The Physical Chemistry of Haemolysis by Surface-active Agents

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A large number of compounds have the property of destroying the selective permeability of erythrocytes, allowing the diffusion of haemoglobin into the external medium, accompanied by partial or complete dispersal of the stromata. Two principal groups of lysins can be distinguished kinetically. Enzymic or bacterial lysins haemolyse at a rate proportional to the lysin concentration, whereas surface-active compounds lyse at rates which are non-linear, frequently exponential, in relation to the lysin concentration (Bernheimer, 1947). It is the purpose of this contribution to suggest a subdivision of the surface-active group, distinguishing between haemolysis by non-ionic detergents and by long-chain ionic compounds. Previous publications have shown that a series of ionic detergents act in a parallel fashion both in penetrating an insoluble monolayer of cholesterol at the air-water interface, and in the haemolytic reaction (Schulman & Armstrong, 1949; Schulman & Rideal, 1937, 1939). Non-ionic lysins, such as the alcohols, do not penetrate cholesterol to any marked extent, and their haemolytic activity is not at present well understood.

There is much evidence to show that red cells possess a partially lipid outer layer (Turner, 1946). Cholesterol occupies a special place in haemolysis, both as an inhibitor of several lysins (Schulman & Rideal, 1937; Ponder, 1944, 1945, 1946), and as an important stroma component (Parpart & Dzeimian, 1940; Turner, 1946; Schmidt-Thomé, 1942). The study of the haemolytic reaction in terms of monolayer processes involving cholesterol and other lipids therefore constitutes a useful approach to the understanding of haemolysis.

DEFINITIONS

It will be convenient here to define some of the terms used in this report. The surface pressure, π , of a solution is defined as the lowering of the surface tension produced by a solute (or an insoluble film). With many surface-active compounds, increasing the concentration in solution decreases the surface tension up to the concentration at which molecular aggregates (micelles) are formed. Beyond this concentration, denoted as the critical micellar concentration or micelle point, the surface tension remains effectively constant, since molecules are not available to accumulate at the interface and the micelles do not lower the surface tension on

their own. This is shown by curves of γ plotted against $\log c$, as in Fig. 3 for sodium dodecylsulphate. Insoluble monolayers spread at phase interfaces may be compressed up to a minimum area per molecule, corresponding to the closest possible packing in the monolayer, usually corresponding to a two-dimensional crystal. Further compression causes the film to collapse with consequent three-dimensional crystallization and destruction of its monolayer structure. The surface pressure at this minimum area per molecule is the maximum pressure possible in the monolayer, and is termed the collapse pressure of the film. If a soluble detergent is introduced into the bulk phase beneath an insoluble monolayer, several processes can occur. If no specific interaction takes place, the soluble detergent can remove (deterge) the insoluble film if the surface pressure of the soluble compound alone is greater than the collapse pressure of the insoluble film. The same process can occur more readily if a specific interaction takes place in the surface between the soluble and insoluble components, when an increase in surface pressure is observed above that of the soluble compound alone, due to the penetration of the soluble molecules into the monolayer. If the interaction pressure is above the collapse pressure of the insoluble film, the molecules in the insoluble film are partially ejected from the interface leaving a mixed associated film of the reacting species, which is more stable than either of the components alone. It can thus be seen that if the plateau of the curve of surface pressure against concentration of a solute is at a pressure below the collapse pressure of an insoluble film, into which the solute molecules are unable to penetrate, no removal or detergence of the insoluble monolayer will take place, however great the concentration of the solute in the underlying solution. On the other hand, removal takes place sharply if the surface pressure of the solute solution can be greater than the collapse pressure of the insoluble film. The haemolytic activity of a compound is taken as $(\pi_H)^{-1}$ (see below). The haemolytic surface pressure (π_H) of a lysin is the reduction in surface tension at the air-water interface produced by the lysin at the haemolytic-index concentration.

MATERIALS AND EXPERIMENTAL METHODS

The non-ionic lysins used were normal aliphatic alcohols (C_4 – C_8) and two vacuum-fractionated polyethylene ethers of normal alcohols. These polymers were heptyl alcohol-11-ethylene oxide [$CH_3(CH_2)_6 \cdot (CH_2 \cdot CH_2O)_{11} \cdot OH$, abbreviated here to C_7 -11EO], and decyl alcohol-8-ethylene oxide [$CH_3(CH_2)_9 \cdot (CH_2 \cdot CH_2O)_8 \cdot OH$, abbreviated to C_{10} -8EO]. The purity of the polymers was judged by the magnitude of the minima in the surface tension/concentration curves of these two compounds. The C_{10} -8EO was almost pure, with a critical micelle concentration of 1.0 g./l. The C_7 -11EO

polymer was somewhat contaminated, with a distinct minimum as shown in Fig. 7. The butanol was a pure specimen, but the amyl and octyl alcohols were contaminated with paraffins. All other materials used were pure. Sodium laurate concentrations are recorded in terms of lauric acid (g./l.), made up to the required pH. Saponin was a sample of 'pure white' from Hopkin & Williams Ltd.

Surface tensions were measured by the drop volume method (Harkins & Brown, 1919), using a glass-tipped tuberculin syringe operated by a micrometer screw. Except with saponin and the unstable solutions mentioned in the text, no appreciable surface ageing was found. Reproducibility was within 0.5–1.0%. All solutions used here

Fig. 1. Graphs of concentration and reciprocal lysis time for A, $C_{11}H_{23}COONa$, pH 7, 37°; B, $C_{12}H_{25}NH_2.HCl$, pH 7, 20°.

were prepared in 0.85% NaCl, buffered with 0.02 M H_3BO_3 , with borax added to pH 7.0 or as otherwise stated.

Haemolysis was studied with thrice-washed human red cells (AB, Rh-positive) suspended in the above medium, the final pH of the suspensions being 6.5–7.0. Tests were made at 20° or as stated, on suspensions of a final red-cell density of 5% volume (about 5×10^8 cells/ml.). The haemolytic end point was observed directly by eye in tubes of fixed size, using a well defined black-on-white background for viewing (Ponder & Yeager, 1930). This method gives reproducible end points corresponding to complete lysis (no turbidity), and to 98% lysis (background just visible), standards being prepared from cells lysed by freezing. Results were plotted as time/concentration (t/c) curves, and the haemolytic index (H.I.) of a compound was taken as that concentration producing complete lysis in 90 min. The H.I. does not represent the free lysin concentration, as some is bound by the cells. The index was chosen for a number of reasons. The t/c curves are steep in this region, and give closely reproducible c values for the index. The t/c curves are not always asymptotic to a limiting c , as is shown by reciprocal plotting. In some cases the reciprocal plots curve off near the index (Fig. 1, curve B) indicative of a two-stage haemolytic process; the first an asymptotic surface process (linear $t/(1/c)$ plot), and the second ill defined. Data given by Ponder (1948) on the effect of cell-suspension density on the

limiting concentration for lysis can be interpreted in terms of a simple adsorption process describable by a Langmuir isotherm, although Ponder adopts a more complicated explanation.

Monolayer interactions were measured at 20° with the standard medium with a compensated Langmuir-Adam trough as previously described (Matalon & Schulman, 1949). The reactants were injected beneath cholesterol monolayers at constant area. The trough was of silica, with a water surface of 300 sq.cm., and a volume of 280 ml.

Fig. 2. The relations between concentration and haemolysis times for A, $C_{11}H_{25}NMe_3I$, pH 7, 20°; B, $C_{11}H_{23}COONa$, pH 7, 20°, concentration scale in g./l. of lauric acid; C, $C_{11}H_{23}SO_3Na$, pH 7.5, 20°; D, $C_{11}H_{23}SO_3Na$, pH 7, 20° (for 98% lysis).

RESULTS

The relation of haemolytic index and haemolytic surface pressure

In Fig. 2 are given the time/concentration relations for lysis by dodecyltrimethylammonium iodide and sodium laurate, and in Fig. 3 the corresponding relations between γ and $\log c$. The laurate solutions in this medium are not stable for long periods at 20°, and the results are approximate only. The $\gamma/\log c$ results for sodium dodecylsulphate (SDS) are also shown in Fig. 3.

Lysis by SDS is complicated by haemoglobin precipitation, which obscures the visible end point. Haemolysis in this system was studied with a photoelectric absorptiometer. At high concentrations of SDS, rapid lysis is followed by haemoglobin precipitation. At the lowest concentrations haemolysis is very slow, and a value for the H.I. of 3.1×10^{-4} M (0.09 g./l.) is obtained. This value was confirmed by additional tests at pH 7.5 and 20°, where no haemoglobin precipitation occurs. These are shown in

Fig. 2, and give an index of $3.45 \times 10^{-4} \text{ M}$, an increase as expected from published data on the pH dependence of SDS lysis (Love, 1950). Haemolysis by sodium dodecylsulphonate is also complex, as the salt precipitates out at $4.7 \times 10^{-4} \text{ M}$. No haemoglobin precipitation was observed, but the time/concentration curves for complete and 98% lysis were unusually separated, the former falling above the precipitation point and the latter below.

The first three columns of Table 1 give a summary of the π_H and H.I. values for all the ionic detergents. The value for sodium taurocholate is also included, as given elsewhere (Pethica & Schulman, 1952).

No particular relation exists between surface pressure, molecular weight and lysis for the ionic detergents.

The non-ionic detergents studied all lyse at higher surface pressures than the ionic compounds.

Fig. 3. Surface tensions in 0.85% NaCl with borate buffer, pH 7, 20°, of A, $\text{C}_{12}\text{H}_{25}\text{NMe}_3\text{I}$; B, $\text{C}_{11}\text{H}_{23}\text{COONa}$, concentration scale in g./l. of lauric acid; C, $\text{C}_{12}\text{H}_{25}\text{SO}_4\text{Na}$.

Fig. 4. Haemolysis by butanol and decyl alcohol-8-ethylene oxide. A, butanol, pH 7, 20°; B, butanol, pH 9.6, 20°; C, butanol, pH 7, 37°; D, $\text{C}_{10}\text{-8EO}$, pH 7, 20°.

Ascribing this displacement to precipitation and noting that with all the other lysins studied the index varies up to 10% between the two end points, the index for complete haemolysis by the sulphonate is taken as $3.5 \times 10^{-4} \text{ M}$. The 98% lysis time/concentration curve for the sulphonate is given in Fig. 2.

Fig. 4 gives the time/concentration relations for haemolysis by butanol and $\text{C}_{10}\text{-8EO}$. As the amyl and octyl alcohol concentrations were not accurately known, the data for these two lysins are recorded in the table only, the haemolysis and surface tensions being correlated from the same stock solutions.

Table 1. Haemolytic and surface characteristics of ionic and non-ionic detergents

	Mol. wt.	H.I.	π_H	$(\cot \alpha) \times 10^{-5}$
$\text{C}_{12}\text{H}_{25}\text{SO}_4\text{Na}$	288	$3.12 \times 10^{-4} \text{ M}$	28.0	3.45
$\text{C}_{12}\text{H}_{25}\text{SO}_3\text{Na}$	272	$3.5 \times 10^{-4} \text{ M}$	—	2.29
$\text{C}_{12}\text{H}_{25}\text{NH}_2 \text{ HCl}$	222	$5.4 \times 10^{-4} \text{ M}$	27.2	0.70
$\text{C}_{11}\text{H}_{23}\text{COONa}$	222	$6.7 \times 10^{-4} \text{ M}$	34.2	0.38
$\text{C}_{12}\text{H}_{25}\text{NMe}_3\text{I}$	355	$14.1 \times 10^{-4} \text{ M}$	23.1	0.28
Sodium taurocholate	556	$360.0 \times 10^{-4} \text{ M}$	29.3*	0.079
BuOH	74	0.39 M	34.3	—
$\text{C}_8\text{H}_{17}\text{OH}$	88	—	36.9	—
$\text{C}_9\text{H}_{17}\text{OH}$	130	—	> 36.0†	—
$\text{C}_7\text{-11EO}$	601	0.037 M	39†	—
$\text{C}_{10}\text{-8EO}$	501	0.001 M	40.8	—

* Micellar at the index concentration.

† A 'saturated' solution slightly lytic, $\pi = 36.0$ dynes/cm.

‡ Non-micellar, see Fig. 5.

In general, π_H rises with increasing molecular weight of the non-ionic lysins. A similar effect has been observed in the lysis of paramecia by similar compounds (Matalon, Molho-Lacroix & Cohen, 1950). The low-molecular-weight alcohols will lyse at pressures of the order of 34 dynes. This general result is corroborated, with two exceptions, by Glassman (1950), with a large range of non-ionic detergents. The fact that non-ionic detergents lyse on attaining pressures of 34 dynes/cm. or above is interestingly confirmed by experiments on haemolysis in mixed solutions of anionic and cationic detergents. Figs. 5 and 6 give the lytic activity and surface tensions of mixed solutions of dodecylamine hydrochloride and sodium taurocholate. At the concentrations used, taurocholate is completely non-lytic (Pethica & Schulman, 1952). The $\gamma/\log c$

Fig. 5. Surface tensions in 0.85% NaCl with borate buffer, pH 7, 20°, of A, $C_{12}H_{25}NH_2HCl$ alone; B, $C_{12}H_{25}NH_2HCl$ with 1:1 sodium taurocholate; C, $C_{12}H_{25}NH_2HCl$ with 2:1 sodium taurocholate.

curves in Fig. 5 indicate the reversible formation of a soluble anion-cation complex with a sharp micelle point. This complex can be considered as a non-ionic detergent. The greatly enhanced lysis in the mixed solutions can be explained simply by the lowering of the surface tension. The π_H values in the mixtures are: 34.6 dynes/cm. (equimolar mixture); 34.1 dynes/cm. (taurocholate 2:1).

An exactly analogous effect was observed in mixtures of C_{12} quaternary ammonium iodide and taurocholate. Lysis in the mixtures is strongly enhanced, but correlation with the surface tension was not made. In a number of mixtures of SDS and C_{12} quaternary amine the surface pressure was raised to approximately 48 dynes/cm., and lysis was extremely rapid. A full analysis of this system was precluded by the slow precipitation of the complex.

The surface-tension lowering, however, is clearly responsible for the increased haemolysis. It is worthy of note here that the heats of mixing of

Fig. 6. Haemolysis at pH 7, 20°, by A, $C_{12}H_{25}NH_2HCl$ alone; B, with added 1:1 sodium taurocholate; C, with added 2:1 sodium taurocholate; D, with added 4:1 sodium taurocholate.

Fig. 7. Haemolytic and surface activity at pH 7, 20°, of heptyl alcohol-11-ethylene oxide. A, lysis time/concentration; B, surface tension/concentration.

dodecylamine hydrochloride solutions with SDS and taurocholate have been measured (Goddard & Pethica, 1951). Zero heat was obtained with taurocholate. The heat of mixing dodecylamine hydrochloride with SDS ($-\Delta H = 8 \text{ kg.cal.} = 33.6 \text{ kJ}$)

is ascribed to precipitation of the complex. Precipitation in the amine-taurocholate system is prevented both by hydration and the steric effect of the hydroxyl groups in the taurocholate molecule.

Additional confirmation of the prime importance of the surface pressure in haemolysis by non-ionic detergents is provided by Fig. 7. The well defined surface-tension minimum in C_7 -11EO solutions corresponds with a sharp maximum in the haemolytic effect of the detergent. The fact that the lytic activity is not completely symmetrical with respect to the surface-tension minimum shows that other factors must also be considered (see Discussion).

Monolayer penetration and haemolysis

Previous reports have shown a parallelism between the rise of pressure obtained in cholesterol monolayer penetration, and haemolysis by a series

into cholesterol at constant area, and at an initial pressure of 4 dynes/cm. The form of the pressure-concentration relation in penetration under these conditions is of a Langmuir isotherm. Fig. 8 shows

Fig. 9. The relation of haemolytic activity to penetration. 5% red cell suspension.

Fig. 8. Penetration of cholesterol at constant area. Isotonic saline substrate, pH 7, 20°. Dodecyl compounds and taurocholate. Initial $\pi = 4$ dynes/cm.

of dodecyl compounds with various ionic head groups and saponin (Schulman & Armstrong, 1949; Schulman & Rideal, 1937, 1939). Ruyssen and co-workers have confirmed a similar parallelism with saponins in the penetration of mixed lecithin-cholesterol and pure cholesterol monolayers (Croes & Ruyssen, 1950; Ruyssen & Loos, 1946). Quantitative interpretation of the constant-area penetration of cholesterol by saponin and cetyl sulphate is difficult, as equilibrium pressure increases are not attained quickly. The pressure increases obtained with the different lysins studied by Ruyssen & Loos (1946) tend to the same value after some time. A study of the characteristics of constant-area monolayer penetration has been undertaken and will be reported in full elsewhere. In penetration by the dodecyl ionic detergents and taurocholate, equilibrium surface-pressure increases are attained rapidly, usually in 10 min. after the injection into the subphase. A series of penetrations were made

Fig. 10. The relation of haemolytic index to cholesterol penetration.

the isotherms for the range of C_{12} compounds and taurocholate, plotted to illustrate the linear Langmuir relation

$$\frac{1}{\Delta\pi} = \frac{1}{\Delta\pi_m} \left(1 + \frac{K}{c} \right), \quad (1)$$

where $\Delta\pi = \pi - 4$, the pressure rise at c ; $\Delta\pi_m = \pi_m - 4$, the pressure rise at $c = \infty$; K is a constant.

$\Delta\pi_m$ is sensibly constant (34–38) and corresponds to an increase of pressure up to the collapse pressure of cholesterol (38 dynes/cm.) which is not markedly affected by penetration with these compounds. In the table are given the reciprocal slopes of the Langmuir plots,

$$\cot \alpha = \frac{\Delta\pi_m}{K} \text{ dynes/m-mole/280 ml.}$$

$\cot \alpha$ is a measure of the free energy of penetration. The reactivity series is in the order:

Previously, the amine was reported as heading the series (Schulman & Armstrong, 1949), a result now ascribed to long-chain impurities in the amine then used. In Fig. 9 the penetrating capacity ($\cot \alpha$) of these compounds is compared with the haemolytic activity. The relation is not linear, but quite regular. The empirical relation

$$\log (\text{h.l.}) = A + B \tan \alpha, \quad (2)$$

where A and B are constants, is obeyed well, and is shown in Fig. 10.

The significance of this series in terms of the stereochemistry of the head groups has been discussed elsewhere (Schulman & Armstrong, 1949). The low activity of the laurate is ascribed to the repression of dissociation in the salt solution used (0.145M).

The effect of pH and temperature on lysis by non-ionic detergents

The surface tension of butanol solutions is not affected by pH, and is lowered at increased temperatures (20–37°). Allowing for the decrease in the surface tension of water at the higher temperature, the surface pressures of the alcohol solutions increase at 37° by a few tenths of a dyne over those at 20° for the range studied. Fig. 4 gives the data for lysis by the alcohol under various conditions. The values of π_H are 34.3 dynes/cm., pH 7, 20°; 27.7 dynes/cm., pH 7, 37°; 31.0 dynes/cm., pH 9.6, 20°. Both increased temperature and pH decrease the stability of the red cell to alcohol lysis.

Lysis by sodium laurate

The table shows that sodium laurate at pH 7, 20°, has a haemolytic surface pressure (π_H) of 34.2 dynes/cm., and could therefore be regarded as belonging to the two groups of detergent lysins by virtue of its surface pressure and penetration effects. Experiments at 37°, where the soap solutions are completely stable, show that laurate lyses principally by penetration. π_H is 24.9 dynes/cm. at pH 7, 37°, lower than the butanol value for the same conditions. At pH 9.6, 37°, π_H for laurate decreases to 18.0 dynes/cm. Suggestions have been made with reference to fat absorption from the intestine that

fatty acids and bile salts form soluble complexes (Verzár & Kuthy, 1929*a, b*). Experiments were accordingly made with equimolecular mixtures of taurocholate and laurate to observe possible phenomena of interest to haemolysis. Fig. 11 gives the surface-tension data, and Fig. 12 the lysis data for laurate and laurate-taurocholate mixtures at 37°, pH 7.0 and 9.6. The enhancement of lysis by the addition of taurocholate is slight at both pH values. At pH 7 there is little evidence of complex formation. At pH 9.6, the $\gamma/\log c$ curves resemble Fig. 5.

Fig. 11. Surface tensions of sodium laurate and mixtures with equimolar sodium taurocholate in NaCl. ○, Laurate alone; Δ, equimolar mixtures of laurate and taurocholate. Upper two graphs, pH 9.6, 37°. Lower two graphs, pH 7, 37°.

It is difficult to envisage a complex between these two anions, unless a dipole-ion interaction occurs, whereas the amine-taurocholate association shown in Fig. 5 is easily interpreted in terms of anion-cation interaction. It would seem that Verzár's data on the solubilization of fatty acids by bile salts is to be explained by solubilization of the acids into the bile salt micelles at the high concentrations used. In Fig. 13 the relative increase of lytic activity (ratio of lysis times) on addition of taurocholate to laurate is compared for different concentrations. At pH 7 the maximum increase occurs exactly at the concentration at which the $\gamma/\log c$ curves for laurate and the mixture cross. The surface pressure at that concentration is 29.0 dynes/cm. which is 1.3 dynes/cm. above π_H for butanol lysis under the same conditions. The enhanced lysis in the mixtures at concentrations below the maximum in Fig. 13 is ascribed to the joint effect of the reduced surface tension in the mixtures and cooperation in penetration by the two detergents. Above the maximum,

the greater surface pressure of the pure laurate solutions commences to outweigh the enhanced penetration as a factor in haemolysis—taurocholate being a weak monolayer-penetrating agent—and

Lysis in the region of the 'mixed' micelle point was too rapid for quantitative study.

Lysis by saponin

Saponins haemolyse at low surface pressures (Ruyssen, Croes & Ommeslack, 1947) but penetrate strongly into cholesterol monolayers to give high surface pressures (Schulman & Rideal, 1937). As pointed out above, equilibrium pressure increases are difficult to obtain in cholesterol penetration by saponin. In these experiments, no stable penetration pressures were observed below 40 dynes, at which pressure the mixed film becomes a strong gel. Penetration with saponin differs from that by C_{12} ionic compounds (Fig. 8) in that the pressure increases proceed rather indefinitely, and that the liquid cholesterol monolayer eventually becomes viscous and then gelatinous. The pressure rises recorded can be greatly in excess of the collapse pressure of cholesterol, and a strong complex is obviously formed. The lytic and penetration activity of saponin have been compared approximately from a Langmuir plot of the $\Delta\pi$ values below 40 dynes, 2–3 hr. after penetration. This gave $\cot \alpha = 2.9 \times 10^6$ m-mole/dyne/280 ml., assuming a mol.wt. of 1250 (Ruyssen, 1950). The haemolytic index in the 5% red-cell suspension is 10^{-4} M. These results are given in Fig. 9 as an extension to the graph, and show that the sequence is maintained. Equation 2 is not obeyed by saponin.

DISCUSSION

The data presented are interpreted as showing that haemolysis by a range of surface-active agents can be described in terms of two basic processes at the erythrocyte membrane surface. The selective permeability and osmotic properties of the cells appear to be the function of a component or complex that is either removed from the interface or broken down above a critical interfacial pressure corresponding to 34 dynes/cm. at the air-water interface. This critical pressure may be regarded as the 'collapse surface pressure' of the component or complex. The collapse is brought about either by a straight detergent effect, as with the non-ionic detergents studied, or by an increase in interfacial pressure due to the ion-dipole or ion-ion interaction in penetration by the long-chain ionic detergents. In the present state of our knowledge, the cell complex involved seems to be a cholesterol-lipo-protein association, unstable above 34 dynes/cm. Cholesterol itself has a collapse pressure of 38 dynes/cm. at the air-water interface. Further investigation may show a similar lysis-penetration relation with ionic detergents in monolayer processes involving other red-cell surface components than cholesterol. It is unlikely that the same reactivity sequence for penetration will apply to monolayers of

Fig. 12. Haemolysis by laurate and laurate-taurocholate mixtures in NaCl. $\circ$, Laurate alone; Δ , equimolar mixtures. Upper two curves, pH 9.6, 37°. Lower two curves, pH 7, 37°.

Fig. 13. The enhancement by taurocholate of lysis by laurate at 37°. t/t_m = ratio of lysis times for laurate and the mixtures (see Fig. 11 for concentrations). A, pH 7.0; B, pH 9.6.

the mixture becomes relatively less lytic as a consequence. At pH 9.6, the enhancement of lysis increases with concentration, over the range studied, as the co-operative penetration and increased surface pressures of the mixture act together.

phosphatidic lipids, as the phosphate groupings will repel the anionic detergents and attract the cationic. Nor would saponin penetration into phospholipins be of the correct order in the series, as it has been shown to penetrate lecithin films weakly (Schulman & Rideal, 1937). Certain analogies to lytic reactions have been observed in mixed cholesterol-lecithin films, in the effect of alkaline earth ions (Croes & Ruysen, 1950). Obviously the elementary cholesterol penetration process would be modified by the cholesterol being contained in a protein-phospholipin-cholesterol complex. Lysis can also be caused by lipolytic enzymes (Hughes, 1935). The protein-phospholipin-cholesterol structure can therefore be broken down both at the phospholipin and cholesterol centres by a suitable choice of haemolysin. Specific protein reagents would also produce a distinctive lysis. For the detergents discussed in this report, the cholesterol is the most likely centre of reaction for ionic detergents and saponin.

The collapse process is envisaged as occurring with non-ionic detergents when the detergent surface pressure exceeds the collapse surface pressure of the cholesterol complex in the cell surface, as with the removal of the monolayers at the air-water interface. Under these conditions, the free-energy gradient allows replacement of the complex by detergent, at a rate determined by the potential barrier at the surface, and the concentration and stereochemistry of the detergent. When the free-energy gradient is established, the rate of entry of detergent molecules into the surface can be written on the basis of the simple collision theory as

$$k = PZe^{-E/RT},$$

where Z is the collision frequency at the surface, P the steric probability factor and E is the energy of the potential barrier. In general, k will increase as the negative free-energy (surface-pressure) gradient increases. P decreases with molecular weight, and since the haemolytic index is taken for an arbitrary constant time for haemolysis (90 min.), the free-energy gradient at the index concentration must therefore be greater for higher molecular weight detergents. This increase in π_H with increasing molecular weight is, in fact, generally found (Table 1). A further consideration is that for a given P and a given surface-pressure gradient, a poorly surface-active substance (i.e. one requiring a high concentration for a given surface pressure) will lyse more rapidly than a strongly surface-active detergent, as Z is smaller in the more dilute system. This is illustrated by the table in the lysis by C_7-11EO and $C_{10}-8EO$. Another illustration is given in Fig. 7. The asymmetry of the t/c curve with respect to the γ/c curve gives two values for π_H , one on each side of the surface-tension minimum. At

the lower concentration, π_H (90 min. lysis) is 39 dynes/cm., recorded in the table. At the higher concentration, where micelle formation has commenced, π_H is 37.6 dynes. Thus the haemolysis proceeds at the same rate at two different concentrations, the lower concentration acting with a greater surface pressure. In the case of lysis by penetrating detergents, lysis occurs when the interfacial pressure is raised to the collapse value of 34 dynes/cm. The form of the lysis-penetration relation in Fig. 9 depends on a number of arbitrary choices, particularly the choice of cell suspension density. The haemolytic activity of saponin increases rapidly with decreasing cell density (Ruysen *et al.* 1947). The haemolytic activity of a good lysin (acting at low concentrations) will be expected to increase more rapidly with decreasing cell density than that of a poor lysin because of the adsorption of lysin by the stroma and released haemoglobin. Reduction of the cell suspension density, therefore, will give a penetration-haemolysis relation progressively nearer to a straight line than the relation in Fig. 9.

Although the division between lysis by detergency and lysis by penetration follows the division of the lysins into non-ionic and ionic with the range of compounds studied in these experiments, overlap may be expected in other compounds. Weak ionic penetrants may lyse by virtue of their lowering of surface tension.

SUMMARY

1. The haemolysis of red blood cells by a number of non-ionic, anionic and cationic reagents has been observed. The surface tensions of solutions of these reagents, and their penetration of cholesterol monolayers, have been measured.

2. Lysis appears to depend on the production of a lowering of interfacial tension corresponding to about 34 dynes/cm. at an air-water interface. This lowering is produced by non-ionic detergents at concentrations at which they give surface pressures greater than 34 dynes/cm., and by ionic detergents at concentrations at which they produce this pressure by penetration of a cholesterol monolayer. Mixtures of cationic and anionic detergents behave like non-ionic reagents. There is a lysis reactivity series with the ionic detergents analogous to their association with cholesterol monolayers.

3. The findings are consistent with the view that haemolysis is due to the collapse of a cholesterol-phospholipin-lipoprotein complex, the cholesterol being the site of attack of saponin and ionic detergents.

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Some Observations on the Absorption and Excretion of 4:6-Dinitro-*o*-cresol (DNOC)

1. BLOOD DINITRO-*o*-CRESOL LEVELS IN THE RAT AND THE RABBIT FOLLOWING DIFFERENT METHODS OF ABSORPTION

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The widespread use of 4:6-dinitro-*o*-cresol* (DNOC) in Great Britain and elsewhere, and the fatal casualties caused by its use amongst agricultural workers have been fully reported by Bidstrup & Payne (1951). A great deal is known about its toxicity (Ambrose, 1942; Spencer, Rowe, Adams & Irish, 1948; Parker, Barnes & Denz, 1951), but no attention has been paid to the possible significance of blood DNOC levels resulting from natural methods of absorbing the substance.

It appeared to us to be essential to rectify this omission for two main reasons. First, a study of blood DNOC levels would give some indication of the possible cumulative effect of DNOC. Secondly, a correlation of blood DNOC levels with early symptoms might be used in a 'monitoring' system to safeguard workers exposed to DNOC. Obviously it was essential to plan some trials on the absorption of

DNOC by human volunteers but before these could be undertaken it was necessary to gain further information from animal experiments on the absorption of DNOC, the significance of the resulting blood levels and the effect of environmental temperatures.

The results of the human trials have now been published (Harvey, Bidstrup & Bonnell, 1951). They have been correlated with environmental studies in the field (Bidstrup, Bonnell & Harvey, 1952) and certain practical recommendations have been made (Harvey, 1952).

The present communication reports the results of the first group of animal experiments, the detailed objects of which were to investigate: (a) the significance of blood DNOC levels following administration of one or more daily doses of DNOC after its absorption through the alimentary canal, the lungs and through the skin of rats and of rabbits; (b) the effect of environmental temperatures on the blood DNOC levels, body temperature, respiratory rate and mortality of the rat and the rabbit.

* 3:5-Dinitro-*o*-cresol in the usage of the Chemical Society, 4:6-dinitro-*o*-cresol in the usage of the American Chemical Society or, systematically, 2-methyl-4:6-dinitrophenol.