2. Brecher, M., Schneiderman, M., *Am. J. Clin. Path.*, 1950, v20, 1079.
3. Nelken, D., *Vox Sang*, 1961, v6, 348.
4. Millican, R. C., *Res. in Burns, Am. Inst. Biol. Sci.*, 1962, #9, 195.
5. Matter, P., Chambler, K., Bailey, B., Lewis, S. R., Blocker, J. G., Jr., Blocker, V., *Ann. Surg.*, 1963, v157, 725.
6. Simonart, A., *Bull. L'Acad. Roy. de Med. de Belgique*, 1962, vVII, 457.
7. Weeden, A. R., Datta, N., Mollison, P. L., *Vox Sang.*, 1960, v5, 523.
8. Atherton, S., Merrill, N., McCarthy, M. D., *Fed. Proc.*, 1960, v19, 195.

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Methionine Deficiency and Fatty Liver in Male and Female Rats.* (29671)

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Liver fat accumulation has been reported in rats made deficient in tryptophan(1), phenylalanine(2), histidine and threonine(3) and methionine(4).

A recent study from our laboratory(5) demonstrated that isoleucine-deficient rats also developed fatty livers resulting exclusively from an accumulation of triglycerides. Investigations into possible reasons for the excessive triglyceride deposition indicated that an appreciable amount of the fat must have risen from increased hepatic fatty acid synthesis.

Fatty liver in rats made deficient in a single essential amino acid seems, therefore, to be a frequent occurrence. Since the condition may arise by a change in one or more of a number of processes involving synthesis of, mobilization to and from, and utilization of the liver fatty acids, an experiment was conducted to see whether a deficiency of another essential amino acid (methionine) would affect liver lipid metabolism as had isoleucine deficiency.

Materials and methods. Because it had been reported previously(6) that fatty liver developed only in methionine-deficient adult female rats, but not males, both male and female Long-Evans rats were used in the following experiments.

Average weight of the females at the beginning of the experiment was 160 g and that of the males 180 g. A complete diet(5) containing purified amino acids in the proportion and amount necessary to approximate 18% of casein was fed to control groups of both sexes. Methionine was omitted from the amino acid mix in the experimental groups. Both control and deficient groups were force-fed (in 2 separate feedings) about 10 g of diet daily for 10 days. During that time, control rats gained weight, whereas deficient animals lost weight. By the end of the experimental period, most of the deficient animals also exhibited unkempt fur, hyperirritability and porphyrin staining about the whiskers and forepaws. On the eleventh day, rats of each group were fed 5 g of their respective diets. Two hours later they were decapitated, and blood was collected and allowed to clot. Serum was then extracted with alcohol:ether (3:1 v/v)(7), and total cholesterol(8) and phospholipid(9) were determined.

Livers were removed and weighed. About 500 mg of slices, 0.5 mm thick, were incubated with 0.3 μc of sodium acetate-2-C^{14} for 3 hours at 37°C in 4.5 ml of Krebs-Ringer bicarbonate buffer (pH 7.3). Gas phase was 95% O_2 and 5% CO_2. Following incubation, the slices were thoroughly washed, saponified with KOH, acidified and extracted into chloroform. An aliquot of the extract was plated and counted in a gas-flow counter (10). A part of the unincubated liver was

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TABLE I. Body and Liver Weights of Control and Methionine-Deficient Rats, and Triglyceride, Phospholipid, Cholesterol, Glycogen and Nitrogen Contents of the Livers.*

Diet	No. rats	Body wt (g)	Liver wt (g)	Liver				
				Triglyceride	Phospholipid†	Cholesterol	Glycogen	Nitrogen
				(mg/g)				
				Female				
Control	5	172 ± 5‡	5.7 ± .1	12.0 ± 4.0	29.2 ± .5	2.35 ± .04	23.6 ± 2.0	29.6 ± 1.4
Without methionine	7	150 ± 2	5.8 ± .2	38.1 ± 7.7	29.5 ± .5	2.74 ± .03	12.4 ± 3.2	30.3 ± .8
				Male				
Control	5	191 ± 5	5.9 ± .1	8.3 ± 1.8	31.7 ±1.5	2.65 ± .04	22.1 ± 6.0	30.6 ± 1.4
Without methionine	6	166 ± 2	5.3 ± .1	24.3 ±10.2	29.8 ± .8	3.32 ± .31	13.8 ± 2.7	32.9 ± 1.8

* Values expressed as wet weight of liver. † Phosphorus × 25. ‡ Mean ± standard error.

extracted as described by Okey *et al*(11). The solvents were evaporated *in vacuo,* and reextracted into petroleum ether. Triglycerides and phospholipids were separated on silicic acid columns(12), and their fatty acid composition was determined by gas-liquid chromatography following methylation of the fatty acids(13). Nitrogen was determined by semi-micro Kjeldahl on a portion of the whole liver after digestion with sulfuric acid. Glycogen was determined by the method described by Colowick and Kaplan(14). Comparison of the results for statistical significance was by "*t*" test(15).

Results and discussion. Table I shows some liver constituents determined in both female and male rats. Methionine-deficient female rats had significantly more liver triglyceride ($P<0.02$) than did their controls. In male rats, however, although average liver triglyceride concentration was nearly 3 times that of the controls, the wide variation among animals negated any statistical significance ($P>0.1$). The other major component affected by methionine deficiency was liver glycogen. In animals of both sexes fed the deficient diet, glycogen was reduced to about one-half that of control levels. On the other hand, liver phospholipid, cholesterol and nitrogen were unaffected in either sex by the amino acid deficiency.

Total liver lipid in methionine-deficient female rats was also significantly higher ($P<0.05$) than in the controls, whereas again, because of range of variability, no differences appeared in the male animals (Table II). Although female rats incorporated significantly more acetate into their liver lipids than did males ($P<0.01$), methionine deficiency had no significant effect on acetate incorporation in either sex. The tendency for the deficient rats to incorporate less label into their lipids than did the controls most likely reflected dilution of the isotope by the large quantity of liver lipid already present. It is possible, however, that incorporation

TABLE II. *In vitro* Acetate-2-C^{14} Incorporation into Lipids of Liver Slices from Control and Methionine-Deficient Rats.*

Diet	No. rats	Total liver lipid (mg/g)	Acetate incorporation	
			(cpm/g)	(cpm/mg fat)
		Female		
Control	5	40.2 ± 3.3†	4908 ± 1200	124 ± 36
Without methionine	7	67.2 ± 8.0	2368 ± 525	35 ± 7
		Male		
Control	5	36.2 ± 1.7	1156 ± 147	32 ± 9
Without methionine	6	54.3 ± 9.9	870 ± 259	16 ± 5

* Values expressed as wet weight of liver. † Mean ± standard error.

TABLE III. Triglyceride Fatty Acids from Livers of Control and Methionine-Deficient Rats.

Diet	No. rats	Fatty acids (wt %) (14:0)*	(16:0)	(16:1)	(18:0)	(18:1)	(18:2)	(20:4)
				Female				
Control	5	1.3 ± .3†	30.3 ± .7	2.0 ± .3	3.6 ± .2	18.8 ± .5	36.7 ± .7	7.6 ± 1.9
Without methionine	6	.6 ± ‡	23.9 ± .9	.7 ±	3.5 ± .1	15.0 ± .5	45.3 ± .7	8.6 ± 1.2
				Male				
Control	5	1.0 ±	26.9 ± .8	1.7 ± .2	2.9 ± .2	18.5 ± .9	40.6 ± .2	6.3 ± 1.1
Without methionine	6	6.7 ±	22.0 ±1.1	.8 ± .1	3.8 ±	17.9 ± .9	45.9 ±1.4	6.2 ± .9

* No. of carbons : double bonds. Some fatty acids, below C_{14}, have been omitted from table.
† Mean ± standard error.
‡ Standard errors below 0.1% were omitted.

may have actually been depressed in these animals, especially in the deficient female rats.

Table III shows the fatty acid patterns of liver triglyceride in the 4 groups of animals. A significantly higher proportion of linoleic acid ($P<0.01$) and a lower percentage of palmitic acid ($P<0.01$) were evident in the methionine-deficient females, as compared with the controls. Methionine-deficient male rats also had a higher percentage of linoleic acid than did the controls ($P<0.05$), suggesting that they may not be entirely protected from the triglyceride accumulation that occurs in the female animals.

Table IV shows the results of cholesterol and phospholipid analyses of the serum. Methionine deficiency significantly reduced cholesterol ($P<0.01$) and phospholipid ($P<0.02$) concentrations in the serum of female rats as compared with control sera. However, these same serum lipids were unaffected by methionine deficiency in the male rats.

TABLE IV. Serum Cholesterol and Phospholipid in Control and Methionine-Deficient Rats.

Diet	No. of rats	Total cholesterol (mg/100 ml)	Phospholipid* (mg/100 ml)
		Female	
Control	5	73 ± 4†	146 ± 5
Without methionine	6	52 ± 4	94 ±15
		Male	
Control	5	73 ± 5	120 ± 5
Without methionine	6	68 ± 4	125 ± 6

* mg/100 ml phosphorus × 25.
† Mean ± standard error.

This study has confirmed the report(6) that a sex difference exists in the relative susceptibility of methionine-deficient rats to accumulate liver fat. Moreover, the experiment demonstrated that important differences occur in the metabolism of hepatic fat by methionine-deficient rats as compared with isoleucine-deficient animals.

The principal effects of isoleucine deficiency on liver lipid metabolism in the rat in comparison with control animals are: (a) a higher rate of acetate incorporation into triglycerides of liver slices; (b) a lower percentage of linoleic acid and a higher proportion of palmitic acid in the accumulated triglycerides; and (c) nearly twice as much liver glycogen(5) In contrast, methionine-deficient female rats showed no increased acetate incorporation into the lipid. Furthermore, the proportion of linoleic acid in the accumulated triglycerides was increased significantly above that of control rats. An increase in percentage of linoleic acid probably occurred as the result of an accumulation of the dietary fat† or of fatty acids mobilized from adipose tissue. Selective retention of linoleic acid by the liver lipids could, perhaps, occur, but to our knowledge, no such selectivity has ever been demonstrated. In addition to the differences in fatty acid metabolism in isoleucine and methionine deficiencies, liver glycogen in methio-

† The diet contained 10% of cottonseed oil with a linoleic acid content of about 52%.

nine-deficient rats was only about half that of control animals, whereas in isoleucine-deficient rats it was higher that in the controls. At the present time, the significance of the glycogen levels in the livers of these animals is not known. However, the higher glycogen concentration in isoleucine-deficient animals could conceivably be related to the increased fatty acid synthesis that these animals appear to show(5).

Therefore, the results from methionine-deficient female rats differ principally from those of the isoleucine-deficient animals in that they provide no evidence for increased hepatic fatty acid synthesis. On the other hand, serum lipids in isoleucine-deficient rats did not differ from those in the controls, whereas in methionine-deficient female rats, they were reduced significantly below control concentrations. This suggests that a principal cause of the triglyceride deposition in these animals may be a decreased ability to transport the lipid from the liver to the blood. Such a mechanism has been proposed (16) to explain the liver triglyceride accumulation(17,18) and lowered plasma lipid concentrations(19,20) seen in female rats treated with ethionine, the ethyl analogue of methionine.

Summary. Male and female rats were made deficient in methionine by force-feeding a methionine-free diet for 10 days. Female, but not male rats developed fatty livers, evidenced by an increase solely in triglycerides. There was no evidence of increased fatty acid synthesis by the deficient animals, as estimated by acetate-2-C^{14} incorporation studies, or by dilution of the triglyceride linoleic acid. Deficient female rats, however, had significantly lower serum cholesterol and phospholipid levels than did controls. The results suggest that methionine-deficient female rats developed fatty livers principally because of impaired transport of lipid from liver to the blood.

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1. Adamstone, F. B., Spector, H., *Arch. Path.,* 1950, v49, 173.
2. Samuels, L. T., Goldthorpe, H. C., Dougherty, T. F., *Fed. Proc.,* 1951, v10, 393.
3. Sidransky, H., Farber, E., *Arch. Path.,* 1958, v66, 119.
4. Forbes, R. M., Vaughan, L., *J. Nutrition,* 1954, v52, 25.
5. Lyman, R. L., Cook, C. R., Williams, M. A., *ibid.,* 1964, v82, 432.
6. Sidransky, H., Farber, E., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 293.
7. Bloor, W. R., *J. Biol. Chem.,* 1928, v77, 53.
8. Sperry, W. M., Webb, M., *ibid.,* 1950, v187, 97.
9. Horecker, B. L., Ma, T. S., Haas, E., *ibid.,* 1940, v136, 775.
10. Williams, M. A., Pertel, R., *Canad. J. Biochem.,* 1964, v42, 558.
11. Okey, R., Shannon, A., Tinoco, J., Ostwald, R., Miljanich, P., *J. Nutrition,* 1961, v75, 51.
12. Lis, E. W., Tinoco, J., Okey, R., *Anal. Biochem.,* 1961, v2, 100.
13. Lyman, R. L., Ostwald, R., Miljanich, P., *Canad. J. Biochem.,* 1964, v42, 365.
14. Colowick, S., Kaplan, N., *Methods of Enzymology,* Academic Press, Inc., New York, 1957, v3, p35.
15. Snedecor, G. W., *Statistical Methods,* Iowa State Coll. Press, Ames, 1956, 5th edition.
16. Robinson, D. S., Harris, P. M., *Biochem. J.,* 1961, v80, 361.
17. Farber, E., Simpson, M. V., Tarver, H., *J. Biol. Chem.,* 1950, v182, 91.
18. Farber, E., Koch-Weser, D., Popper, H., *Endocrinology,* 1951, v48, 205.
19. Harris, P. M., Robinson, D. S., *Biochem. J.,* 1961, v80, 352.
20. Olivecrona, T., *Acta Physiol. Scand.,* 1962, v55, 291.

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