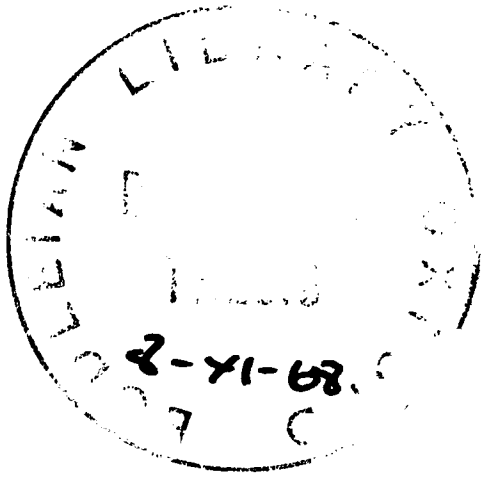


HYDROPHOBIC INTERACTIONS OF PROTEINS

C. F. Louis

Linacre College, Oxford

A thesis submitted to the Board of Biological Sciences

for the degree of

Doctor of Philosophy

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. Rupert Cecil for his much appreciated advice, encouragement and enthusiasm during the course of this work and in the preparation of this thesis.

I would also like to acknowledge the financial support of the Science Research Council.

Abstract

This work is concerned with an investigation of the hydrophobic interactions that are involved in the equilibria of proteins with non-polar surfaces. The strength of hydrogen bonds, as well as salt linkages, will increase in a non-aqueous environment so they have also been considered when studying these equilibria.

The object of this work was to study not only the adsorption of proteins on hydrocarbon surfaces but also the interaction of hydrocarbon molecules with protein molecules in aqueous solution. The work was mostly confined to a study of extracellular proteins as it was found that intracellular proteins tend to unfold irreversibly at hydrocarbon/water interfaces.

Emulsions of hydrocarbons in water were used for studying the adsorption of proteins onto non-polar/aqueous interfaces since this provides the largest surface area for a given volume of hydrocarbon. When a solution of protein was equilibrated with decane an emulsion formed and it was found that a proportion of the protein was lost from solution; this reached a maximum with a given volume of decane and then remained constant. It was discovered that in the presence of alcohols the amount of protein lost from solution reached a maximum with a given volume of alcohol-in-decane solution and then decreased as the volume was further increased. This was thought to be governed by two equilibria;

namely equilibrium of protein between aqueous solution and the interface, and equilibrium of alcohol between decane or aqueous solution and the interface. The alcohol and protein probably form a mixed film at the interface. The volume of decane solution at this maximum is called the "volume at the dip".

In order to investigate this phenomenon in greater detail, the adsorption of various proteins on 0.25% octanol-in-decane (referred to as decane B') emulsions was investigated over a range of protein concentrations and pH values. It was observed with bovine serum albumin and lysozyme that, if the volume of decane B' at the dip was plotted against the initial concentration of protein, then it increased linearly until, at a certain concentration of protein, it became constant. This was connected with the fact that the percentage loss of protein from solution decreased once the volume at the dip became constant. Experiments with insulin and ribonuclease showed no levelling off in the volume of decane B' at the dip as the concentration of protein was increased. The explanation of these results is not clear.

The effect of pH on the adsorption of proteins on decane B' emulsions was found to be related to the conformation or state of aggregation of the protein molecules. The N-F transition of bovine serum albumin, which occurs at pH 4, coincided with a change in the volume of decane B' at the dip; the F form requiring ⅔ the volume of decane B' at the dip as compared to the N form. A further change in bovine serum albumin over the range pH 2 to pH 7 was indicated by the

fact that the percentage loss of protein from solution at the dip rose gradually from 20% at pH 2 to 65% at pH 7. With lysozyme the percentage loss of protein from solution at the dip increased sharply over the range pH 5.5 to pH 6.0 where dimerization of the protein is known to occur. Similarly with insulin, the percentage loss of protein from solution is greater at pH 7 than at pH 2 owing to the increase in molecular weight from 12,000 to 36,000. Ribonuclease, which does not undergo any change in conformation or state of aggregation with pH, showed no change in its surface properties between pH 2 and pH 8. This relationship between properties of proteins in solution and in the presence of decane B' emulsions provides a sensitive method for comparing the solution properties of different proteins.

An investigation of the difference spectra of proteins in equilibrium with emulsions showed that they undergo slight conformational changes but it appears that the extent of the change is related to the volume of decane B'. This suggests that the conformation of the protein must be altered a definite amount every time it comes into contact with the surface of an emulsion. It was found that the addition of an excess of alcohol displaced protein from an emulsion stabilized by protein; the difference spectrum of the protein solution so obtained was almost identical to that of the solution which had been originally in equilibrium with the emulsion. It was also found that lysozyme could be removed from emulsions by re-equilibrating the emulsion with fresh buffer at pH 2.

Besides studying the adsorption of proteins on emulsions, the

combination of decane with protein in aqueous solution was also investigated. When lysozyme, bovine serum albumin or insulin were equilibrated with a given volume of pure decane, then the number of moles of decane bound per mole of protein ($\bar{v}$) increased with decreasing concentration of protein; similar results were obtained when the binding of decanol to bovine serum albumin was investigated. This binding phenomenon is probably controlled by the ratio of the concentration of protein in solution to the volume of decane present i.e. the interfacial area of the decane present. When decane B' was substituted for pure decane, then the binding again increased with decreasing concentration of protein but below a given concentration of protein it decreased dramatically. This result was shown to be linked to the fact that the amount of binding is affected by the amount of emulsion present, i.e. the maximum value of $\bar{v}$ for a given concentration of protein occurs when this concentration is equilibrated with the volume of decane B' at the dip corresponding to this concentration.

The effect of pH on the binding of decane and decanol to bovine serum albumin is closely linked to the N-F transition and $\bar{v}$ was shown to increase between pH 3 and pH 7.

The fact that lysozyme and insulin do not bind decane at 2°C, but do at 22°C, gives weight to Kauzmann's original precept, that the strength of hydrophobic interactions should increase with increasing temperature.

When the percentage of octanol in decane was varied and different

volumes of these solutions were added to a 2 x 10^{-5}M solution of lysozyme at pH 2.0 both the volume at the dip and the percentage loss of protein from solution at the dip remained constant. This effect was only observed when the percentage of octanol in decane was 0.2% or greater. However the sharpness of the dip increased with increasing percentage of octanol in the decane. When this experiment was repeated at pH 7.0, using bovine serum albumin, the results were very different since both the volume of decane solution at the dip, as well as the percentage loss of protein from solution at the dip, varied with the percentage of octanol in decane. The difference between these two sets of results probably lies in the fact that, with a 2 x 10^{-5}M solution of lysozyme at pH 2.0, the volume of decane B' at the dip was still increasing with increasing concentration of protein while, with a 1.1 x 10^{-5}M solution of bovine serum albumin at pH 7.0, the volume remained constant with increasing concentration of protein.

The results obtained with bovine serum albumin provided further insight into the nature of the N-F transition since the amount of protein lost from solution and the volume of decane solution at the dip are identical for a 1.1 x 10^{-5}M solution of bovine serum albumin using a 0.8% octanol-in-decane solution at pH 7.0 or a 0.25% octanol-in-decane solution at pH 2.0. Thus the N form of bovine serum albumin requires approximately 3 times as much octanol at the interface to interact with the protein compared to the F form under similar conditions. This result is in good agreement with that described previously where the N form of

bovine serum albumin required 3 times the volume of decane B' at the dip compared to the F form. These results indicate that a definite stoichiometry between octanol and protein molecules must exist at the interface.

The effect of different alcohols on the relation between the volume of decane solution at the dip and the initial concentration of protein shows that these plots are different from those obtained using decane B'. The effect of secondary alcohols on emulsions stabilized with lysozyme was also investigated but their effect appeared to be similar to those of the homologous primary alcohols.

When the effectiveness of different primary alcohols on preventing emulsion formation was investigated it was found that this effectiveness, which is exhibited by the minimum percentages of alcohol in decane which prevents emulsion formation when 300 μl of decane solution was added to 6 ml. of 2×10^{-5}M lysozyme at pH 2.0, was increased by a factor of 2.5 for each additional $-CH_2$ group on the alcohol. This value is in very close agreement with the results of Cecil and Thomas who investigated the reactivity of the "unreactive" sulphydryl groups of haemoglobin in the presence of primary alcohols. These results suggest that these "unreactive" sulphydryl groups are indeed involved in hydrophobic interactions in the native protein.

Preliminary work with decyl mercaptan, decyl amine and stearic acid showed that they all markedly affected the surface properties of decane emulsions stabilized by lysozyme or insulin.

The specific enzymic activities of lysozyme and ribonuclease, in the presence of emulsions, were investigated and it was found that these enzymes behaved almost identically in the presence of decane emulsions. The enzyme in equilibrium with an emulsion was fully active; the enzyme adsorbed on the emulsion exhibited less than 15% of its specific activity and enzyme desorbed from emulsions retained between 75% and 100% of its specific activity. The fact that the enzyme undergoes a conformational change, as shown by difference spectroscopy, when in equilibrium with decane emulsions, yet undergoes little or no loss of enzymic activity, indicates that enzymes can retain full activity yet be in a different conformational state from that of the native enzyme.

Preliminary results obtained with insulin showed that this protein is fully dissociated into monomers with a molecular weight of 5,700 in 60% ethanol at pH 2.0. The optical rotatory dispersion parameters a_o and b_o, as well as $\triangle$ 37, were shown to be independent of the state of aggregation of the protein but were dependent on the concentration of ethanol present in the insulin solution. The intrinsic fluorescence of insulin in ethanol solution was also investigated and it was found to increase linearly with increasing concentration of ethanol. This would suggest that the tyrosines, which are causing this fluorescence, are not involved in the bonding of the two 5,740 molecular weight monomers in the 11,480 molecular weight units which exist at pH 2.0 in aqueous solution.

CONTENTS

	Page
INTRODUCTION	1
The development of this work.	1
Forces responsible for maintaining protein structure.	2
Structure of proteins.	24
Surface properties of proteins.	25
Interaction of proteins with non-polar solutes.	30
Properties of proteins.	34
EXPERIMENTAL	45
Materials.	45
Apparatus and Methods.	54
RESULTS	67
Surface tension measurements.	67
Preliminary measurements of protein concentration at interfaces.	68
Hydrocarbon in water emulsions.	70
Emulsions of decane containing 0.25% octanol stabilized by different proteins.	74
Displacement of adsorbed protein from emulsions.	81
Conformation of proteins in equilibrium with emulsions.	84
Binding of non-polar compounds by protein in solution.	85

Page

Effect of different alcohols on decane in water emulsions in the presence of proteins. 88

Addition of other polar compounds to decane and their effect on emulsions in protein solutions. 91

The effect of emulsions on the activity of enzymes. 92

Effect of ethanol on some physical constants of insulin. 96

S_{20} values for lysozyme. 99

DISCUSSION 100

Measurement of surface tension. 100

The adsorption of proteins on emulsions. 101

Comparison between different proteins adsorbed on decane emulsions containing 0.25% octanol. 110

Method by which protein is desorbed from emulsions of decane B' stabilized by protein. 116

Conformation of proteins in the presence of emulsions. 117

Binding of non-polar molecules by protein in solution, 118

Effect of different alcohols on decane in water emulsions stabilized by proteins. 124

The effect of emulsions on the activity of enzymes. 132

Effect of ethanol on some physical constants of insulin. 134

REFERENCES 137

INTRODUCTION

The development of this work

The original object of this work was to investigate the hydrophobic interactions which are thought to be involved when proteins are adsorbed on to non-polar/aqueous interfaces. The pendant drop method was found to be the most suitable for the measurement of the interfacial tensions of protein solutions, there being little or no disturbance of surface during the course of the measurement. However other techniques had to be used to determine the amount of protein adsorbed at the interface as it could not be estimated directly in the pendant drop method. Of these techniques the adsorption of proteins on emulsions of decane was found to be the most suitable in that significant amounts of protein were adsorbed at the interface which could then be measured. However the adsorption of proteins on emulsions exhibited some interesting properties, particularly in the presence of alcohols, which appeared to offer some definite experimental facts as to the relative importance of the different factors that govern the adsorption of proteins at non-polar/aqueous interfaces and so this line of work was pursued. An investigation of the binding of decane by proteins in solution, in equilibrium with an emulsion, was also undertaken in order to find out whether it was related to the adsorption of protein on to the emulsions.

Preliminary work was also undertaken on an investigation of the dissociation of insulin from 12,000 to 6,000 molecular weight as this dissociation is thought to involve breaking of hydrophobic interactions.

Forces responsible for maintaining protein structure

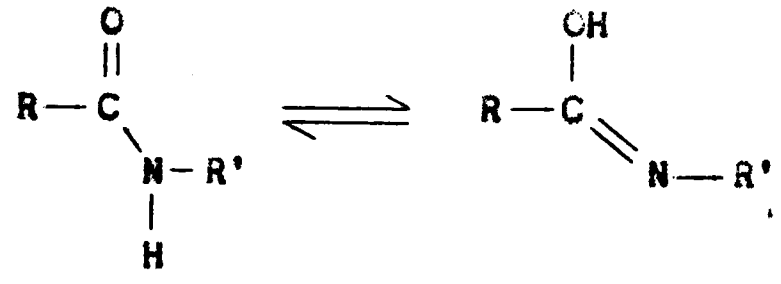
The structure of proteins is maintained by two different types of forces. One is the covalent bond and the other is a series of three different types of forces grouped under the classification of non-covalent bonds. These non-covalent forces are comprised of hydrogen bonds, hydrophobic interactions and electrostatic or salt linkages.

While it is possible to describe the different types of bond that are responsible for maintaining the native structure of any given protein molecule, the predominant types may differ in different proteins.

Covalent bonds.

This type of bond is exhibited in the protein molecule by the peptide and disulphide bonds.

The peptide bond exists in two resonance forms:



This results in two important properties which relate to protein structure. The first is that the six atoms of the peptide group

Cα - CO - NH - Cα, in which Cα refers to the α carbon atoms of adjacent residues in the peptide chain, are coplanar. This coplanarity results from the substantial resonance energy stabilization derived from the maximum interactions of the lone electron pair on nitrogen with the electrons of the - C = O. The C - N bond of the peptide group is 1.32 Å long, corresponding to about 50% double bond character. The second property is that the α-carbon atoms of the peptide group lie trans to the C - N. These two facts are of great importance in determining the structure of native proteins.

By means of the disulphide bond two cysteine residues are held together covalently in independent polypeptide chains or at different points on the same chain of a protein.

Hydrogen bonds.

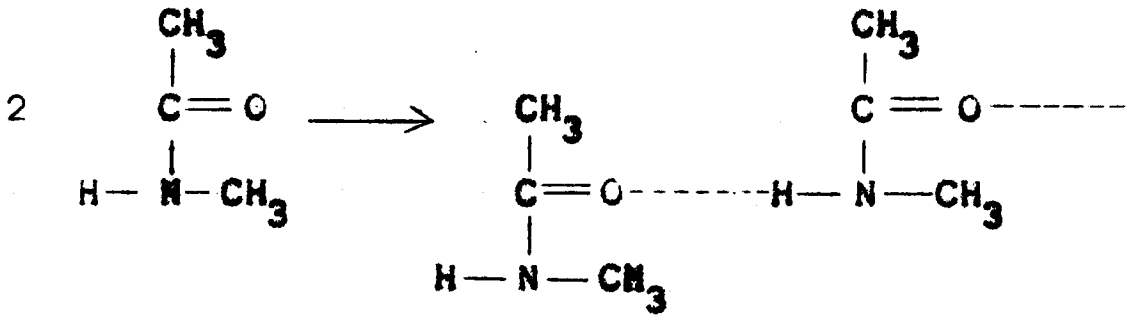
The hydrogen bond is a bond by hydrogen between two atoms, the co-ordination of the hydrogen not exceeding two. Only the most electronegative atoms form these bonds and the strength of the bond will increase with increasing electronegativity of the two bonded atoms. Thus F, O, N and Cl can form hydrogen bonds in a decreasing order of strength from F to Cl. Although it has the same electronegativity as nitrogen, chlorine has a smaller hydrogen bond forming power which may be attributed to its greater diameter. Likewise the ammonium ion and its derivatives such as $[RNH_3]^+$ form stronger hydrogen bonds than ammonia or normal amines because of the increase in electronegativity.

In proteins the hydrogen bond is formed between the hydrogen atoms

of the amide groups and the oxygen atoms of the carbonyl groups of peptide linkages and are assumed to play a basic role in determining the pattern of folding of the polypeptide chains in most structures that have been proposed to account for the X-ray diffraction patterns of both fibrous and globular proteins (Pauling et al., 1951; Blake et al., 1967).

However in an aqueous environment, the evidence for hydrogen bonding between amide and carbonyl groups is not so conclusive.

Klotz (1960) has studied the nuclear magnetic resonance of N-methyl acetamide in both carbon tetrachloride and water:



He was able to show that it is aggregated in carbon tetrachloride but largely dissociated in water, except at high concentration. He calculated that there is little dimer present until a concentration of 7M to 8M had been reached, at which concentration the molar ratio of water to amide is down to about 3. Thus if pure liquid amide is diluted with water the aggregate disappears almost completely after the addition of the minimum number of water molecules required to bond to both - N - H and - C = O of each amide molecule.

Some side chains such as the -OH of serine, the $-NH_2$ of glutamine and the -COOH of aspartic acid are also able to form hydrogen bonds.

For instance the pK of tyrosine in various proteins is abnormally high and this may be interpreted in terms of intramolecular hydrogen bonding of the -OH groups (Shugar, 1952a).

Thus despite the intrinsic instability of hydrogen bonds in aqueous solutions of N-methyl acetamide, there is good reason to believe that some intramolecular hydrogen bonding exists in proteins, even in aqueous solution.

Hydrophobic interactions.

Approximately 40% of the amino acids in proteins have non-polar side chains (e.g. valine, leucine, phenylalanine, proline, tryptophan and tyrosine). These non-polar side chains can form intramolecular micelles analagous to the micelles known to occur in aqueous solutions of detergents. This tendency of the non-polar groups of proteins to adhere to one another has been referred to as a hydrophobic interaction and it is probably one of the more important factors involved in stabilizing the folded configuration in many native proteins.

Solutions of hydrocarbons in water have been used as model systems for the interpretation of the hydrophobic interaction.

Consider:

Hydrocarbon in non-polar solute $\longrightarrow$ Hydrocarbon in water

If the equilibrium constant K is found for this reaction, then the free energy change $\triangle F$ is given by:

$$\triangle F = - RT \ln K$$

The enthalpy change $\triangle H$ can be found calorimetrically and the entropy

change ΔS for the reaction is given by:

$$\Delta S = \frac{\Delta H - \Delta F}{T}$$

Kauzmann (1959) used the thermodynamic values shown in Table 1 to demonstrate that the process for the transfer of a hydrocarbon molecule from a non-polar to a polar environment is exothermic for aliphatic molecules but almost athermal for aromatic molecules. Also the transfer is accompanied by a very large decrease in entropy.

Thus the low affinity of these substances for water, shown by the large positive free energy term, is not caused by an unfavourable energetic situation as would arise if hydrogen bonds were broken when a non-polar molecule was placed in water, rather it is due to a highly unfavourable entropy change.

This large entropy effect when a hydrocarbon is dissolved in water is also exhibited by many organic molecules containing non-polar groups attached to polar groups. For example:

(a) The solubilities of many aliphatic substances, e.g. butanol, ethyl acetate and diethyl ether, in water decreases as the temperature is raised. Thus by Le Chatelier's principle the enthalpy change must be negative ($\Delta H < 0$) for the transfer of a molecule from the non-aqueous phase to water, which combined with the fact that these compounds do not mix with water (free energy $\Delta F > 0$) means that the entropy change on mixing these aliphatic derivatives with excess water must be negative.

(b) The formation of a micelle in water from isolated detergent molecules is not accompanied by a large negative enthalpy change, so

Table 1

Thermodynamic changes in the transfer of hydrocarbons from a non-polar solvent to water (*)

Process	Temp. (°K)	△S (e.u.)	△H (cal/mole)	△F (cal/mole)
CH_4 in benzene ⟶ CH_4 in H_2O	298	-18	-2800	+2600
CH_4 in ether ⟶ CH_4 in H_2O	298	-19	-2400	+3300
CH_4 in CCl_4 ⟶ CH_4 in H_2O	298	-18	-2500	+2900
C_2H_6 in benzene ⟶ C_2H_6 in H_2O	298	-20	-2200	+3800
C_2H_6 in CCl_4 ⟶ C_2H_6 in H_2O	298	-18	-1700	+3700
C_2H_4 in benzene ⟶ C_2H_4 in H_2O	298	-15	-1610	+2920
C_2H_2 in benzene ⟶ C_2H_2 in H_2O	298	-7	-190	+1870
Liquid propane ⟶ C_3H_8 in H_2O	298	-23	-1800	+5050
Liquid n-butane ⟶ C_4H_{10} in H_2O	298	-23	-1000	+5850
Liquid benzene ⟶ C_6H_6 in H_2O	291	-14	0	+4070
Liquid toluene ⟶ C_7H_8 in H_2O	291	-16	0	+4650
Liquid ethyl benzene ⟶ C_8H_{10} in H_2O	291	-19	0	+5500
Liquid m- or p-xylene ⟶ C_8H_{10} in H_2O	291	-20	0	+5800

* Data from Kauzmann (1959).

there must be a large positive entropy change $\triangle S$ occurring when the non-polar groups of the detergent molecules transfer from their aqueous environment to form the oily centre of the micelle.

Butler (1937) and Frank and Evans (1945) showed that the large negative entropy change and small negative enthalpy change on transferring a hydrocarbon moiety from a non-polar to an aqueous environment, were caused by a change in the aqueous phase when the non-polar molecules were added rather than by changes in the non-polar phase. Their reasoning for this explanation was that the entropy change for transfer of a non-polar molecule from a hydrocarbon environment to the vapour obeys Troutons rule, while the transfer of a non-polar molecule from an aqueous environment to the vapour gives an entropy change which deviates widely from Troutons rule. Frank and Evans concluded that when a non-polar substance is dissolved in water, the water molecules adjacent to the non-polar molecules must be in a semi-crystalline structure where there is an increase in hydrogen bonding compared with that in pure water at the same temperature. It follows that hydrophobic bonds can only occur in the presence of water and their properties must be described in terms of the structure of water.

The structure of water. The structural features of liquid water depend primarily on the fact that each oxygen atom can form up to four hydrogen bonds by means of its two hydrogens and the two lone pairs. However as yet no satisfactory theory has been postulated which accounts for all the properties of liquid water. Although all the different models and

theories agree that hydrogen bonding is the most important factor in an understanding of the structure of water, the interpretations and conclusions reached differ in detail.

Originally water was thought to contain polymers, each consisting of a few molecules, but this theory could not explain the observed properties of water (Chadwell, 1927).

Bernal and Fowler (1933) proposed a model for the liquid based on a broken-down ice structure but this was later criticized because it gave a structure whose rigidity was incompatible with the viscosity of water (Pauling, 1959). However the idea of extensively hydrogen-bonded regions and the gradual breakdown of hydrogen bonding with increasing temperature has been confirmed experimentally and has been incorporated into all of the more recent models for the structure of water.

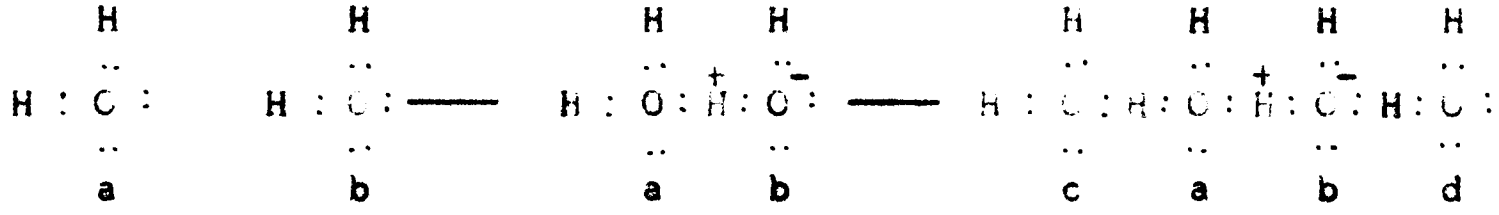
Euken (1948) using dilatometry, considered a model having aggregates of two, four and eight molecules of water but this model was shown to be in contradiction with self-diffusion data (Wang , 1951) which suggested that only one diffusing species exists in water.

Grjotheim and Krogh-Moe (1954) considered liquid water to be composed of a mixture of a highly hydrogen bonded ice like species and non-hydrogen bonded close packed water. A similar treatment by Maggis et al. (1952) assumed a distribution of molecules over five types with 4, 3, 2, 1 and zero hydrogen bonds.

Lennard-Jones and Pople (1951) discussed molecular association in

liquids, arising from lone-pair electron interactions and applied their ideas to the structure of water. They considered that water has very few hydrogen bonds broken at the melting point of the liquid but as the temperature is raised a progressive bending of hydrogen bonds occurs, resulting in a destruction of the repeating ice-lattice arrangement.

Frank and Wen (1957) have considered the partially covalent character of the hydrogen bond. Since the formation of the bond involves a contribution from delocalization energy due to electron overlap (Coulson, 1959) i.e. dipole-interaction energies introducing some covalent character into the bond, large deviations from the co-linearity of the C–H and H---C directions are not allowed. Consider the model:



I II III

II produces a partial charge separation so the two molecules a and b can form additional hydrogen bonds with neighbouring molecules such as c and d in III, more easily than before the formation of the a - b hydrogen bond. Thus the formation of hydrogen bonds in water is considered to be a co-operative phenomenon and bonds are made and broken several at a time, to produce short lived clusters of highly hydrogen bonded regions surrounded by non-hydrogen bonded molecules.

Pauling (1959) proposed a qualitative model for liquid water based on an analogy with the structure of solid gas hydrates where a

non-polar substance sits in a cavity surrounded by a network of tetrahedrally hydrogen-bonded water. Pauling suggested a similar structure for pure water with the non-polar solute molecule at the centre of the cavity being replaced by a non hydrogen bonded water molecule.

The models of both Pople and Pauling have been criticized as being too rigid while the latter has been criticized on the additional grounds that it postulates structures stabilized by long range forces which according to Bernal (1959) are not to be expected in water. Stackelberg (1954) has pointed out that only non-polar molecules are able to participate in gas hydrate formation, because a molecule of even moderate polarity would interact with the surrounding water dipoles and prevent the formation of the polyhedral cage. Thus the occurrence of clathrate type structures may not be frequent in water, although their existence cannot be excluded (Frank and Quist, 1961).

Interpretation of the hydrophobic interaction. Nemethy and Scheraga (1962a, 1962b, 1962c) have used the structure of water as proposed by Frank and Wen to describe hydrophobic interactions. According to this proposal, water exists as either clusters or monomers only, although the individual molecules may be distributed among five classes depending on the number of hydrogen bonds in which they are involved; the half life of these clusters is approximately 10^{-11} seconds.

Frank and Evans have concluded that there is an increase in the ice-like character of water when a non-polar molecule is dissolved in

water. Nemethy and Scheraga used this conclusion in deriving a quantitative treatment of the thermodynamic parameters of liquid water in the presence of dissolved hydrocarbons. These parameters were determined from the difference between the net intermolecular interaction energies of the solute with the non hydrogen bonded water molecules and the interaction energies of the water molecules participating in the formation of hydrogen bonded clusters.

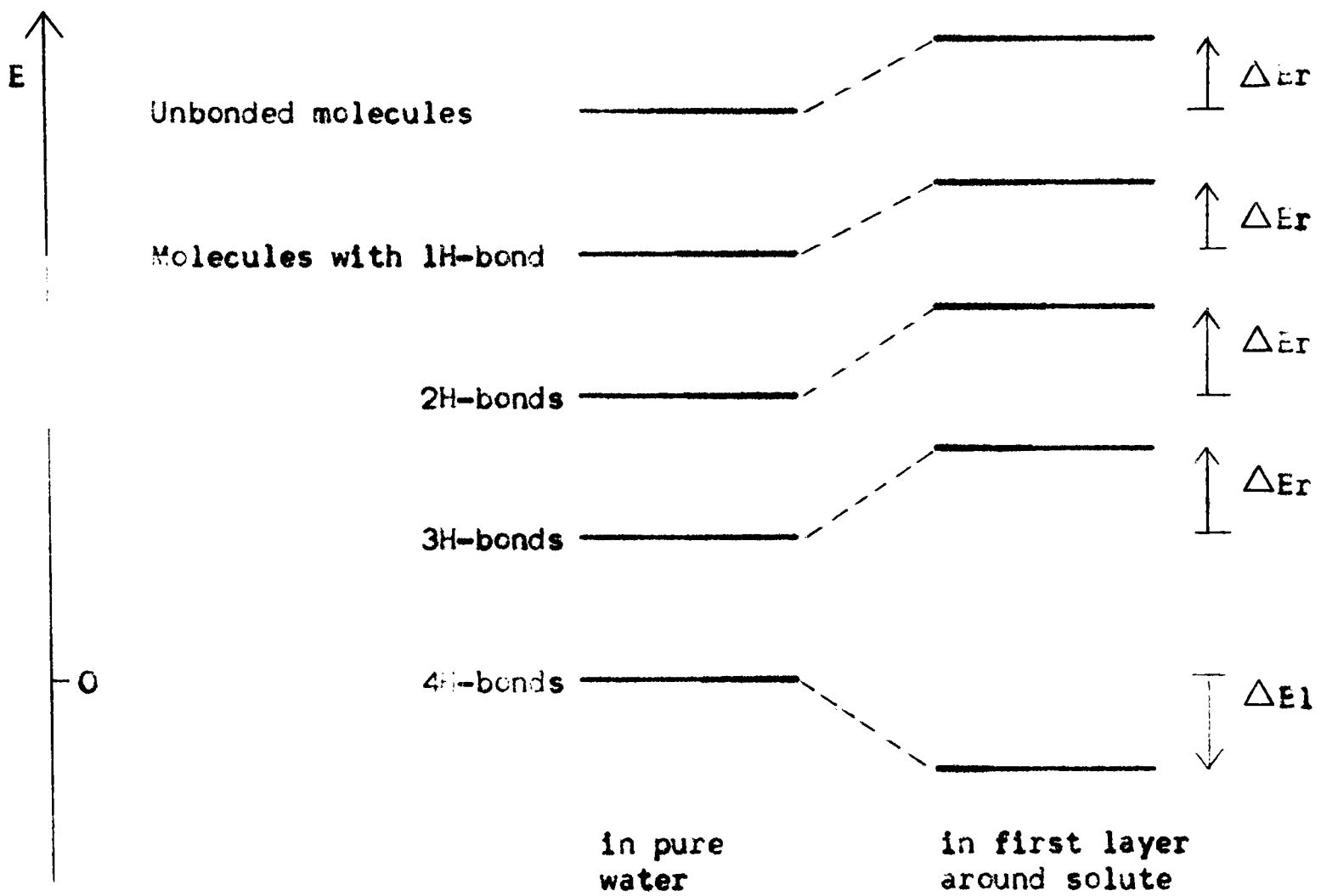
Water that has four hydrogen bonded water neighbours can also have a non-polar neighbour, resulting in an overall drop in the energy of the water molecule by$\triangle E1$, since the van der Waals interaction between the solute and water molecules lowers their free energy. Water molecules with none to three hydrogen bonds also can acquire a hydrocarbon neighbour but this involves replacement of an unbonded water neighbour so a strong water/water dipole interaction is replaced by a much weaker water/hydrocarbon interaction and by dispersion forces so the energy of these species is raised by$\triangle Er$. These energy terms $\triangle Er$ and $\triangle E1$ were then calculated by Nemethy and Scheraga (1962b).

From Figure 1 it may be seen that the effect of adding a non-polar solute to water will be to increase the number of molecules in the lower energy states as compared to that for pure water and thus in the presence of hydrocarbons there will be a greater degree of hydrogen bonding and more ordering of the water structure.

It is observed experimentally that when a hydrocarbon is dissolved in water there is a decrease in total volume. According to Nemethy and

Figure 1

Schematic representation of the energy level changes occurring on transfer of water from the pure liquid to the structure next to a non-polar solute (*)



* Data from Nemethy and Scheraga (1962b)

Scheraga, the hydrocarbon molecules fit into the cavities in the clusters. A similar decrease in the total volume occurs on the formation of crystalline gas hydrates (Bernal and Fowler, 1933) where filling of the cavities by the solute results in a decrease of void space as compared with ordinary ice.

Nemethy and Scheraga consider that if two non-polar side chains of a protein are in water they will have an environment similar to that just described for hydrocarbons in water. When the two non-polar groups approach each other until they are in contact, there will be a decrease in the total number of water molecules in contact with them so this can be considered as a partial reversal of the solution process. Thus the formation of a hydrophobic interaction may be considered as the partial removal of a hydrocarbon from aqueous solution causing an increase of total volume; this is accompanied by a melting of ice-like regions of water which results in a reversing of the signs of the thermodynamic parameters for the solution process of hydrocarbons.

Klotz (1960) disagrees with the arguments used by Nemethy and Scheraga and doubts whether such data, derived from the transfer of hydrocarbon molecules from an aqueous to a non-polar environment, truly reflects changes during formation or disruption of bonds between non-polar groups in proteins. Klotz doubts whether the packing of non-polar side chains attached to a polypeptide framework is comparable to that of freely moving hydrocarbon molecules in non-polar solvents. The volume changes cited by Kauzmann (1959) as characteristic of hydrophobic

bond formation reflect largely the loose packing of volatile molecules in the organic solution irrespective of the nature of the solute, since the volume changes for similar transfers of inorganic molecules such as H_2, N_2 and CO_2 are comparable in magnitude to those for CH_4, C_2H_6 and C_3H_8. Also, as can be seen from Table 2, in both groups of molecules the change in volume ΔV is strongly dependent on the organic solvent that the small molecule is originally dissolved in.

The model adopted by Klotz to describe hydrophobic interactions is that of the crystalline hydrate, formed by many non-polar molecules in water, and the related behaviour of various aliphatic compounds in water. A large number of simple molecules, if dissolved in water under pressure to a sufficiently high concentration, form crystalline hydrates with melting points above $0^{\circ}C$. From Table 3 it can be seen that the heat of hydration ΔH for these molecules is essentially the same despite marked differences in the chemical nature of the non-polar molecule and so the stability of the hydrate cannot depend on the interactions between non-polar molecules and water. Rather, Klotz states that the "crystallization" of the water itself, which occurs in the presence of non-polar molecules, provides the drop in internal energy which stabilizes these inclusion compounds. Thus when referring to the stability of gas hydrates in water, he claims one is observing a change in the water structure which is not due to interaction of non-polar molecules with the water surrounding them, rather one is observing a phenomenon which is inherent in water but which is only observable

Table 2

Volume changes in transfer from non-polar solvent to water at 25°C (*)

Process			ΔV (ml./mole)
CH_4 (hexane)	$\longrightarrow$	CH_4 (water)	-23
C_2H_6 (hexane)	$\longrightarrow$	C_2H_6 (water)	-18
C_3H_8 (pure)	$\longrightarrow$	C_3H_8 (water)	-21
C_6H_6 (pure)	$\longrightarrow$	C_6H_6 (water)	-6
H_2 (CCl_4)	$\longrightarrow$	H_2 (water)	-16
H_2 (C_5F_{12})	$\longrightarrow$	H_2 (water)	-30
H_2 (benzene)	$\longrightarrow$	H_2 (water)	-10
H_2 (methanol)	$\longrightarrow$	H_2 (water)	-10
O_2 (methanol)	$\longrightarrow$	O_2 (water)	-14
N_2 (methanol)	$\longrightarrow$	N_2 (water)	-12
CO_2 (methanol)	$\longrightarrow$	CO_2 (water)	-10
CO (methanol)	$\longrightarrow$	CO (water)	-15
CH_4 (methanol)	$\longrightarrow$	CH_4 (water)	-15

* Data from Klotz (1960).

Table 3

Heats of hydration of non-polar hydrates (*)

Non-polar molecule	$-\Delta H$ (kcal/mole)
Ar	16.6
Kr	13.9 - 16.5
Cl_2	16.0 - 17.7
H_2S	16.5
PH_3	15.0
SO_2	16.6 - 19.0
CH_4	14.5 - 17.0
C_2H_2	15
C_2H_4	15
C_2H_6	15
CH_3Cl	15 - 18.1
CH_3SH	16.6

* Data from Klotz (1960).

when a non-polar group is introduced into water.

Methane and ethane are known to form hydrates and, since these molecules form the side chains in alanine and leucine, Klotz has assumed that when these and other non-polar side chains lie close together in a protein molecule ice-like lattices will form around the residues. This results in patches or ribbons of ice in various regions of a protein molecule and it is this, rather than a negative entropy change, that is responsible for the increased stability of the molecule.

Studies by Frank and Wen (1957) on the apparent molal heat capacity of aqueous solutions of tetra(n-butyl)ammonium bromide show that it is much higher than anticipated from established additivity rules for hydrocarbon molecules. This behaviour is explainable if the large organic cation causes a freezing of the water in its neighbourhood for then extra heat would be necessary to "melt" some of this additional "ice" if the temperature is to be raised. Frank and Wen considered that a cluster is formed when a volume of liquid undergoes a negative energy fluctuation of sufficient magnitude to overcome the disruptive influences of free water molecules at the boundaries of this volume; the cluster dissolves when these surrounding molecules succeed in transmitting into the cluster the necessary energy of melting. A non-polar solute molecule will replace the positioning of some of the free water molecules next to a cluster and it will be relatively incapable of transmitting disruptive influences due to its inability to form electrostatic interactions. Thus an ice-like cluster will form more

readily in a volume of liquid bounded by a non-polar solute particle and, once formed, it should have a longer half-life than "normal" since it has part of its boundary protected from attack. Thus there will be an increase in the overall "ice-likeness" of the solution.

Franks and Smith (1967) have studied the apparent molal volumes of tetra alkyl ammonium bromides in water and his results show that at finite concentrations the apparent molal volumes give negative deviations; this effect becomes increasingly pronounced with increase in the size of the non-polar residues or with decreasing temperature. Franks adopts the suggested interpretation of Wen and Saito (1964), namely that the alkyl groups increase the stability of the hydrogen bonded structure of water. This assumes that the volume occupied by the tetra alkyl ammonium cation will be less than the expansion of bulk water to form cages in order to accomodate this ion since it will be occupying space which would otherwise be void. When the cage system disintegrates due to thermal fluctuations there will be an increase in overall volume of the ion and the structural water surrounding it. This volume will be smaller the longer the half-life of the structured water and so, if two such ions approach each other, the structured water around each of them will become subject to mutual stabilization. This effect should increase with increasing concentration of the tetra alkyl ammonium ion and decreasing temperature, so it can be regarded as the principal contribution to the formation of solid hydrates. There will be a lengthening of the half-lives of both cages and thus a decrease in the

overall volume surrounding the cation. Franks argues that these interactions cannot be identified with hydrophobic bonding as depicted by Scheraga, since this would by definition involve a partial dehydration of alkyl groups and would therefore be expected to give an increase in total volume with increasing concentration of the cation.

Thus Franks' work lends further support to Klotz's view that it is the enthalpy term ΔH of hydration that provides the drop in internal energy which stabilizes non-polar groups in water. This assumes that non-polar groups are never actually in direct contact if they are to stabilize each other. Rather, it is the mutual interaction of the hydration spheres of the non-polar residues which causes an increase in the stability of the non-polar groups in water.

While the interpretations by Klotz and Franks of their own results are acceptable for the systems that they studied the extrapolation of these results to the interaction of non-polar groups with one another such as occurs in proteins, where non-polar groups will be within at least 10 - 20 $\mathring{A}$ of one another, is by no means proven. In dilute solutions detergents such as sodium dodecyl sulphate show negative deviations in the apparent molal volumes with increasing concentration. However at concentrations where micelle formation occurs, it is accompanied by a very large increase in the apparent molal volume of the detergent. It may be considered that only the micelle can be compared with the hydrophobic interaction, while the dilute solutions of detergents such as sodium dodecyl sulphate or tetra alkyl ammonium

bromide, as Franks and Smith (1967) say, cannot be identified with hydrophobic interactions as defined by Scheraga.

Experimental evidence for the nature of hydrophobic interactions. While it is impossible at present to be certain which view is correct, certain facts concerning non-polar groups in proteins can be demonstrated which favour Scheraga's interpretation of the hydrophobic interaction.

(1) X-ray crystallography of globular proteins, such as haemoglobin, myoglobin, lysozyme and ribonuclease crystals, shows no ordered sheets of water surrounding the protein molecule, even though the crystals contain up to 40% by weight of water (Perutz, 1962; Blake et al., 1967; Kartha et al., 1967).

(2) Berendsen (1962) using nuclear magnetic resonance, showed that a model could be made for collagen with columns of water molecules rotating about an axis parallel to the triple helix protein strands. However Chapman and McLaughlin (1967) showed that Berendsen's model was by no means unique in being able to account for the observed changes since the same result is obtained when methanol is substituted for water.

(3) X-ray crystallography of the globular proteins so far elucidated to 2 Å resolution shows that the non-polar groups are for the most part located in the interior of the protein molecule while the more polar hydrophilic groups are on the outside of the molecule in contact with the solvent. This is exactly what Scheraga's theory would predict. While this is not conclusive evidence, since the protein

molecule in solution may be in a different conformation from the protein in the crystal, work by Quiocho et al. (1967) has shown that many of the properties of the molecule in the crystal are similar to those in aqueous solution so the conformation of the protein in the two states must be similar. It is easy to imagine that,by analogy with simple organic compounds, the protein molecule in solution can exist in a large number of conformational states all similar in conformation and free energy but differing in the actual arrangements of individual side chains. The structure obtained from X-ray crystallography is probably one of these conformations and is similar to the structure of the protein in solution.

(4) Privalov and Mrevlishvili (1967) have shown by calorimetry that the hydration of a protein is greater in the denatured than in the native state, indicating that more water is ordered by the denatured protein as its hydrophobic groups are presented to the water; i.e. the hydrophobic groups are on the inside of the molecule in the native form in agreement with Scheraga's interpretation of hydrophobic interactions.

Electrostatic or salt linkages.

It has been suggested that an important factor in maintaining the structure of native proteins is the electrostatic attraction between the positively charged amino or guandino groups and the negatively charged carboxyl groups that are present in proteins.

Consider:

$$R - A^- \text{ (aqueous)} + R' - B^+ \text{ (aqueous)} \longrightarrow R - A^- \cdots {}^+B - R'$$

When RA and RB are separated the A^- and B^+ are surrounded by strongly orientated water molecules which are highly compressed by electro-restrictive forces arising from the electric fields in the vicinity of the charges. When oppositely charged groups come into contact the electric fields no longer pass through so much water so the solvent molecules are much less strongly orientated and compressed. Thus the formation of a salt linkage should be accompanied by a large increase in total volume and entropy so salt linkages, as well as hydrophobic interactions (according to Scheraga), will be stabilized predominantly by entropy rather than enthalpy effects. Salt linkages should be strengthened by lowering the dielectric constant of the medium (by addition of a non-polar substance) while hydrophobic interactions will be weakened by addition of a non-polar substance to the aqueous phase. Conversely electrolytes will tend to strengthen the hydrophobic interaction (by decreasing the solubility of the non-polar groups in water) whereas they will weaken salt linkages by producing a stabilizing ion atmosphere around the charged groups when they are in the dissociated form.

Experimental evidence shows that solvents such as dioxan and ethanol, which are much less polar than water, act as denaturing agents towards protein whereas many electrolytes are inhibitors of denaturation. Thus the hydrophobic interaction is more important than the salt linkage in maintaining the structure of native proteins.

While direct evidence for the electrostatic bond in proteins is

limited, it has been observed that haemoglobin is dissociated by a concentration of 2M potassium chloride into two αβ subunits (Rossi-Fanelli et al., 1961).

Structure of proteins

It is convenient to refer at times to a classification of protein structure that has been proposed by Linderstrom-Lang (1951). This classification has four divisions termed the primary, secondary, tertiary and quaternary structures. The distinctions between these structures may be made in terms of the nature of the interactions necessary for their maintenance.

The primary structure is defined by its covalent bonding, that is, the number and sequence of amino acid residues linked by peptide bonds.

Any ordering of the otherwise flexible chains resulting from the formation of hydrogen bonds between carbonly oxygen and amide nitrogen atoms of the polypeptide backbone is termed the secondary structure.

Interactions by disulphide and non-covalent bonds between different residues on the same or other polypeptide chains results in a three dimensional structure which is termed the tertiary structure.

Individual molecules can associate into dimers or higher polymers and this is the quaternary structure.

The differences between the four types of structure, as outlined by Linderstrom-Lang, are not clear cut, especially between secondary and tertiary structures, but the terminology is useful when describing simple properties of protein molecules.

Surface properties of proteins

Most studies of enzyme structure and function have been made in relatively pure aqueous solutions since the enzymes are most easily studied in this state. While these studies provide a large amount of information concerning enzyme behaviour, the results may be misleading as to the properties of most enzymes in their cellular environment. Few enzymes, apart from the proteolytic ones, function in dilute aqueous solution *in vivo*. Most function either adsorbed at interfaces or in solid state assemblages, such as appear to exist in the mitochondria.

Few enzymes have been studied in such situations and those that have mostly involve films of enzymes at an air/water interface where the adsorption process and molecular weights have been determined, rather than investigating the factors which effect biological activity.

Air/water interfaces.

Early work on protein films at these interfaces was confined to the studies of films formed over large areas, resulting in very dilute surface concentrations (Langmuir and Schaefer, 1939). In this system there is minimal contact between adsorbed molecules and, when these films are compressed, the film pressure increases very rapidly once a surface concentration of about 1 mg. of protein/square metre of interface is reached. When the films become close packed, the thickness corresponds to that of a single polypeptide chain. The original film is such that the free energy of the system of protein and surface of the liquid is minimized, since it allows the maximum number of hydrophilic side chains

to be orientated into the liquid and most if not all the hydrophobic side chains to be projected away from the aqueous phase. This type of unfolding leads to complete loss of enzymatic activity (Cheesman and Schuller, 1954) and the evidence available is that retrieval of the film does not result in the complete recovery of enzymatic activity. James and Hilborn (1968) showed that substances such as urea, which cause an increase in the surface pressure of lysozyme, i.e. increase the spreading of a given surface film of the enzyme, also cause a decrease in enzymic activity when the enzyme is assayed in the presence of these substances. Studies with pepsin (Cheesman and Schuller, 1954) and trypsin (Ray and Augenstein, 1956) have shown that all enzymic activity is lost in dilute films but some is retained in concentrated films.

With protein films formed at air/water interfaces it is found that the pH of the aqueous phase has a considerable influence and that the maximum adsorption is found at the isoelectric point. Presumably at pH values on either side of this point there are repulsive forces, arising from charged protein molecules on the surface (Cumper and Alexander, 1950).

Oil/water interfaces.

There are a number of similarities between protein adsorbed at oil/water and at air/water interfaces. When adsorption occurs at oil/water interfaces where moderate film pressures already exist the proteins apparently do not unfold (Haurowitz et al., 1955). Films of proteins at oil/water interfaces show ageing as do films at air/water interfaces;

this occurs when the film of protein gets thicker with time, probably due to a regrouping,and possible bonding together,of denatured unfolded molecules in the film (Danielli, 1938).

There are also differences between protein adsorption at the two types of interface. In some oil/water systems the surface pressure is so low (e.g. oil/water surface pressures: benzene 35 dynes cm^{-1}, ethyl ether 10 dynes cm^{-1}, butanol 1.6 dynes cm^{-1} as compared with air/water surface pressure 72 dynes cm^{-1}) that there is much less tendency for most proteins to unfold. Thus films adsorbed in these systems are more compact than their counterparts at the air/water interface (Alexander and Teorell, 1939). It is of interest that,since the interfacial forces presumed to exist intracellularly are less than 1 dyne cm^{-1}, little unfolding of protein molecules should occur under biological conditions (Cheesman and Davies, 1954). It has been observed that protein solutions form surface films more rapidly at oil/water than at air/water interfaces (Ghosh and Bull, 1963). Since the solute molecules will diffuse through the aqueous support solution at the same rate, independent of the interface, the difference in the rate of spreading must be due to the difference in the energies of activation of orientation in the two interfaces. Studies with films of synthetic linear polymers indicate that the cohesion between non-polar parts of spread molecules is greater at air/water interfaces compared to oil/water interfaces (Hotta, 1954). Thus the oil molecules at an oil/water interface are considered to penetrate between the non-polar hydrocarbon chains in a looser conformation

of protein in the surface which gives a greater area than for similar protein films at an air/water interface (Cheesman, 1952; Danielli, 1938; James and Augenstein, 1966).

Rather more information has been obtained to date on the biological activity of enzymes on oil/water interfaces than on air/water interfaces. Studies of the adsorption of catalase, trypsin, oxyhaemoglobin and chymotrypsin onto emulsified oil droplets indicate that the extent of adsorption and the retention and recoverability of enzyme activity depends on factors such as the charge on the oil droplets, the stability of the emulsion and the concentration of protein in the film and in the bulk solution (Fraser et al., 1955; Fraser and Schulman, 1956; Ghosh and Bull, 1962). In general the biological activity of an enzyme adsorbed at an oil/water interface increases with increasing concentration of this enzyme at the interface (James and Augenstein, 1966).

The loss of biological activity when enzymes are adsorbed on oil/water interfaces could be due to one of three possibilities:

(a) Unfolding at an interface results in denaturation of the enzyme.

(b) The enzyme may be orientated differently at the interface, depending on the emulsion stabilizer, so that the active centre is facing the oil phase in which the substrate is insoluble. In this case the enzyme would appear inactive on the emulsion, but active on subsequent desorption.

(c) The pH at the surface may be such that the enzymic activity is depressed.

Water/solid interfaces.

The adsorption of proteins onto solids is determined by the porosity of the solid surface and thus the extent to which the protein molecules can penetrate and become enmeshed in the pores or interstices of the solid matrix. Most studies of this type have involved adsorption onto chromatographic resins, glass or porous minerals, the effect on enzymic activity being quite different for the three types of adsorbent.

Clauser and Li (1954) showed that there was no loss of activity when various enzymes were repeatedly adsorbed and eluted on cellulose columns. Little (1953) has reviewed the effects on activity of adsorption followed by desorption which occurs during chromatography.

On glass it appears to depend on the type of adsorption as to whether enzymic activity is retained or not (James and Augenstein, 1966).

Generally adsorption on porous minerals reduces the enzymic activity (McLaren, 1963).

Besides studying the adsorption of enzymes onto solids there are many instances where enzymes have been covalently bound to a support material; e.g. chymotrypsin to carboxycellulose (Micheel and Ewers, 1949), ribonuclease to polyaminostyrene (Grubhofer and Schleith, 1954). Usually the activity of enzymes covalently bound to a support is lowered, but some increases have been reported (Sundram and Crook, 1967) and there are instances where the attached enzyme shows its full free solution activity e.g. papain (Manecke and Gunzel, 1967).

An important reason for differences in enzymic activity measured

in bulk solution and at water/solid interfaces is the different distribution of ions near the solid surfaces which are generally charged (McLaren, 1960). Thus the pH of the surface can differ by as much as 2 pH units from that of the solution (Danielli, 1937).

Interaction of proteins with non-polar solutes

One of the means of studying hydrophobic interactions in proteins is the investigation of their interactions with low molecular weight non-polar solutes. Binding of non-polar solutes to non-polar regions of proteins can have two effects:

(a) The apparent solubility of the solute can be increased, either by adsorption on the surface of the protein, or by incorporation into hydrophobic regions.

(b) The solute can induce conformational changes in the protein by interacting with the hydrophobic regions of the protein.

An example of the first effect is the work of Wishnia (1962), Wishnia and Pinder (1964), who studied the solubility of ethane, propane and butane in solutions of bovine serum albumin, haemoglobin and lysozyme. All these protein solutions increase the apparent solubilities of the hydrocarbons in water and the percentage increase of solubility of the various hydrocarbons in the different proteins is constant over a temperature range of 25°C, indicating that the enthalpy of binding is small and that the increased solubility of the hydrocarbons is due to a favourable entropy change.

Reynolds *et al.* (1967) have studied the binding of long chain alkyl

sulphates and sulphonates to bovine serum albumin. They found that the number of sites which bind sulphates strongly increases with hydrocarbon tail length of the detergent. The sulphonates were found to bind not only to a different number of sites but also with different energies when the length of the hydrocarbon tail was altered. Thus the binding affinities are strongly dependent on the character of the polar portion of the ligand, indicating the importance of salt linkages as well as hydrophobic interactions on the binding of small molecules to proteins. The same group of workers (Ray et al., 1966) studied the binding of various alkanes and alkane derivatives to bovine serum albumin and found that the hydrocarbon moiety of all these substances contributes the major share of the binding energy. Also the binding energy was found to increase markedly with increasing length of the hydrocarbon chain.

Besides studying the binding of simple aliphatic derivatives to various proteins, experiments have been carried out on the binding of fluorescent dyes to proteins. Since the fluorescence of these dyes depends strongly on the polarity of their environment it will be affected when such dyes are bound to a protein. By studying changes in the fluorescence intensity or polarization, information can be obtained regarding the nature of the binding site to which the dye is bound.

Daniel and Weber (1966) studied the binding of 8-anilino-1-naphthalene sulphonic acid magnesium salt to bovine serum albumin and showed that at pH 7 there are 5 strong binding sites on the protein for

this dye. McLure and Edelman (1966) used 2-p-toluidinylnaphthalene-6-sulphonate to study, not only the affinity of this binding of this dye to bovine serum albumin which gives similar results to those obtained with 8-anilino-1-naphthalene sulphonic acid magnesium salt, but also studied the change in fluorescence intensity of 2-p-toluidinylnaphthalene-6-sulphonate in solutions of chymotrypsinogen when the zymogen is activated by trypsin. They found that the increase in fluorescence intensity observed is proportional to the formation of active chymotrypsin (McLure and Edelman, 1967a). Since the fluorescence of the 2-p-toluidinyl-naphthalene-6-sulphonate increased when transferred from an aqueous to a non-polar environment it can be concluded that a hydrophobic region must be exposed in chymotrypsin which is not present in chymotrypsinogen. Kinetic evidence shows that the region is not part of the active site of the enzyme (McLure and Edelman, 1967b).

The other effect that may occur when non-polar molecules are bound to proteins, namely a conformational change in the protein, has been demonstrated by many groups of workers. Wetlaufer and Lovrien (1964) showed that reversible changes in viscosity, optical rotatory dispersion and ultraviolet spectrum of bovine serum albumin at alkaline pH values, and of β-lactoglobulin in the neutral pH range, occurs on the addition of various hydrocarbons. Similarly binding of sodium dodecyl sulphate to bovine serum albumin can cause changes in its conformation as shown by changes in its physical parameters (Ray et al., 1966). It is interesting to note that when less than 8 molecules of detergent are

bound per molecule of protein, the protein is stabilized against the action of urea or guanidine hydrochloride (Lovrien, 1963). Markus et al. (1964) have shown that this protective effect requires the simultaneous binding of the anionic head group to the cationic groups on the surface of the protein, indicating that the detergent acts as a bidentate ligand.

Properties of proteins

The investigation of the hydrophobic interactions of proteins was mostly confined to a study of four extracellular and three intracellular proteins. At the present time more is known of bovine serum albumin, lysozyme, ribonuclease, insulin and haemoglobin than the two intracellular proteins carbonic anhydrase and glutamic acid dehydrogenase. A summary of the properties for these proteins is given in Table 4.

Bovine serum albumin.

Bovine serum albumin is seldom obtained free from contaminants so the molecular weight is still uncertain ± 2,000 (Phelps and Putnam, 1960). Loeb and Scheraga (1956) found that their preparation contained approximately 3% dimer, the molecular weight of the major component being 67,000 compared to an average molecular weight of 69,200. Values as high as 73,000 (Halwer *et al.*, 1951) and 77,000 (Edsall *et al.*, 1950) have been obtained from light scattering experiments. King (1961) showed that there were 0.3 moles of mixed disulphide of mercaptalbumin and cysteine per mole of protein.

Few if any other proteins exhibit such strong affinity for anions and its interactions with ions, especially anions, has been extensively investigated (Foster, 1960; Gurd and Wilcox, 1956). Bovine serum albumin also binds dyes and other non-polar compounds such as fatty acids, more strongly than the other serum proteins (Grollman, 1925).

Results obtained by four independent groups of workers (Tanford, 1952; Weber, 1952; Bjornholm *et al.*, 1952; Jirgensons, 1952) showed

Table 4

Summary of the properties of proteins investigated in this work (*)

	BOVINE SERUM ALBUMIN	HEN EGG WHITE LYSOZYME	BOVINE RIBO-NUCLEASE	BOVINE INSULIN
MOLECULAR WEIGHT	69,200 (1)	14,400 (8)	13,700 (14)	5,740 (18)
SH GROUPS/ MOLECULE	0.7 (2)	0 (9)	0 (15)	0 (19)
SS GROUPS/ MOLECULE	17.3 (3,4)	4 (9)	4 (15)	3 (20)
NUMBER OF POLYPEPTIDE CHAINS	1 (5)	1 (10)	1 (16)	2 (21)
ASSOCIATION WITH pH	-	DIMERIZATION >5.4 (11)	-	DIMER TO HEXAMER >1 (22)
CONFORMATIONAL CHANGE WITH pH	<4; >9 (6)	-	-	-
NUMBER OF AMINO ACID RESIDUES	UNCERTAIN (a) (7)	129 (12)	124 (16)	51 (21)
NUMBER OF AMINO ACIDS IN EACH CHAIN	UNCERTAIN (a) (7)	129 (12)	124 (16)	21,30 (21)
PRIMARY SEQUENCE ELUCIDATED	NO	YES (10)	YES (16)	YES (21)
CONFORMATION IN CRYSTAL ELUCIDATED RESOLUTION:	NO	YES (13) 2 Å	YES (17) 2 Å	NO

*Numbers in parentheses indicate references set below.

Table 4 (continued)

	HUMAN HAEMOGLOBIN	BOVINE GLUTAMIC ACID DEHYDROGENASE	BOVINE CARBONIC ANHYDRASE
OLECULAR EIGHT	64,500 (23)	300,000 (26)	31,000 (29)
H GROUPS/ OLECULE	6 (24)	30-40 (27)	0 (30)
S GROUPS/ OLECULE	0 (24)	0 (27)	0 (30)
UMBER OF OLYPEPTIDE HAINS	4 (25)	6 (28)	-
SSOCIATION ITH pH	-	-	-
ONFORMATIONAL HANGE WITH pH	-	-	-
UMBER OF MINO ACID ESIDUES	534 (25)	2,814 (28)	-
UMBER OF MINO ACIDS N EACH CHAIN	2 x 141 2 x 146 (25)	6 x 469 (28)	-
RIMARY EQUENCE LUCIDATED	YES (25)	NO	NO
ONFORMATION N CRYSTAL LUCIDATED ESOLUTION:	YES (25) 6 Å	NO	NO

REFERENCES

1. Loeb and Scheraga, 1956.
2. Hughes, 1949.
3. Kolthoff et al., 1958.
4. King, 1961.
5. Hunter and McDuffie, 1959.
6. Yang and Foster, 1954.
7. Stein and Moore, 1949.
8. Fromageot and de Garilhe, 1950.
9. Cecil and Wake, 1962.
10. Jolles and Jolles, 1961.
11. Sophianopoulus and van Holde, 1964.
12. Thompson, 1955.
13. Blake et al., 1967.
14. Klainer and Kegeles, 1956.
15. Spackman et al., 1960.
16. Smyth et al., 1963.
17. Kartha et al., 1967.
18. Fredricq, 1956.
19. Ryle et al., 1955.
20. Cecil and Loening, 1960.
21. Sanger and Tuppy, 1951.
22. Teitze and Neurath, 1952.
23. Hill et al., 1962.

24. Allison and Cecil, 1958.

25. Perutz, 1962.

26. Eisenberg and Tomkins, 1968.

27. Hellerman et al., 1958.

28. Appela and Tomkins, 1966.

29. Lindskog, 1960.

30. Lindskog and Malmström, 1962.

(a) Due to uncertainty as to the exact value for the molecular weight of bovine serum albumin (Phelps and Putnam, 1960), Stein and Moore did not give a figure as to the number of amino acid residues in this protein.

that bovine serum albumin undergoes an important structural change at low pH. Subsequent work (Yang and Foster, 1954) showed clearly that all these experiments could be explained in terms of an expansion of the protein molecule as the pH is reduced, rather than by dissociation or association. They found that below pH 4 there was an increase in both the optical rotatory disperaion and intrinsic viscosity of bovine serum albumin in solution and that these changes were extremely sensitive to ionic strength, indicating that electrostatic linkages are involved.

Foster and Aoki (1957) demonstrated that a simple co-operative equilibrium between two species of bovine serum albumin is not sufficient to account for all the experimental facts. They considered that the protein consists of several subunits of fixed conformation linked by peptide bonds; the single polypeptide chain is thus folded into several layers or into several helices. Evidence as to the nature of these subunits was obtained by Foster and Aoki (1958) who found that the expanded "F" form of the protein, unlike the "N" form which exists between pH 4 to 9, does not bind sodium dodecyl sulphate co-operatively. Similar results were later obtained by Wishnia and Pinder (1964) who investigated the binding of gaseous hydrocarbons to bovine serum albumin.

All this evidence supports Foster's model for bovine serum albumin in which the "N" form posseses about 10 strong non-polar binding sites in the interior of the molecule, i.e. most if not all the non-polar side chains of these sites are buried between the separate subunits forming

hydrophobic cores. On lowering the pH of produce the "F" form, these hydrophobic cores are disrupted and approximately 100 weaker binding sites are exposed.

Lysozyme.

Lysozyme is an extracellular enzyme which hydrolyses the muco-polysaccharide in the cell wall of the bacterium Micrococcus lysodeikticus. Purification of this enzyme on ion-exchange and cellulose columns shows that it can contain varying amounts of minor components (Stevens and Bergstrom, 1967).

At least two of the four disulphide bridges are necessary for the biological activity to be retained (Jolles, 1964) and it has been observed that oxidation in air of the partially reduced enzyme resulted in a 60% recovery of the specific activity of the native enzyme (Churchich, 1962).

The tertiary structure of hen egg white lysozyme in the crystalline state which has been resolved at 2 Å resolution (Blake et al., 1967) shows that the polar side chains are all on the surface of the molecule. The non-polar side chains are mostly shielded from the surrounding liquid by the more polar parts of the molecule. There is a deep cleft running up one side of the molecule which has been shown to be the active site. The electron density map showing the change in electron density when tri-N-acetyl glucosamine, an inhibitor of lysozyme, is bound in the protein crystal reveals a change of conformation in the region of the active site.

Ribonuclease.

Ribonuclease is an enzyme which hydrolyses the 3-5' linkage between adjacent residues in ribonucleic acids.

Subtilisin cleaves the native enzyme between alanine at position twenty and serine at position twenty-one in the peptide chain to yield S-protein + S-peptide (Richards and Vithayathil, 1959). Neither of these subunits alone are enzymatically active but if they are mixed together at neutral pH enzymatic activity is recovered.

Reduction of the disulphide bridges by the action of thiols in 8M urea results in complete unfolding of the peptide chain (Harrington and Sela, 1959). However re-oxidation of the reduced ribonuclease in dilute aqueous solution gives complete recovery of the enzymic activity, suggesting that the amino acid sequence contains all the information necessary for correct refolding (White, 1961; Anfinsen and Haber, 1961). Several models have been proposed for the secondary and tertiary structures of ribonuclease in solution (Parks, 1960; Hammes and Scheraga, 1966).

The tertiary structure of this enzyme in the crystalline state has been resolved at 2 Å resolution by Kartha et al. (1967) who have shown that the molecule is roughly kidney shaped with a deep depression in the middle of one side. Because of its shape the molecule has a high proportion of the amino acid side chains on the surface. Consequently some of the hydrophobic side chains, which would normally be shielded from the solvent, lie near the surface. However most of the polar side

chains project outwards into the solvent. The model obtained by Avey et al. (1967) for the structure in the crystal differs in several respects from that obtained by Harker. However Kartha (1967) has shown that the difference between the two groups lie in the interpretation of the electron density maps rather than in the data itself.

Insulin.

Insulin is a polypeptide hormone concerned with the regulation of carbohydrate metabolism.

Two of its disulphide bonds are interchain while the remaining one forms part of a intrachain ring in the shorter chain. Reduction of the disulphide bonds leads to loss of biological activity; reoxidation leads to a partial reformation of the original molecule with recovery of activity (Tsou, 1966; Katsoyannis et al., 1966). An important property of insulin is its remarkable resistance to denaturation. An example of this is that the hormonal activity is not lost after exposure to 5 M urea for up to 24 hours (Phillips, 1952). The protein is insoluble in aqueous solution between pH 4 and 6.

Insulin has a molecular weight of 36,000 at neutral pH; this falls to 12,000 in acid solutions (Teitze and Neurath, 1952). The chemical molecular weight of 5,740 is only observed when organic solvents are added to the aqueous solutions (Harfenist and Craig, 1952) or in concentrated guanidine hydrochloride solutions (Kupke and Linderstrom-Lang, 1954).

Intracellular enzymes.

Carbonic anhydrase, glutamic acid dehydrogenase and haemoglobin are all intracellular proteins.

Bovine carbonic anhydrase is known to consist of two iso enzymes while human carbonic anhydrase has three (Malmstrom et al., 1964). Isoenzymes can be defined as enzymes which catalyse the same reaction but are chemically different and may have different activities. The action of this enzyme is to catalyse the conversion of carbon dioxide to carbonic acid.

Glutamic acid dehydrogenase catalyses the oxidation of glutamic acid to the corresponding imino acid which in turn is hydrolysed spontaneously to α-ketoglutaric acid and ammonia. This enzyme is irreversibly dissociated into 6 subunits of molecular weight 50,000 each by the addition of concentrated guanidine hydrochloride solution (Marler and Tanford, 1964), raising the pH above pH 10 or lowering it below pH 3 (Fisher et al., 1962). The monomers (molecular weight 300,000 each) can undergo linear polymerization which appears to increase with increasing concentration of protein. Only the 300,000 or bigger units are enzymically active (Frieden, 1963).

Haemoglobin is an oxygen carrier which combines reversibly with molecular oxygen and carbon monoxide. Each of the four peptide chains contains one haem group (Perutz, 1962). Two of the cysteine residues of haemoglobin react normally with heavy metal reagents, but the remaining four do so only very slowly; on addition of denaturing agents to

haemoglobin solution these four cysteines react normally (Allison and Cecil, 1958). The protein is unstable in solution below pH 3 to 4 (Allison and Cecil, 1958). Perutz (1962) showed that the structure of haemoglobin in the crystal was compact with the two reactive cysteines on the outside of the native protein while the other four were buried in the interior. The four haem groups lie in separate pockets of the molecule and are not in contact with each other.

EXPERIMENTAL

Materials

n-Decane.

Decane was obtained from British Drug Houses. It was washed three times with 4N sodium hydroxide and then washed repeatedly with distilled water until the washings were no longer alkaline. This decane was washed repeatedly with concentrated sulphuric acid until the acid was no longer yellow after the mixing. It was then distilled and the fraction boiling between 171°-172°C collected. A sample was injected onto a 1% silicone gum rubber gas liquid chromatography column at 100°C; only one major peak was observed and by measuring the peak height it was concluded that the decane was 99.9+ per cent pure. The decane was stored in pyrex bottles at 2°C in the dark.

Samples of decane used were:-

Batch	Referred to in Results section as
2174613	A
0629550	B
0159440	C

n-Heptane.

The purification of heptane, obtained from British Drug Houses, was the same as for decane. The fraction boiling between 98°-99°C was

collected and gas liquid chromatography showed the hydrocarbon to be 99.9+ % pure. The heptane was stored in pyrex bottles at 2°C in the dark.

n-Dodecane.

The purification of dodecane, obtained from British Drug Houses, was the same as for decane. The hydrocarbon was distilled under reduced pressure using a water pump and the major fraction which boiled within a range of 1°C was collected. Gas liquid chromatography showed the hydrocarbon to be 99.9+ % pure. The dodecane was stored in pyrex bottles at 2°C in the dark.

Decan-1-ol, Octan-1-ol, Hexan-1-ol.

These alcohols were obtained from British Drug Houses. They were distilled under reduced pressure using a water pump and the major fractions which boiled within a range of 1°C collected. Gas liquid chromatography on a 15 per cent polypropylene glycol column showed the alcohols to be 99.9+ per cent pure.

Miscellaneous alcohols.

All other alcohols were obtained from Koch-Light Laboratories Ltd., who claimed that they were all at least 98% pure by gas liquid chromatography. No further purification was carried out.

^{3}H n-decane.

^{3}H decane was obtained from the Radiochemical Centre, Amersham. It was diluted with pure decane to give a specific activity of approximately 5 mC/ml. of decane. The washing procedure for pure decane, except for the final distillation, was repeated on the diluted ^{3}H decane to remove

any ^{3}H alcohols which were present as impurities. With early batches this method was successful in producing tritiated decane which had the correct solubility in water (Batches 3 and 4). However with a later batch (Batch 5) this cleaning method was unsuccessful as the decane had far too great a solubility in water. A special purification of this batch was carried out by the Radiochemical Centre, Amersham. This purification was by thin layer chromatography on silica gel in n-hexane: ether:acetic acid (70:30:2). This specially purified ^{3}H decane still showed too great a solubility in water. This larger than expected solubility in water was probably due to ^{3}H lower hydrocarbons present in the decane which were not removed by the thin layer chromatography. The ^{3}H decane was stored desiccated in a pyrex test tube at $2^{o}C$ in the dark.

^{14}C n-decane.

^{14}C decane was obtained from the Radiochemical Centre, Amersham. It was diluted with pure decane to give a specific activity of 0.05 mC per ml. of decane. The washing procedure as carried out on the ^{3}H decane was repeated and ^{14}C decane with the correct solubility in water was obtained. The ^{14}C decane was stored desiccated in a pyrex test tube at $2^{o}C$ in the dark.

^{14}C decan-1-ol.

^{14}C decan-1-ol was obtained from International Chemical and Nuclear Corporation. It was diluted with pure decan-1-ol to give a specific activity of 0.02 mC/ml. The isotopic purity of the ^{14}C decan-1-ol

was claimed to be 99%. No further purification was attempted. The ^{14}C decanol was stored desiccated in a pyrex test tube at 2°C in the dark.

Bovine serum albumin.

Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co. Ltd. Four different samples were used in the course of these experiments as follows:

Batch	Referred to in Results section as
LK 0871	A
MA 1670	B
MH 2470	C

Solutions were made up in pH 7.0 buffer and concentrations estimated from the absorption at 280 mμ. Foster and Sterman (1956) give $E^{1\%}_{1cm}$ = 6.67. It was stored at 2°C in a desiccator.

Fatty acids were removed from bovine serum albumin by the addition of charcoal (Chen, 1967). 0.2 g. of protein was dissolved in 20 ml. of distilled water. 0.1 g. of Norite Sx2 was mixed into the solution and the pH was lowered to 3.0 by the addition of HCl.. The solution was placed in an ice bath and mixed for 1 hour. Charcoal was removed by centrifugation and the clear solution stored at 2°C.

Lysozyme.

Lysozyme (2 x crystallized salt free from hen egg white) was obtained from Worthington Biochemical Co. This enzyme was also obtained from Sigma Chemical Co. (3 x crystallized, dialysed and lyophilized from

hen egg white). Three samples were used in the course of these experiments:

Batch	Referred to in Results section as
Worthington 6JA	A
Worthington 7GA	B
Sigma 96B-8571	C

Solutions were made up in pH 2.0 buffer and concentrations were estimated from the absorption at 280 mμ. Sophianopoulus et al. (1962) give $E^{1\%}_{1cm}$ = 26.35 at pH 5.4. For a given concentration of lysozyme it was found that the optical density at pH 5.4 is identical with that at pH 2.0 and pH 7.0. The protein was stored at 2°C in a desiccator.

Ribonuclease.

Ribonuclease (5 x crystallized from bovine pancreas) was obtained from Sigma Chemical Co. Two samples were used in the course of these experiments:

Batch	Referred to in Results section as
115B-8720	A
17B-8630	B

Solutions were made up in either pH 2.0 or pH 7.0 buffer and concentrations estimated from the absorption at 278 mμ. Sela et al. (1957) give the molar extinction coefficient as E = 9,800. It was stored at 2°C in a desiccator.

Insulin.

Crystalline (zinc) beef insulin was obtained from Boots Pure Drug

Co. Ltd. It was stored at 2°C in a vessel containing a saturated solution of calcium chloride, which maintains a relative humidity of 40% (Handbook of Chemistry and Physics, 1951-52). The moisture content of insulin so stored is 10% (Weitzman, 1962). Two samples were used in the course of these experiments:

Batch	Referred to in Results section as
10931 D	A
12779 N	B

Solutions were made up in 0.01 N HCl and concentrations were estimated from the absorption at 277 mμ. The molar extinction coefficient is given by Cecil and Weitzman (1964) as ϵ = 6,130.

Carbonic anhydrase.

Carbonic anhydrase (ex-bovine erythrocytes, salt free lyophilized, Lot No. 17512) was obtained from Koch-Light Laboratories Ltd. Solutions were made up in pH 7.0 buffer and concentrations estimated from the adsorption at 280 mμ. Nyman and Lindskog (1960) give $E^{1\%}_{1cm}$ = 19.0. It was stored at 2°C in a desiccator.

Glutamic acid dehydrogenase.

Glutamic acid dehydrogenase (ammonium sulphate suspension, Lot No. 6028450) was obtained from C.F. Boehringer Co. Ltd. Solutions were made up in pH 7.0 buffer and concentrations estimated from the absorption at 280 mμ. Olson and Anfinsen (1952) give $E^{1\%}_{1cm}$ = 9.7.

Haemoglobin.

Normal human erythrocytes were kindly made available by the

Biochemistry Department, Radcliffe Infirmary, Oxford. These were treated as described by Allison and Cecil (1958). Carboxyhaemoglobin was obtained by bubbling carbon monoxide through haemoglobin solution for ten to fifteen minutes. Solutions were estimated by using ϵ millimolar = 59.6 at 540 mμ for four Fe atoms per molecule of carboxyhaemoglobin (Allison and Cecil, 1958).

Micrococcus lysodeikticus.

Micrococcus lysodeikticus (Dried cells) was obtained from Sigma Chemical Co. It was stored at 2°C in a desiccator.

Sodium dodecyl sulphate.

Sodium dodecyl sulphate was obtained from L. Light and Co Ltd. The detergent was recrystallized three times from 50% aqueous methanol solution.

Cytidine-2',3'-cyclic monophosphoric acid sodium salt.

Cytidine-2',3'-cyclic monophosphoric acid sodium salt was obtained from Sigma Chemical Co. It was stored at 2°C in a desiccator.

Buffer solutions.

In experiments where some property of a protein was studied with varying pH, the stock solution of protein twice the final concentration, was made up in water and the various pH values were obtained by adding 3 ml. of protein solution to 3 ml. of 0.2 M buffer solution of the required pH. In all experiments, unless otherwise stated, the buffer solutions were 0.1 M.

The buffer solutions were made up as follows:

pH 2.0	0.1 M	:	0.01 M HCl, 0.1 M NaCl
pH 2.0	0.2 M	:	0.02 M HCl, 0.2 M NaCl
pH 2.8	0.2 M	:	0.2 M glycine, 0.029 M HCl
pH 3.0	0.2 M	:	0.2 M glycine, 0.011 M HCl
pH 3.5	0.2 M	:	0.128 M formic acid, 0.072 M sodium formate
pH 4.0	0.2 M	:	0.164 M acetic acid, 0.036 M sodium acetate
pH 4.5	0.2 M	:	0.114 M acetic acid, 0.086 M sodium acetate
pH 5.0	0.2 M	:	0.060 M acetic acid, 0.140 M sodium acetate
pH 5.5	0.2 M	:	0.023 M acetic acid, 0.177 M sodium acetate
pH 6.0	0.2 M	:	0.024 M Na_2HPO_4, 0.176 M NaH_2PO_4
pH 6.24	0.1 M	:	0.021 M Na_2HPO_4, 0.079 M NaH_2PO_4
pH 6.5	0.2 M	:	0.063 M Na_2HPO_4, 0.137 M NaH_2PO_4
pH 7.0	0.1 M	:	0.061 M Na_2HPO_4, 0.039 M NaH_2PO_4
pH 7.0	0.2 M	:	0.122 M Na_2HPO_4, 0.078 M NaH_2PO_4
pH 7.5	0.2 M	:	0.166 M Na_2HPO_4, 0.034 M NaH_2PO_4
pH 8.0	0.2 M	:	0.2 M tris(hydroxymethyl)aminomethane, 0.114 M HCl
pH 8.5	0.2 M	:	0.2 M tris(hydroxymethyl)aminomethane, 0.060 M HCl
pH 9.0	0.2 M	:	0.2 M glycine, 0.035 M NaOH
pH 9.5	0.2 M	:	0.2 M glycine, 0.078 M NaOH
pH10.0	0.2 M	:	0.2 M glycine, 0.128 M NaOH

Scintillator fluid.

Scintillator fluid was made up as follows:

8 grms	2-(4'-tert Butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole (obtained from CIBA Limited)
60 grms	naphthalene
100 ml.	methanol

Add dioxan to 1000 ml.

Other reagents.

All other reagents were analytical grade.

Apparatus and Methods

Estimation of protein concentrations.

Where the protein might have undergone some conformational change affecting the absorption spectrum, concentrations were measured by the method of Lowry et al. (1951). The components of the alkaline copper tartrate reagent were stored as three separate reagents [0.2 N NaOH, 4% (w/v) Na_2CO_3; 0.5 % (w/v) $CuSO_4 \cdot 5H_2O$; 1% (w/v) potassium sodium tartrate] and mixed (in ratios by volume 50:1:1) before use. A volume of 1 ml. of alkaline copper tartrate reagent was used and a sample volume made up to 1 ml. with water if necessary. The sample and reagent were mixed and stood at room temperature for 10 minutes before addition of 0.2 ml. of Folin-Ciocalteau reagent (British Drug Houses Ltd.) diluted 1 part plus 2 parts of water. The extinction at 750 mμ was read in 0.5 cm. cuvettes after a further 30 minutes. Standard curves were prepared using the proteins concerned.

Formation and treatment of emulsions.

In all emulsion experiments, unless otherwise stated, 6 ml. samples of aqueous protein solution were pipetted into 15 ml. Quickfit glass stoppered test tubes. The required volume of hydrocarbon was then added to each tube. These tubes were fixed in clips screwed onto a hardboard frame which covered one side of a bicycle wheel. A string belt was used to connect the outer rim of the wheel with a small electric motor which rotated the wheel approximately 30 times per minute. The tubes, unless otherwise stated, were left on the wheel for 24 hours

during which time an opaque emulsion was formed.

After 24 hours the tubes were removed. Originally the emulsion was removed by spinning the contents of each tube in a plastic tube for 30 minutes at 10.000 r.p.m. in a small bench centrifuge. The plastic tube was removed and held in a solid CO_2/ethanol solution which froze the contents of the tube. The supernatant was then removed by sawing through the frozen test tube just below the level of the supernatant emulsion. The plastic test tube minus top was placed in a glass test tube and allowed to thaw; the contents of the tube were removed and assayed. Later a better technique was evolved for removing the emulsion. In this the emulsion was centrifuged in a Beckman Model L preparative ultracentrifuge using an SW 39 rotor at 38,000 r.p.m. at 2°C for 30 minutes. The tubes were then removed from the rotor and a hole pierced in the bottom of each in turn. The first 4 ml. was retained for assaying and, if desired, the supernatant emulsion collected. Identical results were obtained with both methods but the latter was much simpler and also the supernatant emulsion could be separated and assayed if required.

Formation of emulsions by ultrasonic vibration.

Hydrocarbon emulsions were also formed by ultrasonic vibration using an MSE 100 watt ultrasonic disintegrator. The tip of the probe was positioned just below the surface of the liquid which was to be emulsified and the machine operated on auto; this setting ensures that there is maximum possible acoustic energy at the coupling face of the probe, despite changes in load and acoustic properties of the material

being emulsified.

Measurement of particle size.

The attempted measurement of the particle size distributions of different emulsions was carried out using a Coulter counter model A (Medical). From the particle size distribution, the surface area of the emulsion could be estimated. A 30 μ aperture diameter orifice was used with the counter.

Emulsion particles were photographed using a Gillett and Sibert Ltd., Conference microscope.

Lysozyme activity.

The method of assay of lysozyme is that described by Shugar (1952b) in which the rate of lysis of Micrococcus lysodeikticus cells is followed spectrophotometrically at 450 mμ. The assays were always carried out as follows:

	Test sample	Blank
Suspension of lyophilized M. lysodeikticus 0.1 mg./ml. in pH 6.24 M = 0.1 buffer	2.5 ml.	—
Enzyme approximately 0.015 mg./ml. added at zero time	0.5 ml.	—
pH 6.24 M = 0.1 buffer	—	3.0 ml.

Both the test sample and blank were thermostated at 25 ± 0.1°C.

The decrease in optical density was measured on an external recorder set at 0.1 optical density for a full scale deflection. A straight line was drawn through the initial slope of the optical density change and the slope of this line was recorded (ΔE_{450}/minute).

Ribonuclease activity.

Ribonuclease was assayed by the method described by Crook et al. (1960). This is a spectrophotometric assay based on the change in ultraviolet absorption spectrum resulting from the hydrolysis of cytidene 2':3'-phosphate by ribonuclease to cytidene 3'-phosphate. The assays were always carried out as follows:

	Test sample	Blank
Solution of cytidene 2':3'-phosphate in pH 7.0 m = 0.1 buffer	2.5 ml.	3 ml.
Enzyme approximately 0.2 mg./ml. added at zero time	0.5 ml.	-

Both the test sample and the blank were thermostated at 25 $\pm$ 0.1^{o}C for the assay. The maximum difference in the extinction coefficients is at 284 mμ. The increase in optical density at 284 mμ was recorded on an external recorder set at 0.1 optical density for a full scale deflection. A straight line was drawn through the initial slope of the optical density change and the slope of the line was recorded ($\triangle E_{284}$/10 minutes).

Dialysis.

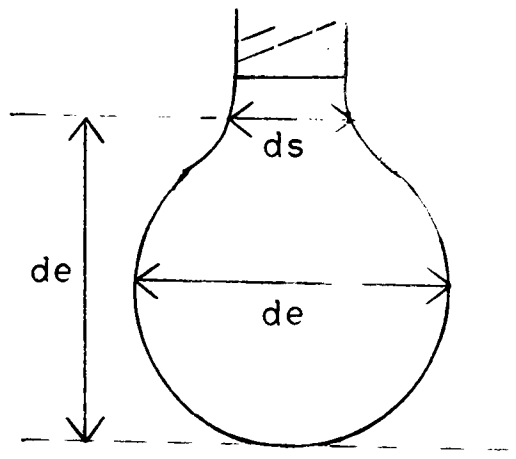
Dialysis was carried out using Visking tubing which had been previously soaked in distilled water for half an hour before the solution to be dialysed was added.

With protein/sodium dodecyl sulphate dialysis, the tubing was first immersed in boiling saturated sodium carbonate solution for half an hour and then soaked in changes of distilled water until all the carbonate had been removed.

Surface tension.

The surface tension of decane/aqueous interfaces was measured by the pendant drop technique (Andreas et al., 1938; Fordham, 1948). A 1 cm. cell, filled with decane, was placed in a thermostated Hilger cuvette holder. The aqueous solution was contained in an Agla syringe, the tip of which projected below the surface of the decane. Measurement of the drop size by means of a travelling microscope was effected as soon as possible after the drop had been formed.

The surface tension is derived as follows:



Tip of Agla.

Drop

The maximum diameter of the drop de is measured. The distance from the bottom of the drop equal to de is calculated and the diameter at this point ds is measured. The shape of the drop can be described by the ratio:

$$S = \frac{ds}{de}$$

S is defined as a function of H and the surface tension T is given by the

equation:

$$T = \frac{gxde^2}{H}$$

Where x = difference in density between the two fluids, g = acceleration of gravity and H is calculated from S using the values given by Fordham (1948).

Ultracentrifugal analysis.

Sedimentation coefficients were measured in standard and wedge cells using a Beckman Model E analytical ultracentrifuge. The protein solutions were centrifuged at 67,770 r.p.m. and the movement of the concentration boundary position measured by taking photographs of it at 16 minute intervals for up to 80 minutes. The distance of these boundaries from the axis of rotation were measured with a microcomparitor. The data was analysed using the equation originally derived by Lamm (1929) which provides a definition of the sedimentation coefficient as the proportionality constant relating the mass transported across a surface per unit time to the concentration at the surface, the area of the surface and the magnitude of the centrifugal field. The sedimentation coefficient is given as:

$$s = \frac{dx/dt}{w^2x}$$

Where x is the distance of the boundary from the axis of rotation at time t, w is the angular velocity and s has units cm./sec./dyne/g or sec. Sedimentation coefficients are recorded in svedbergs (S) where $1S = 10^{-13}$ sec.

Sedimentation coefficients are evaluated from the slope of the line

relating the logarithm of the boundary position to the time. This is based on the alternative form of Lamm's equation:

$$S = \frac{1}{w^2} \cdot \frac{d\ln x}{dt}$$

Molecular weights were determined by the sedimentation equilibrium method using the short column cells designed by Yphantis in the same ultracentrifuge used for sedimentation velocity experiments. In this type of cell a solution of protein attained sedimentation equilibrium within 2 hours spinning at 20,410 r.p.m. Samples of protein were dialysed against suitable buffer for 24 hours at 2°C before analysis. Photographs were taken of protein solutions at equilibrium using the Schlieren optical system. These were measured and the variations of the gradient of protein concentration with distance from the meniscus derived. The data was analysed by using special forms of the sedimentation equilibrium equation in which functions of the two measured variables dc and dr could be linearly related for a homogeneous protein. Suitable forms of the sedimentation equilibrium equations are described by Lamm and by Van Holde and Baldwin (Schachman, 1959).

Lamm has shown that a plot of $\log_e K\left(\frac{dc}{dr}\right)\frac{1}{r}$ against r^2 gives a straight line for a homogeneous protein, since the 2 functions are related by the equation:

$$\log_e K\left(\frac{dc}{dr}\right)\frac{1}{r} = K'Mr^2 + K''$$

Where r is the distance from the centre of rotation, $\frac{dc}{dr}$ is the concentration gradient, M is the molecular weight, K, K' and K" are

constants. K is dependent on the vertical magnification of the Schlieren optical system. $K\frac{dc}{dr}$ is defined as the actual height measured from a baseline on the photograph. K' is the function of a number of constants:

$$K' = \frac{(1 - \bar{V}po)w^2}{2RT}$$

Where $\bar{V}$ is the partial specific volume of the protein, po is the density of the solvent, w is the angular velocity, R is the gas constant and T the temperature. From the two equations it can be seen that the slope of the Lamm plot, K'M, is dependent on the molecular weight and the partial specific volume of the protein at constant temperature, rotor velocity and solvent density.

Van Holde and Baldwin have shown that a plot of $\left(\frac{dc}{dr}\right)\frac{1}{r}$ against $(C - C_R)$ gives a straight line for a homogeneous protein, since the two functions are related by the equation:

$$\left(\frac{dc}{dr}\right)\frac{1}{r} = \frac{M}{K_1}(C - C_R) + \frac{M}{K_2}C_R$$

Where C is the absolute concentration of protein and C_R is the concentration of protein at the meniscus. The term $(C - C_R)$ is thus the concentration of protein relative to the concentration at the meniscus. The slope of this type of plot is $\frac{M}{K_1}$ and the intercept when $C - C_R = 0$ gives:

$$\left(\frac{dc}{dr}\right)\frac{1}{r} = \frac{M}{K_2}C_R$$

Gas chromatography.

Gas chromatography was carried out using a Perkin-Elmer Model F11 gas chromatograph.

The columns used were:

1½% silicone gum rubber on HMDS Chromosorb W.

20% di-isodecyl phthalate on Chromosorb P.

15% polypropylene glycol on Chromosorb W.

15% Apiezon L on Chromosorb P.

20% Polyethylene glycol succinate ester on Chromosorb P.

15% Carbowax on Chromosorb W terminated with 20% terephthalic acid on Chromosorb P.

Liquid scintillation counting.

The radioactivities of both ^{14}C and ^{3}H samples were measured in an IDL Tritium Scintillation Counter. 0.5 ml. of the sample was added to 4.5 ml. of scintillator fluid contained in a clean glass pot. If insoluble protein was present which was found to quench the efficiency of the counting, then 2 ml. of sample was added to 18 ml. of scintillator fluid. This was shaken and stood for 12 hours. The precipitate was removed by spinning at 2,000 r.p.m. for 5 minutes and 5 ml. of the clear supernatant pipetted into each pot for counting.

Determination of sodium dodecyl sulphate concentration.

Sodium dodecyl sulphate was estimated by forming a methylene blue complex of the detergent which is soluble in chloroform (Mukerjee, 1956). The optical density of the dry chloroform solution was read at 655 mμ.

5 ml. of the detergent sample was added to 5 ml. of methylene blue (25 mg./litre) and 5 ml. of chloroform. A blank correction was determined for the dye extracted when no complex is present. With dialysis experiments only the outside concentration of detergent could be measured as the absorption spectrum of the dye is altered by protein.

Purification of water for surface tension determinations.

Distilled, deionized water was added to 1 g. of $KMnO_4$ and 1 g. KOH. The water was distilled and stored in pyrex bottles in the dark.

Treatment of powdered glass.

20 g. of powdered glass was added to a 250 ml. cylinder to which 200 ml. water was added. Repeated decanting of both the light supernatant and heavy particles was carried out. The final product was washed with concentrated sulphuric acid, washed with distilled water and dried in an oven.

When dry, the glass which was to be treated with dichlorodimethyl silane was added to a 5 per cent solution of this compound in carbon tetrachloride. The mixture was shaken for one hour, the glass filtered off and washed with pure carbon tetrachloride to remove any dichlorodimethyl silane which had not chemically conbined with the glass. The glass was dried in an oven before use.

Fluorescence measurements.

Measurements were recorded using either a Zeiss or an Aminco Bowman spectrofluorimeter.

With the Zeiss the slits on the monochromators were set at values

between 0.1 to 0.5 mm. 1 cm. cells were used and all measurements were made relative to a standard fluorescent block. The cell was illuminated from the side and the fluorescence viewed through the bottom.

With the Aminco Bowman 1 cm. cells were used as with the Zeiss but the cell is illuminated through one side and the fluorescence viewed through the adjacent side at 90°.

Measurement of ultraviolet extinctions, absorption spectra and difference spectra.

Ultraviolet extinctions were measured with a Hilger Uvispek and a Unicam SP800 spectrophotometer which was used in conjunction with a Servoscribe recorder in order to obtain an expanded scale for either optical density or wavelength. Quartz cells of path length 1.0 cm. and 0.5 cm. were used. Measurements in the Hilger were at room temperature while all measurements in the Unicam SP800 were made at 25°C using a thermostated cell holder. The Unicam posseses a second cell position immediately in front of the photomultiplyer. This position was used for studying the extinctions of particularly cloudy solutions; this cell position was not thermostated.

Absorption spectra were recorded with the Unicam under the same conditions used for measuring extinctions.

Difference spectra were recorded by first comparing the solution to be examined with a solution of the native protein so that the 280 mμ absorption of the two solutions was identical. The native protein solution was placed in the blank position in the Unicam and the solution

to be examined was placed in the sample cell position. The base line was checked after each series of runs using buffer in both cells.

Measurement of infra-red spectra.

Infra-red spectra were measured by means of a Perkin-Elmer Model 257 grating infra-red spectrophotometer. This machine was able to scan the spectrum of each sample from 2.5 μ to 16 μ.

Measurement of optical rotation.

All measurements were made on a Bendix-Ericson Polarmatic 62 recording spectropolarimeter.

All measurements were made with a 10 mm. cell (volume 0.6 ml.), which was washed dried and carefully reassembled prior to each series of experiments. The cell was thermostated at 25°C. The instrument was calibrated at 18.3 wave numbers using a standard sucrose solution.

The rotation at 20 wave numbers of the empty cell compartment was recorded at the beginning and end of each spectrum for each sensitivity used. The spectrum could then be referred to this internal standard and allowance made for any drift of the instrument during runs. The observed rotation of a protein solution was obtained by recording the rotation of the cell containing the buffer solution only and subtracting this rotation from that of the protein solution.

The specific rotation $[\alpha]_\lambda$ is given by:

$$[\alpha]_\lambda = \frac{\alpha_\lambda \cdot 10}{c}$$

Where α_λ is the observed rotation at wavelength λ and c is the concentration

of protein per ml. of solution. For the Moffit plot $(m')_\lambda \left[\frac{\lambda^2 - \lambda_o^2}{\lambda^2}\right]$ was plotted versus $\left[\frac{\lambda_o^2}{\lambda^2 - \lambda_o^2}\right]$ where λ_o is a constant for each protein (λo = 212 mμ for insulin), λ is the wavelength, $(m')_\lambda = [\alpha]_\lambda \times 1.14 \times \frac{3}{n^2 + 2}$, and n is the refractive index of the buffer in which the insulin is dissolved. The Moffit parameters ao and bo are obtained from the intercept and the slope respectively.

RESULTS

Surface tension measurements

Initial experiments on the estimation of the surface tension of a decane/water interface gave values which decreased with time. It was found that this was due to impurities present in the water which at that time had not been purified as described in methods. When the water was purified a value of 51.2 dynes cm^{-1} at 25°C was obtained and the surface tension did not change when the drop was left hanging in the decane for up to 30 minutes. This is in good agreement with the value of 52.0 dynes cm^{-1} obtained by Aveyard and Haydon (1965).

Attempts were made, by means of a second syringe in the decane, to inject a small amount of protein and surface active agents such as decylamine, on to the surface of the drop but it was found that, unless the initial size of the drop was very small, it fell off as soon as the protein or amine was injected. It was concluded that the only way this problem could be overcome was to dissolve the protein in the aqueous phase, or the amine in the decane, before forming the drop. However it was impossible to determine the concentration of protein or surface active agent at the interface by this technique. Before experiments using proteins or surface active agents at interfaces could be interpreted, the concentration of these substances at the interface had to be determined by other techniques.

Preliminary measurements of protein concentration at interfaces

Hydrocarbon/water interface.

An evaporating dish with a cross sectional area of 51 sq. cm. was taken and 25.5 ml. of water added to give a layer 5 mm. deep; this was covered by a layer of decane 1 mm. thick (i.e. 5.1 ml.). A Conway burette was filled with a solution of bovine serum albumin (1 mg./ml.) in pH 7.0 buffer, the tip of the burette projecting into the aqueous phase. This phase was equilibrated with the added protein solution by means of a magnetic stirrer. Various additions of protein were made and, 15 minutes after each addition, 3 ml. of the aqueous layer was removed and the concentration of bovine serum albumin measured; this was derived from the ultraviolet absorption at 215 mμ where it was found that $E^{1\%}_{1cm} = 231$, giving an increase in optical density of 0.01 on the addition of 0.01 ml. of protein to the 26.5 ml. of water.

It had been expected that there would be no increase in optical density until a monolayer of protein had been formed at the hydrocarbon/water and glass/water interfaces. The readings obtained however, were very erratic, the optical density of the aqueous phase having increased by over 0.04 optical density on the addition of the first 0.01 ml. of protein solution. Careful examination of the aqueous phase after the addition of protein showed that there was some turbidity in the solution, presumably due to the formation of emulsion particles. This would invalidate any optical density measurement at 215 mμ.

In an attempt to increase the accuracy of the measurements the concentration of protein was measured by spectrofluorimetry. Before reading the fluorescence intensity of the protein solutions they were spun at 5,000 r.p.m. for 10 minutes to remove any emulsion present. The bovine serum albumin was excited at 287 mμ and the emission recorded at 350 mμ. The concentrations measured by this technique were still very erratic and no meaningful results could be obtained.

Hydrocarbon coated glass/water interface.

Powdered glass (0.5 g.), treated with a solution of dichloro dimethyl silane, was added to 10 ml. of bovine serum albumin solution at pH 7.0 contained in a stoppered test tube. Another tube was filled with the same consitituents except that the glass had not been treated with dichloro dimethyl silane.

The two tubes were equilibrated on the wheel at 25° for 12 hours. The aqueous solutions were placed in centrifuge tubes and spun at 10,000 r.p.m. for 10 minutes to remove any glass particles suspended in the liquid. It was never possible to remove the suspended glass completely from the protein solution by centrifugation and a correction for scattered light had to be applied in the estimation of the protein concentration from optical measurements. The ratio of the optical density of the protein at 280 mμ to that at 320 mμ was 69 while that for the glass suspension was 2:1. It was thus possible to obtain the protein concentration by the solution of simultaneous optical density equations. It was found that protein was adsorbed on the untreated glass but little

Figure 2. Equilibrium concentration of lysozyme remaining in solution, plotted against the volume of pure decane added to a solution of lysozyme at pH 2.0.

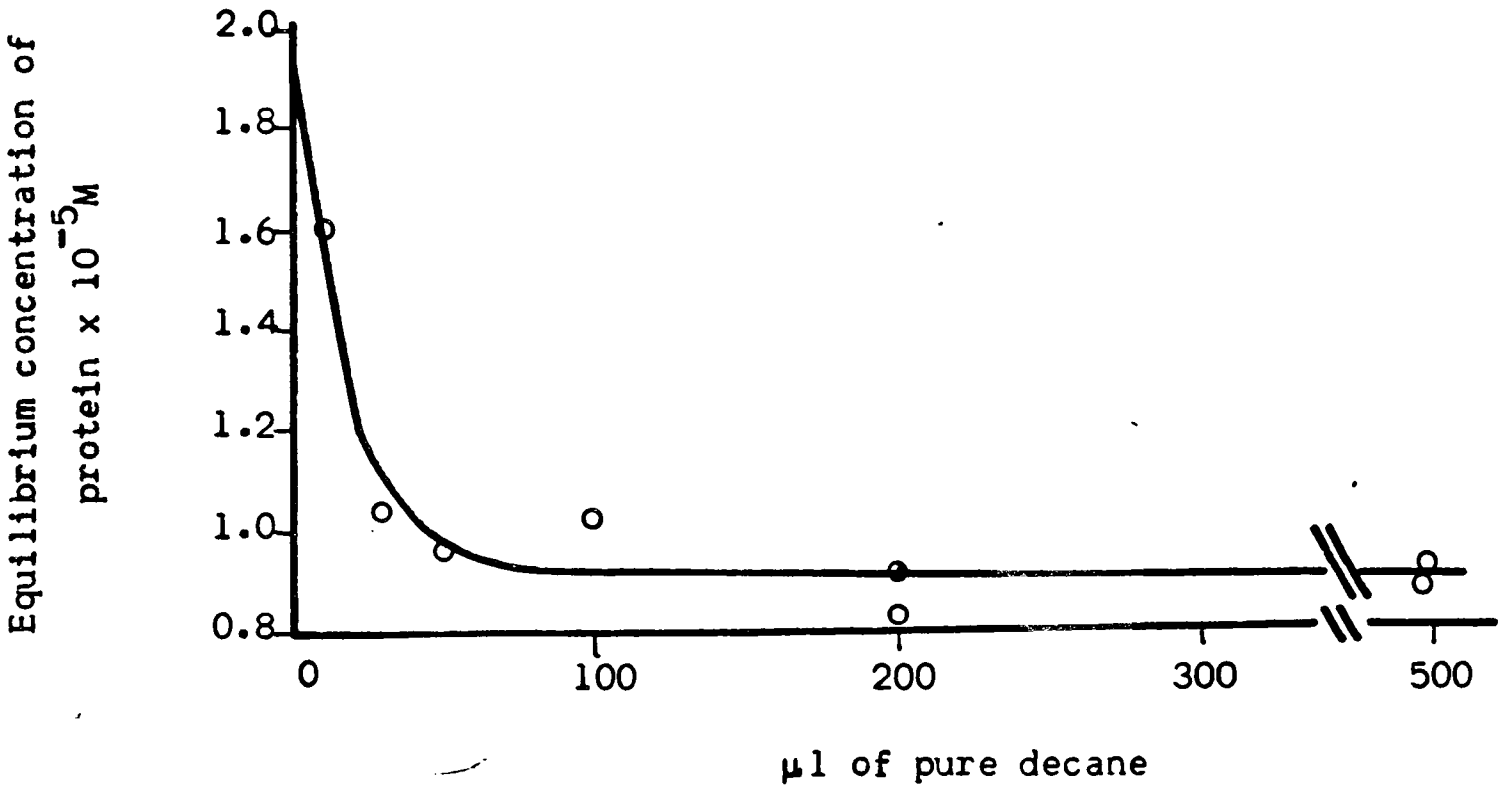
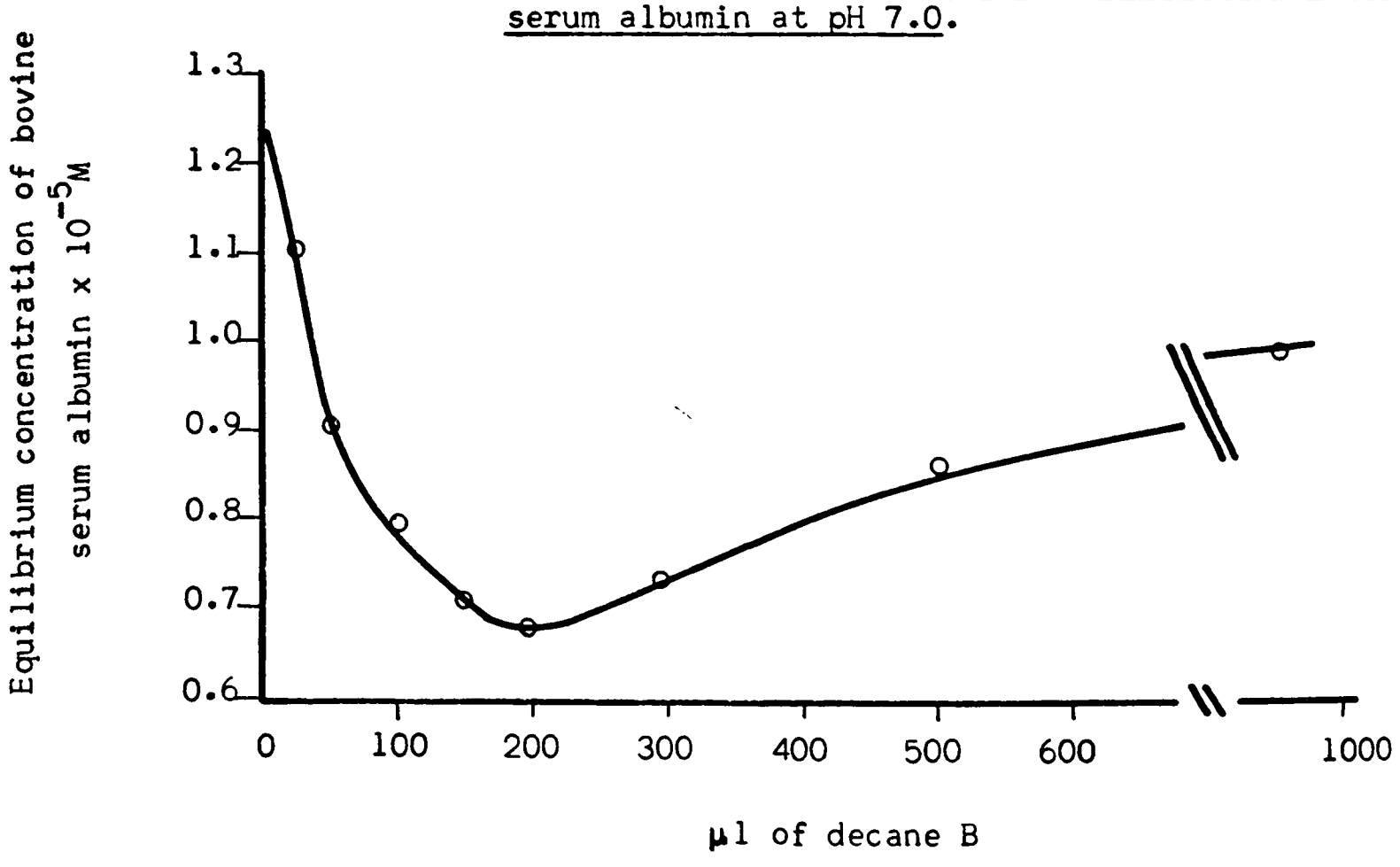


Figure 3. Equilibrium concentration of bovine serum albumin remaining in solution plotted against the volume of impure decane B added to a solution of bovine serum albumin at pH 7.0.



if any adsorbed on the silane-treated glass. This result is in agreement with results obtained by Bull (1956) who found that bovine serum albumin was adsorbed by untreated glass.

Hydrocarbon in water emulsions

In order to determine the amount of protein adsorbed at a hydrocarbon/water interface, the adsorption of protein on decane emulsions in buffered aqueous solution was investigated. The emulsion was formed by adding a given volume of decane to 6 ml. of protein solution and this mixture agitated on a wheel for 24 hours by which time an opalescent emulsion had formed. By measuring the decrease in protein concentration the amount of protein adsorbed by a given volume of decane was found.

Figure 2 shows the effect of increasing volumes of decane on the molarity of lysozyme remaining in solution after the emulsion had been removed by ultracentrifugation. Similar shaped graphs were obtained with insulin, bovine serum albumin and ribonuclease but the volume of decane at which there was no further loss of protein from solution varied from protein to protein. Substitution of heptane or dodecane for decane had no effect on the amount of protein lost from solution.

Effect of impurity on emulsions.

In an experiment with a new batch (B) of decane an anomalous result was obtained. Instead of the type of curve obtained with batch A (decane used in figure 2), it was found that there was a maximum loss of protein from solution when a given volume of decane had been added to a solution of bovine serum albumin as shown in figure 3. A similar result was

obtained with a 2×10^{-5}M solution of lysozyme at pH 2.0 where the maximum loss of protein from solution occurred with 30 μl of decane B. For convenience this volume of decane required to produce the maximum loss of protein from solution will be referred to as the "volume of decane at the dip".

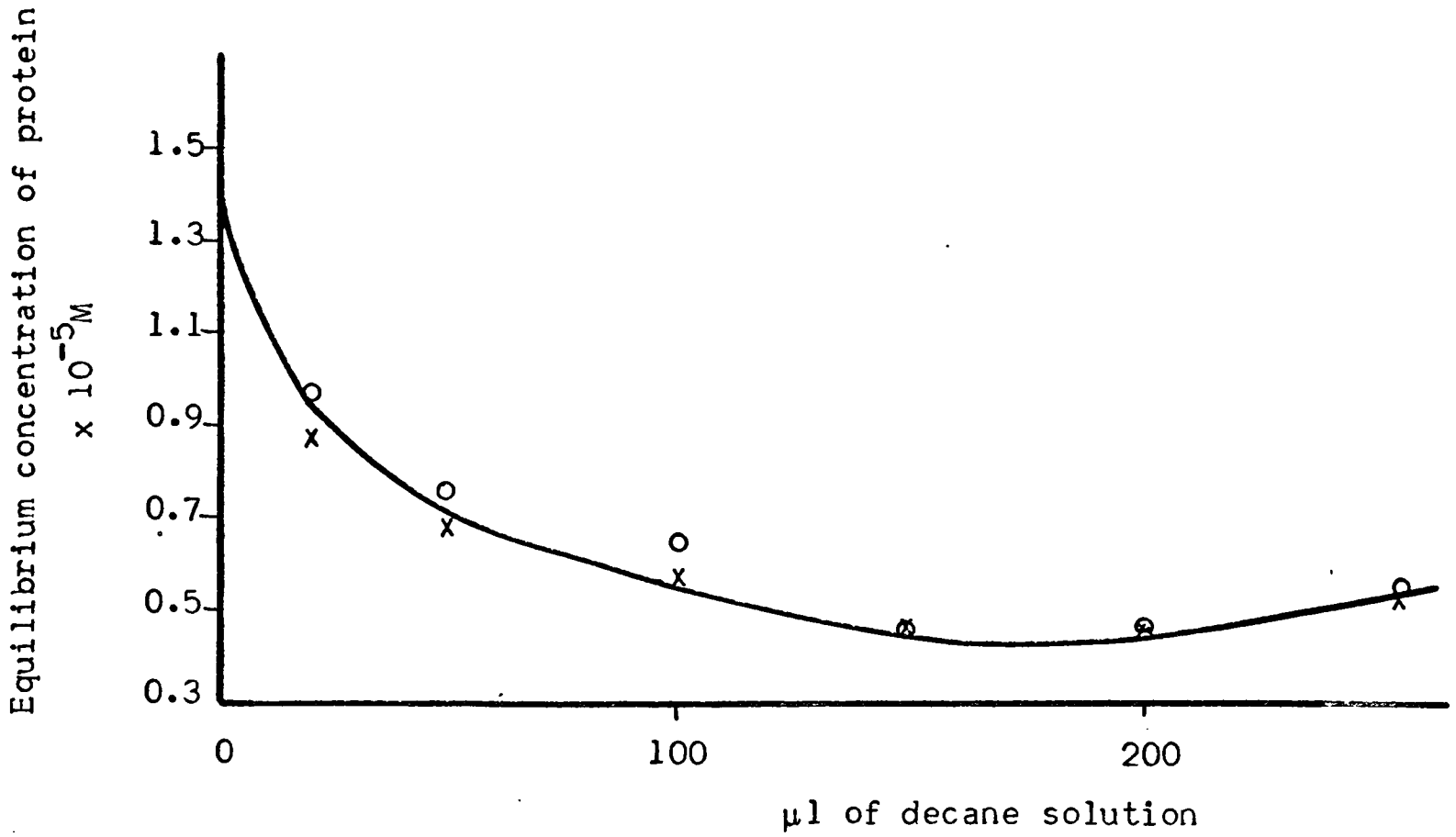
Identification of impurity.

This new batch (B) of decane had been purified four months before it was used so it was possible that an impurity had been produced in the decane due to photo-oxidation. This should have been very unlikely as the decane had been stored at 2°C in a pyrex bottle in the dark. However an attempt was made to identify the impurity which was giving these anomalous results. Since it was competing with the protein for the surface of the emulsion, it was thought to be a long chain carbon compound containing a polar end group.

An infrared spectrum of decane B was recorded and a sharp peak was observed at 2.74 μ corresponding to a free hydroxyl stretching frequency. A smaller peak was observed at 9.5 μ which is characteristic of the hydroxyl bending vibrations of a primary alcohol. This suggested that the impurity in decane B was a primary alcohol.

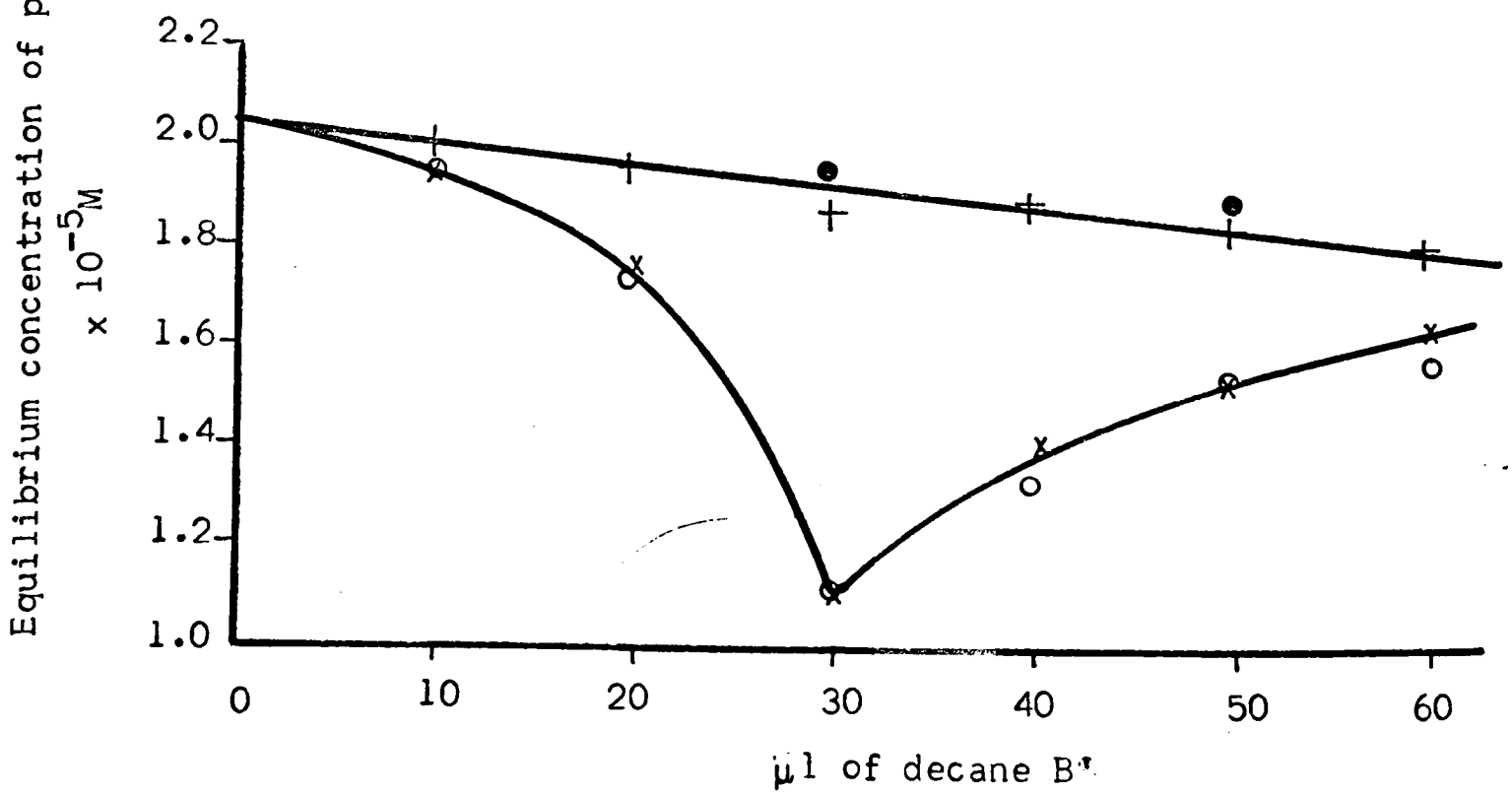
In order to identify the alcohol or alcohols present in decane B 1 μl portions of the decane were injected onto gas liquid chromatography columns. Polypropylene glycol and polyethylene glycol succinate ester columns were found to give the best resolution between different long chain alcohols. When decane B was injected onto both columns in turn,

Figure 4. Equilibrium concentration of protein remaining in solution plotted against the volumes of either impure decane B or decane B' added to a solution of bovine serum albumin at pH 7.0 .



o decane B; X decane B'

Figure 5. Equilibrium concentration of protein remaining in solution plotted against the volume of decane B' added to a solution of lysozyme at pH 2.0 .



+ ultrasonicated only; ● ultrasonicated and stood 24 hours ;

X ultrasonicated then equilibrated 24 hours;

o not ultrasonicated then equilibrated 24 hours.

2 peaks only were observed, one very large which was shown to be due to the decane, the other much smaller emerging some time after the decane peak. In order to identify the smaller peak from decane B, samples of pure alcohols in ether were injected onto both columns and the time taken for their peaks to emerge was recorded. The two sets of results are shown in table 5. The results obtained with the two columns proved that the alcohol present in the decane B was octan-1-ol. Both an Apiezon L column and a carbowax terminated in 20% terephthalic acid column were also tried but the resolution of the alcohols on these columns was poor so no meaningful results could be obtained.

For a given sensitivity setting the area under the peak is propertional to the concentration of the alcohol. Accordingly varying amounts of octanol were dissolved in a given volume of ether and 1 μl portions of each injected onto the polyethylene glycol succinate ester column and the areas compared with that from decane B. In this way decane B was shown to contain 0.25% octanol (v/v).

As a further check that the percentage of octanol in decane had been calculated correctly, dilutions of octanol in decane A were made and their infrared optical densities at 2.74 μ recorded. The percentage of octanol in decane was shown to obey Beers law and the amount of octanol in decane B was found to be 0.25% (v/v).

A solution of 0.25% octanol in decane A (v/v) was made and the effect of this solution,when added to 6 ml. of bovine serum albumin , compared with decane B. The results, figure 4, show that the two decane

Table 5

Gas liquid chromatography of decane B and of samples of pure alcohols

Alcohol	Time, in minutes, for the peaks to emerge after injection (1)	(2)
Alcohol present as impurity in decane B	4.20	5.50
Nonan-1-ol	7.30	8.30
Octan-1-ol	4.20	5.50
Heptan-1-ol	2.30	4.00

Figures in parentheses refer to columns described below.

(1) A polypropylene glycol column, maintained at 130°C with an injection block setting of 4.5.

(2) A polyethylene succinate ester column, maintained at 140°C with an injection block setting of 4.5.

samples are now indistinguishable providing further evidence that the analysis of decane B was correct.

Experiments with decane A, saturated with ethylene, showed no difference from those performed with decane A so any olefine produced on photooxidation of decane would most likely behave in the same way as would pure decane when emulsified in the presence of proteins.

All later batches of decane were checked after purification by gas liquid chromatography and no impurities were detected.

Emulsions of decane containing 0.25% octanol stabilized by different proteins.

As several experiments were carried out with decane B which gave interesting results it was decided to investigate this system in more detail. For this purpose a solution of 0.25% octanol in decane (v/v) was used and is referred to an decane B'.

Time required to reach equilibrium. In previous experiments the protein solution had been equilibrated with decane B' for 24 hours. However to make sure that equilibrium had occurred by that time, 6 ml. of 2×10^{-5}M lysozyme at pH 2.0 was equilibrated with 30 µl of decane B' for varying lengths of time for up to 48 hours. The emulsion was removed by centrifugation after a given time and the concentration of protein in solution measured by the method of Lowry as described. It was shown that equilibrium had been attained after 3 hours so 24 hours equilibration on the wheel was quite sufficient.

Effect of increasing the volumes of the solutions. It was shown that if

the ratio of the volume of decane B' to the volume of protein solution was kept the same then the decrease in protein concentration was always the same irrespective of the actual volumes used. This was shown to be correct if the volumes of either bovine serum albumin, lysozyme, insulin or ribonuclease solutions were varied between 6 ml. and 20 ml., while keeping the ratios of decane B' to the volume of each protein solution the same.

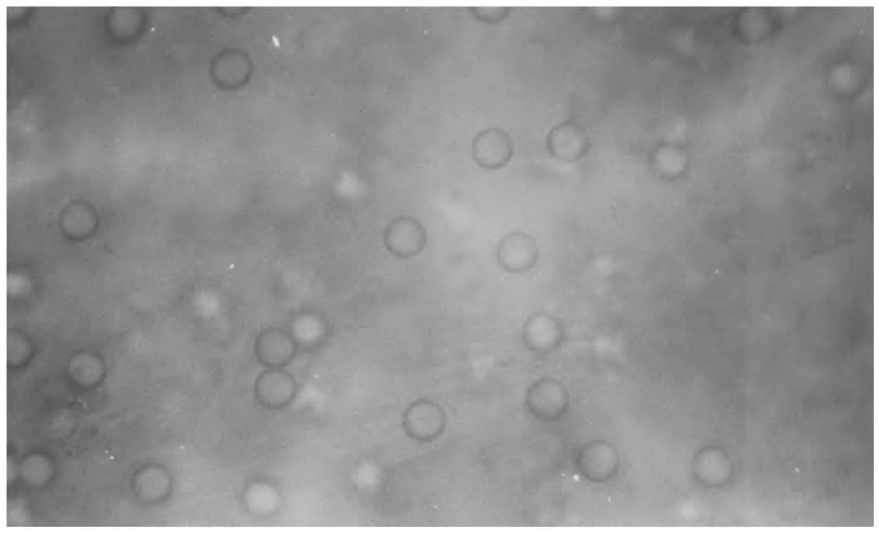
Effect of temperature. All previous experiments were carried out at 2°C. Two experiments, where varying volumes of decane B' were added to 6 ml. of 2×10^{-5}M lysozyme at pH 2.0, were repeated at 25°C and it was found that the loss of protein from solution to the emulsion was identical to that at 2°C.

Thereafter experiments were carried out at 2°C as there is less chance of bacterial growth at this temperature.

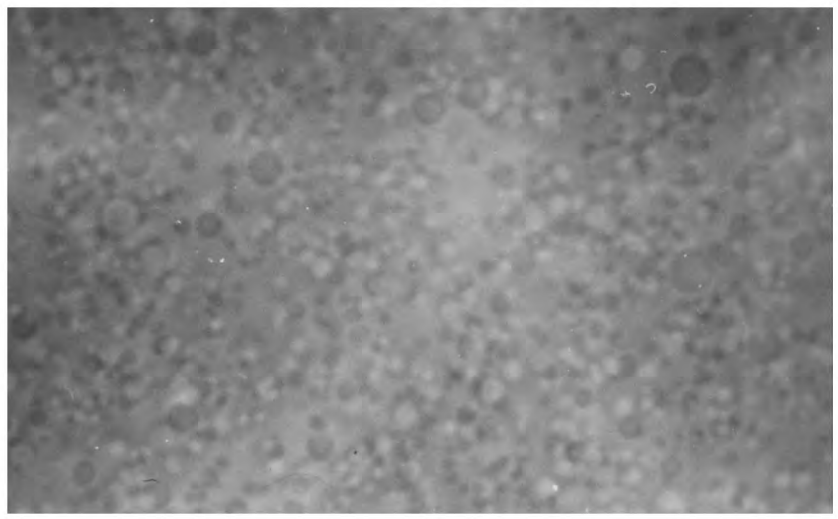
Formation of emulsions by ultrasonic vibrations. It was found that if the contents of a series of tubes containing the same concentration of lysozyme with varying volumes of decane B' were subjected to ultrasonic vibrations for 15 seconds and then equilibrated on the wheel for 24 hours, the volume at the dip was identical to that obtained when there was no prior ultrasonic vibration of the solutions. If the protein concentration was measured immediately after ultrasonic vibration there was a small drop in concentration with increasing volume of decane B' irrespective of whether the tubes were spun immediately or after standing for 24 hours, figure 5. If a mixture of 6 ml. of lysozyme plus the volume of decane B' at the dip

Figure 6. Photographs of decane B' emulsions stabilized by insulin at pH 2.

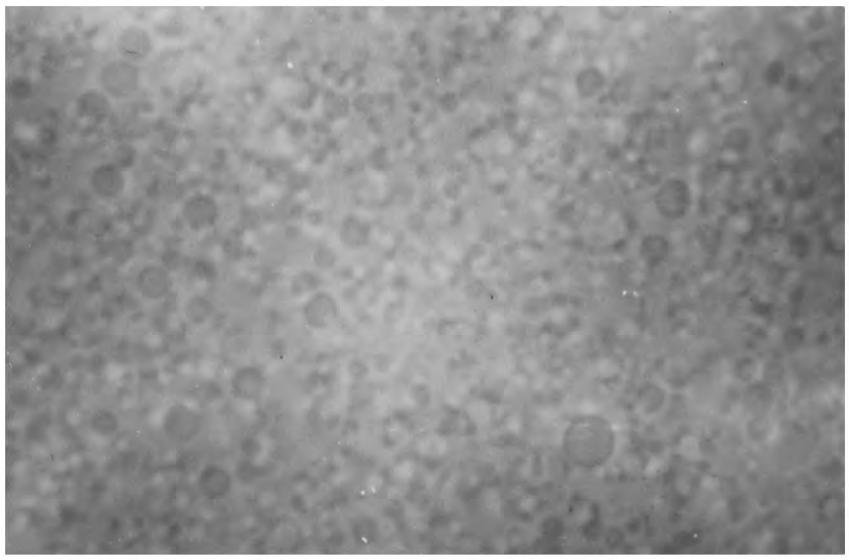
(1) 2.0 μ diameter Dowex polyvinyl latex beads.



(2) Decane B' emulsion formed by equilibrating 6 ml. of 10 x 10^{-5}M insulin at pH 2.0 plus 200 μl of decane B' for 24 hours on the wheel.



(3) Decane B' emulsion formed by subjecting 6 ml. of 10 x 10^{-5}M insulin at pH 2.0 plus 200 μl of decane B' to ultrasonic vibrations for 15 seconds and then equilibrating this mixture on the wheel for 24 hours.



was subjected to ultrasonic vibrations for 15 seconds, then a minimum of 30 minutes agitation on the wheel was required before equilibrium was attained.

Measurement of particle size.

When the particle size distribution was measured using a Coulter counter it was found that no estimation could be made of the number or distribution of particles as no plateau in the count was obtained as the voltage was raised (Blades and Flavell, 1963). This means that the particle size distribution is so large that the counter cannot be used to count the number of particles present.

Photomicrography, though only able to see a very small sample of the total particles present, showed that there was a wide range of particle sizes present, from approximately 2 μ in diameter to mere dots on the photograph which must therefore be less than 0.1 μ in diameter, figure 6.

Similarities between different proteins. When decane B' was added to solutions of different proteins there was a loss of protein from solution which was a maximum for a given volume of added decane B' (referred to as the volume at the dip) as shown previously in figures 3 and 4.

In comparing the different proteins the following quantities were measured:

(a) The volume of decane B' at the dip; i.e. varying volumes of decane B' were added to a given initial concentration of protein.

(b) The equilibrium concentration of protein remaining in solution

Figure 7. Volume of decane B' at the dip , plotted against the initial concentration of bovine serum albumin at pH 7.0 .

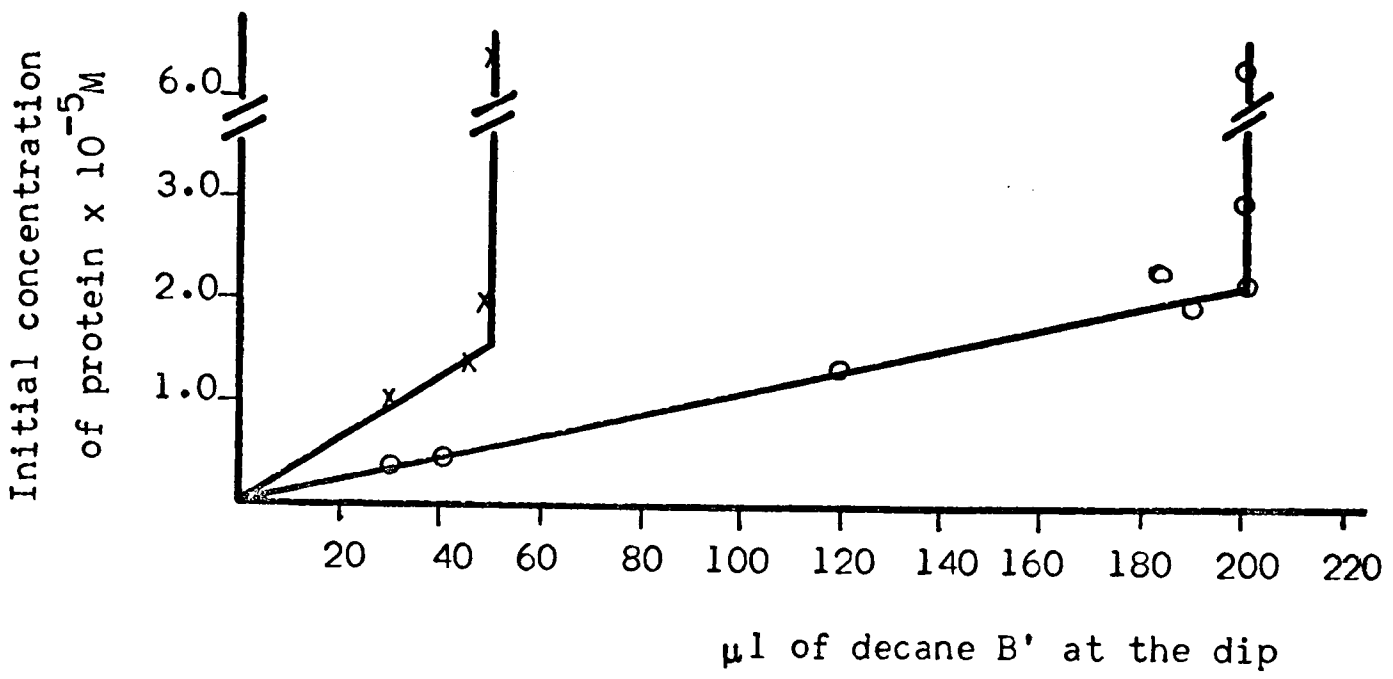
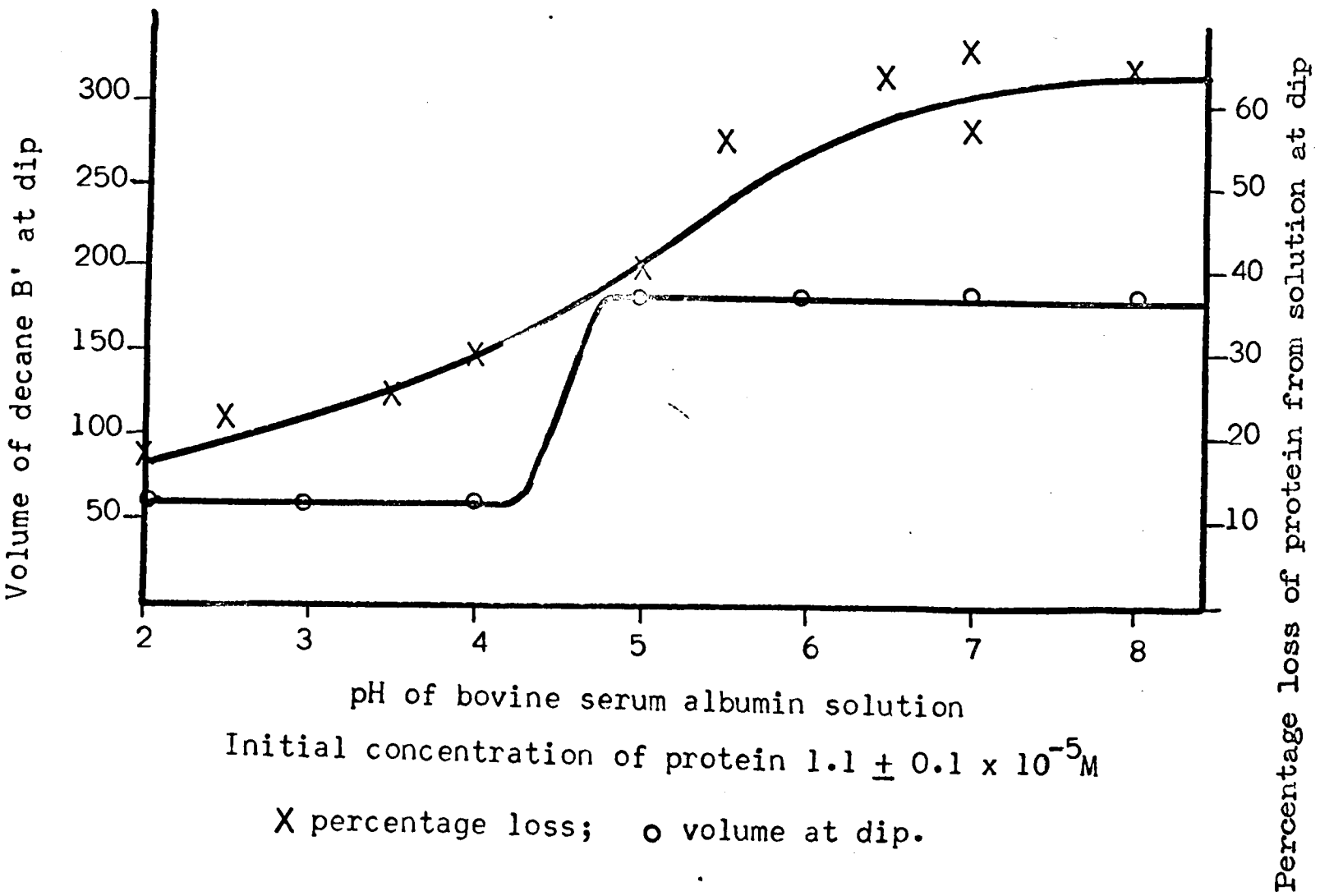


Figure 8. Volume of decane B' at the dip and the percentage loss of protein from solution at the dip , plotted against the pH of the bovine serum albumin solution .



at the dip; i.e. varying volumes of decane B' were added to a given initial concentration of protein.

(c) The volume of decane B' at the dip while varying the pH of the protein solution.

(d) The equilibrium concentration of protein remaining in solution at the dip while varying the pH of the protein solution.

With the four extracellular proteins investigated, namely bovine serum albumin, lysozyme, ribonuclease and insulin and one intracellular protein haemoglobin it was found that the volume of decane B' at the dip increased linearly with increasing concentration of protein. With all the extracellular proteins, except bovine serum albumin, the increase in the volume of decane B' at the dip with increasing initial protein concentration is independent of pH. The amount of protein lost from solution at the dip is independent of pH with ribonuclease but not with the other proteins. With bovine serum albumin and lysozyme it was found that the volume of decane B' at the dip became constant after a certain concentration of protein had been exceeded.

Bovine serum albumin. The changes in the volume of decane B' at the dip with varying concentration of bovine serum albumin at pH 7.0 are shown in figure 7. An earlier batch of this protein (batch A) gave different results in that the volume at the dip became constant at 50 μl instead of 200 μl as shown in the same figure. Unfortunately only a small amount of batch A was available so this difference could not be investigated. The subsequent results with batches B

Figure 9. Volume of decane B' at the dip plotted against the initial concentration of lysozyme at pH 2.0.

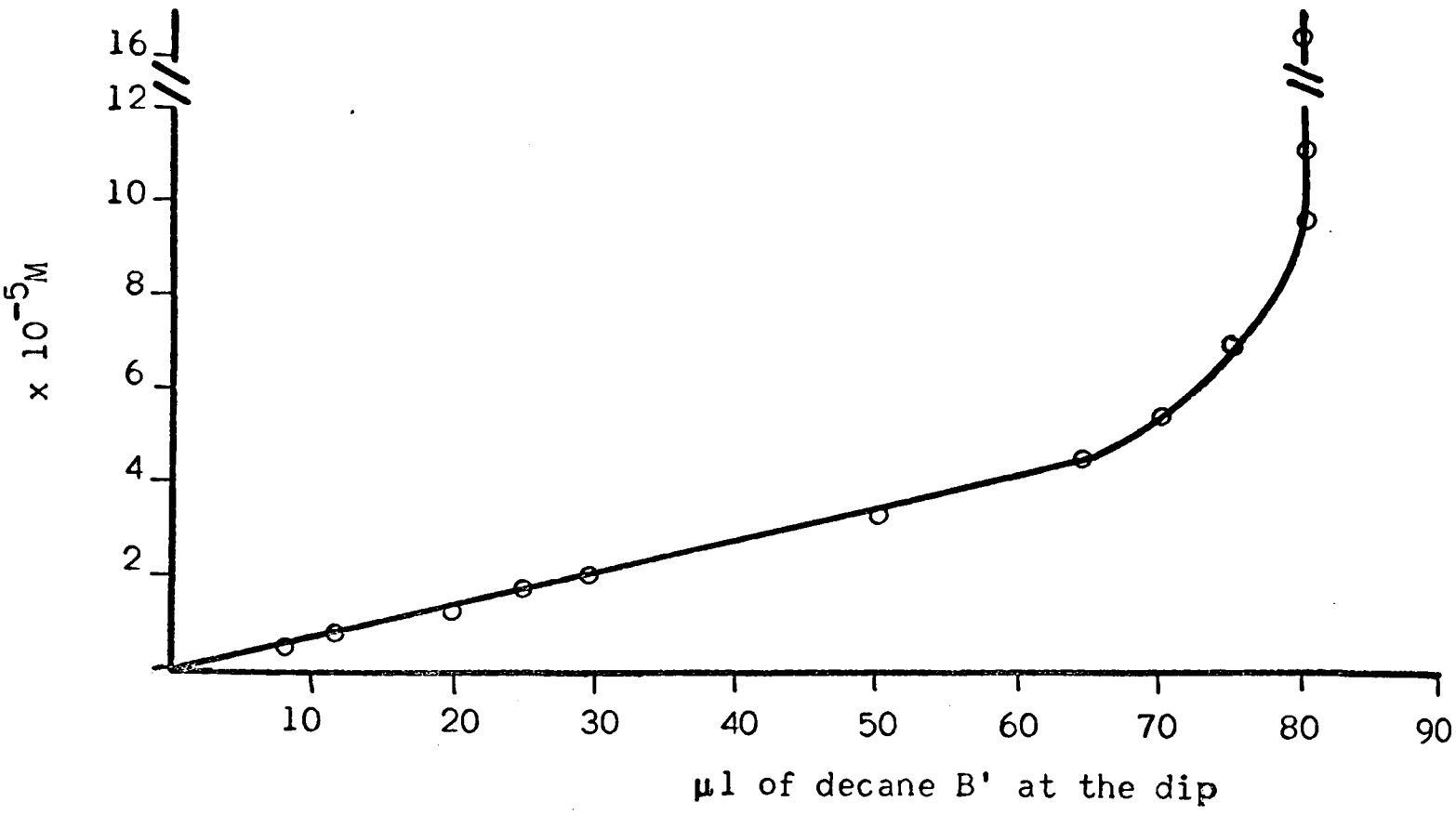
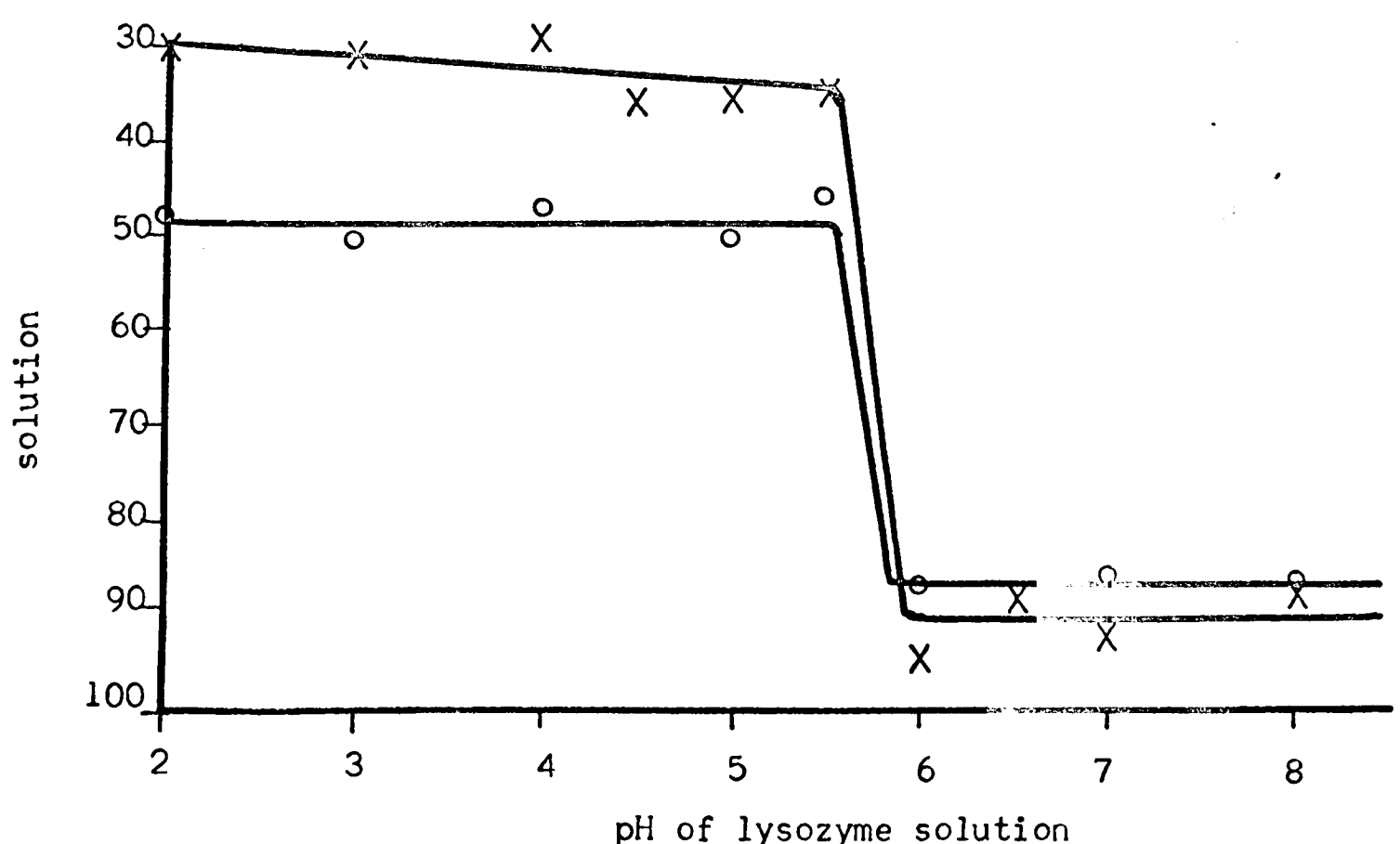


Figure 10. Percentage loss of protein from solution at the dip plotted against the pH of the lysozyme solution.



Initial concentration of lysozyme 1.90 x 10^{-5}M + 30µl decane B' ;
Initial concentration of lysozyme 11.20 x 10^{-5}M + 80µl decane B' .

and C gave results identical with each other.

Besides determining the volume of decane B' at the dip with solutions of this protein at pH 7.0, experiments were carried out over a range of pH values from 2.0 to 8.0. It was found that both the volume of decane B' at the dip and the amount of protein lost from solution at this point varied with pH as shown in figure 8.

It was also observed that the loss of protein from solution at the dip varied, with the initial concentration of bovine serum albumin as shown in table 6.

A sample of bovine serum albumin was defatted by the method of Chen (1967) and it was found to behave identically to the original protein when it was adsorbed on decane B' emulsions.

Lysozyme. The volumes of decane B' at the dip with lysozyme at pH 2.0, with varying initial concentration of protein, are shown in figure 9. Batches A, B and C all gave identical results. As was observed with bovine serum albumin, the amount of protein lost from solution at the dip varies with the initial concentration of lysozyme. Up to a concentration of 7.5×10^{-5}M lysozyme at pH 2.0 this loss is 50% of the initial concentration of protein but by 9×10^{-5}M lysozyme it drops to 20%; above this concentration the loss remains constant at 20%.

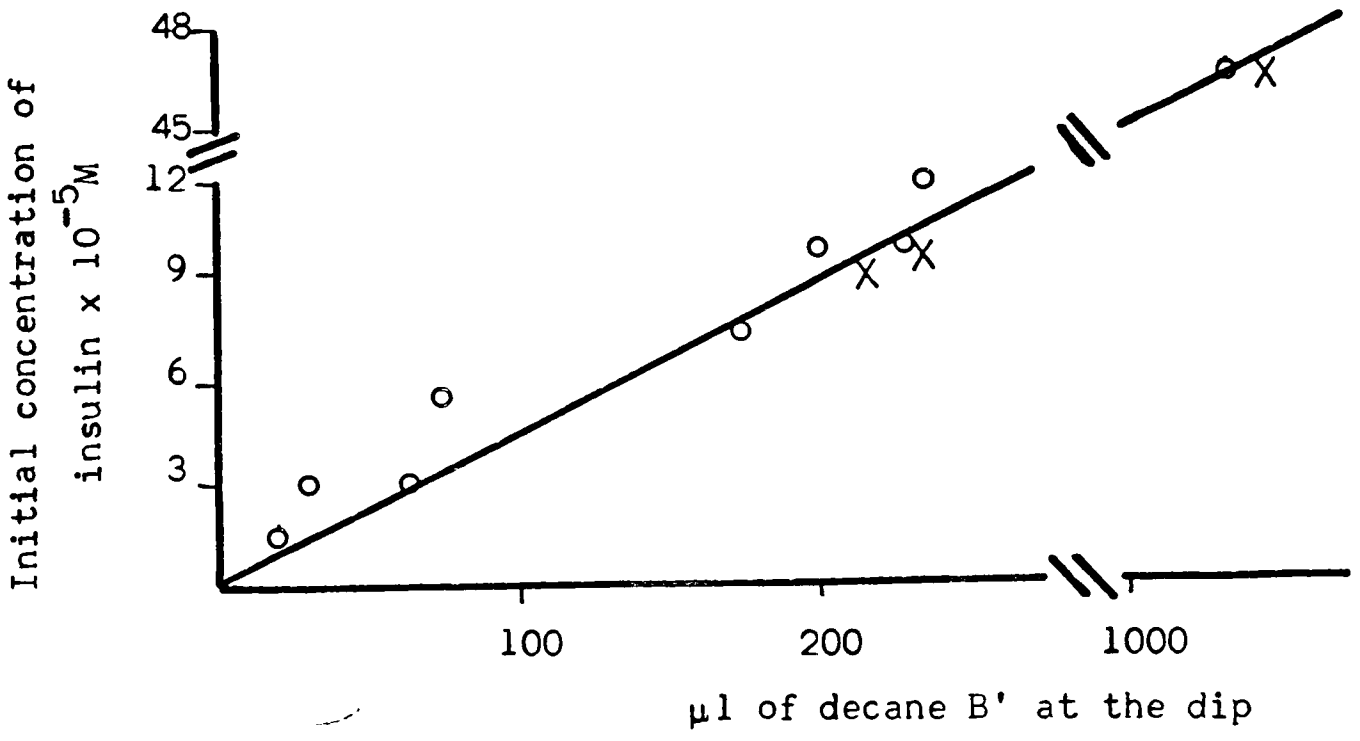
While it has been found that the volume of decane B' at the dip is independent of pH for a given concentration of protein, the amount of protein lost at the dip increases sharply between pH 5.5

Table 6

The amount of bovine serum albumin lost from solution to decane B' emulsion at the dip with varying initial concentration of protein at pH 7.0

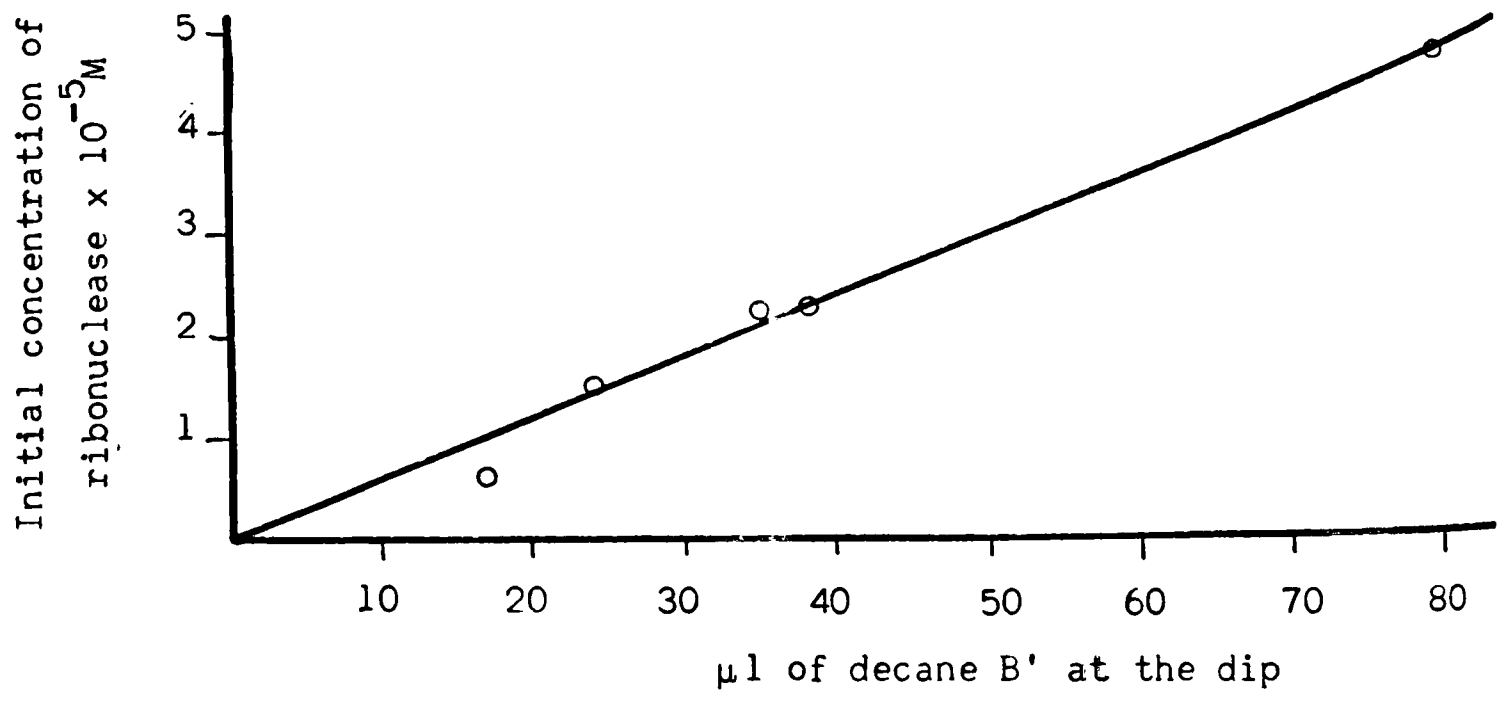
Initial protein molarity x 10^{-5}M	Percentage loss of protein from solution at the dip
0.20	59 %
1.05	55 %
1.15	60 %
4.55	26 %
6.70	25 %
9.00	20 %

Figure 11. Volume of decane B' at the dip plotted against the initial concentration of insulin.



o Experiments at pH 2.0. ;X experiments at pH 9.5 .

Figure 12. Volume of decane B' at the dip plotted against the initial concentration of ribonuclease at pH 7.0.



and pH 6.0. The extent of this change increases with concentration of lysozyme, figure 10.

Insulin. The volumes of decane B' required to give the dip with insulin at pH 2 and pH 9.5 with varying initial concentrations of protein are shown in figure 11. It was found that there was no levelling off in the volume of decane B' up to a concentration of 5×10^{-4} M insulin. While the percentage of protein lost from solution at the dip is 55 ± 5% between pH 2 and 4 irrespective of the initial concentration of protein, between pH 7 and pH 9.5 the loss is 80 ± 5%. Batches A and B gave identical results.

Ribonuclease. The volume of decane B' required to give the dip with ribonuclease was found to be independent of pH between pH 2.0 and pH 7.0 and no levelling off in the volume of decane B' was observed up to a concentration of 5×10^{-5} M protein. These results are shown in figure 12. The percentage of protein lost from solution does not change either with initial protein concentration up to 5×10^{-5} M or with pH between pH 2.0 and pH 7.0. Batches A and B gave identical results.

Intracellular proteins. With both glutamic acid dehydrogenase and carbonic anhydrase there was an immediate flocculation of protein from solution as soon as the decane B' was added. After removing the hydrocarbon and flocculant precipitate by ultracentrifugation, less than 5% protein remained in solution even when a very small volume of decane B' had been added.

Only two experiments were performed with carboxyhaemoglobin. Unlike the two other intracellular proteins mentioned it formed emulsions without any flocculation. With an 8.8×10^{-5} M solution of carboxyhaemoglobin at ph 7.0 the dip was at 110 µl of decane B' and there was a 75% loss of protein from solution at this volume.

Displacement of adsorbed protein from emulsions.

It has been found possible to displace protein adsorbed on emulsions. Table 7 shows the results of experiments with several proteins which had been adsorbed on emulsions. The solutions were equilibrated on the wheel for 24 hours. The initial and final concentrations of protein in solution, as well as the percentage loss of protein from solution to the emulsion, is given. The emulsion was removed from the solution by spinning and resuspended in 6 ml. of the original buffer, unless otherwise stated, together with 0.5 ml. of decanol. When this system was reequilibrated on the wheel for 24 hours a percentage of the protein was displaced from the emulsion and went back into solution. This percentage varied, as would be expected, with the different proteins. In some experiments pure decane was added with the decanol but it was found to have no effect on the percentage of protein displaced from the emulsion.

If a solution of lysozyme at any given pH is equilibrated with the volume of decane B' at the dip a percentage of lysozyme in solution goes onto the emulsion depending on the pH of the solution. If the emulsion is removed by ultracentrifugation and re-equilibrated with 6 ml. pH 2.0

Table 7

The effect of decanol on decane B' emulsions stabilized by different proteins

	(1)	(2)	(3)	(4)	(5)
Initial Protein Molarity x 10^{-5} M	11.76	2.31	2.31	1.30	2.30
Protein Molarity after equilibrium x 10^{-5}M	4.42	1.18	0.37	0.47	0.80
Percentage Loss of Protein to Emulsion	60%	47%	83%	64%	65%
Final Protein Molarity when Separated Emulsion was re-equilibrated x 10^{-5}M	6.54	0.83	1.54	0.32	0.75
Percentage Loss of Protein from Emulsion	85%	87%	87%	39%	50%

Figures in parentheses refer to solutions described below.

Solutions

(1) 6 ml. of insulin at pH 2.0 + 200 µl of decane B'.

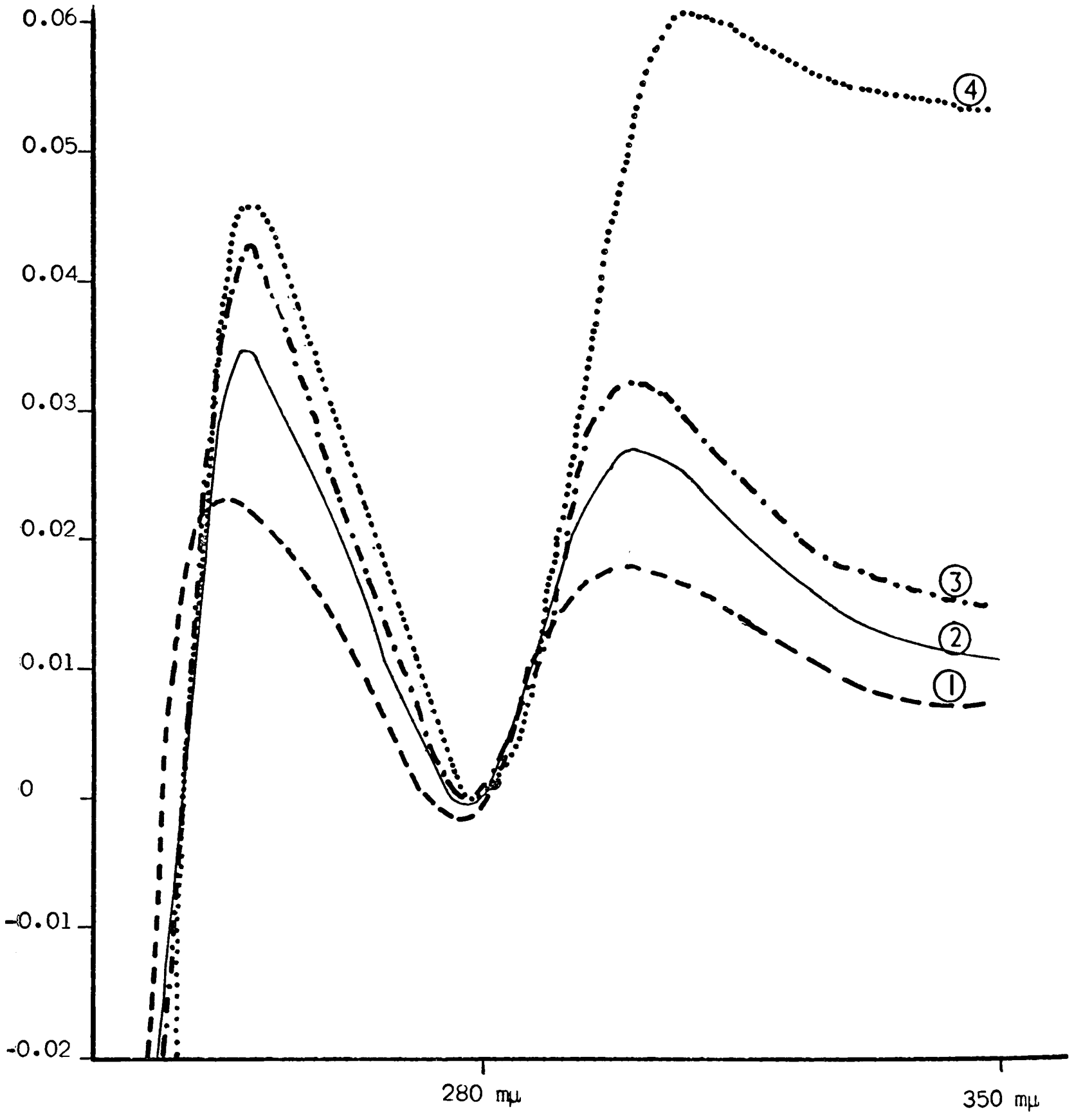
(2) 6 ml. of lysozyme at pH 2.0 + 30 µl of decane B'.

(3) 6 ml. of lysozyme at pH 7.0 + 30 µl of decane B'. The emulsion was re-equilibrated in pH 2.0 buffer.

(4) 6 ml. of bovine serum albumin at pH 7.0 + 200 µl of decane B'.

(5) 6 ml. of ribonuclease at pH 7.0 + 30 µl of decane B'.

Figure 13. Difference spectra of protein solutions in equilibrium with decane B' emulsions.



① Insulin pH 2, concentration 4.72×10^{-5}M.

② Bovine serum albumin pH 7, concentration 0.47×10^{-5}M.

③ Ribonuclease pH 7, concentration 1.64×10^{-5}M.

④ Lysozyme pH 2, concentration 0.49×10^{-5}M.

buffer then it is found that 40 ± 5% of the lysozyme comes off the emulsion while if a pH 7.0 buffer is used less than 10% is removed. Similar results were obtained when the initial concentration of lysozyme was 2×10^{-5}M or 10×10^{-5}M. The amount of protein that comes off the emulsion is independent of the pH of the solution in which the lysozyme originally went onto the emulsion.

Conformation of proteins in equilibrium with emulsions.

Difference spectra. Difference spectra for protein solutions which had been in equilibrium with an emulsion were recorded for insulin, lysozyme, bovine serum albumin and ribonuclease over a range 350 mμ to 240 mμ. Concentrations were in all cases adjusted so that the optical densities at 280 mμ, of both the protein solution to be studied and the native protein, were equal.

With all the proteins investigated there were two peaks, one between 245 mμ and 250 mμ and one between 296 mμ and 305 mμ. The spectra are given in figure 13. While the difference spectra were qualitatively the same in different experiments the absolute values of the optical density of the difference spectra were different. The difference spectra were also recorded for these same proteins when they were removed from the emulsion by addition of the emulsion to buffer and decanol. They were found to be qualitatively the same as for the protein solutions that were in equilibrium with the emulsion as it was formed.

Thus both types of protein undergo similar conformational changes.

Optical rotatory dispersion. In spite of the difference in the absorption

spectra no difference in optical rotation was observed between native bovine serum albumin and that in equilibrium with an emulsion. In a series of experiments the spectra were recorded between 20 and 44 wave numbers and over this range the two types of protein solutions had identical specific rotations.

Binding of non-polar compounds by protein in solution.

Binding of decane. In addition to protein being adsorbed on the surface of an emulsion particle it has been found that decane is bound by protein solutions in equilibrium with an emulsion.

Experiments have been carried out to investigate the binding of decane to various proteins. The decane was present either as pure decane or as decane B' (i.e. a 0.25% solution of octanol in either ^{3}H or ^{14}C decane).

The radioactivity of the solutions is directly proportional to the amount of decane present and, as the solubility of decane in water is approximately $10^{-9}M$, no blank solubility determination is necessary.

Experiments using batches 3 and 4 ^{3}H decane were satisfactory but batch 5 gave erratic results. This was found to be due to an impurity in the ^{3}H decane which was not removed by washing with concentrated sulphuric acid. In order to overcome this problem ^{14}C decane was used which gave the correct solubility in water. Pure samples of ^{3}H and ^{14}C decane gave the same results.

It was found that at $22 \pm 1^{o}C$ decane was bound by bovine serum albumin, lysozyme, insulin and carboxyhaemoglobin but not by ribonuclease;

Figure 14. Moles of decane bound per mole of bovine serum albumin at pH 2.0 , using 10µl of radioactive decane B' and varying the protein concentration .

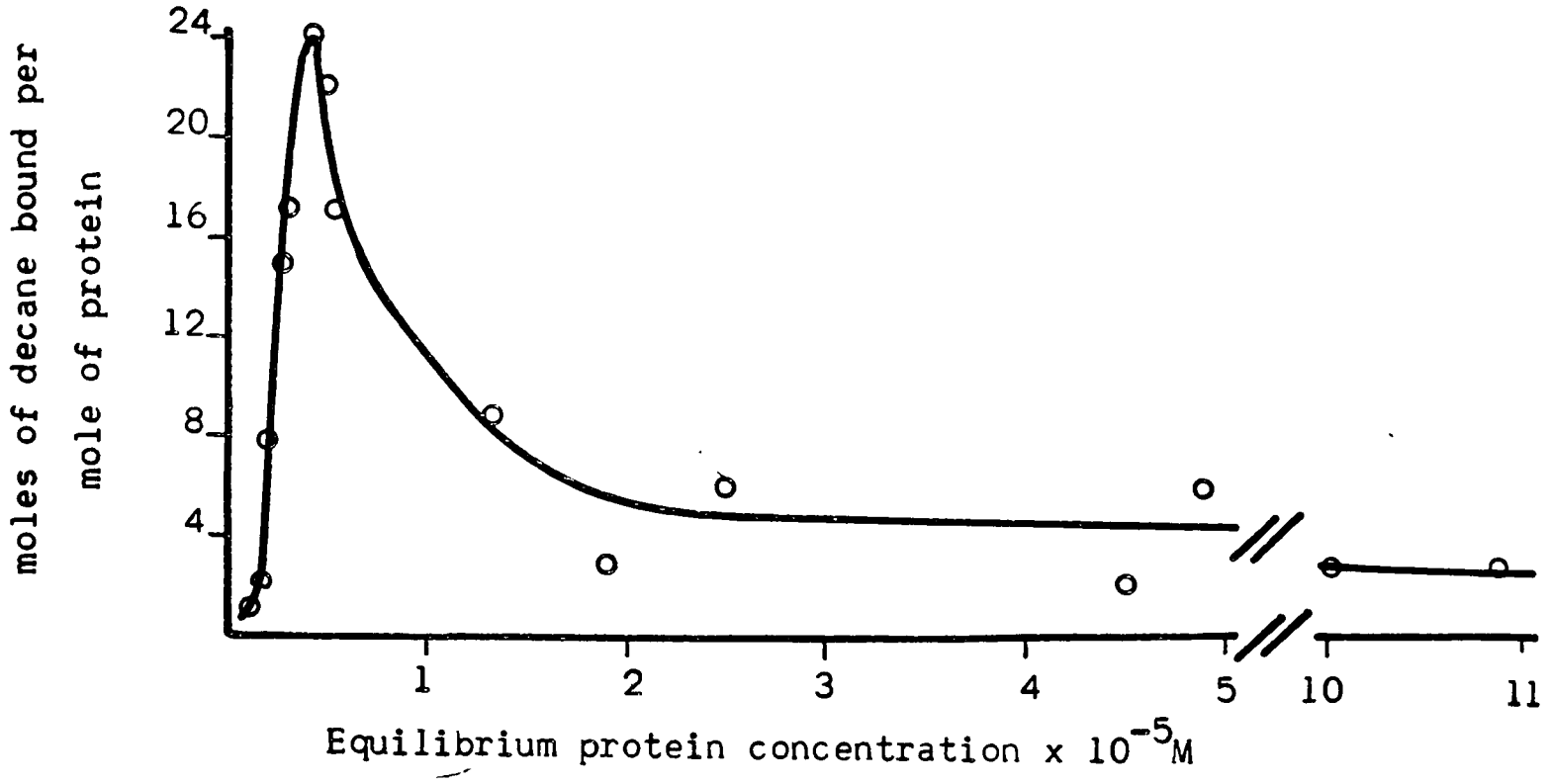


Figure 15 . Moles of decane bound per mole of bovine serum albumin at pH 6.0 , using 10µl of radioactive decane B' and varying the protein concentration .

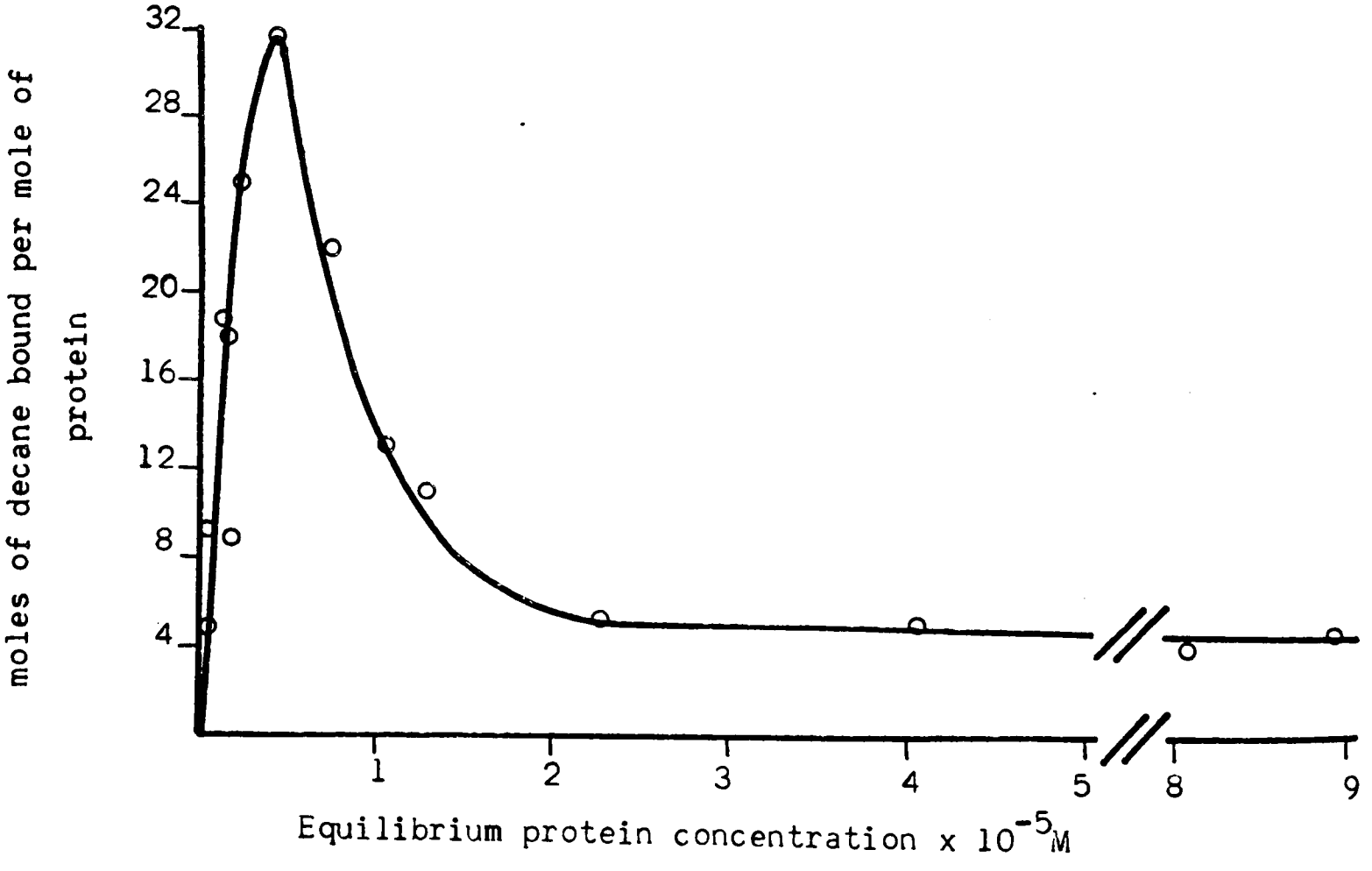


Figure 16. Moles of decane bound per mole of lysozyme at pH 2.0, using 10 µl of radioactive decane B' and varying the concentration of protein.

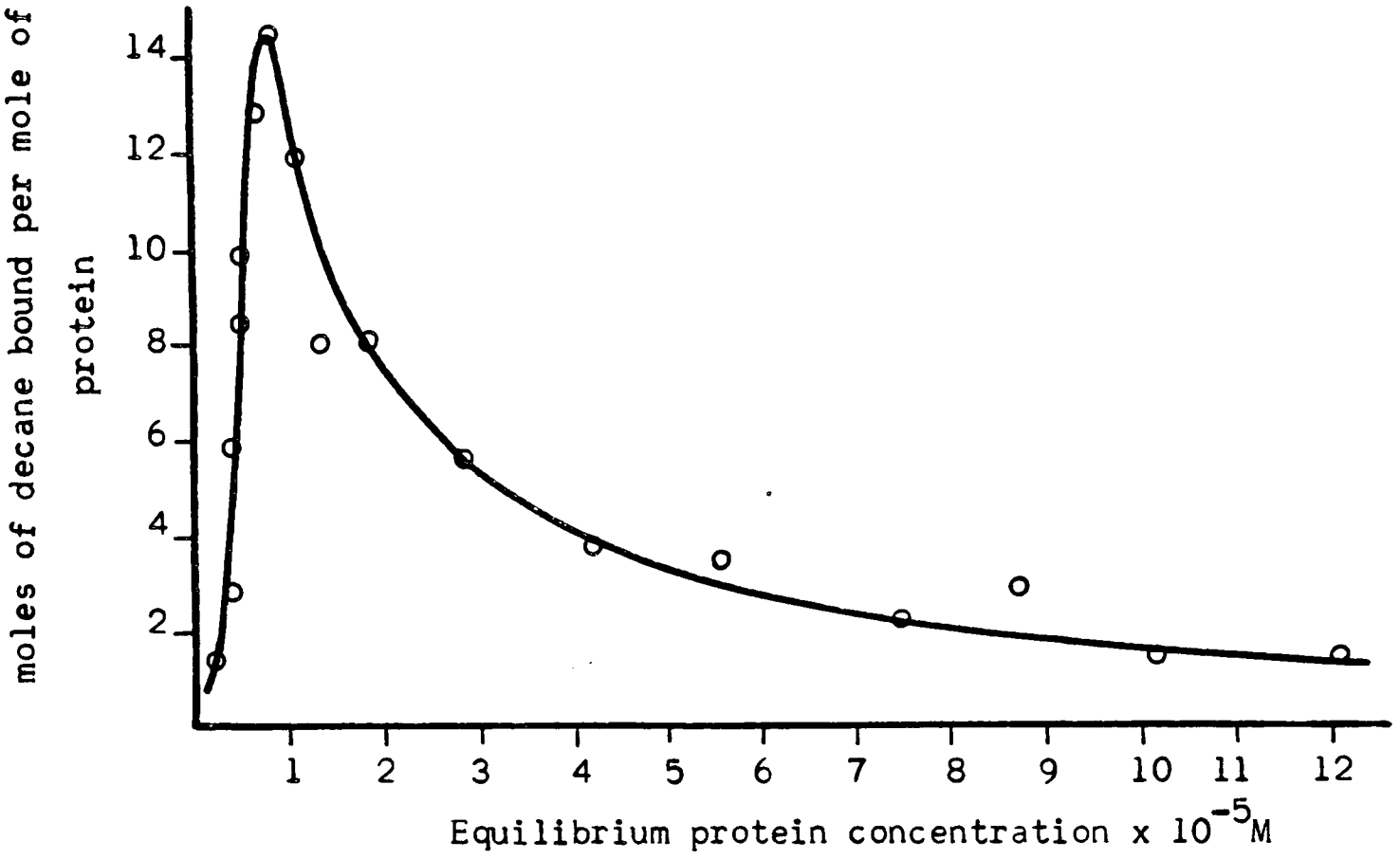
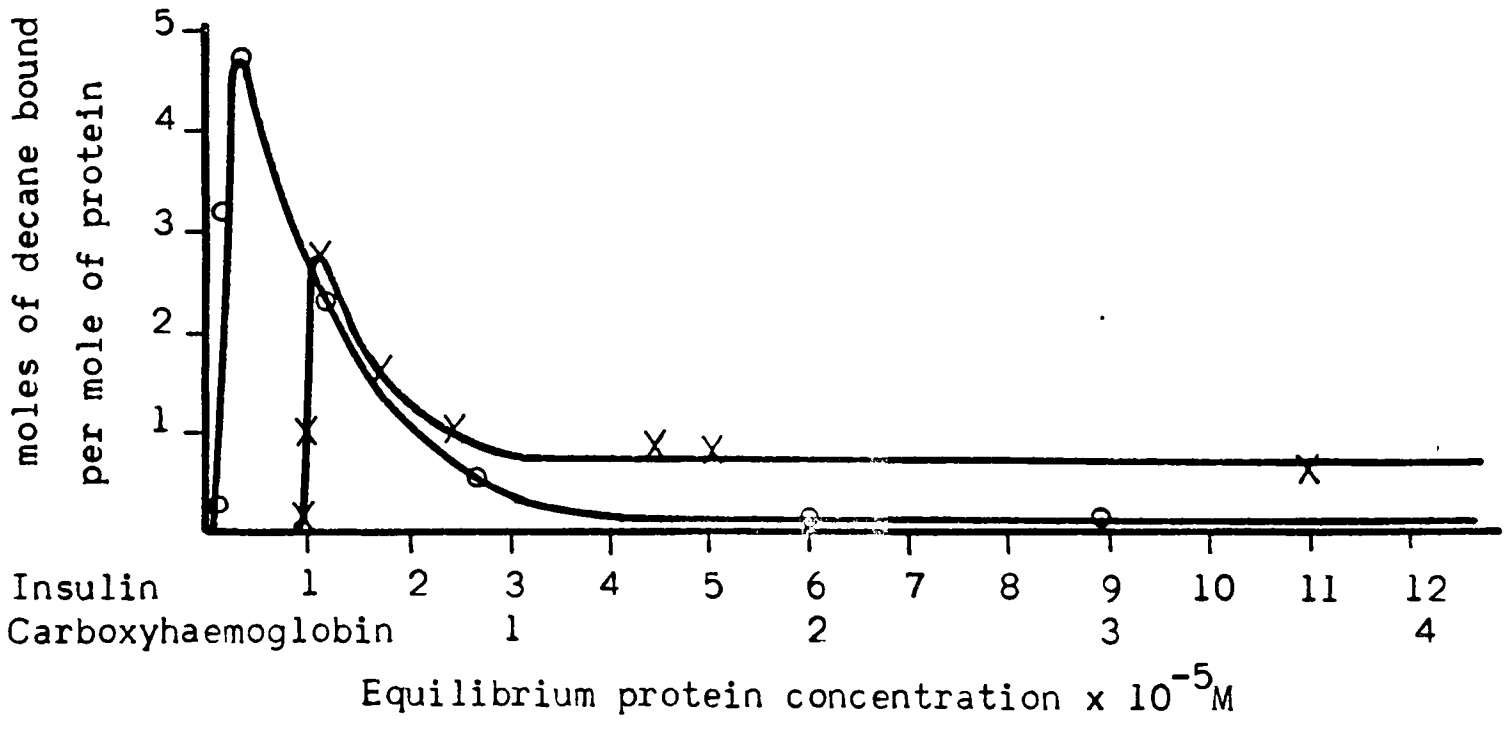
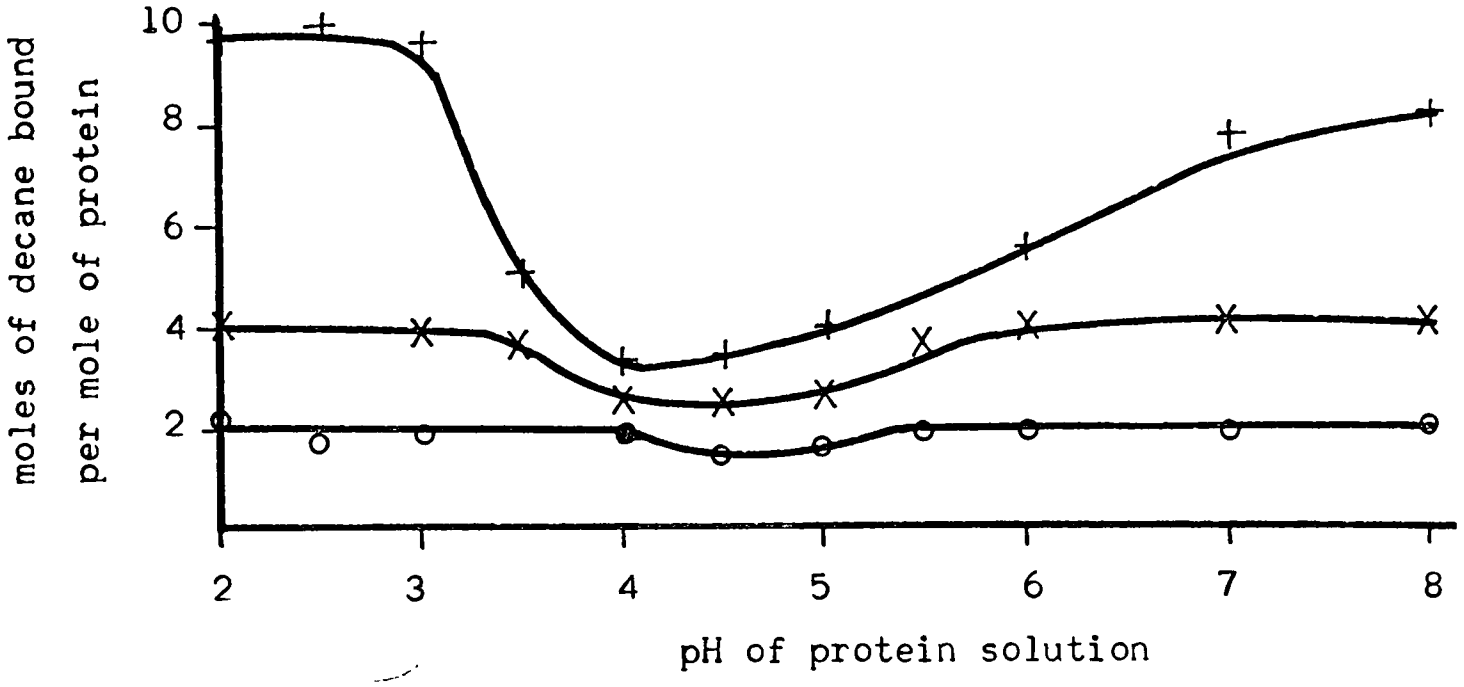


Figure 17. Moles of decane bound per mole of carboxyhaemoglobin at pH 7.5 and insulin at pH 2.0, using 10 µl of radioactive decane B' and varying the concentration of protein.



X insulin; o carboxyhaemoglobin.

Figure 18. Moles of decane bound per mole of bovine serum albumin at pH 7, using 10 µl of radioactive decane B' and varyi the pH and concentration of protein.

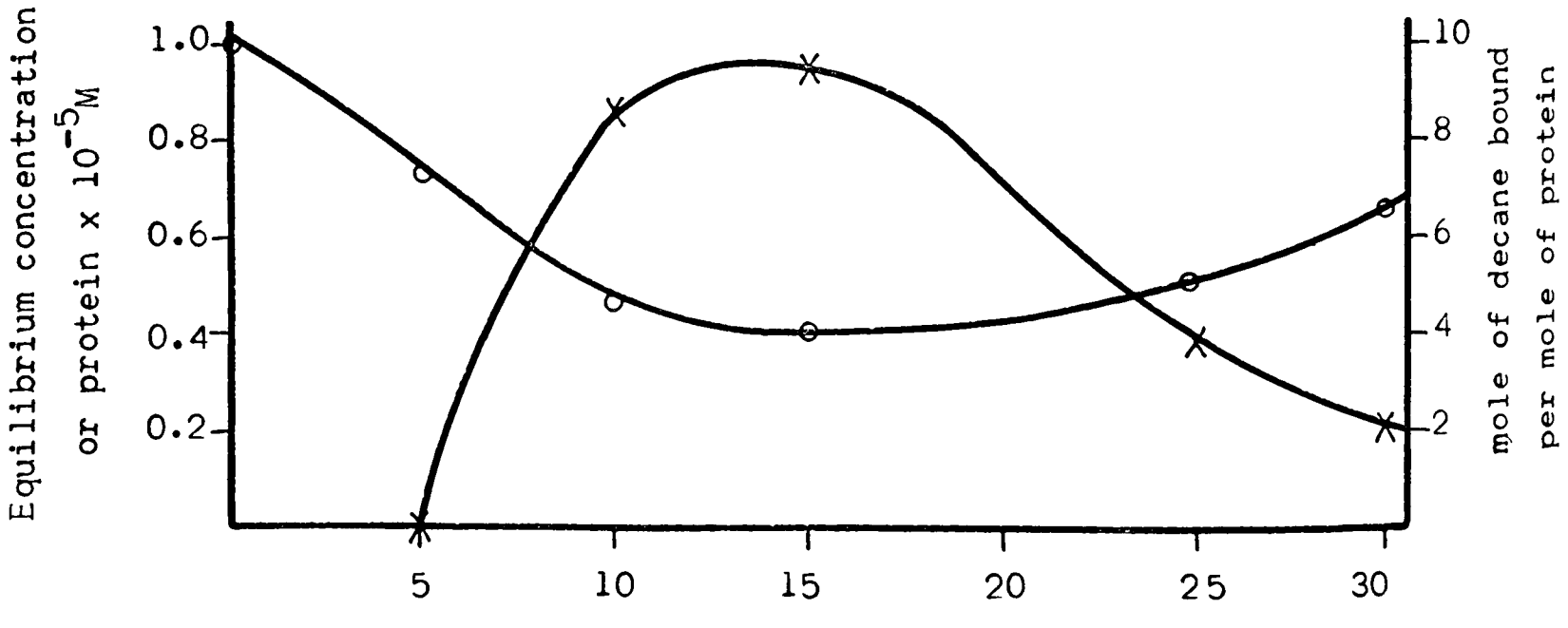


+ initial concentration of protein 1.64×10^{-5}M.

X initial concentration of protein 5.78×10^{-5}M.

o initial concentration of protein 24.3×10^{-5}M.

Figure 19. Moles of decane bound per mole of lysozyme at pH 2.0 and the equilibrium concentration of protein in solution, varying the volume of radioactive decane B'.



Initial protein concentration 1.00×10^{-5}M.

o equilibrium concentration of protein;

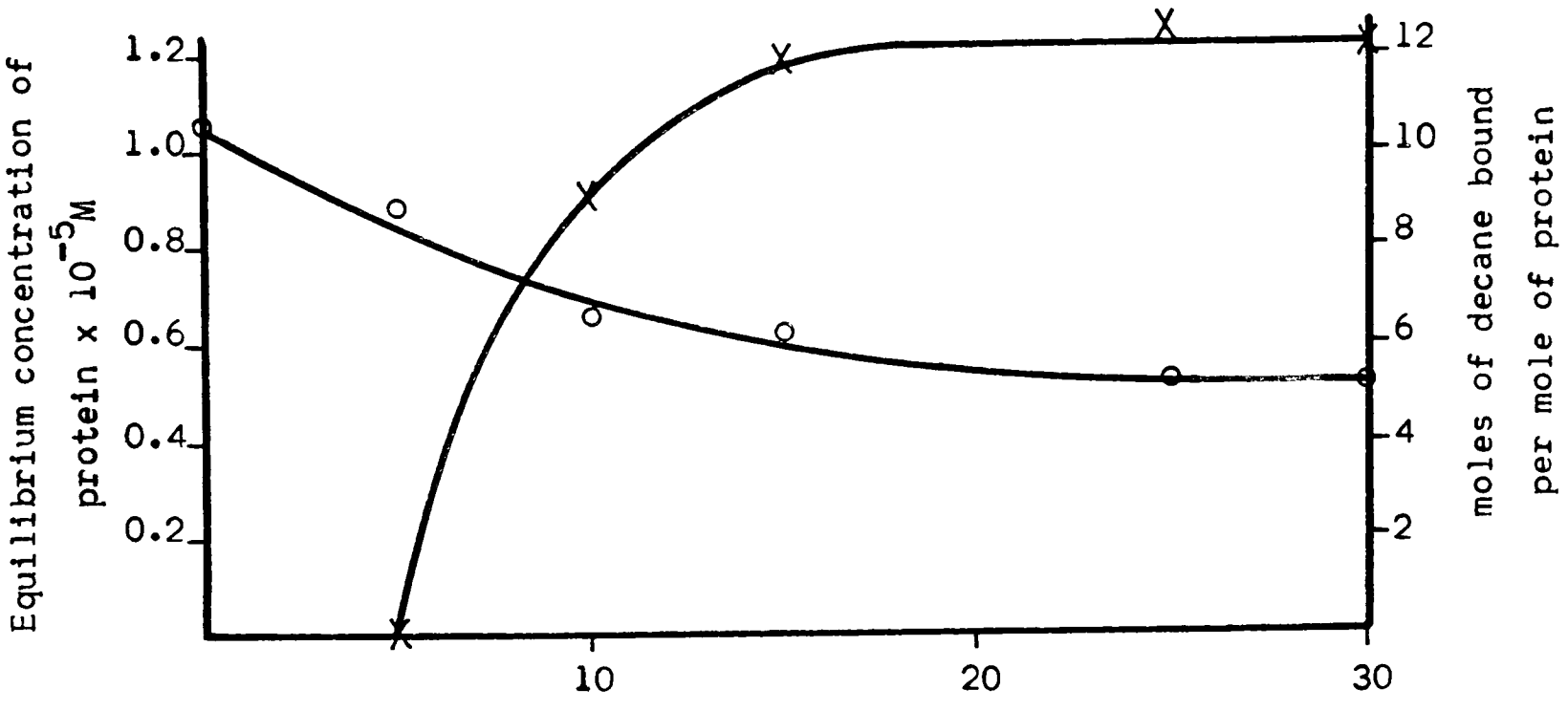
X moles of decane bound per mole of protein.

at 2°C no binding of decane by either insulin or lysozyme was detected. When given amounts of decane and protein solution were equilibrated for varying lengths of time it was found that equilibrium was reached within 5 hours.

When a solution of ^{14}C or ^{3}H decane in pure decane containing 0.25% (v/v) octanol (referred to as radioactive decane B') was used to investigate the binding of decane by various proteins it was found that, for a given volume of decane, the number of moles of decane bound per mole of protein increased with decreasing protein concentration down to about 0.1 mg. protein per ml. of solution whereupon the binding of decane decreased very rapidly with decreasing concentration of protein. In all these experiments 10 µl of this decane were equilibrated with 6 ml. of protein solution at 22°C for 24 hours on the wheel. The results are shown in figures 14 to 17. It was observed that bovine serum albumin differed from the other proteins in that the pH of the solution affected the binding of decane to this protein. Figure 18 shows the binding of given concentrations of bovine serum albumin with varying pH.

An experiment was carried out at room temperature using a solution of lysozyme at pH 2.0 in which the initial concentration of protein was maintained at 1×10^{-5}M and the volume of radioactive decane B' was varied. The result is shown in figure 19 where both the final concentration of protein and the number of moles of decane bound per mole of protein, at this protein concentration, are plotted against the volume of hydrocarbon added. A similar experiment was performed using a solution

Figure 20. Moles of decane bound per mole of lysozyme at pH 2.0 and the equilibrium concentration of protein in solution, varying the volume of radioactive pure decane.

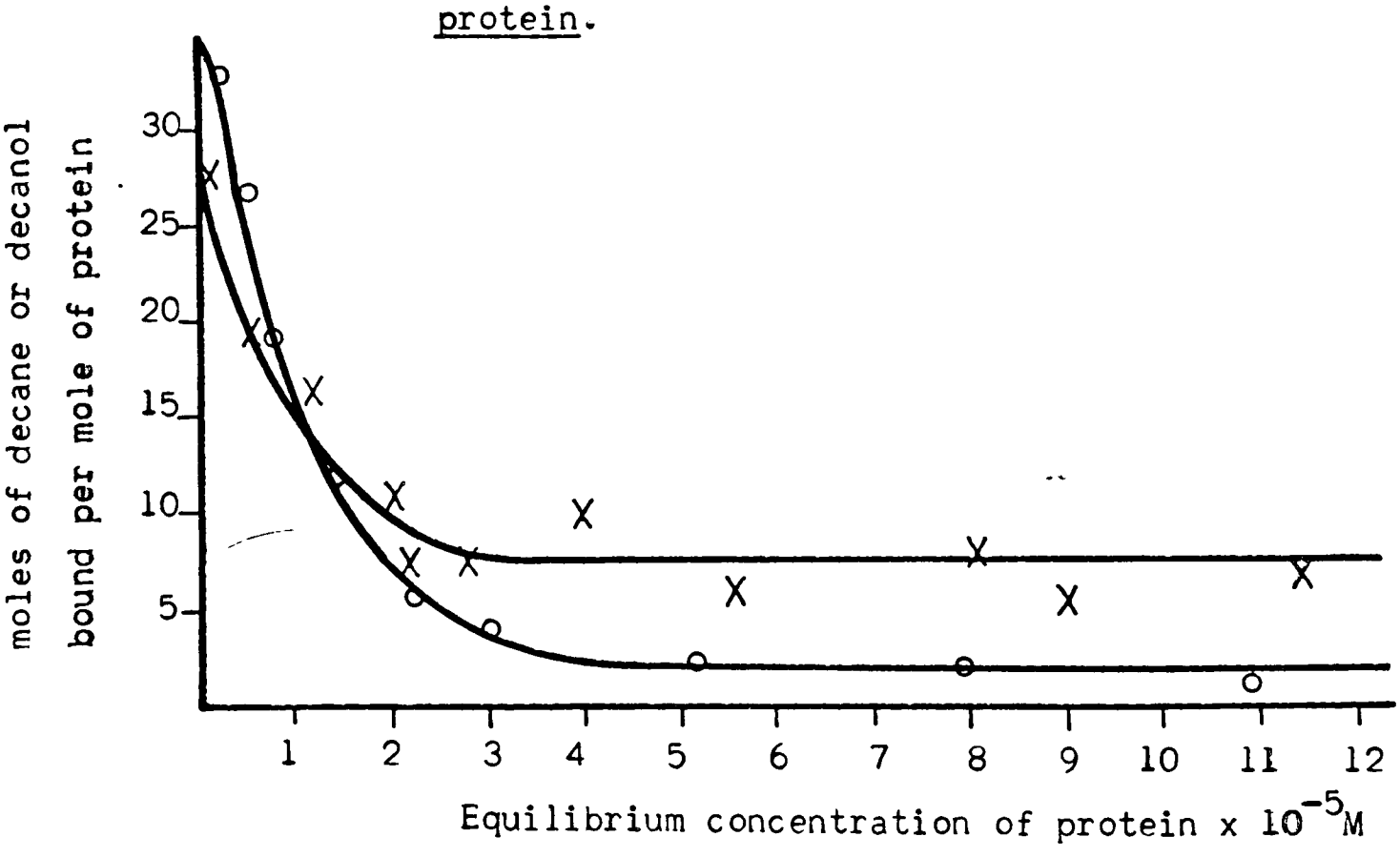


Initial protein concentration 1.04×10^{-5}M

o equilibrium concentration of protein;

X moles of decane bound per mole of protein.

Figure 21. Moles of decane or decanol bound per mole of bovine serum albumin at pH 7.0, using 10 µl of radioactive decane or decanol and varying the concentration of protein.



X decanol; o decane.

Figure 22. Moles of decane bound per mole of lysozyme at pH 2.0, using 10 µl of radioactive pure decane and varying the concentration of protein in solution.

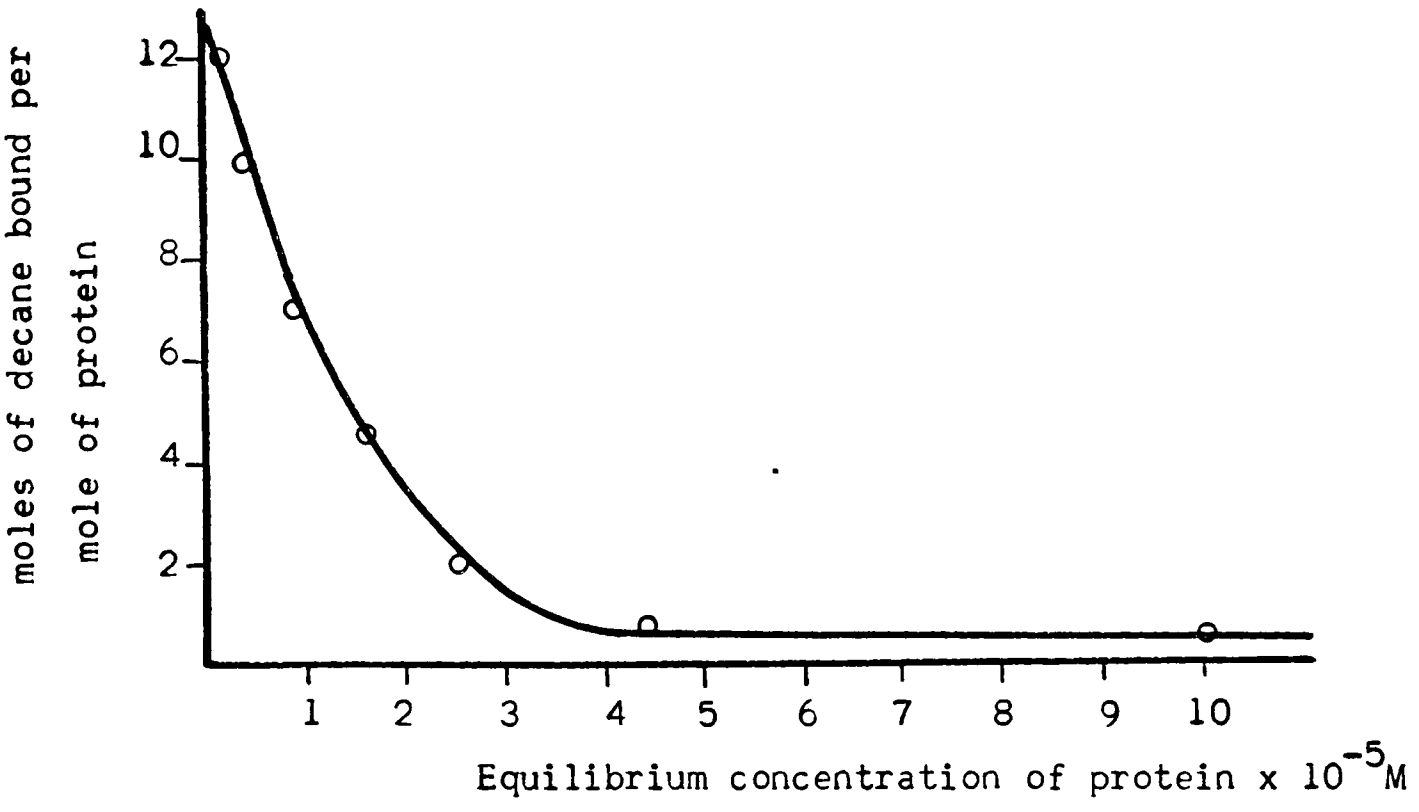
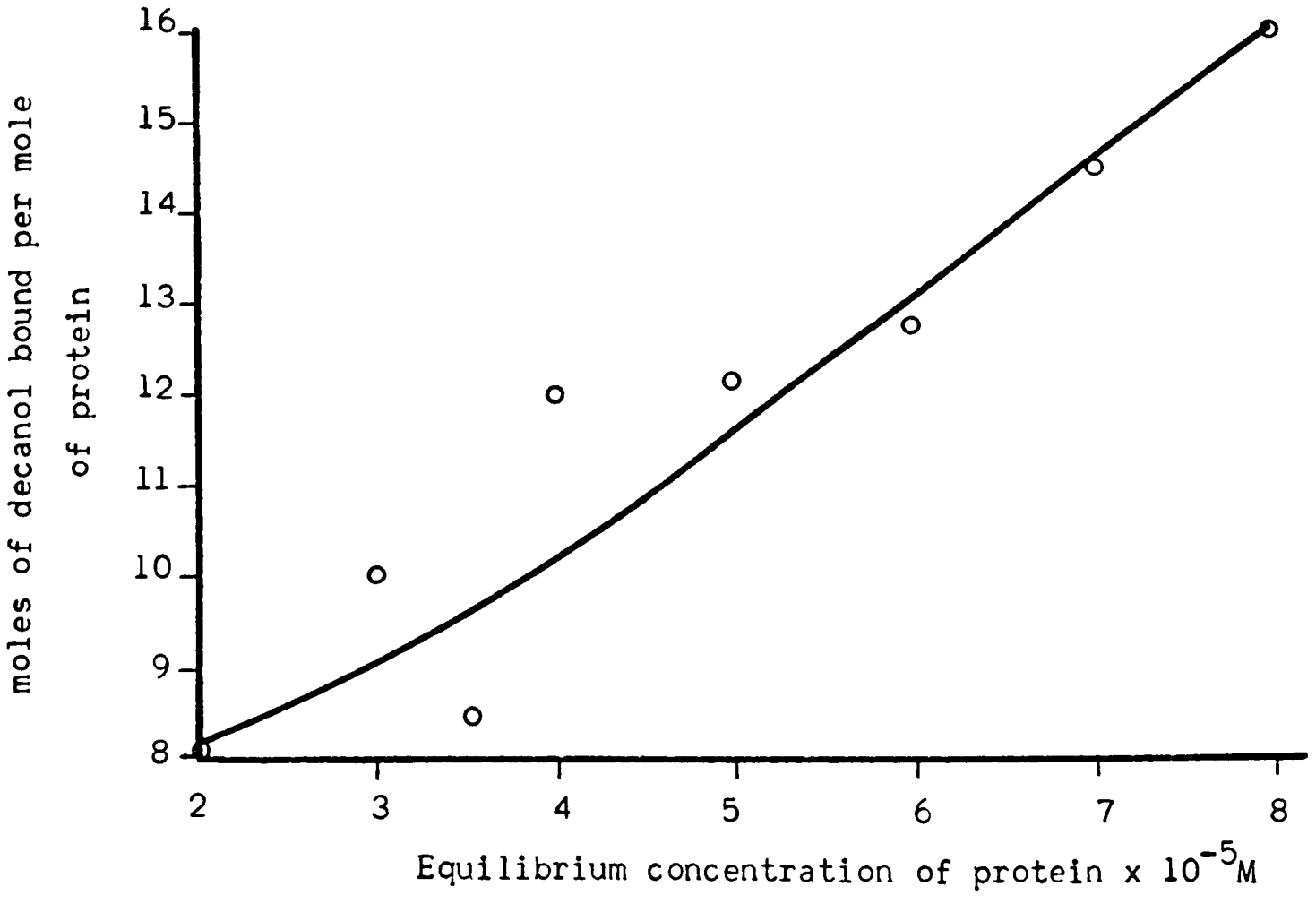


Figure 23. Moles of decanol bound per mole of bovine serum albumin at pH 7.0, using 10 µl of decanol and varying the pH of the protein solution.



of ^{14}C decane in pure decane and the result is shown in figure 20. When the experiment shown in figure 20 was repeated with an initial concentration of 0.75×10^{-5}M, the maximum value of $\bar{V}$ rose to 15 moles of decane/mole of protein.

When pure ^{14}C decane was used to investigate the binding of decane at 22°C to bovine serum albumin at pH 7.0, figure 21, it was found that there was no fall off in the number of moles of decane bound per mole of protein at low concentration of protein. Similar results were obtained with lysozyme at pH 2.0, figure 22, at room temperature.

Binding of decanol. The binding of decanol at 22°C to bovine serum albumin at pH 7.0 has been investigated and is shown in figure 21. In all cases 10 µl of ^{14}C decanol were added to 6 ml. of protein solution. The binding of decanol to a given concentration of bovine serum albumin at varying pH has also been investigated and the results are given in figure 23.

Binding of sodium dodecyl sulphate. The binding of the detergent, sodium dodecyl sulphate, to bovine serum albumin at pH 6.0 was investigated. Binding was studied by dialysis experiments where the concentration of detergent bound by the protein was found by calculating the drop in concentration of detergent outside the sac.

While experiments of this nature have been reported by other workers using the same system (Ray et al., 1966), it was found in this laboratory that true equilibrium of detergent between the inside and the

outside of the sac was never reached, even when the sacs were washed in boiling sodium carbonate solution.

A 10^{-4}M solution of detergent inside and outside the sac was agitated for 24 hours at room temperature; this caused a 30% drop in detergent concentration which was presumably bound to the sac, as no protein was present. To check this the experiment was repeated but no dialysis sac was present and in this case there was only a 4% drop in the concentration of detergent. This experiment was repeated using a 10^{-5}M solution of detergent and a 60% loss was observed.

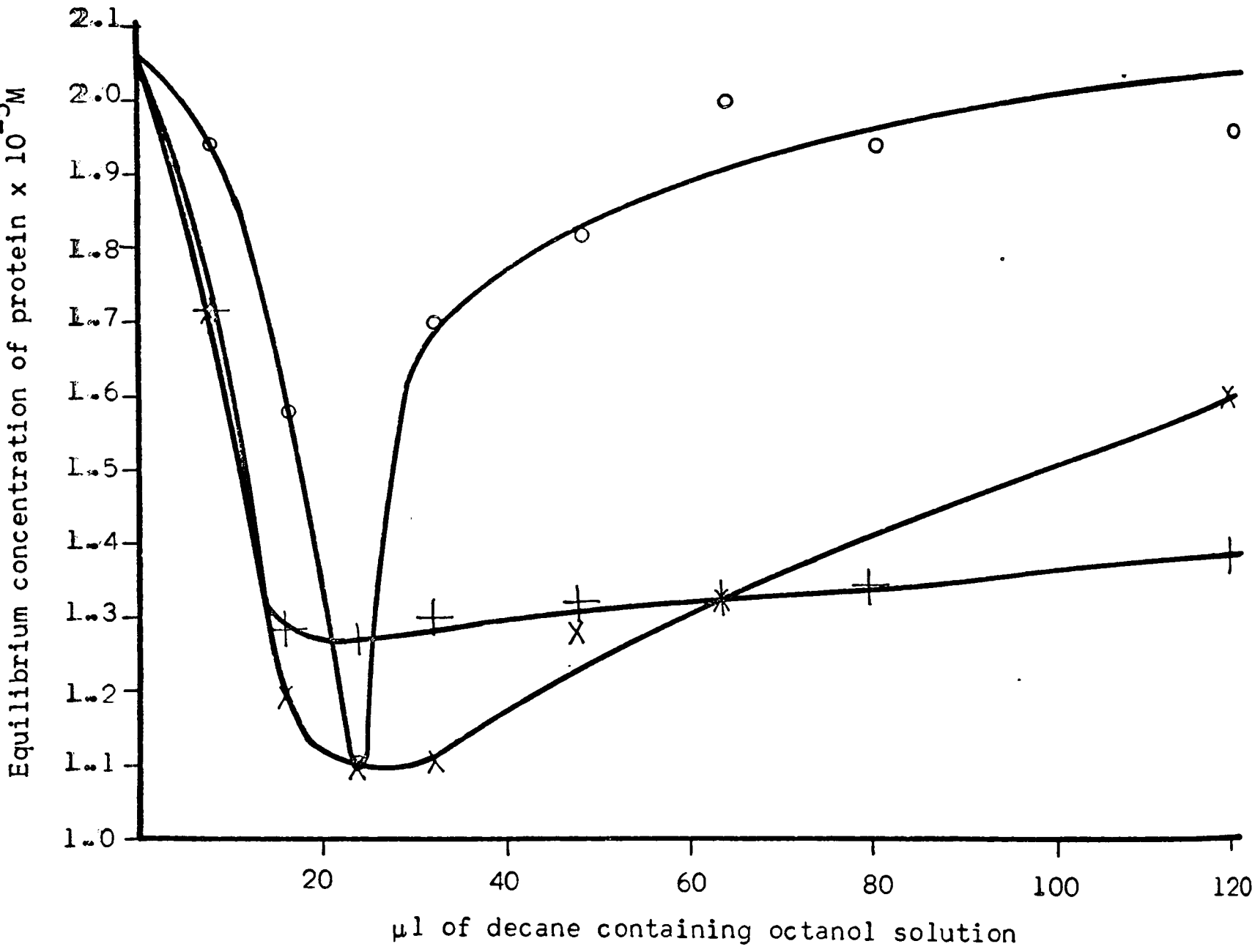
Since it was impossible to attain equilibrium using this technique it was abandoned.

Effect of different alcohols on decane in water emulsions in the presence of proteins.

Lysozyme. In order to extend those studies that were carried out where decane B' was added to different protein solutions, the effect of varying percentages (v/v) of a number of alcohols in decane was investigated.

When decane solutions containing different percentages of octanol were added to solutions of 2×10^{-5}M lysozyme at pH 2.0 it was found that the volume of hydrocarbon solution at the dip was identical irrespective of the percentage of alcohol in the decane. However the actual amount of protein lost from solution decreased when the amount of octanol in decane was below 0.2%. It was also found that the higher the concentration of the alcohol in decane, the sharper was the dip.

Figure 24. Equilibrium concentration of lysozyme remaining in solution , plotted against the volume of different octanol - containing decane solutions added to lysozyme solution at pH 2.0 .



o 1% octanol in decane;

X 0.2% octanol in decane;

+ 0.1% octanol in decane.

Figure 25. Equilibrium concentration of lysozyme remaining in solution when 300µl of decane solution containing varying amounts of propanol was equilibrated with 6 ml. of lysozyme solution .

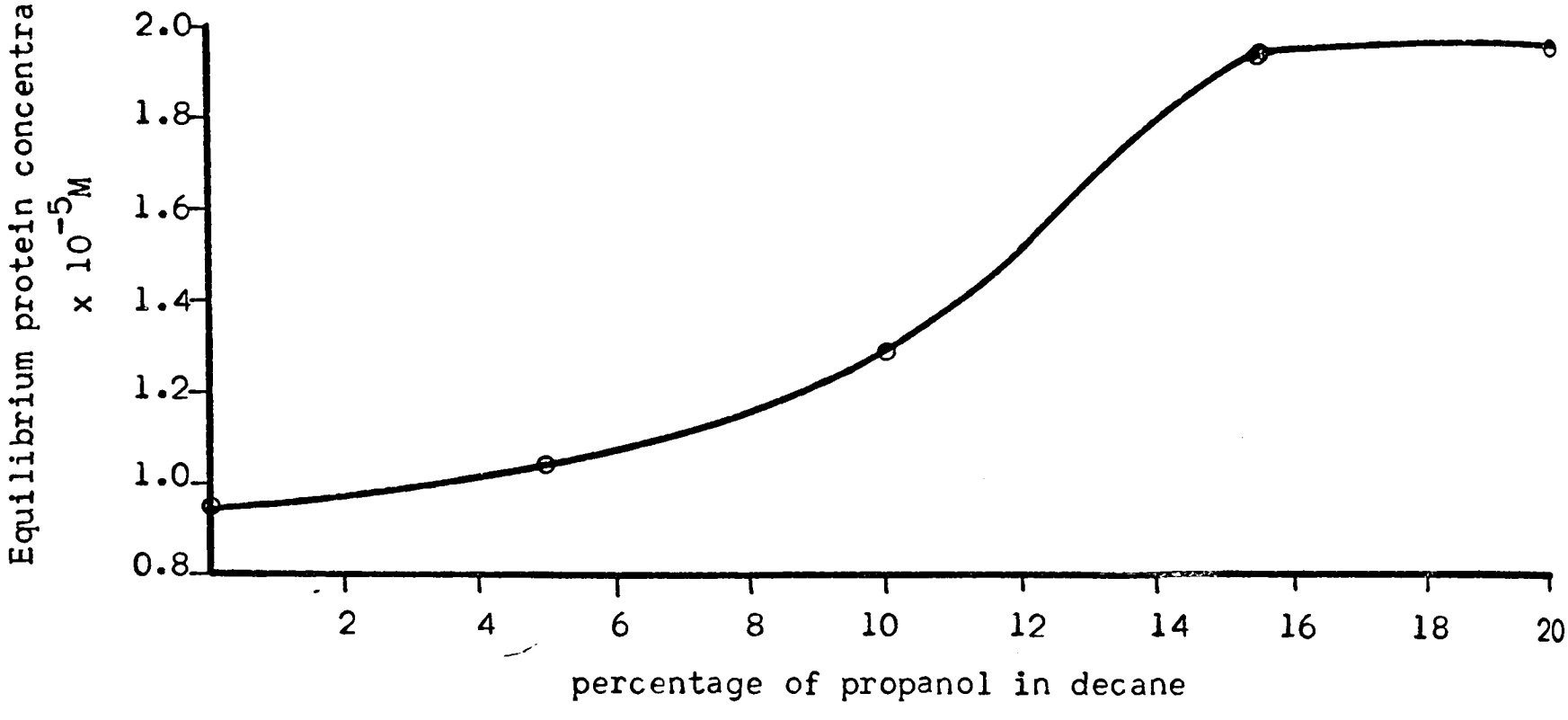
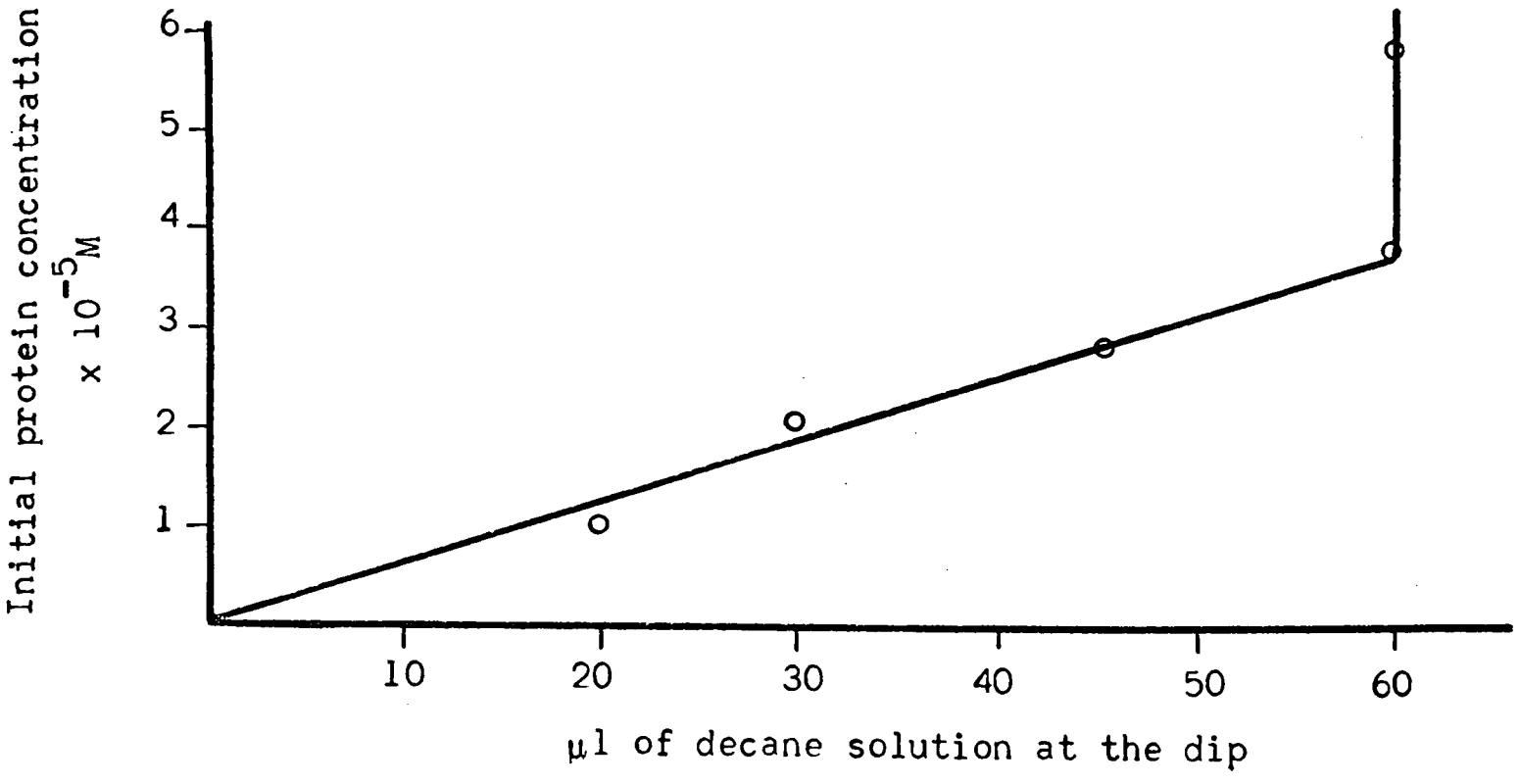


Figure 26. Volume of 5% hexanol-in-decane solution at the dip , plotted against the initial concentration of lysozyme at pH 2.0 .



These results are shown in figure 24. It can be seen from this figure that if a volume of decane much larger than that required to produce the dip were added to a solution of 2 x 10^{-5}M lysozyme at pH 2.0 and if the percentage of alcohol in the decane were large enough, there would be little or no loss of protein from solution. This observation was repeated using all the primary alcohols from methanol to octanol. The type of result obtained is shown in figure 25 where 300 µl of decane containing varying percentages of propanol was added to 6 ml. of 2 x 10^{-5}M lysozyme at pH 2.0. For this alcohol it may be seen that the minimum percentage of alcohol in decane which gave no loss of protein from solution was 15%. The corresponding percentages for the other alcohols are given in table 8.

Table 8

Minimum percentages of alcohol in decane required to prevent loss of protein from solution when 6 ml. samples of 2 x 10^{-5}M lysozyme at pH 2.0 were equilibrated with 300 µl of alcohol-in-decane solutions for 24 hours at 2°C on the wheel.

Alcohol	Percentage of Alcohol
Methanol	90 %
Ethanol	30 %
Propanol	15 %
Butanol	10 %
Amyl alcohol	2.8 %
Hexanol	1.6 %
Heptanol	0.9 %
Octanol	0.3 %

When varying volumes of a 1% solution of decanol in decane were added to a 2×10^{-5}M lysozyme solution at pH 2.0 there was little emulsification and no loss of protein from solution. This behaviour differed from that of the other alcohols investigated as 1% decanol in decane appeared to prevent emulsion formation altogether in the presence of protein.

Before the importance of the percentage of alcohol in decane was realized several experiments had been carried out using varying percentages of different alcohols in decane. When decane, containing 5% hexanol, was added to different concentrations of lysozyme it was found that the volume of the hexanol-in-decane solution at the dip, using lysozyme, differed from experiments where decane B' was used. The volume of 5% hexanol in decane solution was only 60 µl at the dip when it became constant with increasing concentration of protein. This is shown in figure 26 and should be compared with figure 9 where decane B' was used. Up to a concentration of 3.5×10^{-5}M lysozyme the loss of protein from solution to the emulsion of 5% hexanol-in-decane particles was 50% but with 6×10^{-5}M lysozyme this had dropped to 30%.

In order to investigate the effect of secondary alcohols in the emulsions, 4% solutions of hexan-1-ol, hexan-2-ol and hexan-3-ol in decane as well as 2% solutions of heptan-1-ol, heptan-2-ol and heptan-4-ol in decane were equilibrated with solutions of 2×10^{-5}M lysozyme at pH 2.0 and the effect of varying the volume of the decane solution added to the protein solution was investigated. All the alcohols gave similar

Figure 27. Equilibrium concentration of lysozyme in solution when varying volumes of decane containing secondary hexanols were added to the same initial concentration of lysozyme at pH 2.0 .

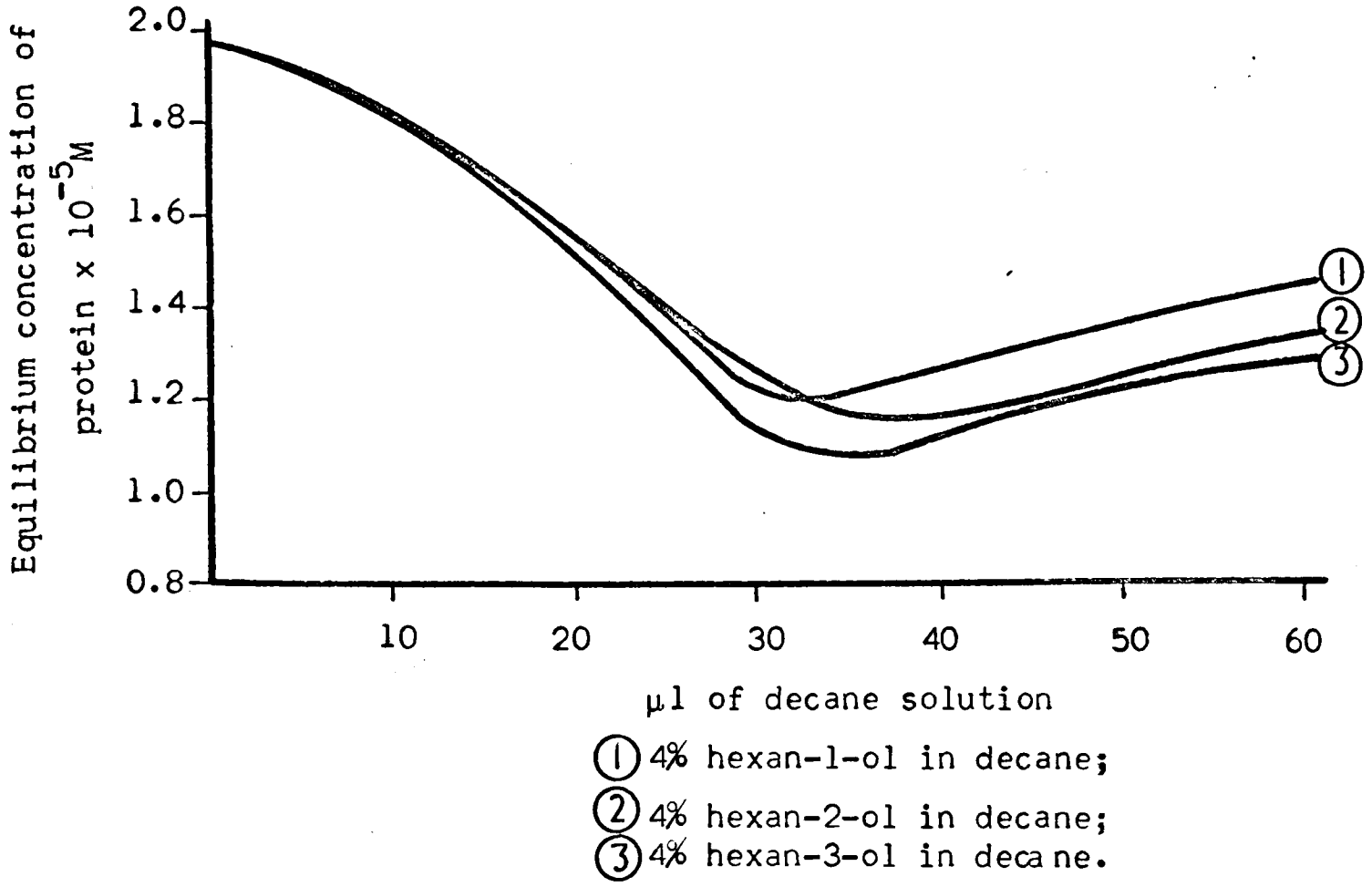


Figure 28 . Equilibrium concentration of lysozyme remaining in solution when varying volumes of decane containing secondary heptanols were added to the same initial concentration of lysozyme at pH 2.0 .

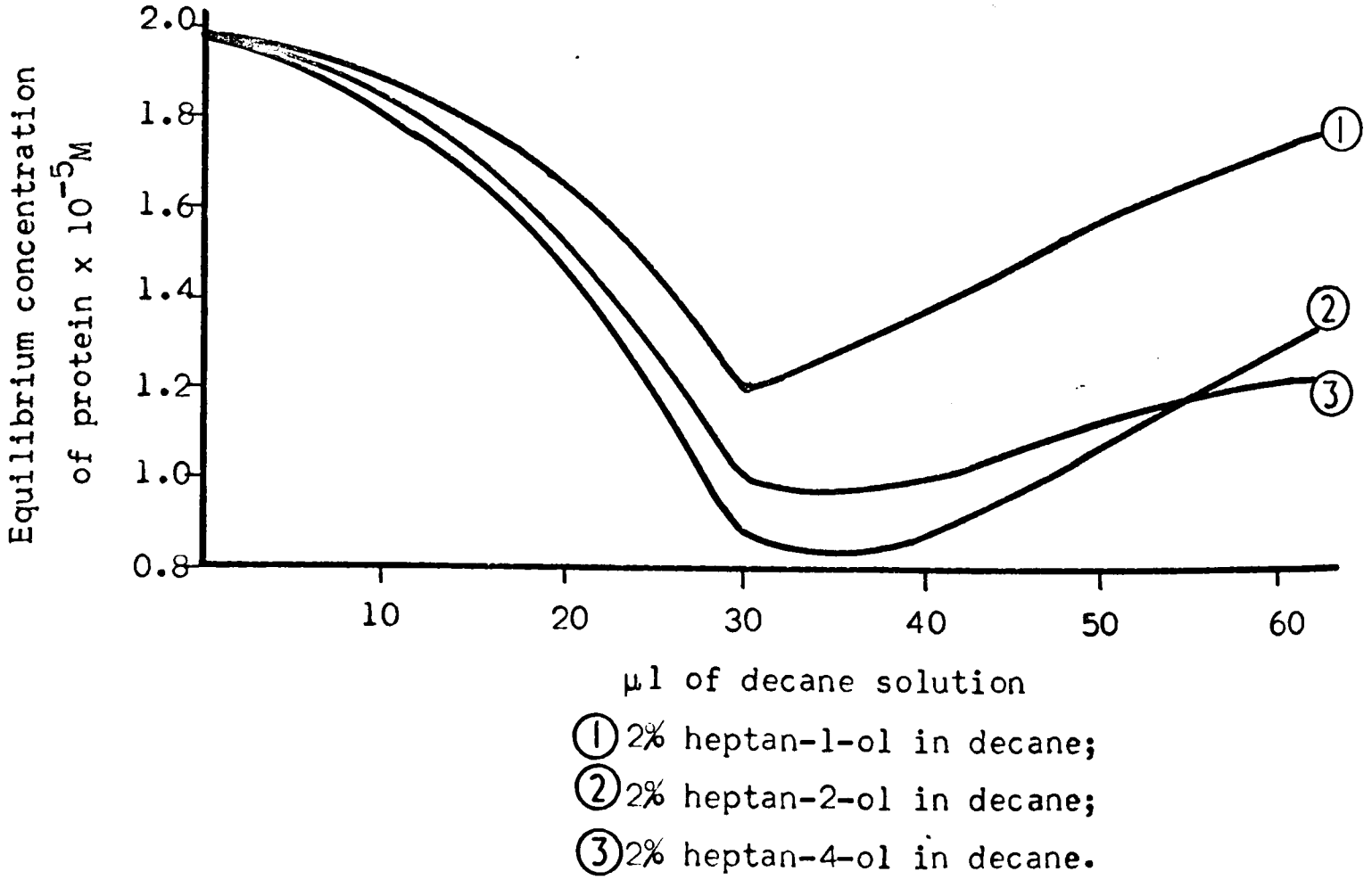
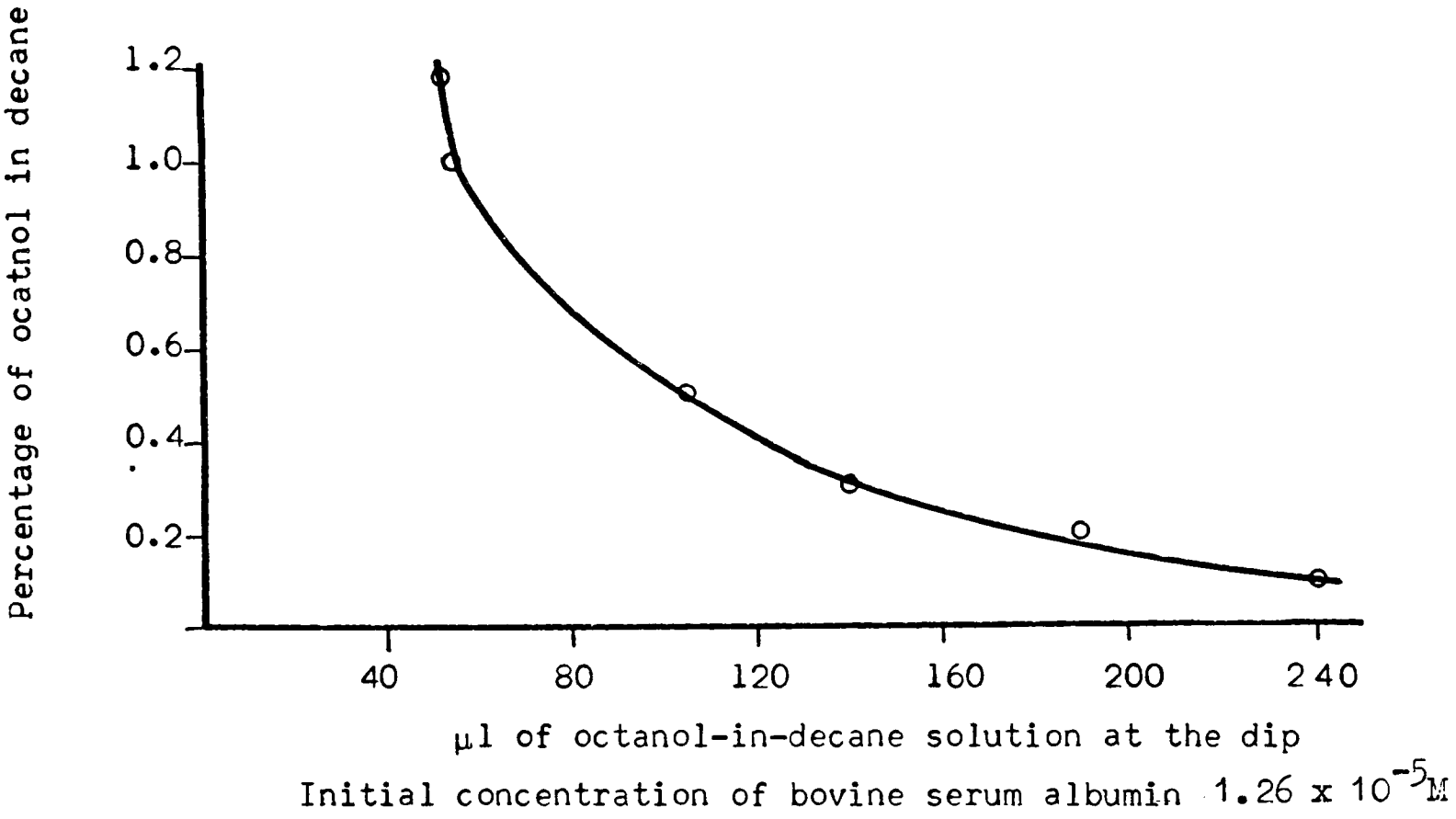
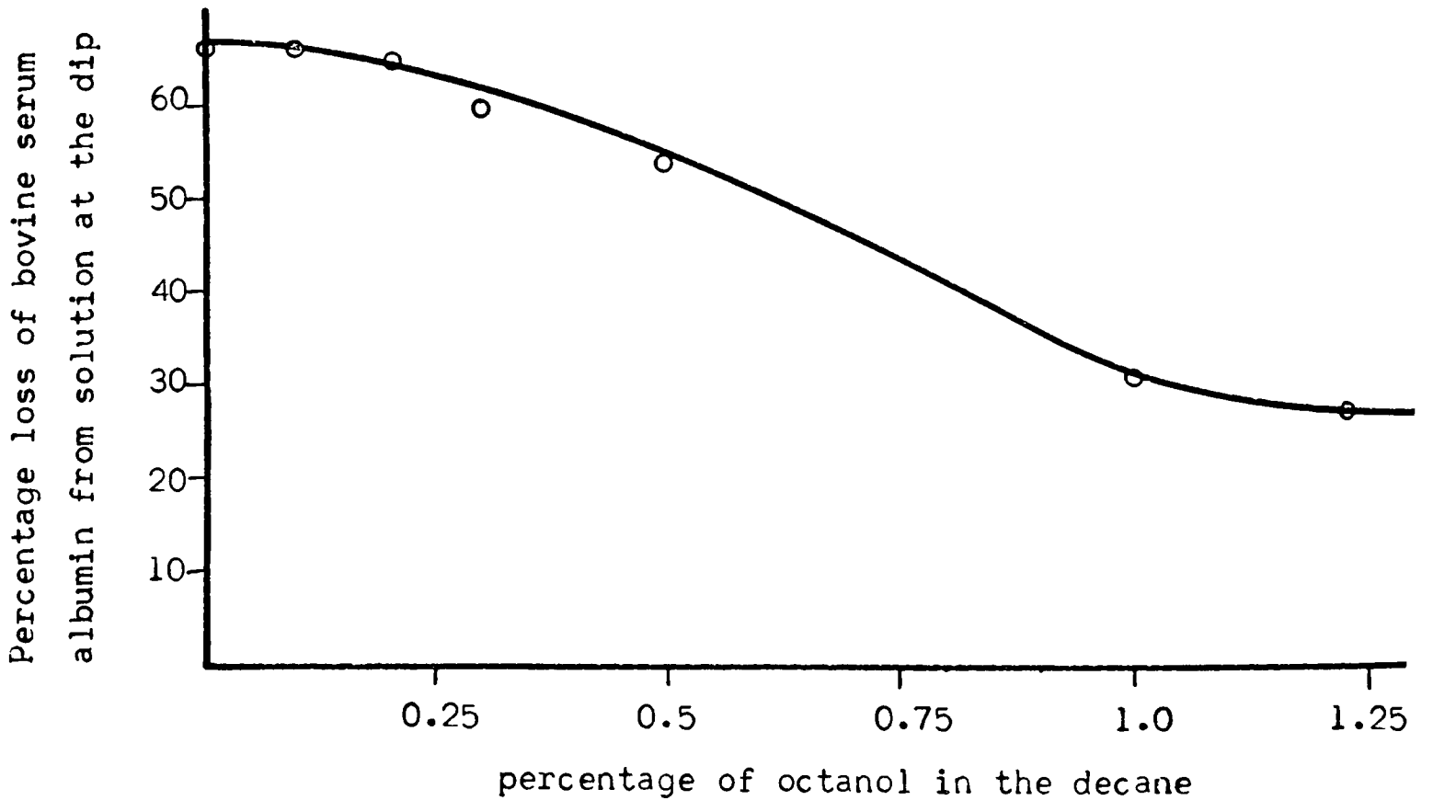


Figure 29. Volume of varying percentages of octanol-in-decane solution at the dip using a constant initial concentration of bovine serum albumin at pH 7.0.



Initial concentration of bovine serum albumin 1.26×10^{-5}M

Figure 30. Percentage loss of bovine serum albumin from solution at the dip at pH7.0 ,plotted against the percentage of octanol in the decane .



Initial concentration of bovine serum albumin 1.26×10^{-5}M

Figure 31. Volume of 3% hexanol-in-decane solution at the dip plotted against the initial concentration of bovine serum albumin at pH 7.0.

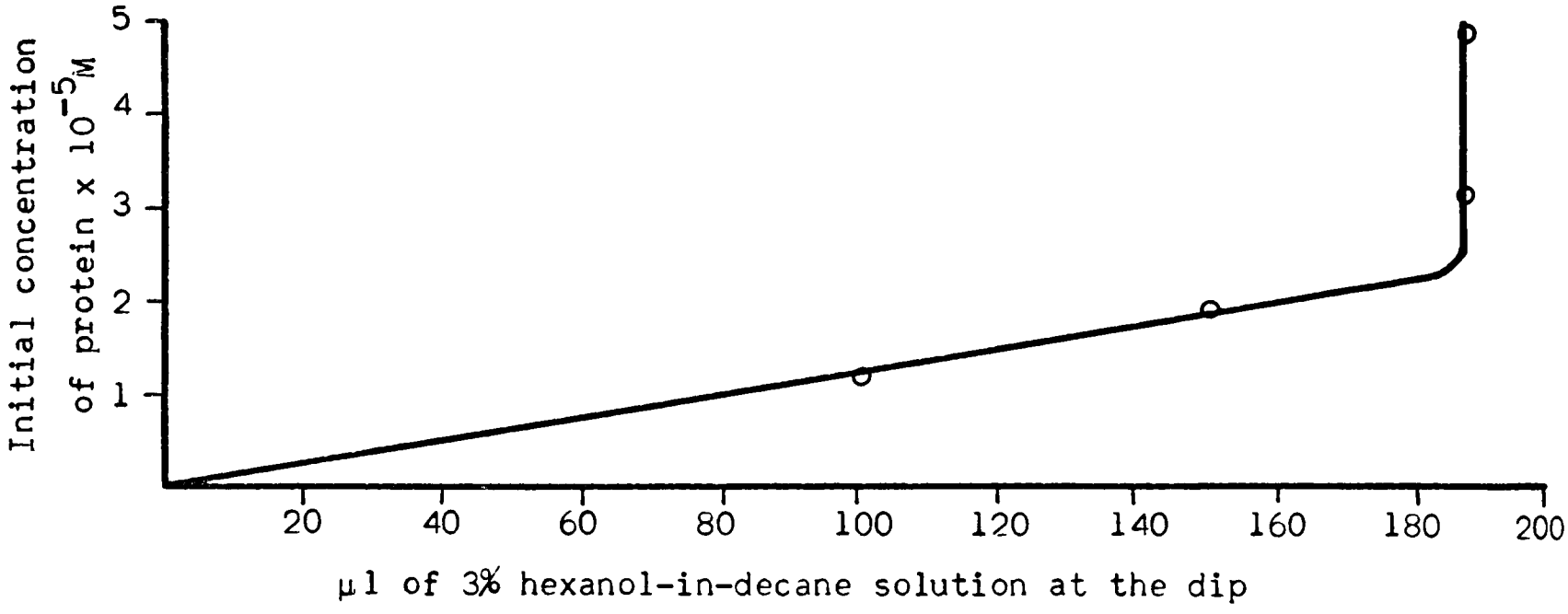
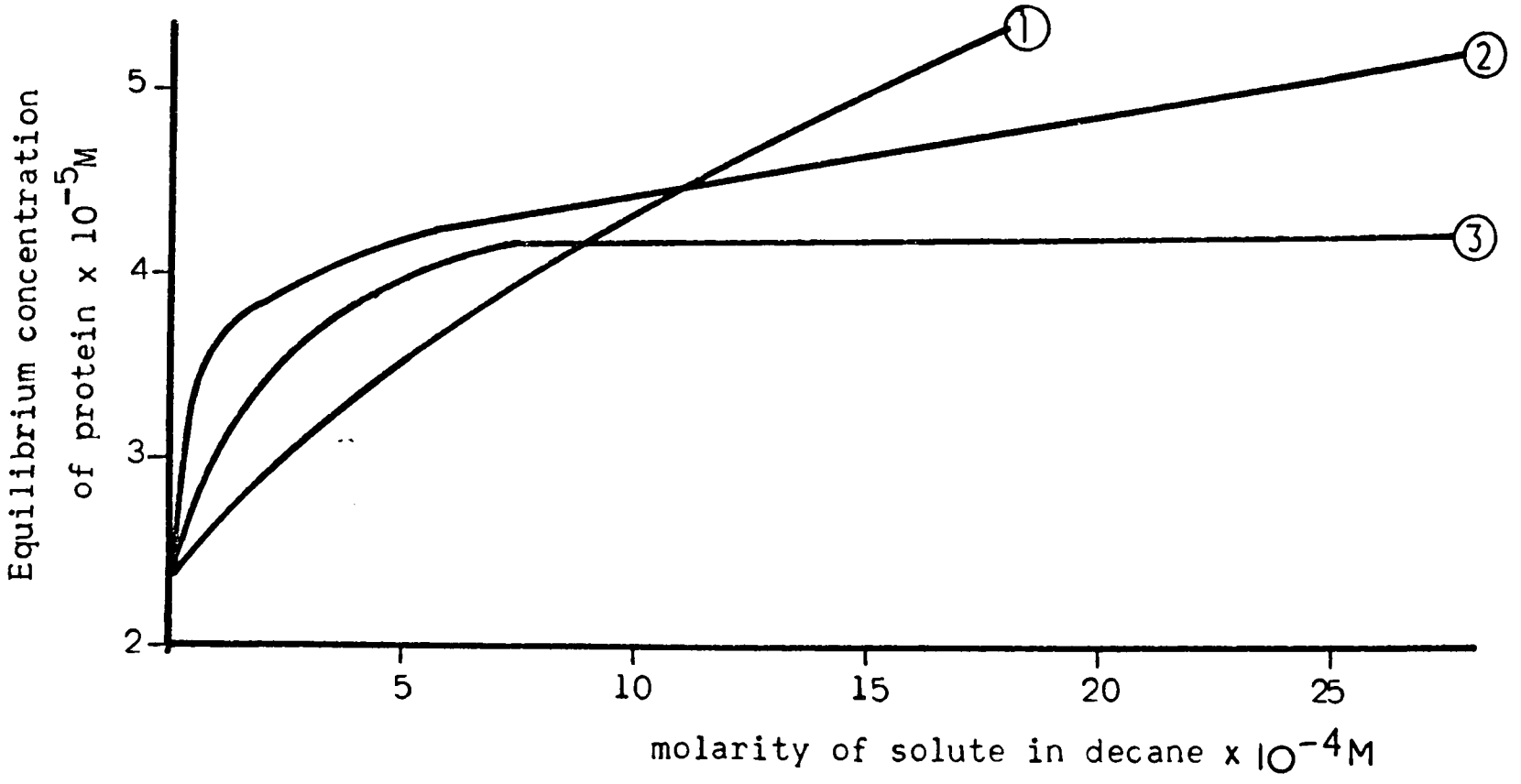


Figure 32. Equilibrium concentration of insulin in solution at pH 9.5, plotted against the molarity of various polar compounds in 300 µl of decane.



Initial concentration of insulin 10.95 x 10^{-5}M

① decylamine; ② stearic acid; ③ decyl mercaptan.

results except there was slightly more loss of protein from solution at the dip using the secondary alcohols, figures 27 and 28.

Bovine serum albumin. The effect of varying the percentage of octanol in decane greatly affected emulsions with bovine serum albumin at pH 7.0. Unlike similar experiments with lysozyme, both the volume of octanol-in-decane solution required to give the dip and the percentage of protein lost from solution at the dip varied with the percentage of octanol in decane. The results are shown in figure 29 and 30.

An experiment was carried out in which varying volumes of a solution of 3% hexanol in decane were added to different concentrations of bovine serum albumin. It was found that the volume of this solution that produced the dip differed from experiments where decane B' was used. The dip became constant at 2.8×10^{-5} M as shown in figure 31; up to this concentration 60% of the protein is lost from solution but after this concentration has been exceeded the percentage falls to 25% at 5×10^{-5} M.

Addition of other polar compounds to decane and their effect on emulsions in protein solutions.

When a 2% solution of decyl mercaptan in pure decane was equilibrated with a 2×10^{-5} M lysozyme solution at pH 2.0 it was found that there was no minimum in the amount of protein lost from solution as the volume of mercaptan-in-decane solution was increased. However there was less loss of protein from solution in the presence of mercaptan; instead of the normal 50% loss of protein from solution when pure decane was used this

dropped to 35% in the presence of mercaptan. A similar decrease was observed when a 10^{-2}M solution of mercaptan in decane B' was equilibrated with a solution of insulin at pH 9.5; instead of the normal 85% loss at this pH when decane B' was used this dropped to 60% in the presence of mercaptan.

This phenomenon was more thoroughly investigated by varying the concentration of mercaptan in decane B' and adding 250 µl of each of these solutions to a solution of 10 x 10^{-5}M insulin at pH 9.5. The loss of protein from solution versus the concentration of mercaptan in decane B' is shown in figure 32. Similar experiments where decylamine or stearic acid were substituted for the mercaptan are also shown in the same figure.

The effect of emulsions on the enzymatic activity of enzymes

Lysozyme.

Micrococcus lysodeikticus suspensions, approximately 0.1 mg./ml. in pH 6.24 buffer, were used as the substrate for comparing the activities of different lysozyme solutions. It was shown that the initial slope of the turbidity change when lysozyme was added to the substrate was linearly proportional to the concentration of lysozyme up to 0.03 mg./ml. in the 0.5 ml. of lysozyme solution added to the 2.5 ml. of substrate. The comparison between different samples of lysozyme in any given experiment were always made using the same micrococcus lysodeikticus

suspension.

As the micrococcus cells tend to clump with time when in suspension, the activity of a lysozyme solution was measured using the same substrate solution at zero time, after 30 minutes and one hour after mixing the micrococcus substrate suspension. The difference between the three activities was ± 1% which is well within the experimental error of the method. Thus different lysozyme solutions could be compared against the same substrate suspension, provided all the measurements were recorded within one hour of adding buffer to the dry cells to make the suspension.

Since the activity of lysozyme on the emulsion particles was to be studied it was important to find out whether the activity of lysozyme on an emulsion in pH 2.0 solution would change with time when it was diluted with pH 6.24 buffer to bring the concentration of lysozyme down to approximately 0.01 mg./ml. To do this an emulsion of pure decane in lysozyme at pH 2.0 was diluted with pH 6.24 buffer and the activities recorded over a period of one hour. A solution of native lysozyme was measured at the same time using the same substrate solution to make sure that any change in activity of the emulsion solution with time was due to the emulsion and not the substrate solution. No difference with time was observed so lysozyme emulsions formed at pH values other than pH 6.24 can be diluted in pH 6.24 buffer for assaying.

The activity of the enzyme on the emulsion was determined from the difference between the activities of the lysozyme stabilized emulsion solution and the emulsion free solution of lysozyme that remained when the

emulsion was removed by centrifugation.

Experiments were carried out adding different volumes of decane B'' to a given concentration of lysozyme solution. It was found that there were no clear differences in the activity of the lysozyme on the emulsion as the volume of hydrocarbon was varied. In some experiments small negative activities were recorded but this was probably due to either the difficulty in measuring the initial rates very accurately or in the insensitivity of the assay procedure itself. The most constant values for activities of lysozyme on emulsions were obtained when 30 µl of decane B' or over 50 µl of pure decane were added to 6 ml. of 2×10^{-5} M lysozyme solution which was then equilibrated on the wheel. In both these cases the activity of lysozyme on the emulsion was less than 10% of the activity of native lysozyme. The activity of the lysozyme on the emulsion particles appeared to be independent of the pH at which the enzyme was adsorbed onto the emulsion.

The solution that was in equilibrium with the emulsion which had been separated by centrifugation, was found to have retained $90 \pm 10\%$ of its original activity.

In experiments where the decane had been subjected to ultrasonic vibrations for 15 seconds before equilibrating, the activities of the lysozyme on the emulsion and in free solution were the same as those in which there had been no prior ultrasonic vibration of the solutions. If a solution of lysozyme in pH 2.0 buffer was subjected to ultrasonic vibrations for the same time, the activity of this solution was unchanged.

It was also found that if the time of equilibrating the decane and lysozyme solutions was varied, the activities were in all cases the same as those in which the solutions had been equilibrated for 24 hours.

There are two ways of removing lysozyme from an emulsion; either re-equilibrating the emulsion in pH 2.0 buffer or re-equilibrating in a buffer plus long chain alcohol. In both these cases the activity of the lysozyme removed from the emulsion is 75 $\pm$ 10% of the activity of native lysozyme.

In several cases the activity of the lysozyme remaining on the emulsion after this second equilibration was estimated and was found to be the same as that on the original emulsion, i.e. less than 10% of the activity of native lysozyme.

Ribonuclease.

2'-3' cyclic cytidine monophosphate solutions of approximately 0.2 mg./ml. of pH 7.0 0.1 M buffer were used as the substrate for comparing the activities of different ribonuclease solutions. As with lysozyme the initial slope of the optical density change was linearly proportional to the concentration of ribonuclease even up to 0.3 mg./ml.. in the 0.5 ml. of ribonuclease added to the 2.5 ml. of substrate.

The activity of ribonuclease on emulsion particles was only studied in experiments where 30 μl of decane B' were added to 6 ml. of 2×10^{-5}M ribonuclease solution and this mixture equilibrated for 24 hours. In these experiments the activity of the enzyme on the emulsion was found to be 15 $\pm$ 10%.

When the emulsion was removed by ultracentrifugation the protein that remained was found to be 100 ± 10% of its original activity.

The ribonuclease that was removed from emulsions by the addition of a long chain alcohol had 100 ± 10% of its original activity.

Effect of ethanol on some physical constants of insulin

From osmotic pressure experiments at pH 2.5 the molecular weight of insulin, by extrapolation to zero concentration, is 12,000 (Gutfreund, 1952). The molecular weight at finite protein concentrations is however, larger the greater the salt concentration (Gutfreund, 1952); in the presence of dioxan insulin has a molecular weight of 6,000 (Fredericq, 1957). Thus both the dissociation from 12,000 to 6,000 and the association from 12,000 to larger molecular weights are governed by hydrophobic forces though the association is also controlled by electrostatic forces since it is dependent on both pH and salt concentration (Gutfreund, 1948).

Some properties of insulin were investigated using various physical techniques in order to study the hydrophobic interactions that may be connected with the dissociation of this protein from 12,000 molecular weight to 6,000 molecular weight by the action of ethanol.

Molecular weight.

At pH 2.0, 0.1 M sodium chloride, insulin at a concentration of 2 mg./ml. of solution was shown to have a molecular weight of approximately 20,000. In the presence of ethanol the molecular weight decreases progressively until at approximately 60% ethanol (v/v) it drops to 5,700.

Figure 33. Molecular weight of insulin at pH 2.0 plotted against the percentage of ethanol in solution .

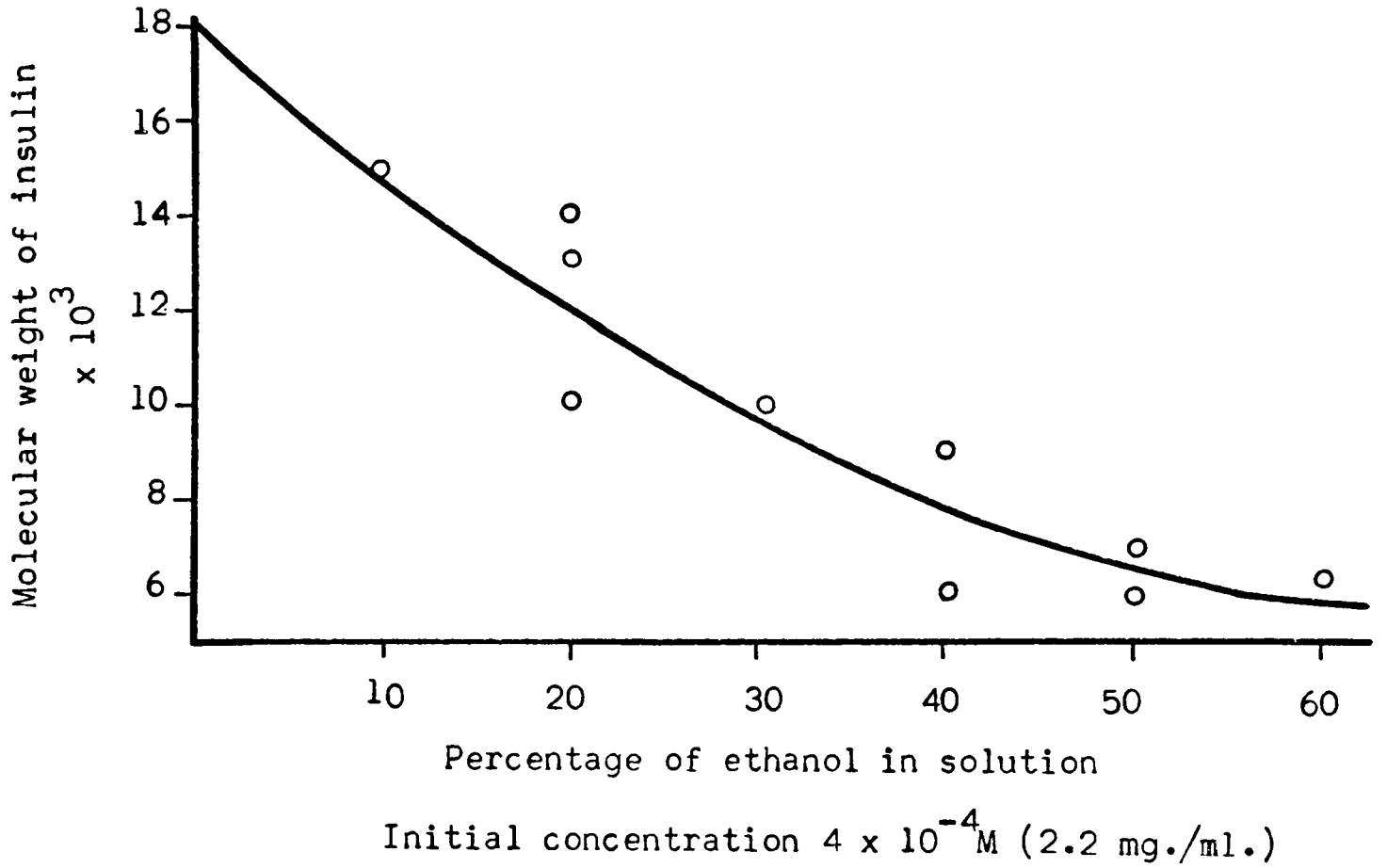
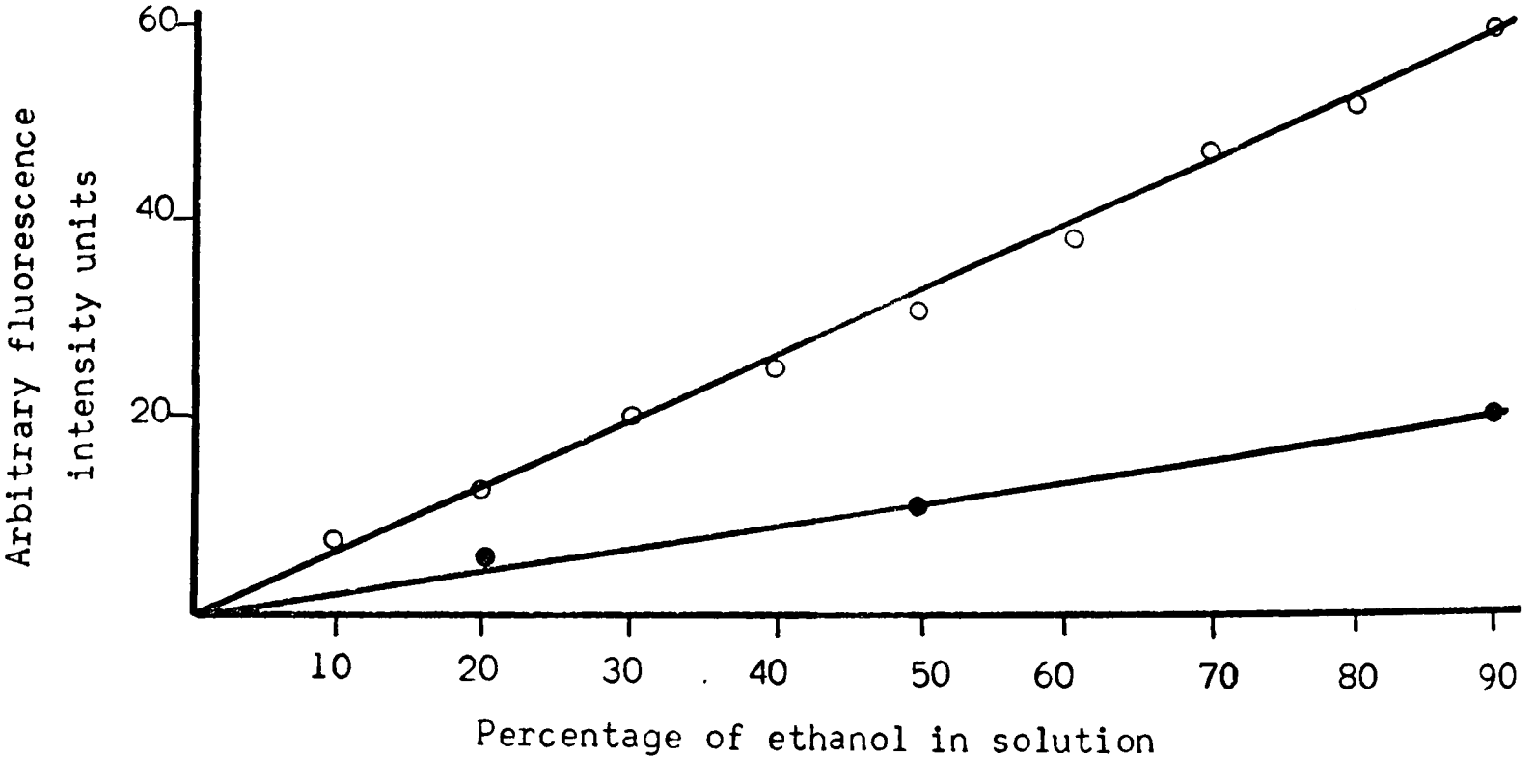


Figure 34. Intrinsic fluorescence intensity of insulin plotted against the percentage of ethanol in solution .



The results are shown in figure 33.

Optical rotatory dispersion.

In order to determine whether the molecular weight change of insulin with varying percentage of alcohol is accompanied by any changes in the optical rotatory dispersion, spectra were recorded at pH 2.0, 0.1 M sodium chloride, at different ethanol concentrations. The insulin concentration was varied in different experiments between 1×10^{-4}M and 3×10^{-4}M.

Insulin exhibits an anomalous dispersion in the region of 37 wave numbers. Since this anomaly causes a displacement in the Moffit plot it is possible to measure its magnitude. This is taken as the vertical displacement of the Moffit plot from its normal (i.e. the extrapolation of the straight line which goes through the points of the Moffit plot from 20 to 34 wave numbers) to its observed value. This difference was measured at 37 wave numbers and is referred to as $\triangle 37$ (Bayley, 1965).

The effect of increasing the percentage of ethanol (v/v) in buffer on ao, bo and $\triangle 37$ of insulin are shown in table 9.

Intrinsic fluorescence.

In order to determine whether the conformation of tyrosine residues of insulin are affected by the dissociation which occurs in the presence of ethanol, the intrinsic fluorescence of insulin at pH 2.0 was measured in solutions of increasing ethanol concentration.

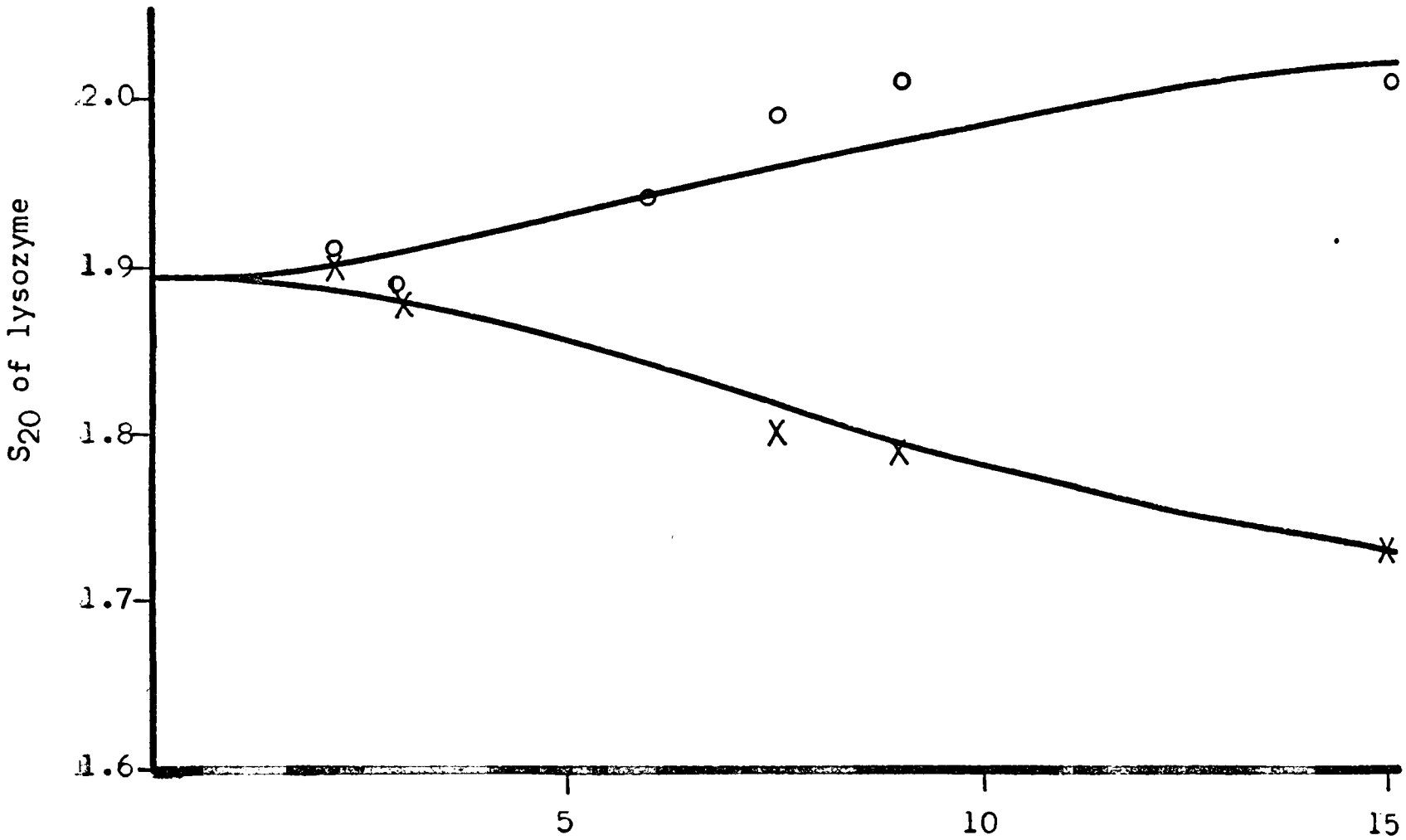
The insulin was excited at 280 mμ and the emission spectra recorded at 305 mμ. The fluorescence intensity was studied at two protein

Table 9

Effect of different percentages of ethanol in pH 2.0, 0.1 M sodium chloride, on the optical rotatory dispersion parameters of insulin.

Percentage of ethanol	$-a_0$	$-b_0$	Δ 37
0 %	129	205	130
10 %	136	220	150
20 %	170	214	158
30 %	168	217	148
40 %	170	240	155
50 %	178	245	173
60 %	182	263	168
70 %	187	265	191
80 %	196	255	185

Figure 35. S_{20} values of lysozyme plotted against the concentration of protein in aqueous solution.



concentrations and the results are given in figure 34. The increase in fluorescence intensity with increasing percentage of alcohol was linear at both protein concentrations up to 90% (v/v) ethanol. This linear increase in intensity with increasing percentage of alcohol was also observed when the intrinsic fluorescence of the amino acid tyrosine was investigated.

S^{20} values for lysozyme

In order to determine whether lysozyme dimerizes above pH 5.5, at protein concentrations below 15 mg./ml., several experiments were carried out to determine the S^{20} values of lysozyme in solution, varying the pH and the concentration of lysozyme. Sophianopoulus and Van Holde (1964) have shown that a 1.5% solution of lysozyme dimerizes reversibly above pH 5.5.

It was found that at pH 7.0 the S^{20} value increases with increasing concentration of protein, while at pH 2.0 it decreases with increasing concentration of protein. The results are shown in figure 35.

DISCUSSION

Measurement of surface tension

The pendant drop technique is one of the most suitable methods for the measurement of the surface and interfacial tensions of protein solutions as there is little or no disturbance of the surface during the course of the measurement. It was intended originally to form a drop of buffer in decane and then apply a known amount of protein to the interface by means of a second syringe. Ghosh and Bull (1963) have measured the interfacial tensions of bovine serum albumin solutions at an octadecane/water interface using this technique. However they were not able to obtain a direct measurement of the concentration of protein at the interface. Instead they estimated the amount of bovine serum albumin adsorbed on an emulsion of octadecane in water of known surface area. The amount of protein adsorbed per square metre of hydrocarbon/water interface, 2.5 mg./metre^2, is in good agreement with the value of 1.8 mg./metre^2 obtained by Cockbain (1956) using decane/water emulsions. Ghosh and Bull obtained values as high as 5 mg./metre^2 in the higher film pressure region which are very much higher than that occupied by a spread monolayer of protein at low pressures but are comparable to those of films spread at high initial pressures, Augenstein *et al*. (1958). Thus it is unwise to compare interfacial tension values directly with the amount of protein adsorbed on hydrocarbon-in-water emulsions under the same conditions.

Both of the early techniques used in this work to estimate the concentration of protein at a non-polar/aqueous interface, namely at liquid decane/water and dimethyl silane coated glass/water interfaces, were unsuccessful as the amount of protein adsorbed was very small and thus difficult to measure. The latter technique was unsatisfactory for a second reason in that the number of methyl groups on the surface of the glass was unknown and it is possible that not all the Si-OH groups had reacted with the dichloro dimethyl silane.

It was clear from these experiments that a liquid hydrocarbon/water interface had to be used and the only way of creating a sufficient surface area was to form an emulsion of hydrocarbon in water.

The adsorption of proteins on emulsions

Formation of emulsions.

In this work emulsions were formed by adding protein solutions and hydrocarbon to test tubes which were then agitated on the bicycle wheel; an emulsion was formed in a few minutes and the system reached equilibrium within 3 hours. This technique has not been used previously in investigating the properties of proteins on emulsions. Cockbain (1956), Fraser *et al*. (1955), and Fraser and Schulman (1956) preformed their emulsions by mechanical stirring while Ghosh and Bull (1962, 1963) used ultrasonic vibrations. In all these cases the protein and hydrocarbon were only equilibrated after an emulsion had already been formed.

Adsorption of proteins on emulsions of pure decane.

When protein is adsorbed from solution onto emulsions of pure decane the particles are stabilized by the formation of a film of protein at the interface. The effect of this protein layer is to reduce the free energy per unit area between the two liquids since pure decane in water, if shaken, will not form an emulsion. Since only a portion of the protein in solution is adsorbed at the interface, figure 2, there must be an equilibrium between protein spread on the interface and protein remaining in aqueous solution, i.e. a difinite concentration of protein will be required in the aqueous phase in order to maintain sufficient protein on the interface to reduce the surface free energy to a level at which emulsification can occur. The actual amount of protein on the interface at any point on the curve will depend on the area of the emulsion surface; this in turn will depend on both the total volume of decane present and the particle size distribution in the emulsion, this latter may vary at different points on the curve. The area of the emulsion surface will continue to increase as the volume of decane increases (not necessarily linearly) until no more protein is lost from solution as more decane is added. At this volume of decane the amount of protein remaining in solution is just sufficient to maintain the layer of protein on the surface. If any more decane is added to the system it will not be covered with a layer of protein and it will not emulsify.

Ghosh and Bull (1962) obtained similar results to those shown in

figure 2, when they studied the adsorption of chymotrypsin on emulsions of octadecane in water.

Adsorption of proteins on decane B' emulsions.

When emulsions of decane B' were formed in the presence of protein it was found that, if the volume of decane B' was varied and the initial concentration of protein was kept the same, then the amount of protein lost from solution reached a maximum as the volume of decane B' was increased and fell again until, with sufficiently large volumes of decane B', no emulsion was formed and no protein was lost from solution, figure 3. Thus the presence of very small amounts of alcohol in the decane markedly effects the properties of pure decane emulsions stabilized by proteins.

The problem of equilibria between protein solutions and decane B' is more complicated than with pure decane because of the presence of an additional surface active component, namely octanol. Decane B', like pure decane, does not emulsify spontaneously in water but does emulsify if equilibrated with protein solution. Octanol does not emulsify in buffer solution or in protein solution; although it is surface active it does not produce a sufficient drop in the interfacial free energy in either case to allow emulsification.

At any point on the curve in figure 3, if no protein were present, the octanol would be distributed between the aqueous and hydrocarbon phases according to the partition function, and there would also be a layer of octanol at the interface. Since figure 3 differs from figure 2

up to the point when no more protein is lost from solution and the extent of this difference increases with increasing concentration of octanol, figures 24 and 29,some octanol must also be present, together with protein, at the interface. Since pure octanol does not emulsify in protein solution it is unlikely that a complete monolayer of octanol is present at the interface. Thus it would seem most likely that there is a mixed layer of protein and octanol at the interface between the emulsion and the aqueous solution. It is probable that there is one ratio of protein to octanol which gives the lowest interfacial free energy so this composition will be normally present at the decane/water interface of an emulsion particle.

As the volume of decane B' is increased the area of the emulsion, and the amount of protein adsorbed at the interface, will increase up to the volume of decane B' at the dip. At this point, as with pure decane, the concentration of protein in the aqueous phase is just sufficient to maintain the mixed protein layer and so no further emulsification occurs. Meanwhile the total amount of octanol in the system, and therefore the amount distributed between the two liquid phases, has increased. As the volume of decane B' is now increased further past the volume at the dip there is even more octanol present in the system, and therefore the amount distributed between the two liquid phases, has increased. Thus with volumes of decane B' in excess of those at the dip, the amount of octanol in the system is sufficient to cause an increase in the ratio of octanol to protein at the interface.

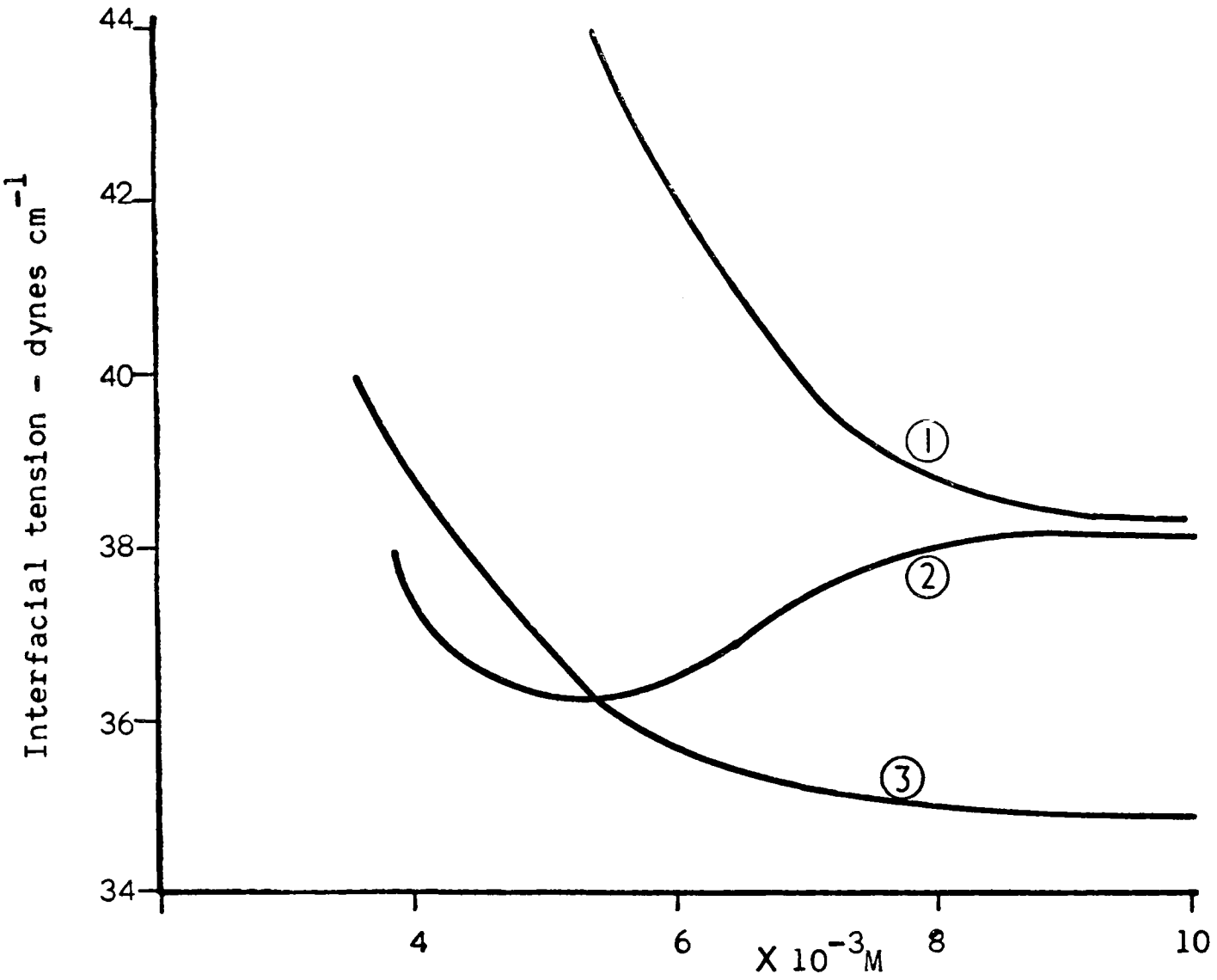
Since protein does not form a layer on an octanol surface this will tend to prevent protein forming a layer on the decane B' surface and thus reduce the amount of emulsion formed.

After the dip the overall concentration of octanol in both liquid phases and the interface increases. For a given initial protein concentration and volume of decane B' less emulsion will therefore be formed. If the concentration of octanol in decane is increased, figures 24 and 29, once again there is less emulsion formed.

There are therefore at least two equilibria controlling the adsorption of protein at the interface, namely equilibrium of protein between solution and the interface, and equilibrium of octanol between decane or aqueous solution and the interface.

Mixed film formation has been the subject of many investigations, especially by Schulman and coworkers (Schulman and Stenhagen,1938; Schulman and Rideal, 1934; Schulman and Cockbain, 1940; Bowcott and Schulman, 1955; Rideal, 1945). In most of these cases a long chain alcohol or cholesterol was present at the interface and a detergent present originally in the aqueous phase. Schulman and Rideal (1934) as well as Carless and Hallworth (1968) have shown that the two substances were reacting stoichiometrically at the interface although by no means all the results for interactions of mixed monolayers indicate that a stoichiometric relationship is involved (Pankhurst, 1942; Hutchinson, 1948b). Rideal (1945) showed that cholesterol or long chain fatty acids, when injected under a protein monolayer, formed a complex with the

Figure 36. Effect of sodium hexadecyl sulphate and of sodium chloride upon the surface tension of sodium dodecyl sulphate (*).



* Data from Miles and Shedlovsky (1944)

① pure sodium dodecyl sulphate.

② sodium dodecyl sulphate + 1% sodium hexadecyl sulphate of the amount of sodium dodecyl sulphate

③ sodium chloride + 1% sodium hexadecyl sulphate of the amount of sodium chloride.

protein and penetrated the monolayer. However when this layer was compressed, it was found that only cholesterol remained at the interface while all the protein went back into solution. Cockbain (1953) obtained similar results with bovine serum albumin adsorbed at a benzene/water interface where the second surface active component was sodium dodecyl sulphate. Schulman and Cockbain (1940) also observed formation of complexes between various alcohols and gelatin at an air/water interface.

Thus the evidence would indicate that proteins form some sort of complex with alcohols and detergents at the interface and this complex must reduce the free energy of the interface by a greater amount than either component alone is able to do.

The only other work that could provide a possible explanation as to what is actually happening at the interface between the decane and the aqueous solution in figure 3 is provided by Miles and Shedlovsky (1944) and Miles (1945) who investigated the surface tensions of both air/water and oil/water interfaces in the presence of surface active materials. They found that a plot of the interfacial tensions versus the concentration of pure sodium dodecyl sulphate, levelled off at a constant value without any minimum. However, when the sodium dodecyl sulphate contained 1% sodium hexadecyl sulphate or 0.5% dodecanol and the previous plot repeated, there was a minimum in the interfacial tension versus concentration of surface active mixture, figure 36. For the double sulphate mixture the actual surface tension for the bulk concentration at which the minimum occurs corresponds to the same

bulk concentration of the mixture of sodium chloride and sodium hexadecyl sulphate. Thus up to the minimum with the double sulphate mixture the sodium dodecyl sulphate is acting as a salt since it can be replaced by sodium chloride. When more of the double sulphate mixture is added, the sodium dodecyl sulphate no longer acts just as a salt but is competing with the sodium hexadecyl sulphate for the interface.

The explanation as to why dodecanol displaces sodium dodecyl sulphate from an interface appears to lie in the fact that dodecanol is more surface active than the detergent as it lowers the interfacial tension by a greater amount. Since the alcohol does not have a charged group it is unlikely that sodium chloride would effect the interfacial tension exerted by this compound. The apparent superiority of the hydroxyl over the sulphate at lowering the interfacial tension at low concentrations of surfacant could be due to hydrogen bonding between the hydroxyl and water which would lower the free energy of the interface by a greater amount than would the sulphate group by electrorestriction of the surrounding water molecules. Thus, at the minimum, the sodium dodecyl sulphate is not exerting any surface activity and the interfacial tension is entirely due to the dodecanol or sodium hexadecyl sulphate. As the maximum effect by the alcohol or sodium hexadecyl sulphate occurs when it is present as a compressed monolayer it is possible to conclude that, at the minimum, the dodecanol or sodium hexadecyl sulphate is

present at the interface in this form.

This work of Miles and Shedlovsky supports the idea that there is a monolayer of octanol present at the interface at the dip with decane B' emulsions stabilized by proteins. However none of the results reported by Rideal (1945), Cockbain (1953) or in this work using decane B', would indicate that this is the case. Rather the evidence indicates the presence of a mixed layer of alcohol and protein at the interface.

The idea of a mixed layer at the interface gives rise to the interesting conclusion that the protein is probably held onto the emulsion particle by a mixture of hydrophobic interactions between non-polar residues on the protein with the decane and hydrogen bonds between the octanol hydroxyl groups with the peptide groups of the protein backbone. These hydrogen bonds will be much stronger than those between the same groups in free solution since they are in a non-aqueous environment.

Estimation of particle size.

Measurement of particle size was attempted using a Coulter counter but it was found that there was a large spread of particle size so no measurement could be made. This fact was further indicated by photographs of the emulsions which showed that there was a spread of particle sizes from approximately 2μ in diameter to less than 0.1 μ. Ghosh and Bull (1962) measured the particle size of preformed emulsions by means of a Coulter counter before the emulsion was equilibrated with protein. Since emulsions of decane B' which were pre-emulsified were

also shown to have a complete spread of particle sizes after equilibration on the bicycle wheel for 30 minutes, it is likely that the final particle size obtained by Ghosh and Bull in their experiments were not the same as those actually measured.

The problem is further complicated by the fact that the particle size of emulsions has been shown to vary with the composition of the emulsion stabilizer. Bowcott and Schulman (1955) showed that emulsions of benzene in water stabilized by mixed films of soap and alcohols can have varying particle size depending on the amount of alcohol present. However this effect was only observed with alcohols which had a chain length of less than 10 carbon atoms since the higher alcohols form a 1:1 complex with the soap and this produces a very rigid film which cannot assume a large enough curvature to form droplets below 1 μ in diameter. Rehfeld (1968) has shown that the particle size of benzene in water emulsions, stabilized by sodium dodecyl sulphate, decreased with increasing concentration of detergent until the critical micelle concentration was reached, after which no further decrease was observed. He also observed that measuring the particle size of emulsions by dilution of a sample and then counting was inaccurate, as the conditions in which the emulsion had been formed were now different. Thus the results obtained by Cockbain (1956) as to the interfacial area occupied per unit weight of bovine serum albumin may be in error as the addition of increasing concentration of sodium dodecyl sulphate to the protein stabilized emulsion is likely to result in a change in the particle size.

Thus the only valid measurements of particle size are those using a direct method under the precise conditions used in the experiment concerned. Unfortunately such measurements have so far proved to be impossible and methods involving addition of other surface stabilizers, or even dilution, are likely to change the size of the particles.

Comparison between different proteins adsorbed on decane emulsions containing ¼% (v/v) octanol.

The fact that the amount of protein adsorbed per unit volume of hydrocarbon does not vary as the absolute volumes of the protein solution and decane are increased means that the phenomenon is not dependent on the amount of water present (beyond that necessary for the formation of the emulsion). This indicates that the loss of protein from solutions is purely a surface phenomenon and is dependent, not on the total volumes of the mixture, but on the ratio of the volume of hydrocarbon to the volume of protein in solution.

Since the amount of protein lost from solution at the dip does not vary with temperature, as would be expected if the adsorption were purely a hydrophobic phenomenon, it indicates that other types of bonding, even on pure decane emulsions may be important in determining the adsorption of proteins at the decane/water interface.

The fact that so little protein was adsorbed on emulsions formed by ultrasonic vibrations could mean either of two things. (a) 15 seconds ultrasonic vibration does not produce fine enough emulsion particles which have a sufficient surface area, so agitation on the bicycle wheel

is necessary to produce smaller particles on which protein is adsorbed. (b) Agitation for at least 20 minutes is necessary for protein to be adsorbed on preformed emulsion particles. Since Ghosh and Bull (1962) had only to mix their emulsions, formed by ultrasonic vibration, with protein solution and then stand this mixture for 90 minutes in order to attain equilibrium in the system, it would seem that 15 seconds ultrasonic vibration is not producing sufficiently fine emulsion particles.

Bovine serum albumin. One interesting fact that emerged from the study of emulsions of decane B' stabilized by bovine serum albumin is that two different batches of this protein gave different relations between volumes of decane B' at the dip versus initial protein concentrations as seen in figure 7. This could have been due to the fact that batch A contained different impurities so its surface properties were different. Both batches B and C of bovine serum albumin gave identical results so these were more likely to be typical.

The exact explanation as to why there is a break in the plots shown in figure 7 is not clear, but it is related to the fact that the percentage loss of protein from solution begins to fall off as soon as an initial concentration of protein of 1×10^{-5}M has been exceeded.

The conformational change which bovine serum albumin undergoes below pH 4 has been extensively investigated (Yang and Foster, 1954) although the mechanism of this change is by no means clearly understood. With decane B' emulsions, stabilized by bovine serum albumin, the volume of decane B' at the dip increases sharply between pH 4 and 5 indicating

that the protein has undergone a change in its surface properties over this range, figure 8. Below pH 4 bovine serum albumin occupies one third the area occupied above pH 4, for a protein solution which was initially 1.1×10^{-5}M, providing of course that the particle size has not changed dramatically with the change of pH. The situation is further complicated by the fact that there is a steady increase in the amount of protein adsorbed at the dip with increasing pH over the range pH 2 to pH 8. Thus it would appear that there is a sharp change in the conformation of bovine serum albumin between pH 4 and pH 5, and possibly a more gradual change over the whole pH range that was studied; this gradual change appeared to cease above pH 7.

The only detailed investigation of the properties of bovine serum albumin on hydrocarbon emulsions has been by Cockbain (1956) who investigated the adsorption of this protein on emulsions of decane in water stabilized by sodium alginate (a polyuronic acid). He concluded that the adsorption was independent of albumin concentration, pH and ionic strength, and that the albumin layer was monomolecular in thickness. The results reported here with decane B' emulsions are clearly different, but this difference could be accounted for by the presence of sodium alginate in Cockbain's emulsions. Cockbain pre-emulsified his decane and obtained a value of 1.75 mg. of protein per metre^2 of decane/water interface. He used an initial concentration of bovine serum albumin of 2.5×10^{-5}M at pH 5.6 and observed a 45% loss of protein from the solution; this value is the same as that observed for decane B' emulsions

at the dip. 190 µl of decane B' was required to produce a 45% loss of protein from 6 ml. of a 2.5 x 10^{-5}M bovine serum albumin solution at pH 5.5 whereas Cockbain used 80 ml. of protein solution with 20 ml. of decane. If the particle sizes are approximately the same in both types of emulsion then only 3.17 ml. of decane B' would be required to produce the same loss of protein from solution compared to the 20 ml. of decane Cockbain found necessary. Using this volume of 3.17 ml. of decane B' the amount of protein per square metre of interface would come to 11.0 mg. This surface concentration of protein is far in excess of a monolayer of protein, indicating that the protein may be adsorbed on decane B' emulsions in some kind of multilayer. However the surface of the emulsions obtained by Cockbain was probably partly covered by sodium alginate.

Lysozyme. With lysozyme the volume of decane B' at the dip did not become constant until a concentration of approximately 9 x 10^{-5}M lysozyme had been exceeded, figure 9. The change in the molecular weight of lysozyme between pH 5.5 and pH 6.0 has been well characterized by Sophianopoulus and Van Holde (1964) and it is not surprising that the surface properties of this protein undergo a sharp change in this region as shown by the sharp increase in the amount of protein lost from solution, figure 10. In comparison with bovine serum albumin, the volume of decane B' at the dip does not change between pH 2 and pH 6.

The extent of the dimerization of lysozyme appears to increase with concentration as is shown by the fact that the percentage of protein

lost from solution at the dip increases slightly with increasing concentration between pH 6 and pH 8. From figure 35, the S_{20} value of lysozyme at pH 7.0 does not appear to increase until a concentration of 2 mg. per ml. (13.5×10^{-5}M) has been exceeded. The fact that the dimerization effects the surface properties of lysozyme even at protein concentrations as low as 2×10^{-5}M (0.3 mg. per ml.) is undoubtedly due to the fact that the intermolecular distances between protein molecules adsorbed at a decane B'/lysozyme solution interface will be much smaller than that pertaining in a solution containing more than 2 mg. protein per ml. of solution.

Insulin. Insulin behaves in a similar manner to the other proteins except that the volume of decane B' at the dip does not become constant with increasing concentration of protein up to the highest concentration used, 4.8×10^{-4}M.

The effect of pH on decane B' emulsions stabilized by insulin is very similar to those stabilized by lysozyme in that the volume of decane B' at the dip does not change but the percentage loss of protein from solution at the dip increases above pH 7.0. At pH 2.0 insulin has a molecular weight between 12,000 and 18,000 while above pH 7.0 insulin can have a molecular weight as high as 36,000 (Creeth, 1953). It is this aggregation which is likely to be responsible for the change in surface properties of insulin above pH 7.

Ribonuclease. Ribonuclease is not known to polymerize between pH 2 and pH 8, even at high concentrations. The results obtained with decane B'

emulsions agree well with this fact since the volume of decane B' at the dip and the percentage loss of protein from solution at the dip do not vary over the pH range studied.

Intracellular proteins. Carboxyhaemoglobin appears to be quite stable in the presence of decane B' emulsions but too few experiments were carried out to discover whether haemoglobin undergoes any change in its surface properties as the pH or concentration of the protein in solution is changed.

The other intracellular proteins investigated are less stable than carboxyhaemoglobin and were denatured in the presence of decane B'. This is not surprising since intracellular proteins contain no disulphide bonds which give increased structural stability to extracellular proteins (Cecil, 1963). Fraser et al. (1955) found that catalase, an intracellular enzyme, is irreversibly inactivated by pure nonane emulsions and also that 90% of the catalase initially in solution was adsorbed irreversibly on the emulsion, indicating that it must unfold on the surface. Adsorption of catalase onto charged surfaces did not lead to total inactivation and in some cases, adsorbed protein could be desorbed in the fully active state.

Summary of results with decane B' emulsions stabilized by proteins. The first observation that can be made concerning emulsions of decane B' stabilized by any of the proteins which were not denatured by decane B' is that the volume of decane B' at the dip is linearly proportional to the initial concentration of protein, except that with bovine serum

albumin and lysozyme it becomes constant as the initial concentration is increased beyond a certain point. The reason for this is not clear.

The other conclusion that can be made is that, if a conformational change occurs in a protein which is dependent on pH, then the volume of decane B' at the dip changes. Also, if the protein associates with pH, then the amount of protein lost from solution at the dip increases; however it is known that bovine serum albumin does not associate with pH yet the amount of protein lost from solution increases with increasing pH, figure 8.

Method by which protein is desorbed from emulsions of decane B' stabilized by protein. Protein may be desorbed from a decane/water interface by the addition of a long chain alcohol such as octanol or decanol. The method by which the alcohol displaces the protein must be by the preferential adsorption of the alcohol to the protein at the decane/water interface, in a similar manner to the mechanism already described to explain the adsorption of proteins on decane B' emulsions; since emulsions of pure alcohols are unstable, the net effect of the addition of alcohols is to break up the emulsions. This is similar to the system studied by Rideal (1945) where he found that the cholesterol displaced protein from an interface when the mixed film was compressed.

The removal of lysozyme from solution by changing the pH of the solution is undoubtedly due to a shifting of the equilibrium between the monomer and dimer on the interface as the pH of the solution is lowered. Since the properties of the protein on the interface are so

closely linked to the pH of the solution, it would indicate that, contrary to the conclusion of Danielli (1938), the pH of the decane/water interface must be close to that of the bulk solution. It is interesting to note that 40% of the protein on the emulsion is removed when it is re-equilibrated in pH 2.0 buffer; this effect is independent of the pH of the solution from which the lysozyme originally went onto the emulsion.

Conformation of proteins in the presence of emulsions. Difference spectroscopy showed that protein in aqueous solution in equilibrium with an emulsion undergoes a conformational change similar to that of the protein removed from the emulsions, figure 13. This indicates that when a given volume of decane is equilibrated with a protein solution, the protein is continually adsorbing onto the emulsion droplets, undergoing a conformational change, desorbing, and going back into solution. This would explain why the protein which is desorbed from emulsion particles by the addition of excess alcohol, has a very similar difference spectrum to the protein that is in solution in equilibrium with the original emulsion droplets.

Elkes et al. (1945) have shown that human haemoglobin, removed from oil in water emulsions, was different in many of its properties from native haemoglobin and solubility and colour tests indicated that the haemoglobin molecule had been broken down, with the separation of parahaematin.

Since the change caused by any given volume of decane or decane B' on the surface properties of a protein appear to be reproducible, the

conformation of the protein must be altered a definite amount every time, when it is in the presence of decane.

Binding of non-polar molecules by protein in solution

When solutions of bovine serum albumin, lysozyme and insulin are equilibrated with decane, specific binding occurs.

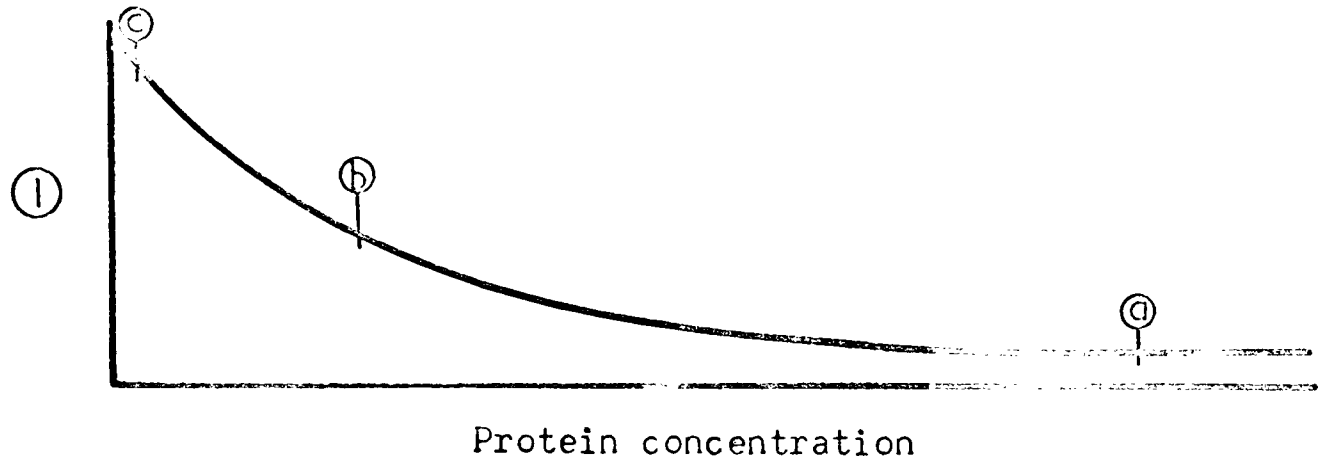
When 10 µl of radioactive decane containing 0.25% (v/v) of octanol was equilibrated with bovine serum albumin, insulin or lysozyme it was found that the binding increased with decreasing concentration of protein. However when the equilibrium concentration of protein was reduced below 0.2 mg. per ml. of solution, the binding of decane by these proteins decreased dramatically. By comparison of the points of maximum binding with the initial and equilibrium concentrations of protein in solution, it may be seen that the concentration of protein which exhibits the maximum amount of decane bound per mole of protein, corresponds to the equilibrium concentration of protein having its dip at 10 µl of decane B'.

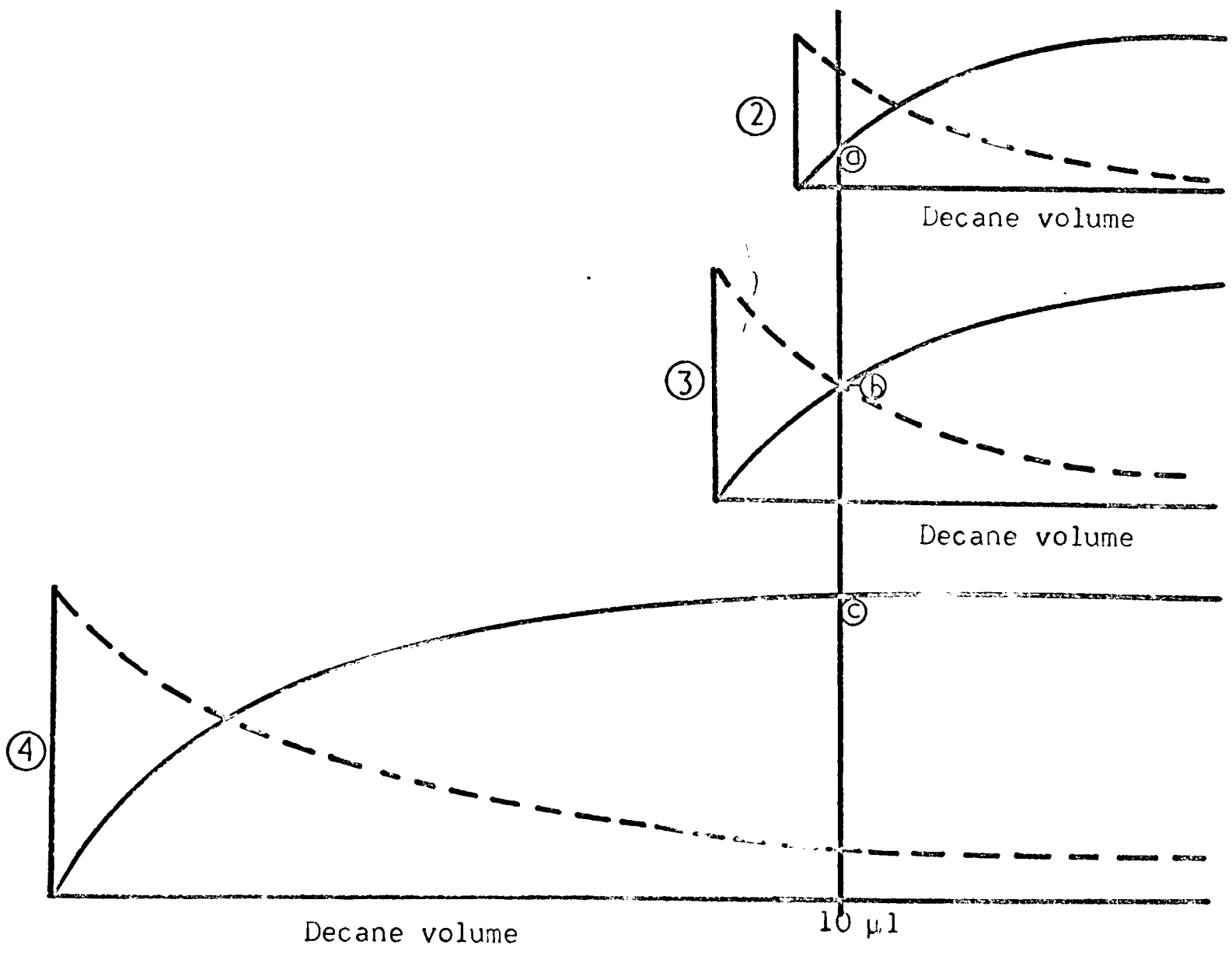
There is therefore a direct relationship between the amount of emulsion formed and the amount of binding. When 10 µl of pure radioactive decane was added to different concentrations of protein, it was found that there was no sharp fall off in the number of moles of decane bound per mole or protein below 0.2 mg. of protein per ml. of solution, figures 21 and 22. The binding of radioactive decanol to bovine serum albumin was also investigated and although the binding was similar

to that using pure decane the increase in binding with decreasing concentration of protein occurred at lower concentrations of protein than it did with pure decane; as with pure decane, the binding showed no falling off below 0.2 mg. of protein per ml. of solution.

The effect of the volume of decane emulsion on the binding of decane to lysozyme was further investigated using a constant initial concentration of protein and varying the volume of decane. It was found that the number of moles of decane bound per mole of protein was closely related to the volume of decane present, as well as the presence or absence of 0.25% octanol in the decane, figures 19 and 20. In the absence of octanol it was observed that, with a constant initial concentration of protein, the binding of decane by lysozyme increased with increasing volume of added decane until the maximum amount of protein had been lost from solution. After this volume of decane had been exceeded there was no further loss of protein from solution and the amount of decane bound per mole of protein was constant. In the presence of 0.25% octanol however, with the same constant initial concentration of protein, the binding of decane by lysozyme increased with increasing volume of decane up to the volume of decane B' at the dip, which was also the point of maximum loss of protein from solution. Once the volume of decane added was greater than that at the dip, the binding decreased as the equilibrium concentration of protein in solution increased. It has also been observed, using pure decane, that the

Figure 37. Moles of decane bound per mole of protein varying the concentration of protein , using 10 µl of decane , figure ① . Points ⓐ , ⓑ and ⓒ on figure ① are represented separately in figures ② , ③ and ④ where the volume of decane is plotted against the equilibrium concentration of protein in solution and the moles of decane bound per mole of protein.





——— moles of decane bound per mole of protein, $\bar{V}$.

– – – – equilibrium concentration of protein in solution.

maximum amount of decane bound per mole of protein, increases with decreasing concentration of protein when the decane is well in excess of the volume required to give the dip when decane B' is used.

These observations provide a description as to why the amount of decane bound per mole of protein decreases with increasing concentration of protein when the volume of decane is kept constant. This is best seen by referring to figure 37 which is a schematic representation of the possible binding curves. Curve 1 shows the variation of decane binding with varying protein concentration using a fixed volume of decane. The situations represented by points a, b and c are shown separately in curves 2, 3 and 4 in terms of the variation of the moles of decane bound per mole of protein with the volume of decane, at fixed initial concentrations of protein. At a the volume of decane present in relation to the equilibrium concentration of protein is well below the volume of decane that is required to give the maximum value of $\overline{V}$ and the maximum loss of protein from solution ; thus $\overline{V}$ in 1 is small at a. At b the volume of decane present is now closer to the volume necessary to give maximum binding for that concentration of protein, so $\overline{V}$ is larger than at a. At c $\overline{V}$ is still larger since the maximum value of $\overline{V}$ when excess decane is present is largest in $4 > 3 > 2$.

When decane B' is used to investigate the binding, a and b are similar to pure decane but c differs. In this case, in figure 4, the binding is much smaller even taking into account the fact that the $\overline{V}$ maximum in 4 will be greater than in 2.

Ray *et al.* (1966) observed a similar phenomenon when they investigated the binding of dodecanol to bovine serum albumin; this increased with decreasing concentration of protein in the same manner as decanol. They considered that this was due to the fact that the alcohol can adhere to more than one protein molecule and that at high protein concentrations this will lead to dimerization of bovine serum albumin. Since there is no evidence to show that dimerization of bovine serum albumin occurs under these conditions (Phelps and Putnam, 1960) this explanation is not satisfactory. The results reported in this work, using radioactive decane, indicate that the binding is related to the ratio of the protein concentration in solution to the volume of decane emulsion i.e. surface area of decane interface. Since decanol has a much higher solubility in water than does decane, the binding of decanol by bovine serum albumin could be explained by a similar reasoning except that the solution concentration of decanol is substituted for the surface area of decane interface. Since there is excess decanol present it is possible that some binding of alcohol by protein also occurs via the interface between liquid decanol and the protein solution.

The results obtained from the binding of decane to lysozyme reveal the intimate relationship between the binding of decane to proteins in solution and the adsorption of proteins on emulsions of decane in water. The most likely mechanism is that protein goes onto an emulsion particle and is adsorbed where it undergoes a conformational change, picks up its

quota of decane, desorbs and goes back into solution with the decane adsorbed on it. There is also the possibility that reaction occurs between protein and decane in solution, although the low solubility of decane in water would suggest that this plays a minor part. Ribonuclease does not bind decane, even though its properties in the presence of decane emulsions are identical to those proteins which do adsorb protein in free solution. This protein is therefore more likely to acquire its conformational change by interaction with decane on the emulsion than in solution. The explanation as to why lysozyme and insulin do not bind decane at 2°C, yet undergo a conformational change, is most probably the same as for ribonuclease.

Evidence against the view that decane only combines with the protein when the protein molecule is adsorbed on an emulsion particle is given by the work of Mohammadzadeh-K. et al. (1967) who carefully avoided obtaining an emulsion, yet bovine serum albumin was still found to bind nonane; this favours the view that protein molecules can bind decane which is in free solution. They also studied the binding of mixtures of hydrocarbons to proteins and showed that a ratio of at least 20% of one hydrocarbon in another was required before the binding of the hydrocarbon present in smaller amount to the protein could be detected. Since the binding of decane and decanol to bovine serum albumin are very similar, figure 21, it is unlikely that any octanol is bound to the protein in the presence of radioactive decane B'. However octanol is very much more soluble in water than is decane so, if binding of decane

is purely a solution process, it is possible that some octanol could be bound by the protein. The experimental evidence, figures 15 and 21 shows that the binding of decane by proteins when equilibrated with decane B' or pure decane is reasonably similar except for the fall off at low concentrations when using decane B', so it is unlikely that any octanol is bound by the protein.

The binding of decane to bovine serum albumin was further investigated by studying the binding at various concentrations of bovine serum albumin at various values of pH, figure 18. It was immediately observed that, as the concentration of bovine serum albumin was reduced so the variation in binding with pH became more pronounced. The result obtained at 1.64×10^{-5}M bovine serum albumin is very similar to that recorded by Ray et al. (1966) when they studied the binding of dodecanol to a 1% solution of this protein. In both cases the binding increases over the range pH 5 to pH 8. At pH 2 and pH 8 bovine serum albumin was found to bind approximately the same amount of decane and it is only over the range pH 3 to pH 8 that the binding varies with concentration of protein. Wishnia and Pinder (1964), when studying the binding of butane and propane to the same protein, found that the N form bound approximately 5 times and 4 times respectively as much hydrocarbon as did the F form. The binding of decanol to bovine serum albumin was also studied at various values of pH, figure 23, and it was found that the binding increased steadily from pH 2 to pH 8.

The fact that binding of decane by lysozyme and insulin occurred

at 22°C but not at 2°C gives weight to Kauzmann's (1959) original precept, that the strength of hydrophobic interactions must increase with increasing temperature.

It is unfortunate that the attempt to measure the binding of sodium dodecyl sulphate to bovine serum albumin did not produce meaningful results as these would have provided information as to the binding of non-polar compounds by this protein in free solution in the absence of emulsions.

Effect of different alcohols on decane in water emulsions stabilized by proteins

Effect of varying percentage of octanol in decane on the dip.

When solutions of decane containing varying percentages of octanol were added to solutions of 2×10^{-5}M lysozyme at pH 2.0 it was found that the volume of decane solution at the dip was the same, irrespective of the percentage of octanol in decane, figure 24.

When this experiment was repeated using a 1.1×10^{-5}M solution of bovine serum albumin at pH 7.0, the results were very different from those obtained with lysozyme. It was observed that both the volume of octanol-in-decane solution at the dip, as well as the percentage loss of protein from solution at the dip, varied with the percentage of octanol present in the decane, figures 29 and 30.

The difference between the results obtained with these two proteins possibly lies in the fact that with a 2×10^{-5}M solution of lysozyme at

pH 2.0, the volume of decane B' at the dip was still increasing with increasing concentration of protein, figure 9. However with a 1.1×10^{-5}M solution of bovine serum albumin at pH 7.0 the volume of decane B' at the dip was not increasing, but was constant with increasing concentration of protein, figure 7.

The results obtained with both these proteins shows that the alcohol can influence not only the volume of decane solution at the dip, but also the amount of emulsion formed both before and after the dip. This supports the explanation that the alcohol is present on the surface of the emulsion both before and after the dip and contradicts the view that only protein occupies the surface of the emulsion either before or after the volume of decane B' at the dip.

If it is accepted that a mixed layer of octanol and protein exists at the interface, then with lysozyme it would appear that increasing the percentage of octanol in decane up to 2% does not effect the nature of the layer present at the dip, figure 24, providing that the composition of the mixed film on the interface has a definite stoichiometric ratio if protein is to be adsorbed. This evidence does not concur with the idea of just two equilibria (of protein and octanol for the interface) controlling the adsorption of protein at the emulsion interface with the solution. It is possible that a further equilibrium is also controlling this process, but the evidence presented in this work would not appear to provide an explanation as to this observation. On either side of the dip in figure 24, the amount of emulsion formed is very dependent on the

amount of octanol originally present in the decane; this evidence supports the mixed layer explanation to account for the adsorption of protein on decane B' emulsions.

With bovine serum albumin, the octanol appears to effect not only the volume of decane at the dip but also the percentage loss of protein from solution as the percentage of octanol in the decane is increased. It may be seen from figures 8, 29 and 30 that both the amount of protein lost from solution and the volume of decane solution at the dip are identical for a 1.1×10^{-5}M solution of bovine serum albumin using a 0.8% octanol-in-decane solution at pH 7.0 or using a 0.25% octanol-in-decane solution at pH 2.0. Thus the N form of bovine serum albumin requires approximately three times as much octanol at the interface to interact with the protein than does the F form under similar conditions. This is in good agreement with a previous observation that with decane B' (0.25% octanol in decane) the N form of bovine serum albumin occupies 3 times the surface area as does the F form (assuming the emulsion has a constant particle size at both pH values), figure 8. These results with bovine serum albumin indicate that a definite stoichiometry between octanol and protein molecules must occur at the interface of octanol-containing decane emulsions stabilized by proteins.

Effect of different alcohols on the dip, varying the concentration of protein.

The results shown in figures 26 and 31 for lysozyme and bovine serum albumin, where 5% and 3% hexanol-in-decane solutions respectively

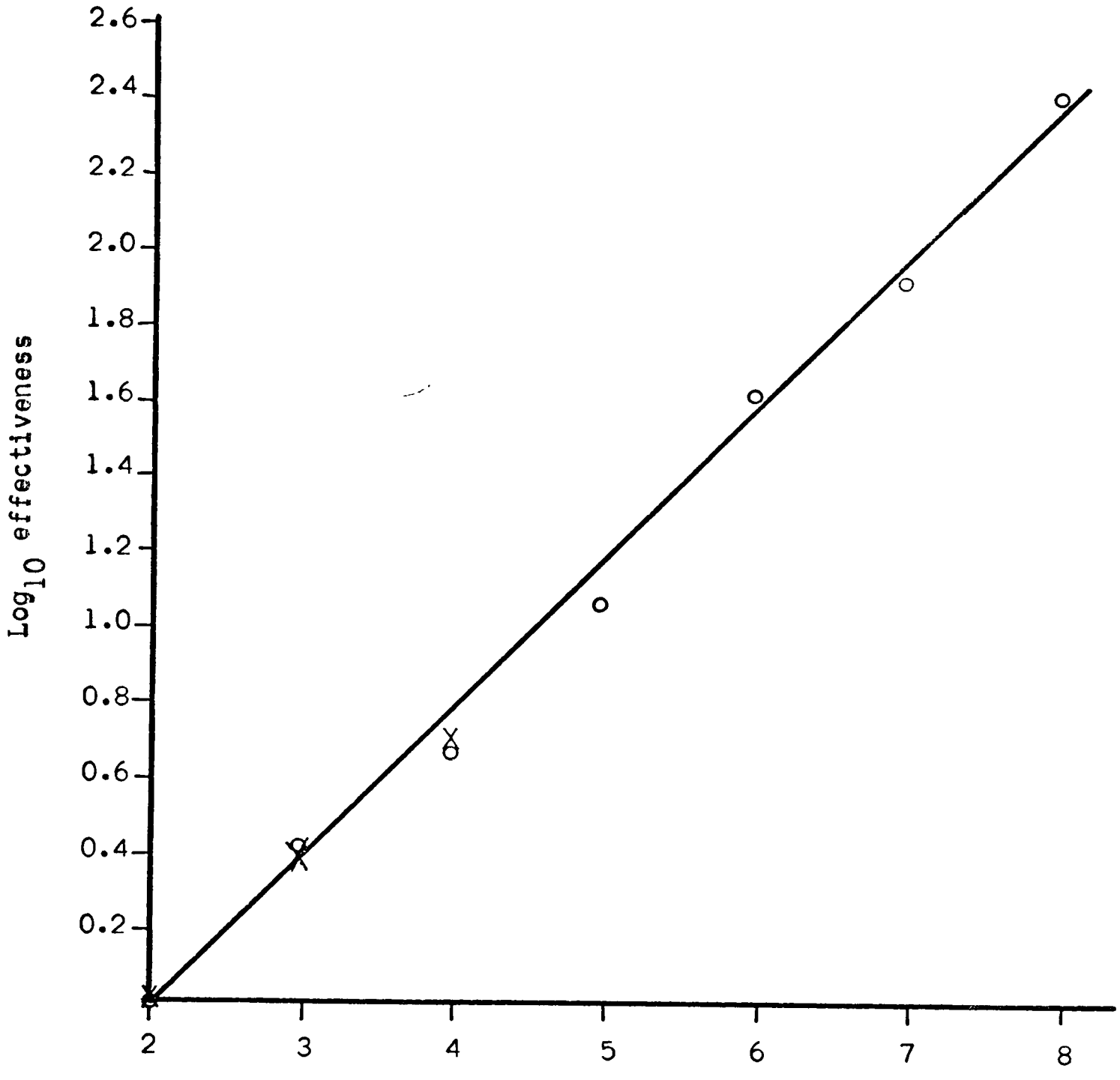
were used, shows the marked effect that changing both the alcohol and the percentage of that alcohol in decane can have on plots of the initial concentration of protein versus the volume of decane solution at the dip. This is as expected, since with different alcohols the stoichiometry of the interaction between alcohol and protein is likely to be different (Pankhurst, 1942) and plots which were obtained using decane B', figures 7 and 9, will be different.

The effect of secondary alcohols on decane emulsions stabilized by lysozyme was briefly investigated using 2×10^{-5}M solutions of lysozyme at pH 2.0, figures 27 and 28. They appear to behave in a very similar manner to the primary alcohols which are also shown in these figures at the same percentage as the secondary alcohols in decane. Slightly more protein is adsorbed on the emulsion at the dip with secondary alcohols than with primary alcohols. This could be due to the fact that if the hydroxyl group is at the interface between the decane and the water, projecting into the aqueous phase, then the secondary alcohols will occupy a larger surface area as they have two hydrocarbon tails and thus for a given mass will be able to interact with more protein than would the homologous primary alcohol.

Comparison of the effectiveness of different alcohols in preventing emulsion formation.

The type of result illustrated in figure 25, where 300 μl of decane containing different percentages of different primary alcohols in decane, provides a method for comparing the effectiveness of different alcohols

Figure 38 . Log_{10} of the effectiveness of different alcohols plotted against the chain length of the alcohol for lysozyme emulsions and the unreactive -SH groups of haemoglobin .



o experiments using emulsions;

X effectiveness of alcohols on the unreactive -SH groups of haemoglobin (Cecil and Thomas, 1965).

on their ability to prevent emulsification in the presence of a 2×10^{-5}M solution of lysozyme at pH 2.0. This effectiveness, which is exhibited by the minimum percentages of alcohol in decane as shown in table 8, may be compared on a molar basis as shown in table 10. Cecil and Thomas (1965) studied the kinetics of the reaction of the "unreactive" sulphydryl groups of human carboxyhaemoglobin with the sulphito-mercuri complex. Because the low reactivity of these groups was thought to be due to their involvement in hydrophobic interactions, the reaction was studied in the presence of different primary alcohols and the logarithm of the rate constant was found to increase linearly with the molar ratio of alcohol to water. The slopes of the lines increased with increasing chain length of the alcohol and these slopes were used to compare the relative effectiveness of these alcohols in increasing the reactivity of the sulphydryl groups; the figures are given in table 10.

A more precise comparison is obtained when the logarithm of the ratio of the effectiveness of the different alcohols, (ethanol taken as unity) plotted against the chain length of the alcohol for both the results reported in this work with the emulsions together with those of Cecil and Thomas (1965), figure 38. From the gradient in figure 38 it may be seen that for each additional $-CH_2$ group, the minimum concentration of alcohol required to prevent formation of emulsion, (i.e. to prevent any loss of protein from solution) was increased by a factor of 2.5 which is identical with the value obtained per $-CH_2$ when comparing the reactivity of the "unreactive" sulphydryl groups of haemoglobin in

Table 10

The effectiveness of different primary n-alcohols on their ability to prevent emulsion formation in the presence of lysozyme and to alter the reactivity of the "unreactive" sulphyaryl groups of haemoglobin

Alcohol	Relative effectiveness - emulsions	Relative effectiveness - SH*
CH_3OH	0.23	0.32
C_2H_5OH	1.00	1.00
C_3H_7OH	2.60	2.40
C_4H_9OH	4.70	5.16
$C_5H_{11}OH$	20.0	-
$C_6H_{13}OH$	40.0	-
$C_7H_{15}OH$	83	-
$C_8H_{17}OH$	250	-

* Data interpolated from Cecil and Thomas (1965).

solutions of different alcohols. The results obtained with decane emulsions provide evidence that the "unreactive" sulphydryl groups of haemoglobin are indeed involved in hydrophobic interactions since they must be in an emulsion type environment. Traube (1891) observed that to obtain a given surface tension for solutions of organic compounds belonging to an homologous series, the concentration required was reduced by a factor of 3.0 for each additional $-CH_2$ group. However Hutchinson (1948a) found that the adsorption of n-alcohols at an oil/water interface did not obey Traube's rule in that they all gave a close packed area of 20 $\mathring{A}^2$ per molecule. However Pankhurst (1942), when studying the penetration of monolayers of aliphatic substances or sterols by aliphatic alcohols of chain length from C_4 to C_8, found that each additional $-CH_2$ added to the hydrocarbon chain decreased the concentration required for equal penetration by about a factor of 4.5.

Addition of other polar compounds to decane and their effect on emulsions stabilized by protein.

The effect of decyl mercaptan, decylamine and stearic acid on emulsions of decane stabilized by proteins is by no means clear. It appears that the addition of these small molecules to pure decane or decane B', which is then emulsified in the presence of protein, reduces the amount of protein adsorbed on the emulsion which could occur in three possible ways.

(a) Decreasing the amount of decane emulsified compared to that in the absence of the mercaptan amine or acid.

(b) Increasing the particle size of the emulsion and thus reducing the surface area available for protein to be adsorbed on.
(c) Complexing or contributing to the effect of the alcohol in the interface so less protein is adsorbed. e.g. mixed disulphide formation.

Since these small molecules cause a reduction in the amount of protein lost from solution in the absence of alcohol, the method proposed in (c) cannot be correct. Either of the first two proposed mechanisms could be responsible and with the evidence available it is impossible to distinguish between the two.

Preliminary experiments were carried out in which different concentrations of mercaptan in decane B' were added to 6 ml. of 1×10^{-4}M insulin solution at pH 9.5. Unfortunately decane B' rather than pure decane was used as this experiment was carried out before the importance of alcohols on decane emulsions stabilized by proteins was realized. The results, figure 32, show that at concentrations above 10^{-3}M mercaptan in decane, only 60% of the protein initially in solution is adsorbed on the emulsion; below this concentration, the amount of protein adsorbed on the emulsion increases until, in the absence of mercaptan, 90% of the insulin originally in solution is adsorbed on the emulsion. With stearic acid and decylamine the amount of insulin remaining in solution appears to increase steadily with increasing concentration of the acid or amine. Fraser *et al*. (1955) showed that the adsorption of catalase at oil/water interfaces stabilized by charged small molecules (such as detergents) is very dependent on the nature and concentration of the small molecule

present. Thus considerably more work is required before any quantitative conclusions can be drawn from this type of experiment.

The effect of emulsions on the activity of enzymes

The results obtained with ribonuclease and lysozyme, adsorbed on emulsions of either decane or decane B', were very similar and the specific activity of both enzymes was less than 15%. When the enzyme solutions that were in equilibrium with the emulsions were separated their specific activities were found to be identical to those of solutions of the native enzymes. When ribonuclease and lysozyme were desorbed from emulsions by the addition of alcohols or, in the latter case, by changing the pH to pH 2.0, their specific activities were 100% and 75% respectively.

It has already been shown how the ultraviolet difference spectra of both these enzymes are changed when decane is emulsified in their presence, figure 13, so the enzyme in equilibrium with the emulsion, although structurally altered, retains most of its specific activity. Moore and Day (1968) have cross-linked lysozyme covalently with phenol-2,4-disulphonyl chloride; this cross-linked enzyme retains full enzymic activity and has approximately the same optical rotatory dispersion as the native enzyme. However Phillips (1968) has indicated that, from the knowledge of the residues involved in the cross-linking, the cross-linking observed could only be achieved at the expense of considerable unfolding of the enzyme molecule. Thus lysozyme and also possibly ribonuclease can undergo some conformational changes without concomitant loss in

enzymic activity.

The low activity of both enzymes on the emulsions could be due to several reasons as mentioned in the introduction.

One possibility is that the active site of the enzyme is orientated towards the hydrocarbon so the substrate cannot reach the active site of the enzyme. This possibility would be difficult to either prove or disprove and from the results reported here one cannot conclude whether the adsorption of both these enzymes on the decane surface is so specific that the active sites are orientated towards the hydrocarbon.

Another possibility is that the pH of the interface may be different from that of the solution. This has been shown to be unlikely since the dimerization of lysozyme between pH 5.5 and pH 6.0 occurs even on emulsions, as shown in figure 10, indicating that the pH of the surface of the emulsion is very similar to that of the solution.

Another likely explanation is that the enzymes are either unfolded to some extent or are in some type of multilayer at the interface, resulting in loss of enzymic activity. It has been shown that both ribonuclease (Harrington and Sela, 1959) and lysozyme (Churchich, 1962) can be reduced with thiols in 8 M urea, to give random polypeptide chains, and then reoxidised under suitable conditions to give active enzyme. It is therefore reasonable to suppose that these enzymes can be partially unfolded on a decane/water interface and then recover their activity after being transferred from the surface to solution. Since only 50% of the ribonuclease and 90% of the lysozyme was desorbed on the

addition of decanol, table 7, the possibility of lower layers of unfolded ribonuclease and lysozyme which cannot refold to enzymically active conformations on the addition of alcohol cannot be ruled out.

This work does not distinguish between the two possibilities that could account for the approximately 10% specific activities of the enzymes when adsorbed on emulsion particles, namely whether 90% of the adsorbed enzyme is totally inactive and the remaining 10% is fully active or whether all the adsorbed enzyme molecules are expressing 10% of their specific activity.

The results reported in this work differ from those reported by other workers. Fraser and Schulman (1956) studied the activity of trypsin on oil in water emulsions and they found that,the more strongly the protein was adsorbed on the emulsion, the greater was the loss in total enzymic activity. Ghosh and Bull (1962), studied the activity of chymotrypsin on octadecane in water emulsions, and found that, at low initial concentrations of enzyme, both the specific activity of the enzyme on the emulsion and in solution were lowered. The activity of lysozyme and ribonuclease in solution, in equilibrium with decane or decane B' emulsions, did not change from that of the native enzyme.

Effect of ethanol on some physical constants of insulin

Fredricq (1957) has shown that insulin has a molecular weight of 7,000 in 20% dioxan and 6,000 in 40% dioxan. Results obtained in this work, where the molecular weight of insulin in varying percentages of

alcohol solutions was estimated, shows that the minimum molecular weight of 6,000 is reached in approximately 60% ethanol. The fact that dissociation occurs in the presence of 60% ethanol supports the view that hydrophobic interactions are involved in the association of insulin monomers.

The effect of methanol on the optical rotatory dispersion spectrum of insulin has already been recorded by Herskovits and Mescanti (1965). The Moffit parameters that they obtained, table 11, are in reasonably good agreement with the values obtained in this work at pH 2.0 in the presence of ethanol, table 9. In 80% ethanol b_o is more negative than in 0.01 M HCl in pure methanol, indicating that insulin contains more α-helix in the former solvent.

Table 11

Summary of optical rotatory dispersion data for insulin in various solvents*

Solvent	pH	$-a_o$	$-b_c$
Aqueous buffer 0.1 M	2.3	150	200
Aqueous buffer 0.1 M	1.9	145	200
Methanol 0.01 M HCl	2.0	255	240

* Data from Herskovits and Mescanti (1965)

Herskovits et al. (1964) have shown that β-lactoglobulin exhibits changes in the value of a_o which may be correlated with the intermolecular

association of the protein. However Tanford (1962) has shown with optical rotatory dispersion studies on small peptides, that the changes in a_o with β-lactoglobulin could result from unfolding of polypeptide chains, irrespective of whether the helical content alters. The fact that insulin is fully dissociated into 6,000 molecular weight monomers by 60% ethanol, yet a_o shows no sign of levelling off, indicates that a_o is dependent on some factor besides the state of aggregation of the insulin monomers.

The effect of ethanol on $\triangle 37$ for insulin differs from the results of Bailey (1965) who found that this constant did not change when measured at pH 2 in aqueous solution, at pH 2 in 10% secondary butanol, or at pH 2 in 20% ethanol. However the value observed in this work with aqueous solutions of insulin at pH 2 for $\triangle 37$ is 130 which is less than the value of 187 recorded by Bailey under the same conditions. Since $\triangle 37$ shows no sign of levelling off, even after 60% ethanol has been exceeded, it indicates that $\triangle 37$, like a_o and b_o, is dependent on some factor besides the state of aggregation of the insulin monomers.

The increase in fluorescence intensity of insulin with increasing percentage of ethanol is linear up to 90%, indicating that the tyrosines responsible for this fluorescence are not undergoing any change in environment over this range of concentration (Steiner *et al.*, 1964). This would suggest that the tyrosines which fluoresce, are not involved in the bonding between the two 6,000 molecular weight monomers in the 12,000 molecular weight units which exist in aqueous solution at pH 2.

References

Alexander, A.E., and Teorell, T. (1939). Trans. Faraday Soc. 35, 727.

Allison, A.C., and Cecil, R. (1958). Biochem. J. 69, 27.

Andreas, J.M., Hauser, E.A., and Tucker, W.B. (1938). J. Phys. Chem. 42, 1001.

Anfinsen, C.B., and Haber, E. (1961). J. Biol. Chem. 236, 1361.

Appela, E., and Tomkins, G.M. (1966). J. Mol. Biol. 18, 77.

Augenstine, L.G., Ghiron, C.A., and Nims, L.F. (1958). J. Phys. Chem. 62, 1231.

Avey, H.P., Boles, M.C., Carlisle, C.H., Evans, S.A., Morris, S.J., Palmer, R.A., and Woolhouse, B.A. (1967). Nature 213, 557.

Aveyard, R., and Haydon, D.A. (1965). Trans. Faraday Soc. 61, 2255.

Bayley, P.M. (1965). D. Phil. Thesis, page 171.

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

Bernal, J.D. (1959). "Hydrogen Bonding", page 6. Ed. Hadzi, D. Pergamon Press, London.

Bernal, J.D., and Fowler, R.H. (1933). J. Chem. Phys. 1, 515.

Bjornholm, S., Barbu, E., and Macheboeuf, M. (1952). Bull. Soc. Chim. Biol. 34, 1083.

Blades, A.N., and Flavell, H.C.G. (1963). J. Clin. Path. 16, 158.

Blake, C.C.F., Mair, C.A., North, A.C.T., Phillips, D.C., and Sarma, V.R. (1967). Proc. Roy. Soc., Ser. B 167, 365.

Bowcott, J.E., and Schulman, J.H. (1955). Z. Electrochem. 59, 283.

Butler, J.A.V. (1937). Trans. Faraday Soc. 33, 229.

Bull, H.B. (1956). Biochim. Biophys. Acta 19, 464.

Careless, J.E., and Hallworth, G.W. (1968). J. Colloid Interfac. Sci. 26, 75.

Cecil, R. (1963). "The Proteins", Volume 1, page 379. Ed. Neurath, H. Academic Press, New York.

Cecil, R., and Loening, U.E. (1960). Biochem. J. 76, 146.

Cecil, R., and Thomas, M.A.W. (1965). Nature 206, 1317.

Cecil, R., and Wake, R.G. (1962). Biochem. J. 82, 401.

Cecil, R., and Weitzman, P.D.J. (1964). Biochem. J. 93, 1.

Chadwell, H.M. (1927). Chem. Rev. 4, 375.

Chapman, G., and McLaughlin, K.A. (1967). personal communication.

Cheesman, D.F. (1952). Biochem. J. 50, 667.

Cheesman, D.F., and Davies, J.T. (1954). Adv. Prot. Chem. 9, 439.

Cheesman, D.F., and Schuller, H. (1954). J. Colloid Interfac. Sci. 9, 113.

Chen, R.F. (1967). J. Biol. Chem. 242, 173.

Churchich, J.E. (1962). Biochim. Biophys. Acta 65, 349.

Clauser, H., and Li, C.H. (1954). J. Amer. Chem. Soc. 76,4337.

Cockbain, E.G. (1953). Trans. Faraday Soc. 49, 104.

Cockbain, E.G. (1956). J. Colloid Interfac. Sci. 11, 576.

Coulson, C.A. (1959). "Hydrogen Bonding", page 339. Ed. Hadzi, D. Pergamon Press, London.

Creeth, J.M. (1953). Biochem. J. 53, 41.

Crook, E.M., Mathias, A.P., and Rabin, B.R. (1960). Biochem. J. 74, 234.

Cumper, C.W.N., and Alexander, A.E. (1950). Trans, Faraday Soc. 46, 235.

Daniel, E., and Weber, G. (1966). Biochemistry 5, 1893.

Danielli, J.F. (1937). Proc. Roy. Soc., Ser. B 122, 155.

Danielli, J.F. (1938). Cold Spring Harbour Symposia Quant. Biol. 6, 190.

Edsall, J.T., Edelhoch, H., Lontie, R., and Morrison, P.R. (1950). J. Amer. Chem. Soc. 72, 4641.

Eisenberg, H., and Tomkins, G.M. (1968). J. Mol. Biol. 31, 37.

Elkes, J.J., Frazer, A.C., Schulman, J.H., and Stewart, H.C. (1945). Proc. Roy. Soc., Ser. A 184, 102.

Euken, A. (1948). Z. Electrochem. 52, 255.

Fisher, H.F., McGregor, L.L., and Power, U. (1962). Fed. Proc. 21, No. 2, 56.

Fordham, S. (1948). Proc. Roy. Soc., Ser. A 194, 1.

Foster, J.F. (1960). "The Plasma Proteins", volume 1, page 179. Ed. Putnam, F.W. Academic Press, New York.

Foster, J.F., and Aoki, K. (1957). J. Phys. Chem. 61, 1369.

Foster, J.F., and Aoki, K. (1958). J. Amer. Chem. Soc. 80, 1117.

Foster, J.F., and Sterman, M.D. (1956). J. Amer. Chem. Soc. 78, 3656.

Frank, H.S., and Evans, M.W. (1945). J. Chem. Phys. 13, 507.

Frank, H.S., and Quist, A.S. (1961). J. Chem. Phys. 34, 604.

Frank, H.S., and Wen, W.Y. (1957). Disc. Faraday Soc. 24, 133.

Franks, F., and Smith, H.T. (1967). Trans. Faraday Soc. 63, 2586.

Fraser, M.J., Kaplan, J.G., and Schulman, J.H. (1955). Disc. Faraday Soc. 20; 44.

Fraser, M.J., and Schulman, J.H. (1956). J. Colloid Interfac. Sci. 11, 451

Fredericq, E. (1957). J. Amer. Chem. Soc. 79, 599.

Frieden, C. (1963). J. Biol. Chem. 238, 3286.

Fromageot, C., and de Garilhe, M.P. (1950). Biochim. Biophys. Acta 4, 509.

Ghosh, S., and Bull, H.B. (1962). Arch. Biochem. Biophys. 99, 121.

Ghosh, S., and Bull, H.B. (1963). Biochim. Biophys. Acta 66, 150.

Grjotheim, K., and Krogh-Moe, J. (1954). Acta Chem. Scand. 8, 1193.

Grollman, A. (1925). J. Biol. Chem. 64, 141.

Grubhofer, N., and Schleith, L. (1954). Z. Physiol. Chem. 297, 108.

Gurd, F.R.N., and Wilcox, P.E. (1956). Adv. Prot, Chem. 11, 311.

Gutfreund, H. (1948). Biochem. J. 42, 544.

Gutfruend, H. (1952). Biochem. J. 50, 564.

Haggis, G.H., Hasted, J.B., and Buchanan, T.J. (1952). J. Chem. Phys. 20, 1452.

Halwer, M., Nutting, G.C., and Brice, B.A. (1951). J. Amer. Chem. Soc. 73, 2786.

Hammes, G.G., and Scheraga, H.A. (1966). Biochemistry 5, 3690.

Harfenist, E.J., and Craig, L.C. (1952). J. Amer. Chem. Soc. 74, 3087.

Harrington, W.F., and Sela, M. (1959). Biochim. Biophys. Acta 31, 427.

Haurowitz, F., Boucher, P., Dicks, M., and Therriault, D. (1955). Arch. Biochem. Biophys. 59, 52.

Hellerman, L., Schellenberg, K.A., and Reiss, O.K. (1958). J. Biol. Chem. 233, 1468.

Herskovitz, T.T., Townedd, R., and Timasheff, S.N. (1964). J. Amer. Chem. Soc. 86, 4445.

Herskovitz, T.T., and Mescanti, L. (1965). J. Biol. Chem. 240, 639.

Hill, R.J., Koningsberg, W., Guidotti, G., and Craig, L.C. (1962). J. Biol. Chem. 237, 1549.

Hotta, H. (1954). J. Colloid Interfac. Sci. 9, 504.

Hughes, W.L. (1949). Cold Spring Harbour Symposia Quant. Biol. 14, 79.

Hunter, M.J., and McDuffie, F.C. (1959). J. Amer. Chem. Soc. 81, 1400.

Hutchinson, E. (1948a). J. Colloid Interfac, Sci. 3, 219.

Hutchinson, E. (1948b). J. Colloid Interfac. Sci. 3, 413.

James, L.K., and Augenstein, L.G. (1966). Adv. Enzymol. 28, 1.

James, L.K., and Hilborn, D.A. (1968). Biochim. Biophys. Acta 151, 279.

Jirgensons, B. (1952). Arch. Biochem. Biophys. 39, 261.

Jolles, P. (1964). Angew. Chem. Internl. Ed. 3, 31.

Jolles, J., and Jolles, P. (1961). C.R. Acad. Sci. 253, 2773.

Kartha, G. (1967). Nature 214, 234.

Kartha, G., Bello, J. and Harker, D. (1967). Nature 213, 862.

Katsoyannis, P.G., Tometsko, A., and Zalut, C. (1966). J. Amer. Chem. Soc. 88, 166.

Kauzmann, W. (1959). Adv. Prot. Chem. 14, 1.

King. T.P. (1961). J. Biol. Chem. 236, PC5.

Klainer, S.M., and Kegeles, G. (1956). Arch. Biochem. Biophys. 63, 247.

Klotz, I.M. (1960). Brookhaven Symposia in Biology 13, 25.

Kolthoff, I.M., Anastasi, A., and Tan, B.H. (1958). J. Amer. Chem. Soc. 80, 3235.

Kupke, D.W., and Linderstrom-Lang, K. (1954). Biochim. Biophys. Acta 13, 153.

Micheel, F., and Ewers, J. (1949). Makromol. Chem. 3, 200.

Miles, G.D. (1945). J. Phys. Chem. 49, 71.

Miles, G.D., and Shedlovsky, L. (1945). J. Phys. Chem. 48, 57.

Mohammadzadeh-K., A., Feeney, R.E., Samules, R.B., and Smith, L.M. (1967). Biochim. Biophys. Acta 147, 583.

Moore, G.L., and Day, R.A. (1968). Science 159, 210.

Mukerjee, P. (1956). Anal. Chem. 28, 870.

Nemethy, G., and Scheraga, H.A. (1962a). J. Chem. Phys. 36, 3382.

Nemethy, G., and Scheraga, H.A. (1962b). J. Chem. Phys. 36, 3401.

Nemethy, G., and Scheraga, H.A. (1962c). J. Phys. Chem. 66, 1773.

Nyman, P.C., and Lindskog, S. (1964). Biochim. Biophys. Acta 85, 141.

Olson, J.A., and Anfinsen, C.B. (1952). J. Biol. Chem. 197, 67.

Pankhurst, K.G. (1942). Proc. Roy. Soc., Ser. A 179, 393.

Parks, J.M. (1960). Brookhaven Symposia in Biology 13, 132.

Pauling, L. (1959). "Hydrogen Bonding", page 1. Ed. Hadzi, D. Pergamon Press, London.

Pauling, L., Cory, R.B., and Bramson, H.R. (1951). Proc. Nat. Acad. Sci. U.S. 37, 205.

Perutz, M.F. (1960). "Proteins and Nucleic Acids", page 3. Elsevier Publishing Company, Amsterdam.

Phelps, R.A., and Putnam, F.W. (1960). "The Plasma Proteins", volume 1, page 143. Ed. Putnam, F.W. Academic Press, New York.

Phillips, D.C. (1968). Science 159, 211.

Phillips, D.M.P. (1952). Biochem. J. 50, 479.

Langmuir, I., and Schaefer, V.J. (1939). Chem. Rev. 24, 181.

Lamm, O. (1929). Arkiv Mat., Astron. Fysik 21 B, No. 2.

Lennard-Jones, J., and Pople, J.A. (1951). Proc. Roy. Soc., Ser. A 205, 155.

Linderstrom-Lang, K.U. (1951). "Lane Memorial Lectures: Proteins and Enzymes", page 58. Stanford Univ. Press, Stanford, California.

Lindskog, S. (1960). Biochim. Biophys. Acta 39, 218.

Lindskog, S., and Malmström, B.G. (1962). J. Biol. Chem. 237, 1129.

Loeb, G.I., and Scheraga, H.A. (1956). J. Phys. Chem. 60, 1633.

Lovrien, R. (1963). J. Amer. Chem. Soc. 85, 3677.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265.

McLaren, A.D. (1960). Enzymologia 21, 356.

McLaren, A.D. (1963). "Cell Interface Reactions", page 1. Ed. Brown, H.D. Scholars Library, New York.

McLure, W.O., and Edelman, G.M. (1966). Biochemistry, 5, 1908.

McLure, W.O., and Edelman, G.M. (1967a). Biochemistry 6, 567.

McLure, W.O., and Edelman, G.M. (1967b). Biochemistry 6, 559.

Malmström, B.G., Nyman, P.O., Strandberg, B., and Tilander, B. (1964). "Structure and Activity of Enzymes". Federation of European Biochemical Societies, Symposium No. 1, page 121. Ed. Goodwin, T.W., Harris, J.I., and Hartley, B.S. Academic Press, New York.

Manecke, G., and Günzell, G. (1967). Naturwissenschaften 54, 531.

Markus, G., Love, R.L., and Wissler, F.G. (1964). J. Biol. Chem. 239, 3687.

Marler, E., and Tanford, C. (1964). J. Biol. Chem. 239, 4217.

Privalov, P.L., and Mrevlishvili, G.M. (1967). Biofizika 12, 19.

Quiocho, F.A., Bishop, W.H., and Richards, F.M. (1967). Proc. Nat. Acad. Sci. U.S. 57, 525.

Ray, B.R., and Augenstein, L.G. (1956). J. Phys. Chem. 60, 1193.

Ray, A., Reynolds, J.A., Polet, H., and Steinhardt, J. (1966). Biochemistry 5, 2606.

Rehfeld, S.J. (1967). J. Colloid Interfac. Sci. 24, 358.

Reynolds, J.A., Herbert, S., and Steinhardt, J. (1967). Biochemistry 6, 937.

Richards, F.M., and Vithayathil, P.J. (1959). J. Biol. Chem. 234, 1459.

Rideal, E.K. (1945). J. Chem. Soc. 423.

Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1961). J. Biol. Chem. 236, 391.

Ryle, A.P., Sanger, F., Smith, L.F., and Kitai, R. (1955). Biochem. J. 60, 541.

Sanger, F., and Tuppy, H. (1951). Biochem. J. 49, 481.

Schachman, H.K. (1959). "Ultracentrifugation in Biochemistry", page 203. Academic Press, New York.

Schulman, J.H., and Cockbain, E.G. (1940). Trans. Faraday Soc. 36, 651.

Schulman, J.H., and Rideal, E.K. (1934). Proc. Roy. Soc., Ser. B 122, 29.

Schulman, J.H., and Stenhagen, E. (1938). Proc. Roy. Soc., Ser. B 126, 356.

Sela, M., Anfinsen, C.B., and Harrington, W.F. (1957). Biochim. Biophys. Acta 26, 502.

Shugar, D. (1952a). Biochem. J. 52, 142.

Shugar, D. (1952b). Biochim. Biophys. Acta 8, 302.

Smyth, D.G., Stein, W.H., and Moore, S. (1963). J. Biol. Chem. 238, 227.

Sophianopoulus, A.J., Rhodes, C.K., Holcomb, D.N., and Van Holde, K.E. (1962). J. Biol. Chem. 237, 1107.

Sophianopoulus, A.J., and Van Holde, K.E. (1964). J. Biol. Chem. 239, 2516.

Spackman, D.H., Stein, W.H., and Moore, S. (1960). J. Biol. Chem. 235, 648.

Stackelberg, M.V., and Müller, H.R. (1954). Z. Electrochem. 58, 25.

Stein, W.H., and Moore, S. (1949). J. Biol. Chem. 178, 79.

Steiner, R.F., Lippoldt, R.E., Edelhoch, H., and Frattali, V. (1964). Biopolym.. Symp. 1, 355.

Stevens, C.C., and Bergstrom, G.R. (1967). Proc. Soc. Exp. Biol. Med. 124, 187.

Sundram, P.V., and Crook, E.M. (1967). Proc. 7th Int. Cong. Biochem. Tokyo, page 801.

Tanford, C. (1952). Proc. Iowa Acad. Sci. 59, 206.

Tanford, C. (1962). J. Amer. Chem. Soc. 84, 4240.

Teitze, F., and Neurath, H. (1952). J. Biol. Chem. 194, 1.

Thompson, A.R. (1955). Biochem. J. 60, 507.

Traube, J. (1891). Justus Liebigs Ann. Chem. 265, 27.

Tsou, C.L. (1966). Kexue Tongbao 17, 241.

Wang, J.H. (1951). J. Amer. Chem. Soc. 73, 510.

Weber, G. (1952). Biochem. J. 51, 155.

Wen, W.Y., and Saito, S. (1964). J. Phys. Chem. 68, 2639.

Wetlaufer, D.B., and Lovrien, R. (1964). J. Biol. Chem. 239, 596.

White, F.H. (1961). J. Biol. Chem. 236, 1353.

Wishnia, A. (1962). Proc. Nat. Acad. Sci. U.S. 48, 2200.

Wishnia, A., and Pinder, T. (1964). Biochemistry 3, 1377.

Yang, J.T., and Foster, J.F. (1954). J. Amer. Chem. Soc. 76, 1588.

Zittle, C.A. (1958). Adv. Enzymol. 14, 319.