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

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RESUME

Lysis of rat and human erythrocytes by synthetic fatty acyl esters of DL-carnitine and choline was compared to hemolysis by synthetic lysolecithin. Stearoyl carnitine was the most effective lysin, whereas 1-palmitoyl-sn-glycero-3-phosphoryl choline and palmitoyl carnitine were more potent than either the shorter-chain acyl esters of carnitine or any one ester in the series of choline esters tried. Even with lower concentrations of any one lysin tested, the rate of hemolysis was very rapid initially and proceeded without a lag period to an extent which was dependent on both the concentration of lysin and the amount of red blood cells present. 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. 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. 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 pre-incubation. 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

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non-specific interaction of the lysis 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. 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. These results are discussed in terms of the mechanism of lysis by quaternary ammonium amphipathic derivatives. The interaction of isolated lipids and complex lipid mixtures with structural protein or Maddy protein was studied under various conditions. Structural protein was able to bind cholesterol, lecithin, lysolecithin and palmitoyl carnitine by a mechanism involving mainly hydrophobic interactions. The complexes thus formed could be dissociated by various lysins to a degree depending on their acyl chain length. Maddy proteins interacted with complex lipid mixtures to form insoluble lipoproteins at pH 4. Lysins depending on their charge and acyl chain lengths could prevent lipoprotein or cause the dissociation of such complexes. The mechanisms whereby lysins interact with intact membrane or reconstituted lipoproteins to release lipids are discussed.

C O N T E N T S

To Professor Pierre Proulx, who has directed this work with valuable guidence and consideration, I express my deep gratitude.

GENERAL INTRODUCTION

In a following section structural models for the biomembrane will be presented. It will be seen that there is considerable disagreement as to which model is correct. Perhaps major difficulties lie in the fact that significant structural differences between different types of biomembranes may occur, the membrane possessing a dynamic character may exist in different structural forms depending on the conditions and finally the methods for isolation and experimental approaches are still too crude to allow unquestionable conclusions. At the present time, all these facts still lend themselves to concrete considerations of the type of membrane used and the limitations of the approach rather than all-embracing abstraction of the membrane. There is the general knowledge however, that lipids and proteins are the major constituents of membranes and that these are associated by both electrostatic and hydrophobic interactions.

Hemolysis studies have proved to be very useful for the study of chemical associations in the membrane, and have contributed to some knowledge of the chemical structure of membranes. The rationale behind chemical hemolysis studies has been that by learning what factors

destroy the architecture of the erythrocyte membrane, one can help reconstitute the membrane in hypothetical terms from the known physico-chemical properties of the lysins and their detailed action on natural and artificial systems. The approach deals with such questions as, "why are certain substances hemolytic, others not? why are certain lysins more potent than others?". Invariably these questions lead to considerations of the mechanism of lytic action and membrane structure. Although the approach is simple and has the advantage of not requiring the isolation of membrane, with regards to structure, it has shared the plight of many others in that only possibilities rather than unequivocal answers are obtained. Detailed explanations for the mechanism of hemolysis by any group of substances would require precise information on the structure of the erythrocyte membrane. Such is not yet available. However, meaningful studies concerning hemolysis can be isolated at least to some extent from precise and complex structural considerations.

A number of naturally-occurring lysins, such as acyl Co A, lysolecithins, and hitherto ill-characterized, acyl carnitines occur or are formed in biological membranes. To further understand their biological role as well as their likely impact on membrane architecture and function,

it is necessary to study in detail their chemical and biologic properties as well as their metabolism.

The present study is one largely concerned with the biological properties of acyl carnitines and lysolecithins as manifested on erythrocyte membrane. Our findings do relate to mechanisms of lytic action and consequently to membrane structure, however, they also lend themselves to discussions on the biological function of these substances.

REVIEW OF THE LITERATURE

STRUCTURAL MODELS OF CELL MEMBRANE

The recent developments concerning cell membrane structure and function are well discussed in the following review articles (1 - 8). The literature in this domain is vast and consequently we will limit our presentation to only some of the important features described.

(A) The Paucimolecular Model

For about half a century a predominant idea has been that the cell membrane is composed of a bimolecular lipid leaflet (Gorter and Grendel model, Fig. 1) arranged with hydrophobic tails apposing each other end to end on the inside and polar heads protruding towards the surface (9). This concept was based on the fact that lipids

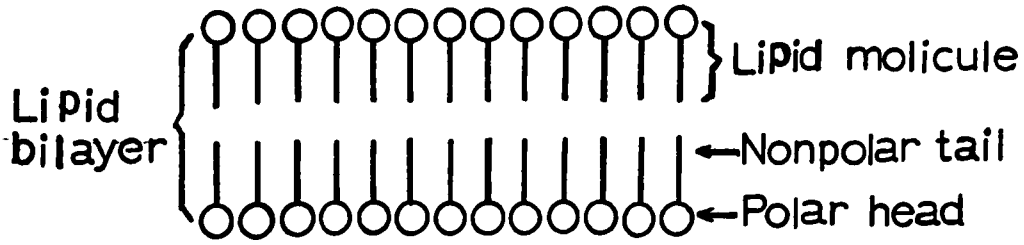


Fig.1 Lipid bimolecular leaflet (1925)

which could be extracted from a known number of red blood cells spread as a monolayer film to an area twice that of the total area of the cells when the surface pressure of the film equalled 30 dynes. Consequently, a double layer of lipid had to exist in the membrane. This method was subjected to considerable criticism from the standpoint that the area of the erythrocytes had been underestimated and that the lipids had not been quantitatively extracted from the cells. It was then shown that the too-small-a-value for the surface area of erythrocytes which they used was compensated for by their incomplete acetone-extraction of lipids from these cells (10). It is known that the area occupied by a monolayer of lipids is dependent on the surface pressure applied. Since there is no way of knowing the surface pressure of lipids inside the membrane, one can not be sure on this basis whether they exist exclusively as a bilayer.

In 1935 Danielli and Davson (11) envisaged a lipid bilayer as having too much surface tension to account for the surface properties of biomembranes which have a surface tension equal to zero. They therefore proposed that lipids were coated with protein which abolished surface tension at the cell surface. It must be added here that pure lipids available at that time were non polar,

and when these were layered over aqueous phases large interfacial tensions resulted. It is known today that bilayers formed with polar lipids have no surface tension. Consequently, protein was introduced in the model for the wrong reasons. The paucimolecular model was then modified by Danielli and Davson(11), to include hydrophilic pores which would account for the permeability of the cells towards small polar molecules (Fig. 2).

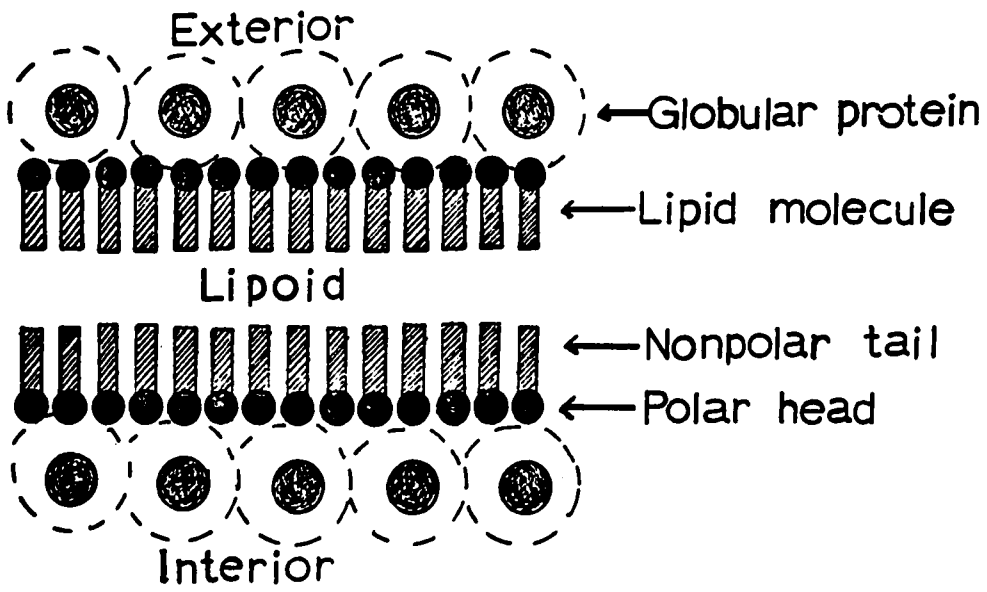


Fig.2 Danielli-Davson membrane structure(1935)

In 1957, Robertson (12) summarized a series of newer observations on cell membranes. On the basis of these observations he specifically modified the Danielli-Davson model and proposed the unit membrane hypothesis representing a universal structure for all biological

membranes (Fig. 3) (13)(14). The differences between

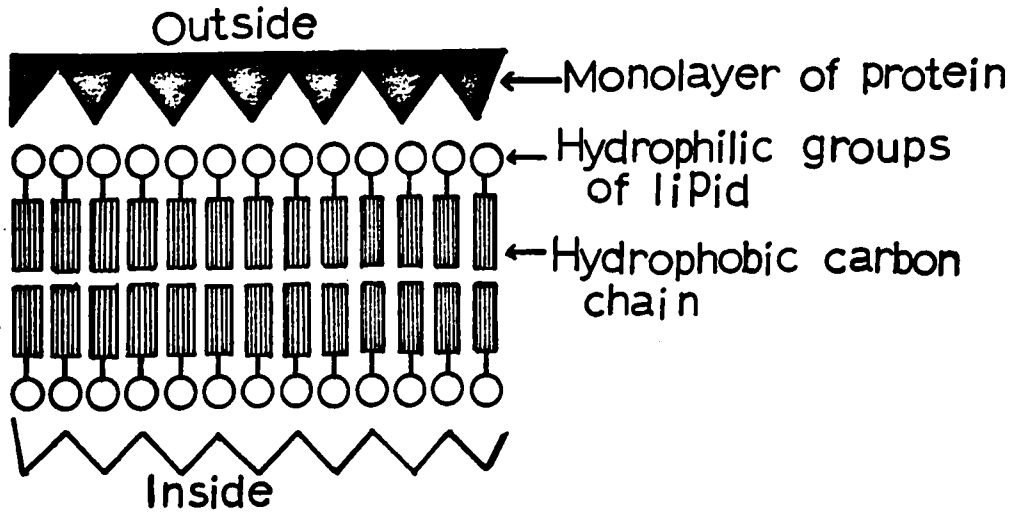


Fig.3 Unit membrane model of Robertson

Danielli-Davson and Robertson models involve three new ideas introduced by Robertson. Firstly, he emphasized the number of lipid layers present in the core of the membrane, and secondly, protein on the outside of membrane had to be in the β extended form because myelin structure could spare only 15 - 20 Å of thickness for each non-lipid monolayer. This thickness is not enough for usual types of globular proteins. Thirdly, as the most emphasized point, there is an important chemical difference or asymmetry between the outside and inside cell surfaces. This unit-membrane hypothesis of Robertson enjoyed almost universal acceptance for several

years. However, questions were raised in the early 1960's about its general applicability (15)(16). By the middle 1960's several authors advocated a virtual rejection of the hypothesis and proposed other models.

(B) Other models

In electron micrographs of osmium-fixed mitochondrial membrane and potassium permanganate fixed, frozen, dried mitochondrial membrane, Sjöstrand (17) observed the triple-layered structure of the membrane elements. On the basis of this observation, he proposed a triple-layered model for the molecular structure of mitochondrial membrane, in which the lipid layer was assumed to be sandwiched between two layers of protein molecules in a stretched out, unfolded configuration. In addition, one layer of globular protein molecules covered the matrix surface of each protein layer and were assumed to contain the respiratory enzymes. Sjöstrand has since modified his model to include (a) lipid bilayers with polar groups protruding at the surface, (b) lipoproteins (c) protein; all of which formed a mosaic structure (18).

Lucy (1964) (19-21) proposed another model on the basis of ultrastructural examination of artificial

systems consisting of various mixtures of phospholipid, cholesterol and saponin, which had been negatively stained. These mixtures revealed that spherical micelles interact with each other to spontaneously form membrane-like structures(Fig. 4). Up to that time it had been believed

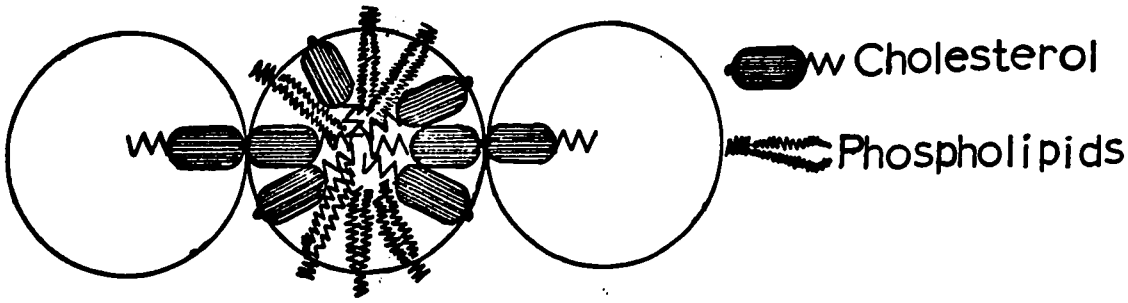


Fig. 4 Globular micellar membrane model by Lucy

that the only thermodynamically stable form of lipids in water was the bilayer structure. He suggested that globular micelles of lipid may be present in cell membranes under appropriate circumstances, and also showed a bimolecular leaflet (Fig. 5) representing biological

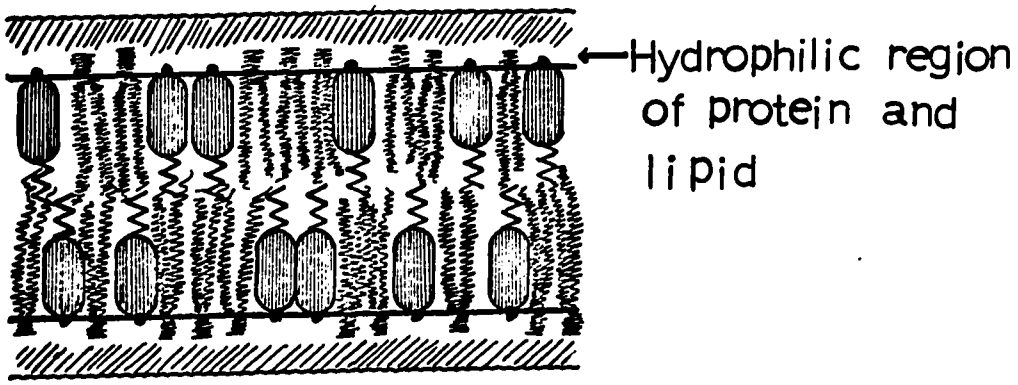


Fig.5 Bimolecular structure of membrane by Lucy (1964)

structure of membrane which may be formed by association of lipids and proteins under certain other circumstances.

With regard to more detailed models for the configuration of specific phospholipid-cholesterol complex in myelin, Finean (22) presented a model (Fig. 6) based on studies with X-ray diffraction and electronmicroscopy of fresh myelin, and described a significant role of cholesterol in promoting the stability of this molecular

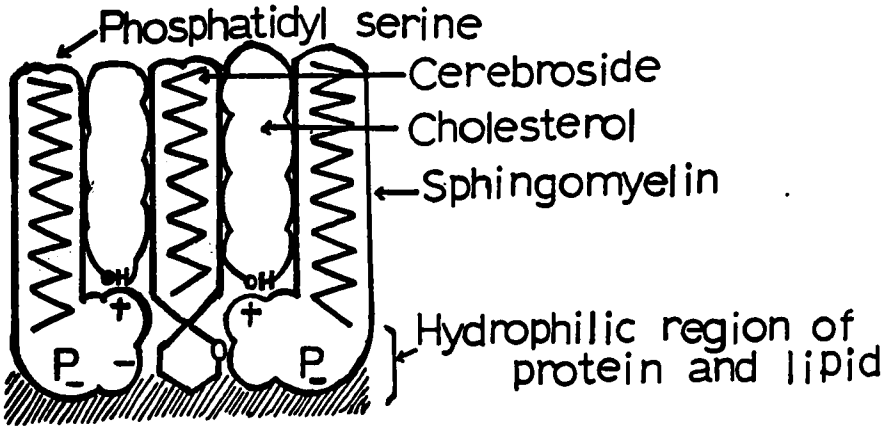


Fig.6 Molecular arrangement of the myelin repeating unit

arrangement. Finean's speculations concerning the possible structure of the phospholipid-cholesterol complex have been modified by Vandenheuvel (23)(24). On the basis of work with molecular models and of X-ray diffraction studies, he proposed another structural model of myelin, which describes more precisely the arrangement

of the lipid counterpart and a possible configuration of the protein component in myelin. He also predicted that London-Van der Waals interactions accounted for interlipid cohesion, and ionic and hydrogen bonding are involved in protein to protein and in protein to lipid bindings. The hydrogen bonding between lipid and protein was mediated by an intercalated monolayer of water.

By 1966, several different models were proposed which involved hydrophobic interactions between lipids and proteins as a predominant feature. Benson (1966) (25)(26) proposed a lipoprotein subunit model for plasma and chloroplast membranes (Fig. 7), in which the hydrocarbon chains of the lipids associate hydrophobically

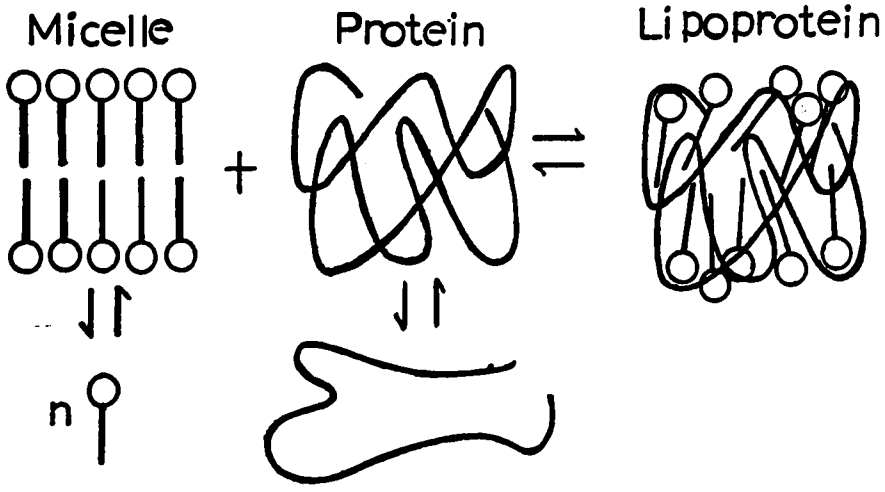


Fig. 7 Subunit plasma membrane by Benson(1966)

with complementary hydrophobic regions within the interior

of the protein.

At this time, newer physical techniques provided some useful information about membrane structures. For instance, optical rotatory dispersion (ORD) and circular dichroism (CD) measurements of the peptide bond absorption band showed the protein was mainly in the α-helical conformation in several different membranes tested. Proton magnetic resonance (pmr)spectra and electron spin resonance studies provided some information about the mobility of the lipid and protein constituents in the membrane. Lenard and Singer (1966) (27) obtained evidence from ORD and CD spectra analyses of membranes from erythrocytes and B. subtilis, which they compared with the spectra obtained for poly-L-lysines in the α- and β-helical, and random-coil conformations. From this comparison they concluded that membrane protein is a mixture of α-helix and random-coil, and lipid-protein interactions are mainly

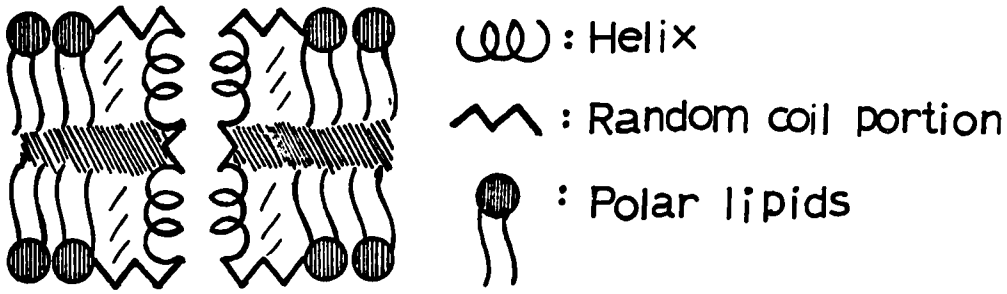


Fig.8 Lenard and Singer membrane model (1966)

hydrophobic in character. They fitted these observations to a new model (Fig. 8).

Other authors arrived at similar conclusions using these same techniques (28-33). These studies suggested that a large portion of protein was in the alpha-helical form and was located in a special environment within the hydrophobic portion of the basic lipid layer, implying a large degree of hydrophobic bonding between protein and lipid or between non-polar portions of adjacent helical regions of protein. Essentially this would mean that large areas of hydrophobic segments of protein would be burried in the membrane core whereas polar regions of protein and lipid would be exposed at the surface. Morowitz and Terry (34) showed however that pronase attacked up to 80 % of mycoplasma membrane protein, indicating that only 20 % was burried in the core. The amino acid composition of pronase sensitive and insensitive regions was identical. The significance of such studies on isolated membrane however would likely depend on the integrity of the isolated membrane and the purity of the enzyme. Electronmicroscopic examination of the membranes would not be necessarily adequate to show integrity.

Pertinent to this is that crude phospholipase C

has been known to cause hemolysis of red blood cells (35), and release phosphoryl choline from erythrocyte ghosts(36). This has been taken as evidence for disclosure of choline groups at the membrane surface. However, Zwaal et al. (37) purified phospholipase C extensively and showed that it was completely devoid of hemolytic activity, but did hydrolyse choline from isolated erythrocyte membranes. Obviously in such studies the importance of dealing with pure enzyme is essential. Furthermore one cannot assume that isolated membranes are intact just on the basis of microscopic examination. It would appear that in intact membranes, the choline group is burried within the core, but is released at the surface during isolation of the membranes. Studies of all types done with isolated membranes must therefore be interpreted with caution.

Glaser et al. (1970) (38) significantly observed that phospholipase C altered the chemical structure and physical state of about three-fourths of the phospholipids in the membrane but no detectable effect on the gross physical state of the protein was seen from CD and pmr measurements. In an experiment with untreated membranes raised to high temperatures, about two fifths the physical state of membrane protein was altered with no indication

of a change in the state of the lipid fatty acid chains. They concluded from these observations that a substantial fraction of the phospholipids and the proteins of the red cell membrane can change structure independently of one another, and finally proposed a mosaic model for the cell membrane (Fig. 9).

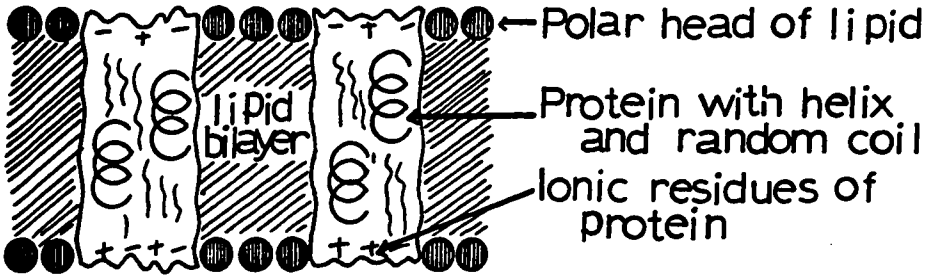


Fig.9 Mosaic membrane model by Glaser et al.(1970)

A similar membrane model (Fig. 10) was presented by Vanderkooi and Green (1970) (39) from the evidence of protein crystallographic studies and other evidence

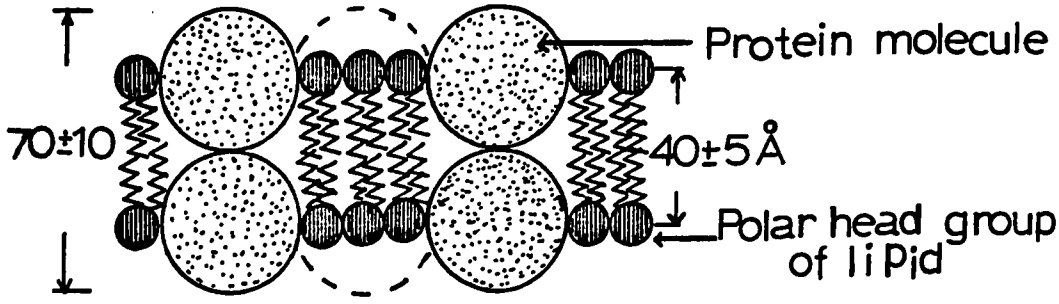


Fig.10 Diagramatic cross section of membrane model by Vanderkooi and Green(1970)

presented by other authors. The essential principle underlying this model is that when membrane proteins polymerize or aggregate, the points of contact between proteins are few, and cavities lined with predominantly nonpolar amino acids are formed. Phospholipid molecules become oriented with the fatty acid chains inserted into the cavities together with cholesterol while the polar heads remain at the surface of the membrane.

(C) Conclusion

Membrane models have been greatly modified since Gorter and Grendel proposed their simple model involving a lipid bilayer. The many other models which have been proposed are either variants of the paucimolecular model or antipodes of this model. We have presented only a few of these and have avoided an intensive critical approach. It may be said however that compromising structural models may be no more accurate than either of the antitheses. It is quite clear that there is presently no generally-accepted model for membrane structure. Although newer techniques, such as ORD and CD have led to observations which oppose the paucimolecular model still newer techniques, such as differential thermal analysis of biomembranes can be used to defend the point

of view that extensive regions of the membrane are made up of bilayers of lipids (40). Perhaps it is the defect of abstraction to belittle the artifacts introduced by isolating and studying biomembranes as well as the essential differences that seem to exist from one membrane to another, and the essentially dynamic character of the membranes the structural features of which cannot yet be studied unimpaired.

MEMBRANE COMPOSITION

(A) General Consideration

Proteins and lipids are the main components of cellular membranes, such as mitochondria, nuclei, endoplasmic reticulum, myelin and plasma membranes. Each type of membrane has very different concentrations of protein, phospholipids and cholesterol (Table 1). Myelin is particularly unusual in its very low protein content, in its high cholesterol content and in its unique content of cerebrosides. Many of the available data on the proteins and lipids of cellular membranes have recently been assembled by Korn (5)(41) and Rogers et al. (42). It is apparent from those data that there

Table 1

Protein and lipid content of membranes

Membrane	Protein/Lipid	Cholesterol/Polar lipid	Major polar lipid
	wt/wt	mole/mole	
Myelin	0.25	0.7-1.2	Cer. PE. PC
Plasma membranes			
Liver cell	1.0-1.4	0.3-0.5	PC. PE. PS. Sph
Ehrlich ascites	2.2		
Intestinal villi	4.6	0.5-1.2	
Erythrocyte ghost	1.5-4.0	0.9-1.0	Sph. PE. PC. PS
Endoplasmic reticulum	0.7-1.2	0.03-0.08	PC. PE. Sph
Mitochondria			DPG. PC. PE. Plas
Outer membrane	1.2	0.03-0.09	
Inner membrane	3.6	0.02-0.04	
Retinal rods	1.5	0.13	PC. PE. PS
Chloroplast lamellae	0.8	0	GalDG. SL. PS
Bacteria			
Gram-positive	2.0-4.0	0	DPG. PG. PE. PGaa
Gram-negative		0	PE. PG. DPG. PA
PPLO	2.3	0	
Halophilic	1.8	0	Ether analogue PGP

From Ann. Rev. Biochem., 38 (1969) p. 268 (ref. 41)

exist differences in the lipid and protein compositions of membranes from different sources.

The observed changes in membrane behavior and properties resulting from a variety of changes in membrane composition has given an insight into the structural role of constituents and ultimately, information on membrane organization. For instance, there is a parallel increase of permeability of glycerol and urea with increasing lecithin (43) and unsaturated fatty acid (44) contents of red blood cells from different sources. The osmotic fragility and glycerol permeability were also affected by partial removal of cholesterol from erythrocytes (45). Cholesterol is also known to increase the capacitance (46)and thickness (47) of lecithin bilayers and condenses expanded liquid monolayers of unsaturated lecithin. Cholesterol also affects the fluidity of acyl chains in the biomembranes as evidenced by differential thermal analysis of isolated membranes (48). Such evidence clearly demonstrates an important functional role of each membrane component.

Since in biological membranes, individual components have combined for various functional and structural roles, and the composition of the membrane varies, it is difficult to accept on this basis that a single

structural model can apply to all cellular membranes.

(B) Lipids

Lipids are perhaps the most characterized components of cell membranes and they may be divided into several groups, i.e., neutral lipids, phospholipids and sterols, such as cholesterol. All cellular membranes contain phospholipids (see Table 1), but the types and amounts of phospholipids vary in different organelles and in different species as can be seen in Table 2 (5).

Table 2

Major phospholipids of plasma membranes

Phospholipid	Myelin	Erythro-cyte	Liver cell	Gram-positive bacteria	Gram-negative bacteria	Halophil bacteria
Cerebrosides	31					
Sphingomyelin	8	30	19			
PE	24	25	15	45	75	
PS	8	15	8		2	
PC	21	30	37			
PG				45	9	
DPG			7		13	
Lysyl PG				10		
Ether analogue of phosphatidyl GP						85

From J. Gen. Physiol., 52 (1968) 263 (ref. 5)

The phospholipids are essential not only to the structural lipoprotein network forming the cellular boundary but also to several enzymes integrated in the membrane or attached as distinct sub-units to this framework. The intimate relationships between phospholipids with membraneous structures and enzymic activity are well discussed by Lester et al.(49) and Van Deenen (1).

There are also marked variations in cholesterol contents of membranes of different subcellular organelles in higher organism (50)(51). Cholesterol is not always present (6)(42) (see Table 1) and the membranes of mitochondria contain almost negligible amounts of this lipid (6), whereas erythrocyte membranes contain higher concentrations of cholesterol (52)(53). Thus, it is clear that sterols are not required for the basic structure, but where present, they seem essential to the structure and properties of the biomembranes (46)(47)(54).

Fatty acid composition is also a major variable; mammalian membranes contain a relatively high proportion of polyunsaturated fatty acids (55)(56), whereas gram-negative bacteria contain the monoenoic type as well as cyclopropane and branched chain fatty acids (57)(58). Gram-positive bacteria contain mainly C 15 and C 17 branched chain fatty acids (59), and in Halophilic bacteria fatty acids are totally replaced by a highly branched

alcohol (60). A more detailed discussion of bacterial lipids was given by Kates (61).

(C) Proteins

It can be surmised that membrane proteins consist of both catalytic and non-catalytic proteins, which are essential to the structure and general functioning of the membrane. Non-catalytic protein, part of which is now designated as "structural protein" by Green and co-workers (62)(63), accounts for about 50 -75 % of the total mitochondrial protein, which is primarily concerned with the structural integrity of the mitochondrion. Structural proteins are usually insoluble in water but soluble in detergents, in acid and in alkaline solutions, and have molecular weight of 20,000 to 30,000 as determined by sedimentation. Mitochondrial structural protein is revealed as a single peak by free-boundary electrophoresis as well as by DEAE-cellulose and Dowex-1 column chromatography. The amino acid analysis of this structural protein indicated that 51 % of the total amino acids had non-polar side chains, while the combined glutamic acid and aspartic acid contents were lower (18 %). This high concentration of non-polar amino acids contributed to the hydrophobic character of structural protein. According to these authors, the concept of the

"structural protein" has general applicability to other membrane systems.

Amino acid compositions of membrane protein from different sources were summarized by Benson (26). All the membrane proteins contained relatively high concentrations of the apolar amino acids up to 56 % of the total amino acids which would account for their hydrophobic character. With respect to the low concentration of sulfhydryl or disulfide group, it is proposed that the absence of disulfide cross-linking in membrane protein offers flexibility and freedom for conformational alteration.

The most detailed studies on membrane protein have been done with erythrocyte ghosts. Richardson et al.(64) separated structural proteins from mitochondria, microsomes, erythrocyte stroma, and chloroplast by similar methods and the resulting proteins are all insoluble at physiological pH. Schneiderman and Junga (65) isolated structural protein from erythrocyte ghosts by a slight modification of the method of Green et al. Triton-X 100 was used as solubilizing agent instead of bile salts. Bakerman et al. (66) solubilized erythrocyte ghosts by anionic and nonionic detergents and also by urea. All structural protein fractions from erythro-

cyte ghosts are heterogeneous, showing a number of bands after polyacrylamide gel electrophoresis.

Zahler and Wallach (67)(68) prepared membrane protein from erythrocyte stroma by solubilization of membrane with 2-chloroethanol-water or methylcellosolve at pH about 2, followed by Sephadex LH-20 gel filtration. The membrane protein isolated is poorly soluble at pH above 5 and very soluble in acid and at more alkaline pH. Neutralization of acid membrane protein solution leads to complete precipitation. Very likely a denatured form of membrane protein as the authors pointed out, this fraction resembles very closely in its properties structural protein isolated by other procedures.

Mitchell and Hanahan (69) solubilized loosely bound protein and lipoprotein fractions from human erythrocyte ghosts using hypertonic saline media. They observed that membrane structure was still maintained after partial solubilization of loosely bound protein and lipoprotein from erythrocyte ghosts, and demonstrated that these entities are needed to stabilize the minimal membrane framework. Their removal causes hemolysis.

Independently, Maddy (70)(71) isolated up to 95 % of the total proteins from ox erythrocyte ghosts by treatment with n-butanol alone. This procedure has also been applied to human erythrocyte ghosts (72) and

several different mammalian erythrocyte membranes (73) with high yields of 80 - 95 % of total membrane proteins. The solubility of Maddy membrane protein is markedly dependent on pH. It is soluble in physiological conditions, pH and ionic strength. The low isoelectric point, between pH 3.7 - 4.8, was attributed to the presence of sialoprotein. Accordingly, the removal of the sialic acid by neuraminidase shifts the isoelectric point towards the alkaline side, i.e., pH 4.8 - 5.3. The influence of this molecule on red cell surface is to make it strongly negative. Cook et al. (74) studied the action of crystalline neuraminidase on human erythrocytes and found that the enzyme did indeed release N-acyl-neuraminic acid with subsequent drop in anionic charge of the cell surface.

Attempts to further define erythrocyte structural protein have been hindered by the difficulty in obtaining homogeneous preparations, and the question of contamination by non-proteineous components is still a recurring problem. Extraction and purification of structural protein from non-proteineous component by variants of the method of Green et al. usually involves drastic steps and finally yields a fraction which is insoluble at physiological pH.

Such a property of membrane proteins has been explained by high content of apolar amino acid and has been attributed to the formation of the structural network of the membrane (62). The procedure used by Maddy, however, does not yield insoluble products and accounts for 95 % of the erythrocyte protein. It is obvious then that if structural protein is not denatured protein its conformation is such that it presents a highly hydrophobic surface to water and contrasts with the hydrophilic character of the Maddy protein.

LIPID - PROTEIN INTERACTIONS IN ARTIFICIAL AND RECONSTITUTED MEMBRANES

The nature of interactions between proteins and lipids in membranes is of primary importance for understanding of membrane structure and organization. Basically two main types of interactions are recognized, i.e., electrostatic and hydrophobic.

It has been shown that basic proteins, such as cytochrome C interact with phospholipid micelles to form stable complexes (75-79). Kimelberg et al. (80)(81) presented evidence that interactions of positively charged proteins, such as lysozyme, cytochrome C, ribonuclease,

and poly-L-lysine with negatively charged phosphatidyl serine vesicles are initiated by electrostatic combination and then stable complexes are formed by hydrophobic association. The electrostatic character of binding is opposed by increasing ionic strength or by decreasing the negative charge of the micelles. Likewise, Quinn and Dawson (82) showed that the interaction of cytochrome C, bovine serum albumin and synthetic oxytocin with monolayers of stearic acid, phosphatidyl choline and phosphatidyl ethanolamine are affected by ionic and nonionic binding. Sweet and Zull (83) found that serum albumin binds to phospholipid liposomes at a pH value below its isoelectric point. A similar finding was obtained with Maddy protein which binds to phospholipids both by ionic and hydrophobic interactions (84)(85). On the other hand, Lenaz et al. (86) and Green et al. (87) showed that interactions between structural proteins and phospholipids are mainly hydrophobic, and an initial ionic interaction was not detected. It is now quite generally agreed that there is at least some hydrophobic bonding between proteins and lipids in the membranes, and experiments on lipoprotein formation convey the idea that such associations occur spontaneously.

HEMOLYSIS STUDIES

Hemolysis can be achieved by a very wide variety of physical and chemical means. The present study is concerned mainly with lysis by surface active substances. Consequently, other types of hemolytic phenomena will be presented very briefly.

Non-chemical Hemolysis

(1) Osmotic shock

Two factors are concerned in osmotic hemolysis (88)(89); the semipermeability of the cellular membrane and osmotic pressure. Red blood cells are highly permeable to H^+, OH^- and HCO_3^-, urea and ammonium salts, and slightly to glucose and amino acids. It is impermeable, or nearly so, to Na^+, K^+ and Ca^{++}, hemoglobin and disaccharides. The contents of the red blood cell exert osmotic pressure equal to that of the surrounding plasma, which is equal to that of a 0.9 % NaCl solution. On placing erythrocytes in a dilute salt solution, some water enters into the cell and swelling results. The volume of the spherical cell is about 1.6 times the initial volume and is known as the critical hemolytic

volume because the cell can no larger increase its diameter without losing hemoglobin (90-92). Since the cell membrane is not elastic, further entry of water exerts sufficient hydrostatic pressure to rupture the cell wall, and hemolysis takes place.

There is some evidence that the membrane becomes less rigid as it ages (93-96); thereby the fragility of the cell is increased. With regard to age, separation of cells by centrifugation (97) reveals that the younger or less dense cells are the most resistant to osmotic lysis, whilst the opposite is found with the older or denser cells (98).

(2) Freezing and Thawing

Two mechanisms are proposed to be involved in the production of hemolysis by freezing and thawing. In the first place, it is quite probable that the formation of ice crystals within or outside the cell membrane simply disrupts the lipid-protein structure by mechanical action of the ice crystals, enabling the cell content to escape after thawing (99). On the other hand, Lovelock (100)(101) reported that the formation of ice crystals is not in itself damaging the red blood

cells. Physical change which appears to be most important in causing damage on freezing is that of the concentration of electrolytes within and outside the cells. Because the formation of ice crystals leads to areas of high electrolyte concentrations in the cell membranes, this leads to a rapid disruption and loss of membrane lipids, particularly phospholipids. Such would imply electrostatic association of phospholipids with other membrane components as the main structural feature.

(3) Mechanical Lysis

Hemolysis of red blood cells occurs by shaking cells with glass beads (102)(103). The susceptibility to this treatment is referred to as their mechanical fragility. The mechanical lysis of erythrocytes is affected by heating (104)(105) and also affected by cell age (106). Mechanical fragility increases as erythrocytes near the end of their life span.

(4) Lysis by UV light and other Radiation

Microscopic observation has shown that all the

red blood cells have became spheres before hemolysis by irradiations of ultraviolet (107), visible light (108), X-ray (109), and γ-ray (110)(111) ensues, implying that this hemolysis is osmotic in nature. Hemolysis is accelerated in solutions of decreasing osmotic pressure and inhibited by the presence of sucrose in the external medium.

The mechanism of radiation-induced hemolysis was summarized as follows: There is an alteration (oxidation) of membrane sulfhydryl groups by direct hit or radiation-produced water radicals (first step); subsequent membrane alterations result in a partial destruction of the natural barrier to potassium loss and sodium entry in isotonic saline (second step); exchange of potassium by sodium proceeds until most of cell potassium is lost (third step) and this process is accompanied by water entry (fourth step); finally the cell swells and ruptures (fifth step).

Chemical Hemolysis

A wide range of chemical substances, that is, organic solvents, ionic and non-ionic detergents, animal venoms and bacterial toxins are well known to

hemolyse erythrocytes in isotonic saline without a change in the external osmotic pressure. At the present time, there is available some information about chemical lytic phenomena, but so many factors, physical and chemical, still remain unsolved.

(1) Lipid Solvents

The cell membrane, we may recall, is constructed with a large proportion of lipids, such as lecithin and cholesterol, which are dissolved in fat solvents, such as alcohols, ethers and chloroform, etc.(88). Therefore, organic solvents cause hemolysis by extracting lipids from the membrane destroying its integrity.

(2) Venoms and Toxins

Animal venoms are well known for their lytic properties (112)(113). Snake venoms contain phospholipase A2 which hydrolyzes the fatty acid ester at position 2 of phosphoglycerides and yields a lysoanalogue. Lysophosphoglycerides, especially, lysolecithin are very lytic. Although it was thought initially that snake venoms lysed red blood cells by direct action of phospholipase A on erythrocytes, there is evidence that purified

phospholipase A does not itself cause hemolysis (114)(115). The direct hemolytic factor, known as a basic protein, might be expect to bind on the negatively charged cell surface and thus alter the permeability of erythrocytes to phospholipase A (114). Alternatively erythrocytes which may be contaminated at their surface with serum lecithin, might be lysed initially by small amounts of lysolecithin formed. Action of phospholipase A on the lecithin of these lysed cells would favour continued lysolecithin formation and further hemolysis. Since phospholipase A is not hemolytic it would seem that the acyl chains are not accesible to this enzyme and would likely be burried in the core of the membrane as proposed in the paucimolecular and other models.

Bee venoms contain phospholipase A and besides, a lytic polypeptide, melittin, which is composed of 26 amino acids with hydrophobic and hydrophilic amino acid sequences (116). Melittin, extremely surface-active, forms micelles in aqueous solution. Its lytic properties are explained by its ability to penetrate and disrupt natural and artificial lipid membranes (117).

The toxins from a number of bacteria are hemolytic. The hemolytic factors secreted by E. Coli appear to be proteins or peptides but are yet ill-characterized (118).

Those of streptococci and staphylococcus have been ascribed to hyaluronidase-like activity (119). The hemolytic alpha-toxin of Cl. welchii was first identified as phospholipase C by Macfarlane and Knight (35). The conclusion was based on the fact that antisera prepared against partially purified alpha-toxin reacted with its antigen and simultaneously destroyed hemolytic and phospholipase C activity of these preparations. Recently, Schulze and Nakamura (120) further recognized the work of Macfarlane and Knight, and further separated three lytic toxins, alpha, delta and theta-toxins among twelve other toxins produced by Cl. welchii. It was just recently shown, however, that highly purified phospholipase C from B. cereus has no hemolytic activity nor does it hydrolyse phospholipids of whole cells but does attack lecithin of membranes that have been damaged by osmotic shock (37). The possibility exists therefore that in crude phospholipase C preparations, the true hemolytic factor is not lipolytic. A more detailed discussion of cytolytic toxins of bacteria was given by Bernheimer (121).

(3) Surfactive Substances

Surfactive substances have been the most extensi-

vely studied of hemolytic agents. These are generally characterized structurally by an elongated apolar portion combined to a polar head group. Chemically, there are two major classes of surface-active agents, the ionic or ionogenic and the nonionic. The ionic class of surfactive agents has two main divisions. If the elongated low affinity portion of the molecule is included in the anion in aqueous solution, the substance is called anionic and vice versa.

(a) Ionic Surfactants

Among the most extensively studied of the ionic detergents are sodium acyl sulfates (122-125), fatty acid salts (126), bile salts (127-129), alkyl amine halides (130), alkylpyridinium halides (131) and quaternary N-alkylpyridinium halides (132), and more recently various lysolecithins (133)(134). Lytic effectiveness of alkyl ionic surfactants on erythrocytes is highly dependent on their paraffinic chain lengths, i.e., increasing their chain lengths, up to C 16 or C 18, increases the extent of hemolysis. This implies that the interactions of lytic surfactants with membrane components are ultimately hydrophobic in nature.

(b) Non-ionic Surfactants

Hemolytic action of non-ionic surfactive agents have been less extensively studied. Typical among these, the aliphatic alcohols and polyoxyethylene glycol monododecyl ethers, have been studied by several investigators (126)(135-137).

The saponins and digitonin are natural glycosides of known lytic activity (127)(138-141). Each saponin consists of a sapogenin which constitutes the aglycon moiety of the molecule and a sugar. The sapogenin may be of a triterpenoid or steroid structure, and the sugar moiety may be glucose, galactose, pentose or methylpentose. Recently, Schlösser (140) as others (126) recognized that the active center of saponin interaction is cholesterol in the membrane as was revealed in the case of the fifteen different saponins used in his studies.

Polyene antibiotics, such as filipins are known to be hemolytic (142)(143), and their properties were extensively studied with natural and artificial membranes (144-146). Filipin affected only those organisms which contained sterols, but were inactive on membranes which lacked sterols. This observation led to the conclusion that filipin interacts only with sterols in the membrane. However interaction with and penetration of certain filipins

into mono and bilayer aggregates does not always require the presence of cholesterol (147).

Mechanisms for Chemical Lysis of Membranes and Related Problems

The process of hemolysis by lytic agents was discussed extensively by Ponder (128), Pethica et al. (126)(148), and Rideal and Taylor (125)(149). Recently, Reman and co-workers (134) recognized five consecutive reactions which may be involved in the lysis of erythrocytes. Starting with an adsorption of the lysin molecules onto the membrane (step 1) there is a subsequent penetration into the structural areas (step 2) where they induce a change in the molecular organization (step 3). This leads to a radical change in permeability of the membrane, and a disturbance of the osmotic equilibrium (step 4). Finally hemoglobin leaks out into the surrounding medium (step 5).

Pethica and Schulman (126), and Pethica and Anderson (150) have discussed the hemolytic properties of a wide range of surface-active lysins in terms of the collapse of a cholesterol-phospholipid-lipoprotein complex in the red cell surface. They stressed that long-

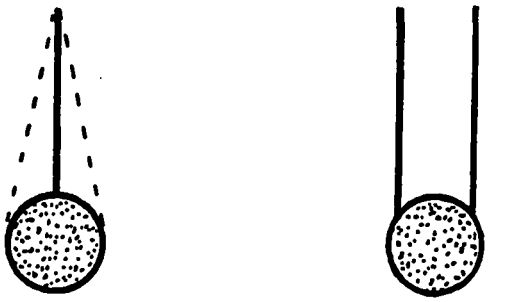
chain ionic lysins and saponin raise the interfacial pressure by reacting with the stroma cholesterol, and non-ionic lysins, such as alcohols will hemolyse the erythrocytes on attaining a concentration sufficient to give a minimum surface pressure of 34 dynes/cm at an air - water interface.

The penetration of lytic long-chain ionic surface-active compounds into the cholesterol-containing monolayers to form a complex has been known from the work of Schulman and Rideal (151). The extent of penetration of lysins into the cholesterol mixed films depended on the molecular structure of the lysins, and decreased with decreasing length of the alkyl group, because the penetration was a result of the interaction of the hydrophobic portion of the long-chain ion and the cholesterol molecule. The possible importance of monolayer penetrability in understanding the mechanism of hemolysis was later emphasized by Pethica and Schulman (126). However, they also considered the possibility that hemolysis may be related to interaction with membrane phospholipids as well as cholesterol.

As discussed previously, polyene antibiotics which react exclusively with biomembranes containing cholesterol will likewise penetrate more readily those

monolayers containing this sterol (143). While it is true that certain lysins, such as saponin, digitonin also react mainly with cholesterol of the membranes (141), cholesterol need not be the main reactive target of all lytic agents. As Salton (152) pointed out, many lytic agents, such as lysolecithin and various detergents are quite effective against bacterial membranes which lack this sterol. He proposed that interactions of the lysins could directly involve protein and phospholipids as well.

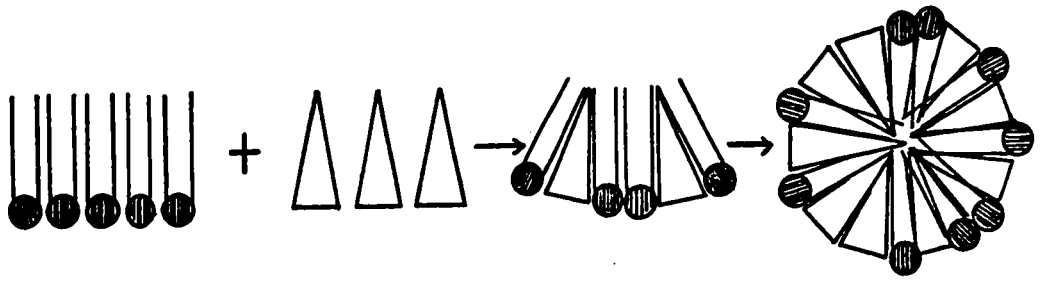
Quite significantly, Haydon and Taylor (153) observed that many lytic agents had a wedge shape, i.e., they possessed a paraffinic chain which was comparatively narrower than their polar head group. Highly lytic lysolecithin, for example, had this shape whereas weakly lytic



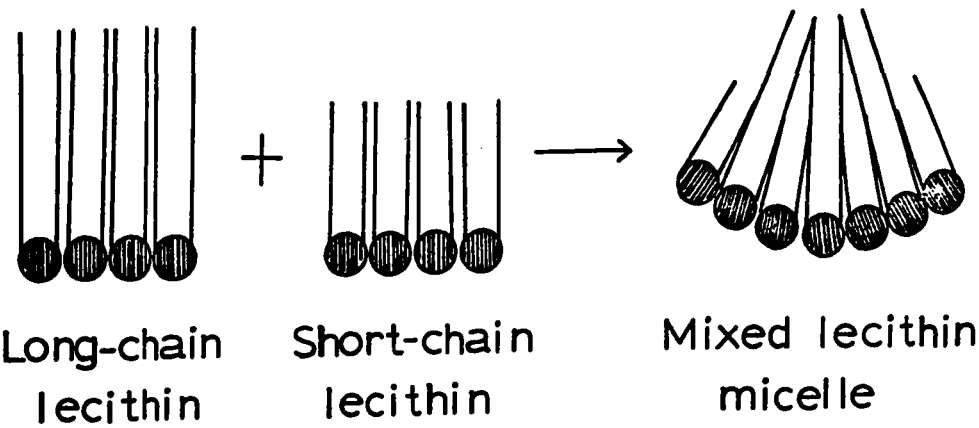
Lysolecithin Lecithin

lecithin did not. When such molecules entered a bilayer, they destroyed the lamellar orientation of the lipid

molecules and caused the formation of micelles. Lecithin with shorter chains also caused disruption of bilayer arrays, because longer acyl chains interspaced



with the shorter chains of lecithin would be attracted together hydrophobically at their protruding ends but were kept separated at their carbonyl ends.



When agents possessing similar charges penetrated lipid layers repulsion between like charges had the same

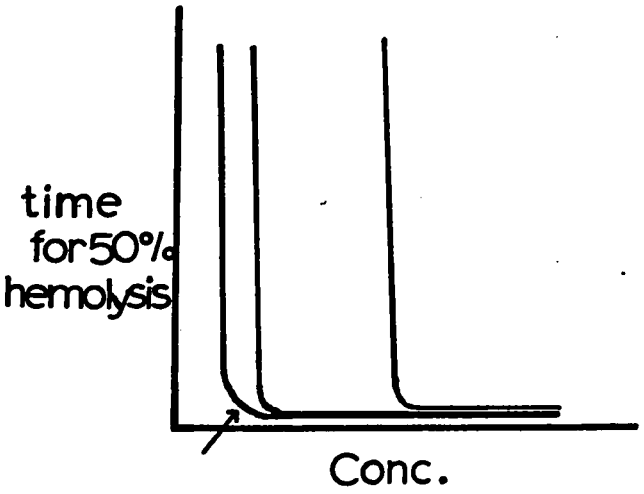
effect as increasing the diameter of the polar head group. Thus according to these authors by considering the shape of the lysin molecules and their charge one could explain their basic lytic character. The significance of this hypothesis is however, highly dependent on the validity of the paucimolecular model of the biomembrane. Since it is currently believed by many, that the extensive regions of the membrane are formed by lipid bilayers, the hypothesis remains attractive. In support of this concept, Bangham and Horne (154) showed that on adding the lysolecithin to smectic dispersions of lecithin and cholesterol their bimolecular lamellar structure was completely destroyed and lipids reaggregated in the form of micro-emulsion particles of a diameter of about 65 Å. Recent studies by Reman et al. (134) revealed, however, that increasing the distance between the phosphate and quaternary N group or removing the hydroxyl group of lysolecithin did not appreciably alter the lytic potency. Thus small variations in the size of the polar head group failed to reveal the importance of the "wedge-shape" principle suggested by Haydon and Taylor. Further studies in which the size of the polar head group as well as the intensity of its charge are varied over a wider range are needed.

Although changes in the size of the polar head group have no pronounced effect, the lytic potency of a compound is largely dependent on the length of its paraffinic chain. In the case of alkylammonium halides or alkyl sulfonates, the optimal chain length is 16 carbons. Recently, Reman et al. (133) showed that with the lysolecithin analogues, the most effective chain lengths are those of palmitoyl and stearoyl derivatives. Above 18 carbons lytic potency falls rapidly whereas the decanoyl lysolecithin is devoid of hemolytic activity. Short chain lecithins were also found to be lytic and in this case the di-undecyloyl derivative was the most active. Unsaturated mono- and diacyl analogues were less active than their saturated analogues. These results as a whole indicate that lytic activity is largely dependent on hydrophobic interaction between lysin and membrane components.

The nature of polar head group, i.e., the degree of charge or polarity may be important from at least one point of view. Firstly, if it is sufficiently strong it will allow isotropic micellar solutions of the lysin to form in the aqueous phase. Assumably the rate of adsorption onto the membrane and subsequent penetration would be favoured by the large surface area of the lysins

when in this form. When the hydrophobicity increases sufficiently relative to the polarity as in the C 20 lysolecithins or C 14 lecithins solutions tend to become anisotropic consequently larger lipid aggregates are formed and the rate of penetration would decrease.

When time for 50 % hemolysis is plotted as a function of lysin concentration, it is seen that the response varies until a maximal effective concentration is reached typical for each lysin. The break of the curve (see arrow) is thought to correspond to the critical micellar concentration (CMC) of the lysin (134).



Thus lytic response varies only with concentrations below the CMC and consequently lysin monomers would be involved in the lytic phenomenon.

The red blood cell surface is known to possess negative charges arising from the carboxyl groups of bound N-acetylneuraminic acid (74)(155). These negative charges could be viewed as greatly facilitating the

adsorption of cationic detergents on the membrane surface. Kondo et al. (131)showed that the negative surface charge of red blood cells was in fact reduced by addition of cationic surfactants. As a consequence, they pictured the outer surface as becoming coated with lipophilic acyl chains which could attract and thus concentrate other lysin molecules at the surface. This would eventually favour penetration of the lysin into the membrane. The action of cationic detergents inside the membrane was explained on the basis of their ionic interaction with acidic groups of lipids rather than cholesterol. Phospholipids and cholesterol would be lost in this manner and resulting changes in membrane protein conformation would cause release of hemoglobin. This argument is weak in that the authors stress only the ionic character of the interaction of lysin with membrane and do not account sufficiently for the well-documented hydrophobic character of the lysin interaction. Furthermore it cannot serve as a general mechanism to explain the action of nonionic or anionic detergents which would not react with or be repelled by the net negative charge at the cell surface, if indeed sialic acid groups play an important role. Furthermore it would not account for the accelerator effect of a number of substances on lysis. Albumin

and lipids which are non-lytic per se, can accelerate or increase the extent of hemolysis when the cell has been exposed to weakly-lytic concentrations of taurocholate (128). It seems therefore that the real determinative of lytic activity is the ability of a substance to penetrate the structural areas of the cell membrane. Thus considerations of shape, size, charge, relative degree of polarity and apolarity of a lysin should be translated not only in terms of their reactivity with structural elements of the membrane but first of all in terms of their ability to penetrate the structural areas of the membrane, and related to this ability would be the lyotropic mesomorphism of the substance.

Naturally-occurring Hemolytic Substances

In practice but for reasons that vary in detail, any substance which is surfactant could be suspected to have hemolytic properties. Lecithin, for example, is a nonlytic membrane constituent, but when erythrocytes are exposed to large concentrations of this phosphoglyceride hemolysis ensues because eventually this lipid will replace large quantities of cholesterol of the membrane (156). It is, however, only very weakly lytic

as compared to its lyso analogue. Lysolecithin, on the other hand, is recognized as a very potent lysin and its properties as well as its metabolism have been extensively studied. We will therefore limit our consideration to this substance mainly.

(1) Metabolism and Possible Function of Lysolecithin

Lysolecithin can be formed by three well characterized enzymatic reactions (157).

Lecithin $\xrightarrow{\text{Phospholipase } A_1}$ 2-lysolecithin (1)

Lecithin $\xrightarrow{\text{Phospholipase } A_2}$ 1-lysolecithin (2)

Lecithin + Cholesterol $\longrightarrow$ 1-lysolecithin + cholesterol ester (3)

Reactions 1 and 2 have a wide distribution in mammalian cells. In so far as they occur in microsomal and mitochondrial membrane fractions, their functioning would have to be coupled with controlling factors which prevent accumulation of their highly lytic products. It may be said however, that there is still the possibility that such fractions may be contaminated with lysosomes which

are rich in phospholipase A activity. Nevertheless, their accumulation could be prevented by reacylation (reaction 4), transacylation (reaction 5) or hydrolysis (reaction 6) which are known to operate in mammalian tissues (158).

Lysolecithin —Acyl Co A→ Lecithin (4)

2 Lysolecithin → Lecithin + Glycerophosphoryl choline (5)

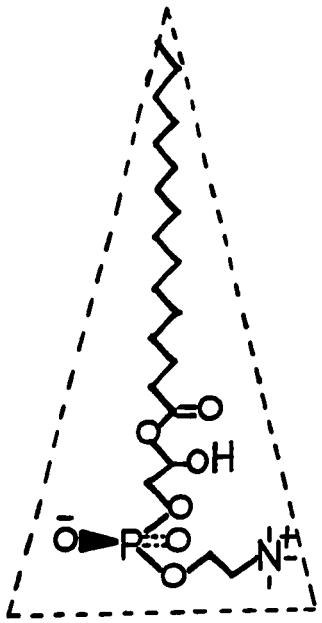
Lysolecithin —Lysophospholipase→ Glycerophosphoryl choline + Fatty acid (6)

Erythrocytes lack a typical phospholipase A, although some activity has been found with phosphatidylglycerol as substrate (159). Nevertheless, lysolecithin bound to serum albumin will readily exchange for lecithin of erythrocyte membrane. Serum lysolecithin could be formed from reaction 3 or possibly from post heparin-lipase-phospholipase A activity (160)(161). Thus accumulation of this lytic substance in erythrocyte membranes would be unavoidable unless reactions 4, 5 and 6

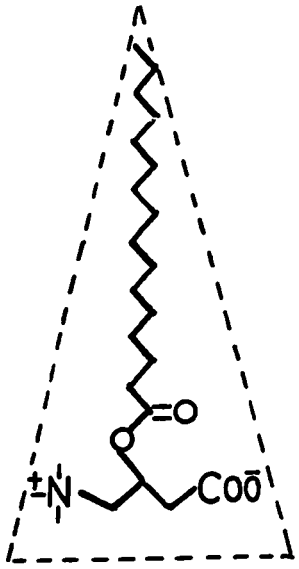
would serve to control the level of this lyso analogue (162). Nevertheless, small amounts of lysolecithin are detected as part of the lipid composition of globular membrane (53). The role of this substance in the membrane remains enigmatic. One can suppose from its highly lytic character, that its presence in the membrane would impose profound but perhaps localized changes in structural architecture which would alter permeability of the membrane.

(2) A Comparison of Lysolecithins and Acyl Carnitines

If one examines the structure of palmitoyl lysolecithin, one sees that it is amphipathic, consequently surfactant, that it has a wedge shape and that at physiological pH it is zwitterionic. Palmitoyl carnitine shows all these properties and therefore, one would expect it to have very similar biological activity. Although the lytic properties of acyl carnitines and mechanisms of action vis à vis the red blood cell membrane are not established, a brief report by Thomitzek et al. did describe the hemolytic properties of palmitoyl carnitine (163). That acyl carnitines might profoundly affect the structure of biological membrane can easily be surmised from the well-established bacteriocidal properties of amphipathic



1-Palmitoyl lysolecithin



Palmitoyl carnitine

quaternary ammonium derivatives and also from the work of Kuttis et al. (164) who showed that palmitoyl carnitine causes a transient swelling of mitochondria.

(3) The Metabolism and Function of Acyl Carnitines

The ubiquitous occurrence of carnitine in nature is well established (165)(166). The routes for biosynthesis of this compound are not completely resolved for all species. The formation of carnitine from γ-butyrobetaine by an hydroxylation reaction was demonstrated in

intact rat (167-169) and in Pseudomonas (170)(171). The precursor of γ-butyrobetaine is γ-aminobutyric acid which can be methylated by S-adenosylmethionine in brain and muscle homogenates (172). Recently, Horne et al. (173) reported that in Neurospora crassa, 6-C^{14}-lysine incorporated into carnitine without dilution of radioactivity, and characterized the lysine metabolites, ε-N-di- and trimethyl lysine and ε-N-trimethyl-δ-hydroxy-lysine as intermediates for carnitine biosynthesis.

Although carnitine is readily degraded by β-oxidation in Pseudomonas and may serve as a source of choline, it appears to be quite stable in mammals (174)(175). A carnitine decarboxylase and its product, β-methyl choline were identified in vivo and in vitro in rats (176)(177) but these results have not been confirmed.

Carnitine serves as a vitamin for certain insect larvae (165), where it is eventually used to form phosphatidyl-β-methylcholine. Phosphatidyl carnitine and its decarboxylated analogue are not formed in mammalian cells (178).

Perhaps the most significant function of carnitine is that concerned with fatty acid oxidation. For several decades it had been known that carnitine greatly stimulated

fatty acid oxidation but the reason for this remained unknown. Later, Friedman et al. (179), Fritz et al. (180) and Bremer (181) discovered that acyl groups could be reversibly transferred from Co A to carnitine as illustrated in the following reaction;

Acyl Co A + Carnitine $\rightleftharpoons$ Acyl carnitine + Co A

The enzyme concerned, carnitine palmitoyltransferase (E. C. 2. 3. 1. 7) has a wide distribution in mammalian tissues and is usually associated with mitochondrial fractions (182)(183). However, its presence in erythrocyte ghosts was also detected and confirmed by independent workers (184)(185).

The stimulatory role of carnitine could be explained thereafter (186)(187). Acyl Co A derivatives formed outside the mitochondria, i.e., on the outer mitochondrial membrane or in the microsomal fraction could not readily penetrate the mitochondrial membrane. Carnitine accepted acyl groups on the inner membrane and the resultant acyl carnitines could readily penetrate the mitochondrion. Once it had crossed the inner membrane, acyl carnitine served to reacylate Co A and β-oxidation ensued. The greater permeability of acyl

carnitines could perhaps be related to their greater lytic activity than acyl Co A and this could closely relate to the transport mechanism involved.

The presence of carnitine palmitoyltransferase in the erythrocyte membrane poses a functional problem since acyl carnitines formed thereby, do not appear to participate in lipid acylating reactions (184) 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 in that their formation in the membrane could induce structural changes which would alter the permeability and the fragility of erythrocytes.

EXPERIMENTAL RESULTS

SECTION I

In this section, experiments were designed to show the lytic properties of amphipathic quaternary ammonium derivatives and to compare their potencies. Investigations were also made to prove the mechanism of lysis by these substances.

PART I. Hemolysis of Rat and Human Erythrocytes by Long-chain Acyl Esters of Carnitine and Choline, and 1-Palmitoyl-sn-glycero-3-phosphoryl choline.

I. Materials and Methods

(A) Materials

(+)-, (-)- or (DL)-carnitine-HCl were purchased from Light and Koch Co., England and used without purification for synthesis of acyl carnitines.

Acyl cholines purchased from Sigma Chemical Co., were purified by recrystalization from ethanol-water (5:1, v/v) with addition of ether.

1-Palmitoyl-sn-glycero-3-phosphoryl choline was prepared by the action of snake-venom phospholipase A from the synthetic dipalmitoyl analogue purchased from

General Biochemicals, Ohio.

Snake-venom (Crotalus adamanteus) phospholipase A was purchased from Sigma Chemical Company.

(B) Methods

(1) Synthesis of Acyl Carnitines

Acyl carnitines were synthesized according to the method of Bremer (188)and slightly modified as follows: 1 gram of carnitine hydrochloride was dissolved in 1.5 ml of trifluoroacetic acid by warming in a 50 ml test tube. 5 equivalents of fatty acid chloride were added and the reaction mixture was mixed vigorously with a mechanical stirrer in a water bath at 50 - 55 °C for 3 or 4 hours. After the acylation reaction was completed, most of the trifluoroacetic acid was removed by rotatory evaporator under reduced pressure. After cooling, 20 ml of ether was added to the reaction mixture and agitated with a glass rod. Ice chips (about 10 ml as water) were added and shaken until dissolved. The reaction mixture was then cooled to -20 °C for an hour. Acyl carnitine precipitated in the water phase and excess fatty acid chloride remained mainly in the upper ether phase. The ether phase was removed and the precipitate of lower phase, washed with cool ether 3-4 times. The precipitated

acyl carnitine was then dissolved in 2-3 ml of ethanol to which 40 ml of ether were added gradually. After standing one hour at 5 °C, the precipitated acyl carnitine was separated by centrifugation and washed once more with cool ether. Fatty acid can be removed completely in this process, but not unreacted carnitine which still contaminated the product. The precipitated acyl carnitine was dissolved in 20 ml of distilled water and adjusted the pH to 7.0. Short-chain acyl carnitines (below 12 carbons) were not precipitated by this step but pure long-chain acyl carnitines (over 14 carbons) were precipitated completely at neutral pH. Short-chain acyl carnitines were purified with the same solvent system as for acyl cholines. The yields are usually 90-95 % on the basis of carnitine used.

The purity of the synthesized acyl carnitines was checked by thin-layer chromatography and elementary analysis. Thin-layer chromatography on silica gel G plates was performed with chloroform-methanol-acetic acid-water (50:25:8:4 , v/v). In this system all the synthesized acyl carnitines gave only one spot (Rf value approximately 0.5) with iodine vapor or Ponceau red stain.

Elementary Analysis

Hexanoyl carnitine Cl. (mol.wt., 295.7), Found: N, 4.78 %

$C_{13}H_{26}NO_4Cl$ requires N, 4.73 %

Octanoyl carnitine Cl.(mol.wt.,323.7),Found:C,55.09;H,9.38;N,4.16%

$C_{15}H_{30}NO_4Cl$ requires C,55.61; H,9.31; N,4.32%

Decanoyl carnitine Cl.(mol.wt.,351.7),Found:C,58.60;H,9.84;N,4.01%

$C_{17}H_{34}NO_4Cl$ requires C,58.00; H,9.67; N,3.98%

Myristoyl carnitine Cl.(mol.wt.,407.8),Found:C,59.79;H,10.03;N,3.14%

$C_{21}H_{42}NO_4Cl$ requires C,61.77; H,10.38; N,3.43%

Palmitoyl carnitine Cl.(mol.wt.,435.8),Found:C,63.48;H,10.52;N,4.25%

$C_{23}H_{46}NO_4Cl$ requires C,63.33; H,10.64; N,3.21%

Stearoyl carnitine Cl.(mol.wt.,463.9),Found:C,64.59;H,10.60;N,3.31%

$C_{25}H_{50}NO_4Cl$ requires C,64.65; H,10.86; N,3.02%

(2) Preparation of 1-Palmitoyl-sn-glycero-3-phosphoryl choline

1-Palmitoyl-sn-glycero-3-phosphoryl choline (1-palmitoyl lysolecithin) was prepared by the action of phospholipase A (E.C. 3.1.1.4) from Crotalus Adamanteus venom on synthetic L-a-dipalmitoyl lecithin.

0.5 g of dipalmitoyl lecithin dissolved in 2 ml of ether in 50 ml round bottomed flask and mixed with

Elementary Analysis

Hexanoyl carnitine Cl. (mol.wt., 295.7), Found: N, 4.78 %

$C_{13}H_{26}NO_4Cl$ requires N, 4.73 %

Octanoyl carnitine Cl.(mol.wt.,323.7),Found:C,55.09;H,9.38;N,4.16%

$C_{15}H_{30}NO_4Cl$ requires C,55.61; H,9.31; N,4.32%

Decanoyl carnitine Cl.(mol.wt.,351.7),Found:C,58.60;H,9.84;N,4.01%

$C_{17}H_{34}NO_4Cl$ requires C,58.00; H,9.67; N,3.98%

Myristoyl carnitine Cl.(mol.wt.,407.8),Found:C,59.79;H,10.03;N,3.14%

$C_{21}H_{42}NO_4Cl$ requires C,61.77; H,10.38; N,3.43%

Palmitoyl carnitine Cl.(mol.wt.,435.8),Found:C,63.48;H,10.52;N,4.25%

$C_{23}H_{46}NO_4Cl$ requires C,63.33; H,10.64; N,3.21%

Stearoyl carnitine Cl.(mol.wt.,463.9),Found:C,64.59;H,10.60;N,3.31%

$C_{25}H_{50}NO_4Cl$ requires C,64.65; H,10.86; N,3.02%

(2) Preparation of 1-Palmitoyl-sn-glycero-3-phosphoryl choline

1-Palmitoyl-sn-glycero-3-phosphoryl choline (1-palmitoyl lysolecithin) was prepared by the action of phospholipase A (E.C. 3.1.1.4) from Crotalus Adamanteus venom on synthetic L-a-dipalmitoyl lecithin.

0.5 g of dipalmitoyl lecithin dissolved in 2 ml of ether in 50 ml round bottomed flask and mixed with

2.8 ml of 0.1 M borate buffer (pH 7.0) containing 10 mg of snake venom phospholipase A and 0.2 ml of $CaCl_2$ (0.2 M) was incubated in a shaking water bath at 37 °C for 3-4 hours. Following incubation the ether was removed in a rotatory evaporator. Lipids were then extracted by the Bligh and Dyer method (189) as follows: 4 ml of incubation mixture was stirred mechanically for a minute with 5 ml chloroform and 10 ml methanol and followed by addition of 5 ml chloroform. After 30 minutes, 5 ml distilled water was added to the mixture and further stirred for 10 minutes. The lower chloroform phase containing lipid after centrifugation was carefully removed and the upper phase washed once again with 10 ml chloroform. The chloroform phases combined together were evaporated in a rotatory evaporator. The products were separated by preparative thin-layer chromatography (190) with the solvent mixture, chloroform-methanol-water (65:35:4, v/v). The separated lysolecithin fraction was eluted by Bligh and Dyer extraction.

The lysolecithin was further purified by chromatography on Sephadex G - 25 purchased from Sigma Chemical Company. 1 gram of Sephadex was suspended in distilled water and stirred. Fine particles were removed by decantation and the sediment washed with acetone several times. The washed Sephadex dried in air was suspended

in chloroform-methanol-water (60:30:4.5, v/v) and allowed to swell for a few hours and then pourred into the small column (0.5-1 cm in diameter). The lysolecithin dissolved in 15 ml of the same solvent passed through the column, and an extra 5-10 ml of solvent was further added to the column. Effluents were collected and dried on a rotatory evaporator under reduced pressure.

(3) Purification of Acyl Choline Chloride

Acyl cholines purchased commercially were purified as follows: 0.5 g of acyl cholines were dissolved in 1 ml of distilled water by warming and then 5 ml of ethanol were added and the mixture cooled in an ice bath. Acyl cholines were precipitated by gradual addition of 40 ml ether. The pure acyl cholines freed from fatty acid and choline were obtained by centrifugation and dried in air.

(4) Preparation of Erythrocytes

The red blood cell suspension used in these experiments was prepared from adult male, human or rat blood. Blood was mixed with 0.4 volume of 0.14 M glucose containing 0.057 M citrate (pH 7.4), and centrifuged

at low speed with a clinical centrifuge for 2-3 minutes. The leucocyte buffy coat was removed and the remaining cells were washed 3 times with isotonic saline (pH 7.4) to further eliminate leucocytes and citrate. The packed red cells were suspended to give a 50 % hematocrit in saline and stored at 5 °C for no more than a day.

(5) Lysin Solutions

All lysin solutions used in these experiments were dissolved in isotonic saline by warming in the case of long-chain acyl carnitines and acyl cholines, and adjusted to pH 7.4. Before use, they were kept in a warm bath (37 - 39 °C) to prevent precipitation.

(6) Measurement of Hemolysis

The lysis test was determined spectrophotometrically at 37 °C. 100 % hemolysis blanks were prepared by adding 25 ul of cells and a known amount of lysin to 3 ml of water in each of two cuvettes (1 cm)* of a Spectronics 505 recording spectrophotometer and allowing the mixture to stand at least 20 minutes. The absorbance was adjusted to 0 and one of the blanks was replaced by another cuvette containing 3 ml of saline to which the same amount

*path length

of cells and of lysin were added. The change in absorbance at 625 mu was recorded as a function of time. The extent of hemolysis was usually complete within 10 - 15 minutes, but was measured over a longer period when required. The percent hemolysis was estimated from a standard curve prepared by mixing different proportions of intact and hypo-osmotically hemolysed cells (Fig. 1) in a 3 ml volume, in which optical density is plotted as a function of percent hemolysis.

Results shown in Part I are the average of 3 experiments.

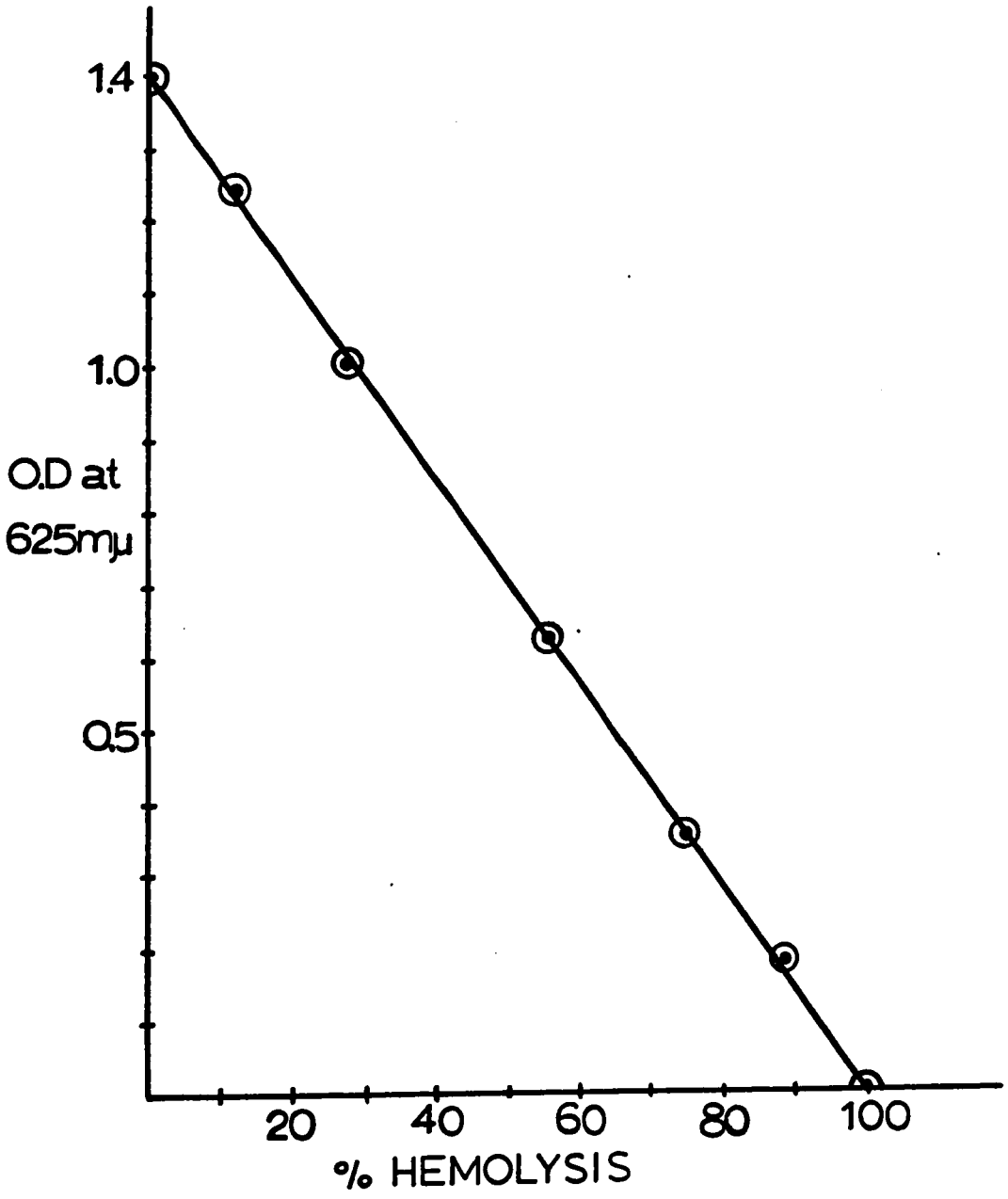


Fig.1. Hemolysis standard curve

II. Results

(A) Hemolysis of Rat and Human Erythrocytes by Acyl-DL-carnitines, Acyl Cholines and 1-Palmitoyl-sn-glycero-3-phosphoryl choline (1-Palmitoyl lysolecithin)

In Fig. 2, results show the susceptibility of rat and human erythrocytes to hemolysis by (1-palmitoyl)-lysolecithin and DL-acyl carnitines with various acyl chain lengths. The time required to give 50 % hemolysis was plotted as a function of lysin concentration.

As can be seen from Figs. 2a and 2b, acyl carnitines are potent lytic agents when either rat or human red blood cells are used. Stearoyl carnitine was the most effective lysin when compared to (1-palmitoyl)-lysolecithin or its homologues of shorter acyl chains.

Human erythrocytes are slightly more susceptible than rat red blood cells to the action of the lysins used, and (1-palmitoyl)-lysolecithin was slightly more potent than palmitoyl carnitine in human erythrocytes but not in rat erythrocytes.

Hemolysis by acyl carnitines with shorter chain length, such as decanoyl carnitine was observed only

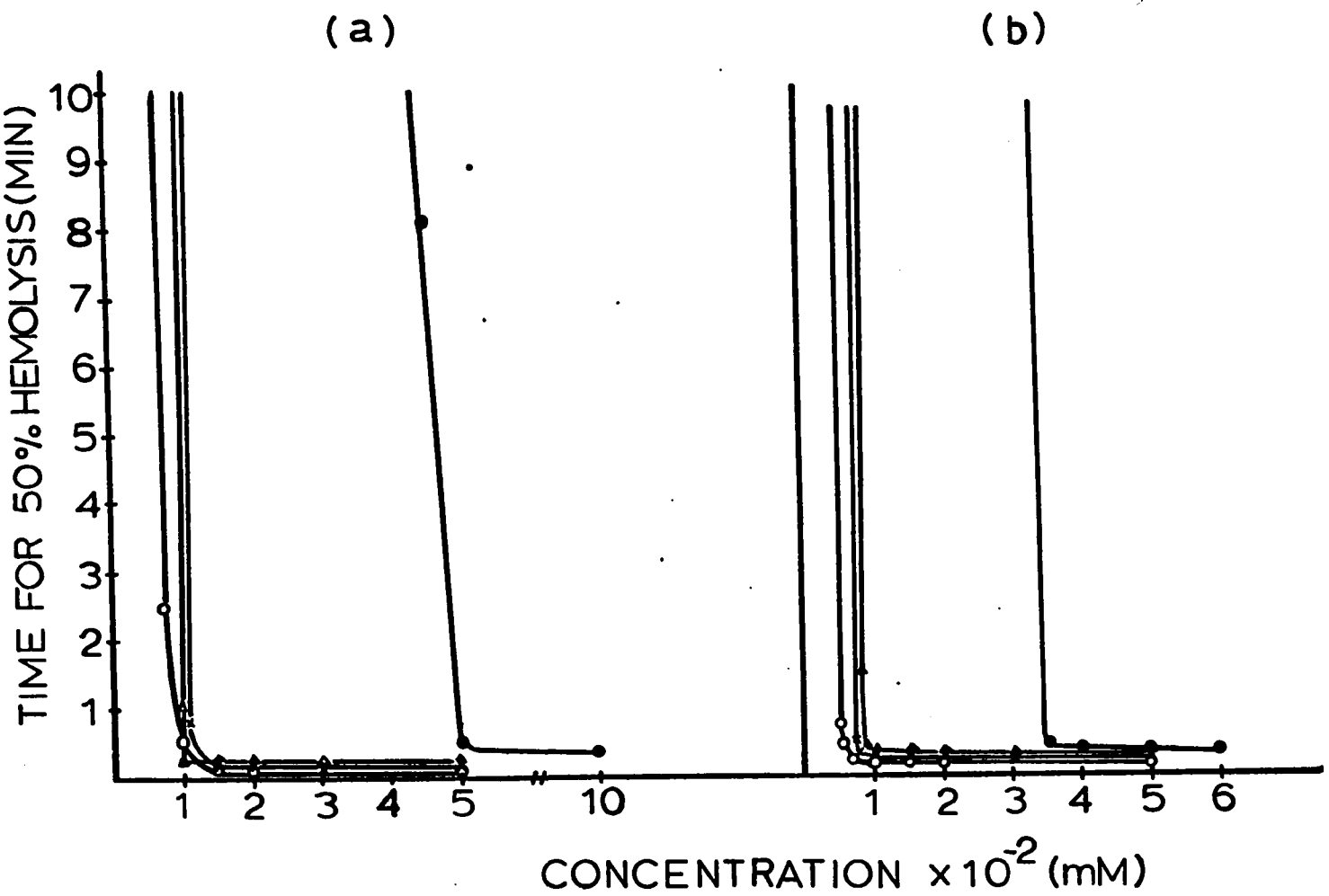


Fig. 2. Hemolysis of (a) rat, (b) human erythrocytes by acyl-DL-carnitines and lysolecithin. o—o, stearoyl carnitine; Δ—Δ, 1-palmitoyl-sn-glycero-3-phosphoryl choline; x—x, palmitoyl carnitine; ●—●, myristoyl carnitine.

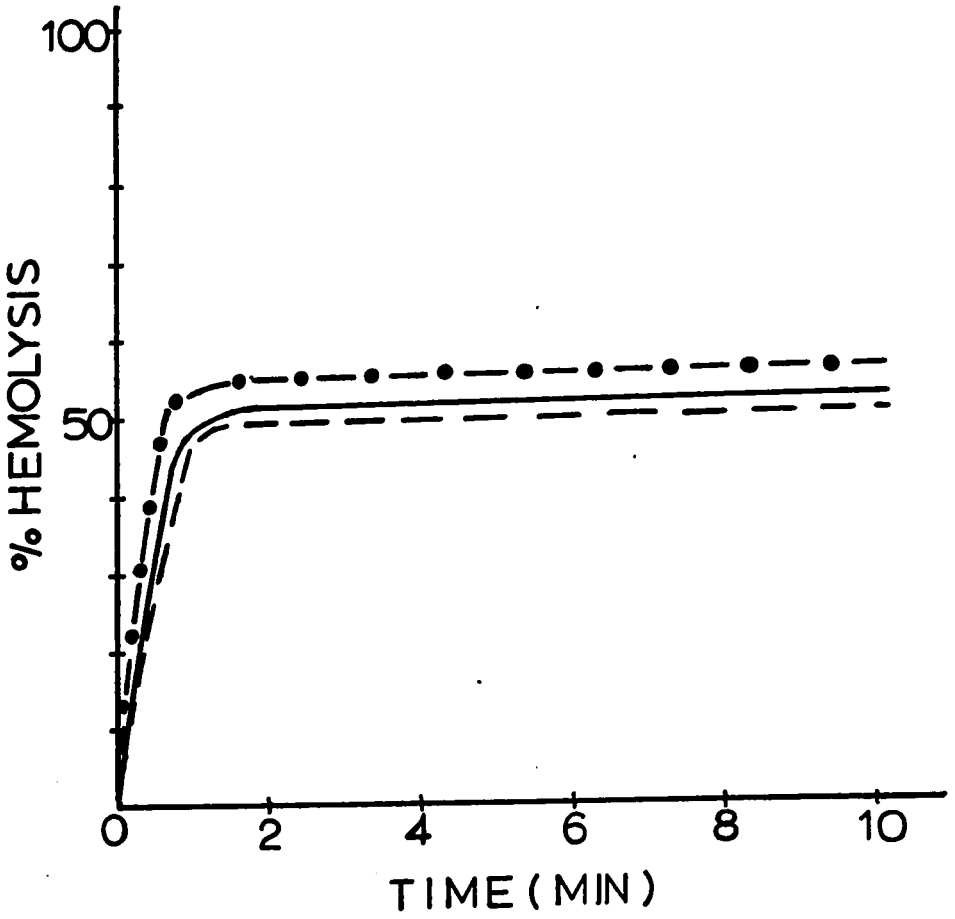


Fig. 3. Hemolysis of rat erythrocytes by stereo-isomers of palmitoyl carnitine.
——, DL-palmitoyl carnitine (1×10^{-2} mM)
●—●, (−)-palmitoyl carnitine (")
– – , (+)-palmitoyl carnitine (")

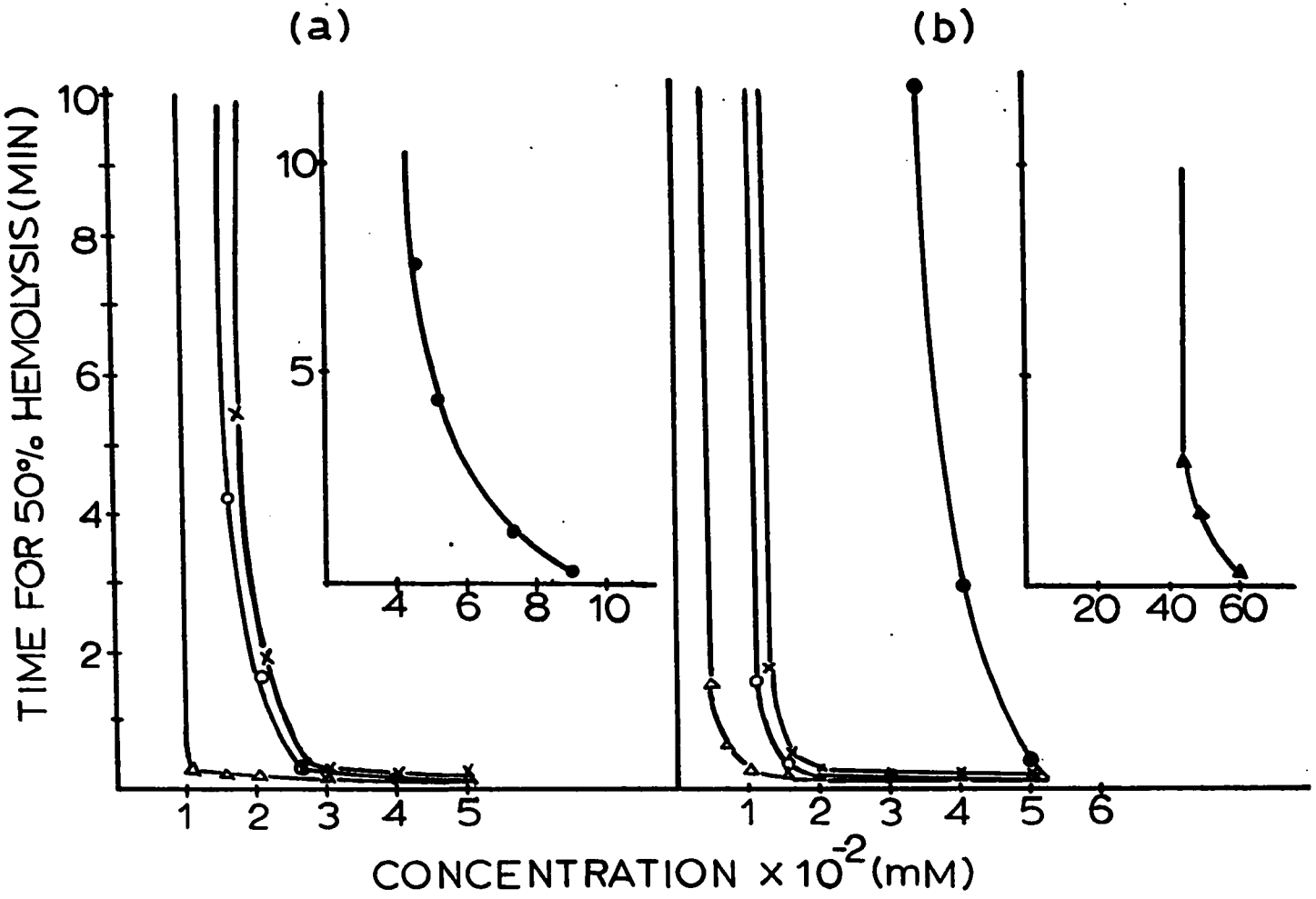


Fig.4. Hemolysis of (a) rat, (b) human erythrocytes by acyl cholines and lysolecithin. Δ—Δ, 1-palmitoyl-sn-glycero-3-phosphoryl choline; o—o, stearoyl choline; x—x, palmitoyl choline; ●—●, myristoyl choline; ▲—▲, lauroyl choline.

with very high concentrations (5-6 x 10^{-3} M), which resulted in less than 10 % hemolysis in 15 minutes. Octanoyl carnitine and hexanoyl carnitine did not hemolyse at these concentrations.

In Fig. 3, the lytic activity of stereoisomers of palmitoyl carnitines are compared. (-)-Palmitoyl carnitine and (+)-palmitoyl carnitine were each about as potent as DL-palmitoyl carnitine.

Results shown in Figs. 4a and 4b confirmed the previous findings of Pethica and Anderson (150) that acyl cholines are potent lytic agents and the effective minimal concentration decreases with lengthening of the acyl chain. In this respect, acyl carnitines and acyl cholines are very similar. Stearoyl choline was the most potent lysin in its series of homologues but less potent than (1-palmitoyl)-lysolecithin, stearoyl carnitine and palmitoyl carnitine when either rat or human erythrocytes were used. Human erythrocytes were also more susceptible than rat erythrocytes to lysis by acyl cholines.

(B) <u>The Effect of Decreasing Concentrations of Lytic Palmitoyl Esters on Hemolysis of Rat Erythrocytes</u>

Figs. 5a - c show the effect of decreasing concentrations of (1-palmitoyl)-lysolecithin, palmitoyl-DL-carnitine and palmitoyl choline when a constant amount of rat erythrocytes was used.

Inspection of the results reveals that even with lower concentrations of either (1-palmitoyl)-lysolecithin, palmitoyl carnitine or palmitoyl choline there is no discernible lag period before the onset of hemolysis. This result differs from that obtained with polyene antibiotics, animal toxins and some non-ionic surfactants on erythrocytes. In the later cases, a lag period which increases as the concentration of lysin is decreased is noticed. In the case of the polyene antibiotics, the rate of binding or penetration into the cell membrane is a rate limiting step in the hemolysis process (143). Such does not appear to be the case with lysins used in the present study where both penetration and hemolysis are rapid.

In palmitoyl carnitine and (1-palmitoyl)-lysolecithin hemolysis, the extent of hemolysis reaches a maximum within few minutes, and does not change appreciably by increasing the incubation time. This phenomenon is in accord with the view that a definite ratio of acyl

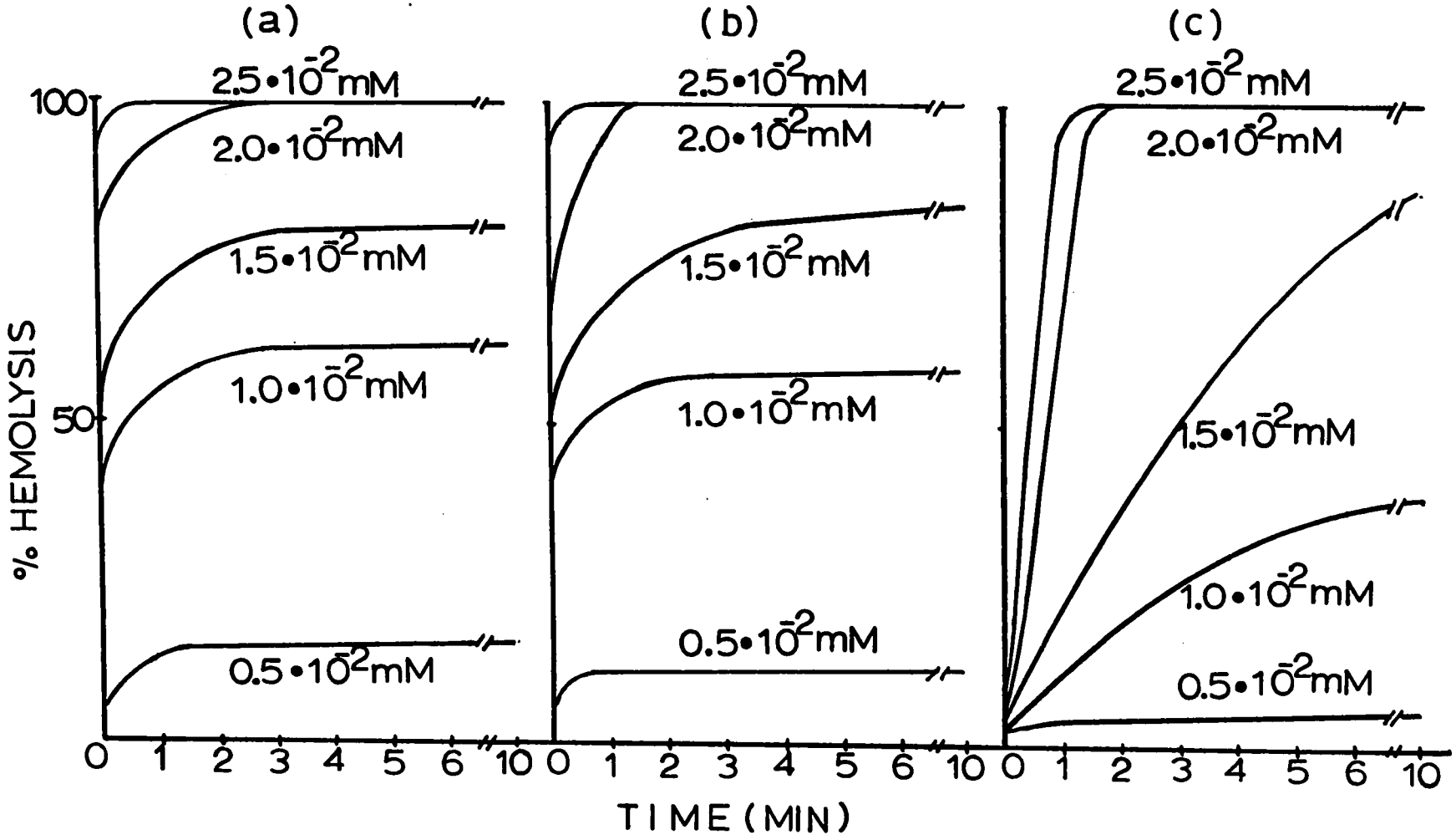


Fig. 5. The effect of decreasing concentrations of lytic palmitoyl esters on hemolysis of rat erythrocytes. (a) 1-palmitoyl-sn-glycero-3-phosphoryl choline, (b) palmitoyl-DL-carnitine, (c) palmitoyl choline.

carnitine and lysolecithin per cell must be attained before hemolysis can occur as shown also by antibiotic hemolysis. The lysis by palmitoyl choline is a little different in that it shows a slightly slower hemolysis rate than other lysins at certain concentrations. Such a difference in lytic properties of palmitoyl choline might be due to its net positive charge which is absent in acyl carnitines and lysolecithins at pH 7.4.

(C) The Effect of Increasing the Amount of Rat Erythrocytes on Hemolysis by a Constant Concentration of (1-Palmitoyl)-lysolecithin, Palmitoyl-DL-carnitine or Palmitoyl choline.

If the concentrations of lysins are kept constant and the amount of red blood cells is increased (Figs. 6a-c) there is a corresponding decrease in the percent hemolysis with acyl lysins tried. The extent of hemolysis by (1-palmitoyl)-lysolecithin, palmitoyl carnitine or palmitoyl choline varies not only with their concentration but also with the amount of cells present. A similar effect with polyene antibiotics has been explained on the basis that the lysin combines with a definite proportion of lipid of the cell suspension. In this case

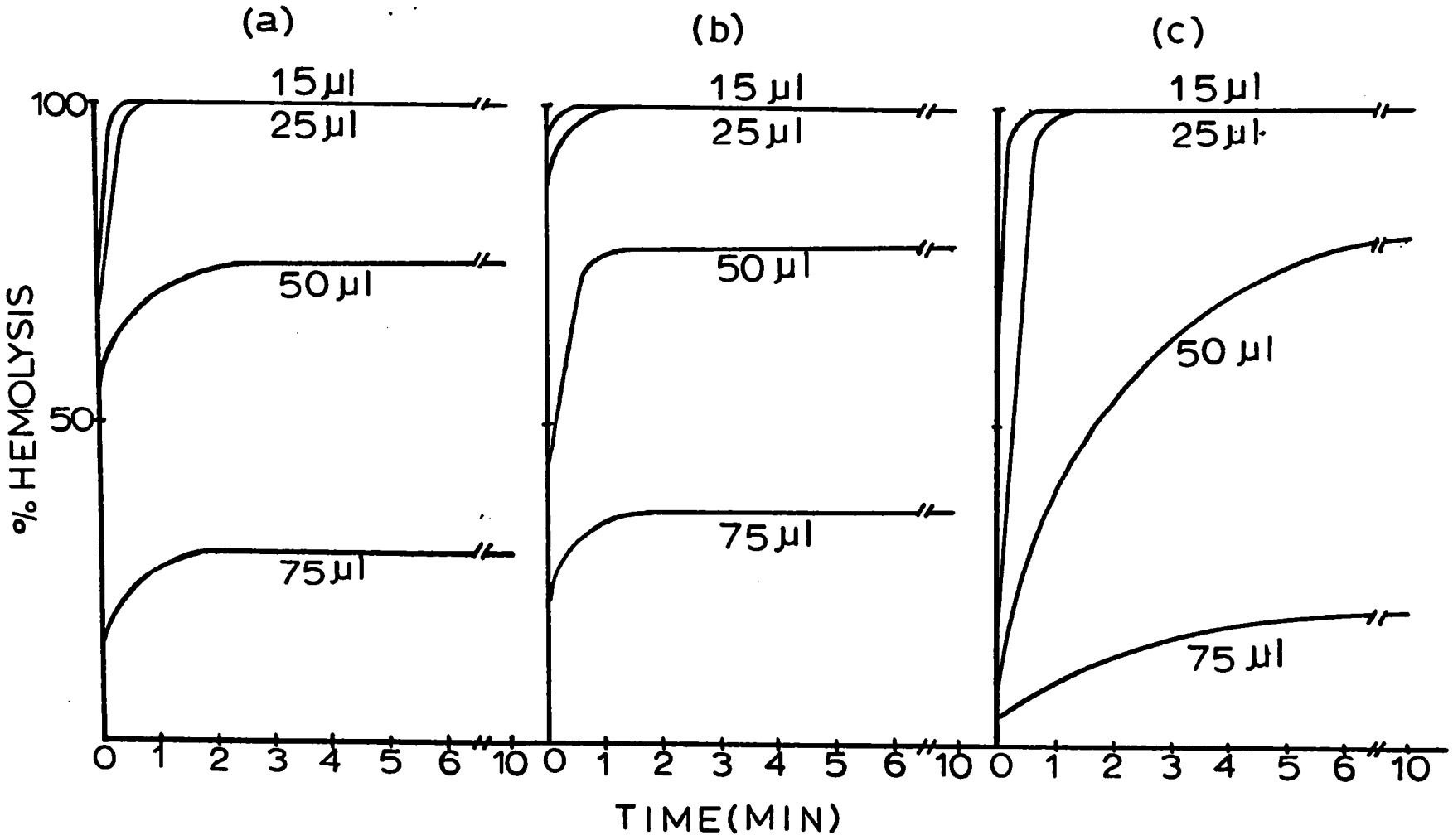


Fig.6. The effect of increasing the amount of rat erythrocytes on hemolysis by $2.5 \cdot 10^{-2}$ mM concentrations of (a) 1-palmitoyl-sn-glycero-3-phosphoryl choline, (b) palmitoyl-DL-carnitine, (c) palmitoyl choline.

complete hemolysis was obtained only when the number of filipin molecules added per molecule of lipid in the erythrocyte membrane was approximately 0.10 - 0.21 under the conditions used.

PART II. The Effect of Membrane Components and Normal Cytosol Constituents on Lysis of Rat Erythrocytes by Palmitoyl-DL-carnitine, Palmitoyl Choline and 1-Palmitoyl-sn-glycero-3-phosphoryl Choline.

I. Materials and Methods

(A) Materials

Synthetic L-a-(dipalmitoyl)-lecithin, phosphatidyl inositol, phosphatidyl serine, sphingomyelin, cholesterol and palmitic acid were purchased from General Biochemicals and purified by preparative thin-layer chromatography and Sephadex chromatography.

Total lipids from rat blood cells and from rat liver were extracted by the method of Bligh and Dyer (189).

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, v/v) as solvent.

Membrane proteins were prepared from erythrocyte ghosts by the method of Zahler et al. (67)(68).

Albumin was purchased from General Biochemicals and before use was freed of fatty acid by acetone extraction.

Glucose, glycerol and glycine were obtained from Fisher Scientific Co., N.J. and L-cystine was from Nutritional Biochemicals Corp., Ohio.

Other materials used were described in the previous part.

(B) Methods

(1) Preparation of Total Lipids

Rat erythrocytes were prepared as previously described. Rat liver was obtained from adult, male rats. 10 ml of packed erythrocytes or 10 g of rat liver sliced into small pieces were homogenized in distilled water and adjusted to a volume to 18 ml. Total lipids were then extracted by the method of Bligh and Dyer described previously.

(2) Isolation of Phosphatidyl Ethanolamine from Total Lipids

Phosphatidyl ethanolamine was prepared from total lipids by preparative thin-layer chromatography as follows; total lipids extracted from liver were applied on the activated silica gel G plates and de-

veloped for an hour at room temperature with chloroform-methanol-water (65:25:4, v/v) as solvent. After staining with Ponceau red solution, phosphatidyl ethanolamine fraction was scraped off and extracted by the method of Bligh and Dyer. Phosphatidyl ethanolamine was further purified by Sephadex column chromatography as previously stated.

Ponceau Red Solution

0.05 g ponceau S red (from Allied Chemical Corp., N.Y) and 0.2 g uranyl nitrate (from J.T. Baker Chemical Co., N.J) were dissolved in 0.01 N hydrochloride solution.

(3) Preparation of Membrane Protein from Rat Erythrocyte Ghosts.

(i) Rat Erythrocyte Ghosts

For the preparation of erythrocyte ghosts the method of Dodge et al. (191) was used as slightly modified by Azen et al. (192) and Zwaal et al. (73).

Rat blood was collected into 0.4 volume of 0.14 M glucose containing 0.057 M citrate (pH 7.4), and centrifuged at low speed with a clinical centrifuge for 2-3 minutes. The fluffy coat was removed by several washings

with isotonic phosphate buffer pH 8.0 at a concentration of 310 mosM made by mixing NaH_2PO_4 0.155 M and Na_2HPO_4 0.103 M. The packed cells were hemolysed in 10 volumes of hypotonic phosphate buffer, 20 mosM (pH 7.4) containing 0.1 mM EDTA, during an hour. The membranes were collected by centrifugation of the hemolysate at 30,000 g for 20 min and washed repeatedly with the hypotonic phosphate buffer until hemoglobin-free ghosts were obtained. The ghost suspensions were frozen over-night at -25 °C and thawed the next day and washed three times with distilled water. Finally, cream-colored stroma was obtained.

(ii) Isolation of Membrane Protein

The packed erythrocyte ghosts were dispersed in water to give a suspension of about 10 mg protein per ml. This was mixed with 5 volume of 2-chloroethanol, leading to a rapid clearing of the membrane suspension. The membrane solutions were concentrated 5-fold under a stream of nitrogen at room temperature. After this step the proportion of 2-chloroethanol is about 95 % and the apparent pH about 2. This solution was applied on the top of a Sephadex LH-20 column.

For gel filtration Sephadex LH-20 was packed into

the column only after previous swelling in 2-chloroethanol-water (9:1, v/v) for 16 hours. Column dimensions were 2.5 x 58 cm. Effluents were collected in on ULTRORAC fraction collector, type 7000 (LKB-PRODUCTER AB SWEDEN) as 5 ml aliquots. The proteins were determined spectrophotometrically on the basis of absorbance at 280 mu. Aliquots containing protein were combined and were dialysed 24 hours against distilled water at 4 °C whereupon the protein 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. (193).

(4) Preparation of Phospholipid Dispersion

Aqueous dispersions of synthetic L-a-(dipalmitoyl)-lecithin, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol and sphingomyelin were prepared by the method described by Bruckdorfer et al. (45).
A known amount of lipid was subjected to ultrasonic dispersion with a Biosonik cell disruptor, at 70 w for 5 min in 10 ml of 30 mM NaCl at room temperature. The dispersions were used without prior centrifugation and adjusted to pH 7.4.

(5) Preparation of Cholesterol and Fatty acid Sols

Cholesterol and palmitic acid sols were prepared essentially by the method of Lee and Tsai (194) as follows: Crystalline cholesterol and palmitic acid were first dissolved in 96 % ethanol (1 mg/ml). 1 ml of the solution was mixed quickly with 10 ml of warm water (about 60 °C). The mixture formed a colloidal dispersion. The alcohol was then evaporated off in a boiling water bath until the volume reduced to 8 ml. After cooling, the final volume was adjusted as required by additionof distilled water. These sols were very stable and did not precipitate for 2 weeks when kept in a refrigerator at 5 °C.

(6) Measurement of % Inhibition of Hemolysis

The extent of hemolysis under the various conditions to be described for the test were determined spectrophotometrically using a Cary 15 recording spectrophotometer.

100 % hemolysed blanks (25 ul cells of 50 % hematocrit) in 3 ml water were prepared as before and the absorbance adjusted to 0. One of the blanks was replaced by another cuvette containing 25 ul red cells in 3 ml isotonic saline (pH 7.4) and the absorbance read. In the all experiments, the cell concentration was adjusted

so that 25 ul red cells gave an O.D of 1.4. For studies of inhibition of lysis, 25 ul of erythrocytes were mixed with increasing amounts of modifier substance and the volume was adjusted to 2.96 ml with saline (pH 7.4) and 0.04 ml of lysin solution which contained enough lysin to give 100 % hemolysis in 10 minutes in the absence of inhibitor. The extent of lysis after 10 minutes was calculated from the % hemolysis standard curve.

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 represented the average of 3 experiments.

II. Results

(A) The Effect of Modifier Substances on the Hemolysis of Rat Erythrocytes by Palmitoyl-DL-carnitine, Palmitoyl Choline and (1-Palmitoyl)-lysolecithin.

As can be seen from Figs. 7a - j 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 lysin was added after admixture of the protective agents with erythrocytes. Lysis by palmitoyl 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.

(B) The Effect of Preincubation and Order of Addition on Inhibition of Palmitoyl-DL-carnitine, Palmitoyl Choline and (1-Palmitoyl)-lysolecithin Hemolysis by Membrane Protein and Lipid.

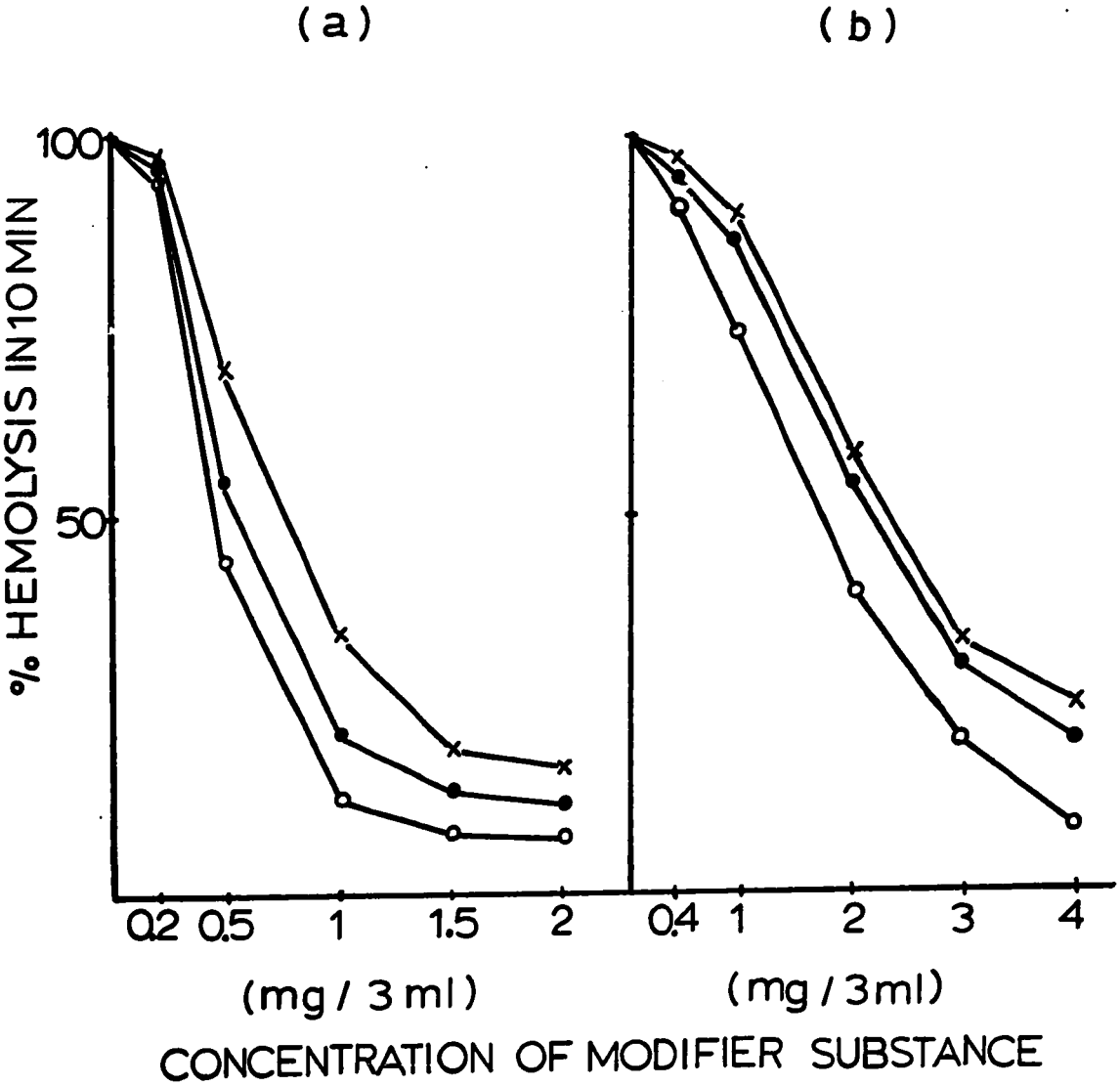
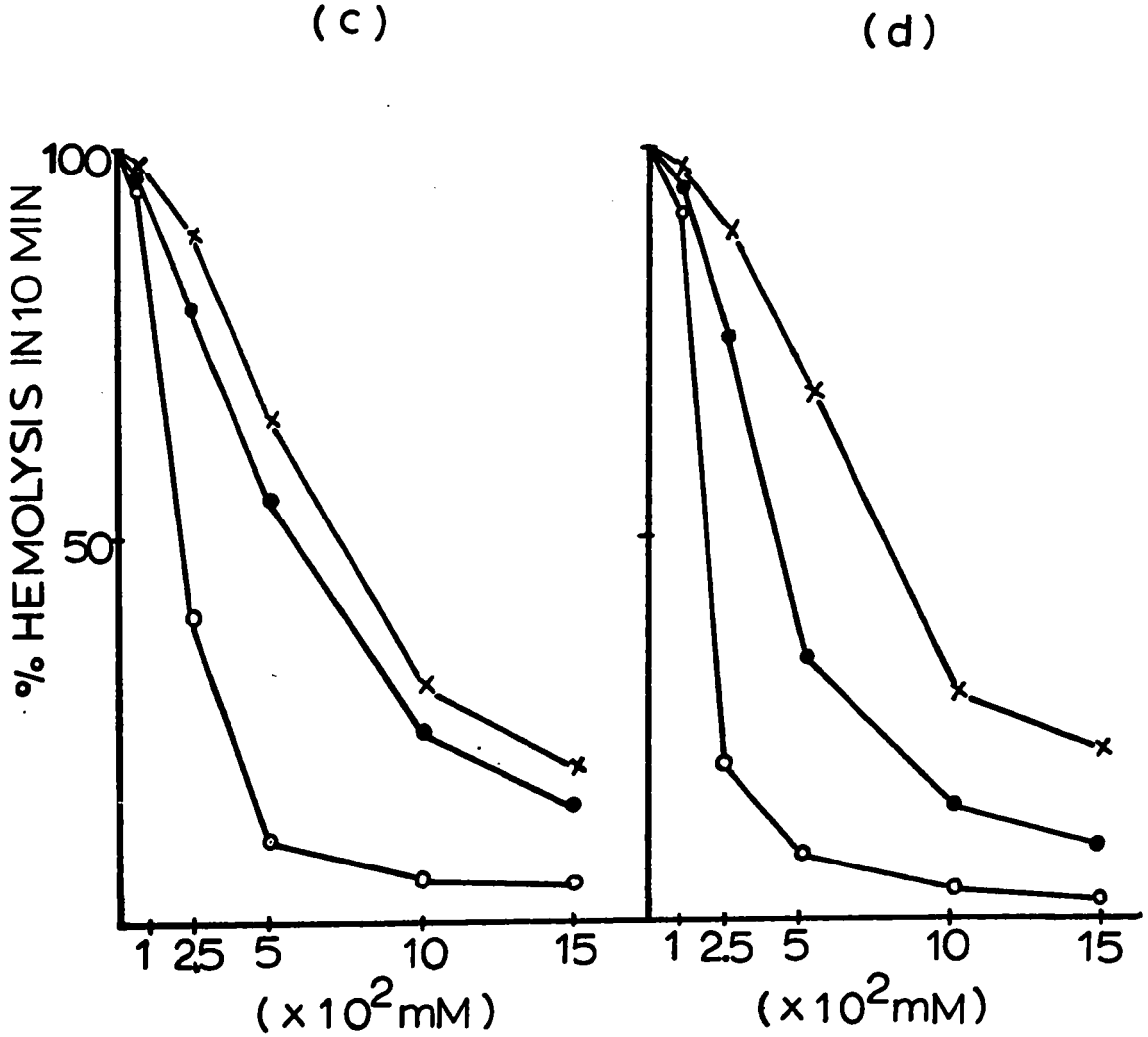
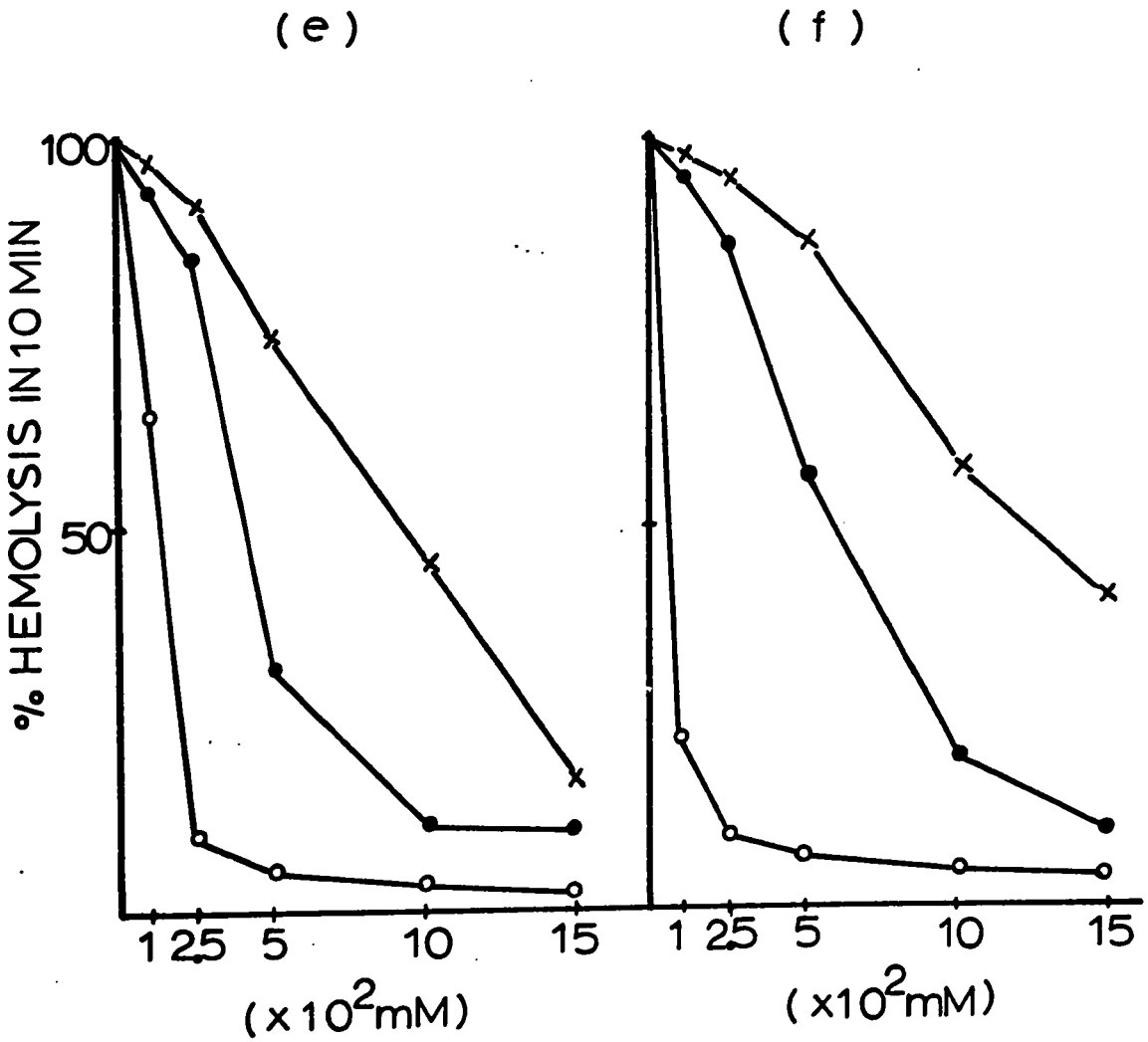


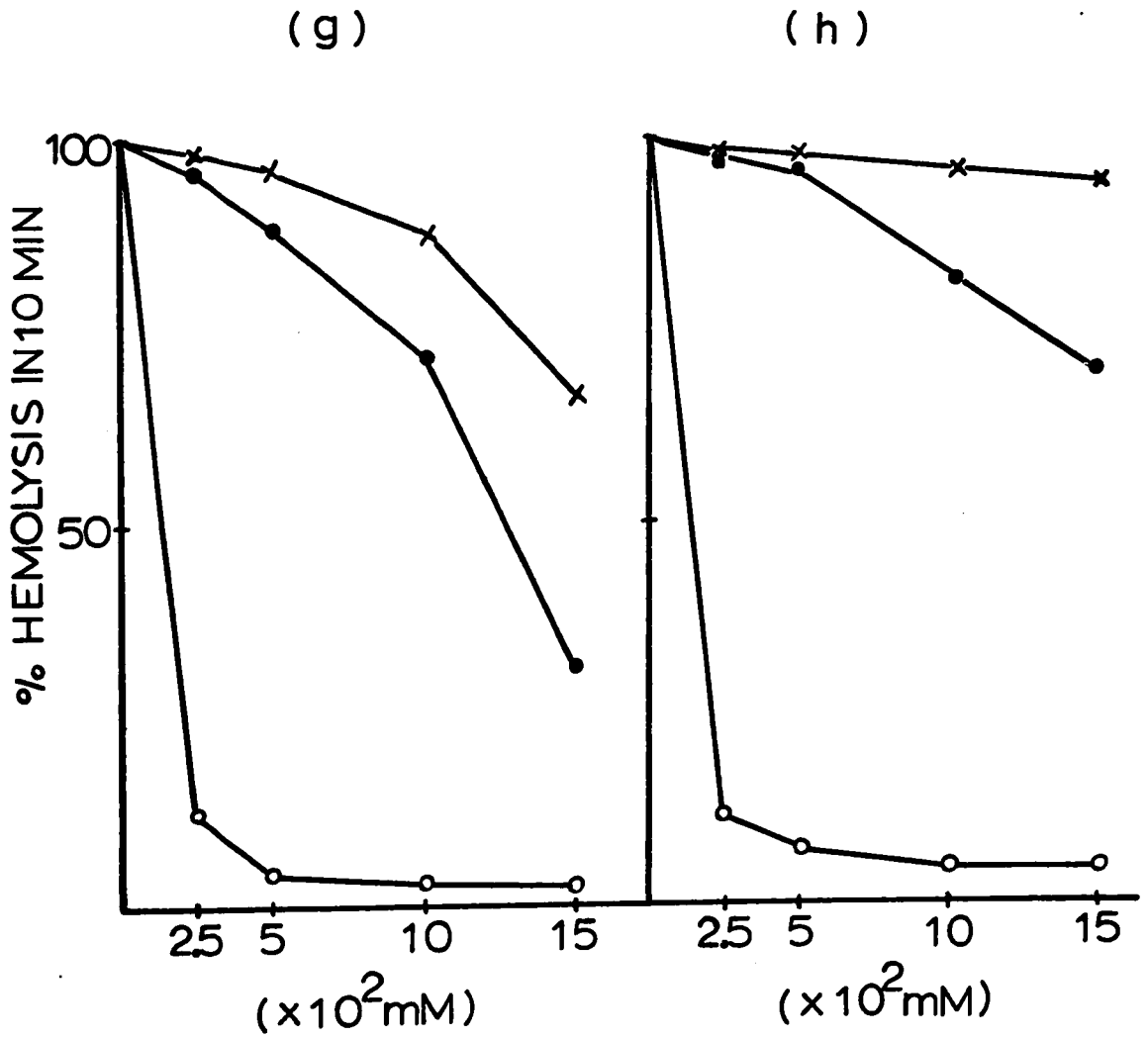
Fig. 7. The effect of modifier substances on lysis of rat erythrocytes by x—x, $2 \cdot 10^{-2}$ mM (1-palmitoyl)-lyso-lecithin; ●—●, $2 \cdot 10^{-2}$ mM palmitoyl-DL-carnitine; and o—o, $2 \cdot 10^{-2}$ mM palmitoyl choline.
(a) albumin, (b) membrane protein



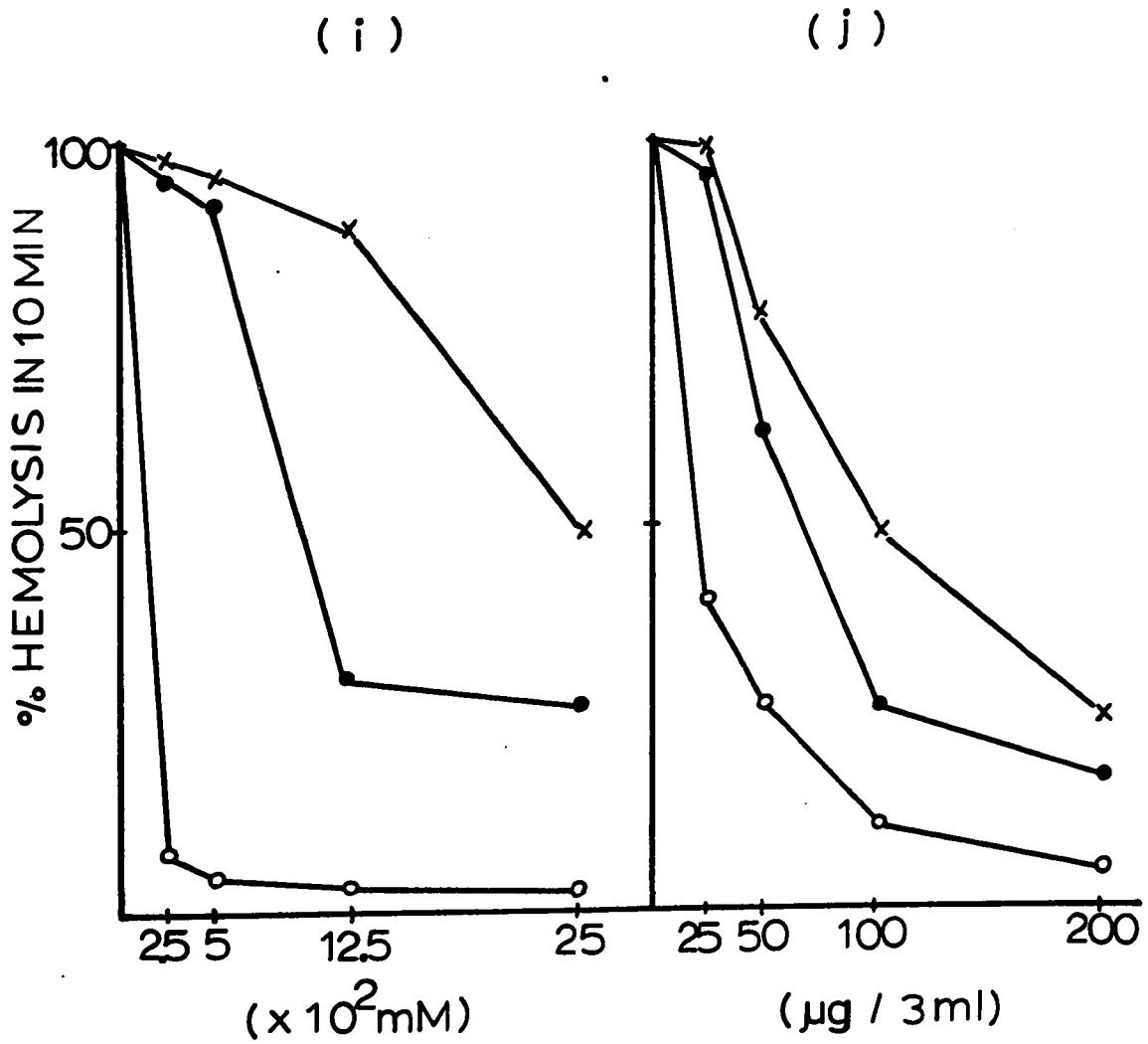
(c), lecithin; (d), cholesterol



(e), phosphatidyl ethanolamine; (f), phosphatidyl serine.



(g), phosphatidyl inositol; (h), sphingomyelin.



(i), palmitic acid; (j), total lipid from erythrocyte membrane

When palmitoyl carnitine in completely lytic concentrations was incubated 10 minutes with either membrane protein, lecithin or cholesterol, prior to addition of erythrocytes (Figs. 8a - c), the inhibition was more pronounced 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. 9a, 9b and 10a - 10c) 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 (1-palmitoyl)-lysolecithin 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

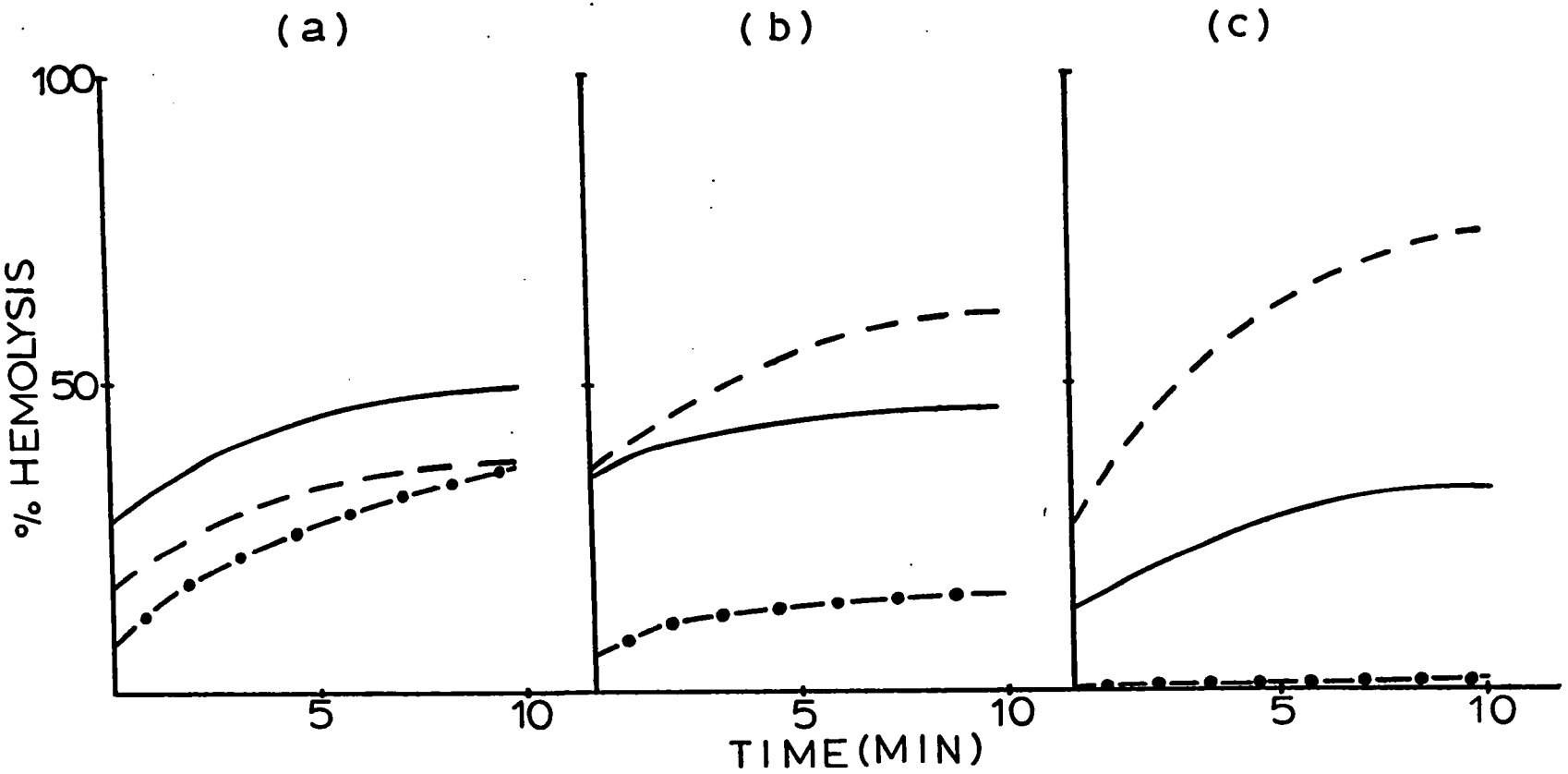


Fig.8. The effect of preincubation and order of addition on inhibition of palmitoyl carnitine hemolysis by lipid and membrane protein. Addition seqence: ——,erythrocytes-inhibitor-lysin without pre-incubation; – – –,erythrocytes incubated 10 min at 37° with modifier substance prior to addition of lysin; ●—●, lysin and modifier substance incubated 10 min at 37° prior to addition of erythrocytes. (a) 2.07mg/3ml 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. 7.

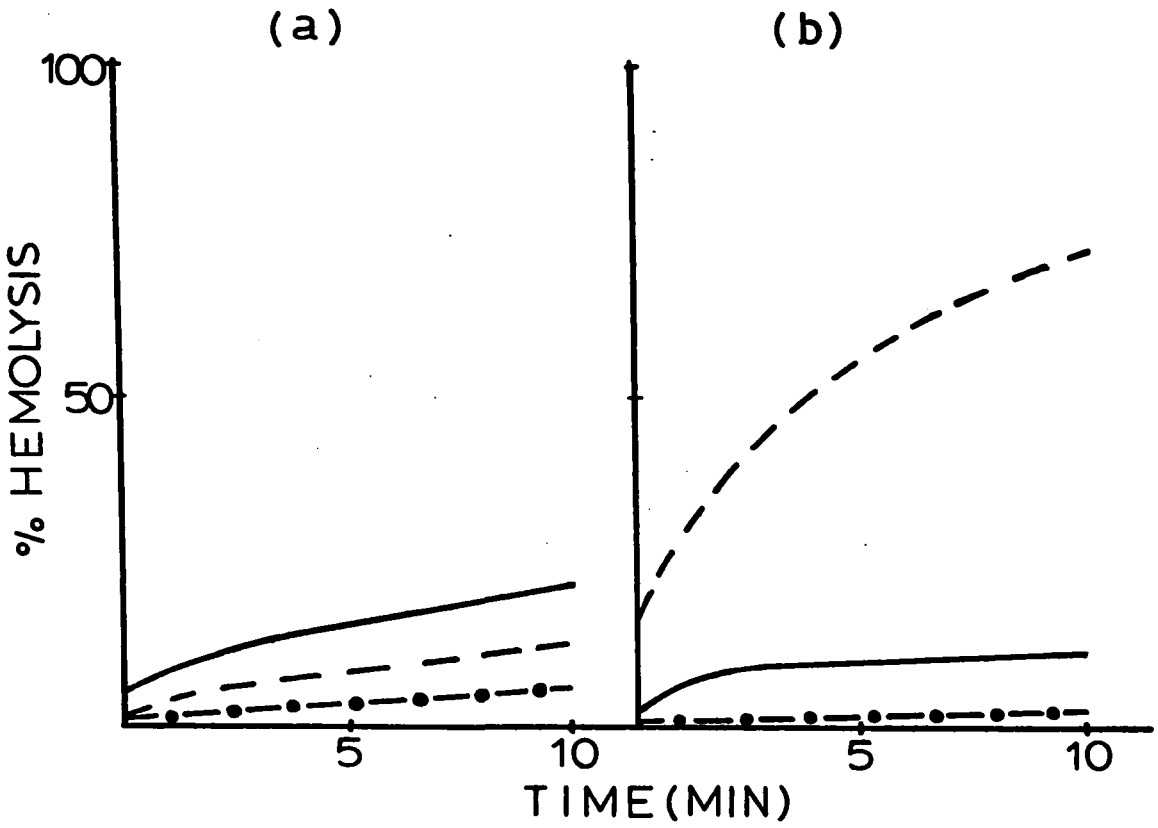


Fig. 9. The effect of preincubation and order of addition on the inhibition of palmitoyl choline hemolysis by lipid and membrane protein. The concentration of palmitoyl choline used was $2 \cdot 10^{-5}$ M. All other conditions were as stated for Fig. 8.

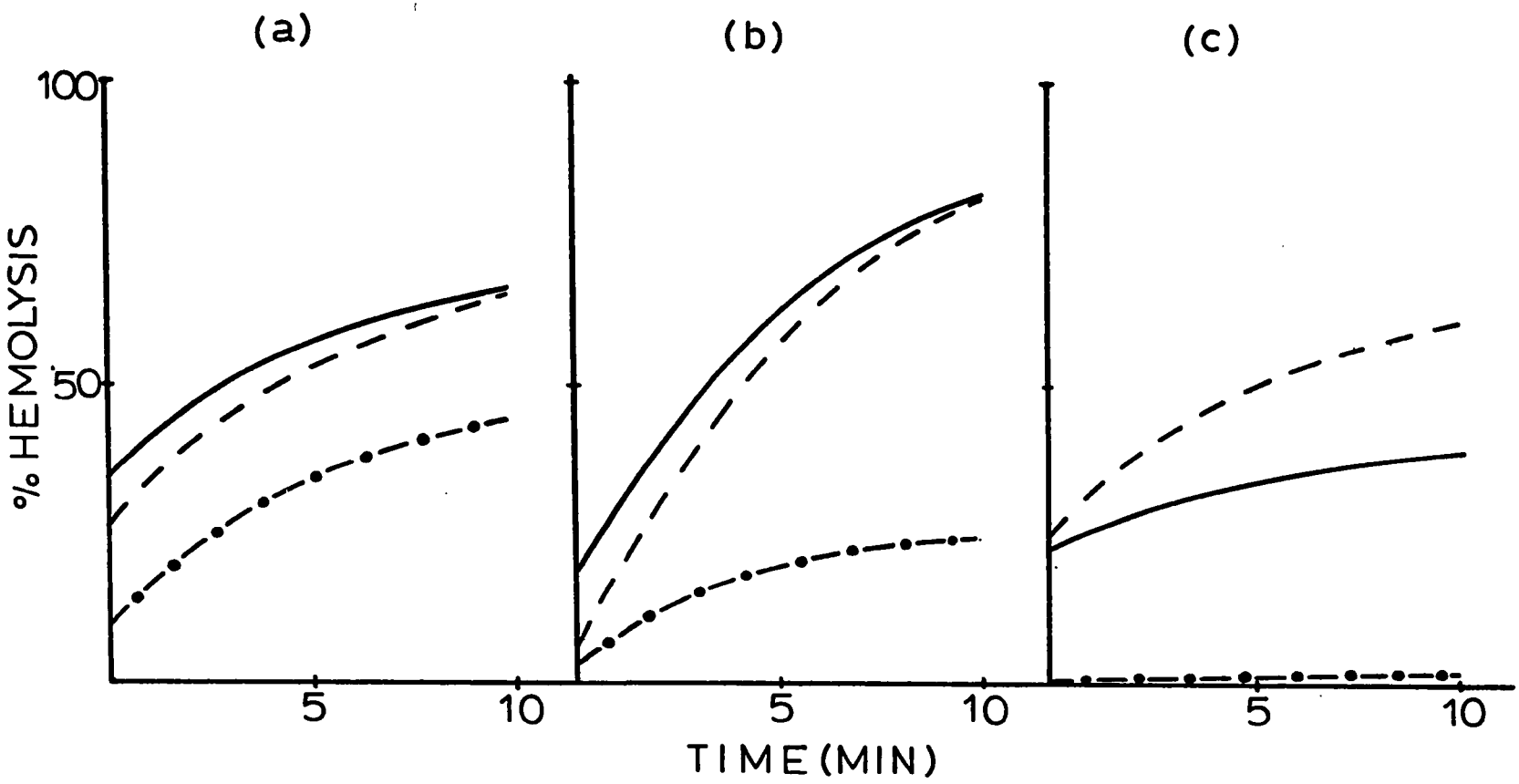


Fig.10. The effect of preincubation and order of addition on inhibition of (1-palmitoyl)-lysolecithin hemolysis by lipid and membrane protein. The final concentration of lysolecithin was $2 \cdot 10^{-5}$ M. All other conditions are as stated for Fig.8.

the inhibition of lysis by lysolecithin.

(C) The Effect of Membrane Protein and Lipid on the Lysis by Sublytic Amount of Palmitoyl-DL-carnitine and (1-Palmitoyl)-lysolecithin.

If erythrocytes were admixed with weakly-lytic concentrations of palmitoyl carnitine (Figs. 11a - 11e) and lysolecithin (Figs. 12a - 12e) 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 (dot-and-dash lines).

(D) The Effect of Various Polar Substances on the Lysis by Sublytic Amount of Palmitoyl-DL-carnitine and (1-Palmitoyl)-lysolecithin.

Hemolysis of cells "sensitized" by prior addition of weakly lytic concentrations of palmitoyl carnitine (Figs. 13a - 13e) and lysolecithin (Figs. 14a - 14e) was also accelerated to different extents by various polar substances tried.

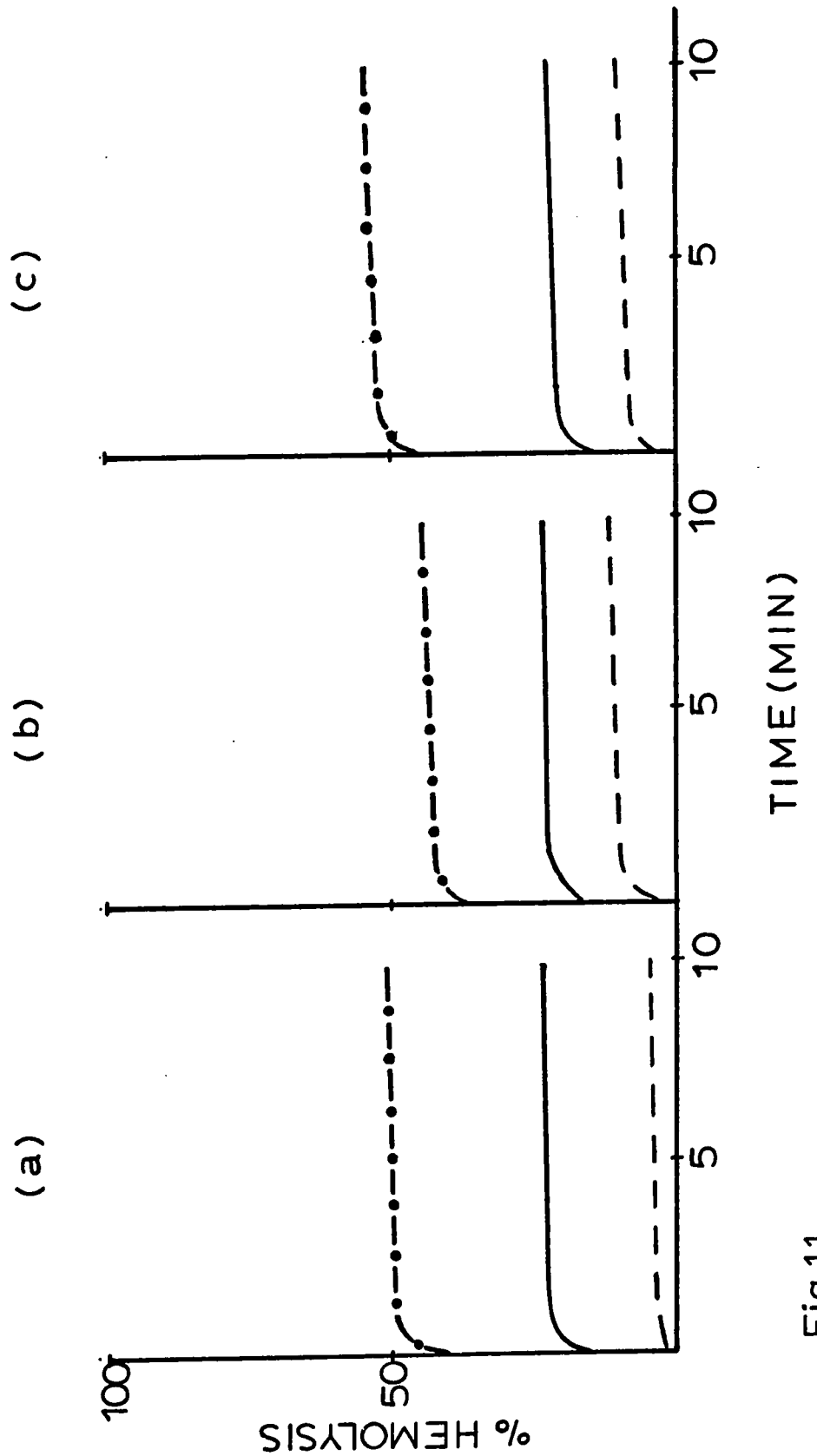


Fig.11.

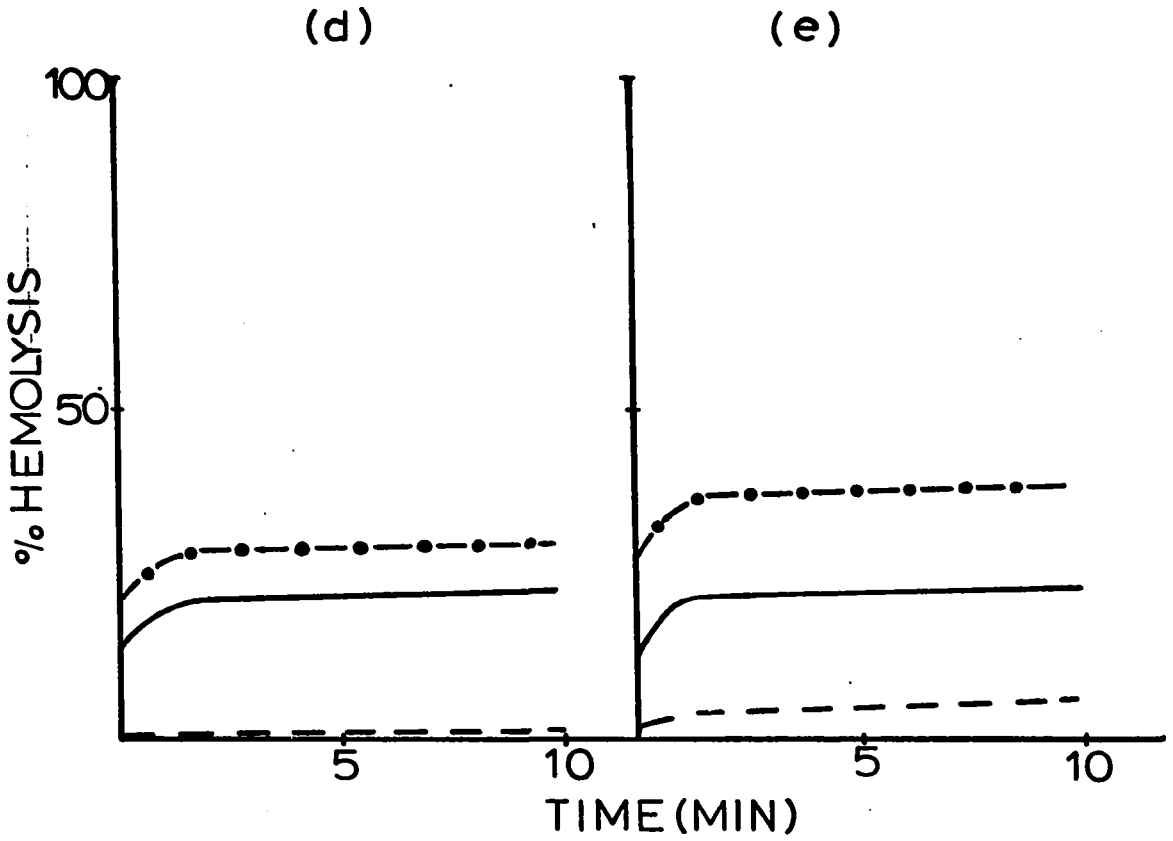


Fig.11. Acceleration of palmitoyl carnitine hemolysis by lipids and membrane protein. Addition sequence without preincubation: ——, erythrocytes–lysin; — — —, erythrocytes–modifier substance–lysin; ●—●, erythrocytes–lysin–modifier substance. (a) 1.035mg / 3ml membrane protein; (b) 0.60mg / 3ml total lipid from erythrocyte membrane; (c) $5 \cdot 10^{-5}$M lecithin; (d) $8 \cdot 10^{-5}$M cholesterol; (e) $2.5 \cdot 10^{-4}$M palmitic acid. The concentration of palmitoyl carnitine used was $5 \cdot 10^{-6}$M.

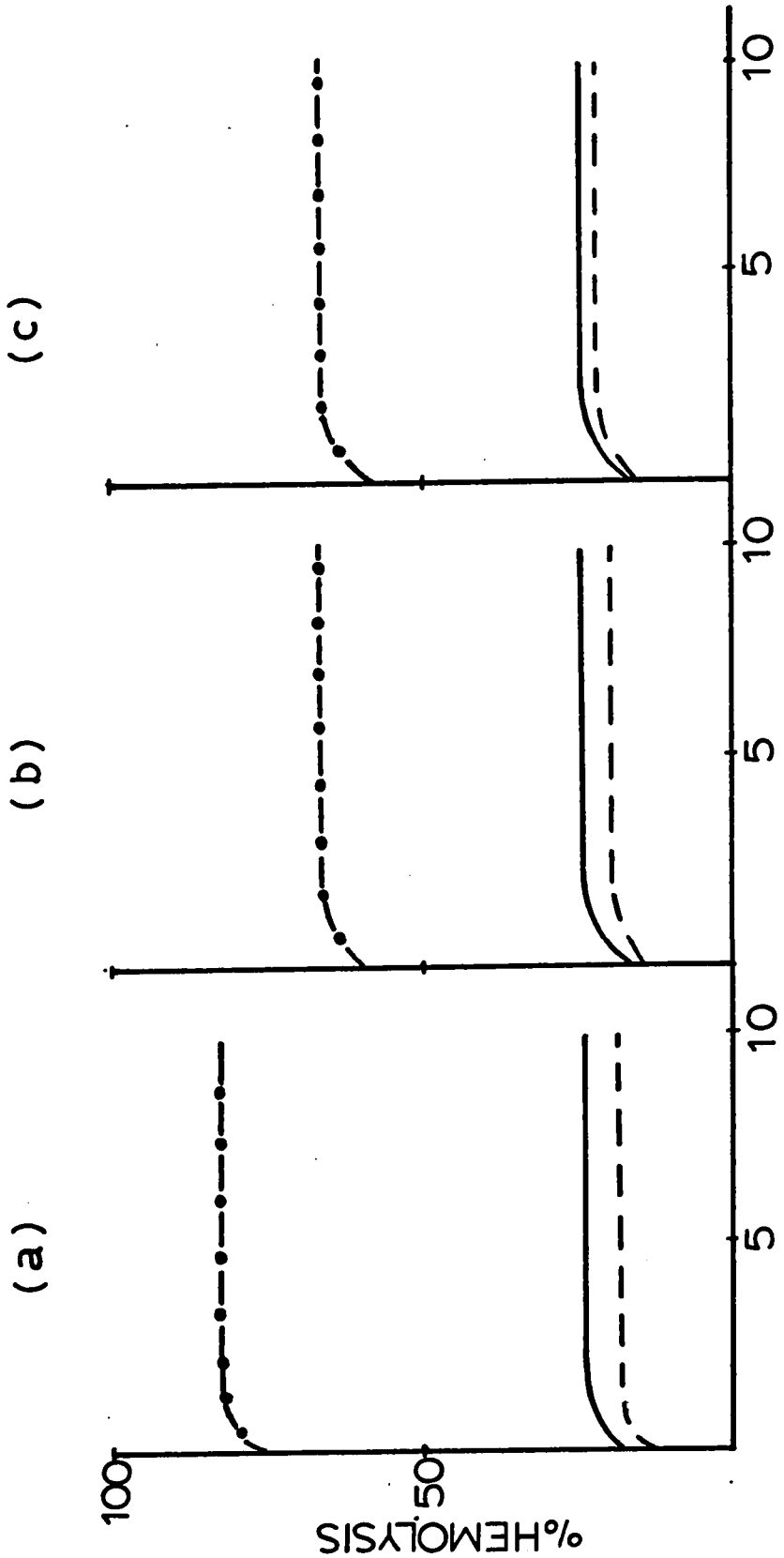


Fig.12.

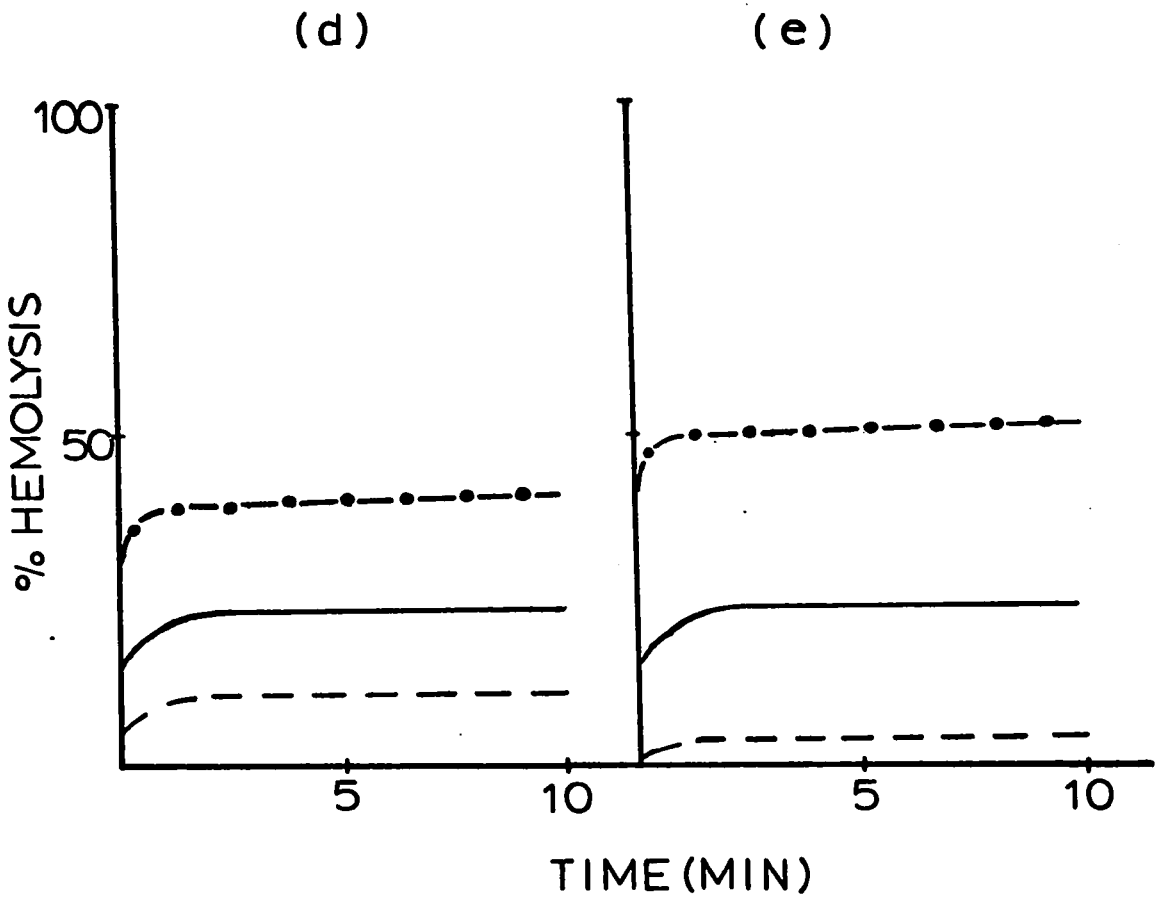


Fig.12. Acceleration of lysolecithin hemolysis by lipids and membrane protein. The concentration of lysolecithin used was $5 \cdot 10^{-6}$ M. All other coditions were as stated for Fig.11.

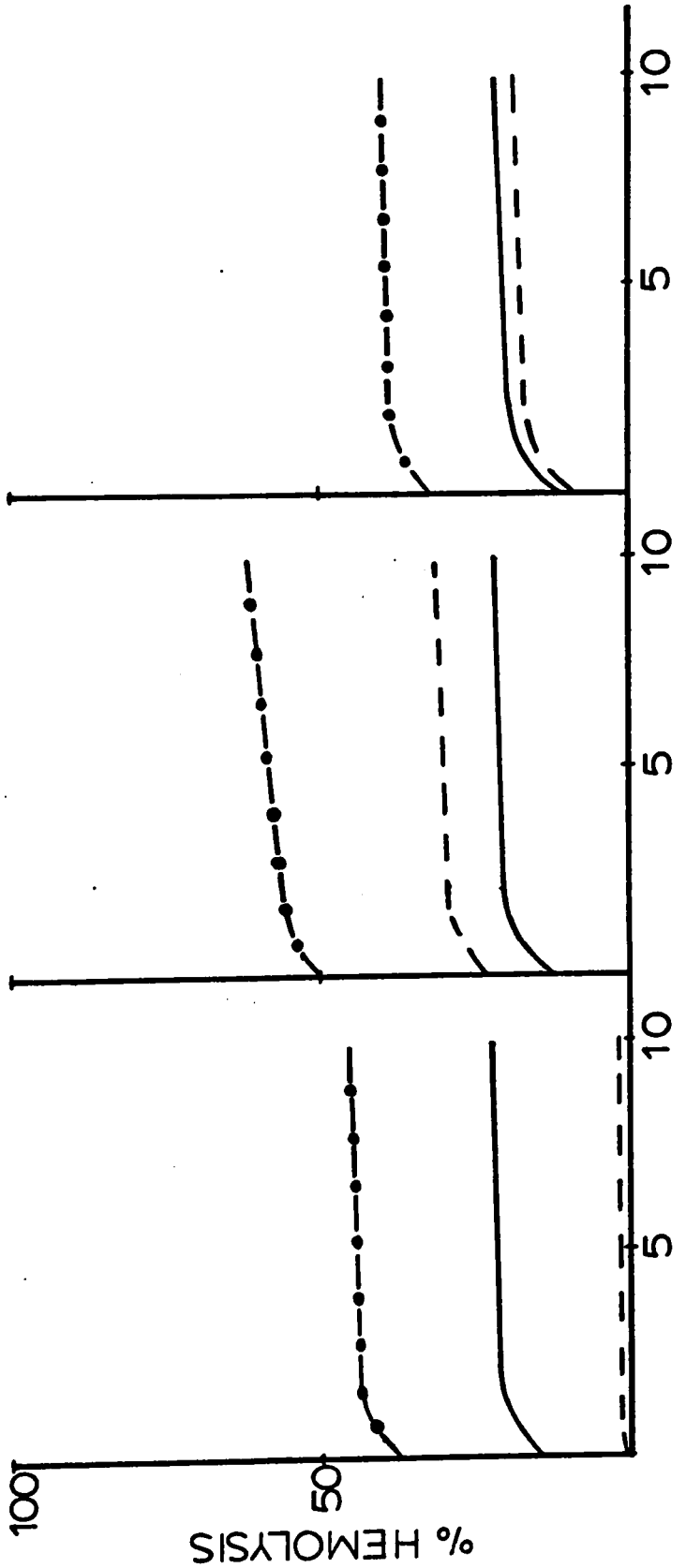


Fig.13.

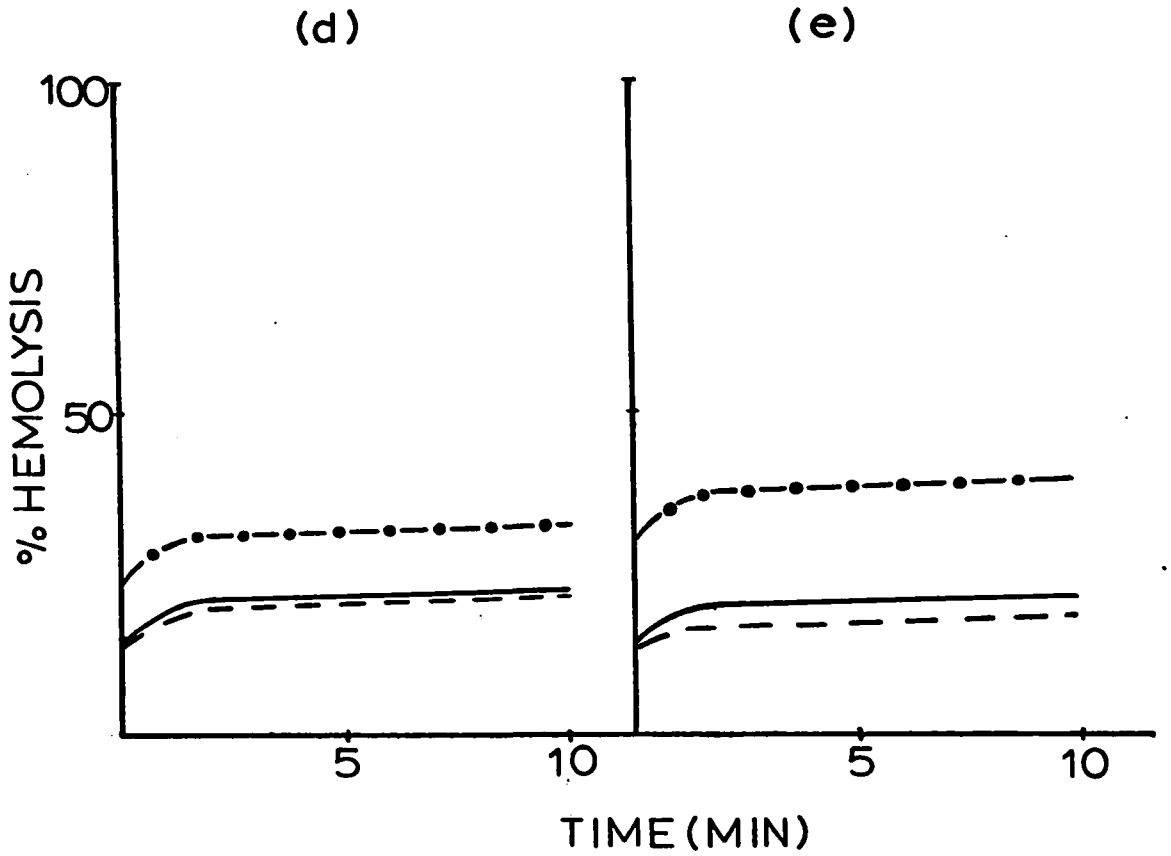


Fig.13. Acceleration of palmitoyl carnitine hemolysis by various polar substances (a) 1.035 mg/ml albumin; (b) $5 \cdot 10^{-4}$M glucose; (c) $5 \cdot 10^{-4}$M glycerol; (d) $5 \cdot 10^{-4}$M glycine; and (e) $2.5 \cdot 10^{-4}$M cystine. All other conditions were as stated for Fig.11.

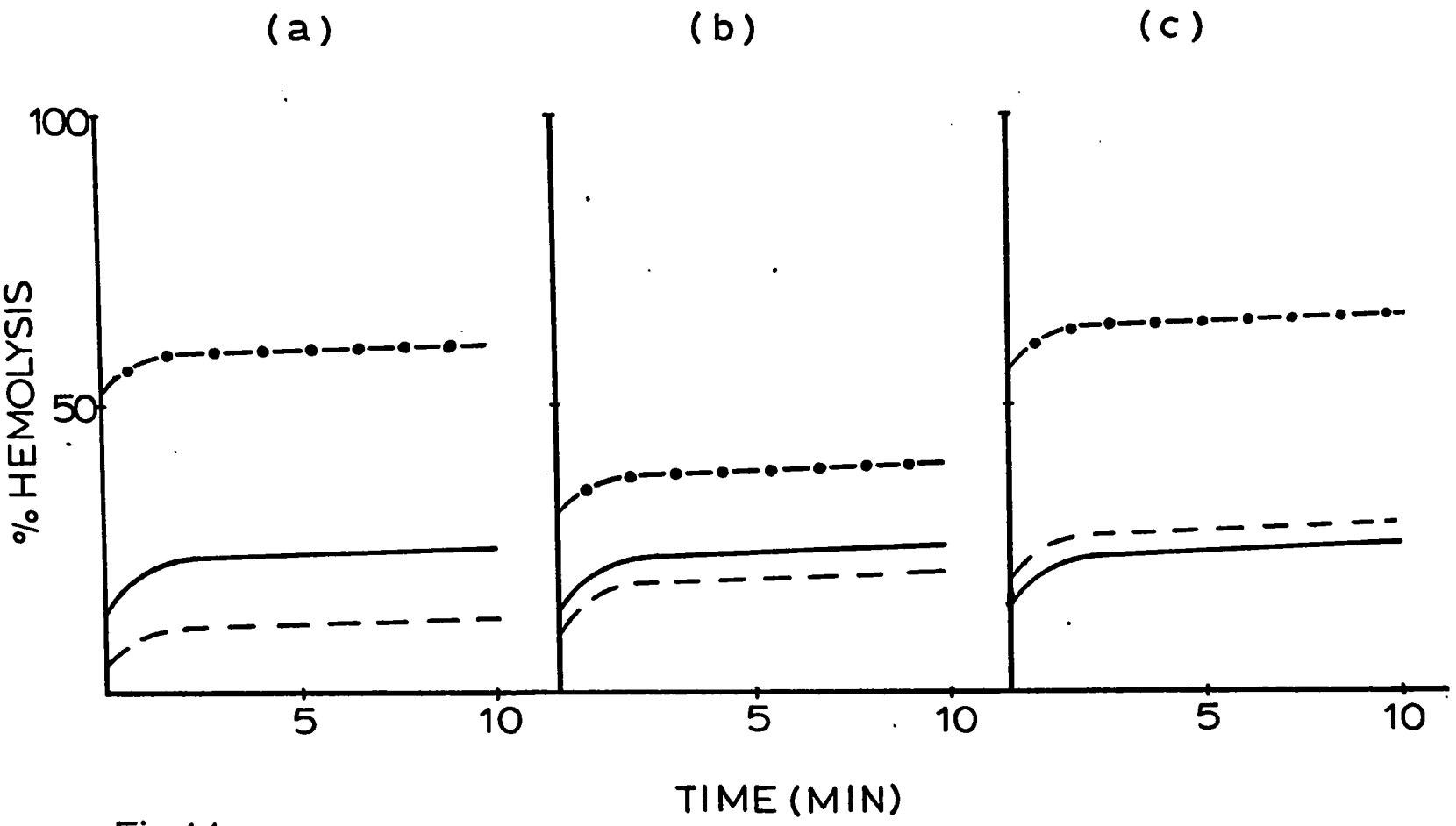


Fig.14.

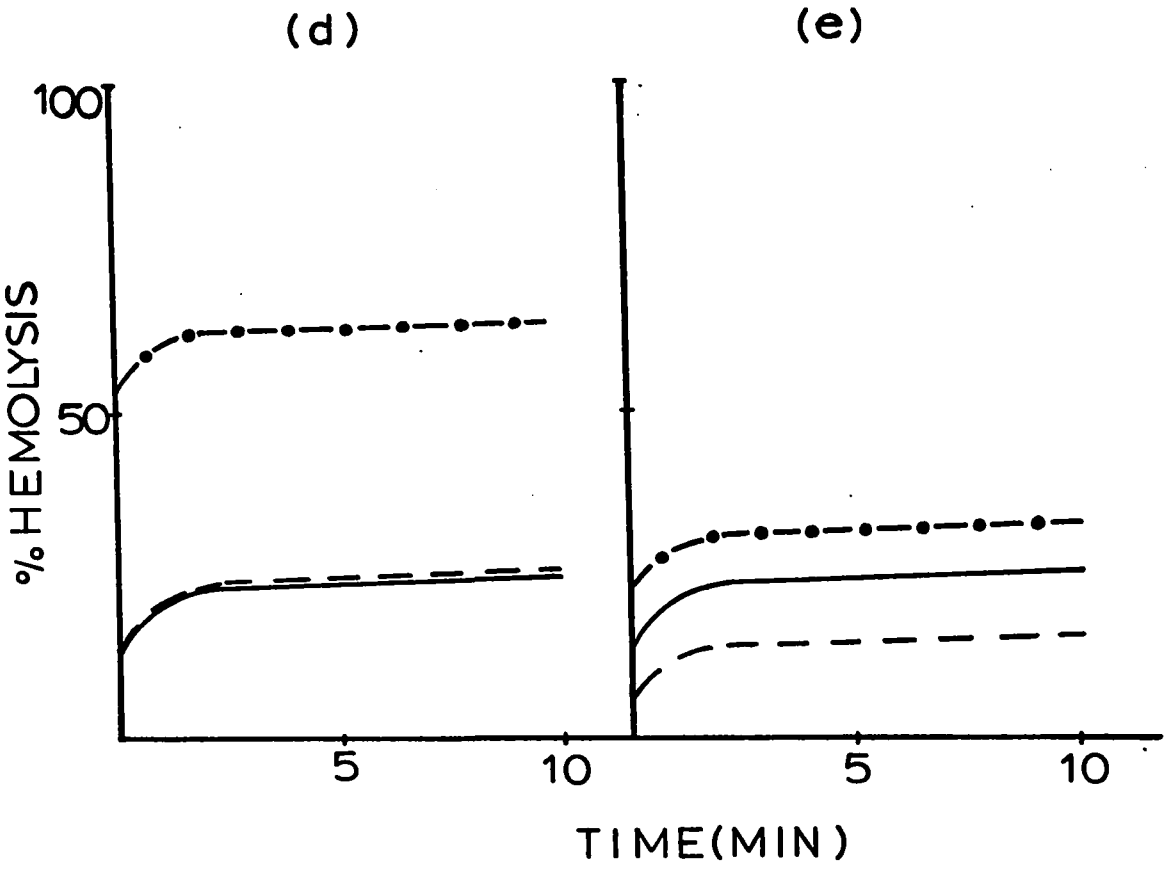


Fig.14. Acceleration of lysolecithin hemolysis by polar substances such as stated for Fig.13. The final concentration of lysolecithin used was $5 \cdot 10^{-6}$M. All other conditions were as stated for Fig.11.

PART III. Release of Phospholipid-P^{32} and Cholesterol-H^3 from Red Blood Cell during Cell Lysis.

I. Materials and Methods.

Rat red blood cells were labelled with ^{32}P-labelled hepatic lipid prepared as stated on page 115 or ^{3}H-cholesterol in vitro. 10 ml (50 % hematocrit) of red blood cells were incubated at 37 °C for 3 hours with 2 ml of total lipid (37,500 cpm/mg/ml) and also with 2 ml of cholesterol-H^3 (200,000 cpm/mg/ml), only a small amount of lysis occurred during incubation. The labelled erythrocytes were washed several times with isotonic saline at pH 7.4 until the supernatant showed only background radioactivity. Finally the packed erythrocytes were diluted to give a 50 % hematocrit.

In order to know the effect of lysins on the membrane lipids during hemolysis, three different weakly-lytic concentrations of lysin were incubated with labelled erythrocytes, i.e., 3 ml (50 % hematocrit) erythrocytes were incubated with 0.2, 1 and 2 u moles of stearoyl-, palmitoyl- and myristoyl carnitines, palmitoyl choline and (1-palmitoyl)-lysolecithin in a final volume 5 ml at 37 °C for 10 min in water bath with gentle shaking. The incubated cells were centrifuged for 5 minutes with a clinical centrifuge at maximum speed. The clear supernatant was separated from precipitated cells.

0.5 ml of supernatant was mixed with scintillation liquid for counting of radioactivity released and the remaining solution was used for measurement of % hemolysis. The precipitated cells were also resuspended in a known volume of saline and counted for radioactivity.

Measurement of Radioactivity

Radioactivity of ^{3}H- or ^{32}P-labelled substances were measured in a Beckman LS 133 liquid scintillation counter. 20 ml glass vials containing 18 ml of 0.6 % 2,5-diphenyloxazole (P.P.O) in toluene were used for this purpose.

The scintillation liquid for measurement of aqueous samples was prepared as follows: 11.1 g of P.P.O were dissolved in a mixture of 1,250 ml toluene and 600 ml ethanol. 18 ml of scintillation liquid were needed for 0.5 ml of aqueous solution. Radioactivity counted was corrected with a standard quench curve prepared with this fluor mixture and the addition of chloroform.

Measurement of % Hemolysis

The % hemolysis was measured against a saline blank with a Cary 15 spectrophotometer at wavelength 625 mu as previously described on page 77.

II. Results

Table 1 shows the release of phospholipid from erythrocytes by weakly-lytic amounts of stearoyl-, palmitoyl- and myristoyl carnitines, palmitoyl choline and (1-palmitoyl)-lysolecithin. The release of labelled phospholipid is proportional to the amount of lysin added, the chain length in acyl carnitines and extent of hemolysis. Similar results were obtained with cholesterol-H^3 labelled cells as shown in Table 2.

Results are the average of 2 experiments.

Table 1

The Amount of P^{32}-Phospholipid released from Red Blood Cell during Hemolysis by Carnitine Acyl Esters, Palmitoyl Choline and (1-Palmitoyl)-lysolecithin.

Lysins (u moles)		Phospholipid bound (ug)	Phospholipid* released (%)	Hemolysis (%)	Recovery (%)
Control I		219.2			100.0
Control II		189.6	11.8	0.0	98.3
Ste-Car.	$2x10^{-1}$	157.6	18.9	3.5	90.8
	$10x10^{-1}$	142.4	35.5	11.0	100.5
	$20x10^{-1}$	81.6	64.4	16.0	101.5
Pal-Car.	$2x10^{-1}$		17.3	2.0	
	$10x10^{-1}$		32.9	9.0	
	$20x10^{-1}$		58.4	13.0	
Myr-Car.	$2x10^{-1}$		15.1	0.5	
	$10x10^{-1}$		30.1	1.0	
	$20x10^{-1}$		46.3	3.5	
Lyso-PC.	$2x10^{-1}$		16.4	2.5	
	$10x10^{-1}$		32.9	10.0	
	$20x10^{-1}$		55.6	13.0	
Pal-Chol.	$2x10^{-1}$		16.2	0.0	
	$10x10^{-1}$		31.0	4.0	
	$20x10^{-1}$		55.1	7.0	

3 ml (50 % hematocrit) erythrocytes (P32-lipid labelled, 8,220 cpm) were incubated with 0.2, 1 and 2 u moles of stearoyl-, palmitoyl- and myristoyl carnitines, palmitoyl choline and (1-palmitoyl)-lyso-lecithin in a final volume of 5 ml at 37 oC for 10 min in water bath with gentle shaking. After centrifugation at 3,000 g for 10 min, the clear supernatant was separated from precipitated cells. 0.5 ml of supernatant was mixed with scintillation liquid for counting and the remaining solution was used for measurement of % hemolysis. The precipitated cells were also resuspended in a known volume of saline and counted for radioactivity.

*Phospholipid released was calculated from counts recovered in the bulk phase but excluded unlabelled phospholipid release.

Table 2

The Amount of H^3-Cholesterol released from Red Blood Cell during Hemolysis by Carnitine Acyl Esters, Palmitoyl Choline and (1-Palmitoyl)-lysolecithin.

Lysins (u moles)		Cholesterol bound (ug)	Cholesterol released (%)	Hemolysis (%)	Recovery (%)
Control I		248.8			100.0
Control II		238.9	3.1	0.0	99.2
Ste-Car.	$2x10^{-1}$	234.0	7.8	6.5	101.8
	$10x10^{-1}$	171.3	25.3	18.0	94.1
	$20x10^{-1}$	150.5	41.8	21.0	102.3
Pal-Car.	$2x10^{-1}$		5.5	5.0	
	$10x10^{-1}$		21.8	10.0	
	$20x10^{-1}$		32.6	15.0	
Myr-Car.	$2x10^{-1}$		4.5	3.0	
	$10x10^{-1}$		21.2	3.5	
	$20x10^{-1}$		29.6	4.5	
Lyso-PC.	$2x10^{-1}$		7.2	3.5	
	$10x10^{-1}$		23.9	11.0	
	$20x10^{-1}$		33.3	16.0	
Pal-Chol.	$2x10^{-1}$		4.8	2.0	
	$10x10^{-1}$		21.3	6.0	
	$20x10^{-1}$		29.8	10.0	

3 ml (50 % hematocrit) erythrocytes (H^3-cholesterol labelled, 49,770 cpm) were used. All other conditions are as stated for Table 1.

III. Discussion

The red blood cell has served as a very useful model for studying the properties of biomembranes and the mechanisms involved with lysis. The hemolytic activity of paraffinic, ionic, surfactive compounds has been related to their ability to penetrate monolayers of cholesterol (143)(151). Such a relationship also exists between the lytic activity of polyene antibiotics, the presence of sterol in the membrane and the ability of these lysins to interact with monolayers containing cholesterol (195-197). Yet other lysins exemplified by a number of organic solvents miscible with water, cause rupture of the membrane possibly by raising the effective value of the dielectric constant within the membrane thereby reducing the stability of the ionic bonds constituting the structural network (198).

Figs. 2a - b and 4a - b show the comparative studies on the lytic potency of both homologuos series of acyl carnitines and acyl cholines with rat and human erythrocytes as test materials. Both series of compounds show an increased lytic activity with an increase in acyl chain length. The results from reman et al. (134) showed that the stearoyl- and palmitoyl lysoleci-

thins were found to be the most active among lysophosphoglycerides with acyl chain lengths ranging from 10 to 20 carbon atoms, whereas the short chain compounds like decanoyl lysolecithin showed no activity at all. Such evidence together with our results suggests some degree of non-polar interactions between quaternary ammonium containing agents and membrane constituents. Similar conclusions can be derived from work performed with anionic detergents (128). Beyond a chain length of 18 carbons, aqueous dispersions become markedly anisotropic when saturated derivatives are used and factors involved with penetration of the lysin into the membrane are likely to intervene. On the other hand, unsaturation tends to decrease lytic potency which again argues in favor of hydrophobic interactions of lysin acyl chains with membrane constituents.

Aside from non-ionic interactions, perturbations in the metal-ligand arrangements or in the ionic bonds involving protein and lipid might also intervene in the lytic process caused by the ionic detergents studied. In the case of acyl cholines which possess a net positive charge this seems more likely since relevant studies by Blaustein (199) and by Feinstein (200) have shown that a number of cationic drugs can displace Ca^{2+} from their

binding to acidic phospholipids. In the case of acyl carnitines which possess little or no net charge at physiologic pH a displacement of Ca^{2+} from acidic phospholipid is not as likely, although lipids, such as lecithin uncharged at physiological pH are capable of binding Ca^{2+} (201)(202). This bears on the observations of Manery (203) that Ca^{2+} has stabilizing effects on biomembranes a point which was not ignored by Burger et al.(204) in their considerations of lytic phenomena.

It is known that (-)-palmitoyl carnitine is the active stereoisomer required as a intermediate in the carnitine-induced stimulation of palmitate oxidation in mitochondria and (+)-palmitoyl carnitine acts as a competitive inhibitor (184). In connection with hemolysis, no differences due to stereoisomerism were noticed and consequently a stereospecific binding to membrane constituent would not be involved in the lytic action of acyl carnitines (Fig. 3).

As our results indicate, acyl carnitines, acyl cholines and lysolecithins have some biological properties which are similar. This might be expected from the fact that they are structurally related. All belong to a group of substances described by Haydon and Taylor (153)

as wedge-shape molecules. Acyl carnitines and lysolecithins are even more closely related since at physiological pH they possess no net charge and their polar head groups are of similar nature and size. Acyl cholines have somewhat lesser lytic potency and this might be related to the fact that they are cationic and possess a smaller polar head group. We hasten to say however that our results discussed so far permit no definite conclusions relating detailed structural features of the polar head group and the lytic potency of nitrogen containing amphipaths. Such studies are in progress in this laboratory. Our results however do show that the acyl chain length is an important feature determining the lytic potency of such compounds. The structural differences in these classes of lysins are small and thereare no very good grounds for inferring a basically very different mechanism of lysis in either of these cases. The fact is, they all cause a release of ^{32}P-labelled phospholipids and ^{3}H-labelled cholesterol from erythrocytes to an extent which matches the degree of resulting hemolysis and their lytic potencies.

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. 7a - 7j). 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 (127) 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. 8 - 10 illustrate that the inhibitory effects of lecithin, cholesterol and membrane protein against hemolysis by palmitoyl carnitine, palmitoyl choline and (1-palmitoyl)-lysolecithin, 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. 8a and 9a) results in a significant increase in inhibition in the case of palmitoyl carnitine lysis, but which is smaller in the case of (1-palmitoyl)-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. 8b) but insufficient to significantly affect the inhibition of lysolecithin hemolysis (Fig. 10b). 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 binding experiments which will be discussed in the next section, have clearly indicated that albumin as well as membrane protein and micellar lipids prepared from erythrocytes, can bind with ^{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 (156). In the case of lysolecithin however, Reman et al. (134) 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 (153) 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 (134). 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. 11 - 14) 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 constituents 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.

The aforementioned discussion has dealt with mechanisms involved with a gross phenomenon resulting in the death of the cell. Nevertheless, similar mechanisms implicating small concentrations of naturally occurring lysins might be concerned in a more refined phenomenon such as the permeability of the cell. In this regard a study of sublytic concentrations of acyl carnitines and other natural amphipaths on the permeability of the red blood cell may be of considerable value. Furthermore, studies possibly implicating the lytic properties of such compounds with release of substances from the cells or from subcellular organelles, e.g. the release of bound acetylcholine or adrenaline, might well define in broader terms the physiological role of acyl carnitine and the enzyme responsible for its formation.

SECTION II

Studies on the Effects of Lysins on Reconstituted Lipid-Protein Complexes.

I. Materials and Methods

(A) Materials

DL-Carnitine-methyl-C^{14} was purchased from Tracerlab, Mass., U.S.A and used for synthesis of palmitoyl carnitine-C^{14} and decanoyl carnitine-C^{14} as described earlier.

Choline-methyl-C^{14}, cholesterol-H^3 and $H_3P^{32}O_4$ were purchased from New England Nuclear Corp., Mass., U.S.A. Choline-methyl-C^{14} was used for synthesis of palmitoyl choline-C^{14} as described for the synthesis of carnitine esters.

Membrane proteins used in this experiment were isolated from bovine erythrocyte ghosts by two different methods. Bovine blood was obtained from a slaughter house in Ottawa.

Triton X-100 from Hartman Leddon Co., Philadelphia, was prepared as a 5% solution by addition of 95 ml of distilled water to 5 ml of Triton X-100 for solubilization of membrane.

2-Chloroethanol was purchased from Eastman Organic Chemicals, New York.

Ammonium sulfate from Schwarz Mann Co., New York, ultra pure was used for protein isolation.

Other solvents and reagents, such as acetic acid, acetone, n-butanol, methanol, toluene, NaOH, urea and sucrose were obtained from Fisher Scientific Co., U.S.A.

Cetyltrimethyl ammonium chloride (hexadecyl ammonium chloride) and sodium dodecyl sulfate were purchased from Fisher Scientific Co. and purified as described for choline esters.

Tris-(hydroxymethyl)-aminomethane was obtained from General Biochemicals, Ohio.

P.P.O (2.5-Diphenyloxazole) and dimethyl POPOP were supplied by Fraser Medical Supplied Ltd., Vancouver.

Dialysis tubing (flat width , $1\frac{1}{8}$ inches) was purchased from Canlab, and washed with distilled water and soaked for 24 hours before use.

(B) Methods

(1) Synthesis of Palmitoyl Carnitine-C^{14}, Decanoyl Carnitine-C^{14} and Palmitoyl Choline-C^{14}.

Palmitoyl carnitine-C^{14} and decanoyl carnitine-C^{14} were synthesized as previously described in Section I. Palmitoyl choline-C^{14} was synthesized as the same method for palmitoyl carnitine.

(2) Preparation of P^{32}-labelled Total Lipid, Lecithin and Lysolecithin.

Total lipid-P^{32}, lecithin-P^{32} and lysolecithin-P^{32} were prepared *in vitro* as follows; rat liver was removed from 3 - 4 weeks old rat and soaked in cold Krebs Ringer bicarbonate buffer at pH 7.4. About 2 gm (wet weight) of liver were cut into small pieces and placed in a 50 ml test tube. 5 mC-P^{32}, 2 ml of 0.1 M acetate buffer pH 7.4 containing 10 mg glucose per ml, and a trace of CDP-choline or CDP were added to the sliced liver. The pH of a mixture was adjusted to 7.4 with 0.1 N NaOH solution. This mixture was incubated for 3 - 4 hours at 37 °C in a water bath with mechanical shaking and oxygenated.

Total lipid was extracted from the incubation mixture as described by the method of Bligh and Dyer. This preparation was used for P^{32}-labelled total lipid in the experiment to follow. A part of this preparation was used for lecithin-P^{32} and lysolecithin-P^{32} isolation.

Lecithin-P^{32} was separated from P^{32}-total lipid by preparative thin-layer chromatography and purified through a Sephadex column as indicated previously.

Lysolecithin-P^{32} was prepared by the action of snake venom phospholipase A as previously described in Part I, Section I.

(3) Isolation of Structural Protein from Erythrocyte Stroma.

Structural protein was prepared from bovine erythrocyte ghosts by the method of Schneiderman and Junga (65) with slight modification. Preparation of erythrocyte stroma was described in Part II, Section I. Packed bovine erythrocyte stroma was suspended in 1.65×10^{-4} M Tris buffer (50 mg/ml) and then sonicated for 5 minutes. An equal volume of 5 % Triton X-100 was added and was sonicated again until the mixture was clear, then centrifuged at 45,000 g at 4 °C for 30 minutes in refrigerated Beta-Fuge model A centrifuge. The supernatant was adjusted to pH 3 with 75 % acetic acid, then brought to 12 % saturation with ammonium sulfate and was stirred at room temperature for 30 minutes. The precipitate was centrifuged at 45,000 g for 30 minutes and washed twice with cold distilled water, then suspended in 0.1 N NaOH-8M urea solution at a concentration representing 50 mg/ml of original stroma. The suspension was sonicated for 5 minutes until clear and centrifuged at 45,000 g at 4 °C for 30 minutes. The pH of supernatant was again adjusted to 3 with 75 % acetic acid and brought to 40 % saturation with ammonium sulfate,

then stirred at room temperature for 30 minutes. The precipitate was now separated by centrifugation under the same condition as above, and washed twice with distilled water, then homogenized in 4 : 1 acetone-butanol (v/v) before being collected on a glass filter. This filtrate was thoroughly washed with same solvent several times followed by addition of 75 % methanol heated to 50 °C, and finally washed with acetone. A white powdered structural protein is thus obtained. Chloroform-methanol (2:1, v/v) extraction of this preparation revealed the absence of lipid after thin-layer chromatography.

(4) Preparation of Membrane Protein from Erythrocyte Stroma by the Maddy Method.

10 ml of bovine erythrocyte stroma (50 mg/ml) was cooled to 0 °C in an ice-water bath and 7.5 ml of n-butanol was added at this temperature. This mixture was shaken vigorously for 5 minutes and kept in ice bath for 15 - 20 minutes. This mixture was subsequently centrifuged at 27,000 g for 10 minutes when it separated into an upper butanol phase containing the lipid, overlying the aqueous protein solution and a thin interfacial film of insoluble protein. The lower aqueous phase was drawn

carefully into tube in an ice bath. This aqueous phase was dialysed in ice cold water until no odour of butanol was detectable and further for 24 hours.

This membrane protein is soluble under physiological conditions of pH and ionic strength, but precipitated between pH 3.7 - 4.8. This membrane protein was kept precipitated at pH 4.0 with 0.1 N HCl addition for future binding experiments.

(5) Methods for Binding Experiments.

Several methods have been known for measurement of binding of protein with other molecules; i.e., equilibrium dialysis, ion exchange, gel filtration, sedimentation by centrifugation and sucrose density gradient ultracentrifugation methods. Among them, equilibrium dialysis, sedimentation and ultracentrifugation methods were adapted for our experiments. The equilibrium dialysis method was used for the measurement of interactions between lysin and protein or lipid micelles, and the sedimentation method for binding of lipid or lysin micelles to structural or Maddy proteins. The ultracentrifugation method was applied to complexes formed between protein and lipid or lysin to determine more clearly their mode of association.

(a) Equilibrium Dialysis

Tris-acetate buffer of pH 7.4, 10 mM, prepared with an enzyme research grade and deionized distilled water, was used as solvent.

A known amount of lysin was mixed with a known concentration of protein or lipid micelles in the dialysis bag, and adjusted to a volume of 5 ml with Tris-acetate buffer. The dialysis bag was placed in a 125 ml erlenmyer flask containing 75 ml Tris-acetate buffer. The flask was sealed with parafilm. A control blank prepared either without protein or lipid micelles was incubated simultaneously. Equilibrium was attained by shaking the flask gently in a water bath at 37 ^{o}C for 24 hours. The inner solution was removed and adjusted to a volume 6 ml in a 10 ml graduated test tube. 0.5 ml of this solution was mixed with scintillation fluid and counted as described previously. The amount of binding was calculated by subtraction of the blank counts from sample counts.

(b) Sedimentation by Centrifugation for Assessing Binding

(i) Studies with Structural Protein

Structural protein (acetone powder) suspended in 0.02 M Tris-acetate buffer, pH 7.4 was homogenized and adjusted to a concentration to 10 mg/ml. 5 mg structural protein was mixed with a known amount of lipid or lysin

solution in a centrifuge tube, and adjusted to a volume of 5 ml with Tris-acetate buffer. Incubation was carried out in a shaking water bath at 37 °C for 30 minutes, then 10 ml warm water (37 °C) was added and the suspension was centrifuged at 10,000 g at room temperature for 10 min. The pellet was washed twice more with 15 ml of warm water to remove unbound lysin or lipid and suspended by addition of few drops of water (total volume less than 0.5 ml) and then added to 18 ml of scintillation liquid for counting. The counts were taken as the amount of lipid or lysin bound to the protein. Recoveries of protein were over 95 %.

(ii) Studies with Maddy Protein

Maddy protein precipitated at pH 4.0 was suspended in 0.05 M acetate buffer and adjusted to a concentration to 10 mg/ml. 5 mg Maddy protein was mixed with a known amount of lipid solution in a centrifuge tube and adjusted to a volume of 5 ml with acetate buffer. Incubation was usually carried out in a shaking ice-water bath for 15 min and the lipoprotein formed was collected by centrifugation at 3,000 g for 10 min and washed twice with distilled water.

For the effect of lysin on reconstituted lipoprotein stability, lipoprotein was mixed with a known concentration of lysin and adjusted to a volume of 5 ml with acetate buffer, pH 4.0 or Tris-acetate buffer, pH 7.0, and then incubated

for 30 min at 37 °C in a shaking water bath. After separation of supernatant and precipitate by centrifugation, the radioactivity was counted in both fractions.

(c) Sucrose Density Gradient Ultracentrifugation

Maddy protein precipitated at pH 4.0 was incubated with lipid or lysin at pH 4. After washings, the pellet was suspended with a few drops of sucrose solution (13 %) by agitating with a glass rod and adjusted a volume of 0.5 ml with sucrose. The suspended sample (0.5 ml) was gently layered over a linear sucrose density gradient containing 3.5 ml in a 4.5 ml polyethylene centrifuge tube. The density gradient varied from 50 % sucrose at the bottom to 13 % sucrose at the top of the tube. The tubes were then loaded in a precooled swinging bucket rotor SW-56, and were centrifuged in a refrigerated Beckman Model L2-65B ultracentrifuge for 2 hours at 120,000 x g.

Each tube was divided into eight fractions by successive removal of 0.5 ml of aliquots with a syringe. Each sample was divided in two fractions, one for measurement of radioactivity due to lipid or lysin and the other for protein estimation.

II. Results

PART I. Studies with Structural Protein.

(A) Binding of Palmitoyl Carnitine-C^{14}, Palmitoyl Choline-C^{14} and Lysolecithin-P^{32} to Protein, Lecithin and Cholesterol by Equilibrium Dialysis.

When 0.1 mM (0.218 mg/5 ml) palmitoyl carnitine (specific activity, 802,752 cpm/mg) was equilibrated with different concentrations of albumin, structural protein, lecithin and cholesterol at pH 7.4 for 24 hours results expressed in Figs. 15 - 18 were obtained.

Binding of palmitoyl carnitine-C^{14} with acetone-treated albumin, structural protein, lecithin and cholesterol by equilibrium dialysis gave direct evidence that this lysin interacts nonspecifically with a variety of substances, some of which form part of membrane. Palmitoyl carnitine binded more avidly with structural protein than with albumin (Fig. 15) and more readily with cholesterol than with lecithin sols (Fig. 16).

Figs. 17 and 18 also demonstrate that palmitoyl choline-C^{14} and lysolecithin-P^{32} bind nonspecifically with lecithin and cholesterol micelles under the same

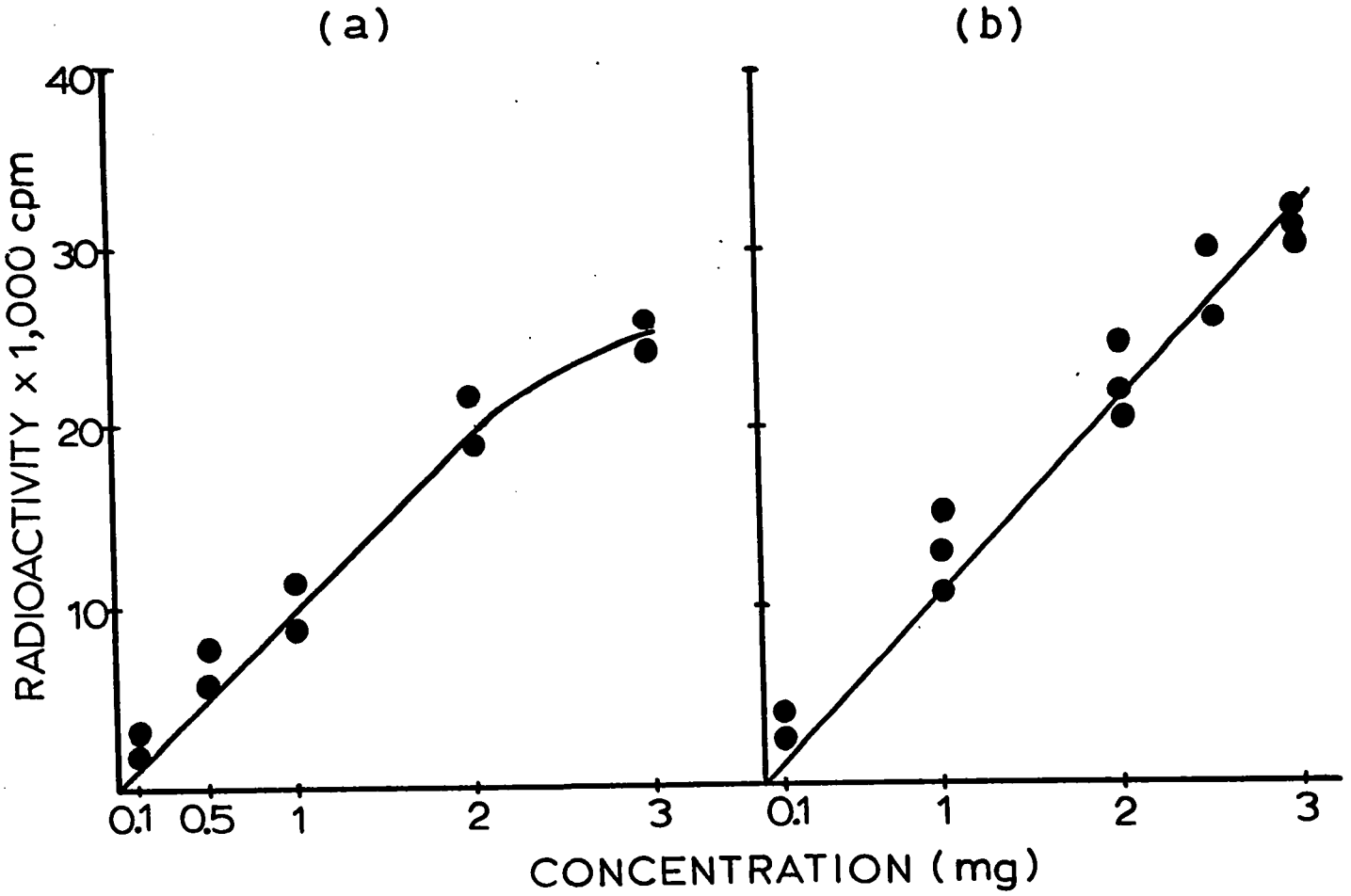


Fig. 15. Binding of Palmitoyl-DL-carnitine-C^{14} with Albumin (a) and Membrane Protein (b) by Equilibrium Dialysis.

0.1 mM (0.218 mg/5 ml) palmitoyl carnitine-C^{14} (Sp.Act., 802,752 cpm/mg) was mixed with different concentrations of albumin or membrane protein in a dialysis tube and placed in 125 ml erlenmyer flask containing 75 ml of 10 mM Tris-acetate buffer, pH 7.4. This mixture was equilibrated with gentle shaking in a 37 °C water bath for 24 hours. The volume inside dialysis bag was adjusted to 6 ml and duplicate 0.5 ml of aliquots were mixed with scintillation liquid for counting.

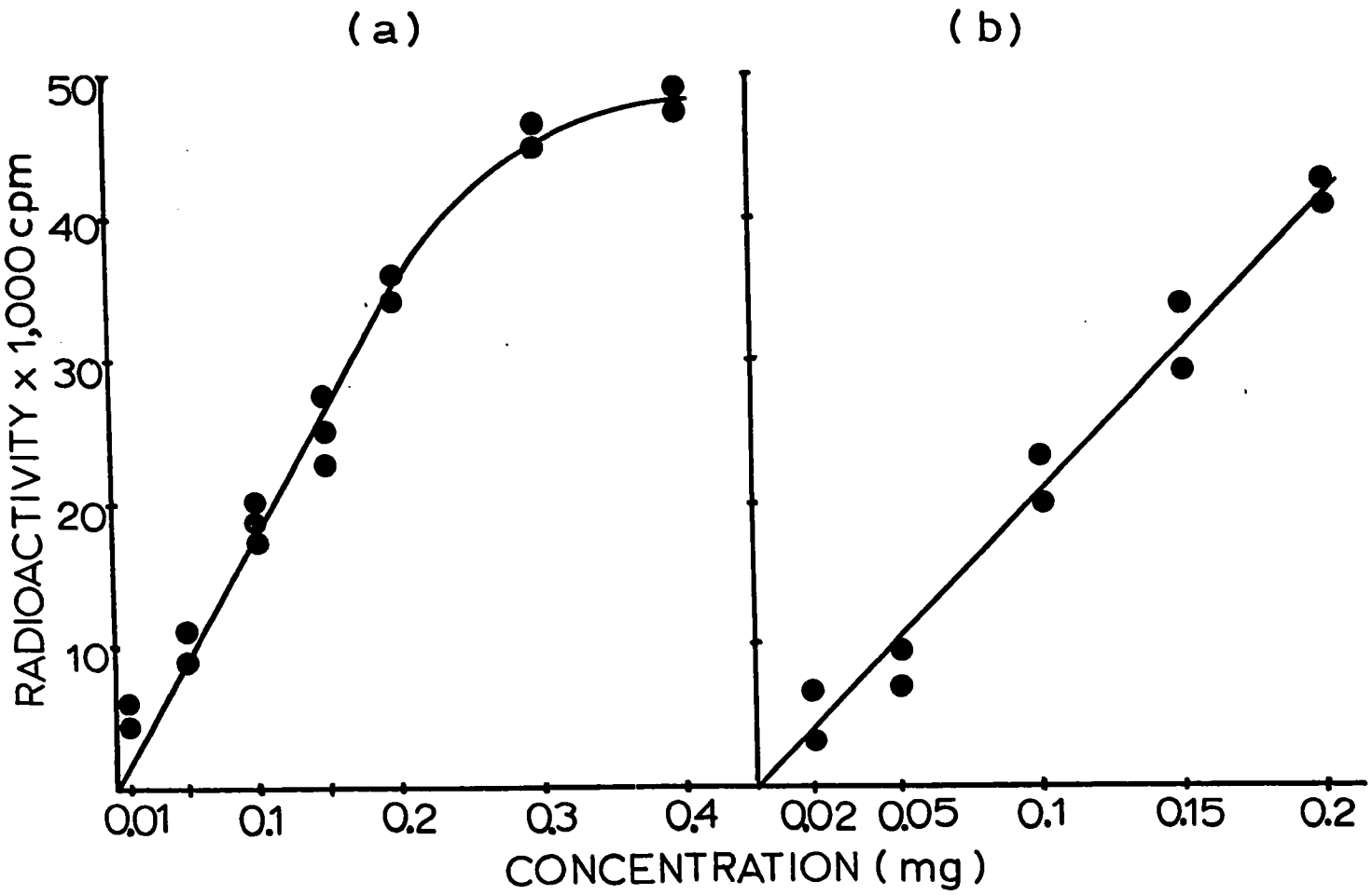


Fig. 16. Binding of Palmitoyl-DL-carnitine-C^{14} with Lecithin (a) and Cholesterol (b) by Equilibrium Dialysis.

All conditions for equilibrium dialysis were as stated for Figure 15.

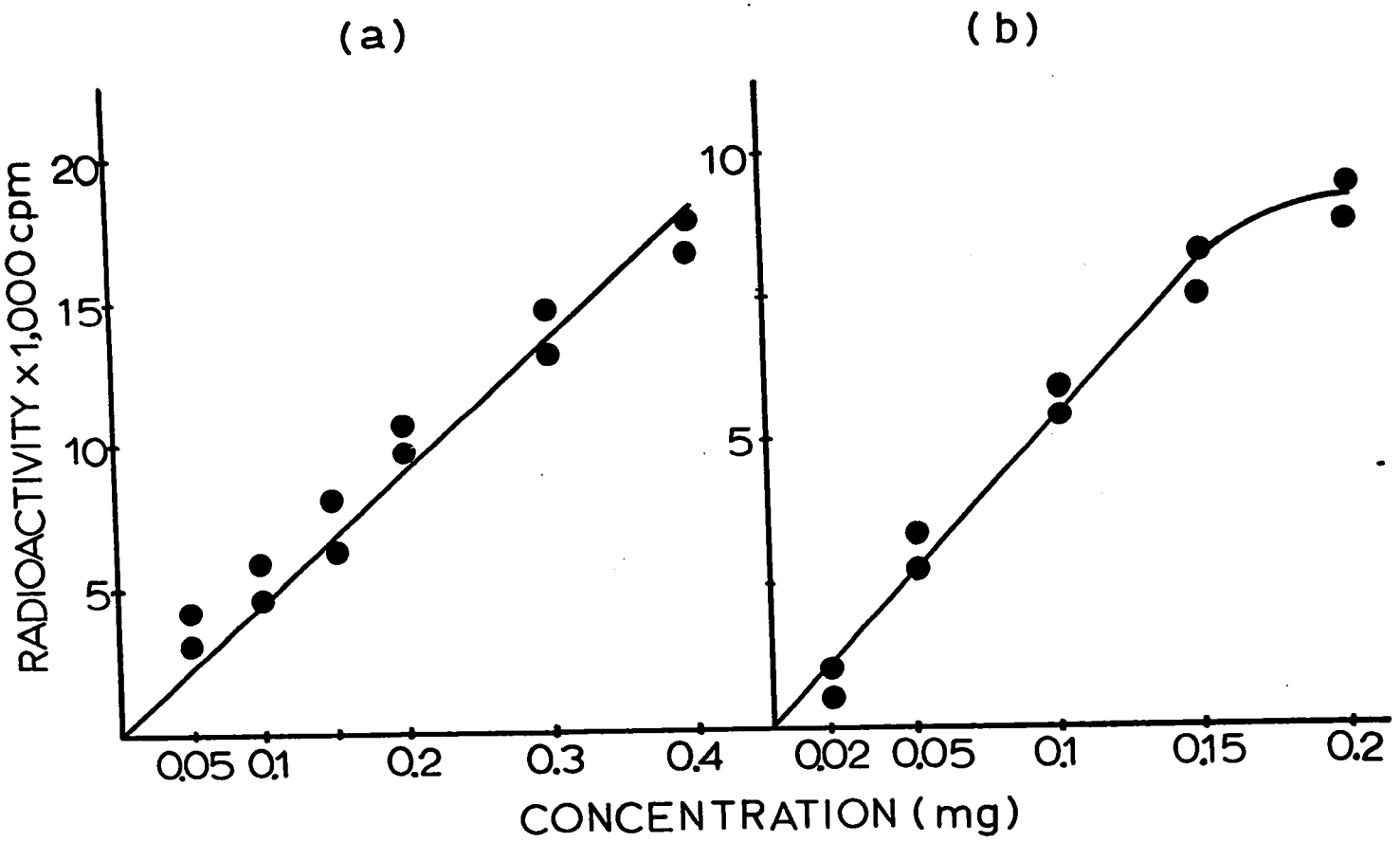


Fig. 17. Binding of Palmitoyl choline-C^{14} with Lecithin (a) and Cholesterol (b) by Equilibrium Dialysis.

The concentration of palmitoyl choline-C^{14} used was 0.1 mM (0.189 mg/5 ml, Sp.Act., 275,132 cpm/mg). Other conditions were as stated for Figure 15.

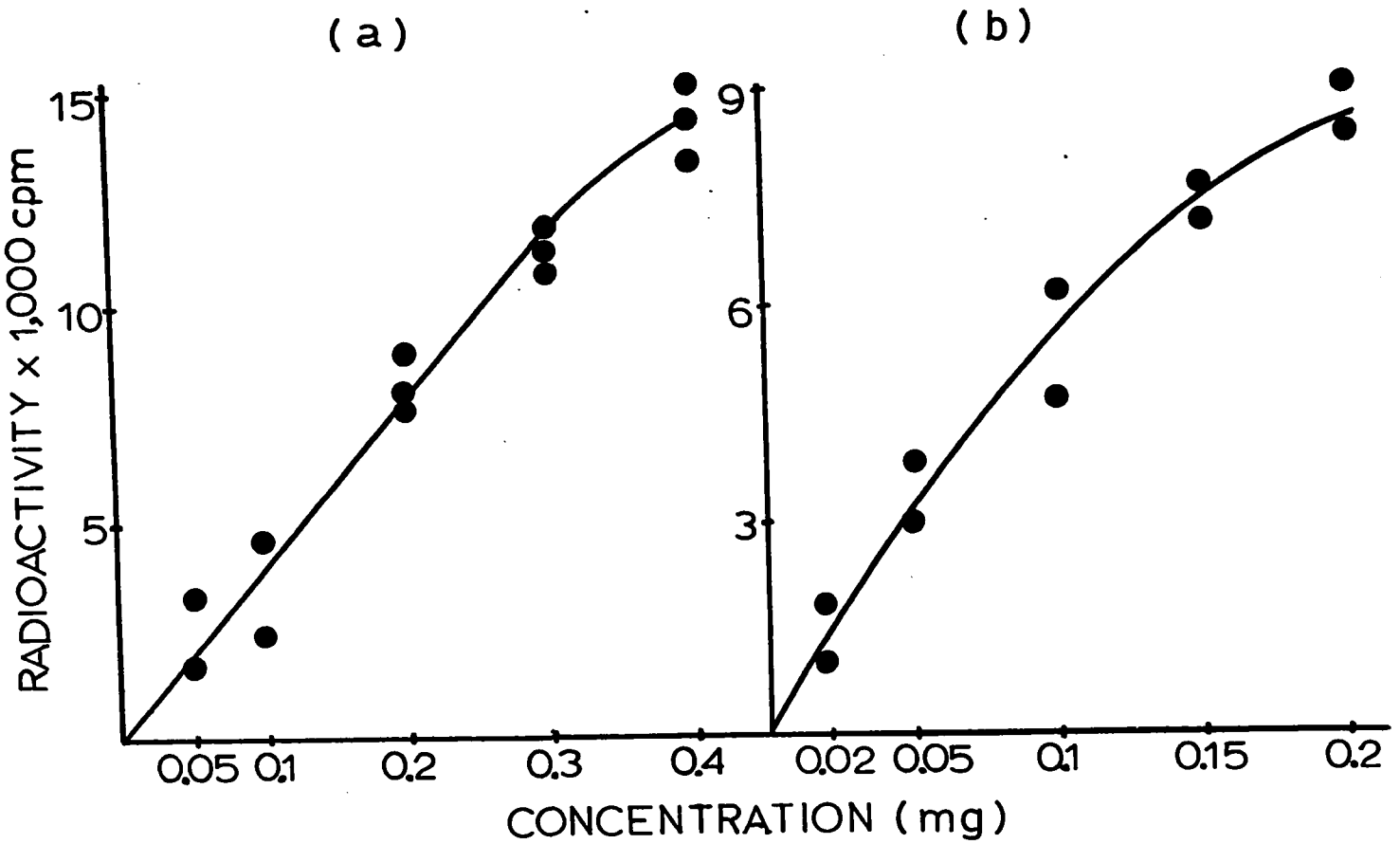


Fig. 18. Binding of Lysolecithin-P^{32} with Lecithin (a) and Cholesterol (b) by Equilibrium Dialysis.

The concentration of lysolecithin-P^{32} used was 0.1 mM (0.266 mg/5 ml, Sp.Act., 212,067 cpm/mg). Other conditions were as stated for Figure 15.

conditions. By comparison 1 mg of lecithin binds with 0.202mg palmitoyl carnitine, 0.16 mg palmitoyl choline and 0.195 mg lysolecithin. Whereas 1 mg cholesterol binds with 0.27 mg of palmitoyl carnitine, 0.2 mg of palmitoyl choline and 0.37 mg of lysolecithin, respectively.

(B) Binding of Lysins to Structural Protein.

As can be seen in Table 3, palmitoyl carnitine, palmitoyl choline and lysolecithin bind to structural protein, when incubated for 30 minutes at 37 °C. By comparison, at pH 7.4, 5 mg of structural protein bound nearly equal quantities of lysolecithin and palmitoyl carnitine whereas palmitoyl choline was bound somewhat more readily. On the other hand, decanoyl carnitine was not bound which indicated a hydrophobic interaction of lysin with protein.

Effect of pH and 1 M NaCl.

Table 4 shows the binding of palmitoyl carnitine-C^{14}, lysolecithin-P^{32}, lecithin-P^{32} and cholesterol-H^{3} to structural protein at pH 4, 7 and 8 with and without 1 M NaCl. As can be seen, increasing the ionic strength

Table 3 *

Binding of Palmitoyl Carnitine-C^{14}, Decanoyl Carnitine-C^{14}, Palmitoyl Choline-C^{14} and Lysolecithin-P^{32} to Structural Protein.

Lysins	Bound Radioactivity (cpm)	Bound (mg)
Palmitoyl Carnitine	104,135	0.08
Decanoyl Carnitine	146	0.00
Palmitoyl Choline	38,310	0.15
Lysolecithin	11,520	0.05

5 mg of structural protein were incubated with 0.2 mg of palmitoyl carnitine-C^{14} (Sp.Act., 1,315,660 cpm/mg), decanoyl carnitine-C^{14} (Sp.Act., 835,000 cpm/mg), palmitoyl choline-C^{14} (Sp.Act., 252,000 cpm/mg) and lysolecithin-P^{32} (Sp.Act., 234,000) in 0.02 M Tris-acetate buffer, pH 7.4 for 30 min at 37 °C water bath in total volume 5 ml. The precipitate washed twice with warm water (37°C) by centrifugation at 3,000 g for 10 min, was mixed with 0.1 ml water and transferred to a scintillation vial for counting.

* Results are the average of 3 experiments.

Table 4 *

Effect of pH and 1 M NaCl on the Binding of Palmitoyl Carnitine-C^{14}, Lysolecithin-P^{32}, Lecithin-P^{32} and Cholesterol-H^3 to Structural Protein.

Substrate	pH					
	4.0		7.0		8.0	
	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
	(ug)		(ug)		(ug)	
Pal-Carn. 500 ug	184.5	194.3	195.2	198.1	161.4	159.6
Lyso-PC. 500 ug	74.2	82.5	65.4	70.1	60.5	65.3
PC. 500 ug	423.9	318.1	60.6	60.3	50.8	55.2
Cholest. 200 ug	147.8	150.2	151.8	153.3	146.4	153.0

5 mg of structural protein were incubated with 0.5 mg palmitoyl carnitine-C^{14} (Pal-Carn., Sp.Act., 1,285,628 cpm/mg), 0.5 mg lysolecithin-P^{32} (Lyso-PC., Sp.Act., 215,900 cpm/mg), 0.5 mg lecithin-P^{32} (PC., Sp.Act., 41,930 cpm/mg) and 0.2 mg cholesterol-H^3 (Cholest., Sp.Act., 49,000 cpm/mg) at pH 4, 7 and 8 with and without 1 M NaCl in a total volume 5 ml. After incubation of 30 minutes, the complexes were washed twice with 10 ml warm distilled water and counted as usual.

* Results are the average of 3 experiments.

had no very marked effects on binding whereas acid pH favoured complex formation especially when lecithin was used as the reactive counterpart. The difference in behavior between lysolecithin and lecithin possessing the same ionic properties was an unexpected but reproducible result. It appeared that mainly hydrophobic interactions were involved between structural protein and the various lipids or lysins used. pH may have increased binding of lecithin by inducing conformational changes in the protein which would increase its hydrophobic character. Had this been the case, cholesterol binding should have also been favoured. However, the four reactants tried, only lecithin would likely form bilayer aggregates in aqueous media and it is therefore possible that changes in protein conformation could favour binding only with this reactant, but a different mode of interaction would have to be involved.

(C) Effect of Lysins on Reconstituted Lipoprotein Complexes.

Fig. 19 shows the effect of palmitoyl carnitine, myristoyl carnitine, decanoyl carnitine and lysolecithin on the release of lecithin-P^{32} and cholesterol-H^3 bound with structural protein.

5 mg of structural protein were incubated at pH

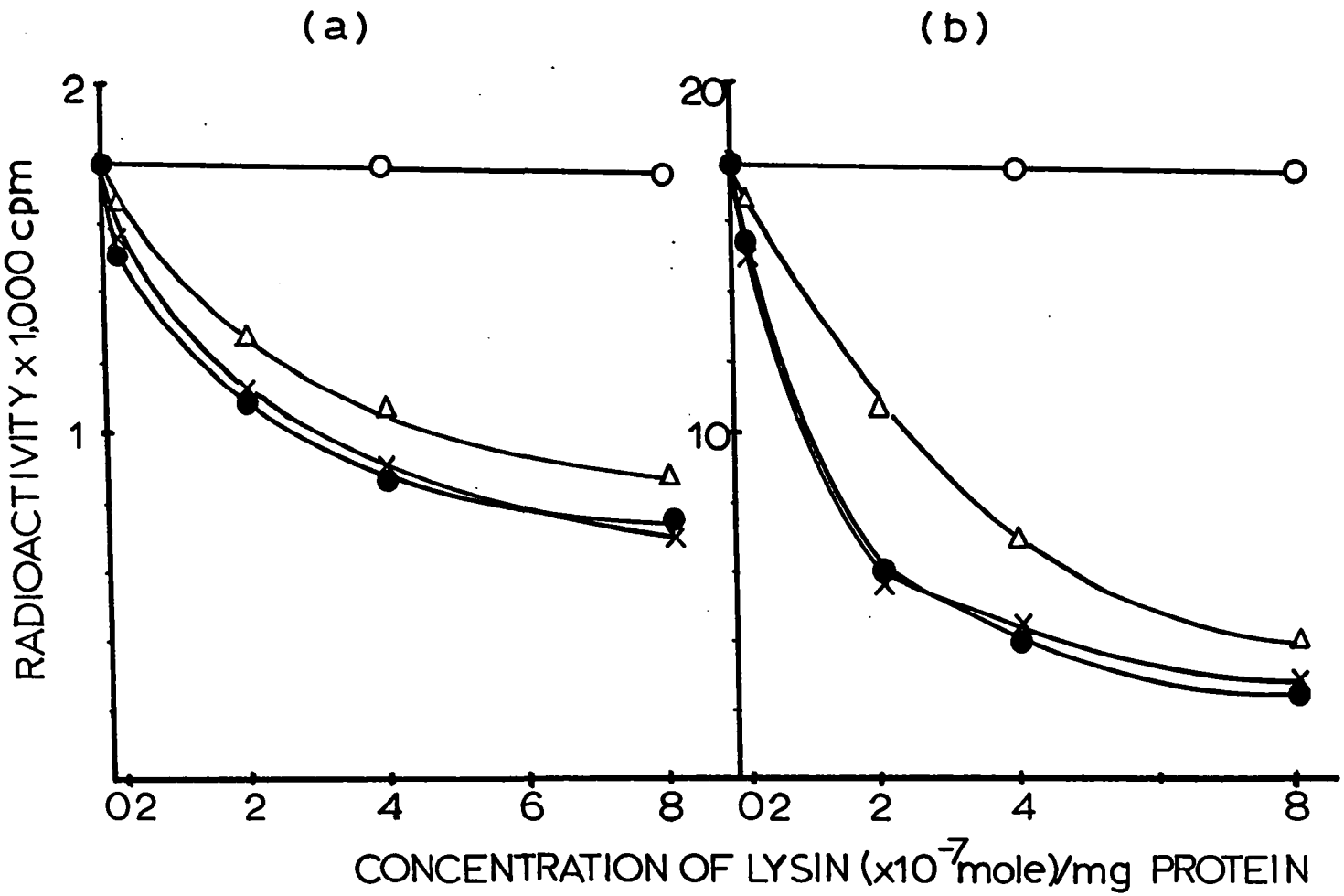


Fig. 19.* **Effect of Lysins on the Binding of Lecithin-P^{32} (a) and Cholesterol-H^3 (b) to Structural Protein.**

5 mg of structural protein were incubated at pH 7.4 with 0.5 mg lecithin-P^{32} (16,235 cpm) and 0.5 mg cholesterol-H^3 (21,950 cpm) at 37 °C water bath for 30 min. The precipitate was washed once with warm water and reincubated for 30 min with different amounts of lysins. After washing, the radioactivity remaining in the precipitate was counted.
●—● ; palmitoyl carnitine, x—x ; (1-palmitoyl)-lysolecithin,
△—△ ; myristoyl carnitine, o—o ; decanoyl carnitine.

*Average of 3 experiments.

7.4 with 0.5 mg lecithin-P^{32} (16,235 cpm) and 0.5 mg cholesterol-H^3 (21,950 cpm) at 37 °C water bath for 30 minutes. The precipitate was obtained by centrifugation and washed. Radioactivity of bound lecithin (a) or cholesterol (b) is represented as the top of the ordinate, respectively. When this lipid-protein complex was again incubated for 30 minutes with different concentrations of lysins, lipid was released from structural protein into the medium proportionally to lysin concentration. Palmitoyl carnitine and lysolecithin showed nearly the same capacity in releasing lipid from lipid-protein complex, but a shorter chain analogue, myristoyl carnitine was less effective and decanoyl carnitine also tried, ineffective. Again the interaction of lysins with artificial lipoproteins causing release of lipid, seemed to be hydrophobic in character.

PART II. Studies with Maddy Protein.*

(A) The Recombination of Membrane Protein with Lipid.

Recombination of total lipid-P^{32} (1 part of rat liver lipid-P^{32} diluted with 4 parts of erythrocyte membrane lipid) with Maddy membrane protein was carried out in 5 ml, 0.05 M acetate buffer at pH 4.0 by incubation in an ice-water bath for 15 minutes. At this pH Maddy protein was almost insoluble and lipoprotein formation could be studied by counting the radioactivity present in the precipitate after centrifugation and washing. After addition of lipid, protein completely precipitated.

Binding of protein (Fig. 20) increased with concentration of lipid until 5 mg of lipid had been added to the incubation mixture. At this point, the lipid-protein ratio in the precipitate was approximately 0.6 and could no longer be increased by further addition of lipid. This value is in good agreement with the original lipid-protein ratio found in erythrocyte ghosts ranging from 0.58 - 0.80. The amount of lipid bound to the protein was estimated by the ^{32}P-counts retrieved in the

*Results shown in Part II are the average of 3 experiments.

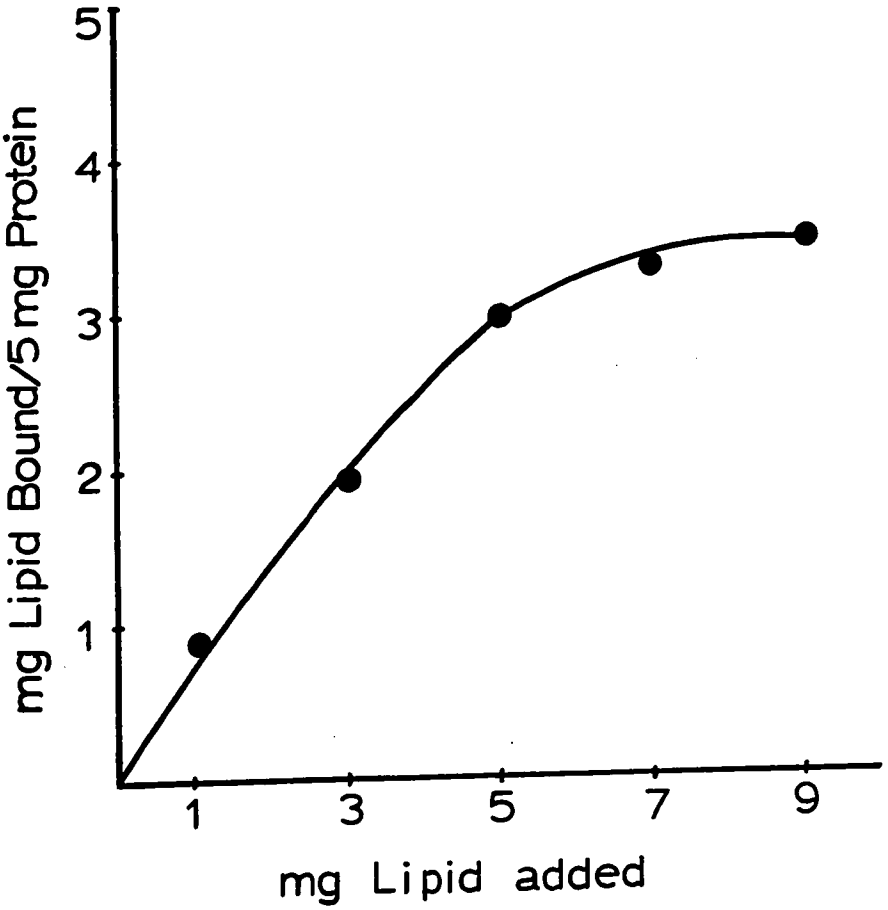


Fig. 20. Binding of Total Lipid-P^{32} with Maddy Membrane Protein.

5 mg of Maddy protein was incubated with different amount of total lipid-P^{32} (1 part of rat liver lipid-P^{32}, diluted with 4 parts of cold erythrocyte membrane lipid, Sp.Act., 33,033 cpm/mg) in a total volume 5 ml with 0.05 M acetate buffer pH 4.0 at 0 °C ice-water bath by gentle shaking for 15 min. The precipitate washed twice with distilled water was counted as usual.

precipitate, and from the specific activity of the lipid calculated on the basis of a total lipid mixture.

It could be argued however that there was selective binding of phospholipids and that counts obtained did not represent total lipids bound. By comparison the lipid composition of bound and total lipid, Figure 21 (lane 1) shows that the lipid binding to Maddy protein is completely non-selective and reflects the composition of the original lipid mixture (lane 5).

(B) Binding of Palmitoyl Carnitine to Apoprotein and to Reconstituted Lipoprotein and Effect of 1 M NaCl.

Results in Table 5 show the binding of palmitoyl carnitine-C^{14} to apoprotein and lipoprotein at pH 4.0 in the presence or absence of 1 M NaCl. In the absence of NaCl, palmitoyl carnitine binding to apoprotein was proportional to lysin concentration. It was somewhat less pronounced than its binding to lipoprotein. When binding experiments were repeated in the presence of higher ionic strength there was only a small decrease in the extent of binding to either apoprotein or lipoprotein. With either

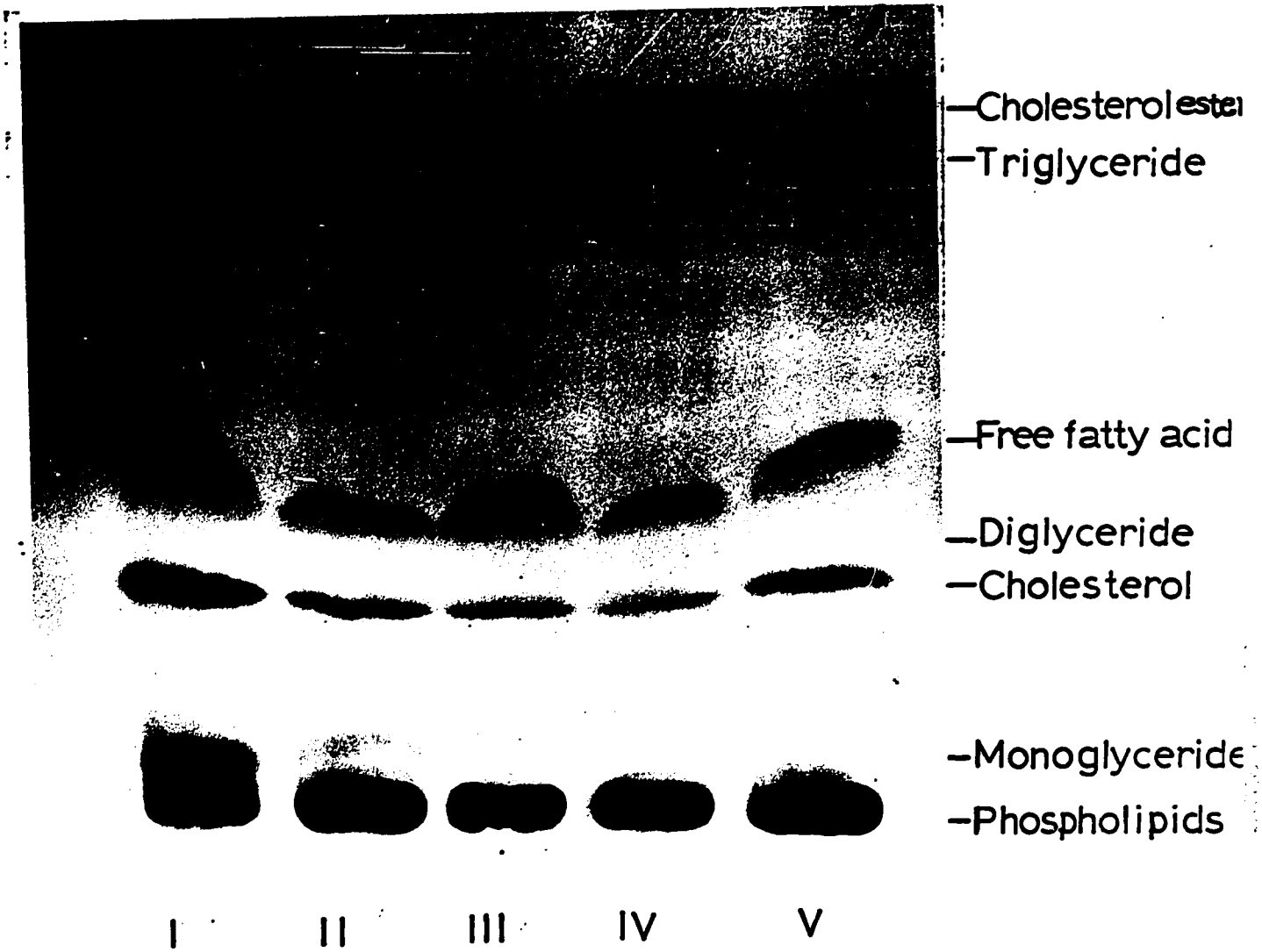
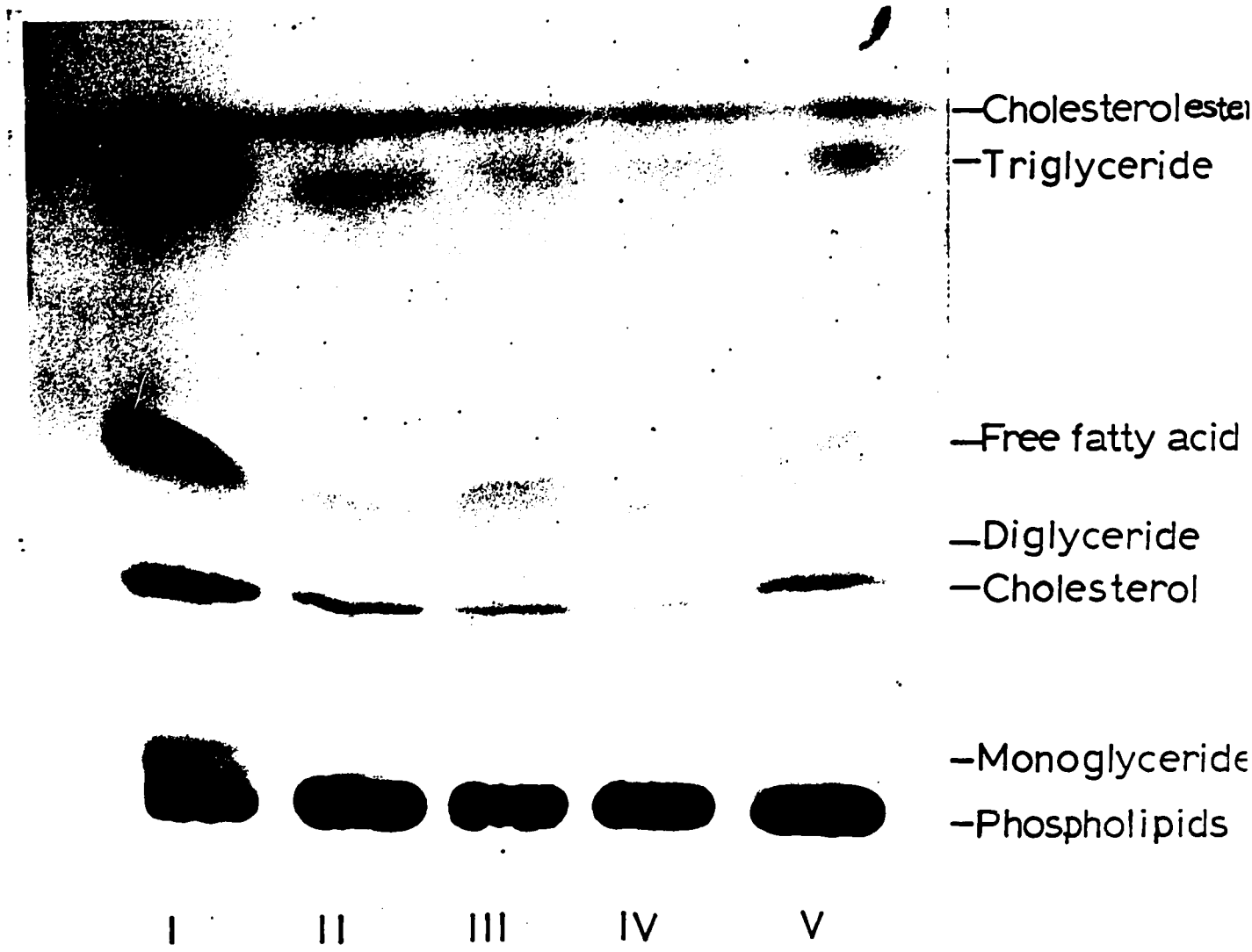


Fig. 21. A Comparison of the Composition of Bound and Unbound Total Lipid.

10 mg Maddy protein were incubated with 10 mg total lipid (liver: stroma, 1:4, w/w) in an ice-water bath for 15 minutes. In samples II, III, IV, lipoprotein complexes, washed twice were incubated again for 30 min with 2 x 10^{-6} moles of palmitoyl carnitine, palmitoyl choline and (1-palmitoyl)-lysolecithin respectively. Supernatant was separated by centrifugation and extracted lipid by the method of Bligh and Dyer and applied on silica gel G plates and developed in the solvent mixture, petroleum:ethyl ether: HAc (70:30:1, v/v). Components were visualized with iodine vapor.

I; Control (reconstituted lipoprotein), II; lipid released by palmitoyl carnitine, III; lipid released by (1-palmitoyl)-lysolecithin, IV; lipid released by palmitoyl choline, V; total lipid (liver:stroma, 1:4, w/w) incubated without protein.



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I; Control (reconstituted lipoprotein), II; lipid released by palmitoyl carnitine, III; lipid released by (1-palmitoyl)-lysolecithin, IV; lipid released by palmitoyl choline, V; total lipid (liver:stroma, 1:4, w/w) incubated without protein.

Table 5

Binding of Palmitoyl-DL-carnitine-C^{14} to Apoprotein and Lipoprotein and Effect of 1 M NaCl.

Palmitoyl Carnitine added (mole)	Radioactivity bound (cpm) (-NaCl)	(+NaCl)
Apoprotein		
5×10^{-7}	3,957	3,246
10×10^{-7}	7,668	6,440
Lipoprotein		
5×10^{-7}	4,866	3,871
10×10^{-7}	9,298	7,122

The incubation mixture contained 5×10^{-7} or 10×10^{-7} moles of palmitoyl carnitine-C^{14} (Sp.Act., 38,709 cpm/mg) and 5 mg Maddy protein in 5 ml of 0.05 M acetate buffer pH 4.0. Lipoprotein was prepared by incubation 5 mg protein with 5 mg total lipid. The reconstituted lipoprotein thus formed were then incubated with 5×10^{-7} or 10×10^{-7} moles palmitoyl carnitine-C^{14} for 15 min with and without 1 M NaCl, and washed twice. The radioactivity bound to the precipitated material after centrifugation was counted as usual.

these reactive counterparts, the binding of palmitoyl carnitine appeared to involve mainly hydrophobic interactions which were not appreciably enhanced by charge in the protein, lipoprotein or lysin. Preliminary experiments with ^{32}P-lysolecithin indicated a similar degree of binding to apo- or lipoprotein which however was enhanced by higher ionic strength. This confirms the hydrophobic character of lysin binding but there are some differences in the precise manner of association depending on the lysin used.

(C) Studies on Lipid-Protein and Lysin-Protein Complexes by Sucrose Density Gradient Ultracentrifugation.

The association of Maddy protein with lipid or lysin was further studied by sucrose density gradient ultracentrifugation at 120,000 x g as described in Methods, 5c, Section II. The density gradient tube was sectioned into eight fractions and all samples were duplicated for measurement of both lipid and protein.

Tube (a) in Fig. 22 represents apoprotein incubated and washed in the absence of lipid. It sedimented

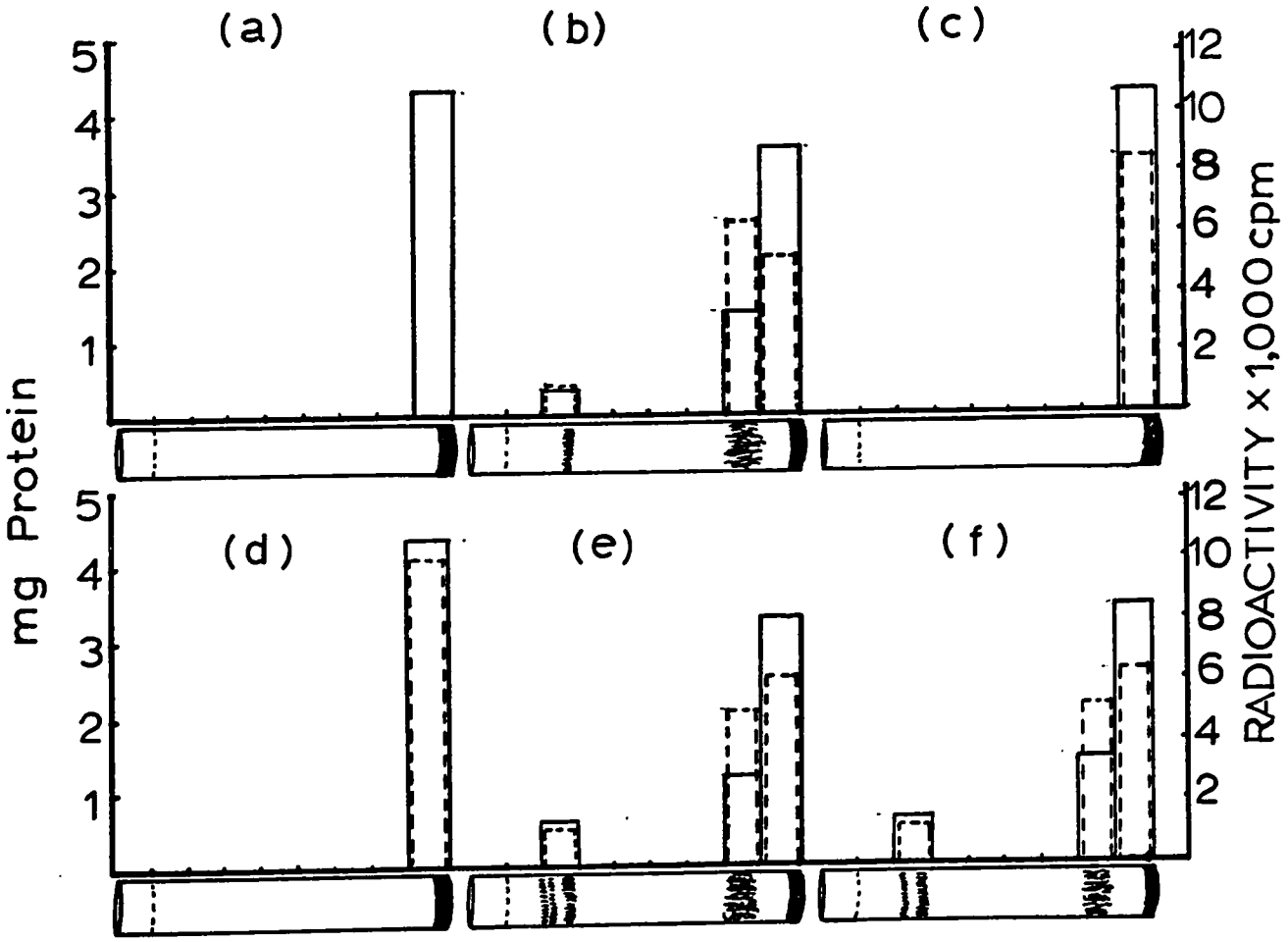


Fig. 22. The Distribution of Lipid and Lysins in the Sucrose Density Gradient of 120,000 x g Sediment after Binding of Membrane Protein with Labelled Lipid and Lysin.

5 mg Apoprotein or apoprotein-lysin complexes prepared by incubation 5 mg protein with palmitoyl carnitine-C^{14} (2×10^{-6} mole, 21,140 cpm) and lysolecithin-P^{32} (2×10^{-6} mole, 14,500 cpm) for 15 min, or lipoprotein prepared by incubation of 5 mg protein with 5 mg lipid-P^{32} (18,540 cpm) and lipoprotein-lysin complexes prepared from lipoprotein with 2×10^{-6} moles palmitoyl carnitine-C^{14} and lysolecithin-P^{32} were suspended in 0.5 ml of 13 % sucrose and were gently layered over a linear sucrose density gradient containing 3.5 ml in a 4.5 ml polyethylene centrifuge tube. The density gradient varied from 50 % sucrose at the bottom to 13 % sucrose at the top of the tube. These samples were centrifuged in a refrigerated Beckman Model L2-65 B ultracentrifuge, rotor SW-56 for 2 hours at 120,000xg. Each tube was divided into eight fractions and protein and radioactivity were estimated with duplicate aliquots.

(a) Apoprotein, (b) Apoprotein + lipid-P^{32} (5 mg, 18,540 cpm),
(c) Apoprotein + palmitoyl carnitine-C^{14} (2×10^{-6} mole, 21,140 cpm),
(d) Apoprotein + lysolecithin-P^{32} (2×10^{-6} mole, 14,500 cpm),
(e) Lipoprotein + palmitoyl carnitine-C^{14},
(f) Lipoprotein + lysolecithin-P^{32}
——; protein, -----; radioactivity.

as a single band of high density, whereas lipoprotein complex (tube b) reconstituted by admixture of protein to total lipid separated into three bands which contained ^{32}P-labelled lipid as well as protein. C^{14}-Palmitoyl carnitine and P^{32}-lysolecithin (tubes c and d, respectively) completely sedimented as did apoprotein implying that they formed very stable complexes with the Maddy protein. Incubation of C^{14}-palmitoyl carnitine (tube e) and P^{32}-lysolecithin (tube f) with unlabelled lipoprotein resulted in a distribution of radioactivity quite similar as that found in (b) except that one or two extra low density bands appeared. From these experiments it was deduced that the binding of lipids to Maddy protein caused the formation of several types of lipoprotein varying in density. Lysins could bind with apoprotein directly and with all reconstituted lipoproteins formed.

(D) The Effect of Lysins on Lipoprotein Formation and Dissociation at pH 4.

(i) The Effect of Charge of the Lysin on the Lipoprotein Formation and Dissociation.

In Fig. 23 results show that with increase of lysin

concentration in the incubation mixture there is corresponding decrease in lipoprotein formation. The effect is much more marked in the case of cationic detergents, such as cetyltrimethyl ammonium chloride (CTA). Palmitoyl carnitine is also positively charged at this pH since titration studies with acetyl and octanoyl carnitines gave pK values of 3.5 and 3.7, respectively. Lysolecithin has no charge at this pH whereas sodium dodecyl sulfate (SDS) would possess slight negative charge.

In Fig. 24 results show that with increase of lysin concentration in the incubation mixture, lipid dissociation from preformed lipoproteins increases proportionally. Again marked effects are seen only with cationic detergents such as CTA, palmitoyl choline and palmitoyl carnitine. Thus a "net" positive charge is an important criterion for detergent action of a surfactive substance at pH 4.

(ii) <u>The Effect of Acyl Chain Length of the Lysin on Lipoprotein Dissociation.</u>

Fig. 25 shows that as the chain length of the acyl carnitines is decreased from C 18 to C 10 there is a corresponding decrease in the ability of the de-

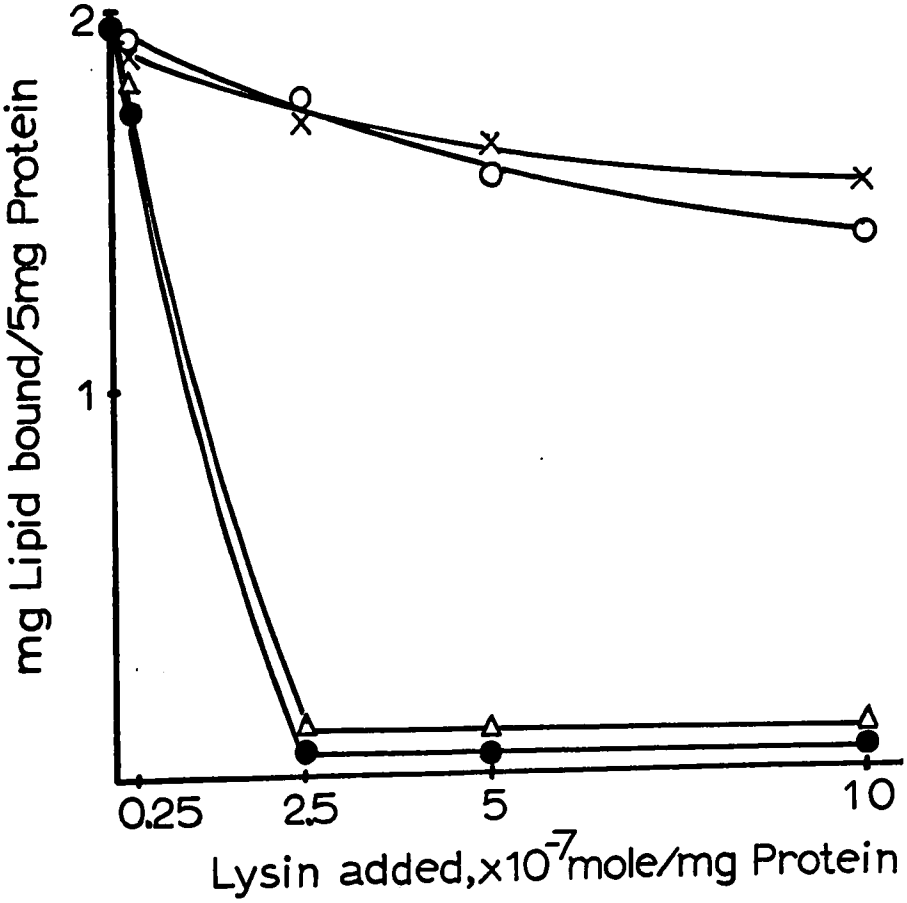


Fig. 23. Effect of Preincubation of Lipid-P^{32} with Palmitoyl carnitine, and Anionic and Cationic Lysins before Addition of Membrane Protein.

3 mg lipid-P^{32} (132,272 cpm) was preincubated with different amounts of palmitoyl carnitine, (1-palmitoyl)-lysolecithin, sodium dodecyl sulfate and cetyltrimethyl ammonium chloride in 0.05 M acetate buffer pH 4.0 at 37 °C in a total volume 4.5 ml for 10 min. After addition of 5 mg membrane protein, the samples were incubated again for 15 min at 37 °C. The precipitate were then washed twice and counted as usual.

●—●: palmitoyl carnitine, Δ—Δ: cetyltrimethyl ammonium Cl,
×—×: sodium dodecyl sulfate, ○—○: (1-palmitoyl)-lysolecithin.

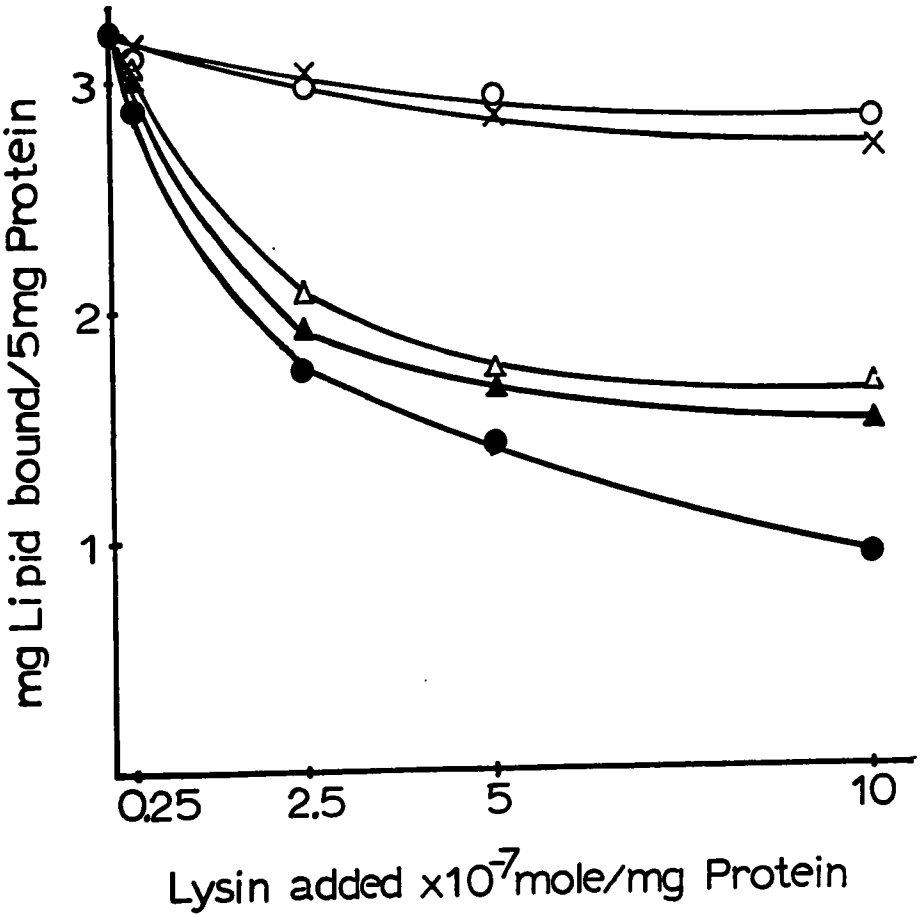


Fig. 24. The Effect of Lysins on Lipoprotein Dissociation at pH 4.0.

5 mg Maddy protein were incubated with 5 mg lipid-P^{32} (75,116 cpm) at 0°C ice-water bath for 15 min in a volume of 5 ml. After washing by addition of 10 ml cold distilled water, lipoprotein complex was reincubated with four different amounts of lysins for 15 min at 37 °C in a shaking water bath. The precipitates obtained were washed twice with warm water and counted as usual.

●—●: palmitoyl carnitine, ▲—▲: cetyltrimethyl ammonium Cl,
△—△: palmitoyl choline, X—X: sodium dodecyl sulfate,
○—○: (1-palmitoyl)-lysolecithin.

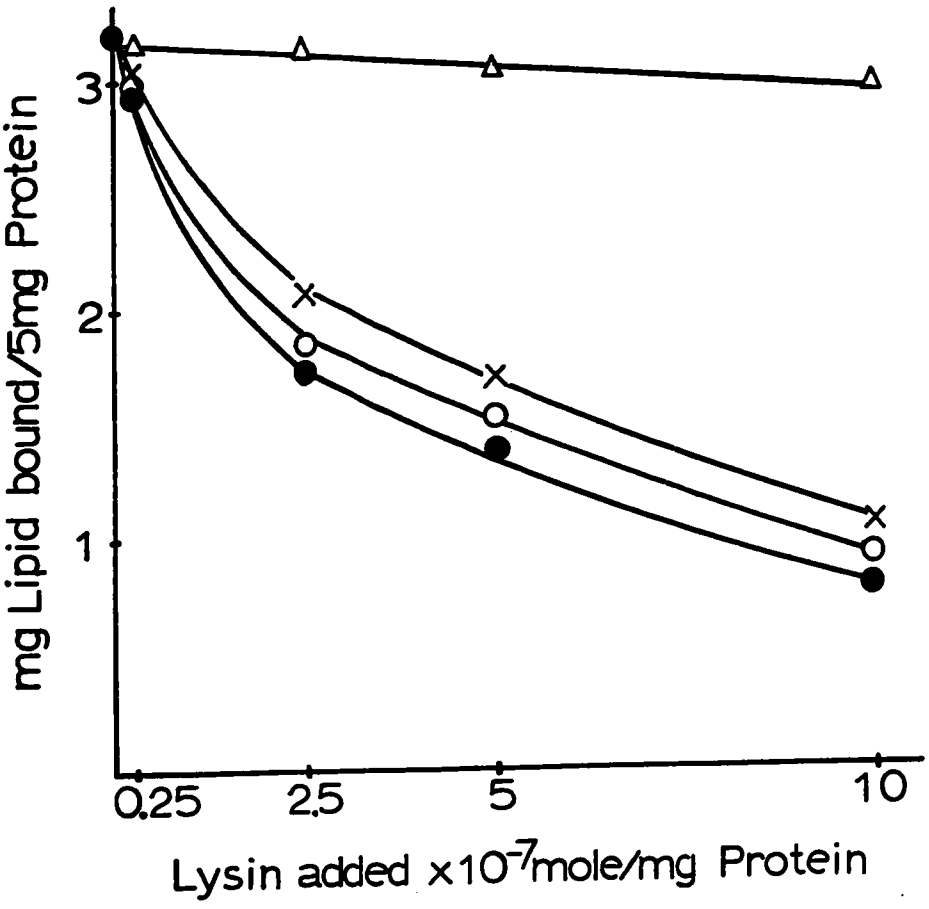


Fig.25. The Effect of Acyl Chain Length of Carnitine Ester on Lipoprotein Dissociation.

●—●; stearoyl carnitine, o—o; palmitoyl carnitine,

x—x; myristoyl carnitine, Δ—Δ; decanoyl carnitine.

Conditions for this experiment are as stated for Fig. 24.

tergent to dissociate lipoproteins. Consequently, there must be a hydrophobic interaction of the lysin with lipoprotein prior to release of lipid.

In Fig. 21 lanes II to IV show that the release of lipids by these detergents is not specific for ^{32}P-labelled components but includes cholesterol and other neutral lipids. The plate shows results obtained at pH 7.4, however essentially the same release pattern was obtained at pH 4, except that in this case, only palmitoyl carnitine of the ionic detergents tried caused appreciable release.

(iii) <u>The Effect of Ionic Strength on the Detergent Action of Various Surfactant Substances.</u>

Results in Table 6 indicate that increasing the ionic strength of the medium markedly impairs the detergent action of the various amphipathic substances tried. At first glance conclusions from such results would dictate that there is marked ionic interaction of lysin with lipoprotein which is impaired at high ionic strength but in fact this is not so as indicated in Table 4 and Table 5. We shall elaborate on this point in the Discussion of this section.

Table 6

Comparative study on Release of Lipid from Lipoprotein Complex by Palmitoyl carnitine, and Cationic and Anionic Surfactants at pH 4.0. Effect of 1 M NaCl.

Lysins (mole/mg protein)	Lipoprotein Radioactivity (cpm) (-NaCl)	(+NaCl)
Control	102,332	99,806
Palmitoyl carnitine, $1x10^{-6}$	36,815	84,549
Sodium dodecyl sulfate, $1x10^{-6}$	74,707	90,612
Cetyltrimethyl ammonium Cl, $1x10^{-6}$	48,566	80,846

5 mg lipid-P^{32} (161,550 cpm) and 1 x 10^{-6} mole/mg protein of lysin were used in this experiment. All other conditions are as stated for Fig. 24, except that in one set of experiments 1 M NaCl was added.

(iv) A Comparison of Detergency of Several Surfactant Substances at pH 4.0 and pH 7.4.

Results summarized in Fig. 26 confirm that detergency is related to charge. For equal concentrations of detergents at pH 4, one sees that only positively charged surfactants are highly effective in releasing lipid from lipoprotein. At physiological pH, although zwitterionic amphipaths are effective, the detergency of negatively charged sodium dodecyl sulfate (SDS) is very much increased whereas that of positively charged cetyltrimethyl ammonium chloride (CTA) is markedly decreased. These effects must correlate with the variation in charge of the protein as a function of pH, as will be discussed later.

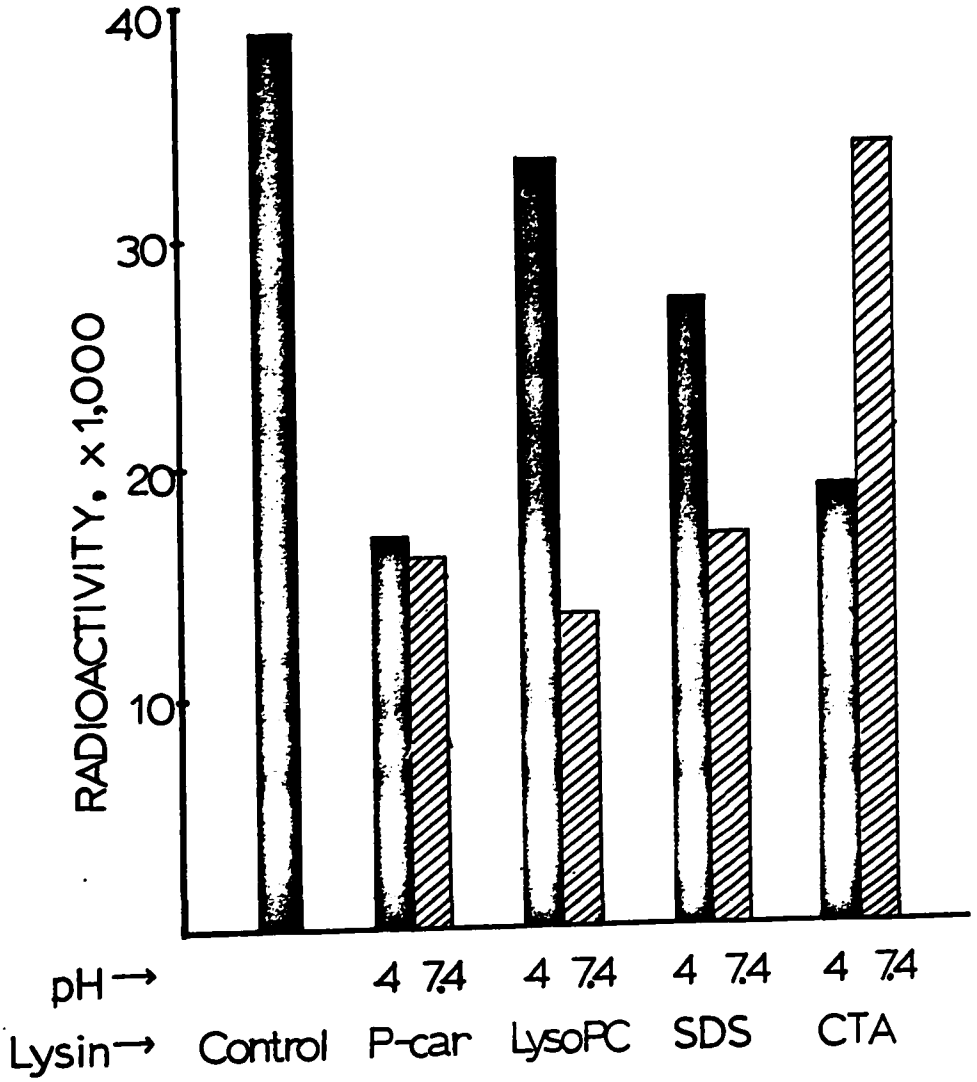


Fig. 26. A Comparison of Detergency of Lysins on Lipoprotein Dissociation at pH 4.0 and pH 7.4. Lipoprotein prepared from 5 mg Maddy protein with lipid-H3 (cholesterol labelled, 75,628 cpm) was incubated with 1x10-6 moles lysins at pH 4 and pH 7.4 in a volume of 5 ml at 37 °C for 30 minutes. The precipitate was washed twice and radioactivity was counted as usual.

III. Discussion

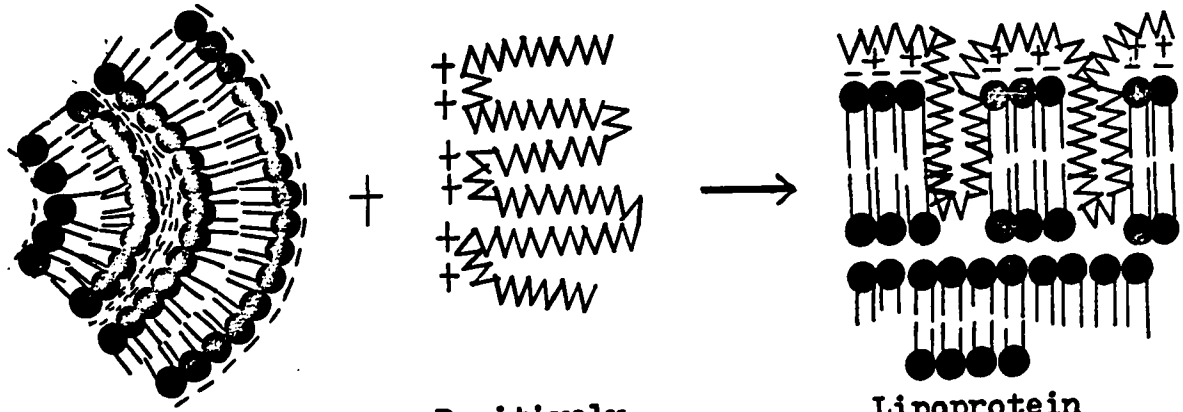
The lipid-binding characteristics of erythrocyte structural protein appear to be quite different from those of Maddy protein described by Zwaal and Van Deenen (84). Structural protein can bind individually, cholesterol, lecithin, lysolecithin and palmitoyl carnitine at pH 4 or pH 7, (Table 4) and therefore does not require a particular charge on the lipid micelles opposite to that of net protein charge. In some cases, increasing the pH to 12 and readjusting the medium to pH 7 has resulted in an increased binding and Fleischer et al (85) explain this effect by an unfolding of the protein at higher pH exposing more hydrophobic sites for combination with lipids. On a few occasions we have not been able to reproduce this effect with erythrocyte structural protein, however in our case a slightly different method for isolation of structural protein was used and isolated lipids rather than lipid mixtures were the reactive counterparts. The binding of lipids or lysins was little affected by ionic strength. Acid pH favoured the binding of the polar lipids but not of cholesterol. This effect was much more pronounced in the case of lecithin which has the same polar head group as lysolecithin but displays quite different lyotropic mesomorphism. The effect

of pH therefore could be attributed to an unfolding of structural protein allowing greater accessibility to the larger lecithin aggregates. At any rate, the binding of lecithin, cholesterol or lysins appeared to result from hydrophobic interactions only. The solubilizing action of lysins on protein-bound cholesterol or lecithin at pH 7 was dependent on acyl chain length but not on the net charge of the detergent. Thus one can conclude from this that the action of the lysin is also largely due to hydrophobic displacement of the lipid from the protein. However, quantitative studies to see whether this displacement occurred on a mole per mole basis were not done in this case.

It seems from our results that structural protein is conformationally different from soluble Maddy protein and must expose relatively larger areas for hydrophobic binding with lipids. This could explain to some extent its complete insolubility in aqueous medium except at extreme pH.

The binding of lipids to Maddy protein pretreated with neuraminidase, positively charged at pH 4 was studied in detail by Zwaal and Van Deenen(84). Their results indicated that lipoprotein formation involved initially

an ionic interaction between the protein and negatively charged lipid micelles. The complex then becomes stabilized to high ionic strength by hydrophobic bonding. Analogous findings were made by Sweet and Zull (83) who studied lipoprotein formation using liposomes and albumin as a model system. Here again, complex formation involved bonding of a dual character. They suggested that an initial ionic interaction was followed by a penetration of apolar segments of protein into the bilayered structure of the liposomes as shown in Scheme I. Very likely, a similar phenomenon was involved in the studies of Zwaal and Van Deenen.



Scheme I. Schematic Representation of Lipoprotein Formation

In the present study, apoprotein was not treated

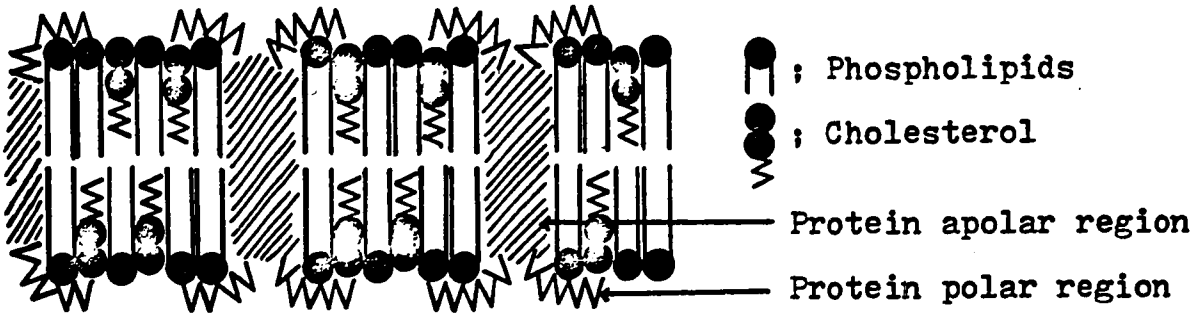
with neuraminidase and consequently at pH 4, which is within the isoelectric pH range, the protein would bear no net positive charge. It is clear however from the work of Zwaal and Van Deenen (84) that sialic acid groups do not play an important role in the binding of lipids, and consequently their presence in our case would not likely alter the binding characteristics of the protein. Accordingly, at pH 4 the initial ionic interaction of lipids could involve positively charged areas of the protein. This conclusion is supported by the fact that addition of positively charged but not of uncharged or negatively charged amphipaths to lipids greatly diminished their interaction with protein (Fig. 23).

That the lipid-protein interaction at pH 4 may be as outlined in Scheme I is further supported by the fact that the action of cationic detergents does not involve a simple mole per mole displacement of lipids. It can be estimated from our results that 1.25 u moles of palmitoyl carnitine in the bulk phase can displace approximately 1.5 mg of lipid (equivalent to approximately 3 u moles of lipid of average Mol.Wt.* 500) from reconstituted

* The average Mol.Wt. of our lipid mixture is based on a cholesterol:phospholipid ratio of approximately 1.0 in erythrocyte (41) and the total lipid composition of rat liver (205), taking into account that this lipid mixture was formed from 4 parts of erythrocyte lipid and 1 part of liver lipid.

lipoprotein, yet on a molar basis apoprotein under similar conditions will bind no more palmitoyl carnitine than 20 % of this amount of lipid released. If the basic structure of the lipoprotein formed does indeed involve bilayers of lipid interupted by apolar segments of protein, the release of lipids by smaller amounts of cationic detergents at pH 4 can be explained by a micellar dispersion of the bilayers. The positively charged micelles formed would be unable to reassociate with protein segments of like charge. Also their mesomorphic character may be less compatible with lipoprotein formation in any case.

When lipoprotein is formed at pH 4 and the insoluble complex is transferred to a medium of pH 7.4, there results no dissociation of lipid and no increase in solubility. Yet this change in pH ought to neutralize positive charges due to imidazole groups in the protein which have been suggested as binding sites for lipid at pH 4 (84). Also the protein as a whole would become more negatively charged. This further implies that once formed, the lipoprotein is stabilized by hydrophobic interactions between lipids and apolar segments of their reactive counterpart. One may picture the structure of the complex at pH 7.4 as in Scheme II.



Scheme II. Possible Structure of Lipoprotein Complex.

Addition of uncharged lysins would result in the formation of micelles with a net negative surface because at pH 7.4 the acidic character of phosphatidyl serine and the inositides would be intensified. Since the protein bears net negative charge, recombination of these micelles would not easily occur. Addition of a negatively charged lysin such as sodium lauryl sulfate would produce essentially the same results. On the other hand, addition of a cationic detergent would result in the formation of positively charged micelles which are freer to recombine with the oppositely charged protein. This would explain why at pH 7.4 cetyltrimethyl ammonium chloride has poorer detergency than at pH 4.0.

As in the case of structural protein complexes formed with Maddy protein the displacement of lipid from lipoprotein by detergents, must also involve hydrophobic

interactions. This is well shown by results obtained in Fig. 25. Whereby it is seen that the detergency of acyl carnitine analogues is highly dependent on the acyl chain length.

Besides possessing positively charged regions which favour binding to oppositely charged lipid micelles at pH 4, the apoprotein not treated with neuraminidase must contain other sites which favour the binding of ionic amphipaths such as palmitoyl carnitine or lysolecithin. These sites need not be sialic acid groups, since the binding of palmitoyl carnitine is not markedly decreased with presence of 1 M NaCl. This implies that hydrophobic sites on the protein are accessible and immediately implicated in this case. The accessibility of such sites to palmitoyl carnitine could easily be explained on the basis of the lyotropic mesomorphic properties of wedge-shaped amphipaths, being quite different from those of lipid mixtures adopting larger aggregate forms in water. The decrease in binding of palmitoyl carnitine at high ionic strength would indicate that apoprotein can bind these lysins in monomer form, since it is well known that this condition also decreases the critical micellar concentration of ionic detergents. The binding of palmitoyl carnitine at pH 4 (Table 5) is somewhat greater with lipo-

protein than with apoprotein. In either case, binding at higher ionic strength is diminished only slightly. It can again be deduced from this that the interaction of lysins with lipoprotein is hydrophobic in character and very likely involves monomeric penetration into the lipoprotein complex.

Our studies at pH 4 (Table 6) also indicated that dissociation of lipid from lipoprotein by palmitoyl carnitine and cationic detergents is markedly decreased at high ionic strength. A decrease in the critical micellar concentration of these detergents could again partly explain these results. However, once lipids have been dispersed by the action of detergent, the net surface charge of micelles formed as well as that of protein would also be affected by this change in ion concentration and at this point no other but more complex considerations could account for this effect.

Concluding Remarks

In the foregoing sections, the lytic properties of acyl carnitines were described in detail and contrasted with lysolecithin and other detergents. It can be deduced from these studies that acyl carnitines and lysolecithins, both naturally occurring in membranes, profoundly affect the structural architecture of the cell surface. This is well illustrated by the fact that low concentrations of these substances, cause hemolysis and death of the cell. In view of these drastic effects, one is perplexed by the fact that these substances can accumulate in small quantities at the cell surface where they are either formed or can permeate via lipid-lipid exchange. Since their presence is compatible with the basic integrity of the membrane, it is obvious that their accumulation must never reach lytic proportions and in fact in the case of lysolecithin, membrane is well equipped with enzymes that can control the levels of this lysin. How palmitoyl carnitine is further metabolized in erythrocyte membrane is not known although the specificity of esterases may include this substance as a suitable substrate. However our own preliminary observations reveal that palmitoyl carnitine or palmitoyl choline are not rapidly metabolized in erythrocytes

and it would seem that esterase intervention is not an important factor. Our studies have also shown that a number of membrane constituents including protein can bind quaternary ammonium amphipaths and in the bound form, these substance lose their hemolytic capacity. One can envisage the possibility that in minute concentrations, palmitoyl carnitine would have no effect on membrane integrity since protein could serve as a suitable titrant of this lysin. One may recall that when erythrocytes are sensitized by pre-exposure to very weakly lytic concentrations of palmitoyl carnitine and lysolecithin, normally, non-lytic substances such as lecithin, cholesterol and normal cytosol constituents acquire a highly lytic character when added to suspensions containing "sensitized" red blood cells. Very likely then, even in sublytic concentrations, these lysins would affect the structure of the membrane. From such evidence, which further experimentation should fruitfully extend, we propose that in non-lytic concentrations lysolecithin and palmitoyl carnitine contribute to the dynamic character of the membrane allowed by certain structural models discussed in our review of the literature. One could envisage the possibility that these naturally-occurring lysins might be intimately

related to permeability phenomena such as the lipid-lipid exchange reactions occurring at the surface of the cell, the entry of fatty acids and/or polar metabolites, or the release of biologically active amines from their granular storage sites. All such possibilities have not been extensively explored as yet and further studies on the physiologic involvement of these amphipaths would seem a fruitful course to follow.

As regards the mechanism of lysis by acyl carnitines and lysolecithin, we have presented evidence indicating that their interaction with the membrane is hydrophobic. There results from their penetration into labelled erythrocytes, a release of ^{3}H-cholesterol and ^{32}P-lecithin proportional to the extent of hemolysis. When constituents such as protein or lipids are isolated and incubated with lysins, complexes are formed which are mainly hydrophobic and non-specific in character. Assumably lysin penetration into the membrane is followed by similar complex interactions which cause release of lipid constituents. The ratio of lipid displaced to lysin added is usually high and consequently a simple mole per mole displacement is not involved. Rather, these results are compatible with the idea that there are extensive regions in the membrane which contain lipid

bilayers and dispersion of these aggregates as micelles by relatively fewer wedge-shaped molecules could account for massive loss of lipids accompanying detergent action.

To further probe the mechanism of lysin action we have used artificial systems constituted by membrane protein and lipids. The difficulties with such studies can be easily assessed, not least among which is the problem of obtaining apoprotein which is not denatured. This, together with the fact that such protein preparations are heterogeneous bear on the significance of the results obtained. Not wanting to belittle these difficulties, we can add however that our studies have included two types of protein preparations obtained from the most recent methodology available. The saliant features of these studies have already been described in detail. With respect to results obtained with Maddy protein we can elaborate further. In this case the action of lysins on reconstituted lipoproteins, resembles quite closely the picture with intact membranes. As in the latter case, the interaction of palmitoyl carnitine and other lysins with reconstituted lipoproteins involves mainly hydrophobic binding. There results from their action, a release of lipid which exceeds the amount of lysin that can interact with the apoprotein.

We propose that the lipoprotein complex formed in our case is constituted by a basic lipid bilayer interrupted in certain regions by apolar segments of proteins. The lysins act by dispersing these bilayer regions and whether or not the micelles formed are freed from the complex depends on the net surface charge of the micelles and of the protein.

Although results with artificial lipoprotein systems must be interpreted with caution, with respect to lysin action at least, such preparations seem to serve as adequate models of the intact erythrocyte membrane. A clearer comprehension from such studies will be obtained only if and when pure membrane apoproteins can be separated from constituent parts without denaturation. Perhaps even more fruitful studies could be made if non-hemolytic reporter groups could be attached to surface proteins of the intact cell, and changes in protein conformation as a result of hemolysin addition, studied spectrophotometrically. Such labelled proteins could then be isolated and further characterized.

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