (Figure 9) without a resultant change in optical rotation or viscosity (when these are compared to the values in acetate buffer). In phase 2,(0.5 - 3M), there is a solubilising effect accompanied by a progressive decrease with salt concentration of specific rotation and viscosity. In phase 3, (greater than 3M), the change in these parameters appears to be maximal and independent of salt concentration (Figures 5 and 8). Great quantities of protein can be brought into solution in this range of concentration, 3 g. protein per 100 ml. being achieved in 3M $CaCl_2$ as compared to slightly over 1 g. per 100 ml. in the extraction medium, 0.1M acetic acid.

In phase 1, since the solubilised collagen molecules are indistinguishable from the acid-soluble native type

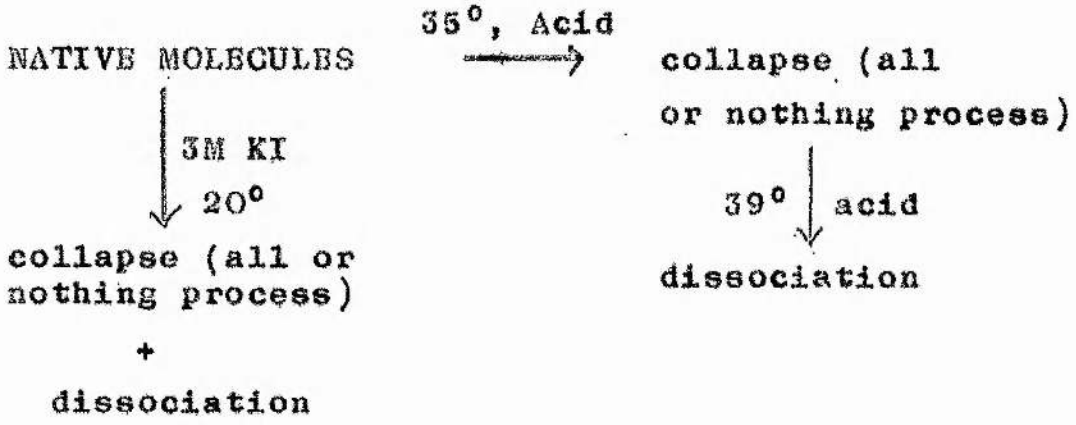
with respect to specific rotation, viscosity and the ability to form fibres when warmed to 37° (KI concentrations below 0.4M) it is most probable that the salts exert an electrostatic effect, i.e. an association of ions with charged groups on the protein. This will have the result of weakening electrostatic interactions between oppositely charged groups and thus allowing dispersion, the process being enhanced by the tendency for osmotic swelling. The physico-chemical mechanism cannot be radically different from the solubilisation of collagen from tissues with neutral NaCl of up to 2M. (Jackson and Bentley, 1960).

In phase 2, specific rotation and viscosity fall, the latter faster than the former as a function of salt concentration, and KI having a more drastic effect than $CaCl_2$. Fibres cannot be formed by heating to 37°, a property of intact native collagen. It is well established that heat denaturation (Doty and Nishihara, 1958) and urea denaturation (Steven and Tristram, 1962b) cause such alterations in physical properties and it is clear that the salts under discussion also cause a denaturation. When calf skin collagen is heated there is a sharp decrease in viscosity in the region of 35° (Figure 9). Doty and Nishihara (1958) also found that the viscosity of calf skin collagen decreased sharply at 35°. They further

noted that, if a solution of collagen was heated at 35.9°, viscosity and specific rotation decreased at the same rate. (This discussion relates solely to acid solutions of collagen.). According to Doty and Nishihara, these parameters would be expected to decrease at different rates were not denaturation a process involving a very rapid and complete breakdown of the structure of whole molecules by an all or nothing process. Engel (1962) has however proposed that the process is more complex. Studying the rate of isothermal transformation of collagen at 35°, he found that whilst viscosity and the initial slope of angular dependence of light scattering (a function of axial ratio) decreased at about the same rate, the weight average molecular weight (light-scattering) decreased more slowly. This was interpreted as implying that after the molecules collapse at 35° their subunits are still somehow bonded together and dissociate only at higher temperatures such as 39°.

In the present work, rate studies following the mixing of native collagen with concentrated KI revealed that both rotation and viscosity changed rapidly, but quantitative measurements could not be made (Section III, 2,(iii)). This in so far as it goes is indicative of the type of denaturation envisaged by Doty and Nishihara (1958), the

more so as dissociation into three subunits appeared to take place at various concentrations of KI (discussed in Section IV,2). Heating collagen under acid conditions to 39° might be expected to hydrolyse the weaker type of covalent bonds suspected to exist in the molecule (e.g. ester links as proposed by Gallop et al., 1959) whereas treatment with neutral salts in the cold could hardly do so. The second stage (dissociation of the collapsed but cross-linked protein) proposed by Engel (1962) should not thus take place during salt denaturation, and so it may be that the subunits produced during the present work are capable of further degradation through the hydrolysis of weak covalent bonds. A summary of these results would appear to be:-



For a variety of salts at 0.6M (phase 2 as defined above) the order of effectiveness for dispersing collagen fibres is:-

Na salicylate > KCNS > KI > NaI > $CaCl_2$ > LiBr > NaBr; $NaNO_3$ > NaF; KCl; NH_4Cl.

Here also KI is more effective than $CaCl_2$. The general order is very similar to the various series summarised in Table 3. There is no evidence at present to suppose that any of these salts have a different mode of action to the others, except in degree. Gallop et al., (1957) assumed that KCNS, being a well known denaturant, had an effect different to that of other salts but it can be seen that salicylate and thiocyanate, both well known denaturants, are merely the most powerful.

It is notable that the 0.4 - 0.5M salt threshold for the denaturation of collagen (Figures 5 and 8) is not repeated for the inhibition of helix formation in quenched gelatin, (von Hippel and Wong, 1962), or in the present work for the lowering of the initial temperature of viscosity drop on heating (Figure 12), i.e. the salts in these cases appear to exert a denaturing effect at quite low concentrations. This implies that the 0.5M threshold does not indicate a change in the properties of the salt but is a measure of the limit of the resistance of native collagen to denaturation; if a second disrupting factor such as heat is extant, salts below the threshold are capable of disruption, i.e. the limit is lowered.

Reversibility of salt denaturation.

In Table 10 the recoveries of specific rotation and intrinsic viscosity of collagen after heat, urea, and salt denaturation are compared, following dialysis or cooling as appropriate. It was noted that while salt denaturation (judged as complete from limiting physical measurements) is highly reversible, it is not completely so and the increase in laevorotation noted by Venkataraman, (1960) and the recovery of the native X-ray pattern noted by Sanathanam, (1959), after removal of salt are probably due to reconstitution of parts only of the molecule. In the present work gelatin with properties approaching those of native collagen could not be obtained (Section III, 2(v) and cf. Tables 5 and 7). Under the conditions used in the present work therefore, KI and urea denaturation are approximately equally reversible, and more reversible than heat denaturation. This result is distinct from that of Boedtker and Doty (1956) who found that gelatins formed by (a) heating ichthyocol in citrate buffer, pH 3.7, to 36.5°, and (b) adding concentrated KCNS to ichthyocol in the same buffer, were identical as regards intrinsic viscosity and light scattering molecular weight.

There is no literature concerning the reversibility of salt and urea denaturation of collagen, but much knowledge

has been gained in recent years concerning the mechanism by which the cooling of hot gelatin results in the reformation of native collagen-type structures. Harrington et al.(1959) using collagenase as a "structural probe" along with a variety of physical techniques were able to suggest that the redevelopment of the collagen-type structure in cooled gelatin could be considered in terms of three distinct steps going to completion at markedly different rates. These are (a) a local rapid configurational change resulting in the establishment of nuclei of poly-L-proline type helices, characteristic of the individual chains of collagen, (b), thereafter a significant proportion of the imino-rich portions of the chains folds into the poly-L-proline configuration, and finally, (c), individual chains associate specifically and a slow increase in particle weight and size results. Flory and Weaver (1959) have also stressed the importance of the formation of poly-L-proline type helices in a rate-determining process during reversion. Harrington and von Hippel (1961) have further postulated that in their step (b) above, the "collagen-fold" is stabilised through the participation of water in doubly bonded hydrogen bridges between adjacent carbonyl groups.

Rice (1960) found that only 14% of a cooled gelatin sample did reconstitute, since only this fraction of the total would yield SLS-type structures on addition of ATP. Altgelt et al., (1961) succeeded in isolating this fraction and found that it consisted of components (ascribed the symbol γ) which had a molecular weight close to that of the tropocollagen monomer. Grassmann et al., (1961) also detected a "γ" component which had a molecular weight close to that of tropocollagen and assumed to contain the other components (3α or 1α plus 1β) connected by covalent bonds. Engel (1962) also gained evidence to support the view that the reversion of hot gelatin is almost entirely due to the γ component, which allows reformation of the collagen structure because covalent bonds maintain the proper register between chains.

The removal of salt by dialysis is difficult to follow physically involving as it does volume change and precipitation; however, some limited conclusions can be arrived at concerning the reformation of some of the collagen-type structure after its removal. First of all, it seems unlikely that treatment with neutral salt at room temperature hydrolyses covalent bonds; The second stage of denaturation as described by Engel (1962), (separation of the components of the collapsed molecule

at 39° in acid,) if this, as seems likely, involves the rupture of weak covalent bonds, would not be expected to occur. During salt denaturation, if there is maximal preservation of covalent bonds, then there will be a high degree of preservation of the correct register of the polypeptide chains and therefore a high recovery of physical properties as compared with heat denaturation. This at any rate is an explanation of the results in Table 10 based on the modern views of reversion as discussed above. This explanation is substantiated to some extent by the fractionation results (Figure 15). The last component to be eluted in 3M KI, thought to be the largest in molecular weight and considered to be similar or identical to the γ component (Grassmann et al., 1961) (see Section IV,2), represents about 60% of the area under the elution curve. Altgelt et al., (1961) found however that the γ component comprises only 8% of the total collagen on heat denaturation in acid. A low rupture of covalent bonds in concentrated salt would be compatible with the high proportion of this high molecular weight component and a high recovery of specific rotation and viscosity after removal of salt (Table 10).

Engel (1962) has noted that other factors which may contribute to recovery of specific rotation and viscosity

are the formation of non-specific aggregates of components, and perhaps the formation of poly-L-proline type conformations giving short unstable regions of high rigidity and rotation.

Enzyme hydrolysis of collagen in KI solution.

Dispersal of collagen in 0.5M $CaCl_2$ has been frequently used to obtain a collagen solution for enzyme studies. (References given in Section I, 2). This concentration of salt is on the threshold for denaturation (Figures 5 and 8). Higher concentrations of salt do not apparently inhibit enzyme hydrolysis (Figure 18) and so dispersal in, say, 3M KI plus enzyme digestion could conceivably be a useful procedure where the object is to prepare peptides in large quantities for further analysis. However this may have no advantage over digestion of solid collagen for long periods of time, or digestion of conventionally prepared gelatins.

Gallop et al., (1957) found that trypsin decreased the specific rotation of ichthyocol by about 8% in 2 hours, and attributed this to digestion of small amounts of gelatin formed by thermal denaturation at room temperature. In the case of the present work, no evidence was obtained to indicate that collagen did deteriorate if left for

short times at 20°. Controls with collagen in 0.2M KI in the viscometer revealed a constant draining time over several hours, and the drop in viscosity after addition of trypsin to this has been attributed to enzymic hydrolysis of intact collagen.

Since, as can be seen in Figure 18 (inset), when collagen is dispersed in 1 - 3M KI the drop in viscosity caused by addition of trypsin (E : S = 1 : 10) is proportional to the initial viscosity of the collagen and has no apparent relationship to the concentration of salt, it is presumed that even at these high concentrations the salt has a negligible effect on the extent of the enzyme digestion.

Mechanism of salt denaturation of collagen.

The primary consideration with respect to the alteration of the properties of native collagen by concentrated KI or $CaCl_2$ is the nature of the intramolecular bonds broken in the process. Possible types of bonds in the collagen molecule can be listed:-

1. α-peptide bonds.
2. Hydrogen bonds.
3. Ionic (salt) links.
4. Other covalent bonds including ester-type links between the hydroxyl of hydroxylysine and the

β- or γ-carboxyl of aspartic or glutamic acid (Gallop et al., 1960), ester links involving carbohydrates (Grassmann et al., 1957), and peptide-like bonds involving the ε-amino group of lysine (Mechanic and Levy, 1959).

Breakage of 1 and 4 is unlikely during salt treatment at room temperature and neutrality. Breakage of ionic bonds must be considered as a possibility during salt denaturation, but it is unlikely to be a major factor since (a) non-protein nitrogen, which must be ionically bound to the protein (Section IV, 4) is not removed by 3M $CaCl_2$ or KBr, (b) Gustavson (1926b) has shown that treatment of hide with such concentrated salts does not alter its reactivity for hydrogen ions, and (c) Weir and Carter (1950) were able to conclude from thermodynamic data that the lowering of the shrinkage temperature of collagen by concentrated neutral salts is not due to breakage of ionic bonds. It must therefore be assumed that the denaturation of collagen by concentrated salts is due primarily to the rupture of hydrogen bonds.

In general there are two ways in which salts could be imagined to break hydrogen bonds in collagen:-

(a) By the binding of salt molecules or ions to specific groups which in the intact protein

are responsible for hydrogen bond cross-links.

(b) By an alteration of the properties of the medium (water) such that existing hydrogen bonds are rendered unstable.

Alternative (a) is that proposed by Gustavson (1956) and (b) was assumed by von Hippel and Wong (1962) for the inhibition of helix formation in gelatin by salts (See Section I, 2). If the former alternative is operative it is difficult to see which grouping could be involved in binding or chelating salt other than the peptide bond, and this has been assumed by Gustavson (1956) von Hippel and Wong (1962), and Bello (1963) during their discussions on possible mechanisms of denaturation of collagen by salts. The results obtained in the present work favour alternative (b), for the reasons now enumerated.

1. In Table 11 the percentage drop in the viscosity of collagen in solution in 1M KI caused by various proteolytic enzymes was shown. This drop was in many cases very nearly the same as that given in the literature for native collagen. Were the peptide bond involved in binding salt molecules or ions in the solutions studied, it is very difficult to imagine how an enzyme could hydrolyse these bonds. Trypsin appears to cause a drop in collagen viscosity even in KI solutions above 3M, when denaturation

is maximal and therefore all sites presumably "chelated" if this is the mechanism of denaturation. It might be objected that salt could be bound in specific areas in the collagen molecule, causing relaxation of structure, and that trypsin for example hydrolyses bonds in other areas causing a further drop in viscosity. However this is not supportable in view of the fact that the enzymes used, in combination, almost certainly attack the entire collagen molecule, i.e. collagenase hydrolyses peptide bonds in the imino-rich "crystalline" regions, (Mandl, 1961), trypsin in the randomised "end-tails" (Hodge and Schmitt, 1960), papain, since it releases little hydroxyproline, in the polar regions (Candlish, 1960) and pronase probably also in the polar regions (Tyson, 1963). Moreover, if a binding mechanism were extant, one would expect the enzymes themselves to become denatured and inactivated, but apparently this does not occur. *

2. von Hippel and Wong, (1962) argued that the linearity of their graph which shows the temperature of mid-point of transition of rotation in cooling gelatin against molarity of added salt excluded a binding mechanism, if the equilibrium constant for the binding reaction was small and the concentration of binding sites is small compared with the concentration of free salt. The same

* See note added in proof, page 158.

argument can be applied in the present work to Figure 12, the graph of the temperature of initial drop of viscosity of collagen in KI as a function of KI concentration. Were the lowering of this temperature in KI due to binding of the salt by the protein, one would expect that by gradually adding salt, all sites could eventually be filled or "titrated" and therefore the amount of salt bound, which would be proportional to the temperature of initial drop in viscosity, when plotted against salt concentration should give a simple adsorption isotherm. However a straight line, Figure 12, is obtained. It might be argued against this that the plots of specific rotation and viscosity of collagen in KI and $CaCl_2$ (these being proportional to the amount of salt bound if binding is operative) against molarity of added salt (Figures 5 and 8) give curves similar to a Type V adsorption isotherm. This would nevertheless indicate a complicated process distinct from simple adsorption (Maron and Prutton, 1958). Bello(1963) has severely criticised the above argument as used by von Hippel and Wong (1962) on the grounds that a deviation from linearity in theoretical graphs occurs at certain values of equilibrium constant and concentration of binding sites. However the values of K_{eq} at which this occurs are high and von Hippel and Wong assumed the

argument only for low values. Bello has also criticised the reasoning of von Hippel and Wong on this point on the grounds that the adsorption isotherm concept may not be applicable to the denaturation of a protein by ion interactions, and that moreover, partial denaturation may "destabilise" the protein toward additional denaturation leading to a greater effect when extra salt is added so that the simple adsorption isotherm graph might not be obtained.

3. It was seen in Section III,2,v that a series of salts at 0.6M varied widely in ability to solubilise collagen. This is essentially the Hofmeister series as usually given (Bull, 1951) for anions (cations show a much smaller diversity in effect), i.e.

$$KCNS^- > I^- > Br^- > Cl^-$$

According to Bull, (1951) the Hofmeister series has its immediate origin in the differences in hydration of the ions which in its turn is a function of the electrostatic field around them and their size. This is taken to be an indication that the solubilisation of collagen by salts is due in the first place to a reorientation of solvent rather than the binding of ions or molecules within a specific distribution of size at which an optimum effect would be manifested.

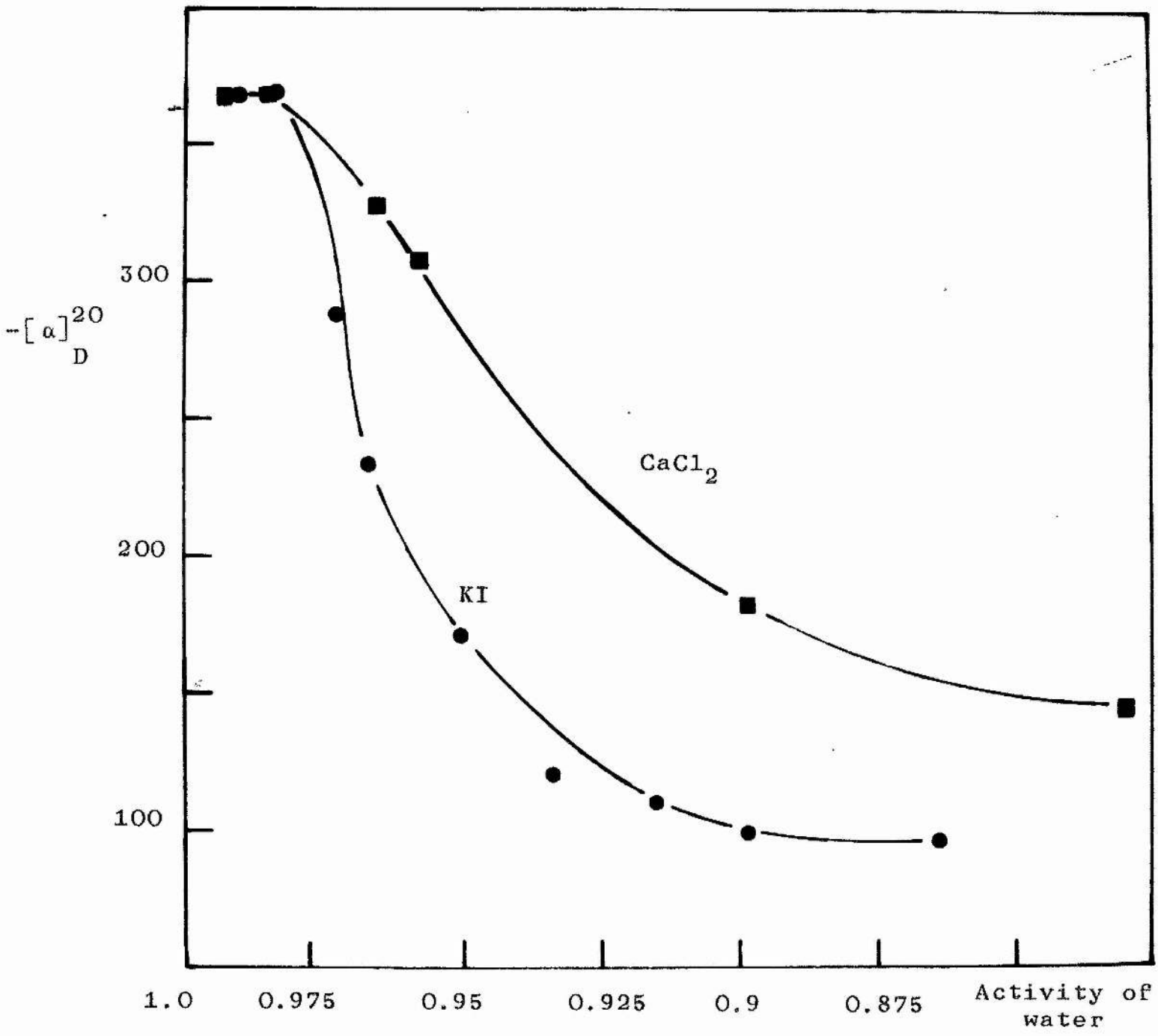


Figure 3I - Specific rotation of collagen in KI and $CaCl_2$ solutions as a function of the activity of water in these solutions.

If the denaturation of collagen by salts is due to an effect on the solvent rather than direct binding, a further conclusion from the present work is that the protein configuration is not rendered unstable by a shortage of water as such but due to some sort of alteration in its properties. It is unlikely that the hydrolytic reactions summarised in Table 11 and Figure 18 would proceed with any facility in a medium where water is scarce. Moreover, a graph in which the specific rotation of collagen in KI and $CaCl_2$ is plotted as a function of the activity of water in these salt solutions reveals that there is no simple relationship between the two. (Figure 31).

The supposition that collagen is denatured by salts through a reorientation of water offers some correlation with the viewpoint put forward by many workers (Rougvie and Bear, 1953; Bradbury et al., 1958; Bsipova, 1958; Harrington and von Hippel, 1961; Berendsen, 1962) that water molecules have an essential structural role in the maintenance of the collagen configuration.

2. The inter- and intramolecular cohesion of collagen.

From the fractionation results in Figure 15 it appears that collagen on denaturation by 3M KI dissociates

into three components. Preparative fractionations were not attempted, being outwith the main problem, namely, the nature of the action of salts such as KI on collagen, but some limited conclusions can be arrived at regarding the nature of these fractions in view of other aspects of the present work and the published results of others.

Boedtker and Doty (1956) interpreted light scattering results with collagen denatured by KCNS or heat as implying the presence of three unequal components. Kessler et al. (1960) also found three components in rat tail tendon collagen plus some low molecular weight material, using column chromatography. Grassmann et al., (1961) using calf skin collagen denatured by heating, found three components by sedimentation. These had molecular weights of 115,000 (α component), 215,000 (β), and 290,000 (γ). Since the three components found in the present work probably travel through the Sephadex gel in order of increasing molecular weight (Section II, 15) it would seem likely that, in order of elution, α, β, and γ-types were obtained. Piez et al., (1961) separated the smallest components (α and β) into two types each ($\alpha_1 + \alpha_2$ and $\beta_1 + \beta_2$) by chromatography on ion-exchange columns; since the separation in the present work depended on molecular weight rather than charge differences, a distinction between the separate types of α and β

components would not be expected.

When no more collagen could be eluted from the Sephadex after a fractionation using 3M KI, about 11% of the total collagen originally applied could not be accounted for, but could however be detected on extrusion of the column. Sephadex has little or no ionising groups and its binding power for proteins is considered to be negligible in media containing concentrated salt (Glazer and Wellner, 1962) so that this retention is probably due to a filtration mechanism. The total picture of the fractionation can then be stated as either (a), the tropocollagen monomer in 3M KI dissociates into 4 components, one of which is too large to pass through the dextran gel, (b), there is in collagen a proportion (ca. 11%) of higher aggregate of collagen molecules which are retained by the gel or (c), some of the collagen molecules do not collapse at all in 3M KI but remain as rigid rods which are retained by the gel. (It was noted in Section III, 2(viii) that undenatured collagen apparently did not pass through the gel under the condidions employed in the present work).

There is no proof to decide between these possibilities in an authoritative manner. At attempt to characterise the retained material by specific rotation and viscosity measurements was unsuccessful due apparently to contamination

with soluble dextran. However it is very likely that it is (b) which is operative, since various workers have described in preparations of soluble collagen small but definite fractions of material of dimensions larger than the tropocollagen monomer. Thus Boedtker and Doty (1956) found that prolonged untracentrifugation of ichthyocol solutions was necessary to remove material which was responsible for artificially high values of light scattering molecular weights. Jackson and Fessler (1958) reported that purified neutral extracts of rabbit skin contained collagen aggregates with dimensions of 4000-6000 x 100-200Å. Such aggregates would correspond to over 10 tropocollagen units and would have molecular weights of several millions. Veis et al., (1960) solubilised gelatin directly from skin and found a component with a molecular weight of 12×10^{-5}. Altgelt et al., (1961) also reported in some preparations of denatured calf skin collagen a component which sedimented more rapidly than tropocollagen itself. Although the presence of such aggregates is well documented, their concentration is always small and the other units in the ichthyocol preparation examined by Boedtker and Doty (1956) were stated to have a very narrow size distribution.

Subsequent to the fractionation of collagen in 3M KI (Figure 15), the fractionation in which the concentration of salt was raised in a stepwise manner (Figure 16) revealed

that undenatured collagen could not be eluted from the gel, but that as the concentration of salt was raised above the denaturing threshold (0.5M) a multi-peak elution curve was obtained which seemed to indicate some sort of complex dissociation. An attempt to clarify this matter was made when collagen was applied to a Sephadex column in acetate buffer, a solvent which was unable to elute any protein, and the elution system switched to 1M KI, when three peaks were obtained (Figure 17). When no more protein could be eluted in 1M KI, another three peaks could be obtained by washing through 2M KI.

To sum up, collagen can apparently only pass through the gel after denaturation. When it is eluted with denaturing salt, the same elution pattern was obtained at 1, 2, and 3M KI. Denaturation is only complete at 3M KI, so that only part of the collagen can dissociate into 3 components in 1M salt, leaving material which can only be dissociated by higher concentrations of salt, for example, 2M, as has been demonstrated. In other words, a sample of soluble collagen is seen as being composed of molecules of varying degrees of resistance to salt denaturation, as the result of more than one layer of strength of cross-linkage between the polypeptide chains of the subunits. Since the specific rotation and viscosity curves representing

the range of denaturation in salt (Figures 5 and 8) are uninflected as far as the methods used demonstrated, it is likely that these layers of strength of cohesion are numerous.

A similar proposal was put forward by Doty and Nishihara (1958) who deduced from studies of the rate of fall of specific rotation and viscosity of collagen when heated that the collagen molecules relaxed in an all or nothing type of transition which did not involve the persistence of intermediate states for any significant length of time. Thus when the denaturation is, say, half complete, half the molecules in the sample have melted and half are still intact. A similar picture was proposed for the denaturation of rat tail tendon collagen by Kessler *et al.*(1960).

If collagen, as proposed in the present work and by the above authors, consists of molecules with apparently similar chemical and physical properties but with varying resistance to denaturation, then this is an example of the "microheterogeneity" of a protein, a definition of which was adopted by Colvin *et al.* (1954) from an older use of the term by Synge (1943). A protein preparation is, then, said to be microheterogeneous if there is experimental evidence for one or more minor differences between individual molecules of the preparation, over a period which is long

compared with the duration of the experiment(s). This definition excludes heterogeneity caused by non-protein components, tautomerism, or degrees of polymerisation. Analysing the available data on protein heterogeneity Colvin et al. came to the conclusion that the microheterogeneity of protein preparations is a normal phenomenon caused by inherent variations which do not appear to consist of discrete steps but rather of continuous changes resulting in a family of molecules. That such a phenomenon existed was implicit in the suggestion made earlier by Tristram (1953) that if certain essential groups in a protein are specifically spaced and orientated, the identity of the remaining residues may not be very important and may reflect the environmental conditions at the time of synthesis. Kauzmann (1956a) has echoed this proposal.

Haurowitz (1956) has provided a theoretical discussion of protein heterogeneity in which he draws conclusions similar to those of Colvin et al., (1954) and discusses how inherent variations in a specific protein might arise. These are,- 1, the presence in a given protein preparation of molecules of different ages, 2, the presence in a preparation of molecules formed at different intracellular or tissue sites, 3, the influence of external factors such as abnormal temperature, poisons, antibodies or the

availability of nutrients. If microheterogeneity exists in collagen, its most likely origin is in age differences, since it is now well known that the properties of this protein in connective tissue are dependent on age. It becomes more insoluble and thus presumably more cross linked with increasing maturity (Harkness, 1961) and Jackson and Bentley (1960) have convincingly argued that at any point of time in a connective tissue the collagen is a continuous spectrum of aggregates of varying degrees of strength of cross-linkage dependent on the time which has elapsed since the constituent molecules were synthesised. These ageing effects are manifested in intermolecular cohesion, but that intramolecular changes can also take place with age has been demonstrated by the fact that the subunits of collagen similarly alter. Thus α-components in neutral salt soluble collagen link up to form β-components in the more mature acid soluble extract (Orekhovitch et al., 1960).

If the concept of continuous or microheterogeneity is entertained for collagen it is necessary to discuss how this can occur, i.e. what entities in the protein are capable of alteration without affecting the properties of the protein significantly. It seems likely that the so-called "non-crystalline" or imino-poor regions may play

a role here. For the degree of crystallinity of collagen, Ghosh (1960) has given 80%, and Haisa (1962) 50%. The non-crystalline regions, with highly polar amino acid sequences (demonstrated by Hodge and Schmitt, 1960, to occur at five discrete regions along the length of the tropocollagen unit) although not in the form of the highly ordered triple helix proposed by Rich and Crick (1956), (Bear, 1952) must nevertheless be rigid, since the whole molecule is rigid (Boedtker and Doty, 1956). In other words, they must be to some extent cross-linked, and different degrees or types of cross links in these regions could be one cause of microheterogeneity. Other causes which it is possible to imagine are differences in the overall proportion of highly ordered poly-L-proline-type or trihelix-type conformations, or differences in, or different extents of, the various unusual linkages proposed from time to time to exist in collagen, e.g. ester bonds and "unusual" peptide bonds (reviewed by Steven and Tristram, 1962c, and Harrington and von Hippel, 1961).

The overall picture of the dispersion of precipitated acid soluble calf skin collagen by KI or $CaCl_2$ is thus seen as the following:-

Below 0.5M salt, dispersion takes place without denaturation since the disruptive power of the salts is

insufficient to break any of the intramolecular bonds unless a second disruptive factor such as heat is introduced, when this threshold is lowered. As the concentration of the salt causing dispersion is increased from 0.5M to 3M, an increasing proportion of the molecules in the sample of collagen brought into solution dissociates in a fast reaction to yield three subunits, leaving a decreasing proportion undissociated until the concentration of the denaturant becomes in turn critical for their cohesive forces. The effect of the salt is seen as being exerted through a modification in the properties of the water in the solution.

3. Effect of physiological substances on the resistance to dispersion of thermally reconstituted collagen.

(i) General discussion of results.

At first sight it is peculiar that collagen should precipitate to form fibres when warmed to 37° at pH 4-9, in view of the well known solubilisation (gelatinisation) of the protein on heating. However it can presumably be visualised as the normal phenomenon of coagulation in the isoelectric range; warming speeds up the process but is not responsible for it. Such a general concept is inherent in the mechanism proposed by Hodge and Schmitt (1960) for fibre formation, namely, a quarter staggering arrangement of tropocollagen monomers caused by interaction of acidic and basic groups along the polypeptide chains.

Below the 4-9 pH range collagen is completely denatured at 37° as judged by viscosity curves (Figure 9; Doty and Nishihara, 1958). It seems most probable that warming above room temperature at both acid and near-neutral pH causes a relaxation of the protein structure; in acid, however, the highly protonated polypeptide chains do not interact and the relaxation goes to completion at 35°. In the range 4-9, increased random thermal movement results in a large number of interactions between chains

which have a balance of ionic groups and can thus form fibres. Above 50°, the disruptive effects of heat are sufficient to overcome these cohesive forces, and the collagen returns into solution in the form of random chains. Bello and Bello have recently (1963) put forward the same type of argument when discussing fibrogenisis in vivo, namely, that since the process takes place at 37°, it is probable that newly synthesised collagen molecules may not have to be as highly organised as possible to form connective tissue fibres.

From Tables 12, 13, 14 and 15 it is apparent that the substances which, when in the gelling medium, cause collagen to become more resistant to dispersion by KI or urea as compared to controls, can be divided into three classes:-

1. Anions at the "salting-out end" of the Hofmeister series.
2. Amino acids.
3. L-ascorbic acid and related enols.

These three classes are discussed separately in the following three sections.

An ascorbic acid-collagen interaction seemed to be the most interesting possibility which arose from the preliminary work, and so the bulk of subsequent experiments were performed using this substance.

It is noticeable that none of the polysaccharides tested showed any power to delay the dispersion of fibres. Since much evidence has been presented (reviewed in Section I,3) that some polysaccharides, notably chondroitin sulphate, interact with collagen in tissues, this negative result may represent merely a failure to find the correct conditions for such an interaction to be manifested. Other conditions which may be tried in the future are different buffers, different ionic strengths, and longer periods, perhaps weeks, to allow fibres to form.

Since there is a good case (reviewed in Section I,3) for supposing that the process of thermal reconstitution of collagen is similar to its polymerisation or fibrillation in vivo, the main interest of this work lay in the possibility that substances shown to delay dispersion might have a similar action in connective tissue. The reasons for supposing that there may be a stabilising factor or factors for collagen in vivo are as follows. Collagen after its initial polymerisation into fibres is known to become gradually more cross-linked, insoluble, and stable (Harkness, 1961) and it seems likely that it might be susceptible, before maturation is well advanced, to redispersion by cathepsins, denaturing metabolites such as urea, and salts. It is worthwhile therefore to look for

an agent which might bind collagen fibres, invest them, or otherwise stabilise them to prevent redispersion during the considerable period of maturation. (Verzar, 1962, found that by the criterion of hydroxyproline release scar tissue became identical with that of the rest of the body only after 10-15 years in a host 50 years old). As discussed above polysaccharides have often been assigned such a stabilising role, although in the present work this could not be demonstrated. Anions, amino acids, and ascorbic acid are specifically discussed in the following sections.

In the absence of an electron microscope, it was impossible to tell if all the fibres obtained in this work possessed the native-type 640Å longitudinal periodicity of collagen, normally obtained on thermal reconstitution in dilute media (Gross et al., 1955). It is probable that the coarse, easily dispersed fibres sometimes formed, e.g. in 0.2M LiBr were of a much more disorganised type.

(ii) Influence of Anions.

For effectiveness in preventing the redispersion of collagen fibres, a Hofmeister series of

$$SO_4^{2-} > HPO_4^{2-} > F^- > \text{citrate}^{3-} > Cl^-$$

for anions in the concentration range 0.05 - 0.2M is obtained. The Hofmeister series has its immediate origin in the differences in hydration of the various ions (Bull, 1951). The experiments described here in fact demonstrate the powers of the anions to salt-out this particular colloidal system. Water is orientated by them in such a way that the solvation of the protein is decreased and its stability as a consequence increased (Gustavson, 1956). At concentrations below 0.05M, KI, which was used to disperse collagen fibres in competition with the anions above, accelerates fibre formation as compared to NaCl (Figure 14). It is evident that the qualitative effect of salts on collagen fibres is highly dependent on concentration. This was also noted by Bensusan (1960) who found that a lack of correlation between his own results and those of Gross and Kirk (1958) could be explained by the fact that the latter workers used phosphate buffers which were competing with anions such as iodide and thiocyanate, lowering their effective concentration, and thus causing an acceleration in fibre formation at the same molarities as Bensusan observed a decrease in rate. Wood (1960a) pointed out that the nature of the solvent buffer may affect the characteristics of fibre formation, and that this is true can be readily

seen from the above series, e.g. fibres formed in citrate buffer at 0.2M will be more soluble than those formed in the same concentration of phosphate buffer, other things being equal. Courts (1962) found that citrate promoted helix formation in gelatin. This may possibly be a related phenomenon to the one studied here (the stabilisation of collagen fibres) involving the possibility of intramolecular hydrogen bond formation through the abstraction of water of solvation.

Fluoride and sulphate have been noted by Bello and Bello (1962) to be antidenaturants. Gustavson (1926b) showed that sulphate had a conserving action on hide powder since this was less soluble in it than in pure water.

(iii) Influence of Amino acids.

The effects of amino acids and salts on the subsequent resistance to dispersion of collagen after incubation at 37° with them are quite different. Although quite low concentrations of any of the amino acids tested (10^{-4}M) appear to confer some resistance to dispersion by urea, this effect is reversed or becomes negligible at about 10^{-3}M (Figure 21), i.e. the dispersion time/concentration curve is parabolic for amino acids whereas for salts it is sigmoid and furthermore salts are only

effective under the same conditions at much higher concentrations (Figure 20).

The mechanism of action of amino acids must then be different from that of salts, for which a salting-out effect has been proposed (Section IV, 3,(ii)).

The fact that all amino acids tested had a broadly similar action implies that their effect is a function of groups which are common to them all, i.e. the α-amino and carboxyl groups, which at pH 7.4 will be largely ionised. If fibre formation is visualised as a case of the coagulation of a protein in the isoelectric range, it seems most probable that these groups help to decrease the net charge of the collagen. In the region where the stability is decreased in the presence of some of the amino acids (greater than 10^{-3}M) the amino acids presumably interfere with the fibre forming tropocollagen interactions by competing for charged groups at specific sites on the molecule responsible for the quarter staggering arrangement (Hodge and Schmitt, 1960).

Steven and Tristram (1962a) found that in all the collagens they examined there were present physically associated amino acids and small peptides which constituted about 2% of the total nitrogen present in the collagen sample. The present work raises the question as to whether these amino acids associated with the collagen have a

cross-linking or stabilising action *in vivo*. The amino acid in highest concentration in the non-protein nitrogen was serine but no special effect for this could be demonstrated with the conditions used in the present work, (Figure 21). A mixture of amino acids corresponding proportionately to the composition of Steven and Tristram's non-protein nitrogen was tested in the same way as for individual amino acids. (Figure 22). A type of curve similar to that obtained for individual amino acids was obtained. If the total molarity of amino acid is considered the small stabilising effect is reversed at about 7×10^{-4}M amino acid. The evidence for and against a specific biological role for the non-protein nitrogen of Steven and Tristram is discussed fully in Section IV, 4.

(iv) Influence of L-ascorbic acid.

In the present work ascorbic acid was added to collagen solutions to give for the acid final concentrations of 0.1 - 1.5 mg. per 100 ml. Figures which have been obtained for tissues are:- 0.2 - 0.7 mg. per 100 ml. for human whole blood (Osborne *et al.*, 1948), 5 - 29.5 mg. per 100 ml. for human aqueous humor (Huber, 1959), 0.09 mg. per 100 ml. for the tissue fluid of healing areas in guinea pigs (Schilling *et al.*, 1953), and 6.2 mg. per 100 ml. for guinea pig tendon after wounding (Abt *et al.*, 1960).

Since the concentrations used in the present work were within the physiological range and there have been suggestions in the literature that collagen interacts directly with ascorbic acid, possible inferences from this work concerning *in vivo* processes will be discussed in some detail.

Gould (1960a) in a comprehensive review of the relationship between ascorbic acid and connective tissue states that "their interaction has been demonstrated to be a specific and direct one" but that "little can be said equivocally regarding specific mechanisms by which ascorbic acid may control or regulate collagen biosynthesis". It has been proposed that ascorbic acid is a cofactor in the hydroxylation of proline to hydroxyproline (Robertson *et al.*, 1959). However Mitoma and Smith (1960) have presented evidence that this hydroxylation is not decreased in ascorbic acid deficiency, and suggest that in scurvy the maturation of the fibroblast is impaired. Scorbutus has also been associated with an abnormal ground substance (Gersh and Catchpole, 1949). The biochemistry of the vitamin is further complicated by its implication in the respiratory chain (Kersten *et al.*, 1958) and in tyrosine metabolism (Zannoni and La Du, 1959).

Moreover there exists a body of evidence regarding

a possible role for ascorbic acid extracellularly at the site of collagen fibre formation, especially in healing wounds. Klein (1938) reported that in the chick embryo extracellular granules of an ascorbic acid-like substance were present and associated with collagen fibres. Schilling et al., (1953) found higher levels of ascorbic acid in the tissue fluid of healing areas than in the serum. Boyd (1955) found that the healing of corneal wounds was to some extent dependent on the concentration of ascorbic acid in the cornea and aqueous humour, Abt et al., (1960) reported increased concentrations of ascorbic acid in connective tissue during wound healing. Ksab'yan (1960) found that after production of a skin wound ascorbic acid accumulated in the protein mass at the bottom of the wound. Rauch (1949) and Personn (1953) claimed that direct application of ascorbic acid to a wound surface accelerated the rate of healing. Bourne (1956) has proposed that ascorbic acid may participate in linkages in collagen, and very recently Cmuchalova and Chvapil (1963) have found that in the carrageenan granuloma, changes in ascorbic acid correlate with collagen changes rather than cell changes, and suggest that ascorbic acid participates in the control of the structural stability of collagen fibres.

If it is accepted that the thermal reconstitution of collagen resembles the process of fibre formation at the fibroblast surface, then the present work supports the concept of an extracellular role for ascorbic acid; that is, that it interacts with collagen fibres or otherwise influences their formation such that they are more resistant to dispersion than they would be in its absence. The likely existence of such a stabilising agent was discussed in general terms in Section IV,3,(i). It is of much interest that Gould (1960b) demonstrated that on scorbutogenisis neutral salt soluble collagen in polyvinyl sponge implants in guinea pigs disappeared, and moreover there was a slow disappearance of insoluble collagen as well.

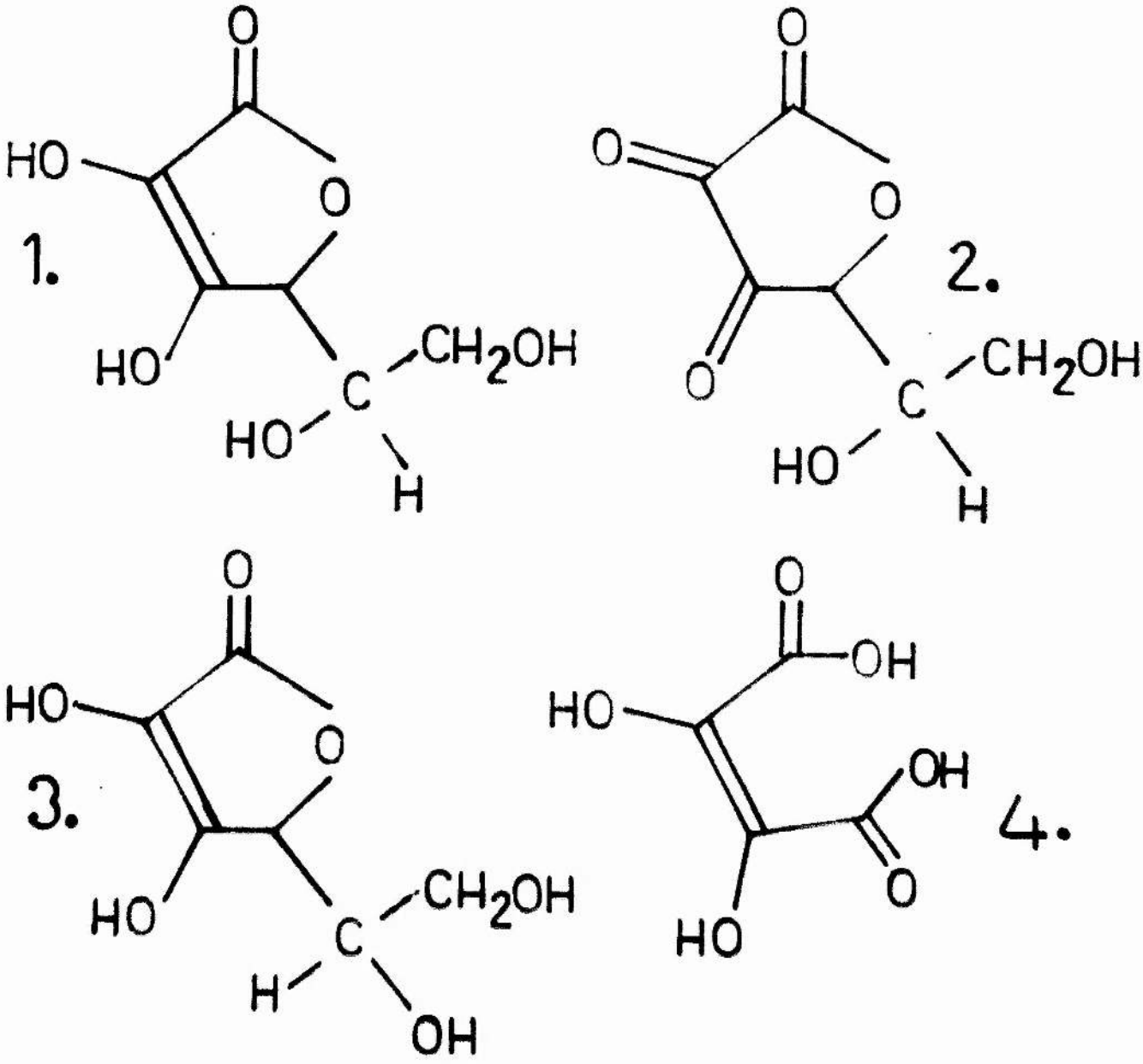
In view of the plethora of functions proposed for ascorbic acid, it would seem likely that, in repair tissue at any rate, it has at least two functions, one a basic intracellular one as a reducing agent, the other a secondary extracellular one to interact with collagen fibres. This is little more than an echo of the classical view of Wolbach and Howe (1926) - that the antiscorbutic agent is responsible for the setting or gelling of a liquid product - combined with the suggestion (Williams, 1959) that since the mucopolysaccharide and collagen

biosyntheses are probably dissimilar, and scorbutus appears to affect both, then ascorbic acid must be involved at a very basic intracellular level. In the context of the first suggestion it is notable that Stein and Wolman (1958) gained evidence indicating that the necessary materials for repair were present in a scorbutic wound but could not somehow be utilised; also Abt et al., (1960) doing collagen determinations (as hydroxyproline) could find no difference in scar tissue between scorbutic and normal animals.

A dual function for ascorbic acid might explain the long-known phenomenon of completely healed wounds breaking open upon the onset of scurvy (Lind, 1753). The ascorbic acid in the scar connective tissue in such a case is being mobilised to fulfil the more biologically important function in the cells. Again an observation of Wolbach and Howe (1926) can be cited, that is, that the factor which is needed to mediate the setting of liquid product (and which is absent in scurvy) was liberated during the destruction of tissues.

Ascorbic acid slightly decreases the fraction of collagen precipitable by warming a solution of it to 37° at pH 7.4 (Figure 25), but a similar equilibrium system is presumably not in existence in vivo since fibres will

Figure 32 - Formula of L-ascorbic acid and substances with similar structural features to it.



1. L-ascorbic acid.
2. dehydro-L-ascorbic acid.
3. D-isoascorbic acid.
4. dihydroxymaleic acid.

be extruded away from the fibroblast surface as new collagen is synthesised.

L-ascorbic acid, D-isoascorbic acid, and dihydroxy-maleic acid appear to confer resistance to salt dispersion in collagen, whereas dehydro-L-ascorbic acid is ineffective (Figure 27). The first three have in common the C - OH / C - OH grouping which in the enolic form is responsible for the acidic nature of ascorbic acid.* Dehydro-L-ascorbic acid relieves the symptoms of scurvy (Gould and Shwachman, 1943) but its possible reduction after administration must be considered. In view of the large relative rise with pH of resistance to urea dispersion in collagen fibres (Figure 24) it seems likely that this stabilisation is due to the increasing availability of the negative charges of the ascorbic acid molecules as ionisation becomes complete above the pK, quoted by Zaides (1962) as 4.1.

* See Figure 32 for formulae.

4. <u>The non-protein nitrogen of collagen.</u>

It has been established in this work that the non-protein nitrogen bound to collagen (Steven and Tristram, 1962a; Sections I,4 and III,4) is not the result of hydrolysis of weak bonds since it could be obtained from collagen extracted from skin with 3M $CaCl_2$, pH 7, at room

temperature, by means of a further manipulation under cold neutral conditions, i.e. precipitation of the protein from salt with acetone. This treatment can hardly be responsible for hydrolytic reactions. Other factors which might possibly result in the formation of amino acid artefacts, such as co-precipitation during preparation or enzymic hydrolysis have been discussed and discounted by Steven and Tristram (1962a).

Since a hydrogen bond breaker (3M $CaCl_2$) was not capable in itself of removing significant proportions of non-protein nitrogen, and if it can be ruled out that the material is covalently bonded, it is evident that the amino acids and small peptides are bound to the protein by ionic or salt links. Since different preparations yield non-protein nitrogen fractions with different compositions there must be variations in the strength of binding among the amino acids.

Table 16 shows that hydroxyproline and proline did not occur in any of the chromatograms obtained for non-protein nitrogen preparations. Steven and Tristram found these in small amounts using the rather more sensitive method of chromatography of the dinitrophenyl derivatives of the amino acids. They also found small amounts of

tyrosine, this being undetected in any of the present preparations. The predominant amino acids in the chromatograms were glycine, serine, alanine, leucine and valine; a similar type of pattern was also observed by Steven and Tristram.

Spots with very low mobilities, due probably to peptides small enough to stain with ninhydrin, were observed in Preparations 1, 5, 6, and 7 (Table 16).

Other work of interest in this context has been that of Zahn and Meienhofer (1955) who demonstrated the binding of free amino acids and peptides to wool, and that of Schneider, Bishop and Shaw (1960) who found the same phenomenon to apply to wheat gluten. Motomura (1961) found a number of free amino acids in dental hard tissues. Verzar (1960) found that both free and peptide-bound hydroxyproline was liberated from steer hide collagen during thermal contraction. Synge (1953) predicted the general occurrence of such materials in protein systems.

The unequivocal demonstration of non-protein nitrogen in collagen raises a number of important points. First of all, its presence must be kept in mind during the design and interpretation of experiments on the protein. Bearing in mind that Steven and Tristram (1962) found tyrosine to comprise 8% of their non-protein nitrogen on a molar basis (there being traces only in total collagen)

it is interesting to note that Hodge et al., (1960) regard the tyrosine-rich material liberated from collagen by trypsin as being derived from the randomised "end-tails" which interact to form fibres, whereas Kuhn et al., (1961) consider that trypsin removes only tyrosine-rich impurity from the system. It is at least possible that non-protein nitrogen contributes to these fractions and should be considered in an attempt to resolve the controversy. Since it was shown in the present work that non-protein nitrogen is released under acid and near-neutral conditions by an ion-exchanger, it is evident that its presence should always be suspected during column fractionations of collagen Kessler et al., (1960) in fact found a forepeak of low molecular weight material during column fractionation of rat tail tendon collagen. During such manipulations as dialysis, electrodialysis, electrophoresis, and precipitation with ethanol or acetone, the possible release of non-protein nitrogen should be kept in mind and checked with controls.

Secondly, since we must assume that the non-protein nitrogen is associated with collagen in connective tissue, a possible biological function for the material must be considered. It was shown in the present work that when

fibres were thermally reconstituted in the presence of various amino acids, these fibres were slightly more resistant than controls to urea dispersion (Section III, 3,(iii)). The resistance however was comparatively low and appeared to be non-specific among the amino acids tested, i.e. although serine was invariably in high concentration in the non-protein nitrogen it was not markedly more effective as a stabilising agent than the others. Again, it must be remembered that optimum conditions for cross-linking or other stabilising processes may not have been obtained in the present work.

While bearing all these points in mind, it is interesting to note that a whole series of non-protein constituents have been demonstrated in collagen and gelatin. Apart from amino acids (Steven and Tristram, 1962a) there have been shown to be such ions as fluoride (Bartlett, 1961), aldehydes (Landucci et al., 1958), nucleic acids (Venet and Pouradier, 1959) and of course sugars (Consden, 1953). It appears that collagen can very effectively bind a variety of non-protein substances. Collagen after its synthesis in the molecular form must be transported to the cell surface, shed into the extracellular space where it polymerises (Porter and Pappas, 1959) and then be further organised sterically into the typical connective tissue fibre, having a high tensile strength. During this

process the polyfunctional groups in the molecule, especially outwith the protection of the cell membrane, come into contact with a large variety of other substances, some of which may be bound, the protein in this respect perhaps acting in a manner akin to that of an ion-exchanger.

There is no definite evidence at the moment to decide whether the amino acids and peptides examined here come into the category of fortuitously bound substances or have some definite chemical or biological function.

SUMMARY

1. The literature concerning the dispersion of collagen by concentrated salt solutions has been reviewed; the literature concerning the reconstitution of native type collagen fibres has been briefly reviewed, as has recent literature on the properties of the substrate for this work, calf skin collagen.

2. It has been shown that the dispersion of collagen by KI and $CaCl_2$ involves, above a threshold concentration of salt, a highly reversible denaturation of the protein.

3. This denaturation has been shown to result in the dissociation of collagen into three subunits at various concentrations of salt. It has been proposed on the basis of these fractionations that the collagen employed consists of a population of units with varying degrees of resistance to depolymerisation, and therefore varying degrees of intramolecular cohesion.

4. It is considered that the available evidence favours as the mechanism of denaturation the reorientation of water in the solvent by KI or $CaCl_2$ rather than the direct binding of salt molecules or ions to the protein.

5. Collagen fibres thermally reconstituted in the presence of various physiological substances have been shown to have different degrees of resistance to redispersion by KI and urea. Substances which appear to render the collagen more resistant are some anions, all amino acids tested, and L-ascorbic acid.

6. The effect of ascorbic acid and related substances on the resistance to redispersion of thermally reconstituted collagen has been studied in some detail and it is considered that the stabilising effect of this substance is due to its ionising enol grouping. The literature concerning a possible interaction of L-ascorbic acid with collagen in vivo has been reviewed and it has been proposed that the acid may have both an intracellular and extracellular function in repair tissue.

7. It has been demonstrated that non-protein nitrogen can be prepared from collagen in a number of ways, some of which do not involve extremes of pH or temperature throughout the entire manipulation of the collagen. Evidence for and against the assigning of a specific biological function to this non-protein nitrogen has been discussed.

BIBLIOGRAPHY

(An asterisk indicates that the original publication was not consulted).

Abt, A.F., von Schuching, S. and Roe, J.H. (1960) J. Nutrition 70, 427.

*Alexander, P. (1957). Ann. New York Acad. Sci. 53, 653.

Altgelt, K., Hodge, A.J. and Schmitt, F.O. (1961). Proc. Natl. Acad. Sci. U.S. 47, 1914.

Astbury, W.T. (1961). J. Internat. Soc. Leather Chemists 45, 186.

*Bakerman, S. (1961). J. Gerontol. Abs. 16, 388.

*Bartlett, J.C. (1961). Analyst 86, 200.

*Bazin, S. and Delauney, A. (1958). Ann. Inst. Pasteur 94, 7.

Bear, R.S. (1952). Adv. Protein Chem. 7, 69.

Bello, J. and Bello, H.R. (1962). Nature 194, 681.

Bello, J. and Bello, H.R. (1963). Nature 197, 77.

Bello, J. (1963). Biochemistry 2, 276.

Bensusan, H.B. (1960). J. Amer. chem. Soc. 82, 4995.

Bensusan, H.B., Mumaw, V.R. and Scanu, A.W. (1962) Biochemistry 1, 215.

*Berendsen, H.J.C. (1962). J. Chem. Phys. 36, 3297.

Bigelow, C.C. and Geschwind, I.I. (1961). Comp. rend. Lab. Carls. 32, 87.

Blout, E.R. and Fasman, G.D. (1958) in "Recent Advances in Gelatin and Glue Research", p. 271. Ed. by Stainsby, G. Pergammon Press, London.

Boedtker, H. and Doty, P. (1956). J. Amer. chem. Soc. 78, 4267.

Bourne, G.H. (1956) in "The Biochemistry and Physiology of Bone", p. 539. Ed. by Bourne, G.H., Academic Press, New York.

Bowes, J.H., Elliot, K.G. and Moss, J.A. (1953) Biochem. J. 61, 143.

Boyd, T.A.S. (1955). Brit. J. Ophthalmol. 39, 202.

*Bradbury, E.M., Burge, R.E., Randall, J.T. and Wilkinson, G.R. (1958). Disc. Faraday Soc. 25, 173.

Bull, H.B. (1951) "Physical Biochemistry". John Wylie and Sons, New York.

Burge, R.E. and Hynes, R.D. (1959). J. mol. Biol. 1, 155.

Candlish, J.K. (1960) Honours thesis, St. Andrews University.

Carpenter, D.C. and Lovelace, F.E. (1935). J. Amer. chem. Soc. 57, 2337.

Chmuchalova, B. and Chvapil, M. (1963). Biochim. Biophys. Acta, 69, 565.

Cohen, C. (1955a). J. biophys. biochem. Cytol. 1, 203.

Cohen, C. (1955b). Nature 175, 129.

Colvin, J.R., Smith, D.B. and Cook, W.H. (1954). Chem. Revs. 54, 687.

Consden, R. (1953) in "Nature and Structure of Collagen", p. 196. Ed. by Randall, J.T. Butterworths, London.

Courts, A. (1958) in "Recent Advances in Gelatin and Glue Research", p. 145. Ed. by Stainsby, G. Pergammon Press, London.

Courts, A. (1961) Biochem. J. 81, 356.

Courts, A. (1962) Biochem. J. 83, 124.

Cowan, P.M., McGavin, S. and North, A.C.T. (1955). Nature 176, 1062.

*Deasy, C. (1960). J. Amer. Leather Chem. Assoc. 58, 258.

*Delaunay, A., Bazin, S. and Henon, M. (1956) Bull. Acad. nat. Med. (Paris) 140, 233.

Doty, P. and Nishihara, T. (1958). In "Recent Advances in Gelatin and Glue Research", p.92. Ed. by Stainsby, G. Pergammon Press, London.

Dweltz, N.E. and Mahadevan, V. (1961). Biochem. J. 81, 134.

Engel, J. (1962). Arch. Biochem.Biophys. 97, 150.

*Esipova, N.G. (1957). Biofizika 2, 455.

Fessler, J.H. (1960a). Biochem. J. 76, 124.

Fessler, J.H. (1960b). Biochem. J. 76, 452.

Gallop, P.M., Seifter, S. and Meilman, E. (1957). J. biophys. biochem. Cytol. 3, 545.

Gallop, P.M., Seifter, S. and Meilman, E. (1959). Nature, 183, 1659.

*Gallop, P.M., Seifter, S., Lukin, M., and Meilman, E. (1960) J. Amer. chem. Soc. 235, 2619.

*Gersh, I. and Catchpole, H.R. (1949). Ann. J. Anat. 85, 457.

*Ghosh, S. (1960). Bull. cent. Leath. Res. Inst. Madras 6, 329.

*Giffee, J.W., Firch, J.G., Campbell, J.N. and Kirvaitis, J.S. (1961) Bull. cent. Leather Res. Inst. Madras 8, 93.

Glazer, A.N. and Wellner, D. (1962). Nature, 191, 862.

Gould, B.S. and Shwachman, H. (1953). J. biol. Chem. 151, 439.

Gould, B.S. (1960a). Vitamins and Hormones 18, 89.

Gould, B.S. (1960b). Fed. Proc. 19, 149.

Grant, W.H. and Alburn, H.E. (1960). Arch. biochem. biophys. 89, 262.

*Grassmann, W., Hannig, K., Endres, H., and Riedel, A.(1956) Z. physiol. Chem. 306, 123.

Grassmann, W., Hofmann, V., Kuhn, K., Hormann, H., Endres, H., and Wolf, K. (1957) in "Connective Tissue" (Council Intern. Org. Med. Sci. Symposium), p.157. Ed. by Tunbridge, R.E. Blackwell, Oxford.

Grassmann, W., Hannig, K. and Schleyer, M. (1960). Z. physiol. Chem. 322, 71.

*Grassmann, W., Hannig, K. and Engel, J. (1961). Z. physiol. Chem. 324, 284.

Green, N.M. and Lowther, D.A. (1959). Biochem. J. 71, 55.

*Gross, J., Highberger, J.H. and Schmitt, F.O. (1952) Proc. Soc. exptl. Biol. Med. 80, 462.

Gross, J., Highberger, J.H. and Schmitt, F.O. (1955). Proc. natl. Acad. Sci. U.S.41, 1.

Gross, J. (1956). J. biophys. biochem. Cytol. 2, 261.

Gross, J. (1958a). J. Exptl. Med. 108, 215.

Gross, J. (1958b). Nature 181, 556.

Gross, J. and Kirk, D. (1958). J. biol. Chem. 233, 355.

*Gustavson, K.H. (1926a) J. Amer. Leather Chem. Assocn. 21, 206

*Gustavson, K.H. (1926b). Colloid Symposium Monograph 4, 79.

Gustavson, K.H. (1956). "The Chemistry and Reactivity of Collagen". Academic Press, New York.

Gustavson, K.H. (1957). In "Connective Tissue" (Council Intern. Org. Med. Sci. Symposium), p.185. Ed. by Tunbridge, R.E. Blackwell, Oxford.

*Haisa, M. (1962). Bull. Chem. Soc. Japan 35, 769.

Hannig, K. and Engel, J. (1961). Leder 12, 213.

Harkness, R.D. (1961). Biol. revs. 36, 399.

*Harrington, W.F. and Schellman, J.A. (1957). Comptes rend. Lab. Carls. 30, 167.

Harrington, W.F. and Sela, M. (1958). Biochim. Biophys. Acta 27, 24.

Harrington, W.F. (1958). Nature 181, 997.

Harrington, W.F., von Hippel, P.H. and Mihalyi, E. (1959). Biochim. Biophys. Acta, 32, 303.

Harrington, W.F. and von Hippel, P.H. (1961). Arch. Biochem. Biophys. 92, 100.

Harrington, W.F. and von Hippel, P.H. (1961). Adv. Prot. Chem. 16, 1.

Haurowitz, F. (1956). J. cell. comp. Physiol. 47, Supp.1, 10.

Highberger, J.H., Gross, J. and Schmitt, F.O. (1950) J. Amer. chem. Soc. 72, 3321.

Hodge, A.J. and Schmitt, F.O. (1958). Proc. natl. Acad. Sci. U.S. 44, 418.

Hodge, A.J. and Schmitt, F.O. (1960). Proc. natl. Acad. Sci. U.S., 46,186.

Hodge, A.J., Highberger, J.H., Deffner, G.G.J. and Schmitt, F.O. (1960). Proc. natl. Acad. Sci. U.S.46,197.

*Houck, J.C. (1962). Surgery 51, 632.

Huber, A. (1959) in "Scientific Tables", p. 363. Ed. by Geigy, J.R. Geigy Pharmaceutical Co., Ltd., Manchester.

Jackson, D.S. (1953) Biochem. J. 54, 638.

Jackson, D.S. and Fessler, J.H. (1955). Nature 176, 69.

Jackson, D.S. and Fessler, J.H. (1957) in "Connective Tissue" (Council Intern. Org. Med. Sci. Symposium), p.57. Ed. by Tunbridge, R.E. Blackwell, Oxford.

Jackson, D.S. and Bentley, J.P. (1960). J. biophys. biochem. Cytol., 7,37.

Kahn, L.D., Carroll, R.J. and Witnauer, L.P. (1961). Biochim. Biophys. Acta 50, 592.

*Katz, J.R. and Wienhoven, J.F. (1933). Rec. trav. chim. 52, 487.

Kauzmann, W. (1956a). J. cell. comp. Physiol. 47, Supp. 1, 29.

Kauzmann, W. (1956b). J. cell. comp. Physiol. 47, Supp. 1, 113.

Keech, M.K. (1961). J. biophys. biochem. Cytol. 9,193.

*Kersten, H., Kersten, W. and Staudinger, H.G. (1958) Biochim. Biophys. Acta 27, 599.

Kessler, A., Rosen, H. and Levenson, S.M. (1960). J. biol. Chem. 235, 989.

*Klein, L. (1938). Anat. Anz. 87, 14.

*Kraemer, E.O. (1938). J. Ind. Eng. Chem. 30, 1200.

*Kroner, T.D., Tabroff, W., and McGarr, J.J. (1955). J. Amer. chem. Soc. 77, 3356.

*Ksab'yan, G.S. (1960). Ark. Patol. 18, 91.

*Kuhn, K., Grassmann, W. and Hofmann, U. (1960). Naturwiss. 47, 258.

Kuhn, K., Kuhn, J. and Hannig, K. (1961). Z. physiol. Chem. 326, 50.

Kuhn, K. (1962). Collagen Currents 3, 253. (Abstract of lecture).

*Kuntsel, A. (1937). in "Stiasny Festschrift", p.191. Roether, Darmstadt.

Landucci, J.M., Pouradier, J. and Durante, M. (1958) in "Recent Advances in Gelatin and Glue Research", p.62. Ed. by Stainsby, G. Pergammon Press, London.

Lathe, G.H. and Ruthven, C.R.J. (1956). Biochem. J. 62, 665.

Leach, A.A. (1960). Biochem. J. 74, 61.

*Lind, J. (1753) in "A Treatise on Scurvy" Millan, London.

Lowry, O.H., Roseburgh, V.J., Farr, A.L. and Randall, R.J. (1951) J. biol. Chem. 193, 205.

Manahan, J. and Mandl, O. (1961). Biochim. biophys. res. Comm. 4,368.

Mandl, I. (1961). Adv. Enzymol. 23, 103.

Maron, S.H. and Prutton, C.F. (1958) in "Principles of Physical Chemistry" p.217 MacMillan, New York.

Martin, G.R., Mergenhagen, S.E. and Scott, D.B. (1961). Biochim. Biophys. Acta 49, 245.

Mechanic, G.L. and Levy, M. (1959). J. Amer. chem. Soc. 81, 1889.

Mitoma, C. and Smith, T.E. (1960). J. biol. Chem. 235,426.

*Motomura, S. (1961). J. Biochem. Tokyo 50, 172.

*Nageotte, J. (1927). Compt. rend. soc. biol. 96, 172.

*Nemeth-Csoka, M. (1960). Acta Histochemica 9, 282.

Nemetscheck, T. and Gansler, H. (1961). Ztschr. Naturforsch. 166, 496.

*Neumann, R.E. and Logan, M.A. (1950). J. biol. Chem. 184, 103.

*Nordwig, A., Hormann, H., Kuhn, K., and Grassmann, W. (1961) Z. physiol. Chem. 325, 242.

*Ohlwiler, D.A., Jakiewicz, M.J., Butcher, H.R. and Brown, J.B. (1960). Surg. Forum 10, 301.

Orekhovich, V.N. and Shpikiter, V.O. (1957) in "Connective Tissue" (Council Intern. Org. Med. Sci. Symposium), p. 281. Ed. by Tunbridge, R.E. Blackwell, Oxford.

*Orekhovich, V.N., Shpikiter, V.O., Mazourov, V.I., and Kounina, O.V. (1960). Bull. soc. chim. biol. 42, 505.

*Osborne, L.S. (1942). J. Lab. clin. Med. 27, 1135.

Partington, F.R. and Wood, G.C. (1963). Biochim. Biophys. Acta 79, 485.

Patterson, J.W. (1950). J. biol. Chem. 183, 81.

Personn, B.H. (1953). Acta Soc. Med. Upsaliensis 58, Supp. 2.

*Pfeiffer, P. and Wurgler, J. (1926). Z. physiol. Chem. 97, 128.

Piez, K.A. and Gross, J. (1960). J. biol. Chem. 235, 995.

Piez, K.A., Lewis, M.S., Martin, G.R., and Gross, J. (1961) Biochim. Biophys. Acta, 53, 596.

Piez, K.A., Eigner, E.A. and Lewis, M.S. (1963). Biochemistry 2, 58.

Porath, J. and Flodin, P. (1959). Nature 183, 1657.

*Porath, J. (1959). Clin. Chim. Acta 4, 776.

Porath, J. (1960). Biochim. Biophys. Acta 39, 193.

*Porter, K.R. and Pappas, G.D. (1959). J. biophys. biochem. Cytol. 5, 153.

Ramachandran, G.N. and Kartha, G. (1955). Nature 176, 593.

*Rauch, S. (1949). Deut. med. Wochschr. 74, 863.

Redfield, R.R. (1953). Biochim. Biophys. Acta 10, 344.

Rice, R.V. (1960) Proc. natl. Acad. Sci. U.S. 46, 1186.

Rich, A., and Crick, F.H.C. (1955). Nature 176, 915.

*Rich, A. and Crick, F.H.C. (1961). J. Mol. Biol. 3, 483.

Robertson, W. van B., Hewitt, S. and Hermann, C. (1959). J. biol. Chem. 234, 105.

*Rougvie, M.A., and Bear, R.S. (1953). J. Amer. Leather Chem. Assoc. 48, 735.

Sanathanam, M.S. (1959). Proc. Ind. Natl. Acad. Sci., 49A, 215.

Saunders, P. (1956). Personal communication to Courts(1958)

Schellman, C. and Schellman, J.A. (1958). Comptes rend. Lab. Carls. 30, 462.

Schilling, J.A., Radakovich, M., Favata, B.V., Filer, L.J. and Jespersen, H.W. (1953). Surg. Gynecol. Obstet. 97, 434.

Schleyer, M. (1962). Z. physiol. Chem. 329, 97.

Schmitt, F.O., Gross, J. and Highberger, J.H. (1953). Proc. natl. Acad. Sci. U.S. 39, 459.

*Schneider, R., Bishop, H. and Shaw, B. (1960). Brit. J. Pharmacol. 15, 219.

Schrohenloher, R.E., Ogle, J.D., and Logan, M.A. (1959) J. biol. Chem. 234, 58.

Smith, E.L. and Kimmel, J.R. (1957). Adv. Enzymol. 19, 267.

*Soejima, M. and Shimura, K. (1961). Koso Kagaku Shimpojiomu 14, 229.

*Stein, O. and Wolman, M. (1958). Brit. J. exptl. Pathol. 39, 418.

Steven, F.S. and Tristram, G.R. (1962a) Biochem. J. 83, 240.

Steven, F.S. and Tristram, G.R. (1962b). Biochem. J. 85, 207.

Steven, F.S. and Tristram, G.R. (1962c). Ann. Rep. Chem. Soc. 58, 367.

*Synge, R.L.M. (1943). Chem. Revs. 32, 135.

*Synge, R.L.M. (1953) in "The Chemical Structure of Proteins" p. 43. Ed. by Wolstenhome, G.E.W. and Cameron, M.P. Churchill, London.

*Thomas, A.W. and Kelly, M.W. (1927). Ind. Eng. Chem. 19, 477.

Todd, A. (1960) in "A Laboratory Manual of Analytical Methods in Protein Chemistry" p.245. Ed. by Alexander, P. and Block, R.J. Pergammon Press, London.

Tristram, G.R. (1953) in "The Proteins" vol. I, part A, p. 181. Ed. by H. Neurath and K. Bailey. Academic Press, New York.

Tristram, G.R. (1961). Personal communication.

Tyson, I. (1963). Personal communication.

Urnes, P. and Doty, P. (1961). Adv. Protein Chem. 16,401.

Veis, A., Anesey, J. and Cohen, J. (1960). J. Amer. Leather Chem. Assoc. 55, 548.

*Venet, A.M. and Pouradier, J. (1959). Sci. Ind. Phot. 30, 406.

Venkataraman, S. (1960). Proc. Ind. natl. Acad. Sci. 52A, 80.

*Verzar, F. (1960). Gerontologia, 4, 104.

von Hippel, P.H. and Wong, K.Y. (1962). Biochemistry 1, 664.

*Weir, C.E. and Carter, J. (1950). J. Research natl. Bur. Standards 44, 599.

*Williams, G. (1959). Brit. J. exptl. Pathol. 40, 176.

*Wilson, J.A. (1928) in "The Chemistry of Leather Manufacture vol. 1, p.232. Chemical Catalog. Co., New York.

Wolbach, S.B. and Howe, P.R. (1926). Arch. Pathol. Lab. Med. 1, 1.

Wood, G.C., and Keech, M.K. (1960). Biochem. J. 75, 588.

Wood, G.C. (1960a). Biochem. J. 76, 598.

Wood, G.C. (1960b). Biochem. J. 76, 605.

Wood, G.C. (1962). Biochem. J. 84, 429.

Yang, J. T. (1961). Adv. Protein Chem. 16, 323.

*Zahn, H., and Meienhofer, J. (1955). Textile Res. 25, 738.

*Zaides, A.L. (1962). Biofizika S.S.S.R. 7, 263.

Zannoni, V.G., and La Du, B.N. (1959). J. biol. Chem. 234, 2925.

Note added in proof.

The argument concerning enzymes in Section IV,1 appears on reperusal to be presented, through the desire for brevity, in such a way as to be capable of being misunderstood.

The essential point made is that the extent of enzyme digestion of collagen does not seem to be affected by salt, i.e. the percentage drop in viscosity caused by trypsin is related not to salt concentration but to the initial state of the collagen; also the percentage drop in viscosity caused by various enzymes in 1M KI is the same as that obtained when salt is absent. If salt were being bound to the collagen during salt denaturation, it would seem likely that as the denaturation became maximal in concentrated salt, i.e. nearly all the binding sites were filled, the bulky enzyme molecules would not be able to approach the few remaining hydrolysable sites due to the blocking effect of the bound salt. However this inhibition apparently does not occur. Moreover, one would in considering a binding mechanism have to bear in mind that salt denaturation is not specific for collagen as a protein (cf. Bigelow and Geschwind, 1961) so that in concentrated salt at least some of the enzymes used might be expected to bind quantities of salt, causing inactivation by shielding the active site.

It is not claimed that this highly mechanical picture can be comprehensive in discounting ion or salt binding; for example it ignores such possible two-stage mechanisms as the binding of ions at low salt concentrations making the protein susceptible to, say, a shortage of solvent water at higher denaturing salt concentrations, or the possibility that the salt is chelated in some sort of "loose" manner which allows displacement or competition by enzymes. However it seems a reasonable picture in terms of present knowledge.