J. Lipid Research, January, 1963
Volume 4, Number 1

The biochemistry of plasmalogens: II. Hemolytic activity of some plasmalogen derivatives*

EUGENE L. GOTTFRIED and MAURICE M. RAPPORT†

Departments of Medicine and Biochemistry, and the Unit for Research in Aging, Albert Einstein College of Medicine, Yeshiva University, New York 61, N. Y.

[Manuscript received September 10, 1962; accepted October 29, 1962.]

SUMMARY

A series of lysophosphatides was prepared from the plasmalogens phosphatidal choline and phosphatidal ethanolamine, and their hemolytic activity compared with that of α-palmitoyl lysolecithin. The nature of the linkage of the hydrocarbon chain (acyl ester, α,β-unsaturated ether, or alkyl ether) did not appreciably affect lytic activity. Unsaturation in the hydrocarbon chain of lysolecithins decreased hemolytic activity considerably; lysocephalins were significantly different in this respect. Sheep erythrocytes were much more sensitive to differences in lysolecithin structure than human or rabbit erythrocytes.

Although hemolytic activity is a well-known property of lysophosphatides, particularly lysolecithins, a systematic comparison of the activity of a series of well-characterized analogues of lysolecithin has not been available. A number of these analogues have recently become more accessible by using pure phosphatidal choline as a common source; viz., lysophosphatidal choline (α-1-alkenyl lysolecithin), the corresponding saturated ether (α-alkyl lysolecithin), and β-acyl lysolecithin. It is thus possible to examine a number of compounds with closely related but distinctive structures.

An understanding of the relation between chemical structure and hemolytic activity is important for two reasons: (1) lytic activity may be a tool of considerable value for studying the molecular organization of cell membranes and other lipid-containing aggregates, and (2) it is essential to know to what degree this hemolytic activity is useful for the characterization of unknown chemical structures.

* This work was supported by PHS Research Grants A-2965, B-1570, H-3838, and M-2562, National Institutes of Health, U. S. Public Health Service. A preliminary report of this work was presented at the annual meeting of the American Society of Biological Chemists, Atlantic City, New Jersey, April 1962 (1).

† American Cancer Society Professor of Biochemistry.

In this paper, we present a quantitative comparison of the activity of ten different lysolecithins and four lysocephalins (ethanolamine derivatives) on erythrocytes from three species: man, rabbit, and sheep.

MATERIALS

Synthetic dipalmitoyl L-α-lecithin was obtained from the California Corporation for Biochemical Research; egg lecithin from the Sylvana Chemical Co., Millburn, New Jersey; and snake venoms from the Ross Allen Reptile Institute, Silver Springs, Florida.

α-Acyl Lysolecithins (Fig. 1, III*a*). Egg lysolecithin was obtained from chromatographically pure commercial egg lecithin by hydrolysis with *Naja naja* venom in moist diethyl ether containing Ca^{++} (2). Palmitoyl lysolecithin was prepared similarly from synthetic dipalmitoyl L-α-lecithin (Fig. 1, I*a*). Yeast lysolecithin was a natural product isolated by silicic acid chromatography of a partially purified yeast lecithin prepared by the method of Hanahan and Jayko (3). Vegetable lysolecithin was isolated in the same manner from a vegetable phospholipid mixture (Azolectin, American Lecithin Co.).

Lysophosphatidal choline (α-1-alkenyl lysolecithin, Fig. 1, IV*a*) and *β-acyl lysolecithin* (V*a*) were derived

from pure phosphatidal choline (II*a*) (2). *Naja naja* venom was used to cleave the fatty ester chain, producing lysophosphatidal choline. β-Acyl lysolecithin was produced by hydrolysis of phosphatidal choline with 90% acetic acid at 37° for 60 min to cleave the 1-alkenyl ether.

Lysophosphatidal ethanolamine (α-1-alkenyl lysocephalin, Fig. 1, IV*b*) was isolated from beef muscle

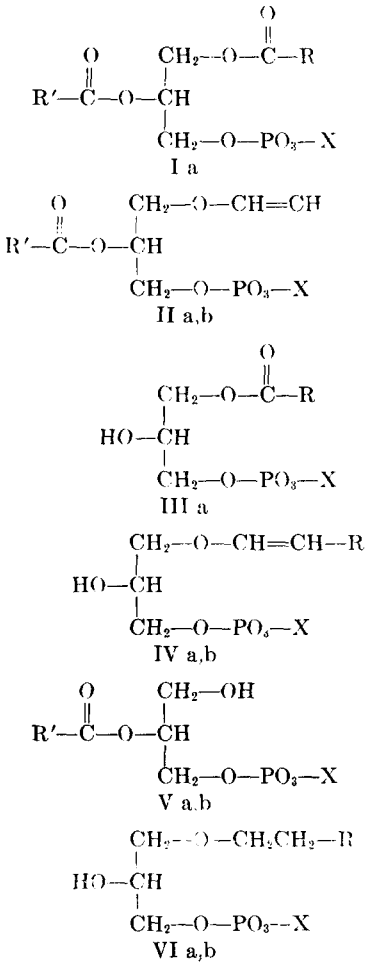

FIG. 1. Structure of various phosphatides. R and R′ represent hydrocarbon chains; X = (a) choline, or (b) ethanolamine.

(4). *β-Acyl lysophosphatidyl ethanolamine* (V*b*) was obtained by acid hydrolysis of a highly purified brain phosphatidal ethanolamine preparation (Fig. 1, II*b*)[1] with 90% acetic acid for 60 min at 37°. To prevent yellowing, acetic acid was removed immediately at low temperature in a rotary evaporator, and the product was stored in ethanol solution in the dark at −10°.

[1] M. M. Rapport and N. F. Alonzo. In preparation.

METHODS

Column Chromatography. The lysophosphatides were purified by fractionation on columns of silicic acid (Unisil, Clarkson Chemical Co., 200–325 mesh) at 5°. Discontinuous gradient elution with mixtures of chloroform and methanol was carried out as described by Hanahan *et al.* (5). Yeast and vegetable lysolecithins were fractions obtained from silicic acid–Celite columns with mixtures of methanol and ethanol (6). Further purification of the choline phosphatides after column fractionation was obtained by precipitation from concentrated solutions in ethanol or methanol with 20 volumes or more of diethyl ether or acetone, respectively.

Hydrogenations were carried out at 25° in 95% ethanol for 60–120 min, using platinum oxide or 5% palladium on charcoal as catalyst, in a microhydrogenation apparatus similar to that described by Farquhar *et al.* (7). Hydrogenation was used for preparation of α-alkyl lysolecithin (Fig. 1, VI*a*) and α-alkyl lysocephalin (VI*b*) (from the corresponding 1-alkenyl compounds), saturated lysolecithins (from the lysophosphatides of yeast and vegetable origin), and reduced β-acyl lysolecithin.

Methods for determination of phosphorus, ester groups, unsaturation by bromine addition, and α,β-unsaturated ether groups have been described (2).

Gas-Liquid Chromatography. Methyl esters of the fatty acyl chains of the lysophosphatides were prepared by the method of Stoffel *et al.* (8), omitting the sublimation step. Gas-liquid chromatography was performed with a 6-ft column of 20% diethylene glycol succinate polyester on 80–100 mesh Gas-Chrom P at 175° or 185°, using a Barber-Colman model 15 chromatograph equipped with an argon ionization (Sr^{90}) detector.

Thin-Layer Chromatography. Plates of 200 x 200 mm were prepared with Silica Gel G (Merck) containing calcium sulfate, using a Desaga spreader (9). Development was carried out at room temperature in rectangular tanks (26 x 26 x 7 cm interior dimensions) lined on three sides with Whatman #1 filter paper, using a solvent mixture of chloroform–methanol–water 75:25:4. The following methods of detection were used: (1) iodine vapor, (2) charring in an oven at 160° after spraying with 50% sulfuric acid, (3) examination under ultraviolet light after spraying with 0.5% iodine in chloroform followed by dilute (0.001%) 2′,7′-dichlorofluorescein in ethanol.[2] Plasmalogens and free aldehydes were identified from the deep purple

[2] R. B. Brandt and H. Schneider, private communication.

spots formed on spraying successively with 1% mercuric chloride, Schiff reagent, and 5% sodium bisulfite. Each of the lysophosphatides tested at the 50-μg level showed only a single spot, the lysolecithins having an R_f value of about 0.10, and the lysocephalins an R_f value of 0.30–0.35. No significant change in R_f was observed with differences in linkage or position of the hydrocarbon chain or in degree of unsaturation.

Erythrocytes. Pooled blood from Merino sheep was collected in modified Alsever's solution (10). Ear vein blood from individual New Zealand white male rabbits was also collected in modified Alsever's solution, as was venous blood from healthy human volunteers. Comparison of red cells stored in modified Alsever's solution with cells from freshly drawn blood collected in tubes containing disodium EDTA as anticoagulant showed no difference in sensitivity to lysis by lysolecithin as a result of the addition of preservative. With storage at 4°, however, cells in Alsever's medium remained stable to the action of lysolecithin for periods up to 3 weeks, while cells treated with EDTA quickly deteriorated.

Preparation of Standard Erythrocyte Suspension. Red blood cells were washed three times with 20 volumes of isotonic, phosphate-buffered saline, pH 7.4, then separated by brief centrifugation and resuspended in a volume of buffered saline sufficient to make a final count of 3.0×10^5 cells/mm^3. Red cell counts were performed with a Spencer "Bright-Line" hemacytometer.

Preparation of Lysolecithin Solutions for Assay. Solutions in isotonic, phosphate-buffered saline, pH 7.4, were prepared in concentrations of 10–100 μg of lysolecithin/ml by adding 9 volumes of saline to the appropriate quantity of lysolecithin dissolved in a small volume of absolute ethanol. While the lysolecithins retained complete hemolytic activity when stored in organic solvents in the cold and protected from light and oxygen, the saline solutions of some preparations lost activity rapidly (2–3 hr). For this reason, all dilutions in saline were made directly before assay (within 10 min).

Determination of Hemolytic Activity. To a series of 12 x 75 mm tubes previously calibrated for optical uniformity, increasing quantities of phosphatide were added in saline solution, and the volume adjusted with additional buffered saline to 0.40 ml. To each tube, 0.10 ml of erythrocyte suspension (3.0×10^7 cells) was added. The contents were mixed and the tubes were placed in a 37° shaking water bath (Research Specialties Co., model #2156) for 15 min. Additional buffered saline was then added to bring the volume of each tube to 1.0 or 1.5 ml; the tubes were centrifuged briefly,

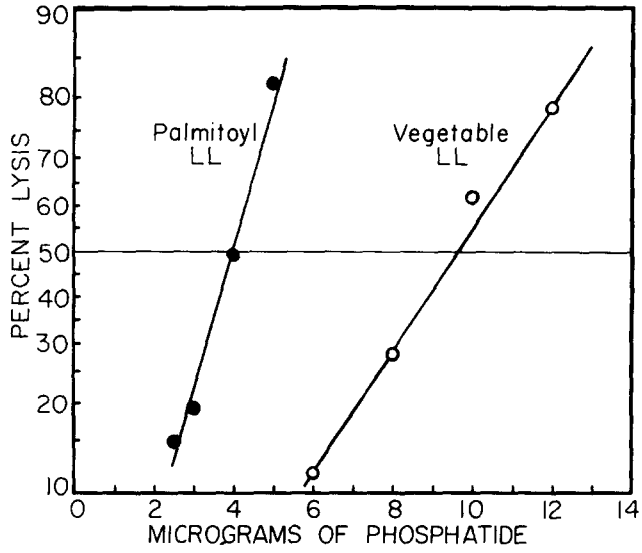

FIG. 2. Lysis of human erythrocytes by α-palmitoyl lysolecithin (solid circles) and vegetable lysolecithin (open circles).

and the absorbancy of the supernatant solution was determined at 540 mμ with a Coleman Jr. model 6A spectrophotometer. Determination of the absorbancy for 100% hemolysis was made by using control tubes containing distilled water instead of saline. In the system described, lysis proceeds rapidly at first, then ceases after about 10 min; stable readings may be obtained after a 15-min incubation period.

The amount of phosphatide required for 50% lysis (the L_{50} value) is readily determined by graphical interpolation. The plot of percentage lysis versus concentration of lysolecithin takes the form of a sigmoid curve (11). If the data are plotted in a logarithmic unit (logit, probit) form, the curve is converted to a straight line (Fig. 2). In order to minimize the effects of biological variation in the red cells, each day's experiments were evaluated with reference to the activity of a standard preparation of lysolecithin. The reference standard chosen was α-palmitoyl lysolecithin prepared from synthetic dipalmitoyl L-α-lecithin. In the experiment illustrated in Fig. 2, the L_{50} value for palmitoyl lysolecithin is 4.0 μg, while this value for vegetable lysolecithin is 9.6 μg.

RESULTS

Characterization of Lysophosphatides. Choline and ethanolamine lysophosphatides were characterized by analyses for phosphorus, ester groups, α,β-unsaturated ether groups (iodine uptake), unsaturation (bromine uptake), and fatty acid composition (Table 1). Each of these materials was chromatographically pure. The

TABLE 1. ANALYSES OF THE LYSOPHOSPHATIDES

Lysophosphatide	P	Molar Ratio			Fatty Chains					
		Ester:P	I_2:P	Br_2:P	16:0	16:1	18:0	18:1	18:2	Other
	%				*Mole %*			*Mole %*		*Mole %*
I. Lysolecithins										
α-Palmitoyl	6.28	0.98		<0.05	100					
Egg	5.70	0.99		0.08	76		20	3		
Yeast	6.00	0.97		0.92	4	70		24		
Reduced yeast	5.71	1.02		<0.05						
Vegetable	5.66	1.00		1.38	20		5	11	62	
Reduced vegetable	5.92	1.00		0.06						
β-Acyl	5.80	1.00		2.07	3	1		15	62	20:3, 8%; 20:4, 10%
Reduced β-acyl	5.67	1.05		<0.05						
α-1-Alkenyl (lysophosphatidal choline)	6.06	0.03	0.93	0.89	(59)		(21)			17:0 (br, ante-iso), 9%
α-Alkyl (reduced lysophosphatidal choline)	5.90	<0.03	<0.03	<0.05						
II. Lysocephalins (ethanolamine lysophosphatides)										
β-Acyl	5.68	1.02		2.22	2		1	51	2	20:1, 13%; 20:3, 5%; 20:4, 7%; 22:4, 3%; 22:6, 9%
Reduced β-acyl	5.45	1.01		0.10						
α-1-Alkenyl (lysophosphatidal ethanolamine)	6.34	0.03	0.89	0.85						
α-Alkyl (reduced lysophosphatidal ethanolamine)	6.49		<0.03	0.08						

α-acyl lysolecithins were composed chiefly of C_{16} and C_{18} fatty acids. Both synthetic palmitoyl lysolecithin and egg lysolecithin were saturated, while yeast and vegetable lysolecithins contained predominantly unsaturated hydrocarbon chains. The major fatty chain of yeast lysolecithin was $C_{16:1}$; of vegetable lysolecithin, $C_{18:2}$. Both β-acyl lysolecithin and β-acyl lysocephalin were highly unsaturated, with an average of 2.1 and 2.2 double bonds, respectively. Each contained a large number of different hydrocarbon chains, including polyunsaturated fatty acids. The main fatty acid component of β-acyl lysolecithin was $C_{18:2}$, while β-acyl lysocephalin contained 50% of $C_{18:1}$. The composition of the aldehydogenic chains of lysophosphatidal choline, shown in parentheses, is based upon the analysis of the starting material (2); the chains are almost completely saturated, except for the α,β-unsaturated ether group of the plasmalogen, and C_{16} predominates.

Hemolytic Activity of Lysolecithins. Hemolytic activities of the choline-containing lysophosphatides, relative to that of palmitoyl lysolecithin, are listed in Table 2. The amount of palmitoyl lysolecithin required for lysis of 50% of the red cells (L_{50} value) was determined for each day's experiments. The L_{50} value for this reference compound was 4.34 ± 0.50 μg with human red cells, 3.95 ± 0.43 μg with rabbit cells, and 1.98 ± 0.27 μg with sheep cells. The smaller L_{50} value obtained for sheep erythrocytes might reflect the smaller cell surface area—approximately half that of human or rabbit erythrocytes (12)—but other factors must be considered as well (cf. Discussion).

Relative hemolytic activity of the lysophosphatides was determined by comparing L_{50} values with the L_{50} value of palmitoyl lysolecithin; relative activity in per cent was expressed as the reciprocal of molar L_{50} ratio (based on phosphorus content) multiplied by 100.

The lysolecithins tend to fall into three groups with respect to hemolytic activity. The saturated α-acyl lysolecithins of egg, yeast, and vegetable origin had relative hemolytic activity ranging from 85% to 120% with the three red cell types; the over-all average was 100% for this group. Lysophosphatidal choline (α-1-alkenyl lysolecithin) had an activity of 80–95%, while its saturated alkyl ether derivative was active at the 100–115% level. On the other hand, the unsaturated lysolecithins of yeast and vegetable origin had much less activity—40–50% with human and rabbit erythrocytes, and only 25% with sheep erythrocytes. β-Acyl lysolecithin, the most unsaturated lysolecithin, showed very low activity with sheep erythrocytes—only 8%. Hydrogenation resulted in a marked increase of hemolyzing power, particularly with sheep erythrocytes. Fully saturated β-acyl lysolecithin

showed a 20-fold increase in activity over its unsaturated precursor and was thus even more active than the reference standard, α-palmitoyl lysolecithin. With human and rabbit cells, saturated β-acyl lysolecithin was about as active as the saturated α-acyl lysolecithins (85–100%).

It is clear from these figures that sheep erythrocytes are a more sensitive indicator of structural changes in the lysophosphatides than human or rabbit erythrocytes.

Hemolytic Activity of Lysocephalins. Because of limited solubility in the saline medium employed, this series of lipids was more difficult to study than the corresponding choline compounds. Solubilization was achieved by substitution of methanol for ethanol in the initial step and by careful warming. While this procedure yielded nearly clear solutions with the unsaturated materials, preparations of the saturated derivatives—especially β-acyl lysocephalin—showed definite turbidity.

Table 3 shows the relative activity of the ethanolamine phosphatides. With sheep cells, the relative activity of α-alkenyl and α-alkyl lysocephalins was 110% and 115%, respectively, in the same range as the choline analogues. Although β-acyl lysocephalin was less active than the reference standard (70%), it was ten times as active as β-acyl lysolecithin. Hydrogenation reduced hemolytic activity so that reduced β-acyl lysocephalin was only one-third as active on sheep cells as reduced β-acyl lysolecithin. This is in contrast to the effect of hydrogenation in the lysolecithin series. With human erythrocytes, alkenyl and alkyl lysocephalins showed only one-third the activity of the corresponding lysolecithins, whereas β-acyl lysocephalin and β-acyl lysolecithin had the same activity. Saturated β-acyl lysocephalin did not produce 50% lysis in the concentrations tested (up to 100 μg/0.5 ml of reaction mixture) but reached a plateau at 25–35% lysis. This phenomenon may be explained by the limited dispersion of this material in the aqueous medium, which gave concentrations adequate for lysis of sheep erythrocytes but insufficient to lyse 50% of human red cells, which are more resistant.

TABLE 2. ACTIVITY OF CHOLINE LYSOPHOSPHATIDES

Lysolecithin	Relative Activity* Against Erythrocytes		
	Human	Rabbit	Sheep
Saturated α-acyl			
Palmitoyl	100	100	100
Egg	115	85	105
Reduced yeast	95	85	115
Reduced vegetable	95	95	120
Saturated β-acyl	100	85	150
α-1-Alkenyl	80	85	95
α-Alkyl	100	100	115
Unsaturated α-acyl			
Yeast	40	40	25
Vegetable	50	40	25
β-Acyl	35	35	8

* Relative Activity = Hemolytic activity relative to that of palmitoyl lysolecithin (expressed in per cent), derived from reciprocal molar L_{50} ratio. The L_{50} value is that quantity of phosphatide giving 50% lysis in the test system used (see text). The absolute L_{50} values for palmitoyl lysolecithin, used as a reference here, were 0.0086, 0.0081, and 0.0041 μmoles for human, rabbit, and sheep erythrocytes, respectively.

DISCUSSION

The results of this study indicate that in lysophosphatides the nature of the linkage of the hydrocarbon chain to glycerol (viz., acyl ester, α,β-unsaturated ether, or alkyl ether) does not materially influence hemolytic activity. In contrast, unsaturation of the hydrocarbon chain of the choline lysophosphatides is associated with a considerable reduction of activity. This effect of unsaturation is almost negligible when the unsaturation is present at the polar end of the molecule, as in the lysoplasmalogens. Low activity in the unsaturated lysolecithins possibly results from greater bending and consequent bulkiness of the hydrocarbon chain, which reduces penetration into the protein-lipid leaflets of the cell membrane. Comparison of the hemolytic activity of ethanolamine lysophosphatides with the corresponding choline analogues does not yet permit any generalization regarding the influence of the polar portion of the molecule. It is clear, nevertheless, that physical state as well as chemical configuration plays an important role in this system.

It is interesting that the largest difference in activity (with sheep erythrocytes) was found between saturated and unsaturated β-acyl lysolecithins. Evidently, although linkage of the hydrocarbon chain in the β-position is favorable for hemolytic activity, un-

TABLE 3. ACTIVITY OF ETHANOLAMINE LYSOPHOSPHATIDES

Lysocephalin	Relative Activity*	
	Human	Sheep
α-1-Alkenyl	30	110
α-Alkyl	35	115
β-Acyl	45	70
Saturated β-acyl	<5	55

* Relative Activity = Hemolytic activity relative to that of palmitoyl lysolecithin (expressed in per cent), derived from reciprocal molar L_{50} ratio.

saturation decreases activity even more effectively than with α-chain analogues. The possible influence of polyunsaturation in the chains of the β-acyl lysolecithin preparation must, of course, be considered.

Since the possibility has been raised of migration of the acyl chain of β-acyl lysolecithin to the α-position on silicic acid columns, and because column chromatography was necessary to obtain a pure product, contact time with the silicic acid was minimized by using a rapid flow rate, and the columns were maintained at 5°. Assays of hemolytic activity before and after column fractionation revealed a change in activity of only a few percent, suggesting that little or no migration had occurred.

Earlier studies (13, 14) of red cell lysis with egg lysolecithin suggested that the amount of lipid necessary for complete lysis is just sufficient to form a monolayer on the surface of the cells, or to "combine" with the free cholesterol in the cell membranes. The relationship of the L_{50} value for the saturated α-acyl lysolecithins to surface area and to cholesterol content would tend to fit either of these hypotheses, but the anomalous behavior of the unsaturated lysolecithins—especially β-acyl lysolecithin—suggests that the relationship is much more complex. Aggregate formation, partition between aqueous and lipid phases, and the role of other variables in membrane architecture (protein composition, lecithin vs sphingomyelin content, ratio of saturated to unsaturated fatty acids, etc.) are yet to be evaluated.

Assays of hemolytic activity are usually performed either by determination of the time required for complete lysis of a red cell suspension or by measurement of the degree of lysis after a fixed time interval (11, 13, 15, 16). The test system in the present study uses the latter procedure to determine the amount of lytic agent required to produce 50% lysis (the L_{50} value) of a fixed number of cells. This method offers a number of advantages over the measurement of lysis time. The L_{50} value is found by interpolation from a curve determined by several points, instead of using either a single point or the less precise method of extrapolation. Measurement of time intervals is not critical because the extent of hemolysis does not change appreciably after the initial 15-min reaction period. The L_{50} value indicates the *average* sensitivity of the entire cell population, whereas an end point of 100% lysis depends primarily on the most resistant cells.

The use of one preparation of lysolecithin as a reference standard provides a convenient means of comparing the results of different experiments. A control is introduced to minimize the effects of day-to-day biological variation in the red cells. Different lysophosphatides may be compared by relating their activity to this common reference point. In addition, one may evaluate quantitatively the effects of changes either in the test object (cell species, prior treatment of cells, disease states, etc.) or in experimental conditions (added substances, concentration of erythrocytes, temperature, etc.).

The authors are indebted to Phyllis Rosen for her capable technical assistance.

REFERENCES

1. Gottfried, E. L., and M. M. Rapport. *Federation Proc.* **21**: 293, 1962.
2. Gottfried, E. L., and M. M. Rapport. *J. Biol. Chem.* **237**: 329, 1962.
3. Hanahan, D. J., and M. E. Jayko. *J. Am. Chem. Soc.* **74**: 5070, 1952.
4. Rapport, M. M., B. Lerner, N. Alonzo, and R. E. Franzl. *J. Biol. Chem.* **225**: 859, 1957.
5. Hanahan, D. J., H. Brockerhoff, and E. J. Barron. *J. Biol. Chem.* **235**: 1917, 1960.
6. Rapport, M. M., and N. Alonzo. In *Biochemical Problems of Lipids*, edited by G. Popják and E. Le Breton, London, Butterworths Scientific Publications, 1956, p. 69.
7. Farquhar, J. W., W. Insull, Jr., P. Rosen, W. Stoffel, and E. H. Ahrens, Jr. *Nutrition Revs.* **17**: (suppl.), August, 1959.
8. Stoffel, W., F. Chu, and E. H. Ahrens, Jr. *Anal. Chem.* **31**: 307, 1959.
9. Mangold, H. K. *J. Am. Oil Chemists Soc.* **38**: 708, 1961.
10. Wadsworth, A. B. *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, 3rd edition, Baltimore, Williams and Wilkins, 1947, p. 228.
11. Collier, H. B. *J. Gen. Physiol.* **35**: 617, 1952.
12. Spector, W. S. *Handbook of Biological Data.* Philadelphia, W. B. Saunders, 1956, p. 275.
13. Gorter, E., and J. J. Hermans. *Rec. trav. chim. Pays-Bas* **62**: 681, 1943.
14. Collier, H. B., and H. L. Chen. *Can. J. Research, Sect. E.* **28**: 289, 1950.
15. Ponder, E., and R. T. Cox. *J. Gen. Physiol.* **35**: 595, 1952.
16. Greisman, S. E. *Proc. Soc. Exptl. Biol. Med.* **101**: 117, 1959.