Lipid metabolism in fatty liver of lysine- and threonine-deficient rats

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ABSTRACT Rats were fed a low protein diet deficient in and supplemented with lysine and threonine. Liver lipids contained more lecithin, sphingomyelin, and free fatty acids, and less amino phospholipids in the deficient rats. No variations in fatty acid composition of choline- and ethanolamine-containing phospholipids were found; only palmitic acid was increased in the serine-containing phospholipids of the deficient animals. The incorporation of acetate-^{14}C into phospholipids, but not into other liver lipids, was lower in deficient rats.

In the plasma of deficient rats the concentration of esterified fatty acids and phospholipids was lower, of free fatty acids higher, than in the controls. The fatty acid composition of depot fat differed from that of liver neutral fat both in deficient and supplemented animals.

The results presented establish that multiple metabolic defects resulting from lysine and threonine deficiency accompany the fatty liver. The design of the experiments does not permit conclusions to be drawn regarding the causal relationship between the various alterations in lipid metabolism and the fatty liver.

KEY WORDS amino acid deficiency · fatty · liver · phospholipids · metabolism · composition · adipose tissue · depot fat · plasma lipids · rat

GROWING RATS fed low protein diets deficient in essential amino acids exhibit liver lipid infiltration of the periportal type when fed these diets either ad lib. (1–3) or by force feeding (4, 5). Little is known about this type of lipid infiltration, although it is possible that inadequate protein synthesis in the deficiency state may cause fundamental damage to the enzyme systems related to lipid metabolism and simultaneously lead to an increased content of lipids in liver cells.

Abbreviations: FFA, free fatty acids; EGP and SGP, ethanolamine and serine glycerophosphatides respectively.

The aim of the present investigation is to investigate the fatty liver induced by a diet deficient in lysine and threonine in regard to (*a*) the distribution of lipid among the various lipid classes (with particular reference to the phospholipids) of liver, (*b*) the fatty acid composition of these lipid classes, and (*c*) certain alterations of lipid metabolism that accompany the fatty infiltration of the liver.

METHODS

Animals and Diets. Weanling male rats of the Wistar strain weighing 44 ± 2 g, from our inbred colony, were divided into two groups. One group was fed on a low protein rice diet deficient in lysine and threonine. The other group received the same diet but with lysine and threonine supplementation. The composition of the diets, which were isonitrogenous, is reported in a previous paper (3). The degree of supplementation with the limiting essential amino acids was that recommended by Rosenberg, Culik, and Eckert (6) to obtain both better growth and a physiological level of liver lipids. The dietary fat contained a high level of linoleic acid and a small quantity of unsaturated fatty acids of the linolenic acid family; arachidonic acid was absent. At the end of the experimental period of 6 weeks, analyses of liver, plasma, and depot fats were made, and in vivo studies were made on the incorporation of acetate-^{14}C into the fatty acids of liver neutral fat and liver phospholipids.

Liver Lipid Studies

Analytical Determinations. Rats from both the amino acid-deficient and control groups were killed by decapitation. The livers were excised and pooled in groups of three, and the total lipids from each pool were extracted and purified according to Folch, Lees, and

Sloane Stanley (7). The yield of purified lipid was determined by weighing. Aliquots of the total lipid extracts were then analyzed for proteolipid protein according to Davenport (8), and for total and free cholesterol according to Sperry and Webb (9). Neutral lipids were separated by a batch silicic acid procedure (10). Determination of phosphorus (11) demonstrated the complete separation of phospholipids from neutral fat; the recovery of phospholipids was practically complete. Aliquots of the separated phospholipids were assayed for aminonitrogen phospholipids (12, 13), plasmalogens (14), and alkali-labile and alkali-stable phospholipids (15). The total phospholipids were separated on alumina columns into three fractions: choline-containing phospholipids, ethanolamine-containing phospholipids, and serine-containing phospholipids, by a combination of the methods of Long and Staples (16) and of Long, Shapiro, and Staples (17). The alumina column procedure was used since it produced a reproducible and rapid method of separation of phospholipid classes with little destruction of lipid. The yield of each class was estimated by determination of lipid phosphorus. The nitrogen base composition of the three fractions was checked by paper chromatography of the acid hydrolysate (18). The first fraction contained only choline, the second only ethanolamine, and the third only serine.

Preliminary experiments indicated that the second fraction contained about 90% of the plasmalogen content, in accordance with the general distribution found for plasmalogens in most tissues (19), although the plasmalogen content of liver is very low both as a total amount and as a percentage of the phospholipids.

Free fatty acids (FFA) were extracted from a petroleum ether solution of the neutral fat fraction with 0.05 N aqueous KOH, and the alkaline extract was reextracted with petroleum ether after acidification (20).

Aliquots of total lipids, of phospholipids, and of the individual phospholipid fractions were saponified in methanolic KOH and in each case the amount of total fatty acids was determined by titration (21), as was the amount of free fatty acids obtained by the extraction procedure described above.

Fatty acids obtained by saponification were esterified by refluxing in dry methanolic HCl (22). In addition fatty acid methyl esters were prepared by methanolysis of total phospholipids and the individual phospholipid classes (23). The methyl esters were analyzed by gas–liquid chromatography on a Beckman GC-2A apparatus equipped with a thermal conductivity detector. A column of 20% diethylene glycol succinate polyester on Chromosorb W was used at 212°C. Helium was the carrier gas with an inlet pressure of 27 psi. Peaks were identified and quantitatively determined as previously described (3).

Incorporation of Acetate-^{14}C In Vivo. Twenty rats, 10 of each group, were injected intraperitoneally with 20 μc per 100 g body weight of sodium acetate-1-^{14}C (specific activity 373 mc/mmole). After 3 hr (2) the animals were killed by decapitation, and the livers were removed; total lipids were extracted, and neutral lipids were separated from phospholipids as described above. Fatty acids of neutral fat and phospholipids were prepared (10) and determined by titration (21). Fatty acids obtained from each fraction (neutral fat and phospholipids) were dissolved in chloroform to give the same amount of fatty acid per unit volume of solution, and a constant volume of each solution was assayed for radioactivity. Samples were mounted on aluminum planchets and dried under an infrared lamp, and their radioactivity was counted at infinite thinness with a windowless gas flow counter (Tracerlab Inc., Boston, Mass.).

Plasma Lipid Studies

Analytical Determinations. After 6 weeks of experimental feeding, 12 animals each from the control and amino acid deficient group were starved for 10 hr. The rats were then lightly anesthetized with ether, their abdomens opened, and samples of blood collected from the abdominal aorta with a syringe and transferred to heparinized tubes. The tubes were cooled and centrifuged at 4°C. Samples of plasma were analyzed for FFA according to Dole (21), lipids were extracted according to Robinson and Harris (24), and determination of lipid phosphorus (11) and fatty acid esters (25) made.

Adipose Tissue Studies

Perirenal adipose tissue was removed from rats of both groups. The lipids were saponified and the fatty acid methyl esters were prepared and analyzed as described above.

Statistical analyses of the experimental data were carried out by the "t" test; values of $P < 0.05$ were considered to be significant.

RESULTS

Liver Lipid Studies

Analytical Determinations. Table 1 shows the response of liver lipid to a diet deficient in lysine and threonine. From the data presented it is clear that the excess lipids in the fatty livers consisted primarily of neutral lipid, there being an increase both of glycerides and, to a smaller extent, of cholesterol esters, as previously observed (3). FFA were also elevated ($P < 0.01$). The amount of total phospholipids, of proteolipid protein, and of plasmalogens was not changed; nevertheless there was a significant change in the relative proportions of the various phospholipid classes in the deficient animals by comparison with the supplemented ones.

TABLE 1 EFFECT OF LYSINE AND THREONINE DEFICIENCY ON LIVER LIPIDS OF RATS AT 6 WEEKS FROM WEANING

	Supplemented	Deficient
	mg/g	
Total lipids	51.8 ± 4.7 (8)	116.0 ± 24.6 (8)*
Phospholipids	34.3 ± 1.9 (8)	35.7 ± 2.9 (7)
Neutral fat	17.4 ± 4.8 (8)	73.6 ± 14.9 (7)*
Total cholesterol	2.4 ± 0.3 (7)	4.7 ± 0.6 (7)*
Free cholesterol	1.4 ± 0.3 (7)	1.6 ± 0.3 (7)
Proteolipids†	2.10 ± 0.1 (8)	2.25 ± 0.2 (8)
Plasmalogen	0.44 ± 0.2 (5)	0.54 ± 0.2 (5)
Alkali-stable phospholipids	1.68 ± 0.17 (5)	3.01 ± 0.1 (5)*
Alkali-labile phospholipids	29.6 ± 2.7 (5)	32.8 ± 2.3 (5)
Sphingomyelin‡	1.24 ± 0.17 (5)	2.40 ± 0.2 (5)*
Phospholipids containing free amino-nitrogen§	12.6 ± 0.4 (4)	10.8 ± 0.8 (4)*
	μeq/g	
Free fatty acids	2.8 ± 0.4	8.3 ± 2.0 (5)*

Values are means ±SD; number of determinations in parentheses.

* Significant difference from control ($P < 0.01$).

† Proteolipid protein determined according to Davenport (8).

‡ Calculated: milligrams of alkali-stable phospholipids minus milligrams of plasmalogens.

§ μmoles of free amino nitrogen × 0.750.

Although the total amount of phosphoglycerides (as represented by the total alkali-labile phospholipids), appears to be similar in the two groups, phospholipids containing free amino-nitrogen were significantly decreased ($P < 0.01$) in deficient animals. By difference an increase in the lecithin fraction, and (or) of other ninhydrin-negative phospholipids may be deduced.

The alkali-stable phospholipids were increased in deficient animals ($P < 0.01$), although the plasmalogen content was constant. By subtraction of the plasmalogen content from alkali-stable phospholipids a reliable measure of sphingomyelin can be obtained (26). Hence it is apparent that sphingomyelin was present in higher concentration in the livers of deficient animals ($P < 0.01$).

This modified distribution of lipid among the various phospholipid classes is also evident from the results of chromatography on alumina columns (Table 2). The recovery of P after the chromatographic separation was 85–95%. The choline-containing fraction increased significantly in the deficient animals ($P < 0.05$), while fractions containing ethanolamine and serine glycerophosphatides (EGP, SGP) did not change. Table 2 also shows the calculated values of phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine, and nitrogen-free phospholipids. These values indicate an increase of lecithin and of sphingomyelin in deficient animals.

The indirect values for nitrogen-free phospholipids (inositides + cardiolipin + phosphatidic acids), calculated from total alkali-labile phosphoglycerides minus lecithin minus amino phospholipid, suggest an increase of this fraction in deficient animals.

In Table 3 the fatty acid composition of total phospholipids and of the three fractions isolated by separation on alumina columns is reported. The fatty acid composition of phospholipids obtained with the silicic acid procedure is similar to that of phospholipids obtained with acetone precipitation (3). In both cases the level of 22:6 is lower in deficient rats ($P < 0.01$).

Choline- and ethanolamine-containing phospholipids did not change in fatty acid composition with the deficiency, but in SGP the percentage of palmitic acid increased. The data show, as do other chromatographic techniques (27, 28), that in general palmitic acid is much higher, 22:6 lower, in lecithin than in cephalins. The decrease of 22:6 in total phospholipids from deficient animals appears, then, to arise primarily from the increase in ratio of lecithin to cephalins in the over-all phospholipid pattern.

In Vivo Incorporation of Acetate-^{14}C. The effect of lysine and threonine deficiency on incorporation of acetate-^{14}C into liver lipid in vivo is shown in Table 4. The total incorporation of radioactivity into fatty acids of neutral lipid (glycerides, free fatty acids, and cholesterol esters) and of phospholipids was measured. The net incorpora-

TABLE 2 EFFECT OF DIETS DEFICIENT IN AND SUPPLEMENTED WITH LYSINE AND THREONINE ON LIVER PHOSPHOLIPID FRACTIONS

	Supplemented	Deficient
	mg/g liver	
Total phospholipids	34.8 ± 1.6	36.0 ± 2.2
Choline-containing fraction*	19.6 ± 0.7	22.9 ± 1.2†
Ethanolamine glycerophosphatides (EGP)*‡	13.9 ± 1.2	13.0 ± 0.5
Serine glycerophosphatides (SGP)*	0.5 ± 0.0	0.5 ± 0.2
Sphingomyelin§	1.2	2.4
Lecithin‖	18.3	20.5
Phosphatidyl ethanolamine¶	12.0	10.2
Nitrogen-free phospholipids**	1.7	2.8
Lecithin/phosphatidyl ethanolamine	1.5	2.0

* Mean values for three samples of phospholipids (each sample obtained from a pool of three livers). Values calculated from percentage of lipid P recovered from alumina columns.

† Significant difference ($P < 0.05$).

‡ Plasmalogens are mainly in this fraction.

§ Alkali-stable phospholipids minus plasmalogens (data from Table 1).

‖ Choline-containing fraction minus sphingomyelin.

¶ Tentative data calculated by means of the formula: mean values of free amino-nitrogen phospholipids (Table 1) minus mean values of SGP (Table 2).

** These data are only tentative, calculated by the formula: (mean values of EGP + mean values of SGP) − (mean values of phospholipids containing free amino-nitrogen).

TABLE 3 FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS AND OF INDIVIDUAL PHOSPHOLIPID FRACTIONS FROM LIVERS OF RATS FED DIETS DEFICIENT IN AND SUPPLEMENTED WITH LYSINE AND THREONINE

Fatty Acid	Total Phospholipids		Choline-Containing Fraction		Ethanolamine Glycerophosphatides		Serine Glycerophosphatides	
	S	D	S	D	S	D	S	D
	wt % calculated from peak areas							
14:0	0.2	0.2	0.2	0.4	0.2	0.3	1.0	1.0
16:0	24.1	26.8	30.8	30.6	16.8	18.6	13.9	19.5*
16:1	1.9	1.5	2.8	2.2	1.9	1.5	2.5	3.8
18:0	21.6	22.5	16.2	16.7	25.6	25.7	20.5	23.0
18:1	6.8	7.7	8.3	8.6	5.9	6.4	10.0	11.9
18:2	11.3	12.0	11.4	10.1	11.5	11.3	8.2	8.4
20:3	1.0	0.8	1.1	tr.	1.8	0.7	0.6	tr.
20:4	16.2	14.7	16.8	17.6	17.0	16.6	16.1	13.8
20:5	0.6	0.6	1.0	0.7	0.8	0.7	1.1	0.9
22:5	1.3	1.4	0.7	0.7	1.9	1.4	1.9	tr.
22:6	14.7	11.4†	11.0	12.7	16.5	16.6	20.5	16.3

S, supplemented; D, deficient. Values are the means of data from five preparations (total phospholipids), or four preparations (EGP, SGP, and choline-containing phospholipids).
* Significant difference, $P < 0.05$.
† Significant difference, $P < 0.01$.

TABLE 4 INCORPORATION OF ACETATE-^{14}C INTO FATTY ACIDS OF NEUTRAL FAT AND PHOSPHOLIPIDS IN LIVER OF RATS FED DIETS DEFICIENT IN AND SUPPLEMENTED WITH LYSINE AND THREONINE

	Supplemented	Deficient
Body weight, *g*	159.7 ± 12.1	77.7 ± 3.3
Liver weight, *g*	5.7 ± 0.1	2.6 ± 0.4
Fatty acids of liver neutral fat		
Content, *mg/g liver*	14.2 ± 4.0	49.4 ± 17.0*
Specific activity, *cpm/mg*	199.1 ± 45.8	68.9 ± 14.0*
"Total activity"† × 10^{-3}	9.7 ± 3.1	11.3 ± 3.5
Fatty acids of liver phospholipids		
Content, *mg/g liver*	16.0 ± 1.5	14.2 ± 1.7
Specific activity, *cpm/mg*	320.0 ± 52.8	105.0 ± 28.0*
"Total activity"† × 10^{-3}	14.6 ± 4.1	3.7 ± 1.2*

* Significant difference, $P < 0.01$.
† $\frac{\text{Specific activity} \times \text{fatty acids/total liver}}{\text{Body weight}/100}$.

tion of labeled acetate into the fatty acids of neutral fat was unchanged in the livers of the deficient animals. However, there was a pronounced decrease in incorporation of ^{14}C into phospholipid fatty acids in the deficient rats ($P < 0.01$).

Plasma Lipid Studies

Analytical Determinations. After 6 weeks of dietary treatment, the plasma from the blood of abdominal aorta contained more FFA ($P < 0.01$) in deficient animals (Table 5). On the other hand there was a statistically significant decrease of esterified fatty acids ($P < 0.01$) and of total phospholipids ($P < 0.05$) in the plasma of deficient animals. Total plasma cholesterol has also been found to decrease in the deficiency state (29).

Depot Fat Studies

In Table 6 are recorded the fatty acid compositions of dietary and of depot fat and of liver neutral fats in the two groups of animals. Also shown is the composition of the lipids accumulated in the liver, calculated according to Horning, Williams, Maling, and Brodie (30). The fatty acid composition of the perirenal adipose tissue fat was the same in both groups of animals. The fatty acid composition of liver neutral fat was different in the two groups: besides the increase of 18:2 in deficient rats previously described (3), there was also a decrease of 16:0. The calculated fatty acid composition of the fat accumulated in the liver in this deficiency state indicates that it is not derived directly from depot fat. The reliability of this calculation in long-term experiments of this kind has, however, yet to be established. If we consider linoleic acid as a reference physiological tracer (30), it appears that in the neutral fat of the normal livers its percentage is the same as in depot fat; however, the percentage of 18:2 in the accumulated fat (25%) is different both from the percentage in dietary fat (37%) and in depot fat (17%). The mechanism by which 18:2 is increased in liver neutral fat is undoubtedly complex, and although it

TABLE 5 EFFECT OF LYSINE AND THREONINE DEFICIENCY ON PLASMA LIPIDS OF RATS

	Supplemented	Deficient	*P* for difference
	mg/100 ml plasma		
FFA	10.1 ± 1.3	13.4 ± 1.9	<0.01
Esterified fatty acids	190.3 ± 24.2	149.0 ± 38.7	<0.01
Phospholipids	131.1 ± 16.7	105.7 ± 8.4	<0.05

TABLE 6 FATTY ACID COMPOSITION OF DIETARY LIPIDS, PERIRENAL ADIPOSE TISSUE LIPIDS, AND LIVER NEUTRAL FAT IN DEFICIENT AND CONTROL RATS

Fatty Acid	Dietary Lipids	Perirenal Lipids*	Liver Neutral Fat		Accumulated Fat (Calculated)†
			S	D	
	wt % calculated from peak areas				
14:0	1.3	2.2	0.9	1.1	1.1
16:0	10.1	32.9	31.9	25.9	24.0
16:1	4.4	9.7	5.7	5.7	5.8
18:0	2.5	4.0	5.7	6.7	7.0
18:1	33.7	32.1	30.9	30.8	30.7
18:2	37.9	17.3	17.7	23.2	24.9
20:1	3.6	1.3	1.9	1.2	—
20:3	1.3	—	0.5	0.3	—
20:4	—	—	2.8	2.6	—
20:5	3.0	—	0.3	1.5	1.8
22:6	1.5	—	3.3	2.9	—

* Mean of both groups of rats (deficient and supplemented).

† $= \frac{A \cdot B - C \cdot D}{A - D}$ in which A = total fatty acid content of neutral fat from deficient rats (73.6 mg/g liver); B = fatty acid as percentage of total fatty acids in liver neutral fat from deficient rats; C = fatty acid as percentage of total fatty acids in liver neutral fat from supplemented rats; and D = total fatty acid content of neutral fat from supplemented rats (17.4 mg/g liver).

appears from these findings that the diet is a conspicuous source of accumulated fat, these data must be interpreted with caution.

DISCUSSION

Metabolic defects that singly or in combination might account for the pathogenesis of fatty liver are (*a*) increased triglyceride synthesis in the liver; (*b*) decreased fatty acid oxidation in the liver; (*c*) increased uptake by the liver of arterial blood lipids (chylomicrons and FFA); (*d*) decreased secretion of lipoproteins from liver into the blood stream.

In the case of fatty liver induced in growing rats fed a lysine- and threonine-deficient diet, the studies presented in this paper do not indicate a single metabolic lesion, but rather the sum of a number of effects. The most noteworthy alterations found in rats after 6 weeks on a lysine- and threonine-deficient diet are (*a*) an increase of FFA in the blood and in the liver; (*b*) a decrease of blood lipids of hepatic origin (esterified fatty acids and phospholipids); (*c*) a modification of the distribution of liver phospholipid among the various phospholipid fractions, which might be taken as an expression of an alteration in the relative rates of metabolism of lecithin, cephalins, and sphingomyelin; (*d*) an altered incorporation of acetate-^{14}C into the fatty acids of liver phospholipid while acetate incorporation into the fatty acids of neutral fat was unchanged.

These observations do not exclude the possibility that the mechanism of steatosis involves an impairment of liver lipid oxidation. Indeed there are indications that protein deficiency (31–33) and threonine deficiency (34) can damage oxidation processes in the rat liver. Nevertheless, other work (35–37) indicates that this mechanism alone cannot account for the development of fatty liver.

A comparison of some of our results with those of Yoshida and Harper (2) in threonine imbalance and of Lyman, Cook, and Williams (5) in acute isoleucine deficiency indicates that metabolic lesions in these fatty livers (which are in any case of periportal type and depend on essential amino acid deficiency or imbalance) could be different from those observed by us.

Thus after 3 hr the amount of ^{14}C from injected acetate-1-^{14}C incorporated in both neutral fat and phospholipid of liver was significantly greater in deficient rats than in those fed the threonine-supplemented diet (2). Also, the incorporation of acetate-^{14}C into triglycerides and phospholipids is increased in liver slices of isoleucine-deficient rats, in comparison to supplemented ones (5). In the same animals no statistical variation in fatty acid composition of phospholipids was observed (5).

In addition, our results on liver lipid classes, and in particular the increases in choline containing phospholipids (lecithins and sphingomyelin) in deficient rats, could be correlated with the well-known negative lipotropic effect of choline on lysine and threonine fatty liver (38).

Although the data presented in the present paper establish that multiple changes in lipid metabolism accompany the development of the fatty liver resulting from a lysine- and threonine-deficient diet, the design of the experiment does not permit conclusions to be drawn regarding the causal relation of these changes to the fatty infiltration. However, the data should serve as starting points for further experiments especially designed to relate specific changes in lipid metabolism to the genesis of this type of fatty liver.

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REFERENCES

1. Singal, S. A., S. J. Hazan, V. P. Sydenstricker, and J. M. Littlejohn. *J. Biol. Chem.* **200:** 875, 1953.
2. Yoshida, A., and A. E. Harper. *J. Biol. Chem.* **235:** 2586, 1960.
3. Viviani, R., A. M. Sechi, and G. Lenaz. *J. Lipid Res.* **5:** 52, 1964.
4. Sidransky, H., and E. Farber. *Arch. Pathol.* **66:** 135, 1958.
5. Lyman, R. L., C. R. Cook, and M. A. Williams. *J. Nutr.* **82:** 432, 1964.
6. Rosenberg, H. R., R. Culik, and R. E. Eckert. *J. Nutr.* **69:** 217, 1959.
7. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **226:** 497, 1957.

8. Davenport, J. B. *Biochem. J.* **90:** 116, 1964.
9. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
10. Marks, P. A., A. Gellhorn, and C. Kidson. *J. Biol. Chem.* **235:** 2579, 1960.
11. Bartlett, G. R. *J. Biol. Chem.* **234:** 466, 1959.
12. Brady, C. J. *Biochem. J.* **91:** 105, 1964.
13. Moore, S., and W. H. Stein. *J. Biol. Chem.* **176:** 367, 1948.
14. Rapport, M. M., and B. Lerner. *Biochim. Biophys. Acta* **33:** 319, 1959.
15. Dawson, R. M. C. *Biochem. J.* **75:** 45, 1960.
16. Long, C., and D. A. Staples. *Biochem. J.* **78:** 179, 1961.
17. Long, C., B. Shapiro, and D. A. Staples. *Biochem. J.* **85:** 251, 1962.
18. Marinetti, G. V., D. J. Scaramuzzino, and E. Stotz. *J. Biol. Chem.* **224:** 819, 1957.
19. Marinetti, G. V., J. Erbland, and E. Stotz. *Biochim. Biophys. Acta* **30:** 642, 1958.
20. Böttcher, C. J. F., F. P. Woodford, E. Boelsma-Van Houte, and C. M. van Gent. *Rec. Trav. Chim.* **78:** 794, 1959.
21. Dole, V. P., *J. Clin. Invest.* **35:** 150, 1956.
22. James, A. T. *J. Chromatog.* **2:** 552, 1959.
23. Farquhar, J. W. *J. Lipid Res.* **3:** 21, 1962.
24. Robinson, D. S., and P. M. Harris. *Biochem. J.* **80:** 361, 1961.
25. Stern, I., and B. Shapiro. *J. Clin. Pathol.* **6:** 158, 1953.
26. McIlwain, E., and R. Rodnight. *Practical Neurochemistry.* Churchill Ltd., London, 1962, p. 103.
27. MacFarlane, M. G., G. M. Gray, and L. W. Wheeldon. *Biochem. J.* **77:** 626, 1960.
28. Getz, G. S., W. Bartley, F. Stirpe, B. M. Notton, and A. Renshaw. *Biochem. J.* **80:** 176, 1961.
29. Viviani, R., and A. M. Sechi. *Experientia* **19:** 188, 1963.
30. Horning, M. G., E. A. Williams, H. M. Maling, and B. B. Brodie. *Biochem. Biophys. Res. Commun.* **3:** 635, 1960.
31. Artom, C. *J. Biol. Chem.* **205:** 101, 1953.
32. Fritz, I. B. *Am. J. Physiol.* **190:** 449, 1957.
33. Frunder, H., and W. Friedel. *Z. Physiol. Chem.* **295:** 77, 1953.
34. Arata, D., C. Carrol, and D. C. Cederquist. *J. Nutr.* **82:** 150, 1964.
35. Fritz, I. B. *Physiol. Rev.* **41:** 52, 1961.
36. Recknagel, R. O., and D. D. Anthony. *J. Biol. Chem.* **234:** 1052, 1959.
37. Henderson, J. F. *J. Lipid Res.* **4:** 68, 1963.
38. Shils, M. E., R. De Giovanni, and W. B. Stewart. *J. Nutr.* **56:** 95, 1955.