SPECIFIC INTERACTIONS OF PROTEINS AND PROTEIN MODEL SYSTEMS.

A Thesis submitted by

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SUMMARY

After an introduction, where the development of the idea of the paramount importance of the tertiary structure of globular proteins is briefly traced - the main section of the thesis gives an account of two parallel lines of research.

1. The natural protein system - bovine serum albumin. An investigation of its unique binding ability from the point of view of the tertiary structure was made, using the technique of "Spot Line Chromatography". Particular attention was paid to a recently noted effect, that the binding ability of Serum Albumin "decays" when the protein moves across filter paper. The protein was treated with various denaturing agents and the effects produced in binding and decay noted. A point of particular interest was that urea appeared to "renature" heat treated bovine serum albumin. This has been explained on the basis of urea, refolding the polypeptide chain. Chemical modifications of the protein were carried out and various effects noted. The reaction with a bifunctional fluoro reagent stabilised the tertiary structure and significantly reduced binding decay.

The general conclusions reached were that: (a) binding of anions was due to separation of positive from negative centres on the protein molecule. (b) Decay was due to a partial refolding of the molecule different from normal denaturation, possibly initiated by disulphide transfer

reactions and reducing the charge separation.

2. "Synthetic protein" models were made with the idea of imitating a tertiary structure directly, using systems quite different from polyamino acids. These included:-

(a) Solutions and films of polyvinyl alcohol.

(b) Solutions of chemically modified polyvinyl alcohol.

(c) Colloidal solutions of partially hydrolysed long chain esters of polyacrylic acid.

Diffusion of dyes through polyvinyl alcohol films was found to be strongly solvent and temperature dependent. It has been proposed that this is due to a selective lowering of the glass transition temperature of the polymer by solvent - a comparison with protein denaturation has been made. Dye interaction with the chemically modified polyvinyl alcohol has also been studied.

Strong solvent dependence has also been found on the scattering of colloidal solutions of the partially hydrolysed long chain polyacrylates. This is believed to be a result of the change in shape of the polymer with alteration in solvent composition. Treatment with ultraviolet radiation in mixtures of ethanol/water has indicated a slight ability to retain a specific structure.

In an attempt to mimic the binding power of serum albumin, a system was prepared which would give a separation of charges. Water soluble samples, when investigated by spot line

chromatography produced loops in a line of anionic dye and migrated towards the anode - i.e. behaved as a macroanion. This very interesting system also appeared to lose its dye binding ability with standing. This is an effect analogous to protein decay and probably due to refolding or aggregation of the polymer molecules so as to eliminate charge separation.

Also included in the above main section are short reviews on e.g. the occurrence, function and binding ability of serum albumin, protein denaturation, synthetic protein models, glass transition temperature in polymers, at points where they are relevent to a clearer understanding of the problem. Most of the experimental details are also included in the above section. More specific details of methods and an account of work on another example of protein interaction, viz., the esterase ability of serum albumin, is given in an Appendix, along with attempts to make a model esterase system.

INTRODUCTION.

The biological significance of globular proteins seems to be critically dependent on the folding of the polypeptide chains, and can hardly be understood at all from simple amino acid composition, or even sequence data, alone. The development of this idea of tertiary structure in proteins will be considered at this stage and its significance as an abstract idea discussed with respect to possible protein model systems.

The Tertiary Structure of Proteins.

It is now generally accepted, that the predominant chemical linkage in proteins is the α-peptide linkage, that is, an amide bond between α-amino and α-carboxyl groups of adjacent amino acid residues. The peptide concept of protein structure was proposed independently by Emil Fischer and F. Hofmeister in 1902. There was no mention at this early stage of any folding or arrangement of the resulting polypeptide chain. It was assumed that probably the curious properties of proteins would be completely revealed once the amino acid sequence was worked out.

Knowledge of sequential arrangements of amino acid residues began to appear in the early 1930's. In addition to these, there also began to appear a number of attempts to find regularity in the order of amino acid residues in proteins. One such attempt was Bergmann's periodicity hypothesis, which in light of modern work is now considered to be incorrect.

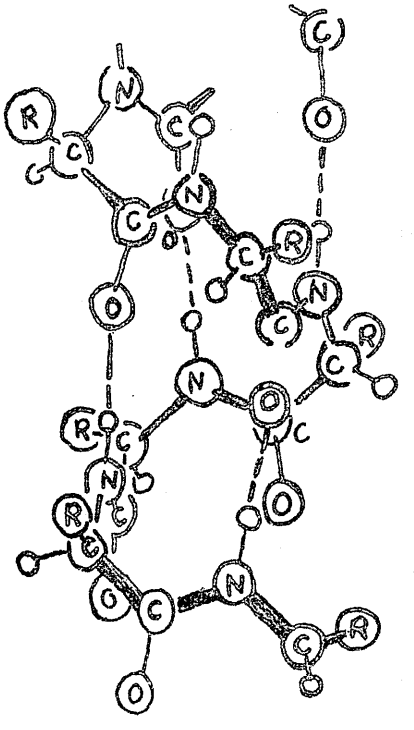
A milestone in this field was Sanger's[1] brilliant elucidation of the complete amino acid sequence of the protein insulin, by purely chemical means. This led to a spate of this type of work culminating in the recent elucidation of the structure of ribonuclease[2], and cytochrome C[3].

The idea of making synthetic polypeptides (discussed in a later section) was initiated also by Fischer, who synthesised an octadeca peptide. Nowadays advanced syntheses of naturally occurring polypeptide hormones have been accomplished, e.g. Du Vigneaud's work on oxytocin and vasopressin, the synthesis of the A[4] and B chains of insulin, and the total synthesis of adrenocorticotrophic hormone[5].

From X-ray diffraction studies on fibrillar proteins, e.g. silk and keratin, there have emerged several facts which are extremely useful in attacking the structure of globular proteins. Firstly the deduction that there are only a few characteristic configurations for the polypeptide chain, and the existence in keratin of two discrete configurations the α and β forms. Utilising these data, Pauling and Corey[6] have suggested two possible extended configurations for polypeptide chains "the pleated sheets", these structures lead to planar sheets possessing a regular folded or pleated arrangement. They seem to explain adequately the known facts of the β-keratin structure. The folded α form has been shown to possess a helical structure. This idea was suggested by Huggins[7] in 1943, who proposed a model of three amino acid residues per turn, alternate turns being bonded together by

intramolecular hydrogen bonds between N-H and C=O groups. A similar suggestion was put forward in 1949 by Bragg[8], for the chains in native globular proteins. Such a structure has been shown to be inadequate.

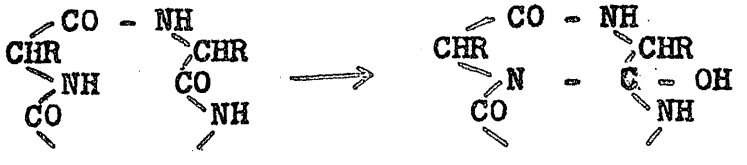
Pauling and Corey made an exhaustive survey of possible structures based on bond angles and bond distances, with the additional assumption that each nitrogen atom forms a hydrogen bond with a carbonyl oxygen atom of another residue. The structure best fitting their requirements is the so called α-helix. (A part of which is shown diagrammatically below).



In this helix, each NH group is hydrogen bonded to the carbonyl oxygen of the third residue from it. There are 3.7 residues per turn of the helix (previous authors of helical models had assumed, for no apparent reason, that this number must be

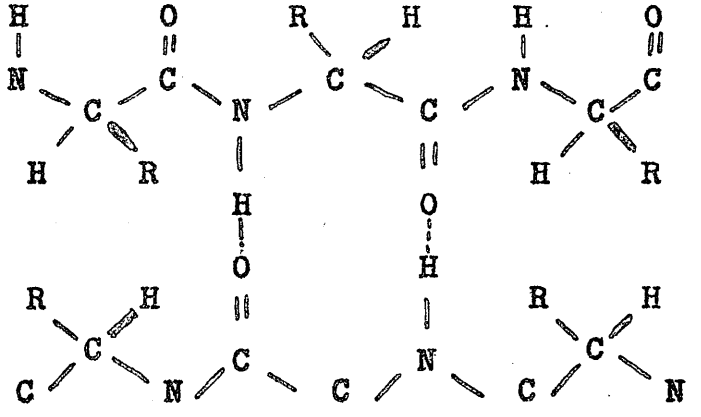
integral) and the pitch corresponds to 1.5 Å per residue. The hydrogen bonds run approximately parallel to the fibre axis. It would appear that the α-helix is the configuration adopted by α-keratin and also by certain synthetic polypeptides of high molecular weight, such as polyglutamate esters. It should be noted that the presence of various R groups in actual proteins complicates the picture and may lead to some modification of the coiled structure. In the derivation of the α-helix the only consideration that was given to these side chain groups was that there be adequate room for them. It seems very remarkable that a structure so derived could be correct for even synthetic polypeptides. This then is the picture of the three dimensional structure of the fibrillar proteins, but what about their globular counterparts, whose structures are undoubtedly much more complex?

Assuming a folded structure, it is of interest to find out the nature of the folding. Many ideas have been proposed, some with little experimental proof. One type, of considerable historical interest, is the cyclol structure proposed by Wrinch[9] in the late 1930's. This was a proposal that in native proteins, the amino acid residues are linked into rings through an N-C bond arising by a hydrogen shift from an NH group to a CO as shown below.



By means of this concept, one can build up many planar ring structures of high symmetry. Such layers might stack up to yield essentially globular structures, which could dissociate reversibly. However it does appear, that all the experimental evidence for the cyclol structure has been disproved.

A folding similar to that proposed in the cyclol structure, but involving hydrogen bonding between the CO and NH groups might not be subject to some of the criticisms of the cyclol structure. As one of the simplest postulates, one might assume extended chains running across the molecule and doubling back in a plane or pleated sheet, bonded laterally by interchain hydrogen bonds, shown diagrammatically below.



Coulombic forces would probably be of importance in holding this structure together; possibly also -S-S- bonds. It has been suggested that proline residues might serve the function of "hinges" at the folds, as these residues do not fit smoothly into an ordinary peptide chain but tend to cause it to double back. Polar R groups might tend to lie on one side of the planes, nonpolar groups on the other. From this one could easily understand the formation of layers with one

polar and one non-polar surface. The thickness of this layer, approximately 9 Å, is what is usually observed for protein monolayers in surface studies. If proteins were essentially sheets composed in this way, they would tend to combine in solution in such a way as to cover up their non-polar surfaces. A minimal water soluble protein molecule on this basis, would be analogous to a double layer. This process could continue, leading to stacked structures with polar outer surfaces. These structures however are not in complete accord with various physicochemical data for proteins.

Pauling and Corey suggested that the α-helix may be the basic configuration in at least some if not all globular proteins. Certain features of the X-ray diffraction patterns of certain globular proteins are in excellent agreement with this idea e.g. the strong 1.5 Å reflection predicted by the α-helix, but not accounted for by any other proposed configuration. With the development of improved methods of X-ray analysis the complete three dimensional structures of the proteins haemoglobin,[10] and myoglobin,[11] have been elucidated. This work showed the completely unpredictable way the polypeptide chain was folded into a highly coiled and specific structure - the tertiary structure.

The ability of globular proteins to retain a highly specific chain conformation, in solution, is something which sets them apart from other natural macromolecules, e.g. rubber and polysaccharides, which in suitable solvents undergo continuous folding and unfolding and possess no definite

tertiary structure. The modern view is that the tertiary structure of globular proteins is of paramount importance: it is essential for the biological activity of those proteins which act as enzymes, hormones, antigens or antibodies: e.g. haemoglobins from different species, although differing somewhat in amino acid sequence, have virtually the same tertiary structure and the function of the protein in the various species is always the same - viz. the transport of oxygen.

The question one may now ask is, how does the tertiary structure come about? In the light of modern biosynthetic evidence it seems that once the basic polypeptide chain is made, this (because of the order of the amino acid residues) spontaneously folds into a highly coiled specific structure, the stability of which is maintained by a number of factors. Among these may be mentioned: (1) the hydrogen bond - so important in stabilizing the α-helix.

(2) Coulombic attractions between negative and positive groups.

e.g. $-\overset{+}{N}H_3 \cdots\cdots \overset{-}{C}O_2$

(3) Direct ester linkages - formed between alcohol groups of serine and threonine with available carboxyls.

(4) The disulphide bridge, both intra and inter chain. This undoubtedly maintains the specific conformations of many globular proteins by forming cross links between different parts of the polypeptide chains.

With the elucidation of the structure of myoglobin, one can say with greater certainty, which forces are involved in

stabilisation. In myoglobin about 70% of the peptide chains, the straight parts, are composed of an α-helix, the kinks being caused by proline residues. The resulting structure is so closely folded that there is very little space for water molecules inside the globular parcel. It was thus concluded that the specific chain conformation was maintained through attractions between side chains of amino acid residues in adjacent peptide chains, with dipole induction of polar side chains and Van der Waal's forces between the non-polar side chains of valine, leucine, isoleucine and phenylalanine.

This concept of non-polar interactions can be explained very briefly as follows. In aqueous solutions of the protein those non-polar side chains are surrounded by water molecules, which tend to combine with other water molecules by means of the hydrogen bond. The mutual attraction of the water molecules causes a repulsion of these non-polar groups from the water, and therefore they will coalesce with groups of the same kind forming hydrophobic regions. It is highly probable that in all the globular proteins the maintenance of the unique conformation depends to a great extent on these "hydrophobic bonds", which originate from deep within the molecule.

Modern views on the biosynthesis of proteins suggest that the mechanisms whereby these molecules are assembled are all concerned with linear arrangements and sequences, e.g. the genetic information relayed from D.N.A. to messenger R.N.A. is contained in a linear arrangement of groups of three

heterocyclic bases. This linear code is then "translated" into a three dimensional end product or further information carrier - the globular protein. From this, it can be seen why the tertiary structure of proteins has assumed such paramount importance, as a clear understanding of this structure, in each case, would yield a wealth of information on enzyme and hormone action, how an antigen "recognises" its antibody, and perhaps even the origin of life itself.

Molecular Biology and Macromolecular Chemistry.

As the functions of biopolymers become increasingly understood in terms of physicochemical ideas, so the possibility emerges of synthesising other molecules, with perhaps quite different structures, that will perform similar functions.

Thus macromolecular chemistry, which at present is almost exclusively concerned with the purely architectural functions of natural and synthetic polymers, has an enormous potential growth towards the development of synthetic systems, which will exhibit many of the properties of the natural macromolecule. The search would be for methods of constructing systems in such a way, that the end product would exhibit such features as enzymic activity, selective interaction with small molecules and other macromolecules, or perhaps in the not too distant future - the power of partial self replication.

Specific Interactions of Proteins and Protein Model Systems.

One of the oldest and most important ideas in this field is the "Lock and Key hypothesis". This is explained rather well by looking at almost any enzyme system, the substrate specificity of which can be explained by assuming that there is a region, (or regions) on the protein surface, which form some sort of complementary "image" of the shape of the substrate molecule. A similar sort of correspondence could account for immunological reactions. One can perhaps summarise the key functions of the soluble proteins, by saying that a protein can hold, and act on information about other molecules: it is the central general molecular control device; and the control element always depends on the protein holding some sort of "image" of the molecules which it can control. If this "image" can be interpreted fairly literally then the fundamental property of matter, which would allow the structure of such a molecule to be significant to another molecule would be the fall in potential energy when two molecular surfaces fit closely to each other.

For the protein chemist, then, two subjects are of particular interest.

(1) Protein - small molecule interactions and protein - protein interactions.

(2) The construction of non-protein models, which can mimic, in even the slightest way, the "Lock and Key" control mechanism in proteins.

In the research described here, Part 1 is concerned with the first subject and Part 11 with the second, in particular, attempts made to construct a system to interact specifically with small molecules.

DISCUSSION

(Part 1)

Choice of System

Apart from being readily available, serum albumin was chosen for this work, because it is a well studied protein, and in particular with the following points in view:-

1. The amino acid composition[12] has been established as have the molecular weight and general shape and size of the molecule.

2. The molecule is particularly interesting in that it interacts prolifically with small molecules and ions, both anionic and neutral. A great deal of study has been done on these interactions particularly by Klotz[13] and co-workers who have proposed many theories as to the mode of binding and the structure of the protein. The possible implications of this binding ability with respect to the biological function of plasma albumin are manifold, and often obscure.

3. Finally a technique called Spot Line Chromatography has been developed[14] and introduced recently. This technique can readily study protein:- dye interactions and it was found that it worked very well with serum albumin, and also showed a very interesting hitherto unexplained effect, which could have great biological significance.

Figure 2.

Equal spots of B.S.A. encountering a dye line of Biebrich Scarlet, at pH 6.8.

All 300 µg. spots of B.S.A.

Spots are placed different distances from the dye line so that the effect on loop area due to the protein covering different areas of paper can be studied.

Spot Line Chromatography

This is a method for studying interactions between soluble proteins and dyes. It depends on measuring the movement of dye molecules caused by moving protein and usually a paper electrophoresis technique like the original one of Kunkel and Tiselius[15], is employed.

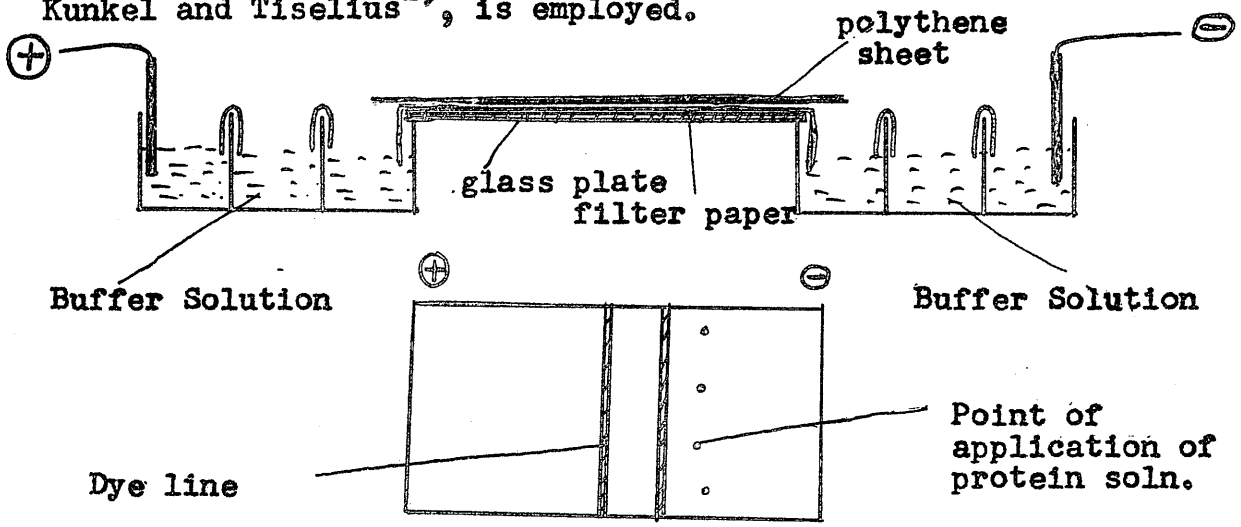
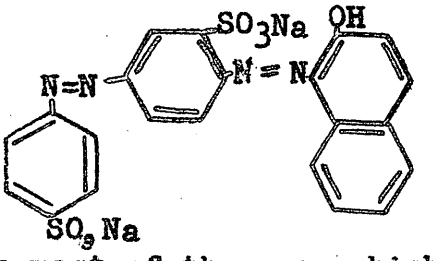


Figure 1.

The filter paper, or any other supporting medium, is placed on the glass plate as shown (Fig. 1). The dye under examination is drawn as a fine uniform line at right angles to the direction of movement of the protein. The technique is akin to a chromatography because there is a distribution of dye between a moving phase (the protein solution) and a stationary one (the filter paper). The protein is moved through the dye, usually by electrophoresis, but it can be done by siphoning. If there is binding of the dye by the protein, a distortion or "loop" as it has been called, is produced in the dye line. (Fig. 2), shows the effect that is obtained, when spots of bovine serum albumin move through a line of the water soluble anionic azo dye Biebrich Scarlet.

It has been shown[14] that the area of the loop formed in the line of dye is directly proportional to the weight of protein used, with a minimum threshold weight of protein below which loop formation does not occur. It was also shown that the loop area produced seems to depend on the area of paper covered by the protein before it reaches the dye line (Fig. 2). For the technique to work efficiently, it is essential that the dye is bound reasonably strongly to the paper. Biebrich Scarlet fulfils this condition very well,



and was used in most of the runs which will be described later.

The binding of non-coloured materials to serum albumin can also be studied by this technique. There is only a limited number of binding sites on the serum albumin molecule and hence if there is competition between the non-coloured material and the dye, for these available binding sites, then this competition will be shown by a reduction in the size of the loop formed in the dye line.

Other techniques which have been used to study protein-dye interactions include equilibrium dialysis, partition analysis, and measurement of the shift in absorption spectrum of a dye when it is bound to protein. All are very accurate and give good quantitative results. They have the drawback of using relatively large quantities of material and it

takes time to get many results on chemically modified, or physically treated protein. Spot line chromatography has the advantage that a wealth of semi-quantitative data, on modified and native protein can be collected quite quickly. The beauty about the procedure is that the results are directly observable, conclusions can be reached as soon as the run is completed. Furthermore it is not a difficult matter to carry out several similar experiments at the same time. Also spot line chromatography requires small amounts of protein, usually about 200 µg - 300 µg, but good loops can be produced with as little as 50 µg of bovine serum albumin.

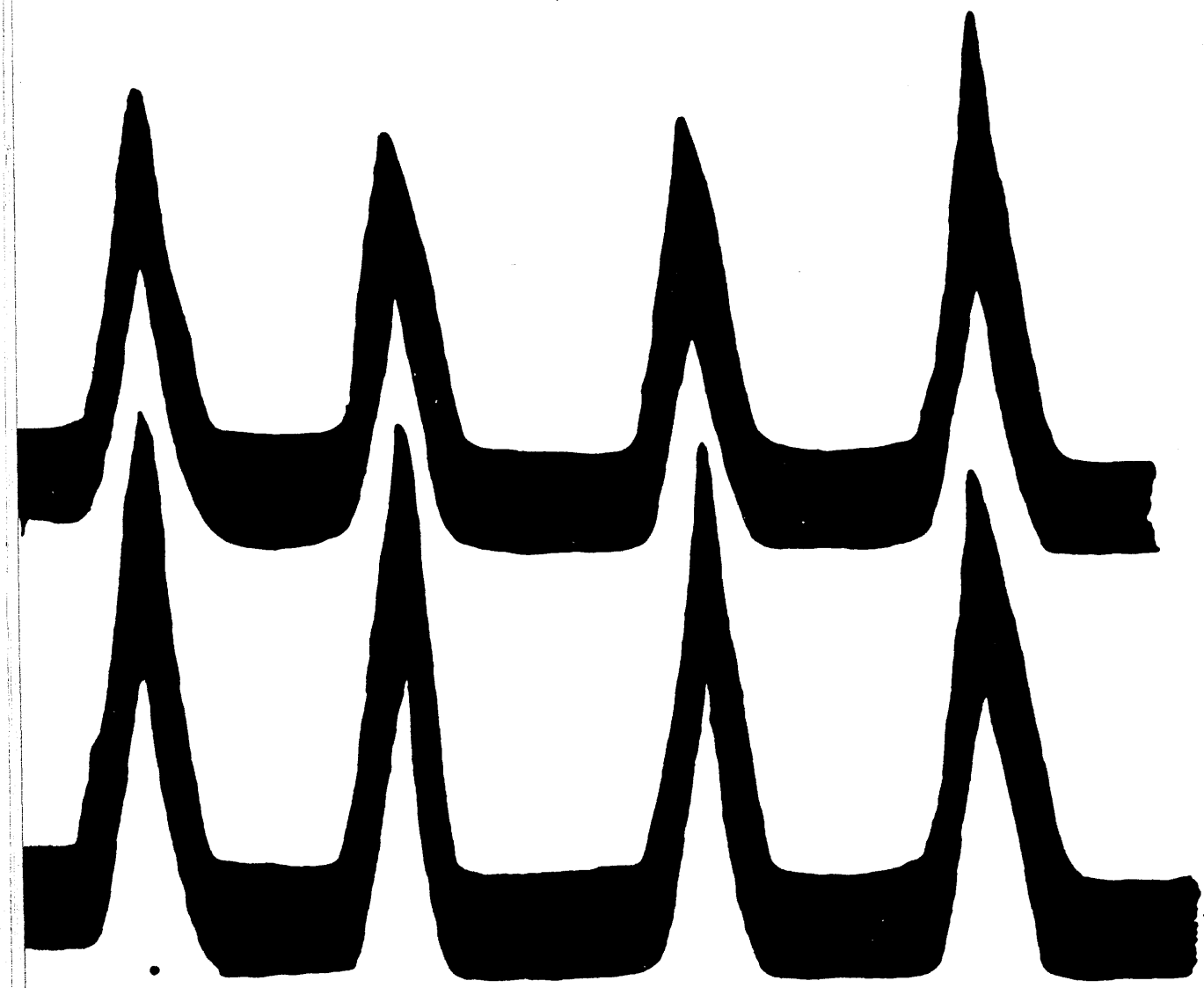
The accuracy of results obtained from spot line chromatography is quite good. The binding constant of bovine serum albumin for the dye biebrich scarlet has been found and the value agreed quite well with data for methyl orange worked out by others[16]. The dyes have quite close structural relationship and the binding sites are probably the same in each case, so a comparison of results seems valid.

(see p. 26)

It was noted[14] that loop area was dependent on the area of paper covered by the protein before it encountered the dye. This reduction in binding ability has been called "binding decay" and will be discussed in much greater detail later. It was thought that perhaps this effect was some artifact of the technique and did not represent anything unique about bovine serum albumin. To settle the question, the technique was applied to studying the interaction between

Figure 3.

Run with D.N.A. and Safranine O, at pH 6.8.



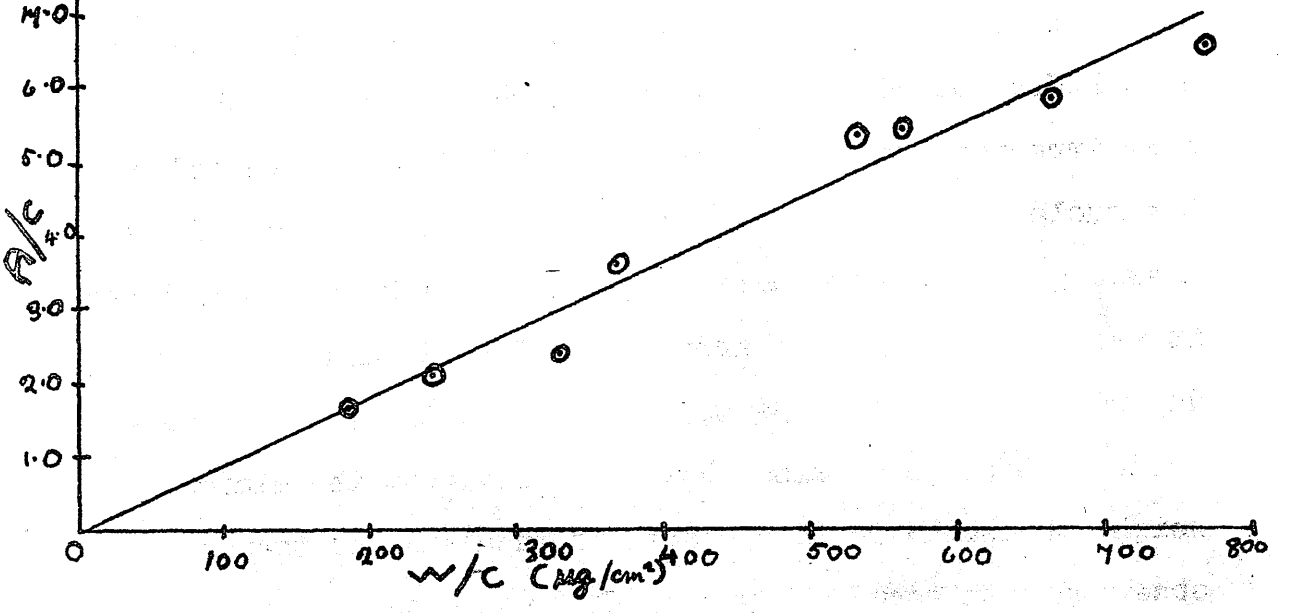
Two dye lines used here to examine the decay effect. (In serum albumin system loops in second line are always smaller than those in first). In D.N.A. system very little difference.

All spots, 300 µg of D.N.A.

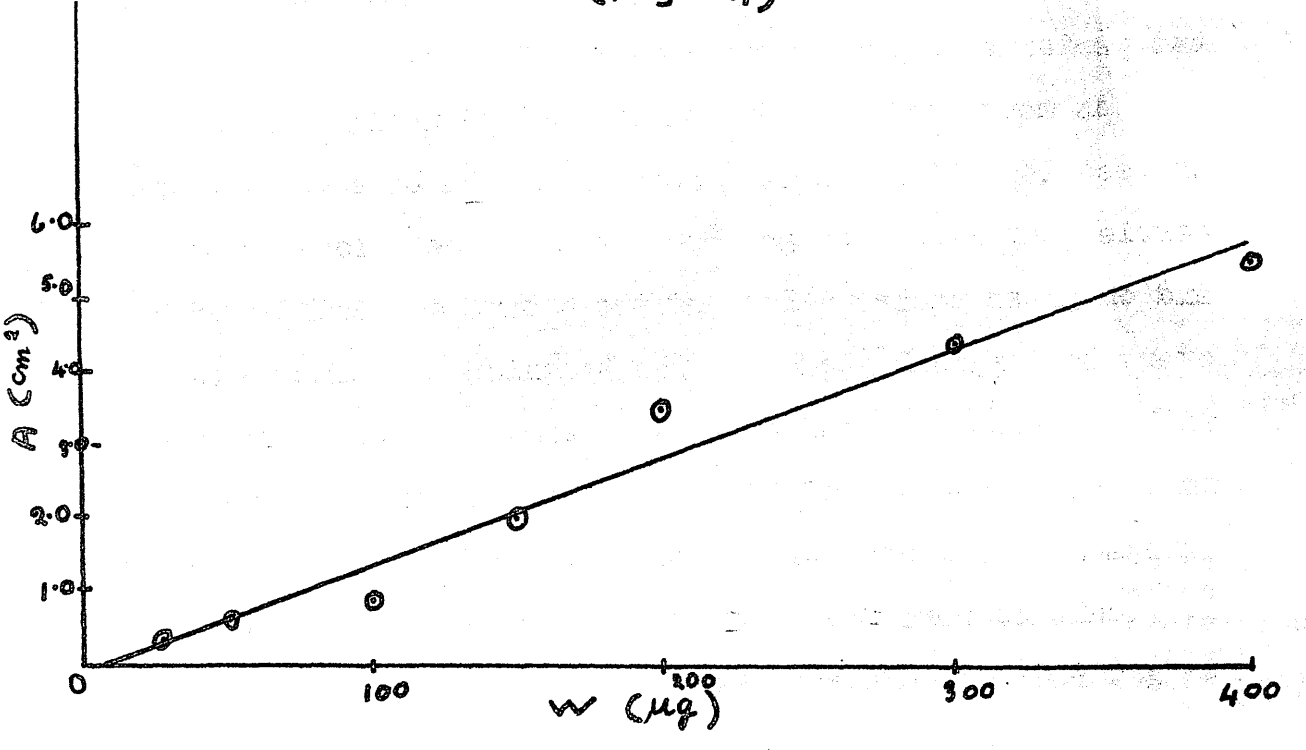
A denotes area of loop (cm^2),

W is weight of protein (μg) and

C is area of paper swept out by protein (cm^2).



(Fig 4)



(Fig 5)

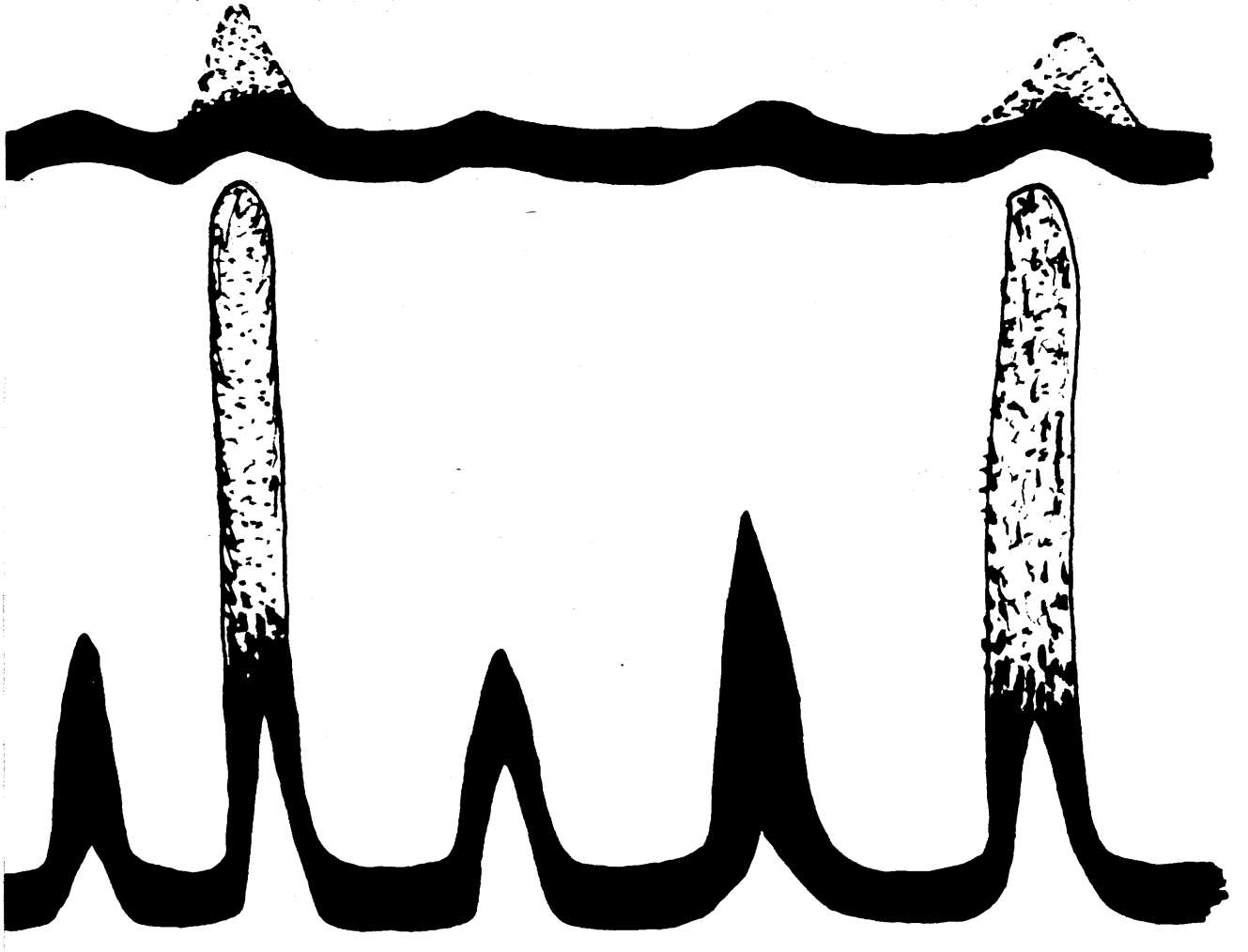
D.N.A. and the cationic dye Safranine O. The binding in this case is probably not specific, but simply electrostatic attraction. It was found that (Fig. 3) the loops produced when D.N.A. moved through a line of Safranine were the same size irrespective (within reason) of distance travelled by the nucleic acid before it entered the dye. When A/C was drawn against W/C and also A against W, as Figs. 4 and 5 show, it was obvious that the area of the loop formed does not depend on the area of paper swept out by the spot of nucleic acid. These plots also showed clearly that the minimum weight threshold in this system is much smaller than was observed with serum albumin. This would appear to be conclusive evidence that "decay" is not a fault of the technique, but pertains to the serum albumin molecule.

As mentioned already, the supporting medium for this process is filter paper. However it can be successfully carried out in starch gel, on strips of cellulose acetate, and on glass paper. None of these have any real advantage over the original choice. The technique has also been tried with no supporting medium at all, simply a thin film of buffer, in a cell made out of two microscope slides. This used extremely small amounts of protein, about 5 μg, but practical difficulties were very great and it was extremely tedious to get reproducible results.

Spot line chromatography was applied to studying albumins from different species e.g. bovine, human, equine and porcine.

Figure 6.

Comparison of binding power of various albumins, bovine, human and porcine, at pH 6.8.



100 µg. B.S.A.

100 µg. H.S.A.

100 µg. P.S.A.

200 µg. B.S.A.

200 µg. H.S.A.

Loops produced by equine albumin very similar to those of bovine albumin.

As Fig. 6 shows there is very little difference in the binding ability of the protein from human, cow or pig. However the vast "flame" on the loops produced by the human albumin is indicative of different classes of binding sites on this molecule which adsorb the dye much more strongly.* Finally it should be noted that the decay effect operates in all of these proteins; thus being a general effect.

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* It had already been shown[17], that although the binding of methyl orange to human serum albumin and bovine serum albumin was the same at pH 6.8 it was higher with human serum albumin at pH 9.0. The method of investigating the dye binding here viz., spot line chromatography may expose these other binding sites at lower pH (6.8) than usual.

Serum Albumin

1. Occurrence. 2. Function. 3. Structure and Binding Ability.

1. The general term albumin applies to any globular protein which is soluble in water at its isoelectric point. The blood plasma contains about 6.5% protein in solution, of which the major fraction is albumin. Albumins from different species, especially bovine and human, have been crystallised by salting out with ammonium sulphate. A more recent method of isolation involves the use of ethanol-water mixtures at low temperature[18].

There is a slight difference in the amino acid composition of albumins from different species, e.g. the bovine serum protein differs from the human one in the amount of aliphatic, aromatic and hydroxy amino acids. Also as Klotz[19,20], has shown bovine serum albumin must be slightly different from human serum albumin because Cu^{2+} interacts differently. However, overall features like molecular weight, acidity and basicity are about the same for the proteins.

2. The question of the function, or functions, of serum albumin is still the subject of much speculation. It contributes most of the blood's osmotic pressure[21]. However individuals are known who have very little serum albumin, and although the osmotic pressure of their blood is about one third of normal, they are otherwise healthy. Another role which has been proposed for this protein, is that it provides a

nutritional reserve.

It has been suggested[22] that the function of serum albumin is to act as a vehicle for the transport, round the system of fatty acids. From the data on the interactions of serum albumin with small anions, and various neutral molecules, (mentioned in greater detail later), the idea of this protein as a "transport vehicle" has been extended greatly. In addition to ionic material being transported, because of the solubilising effect exerted on water insoluble materials, e.g. lipids, steroids etc., these are also believed to be carried round the system, by this protein.

Further speculation, indicated by research described here, is that serum albumin may have a built in mechanism which allows the release of the molecule being transported, at its required site of action.

3. The molecular weight of 66,000 for plasma albumins has now been found by a variety of methods including X-ray diffraction,[23a] osmotic pressure, and sedimentation - diffusion.[23b] Light scattering usually yields a somewhat higher value, typically 70,000 - to 75,000, presumably because of the presence of dimer. The frictional ratio 1.30 and the intrinsic viscosity 0.035, indicate the molecule to be somewhat asymmetric or swollen. It has been shown[23c] that these data can be interpreted on the basis of a parcel, of dimensions 145 Å x 45 Å x 32 Å.

The amino acid composition of the albumins is known[24].

Points of particular interest which stand out are:-

(a) The very high value for cystine residues and

(b) the very low value for cysteine residues.

The amphoteric nature of the macromolecules is influenced by the large number of imidazole, phenolic and guanidyl groups of the amino acid residues. The dissociation constants of the supposedly free carboxyl, amino and phenolic groups were found to be abnormal; it seems that these groups participate in some kind of internal binding in the molecule[25], presumably by hydrogen bond, type interactions.

There is no evidence which is incompatible with the model of serum albumin, as a single polypeptide chain coiled into a specific structure, stabilised in three dimensions by seventeen disulphide bonds, hydrogen bonds, and hydrophobic interactions, certainly this is an exceptionally long chain. A subunit smaller than 66,000 has been proposed[26] on the basis of molecular weight studies of albumin oxidised with performic acid to break disulphide bonds. That mild oxidation produces subunits seems unlikely on the basis of the results obtained by others[27,28].

It has been shown[29,30] that both human and bovine serum albumin contain 0.68 sulphydryl group per molecule. The fraction of the albumin with the free -SH group has been called mercaptalbumin. This protein dimerises readily, by direct reaction with mercury or by standing on its own. The reaction with mercury can be formulated thus:-

$$2\ \text{ALB-SH} + Hg^{++} \longrightarrow \text{ALB-S-Hg-S-ALB} + 2H^{+}$$

Upon treatment with cysteine, the molecule reverts to its original molecular weight. Apart from this evidence of inhomogeneity, there is much evidence to show that there are more than two or three types of albumin in a pure preparation. It seems plausible that many different kinds of albumin molecules exist in any preparation, but they do not differ much in their molecular size, shape of chemical composition[31].

The seventeen or so, cystine residues in serum albumin presumably represent that number of intramolecular disulphide bonds, which form cross links between different parts of the single polypeptide chain. That the disulphide bonds were intramolecular was proved by showing that the molecular weight of the protein remained unchanged even after complete reduction of the protein[32]. If any of the dithio bonds had been interchain their cleavage would have been accompanied by a decrease in molecular weight.

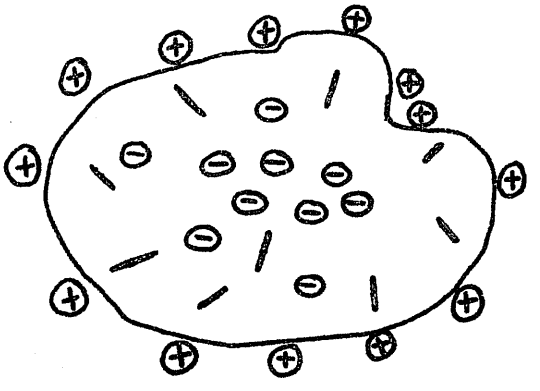
As some of the dithio bridges are buried inside the globular protein, they will be inaccessible to the reducing agents used. For complete reduction, it is essential to treat the molecule in such a way as to loosen the structure (e.g. by concentrated urea[32,33] or detergents) to allow access to the reagent.

Although the numerous -S-S- bonds in the serum albumin molecule are important for maintaining the tertiary structure they do not prevent flexibility of the polypeptide chain, as shown by reversible expansion of the molecule, on acidification[34,35]. The resulting open structure is easily adaptable to induced

changes produced by various agents, which in most other proteins would cause irreversible denaturation. An open flexible structure is also suggested by the increase of the reduced specific viscosity with concentration[36], and the ease with which the very diverse physiological interactions take place[37].

Because of the almost unique binding ability of this protein, which is a central part of the research being described here, any theory which is proposed for the structure will need to take the unusual affinity of this macro anion for other anions into consideration. It is perhaps useful at this stage to indicate two theories for the overall structures which have been suggested (Figs. 7 and 8). These will be discussed more fully in a later section in the light of new experimental results.

1. A loose structure in which positive groups are on the surface and carboxyl groups are buried within the molecule as shown diagramatically below.



— Represents -S-S-bridges

Figure 7.

2. A structure in which there are closely grouped clusters of positive charges in an adaptable hydrophobic environment on the surface, and an absence of similar clusters of negative charges, e.g.

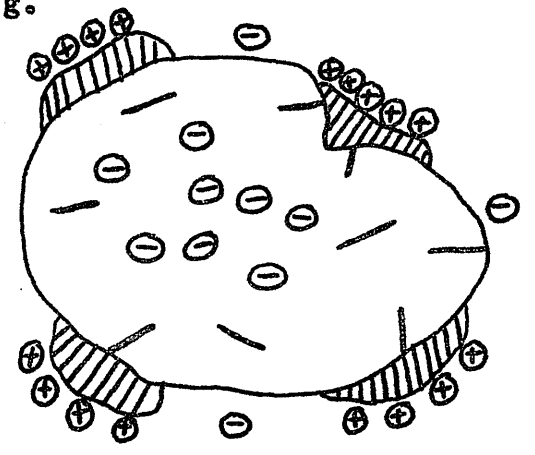


Figure 8.

— Represents -S-S- bridges.

Investigation of Interactions of Small Molecules with Bovine Serum Albumin.

In this work the technique of spot line chromatography was mainly used to study the interactions of bovine serum albumin with the anionic dye, biebrich scarlet.

Experiments were initially done to become familiar with the technique and also to check that the area of the loop formed (A) is proportional to the weight of the protein used (W). It was also checked that A depends on the area of paper (C) cm^2 swept out by the protein before it encountered the line. These results were obtained by running known weights of bovine serum albumin through a line of beibrich scarlet and noting the distance travelled. Plots of A/C versus W/C gave a straight line which cut the W/C axis at about 20 $\mu g.cm^2$ indicating that this is the minimum threshold of weight that will produce loops. Plots of A against C also confirmed the initial findings.

A formula has been derived for the area of the loop produced in a dye line, in terms of known, or calculable quantities[14]. If there are several classes of independent binding sites on the protein and n_i sites with an apparent binding constant K_i then the relationship:-

$$\frac{[X_B]}{[P]} = [X]\sum_i \frac{n_i K_i}{1 K_i[X]}$$ (1) is approximately true[22,38].

where $[X_B]$ is the number of moles of bound dye in a uniform spot with a protein concentration of $[P_o]$

It has been shown[14] that for spot line chromatography under ideal conditions.

$$A = \frac{W \sum_i n_i K_i}{t.m \quad 1+K_i[X]} \qquad (2)$$

Where t is the effective thickness of the buffer in the supporting medium, m is the molecular weight of the protein, and $[X]$ is the concentration of dye in the band.

If some of the unbound dye molecules are adsorbed by the supporting medium and $[X^1]$ is the concentration available for binding by the protein, then $[X^1]$ must be put for $[X]$ in (1) so that from (1) and (2) one obtains.

$$A = \frac{[X^1]}{[X]} \frac{W}{t.m} \sum_i \frac{n_i K_i}{1+K_i[X^1]} \qquad (3)$$

And if the assumption is made that each site binds equally strongly i.e. $K_1 = K_2 = K_3 = K_i$

Expression (3) becomes

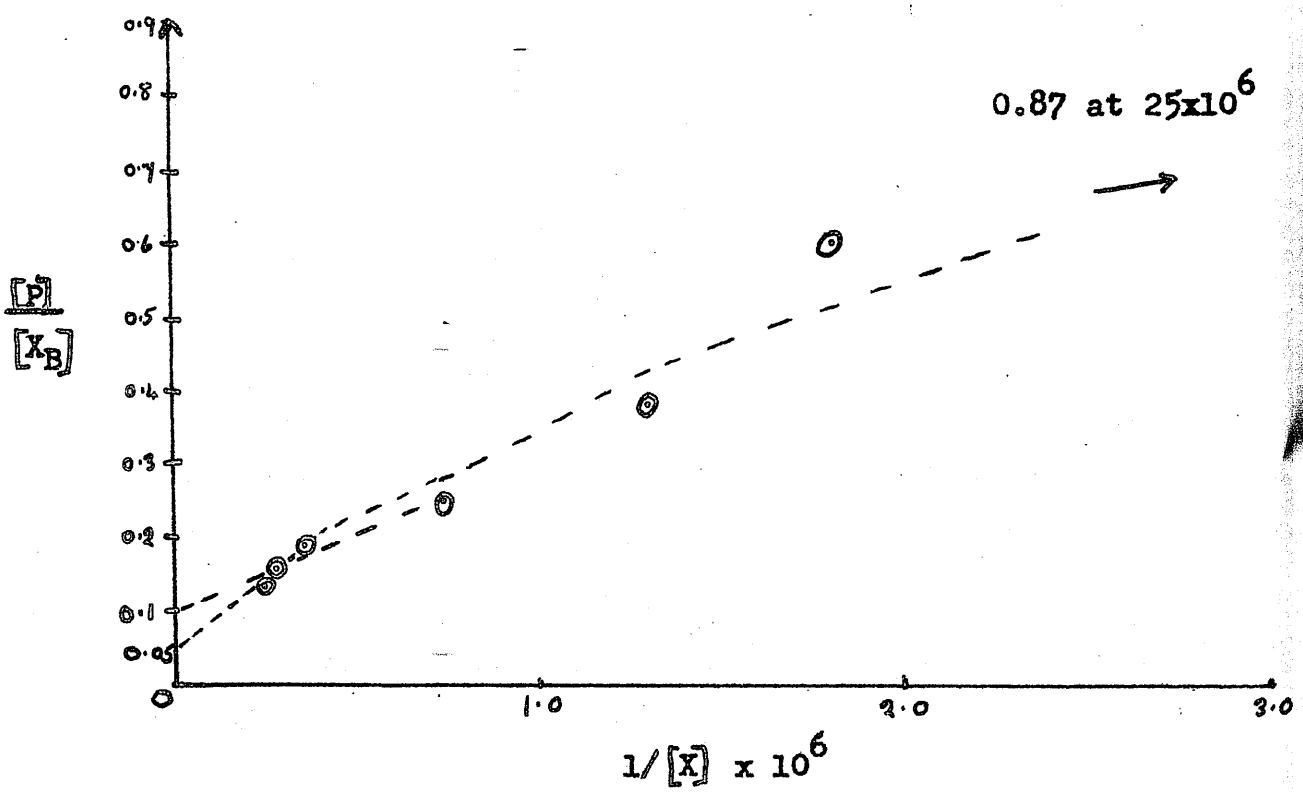
$$A = \frac{[X^1]}{[X]} \cdot \frac{W}{t.m} \quad \frac{nK}{1+K[X^1]} \qquad (4)$$

From which

$$A = \frac{-[X]\,A}{A[X][X^1] - [X^1]\frac{W}{t.m}.n} \qquad (5)$$

Figure 9

Calculation of number of binding sites and binding constant from equilibrium dialysis data.



At high dye concentration (7 - 10) x 10^{-5}M n = (10 - 20)

At low dye concentration (1 - 4) x 10^{-5}M n = (2-- 5).

Taking a value of n, as n = 10, K = 4.0 x 10^{5}.

The technique of equilibrium dialysis was used to calculate the number of binding sites on serum albumin, for biebrich scarlet in the following way. By assuming that each site binds independently, equation (1) can be reduced to

$$\frac{[X_B]}{[P]} = \frac{[X]nK}{1+K[X]} \qquad (6)$$

which can be written in the form

$$\frac{[P]}{[X_B]} = \frac{1}{nK[X]} + \frac{1}{n}$$

If $\frac{[P]}{[X_B]}$ is plotted against $\frac{1}{[X]}$

the values of n and K are readily found. Figure (9) shows the results, which indicated that at low dye concentration there were about 2-5 binding sites. As the dye concentration is increased, the number of sites seems to be about 10-20. Taking a value for the number of binding sites as 10, the value of the binding constant (K) was found to be 4.0×10^5. By now using spot line chromatography and running two spots of bovine serum albumin through a broad band of dye another estimate was made of the binding constant K. This was found to be 1.7×10^5 which agrees reasonably well with the value already found by equilibrium dialysis and, with the values calculated by Klotz and co-workers[16], for the binding of methyl orange and other dyes.

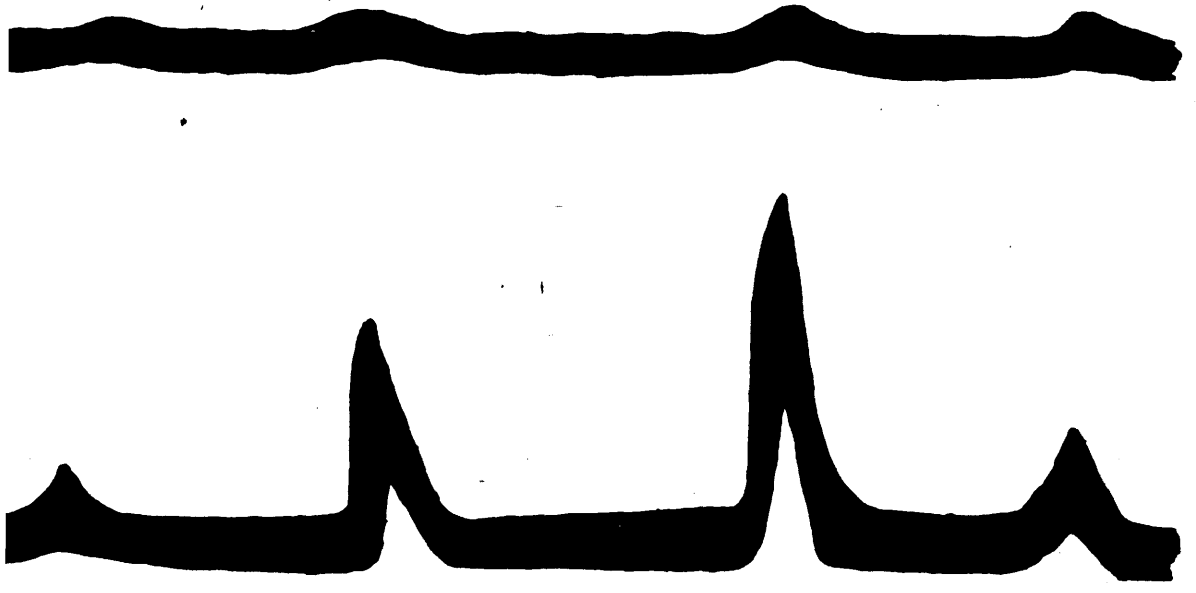
The binding of beibrich scarlet by bovine serum albumin could also be conveniently demonstrated by doing ordinary column chromatography on cellulose powder. A column was made up in the usual way. The dye, in buffer solution was introduced at the top of the column. By eluting with buffer the dye travelled only a few millimeters. The solvent was then changed to a dilute solution of bovine serum albumin in buffer. This rapidly eluted the dye. This experiment not only demonstrated dye binding ability, but also vividly illustrates the power of blood as a selective solvent.

The idea of serum albumin solutions as sophisticated chromatographic solvents was extended to a resolution of D.L. tryptophan, again using a cellulose column. It is known[39] that serum albumin preferentially binds the L isomer of this amino acid, and it was hoped to use this to effect a practical separation. The main difficulty was that the tryptophan was not strongly adsorbed on the column, and eluting it, brought it all off, too quickly. Also the solutions, by necessity, were so dilute that it was very difficult deciding if they possessed any optical rotation. Using spot line chromatography, where the "line" is formed of a dilute solution of tryptophan and bovine serum albumin is electrophoresed through it, it was possible to show the preferential binding of the L isomer. After electrophoresis the paper was carefully removed and gently dried. It was then sprayed with a modified ninhydrin reagent, to bring up the position of the amino acid line and the protein spot. The results were

Figure 10.

Spot Line Chromatography, using cellulose acetate and paper, at pH 6.8.

(The two spots on left, are run almost to the first dye line on cellulose acetate, then on paper).



200 µg.
B.S.A.

200 µg.
B.S.A.

200 µg.
B.S.A.

200 µg.
B.S.A.

rather blurred but two loops, one inside the other could just be seen. The larger was due to the binding of the L isomer.[39] The other, which was very tiny and vague was due to the D isomer. The idea of making this a practical proposition was discarded.

As mentioned previously, plots of A against C indicated that bovine serum albumin loses its ability to bind dye as it moves across filter paper. This loss in binding ability is equivalent to 20 µg of protein per cm^2 of paper traversed. As shown (page 16) this does not appear to be an artifact of the technique. Since a major function of serum albumin may well be "general transport", any factors affecting serum albumin binding are of interest: the decay could for example be a specific molecular mechanism for dropping bound hormones etc., at particular sites in the body, similar to the mechanism by which haemoglobin suddenly drops oxygen molecules at low concentrations of oxygen. The effect produced on the protein by the cellulose of the supporting medium in vitro, may be paralleled by interactions with cell walls of vital organs, or blood vessels in vivo. It is certainly very tempting to present this idea although strict experimental proof has not been forwarded.

Spot line chromatography runs were done on other supporting media and it appears that the process of decay occurs in them all, indeed from results using cellulose acetate, it seemed to be much more rapid on this material (Fig. 10) than on filter paper. It had been shown in the original paper[14] that the protein had to be moving across the paper before it could

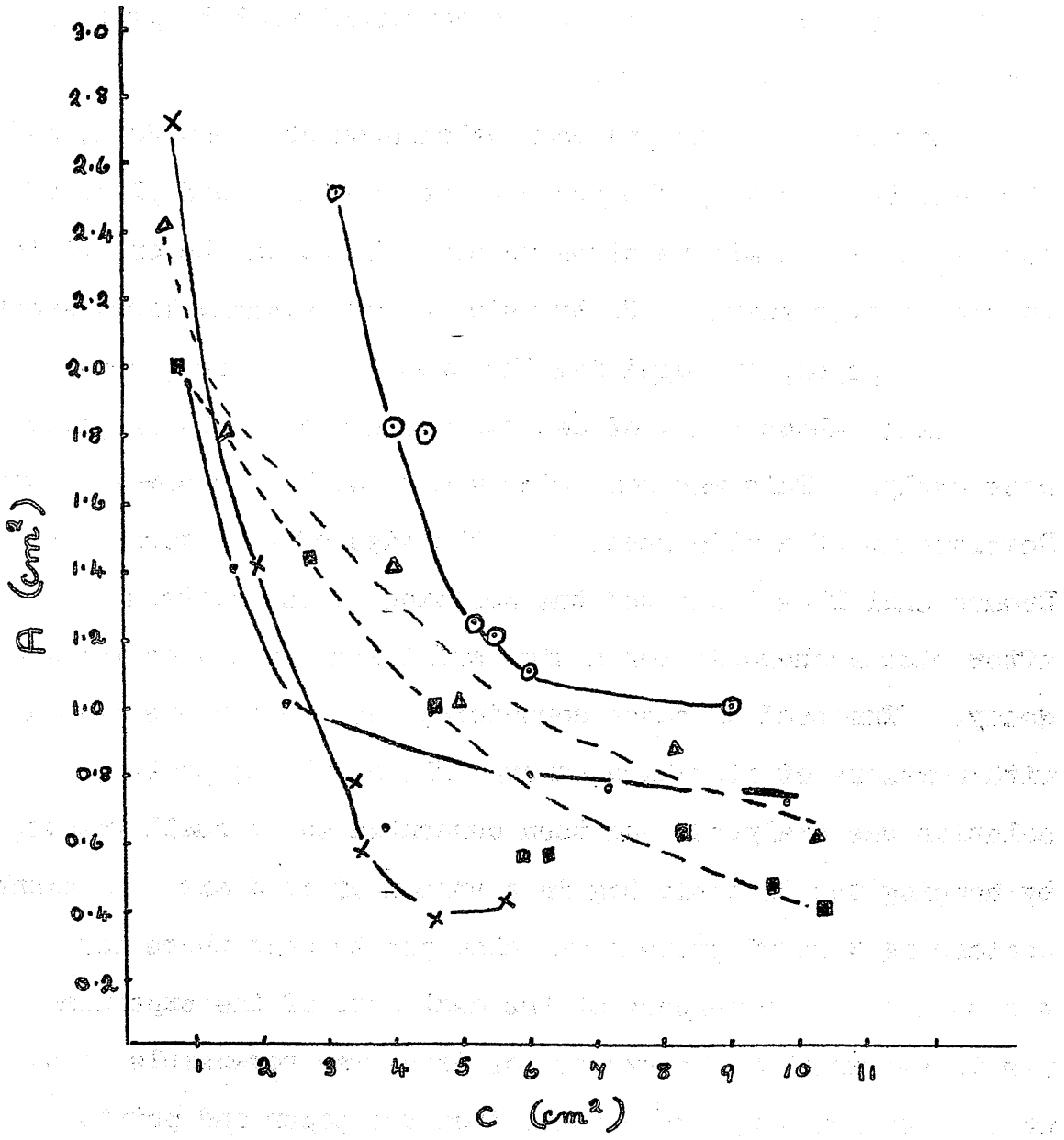
decay. The initial suggestion was that decay was due to an adsorptive interaction between the protein and the supporting medium. This was really based on the negative evidence that no other mechanism could be discovered.

The decay cannot be due to the loss of a loosely bound small molecule or ion, because prolonged pre-dialysis had little effect on decay patterns. Also it cannot be due to dissociation of different components within the protein spot, or electrophoretic separation of the components, for the binding power was not restored when "decayed" spots were reversed in their tracks, as would have been expected if separation was an important factor. Also it appears that a dilution effect, if operating at all, cannot account for the whole of the decay. A small amount of protein is lost by permanent adsorption on the paper[15], usually about 2 $\mu g/cm^2$ of paper, but decay cannot be completely due to this, as no heavier protein "tails" were observed in the regions where the protein decayed rapidly, than elsewhere. It cannot be due to denaturation, because this gives rise to very heavy "tails" on developing the paper for protein with bromophenol blue.

It was decided now to study the effect of temperature on decay. This was done using varying amounts of protein, travelling different known distances, through two dye lines, the filter paper being kept at constant temperature by means of a block, placed on top of the polythene, through which water, at a known temperature, can be circulated. Plots of A against C were drawn in each case. The temperature range

Figure 11.

Investigation of effect of temperature on binding decay.



All runs done at pH 6.8.

⊙ = 25°C

× = 30°C

▣ = 35°C

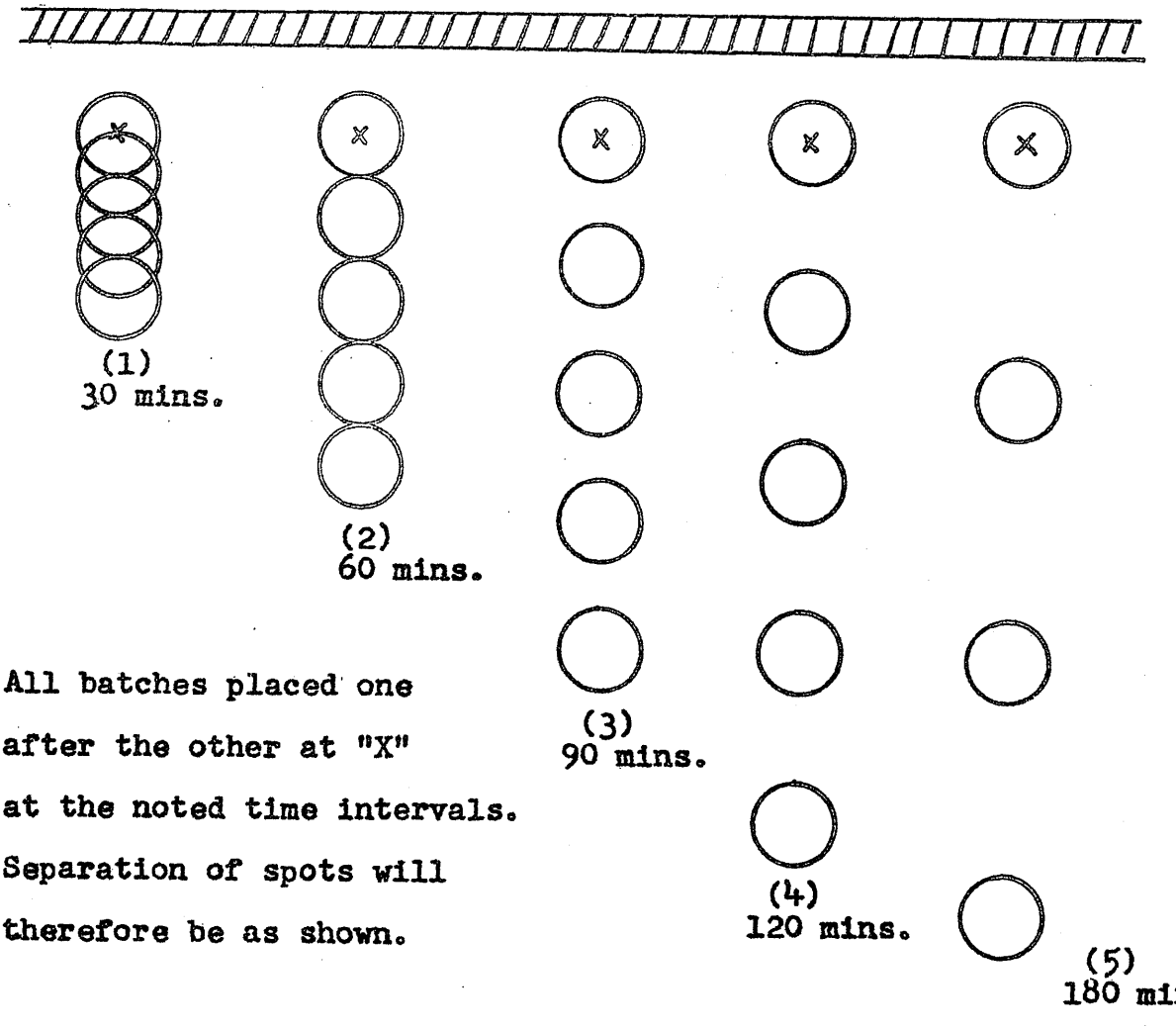
△ = 40°C

○ = 45°C

from 0 to 45°C was covered. As (Fig 11) shows, the plots had all similar shape, and it was concluded that temperature had little effect on decay.

The rate of decay has been calculated at about 20 $\mu g/cm^2$ of paper traversed by the protein, or in other words, 1 mg of bovine serum albumin requires to cover 50 sq.cm. before it is in the decayed state. So, by using a sufficiently large sheet of filter paper, and applying the protein as a line, about 1 cm. wide, about 5 mg. of decayed protein could be prepared conviently. This was done with 4 mgs. of bovine serum albumin. Development of a thin strip from the edge of the paper with Bromophenol Blue indicated the position of the protein, after electrophoresis for a time sufficient to induce maximum decay. The band of paper containing the protein was eluted with portions of distilled water. The resulting protein solution was dialysed, and then concentrated to small volume, by hanging the dialysis bag in a stream of cold air. A blank containing 4 mg of protein was also put through these last two stages. The purpose of the next part of the experiment was to see whether the process of decay was reversible, i.e. whether by removing the protein from the paper and putting it back on later, it would have recovered its initial binding ability. Results from spot line chromatography runs were vague, as the loops produced were very tiny. No definite conclusion was reached. Possibly the process of decay is reversible, but the work-up technique allows irreversible denaturation with resulting loss in binding ability.

So far the cause of decay has been postulated as due to paper - protein interaction. After some thought it was decided that a key experiment would be a "train" run, i.e. a run in which a number of spots follow each other through two dye lines. The question of interest is whether separation of the spots from each other, affects the loop area. Fig. (12) shows the way in which the protein spots will encounter the dye lines.

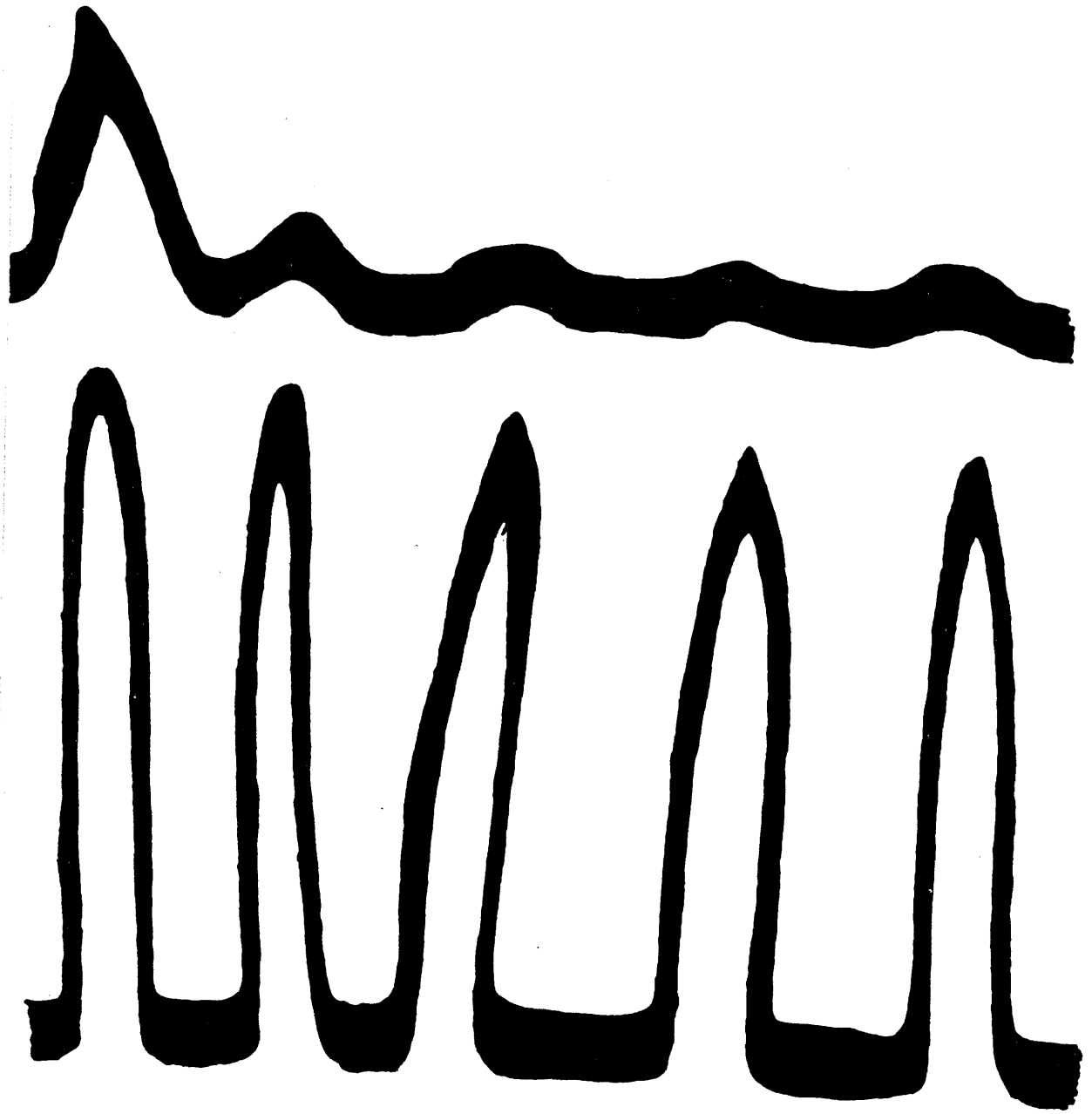


All batches placed one after the other at "X" at the noted time intervals. Separation of spots will therefore be as shown.

Figure 12.

Figure 13.

"Train Run" at pH 6.8, with 500 µg. lots of B.S.A. put on as shown in five batches.



100 µg spots separated by 30 mins. (1)

100 µg spots separated by 60 mins. (2)

100 µg. spots separated by 90 mins. (3)

100 µg. spots separated by 120 mins. (4)

100 µg. spots separated by 180 mins. (5)

It was decided to use 500 µg of bovine serum albumin, applied in five lots of 100 µg. Using a 5% protein solution the diameter of a 100 µg spot was found to be one cm., and under the conditions of electrophoresis used, the rate of movement is 1cm./hour. Thus to obtain the separation noted in Fig.(12) the spots have to be put on at intervals of half an hour, one hour, two hours, and three hours, for spots one to five respectively.

It was found, as Fig. (13) shows that spot (1) gave a loop in the second dye line, about the same size as a 500 µg blank, i.e. incompletely decayed. The area of the loop formed by spot (2) is much smaller than this, but indicates that the protein is not so completely decayed as spots (3), (4), and especially (5).

That a 500 µg portion of protein should behave differently when split up into five separate spots - and it is the separation that seems to be crucial - is rather remarkable, and throws considerable light on the mechanism of decay. In some way the protein spot is a unit, this implies that the protein molecules interact with each other. The interaction is a protective one - when the molecules are all together they decay more slowly.

Now the evidence already discussed, that decay involves interaction with the supporting medium, is fairly strong. That decay occurs with other supporting media than cellulose makes it improbable that this interaction is very specific.

Rather it seems like an abrasive effect on the protein as it passes through the ramifying channels of the supporting medium - something akin to interface denaturation.

The following mechanism incorporates both the general ideas of protein - supporting medium interaction and protein self protection as providing an explanation of the decay phenomenon.

Imagine a spot of protein just applied to the moist filter paper in a spot line chromatography run. There will be a bottom layer of protein molecules which will come into very close contact with the supporting medium, these will be most likely to undergo any induced changes. In other words, they will decay first, but they will have a protecting influence on the rest of the protein molecules, which will never, unless the protein is moving, come into intimate contact with the paper. This explains why a protein spot which is not moved (e.g. by electrophoresis or siphoning) does not decay nearly so much. As the protein moves, fresh molecules become the bottom layer, are decayed but exert their protecting influence on the rest. These "bottom layer" molecules are then either permanently adsorbed on the paper, (would account for the 2 $\mu g/cm^2$ tail) or are regenerated sufficiently, when they are desorbed from the paper, to move off with the others.

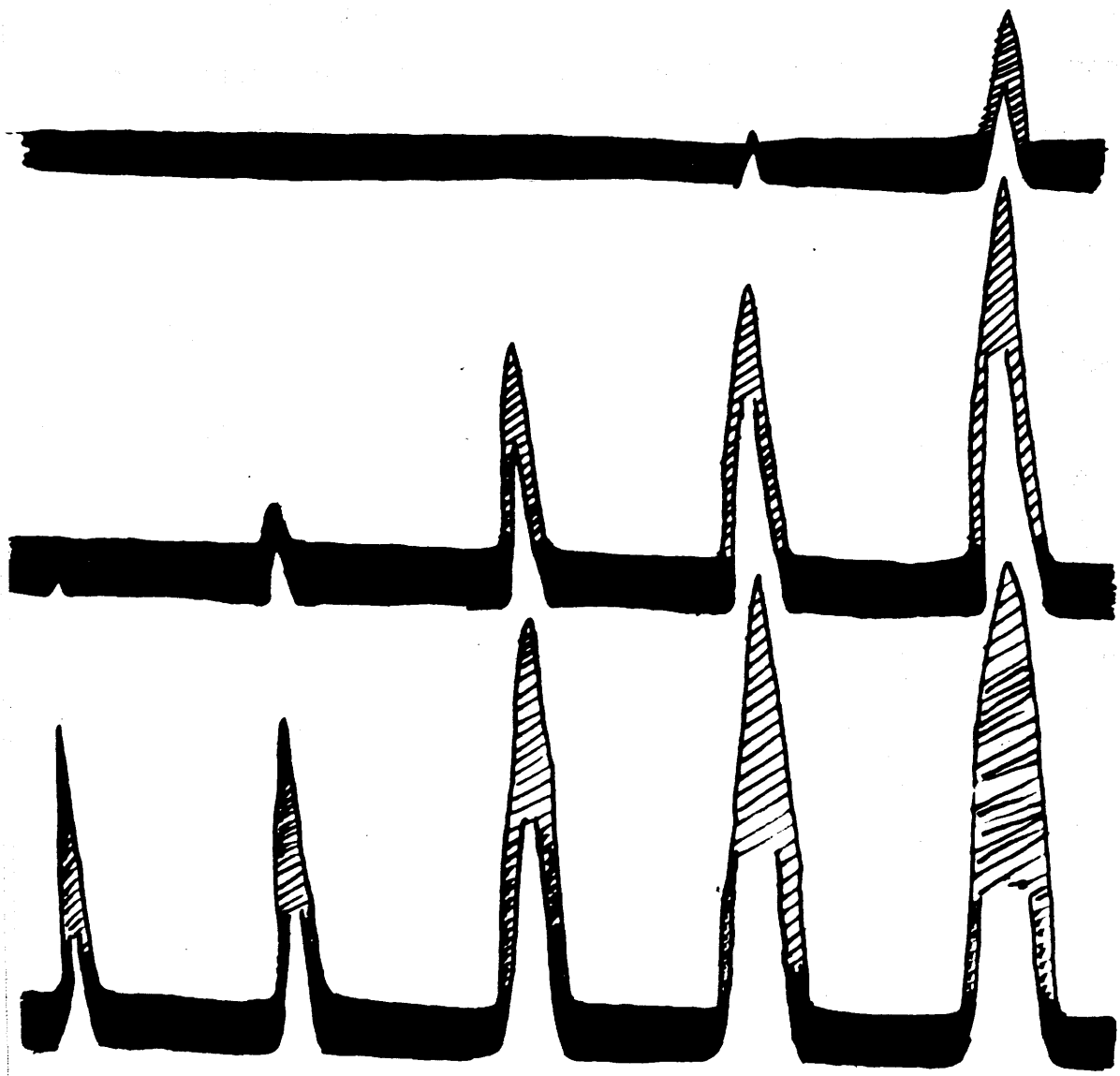
The otherwise paradoxical results of the "train Run" can now be understood. The rate of decay is determined by the total area of paper covered by the total protein. Thus,

when the five 100 µg batches are close together the effective concentration of protein that is in actual contact with the paper is low; in other words the fraction of the total number of protein molecules actually in contact with the paper is small; hence the rate of decay is slow. As the spots separate, so the fraction of protein molecules in contact with the paper increases, with consequent increase in the rate of decay.

It is known that certain agents protect serum albumin against heat denaturation, by forming a protecting influence round the molecule: such a compound is sodium caprylate used at about 0.2M. This presumably exerts its influence by being bound to the protein molecule[40]. It may be, that treatment of the protein with different concentrations of caprylate, during spot line chromatography runs would protect it against decay. Consequently a number of two line runs were done using different concentrations of caprylate in the protein solution. At a caprylate concentration of 0.2M a spot of bovine serum albumin would not produce a loop in a line of biebrich scarlet. This suggested that the caprylate had been preferentially bound at the dye binding sites. By lowering the concentration of caprylate, loops were produced. On one occasion a run was done and the loop produced in the second line was larger than that in the first. Now, this could be due to a reduction in decay, but an alternative explanation is the following. The protein with bound caprylate passes through the first line and because of the reduction in

Figure 14.

Spot Line Chromatography run using B.S.A. at pH 2. KCl/HCl buffer, dye biebrich scarlet. Protein now moves to cathode.

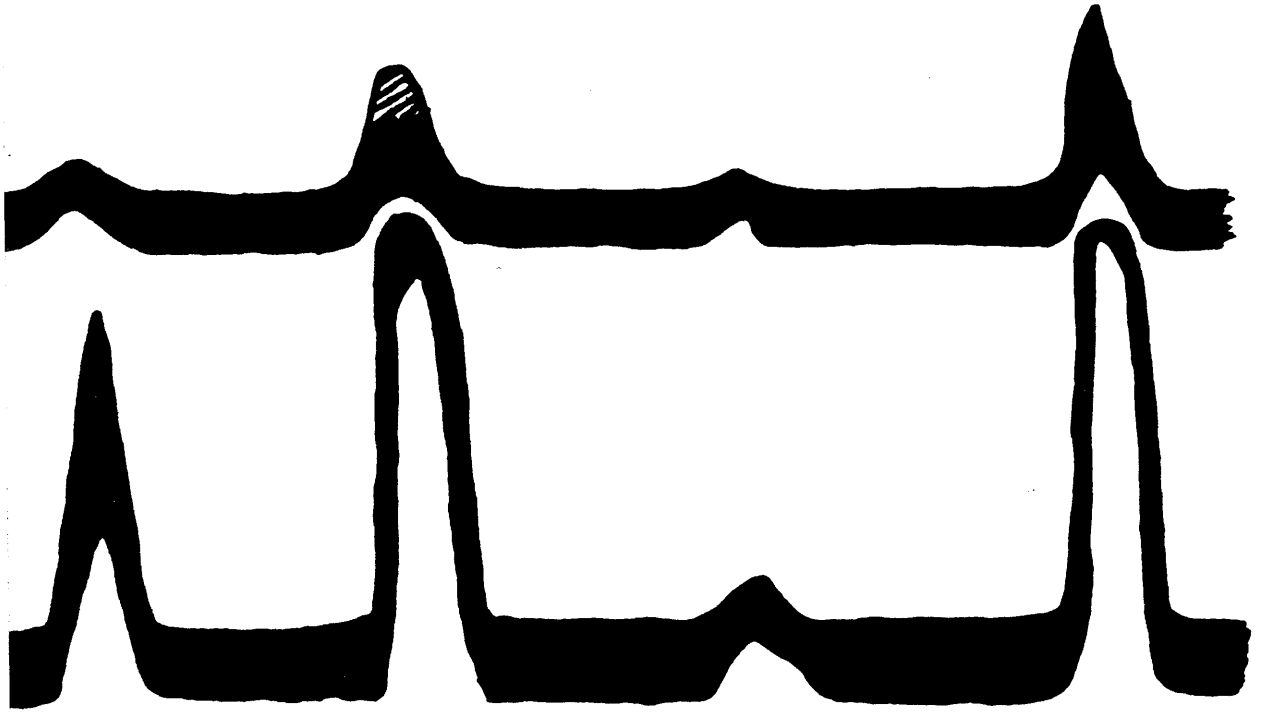


available binding sites it gives a smaller loop than usual in this line. Under the conditions of electrophoresis, caprylate ions are desorbed from the protein, and rapidly move off ahead of it. By the time the protein encounters the second line, all, or nearly all the caprylate ions have come off. This results in an increase in the number of available binding sites; the loop produced in the second line will be larger than that in the first, even although decay may still be going on. These experiments confirm, that the changes involved in decay are unlike those produced when the protein undergoes heat denaturation.

The pH of the buffer used in all the runs previously mentioned has been 6.8 but typical decay patterns have been obtained at pH 5.0 and 8.6. At this latter pH, loops were not as clear as at 6.8 and the rate of decay appeared to be faster. This was probably due to the protein moving faster at this latter pH and as loop formation is due to a distribution between dye on the paper, and bound to the protein; this increased speed of movement will tend to make the loops more blurred. At all the pH values mentioned, the protein has been anionic. It is known that anion binding is greatly enhanced at a pH below the isoelectric point of the protein [41,44], i.e. when it is cationic; dye binding under these conditions is probably not very specific and involves mainly electrostatic interactions. Spot line chromatography runs have been done using biebrich scarlet and a buffer of pH 2.0. Very good loops were formed (Fig. 14), with as small quantities as 5 µg of

Figure 15.

Examination of binding ability of B.S.A. which has been treated with (a) heat (b) alkali. Run done at pH 6.8.



300 µg. B.S.A. 40°C for 17 hours.

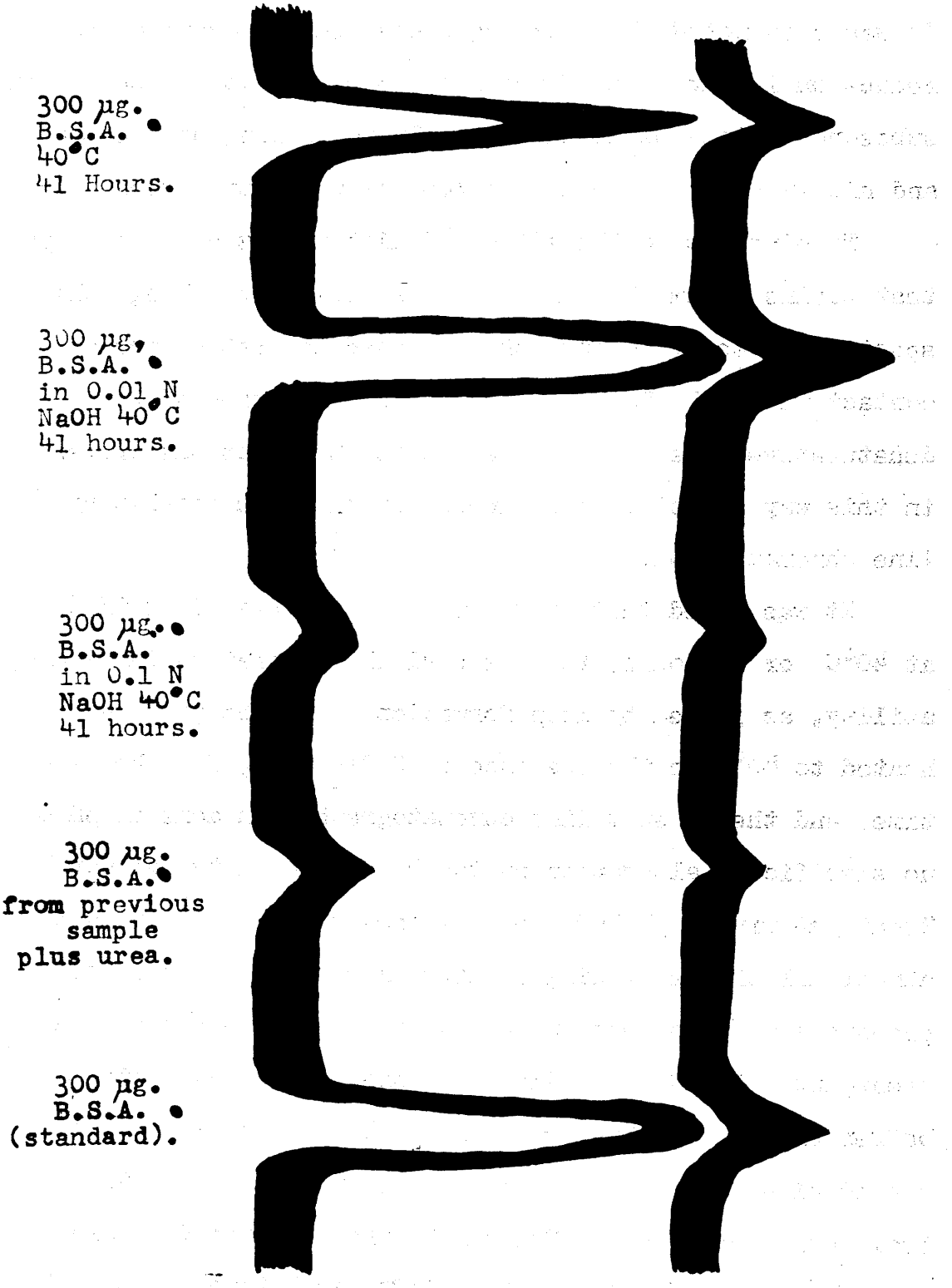
300 µg. B.S.A. 40°C in 0.01NNaOH for 17 hours.

300 µg. B.S.A. 40°C in 0.1N NaOH for 17 hours.

300 µg. B.S.A. (standard).

Figure 16.

Examination of binding power of B.S.A. treated with (a) heat (b) alkali. Run done at pH 6.8.



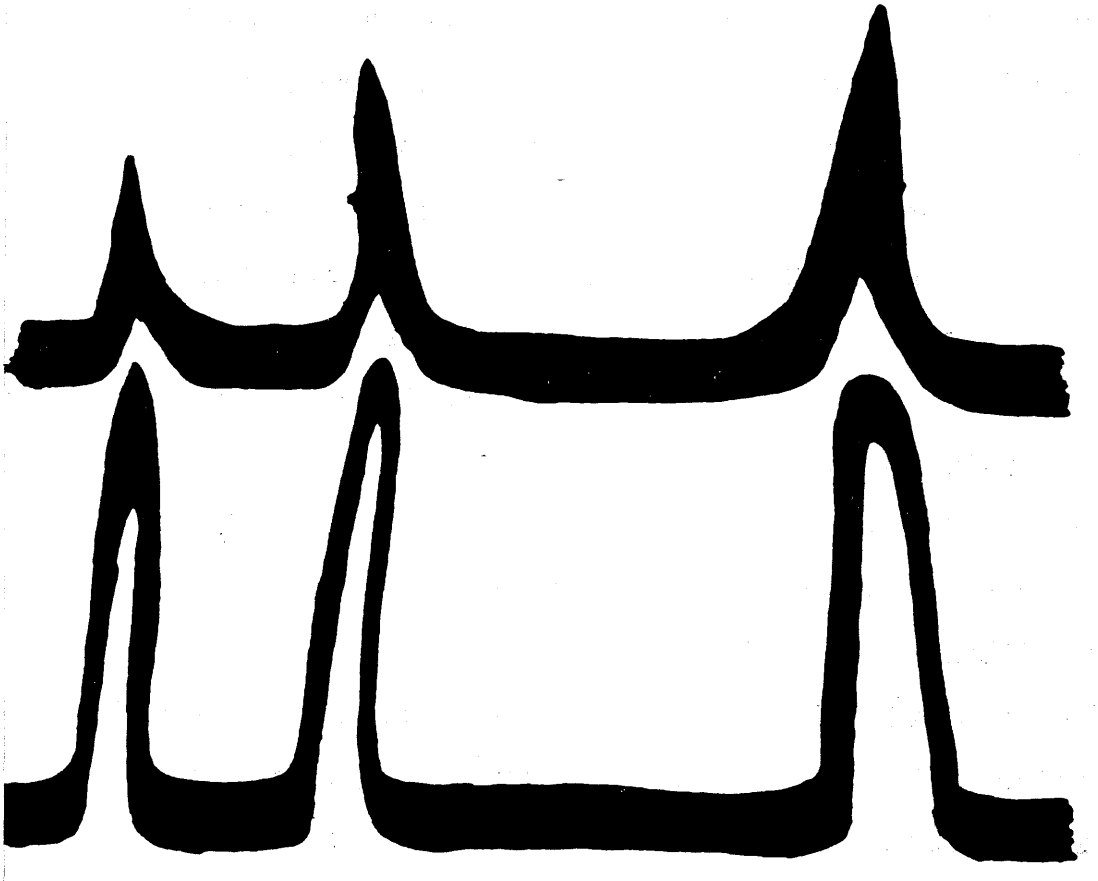
bovine serum albumin a quantity which would not even have produced a "blip" in a line of biebrich scarlet at pH 6.8. It was also noted that binding decay did not occur at this pH, reduction in the size of the loops produced in the second and subsequent lines being due entirely to tailing on the paper, and nicely shown at about 2 $\mu g/cm^2$ of paper traversed.

Turning now to the effect of high pH, it has been reported[42] that bovine serum albumin loses its ability to change the spectrum of methyl orange when exposed to heat or prolonged contact with 0.01N NaOH. This has been attributed to a denaturative effect. It was decided to treat the protein in this way and study the binding of biebrich scarlet by spot line chromatography.

It was noted that if the protein was heated at pH 6.8 at 40°C for 17 hours, there was slight alteration in binding ability, as judged by loop formation. If the protein was heated to 40°C in the presence of 0.01N NaOH, for the same time, and then a spot line chromatography run done at pH 6.8, no significant alteration in the binding ability was noted. Treatment with 0.1N NaOH for the same time at 40°C removed almost all of the binding ability (Fig. 15). The protein preparations were then subjected to another 24 hours of heat treatment at 40°C. As Fig. (16) shows, the one heated in buffer pH 6.8, had lost some of its binding ability, but that heated with 0.01N NaOH had undergone little or no alteration, from native material. The sample treated with 0.1N NaOH had very little binding ability left, as before. The solution

Figure 17.

Examination of dye binding ability of B.S.A. which has been heated at 55°C, in 0.01N NaOH solution, for 48 hours. Run done at pH 6.8.



200 µg. B.S.A.

300 µg. B.S.A.

500 µg. B.S.A.

of the protein in 0.01N NaOH was then heated at 55°C for a further 48 Hours. As Fig. (17) shows the product was still able to bind efficiently.

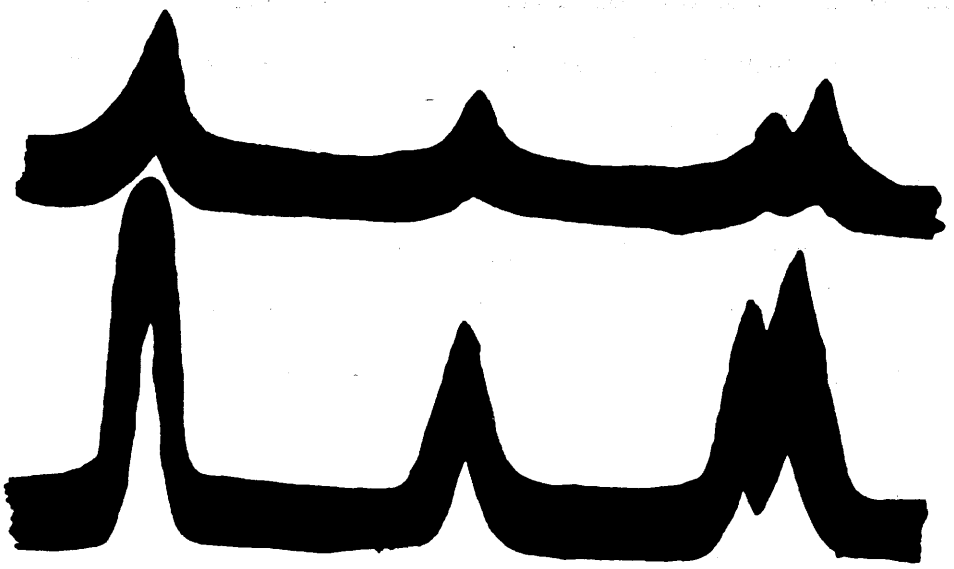
There is a discrepancy here with previous work where the technique used to study the dye binding was an examination of the absorption spectrum of the dye, free and bound to the protein. This technique employed the protein in bulk solution, and not as in spot line chromatography, on filter paper. This might be the important difference. Although denaturation by base may have occurred, the elasticity of the albumin molecule allows this to be reversed, when the protein, on the paper, is returned to pH 6.8. The effect of 0.1N NaOH upset the charge balance on the protein too much, and in this case structural alterations were not reversible, as shown by the very small binding ability.

Having mentioned the effect of both acid and base on the dye binding ability of the protein, it should be said that spot line chromatography runs have been done at a pH very close to the isoelectric point of the protein, where the protein is kept stationary, and the line of dye moved through it. The dyes, eosin and naphthol Green B were very useful in this connection because they migrated quite rapidly. With the whole paper soaked in eosin, naphthol green B, and mixtures of these two dyes, and using spots and small lines of serum albumin and whole blood some interesting effects were noted. The runs with whole blood, eosin and naphthol green B may be particularly interesting, because they show a visual separation

Figure 18

Comparison of binding power and rate of decay of deaminated B.S.A. with untreated material.

Run done at pH 6.8.



300 µg. B.S.A.

300 µg. deaminated B.S.A.

500 µg. deaminated B.S.A.

of the protein components. This technique could perhaps be developed as a fingerprint test in pathological work.

To shed further light on:-

(a) the initial dye binding, and

(b) the binding after running on paper i.e. decay, various chemical modifications, of bovine serum albumin, were effected. Specific reactions being used so that the groups undergoing reaction would be known.

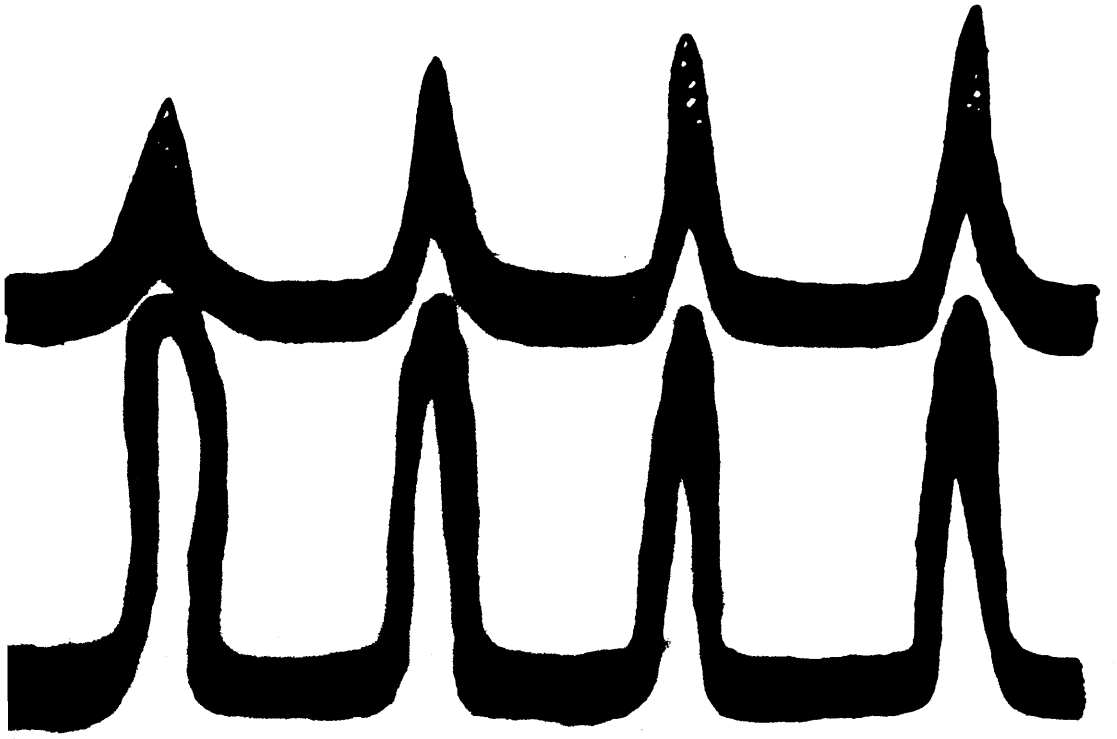
It has been reported[43,44], that the binding of small anions involves mainly the ϵ amino groups of lysine residues. This was shown because serum albumin loses most of its binding ability at pH 12 where $-NH_3^+$ groups are changed to uncharged $-NH_2$ groups. The more basic guanidine group of arginine residues is also known to be involved. The ϵ amino groups will thus present the first point of attack.

Proteins undergo reaction when treated with sodium nitrite at about pH 4 at 0°C. Among other things, deamination is known to occur[45]. Consequently, bovine serum albumin was deaminated and samples from the reaction solution used in a two line spot line chromatography run, as shown in Fig. (18). The overall size of the loops, is, as expected much smaller than for native material, the binding ability has fallen to about 25% of its original value. As can be seen, the protein is still able to decay.

It was next decided to treat samples of bovine serum albumin with varying quantities of aqueous formaldehyde and examine the dye binding ability of the product in each case

Figure 19.

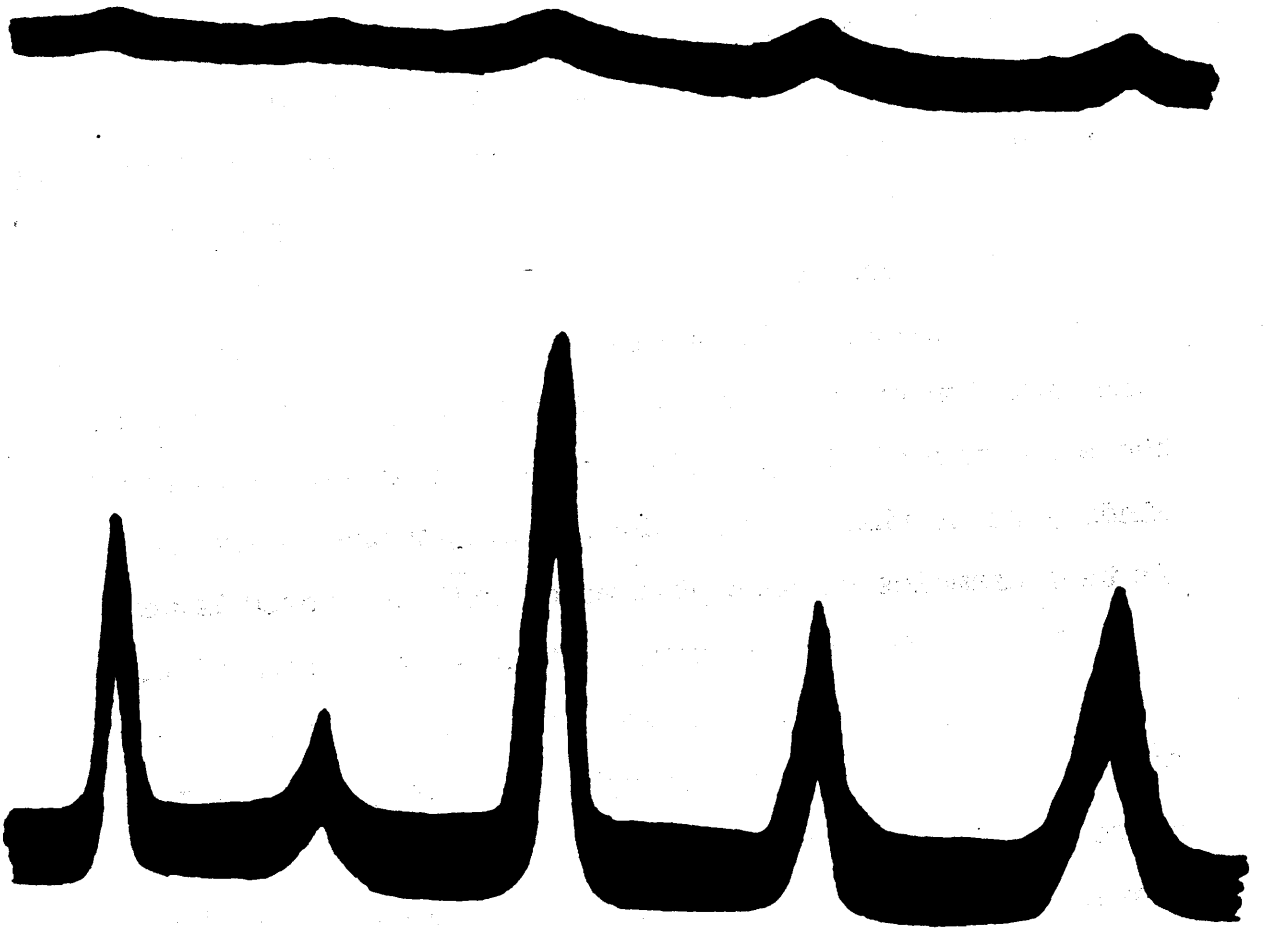
Binding ability of B.S.A. treated with aqueous formaldehyde at pH 6.8.



300 µg. B.S.A. 0% HCHO. | 300 µg. B.S.A. 1% HCHO. | 300 µg. B.S.A. 5% HCHO. | 300 µg. B.S.A. 10% HCHO.

Figure 20.

Comparison of binding ability and rate of decay of acetylated B.S.A. with untreated material. Acetylation done by treating 50 mg. of B.S.A. with 0.06 ml acetic anhydride in half satd. sodium acetate solution. Run done at pH 6.8.



100 µg. B.S.A.

100 µg. acetylated B.S.A.

200 µg. B.S.A.

200 µg. acetylated B.S.A.

300 µg. acetylated B.S.A.

by spot line chromatography. Formaldehyde is known to cross link proteins and it also reacts with ϵ amino groups, (Fig.19) shows the results. As noted, loop area in the first line falls with increasing concentrations of HCHO. This is to be expected from a reduction in the number of binding sites. Also those in the second line increase in size with increasing HCHO concentrations. (Decay is still operating although it may have been reduced slightly). Notice also the flame effect in the loop produced by the protein treated with the highest concentration of HCHO, these indicate slight alteration in the structure of the protein giving rise to new classes of binding sites which retain the dye longer than normally. Further examples of this phenomenon will be quoted later.

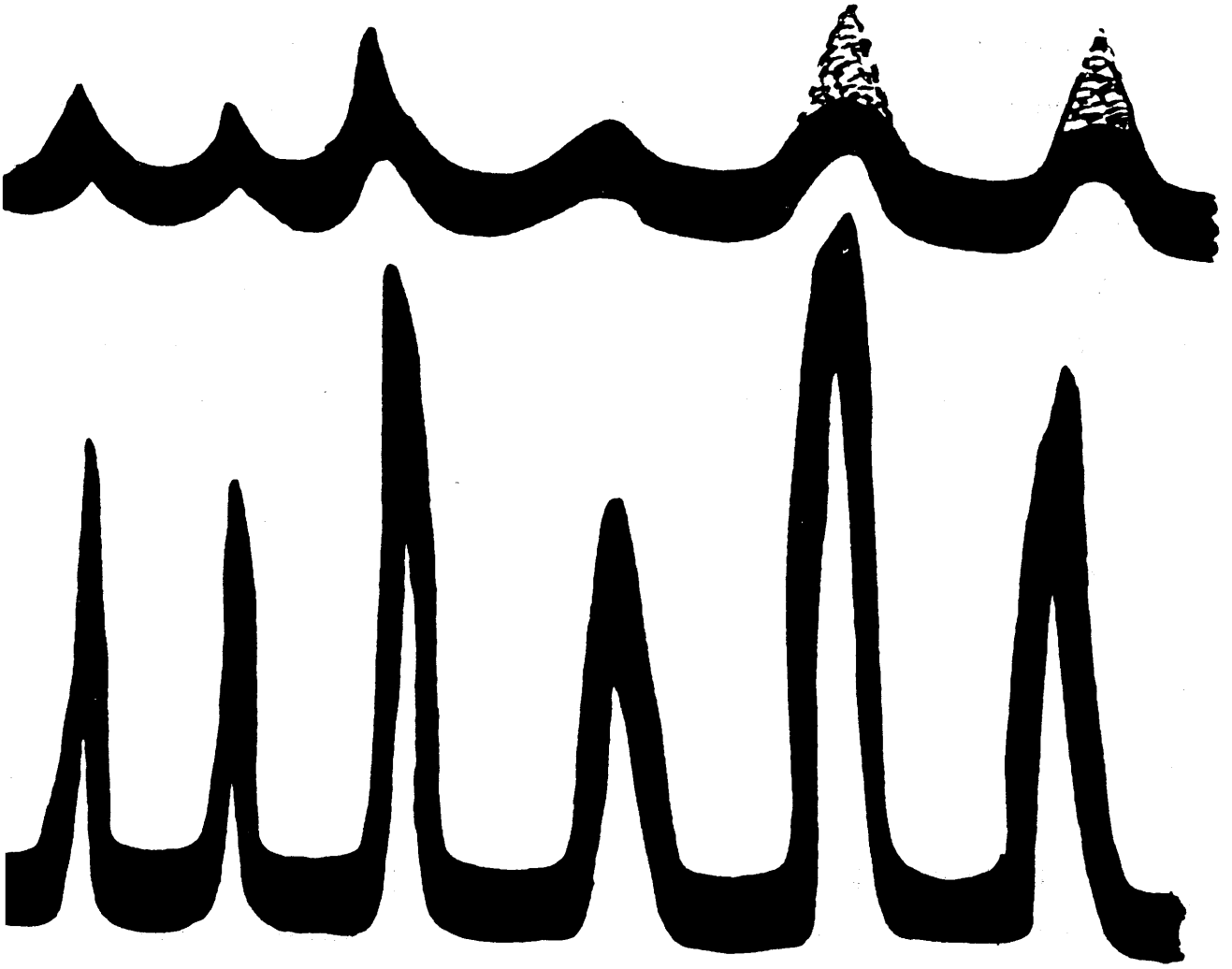
The ϵ amino groups were acetylated by treatment with acetic anhydride in an alkaline solution of the protein at $0^{\circ}C$ [46]. Spot line chromatography runs using the acetylated protein gave smaller loops in the first line, than standard spots, but all the loops in the second line, as noted (Fig. 20) were equal, indicating that decay was still occurring, but still more slowly. It has been shown by Klotz [44] that acetylation of the amino groups reduces binding, so this is really confirmatory. The protein has also been treated with succinic anhydride. It was observed that the protein, because of the increase in CO_2H groups moved faster at pH 6.8 than standard material, but nothing dramatic in its dye binding ability was noted.

Figure 21

Comparison of binding ability and rate of decay.

Guanidinated B.S.A. with standard material.

Run done at pH 6.8.



100 µg. B.S.A.

100 µg. guanidinated B.S.A.

200 µg. B.S.A.

200 µg. guanidinated B.S.A.

300 µg. B.S.A.

300 µg. guanidinated B.S.A.

It is known[44] that the ε amino groups are essential for dye binding ability only in so far as they provide a cationic site. The conversion of these to the more basic guanidinium group has very little effect on the binding. However it was thought that it might have some effect on the decay phenomenon. Consequently a sample of bovine serum albumin was guanidinated, by treatment with o-methyliso-urea at pH 10[47]. A two-line spot line chromatography run using the product Fig. (21) showed very little deviation in binding ability, or decay, from untreated material. (Binding ability slightly reduced).

It was next thought that the process of decay, might be initiated by hydrogen transfer from the solitary sulphydryl group, to a nearby dithio bridge, with resulting alteration of conformation.

If the sulphydryl group is blocked, such a hydrogen transfer process would cease and the decay should be halted. Samples of bovine serum albumin were treated with iodoacetate, and p-chloromercuribenzoate[48], both specific reagents for sulphydryl groups in proteins, via the reactions shown below.

1. $P\text{-}SH + ICH_2CO_2^- \xrightarrow[25^\circ C]{pH7\text{-}8} P\text{-}S\text{-}CH_2CO_2^-$

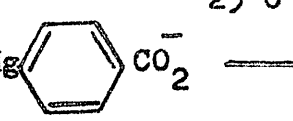
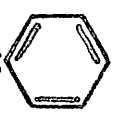
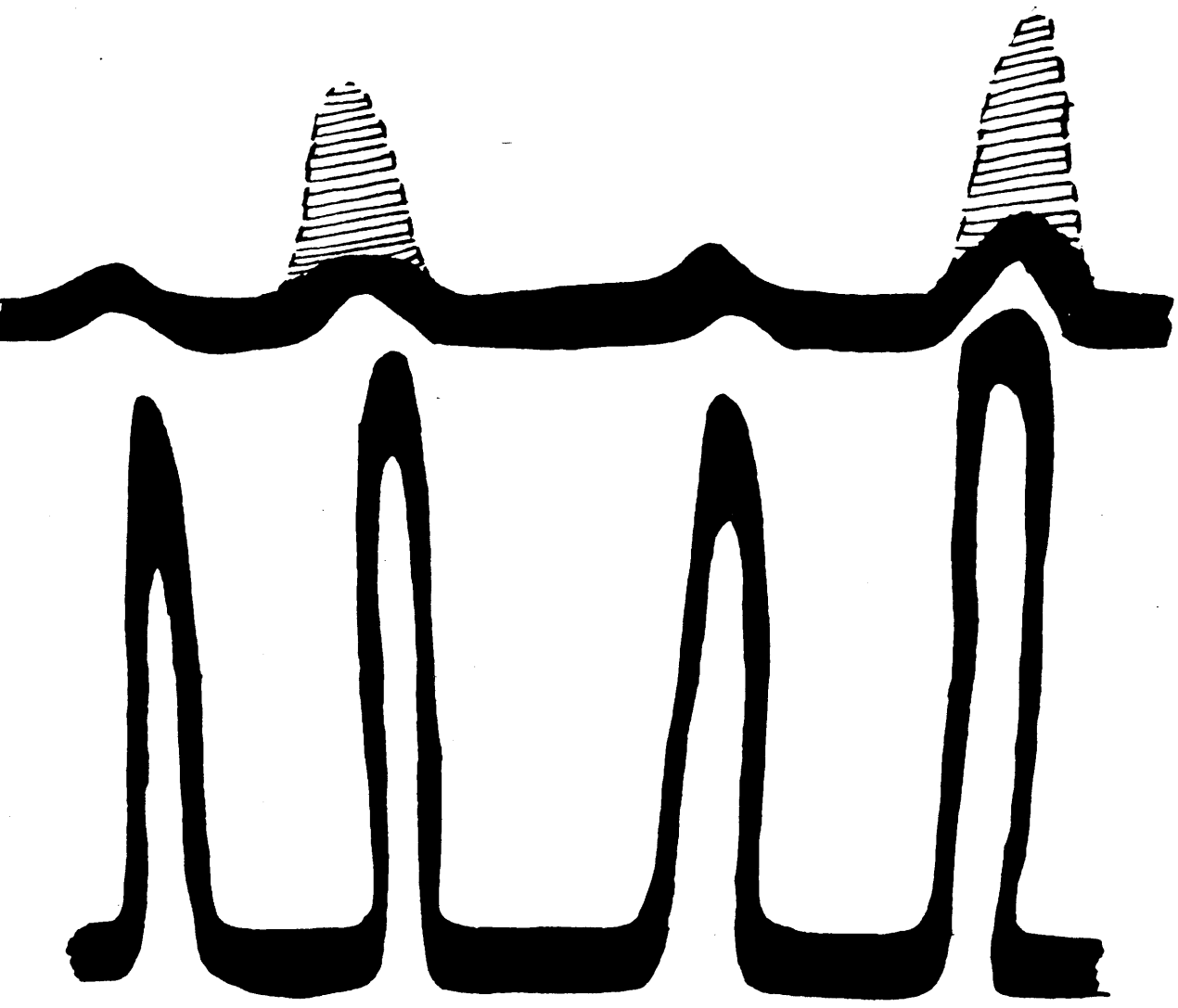
2. Prot.-SH +  → Prot.-S-Hg  + HCl

Figure 22

Comparison of binding power and rate of decay of iodoacetate treated B.S.A. with untreated material. Run done at pH 6.8.



200 µg. B.S.A.

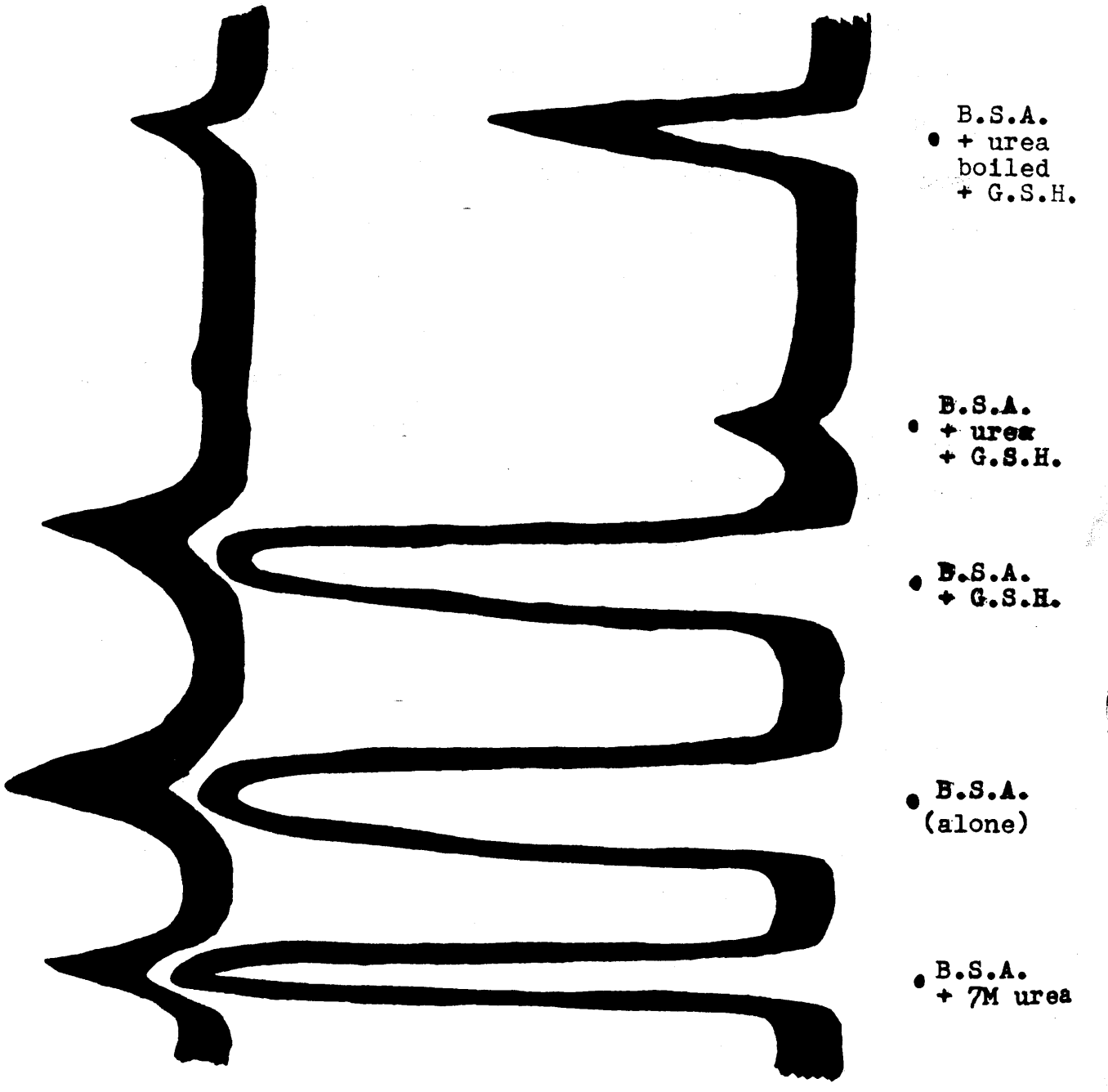
200 µg. iodoacetate treated B.S.A.

300 µg. B.S.A.

300 µg. iodoacetate treated B.S.A.

Figure 23

Study of the binding power and rate of decay of the products of reduction of B.S.A. with glutathione (GSH) in the presence and absence of urea (7M). Run done at pH 6.8. (All spots about 500 µg.)



Two line spot line chromatography runs were then done in each case. No significant reduction in decay was observed with either, indeed treatment specially with iodoacetate, increased the overall binding ability, as can be seen in Fig. (22). Notice also the large "flame" effect on the loops produced in the second line by the treated protein. Presumably the mere steric effect of blocking the -SH group results in slight structural rearrangements near to the -SH. This process apparently reveals new classes of binding sites, which retain the dye much longer, as indicated by large "flames" on the loops in the second line. This process of structural rearrangement on the blocking of the -SH group is known to occur in some other protein systems, e.g. G-actin [49].

As noted in the review on the structure of serum albumin, the molecule has many (approx 17) dithio bridges which tend to hold the structure together. As shown, complete reduction is only possible after treatment with, e.g. concentrated urea solution which opens up the structure for attack. Hird [50,51] has studied the reduction of bovine serum albumin using glutathione (G.S.H.) in the presence and absence of urea. Reduction of bovine serum albumin was carried out by the methods given and the dye binding ability of the resulting products studied by spot line chromatography as shown in Fig. (23). The sample which was reduced in the absence of urea, showed very slight reduction in binding ability from standard. On the other hand, reduction in the presence of concentrated urea practically eliminated the binding power.

The resulting alteration in conformation, on reducing all the dithio bridges is presumably the cause of this drop in binding power. It is also interesting to note that:-
(a) bovine serum albumin treated with concentrated urea shows little reduction in binding power from standard.
(b) the sample which was reduced in the presence of concentrated urea appears to withstand a few seconds boiling. This must be a very good example of the flexibility and resistance of this intriguing protein.

Before going on to consider in greater detail possible mechanisms of binding and decay there follows a brief review of those aspects of protein denaturation, which may be relevent.

Denaturation of Proteins.

The irreversible coagulation of egg white upon heating is a well known phenomenon. Similar drastic changes can be brought about by treating proteins by other relatively gentle means. These include such physical methods as shaking, stirring, treatment with ultra-violet, and ultra sonic waves, and, by using chemical agents such as, urea, guanidine salts, detergents etc. These changes, referred to as denaturation, are usually accompanied by a loss of the biological properties of the protein; enzymes lose their catalytic activity, hormones their physiological action, and antibodies their ability to combine with antigen.

A general feature of all these reactions, is that the proteins lose their original solubility, in most instances they become insoluble at their isoelectric range, an exception is collagen which becomes soluble when heated with water. Also the protein loses the ability to form true crystals.

The earliest theories proposed that the action of heat, was to dehydrate the protein molecule, or to establish peptide linkages between some of the free amino and carboxyl groups. It was also thought that the reverse reaction, namely the cleavage of peptide bonds by heat, was possible. Dilatometric measurements[52] showed however that denaturation was not accompanied by any noticeable change in volume. The hydration of denatured proteins in humid air is only slightly lower than that of native proteins; their ability to bind water is of the same order of magnitude[53].

The first modern theory of denaturation proposed that the process consists of a rearrangement of the peptide chains in the protein molecule, initiated by rupture of the weak links which hold these chains together, by the denaturation agent. As an extension to this it appears that denaturation consists of an alteration in the highly specific coiled conformation of the native protein, the closely folded peptide chains are unfolded and/or refolded[54]. The particular method of denaturation will determine whether the disrupted peptide chains remain in the unfolded state, whether they are refolded to give the original specific pattern, or whether there will result some other pattern different from the

original structure. Obviously the extent of denaturation, can vary from slight structural changes, to complete rearrangement of the polypeptide chains.

Denaturation leads to an enhancement in the reactivity of various groups[55], over that in the native protein, e.g. ovalbumin gives no test for sulphydryl in the native state but responds upon denaturation. Some proteins, will not react completely with 2,4-dinitrofluoro benzene, (a reagent for ϵ amino groups), while native, but react completely upon denaturation. These results, and also the fact that denaturated proteins suffer ready proteolytic attack, all tend to suggest that in the native protein, these groups are either buried inside the globular structure, or masked in some other way, and that denaturation causes an exposure of the groups sufficiently for reaction. The increase in viscosity usually accompanying denaturation also supports the idea of unfolding[52].

Since changes in the highly specific native conformation involve numerous hydrogen bonds, it is not surprising that denaturation causes a shift in the value of the carbonyl absorption in the infra red[56], and also causes a shift of the isoelectric point towards higher pH values[57]. For these reasons also the rate of denaturation depends greatly on pH and temperature. It is low at the isoelectric point of the protein and increases in acid or alkaline solution, and markedly with rise in temperature[58,59]. Denaturation as brought about by the variety of agents already mentioned

probably does not proceed in each case by the same mechanism. A number of reagents and conditions, and the general effects produced will now be considered very briefly.

Heat denaturation causes the protein to remain in the Zwitterionic state. Hydrogen bonds between the peptide chains are cleaved by the increased thermal motion, of the chains, and bonds between hydrophobic groups may "melt". The observed insolubility of the heat treated protein is probably caused by disulphide interchange reactions and by the resulting formation of new intermolecular disulphide bonds resulting in aggregation[60,61,62].

This view is based on the fact that if a protein is treated with iodoacetate[63], or p-chloromercuribenzoate[64], which are both specific blocking reagents for -SH groups, then the coagulation is inhibited. The sulphydryl group probably initiates the disulphide interchange reactions and on being blocked, can no longer do so. It is also in agreement with the fact that collagen which is free of cystine residues, is converted into soluble gelatin after heating. As long as the changes induced by the denaturing agent leave the disulphide bonds intact, the process is reversible.

The effect of mineral acid is to convert carboxylate anions, in the protein, into uncharged carboxylic acid functions, leaving the positively charged ammonium groups unchanged. The mutual electrostatic repulsion of these ammonium groups causes subsequent unfolding and expansion of the molecules. These postulates have been put forward by an examination of

the specific rotation of a protein in acid solution[65,66]. Similarly treatment of the protein with alkali causes unfolding due to the mutual repulsion of the negatively charged groups. If the added acid or alkali is neutralised, the protein is reconverted to its amphoteric state, although probably some of the original chain conformation may be changed[67].

Denaturation brought about by shaking or stirring is really surface denaturation. When a solution of egg albumin is shaken fibrous particles are formed in the solution. This is due to denaturation, causing unfolding at the water-air interface followed by aggregation.

Denaturation has been shown also to occur if solutions of globular proteins are spread on surfaces. Monolayer formation occurs here with drastic unfolding of the compact native structure[68]. Ultra sonic waves are effective probably because of the local extremes of heat and pressure which they create as they travel through the protein solution. X-ray or γ radiation cause rupture of peptide chains and also disulphide interchange reactions[69].

The peptide chains of the closely folded native protein cannot unfold unless water flows into the space between the chains. Therefore dry protein preparations are much more resistant to heat denaturation than proteins in aqueous solution. For the same reason concentrated protein solutions are more stable than dilute ones.

The most interesting denaturing agents are those substances which are neutral and apparently indifferent such

as urea, some acid amides, guanidinium salts and detergents. Urea is neither acid nor basic it is neither toxic nor surface-active, yet high concentrations, (6-8 Molar), of urea denature protein at room temperature as judged by such criteria as increases in viscosity, optical rotation etc. This has been attributed[70] to urea having in it the structure -CONH- which enables it to form hydrogen bonds with the peptide linkages and thus to compete with the intrachain hydrogen bonds which maintain the protein in its native conformation. It is thought that urea molecules will tend to force their way between the closely folded peptide chains and would thus tend to rupture bonds responsible for their mutual attraction. This idea has now been somewhat superseded for recent investigations[71] have revealed the relatively low energy of hydrogen bonds in aqueous solutions and it has been suggested therefore that urea breaks hydrophobic rather than hydrogen bonds[72,71,73,74], and that the high concentration of urea required for denaturation disrupts the hydration lattice of proteins[75,76]. Changes in hydration may also explain why denaturation of some proteins by urea takes place more readily at 6°C than at higher temperatures[77]. Denaturation by urea may also come about for the following reason. In concentrated urea solutions the following equilibrium exists:-

$$H_2NCONH_2 \rightleftharpoons NH_4^+ + NCO^-$$

the cyanate ion could form carbamylates with amino groups or sulphydryl groups[78], and thus may cause irreversible changes. Urea may interfere with the formation of hydrogen bonds

between water molecules and carboxyl, amino and other polar groups which are necessary[79] for the native structure of the protein. Whatever the mechanism of the action of urea may be, it can also give rise to disulphide interchange and to the formation of new intermolecular disulphide bonds[80]. This phase of denaturation is preceded by an increase in the number of detectable sulphydryl groups[81,82].

Several proteins are dissociated by urea into smaller subunits, e.g. haemoglobin M.W. 68,000 and edestin M.W. 212,000 are split into units of M.W. 34,000 and 49,500 respectively[83]. It is not yet clear why some proteins dissociate in urea solutions, whereas others form aggregates held together by intermolecular dithio bonds.

The action of detergents in denaturing proteins seems similar, but slightly distinguishable from that of urea or guanidinium salts. Detergents are usually considered as dissociating agents[84], but association in detergents has also been observed[85]. There is also direct evidence that detergents are bound to protein[85,86]. An excellent comparison of the effects of urea and detergents has been made on a recent study of thyroglobulin[87]. Detergent here, causes dissociation but the helical configuration of the subunits is undisturbed. In urea and guanidine hydrochloride all order is lost, both dissociation and unfolding being more complete than in detergent.

Denaturation and coagulation of certain proteins are inhibited by concentrated solutions of glucose and other sugars.

Heat coagulation of serum albumin is prevented by small amounts of alkali salts of fatty acids[40], maximum protection being obtained by acids having six to eight carbons in their chain. Protection is also obtained by anionic dyes such as congo red, urea and certain other polar substances. The action of these compounds, may be due to their adsorption to the globular protein particles and to the formation of large hydrophilic complexes, in which the protein is coated by the adsorbed molecules and thus prevented from forming aggregates with other protein molecules.

If drastic methods are used, and, if the molecular weight of the native protein is very high, denaturation is usually an irreversible process. If, however, mild methods of denaturation are used, almost complete renaturation is observed in some of the smaller proteins e.g. a trypsin inhibitor from soy bean[88], and in chymotrypsinogen[89]. Similarly the denaturation of the enzyme ribonuclease by sulphydryl compound is reversible as judged by the protein regaining its enzymic ability[90]. It seems that in all cases of renaturation the chain conformation is so stable that almost complete reversion to the native state takes place at low temperatures.

Does denaturation proceed in stages or is it an "all or none" process? The latter hypothesis has been favoured by many workers, but evidence appears to indicate that denaturation takes place stepwise through many intermediates[64]. The ease of reversibility being determined by the particular protein

and by the distance passed along the denaturation pathway, the farther down the path, the less likely reversal will be.

The kinetics of denaturation are very difficult to study because the process is complicated by so many factors. However it is predictable that in the not too distant future this difficult problem will be clarified.

In view of the fact that most of the work done in this research has been on serum albumin, and also that a curious effect by a powerful denaturing agent has been noted, it seems advantageous to mention, in greater detail, the denaturative effects of various agents produced on this protein.

It is well known that if a neutral solution of serum albumin is heated, even for a few seconds at 65°C, irreversible coagulation occurs, and the protein is considered to be denatured.[40] It has been shown however that if serum albumin is heated in the presence of anions of long chain fatty acids, especially caprylate, the protein is stabilised against heat denaturation. The binding of the caprylate ion is able to maintain the native specific conformation. If the heat treatment is carried out in slightly acidic or basic solution, the protein, although denatured, as judged by the increase in viscosity, etc., may not coagulate. This is probably because of the excess charge on the molecule preventing aggregation.

The expansion and aggregation produced when serum albumin is exposed to acid has been studied in detail by several

workers[91,92]. The changes which occur in the pH region of approximately 3, are called N--F transitions. It has been proposed that there are four regions in the molecule which behave as if they were structural sub-units linked together, giving three regions of greater flexibility which allow the molecule to undergo intrachain dissociation association equilibrium. Another view[93] of this low pH expansion proposes that about 35% of the polypeptide chain unravels, while the rest of the molecule remains unchanged. It is suggested[91] that the aggregation is initiated by liberation of bound fatty acid, which causes structural modification of the protein, allowing disulphide exchange reactions to cause aggregation.

The mechanism of urea denaturation of protein has already been mentioned with the ideas of unfolding, and dissociation of the native structure. With serum albumin the picture is different. Many workers using techniques like changes in optical rotation, viscosity and light-scattering methods have proposed the idea that serum albumin molecules do not unfold in concentrated urea, only swell. It has also been shown that denaturation of serum albumin by concentrated urea is reversible[94]. The ease of reversal is due to the large number of inter helix dithio links[95], which are responsible for the compact nature of the native molecule. As long as denaturation does not break these bonds, the original compact structure is regained easily upon removal of the denaturing agent. If the treatment with urea is prolonged, slow intermolecular cross linking occurs and denaturation becomes irreversible.

Recent work[96] using a study of the dielectric dispersion of serum albumin presents very good evidence for very little alteration in the coiled structure of serum albumin in concentrated urea; ovalbumin when studied by this technique showed aggregation.

As mentioned in the general discussion on denaturation, urea can protect proteins against heat denaturation. This has been attributed to the formation of large hydrophilic complexes which isolate, so to speak, each protein molecule, and so prevent aggregation. Thus urea can on the one hand, unfold and dissociate, and on the other, protect against these very same changes.

It has been reported[42] that if serum albumin is exposed to heat, or dilute sodium hydroxide solution, or concentrated urea, it loses the power to interact with the anionic dye methyl orange. This has been attributed to a denaturation effect. However it has been found during this research, that if a solution of bovine serum albumin is heated for a few seconds at 65°C till coagulated, it is possible by adding crystalline urea to redissolve the presumably denatured protein. It would appear that the protein after undergoing this treatment has regained some of its initial folding, judging by the fact that its dye binding capabilities are not drastically altered. (This is very interesting as it is akin to "unboiling" an egg and will be discussed in fuller detail later).

In conclusion it would appear that denaturation by any

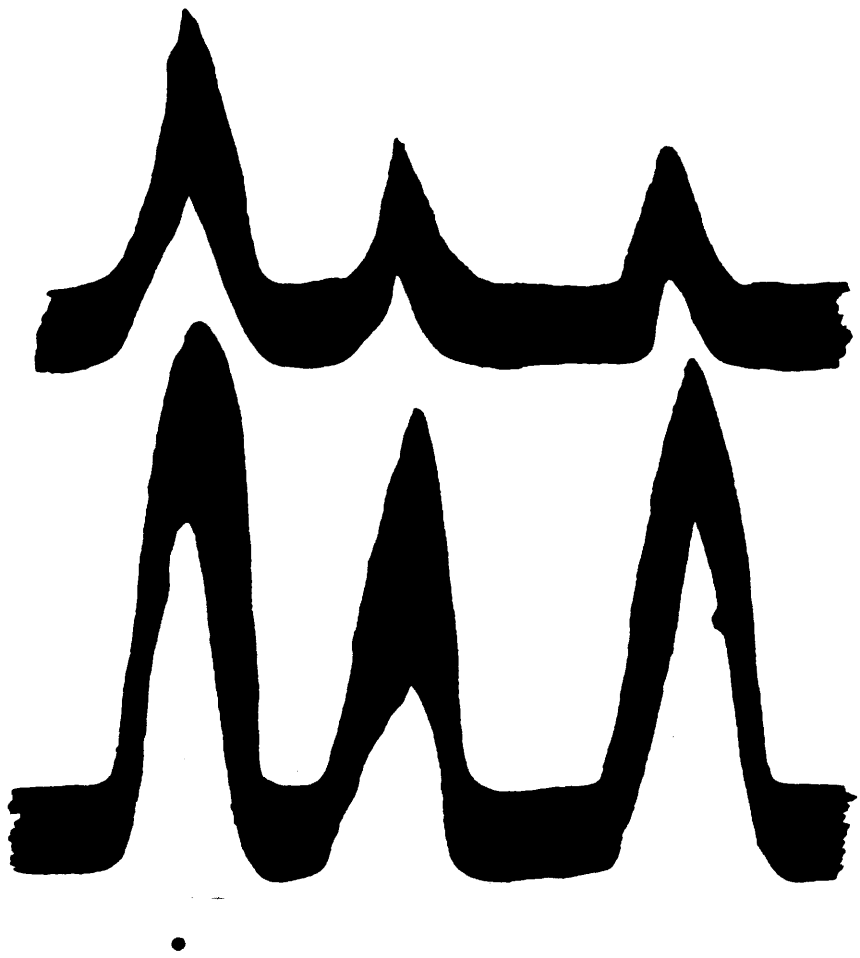
of the means already described is a process involving an alteration in the folded structure of the peptide chains, in the protein molecule, together with an associated alteration in the spatial relations of the various amino acid residues. and their interactions with one another and with solvent. Depending on the protein and the agent used, the process can be reversible. In fact it is more a transition of the physical kind than a definite chemical reaction involving covalent bonds.

As shown on page (41) bovine serum albumin when treated with concentrated urea, and subsequently studied by spot line chromatography indicates very little alteration in binding power, whereas other workers[42], studying the effect of urea on the binding of methyl orange to serum albumin, concluded that urea stopped the binding of dye. When methyl orange binds to serum albumin there is a marked change in the absorption spectrum of the dye, compared with the free dye. In the presence of 5M urea the dye absorption spectrum was not displaced. It was concluded from this, that denaturation had occurred, with loss in binding ability. From the review on denaturation it was noted that serum albumin is a very flexible molecule and can resist the effect of concentrated urea, upon removal of the urea, the protein reverts more or less to its native state. In spot line chromatography the removal of urea from the protein is made easy, charged protein

Figure 24

Examination of binding power and rate of decay of B.S.A. treated with 7M urea solution and then dialysed.

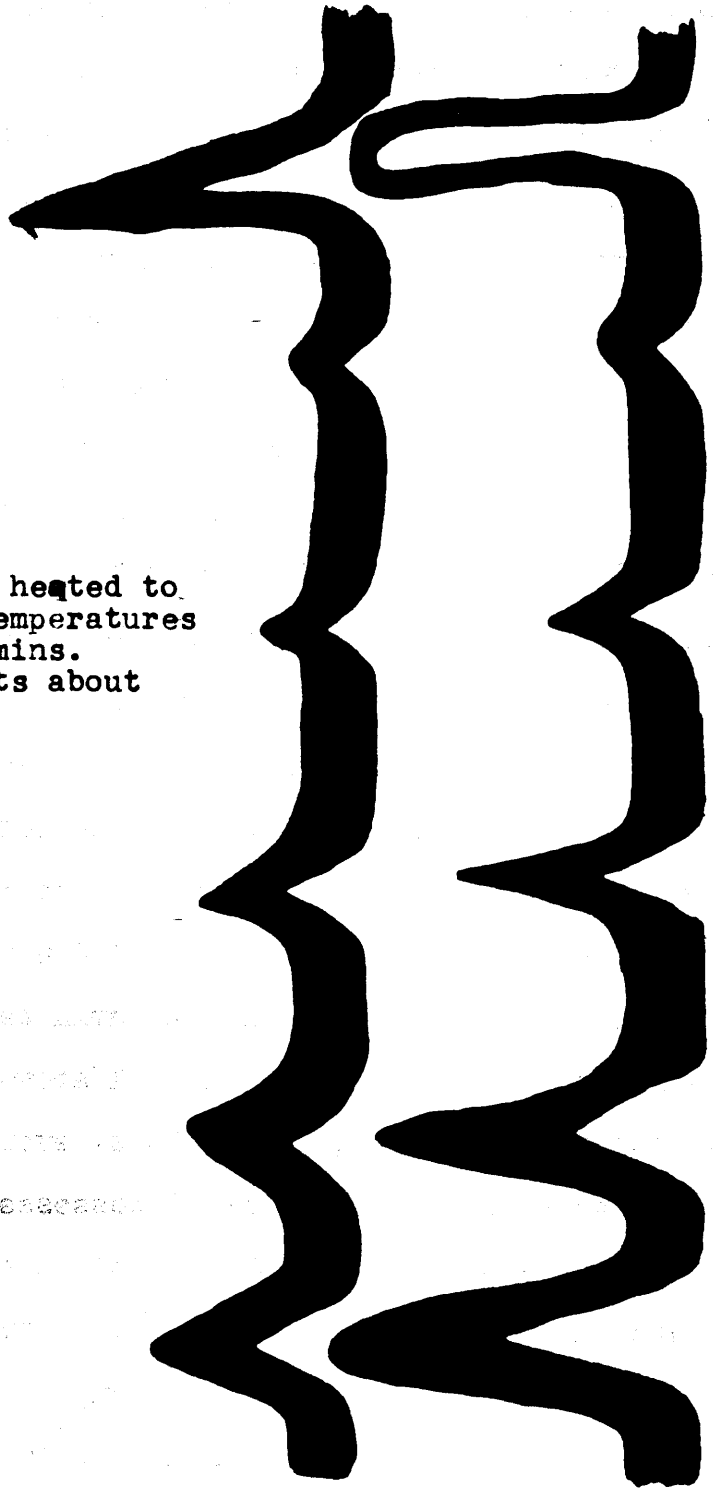
Run done at pH 6.8.



Equal volumes of the treated protein solution (≒ 500 µg. B.S.A.).

Figure 25

Examination of binding power of B.S.A. which has been treated with 7M urea solution and then heated to various temperatures. Run done at pH 6.8.



• heat denatured, urea added.

• 90°C

• 80°C

• 70°C

• 60°C

• 50°C

Samples heated to noted temperatures for 10 mins. All spots about 300 µg.

moves off ahead of the urea. This presumably leaves the protein, more or less in its native state ready to bind when it encounters the dye line. A number of two line spot line chromatography runs have been done in which the protein has been in contact with the urea, for up to two days. The protein solutions have also been dialysed first, to remove the urea. All these runs show that the binding power of serum albumin is only slightly reduced. In a few experiments large "flames" on the tip of the loops indicated that probably the protein had not returned exactly to its initial native conformation. (Fig. 24) show the type of results that were obtained. Note that in all cases the protein can still decay.

Spot line chromatography runs cannot be used to study the binding of heat coagulated bovine serum albumin, the protein sticks to the supporting medium and will not move by electrophoresis. Bovine serum albumin which was treated with caprylate could be heated above 65°C without coagulating and the binding then studied (but this does not denature). However it was found that bovine serum albumin can be made up in 6M urea solution and then heated to well above 65°C without coagulation occurring. As Fig. (25) shows, even protein which has been heated to 90°C with urea, still possesses the ability to bind dye. Some alteration to the protein has occurred under these drastic conditions, for the loops formed by the 80°C and 90°C treated protein are very triangular and indicate rather heavy tailing on the paper. This has been confirmed by developing for protein with bromophenol blue.

An even more interesting experiment is the following. A sample of bovine serum albumin in neutral (pH 6.8) buffer was heated for 30 seconds at 70°C in a water bath till coagulation of the protein had occurred. The protein should now have been well and truly heat denatured. However it was found that when crystalline urea was added, and the white mass stirred, a clear solution resulted. The amount of urea needed was such as to give approximately 7M solution. This is presumably due to the dispersive nature of the urea on the hydrophobic protein. A sample of this solution was then used in a usual two line spot line chromatography run. As Fig. (25) shows, the loops produced in both lines were very clear and of good size, indicating that the protein was still able to bind efficiently.

Thus the curious position is, that as far as serum albumin is concerned, urea is here not only not fulfilling its usual function, as a powerful denaturing agent , not only can it protect protein molecules against denaturation , but also it can be seen now, that it can regenerate denatured protein, into something like its original conformation. (cf. regaining of initial conformation by reoxidation of fully reduced protein [90]).

90

Theory for Action of Urea.

When a protein binds a small molecule this can either lead to (a) protection or (b) disruption of the structure. An example of protection is the binding of caprylate anion by serum albumin stabilising the structure against heat

denaturation[40]. Detergents, on the other hand, although at first not causing any structural alterations when bound in higher concentration are powerful disruptive agents[84]. Thus concentrated urea solutions usually function as denaturing agents because they disrupt the tertiary structure. However if the native conformation of the protein is fundamentally very stable, as would appear to be the case with serum albumin then when urea is bound to the protein it will tend to exert a protective effect. As mentioned previously, irreversible denaturation changes are usually associated with disulphide interchange reactions producing aggregation.

i.e. $n[\text{Prot}] \rightleftharpoons [\text{Prot}]_n$

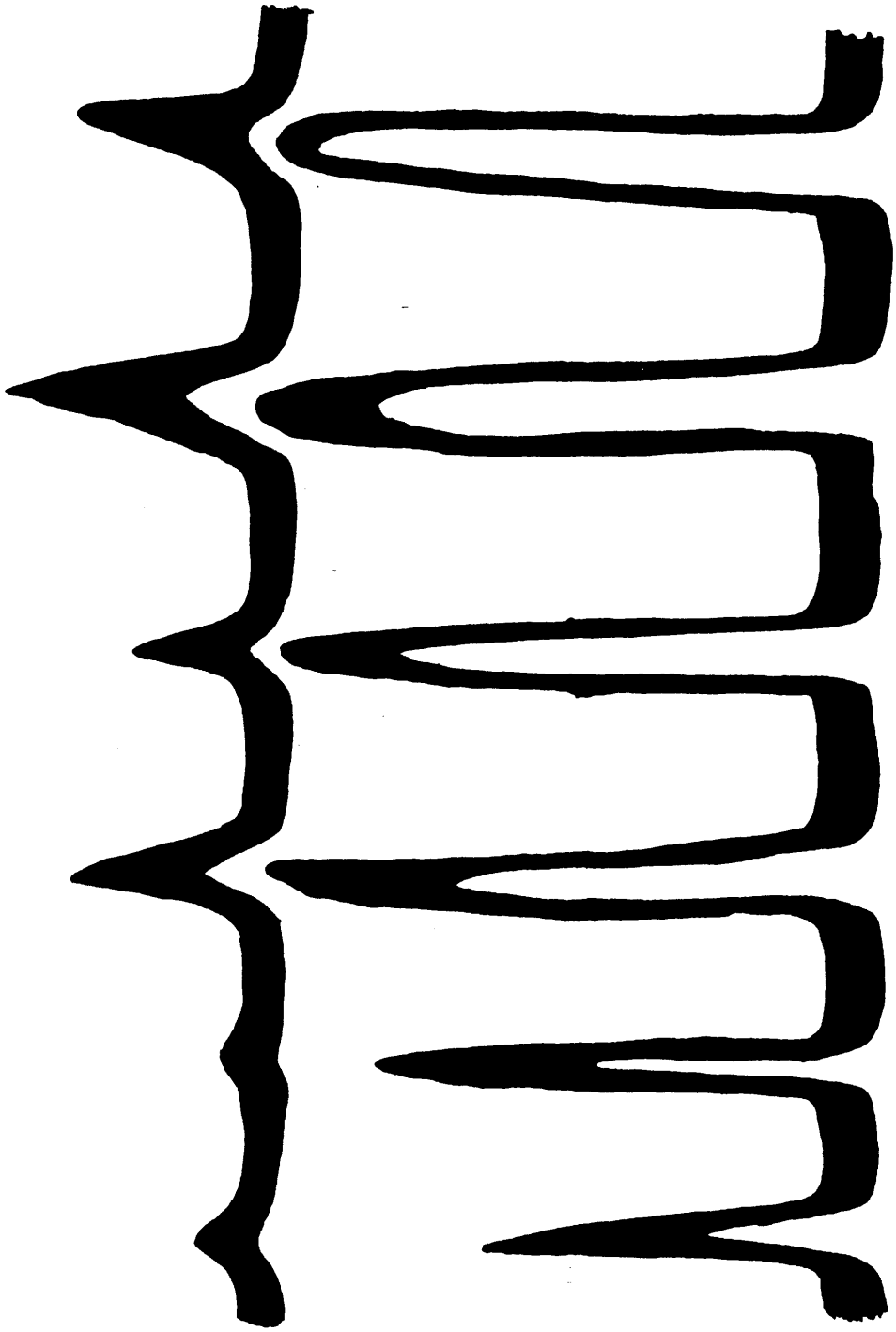
Assume that this reaction is reversible if there is a rigid structure stabilised by many disulphide bridges. Then the regenerative effect of concentrated urea, on serum albumin can be explained by suggesting that urea tends to displace the above equilibrium to the left, because of the hydrophilic sphere of influence tending to isolate each protein molecule. Once disentangled from other molecules, the inherently stable tertiary structure is regained.

Further Studies on the Mechanism of Binding Decay in Serum Albumin

It is now thought that the mechanism of the process involved a loss of $-\overset{+}{N}H_3$ binding sites. As mentioned previously it has been suggested that the surface of the serum albumin molecule contains positively but not negatively charged groups[97,98]. Binding decay could come about by a neutralisation of $-\overset{+}{N}H_3$

Figure 26

Spot line chromatography run to compare the binding ability, and rate of decay of B.S.A. treated with fluorodinitronenzene with untreated B.S.A. Done at pH 6.6.



• 300 µg. B.S.A.

• 300 µg. (DNFB) B.S.A.

• 200 µg. B.S.A.

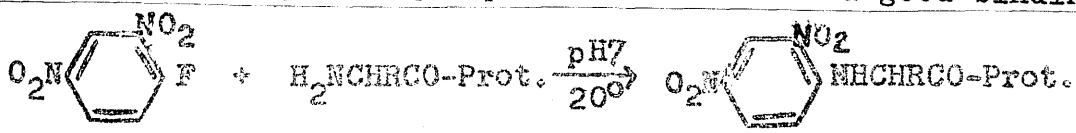
• 200 µg. (DNFB) B.S.A.

• 100 µg. B.S.A.

• 100 µg. (DNFB) B.S.A.

by CO_2^- caused by a slight alteration in the structure of the protein. It has been noted[99] that heating converts lysine in other proteins into a biologically inactive form, presumably by the formation of a peptide link between the ϵ amino group of lysine, and a carboxyl group of glutamic or aspartic acid. Decay could also come about by the structural alteration, (produced by the interaction with the supporting medium), causing the binding sites to become folded inside the molecule. It was decided that if the ϵ amino groups could be reacted in such a way as to hinder these or any other changes, then this should markedly reduce decay.

Bovine serum albumin was treated with 2,4-dinitrofluorobenzene (2,4-D.N.F.B.), the reagent which Sanger used so brilliantly in the elucidation of the structure of insulin. This was known to react, under the mild conditions used, specifically with free amino groups. A spot line chromatography run (Fig. 26) shows that the decay seems to be slightly reduced, as is also the initial binding. The drop in initial binding is presumably because the reaction, as shown below, converts a primary amino group into a secondary one, which because of attached dinitrophenyl group will not be such a good binding site.



The reduction in decay may simply be due to the bulky group being introduced hindering slight conformational changes. There is also the possibility of interactions of the nitro groups with polar groups in the protein, and also between the

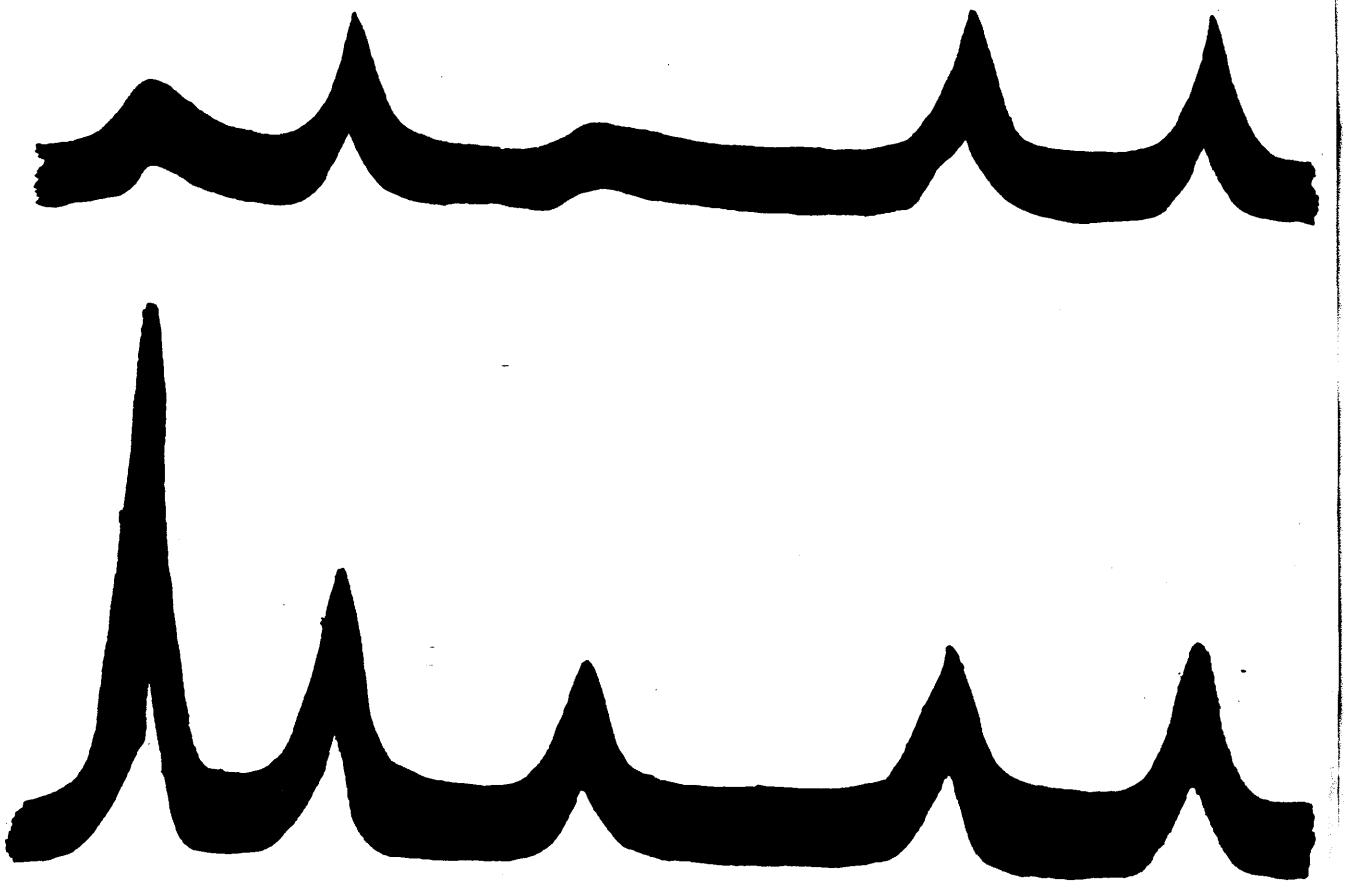
benzene ring and the hydrophobic side chains, stabilising the structure against induced changes.

When protein spots were run on paper for different times in order to produced different stages of decay, then it was noted that the spot which had moved farthest i.e. had decayed most, took the longest time to develop a yellow colour with 2,4-D.N.F.B. The reaction was done by carefully placing a filter paper which had been treated with an acetone solution of the reagent, on the filter paper of the electrophoresis set up, and noting the time taken to produce the yellow colour, indicative of reaction. Spots newly placed on paper produced a yellow colour within a few seconds, but ones run to induce maximum decay took several minutes. Also, colour produced by decayed spots did not appear to be so intense as that produced by native protein. These results would suggest that there is a reduction in the number of cationic binding sites which are available on the surface.

During a search of the literature it was found that the bifunctional reagent p,p'-difluoro-m,m'-dinitrodiphenylsulphone had been used for interaction with bovine serum albumin[100]. It had been found that the protein treated in this way, was stabilised against heat denaturation probably by the introduction of several covalent linkages stabilising the tertiary structure. It was thought that perhaps this reagent might "lock" the structure sufficiently to prevent decay. The bifunctional reagent is known to attack two ϵ amino groups simultaneously with resulting cross link formation.

Figure 27

Comparison of binding ability of $(FNPS)_{10}$B.S.A. with untreated material. Run done at pH 6.6.



200 µg. B.S.A.

200 µg. $(FNPS)_{10}$B.S.A.

200 µg. B.S.A.

200 µg. $(FNPS)_{10}$ B.S.A.

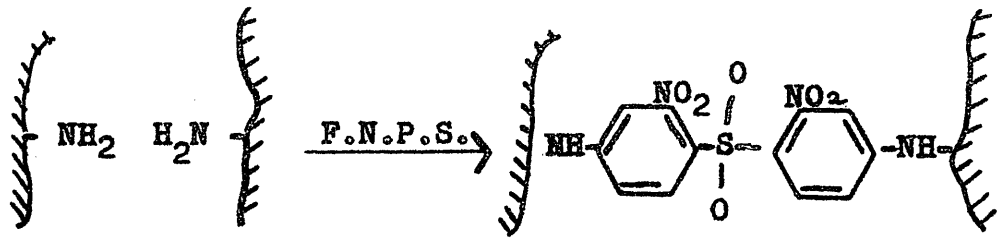
A gift of 500 mg of F.N.P.S. was obtained, and using it a sample of bovine serum albumin was treated by the method given. The resulting compound is $(FNPS)_{10}$ bovine serum albumin. A two line spot line chromatography run was done on the yellow product (Fig. 27). It was noted that the modified protein moved faster during electrophoresis than native material. This is understandable because the protein will have less positive charges on it. The area of the loop produced in the first line by the $(FNPS)_{10}$ bovine serum albumin at the close range is only about half the area of that produced by an equal weight of untreated bovine serum albumin. This is because up to 20 of the initial $\overset{+}{=}NH$ centres have been modified. Very little decay will have occurred when these spots encounter the line. As can be seen the picture in the second line is reversed, now the $(FNPS)_{10}$ bovine serum albumin produces a much bigger loop than the untreated protein, (0.30 cm^2 to 0.15cm^2). The loop produced by the untreated protein is like a typical decay one. Note that the loops produced in the first line by the long range $(FNPS)_{10}$ bovine serum albumin spots are bigger than that by an equal weight of untreated protein from the same distance, 0.67cm^2 to 0.36cm^2. This can be explained as follows. The untreated 200 µg spot will have about 80 ug in the decayed state before it reaches the dye line, hence loop area will be small. The treated protein has lost very little of its ability, cf. 0.75cm^2 for short range spot, 0.67cm^2 for long range spot. The loop produced by the untreated protein in the second line is almost negligible in area,

whereas that produced by $(FNPS)_{10}$ bovine serum albumin is still slightly less than half of its initial area $0.26cm^2$.

Immediate Conclusions of "Scaffold" Experiment:-

The immediate importance of this experiment is that this is the first time, that a means has been found, to reduce significantly the decay effect, and this adds greatly to the general theory put forward below.

The reaction with fluoronitrophenylsulphone was done at low protein concentration to prevent intermolecular cross linking occurring, with the production of insoluble protein aggregates. As the protein was attacked by several reagent molecules (about 10) the effect is to construct a molecular scaffold - by introducing several covalent linkages at different places in the molecule - which will hold the protein chain in a strict conformation and the resulting inflexibility will greatly reduce any distortion or collapse, which would otherwise occur. Under the conditions used the reaction can be formulated thus.



As the binding decay is markedly reduced by the above "scaffolding" of the protein, one can conclude quite confidently that decay in serum albumin is associated with the natural flexibility of the molecule.

Theories of binding of Small Molecules and Structure of Serum Albumin.

As mentioned previously serum albumin has a unique ability to combine with small anions and neutral molecules, even when it is itself an anion. It binds cations no more strongly than a normal corpuscular protein.

It seemed initially that the binding power of serum albumin might be due to its symmetric structure. This does not appear to be the case, for egg albumin and insulin which have smaller axial ratios than serum albumin, show decreased, rather than increased binding [101, 102].

Because binding decreases in the region of pH 12, it has been suggested [43] that cationic residues, presumably ϵ amino groups of lysine residues and guanidinium groups of arginine residues play an important role. However mere possession of these groups is not sufficient for binding ability, because γ globulin, a non binding protein, has very substantial quantities of arginine, lysine and histidine residues. Indeed lysozyme which only binds feebly [103] contains as large a percentage of basic amino acids as does serum albumin. It has therefore been suggested, that one or more residues must be in proper juxtaposition to the quaternary nitrogen to supply the additional specific attraction to ensure binding. This however seems unlikely since anions of all shapes and sizes, organic and inorganic, bind to serum albumin.

It has been proposed [104, 44] that the cause of binding in serum albumin is due to interactions between hydroxyl groups and carboxyl groups, within the molecule. The net result

of this will be a decrease in the number of carboxyl and hydroxyl groups, which can combine with cationic $\equiv\overset{+}{N}H$ loci, and hence to increase the number of free cationic nitrogens, the proposed anion binding centres. A binding index has been proposed to measure the efficiency of any protein, this is $\frac{\Sigma[\equiv\overset{+}{N}H]}{\Sigma[CO_2^-]-\Sigma[OH]}$ This value for serum albumin is 29. Next best is β-lactoglobulin which has a value of 4.6.

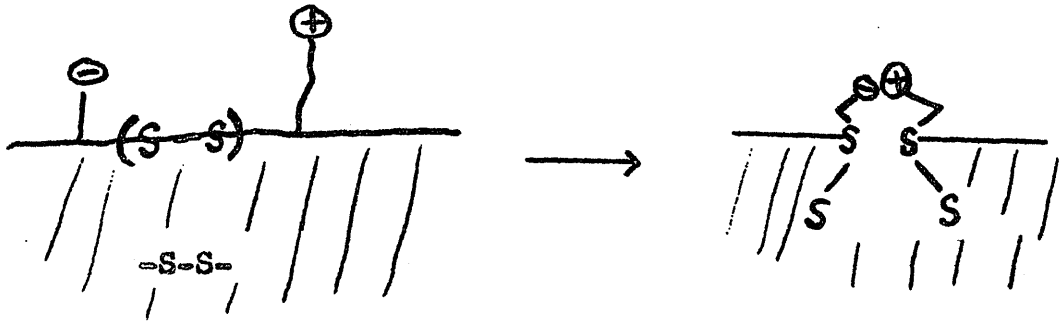
An alternative theory for binding has been put forward[105]. This visualises the region of anion binding as one where a local excess of positive groupings is backed by a local excess of hydrophobic side chains of leucine, valine etc. Because of the low dielectric constant of this microphase the positive charge is protected from the general field of the protein, and is not greatly affected by the net charge on the protein as a whole. To account for the absence of sites of adsorption for cations it must be supposed that the negative groups on the protein are more diffusely arranged and lack the hydrophobic environment, which the positive centres have.

Because serum albumin binds neutral molecules, e.g. sulphonamides[106], steroids[107], detergents[85,86], neutral dyes[108] etc., as well as anions, is a good indication that most probably the hydrophobic side chains of leucine, isoleucine, valine etc., are also involved in the binding process.

These above, are the two mains ideas which have been proposed to explain the binding power of serum albumin. The work described here cannot favour one or another. The important

point about both of these suggestions is that they postulate a resilient structure for the molecule, which separates centres of positive charge, from centres of negative charge: this simple statement explains the phenomen of binding and at this stage it is perhaps the best model. Perhaps the best way of approaching the problem is therefore, not an immediate concern with the exact position of the centres of positive and negative charge, but rather a closer examination of the reasons why the serum albumin molecule is so resilient.

Undoubtedly the main factor which contributes to the resilience of this molecule is the high number (approximately 17) of disulphide bridges, which in the maintenance of the specifically folded tertiary structure, form some sort of network. In particular, the binding decay is seem as some kind of breakdown, or rearrangement in this network, allowing the separated charges to collapse together with resulting removal of charge, and decrease in binding ability. Such a change is shown diagramatically below.



It has already been mentioned (page 55) that concentrated urea solutions appear to renature heat coagulated serum albumin - with respect to binding ability but do not alter the decay effect very much. Thus in a sense it would appear that the changes involved in decay are "more severe" than those brought about by heat denaturation. Yet it was shown that there was no evidence for more extensive denaturation occurring in regions of rapid decay than elsewhere. Therefore it would seem that whatever changes do take place, they are different from those induced during normal denaturation.

This dependence on the disulphide bridges was mentioned earlier when it was thought that the solitary sulphydryl group was responsible for initiating the changes leading to decay. However disulphide interchange reactions are quite possible without the participation of the -SH group cf. the role played by these in denaturative coagulation and by independent work on polymer systems. e.g. the polysulphides[109], where the interchange of chain segments is known. Denaturative coagulation can be considered to be due to the effect of <u>intermolecular</u> disulphide interchange reactions, contrasted with decay which is possibly due to some <u>intramolecular</u> reaction involving the disulphide bonds. The rearrangement might even be, as was suggested previously, (p.28), some special biomolecular mechanism which happens to be set off, by the protein being exposed to large surface areas, under the conditions of paper electrophoresis.

The idea of decay being due to a neutralisation of charge

i.e. $\equiv\overset{+}{N}H$ by CO_2^- is supported by the following observations:-

(a) The electrophoretic mobility of the decayed protein is about the same as the native material, suggesting little alteration in net charge on the molecule.

(b) At a pH below the isoelectric point, serum albumin does not appear to exhibit binding decay. A possible explanation is that neutralisation will be hindered because the carboxyl groups will now be in the unionised CO_2H form and hence, will not be available.

DISCUSSION

(Part 2)

The first part of the discussion was concerned with the interactions of the natural macromolecular system, serum albumin with various small molecules. In this second part, the approach is to search for a synthetic polymer which will have a reasonably specific molecular surface, (i.e. some sort of tertiary structure). If this could be achieved it is possible that it might lead to the imitation of other protein like functions (in particular, specific catalytic activity and specific binding ability).

Protein Model Systems

Since the days of the earliest studies on proteins, attempts have been made to produce synthetic models. The ideas behind the synthesis of such compounds are continually changing, with changing views on the structure and properties of the natural macromolecule.

A brief review now follows on several of the most important type of protein models, showing the points of protein structure that each can clarify and where each fails.

Polypeptides:-

These are probably the best known protein models, being introduced about 1900 by Fischer [110]. They were important in helping to demonstrate that proteins consisted of long chains of amino acid residues linked through the peptide bond. Since these early days modern methods of peptide synthesis have made possible the preparation of special peptides of low

molecular weight and known chemical structure, for use as models in the examination of several physical and chemical properties, of proteins, e.g. the acid-base relationships in proteins[111]. They have also been used to clarify the results of reacting various reagents with proteins (e.g. the study of the iodination of tyrosine residues[112]). They have also been used as final proof of the structures of several physiologically important substances such as glutathione[113] and carnosine[114].

However, although polypeptides have indeed proved valuable models in structural elucidation, they have the limitation that they all have too short a chain to possess a definite tertiary structure and exist in solution as no more than a random coil. They are also difficult to study, because as the molecular weight increases so the solubility in water decreases.

Polyamino Acids:-

Unlike polypeptides, which are built up by a laborious step-wise method these are synthesised in a one step polymerisation. Attempts to polymerise natural amino acids were recorded[115] even before the existence of the peptide bond in proteins had been clearly established. The main object of these early attempts was the synthesis of protein models from the structural units of the protein molecule.

Discouraged, with the meagre success obtained in the preparation of polymeric material from amino acids, chemists searching for protein models turned therefore to the now classical methods of polypeptide synthesis. However recent

refinements in the chemical technique of polymerisation, and the development of new physical methods in polymer chemistry have led to a renewal of interest in the polymers of the amino acids. The methods of synthesis of α amino acid polymers were re-examined and a systematic study of their physical and chemical properties undertaken. Thus, synthetic polyamino acids tend to fill the gap between the synthetic low molecular weight models (polypeptides) on the one hand, and the naturally occurring high molecular weight proteins on the other.

Most of the known poly α amino acids suffer from the disadvantage that they cannot be studied in aqueous solution in view of their low solubility. Therefore they constitute synthetic models only for water insoluble proteins such as keratin and silk fibroin.

A small number of acidic and basic water soluble poly amino acids have been synthesised, and as such could play a useful role in the elucidation of the structure and properties of water soluble proteins. Among these may be mentioned poly L-glutamic acid, which has been shown to be identical with the polypeptide of molecular weight 50,000 isolated from the capsule of Bacillus anthracis[116], (natural is poly D-glutamic acid).

Like proteins, some of these polyamino acids are enzymatically hydrolysed, e.g. poly L-lysine is hydrolysed rapidly by trypsin. X-ray studies on the solid materials and infrared studies on the solids and solutions show a folded chain with hydrogen bonding between the C=O and N-H. The structure of

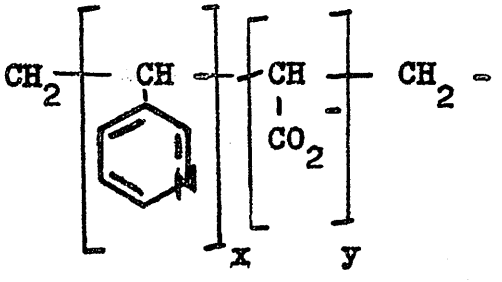
the water soluble polyamino acids in solution is either a random[117] coil or at best an α-helix, and so although they are useful in certain aspects they are limited in their power to solve the complex properties of globular proteins. Most of the known biological and other properties of synthetic and naturally occurring polyamino acids are contained in an excellent review[118].

Polyelectrolytes:-

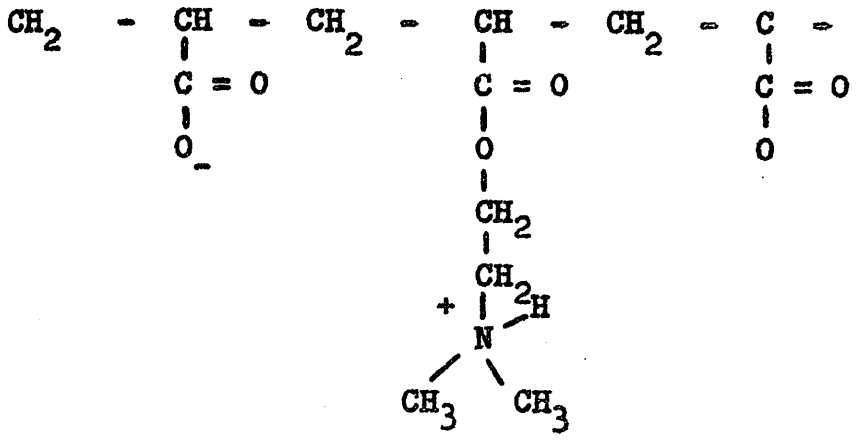
Basic and acidic, polyelectrolytes have been synthesised, and the resulting polymers used as protein models. Examples of such type of compounds are polyvinylamine[119,120] and polyacrylic acid. Much more interesting, because they contain carboxyl and amino groups, like proteins, are the synthetic polyampholytes, both random copolymer type and regular. Examples of each type are numerous and only a few will be shown.

(a) The random copolymer type, synthesised from vinyl pyridine and acrylic acid[121].

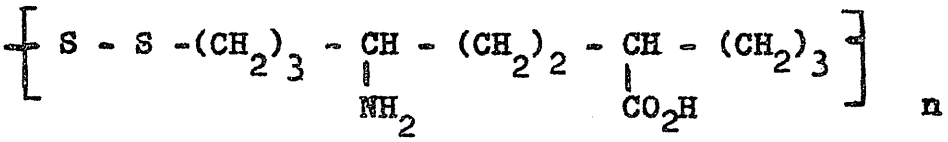
e.g.



and another example, also a random copolymer, which has been prepared[122].



(b) A regular polyampholyte has also recently been synthesised[123], this has the following structure.



Such models are useful, because titration studies on them can help to give a clearer understanding of protein structure. However even these, like all the models mentioned previously have one big failing, viz., the lack of a definite tertiary structure.

<u>The Need for a Precise Surface</u>:-

For any synthetic macromolecular system to exhibit any of the more complex properties of proteins, it is probably essential that they should possess a definite three dimensional shape. The specificity of enzyme activity is probably due to the macromolecule having on its surface, regions which are in some sense "complementary images" of the shape of the substrate molecule. One approach to this problem is to devise a means of printing the impression of a molecule on to a polymer. Pauling's early antibody theory involved this concept. The

suggestion was that the shape of the antigen determined the resulting folding of the γ globulin antibody.

It has been found[124] that when silica gel is formed in the presence of certain dyes, and subsequently extracted to remove the dye, the remaining gel has a strong and specific adsorption capacity for the dye that had been present during its formation. Gels made in the presence of butyl orange were much better adsorbents for that dye, than for propyl orange, and vice versa. Because of this selectivity in their adsorptive power, or "molecular recognition" ability these gels have been called synthetic antibodies.

Silica gel has an irregular three dimensional network structure in which silicon atoms bound to oxygen, occupy much the same position with respect to each other as do hydrogen bonded oxygen atoms in water. When the silica gel network is still incompletely connected, it is flexible enough to conform to the subtle differences in shape presented by different kinds of guest molecule. After the cross linking is complete and the gel dried, the guest can be extracted, leaving a phantom of its shape. The guest molecule is thus used as a template for guiding the polymerisation of the host. This work has recently been extended and silica gels have been prepared which are specific adsorbents for different heterocyclic bases[125].

These models are all inorganic and although they are the only successful ones to date, synthetic organic polymers, employing these concepts would be a truer picture of natural

biological systems.

Attempts to form Molecular Impressions in Organic Polymers:-

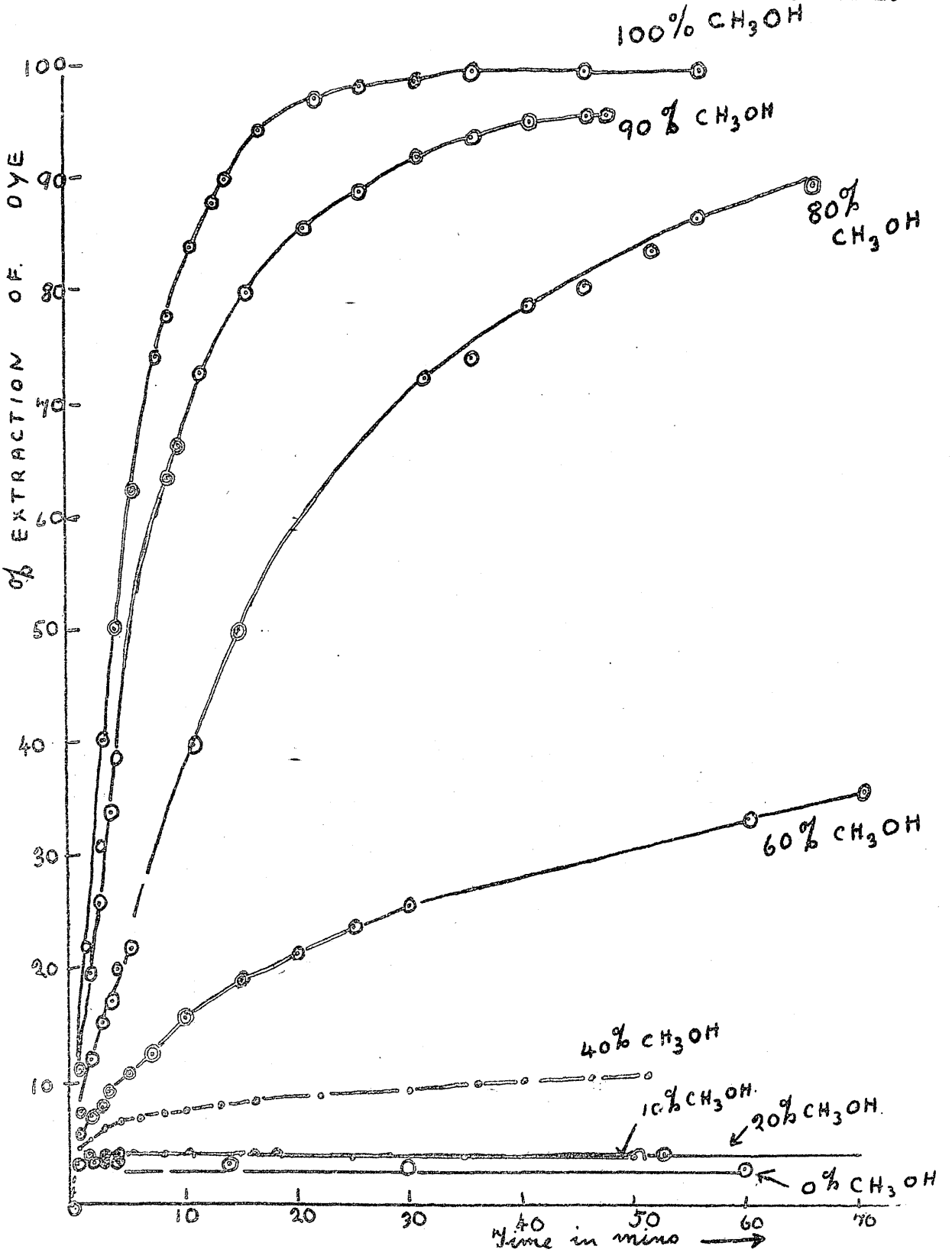
The system chosen was polyvinyl alcohol, This interesting polymer, is water soluble, undergoes a variety of reactions very readily, and there is good evidence that it can accept guest molecules in some inner structure, (e.g. it gives a characteristic colour with iodine, cf. starch-iodine reaction) [126], and gels in the presence of azo dyes of the Congo Red type).

By analogy with the work on silica gel, it seemed that the best way of going about the problem was to form a film of the polymer in the presence of some suitable small molecule, which would have a definite shape. The one chosen was the water soluble triphenyl methane type, dyestuff, brilliant green. Then, by careful extraction of the dye, with a suitable solvent there may be left in the film, some sort of impression of the brilliant green molecule, and this may be stable long enough for the polymer to "recognise" the dye again.

The type of experiments that were done were as follows. Aqueous solutions of polyvinyl alcohol, about 2% by weight, with a slight trace of dye, (about 25 mg of dye per gram of polymer) were cast into thin films on clean glass plates. When required the film was cut into pieces a few centimeters square, and these studied. Using a "Spekker" the concentration of dye in the film was noted. Under known gentle conditions all the dye was then extracted from the film, this amount was noted. The film was then redyed and the process repeated. It was a general conclusion that films of polyvinyl alcohol

Figure 28.

Rate of extraction of Brilliant Green from films of P.V.A. with mixtures of methanol and ethanol.



Extraction of Brilliant Green from a film of P.V.A. with mixtures of C_2H_5OH and CH_3OH. At room temperature.

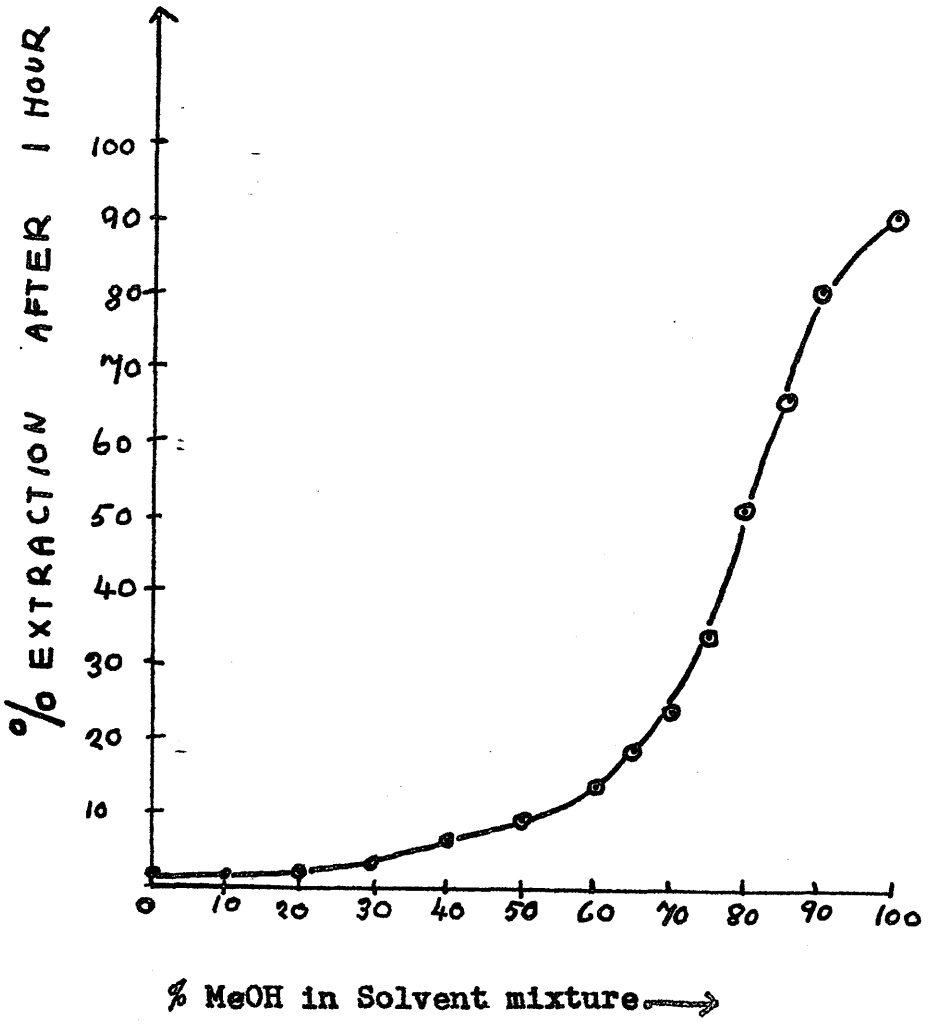
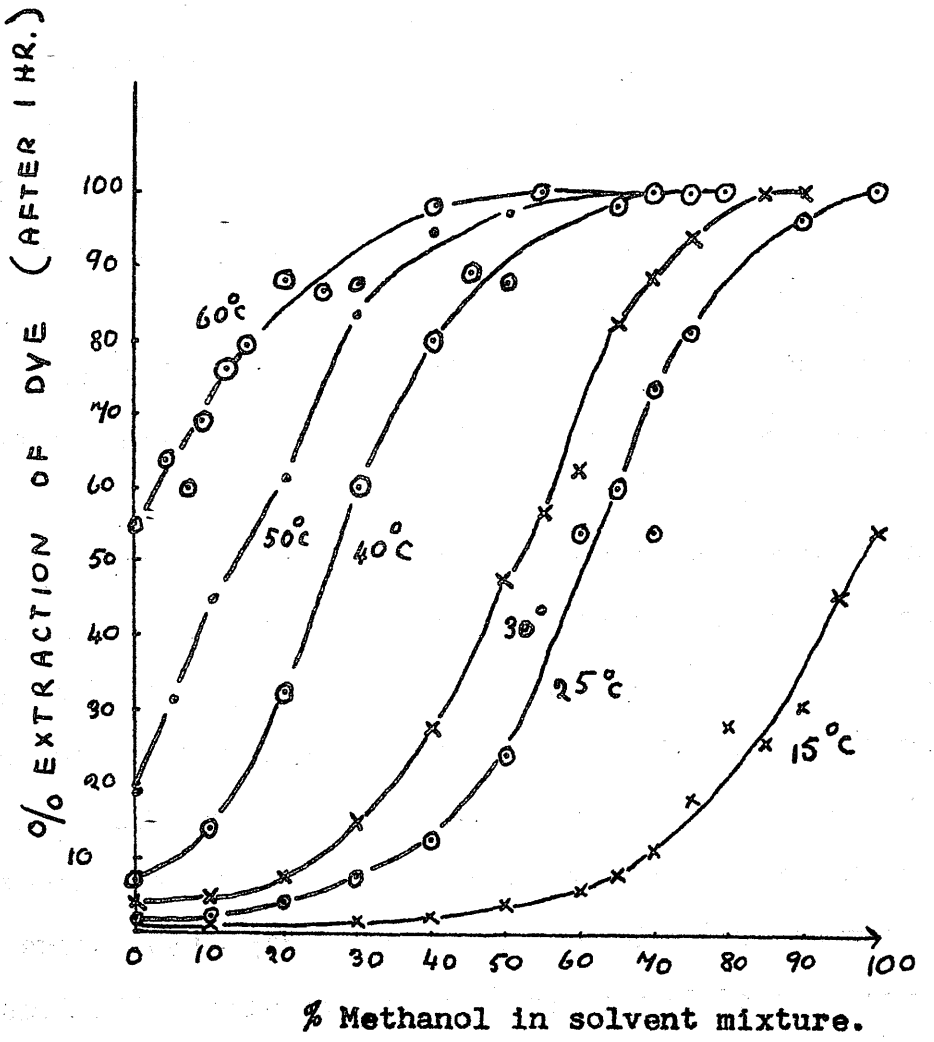


Figure 29.

Figure 39

Effect of temperature on the extraction of Brilliant Green from films of P.V.A. with mixtures of methanol and ethanol.



did not seem to exhibit any "memory" effect for the dye brilliant green. It was decided to try and make the structure of the polymer more rigid, by slight cross linking with formaldehyde before, during, and after treatment with the dye. However, although this treatment did produce alterations in the appearance of the film (went opaque and felt more rubbery), it did not improve its ability to recognise the dye.

Earlier unpublished work by Cairns-Smith had indicated (Fig. 28) a strong ethanol/methanol dependence on the rate of extraction of dye from films of polyvinyl alcohol. This seemed relevent to the problem under consideration.

Experiments on the extraction of dyes were done initially at room temperature, the dyed film being treated with the solvent under examination for one hour with shaking of the vessel. As can be seen from the graph in (Fig. (29) methanol is much more efficient at removing dye from the polymer film, than ethanol, at room temperature. Runs have also been done to examine how the extraction of dye by the different solvent mixtures, depends on temperature, Fig. (30) shows these results. Pure ethanol, begins to extract dye quite efficiently above $50^{\circ}C$; so the phenomenon is probably not simply due to a difference in the size of the ethanol and methanol molecules, as at first sight perhaps seems likely. In order to provide a satisfactory explanation for these phenonena, it is most convenient to discuss them in terms of the Second Order Transition in polyvinyl alcohol, and thus a very brief review of this subject follows.

Second Order Transition in Polymers:-

This has also been called the glass transition. It is, as the name suggests, a definite temperature, or narrow temperature range below which an amorphous polymer is considered to be in its glassy state. It is usually denoted by the symbol T_g or T_m. It is the temperature below which an elastic, rubber like material suddenly becomes brittle.

To understand the factors involved in this transition it is necessary to examine the long polymer chains. These are more or less coiled on themselves and intertwined in an ever changing manner. If cross linked, they are also connected to each other at several points. At temperatures above absolute zero the chains are in continous, complex thermal motion, e.g. combinations of bending and twisting. As the temperature is raised these motions become more violent, the chains tending to move further apart, subject however to the constraint of cross linking. When the temperature is raised high enough the chains will have moved sufficiently far apart, or uncoiled enough so that segments of the chain, or perhaps groups attached to the chains, will now be able to rotate about their bonds without any steric hinderance. It is this point, the onset of free rotation, that appears to correspond to the second order transition.

Because of the ability of the polymer chains to slip past each other, and segments to rotate, physical and mechanical properties change dramatically at the glass transition temperature. It is by measuring the rate of change of these

properties with temperature that the glass transition point is determined experimentally. Methods used have included an examination of refractive index[127], volume changes[128], the thermal elongation with strain[129], and many others.

It has been shown[130] that increased cross linking in an amorphous polymer causes an increase in the glass transition temperature, presumably by decreasing the extent of possible rotation, because the greater number of binding points increases the amount of entanglement. Owing to this, a cross linked polymer has to be heated to a higher temperature than a non cross linked one, before the chains will be far enough apart to start rotating.

It has been suggested[130] that swelling agents and plasticisers should reduce hinderance to rotation hence decreasing the value of the glass transition temperature. An objection to this argument has been put forward on the grounds that low molecular weight compounds would not reduce steric hinderance[131]. However it is probable that these compounds would indeed reduce the steric hinderance, by separating the chains, thus permitting them to rotate with less interchain interference. It is certainly well known that swelling agents do produce a lowering of the glass transition temperature determined experimentally[132,133].

Explanation of Solvent Specificity in terms of Glass Transition

The glass transition temperature for amorphous polyvinyl alcohol has been shown to be around 85°C. The temperature at which the dyed films are formed is well below 85°C, so there will be no chain rotation and hence the dye molecules will be held firmly in the polymer network. Methanol can remove most of the dye from the film by extracting at room temperature, while the amount extracted by ethanol under the same conditions is negligible. As mentioned earlier the effect of swelling agents on polymers is to lower the glass transition temperature. This is considered to be the mechanism of action of methanol. Because of its good solvent power, it is able to push the chains far enough apart to remove steric hinderance at around room temperature. Whenever the frozen structure is released, the dye molecules are freed and are readily accessible to solvent molecules. The solvation (swelling) effect of ethanol is insufficient to lower the glass transition as far as room temperature. The great specificity in the reaction of the polymer to these two solvents, then is due to the chance that the T_g (in presence of ethanol) is higher than room temperature, which is higher than T_g (in presence of methanol).

That methanol is able to swell the polymer network is also demonstrated by experiments to dye the film initially, using dye solutions in methanol and ethanol respectively. It was noted that when the treatment was done in ethanol,

even at 40°C the film picked up very little dye*. On the other hand however, doing the treatment in methanol at the same temperature, the polymer film picked up the dye rapidly.

This selective lowering of the glass transition temperature of polymers by solvents could be important in biological systems. It could be akin to the lowering of the temperature at which denaturation of proteins, by organic solvents, becomes appreciable, e.g. proteins denature much more rapidly in ethanol at 20°C than in water at 20°C. It is possible to keep a protein in ethanol, without any sign of denaturation, only if the temperature is low enough (about -5°C). More generally, effects like this and others where changes in the concentration of small molecules alter dramatically molecular diffusion through macromolecular membranes could be of great interest as models of protein membranes.

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* If the polymer film was treated with formaldehyde to produce a cross linked more open structure, it was found that the film could be successfully dyed in ethanol solution. The dye this time would be able to get in between the chains .

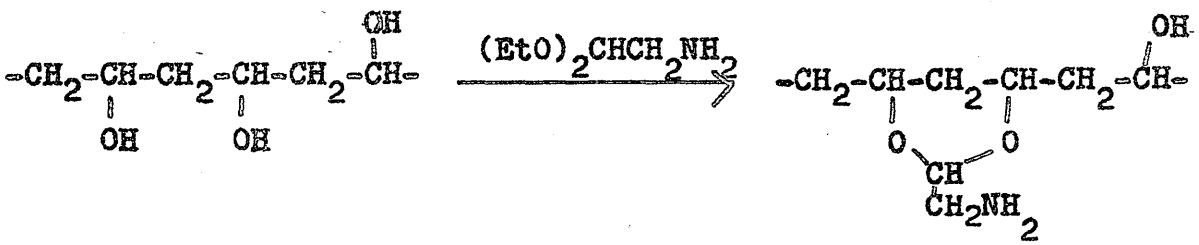
Further Experiments with Polyvinyl Alcohol:-

Polyvinyl alcohol can undergo a variety of common reactions. Use has been made of this in the following two approaches, in an attempt to make some kind of serum albumin model.

(a) Introduction of basic residues into the polymer chain.

(b) Introduction of acidic residues into the macromolecule.

(a) By analogy with previous work[134] it was decided that a possible method of synthesis of the required type of compound would be an ester exchange reaction of the polymer with amino acetal as shown below.

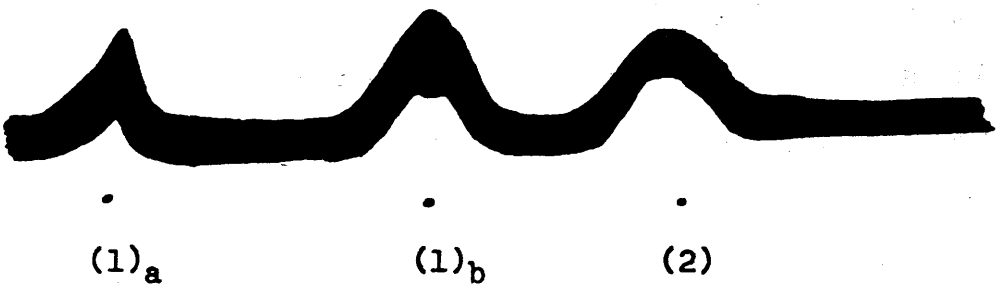


Conditions would be used so that not all the hydroxyl groups would be substituted. It is also interesting to note that this reaction would add stability to the polymer chain, because adjacent hydroxyl groups need not be involved in the reaction. It is possible that groups at distant parts of the chain would react, the resulting slight cross links providing stabilisation.

Various conditions were tried with varying degrees of success. Finally, using an acid catalyst and heating for a few hours the reaction seemed to have gone. During dialysis of the reaction mixture a gel was obtained indicating that probably cross links had been introduced. The extensively

Figure 31

Spot line chromatography in buffer pH 6.8. using modified samples of P.V.A. Dye line of biebrich scarlet. Migration of polymers towards cathode.



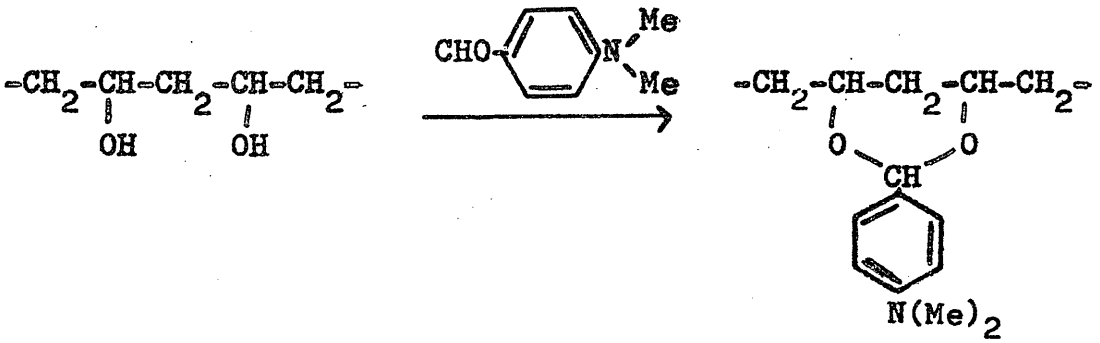
$(1)_a$ Spot from reaction of P.V.A. with aminoacetal after dialysis and concentration. (About 300 µg.)

(2) Spot from reaction of P.V.A% with p-dimethylamino-benzaldehyde. Spot goes about 2cm. then precipitates.

$(1)_b$ Same as $(1)_a$. About 500 µg.

dialysed aqueous solution of the polymer was then concentrated. The ability of the polymer to bind dye was studied by the method of spot line chromatography, using exactly the same technique as was applied to the study of proteins, only this time moving the polymer by electrophoresis, through a dye line. Doing the run at neutral pH as Fig. (31) shows, (where the polymer migrates, as expected, to the cathode) that loops were produced in a line of biebrich scarlet. This binding is not specific but probably electrostatic (cf runs with bovine serum albumin at low pH).

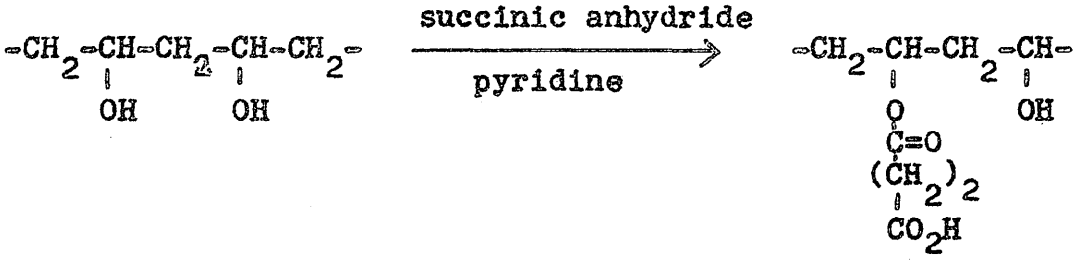
Using an amino aldehyde an alternative method of introducing basic residues into the polymer chains is as follows.



This material also, as Fig. (31) shows was able to bind dye. This polymer was difficult to deal with because it was not very soluble in buffer at neutral pH and precipitated on to the paper after a time. Electrophoresis could be done with less precipitation in decinormal HCl, but the dye moves rather rapidly here. Like the previous example, dye binding ability in this material is not specific. However they are both

quite interesting cases in that they confirm that a $\equiv N^{+}$- centre can be an anion binding site in a synthetic, as well as a natural system.

(b) When polyvinyl alcohol is treated with acetic anhydride in pyridine, polyvinyl acetate results. Using succinic anhydride the following type of compound could be obtained:-

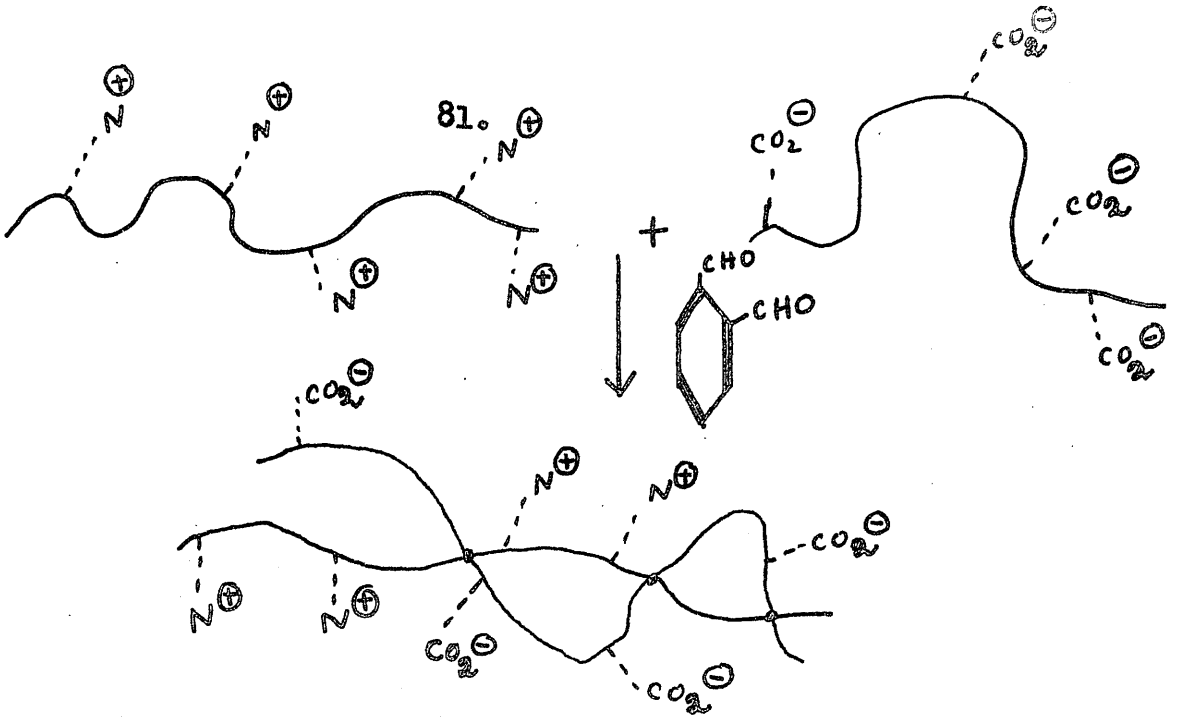


A compound of this type was prepared.

(a) the polymer was soluble in dilute base, and

(b) it gave vigorous evolution of CO_2 with sodium bicarbonate solution.

As mentioned in the section on proteins serum albumin possesses its unique binding ability probably because of separation of charge. If the two types of polymer, i.e. the basic and acidic could be linked together (e.g. by using a cross linking agent) then, if an excess of the carboxyl containing polymer was used, it is possible that a material could be obtained which would still have some separation of positive and negative sites. Like serum albumin, a macroanion would be formed which would possess the ability to bind dye at the cationic centres.



Aqueous solutions of the acidic and basic polymers, and mixtures of them were treated with different concentrations of a cross linking agent o-phthal-dialdehyde, with and without heating[136]. Although cross linking did occur, (it was so great in some cases as to produce a stiff gel) by using spot line chromatography and passing the polymer through a line of biebrich scarlet, no evidence could be obtained for anionic material possessing the ability to produce a loop in the dye line. This could be because (a) the different types of chain would not inter cross link or, more probably, (b) there was complete neutralisation of all the positive sites.

Globular Protein Models:-

As was mentioned previously one of the key features of globular proteins is their ability to retain, even in solution, a specific three dimensional, or "tertiary" structure, and the many complex properties of these molecules are doubtless concerned with this tertiary structure. The

protein models discussed, in the first pages (66-71) of this section (with the exception of the silica gel work) have all had the serious limitations that they do not really take this fact into account. The opposite approach is attempted here, viz., to make a "tertiary" structure directly, without necessarily using a system which basically resembles a protein. The essential thing being, the final folded structure, which may exhibit some of the properties of a protein.

A test for self folding of a macromolecule is sensitivity to solvents, in extreme cases self folding may give rise to phenomena like denaturation e.g. polymethacrylic acid is precipitated from hot aqueous solution, but redissolves readily when the solution is cooled. Likewise it has been deduced from viscosity measurements that a copolymer of styrene and maleic acid [135] changed its shape on exposure to different solvents.

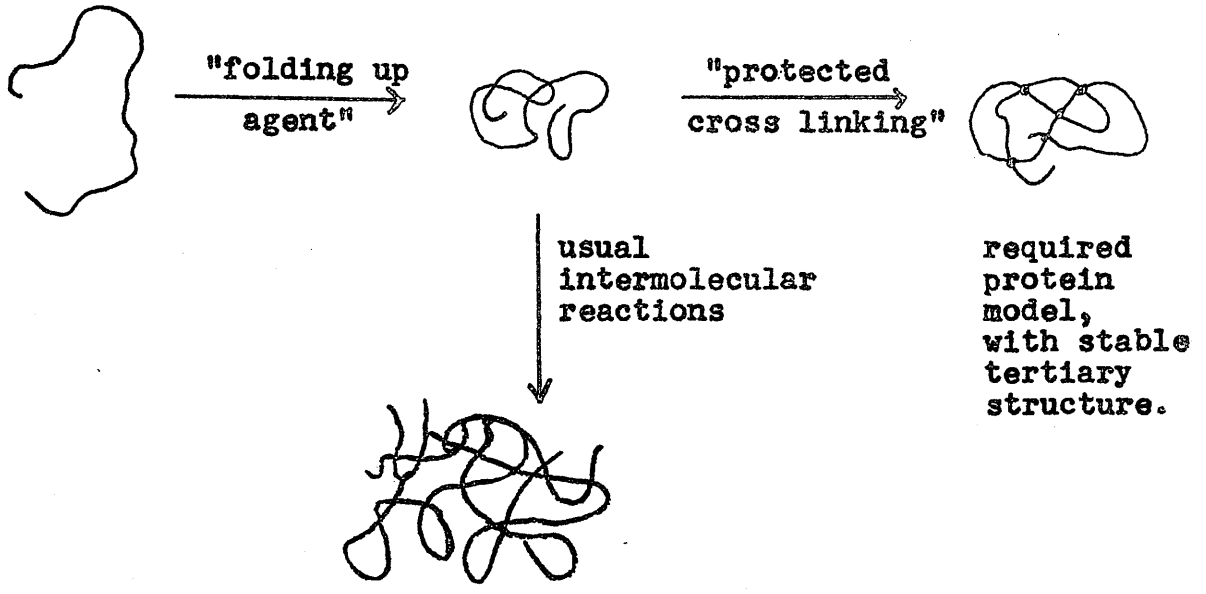
The approach is therefore as follows:-

(1) Synthesise a polymer.

(2) Allow the polymer to fold up. This can be done by putting the polymer in a bad solvent, or alternatively by using a "polysoap" type compound.

(3) Cross link the resulting system without coagulation, i.e. form some internal stabilising structure comparable with the system of -S-S- bridges in proteins. The idea is to try to get the individual macromolecules to undergo intramolecular cross linking, not as is usually the case in cross linking

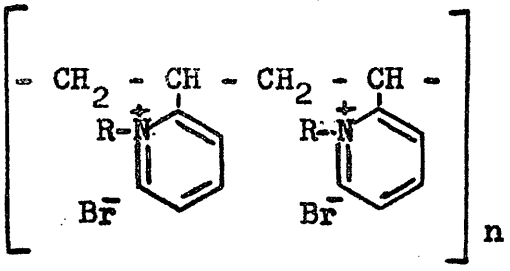
reactions, general intermolecular reactions resulting in aggregation. Hence the use of the prefabricated micelles of the "polysoaps" compounds. Such a procedure is indicated diagramatically below.



Polysoaps:-

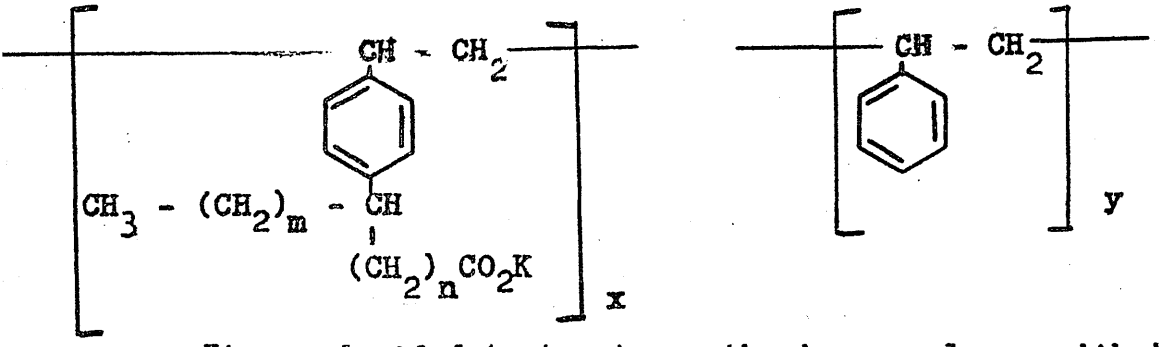
These consist of polymer molecules with chemically attached soap like molecules. Most polysoaps behave like prefabricated micelles in that they have a compact structure and solubilise hydrocarbons[137]. Both cationic[137,138,139] and anionic polysoaps are known[140,141]. Examples of the type of structures encountered for each class are shown below.

(a) Polysoap from reaction of dodecyl bromide with poly-2-vinyl-pyridine.

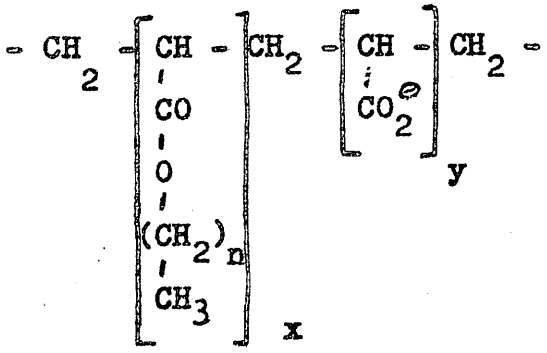


where R denotes the dodecyl radical.

(b) Polysoap from polystyrene and long chain fatty acids exemplified by the following.



It was decided to try to synthesise a polymer with the general formula shown below. By analogy with the polysoaps mentioned earlier, such a molecule should have a compact structure.



Probable routes to such a compound would be:-

(1) Start with polyacrylic acid, and attach the long side chain, by esterification with the required alcohol.

(2) A copolymerisation of acrylic acid with a long chain acrylic ester.

(3) Start with a long chain acrylic ester, polymerise this, then partial hydrolysis to give the required compound.

In the first method, polyacrylic acid can be readily prepared in aqueous solution. It is difficult to get the polymer out of solution, and probably the best way is to evaporate to dryness and grind up the resulting polyacrylic acid film. Esterification with a long chain alcohol is severely hampered because polyacrylic acid is insoluble in the usual solvents for this type of reaction. Because of the practical difficulties this approach was not pursued further.

Higher alkyl acrylates are readily synthesised from methyl acrylate by a process of ester exchange[142,143,144] in general acid catalysed, but also by basic catalysis if the alcohol used is sensitive to acid. Acrylates containing other functional groups are also readily available by this method[145]. The general reaction can be formulated thus:-

$$CH_2 = CHCO_2CH_3 + ROH \longrightarrow CH_2 = CHCO_2R + CH_3OH$$

Starting with stearyl alcohol and using the above method, stearyl acrylate was prepared. This was obtained as a soft white solid m.p. 35°C, literature value (36°C) and the boiling point 152°-157°C at 0.03 mm., literature value (145°-169°C at 0.1 mm.).

The infrared spectrum had carbonyl absorption at 1723 cm^{-1} indicating an α-β-unsaturated ester. Using the technique of suspension polymerisation[146], an attempt was made to copolymerise this monomer with acrylic acid. The polymer was obtained as a creamy mass which hardened to a solid mp 55°C. The material was soluble in benzene, ether, chloroform and insoluble in water, methanol and ethanol. An examination of the infrared spectrum, taken in nujol mull, indicated carbonyl absorption about 1740cm^{-1} characteristic of an ester group. No sign of carboxyl groups could be found. This was borne out by the polymer being insoluble in water, and failing to give effervescence with sodium bicarbonate solution. It was thus concluded that the polymer was polystearylacrylate. Various attempts were made to try to copolymerise these two monomers but no water insoluble polymeric material were obtained with significant carboxyl groups. It is quite possible that acrylic acid undergoes polymerisation to give a polyacrylic acid, and that the much more slowly polymerisable acrylate ester does not enter the growing poly acid chain.

It is still possible to get a compound of the required type by a partial hydrolysis of the above polystearyl acrylate. It is known[147] that acrylate esters can be readily hydrolysed employing basic conditions, to yield alkali salts of polyacrylic acid. Several small samples of polystearyl acrylate have been treated for varying times with potassium hydroxide, either in alcohol or in aqueous solution. It was found that the reaction

went quite quickly and gave a fully hydrolysed polymer, in addition to the long chain alcohol after long periods of reaction. It proved very difficult to characterise the products of partial hydolysis because of incomplete solubility in the solvents used.

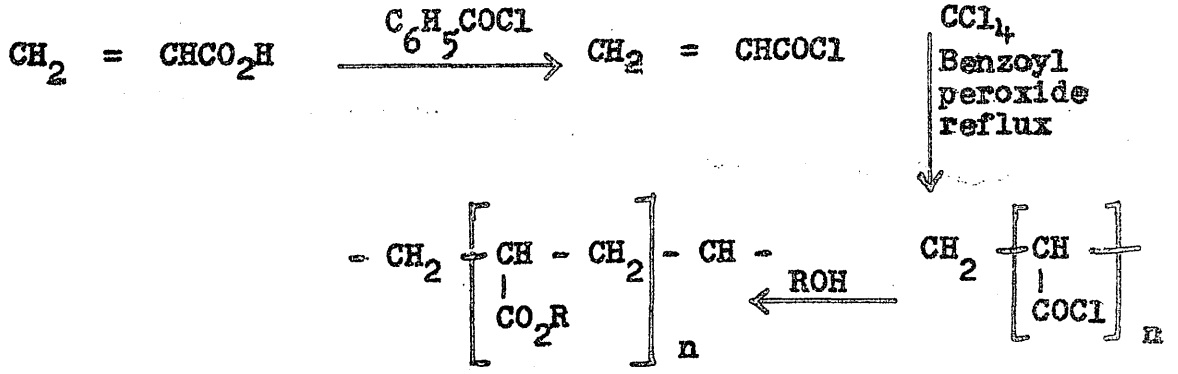
Saturated alkyl chains in polymers can be cross linked by high energy radiation[148], but something more practicable was desired, consequently it was decided to use a long chain alcohol which contained a reactive centre. The one used was oleyl alcohol which has a cis double bond in the chain. It was hoped that by treating the polymer in different solvent systems, with ultra violet radiation, slight cross linking would occur, via these double bonds and hence the three dimensional structure which the polymer had adopted in these various solvents, would be maintained.

Oleyl acrylate was prepared by the method used for stearyl acrylate. The compound was obtained as a fairly viscous colourless oil boiling point 142-140°C at 0.02 mm pressure (literature value 185-195°C at ½ mm.). The infrared spectrum had carbonyl absorption at 1723 cm^{-1} indicative of an α,β-unsaturated ester. This compound like stearyl acrylate was kept at -15°C till required for polymerisation. The polymerisation reactions on these compounds were carried out within one hour of preparation. Oleyl acrylate was polymerised both by suspension and by solution methods. In both cases the

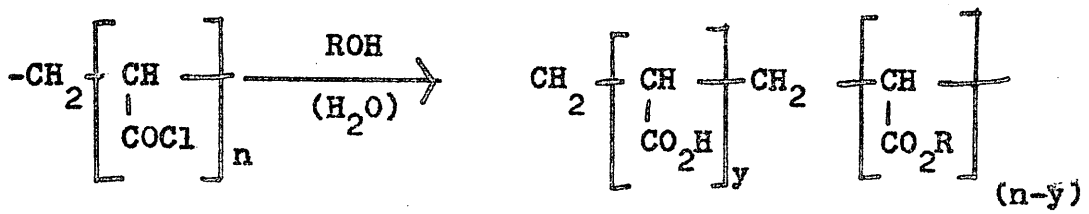
product was obtained as a soft sticky, off white mass, soluble in ether, chloroform and benzene but insoluble in methanol and ethanol. (On one occasion a polymerisation was inadvertently given too vigorous conditions. The polymer this time was soft and rubbery looking. On attempting to dissolve the material in chloroform and benzene, gels were formed. This indicated that during the polymerisation cross linking had formed with resulting insolubility).

It is also interesting to note that the polymer from oleyl acrylate is soft and rubbery whereas the corresponding polymer of stearyl acrylate is a hard easily powdered material. This would indicate that the shape of the actual long alkyl chains is important in determining the physical nature of the polymer, as in oils and fats and rubber.

Another method of preparing this compound was investigated. This involved preparation of the acid chloride of acrylic acid[149], polymerisation[150] of this and reaction of the resulting polyacid chloride with oleyl alcohol as formulated below:- (R = $C_{17}H_{35}$).



Initially, this method was considered as a direct route to the "polysoap" type compound required, by reaction of the poly-acid chloride with a limited quantity of the alcohol in moist conditions as shown below.



Because the acid chloride reacted more quickly with the alcohol function than with water, the polymer obtained was, as far as could be judged a polyester, with no carboxyl groups present.

The polymer, prepared by this method appeared to be essentially similar to that prepared by the other two methods.

Attempts were now made partially to hydrolyse polyoleyl acrylate. The reagent used was ethanolic potassium hydroxide using a quantity calculated to bring about 50% hydrolysis (about 10% KOH by weight of polymer). Typical conditions used were, to reflux a mixture of the polymer with the ethanolic KOH till the polymer had just gone into solution, about one hour. It should be mentioned again, because of the soap like nature of these partially hydrolysed products, they are extremely difficult to characterise and isolate in solid form, consequently they were examined in most cases in solution.

The method used to study the ability of this polymer to change its shape depending on its solvent environment, was to examine the turbidity of solutions of the polymer in various solvent systems, and try to relate changes in turbidity to changes in the shape and state of the macromolecular chains.

The apparatus used to measure the turbidity (or scattering) was the Spekker absorptiometer. This, provided much useful comparative data. There now follows a discussion of typical experiments.

When polyoleyl acrylate is partially hydrolysed there results a yellowish ethanolic solution. In the first case, this was filtered, concentrated, and the resulting syrupy product poured into distilled water to give a cloudy white, solution, concentration about 20 mg/ml. It proved extremely tedious to attempt to separate the other product of hydrolysis, oleyl alcohol, from the ethanolic solution containing the partially hydrolysed poly acrylate. This in fact was not achieved. Owing to the insolubility of oleyl alcohol in pure water, a certain amount of the observed turbidity in pure water, and in solutions containing more than 80% water must be due to this material. This has been shown not to affect measurements taken in solvent mixtures, containing less than this % water, oleyl alcohol being completely miscible in them all.

In experiments in which the starting point was pure ethanol, the reaction mixture was diluted with ethanol to known volume, (concentration of polymer about 10 mg/ml) and the various solvent mixtures were prepared using this clear solution. Two types of procedures were adopted.

(I) Starting with the polymer in water a study of the effect produced on the turbidity by proceeding, to high concentrations of ethanol.

Figure 32

Effect of adding various solvents to an aqueous solution of partly hydrolysed polyoleyl acrylate. Changes in turbidity measured.

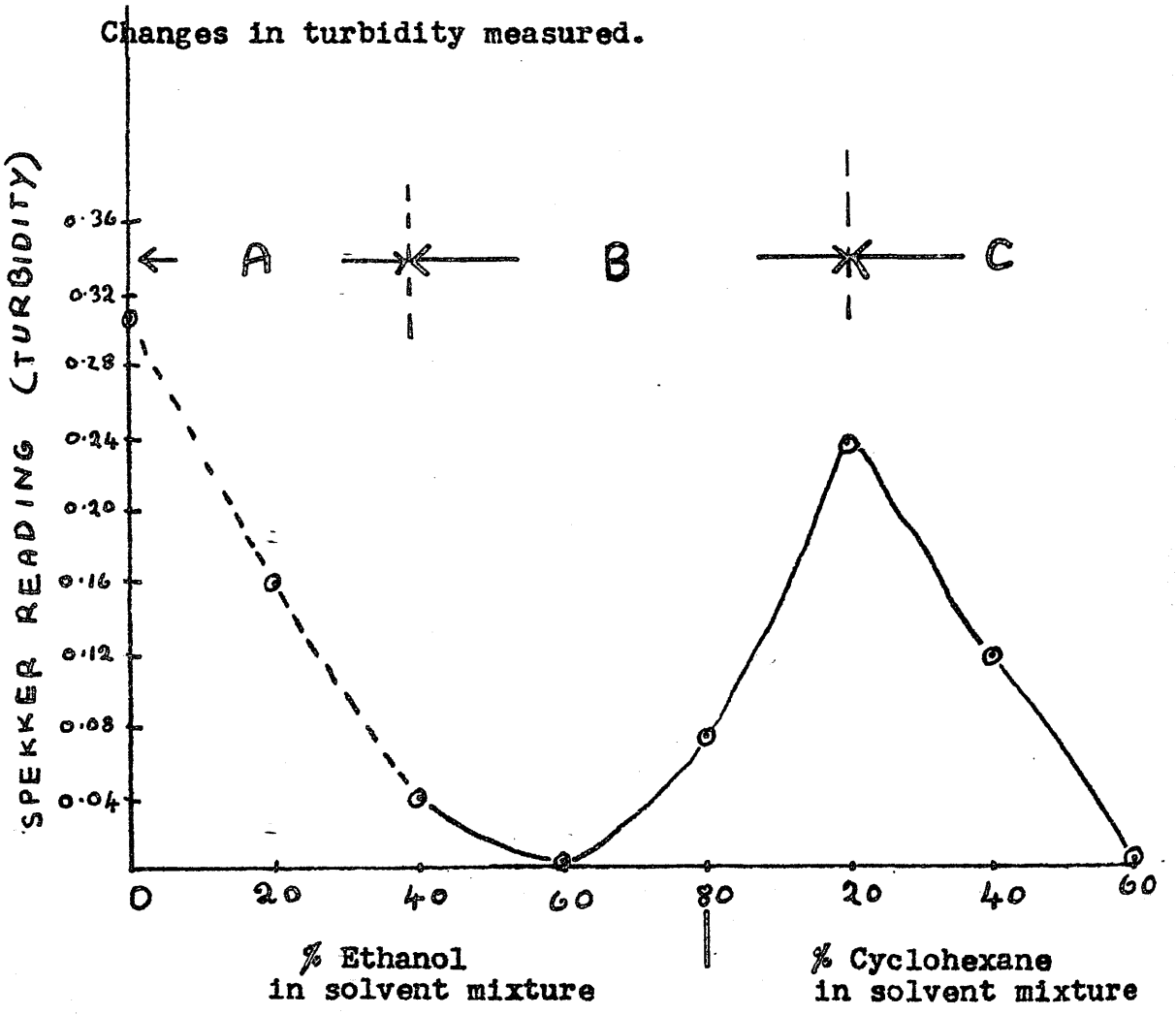


Table for Figure 32

Solvent Composition	% Ethanol	Spekker Reading	% Butanol
4ml H_2O	0	0.310	
3ml H_2O, 1ml EtOH	20	0.161	
2ml H_2O, 2ml EtOH	40	0.040	
1ml H_2O, 3ml EtOH	60	0.008	
4ml EtOH	80	0.075	
3ml EtOH, 1ml Butanol	60	0.237	20
2ml EtOH, 2ml Butanol	40	0.119	40
1ml EtOH, 3ml Butanol	20	0.000	60

1ml of the aqueous polymer solution was made up to 5ml with the solvent mixtures listed above in each case.

Figure 33

Effect of adding various solvents to an ethanolic solution of partly hydrolysed polyoleyl acrylate. Changes in turbidity measured.

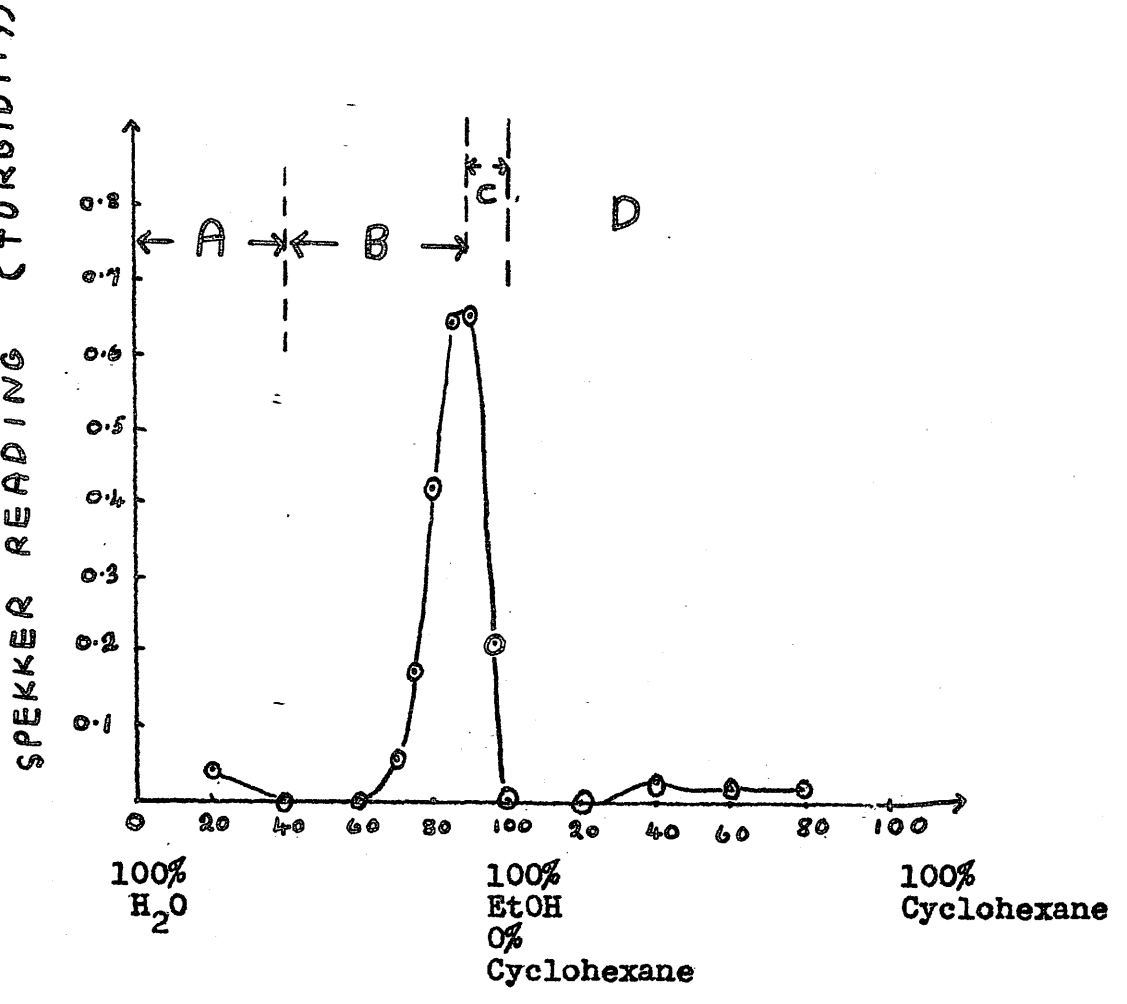


Table for Figure 33

Solvent Composition	$\%H_2O$	Spekker Reading	
4ml of EtOH	0	0.000	
3.5ml of EtOH, 0.5ml H_2O	10	0.652	
3.25ml EtOH, 0.75ml H_2O	15	0.644	
3ml EtOH, 1ml H_2O	20	0.420	
2.75ml EtOH, 1.25ml H_2O	25 25	0.174	
2.5ml EtOH, 1.5ml H_2O	30	0.057	
3.75ml EtOH, 0.25ml H_2O	5	0.210	
2ml EtOH, 2ml H_2O	40	0.000	
1ml EtOH, 3ml H_2O	60	0.000	
4ml H_2O,	80	0.042	
			% Cyclohexane
3ml EtOH, 1ml C_6H_{12}		0.000	20
2ml EtOH, 2ml C_6H_{12}		0.024	40
1ml EtOH, 3ml C_6H_{12}		0.015	60
4ml C_6H_{12}		0.015	80

1ml of the ethanolic polymer solution was made up to 5ml with the solvent mixtures listed above in each case.

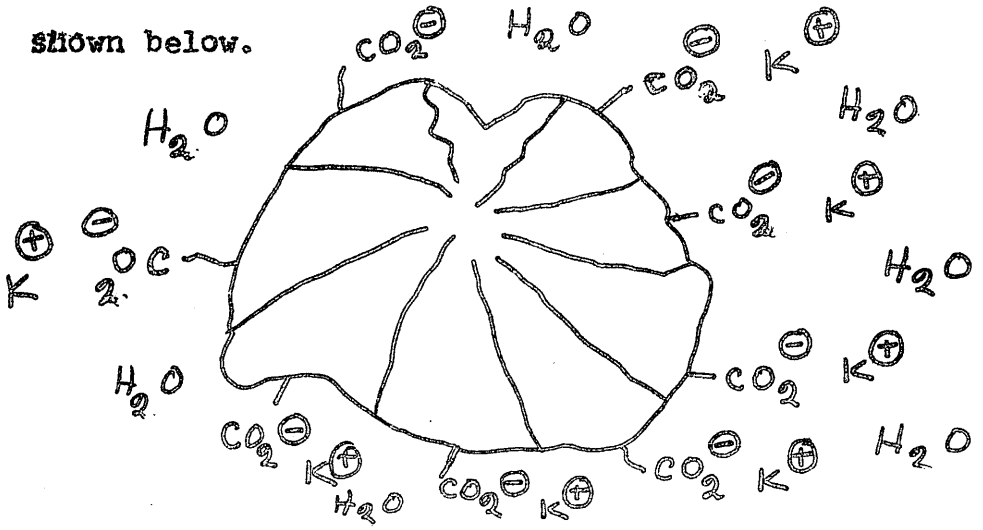
(2) Starting with the polymer dissolved in ethanol, (incidentally this would allow measurements to be made in very hydrophobic solvents, like cyclohexane or benzene) study the way the turbidity of the solution changes on going to high concentration of water, in one direction, and cyclohexane in the other.

Portions (1 ml) of the original polymer solutions (either in water, or in ethanol) were made up in various ethanol-water mixtures, to a total volume of 5 ml in each case. After a time, arbitrarily taken as fifteen minutes, the turbidity of the solution was estimated by taking a "Spekker" reading. These readings in each case were plotted against solvent composition as shown in Figs. (32) and (33). All the solutions were prepared in the same way (with respect to whether ethanol or water was added first). The tables accompanying each figure show how these solutions were prepared. Both these diagrams have been separated, in each case into three regions A, B and C, for ease of discussion. The comparisons which are being made are of a qualitative nature only, but are nevertheless very interesting.

Region A:-

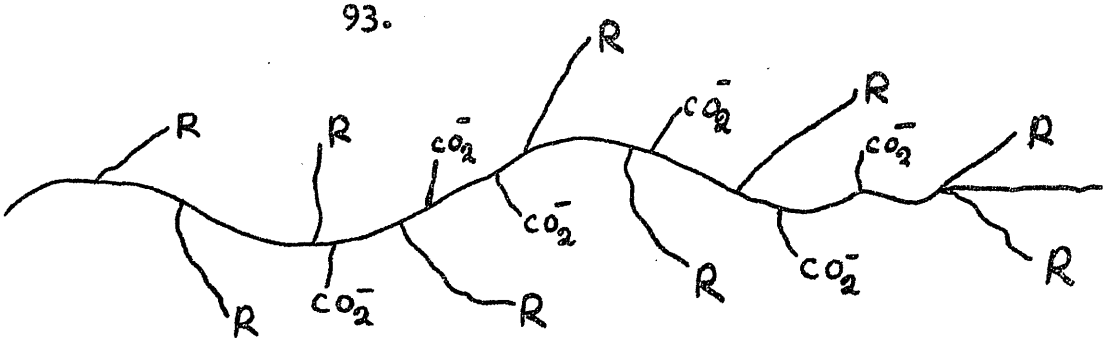
As noted, in both figures this ranges from a solvent composition of 40% ethanol, 60% water, where the solution is clear, or almost so, to 80% water, 20% ethanol where it is becoming quite turbid. It is proposed that this turbidity is produced by an aggregation of coiled polymer molecules into super associated particles of the general sort of structure

shown below.



i.e. the polymer in the poor solvent is so folded that the polar carboxylate anions will be directed towards the polar environment, the water, while the long hydrophobic side chains will be buried inside the structure, to be as far away from the water as possible. Turbidity like that found in ordinary soap solutions is due to large regions of the solution having different refractive indices, which could be the case with the structure illustrated above.

The region of the graphs where the turbidities are very small, round 50% ethanol, 50% water, solvent composition, can perhaps be understood by imagining the effect of adding ethanol on the structure already proposed for the polymer, in high concentrations of water. Ethanol being more hydrophobic than water, will when added, tend to solubilise the long hydrocarbon side chains. At a high enough concentration, scattering will be reduced because aggregations will be broken down into the following type of structure, which will have a sheath of mutual solvent both for the carboxylate anions and also the hydrocarbon chains.

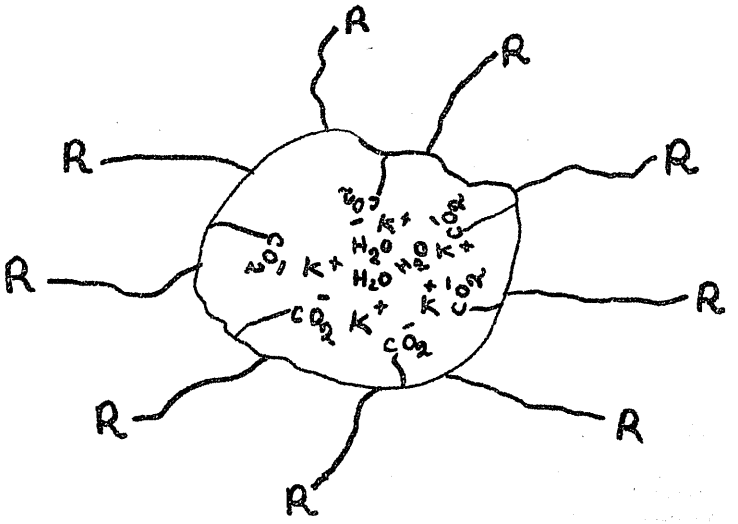


(R denotes the long alkyl chains).

Scattering may be reduced also, because in the above mentioned extended structure there are no longer large hydrocarbon regions clustered together, of different refractive index from the surroundings, as in the coiled or aggregated structure.

Region B:-

This extends from the region where the solutions are clear to where they are turbid again about 90% ethanol, 10% water in Fig. (33); 80% ethanol, 20% water in Fig. (32),(actually to 20% butanol, 60% ethanol, 20% water). This increased scattering would again suggest a change in shape of the polymer molecule. At the points of maximum turbidity a coiled and aggregated structure of the following type may occur.



The hydrophobic regions of the polymer molecules will tend to be directed outwards to the hydrophobic solvent environment. The polar carboxylate anions will be directed inwards. The whole structure tending to be stabilised by the water molecules within.

This idea of the water molecules stabilising the structure is suggested because as the solvent mixture becomes more anhydrous, (Region C in both cases), the turbidity drops again. It is suggested that this is due to the aggregates breaking down into small soluble entities, because of such a dehydration effect. In figure (33) this is extended on into region D.

An interesting point now to consider is the effect of temperature on turbidity. If, as was proposed, turbidity is caused by an aggregation or folding of particles, then one would think that the effect of heat may be to lessen the chances of aggregation and so the turbidity should be less. Measurements were done for several solvent compositions. The solutions were prepared and heated for a known time at $40^{o}C$. Turbidities taken immediately were smaller than those at room temperature. e.g. a solution which at room temperature gave a Spekker reading of 0.65 gave after heating for 15 mins, a reading of 0.45.

Thus, from the experiments discussed it would appear that this polymer system does exhibit an ability to change its shape depending on the nature of its solvent environment. Even though the particular explanation given is speculative

the observation of changes in scattering indicate same size and/or shape alteration in the molecule. The question now is whether it is possible to treat the system in some way so as to "freeze" the structures obtained in the various solvent systems.

As mentioned previously the approach to this problem was to treat the polymer solutions with ultra violet radiation which may initiate a few cross links, enough to stabilise the structure. The main idea was to treat the polymer in, (a) a coiled up state and (b) an extended state, by choosing the correct solvent system, and see if after this treatment the polymer is able to resist changes in the solvent composition, which would otherwise change its shape, and hence the turbidity of the solution.

Consequently, samples of the polymer were irradiated when in a solvent composition of 15% water, 85% ethanol, i.e. when believed to be in the highly coiled or aggregated state, and also when in 50% water, 50% ethanol i.e. the extended state. The source of radiation was a powerful mercury arc (2537 Å) and times of radiation varied from two to twenty four hours. After irradiation the turbidities in each case were measured and compared with a non irradiated blank of the same solvent composition. A comparison was also made of the way the turbidity of each of these solutions changed upon addition of small portions of water, with a blank in each case. As far as could be judged the behaviour of the irradiated samples was very like the blanks and thus it is possible to draw the following conclusions from these experiments.

(a) The treatment has been ineffective in causing cross linking.

(b) Cross linking has occurred, but it has not been effective in maintaining the structure in the original state.

(c) The technique used to study the changes in turbidity may not be sensitive enough to measure any effect that cross linking would produce.

Probably the isolated double bond in the chain was not sufficiently sensitive to ultra violet radiation and hence any tendency to cross link was slight. Although the polymer in solution did absorb slightly round 250 mμ the effect was not very great. Something had to be introduced into the chain which would absorb much more strongly at this wavelength.

Such a grouping would be a system of conjugated double bonds. The presence of these need not interfere with the polymerisation process. Oleyl alcohol has one double bond in the chain, linoleyl alcohol is the corresponding alcohol, with two (9-12) and (10 - 12) double bonds. It can be prepared by reduction of corn seed oil, methyl linoleate, using sodium in butanol [151]. It should also be possible to prepare it, probably as a mixture of isomers, (but this would not matter) starting with oleyl alcohol by a process involving bromination then dehydrobromination. Using this method the compound was obtained as a pale yellow oil, solidified about 10°C, which was not purified further but immediately reacted with a sample of polyacrylyl chloride which had just been prepared. The

Figure 34

Effect of U.V. radiation on change in turbidity of an ethanolic solution of polylinoleyl acrylate, upon adding various amounts of water.

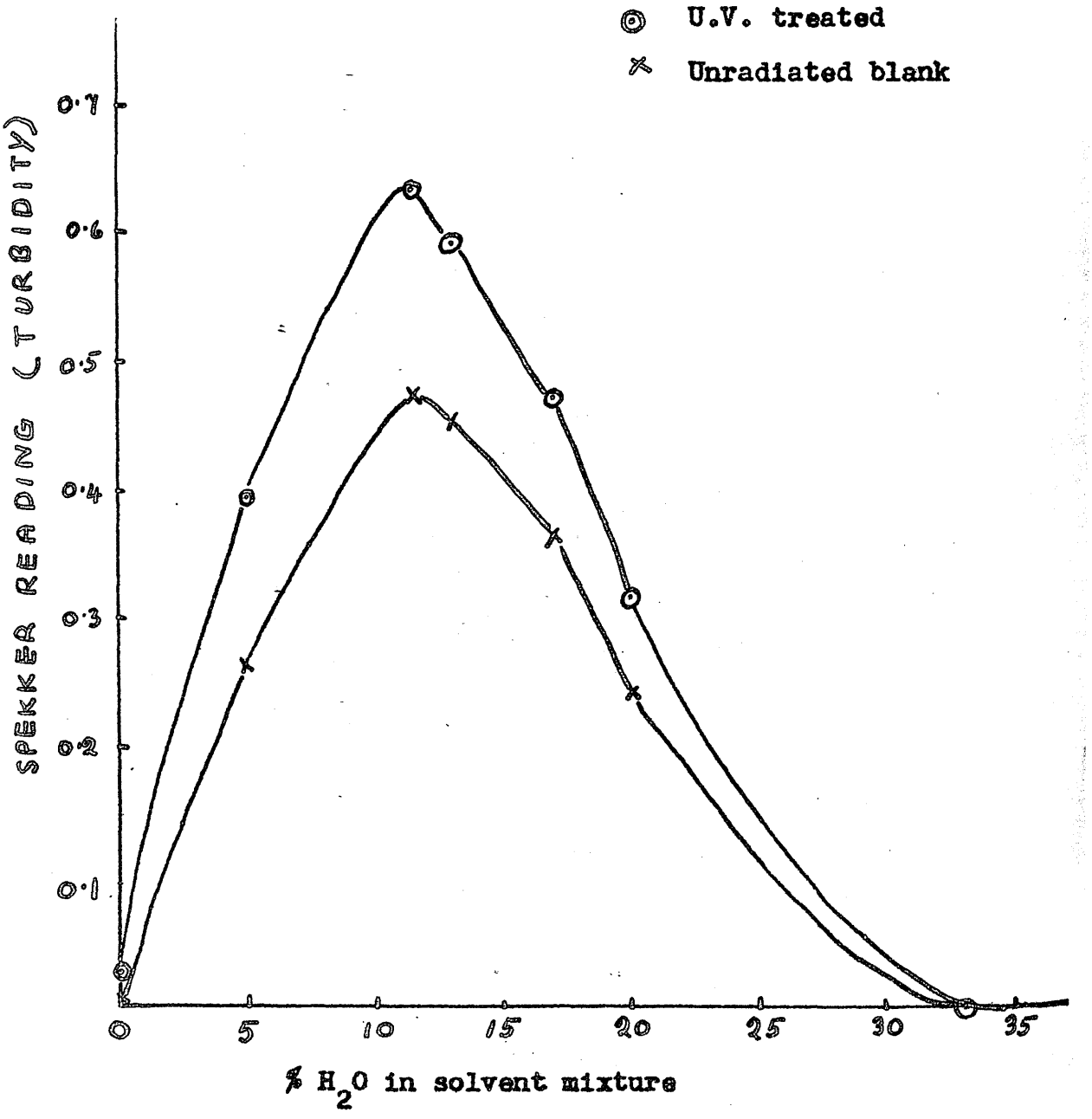
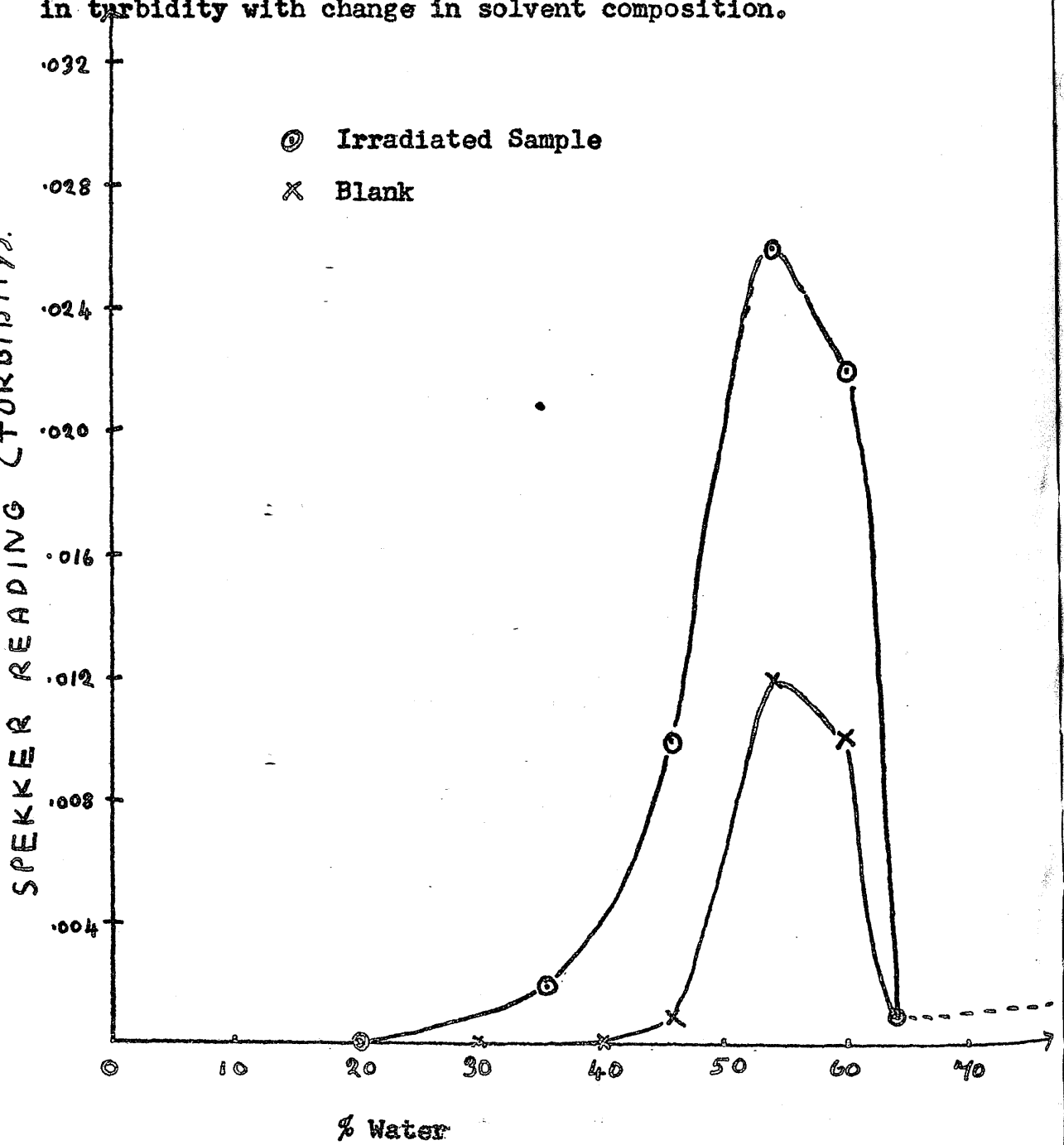


Figure 35

Radiation of partly hydrolysed polylinoleyl acrylate at a solvent composition of 20% H_2O, 80%EtOH. Followed change in turbidity with change in solvent composition.



(Cannot go beyond 70% water because of complete coagulation of polymer).

resulting polylinoleyl acrylate was obtained as a soft, sticky yellowish mass very similar to the product obtained from oleyl alcohol. The infrared spectrum showed carbonyl absorption at 1740cm^{-1} characteristic of an ester. The ultra violet spectrum taken in cyclohexane showed strong absorption in the required area especially at 235 mμ, 260 mμ, 270 mμ and 281 mμ. ϵ values of these λ_{max} were not obtained. By exactly similar methods as applied to polyoleyl acrylate, this polymer was partially hydrolysed and attempts made to cross link it in different solvent environments.

The polymer was treated with ultra violet radiation in ethanol solution and in a mixture of ethanol and water of known composition. The radiation source was the mercury arc used previously and the time given was about 24 hours. It was noted that whereas the solutions before treatment were pale yellow, after radiation they were colourless. The ability of the polymer to resist changes induced by altering the solvent composition was compared in each case with the non irradiated samples. This time as Figs. (34, 35) show the ultra violet radiation did produce some effect, namely to cause the treated sample to be slightly more turbid with each addition of water, than the blank. If the solvent composition is changed too much, the effect ceases and the turbidity follows the blank sample.

A possible explanation for this effect, using earlier arguments is that the ultra violet radiation does produce a slight cross linking effect, with consequent aggregation or folding, of

particles and increase in turbidity. The effect produced by this treatment is slight and not nearly so dramatic as was hoped. Consequently it was decided not to further pursue investigations into the system.

Serum Albumin Model:-

Remembering the almost unique ability of serum albumin to interact with small molecules, the approach chosen was an attempt (like the one described previously using modified polyvinyl alcohols) to make an anionic macromolecule which will interact with anions. The ability of any water soluble material to bind anions would be studied using spot line chromatography, as used in the protein studies.

It was decided that a suitable method might be to use the reactive polyacrylyl chloride prepared earlier. The problem would be to devise some means to react this, with a material like a diamine, so that the product would not be so cross linked as to be hopelessly insoluble. The idea of adding a dilute solution of the poly acid chloride, very slowly, to a large excess of a solution of the diamine was tried, unsuccessfully, the white resulting polymer being as far as could be judged, hopelessly insoluble in all the usual solvents. Several more attempts were tried all of which proved unsuccessfull.

Polyacrylyl chloride is soluble only in anhydrous dioxane, or phosphorus oxychloride, and the next idea was to do the reaction in the presence of a large excess of a neutral inert

Figure 36

Spot line chromatography using samples from reactions of polyacrylyl chloride (P.A.C.) with trimethylene, and hexamethylene diamine (3 D.M. and 6 D.M.) in the presence of P.V.P. Run done at pH 6.8.

(P.A.C. 3 D.M. P.V.P.) ≒ 1 mg.

(P.A.C. P.V.P. 6 D.M.) ≒ 1 mg.

(P.A.C. P.V.P. 6 D.M.) ≒ 750 µg.

macromolecule, e.g. polymethyl methylacrylate, - the increased viscosity may have the effect of slowing down the cross linking reaction and allowing soluble material to be obtained. Polyacrylyl chloride was thus reacted with trimethylene diamine, but again hopeless cross linking occurred.

When this suggestion failed, it was thought that the idea would have to abandoned. However as a last resort, it was decided to do the reaction in an aqueous medium, in the presence of excess water soluble indifferent macromolecule to reduce the diffusion of the polyacrylyl chloride, and so reduce the cross linking reaction. The one chosen was polyvinyl pyrrolidone. Because of the reactivity of the amino group, (cf. with hydroxyl groups) not many of the acid chloride groups, will be hydrolysed, on the other hand, the presence of the water may reduce cross linking by competing with the diamine.

Polyacrylyl chloride was reacted in this way with several diamines. The solutions obtained were extensively dialysed then concentrated. The ability of the products to interact with anionic dye was then checked by doing a spot line chromatography run, at neutral pH and seeing if anything would migrate to the anode, i.e. behave as an anion and yet produce a loop in the dye line. As Fig. (36) shows this has been achieved. The amounts of polymer that were required to produce these loops were much greater than the amount of serum albumin needed to produce loops of comparable areas.

Another interesting point is, that if this colourless solution was left standing for a time (24 hours) it went cloudy and completely lost the ability to produce a loop in the dye - resembles protein decay. This happened in all the cases studied, i.e. the products from tri-tetra- and hexa-methylene-diamine.

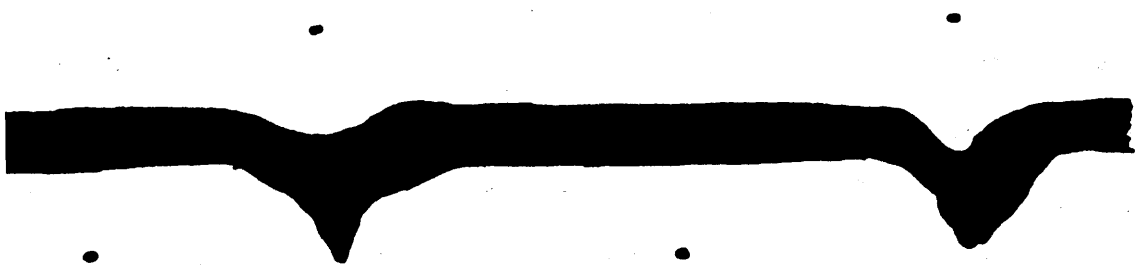
It would also be useful to know the mechanism of binding and the nature of the binding sites. As mentioned, the intention was that the polyvinyl pyrrolidone should be present merely to increase the viscosity of the solution, it did not seem likely that it would be involved in the reaction. However, it was found that if the reaction was carried out in the absence of polyvinyl pyrrolidone, other things being equal, no evidence could be found for anionic binding material being produced. One must therefore conclude that polyvinyl pyrrolidone is somehow involved in the structure of the anion binding model.

It is known[152] that polyvinyl pyrrolidone itself interacts specifically with small molecules. The activity is about one-third of that of the natural system. It was at first thought therefore, that the anion binding ability of the product mentioned above was due purely and simply to the polyvinyl pyrrolidone. This was shown to be incorrect, in the following way.

The usual procedure for doing spot line chromatography was followed. A solution of polyvinyl pyrrolidone in buffer was

Figure 37

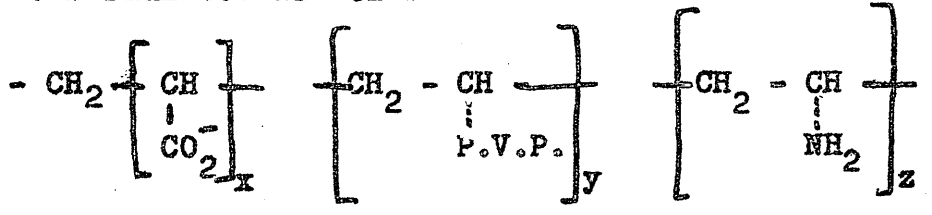
Spot line chromatography using polyvinylpyrrolidone. (P.V.P.) P.V.P. migrates to cathode and binds dye. No evidence for anything migrating to anode and interacting with the dye line. Run done at pH 6.8.



All four spots 500 µg. of P.V.P.

used in place of protein. Spots of the polymer solution were then placed, close to the dye line, and on either side of it. The experiment was then carried out in the usual way. It was found, (as was expected), that the polymer moved by electroomosis towards the cathode. The spot, which encountered the line produced a loop therein, indicating binding ability. Most important however, was the fact that there was no evidence of any material moving towards the anode, i.e. behaving as an anion. Certainly, the spot which would have to cross the dye line gave no sign whatever of loop formation. Fig. (37) shows these results.

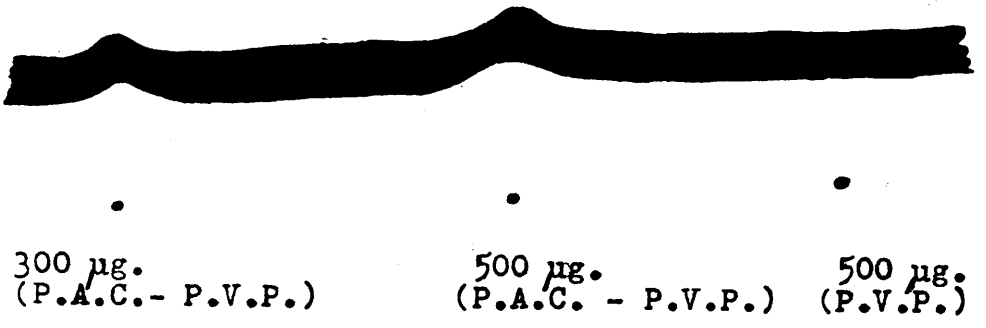
The following explanation is proposed for the observed binding ability. It would appear that an anionic graft copolymer has been formed, with the polyvinyl pyrrolidone chemically attached in some way. The product would probably be formulated as below:-



The question as to the nature of the binding sites can not yet be answered fully. There are two possibilities:-
(1) The dye is bound at sites on the pendant polyvinyl pyrrolidone chains, very probably the same sites that gives rise to the known affinity of this polymer for small molecules.
(2) The binding is at charged amino group centres. If any of these have been introduced into the system.

Figure 38

Spot line chromatography using samples from the reaction of polyacrylyl chloride (P.A.C.) with polyvinyl pyrrolidone (P.V.P.). Run done at pH 6.8.



An attempt was made to treat polyvinyl pyrrolidone in aqueous solution with polyacrylyl chloride to see if any anion binding material would be produced. (This would have cleared up the confusion about the nature of the binding sites). However after dialysis, concentration and examination of the reaction mixture by spot line chromatography little evidence for binding could be found. (Fig. (38) shows the results that were obtained.*

Not only does this system mimic serum albumin in its specific interaction with anions, but it also resembles the natural system in that it loses this binding ability. A possible explanation of this very interesting fact is as follows:-

In the discussion on the binding ability of serum albumin the conclusion was reached that the unique ability to interact with small anions was due primarily to the positive and negative groups being held rigidly apart.

Because the synthetic system is not rigid it will be able to assume a variety of different conformations. In particular, the one which possesses binding ability (if this is due to the charge separation) may not be particulary stable.

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* It might be that without the diamine, the "charge separated structure" is even more unstable and rearrangement occurs before the binding ability could be examined.

Therefore on ageing the chains will wriggle and interact, in order to reduce this charge separation, and thereby increase the stability of the system. The net effect of this, would be that the polymer would tend to aggregate, and the binding sites would be removed. This idea is substantiated by the observation that the polymer solution gradually becomes cloudy - when it is cloudy no dye binding can be observed.

APPENDIX.

Part 1. Further Details of Experimental Procedures.

Methods used in Protein Studies

As mentioned in the first part of the Discussion, the technique which was most often used in this research, to study protein - dye interactions was Spot Line Chromatography. The actual procedure used was Method (3) given in the original paper[14]. Briefly, this is as follows. The apparatus for paper electrophoresis, shown diagramatically on page 13 was set up. The dye was applied by cutting 0.5 cm. wide strips from the filter paper, soaking these in the dye solution in buffer, of known concentration (in the case of biebrich scarlet it was about 0.0025%), for a known time (usually about one minute). The strips were then placed in position on the glass plate together with the rest of the paper soaked in pure buffer. By applying the dye line in such a manner, closely comparable dye lines could be obtained in different runs. A thin (0.005") polythene sheet was placed over the paper, and all air bubbles were carefully pressed out. The level of buffer in each of the troughs was made the same, by using a simple siphon device. The system was left for at least 30 minutes to equilibrate. Pin holes were then made in the polythene sheet known distances behind the dye line. Small measured volumes of the protein solution were placed on the pin holes using an Agla micrometer syringe. Most of the protein then soaked into the paper. Any protein remaining on the surface of the polythene was washed in with a small volume of buffer solution. A potential

gradient of between 5 and 10 volts per cm., was then applied until the protein spots had overtaken the dye completely. This time varied from about 6 hours, for a single line run, to 24 hours for a large two or three line runs.

In all the runs described in this research, unless otherwise mentioned, the buffer solution used was a phosphate one. (pH 6.8: 0.055 M Na_2HPO_4 - 0.044 M KH_2PO_4). The salts used were AnalaR grade, and the solutions were made up in distilled water. Other buffers used included a borate buffer (pH 8.5: 0.022 M H_3BO_3 - 0.0088 M NaOH), diethyl barbiturate (pH 8.6: 0.05 M sodium diethylbarbiturate - 0.01 M diethylbarbituric acid). A chloride buffer pH 2.0 (0.01 M HCL, 0.05 M KCl) and an acetate buffer pH 4.95 (0.06M HAc.-0.14MNaAc.) [53] were also used. The paper used for the electrophoresis, unless otherwise stated, was Whatman No. 1 chromatography paper. Crystalline bovine serum albumin, and Fraction V albumin were obtained from The Armour Laboratories. Samples of porcine, human and equine albumins (Fraction V powder) were obtained from Lights Ltd. Protein solutions were prepared as required by dissolving a known quantity, usually about 50 mg., in the relevent buffer and making up the solution to 1ml. These solutions were stored between 0° - 5°C. They were discarded if not used after one week.

The areas of loops formed in the spot line chromatography runs were estimated by measuring the area of a closely fitting triangle in each case.

Interaction of the Dye Safranine with D.N.A.

In this work, spot line chromatography was used in essentially the same way as for the serum albumin - biebrich scarlet system; however provision was made for the different charges on the molecules. A 5% solution of D.N.A. or R.N.A. was made up in phosphate buffer pH 6.8. The nucleic acid had a negative charge at this pH and consequently, during electrophoresis, migrated to the anode. The cationic dye, safranine was applied to the paper in the normal manner. Spots containing known weights of nucleic acid (10 µg. - 500 µg.) were placed known distances behind the dye line, and electrophoresis carried out. It was found that the runs took much less time than the protein ones - they were usually over within three hours. By running equal weights of D.N.A. through two dye lines, from different distances, in the usual way, the important conclusion was noted, that "decay" did not appear to operate in this system. Runs were also done using other cationic dyes, e.g. Gentian Violet, Methylene Blue and Brilliant Cresyl Blue. All showed the same effects, but were inclined to diffuse on the paper rather more than did safranine.

Use of Equilibrium Dialysis[161]-

This technique was used to calculate the number of binding sites for biebrich scarlet on bovine serum albumin. The procedure adopted was as follows. Firstly, using the Spekker absorptiometer the optical densities of a range of concentrations of biebrich scarlet solutions in phosphate buffer were noted. A graph of optical density against dye concentration was drawn.

From this graph the concentration of unknown solutions of biebrich scarlet could be readily determined. The dialysis were carried out in "18/32" visking sausage tube, in a bath maintained at 20°C. Runs were done until two coinciding optical densities for the outer dye solution were obtained, thus indicating that equlilibrium had been reached. Using the weight of protein, the initial and final dye concentrations, in each case, the number of binding sites on bovine serum albumin was calculated.

Effect of Alkali and Heat on the Dye Binding Ability of Serum Albumin.

In these experiments the procedure adopted was as follows. Three solutions, each containing 50 mg/ml of crystalline bovine serum albumin were prepared.

(a) Protein dissolved in phosphate buffer, pH 6.8.

(b) Protein dissolved in 0.01 M NaOH solution.

(c) Protein dissolved in 0.1 M NaOH solution.

The three lightly stoppered tubes containing these solutions were placed in a water bath maintained at 40°C by a thermostat; they were left for 17 hours. A double-line, spot line chromatography run was then done using 300 µg samples of these solutions, along with a 300 µg sample of untreated protein. The run was done in phosphate buffer at pH 6.8. The results were noted. The three solutions were than returned to the water bath at 40°C, and left for a further 24 hours. Samples were again removed for another double-line run, carried out

in the usual way. The solution of bovine serum albumin in 0.01 M NaOH was then heated in the water bath for a further 48 hours at 55°C. Samples were removed and the binding ability of the treated protein examined as before. The results were again noted.

An important point, which was checked before any of the above mentioned runs were carried out, was that the small amounts of alkali, introduced on to the paper along with the protein, did not produce any anomalous results. It was concluded from the fact that small volumes of dilute NaOH alone did not produce any effect in the dye lines, that the buffer neutralised the small amount of NaOH put on with the protein, quite effectively.

Investigation of Binding Ability of Serum Albumin at low pH:-

The buffer used for the spot line chromatography runs in this work was a KCl/HCl one, (0.01 M HCl-0.05 M KCl) of pH 2.0. The procedure used was normal, except that the pin holes were placed so that the protein, which was acidic and hence migrated to the cathode (not the anode as before), would still encounter the dye lines. The protein moved rapidly at this pH, runs being complete in about three hours. It was noted that much smaller quantities of protein, than used at pH 6.8, would still give successful loops, at this pH.

Figure 39.

Spot line chromatography at isoelectric point of serum albumin. Dye line moves through the protein spots from right to left.

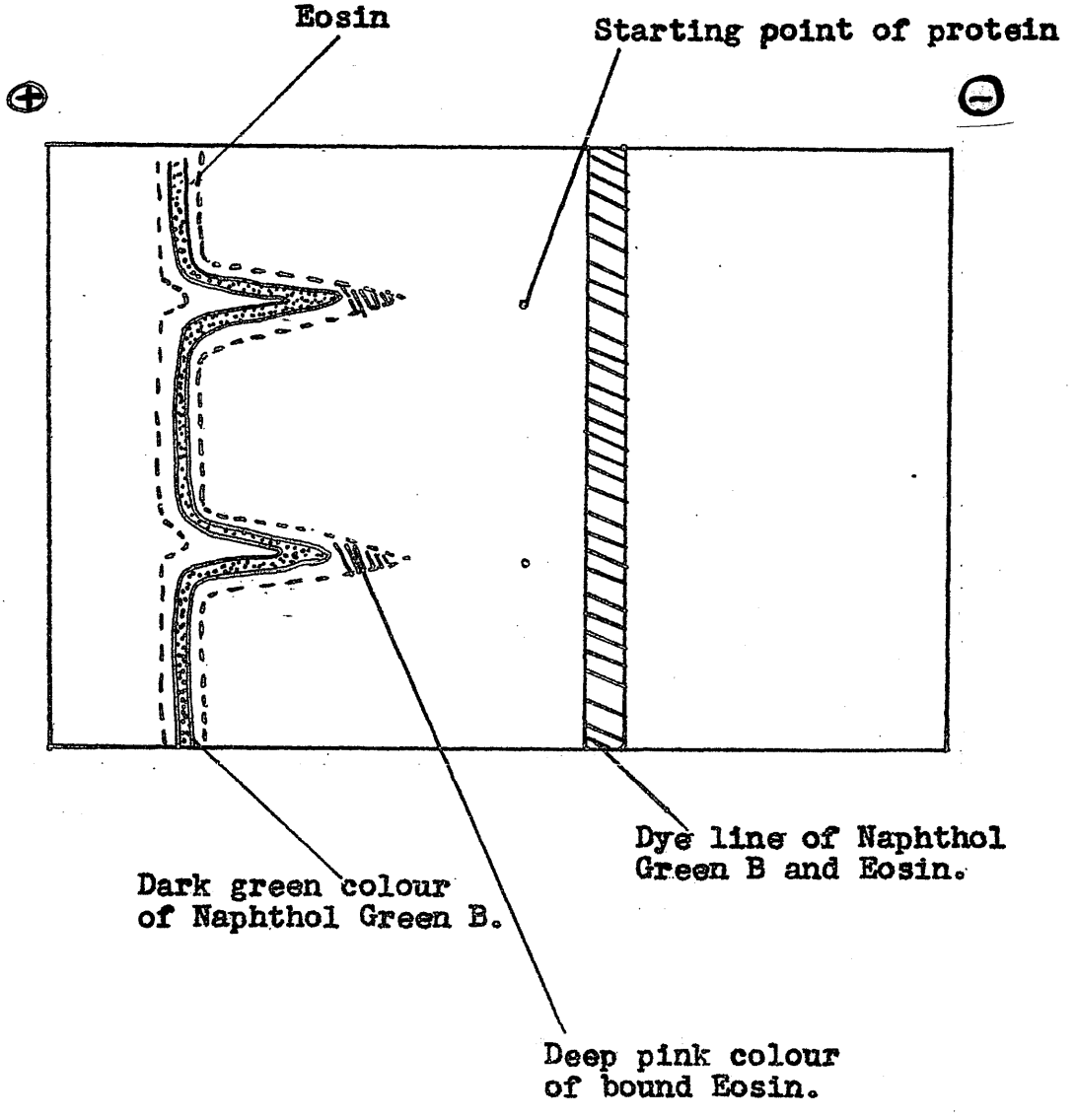
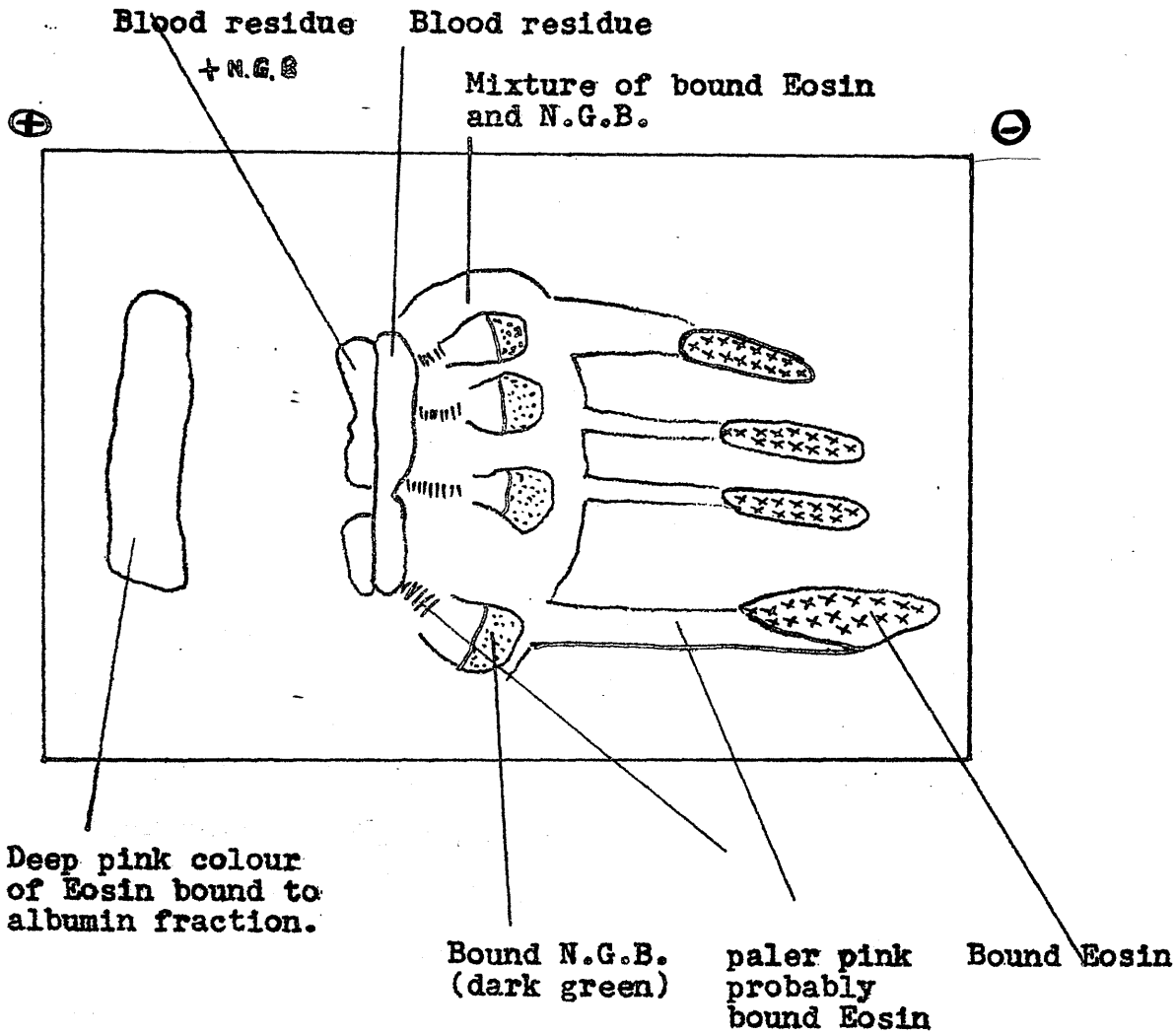


Figure 40.

Spot line chromatography at pH 4.95 using a line of whole blood and the filter paper soaked in a solution of the dyes Eosin and Naphthol Green B. The dyes appear to interact with various protein constituents and produces a visual separation.



Spot Line Chromatography at the Isoelectric Point of Serum Albumin.

The buffer solution used was an acetate one pH 4.95. (0.06M HAc.-0.14MNaAc.) Spots of serum albumin were put on the paper, and lines of various dyes moved through them. In this way, runs were done using lines of naphthol green B, and Eosin*, (which were both known to move rapidly by electrophoresis) and very clear, inverse loops were formed. Fig. (39) shows diagramatically typical results.

A run was done in which the whole paper was soaked in a mixture of the above mentioned dyes. A thin slit was carefully made in the polythene sheet and a line of freshly drawn blood put on the paper, and electrophoresis carried out for a few hours. Fig. (40) shows the curious effect that was obtained.

The general effect is that a rapid separation of the various protein components appears to occur and these show up by an alteration in the appearance of the dye. This experiment was repeated with different types of dye, both anionic and cationic. The same general effect of visual separation of components was obtained in each case.

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* Eosin is also useful in this work because when it is bound as an anion there is a very pronounced change in colour. (becomes deep pink).

Effect of Chemical Modification on Binding Ability of Serum Albumin:-

All the chemical reactions carried out on serum albumin were done by the respective methods quoted in the literature, to give selective reactions and hence need not be described here. The following procedure was, however, used. After dissolving the crystalline protein at the recommended pH and temperature, the reaction was carried out. Instead of then isolating the modified protein in crystalline form, what was usually done was to dilute the reaction mixture to a known volume, with buffer solution, or distilled water. In this way solutions of known protein concentration were readily available. In spot line chromatography runs on these products a known weight of protein could than be placed on the paper using the micrometer syringe. In each case it was checked that excess of reagent, (or in some cases the acidic or basic solution in which the reaction was carried out) did not produce any anomalous effects in the dye line, which might have been mistakingly attributed to the modified serum albumin.

In several instances e.g. deaminated serum albumin, serum albumin treated with 2,4-dinitrofluorobenzene, and p,p'-difluoro-m,m'-dinitrodiphenylsulphone, the product was brightly coloured and its progress across the paper during electrophoresis, could be followed clearly. Also, in the latter two cases, small molecular weight by-products could be seen moving off ahead of the main protein spot and not causing any effect in the dye lines.

In the reaction for the conversion of ε amino groups to guanidinium groups, the protein was treated with o-methyl-iso-urea prepared from the corresponding hydrochloride, not the acid sulphate as quoted[47]. The preparation of the hydrochloride from cyanamide by treatment with HCl gas in methanol[154] seemed simpler and more convenient. Other conditions used in this reaction were as given.

Effect of Concentrated Urea and Heat on Binding Ability of Serum Albumin:-

A typical treatment was carried out as follows. 100 mg. of crystalline bovine serum albumin were dissolved in phosphate buffer, pH 6.8. About 800 mg. of urea was added in portions to this solution till an almost saturated solution was obtained. The volume was then made up to 2ml. by addition of a little phosphate buffer. The urea concentration in this solution was thus about 7M. Samples of the bulk protein solution were heated in a water bath for 10 minutes at noted temperatures (ranged from 50°C - 90°C). After cooling to room temperature equal volumes of the protein solution were placed on the paper and a "two-line" spot line chromatography run carried out.

In examining the effect of concentrated urea alone, the protein was made up in 6-7M urea in phosphate buffer, pH 6.8, and allowed to stand for up to two days. Samples were withdrawn and spot line chromatography runs done. Dialysis was done by transferring the urea protein solution to a visking sausage tube and leaving it in running water for periods up to

72 hours. Samples were removed and spot line chromatography runs done.

Polymer Studies

Search for a "Memory Effect" in Films of Polyvinyl Alcohol.

Polyvinyl alcohol solutions (2% in distilled water) were prepared. A small amount of the dye brilliant green (25mg/1gm. of polymer) was then added. The solution was cast on a large sheet of clean dry glass. The resulting thin film was cut up and kept in a dessicator till required. Several experiments were carried out, the one described is typical. Firstly a calibration curve for the dye was obtained by measuring the optical density of a range of concentrations, with the "Spekker".

A piece of dry, dyed, polyvinyl alcohol film was quickly weighed and put into 10 ml of anhydrous methanol at 40°C. It was left for 15 minutes to extract all the dye. A Spekker reading was taken of the methanol solution; this was noted. The film was carefully dried and treated with a concentrated solution of brilliant green in methanol for 15 minutes. The film was removed, surface dye washed off with ethanol, dried and then treated with anhydrous methanol as before. The Spekker reading obtained was less than before (0.087 as against 0.095). This process of dyeing and extracting the dye was repeated several times. The Spekker reading of the solution soon dropped to a constant value, (0.066).

The polymer film was treated with formaldehyde as follows:- a piece of 2% polyvinyl alcohol film was placed in methanol containing 10% aqueous formaldehyde for 2-3 hours at 45°C. After this procedure the film was slightly opaque and felt

rubbery. The procedure mentioned above, for the dyeing, and extraction of the film was then carried out. No increased effect was noted.

Extraction of Brilliant Green from Polyvinyl Alcohol using different Solvent Compositions:-

For these experiments anhydrous AnalaR methanol and ethanol were used throughout. The following is typical of the general procedure used.

Into each of ten clean, dry test tubes, was placed a known weight of dry, dyed polyvinyl alcohol film, (about 2% as before). Along with 10 ml. of a mixture of methanol and ethanol of known proportions. The stoppers were then placed in the tubes, which were then put in a shaker, in a water bath, maintained at a known temperature, for one hour. Each sample was then withdrawn, shaken by hand for 5 seconds, had one drop of glacial acetic acid added to it (to prevent fading of the colour), and the optical density noted using the Spekker. From the calibration curve already prepared, the amount of dye extracted by the solvent was then calculated. Plots of the percentage extraction against percentage methanol in the solvent mixture were then drawn for each set of results.

Chemical Modifications of Polyvinyl Alcohol:-

(a) Introduction of Basic residues.

This was carried out in the following way.

A solution of polyvinyl alcohol (2g.) in water (30ml) was prepared. Concentrated HCl (2ml) was added and the mixture

warmed to 70°C on a steam bath. Aminoacetal (1g.) was added slowly, stirring gently all the time. After a few minutes the solution became dark red. The mixture was stirred for about 6 hours at 70°C. It was then cooled and poured into Visking sausage tubing and dialysed against running water for two days. The solution was then concentrated, and the dye binding ability examined by spot line chromatography. A similar reaction has been carried out with p-dimethylamino-benzaldehyde. Both these products produced as expected, small loops in a line of biebrich scarlet, as they migrated to the cathode, in a spot line chromatography run.

(b) Introduction of Carboxyl Groups.

Polyvinyl alcohol (5g.) succinic anhydride (10g.) and pyridine (25ml.) were stirred under reflux for about 3 hours, till all material had gone into solution. The resulting yellowish, viscous solution was cooled, and poured into visking sausage tubing, and dialysed against running water for 36 hours. The solution was then concentrated to 25 ml. The modified polymer was obtained (by pouring this solution into 100 ml. of 6N HCl) as a yellow brown "toffee like" mass (6g.)

Attempts were made to combine (a) and (b) type compounds by treating mixtures of their solutions, under different conditions, with a cross linking agent. When judged by spot line chromatography these attempts appeared to be unsuccessful.

Preparation of Long Chain Polyacrylates:-

As the reaction conditions used for stearyl acrylate, oleyl acrylate, and linoleyl acrylate were very similar, only one experiment for each method of polymerisation need be described.

(a) Suspension Polymerisation:-

The appropriate acrylate ester (10g.) with α,α'-azodiisobutyronitrile (0.12g.), polyvinyl alcohol (2ml. of a 5% solution), $Na_2HPO_4.12H_2O$ (1g.), and water (20ml.) were placed in a three-necked flask fitted with an efficient stirrer, reflux condenser, thermometer, and a nitrogen inlet. The contents were heated to 90°C under nitrogen for 4-6 hours. After this time, heating and stirring were stopped. The soft solid obtained, was removed from the aqueous layer, gently dried and dissolved in a suitable solvent e.g. ether, benzene or chloroform. The polymer was purified, by slowly adding it, in solution to a large excess (5 times) of cold methanol, whereupon precipitation occurred. This was repeated three times. The weight of polymer obtained by this method was around 6g. in each case. With the exception of polystearyl acrylate, which could be ground to a fine powder, all the polymers obtained were off-white, soft, sticky solids.

(b) Solution Polymerisation[155].

(1) Oleyl acrylate (5g.), was refluxed in ethyl acetate solution (50ml.), with benzoyl peroxide (100 mg.) as initiator, for 3-4 hours. The bulk of the solvent was then removed under reduced pressure. The resulting concentrated solution was

poured slowly into a large excess of rapidly stirred, cold methanol. This precipitated the polymer as a soft white mass. This process was repeated to effect further purification. Weight of polymer obtained was about 3g.

(11) Acrylyl chloride (12g.) (prepared by reaction of acrylic acid with benzoyl chloride) was refluxed in anhydrous carbon tetrachloride (120ml.), with benzoyl peroxide (100 mg.) as initiator for 15-36 hours. The carbon tetrachloride, plus remaining monomer was then carefully distilled off. There was a residue left adhering to the glass of the flask. This was the very unstable polyacrylyl chloride (about 5g.). The polymer was dissolved as quickly as possible in dry dioxane (25ml.). Excess oleyl alcohol (15gm.) in dioxane (10ml.) was added to the polyacrylyl chloride. The mixture was then warmed gently till the production of HCl had ceased, about 1 hour. The yellow dioxane solution was concentrated then poured slowly into cold stirred methanol, as above, to precipitate the polyoleyl acrylate.

Serum Albumin Models:-

(a) Reaction of polyacrylyl chloride with trimethylene diamine.

Acrylyl chloride (12g.) was polymerised as described above to give 6gm. of a pale yellow solid which was dissolved in anhydrous dioxane. A large excess of trimethylene diamine was dissolved in dioxane and rapidly stirred. The solution of polyacrylyl chloride in dioxane was then added dropwise over a period of 2-3 hours. On addition of each drop, white material was precipitated. This material was filtered, washed with ethanol and water, then dried in an air oven at 50°C. 5.5gm. of polymer were obtained. (I.R. spectrum taken in nujol mull showed a broad band round 1670 $cm.^{-1}$, indicative of -CONH-). The polymer was extremely insoluble in all solvents tried and could certainly not be studied by spot line chromatography.

This reaction was repeated using an excess of polymethyl-methacrylate dissolved in the diamine solution. However again only cross linked insoluble polymer was obtained.

(b) Reaction of polyacrylyl chloride with diamines in water in presence of polyvinylpyrrolidone.

A solution of polyacrylyl chloride (about 5g.) in anhydrous dioxane (50ml.) was prepared as described above. This solution was divided into two portions (A) and (B). A solution of the diamine (large excess) in water (100ml.) was prepared. Polyvinylpyrrolidone (3g.) was also dissolved in this solution. The solution was stirred rapidly and portion (A) was added dropwise over a period of 2-3 hours. The resulting cloudy solution was poured into visking sausage tubing and dialysed

extensively against running water to remove excess diamine.

Portion (B) was added dropwise to a solution of the diamine alone in water. The solution was then dialysed as above.

Both solutions were then concentrated on a rotary evaporator. Samples were then introduced behind a dye line of biebrich scarlet in a spot line chromatography run at pH 6.8. Electrophoresis was then carried out in the usual way. The results were noted.

(c) Reaction of polyacrylyl chloride with polyvinylpyrrolidone.

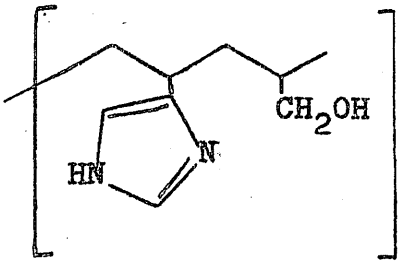
Polyvinylpyrrolidone (3g.) was dissolved in water (50ml.). Polyacrylyl chloride (3g.) in dioxane (20ml.) was added dropwise to the rapidly stirred aqueous solution. After addition, the mixture was stirred for a further 3 hours. The solution was dialysed and concentrated as before. A spot line chromatography run was done at pH 6.8 using samples of the solution and a dye line of biebrich scarlet.

Part 2. (1) Model Esterase Systems.

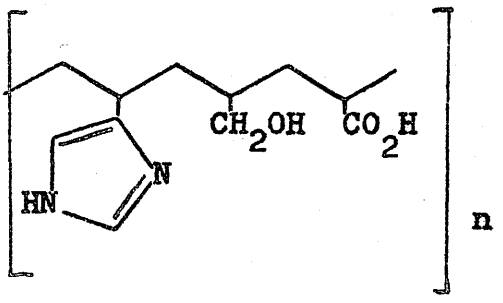
Overberger[156] has given an excellent review on the methods used for synthesis of compounds which possess the ability to catalyse the hydrolysis of esters, i.e. synthetic esterase compounds. A very recent compound which has been made is a pentapeptide which has as its last three amino acid units, L-seryl-L-histidyl-L-aspartic acid[157,158]. It has been shown that histidine, serine and aspartic acid are involved in the active centre of the enzyme chymotrypsin. It is also known that the above sequence of amino acids in the synthetic pentapeptide is present in the enzyme phosphoglucomutase[159].

In chymotrypsin these three groups come into proximity by the way the polypeptide chain is folded in three dimensions, i.e. determined by the tertiary structure, whereas, in the model system and in phosphoglucomutase, they are together because they are adjacent in the chain. Both alternatives, apparently can give rise to a molecule which possesses esterase activity.

Overberger[156] has described the synthesis of polymers of the following type.

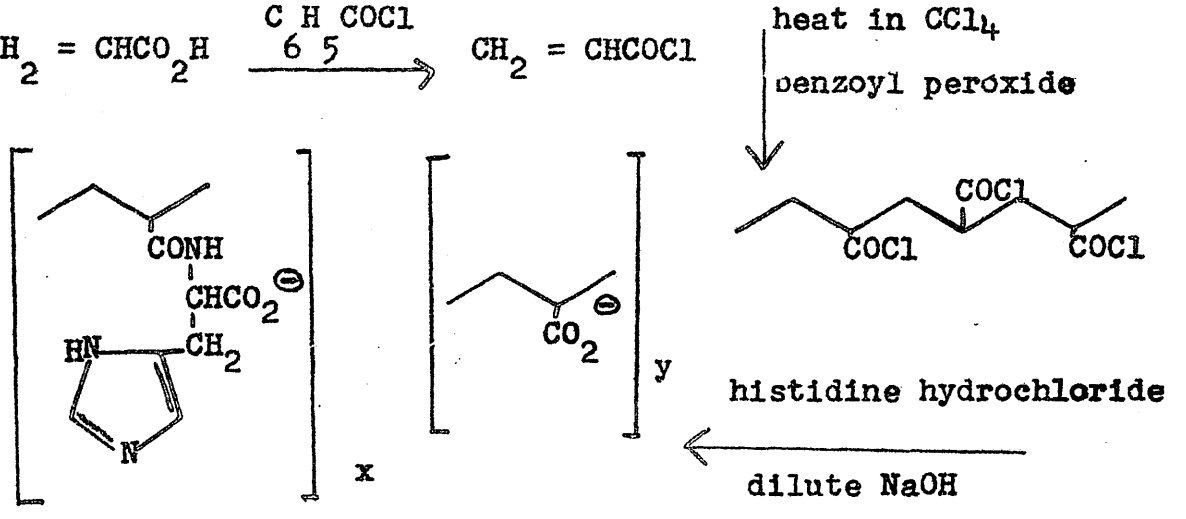


and



These models are like chymotrypsin in that they are macromolecules, but the active groups are adjacent on the chain, no specific folding of the chain is required to bring them into juxtaposition as occurs in the natural system.

The idea described here, is to start with a polymer chain and attempt to introduce imidazole and carboxyl groups at random on to it, and see if the resulting compound has any esterase activity. This type of compound could possibly be made by reacting a limited amount of the amino acid histidine with polyacrylyl chloride, and allowing unreacted acyl chloride groups to be hydrolysed to carboxyl groups, in the scheme outlined below



Polyacrylyl chloride (1g.) was prepared in the usual way and dissolved in anhydrous dioxane (10ml.). A solution of NaOH (5g.) in water (50ml.) was prepared. Histidine hydrochloride (1g.) was then dissolved in this solution. The polyacid chloride solution was added slowly to the stirred aqueous solution. After addition, the mixture was stirred overnight. The mixture was then extensively dialysed against running

water and concentrated to give a solution of polymer concentration 12.5 mg./ml.

The esterase activity of this solution was examined by investigating its ability to catalyse the hydrolysis of p-nitrophenyl acetate. (This ester is generally used because the rate and extent of hydrolysis can readily be studied by following the production of the yellow colour due to the nitrophenolate anion).

1ml. of the above polymer solution (12.5 mg.) was added to borate buffer, pH 8.5 (3ml.), then 1ml. of an acetone solution of p-nitrophenyl acetate (1.5mg.) was added. A blank consisted of borate buffer pH 8.5 (4ml.) with 1ml. of the p-nitrophenyl acetate solution. The reaction was carried out at 20°C. Immediately after addition, and mixing of the p-nitrophenyl acetate solution a "Spekker" reading was taken to measure the intensity of the colour produced. Readings were taken on each solution at intervals of a few minutes for about one and half hours. A reading taken 24 hours later was equivalent to 100% hydrolysis of the ester. Using the data obtained, the percentage hydrolysis (catalysed by the polymer solution) after an arbitrary time, taken as 15 minutes, was calculated. This was found to be 18%. With a solution of histidine, equivalent to the amount calculated to be present in the polymer exactly the same procedure was followed. The amount of hydrolysis after 15 minutes was found to be 32%. Thus it would appear that the idea of introducing imidazole

groups at random on to a polymer does not give it dramatic esterase ability.

(11) Study of Esterolytic Activity of Serum Albumin.

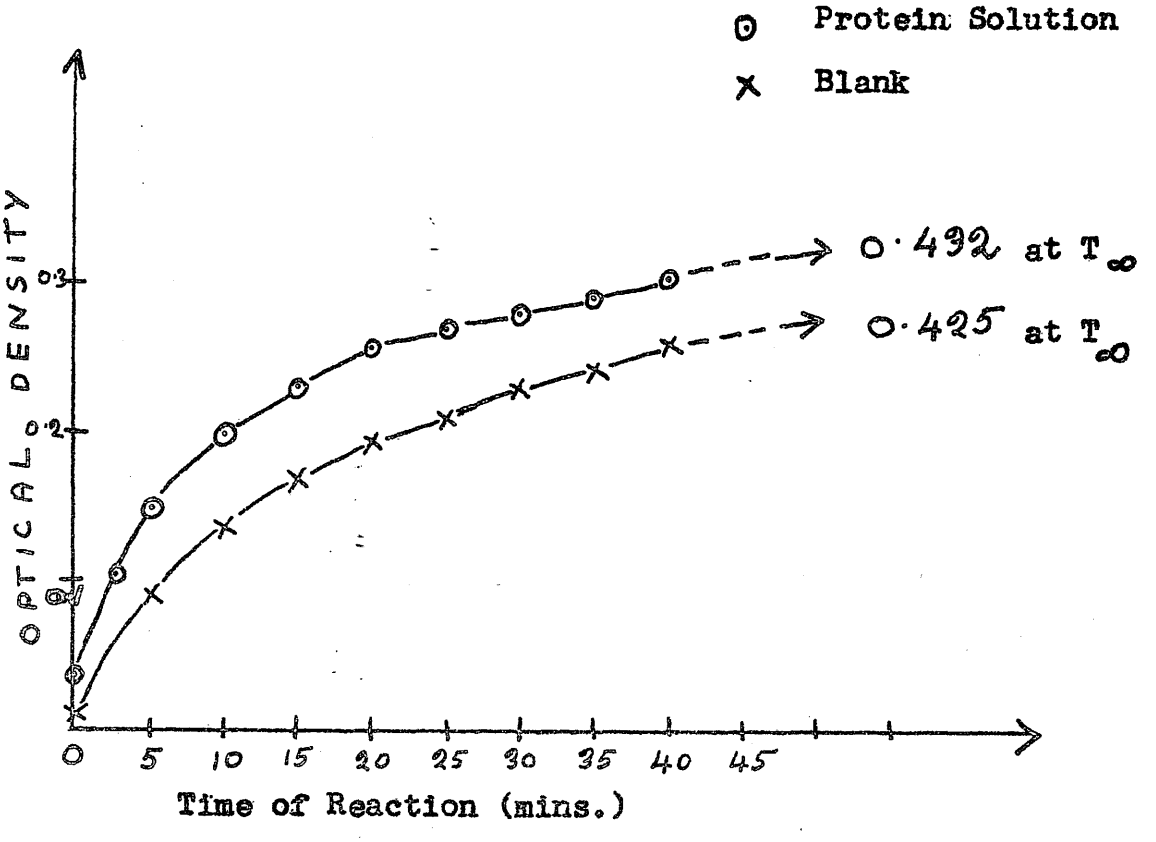
Since it has been found that the imidazole groups of histidine residues catalyses, to some, extent, the hydrolysis of nitrophenyl, and other esters, it is not surprising that some proteins have been shown to possess esterolytic activity. In particular it has been shown that human and bovine serum albumin preparations could hydrolyse fatty acid β-naphthyl esters, and the ability seemed to be inseparable from the protein [160]. This phenomenon is another example of protein interactions and as such is relevent to the general problem.

(a) Qualitative Work.

A few drops of a 5% solution of crystalline bovine serum albumin in veronal buffer pH 8.6, was incubated at 40°C, with a few drops of a methanol solution of β-naphthyl acetate, for about 15 minutes. A blank consisting of the same amount of β-naphthyl acetate in buffer was heated at 40°C for the same time. A drop from each solution was then removed and spotted on filter paper. The paper was sprayed with a freshly prepared aqueous solution of diazotised o-dianisidine (Fast Blue B salt). The spot from the protein solution immediately gave a violet colour, indicating that hydrolysis of the ester, and release of β-naphthol had occurred. The other did not produce a purple colour, only a faint pink. This work confirmed the original findings. By carrying out the same

Figure 41

Examination of esterase ability of crystalline bovine serum albumin (4mg./5ml.). 1.5mg. N.P.A. (1 cm. cell, blue filter.)



0.432 represents 100% hydrolysis of N.P.A.

∴ Hydrolysis produced by protein after 15 mins.

$$= \frac{0.062}{0.432} \times 100$$

$$= \underline{14\%}$$

procedure with iodoacetate treated serum albumin (-SH blocked) the activity appeared to be increased, again in agreement with the original work.

(b) Quantitative Work.

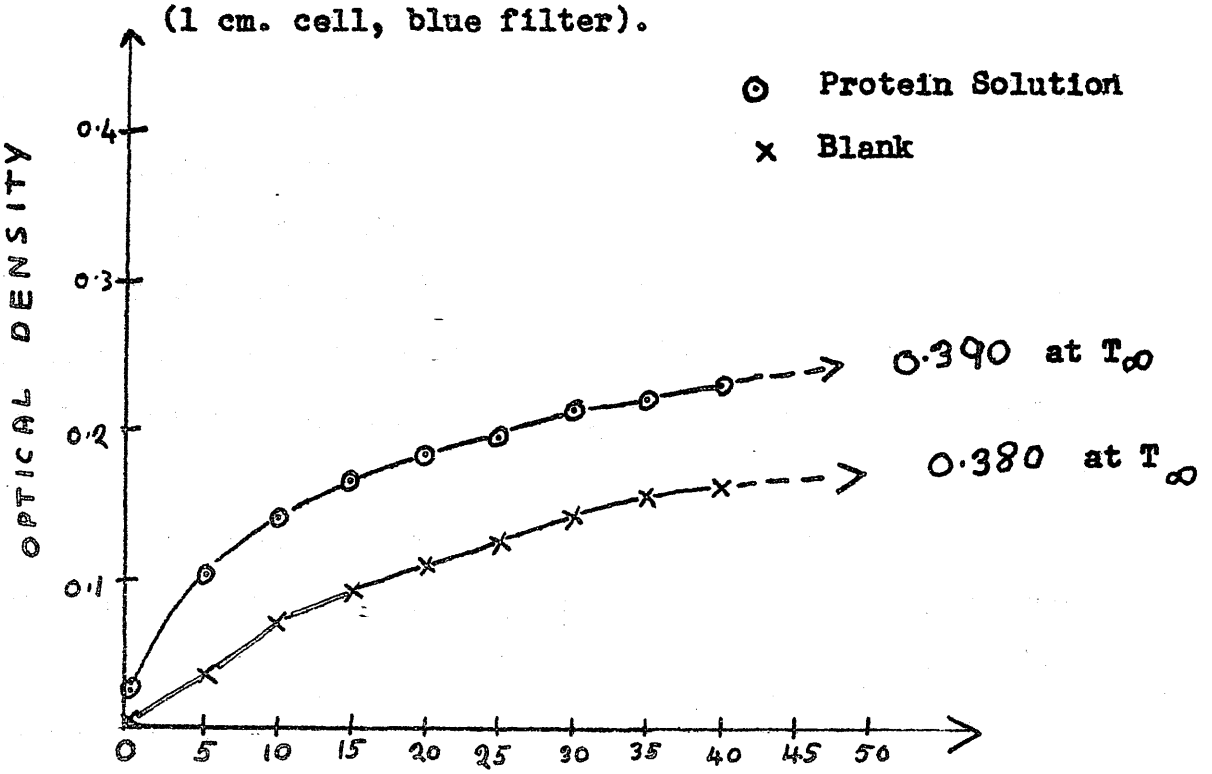
Some work was done to study the ability of serum albumin to catalyse the hydrolysis of p-nitrophenyl acetate. The effect of blocking the -SH group and of heat and concentrated urea solution, on this ability was also noted.

In all the experiments described, the ester was used in the form of an acetone solution. 1ml. (1.5mg.) being used in each case. 4mg. of bovine serum albumin, or modified serum albumin were used and the reaction was carried out in borate buffer pH 8.5 at 20°C. The procedure was as follows.

Into each of two test tubes was placed the required amount of buffer solution. To one was added 1 ml. of bovine serum albumin, (4mg.) in buffer solution. The acetone solution of the ester was then added in both cases. The yellow colour produced by the nitrophenolate anion was then measured using the Spekker, at intervals of a few minutes. A reading 24 hours later was taken as corresponding to 100% hydrolysis of the ester. A plot of "drum reading" (optical density) against time was drawn. The amount of enzymic hydrolysis after 15 minutes was found to be 14%. Fig. (41) shows the plot that was obtained.

Figure 42.

Examination of esterase ability of iodoacetate treated bovine serum albumin, (4mg./5ml) 1.5 mg. N.P.A. (1 cm. cell, blue filter).



0.385 represents 100% hydrolysis of N.P.A.

∴ Hydrolysis produced by protein after 15 mins.

$$= \frac{0.072}{0.385} \times 100$$

$$= \underline{19\%}$$

Blocked -SH Group.

Crystalline bovine serum albumin (4mg.) was treated with iodoacetate in the normal way to block the -SH group. A blank contained the same amount of iodoacetic acid as had been used to react with the protein (3mg.). The esterolytic activity of the product was then determined exactly as in the previous experiments. Fig. (42) shows the plot that was obtained. The amount of enzymic hydrolysis after 15 minutes was found to be 19% i.e. 5% greater than native crystalline bovine serum albumin.

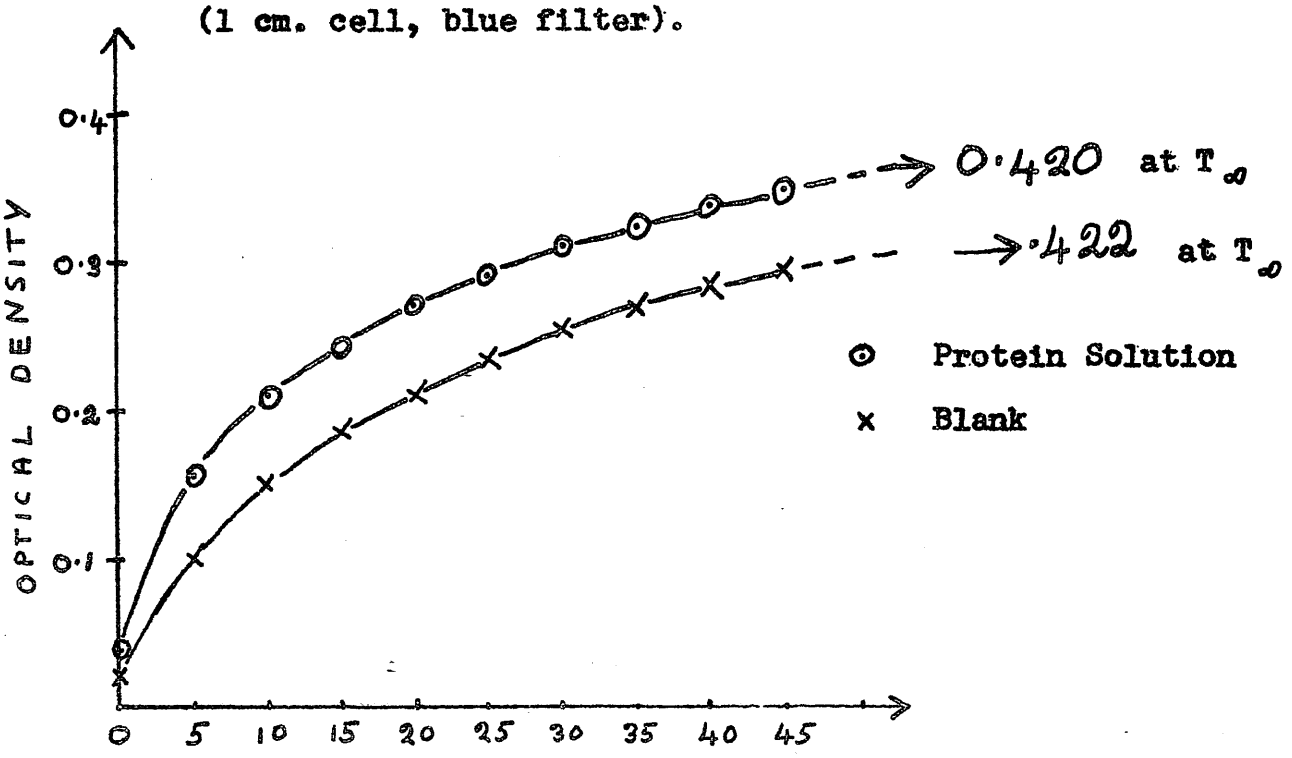
The effect of introducing a relatively bulky group would appear to alter the configuration in the vicinity of the -SH group, in such a way as to expose new esterolytic sites presumably "activated" histidine residues. It should also be noted that blocking the -SH group slightly increased the dye binding ability of the protein, as judged by spot line chromatography. This need not have been due to exposure of the same sites as mentioned here, but probably a similar sort of mechanism was operating.

Urea Treatment;

Crystalline bovine serum albumin (4mg.) was dissolved in a few drops of borate buffer, pH 8.5. Crystalline urea (460mg.) was then added to this solution. When the urea had gone into solution, the volume was made up to 1 ml. with more borate buffer. The clear solution was left overnight at room temperature along with a solution of 460 mg. urea/ml. borate

Figure 43

Examination of esterase ability of serum albumin (4mg./5ml.), treated with 7.5M urea, oovernight. 1.5mg. N.P.A. (1 cm. cell, blue filter).



Time of Reaction (mins.)

0.420 represnts 100% hydrolysis of N.P.A.

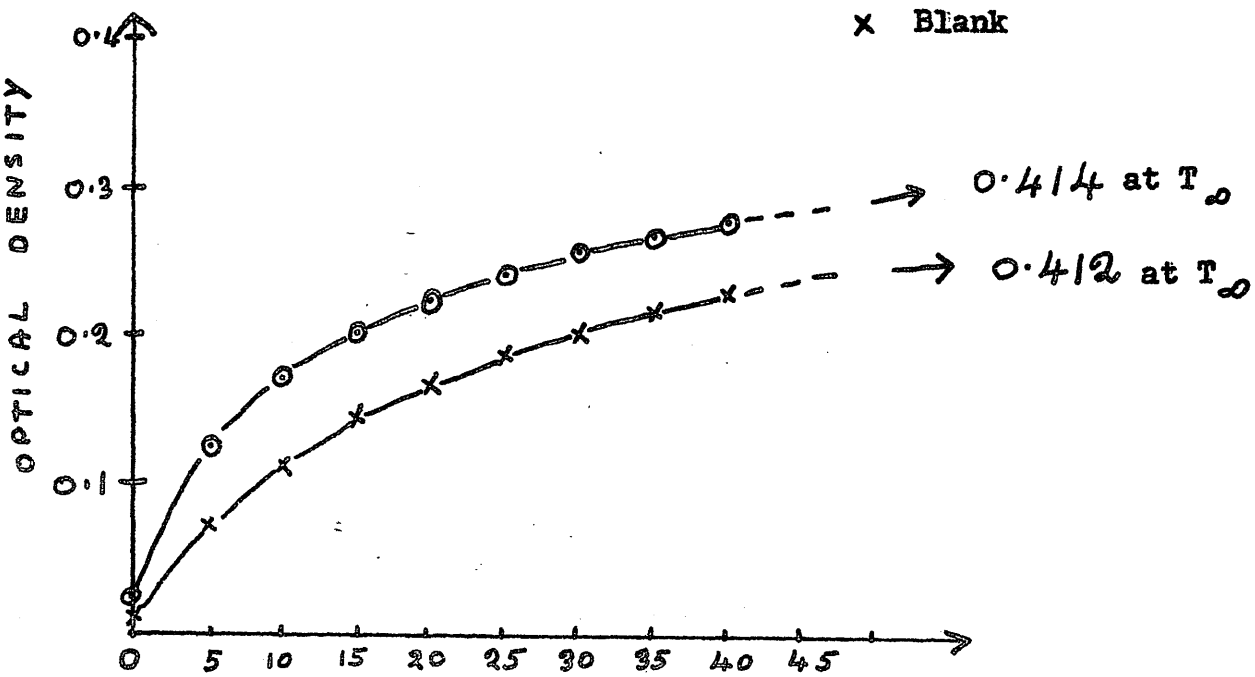
∴ Hydrolysis produced by protein after 15 mins.

$$= \frac{0.058}{0.420} \times 100$$

$$= \underline{14\%}$$

Figure 44.

Examination esterase ability of serum albumin heated to 70°C for 30 mins. (4mg./5ml.) 1.5 mg. N.P.A. (1 cm. cell, blue filter).



0.413 represents 100% hydrolysis of N.P.A.

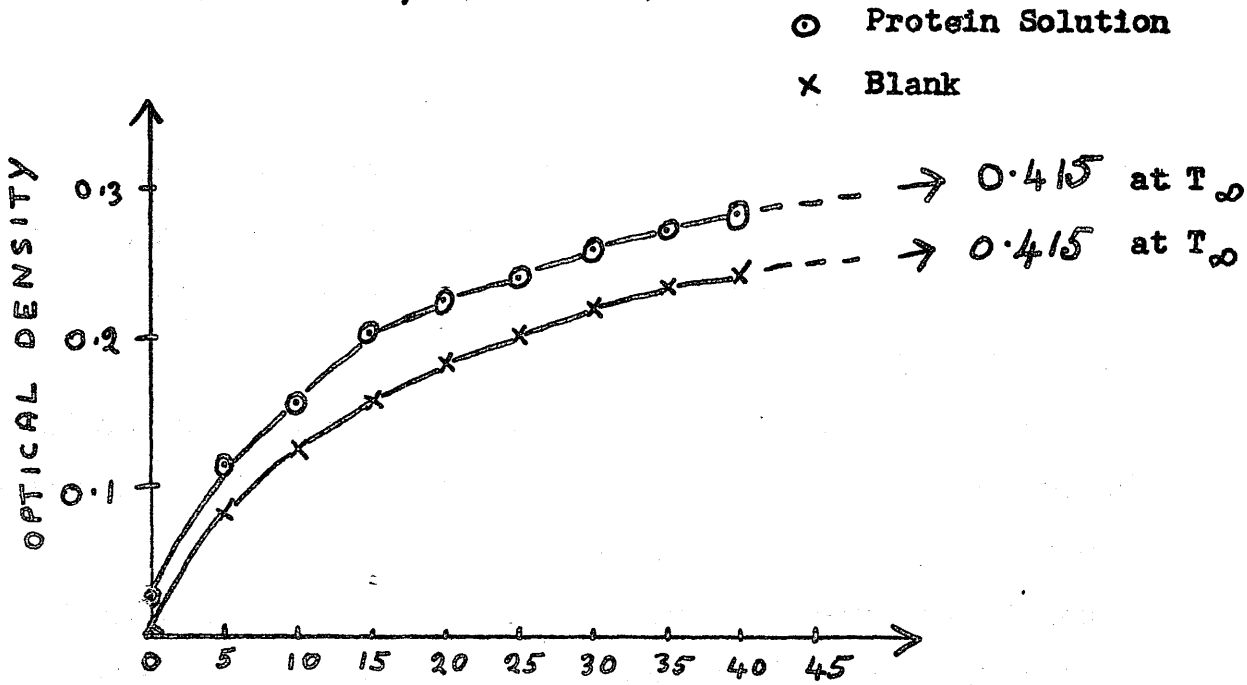
∴ Hydrolysis produced by protein after 15 mins.

$$= \frac{0.059}{0.413} \times 100$$

$$= \underline{14\%}$$

Figure 45

Examination of esterase ability of serum albumin heated to 90°C for 90 mins. (4mg./5ml.) 1.5 mg. N.P.A. (1 cm. cell, blue filter).



0.415 represnts 100% hydrolysis of N.P.A.

∴ Hydrolysis produced by protein after 15 mins.

$$= \frac{0.046}{0.415} \times 100$$

$$= \underline{11\%}$$

buffer, as blank. The esterolytic activity of the treated protein was then determined as before. Fig. (43) shows the graph obtained. The amount of hydrolysis after 15 minutes was 14% which is the same as for native material.

It would thus appear that concentrated urea does not affect this property of the protein. Due to the resilience of the molecule any changes which may have been produced in the configuration will have been reversed on dilution.

Effect of Heat.

A solution of bovine serum albumin 4mg./ml. in borate buffer pH 8.5 was heated in a water bath at 70°C for 30 minutes. The esterolytic activity of the treated protein was then determined in the usual way. The results are shown in Fig. (44). The amount of hydrolysis after 15 minutes was 14%, which is the same as for standard material. When the heat treatment was 90°C, for 90 minutes the subsequent amount of hydrolysis after 15 minutes was 11% which is a drop of about 3% from standard material. Fig. (45) shows the results that were obtained.

Thus, in conclusion it would appear that crystalline bovine serum albumin exhibits a slight non specific esterase ability. This may come about because the chains are so folded, that a few histidine residues are activated by being in juxtaposition to hydroxyl and carboxyl groups, thus forming a hydrolytic site, merely by chance.

Due to the natural rigidity of the molecule, denaturative conditions affect this property only slightly. The question of whether the esterolytic activity of serum albumin has a positive biological function is still a matter of speculation.

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