CXCV. THE INTERACTION BETWEEN PORPHYRINS AND LIPOID AND PROTEIN MONOLAYERS

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IN many porphyrin-protein combinations, e.g. in the haemoglobins, cytochrome *c* [Keilin, 1925] and horseradish peroxidase [Keilin & Mann, 1937], there exists a stoichiometric relation between the porphyrin and the protein, indicating a direct chemical combination, which in the case of cytochrome *c* is effected through a thioether linkage in those side chains which in the free porphyrins contain the vinyl groups [Theorell, 1938].

On the other hand, phenomena such as photosensitization, action on paramoecia or transport in blood serum must be attributed to the ability of the porphyrins to form "complexes" or "adsorption" compounds with lipins and proteins. The monolayer technique permits us to examine in some detail the factors which are operative in the formation of such compounds, such as identification of the polar groups involved and the effect of changes in the structure of the non-polar or hydrophobic portions of the molecule or the van der Waals forces of interaction, to which in great measure the stability of the compounds formed is due. A further point of interest is that in extended monolayers of proteins the operation termed denaturation has taken place and whilst the polar groups of the side chains are immersed in the substrate and therefore accessible to reaction, their relative disposition to one another and to those in the neighbouring molecules differs from that in the globular native proteins and this may affect their reactivity.

The solubility of the different porphyrins depends on the number and nature of the polar groups attached to the porphyrin skeleton. In some cases the solubility is sufficiently low for monolayers to be obtainable. The properties of such monolayers have been recently examined by Alexander [1937]. In this investigation their reactions with monolayers of materials of biological importance are described with the object of obtaining a comparison of their behaviour with the same system *in vivo*.

EXPERIMENTAL

The monolayers were spread on the surface of various substrates in a Langmuir trough from suitable solvents, or in the case of proteins, both from solution [Gorter & Grendel, 1926; Mitchell, 1937] or from the solid [Hughes & Rideal, 1932]. The surface pressures of the monolayers F , surface tensions of the solutions γ and phase boundary potentials ΔV were measured in the usual manner. The porphyrins were dissolved as sodium salts in weak alkali (0.01–0.001*N*) and only freshly prepared solutions were employed. The reacting substance was injected under the monolayer and mixed with the substrate [Schulman & Rideal, 1937] and any penetration or adsorption followed by noting the changes in pressure and potential at constant area. Since many of the substances injected were capillary active it was necessary to determine the change in surface

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tension ($\Delta\gamma$) of the solution behind the floating barrier of the Langmuir trough so that the change in pressure on penetration could be evaluated from the expression

$$F = F_{\text{obs}} + \Delta\gamma.$$

In general the monolayers were compressed to an initial pressure of $F = 6$ dynes/cm. and the reactions were carried out at room temperature (17–23°).

If a small amount of a not too soluble porphyrin be injected into a solution, it will gradually accumulate at the surface passing through a monolayer stage and eventually build a solid layer several molecules thick, with a pressure increase in some cases of more than 20 dynes/cm. which is far higher than the pressure at which the corresponding monolayer collapses. Sobotka [1937, 1] considers such layers to be multilayers with definite structure, but there seems to be no reason to assume that they represent anything but a surface crystallization, the tendency of the flat rings to associate being very strong. Indeed, all other multilayers examined always reveal the presence of crystals [cf., e.g., Stenhagen, 1938, 1]. The rate of formation of these surface-skins of various porphyrins is shown in Fig. 1. That diffusion plays a large part in governing the rate is shown

Fig. 1. Surface skin formation of different porphyrins when injected in concentration of 5 $\mu\text{g./ml.}$ under a clean water surface $M/25$ phosphate buffers.

by the slow rate of building up by the colloidal solution of protoporphyrin in contrast to the rapid rate revealed by the more disperse bilirubin. Both the meso derivatives in which the vinyl groups have been replaced by ethyl groups and the porphyrin esters are too insoluble to be used for this purpose. In Fig. 2 are shown the results of injection of various porphyrins in concentrations of 5 $\mu\text{g./ml.}$ under a cholesterol monolayer at 6 dynes/cm. spread on $M/25$ phosphate buffers at pH 7.2 and 8.2 respectively. Bilirubin, which in the absence of

the cholesterol monolayer, forms a solid skin, which exerts a pressure of $F = 15$ dynes at $pH\ 7.2$ and 3 dynes at $pH\ 8.2$, caused a rapid rise in pressure to 30 dynes and a fall in surface potential of 70 mV. of a cholesterol monolayer at the former pH . The values of 13 dynes and 30 mV. being obtained at $pH\ 8.2$. The monolayer remains liquid in both cases.

Fig. 2. Effect of pH on penetration into cholesterol on phosphate buffers.

In alkaline solutions, especially when exposed to light, bilirubin undergoes rapid autooxidation. The oxidation product is not capillary active and is not adsorbed by, nor does it penetrate, monolayers of cholesterol. It will be seen that both protoporphyrin and haemin penetrate films of cholesterol quite strongly, the former penetrating more rapidly at $pH\ 8.2$ than at 7.2 , this being similar to its behaviour in accumulation at a free surface (Fig. 1). Haematoporphyrin and coproporphyrin, which in the physiological pH range exhibit but little capillary activity, show but a small penetration of cholesterol monolayers, and scarcely any change in surface potential.

Bilirubin in a concentration of $5\ \mu g./ml.$ injected under monolayers of cetyl alcohol, stearic acid, stearamide or methyl stearate penetrates these films in the same way as it does monolayers of cholesterol, the penetration being stronger at $pH\ 7.2$ than at 8.2 . The ester shows the smallest rise in pressure, 23 dynes at $pH\ 7.2$, and a change in surface potential of 160 mV. at $pH\ 7.2$ and 20 mV. at $pH\ 8.2$. The potentials of the cetyl alcohol and the stearic acid monolayer remain practically unchanged and the state of the monolayers remains unaltered, corresponding to that of the original monolayer at the corresponding pressure. Cetyl alcohol is penetrated rather slowly by protoporphyrin, giving a pressure of 20 dynes after 30 min., with a very small change in potential. With octadecylamine

Fig. 3. Penetration of bilirubin into octadecylamine monolayers.

Fig. 4. Penetration of porphyrins into amine and acid monolayers.

monolayers, on the other hand, the behaviour is entirely different and the penetration by bilirubin is in fact somewhat stronger at the more alkaline reaction in spite of the reduced capillary activity (Fig. 3). The potential of the amine monolayer is reduced some 100 mV. The oxidized bilirubin shows a comparatively weak penetration.

Protoporphyrin and haemin also show very strong penetrations of amine monolayers, giving stable pressures of 44–48 dynes (Fig. 4), and reductions in potential of 400 and 200 mV. respectively. The haematoporphyrin penetrates more slowly; the pressure rises to 40 dynes after 20 min., but no change in the potential occurs. Coproporphyrin I penetrates less than haematoporphyrin, the penetration being very weak at *pH* 8 and increasing with decrease in *pH* down to 5.5 (20 dynes after 15 min.) after which it decreases owing to complete insolubility of the haematoporphyrin.

The 2,4-dimethyl-5-carbethoxypyrrole-3-acrylic acid was found to penetrate an amine film slowly. If the amine is spread at an area of 50 sq. Å. per molecule on a phosphate buffer *pH* 6.0 (non-coherent film) and 30 µg/ml. of the acrylic acid be injected, it is found that after 15 hr. the amine is converted into a weak solid, uniform film, exerting a pressure of 6 dynes, probably due to penetration and subsequent surface polymerization of the acrylic acid.

In mixed octadecylamine-stearic acid monolayers, strong polar interaction occurs, as shown by Marsden & Schulman [1938]. If bilirubin is injected under such a monolayer, it is found that the penetration at *pH* 7.2 and 8.2 is reduced to the same value as obtained with the stearic acid alone and there is no change in the surface potential of the mixed film. Similar results are observed with haematoporphyrin.

Protein monolayers

Bilirubin in a concentration of 5 µg./ml. rapidly increases the pressure of a gliadin monolayer at *pH* 7.2 to 20 dynes. Identical results are obtained with and without 0.17*M* NaCl present. The monolayer becomes rigid and behaves like a "tanned" skin, which, however, is not uniform as revealed by the non-uniformity of the surface potential which decreases. At *pH* 8.2 no tanning occurs, the monolayers remain liquid and there is only a slight increase in pressure (Fig. 5), and no change in the surface potential. Injection of protoporphyrin at *pH* 7.2 causes only a slow rise in pressure, a pressure of 20 dynes being obtained only after 2 hr. with a slight change in surface potential. At *pH* 8.2 the "tanning" takes place more rapidly and there is a fall in the surface potential. With haemin at *pH* 7.2 and 8.2 the behaviour is like that of the protoporphyrin at *pH* 8.2, but the surface potential increases by 100 mV. This is in accord with the fact that the surface potential of haemin monolayers is higher than that of protoporphyrin [Alexander, 1937].

The tanned films obtained are rapidly dispersed by soaps, in contrast to protein films tanned by tannic acid [Schulman & Rideal, 1937]. Haematoporphyrin shows only a weak adsorption to gliadin films at 6 dynes pressure, and with coproporphyrin and turacin (copper-uroporphyrin) no interaction is observed.

Serum albumin and other protein monolayers are interacted with by injections of porphyrins much in the same manner as gliadin, and no specificity in the different proteins was observed. The behaviour of the porphyrins with proteins was examined further by carrying out some electrophoresis experiments with electrophoretically isolated human serum albumin and different porphyrins, using the micro apparatus of Tiselius [1938]. The isoelectric point of

human serum albumin at 0° is 4.64 [Stenhagen, 1938, 2]. 0.5 % protein solution was used. In the place of the photographic plate, a spectrometer with a long vertical slit was employed, thus permitting a direct examination of the absorption spectrum of the solution in different parts of the electrophoresis U-tube to

Fig. 5. Gliadin monolayers on *M*/25 phosphate buffers; porphyrins in concentration 5 $\mu\text{g./ml.}$ injected.

be made. The Toepler "Schlieren" bands appeared as dark horizontal lines. In a phosphate-buffer at $\text{pH } 7.0$, $\mu = 0.1$, it was found that bilirubin in a concentration of 80 $\mu\text{g./ml.}$ was entirely absorbed on the protein [cf. studies by Bennhold, 1932], only one boundary on each side being observed. At $\text{pH } 8.2$, however, part of the bilirubin was found to migrate in front of the serum albumin. At $\text{pH } 7.0$, the migrating boundaries were markedly convex in the direction of migration. This indicates an increase in viscosity of the protein solution, as a similar phenomenon occurs with the very viscous solutions of thymonucleic acid [Theorell & Stenhagen, 1939]. Protoporphyrin in concentration 50 $\mu\text{g./ml.}$ is entirely absorbed by a 0.5 % serum albumin solution, there being no sign of free migrating porphyrin, but haematoporphyrin under the same conditions is only partially adsorbed, free porphyrin being found to migrate in front of the protein. In no case was more than one protein boundary on each side observed, and the free porphyrin boundary was very diffuse.

Hill & Holden [1926] concluded from absorption spectrum analysis that protoporphyrin and globin combine in neutral solution and Haurowitz & Waelch [1929] observed the same for haematoporphyrin and horse serum. Holden [1937] reinvestigated the problem and concluded from examination of the ultraviolet absorption spectra that protoporphyrin and haematoporphyrin

can combine with horse serum. The electrophoresis experiments support this but show that the haematoporphyrin-serum albumin compound is weaker than the protoporphyrin one. Thus we note a parallelism between the absorption as observed in bulk and the adsorption to protein films, in spite of the different states of the protein which suggests that forces of the same type are operative.

DISCUSSION

It seems clear that, with the exception of the amine group, there is only a weak interaction between groups such as —OH , —COOH , —COOCH_3 , —CONH_2 and the reactive groups in the porphyrins, and that for monolayers containing these groups the hydrophobic portions of the reacting molecules play a governing part in complex formation as the stability increases with decreasing water solubility of the penetrating molecule. The marked differences with small changes in pH observed with bilirubin may have a biological significance.

Bennhold [1932] showed that bilirubin in blood is absorbed by the albumin fraction of the serum protein; and Pedersen & Waldenström [1937] showed by means of electrophoretic and ultracentrifugal studies that this absorption is specific for serum albumin and that it does not occur with egg albumin. They attributed this difference to the different amino-acid distributions in the two proteins. From the behaviour of bilirubin towards amine films, we may conclude that the coupling takes place via the primary amino groups in the proteins and the phenolic group in the bilirubin. This is further supported by the fact that in the presence of protein involving a change of the —OH to a C=O group [see Fischer & Orth, 1937] bilirubin is stable against oxidation. The percentage of lysine which has a free amino group in the ϵ -position is much higher (13.2 %) in serum albumin than in egg albumin (3.8 %) and in serum globulin. The actual distribution of the amino groups in the protein molecules with respect to neighbouring acid groups appears to be important, for we have shown that if the amino groups are allowed to interact with acid groups (as in the mixed amine-acid films) the reactivity of the amine groups is very much reduced. This factor, together with the relatively small proportion of amino groups present, can be advanced in explanation of why there is no apparent specificity in the adsorption of bilirubin by protein monolayers. The process of spreading a protein involves an unfolding and reorientating of the protein molecules, and bringing chains of different molecules in close proximity to one another, which upsets the original distribution of polar groups. We have also not been able to reproduce the adsorption experiments by Grollman [1925] with phenol red and serum and egg albumin with monolayers of the proteins.¹

The experiments show that the bilirubin-amine interaction is stronger at pH 8.2 than at pH 7.2. Pedersen & Waldenström [1937] have shown that in the bile the bilirubin is adsorbed on to a colloidal carrier of unknown nature, which increases the solubility of the bilirubin. If the bilirubin-carrier coupling is dependent on interaction with amine groups in the carrier, a decrease in pH will tend to weaken this interaction, and at the same time decrease the solubility of the bilirubin and increase its complex formation tendency with cholesterol. In the complex process of the formation of gall stones of the mixed type, containing bilirubin-calcium-cholesterol-protein, we might therefore expect that a decrease in pH will increase the rate of stone formation. In many infections of the gall

¹ On the other hand selectivity has been observed when the various native proteins are injected under monolayers of certain lipoids in some recent experiments by Schulman & Rideal.

bladder the pH is decreased and some evidence in the literature shows [Sobotka, 1937, 2] that gall-stone formation tendency is increased with decrease of the pH in the gall bladder.

SUMMARY

It is shown that bilirubin and the capillary-active porphyrins strongly penetrate lipid monolayers and that this penetration involves but a weak polar interaction and is largely influenced by the interaction of the hydrophobic portions of the molecules. Specific interaction is found, however, to occur with amine monolayers, the strength of the interaction being independent of the capillary activity of the reactant. Protein films interact with and are tanned by the more capillary active porphyrins, but in some cases no adsorption of the porphyrins occurs in spite of strong reaction with amine films. It is shown that this is due at least in part to polar interaction within the protein monolayer itself, as the reactivity of the amine group is negligible in mixed amine-acid films, where strong polar attraction between the basic and the acid groups occurs. Some electrophoresis experiments in porphyrin-serum albumin mixtures in bulk solution are described and compared with the data obtained in monolayers. Possible biological implications of the results are discussed.

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