The Phospholipids of Liver, Plasma, and Red Cells in Normal and Cholesterol-Fed Anemic Guinea Pigs[1] (34890)

R. OSTWALD, W. YAMANAKA, AND M. LIGHT
(Introduced by R. L. Lyman)
Department of Nutritional Sciences, University of California, Berkeley, California 94720

Dietary cholesterol (C) produces in guinea pigs (GP), a hemolytic anemia which is accompanied by pathological changes in the liver and other tissues and by changes in the morphology of the red blood cells (RBC) (1–3).

It is known that the stability of RBC may be influenced by their lipid composition, that lipids exchange between plasma and RBC and that the liver plays a controlling role in determining the lipid composition of plasma (4–6). The amount of C, the ratio of C to phospholipids (PL), the amounts of individual phospholipid classes, and the proportions of certain fatty acids (FA) have all been reported to affect the shape, permeability, and stability of red cells (4, 7, 8).

We have previously reported that the level of total and unesterified C and the ratio of C to PL are greatly increased in liver, plasma, and RBC from C-fed, anemic guinea pigs (9). We are now reporting on the distribution and FA composition of individual PL of these tissues from normal and anemic GP. The results are compared with data from other species which are not as susceptible to injury by C as GP and are discussed in relation to the contribution which the changes in PL composition observed in the anemic GP may make to the histopathology of the liver, the changed morphology of the RBC and the hemolytic process.

Materials and Methods. Groups of young male guinea pigs were fed a semipurified diet with or without the addition of 1% cholesterol (Table I). They were sacrificed after 10–12 weeks. RBC of the C-fed animals were then less than 3 million/mm^3. Lyophilized livers, plasma, and wet-packed RBC were extracted with chloroform–methanol (2:1 for livers and plasma; 1:1 for RBC). The PL were isolated by silicic acid column chromatography (12) and separated into their major components by thin-layer chromatography (TLC) (13). A wash of the silicic acid column with 2-butanone prior to the elution of RBC–PL was found to remove heme pigments which may otherwise interfere with the subsequent TLC procedures. Phosphatidylinositol (PI) and phosphatidyl serine (PS) of liver and plasma as well as phosphtidyl ethanolamine (PE) and PS of RBC were not well separated on TLC and were, therefore, combined for P and FA analyses. Liver and plasma PL classes were transmethylated with 2% H_2SO_4 in

TABLE I. Composition of Control Diet.[a]

Component	g/100 g
Casein	30.0
Cornstarch	20.0
Cerelose	7.9
Sucrose	8.0
Cottonseed oil	9.5
Solka floc	10.0
Alfalfa meal	5.0
Salt mix[b]	7.5
Ferric citrate	0.10
Choline bitartrate	0.36
Ascorbic acid	0.20
Arginine	0.30
B-vitamin mix[c]	0.5
Fat-sol. vitamin mix[c]	0.5

[a] Cholesterol diet had 1 g cholesterol/100 g diet added.

[b] Salt mix GP S1 (10); trace minerals were added, mg/kg salt mix: $KCr \cdot (SO_4)_2 \cdot 12H_2O$: 128.0; $Na_2MoO_4 \cdot 2H_2O$: 6.67; $NiCl_2 \cdot 6H_2O$: 2.67; Na_2SeO_3: 2.67 (11).

[c] (10).

[1] Supported in part by Public Health Service Research Grant AM 08480 from National Institute of Arthritis and Metabolic Diseases. Portions have been presented at the Federation Meeting 1968.

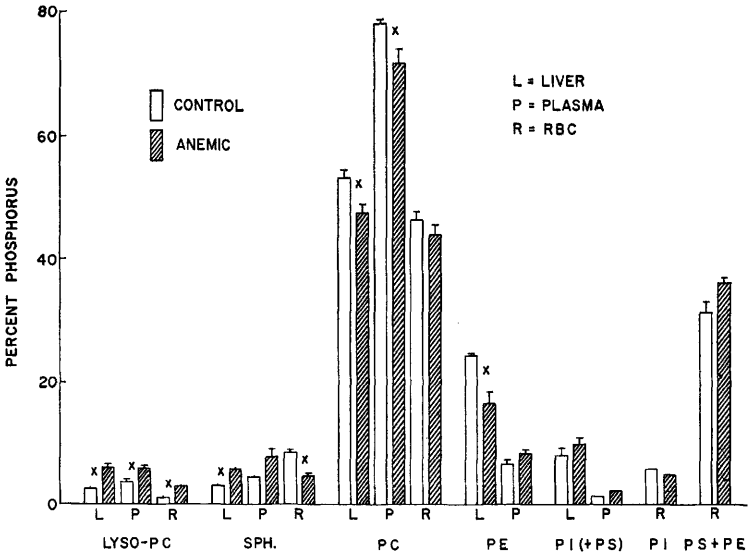

FIG. 1. Phospholipid composition of liver, plasma and RBC, in control and cholesterol-fed, anemic guinea pigs. Bars are means of group of 4–6 animals. Vertical lines are half SE of means. X indicates that the difference between the control and anemic group was significantly different at $p < .05$. The phospholipid fraction of each animal was separated by TLC, P of each fraction was determined in duplicate. Phosphorus recovery, *e.g.*, sum of P recovered compared to P applied to the TLC plate was 85–95%. The amount of PS in liver was very small compared to PI. Preliminary separations of RBC-PL using $CHCl_3$; CH_3OH; H_2O (10:8:0.08) indicated that they contained approximately equal amounts of PS and PE. More recent experiments (unpublished results) using a modified technique for transmethylation, indicate that the changes of plasma Sph and PC in response to C-feeding may be more pronounced than presented here. The changes of lyso-PC and PC expressed as Δ mole % were:

	liver	plasma	RBC
lyso-PC	+6.1	+3.9	+3.3
PC	−7.5	−7.5	−2.5

methanol at 70–80° for 1–1.5 hr, followed by elution from the silica gel with absolute and 95% methanol. RBC-PL were transmethylated for 2 hr in screw-capped tubes sealed with Teflon tape in order to assure complete reaction of the large amounts of sphingomyelin (Sph). Fatty acid methylesters were extracted into petroleum ether and analyzed by gas–liquid chromatography. The amount of each PL class was determined by phosphorus analysis of the remaining aqueous layer (14).

Results. The relative distribution of PL classes is shown in Fig. 1. Phosphatidylcholine (PC) was the largest component in all three tissues, particularly so in plasma. Liver and RBC-PL were also characterized by high levels of PE and those of plasma by very low levels of PI + PS. The distribution of the PL classes in tissues from C-fed anemic guinea pigs differed from that of controls, in that the proportions of lyso-PC increased significantly at the expense of PC in all three tissues of C-fed animals. The proportion of Sph increased in liver and plasma but decreased in RBC and PE decreased in liver.

In plasma and RBC the total amount of each PL class increased in the C-fed anemic animals, except the amount of Sph in RBC which remained unchanged (Table II). The total content of lyso-PC and Sph in liver (mg/liver) increased in spite of the decreases of total PL. In terms of grams of PL/100 g liver, however, the decreases of total PL just outbalanced the proportional increases of these two PL.

The FA composition of lyso-PC differed in the three tissues (Table III). Liver lyso-PC was highest in 18:0 and 18:2, that of plasma

TABLE II. Amounts of Total PL and PL Classes of Liver, Plasma, and RBC in Control and C-fed Anemic GP.[a]

	Lyso-PC	Sph	PC	PI + PS	PE	Total PL
			Liver[b] (g/100 g wet wt)			
Control	0.087	0.145	2.53	0.372	1.149	4.56
C-fed	0.093	0.134	1.10[d]	0.229[e]	0.387[d]	2.32[d]
+C/—C[c]	1.1	0.9	0.4	0.61	0.34	0.51
			Liver (mg/liver)			
Control	12	19	349	50	158	740
C-fed	23[d]	35[d]	287	62	101	600
+C/—C	1.9	1.8	0.8	1.2	0.6	0.82
			Plasma (mg/100 g wet wt)			
Control	0.9	1.5	26.9	0.5	2.2	34.2
C-fed	4.5[d]	8.5[d]	87.9[d]	2.6[d]	9.7[d]	119.9[d]
+C/—C	5.3	5.5	3.3	5.5	4.3	3.5
	Lyso-PC	Sph	PC	PI	PS + PE	Total PL
			RBC (mg/100 ml)			
Control	2.1	22.3	121.4	14.9	81.9	261
C-fed	9.8[d]	23.0	212.5[d]	23.0	173.8[d]	482[d]
+C/—C	4.7	1.0	1.7	1.5	2.1	1.8

[a] Values are means of groups of 4–6 animals fed the control diet (—C) or the diet supplemented with 1% cholesterol (+C) until the blood count in the latter group had fallen to <3 million/mm^3. For abbreviations see Methods section.

[b] Liver weights (g): —C: 15.06 ± 1.54; +C: 25.68 ± 3.51.

[c] Ratio of means for C-fed and control group.

[d] Differences between groups were statistically significant at $p < .01$.

[e] Differences between groups were statistically significant at $p < .05$.

had more 16:0 and 18:1 than liver and that of RBC was very high in 18:0 but low in 18:2. The response of the FA composition of lyso-PC from liver and RBC to dietary C was an increase in unsaturation. In liver, the proportion of 18:1 increased, in RBC that of 18:2. The composition of plasma lyso-PC remained essentially unchanged.

RBC-Sph had a very high content of 16:0 and of long-chain FA (> 20 C) in both groups.

The FA compositions of liver and plasma PC were very similar, both containing high levels of 18:0 and 18:2. RBC-PC differed from these by lower proportions of 18:2 and higher levels of 18:1. C-feeding produced in PC of RBC a decrease of 18:0 and 18:2 compensated by increases in 18:1 and 20:4. The sum of 18:0 plus 18:1 and 18:2 plus 20:4 remained unchanged. (In RBC-TPL similar changes were accompanied by a decrease of higher PUFA.) The composition of liver PC remained unchanged, while that of plasma PC tended to become more like that of RBC by increases in the proportion of 16:0 and a decrease in 18:0 in the C-fed animals.

The major FA of liver PE and PI were 18:0 and 18:2 as was the case in all liver PL classes. Plasma PE was strikingly low in 18:0 but high in 20:4 compared to other plasma PL and compared to liver PE. The PS + PE fraction of RBC had a high proportion of 18:0 and appreciable amounts of long-chain unsaturated FA. Plasma and RBC-PE were the only PL of normal GP which had more than 10% of 20:4. C-feeding had no effect on the composition of PE from plasma and RBC. In the PI fraction of liver there was a signifi-

TABLE III. Fatty Acid Composition of Phospholipid Classes of Liver, Plasma, and RBC in Control and C-Fed, Anemic Guinea Pigs.[a]

	Weight % total methylester measured[b]													
	16:0[c]		18:0		18:1		18:2		20:4		Others		TFA[d]	
	Control	C-fed	Control	C-fed	Control	C-fed	Control	C-fed	Control	C-fed	Control	C-fed	Control	C-fed
													Liver (mg/100 g wet wt)	
Lyso-PC[e]	10.5	12.3	38.8	26.9[f]	5.4	11.6[f]	35.6	42.7	3.5	3.0	3.6[g]	0.7	55	47
PC	15.9	17.3	30.5	25.1	4.9	7.0	45.2	44.6	2.6	4.6			1742	771[f]
PE	7.9	10.3	37.1	32.6	4.4	6.5	39.0	37.8	10.4	11.6			756	271[f]
PI (+PS)	8.5	5.8	38.3	38.7	6.2	8.3	33.1	28.9	8.1	17.8[f]	2.9[h]	2.2	215	160[f]
													Plasma (mg/100 g wet wt)	
Lyso-PC	20.2	20.2	19.0	25.2	19.1	12.7	33.1	38.0	3.7	2.6			0.4	2.3[f]
PC	15.0	20.2[f]	31.3	23.0[f]	6.3	8.7	42.1	41.1	4.0	4.8			18.8	61.5[f]
PE	8.5	12.4	13.0	9.5	4.8	11.7	33.4	35.1	28.0	24.1	7.4[g]	7.1	1.6	6.8[f]
													RBC (mg/100 ml)	
TPL-FA	12.7	12.7	29.9	27.0[f]	6.5	9.4[f]	17.3	14.5[ff]	15.5	20.3[ff]	14.2[k]	10.8[f]	183	338[f]
Lyso-PC	18.4	22.0	57.4	44.8[f]	4.1	6.9	7.0	14.8[ff]	1.6	2.1	6.0[k]	3.5	1.1	5.0[f]
Sph	59.7	65.5	8.6	7.6	2.1	0.9	2.1	0.9	5.2[n]	4.3	17.3[m]	16.0	8.5	8.7
PC	21.9	20.4	27.2	21.1[f]	10.7	15.5[f]	29.3	21.1[f]	4.3	12.7[f]	2.0[k]	3.0	85.0	148.7[f]
PS + PE	4.4	4.4	41.8	41.2	4.5	5.0	9.4	7.4	25.1	28.9	11.1[p]	8.8	57.3	121.7[f]

[a] Values are means of groups of 4–9 animals fed the control diet (—C) or the diet supplemented with 1% cholesterol (+C) until the blood count in the latter group had fallen to <3 million/mm³. The sums of fatty acids presented do not add to 100% because minor components are not listed. The PI + PS fraction of plasma and the PI fraction of RBC represented less than 5% of total P. Their fatty acid composition is, therefore, not presented here. The Sph-FA of liver and plasma were not determined. SE are not presented for reasons of space economy.

[b] The absolute amounts of each FA (mg/100 ml or mg/100 g) are not presented here for reasons of space economy. They can be calculated from: per cent FA × mg TFA/100 ml (or per 100 g).

[c] Number of carbon atom : number of double bonds.

[d] Total amount of fatty acids in PL-class, calculated from: mg P/100 ml (or 100 g) × 0.51, 0.38 or 0.70 for lyso-PC, Sph, or the other PL respectively.

[e] For abbreviations see Methods section.

[f] Differences between groups were statistically significant at $p < .01$.

[ff] Differences between groups were statistically significant at $p < .02$.

[g] Retention times <16:0.

[h] Retention times <18:2.

[k] Retention times >20:4; there were three major peaks which were tentatively identified by hydrogenation to be 24:0, 24:1, and 22:6.

[m] Retention times >20:4; three major peaks, tentatively identified as 24:0, 24:1, and 24:2.

[n] Retention time 20:4, but was mostly overlapping peak of 22:0.

[p] Retention time >20:4, tentatively identified as 22:6.

cant increase of the proportion of 20:4.

The amounts of each FA (mg FA/100 ml) of the individual phospholipids of plasma and RBC (except RBC-Sph) increased due to the large increase of total PL. The amount of 20:4 in TFA and PC of RBC, for instance, increased by a factor of 2 and 5 respectively. In livers, the amounts of each FA in a phospholipid fraction changed in the same direction as did that phospholipid (Table II) because, with three exceptions, the FA composition remained constant (Table III). The amount of 20:4 in the PI (+ PS) fraction doubled in spite of a decrease of this fraction and the amounts of 18:0 and 18:1 of lyso-PC decreased and increased, respectively, in spite of an unchanging amount of lyso-PC.

Discussion. Tissue phospholipid composition of normal guinea pigs and other species. Two reports of the composition of RBC-PL of guinea pigs by Nelson (15) and Condrea (16) give data similar to ours. Two others (17, 18) are not comparable because of differences in methodology. A report on the PL of GP liver subcellular fractions (19) indicates an FA distribution resembling our data on whole liver PL.

The PL compositions of liver and plasma appear to be characteristic for these tissues in most species. The distribution of PL classes in liver of rats (13) and hamsters (20), for instance, are very similar to those reported here for GP. In plasma, however, the lyso-PC of GP (4% of TPL) was low compared to the series: dog (8%), pig (12%), cat (14%), rabbit (20%), and rat (23%) (18).

Greater species variations have been observed in respect to RBC-PL. The ratio of PC/Sph has been reported to increase from 0.02 to 2.2 in the series sheep, ox, pig, man, rabbit, and rat. In the normal GP this ratio was 5.7, in the C-fed GP it increased further to 8.9. van Deenen (4) has pointed out that the permeability to urea and glycerol and the rate of hemolysis in glycerol increases in the same order as this index of RBC composition. Earlier, Turner (21) had suggested that RBC with high PC content are more susceptible to lysis by snake venom than ruminant cells which have little or no PC. According to this characterization the GP RBC would have high permeability and susceptibility to lysis and this would be accentuated in the C-fed GP. This may provide a rationale for their C-induced anemia.

The fatty acid composition of GP-PL classes has not previously been reported. Our data show that GP-PL were low in 20:4 and high in 18:2 as compared to those reported for rats (7, 22, 23). An exception was RBC-PE which contained as much 20:4 as did those of rats. We have previously reported that C-ester of GP liver and plasma also are extremely low in 20:4 compared to rats and have discussed the implications of this observation for the greater susceptibility of the GP to injury by excess dietary C (9). On the basis of the reports of Glomset (24) and Akiyama (25) on plasma and liver lecithin cholesterol acyltransferase (LCAT) it is now clear that these low levels of CE-20:4 are a consequence of the low levels of PC-20:4. The reason for the species-specific low levels of 20:4 in all GP tissues remains to be elucidated.

Response of PL composition to dietary cholesterol. McCandless (26) and Zilversmit (27) reported that the sphingomyelin content of aorta, liver, and plasma increased in C-fed rabbits at the expense of non-choline phosphatides. Sheltawy (28) reported a similar shift in liver mitochondria of C-fed rats. In view of the increases of sphingomyelin in liver and plasma from our C-fed GP, this response to dietary C seems to be quite general, although its physiological significance is not clear.

The mechanism for the increase of the proportion of TPL-20:4 in the RBC of C-fed anemic guinea pigs accompanied by the decrease of higher PUFA may be analogous to that proposed by Witting (29) for similar effects observed in antioxidant-deficient rats. The demand for PUFA by the guinea pig may be increased because the need to transport excess C increases the requirement for essential FA (30). The FA composition would then be a composite effect reflecting the increased quantities of PUFA formed in response to the increased demand and the preferential peroxidative destruction of the most highly PUFA.

It has been shown that the lipid composi-

tion of plasma affects that of RBC (4). The large increases of plasma C and PL and the changed plasma PL composition in the anemic GP may therefore be responsible for the lipid changes observed in their RBC. Since not all PL are exchanged intact (31) it is not surprising that the changes in the RBC lipids differ from those in plasma.

The lipid composition of reticulocytes and "young" RBC differ from that of "old" cells (32). Young RBC contain more TL, C, and P per cell and higher proportions of 16:0 and 20:4, while 18:0 and 18:2 are lower than in old cells. The circulating cells of C-fed, anemic GP included up to 25% reticulocytes and presumably a large proportion of young erythrocytes. These may contribute to the increase of PL and the changes in the proportions of 18:2, 20:4 and 18:0 which we have observed (Table III). It is, however, unlikely that all the changes of RBC lipids in the C-fed anemic GP were due to this factor because it could not account for the observed changes in the proportions of PL classes (Fig. 1).

Relevance of changes in PL composition to anemia in C-fed GP. Neerhout (33) who reviewed studies concerned with the lipid composition of RBC in a number of human anemias could not find a consistent pattern. Westerman (5) has described changes of lipid composition of RBC from patients with Zieve's syndrome which are similar to those found by us in anemic guinea pigs. However, the problem whether the lipid changes are a cause of the anemia, *e.g.*, leading to the destruction of RBC or whether they are a reflection of the reticulocytosis has not been resolved.

Lyso-PC is a known cytotoxic and hemolytic agent (34, 35). Furthermore, Klibansky (36) has shown that the shape of rabbit RBC *in vitro* depends on the amount of lyso-PC in the medium. The doubling of the content of lyso-PC in liver, the almost 5-fold increases of this PL in plasma and RBC and the decrease of the ratio of PC/lyso-PC from 35 to 14 in RBC of C-fed anemic guinea pigs, may, therefore, be contributing factors to the pathological changes in the liver and to the decreased stability of the RB.

The increase in 20:4 observed in the PL of liver and RBC may lead to the accelerated destruction of RBC and other cells because of its susceptibility to oxidative destruction. The anemia in vitamin E-deficient monkeys, for instance, has been interpreted as evidence that the integrity of the RBC membrane depends in some way on the presence of unaltered polyunsaturated FA (37). Although an increase of dietary tocopherol does not prevent the anemia in the C-fed GP (unpublished results) one might postulate a localized antioxidant deficiency in the compartmentalized pools of membrane lipids.

The relationship of the liver to the anemia may be 2-fold. Since the liver is the site of synthesis of plasma lipoproteins (6) liver injury is likely to affect both the amounts and the composition of plasma lipoproteins. Furthermore, the normal liver may be directly involved in the homeostatic maintenance of RBC. Neerhout (33) studied hyperlipemic patients with or without liver disease and found that the degree of the lipid abnormalities of RBC correleated with the severity of the involvement of the portal area but not with nonspecific hyperlipemia.

Summary. The PL classes and their fatty acid compostion were determined in liver, plasma, and RBC from normal and C-fed anemic GP. Compared to other species, guinea pig RBC had low amounts of Sph and high amounts of PC. Most PL of GP had very low levels of 20:4 and lower ratios of 20:4/18:2 and 16:0/18:0 than those of rats and other species which are less susceptible to injury by excess dietary cholesterol. The following changes have been observed in the C-fed anemic GP: Increased proportions and amounts of lyso-PC in the three tissues compensated by nearly equivalent decreases of PC; increased proportions of Sph in liver and plasma; decreased proportions of Sph in RBC accompanied by an increase of PC/Sph ratio; and increased amounts and proportions of PC-20:4 in RBC and PI-20:4 of liver. The possible significance of these changes for the development of the anemia have been discussed.

The authors thank M. Nakanishi and R. Yuan for some of the red cell analyses, and Dr. R. Okey

for stimulating comments on the manuscript.

1. Okey, R., and Greaves, V., J. Biol. Chem. **129**, 111 (1939).

2. Yamanaka, W., Ostwald, R., and French, S., Proc. Soc. Exp. Biol. Med. **125**, 303 (1967).

3. Yamanaka, W., Winchell, H., and Ostwald, R., Amer. J. Physiol. **213**, 1278 (1967).

4. van Deenen, L. L., *in* "Progress in the Chemistry of Fat and Other Lipids" (R. Nolman, ed.), Pergamon Press, New York 8, 1 (1965).

5. Westerman, M., Balcerzak, S., and Heinle, E., J. Lab. Clin. Med. **72**, 663 (1968).

6. de Jong Bungenberg, J. J., and Marsh, J., J. Biol. Chem. **243**, 192 (1968).

7. Farnsworth, P., Danon, D., and Gellhorn, A., Brit. J. Haematol. **11**, 200 (1965).

8. Roelofsen, B., de Gier, J., and van Deenen, L., J. Cell. Comp. Physiol. **63**, 233 (1964).

9. Ostwald, R., and Shannon, A., Biochem. J. **91**, 146 (1964).

10. Hooper, M. C., M.S. Thesis, University of California, Berkeley, (1967).

11. Liu, K., Typpo, J., Lu, J., and Briggs, G., J. Nutr. **93**, 480 (1967).

12. Okey, R., Shannon, A., Tinoco, J., Ostwald, R., and Miljanich, P., J. Nutr. **75**, 51 (1961).

13. Skipski, V., Peterson, R., and Barclay, M., Biochem. J. **90**, 374 (1964).

14. Bartlett, G., J. Biol. Chem. **234**, 466 (1959).

15. Nelson, G., Biochim. Biophys. Acta **144**, 221 (1967).

16. Condrea, E., Mammon, Z., Aloof, S., and de Vries, A., Biochim. Biophys. Acta **84**, 365 (1964).

17. Matsumoto, M., J. Biochem. Japan **49**, 11 (1961).

18. Nelson, G., Lipids **2**, 323 (1967).

19. Parkes, J., and Thompson, W., Biochim. Biophys. Acta **196**, 162 (1970).

20. Youngs, J., Comp. Biochem. Physiol. **9**, 257 (1963).

21. Turner, J., Anderson, H., and Gondal, C., Biochim. Biophys. Acta **30**, 130 (1958).

22. Lyman, R., Shannon, A., Ostwald, R., and Miljanich, P., Can. J. Biochem. **42**, 365 (1964).

23. Clement, J., and Di Costanzo, G., Arch. Sci. Physiol. **22**, 1 (1968).

24. Glomset, J., Biochim. Biophys. Acta **65**, 128 (1962).

25. Akiyama, M., Minari, O., and Sakagami, T., Biochim. Biophys. Acta **137**, 525 (1967).

26. McCandless, E., and Zilversmit, D., Arch. Biochem. Biophys. **62**, 402 (1956).

27. Zilversmit, D., Sweeley, S., and Newman, H., Circ. Res. **9**, 235 (1961).

28. Sheltawy, A., Biochem. J. **95**, 561 (1965).

29. Witting, L., Lipids **2**, 109 (1967).

30. Peifer, J., Holman, R., Arch. Biochem. Biophys. **57**, 520 (1955).

31. Mulder, E., and van Deenen, L., Biochim. Biophys. Acta **106**, 348 (1965).

32. van Gastel, C., van den Berg, D., de Gier, J., and van Deenen, L., Brit. J. Haematol. **11**, 193 (1965).

33. Neerhout, R., J. Lab. Clin. Med. **71**, 438 (1968).

34. Gottfried, E., and Rapport, M., J. Lipid Res. **4**, 438 (1963).

35. Thompson, R., *in* "Metabolism and Physiological Significance of Lipids," (R. Dawson and D. Rhodes, eds.), p. 541. Wiley, London (1964).

36. Klibansky, C., and de Vries, A., Biochim. Biophys. Acta **70**, 176 (1963).

37. Dinning, J., Physiol. Rev. **42**, 169 (1962).

Received Mar. 30, 1970. P.S.E.B.M., 1970, Vol. 134.