REVIEW ARTICLE

BEHAVIOUR OF PROTEINS AT INTERFACES

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Speculation concerning the biological importance of thin films of proteins began as early as 1870 when Ascherson[1] noted that a tough skin of protein formed spontaneously around oil droplets suspended in egg albumin solutions and suggested such droplets as models for living cells. Devaux[2], in 1903, was the first to spread proteins at the air: water interface. He observed that such films could be collapsed to form artificial insoluble fibres, the "Devaux effect". About the same time Ramsden[3] noted that egg albumin is precipitated from solution after being present at an air: water interface. Thus it was early recognised that a considerable change in the properties of protein molecules occurs on their adsorption at interfaces. Wu and Ling[4] later studied this phenomenon of "surface denaturation", finding that shaking solutions of egg albumin, oxyhaemoglobin and methaemoglobin in air leads to an irreversible loss of solubility of these proteins. Langmuir and Waugh[5] calculated that an increase of film pressure of 15 dynes/cm., i.e., great enough to collapse the protein films, should increase the solubility of the film by a factor of 10^{95}, if the adsorption of the protein on to the water surface followed a simple Gibb's isotherm. Apparently then, the loss of solubility of proteins on adsorption is due to some drastic structural change in the molecules.

This review undertakes to consider what is known of the nature of these structural changes that occur on adsorption of proteins at air: water, oil: water, solid: water and the perhaps biologically more important lipid: water interfaces. Secondly the effects of adsorption on the activity of enzymes and other biologically active proteins will be discussed. Finally these physico-chemical studies will be related to the problems involved in the study of the activity of intracellular enzymes, especially those "surface enzymes" associated with the membrane, nucleus, mitochondria, endoplasmic reticulum (microsomes) and other morphological entities of the living cell. Several excellent reviews[6–9] of the physical chemistry of protein monolayers, both of the older and more recent literature, are extant. Among those, Bull[7] has discussed many of the surface chemical techniques employed in the study of spread monolayers of protein which are not elaborated here. The papers of Rothen[8] and Cheesman and Davies[9] discuss the effects of spreading at interfaces on biological activity and the latter in particular dwells on the structure of protein monolayers. Now attempts at building up more complex systems, chiefly studies on enzyme-lipid interactions, can be discussed, and with the advent of the technique of differential centrifugation, these systems can be compared with the simpler ones isolated from living cells.

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Adsorption of Proteins at Interfaces

Protein Structure and Denaturation

Before attempting to discuss the development of the topic of the adsorption of proteins at interfaces it is perhaps best to prejudice the reader with a simple picture of the modern concepts of protein structure and "denaturation", so that the development may be seen in a better light. One of the most useful of such pictures for our purposes is that of Kauzmann[10,11] on protein denaturation, following the studies of Pauling and others[12] on the structure of "native" proteins.

Briefly, the structure of a native globular protein is considered under three aspects: (1) the "primary" structure or linkage of amino acids (for example, 51 in insulin, 288 in ovalbumin) through peptide bonds to give a long polypeptide chain; (2) the "secondary" or helical structure of this long chain stabilised by numerous hydrogen bonds between the amide H-atoms of peptide bonds and O-atoms of the peptide carbonyl groups; (3) the "tertiary" structure or folding of the helix into a globular form, a structure stabilised partly by hydrogen bonding between the folds of the helix and by van der Waal's forces between the non-polar groups. These hydrophobic bonds are the result of the tendency of the non-polar side-chains such as the benzyl groups of the phenylalanines, the butyls of the leucines, propyls of the valines and indoles of the tryptophanes, to adhere to one another because of their low affinity for water. Salt linkages between positively charged lysine and arginine residues and negatively charged glutamic and aspartic acid residues may also play some role in holding the molecule in a specific "tertiary" configuration, though, according to Jacobsen and Linderstrøm-Lang[13] they are not numerous in most proteins. More important are probably disulphide links both in the secondary (between the coils of the helix) and tertiary (between folds of the helix) structures[14,15].

The tertiary structure is the most labile, and probably for many proteins changes in it are reversible under mild conditions. Such things as hydrogen-bonding agents, organic solvents and detergents (though their primary binding on to proteins is probably through polar groups of the protein) can alter the tertiary structure. Hydrogen-bonding agents such as urea and guanidine, by competition for the intramolecular H-bonds, serve to modify both the secondary and tertiary structures considerably, but in some examples reversibly, if conditions are not too extreme. In all but a few instances heat effectively and irreversibly destroys the specific secondary and tertiary configurations of the protein molecules. Any process that succeeds in doing this without proteolysis or damage to the primary structure is generally called a "denaturation". The course of the action of denaturing agents on proteins may usually be followed by measuring either the loss of solubility at the isoelectric point, the loss of biological activity of the protein, or both. Loss of the primary structure or proteolysis can be induced, for example, enzymatically or by digestion with acid or alkali. With this simple picture of protein structure and

denaturation in mind we can go on to consider the changes in structure that occur when proteins are adsorbed at various interfaces.

Films of Protein Spread at the Air: Water Interface

One of the first steps in the study of spread monolayers of proteins was to measure the thickness of the films. It was soon discovered by optical methods and direct measurement[6] of the thickness of the layers deposited on top of one another on conditioned metal slides by the Langmuir-Blodgett technique[7] that the films were only 8 to 10 Å thick, the average length of an amino acid residue. These layers were prepared from films spread at zero surface pressure to allow maximum structural rearrangement. Since the globular molecules in solution were about 40 to 80 Å in diameter and no dissociation had occurred, it was clear that a considerable intramolecular unfolding had taken place, a "surface denaturation". What is more, the amino acid residues were oriented in the monolayer, for if the film was deposited on the plate such that the top side of the film was exposed, it was found to be hydrophobic (not wetted by water). If the deposit was made such that the bottom side of the film was exposed it was found to be wettable or hydrophilic. Thus the orientation of the residues in the film appeared to be similar to that in fatty acid films, the polar groups anchored in the aqueous phase, the non-polar groups in the air. These conclusions were supported by surface potential measurements and interaction studies[16]. The former showed that the orientation could be changed by compressing the films. At low pressure the amino acids lay nearly flat in the interface; in highly compressed films their angle of tilt reached 90°.

In the region of low compression, 0 to 1 dynes/cm., the film molecules act like a 2-dimensional gas and a molecular weight can be obtained from the force-area curves. In general, where there is no dissociation of the molecule on spreading, these molecular weights agree closely with those obtained by other physical methods[7]. Above 1 dyne/cm. a coherent film is obtained whose surface viscosity and elasticity increase sharply as the film is compressed. Above 15 dynes/cm. a time-dependent collapse of the films occurs. With a dark field ultramicroscope long faint striations or folds parallel to the compressing slide[17] are seen at the onset of collapse. Finally, at complete collapse visible fibrils appear and these can be collected in the form of an artificial fibre—the "Devaux effect". Not all proteins exhibit this effect. Most "gelled" films below the collapse pressure generally show marked hysteresis effects on compression, decompression and recompression of the films[7,18].

Several lines of evidence suggest that surface denaturation, once thought to be the most drastic form of denaturation[6], does not always succeed in destroying the tertiary and secondary structures entirely, leaving only a fully extended two-dimensional primary structure. For example, some proteins first heat denatured or treated with the Ställberg-Teorell spreading solution exhibit larger limiting areas than films spread from native proteins[9,19]. Also ultra-violet light has been shown to expand fully spread ovalbumin films[44] and it has been found that the Devaux

fibres prepared from collapsed films of proteins denatured by different agents before spreading possess widely varying morphological properties and tensile strengths[19]. Thus it is likely that these denaturing agents succeed in attacking bonds not broken when the native molecules unfold at the air:water interface[9,19]. The visco-elastic properties of various proteins, and of the same protein treated by different denaturing agents, are seen to vary considerably by the different expansion patterns they give with indicator oils also[7]. Thus, despite the great similarities in the force area curves for proteins other properties of the films may vary considerably[9].

Most of the topics mentioned in this section have been more elaborately reviewed by Bull[7] and Cheesman and Davies[9].

Interactions of Protein Monolayers with Soluble Substances in the Sub-phase

The study of fully spread monolayers of protein, though of great interest in structural studies, has not much direct bearing on the behaviour of proteins at interfaces of biological interest. Of closer relevance are the studies of interactions of proteins at interfaces: (1) of protein monolayers with soluble substances in the sub-phase and (2) of soluble proteins in the sub-phase with insoluble monolayers of other substances, the lipids being probably of most interest. This section deals with the former topic and the succeeding sections with the latter.

Schulman and Hughes[20] early reported that spread monolayers of casein could be digested by both chymotrypsin and pepsin under appropriate pH conditions. The thickness or extent of spreading of the casein films is not known, and these experiments are complicated by the fact that the enzymes are film-forming also. Schulman[21] later studied the interaction of amphipathic substances with protein monolayers coming to the general conclusions that if the molecules in the sub-phase have hydrophilic "heads" and hydrophobic "tails", i.e., are surface-active, three situations could arise: (1) if there is no association between the polar heads of the injected molecules and polar groups in the film there will be no alteration in the film's characteristics, e.g., no change in either film pressure or in surface potential; (2) if there is association between these polar groups but no association between the hydrophobic tails of the molecules in the sub-phase and the non-polar groups of the protein then there is an adsorption of the substance on to the protein with a consequent change in surface potential; (3) if there is association of both polar and non-polar groups of both molecular species then the molecules in the sub-phase penetrate the protein film giving rise not only to changes in surface potential but also to increases in surface pressure. Penetration of the hydrophobic tails can be prevented if the film molecules are tightly packed by compression before injection of the amphipathic substances into the sub-phase, or, in the case of a mixed film, compression may result in the squeezing out of the component with the lowest collapse pressure as the surface pressure increases. These systems thus act as models for the action of haemolytic and agglutinating agents on red blood cells. Lytic agents were found to penetrate either cholesterol films or disperse

protein (gliadin) films and are capillary or surface-active. Agglutinating or sensitising agents adsorb only on protein films, exhibiting no penetrating properties and no appreciable surface activity. Thus the red blood cell membrane, a lipoprotein complex probably containing cholesterol, is likely to be structurally altered by penetrating lytic agents, perhaps even partially dispersed, while the agglutinating agents merely adsorb on the membrane.

With mixed monolayers of two species of surface-active substance one of two phenomena can occur on compression of the films: (1) one component may displace the other from the interface usually at the collapse point of the displaced material or (2) both components may remain as a stable mixed film collapsing as a complex, at pressures above the collapse points of either component. More stable complexes are found with penetrating substances which show association between both polar and non-polar portions of the film molecules. Thus penetrating molecules cannot always be displaced from the interface but remain in strong association with the film molecules.

There are several other instances in which there is found a correlation of the association (adsorption with or without penetration) or non-association of various structurally similar substances in the sub-phase with monolayers of proteins and the biological activity of these compounds. For example, Rideal and Schulman[22] studied the adsorption of a series of compounds related to stilboestrol on monolayers of gliadin. They found a good correlation of the rates of adsorption on the protein, as measured by changes in surface potential, and oestrogenic activity, suggesting that the event of oestrus is initiated by the adsorption of the hormone at a protein surface. This and other examples have been reviewed by Cheesman and Davies[9], and the general interactions of protein films with substances dissolved in the sub-phase have been reviewed by Schulman[23].

Adsorption of Proteins at Clean and Lipid-Stabilised Oil: Water Interfaces

Studies of spread monolayers of proteins at the oil: water interface are considerably less numerous than those at the air: water interface because of the technical difficulties encountered. Alexander and Teorell[24] and Cumper and Alexander[25] have made force-area measurements on proteins spread at non-polar oil: water interfaces, finding the major difference from films at the air: water interface to be a larger specific area. The hydrophobic tails of the amino acid residues no longer cohere at low pressure; the chief van der Waal's forces to be considered are between these groups and the oil. Davies[26] has used a vibrating plate technique to measure film surface potentials at the light petroleum: water interface. Cheesman and Davies[9] have discussed the orientation of the amino acid residues at the oil: water interface and the visco-elastic properties of protein films in some detail, basing their discussion chiefly on data obtained for films of poly-α-amino acids and their copolymers.

An interesting, if somewhat neglected, method employed in the study of the adsorption of proteins to oil droplets in aqueous suspension is the

microscopic electrophoretic mobility technique developed by Abramson, Moyer, Gorin[27] and others. These workers followed the changes in electrophoretic mobility with pH of proteins adsorbed on minute particles of paraffin wax, pyrex glass, carbon, collodion, quartz, and kieselguhr, by microscopic observation, using reflected light, of the rate of movement of the particles across a measured field. Most studies were concerned with adsorption of protein on "inert" particles where the surface protein is electrokinetically equivalent to the bulk globular protein under the same environmental conditions of pH, ionic strength and buffer type. Apparently adsorption on these solid surfaces took place without unfolding of the protein. This type of study was thus chiefly concerned with the elucidation of the properties of the adsorbed protein, the effects of the underlying surface tending to be neglected. In some examples where deviation from the bulk behaviour was noted the results were explained by a partial unfolding or structural rearrangement of the protein which presents a different array of polar groups to the shear boundary layers that determined the mobility[27]. Cumper and Alexander[28] applied the method to a study of the adsorption of proteins (like bovine γ-globulin, haemoglobin, and insulin) on to suspended droplets of non-polar and polar hydrocarbon derivatives like mineral oil, oleyl and cetyl alcohols. In a number of instances the surface isoelectric point for the protein-coated particles coincided with the bulk isoelectric point of the protein, suggesting that the protein in the shear boundary layer was in the globular form. Since this behaviour was closely observed only in protein solutions of higher concentration it is quite likely that a layer of native protein was adsorbed on a layer of unfolded protein. Cumper and Alexander have elsewhere[29] presented a dynamic picture of proteins adsorbing, unfolding and spinning off oil : water interfaces in a denatured form. With some particles, especially cetyl alcohol, a marked shift in isoelectric point was observed, and on either side of this the mobility of the particles depended to a certain extent upon the nature of the particles. Seaman and Fraser[30] have observed differences in apparent isoelectric point for trypsin, even on the non-polar paraffin wax particles in the presence of excess protein, of as much as 6 pH units. Cumper and Alexander[28] have explained such observations as being due either to a specific chemical reaction between the proteins and particles, or as the result of incomplete coverage of the surface. There is thus considerable difficulty in interpreting the results of these mobility measurements, though no extensive study of any one system has been made. Among some of the possible factors that would have to be considered are the variation in the amount of protein adsorbed with changing conditions of pH, salt concentration, or buffers, and the adsorption or loss of trace ions as conditions are altered. The method is a very sensitive one and worthy of fuller exploration. It has found more successful use, in combination with other methods, in the study of the adsorption of the enzyme trypsin at various lipid-stabilised oil : water interfaces[31], as will be discussed later.

The first extensive studies of the interaction of soluble proteins with lipid interfaces were made by Elkes, Frazer, Schulman and Stewart[32],

using what we shall call the "emulsion technique". These authors studied the adsorption of oxyhaemoglobin (and albumin) on emulsified mineral oil droplets stabilised with the cationic detergent, hexadecyl trimethyl ammonium bromide (C_{16}TAB) and the anionic detergent sodium hexadecyl sulphate ($SC_{16}S$) over a wide range of pH at constant ionic strength. They found that adsorption of the protein resulted in flocculation of the droplets and occurred when the protein and interface were oppositely charged, e.g., on the acid side of the isoelectric point with the negatively charged interface ($SC_{16}S$). The amount of protein required to cause maximum clarification of the subnatant fluid, that is flocculation of all the droplets, corresponded to that needed to give a firmly gelled monolayer at the air: water interface. This was 2·5 mg. per sq. metre surface area. With less protein than this the droplets remained discrete because the surface charge had not been neutralised, and with more protein (25 mg./M^2) in the sulphate system, the droplets did not flocculate either, because of further adsorption of protein in the form of multilayers reversing the sign of the charge on the droplets. Further, this surface haemoglobin could be desorbed by altering the pH of the medium so that the protein and interface came to have the same sign of electrical charge. The protein was thus repelled from the surface. This reversible adsorption-desorption resulted in the denaturation of the haemoglobin to parahaematin which was shown by changes in the absorption spectrum and by solubility tests. Ultracentrifugal analysis of the desorbed protein showed it had the same molecular weight as oxyhaemoglobin, indicating that no association or dissociation had occurred.

In these experiments some of the denaturation could have taken place through the bulk interaction of the partially soluble detergents and haemoglobin. Nevertheless, that surface denaturation does occur has been confirmed[31,33], using a water-insoluble anthracene long-chain sulphonate as stabiliser. Here it required 10 mg./M^2 of proteins (haemoglobin, catalase, trypsin) to neutralise the surface charge and amounts above 15 to 20 mg./M^2 could be desorbed in the native forms, the absorption spectrum of oxyhaemoglobin being still present, and the enzyme activities still considerable. The outer multilayers of proteins were firmly bound and could not be washed off with washes of the same medium as that used in adsorption. The formation of these multilayers without loss of the globular structure, or at most only slight reversible changes in the molecules, presents an interesting physico-chemical problem, for it is difficult to account for such strong adsorption when the surface charge has been neutralised. Possibly the layers of proteins are strongly polarised, the polarisation being induced by the specially oriented lipoprotein layers underneath. Such forces have been suggested by Rothen[8] to account for the build-up of multilayers of insulin 450 Å thick on top of a layer of protamine 30 to 50 Å thick, and the fact that slides covered with barium stearate and "conditioned" with uranyl ions can hold large numbers of layers of protein transferred to them by the Langmuir-Blodgett technique[7]. Without being conditioned they can apparently hold only one layer of protein[8].

It is to be noted that in the adsorption of proteins on the anthracene long-chain sulphonate stabilised oil droplets[31,33] considerably more protein was required to neutralise the surface charge to give flocculation than previously reported for the sulphate system[32]—10 mg./M^2 as opposed to 2·5 mg./M^2. This amount of protein is equivalent to ten fully spread layers of protein, and must be thought of as being in the form of multilayers. These inner layers of protein are irreversibly denatured. It is not likely that all of the sulphonate stabiliser remains at the oil : water interface but probable that layers of protein-detergent-like complex are formed. It is possible too, that some of the adsorbed protein is actually solubilised in the oil phase through this complex formation, for it was not possible to desorb the protein entirely. Such oil-soluble complexes have been reported previously by Pankhurst[34]. Putnam[35] has reviewed the bulk interaction of proteins with soluble ionogenic detergents. The amount of detergent bound increases with the concentration of detergent present, at first approximately linearly as a few ions are bound on the surface of the protein, then logarithmically as the molecules are "wedged open" and unfolded, exposing many more binding sites, until all the sites are saturated. Friend, Harrap and Schulman[36] have followed the unfolding by the light-scattering technique, studying bovine serum albumin-sodium dodecyl sulphate (SDS) interaction. Few, Ottewill and Parriera[37] have found that dodecyl trimethyl ammonium bromide (DTAB) binds to this protein at relatively high protein and detergent concentrations giving a sudden increase in molecular asymmetry (as measured by viscosity increases) after six molecules of DTAB per molecule of protein have been bound. These complexes dissociate on dilution and are affected by strong salt concentrations, whereas the formation of the protein-SDS complexes, though affected by salts[36], is virtually irreversible[35]. These observations were paralleled at the oil : water interface[31,33] where interactions with quaternary ammonium ions were reversible, but not so interactions with long-chain sulphates and sulphonates. The former are thought to bind through the carboxyl groups of the protein[37], the latter through the free amino groups, but these may not be the only binding sites[35].

Interaction of Proteins with Lipid Monolayers

Doty and Schulman[38] and Matalon and Schulman[39] studied the interaction of lipid monolayers with soluble proteins injected into the subphase. The lipid films were held at 14 dynes/cm. pressure, the collapse pressure of protein films, to prevent spontaneous spreading of the protein. The interactions of serum albumin, haemoglobin and globulins with monolayers of cephalin, cholesterol, cardiolipin, stearyl choline and a long-chain C_{22}-sulphate were studied. The experiments were carried out over a wide pH range using a Langmuir surface balance; (1) allowing the film pressure to increase from 14 dynes/cm. at constant film area and (2) allowing the area of the film to increase at constant pressures above 14 dynes/cm. Phenomena similar to those encountered at the oil : water interface were observed. For example, the interaction of the proteins on the acid side of their isoelectric points was strong with negatively

charged films. If the pH of the sub-phase was then made alkaline the protein, now having the same charge as the lipid, could be nearly all ejected from the film. Some "bound protein" remained where the penetration of the protein into the lipid had been strong. With the non-ionogenic cholesterol there is only weak association. In mixed films compression above the collapse pressure of the protein results in ejection of the protein[40]. The pH range where strong interaction took place at the air : water interface was found to coincide with the range for flocculation of oil : water emulsions stabilised with the same, or similar, lipids[38,39].

One of the lipids studied by this method, cardiolipin, is the main component, with the sensitising lipids lecithin and cholesterol, of the specific antigen used in the Kahn, Kline, Eagle, Mazzini and Wasserman diagnostic tests for syphilis. In all but the Wasserman test a positive test is given by a flocculation, visible to the eye or microscopically, of the antigenic lipid particles in the presence of syphilitic serum due to the adsorption on them of the specific antibody protein whose production in the human body is induced by the presence of the spirocaete *Treponema pallidum*. Normal human serum under the conditions of the tests does not give the flocculation. Doty and Schulman and others[38] have tried to demonstrate this specific interaction by the monolayer penetration technique without success. They injected small quantities of normal and sylphilitic sera underneath films of cardiolipin and mixtures of this lipid with cholesterol and lecithin. The same penetration was observed by both types of sera, however, perhaps because of the very great dilution of the sera. The specific adsorption has been followed under conditions more nearly approximating those of the clinical test by Eagle[41] using the microscopic electrophoretic mobility technique described above.

Unfortunately very little work has been done to develop further the use of the monolayer penetration technique in the study of lipoprotein interactions at interfaces. There is especially a need for a method to quantitate the results, being able to measure exactly how much protein adsorbs on to the lipid film. A step in this direction has recently been taken by Eley and Hedge[42,43] who have adopted the technique of injecting the protein solutions at the lipid interface 1 mm. or so beneath the film. They claim that the proteins spread rapidly beneath the film without appreciable loss of protein to the bulk phase. However, they have made the injections of protein under lipid films held well below the collapse pressure of proteins (2 and 10 dynes/cm.) so that spontaneous spreading of the protein took place. The final pressures of the "penetrated films" rarely rose above 14 to 16 dynes/cm. Thus their reports of demonstration of interactions between lecithin, cholesterol and uncharged stearic acid (on acid substrate) with bovine plasma albumin, fibrinogen, lysozyme and insulin must be interpreted with caution. Many of these reported interactions do not occur when the lipid films are held above 14 dynes/cm.[38,39]. The fact that the proteins still unfold at an interface covered with a lipid film at 2 to 10 dynes/cm. pressure does not prove there is interaction between the lipid and protein. The unfolding process does however, present ample opportunity for association between the polar

and non-polar groups to occur and one could wish to know more about the properties of these mixed films. In any case these authors have not claimed that the interaction energies are strong.

As these interesting systems warrant further investigation and development so do the effects of ultra-violet light on lipoprotein films. Kaplan and Fraser[44] have observed that strong doses of ultra-violet light first expand albumin films before causing them to dissolve in the substrate at constant film pressure, presumably due to proteolysis. As noted above this expansion is due to a rupture of bonds not broken in the unfolding processes on adsorption. A dose of ultra-violet light that is strong enough to solubilise the protein film was found to have no effect on a film of "cephalin" (chiefly phosphatidyl ethanolamine) but when a lipoprotein film was formed by allowing catalase to penetrate the cephalin at 20 dynes/cm. the entire film was solubilised by the same dose of ultra-violet light[45]. The complex formation between the lipid and protein must have been strong indeed.

In summary of the interactions of proteins with lipid covered interfaces, either oil : water or air : water, where the interfacial tension has already been considerably reduced by the presence of the lipid, one has to consider chiefly the association of the polar and non-polar groups of both molecular species. Many factors which affect these associations can be elucidated by bulk studies, for example, effects of pH, salt concentration, dielectric constant, specific interaction between polar groups, which alter the hydrogen bonding, ion-dipole attractions and van der Waal's forces between the lipid and protein. In other words, one must recognise all the interactions that occur in the bulk phase together with the special steric restrictions imposed by the alignment of the lipid molecules at the air : water or oil : water interface. The orientation and packing of the lipid molecules can be varied considerably, from the two-dimensional gaseous to the solid states, by compression of the films or addition of lipids of other types which associate with that already present, changing the properties of the film. Thus with highly compressed or tightly packed lipid monolayers the penetration of the non-polar groups of the protein may be prevented, and the unfolding of the protein that usually occurs thereby greatly limited, unless possibly there is a high density of polar groups which strongly react with numerous polar groups on the protein. These films would resemble the immobile solid : liquid interfaces where the solid surface greatly restricts movement in the adsorbed film.

Effect of Adsorption on Biological Activity of Proteins

Biological Activity at Air : Water and Oil : Water Interfaces

Surface denaturation, or the non-proteolytic structural modification that occurs on adsorption at air : water or oil : water interfaces, affords excellent conditions for determining the contributions of the secondary and tertiary structures of the intact protein molecule to its biological activity. This is an important point in the light of some recent experiments on the controlled digestion of large portions of the amino-acid residues of certain proteins with proteolytic enzymes in bulk solution.

Rogers and Kalnitsky[46] for example, report that 17 amino acid residues of ribonuclease can be removed without loss of its enzymatic activity. And, most striking of all, Hill and Smith[47] have shown that no less than 120 of the original 180 amino acid residues of the enzyme papain can be removed without loss in activity, though a considerable loss of stability of the protein was noted. Such findings would seem to indicate that only a relatively small portion of the molecule near the active centre need remain structurally intact for the protein to exhibit its enzymatic activity. Also a considerable portion of the amino acid residues are not essential in preserving this specific structure but probably do act to stabilise the entire molecule. On the other hand, adsorption of the protein at air: water and oil: water interfaces, accompanied by drastic overall structural unfolding will be seen to have profound effects on the biological activity, showing that the "native" secondary and tertiary structures of at least part of the molecule are essential for its activity. This fact is also shown by the effects of other denaturing agents in bulk solution, such as heat, ultra-violet light, and urea. Thus catalase and other large enzymes are inactivated by urea[48] and heat[49] while the smaller, more stable enzymes like ribonuclease and trypsin can lose their activities reversibly in the presence of heat[50,51] or urea[52,53].

The effects of spreading proteins at air: water and oil: water interfaces on their biological activity have been previously reviewed by Rothen[8] and Cheesman and Davies[9]. From the outset it was noted that adsorption of enzymes on to these mobile interfaces generally led to a great reduction in their activities. Thus Gorter[54] found that films of pepsin spread at pH = 2·85, and of trypsin spread at pH = 7·00, then removed from the surface with a silk net, still retained as much as 80 per cent activity when tested under appropriate conditions. Langmuir and Schaefer[55] deposited films of pepsin, urease and catalase on metal slides, finding that they retained only about 5 per cent activity. The catalase films were 23 Å thick, and thus not completely unfolded. A few years later Sobotka and Block[56] reported that films of saccharase deposited on slides retained full activity. Here again the protein was not unfolded, the films being 45 Å in thickness. More recently, Hayashi and Edison[57], Hayashi[58] and Kaplan[59] have reported that spread monolayers of pepsin-albumin complexes, actomyosin and catalase respectively, retain a certain fraction of their enzymatic activity. In each case however, closer investigations[60,9,61] have subsequently revealed that if films of these enzymes were spread carefully under no compression for long times to obtain homogeneous films 8 to 10 Å in thickness, no activity could be recovered. The same has been found for pepsin[62], and trypsin too, spread at air: water or oil: water interfaces, loses its activity completely and irreversibly[8,31]. It is now generally agreed that this is the case for most enzymes spread at these interfaces, so that we may now say that at least part or, in some cases, possibly all, of the secondary and tertiary structure of the enzyme molecule is essential for its function.

One difficulty attending measurements of enzyme activities of spread monolayers is that the protein must be collected for assay, either by

deposition on slides, draining the substrate away leaving the film spread on filter paper, collapsing the films to form insoluble fibres and testing their activity or redissolving the fibres in other media, etc. In all these procedures it is tacitly assumed that processes subsequent to the spreading of the enzyme film do not lead to renaturation. There is thus a need for assaying the enzyme *in situ*, but the dangers of contaminating the film with unspread globular protein from the sub-solution are great.

Harkins and colleagues[63] have deposited films of catalase 55 Å thick on conditioned metal slides which exhibited only 5 to 10 per cent activity. They reported that this layer adsorbed first, a layer of antibody protein which gave no further decrease of activity and second, another layer of enzymes only 10 Å thick possessing no activity. The first layer, though it had lost much of its enzymatic activity had apparently retained high immunological specificity. Danielli and others[64], on the other hand, failed to demonstrate the reaction of pneumococcus type II polysaccharides with spread films of the specific antibody proteins. Bateman and others[65] reacted spread egg albumin films transferred to slides with rabbit antisera, but the films were 14 to 20 Å thick, suggesting incomplete spreading. Rothen and Landsteiner[66] however, claim to have obtained immunological specificity with spread films of egg albumin, heat denatured egg albumin, human and horse serum albumins, horse serum globulins, etc., where all the films were carefully spread at the air : water interface to 8 to 10 Å thickness. The globulins lost their ability to react with antibody rapidly on standing while the egg and horse serum albumins retained this property even if left for sixteen hours under no compression. This certainly suggests that at least a few immunological reactions are probably dependent only on the primary structure of the protein or on a very stable or reversibly lost secondary structure.

Rothen and colleagues (see [8])* have also tested the effects of surface denaturation on various protein hormones, being careful to spread the films to 7 to 9 Å thickness. They found that films of *metakentrin*, a gonadotrophic hormone of the anterior lobe of the pituitary gland, and the oxytocic hormone of the posterior lobe of the pituitary, lost 95 to 100 per cent activity on spreading, redissolving and testing for hormonal activity. Insulin, however, retained full activity after having been spread at the air : water interface. Possibly this small molecule can undergo reversible unfolding. It would be interesting to see this observation repeated. Rothen[8] reports also that all these hormones retain their ability to react with specific antisera when spread at the air : water interface and deposited on conditioned plates. In these transfer experiments it is important that the layers recovered should contain only protein unfolded to a uniform thickness, and not be contaminated with unspread globular material. On such considerations Dean, Gatty and Stenhagen[67] have called into question the techniques employed in obtaining these results. However, a generalisation of the usually accepted view that unfolding on adsorption at the air : water and oil : water interfaces leads to a complete

* This review contains a description of the technique of measuring thicknesses of deposited films using elliptically polarized light.

and irreversible loss of biological activities of proteins awaits further experimentation.

Enzyme-Lipid Interactions at Interfaces

In view of the findings that many intracellular enzymes are associated with lipids and nucleic acid in various particulate fractions like the membrane, nucleus, mitochondria, or microsomes, the study of enzyme-lipid interactions at interfaces is likely to yield more practical results from a biological point of view than are studies at the clean air : water and oil : water interfaces. Some early studies at lipid covered interfaces have shown that the packing of the lipid molecules in the film is important in enzyme-lipid interactions. Rideal[68] showed that the rate of digestion of lecithin monolayers by black tiger snake venom in the sub-phase changes on compression of the films. Similarly Schulman[69] showed that the orientation and packing of the long-chain esters at the oil : water interface is a prime factor in the rates of their hydrolysis by pancreatin. However, little is known of the nature of these enzyme surface lipid interactions, for example, to what extent the enzyme unfolds on adsorption at the substrate-lipid surfaces.

Elkes and Frazer[70,71] found that a lecithinase of *Clostridium welchii* type A toxin caused flocculation of the chylomicrons in lipaemic sera due to interference with the stabilising phospholipid film. This enzyme also creamed lecithin stabilised emulsions, but had no effect on intestinal emulsified fat, likely stabilised by a fatty acid-glyceride-soap film. The study of the adsorption of enzymes on lipid stabilised oil droplets by the "emulsion technique" has been extended considerably by Fraser, Kaplan and Schulman[33] and Fraser and Schulman[31]. They have followed the adsorption of the enzymes catalase and trypsin at oil : water interfaces stabilised with water-insoluble lipids so as to minimise the bulk interaction. Changes in the activity of the large (M.W. = 225,000) haem-enzyme catalase in the decomposition of hydrogen peroxide, and of the small (M.W. = 17,000) basic protein trypsin in the hydrolysis of benzoyl arginine ethyl ester were followed. Catalase is reported to have an iso-electric point of 5·7[72], while that for trypsin is nearly pH = 11·00[73]. Trypsin lacks a prosthetic group also, so that the two enzymes are of vastly different natures. The water insoluble stabilisers used were an anthracene-C_{22}-long-chain sulphonate, cephalin (chiefly phosphatidyl ethanolamine) mixed sodium lauryl phosphates, oleic acid-glyceryl mono-oleate, and mixed films of octadecyl trimethyl ammonium bromide (C_{18}TAB) with various long-chain and cyclic alcohols. The adsorption was followed over a wide range of conditions.

Briefly, the technique was to add appropriate amounts of buffered emulsion to dilute solutions of the enzymes, separate the oil droplets with their adsorbed protein by centrifugation, wash them in buffer, resuspend and assay them (also the subnatant fluid and washes) for enzymatic activity. The washed globules could be resuspended in medium in which the protein was desorbed from the interface, the enzyme being released into the bulk phase, then assayed for activity. Thus measures were had

of the amount of protein that adsorbed per unit of surface area of emulsion, the reduction of activity on adsorption, and the reversibility on desorption.

By the appropriate choice of stabilisers it was found possible to obtain these two enzymes adsorbed on emulsion droplets in the form of monolayers and multilayers varying in state from fully active to completely and irreversibly inactive enzyme. It was found that the loss of activity of the enzyme on adsorption could be related to the degree of unfolding of the protein which was dependent on the charge on the protein and interface, pH, polar group specificity and steric factors. Provided that the unfolding process was not allowed to go to completion, desorption of the enzyme from the interface, by charge reversal or displacement with a surface-active agent, resulted in partial to complete restoration of activity. In the extreme cases, catalase could be adsorbed on the positively charged $C_{18}TAB$ + *n*-lauryl alcohol-stabilised interface at pH = 8·3 without loss in activity, while both enzymes adsorbed on the sulphonate-stabilised interface at pH = 4 to 5 were in the form of completely inactive monolayers. Desorption of the protein in the former case occurred with full retention of activity, while none could be regained in the latter. However, in the sulphonate system if excess protein were present it adsorbed strongly in the form of multilayers (over 100 mg./M^2) which possessed considerable activity and yielded active enzyme on desorption. The more protein that adsorbed the greater the activity recovered on desorption. A great number of intermediate forms between these extreme cases were demonstrated. Both enzymes lose their activities irreversibly at the clean air : water and oil : water interfaces as mentioned above.

These surface interactions were compared to the bulk interactions of the enzymes with the soluble lipid analogs SDS and DTAB. As in the bulk phase, the action of the quaternary ammonium compounds was much milder than the action of long-chain sulphates and sulphonates. Wills[74] has recently studied the latter for a wide range of enzymes, suggesting that they are inactivated by adsorption on to micelles. However, several instances in the above papers seemed to indicate that the unfolding of the proteins through the binding of detergent could take place well below the micelle point, a picture more in line with the mechanisms of Putnam[35].

In the case of the adsorption of trypsin on the ionogenic (charged) lipid interfaces observations by the "emulsion technique" were paralleled with the microscopic electrophoretic mobility technique. For example, in the presence of increasing amounts of trypsin at pH = 4·0, the high negative mobility of the long-chain sulphonate-stabilised droplets decreased rapidly to zero when 10 mg. protein/M^2 surface area had been added. Then the mobility slowly reversed in sign taking a constant small positive value above 15 to 20 mg./M^2, approaching that for trypsin itself at this pH. All of the protein added, at least up to 25 mg./M^2, resided at the interface. Another use of this technique was to follow the pH mobility curves for the droplets in the presence of just enough protein to

neutralise the surface charge at pH = 4·0. The mobility of the sulphonate-stabilised droplets is almost constant over the pH range 3 to 12. In the presence of trypsin it decreases by an amount which is proportional to the bulk mobility of the protein, i.e., the amount of protein that adsorbed was proportional to the net charge on the protein. The same was found to hold roughly for adsorption on the lauryl phosphate interface, but quite a different effect was observed at the oleic acid-glyceryl mono-oleate-stabilised oil : water interface. Here as the pH of the medium was lowered the carboxyl groups of the fatty acid were titrated so that at pH = 4·0 the mobility was nearly zero. As expected, there was no measurable adsorption of trypsin at this pH. However, at hydrogen-ion concentrations above pH 8·0 where the droplets had a constant high negative mobility (all the carboxyls were ionised) the positively charged trypsin did not adsorb either. Only in the narrow pH range 5 to 8 did appreciable adsorption occur. These results were confirmed by direct measurement of adsorption using the "emulsion technique". Apparently there must be both charged and uncharged carboxyl groups in the interface to react with the polar groups on the protein. Perhaps special steric requirements are thus fulfilled.

Enzymes at Solid: Water Interfaces

Mention should be made at this point of the adsorption of enzymes at solid : water interfaces, though more work has been done in this connection in the field of chromatography of proteins[75] than with a view to testing the effects of adsorption on enzymatic activity. Talibudeen[76] has studied the adsorption of several proteins and amino acids on montmorillonoid clays by X-ray diffraction methods. The information obtained was used to calculate the thickness of one and two layers of protein which were shown to correspond to the thickness of one and two close-packed polypeptide chains of the β-keratin type, that is, spread monolayers 6 to 9 Å thick. It is doubtful whether enzymes could exhibit activity under these circumstances. Adsorption on these negative surfaces was stronger when attempted at acid pH values where the proteins bore strong positive charges. More than three layers of spread protein could be adsorbed on these clays.

McLaren[77–79] has studied the adsorption of enzymes on negatively charged kaolinite particles, finding that the adsorption led to a reduction or loss in activity of lysozyme, pepsin, trypsin, or chymotrypsin. The adsorption apparently involved both an ion exchange with adsorbed metal cations, and simple adsorption on the external surface of the particles. Some activity of lysozyme and trypsin could be regained on elution (desorption) of these enzymes with ethylamine. Chymotrypsin was still capable of digesting adsorbed heat-denatured lysozyme, but not adsorbed native lysozyme. Apparently, then, the adsorption of these enzymes to kaolin, under the conditions employed, does not radically modify their structure. This adsorption can thus be compared with that to the milder-acting lipid-stabilised oil : water interfaces. The dense packing and immobility of the "adsorbent" molecules probably prevents

extensive penetration and unfolding from occurring. Thus solid surfaces are of more use in the chromatographic separation of enzymes.

Partially Active Enzymes

In any study of the action of denaturing agents in reducing the activity of enzymes one of the problems that arises is whether or not the effect of the agents is "all-or-none" or whether there are intermediate stages in the action giving rise to partially active enzymes. Thus, though the loss in activity of an enzyme parallels its general structural unfolding as measured by changes in viscosity, solubility, birefringence of flow, exposure of –SH and phenolic –OH groups, etc., the activity measurements do not reveal whether one is following displacement of an equilibrium between active and inactive forms of an enzyme, or of an equilibrium between intermediate forms of the enzyme having only partial activity. For example, Harris[53] has found that the inactivation of trypsin in solution by high concentrations of urea is paralleled by a great increase in viscosity. This he states is due to unfolding of the molecules, and he postulates a reversible equilibrium between active and inactive forms.

A virtually unexplored approach to the problem of the action of denaturing or "unfolding" agents, as opposed to specific inhibitors, may be found in the study of the kinetics of partially denatured enzymes. The inhibition by sodium dodecyl sulphate (SDS) of many enzyme activities, though it takes place at fairly low concentrations[74], is accompanied by unfolding of the protein molecules[36]. It may be easily seen from the inhibition curves (conc. SDS vs. enzyme activity) in the literature[31] that a normal type of competitive or non-competitive inhibition does not take place. The usual linear plot of reciprocal of activity vs. concentration of inhibitor is found in these examples to give a hyperbolic curve[31]. This suggests that the effect of SDS is not through an action at the active centre, and it is logical to associate it with the unfolding that occurs.

If strained configurations of enzyme molecules can work at lower efficiency, exhibiting partial activity, then it is possible that kinetic and thermodynamic studies will enable one to differentiate between losses in activity by a drastic (all-or-none) or a slight modification of the structure of the active centre. For example, one might expect the combination of the substrate with the enzyme to be much more difficult and hence would predict an increase in K_m for this reaction. No such measurements have been made, either in bulk or for enzymes adsorbed at lipid-stabilised or solid interfaces. Fraser, Kaplan and Schulman[33] have observed that the activation energy for the catalase-H_2O_2 reaction (the overall reaction, followed by the manometric method) increased by 10 Kcal./mole on adsorption of the enzyme at a cephalin-stabilised olive oil : water interface. They took this as an indication of the action of a partially active form of catalase. The surface enzyme had only one-eighteenth the activity of the bulk enzyme. Desorption of the enzyme, though it did not restore the catalase to full activity, did result in a return of the activation energy to its original level. This system was thus a good model for the intracellular yeast catalase studied by Fraser and Kaplan[49]. The yeast enzyme

possesses a low activity and high activation energy compared with crystalline catalase in bulk. Treatment of the yeast cells with various physical and chemical agents or extraction from the cells converts or alters the enzyme to the bulk state. This was considered to come about through a desorption of the partially unfolded normal enzyme from some interface within the cell accompanied by its renaturation. The argument that an unfolded enzyme is expected to exhibit a high activation energy for substrate decomposition[49] was based on Eyring's picture of protein denaturation and is too involved to be reproduced here. However, it is well to point out that there are many possible causes for a change in activation energy for an enzyme-substrate reaction on adsorption of the enzyme at an interface, for example, the interfacial pH might be considerably different from the bulk pH, the active centres of the enzymes may become partially buried on adsorption so that the substrate no longer has free access to them, and, if water takes part in the reaction, changes in hydration of the protein or of dielectric constant may have occurred. McLaren[80] reports, for example, that the optimum pH, and the pH for half-maximal activity are 2 pH units higher for chymotrypsin adsorbed on kaolinite particles than in bulk solution, indicating that the H^+ concentration at the interface is 100 times that of the bulk concentration. Thus a method of dealing with the problems of partially active enzymes by kinetic and thermodynamic measurements, though fascinating and very worthwhile, must be approached with caution.

There are indications that partially active forms of enzymes can exist in relatively simple systems. Hammond[81] has found that the loss of activity of ficin in hydrolysing benzoyl arginine ethyl ester at alkaline pH is due to the titration of a positive group. On shifting the pH back to the optimum full activity is regained. However, if dimercaprol (BAL) is present only 70 per cent of the activity is recovered at any time. Hammond suggests that this is due to a structural change in the ficin molecules probably resulting from the scission of –S–S– links (with unfolding?). Alkaline pH is known to accelerate the breakage of these bonds by thiol compounds[82]. Citri[83] has found that adsorption of the purified α-penicillinase from *B. cereus* on pyrex glass results in its conversion to the γ form, resembling the bound intracellular enzyme in its antigenic properties and sensitivity to inactivation by iodine[84]. Though little decrease in activity is observed on adsorption to the glass, an increase of activity is observed on desorption in the presence of gelatin.

The State of Intracellular Enzymes

Interfaces in Biological Systems

Cytologists and cell physiologists have long recognised the importance of interfaces in biological systems. Danielli[85] has pointed out that many reactions involving the major components of living tissues, fats, proteins, nucleic acids and carbohydrates, must take place at interfaces. The great majority of neutral fats, for example, are so insoluble in water that the final steps in their synthesis and initial steps in their utilisation must

occur in the interior of the lipoid phase or at the lipid: water interface. That *only* water soluble enzymes can act at the interface, however, is now less likely with the discovery that some lecithinases can operate in organic solvents[86], for example, 80 to 90 per cent ethanol or "wet" ether. The very special orientation of proteins in combination with lipids in the plasma membrane, and membranes of the nucleus, mitochondria and cytoplasm (endoplasmic reticulum) gives rise to unique properties concerned with the transfer of materials and substrates to the proper localities of the cell where they are stored or utilised.

Membranous and other interfacial regions of living cells differ from homogeneous phases with which they are in contact. The pH, ionic strength, oxidation-reduction potential, ratio of univalent to polyvalent ions all may be considerably different from the neighbouring bulk phase. Special forces due to the orientation of the molecules at these interfaces must be considered. Changes in orientation through the penetration of other substances into these layers can alter the rates, activation energies and entropies of chemical or enzymic reactions taking place there[87]. With the great variety of substances to be found in a living cell there are multitudes of possible "micro-environments" in which enzymes can act. To speak of an "intracellular pH" or the "concentration of ions" in a cell in the same manner as in homogeneous solution is not only misleading but quite ridiculous.

Nor must one fall into the danger of thinking of the cell as a static system. New functions come into the fore as the cell grows, divides, differentiates, encapsulates, or reacts to changes in its environment in any way. In view of this constant change we might expect the phenomena of reversible denaturation and "unmasking" of enzyme activities along with the synthesis of "new" enzymes to play an important role in the life of the cell. Meyer, Mark, Goldacre and Lorch[88] have suggested that the reversible folding and unfolding of protein molecules plays an important role in amoeboid movement, cytoplasmic streaming and muscular contraction. Local variations in intracellular pH may help to bring about these changes. It is possible that combination of already synthesised enzymes with macromolecules and interfaces, like ribonucleic acid (RNA) and mixed lipid complexes results in many cases in the temporary "masking" of enzymes. Thus RNA[89] or polysaccharides[90] can act as enzyme inhibitors in certain cases. Subsequent changes in physiological state may serve to unveil their activities, giving rise to different pathways of metabolism of certain materials. It is the hope of the surface chemist to be of some aid to the cell physiologist in elucidating these states and changes in states of intracellular enzymes. Perhaps what is needed at this stage of our knowledge is a Frey-Wyssling[91] with an enzymological bias.

Enzymes Associated with Cell Structures

Since the classical work of Claude[92], Schneider and Hogeboom[93] and others on the isolation of particulate cell components by the technique of differential centrifugation of homogenates, it has become increasingly clear that a large number of enzymes are not haphazardly distributed

in the cytoplasm. They are to be found localised in association with lipids and nucleic acids on such morphologically distinct structures as the plasma membrane, nucleus, mitochondria, microsomes, Golgi apparatus, zymogen and pigment granules, and chloroplasts. It has become clear also that the processes of metabolism are controlled in part by the spatial relations between the enzymes and their substrates, the metabolites. A brief survey will be made of the various enzymes known to be associated with cell particulates, and the effect this binding has on their activities. A review by Schneider[94] will be found to be more comprehensive on the former topic.

(*a*) *The Cell Membrane*

There are many aspects of the study of the plasma membrane of the cell, all of which must be related ultimately to the structure of this complex of lipids and proteins and its function in controlling the passage of materials in and out of the cell. Only the slightest of references to these can be made here. By direct chemical analyses of isolated cell membranes[95], studies of the effects of lytic agents, digestive enzymes[96], detergents and other agents, the use of the microscopic electrophoretic mobility technique in determining the ion-spectra for surface charge reversal by adsorption of polyvalent ions[97], the plasma membranes of red blood cells and bacterial protoplasts have been characterised. Though much work has been done along these lines there is a long way to go towards working out the exceedingly complicated structure of a "simple" cell membrane and to relate this with its functions in "facilitated"[98] or "activated"[99] transport of metabolites.

Sizer[100] studied the properties of yeast invertase which is associated with the cell surface. He found that the activity was the same whether or not the enzyme was associated with the cell. The activation energy for inversion of sucrose was identical for both states of enzyme also. Later Wilkes and Palmer[101] found that the pH activity curves were identical, so that the enzyme is probably directly exposed to the extracellular pH. McLaren[80] however, has recently claimed that these data show a slight surface pH effect due to the negative charge on the yeast cell. Apparently the association of invertase with the cell surface does not affect its activity. We are unfortunately ignorant of the structure of this surface enzyme, but the above data suggest that it is probably in the globular form. Rothstein[102] has reviewed the surface enzymology of the yeast cell (containing invertase, lactase, maltase and phosphatases) suggesting that it is a multimembranous, compartmentalised system. This is a much more complicated picture than that presented for the membranes of red blood cells and bacteria. Even the latter contain adaptively formed "permeases"[103] which concentrate certain substrates rapidly in the cells.

(*b*) *Nucleus and Mitochondria*

Both these elements of the cell are structurally very complex, containing their own enzymic environments within limiting membranes. Little is

known of the states of nuclear enzymes. Much more work has been done on the mitochondria. Half their total mass appears to consist of soluble proteins found in the interlamellar spaces, among which are the enzymes glutamic dehydrogenase, fumarase, ribonuclease, deoxyribonuclease, while the cytochrome oxidase, cytochrome c, DPN-cytochrome c reductase and succinic dehydrogenase are bound to the lipid double membranes. Contrary to previous notions, all of the Krebs tricarboxylic acid cycle enzymes do not reside in the mitochondria, some being found in the soluble cell fraction[94]. Some enzymes concerned in fatty acid oxidation are also localised on the mitochondria[94]. Meyers and Slater[104] have recently examined isolated mitochondria for ATP-ase activity and have come to the conclusion that four of these enzymes are present, with different pH optima. As with many other mitochondrial enzymes, physical disruption, in this case freezing and thawing or ageing, results in an activation of some of the forms. Surface-active agents also "unmask" latent ATP-ase activity of mitochondria[105]. One can attempt to generalise that where these enzymes are bound to lipoprotein or to the strongly acidic RNA they probably exhibit only part of their potential activity when supplied with excess substrate. Many agents can act to release these bound enzymes, leading to a "renaturation" with increases in activity in some cases. Loss of co-factors or if the activity depended strongly upon the structural intactness of the lamellae, would result in the loss of certain activities on disruption of the mitochondria.

(*c*) *Microsomes*

Recent electron microscopic studies[106,107], have revealed that the smallest sedimentable cell particles, the microsomes, are comprised of electron dense spheres of nucleoprotein 20 to 150 mμ in diameter, adsorbed on lipoprotein double membranes found in the cytoplasm, the "endoplasmic reticulum". These membranes carry the enzyme activities found to be associated with this fraction. The lipids comprise 45 per cent of the fraction and include lecithin, cephalin, inositol phospholipids and cholesterol-like steroids[108]. Treatment of suspensions of microsomes with strong solutions of sodium deoxycholate solubilises the lipoprotein membranes and allows the small nucleoprotein particles to be sedimented[109]. These consist of as much as 60 per cent RNA. The high interest in the microsome fraction lies in the fact that it is probably chiefly responsible for the synthesis of the cytoplasmic proteins. The nucleoprotein particles are likely concerned in the actual stages of peptide bond formation linking up the "activated amino acids" from the soluble fraction of the cytoplasm[110]. The most mysterious stage in the synthesis at the moment is how the polypeptide chains fold up to produce globular proteins with specific functions, superficially an opposite process to surface denaturation. One may speculate that the lipoprotein membranes, through some adsorption-desorption mechanism, possibly with the help of a substrate-like substance (the "inducer" in the case of adaptive enzyme formation), are functional in the folding-up process. It is interesting to note in this connection that several adaptively formed enzymes are found

associated with the microsome fraction from bacterial cells[111]. With the adaptive formation of β-galactosidase in *E. coli* and of catalase in yeast Bonner[112] and Bonner and Kaplan[113] have suggested that these enzymes are piled up in layers at their sites of synthesis. Their activities can be unmasked by suitable treatment of the cells.

Many cases of increases of microsomal enzyme activities on solubilisation or release from the sedimentable components have been reported. Rat liver aryl sulphatase, for example, is released by cationic and non-ionic surface-active agents with increased activity[114]. The microsomal alkaline phosphatase and xanthine oxidase of cow's milk are released with considerable activity increases by various physical and chemical agents from the surface of the milk fat globules[115,116]. What role reversible folding and unfolding of the protein molecules has to play in these phenomena has yet to be shown. The observations are highly suggestive in the light of the studies of adsorption of enzymes at lipid-stabilised oil:water interfaces discussed above[31,33].

Changes in Physiological States of Cells

It is evident from the discussions on partially active enzymes and the various enzymes associated with cellular particulate fractions that many intracellular enzymes, by virtue of being combined with lipids, nucleic acids, polysaccharides or other components, perhaps at some interface, could be expressing only part, or possibly none, of their potential activity. Splitting, or changes of physical state of these complexes by some environmental change can lead to expression of increased or full activity. Such processes would thus partake in, if not "cause", a change in physiological state of the cell. In these we have possible mechanisms for the evocation or "unmasking" of intracellular enzyme activities alternative to "de novo" synthesis of enzymes. To what extent such mechanisms play a part in the life of the cell remains to be seen. Cheesman and Davies[9] have called attention to the findings of the cytologists Runnström[117] and Holtfreter[118] in this connection. The former demonstrated the release at the cell surface of a powerful protease immediately after fertilisation of sea-urchin eggs. He suggested that it was held in the inactive form in a complex with a heparin-like polysaccharide such as that demonstrated in the jelly coat of these eggs by Immers and Vasseur[90]. The protease could release other enzymes, triggering a new chain of metabolic pathways. Holtfreter[118] found that interaction must occur between yolk platelets and the lipochondria of the amphibian egg before development can proceed. He suggested that the membranes of the lipochondria contain proteases in the inactive form which became activated by this interaction, initiating the new chain of processes required for development.

A number of other instances of unmasking of intracellular enzyme activities by treatment of intact cells with various physical and chemical agents are known. Reference has already been made[49] to the case of yeast catalase studied by Kaplan[119]. The hypothesis was proposed that the normal enzyme exists adsorbed at some intracellular interface in a partially unfolded state where it exhibits only one-eighteenth of its potential

activity, a high activation energy for substrate decomposition and resistance to inactivation by heat and ultra-violet light. Treatment of the cells with ultra-violet light, grinding for extraction, by toluene, chloroform or the homologous series of aliphatic alcohols, aldehydes and ketones, etc., "alters" the catalase to the bulk state. Even if the enzyme remains *in situ* it has all the properties of crystalline catalase in solution. This "alteration" was thought to be due to a desorption of the enzyme from the interface with a consequent folding up of the molecules into the more active globular form. Such phenomena have also been observed in the case of β-galactosidase of *E. coli*[112], and other instances may occur[119].

The difficulty in the interpretation of such observations comes when it is realised that all these treatments of cells result in the breakdown of the specific permeability properties of the cell membrane, permitting equilibration of the cell contents with the external suspension medium and resulting in the "death" of the cell as measured by loss of viability. Thus Few, Fraser and Gilby[120] have proposed that the observed 4-fold increase in catalase activity and changes in activation energy for H_2O_2 decomposition of *Micrococcus lysodeikticus* on treatment with "altering agents" could be accounted for if the intracellular enzyme were acting at an effective pH of 4·6, lower than the pH of the external medium by two units. Chance[121], using spectrophotometric techniques on intact cells, previously concluded that the physical state of this bacterial catalase was essentially identical with that of the purified enzyme in solution. In the case of the yeast catalase however, it is unlikely that pH changes or increases in the permeability of the cells to hydrogen peroxide can account for all the observed changes in the properties of the enzyme, either qualitatively or quantitatively[119,49]. The interfacial hypothesis remains the most likely explanation. The effects of ultra-violet light[122] and ionising radiations[123] in the alteration of yeast catalase suggest that the enzyme is in close association with RNA, which is in line with the suggestion of Bonner and Kaplan[113] that the adaptively formed catalase of anaerobic cells is "piled up" at its site of synthesis. One can thus speculate that the "interface" involved in alteration is provided by the microsomes.

Finally, attention should be drawn to the effect of homologous series of alcohols, aldehydes and ketones in altering yeast catalase activity[124]. The effectiveness of these agents in "altering" the enzyme was found to correspond to their ability to lower the surface tension at the air : water interface, increasing approximately three-fold for each $-CH_2$ group added on the chain, thus following Traube's rule. This relation between alteration and surface activity was taken as further indication that the phenomenon of alteration involved desorption of the enzyme from some intracellular interface through the action of these agents[124]. Another possibility is that this type of alteration occurs by indirect action on the cell membrane, breaking down its permeability properties allowing equilibration of the cell contents with the external buffer medium. Not only would changes in pH and ion concentrations shift the observed enzyme activities and activation energies for substrate decomposition but also they would allow the loosening or splitting of various complexes of proteins

with lipids and nucleic acids, further enhancing the alteration of the properties of the enzymes. Thus "alteration" may be another of the numerous examples of the result of the collapse of lipoprotein cell membranes by surface-active agents[125]. The phenomenon is also related to the augmented action of narcotics with increasing chain length. Whether the mechanism of action of such compounds involves a change in surface activity, according to Traube, Warburg and Clark, or in fat solubility, according to Meyer and Overton, remains unsettled[126]. Dethier, reviewing this topic[127], points out that it is fundamentally a change in thermodynamic activity which changes with chain length.

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