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STUDIES ON THE MECHANISM OF HEMOLYSIS BY ACYL CARNITINES, LYSOLECITHINS AND ACYL CHOLINES

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SUMMARY

1. The effect of isolated membrane components and normal cytosol constituents on lysis of rat erythrocytes by synthetic, palmitoyl-D,L-carnitine, palmitoyl choline and (1-palmitoyl)-lysolecithin were studied.

2. A number of these constituents were found to act as modifiers of hemolytic activity. Their action as inhibitors or accelerators were highly dependent on the order with which erythrocytes, lysin and modifier substance were added to constitute the lysis test.

3. Preincubation of the lysin with any one membrane component prior to erythrocyte addition resulted in an inhibition greater than in the corresponding case with a similar addition sequence and no preincubation. On the other hand, preincubation of erythrocytes with various membrane constituents prior to lysin addition did not increase inhibition except in the case of membrane protein. It was concluded that the inhibitory effects obtained resulted from a non-specific interaction of the lysins tested with the various membrane constituents added. In the case of added membrane protein, protection against lysis would also result from its interaction with the cell surface.

4. When membrane and cytosol constituents were each added to erythrocytes immediately after cell exposure to a weakly -hemolytic concentration of lysin, this resulted in an increase rather than a decrease in the extent of hemolysis.

5. These results are discussed in terms of the mechanism of lysis by quaternary ammonium amphipathic derivatives.

INTRODUCTION

The presence of carnitine palmityl transferase in the erythrocyte membrane[1] poses a functional problem since acyl carnitines formed thereby, do not appear to participate in lipid acylating reactions[2] and as yet there is no known relationship linking the activity of this acyl translocating enzyme with any metabolic pathway in erythrocytes. On the other hand, the physiologic function of amphipathic carnitine esters might relate to their highly lytic activity[3,4] in that their formation in the membrane could induce structural changes which would alter the permeability and the fragility of erythrocytes.

Acyl carnitine and lysolecithins are similar in some of their chemical and biological properties. Both belong to a group of substances described by HAYDON AND

TAYLOR[5] as wedge-shape molecules, and possess polar headgroups which are similar in nature and size. Their lytic potencies are comparable[4]. It would seem therefore that the mechanism of lysis by acyl carnitines would be very similar to that by lysolecithins.

The sequence of events leading to chemical lysis of erythrocytes has recently been summarized by REMAN *et al.*[6]. There is first an adsorption of the lysin onto the membrane followed by its penetration. This results in a disorganization of the structural arrays, a change in permeability, a disturbance in osmotic equilibrium and finally a loss of hemoglobin.

Although the interaction of certain lysins with cholesterol[7,8] appears to be at the basis of the lytic phenomenon, paraffinic quaternary ammonium derivatives might involve a variety of interactions. At any rate, they are quite lytic against membranes containing no cholesterol[9]. It would appear that the interaction of acyl carnitines and lysolecithins is at least partly hydrophobic in character since their effectiveness as hemolysins increases as the acyl chain is lengthened to C_{18} (refs. 4, 6). In fact, there is increasing evidence that hydrophobic interactions between lipid and protein exist in the membrane[10,11]. However, these interactions *per se* may not be sufficient to impart complete structural stability to the membrane since environmental conditions favoring an increase in dielectric constant within the membrane result in rupture of the cell[12]. Furthermore, the possible stabilizing effects of divalent cations in the membrane have not been overlooked by some authors[13]. Thus the lytic interactions of acyl carnitines and lysolecithin with membrane might involve disruption of structural hydrophobic bonds between protein and lipid, but the possibility that stabilizing polar interactions are also affected by the lysins is not precluded.

In the case of acyl carnitines and lysolecithins at least, one could picture the determinative of lytic acitvity more simply as the ability of the lysin to penetrate the structural architecture of the membrane rather than its specific reactivity with any of the structural constituents. Serum which usually protects against lysis by taurocholate mainly because of its cholesterol but also because of its protein content[14] will accelerate the lysis of erythrocytes previously exposed to this agent. These results together with those presented in this study suggest that once the structural bonds become sufficiently accessible, as is the case with cells pretreated with weakly-lytic concentrations of certain hemolysins, a variety of non-specific interactions becomes possible between structural elements and non-lytic substances, including, even normal membrane constituents, which enhance hemolysis.

MATERIALS AND METHODS

Palmitoyl-D,L-carnitine, palmitoyl choline and 1-palmitoyl-*sn*-glycero-3-phosphoryl choline were prepared as previously described[4]. Synthetic L-α-(dipalmitoyl)-lecithin, phosphatidyl inositide, phosphatidyl serine, sphingomyelin and cholesterol were purchased from General Biochemicals Inc. Total lipids from rat blood red cells and from liver were extracted by the method of BLIGH AND DYER[16]. Phosphatidyl ethanolamine was isolated from rat-liver-total lipids by preparative thin-layer chromatography on activated silica gel G plates with chloroform–methanol–water (65:25:4, by vol.) as solvent. Proteins from rat erythrocyte ghosts were prepared by subjecting 2-chloroethanol solutions of membrane to chromatography on Sephadex LH-20 (ref. 17).

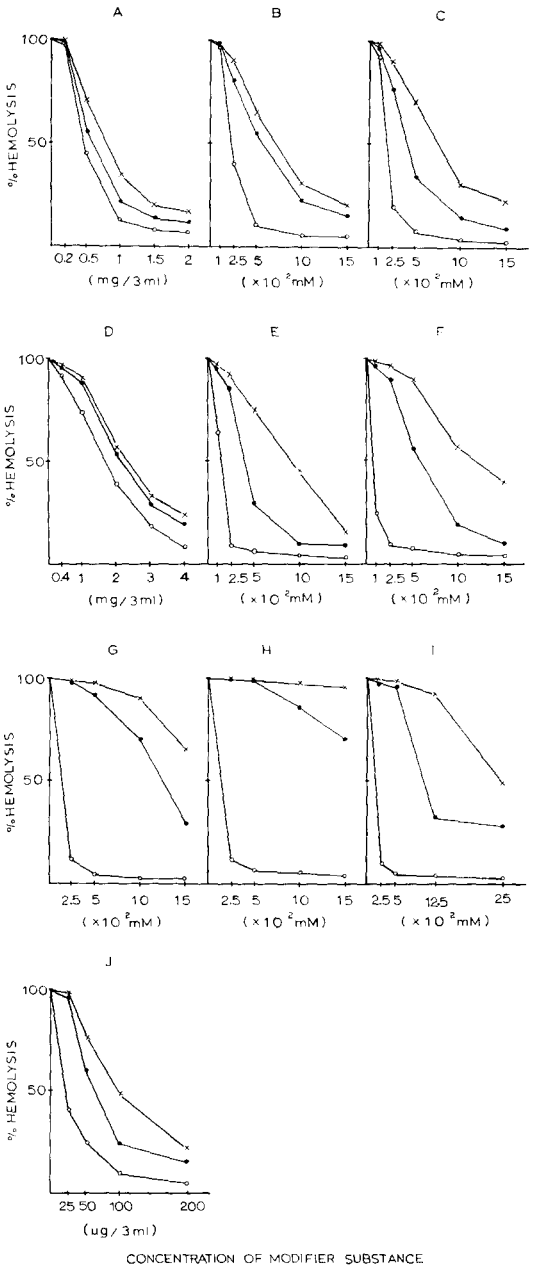

Fig. 1. The effect of modifier substances (A) albumin, (B) lecithin, (C) cholesterol, (D) membrane protein, (E) phosphatidyl ethanolamine, (F) phosphatidyl serine, (G) phosphatidyl inositol, (H) sphingomyelin, (I) palmitic acid and (J) total lipid from erythrocyte membrane on lysis of rat red blood cells by ×—×, $2 \cdot 10^{-2}$ mM (1-palmitoyl)-lysolecithin; ●—●, $2 \cdot 10^{-2}$ mM palmitoyl-D, L-carnitine; and ○—○, $2 \cdot 10^{-2}$ mM palmitoyl choline. 25 μl of erythrocytes (hematocrit 50%) were mixed with increasing amounts of modifier substance. The volume was adjusted to 2.96 ml with saline (pH 7.4) and 0.04 ml of lysin were added, and the extent of lysis after 10 min was measured spectrophotometrically. The concentration used for each lysin caused 100% hemolysis within 10 min in the absence of inhibitor.

Aqueous dispersions of synthetic lecithin, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, sphingomyelin and lysolecithin were prepared by sonication in 30 mM NaCl as described by BRUCKDORFER *et al.*[18] but without subsequent centrifugation, and adjusted to pH 7.4. Aqueous cholesterol and palmitic acid dispersions were prepared by the method of LEE AND TSAI[19]. Prior to use, albumin was

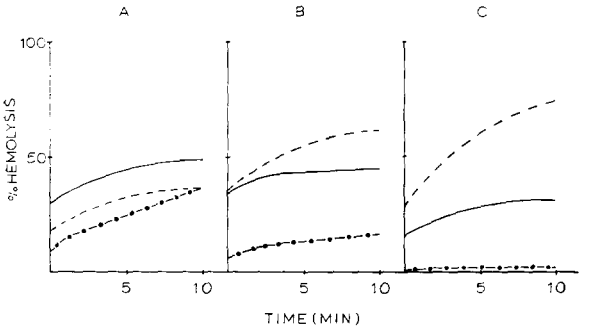

Fig. 2. The effect of preincubation and order of addition on inhibition of palmitoyl carnitine hemolysis by lipid and membrane protein. Addition sequence: ———, erythrocytes–inhibitor–lysin without preincubation; – – – –, erythocytes incubated 10 min at 37° with modifier substance prior to addition of lysin; ●—●, lysin and modifier substance incubated 10 min at 37° prior to addition of red blood cells. (A) 2.07 mg/3 ml membrane–protein; (B) $5 \cdot 10^{-5}$ M lecithin; (C) $4 \cdot 10^{-5}$ M cholesterol. The concentration of palmitoyl carnitine used was $2 \cdot 10^{-5}$M. Conditions for the lysis test are stated for Fig. 1.

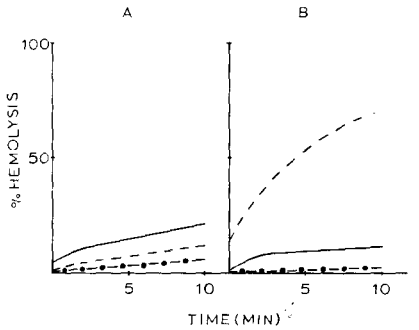

Fig. 3. The effect of preincubation and order of addition on the inhibition of palmitoyl choline hemolysis by lipid and membrane protein. The concentration of palmitoyl choline used was $2 \cdot 10^{-5}$ M. All other conditions were as stated for Fig. 2.

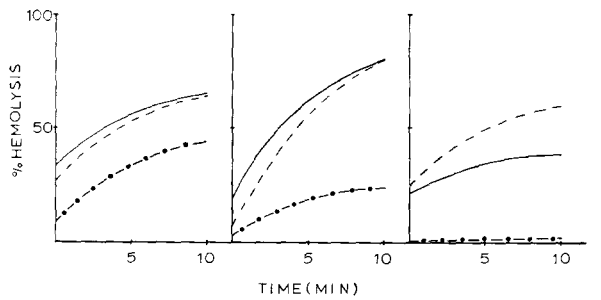

Fig. 4. Effect of preincubation and order of addition on inhibition of (1-palmitoyl)-lysolecithin hemolysis by lipid and membrane protein. The final concentration of lysolecithin was $2 \cdot 10^{-5}$M. All other conditions are as stated for Fig. 2.

freed of fatty acid by acetone extraction. It was then dissolved in water and adjusted to pH 7.4. Membrane protein isolated as an acidic, 2-chloroethanol eluate was dialysed 24 h against distilled water at 4° whereupon it precipitated. The precipitated protein was isolated by centrifugation and finally dissolved in 6 M urea. The final membrane protein concentration was estimated by the method of LOWRY *et al.*[20].

Rat erythrocytes were treated with citrate, washed and suspended to give a 50 % hematocrit in saline pH 7.4 as described previously[4]. All lysin solutions were dissolved in saline. These as well as all other reagents used in the lysis tests were adjusted to pH 7.4. The extent of hemolysis under various conditions described in the test, was followed spectrophotometrically[4] using a Cary 15 spectrophotometer. In each of the figures, the curves were drawn by considering the recorded changes in optical density as a function of time or as a function of additive concentration and represents the average of 3 experiments. The percent hemolysis was estimated from a standard curve prepared by mixing different proportions of intact and hemolysed cells.

RESULTS

As can be seen from Figs. 1A–1I various membrane constituents and albumin at increasing concentrations, progressively inhibit the hemolysis produced by either palmitoyl carnitine, palmitoyl choline and (1-palmitoyl)-lysolecithin provided the

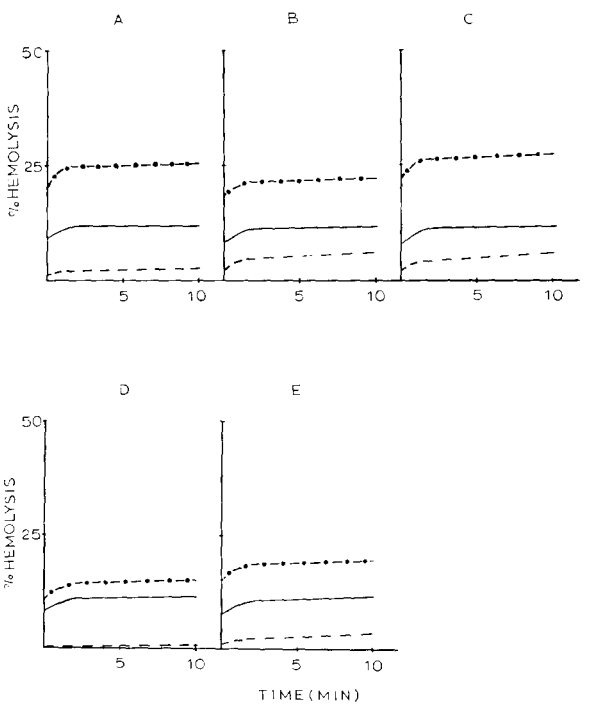

Fig. 5. Acceleration of palmitoyl carnitine hemolysis by lipids and membrane protein. Addition sequence without preincubation: ———, erythrocytes–lysin; – – – –, erythrocytes–modifier substance–lysin; ●—●, erythrocytes–lysin–modifier substance. (A) 1.035 mg/3 ml membrane protein; (B) 0.60 mg/3 ml total lipid from erythrocyte membrane; (C) $5 \cdot 10^{-5}$ M lecithin; (D) 8.10^{-5} M cholesterol; (E) $2.5 \cdot 10^{-4}$ M palmitic acid. The concentration of palmitoyl carnitine used was $5 \cdot 10^{-6}$ M.

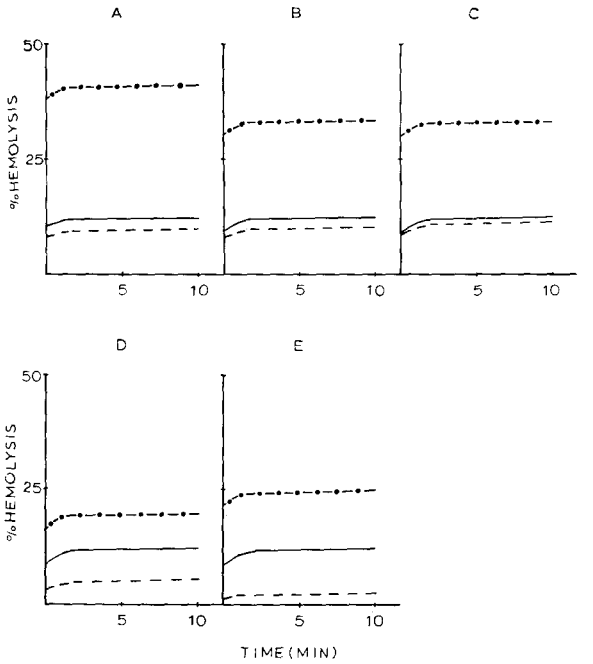

Fig. 6. Acceleration of lysolecithin hemolysis by lipids and membrane protein. The concentration of lysolecithin used was $5 \cdot 10^{-6}$ M. All other conditions were as stated for Fig. 5.

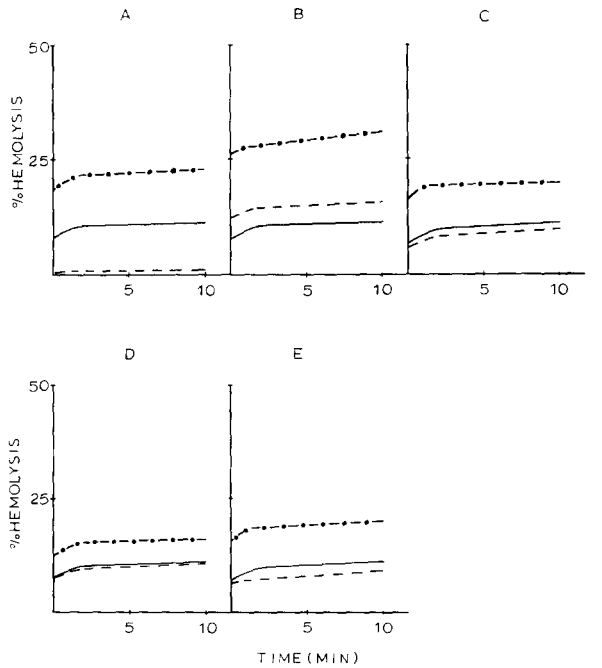

Fig. 7. Acceleration of palmitoyl carnitine hemolysis by various polar substances (A) 1.035 mg/ml albumin; (B) $5 \cdot 10^{-4}$M glucose; (C) $5 \cdot 10^{-4}$M glycerol; (D) $5 \cdot 10^{-4}$M glycine; and (E) $2.5 \cdot 10^{-4}$M cystine. All other conditons were as stated for Fig. 5.

lysin is added after admixture of the protective agent with erythrocytes. Lysis by palmityl choline was the most susceptible to the action of the various inhibitors tested whereas lysis by lysolecithin, the least susceptible. Although quite effective against hemolysis by palmitoyl choline, phosphatidyl inositol and sphingomyelin did not so markedly protect against lysis by lysolecithin and by palmitoyl carnitine under these conditions.

When palmitoyl carnitine in completely lytic concentrations was incubated 10 min with either membrane protein, lecithin or cholesterol, prior to addition of erythrocytes (Figs. 2A–2C), the inhibition was more prononuced than without preincubation. If on the other hand red blood cells were first incubated with lecithin and cholesterol prior to addition of lysin, the inhibitory effect was appreciably diminished. It was enhanced, however, when erythrocytes were preincubated with membrane protein.

The effects of various hemolytic modifier substances on lysis by palmitoyl choline and 1-palmitoyl lysolecithin turned out to be quite similar in both these cases (Figs. 3A, 3B and 4A–4C) but differed slightly from those on lysis by palmitoyl carnitine. With the choline-containing hemolysins, their preincubation with either membrane protein, lecithin or cholesterol increased the inhibitory effect to various extents as was also the case for palmitoyl carnitine lysis. However, preincubation of the erythrocytes with lecithin, prior to lysolecithin or palmitoyl choline addition, resulted in a slightly more pronounced rather than the decreased inhibition noted for palmitoyl carnitine. Preincubation of membrane protein with red blood cells slightly increased the inhibition of lysis by lysolecithin.

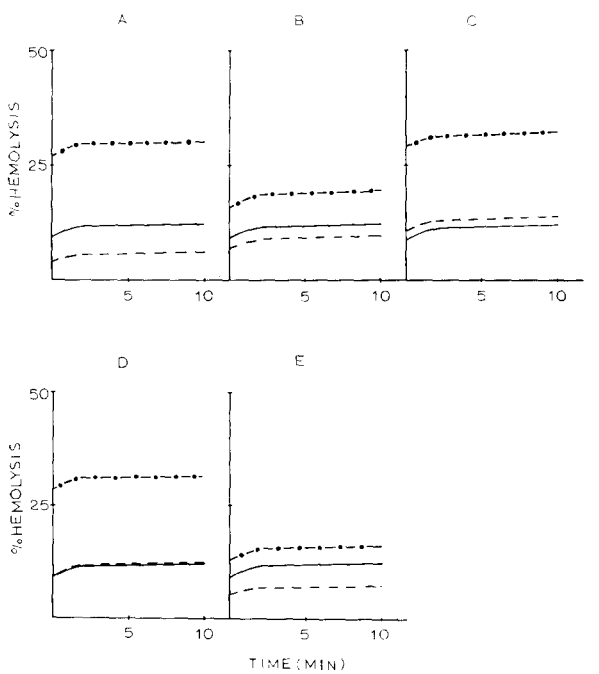

Fig. 8. Acceleration of lysolecithin hemolysis by polar substances such as stated for Fig. 7. The final concentration of lysolecithin used was $5 \cdot 10^{-6}$ M. All other conditions were as stated for Fig. 5.

If, on the other hand, erythrocytes were admixed with weakly lytic concentrations of palmitoyl carnitine (Figs. 5A–5C, dot-and-dash lines) lysolecithin (Figs. 6A–6C, dot-and-dash lines) prior to the addition of either membrane protein, membrane total lipid, lecithin, cholesterol, or palmitic acid, these hemolysis modifiers acting as inhibitors under other conditions (broken lines) now acted as accelerators of hemolysis. Furthermore, with cells 'sensitized' by prior addition of weakly-lytic concentrations of palmityl carnitine (Figs. 7A–7E) and lysolecithin (Figs. 8A–8E), an accelerator effect by several polar substances was noted.

DISCUSSION

It is clear that a number of membrane constituents and serum albumin are quite effective in protecting against lysis by the amphipathic quaternary ammonium compounds tested (Figs. 1A–1J). Differences in the extent of lysis inhibition might be attributable to various factors including the extent of interaction of the protective agent with the cell membrane and/or with lysin under those experimental conditions used. Indeed, PONDER[15] was able to distinguish between two types of protective agents, one which combines with the lysin in the bulk phase and inactivates its hemolytic capacity, and the other which combines with the surface of the red blood cell making it more resistant to lysis. In the first case, preincubation of the lysin with the protective agent will increase the inhibition of lysis whereas in the second instance, inhibition is increased by preincubation of the protective agent with red blood cells prior to lysin addition.

Figs. 2–4 illustrate that the inhibitory effects of lecithin, cholesterol and membrane protein against hemolysis by palmitoyl carnitine, palmitoyl lysolecithin and palmitoyl choline, result at least partly from an interaction of the protective agent with the lysin. Preincubation of these modifier substances with lysin prior to their contact with erythrocytes does increase the inhibition of lysis. On the other hand, preincubation of red blood cells with cholesterol, affords less protection against each of the hemolysins added last. In this case, some of the inhibitor is probably removed by the erythrocytes and less is available for combining with the lysin. In any case, interaction of cholesterol with membrane would not account for its inhibitory effect.

Preincubation of erythrocytes with membrane protein prior to lysin addition (Figs. 2A and 3A) results in a significant increase in inhibition in the case of palmitoyl carnitine lysis, but which is smaller in the case of lysolecithin lysis. It appears that part of the protective effect of membrane protein may be explained by its interaction with the surface of the cell making it less susceptible to lysis.

Just as for cholesterol, preincubation of red blood cells with lecithin likely results in some lipid uptake. The amount removed could be sufficient to decrease the inhibitory effect of lecithin against lysis by palmitoyl carnitine (Fig. 2B) but insufficient to significantly affect the inhibition of lysolecithin hemolysis (Fig. 4B). This difference in results could be further explained by a permeability of lecithin micelles to lysolecithin greater than to palmitoyl carnitine. Further investigations are needed to clarify this point.

More detailed results obtained from equilibrium-dialysis experiments being pursued in this laboratory, have clearly indicated that albumin as well as membrane protein and micellar lipids prepared from erythrocytes, can bind [^{32}P]lysolecithin and

^{14}C palmitoyl carnitine. The protective effect obtained with a number of substances may therefore be taken as qualitative evidence that palmitoyl carnitine, palmitoyl choline and (1-palmitoyl)-lysolecithin can combine rather non-specifically with a variety of membrane constituents. There is no apparent reason for inferring their specific interaction with cholesterol or any particular constituent of the membrane.

Lytic lecithins may exert their disruptive activity by provoking massive loss of cholesterol from the erythrocyte membrane[21]. In the case of lysolecithin however, REMAN *et al.*[6] conclude that the small amounts of lysin required to rupture the cell, would be incapable of releasing such quantities of cholesterol from membrane by an exchange reaction.

The mechanisms suggested by HAYDON AND TAYLOR[5] whereby bimolecular lipid lamellae are dispersed into micelles by the action of various lytic substances which they classify into several groups, attract by their simplicity. Applied to the paucimolecular model of membrane structure, they could explain the lytic action of wedge-shaped amphipaths such as the acyl carnitines and lysolecithins, as well as that of lecithins possessing acyl chains of intermediate length. However, if the wedge-shape principle does apply, one would expect that varying the size of the polar headgroup in lysolecithins, would have quite noticeable effects on lytic potency. Although, altering the acyl chain length profoundly affects lytic activity in the lysolecithin series, removal of the 2-hydroxyl group from the glycerol residue, or increasing the distance between the quaternary ammonium and phosphate groups has little or no effect on lytic potency[6]. The yet-lacking, definite evidence favoring the wedge shape of certain lysins as being related directly to their hemolytic activity, invites such further studies in which the size of the polar head-group would be varied over a wider range.

Membrane constituents as well as a number of polar metabolites can act as accelerators of hemolysis by the various paraffinic quaternary ammonium derivatives tested (Figs. 5–8) provided the lysin is added first. It seems therefore that once the lysin penetrates the membrane, disorganization ensues such that structural bonds become exposed to the disruptive action of a variety of substances. One can thus picture the lysis of a single cell as resulting from a chain reaction involving the penetration of the lysin into the structural areas of the membrane, the disruption of orderly lipid-protein arrays, followed by the release of membrane and cytoplasmic constitutents which are now free to combine randomly with vicinal structural components and cause further, the disruption of the membrane.

At present it is difficult to explain why the various hemolytic modifier substances tested do not accelerate rupture of the cells when added just prior to the lysin. Added in this order they are usually inhibitory or without appreciable effect. One likely possibility is that weakly-lytic concentrations of lysin added first, only the more susceptible cells become lysed; others would absorb a certain sublytic amount of lysin and become 'sensitized'. The accelerating hemolytic effect of the modifier substances tested would likely result from their action on these sensitized cells. When modifier substance is added first, the surface of the erythrocyte membranes would become rapidly coated with this additive and this might be sufficient to prevent or retard penetration of the lysin into the more resistant cells but not into the more susceptible cells. Studies on the combined action of lysins and hemolytic modifiers on different types of erythrocytes separated according to age or characterized by certain abnormalities, would likely help clarify this point.

ACKNOWLEDGEMENT

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