BBA 75095

ENHANCING ACTION OF SYNTHETIC AND NATURAL BASIC POLYPEPTIDES ON ERYTHROCYTE-GHOST PHOSPHOLIPID HYDROLYSIS BY PHOSPHOLIPASE A

C. KLIBANSKY, Y. LONDON, A. FRENKEL AND A. DE VRIES

Rogoff-Wellcome Medical Research Institute, Department of Experimental Biology, Tel Aviv University Medical School, Beilinson Hospital, Petah Tikva, and Department of Biophysics, The Weizmann Institute of Science, Rehovoth (Israel)

(Received July 10th, 1967)

SUMMARY

The natural basic polypeptide, Gramicidin S, and the synthetic basic α-amino acid copolymers, poly-ornithine–leucine, poly-ornithine–leucine–alanine and poly-lysine–leucine, similarly to the direct lytic factor of Ringhals cobra venom, render the phospholipids in osmotic erythrocyte ghosts susceptible to the hydrolytic action of *Vipera palestinae* phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4). The synthetic basic α-amino acid polymers, poly-ornithine, poly-lysine and the copolymer poly-ornithine–valine were inactive in this respect.

The basicity of the polypeptides, by promoting electrostatic attraction, is held responsible for their attachment to the membrane, whereas the lipophilic side chains are invoked in the facilitation of the approach of the phospholipase to the phospholipid substrate situated inside the membrane.

Gramicidin S and the synthetic basic copolymers active in facilitating the splitting of phospholipids are strongly hemolytic. The basic polyamino acids, which are inactive in enhancement of phospholipid splitting, and the Ringhals cobra direct lytic factor have little hemolytic activity.

INTRODUCTION

Hemolysis by cobra venom involves, in addition to its phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4), the so-called direct lytic factor, which, in itself weakly hemolytic, is able to facilitate the action of the phospholipase on the erythrocyte membrane phospholipids[1]. The recently isolated Ringhals cobra direct lytic factor was found to be a single-chain basic polypeptide with a molecular weight of about 7000, and containing hydrophobic side chains in addition to basic ones[2]. With the aim of obtaining information on the mode of action of direct lytic factor on the erythrocyte membrane, a study of the hemolytic and membrane phospholipid splitting-enhancing action of some synthetic model polypeptides was undertaken. The natural basic peptide antibiotic, Gramicidin S, was similarly studied. The inability of *Vipera palestinae* phospholipase A to hydrolyze erythrocyte-ghost phospholipids unless the

ghost membrane is damaged, for example by direct lytic factor[1], was utilized for studying the action of the model compounds.

MATERIALS AND METHODS

Basic polymers and basic copolymers of amino acids were prepared in the Department of Biophysics, The Weizmann Institute of Science[3]. The following substances were used: poly-Orn, poly-Orn–Leu, poly-Orn–Leu–Ala, poly-Lys, poly-Lys–Leu and poly-Orn–Val. Molecular weights of the polymers, as estimated in the analytical ultracentrifuge Spinco Model E, were respectively, 22000, 47000, 11320, 7680 and 53800. The molecular weight of poly-Orn–Val was not determined. Gramicidin S was kindly provided by Dr. L. BICHOWSKY, The Weizmann Institute of Science, Rehovoth, Israel. The phospholipase A fraction from *V. palestinae* venom and direct lytic factor preparation from Ringhals cobra venom were obtained by procedures described previously[2,4].

Blood collection from normal human subjects and separation of erythrocytes was performed as described previously[5]. Osmotic ghosts were prepared by adding 3 volumes of distilled water to 1 vol. of washed packed erythrocytes with continuous stirring for 10 min. Following refrigerated centrifugation at 3000 × *g* for 10 min the ghosts were resuspended in the same medium and then counted in a Neubauer counting chamber under the phase microscope. In one series of experiments gradual osmotic hemolysis was applied[6].

Hemolytic activity of polyamino acids was estimated on samples of washed erythrocytes in phosphate-buffered saline (pH 7.4), containing $6 \cdot 10^8$ cells per ml by determination of hemoglobin released during incubation for 2 h at 37°. The conventional acid hematin or benzidine procedure[7] was applied.

Phospholipid hydrolysis in osmotic erythrocyte ghosts by phospholipase A in the presence of polyamino acids was measured by quantitative chromatographic determination. The phospholipids were extracted from the ghosts with chloroform–methanol (2:1, v/v), after spinning them down at 10000 × *g* in a Sorvall centrifuge model RC-2 rotor SS-34. The phospholipids were separated by two-dimensional thin-layer chromatography on silica gel without binder (Camac). Chromatoplates (20 cm × 20 cm) of 500 mμ thickness were prepared using a Desage apparatus. The plates were subjected to ascending chromatography with chloroform–methanol–water (65:25:4, v/v) as the first developing mixture[8], and chloroform–methanol–30 % NH_4OH (14:6:1, v/v) as the second developing mixture[9]. For identification of phospholipids on the chromatogram appropriate markers were used. In addition choline-containing phospholipids were detected by the Dragendorff procedure[10], secondary amide bonds in sphingomyelin by the method of BRESLER[11], and free amino groups by ninhydrin staining. Phosphatidylinostitol was not separated, similarly to the results obtained with thin-layer chromatography of erythrocyte phospholipids by BRADLOW *et al.*[12]. For localization of the phospholipids in the quantitative analytic procedure I_2 vapor was used. After the I_2 had evaporated from the plate, the outlined spots were scraped directly into digestion flasks, phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine being collected into one flask, and sphingomyelin and the lyso-compounds of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine evolved in the reaction, into a second. Blanks of silica gel of sizes corresponding

to the two collected spots were determined. The material was digested with conc. H_2SO_4–70 % $HClO_4$ (3:2, v/v). Phospholipid phosphorus was estimated by the method of BERENBLUM AND CHAIN[13]. The percentage hydrolysis was calculated by determination of phospholipid phosphorus of the substrate (phosphatidylethanolamine *plus* phosphatidylcholine *plus* phosphatidylserine) acted upon by phospholipase A, in the absence of polyamino acids (control, taken as 100 % substrate) and in their presence. The estimation of sphingomyelin and lysocompounds was carried out for recovery calculations. Total phospholipid phosphorus was also determined in all chloroform–methanol extracts in order to verify that no phospholipids were lost during the procedure prior to chromatography. In another series of experiments the hydrolysis of phospholipids was followed by quantitative determination of unesterified fatty acids (DOLE[14] procedure) evolved in the reaction. The percentage hydrolysis was calculated from the amount of unesterified fatty acids released in the reaction system and the amount of fatty acid present in phosphatidylethanolamine *plus* phosphatidylcholine *plus* phosphatidylserine, the phospholipid phosphorus of these together was assumed to represent 70 % of the total phospholipid phosphorus of the erythrocytes.

Solubility of the basic polymers and copolymers in lipid solvents was examined qualitatively by applying one-dimensional thin-layer chromatography and using the following solvent systems: chloroform–methanol–water (60:35:8, v/v/v), chloroform–methanol–30 % ammonia (14:6:1, v/v/v) and propanol–water (7:3, v/v).

RESULSTS

Hemolytic activity of basic polymers and copolymers of amino acids

All basic polymers and copolymers of amino acids examined caused hemolysis on incubation with washed erythrocytes, the extent of hemolysis increasing with increasing polymer concentration (Fig. 1). The rate of hemolysis produced by the copolymers was higher than that by the polymers (Fig. 1). The strongest hemolytic substances were poly-Orn–Leu, poly-Orn–Leu–Ala and poly-Lys–Leu. The hemolytic activity of poly-Orn–Val was only slightly higher than that of poly-Orn or poly-Lys. Gramicidin S was strongly hemolytic, as were the copolymers. Direct lytic factor had only a very weak hemolytic activity. It is noteworthy that the percentage values for hemolysis obtained with the above substances varied widely from blood to blood, but the difference in the hemolytic activity between the various polymers and copolymers of amino acids was always observed.

Effect of basic polymers and copolymers of amino acids on splitting of erythrocyte-ghost phospholipids by phospholipase A

The results of the hydrolysis experiments, obtained by measuring either decrease in ghost phospholipid substrate (Table I) or unesterified fatty acid release (Table II), show that whereas *V. palestinae* phospholipase A was unable to induce phospholipid hydrolysis in erythrocyte ghosts, it was able to do so in the presence of poly-Orn–Leu, poly-Orn–Leu–Ala or poly-Lys–Leu. Gramicidin S and direct lytic factor had a similar effect, the latter having the strongest activity. Poly-Orn, Poly-Lys and poly-Orn–Val were ineffective. Fig. 2 illustrates the effect of poly-Orn–Leu.

For the amount of ghosts used, $2 \cdot 10^9$ cells, at constant peptide concentration the percentage of phospholipid splitting did not change significantly when increasing

the amount of phospholipase above 200 μg. When erythrocyte ghosts were incubated with a constant amount of phospholipase A and increasing amounts of active copolymer, the percentage of phospholipid splitting increased. In a representative experiment in which $2 \cdot 10^9$ cells were incubated with 400 μg phospholipase A and 500 μg,

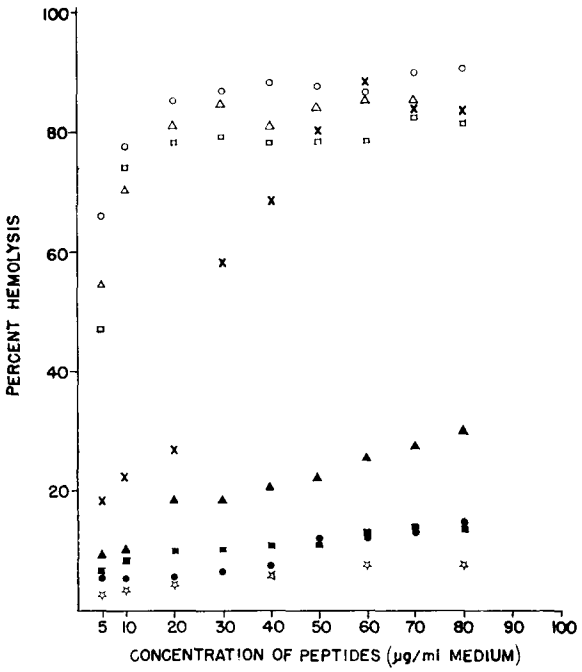

Fig. 1. Hemolytic activity of synthetic and natural basic peptides. ○, Orn–Leu; △, Lys–Leu; □, Orn–Leu–Ala; ×, Gramicidin; ●, Orn; ▲, Orn–Val; ■, Lys and ✱, direct lytic factor.

TABLE I

EFFECT OF BASIC POLYMERS AND COPOLYMERS OF AMINO ACIDS ON ERYTHROCYTE-GHOST PHOSPHOLIPID SPLITTING BY *V. palestinae* PHOSPHOLIPASE A, MEASURED BY PHOSPHOLIPID ANALYSIS

Incubation system: $2 \cdot 10^9$ erythrocyte ghosts per ml medium containing 500 μg polyamino acid and 400 μg (protein content, LOWRY reaction) phospholipase A. Incubation time, 2 h at 37°. For abbreviations and calculation of percentage splitting, see METHODS.

Substance added	*Phospholipid phosphorus* (μg)		% *Splitting*
	Phosphatidyl-ethanolamine plus phosphatidylcholine plus phosphatidylserine	*Sphingomyelin plus lyso compounds*	
	6.20	3.29	0
Poly-Orn	6.48	2.92	0
Poly-Lys	5.99	3.55	3
Poly-Orn–Val	5.89	3.60	5
Poly-Orn–Leu	3.90	5.69	38
Poly-Orn–Leu–Ala	3.89	5.56	38
Poly–Lys–Leu	3.97	5.57	36
Direct lytic factor	2.49	7.07	60
Gramicidin S	3.66	5.68	41

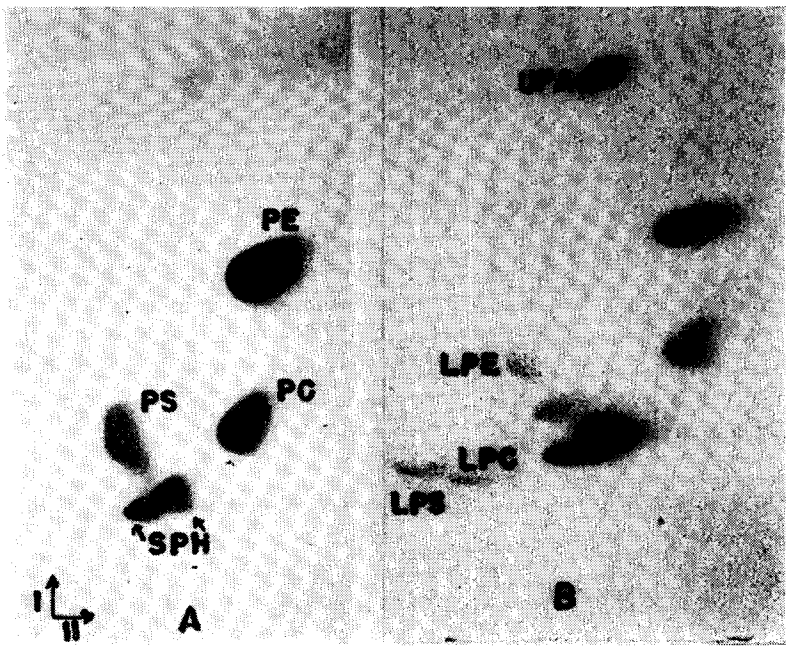

Fig. 2. Enhancement of phospholipid splitting by poly-Orn–Leu. Two-dimensional thin-layer chromatography of erythrocyte-ghost phospholipids. A, treated with *V. palestinae* phospholipase; B, treated with *V. palestinae* phospholipase and Poly-Orn–Leu. Chromatogram of erythrocyte-ghost control was identical to A. Reaction system as in Table I. The plates were developed with Solvent I (chloroform–methanol–water, 65:25:4, v/v) followed by Solvent II (chloroform–methanol–30% NH_4OH, 14:6:1, v/v). Abbreviations are: PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; LPC, lysophosphatidylcholine; UFA, unesterified fatty acids. The non-marked spots in B are analogous to the phospholipids marked in A.

TABLE II

EFFECT OF BASIC POLYMERS AND COPOLYMERS OF AMINO ACIDS ON ERYTHROCYTE-GHOST PHOSPHOLIPID SPLITTING BY PHOSPHOLIPASE A, MEASURED BY UNESTERIFIED FATTY ACID RELEASE

Incubation system: $8 \cdot 10^9$ erythrocyte ghosts per 4 ml medium containing 2000 μg polyamino acid and 1600 μg (protein content, LOWRY reaction) phospholipase A. Incubation time, 2 h at 37°. The value of unesterified fatty acid evolved in the control containing phospholipase but no peptide, was substracted from each value of the above experiments. For abbreviations and calculation of percentage splitting see METHODS.

Substance added	*Unesterified fatty acid*	% *Splitting*
Poly-Orn	0	0
Poly-Lys	0.05	2
Poly-Orn–Val	0.09	4
Poly-Orn–Leu	0.63	30
Poly-Orn–Leu–Ala	0.92	43
Poly-Lys–Leu	0.87	41
Direct lytic factor	1.83	87
Gramicidin S	0.94	44

1000 μg or 1500 μg poly-Orn–Leu, the percentage hydrolysis of the phospholipase A –substrate (phosphatidylethanolamine *plus* phosphatidylcholine *plus* phosphatidylserine) was 27, 61 and 77, respectively.

In order to examine whether the active copolymers remove phospholipid from the erythrocyte ghosts and thus the phospholipase might act in the medium, $8 \cdot 10^9$ ghosts were incubated with 2000 μg poly-Orn–Leu in a 2-ml suspension for 2 h at 37°. The supernatant obtained after centrifugation at 15000 $\times$ *g* for 10 min was freeze-dried and examined for phospholipid. Out of a total of 88 μg of erythrocyte-ghost lipid phosphorus, 0.4 μg was released into the medium containing the copolymers, whereas in the control, 0.5 μg was released. Furthermore, phospholipid was not released into the medium when ghost-phospholipid hydrolysis was produced by the joint action of basic copolymer and phospholipase A. The recovery of phospholipid phosphorus in the erythrocyte ghosts after hydrolysis had occurred was in agreement with these findings as may be seen from the data in Table I.

Attempt to induce erythrocyte-phospholipid hydrolysis by phospholipase A during gradual osmotic hemolysis

10 ml of phosphate-buffered 0.15 M NaCl solution containing $4 \cdot 10^9$ washed erythrocytes and 800 μg phospholipase A were dialyzed against 0.038 M NaCl solution for 2 h at 37°. A control without phospholipase was run. No split products were detected in the ghosts on thin-layer chromatography.

Solubility of basic polymers and copolymers of amino acids in lipid solvents

Thin-layer chromatography using various lipid solvents showed movement of only those synthetic peptides which had hemolytic and membrane phospholipid splitting-enhancing activity (according to Table I). Fig. 3A illustrates the movement of poly-Orn–Leu, poly-Lys–Leu and poly-Orn–Leu–Ala; poly-Orn, poly-Lys and poly-Orn–Val remained at the application point. As seen in Fig. 3B, Gramicidin S moved well in the lipid solvent whereas direct lytic factor stayed at the starting point.

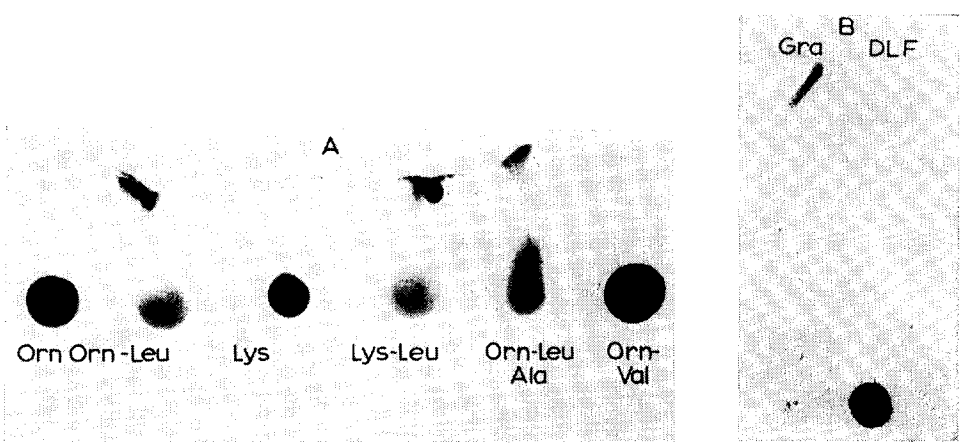

Fig. 3. Thin-layer chromatography of synthetic (A) and natural (B) basic peptides. Gra, gramicidin and DLF, direct lytic factor. Solvent: chloroform–methanol–water (60:35:8, v/v). Ninhydrin staining.

DISCUSSION

It appears that the common characteristic of the presently studied synthetic and natural basic polypeptides which determines their ability to facilitate membrane phospholipid splitting by *V. palestinae* phospholipase A depends both on their basicity and the possession of lipophilic groups. Fulfilling these two criteria among the polypeptides studied are, in addition to the natural compounds, cobra direct lytic factor and Gramicidin S, the synthetic copolymers poly-Orn–Leu, poly-Lys–Leu and poly-Orn–Leu–Ala. The synthetic polymers composed of basic amino acids only, poly-Lys and poly-Orn, were inactive.

In previous studies it was demonstrated that basic polyamino acids are adsorbed to the surface of erythrocytes[15], as they are to various other cells[16]. It is reasonable to assume that one condition for the phospholipid splitting-enhancing action of the basic polypeptides is their becoming attached to the erythrocyte ghost by virtue of their positive charge. However, the mere attachment of the polypeptides appears to be insufficient for the facilitation of phospholipase action on the membrane, and a decisive additional function must be ascribed to their lipophilic groups. It is an attractive idea to relate the membrane phospholipid splitting-facilitating action of the lipophilic groups of the polypeptides to their penetration into the lipid structure of the membrane[17], thereby enabling the phospholipase to reach the phospholipid substrate. In agreement with this interpretation would be the observation that for the membrane phospholipid splitting-facilitating action of cobra direct lytic factor, its simultaneous presence with phospholipase is required, pretreatment of the membrane with direct lytic factor and subsequent removal by heparin prior to phospholipase addition being ineffective[1]. Thus, the polymer's basicity is responsible for its approach and its lipophilic property for its paving the way for the enzyme. An analogy to the presumed penetration of the lipophilic groups of the basic polypeptides into the cell membrane may be found in the property of Gramicidin S and of the active synthetic copolymers to move in lipid solvents on thin-layer chromatography. The inability of direct lytic factor to dissolve under these conditions might point to its lipophilic groups not being oriented to the outside of the molecule. In that case, action of the lipophilic groups of direct lytic factor on the cell membrane might presuppose a conformational change in the peptide molecule when it becomes attached to the cell.

Modern concepts of cell membrane structure appear to be consistent with the hypothesis of the importance of the lipophilic group of the basic polypeptides in determining their action on the membrane. One common denominator of the various models proposed for cell membrane structure[18–21] is the orientation of the lipophilic groups of the membrane phospholipids away from the cell surface. It has been proposed[22] that the predominant force binding the protein envelopes to the phospholipids in the cell membrane is hydrophobic bonding of the non-polar side chains of the protein to the lipophilic portions, *i.e.* the fatty acid residues, of the phospholipids. Possibly, the lipophilic groups of the active copolymers disturb these protein–phospholipid bonds in the membrane, thus making the phospholipids available to the enzyme action. The importance of the lipophilic groups of the basic natural and synthetic polypeptides for their action on the cell membrane is strengthened by the observation previously made that saponin, which causes a rearrangement in the mem-

brane lipids[23,24], is also able to facilitate the action of *V. palestinae* phospholipase on ghost phospholipids.

The hypothesis of the specificity of the mode of action of the active peptides being determined by their lipophilic groups is further supported by the negative result of the experiment in which erythrocytes were subjected to gradual osmotic hemolysis in the presence of phospholipase. From previous studies by us[25] and others[26] it is known that large molecules, such as albumin, fetal hemoglobin and even ferritin, are able to penetrate the erythrocyte membrane during the process of hemoglobin release through pores kept open temporarily by suspending the cell in a medium of gradually increasing hypotonicity. Such pores, as measured by SEEMAN[26], may attain a width of 300 Å. If phospholipase did penetrate during the gradual osmotic hemolysis, the absence of membrane phospholipid splitting would indicate that contact of the enzyme with the phospholipids requires specific rearrangement in the membrane lipids, such as is brought about, presumably, by the lipophilic moieties of the active basic polypeptides, or by saponin[27].

Finally, the lack of parallelism between the strong facilitating action in the splitting of membrane phospholipids and the relatively weak hemolytic action of direct lytic factor indicates that the route by which phospholipase is enabled to reach the membrane phospholipids is different from that by which hemoglobin escapes from the cell. The dual property, strong hemolytic and strong phospholipid splitting-facilitating action, of Gramicidin S and the synthetic basic copolymers would then point to their action on the membrane being of a two-fold nature. The difference in hemolytic activity between direct lytic factor on the one hand, and Gramicidin S and the synthetic basic copolymers on the other, is not easily interpreted. Possibly, it is related to a difference in steric configuration: whereas the synthetic polyamino acids are linear chains[17] and Gramicidin S is a cyclopeptide[28], direct lytic factor is a chain folded upon itself due to three S–S bonds[2].

ACKNOWLEDGEMENTS

The authors are indebted to Dr. L. BICHOWSKY-SLOMNICKI of the Department of Biophysics, the Weizmann Institute of Science, Rehovoth (Israel) for helpful discussion. The valuable technical assistance of Mr. Z. OSSIMI is also appreciated.

REFERENCES

1 E. CONDREA, A. DE VRIES AND J. MAGER, *Biochim. Biophys. Acta*, 84 (1964) 60.
2 S. ALOOF-HIRSCH, A. DE VRIES AND A. BERGER, *Biochim. Biophys. Acta*, in the press.
3 E. KATCHALSKI AND M. SELA, *Advan. Protein Chem.*, 13 (1958) 243.
4 C. KLIBANSKY, E. CONDREA AND A. DE VRIES, *Am. J. Physiol.*, 203 (1962) 114.
5 A. DE VRIES, C. KLIBANSKY, M. DJALDETTI AND C. KIRSCHMANN, *New Istan. Contr. Clin. Sci.*, 6 (1963) 77.
6 D. DANON, *J. Cellular Comp. Physiol.*, 57 (1961) 111.
7 J. V. DACIE AND S. M. LEWIS, *Practical Haematology*, Churchill, London, 1963.
8 G. ROUSER, G. KRITCHEVSKY, C. GALLI AND D. HELLER, *J. Am. Oil Chemists' Soc.*, 42 (1965) 215.
9 S. DE BOHNER, E. F. SOTO AND T. DE COHAN, *J. Chromatog.*, 17 (1965) 513.
10 W. D. SKIDMORE AND C. ENTENMAN, *J. Lipid Res.*, 3 (1962) 471.
11 A. E. BRESLER, *Biochim. Biophys. Acta*, 39 (1960) 375.
12 B. A. BRADLOW, R. RUBINSTEIN AND J. LEE, *S. Afr. J. Med. Sci.*, 29 (1964) 41.
13 J. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
14 V. P. DOLE, *J. Clin. Invest.*, 35 (1956) 150.

15 A. NEVO, A. DE VRIES AND A. KATCHALSKY, *Biochim. Biophys. Acta*, 17 (1955) 536.
16 L. BICHOWSKY-SLOMNICKI, A. BERGER, J. KURTZ AND E. KATCHALSKI, *Arch. Biochem. Biophys.*, 65 (1956) 400.
17 E. KATCHALSKI, *Harvey Lectures, Ser.*, 59 (1963/64) 243.
18 J. F. DANIELLI, in J. F. DANIELLI, K. G. A. PANKHURST AND A. C. RIDDIFORD, *Surface Phenomena in Chemistry and Biology*, Pergamon Press, New York, 1958, p. 246.
19 H. L. BOOIJ AND H. G. BUNGENBERG DE JONG, in L. V. HEILBRUNN AND F. WEBER, *Biocolloids and Their Interactions, Protoplasmatologia I*, 2, Springer, Vienna, 1956, p. 3.
20 J. D. ROBERTSON, *Biochem. Soc. Symp. Cambridge, Engl.*, 16 (1959) 3.
21 J. L. KAVANAU, *Structure and Function in Biological Membranes*, Vol. 1 and 2, Holden-Day, San Francisco, 1965.
22 D. E. GREEN AND A. TZAGOLOFF, *J. Lipid Res.*, 7 (1966) 587.
23 A. W. CUTHBERT, *Pharmacol. Rev.*, 19 (1967) 59.
24 L. L. M. VAN DEENEN, in R. T. HOLMAN, *Progress in the Chemistry of Fats and other Lipids*, Vol. 3, Part 1, Pergamon Press, 1965.
25 C. KLIBANSKY, A. DE VRIES AND A. KATCHALSKY, *Pathol. Biol. Semaine Hop.*, 8 (1960) 2005.
26 P. SEEMAN, *J. Cell Biol.*, 32 (1967) 55.
27 R. R. DOURMASHKIN, R. M. DOUGHERTY AND R. J. C. HARRIS, *Nature*, 194 (1962) 1116.
28 R. L. M. SYNGE, *Biochem. J.*, 39 (1945) 363.