Choline-deficiency fatty liver: impaired release of hepatic triglycerides

B. LOMBARDI, P. PANI, and F. F. SCHLUNK

Department of Pathology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213

ABSTRACT After intravenous injection of palmitate-1-^{14}C to rats fed a choline-deficient (CD) or choline-supplemented (CS) diet for 15–18 hr, liver triglycerides became labeled very rapidly. In CS, but not in CD rats, there was a considerable loss, with time, of radioactivity from liver triglycerides. At the same time, significantly less radioactivity appeared in plasma triglycerides of CD rats than of CS animals. No difference was seen in the triglyceride content of microsomes isolated from the liver of rats fed the two diets. The lower radioactivity in plasma triglycerides of CD rats was essentially due to a lower level and specific activity of very low density lipoprotein triglycerides.

After intravenous injection of Triton and labeled palmitate, considerably less radioactivity accumulated in plasma triglycerides and phospholipids of CD rats than of CS animals. Post-Triton hyperphospholipidemia was also less pronounced in CD rats.

It was concluded that the fatty liver observed in CD rats results from an impaired release of hepatic triglycerides into plasma.

KEY WORDS choline deficiency · rat · pathogenesis · fatty liver · triglycerides · phospholipids · liver · microsomes · plasma · lipoproteins · chylomicra · intestine · palmitate incorporation · Triton · triglyceride secretion

IT HAS BEEN WELL ESTABLISHED, in recent years, that several of the experimentally-induced fatty livers result from a block in the release into plasma of hepatic TG as a moiety of plasma lipoproteins (1–3). Indeed, in fatty livers of this type it has been possible to show that the block precedes or accompanies in time the onset of TG accumulation in the liver. In the case of the fatty liver induced by choline deficiency, there is a considerable body of evidence in the literature indicating that the transport of fatty acids from the liver to peripheral tissues is impaired (4, 5; ref. 1 should be consulted for a critical appraisal of the earlier work in this field). However, this evidence has generally been obtained in animals that had been fed a CD diet for several days or weeks and, therefore, had either a well established or a very severe fatty liver. Thus, the possibility that an impaired release of hepatic TG, as a moiety of plasma lipoproteins, may be a consequence rather than the cause of the choline-deficiency fatty liver has not been ruled out, since profound metabolic and morphologic alterations can result from the prolonged presence of unphysiologic quantities of fat in the liver cell. Indeed, accumulation of TG in the liver starts quite promptly upon feeding a CD diet to rats, and the resulting fatty liver is certainly evident within the first 24 hr of feeding (6, 7).

In the last few years, some attempt has been made to show an impairment in the release of hepatic TG within the first 24 hr of choline deficiency in the rat. The results, however, were either inconclusive or negative. Thus, Lombardi and Recknagel (8) studied this problem in Tritonized rats, but the results were inconclusive because of the difficulty of applying the Triton test (see Discussion) to animals consuming a high fat diet. To avoid this difficulty, Recknagel and Goshal (9) applied the test to rats fed a CD fat-free diet. They thus obtained evidence of a block in hepatic TG secretion, but only in rats fed for a minimum of 4 days. Lombardi and Schotz (10) studied the incorporation into liver and plasma TG of palmitate-1-^{14}C injected into rats fasted for 24 hr after having been fed a CD fat-containing diet for 1 day. The results obtained led to the conclusion that release of hepatic TG was not impaired. However since that experiment was performed, it has become known that fasting exerts a lipotropic action, in that it promotes the removal of TG deposited in the liver of CD rats (9). This fact, therefore, cast some doubts on the conclusion reached by the latter

Abbreviations: TG, triglycerides; PL, phospholipids; VLDL, LDL, HDL, very low density, low density, and high density lipoproteins; CD, choline-deficient; CS, choline-supplemented; SA, specific activity; TA, total activity.

investigators, as well as on some previous results obtained by Lombardi and Recknagel (8).

In view of the uncertainty still remaining, we decided to reinvestigate the problem by means of tracer experiments and a novel application of the Triton test. The results show that an impairment in the release of hepatic TG into plasma is indeed operative in rats with an incipient fatty liver due to choline deficiency.

MATERIALS AND METHODS

General Procedures

Male rats of the Sprague-Dawley strain (Sprague-Dawley, Inc., Madison, Wis.) weighing 100–110 g were used. Preparation of the animals for the experiments, preparation of the choline-supplemented (CS) and of the choline-deficient (CD) diets (11), and measurement of diet intake were performed as previously reported (6, 12). Previous results (6) had indicated that TG accumulation in the liver of rats under the CD regimen is first evident after 6–8 hr of feeding. The rate of accumulation, however, was somewhat slower during the first 15 hr than thereafter. Therefore, all of the experiments reported in this paper, with one exception, were started after 15 hr of feeding with the animals having access to the diet up to the time of sacrifice. Rats fed the CD diet represented the experimental animals and those fed the CS diet, the controls.

Administration of Radioactive Compounds and of Triton

Labeled palmitic acid, as an albumin complex (13), was injected into a saphenous vein under light ether anesthesia. The following were used: palmitic acid-1-^{14}C (47 mc/mmole, Nichem, Inc., Bethesda, Md.) and palmitic acid-9,10-^{3}H (256 mc/mmole, Nuclear-Chicago Corporation, Des Plaines, Ill.), Triton WR-1339, oxyethylated *t*-octylphenol formaldehyde polymer (Winthrop Laboratories, New York), was also injected into a saphenous vein under light ether anesthesia. A 20% solution (w/v) in saline (0.9% NaCl) was used.

Collection of Tissue Samples

5 min before sacrifice, 5 mg of pentobarbital per 100 g of body weight was injected intraperitoneally. Samples of blood, liver, and intestinal mucosa were obtained, stored, and prepared for analysis as previously reported (6, 12).

Isolation of Liver Microsomes

10% liver homogenates, prepared in 0.25 M sucrose–0.002 M EDTA at pH 7.4, were centrifuged at 4°C in an automatic Sorvall SS centrifuge for 20 min at 15,000 *g* to sediment cell debris, nuclei, and mitochondria. The upper fatty layers were carefully removed, and the supernatant fractions were decanted and centrifuged in a Spinco model L ultracentrifuge at 4°C, for 60 min at 100,000 *g*. The microsomal pellets so obtained were resuspended in sucrose–EDTA medium, and then centrifuged as above. The final pellets were finely resuspended in saline by means of several strokes in a Potter–Elvehjem homogenizer.

Separation of Serum Lipoproteins

Lipoproteins were separated (14) by flotation in a Spinco ultracentrifuge as previously described (12, 15). 3-ml aliquots of pooled sera, from two CD or two CS rats, were used. Chylomicra were obtained after three consecutive centrifugations at 10,000 rpm, 10 min each time. After the first and second centrifugation the tubes were rotated 120° inside the rotor to float chylomicra adhering to the tube wall. Chylomicra, however, were isolated only once, after the third centrifugation. No clear-cut separation between chylomicra and VLDL was achieved by this method.

Other Analytical Procedures

Total lipids were extracted (16) with chloroform–methanol 2:1 from suitable aliquots of liver, liver microsomes, plasma, serum lipoproteins, and intestinal mucosa homogenates. Triglycerides (17) and phospholipids (18) were determined after separation of the total lipids by chromatography on columns and thin-layers of silicic acid (15). Aliquots of the eluates were pipetted into counting vials, and the solvents were evaporated under a stream of N_2. 15 ml of a toluene scintillation mixture (4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene per liter of toluene) were added to the vials, and radioactivity was measured in a Packard liquid scintillation spectrometer. Phospholipid samples were dissolved in 0.5 ml of methanol before addition of the scintillation mixture. Aliquots of the palmitate–albumin complexes were also extracted and counted.

The results were calculated and expressed as follows: (*a*) plasma lipids, mg/100 ml; liver lipids, mg/100 g of body weight; liver microsome TG, mg/100 mg of microsomal proteins (19); intestinal mucosa TG, μg/mg of DNA (20); (*b*) specific activities, (cpm/mg) × (body weight ÷ 100) × (100 ÷ cpm injected); (*c*) total activities, liver: (cpm/mg) × (mg/liver) × (100 ÷ cpm injected); plasma: (cpm/mg) × (mg/rat) × (100 ÷ cpm injected), using a value of 5.46 or 5.40 ml/100 g of body weight for the plasma volume of CS or CD rats, respectively (12). In the experiments on Tritonized animals, total activities were calculated on the basis of the radioactivity of measured eluate aliquots of TG and PL isolated from samples of liver or plasma of known weight or volume. Differences between the means were checked by student's "*t*"-test and regarded as significant if $P \leq 0.05$.

RESULTS

Six experiments were performed in rats which had been fed the diets for 15–21 hr, and one experiment in animals fed for 8–9 hr. There was no difference between CS and CD rats in either liver weight or diet intake. The average liver weight was 6.08 (5.20–7.69) g in CS rats and 6.37 (5.20–7.55) g in CD animals (mean of 86 animals on each diet, range in parenthesis). Corresponding values for diet intake were 13.9 (11.4–17.0) g and 14.0 (12.9–17) g (mean of 39 rats on each diet). Table 1 contains data on lipids of liver, liver microsomes, plasma, and intestinal mucosa. In CD rats liver TG were consistently higher (2.1–2.7 fold) than in CS animals, while liver PL were slightly lower in one experiment. No significant difference was observed between CS and CD rats, in the TG concentration of liver microsomes, plasma, or intestinal mucosa.

Incorporation of Palmitic Acid into TG of Liver, Plasma, and Intestinal Mucosa (Expt. 1)

Palmitic acid-1-^{14}C (2.5 μc/rat) was injected into CS and CD rats, and groups of animals were sacrificed 5, 10, 30, 60, and 120 min thereafter. The specific and total activity of liver and plasma TG and the specific activity of intestinal mucosa TG were determined.

The SA time curves for liver, plasma, and intestinal mucosa TG are shown in Fig. 1. The SA of liver TG rose quite rapidly and reached a maximum value at 10 min. During the 10–30 min interval it declined quite rapidly in CS animals (from 0.296% to 0.136%) but only slowly in CD rats (from 0.158% to 0.123%). It then increased slowly with time in both CS and CD rats. This later increase was most likely due to recycling of the injected label (21). At its peak the SA of liver TG in CS animals was almost double (187%) that in CD rats. The SA of plasma TG was quite low during the first 10 min then rose very sharply to a maximum value at 30 min, and declined thereafter quite rapidly. At its peak the SA of plasma TG in CD animals was only 48% that in CS rats.

The SA of intestinal mucosa TG reached a maximum within 10 min and declined thereafter. At no point was a significant difference observed between CS and CD rats.

The correlation between the SA of plasma TG, on one hand, and that of liver and intestinal mucosa TG, on the other, is also shown in Fig. 1. In both CS and CD rats such a correlation suggests a precursor–product relationship (22) between the hepatic and mucosal TG and plasma TG. The fact that the SA of liver TG

TABLE 1 LIPIDS OF LIVER, PLASMA, AND INTESTINAL MUCOSA OF RATS FED A CHOLINE-SUPPLEMENTED (CS) OR A CHOLINE-DEFICIENT (CD) DIET

Expt. No.	Duration	Diet	No. of Rats		
	hr			*Triglycerides*	
1	15–17	CS	15	Liver, mg/100 g of body weight	52.5 ± 3.2
		CD	15		112.2 ± 7.5*
2	15–16	CS	10		48.7 ± 6.6
		CD	10		131.2 ± 8.0*
3	15–16	CS	10		45.2 ± 2.9
		CD	10		124.5 ± 6.5*
4	15–16	CS	12		60.4 ± 3.1
		CD	12		137.7 ± 7.8*
5	8–9	CS	12		n.d.†
		CD	12		n.d.
6	15–18	CS	24		50.4 ± 2.1
		CD	24		124.8 ± 5.9*
7	15–21	CS	15		n.d.
		CD	15		n.d.
2		CS		Liver microsomes, mg/100 mg of microsomal proteins	1.33 ± 0.13
		CD			1.53 ± 0.10
1		CS		Plasma, mg/100 ml	54.2 ± 4.5
		CD			45.4 ± 4.7
1		CS		Intestinal mucosa, μg/mg DNA	474 ± 70
		CD			465 ± 75
				Phospholipids	
4		CS		Liver, mg/100 g of body weight	143.5 ± 4.3
		CD			118.2 ± 3.5*

Each value represents the mean ± SEM.
* $P < 0.05$.
† n.d., not determined.

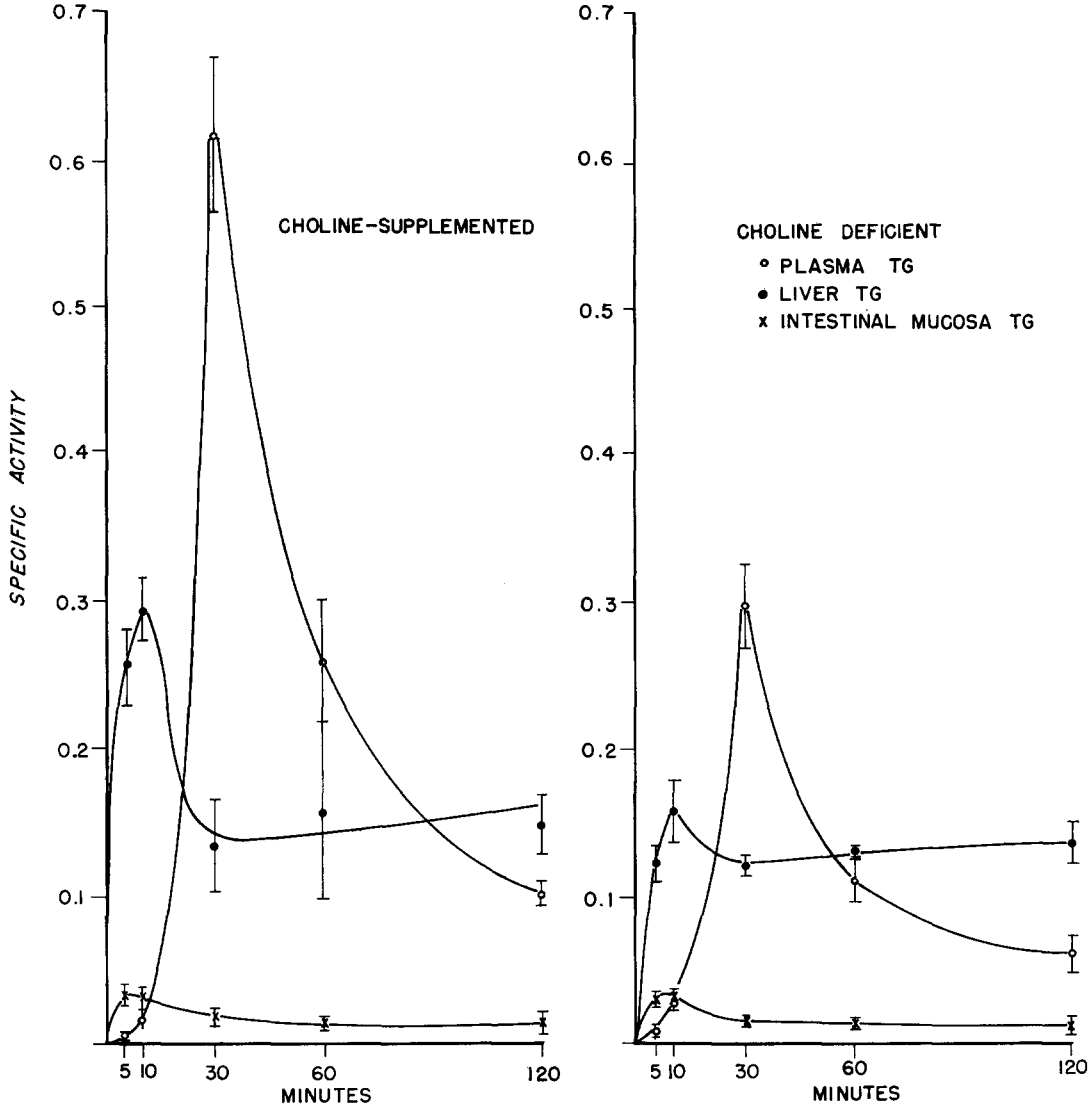

FIG. 1. Specific activity of liver, plasma, and intestinal mucosa TG after injection of palmitic acid-1-^{14}C to rats fed a choline-supplemented or a choline-deficient diet for 15–17 hr (expt. 1). Each point represents the mean ± SEM of three rats.

at its maximum was lower than the maximal SA attained by plasma TG is undoubtedly a reflection of the heterogeneity of the liver TG pool (see Discussion). In both CS and CD rats the SA of plasma TG at its maximum was approximately double the maximal SA of liver TG, while it was about 18- and 9-fold, in CS and CD rats respectively, as high as the SA of intestinal mucosa TG at its peak. It seems, therefore, that in both CS and CD rats far more of the radioactivity in plasma TG was contributed by the liver than by the intestine.

The time curves for total radioactivity in liver and plasma TG are shown in Fig. 2. A larger fraction of injected ^{14}C was present in liver TG of CD rats than in those of CS animals at all points except the first one. The difference was not significant during the first 10 min, but was highly so afterwards, and was essentially due to a considerable loss of radioactivity from liver TG of CS rats (especially during the 10–60 min interval) but only a minor loss from liver TG of CD rats. The fraction of injected ^{14}C present in plasma TG during the first 10 min was somewhat, but not significantly, higher in CD than in CS rats. In both CS and CD animals it was quite small at the 5 min point but rather substantial at 10 min. This observation contrasts with the finding of only negligible amounts of radioactivity in plasma TG 10 min after the injection of palmitic acid-1-^{14}C to fasting or glucose-fed rats (23, 24). This difference may be explained by a faster turnover of hepatic and plasma TG in rats ingesting a high fat (15%) diet, as in the present study. At the 30, 60, and 120 min points significantly higher fractions of injected ^{14}C were present in plasma TG of CS rats than of CD animals.

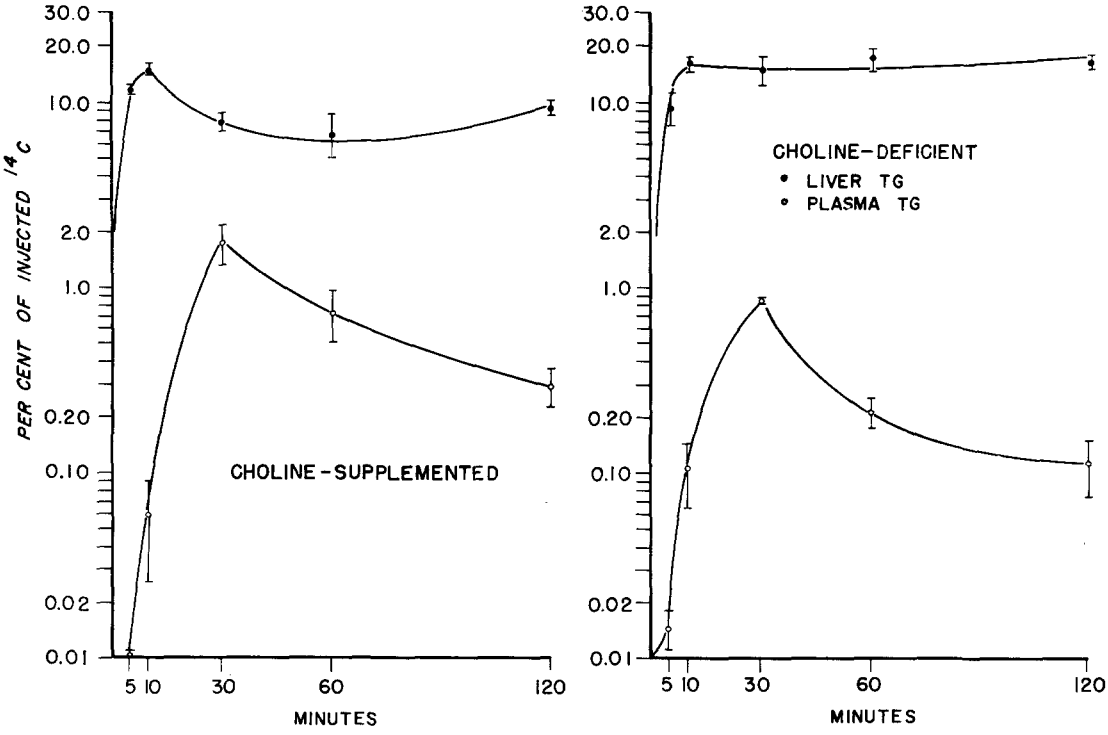

FIG. 2. Total activity of liver and plasma TG after injection of palmitic acid-1-^{14}C to rats fed a choline-supplemented or a choline-deficient diet for 15–17 hr (expt. 1). Each point represents the mean ± SEM of three rats.

Serum Lipoprotein TG (Expt. 3)

Lipoproteins were isolated from sera of CS or CD rats sacrificed 30 min after the injection of 5 μc of palmitic acid-9,10-^{3}H. The concentration, SA, and TA of TG was determined in the various fraction (Table 2).

The only significant ($P < 0.025$) difference in the concentration of TG was seen in the VLDL, the concentration in CD rats being 54% that of the same fraction in CS animals. However, the concentration of TG in whole serum and in chylomicra was also reduced, even though not significantly, in CD rats to 75% ($P > 0.4$) and 85% ($P > 0.5$), respectively, of those in CS rats.

The SA and TA of TG in whole serum, chylomicra, VLDL, and LDL of CD rats were significantly lower (16–40%) than those of CS animals. The TA, but not the SA, of HDL TG was also lower. In both CS and CD rats the SA of VLDL TG was the highest of any fraction. Over 70% of the radioactivity of whole serum TG was carried by VLDL, and no more than 10% by chylomicra. It is likely, however, that the lower radioactivities of chylomicra and of TG of LDL and HDL observed in CD rats was just a reflection of the lower SA of VLDL TG, in view of the imperfect separation between chylomicra and VLDL (see Methods), and of the exchange of TG that occurs in circulating lipoproteins (25). Recovery of TG after fractionation of whole serum was 84% in CS rats and 77% in CD animals; corresponding values for TA were 93 and 89%. In both CS and CD rats chylomicron TG contributed approximately 35% of the whole serum TG pool, yet their SA was quite low. It would seem, therefore, that whenever similar studies are performed in animals fed ad libitum more accurate results may be obtained if the chylomicron fraction is removed prior to analysis of plasma or serum TG.

The SA of liver TG was somewhat higher in the CD animals than in controls. However, 30 min after the injection of the label, more than three times as much radioactivity persisted in liver TG of the CD rats than in those of the controls.

Plasma TG and PL in Tritonized Animals

In expt. 4, 50 mg of Triton and 5 μc of palmitic acid-9,10-^{3}H were simultaneously injected intravenously into CS and CD rats, and groups of animals were sacrificed 20, 40, and 60 min thereafter. TA in plasma and liver TG and PL was determined. As can be seen from Fig. 3, more radioactivity accumulated with time in plasma TG and PL of CS rats than of CD animals. Thus 60 min after injection of Triton and palmitic acid 6.30% of the injected radioactivity was present in plasma TG of CS animals, but only 1.94% in plasma TG of CD rats; corresponding values for plasma PL were 0.42% and 0.25%. The TA–time curves for liver TG and PL are shown in Fig. 4. A larger fraction of the injected dose was present at each of the three time intervals in liver TG of CD rats than in those of controls. In the 20–60 min

TABLE 2 CONCENTRATION AND RADIOACTIVITIES OF SERUM LIPOPROTEIN TRIGLYCERIDES OF RATS FED A CHOLINE-SUPPLEMENTED (CS) OR A CHOLINE-DEFICIENT (CD) DIET

Diet	Fraction	Triglycerides		
		Concentration	SA	TA
		mg/100 ml		
CS	Whole serum	59.14 ± 4.77	0.798 ± 0.047	2.543 ± 0.156
CD		44.63 ± 10.01	0.225 ± 0.033*	0.505 ± 0.073*
CS	Chylomicra	20.21 ± 5.81	0.209 ± 0.046	0.199 ± 0.055
CD		16.69 ± 5.34	0.061 ± 0.012*	0.048 ± 0.010*
CS	VLDL	24.74 ± 4.57	1.744 ± 0.254	2.085 ± 0.144
CD		13.40 ± 1.44*	0.476 ± 0.063*	0.351 ± 0.066*
CS	LDL	2.42 ± 0.07	0.508 ± 0.069	0.066 ± 0.008
CD		2.54 ± 0.22	0.194 ± 0.012*	0.027 ± 0.003*
CS	HDL	1.10 ± 0.18	0.328 ± 0.045	0.018 ± 0.001
CD		0.78 ± 0.13	0.307 ± 0.044	0.012 ± 0.001*
CS	d > 1.210	1.10 ± 0.13	0.156 ± 0.017	0.0090 ± 0.0003
CD		0.91 ± 0.10	0.207 ± 0.028	0.0097 ± 0.0001
CS	Liver		0.165 ± 0.208	7.34 ± 0.45
CD			0.190 ± 0.008*	23.40 ± 0.99*

Each value represents the mean ± SEM. For units of specific and total activity, see last paragraph of Methods.
* $P < 0.05$.

interval liver TG radioactivity decreased 3.0% (from 18.8% to 15.8%) of the injected dose in CD rats and 6.2% (from 14.3% to 8.1%) in CS animals. A slightly higher fraction of injected label was present in liver PL of CS animals than of CD rats. However, in both CS and CD animals there was very little, if any, loss of radioactivity with time from liver PL. Triton had no apparent effect on the concentration of hepatic TG (Table 1), as previously reported (1, 8, 26, 27).

Fig. 5 shows the results obtained in a similar experiment (expt. 5) in which 50 mg of Triton and 2 μc of palmitic acid-9,10-^{3}H were injected into rats fed the CS or CD diet for 8–9 hr, rather than for 15–16 hr as before. Only the TA of plasma TG was determined. A marked difference between controls and experimental rats in the percentage of injected ^{3}H accumulated in plasma TG was again evident. The choice of time intervals of 15, 30, and 45 min resulted, however, in a less linear response than that seen in the previous experiment.

In the last two experiments, 50 mg of Triton was injected intravenously into each CS or CD rat, and groups of animals were killed either after 0, 1, 2, and 3 hr (expt. 6) or after 0, 3, and 6 hr (expt. 7). The concentration of plasma PL was determined at each time interval. As can be seen from Fig. 6, plasma PL increased linearly with time, the rate of increase being slightly, but definitely, higher in CS animals than in the deficient ones. Thus, in CS animals the average hourly increase was 88.3 and 58.5 mg/100 ml of plasma in expts. 6 and 7, respectively. Corresponding values in CD animals were 56.0 and 43.8 mg/100 ml of plasma. The reason for the difference in the rates obtained in CS rats in the two experiments was not readily apparent.

DISCUSSION

A time study of the incorporation of labeled fatty acid into liver and plasma TG had been previously used by several investigators in order to assess the release of hepatic TG into plasma in a variety of physiologic and pathologic conditions (10, 21, 23–25, 28–32). A measure of this release is in fact given by the extent at which newly synthesized and labeled TG are transferred from the liver to the plasma.

As shown in Figs. 1 and 2, after injection of palmitic acid-1-^{14}C the TG of liver became labeled very rapidly in both CS and CD rats. Initially, a similar percentage of the injected ^{14}C was present in liver TG of control and experimental animals, which indicates a comparable uptake of label by the respective livers as observed also by others (33, 34). However, while in controls there was a considerable disappearance, with time, of radioactivity from liver TG, the loss was only minor in the CD animals (see also Fig. 4). Concomitantly, significantly less radioactivity was found to appear in plasma TG of CD rats than of CS animals. An impaired release of hepatic TG into plasma, in CD rats, is thus indicated by these findings.

At least two distinct pools of TG have been identified in rat liver (23–25). One, a storage pool associated with the hyaloplasm of hepatocytes, contains the bulk of hepatic TG and constitutes the floating fat observed upon

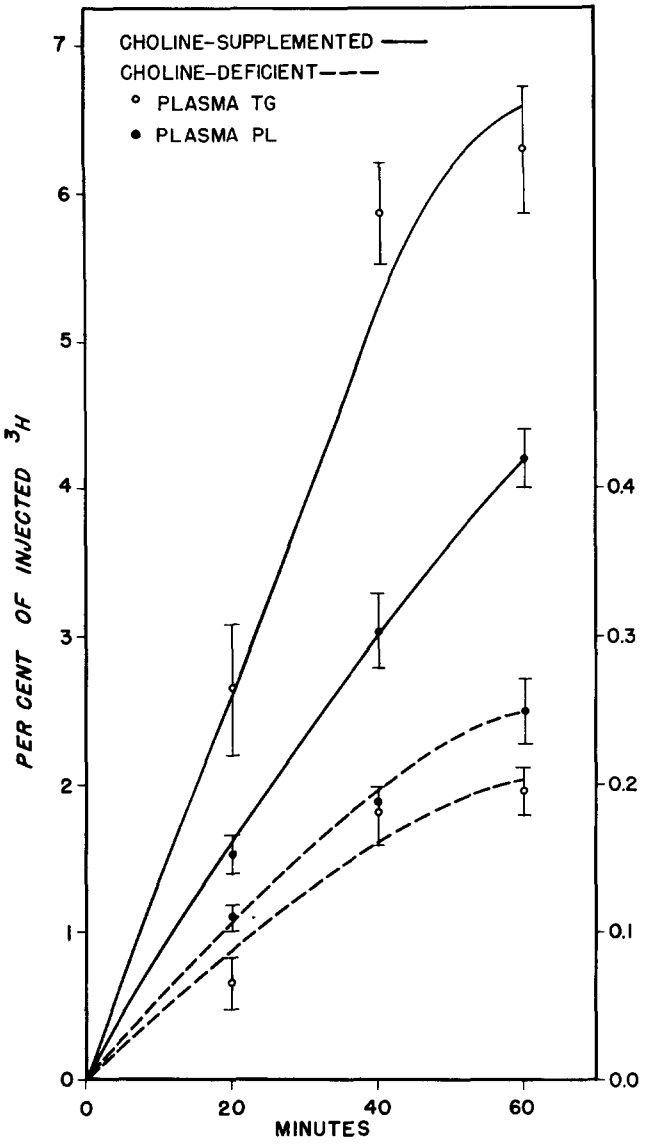

FIG. 3. Total activity of plasma TG and PL at three time intervals after injection of Triton and palmitic acid-9,10-^{3}H to rats fed a choline-supplemented or a choline-deficient diet for 15–16 hr (expt. 4). Each point represents the mean ± SEM of four rats. Left ordinate, TG; right ordinate, PL.

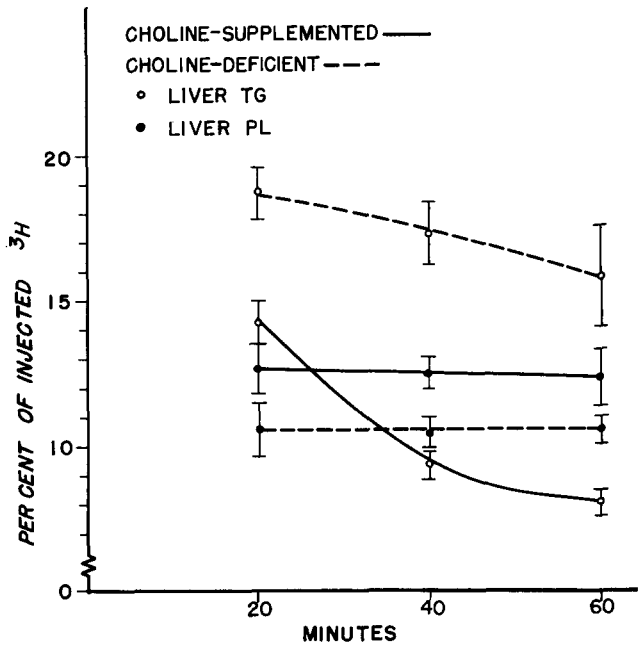

FIG. 4. Total activity of liver TG and PL at three time intervals after injection of Triton and palmitic acid-9,10-^{3}H to rats fed a choline-supplemented or a choline-deficient diet for 15–16 hr (expt. 4). Each point represents the mean ± SEM of four rats.

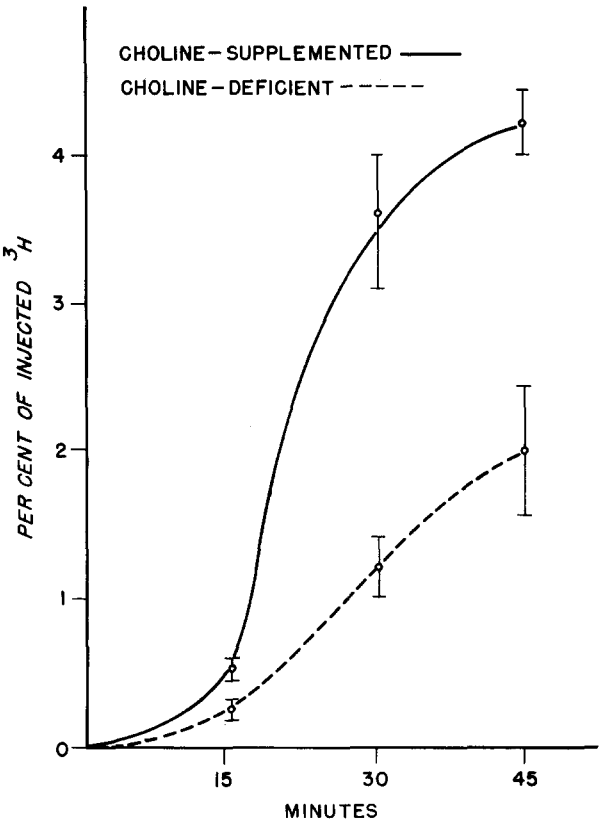

FIG. 5. Total activity of plasma TG at three time intervals after injection of Triton and palmitic acid-9,10-^{3}H to rats fed a choline-supplemented or a choline-deficient diet for 8–9 hr (expt. 5). Each point represents the mean ± SEM of four rats.

centrifugation of liver homogenates. The second, an active pool, is associated with the endoplasmic reticulum (microsomes) of hepatocytes, and is the site at which active esterification of fatty acids to TG takes place. The latter is also the precursor pool of plasma TG (23, 24, 28). In CD rats, TG have been shown to accumulate in the storage pool (35). Thus, the finding that the SA of whole liver TG was initially only about half that in CS animals (Fig. 1) can be accounted for by dilution of TG newly synthesized in the microsomal pool with those already accumulated in the storage pool (Table 1). On the other hand, no difference was found in the TG content of microsomes isolated from livers of ex-

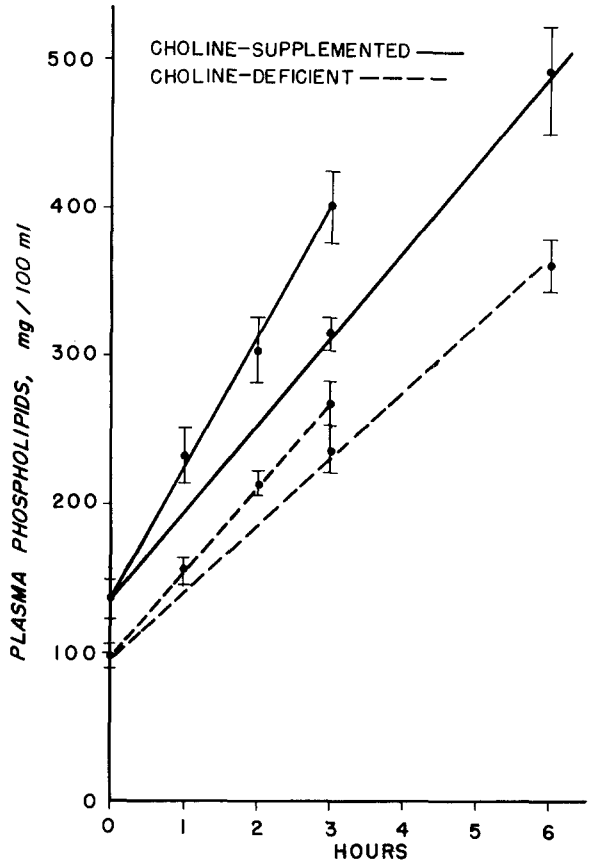

FIG. 6. Hyperphospholipidemia after injection of Triton to rats fed a choline-supplemented or a choline-deficient diet for 15–21 hr (expts. 6 and 7). Each point represents the mean ± SEM of five or six rats.

perimental and control rats (Table 1). It can, therefore, be excluded that the lower SA and TA of plasma TG, observed in CD rats, were the consequence of an expansion of, and dilution in, the precursor pool.

An impairment in the release of hepatic TG is usually reflected by lower concentrations of plasma TG (8, 26). Even though CD rats tend to have somewhat lower levels of plasma TG than CS animals, the difference has never been significant (6, 8, 12, and Table 1). VLDL constitute the major vehicle for the release of hepatic TG into plasma (25, 29, 30). However, in CD rats consuming a high fat diet, a lower level of VLDL TG may not be detected in an analysis of whole plasma TG, a large fraction of which would be represented by chylomicron TG. Indeed, when various classes of plasma lipoproteins were analyzed, a significant decrease in circulating VLDL TG was found in CD rats (Table 2). In a previous study (12), a lower concentration of VLDL protein, but not of chylomicron protein, was also found. It seems, therefore, very likely that the decrease in the concentration of VLDL TG and protein, observed in CD rats, reflects a true decrease in the level of this lipoprotein as indicated by similar findings in other experimental conditions (15, 36).

In both CS and CD rats, after administration of palmitic acid-9,10-^{3}H more than 70% of the radioactivity present in total plasma TG was found in VLDL TG (Table 2). This finding is certainly in keeping with the role played by VLDL in the release of hepatic TG into plasma—the more so, since the above value is probably a minimum one in view of the exchange of TG that occurs among circulating lipoproteins (25). In CD rats both the SA and TA of plasma TG were significantly lower than those in CS animals, the difference being essentially a reflection of the corresponding SA and TA of VLDL TG. Thus, these findings are also consistent with an impairment in the release of hepatic TG into plasma of CD rats.

The ability of the liver to release TG into plasma has also been assessed in a variety of conditions by means of Triton (8, 26, 27, 37), a nonionic detergent. Upon intravenous injection of Triton into experimental animals, a marked hyperlipemia (mainly hypertriglyceridemia) ensues (38, 39). This effect results from a block in the exit of lipids from the plasma compartment (1) due to alterations in the physicochemical properties of plasma lipoproteins (40). In fasting animals, in which the liver is the major if not the only source of plasma TG (41), the post-Triton hypertriglyceridemia affords a direct measure of hepatic TG release into plasma. Indeed, the Triton test has been successfully used in the study of fatty livers that can be induced in fasting animals (8, 26, 27). However, its application to the study of hepatic TG secretion in fed animals, especially in CD rats, presents a serious difficulty. This difficulty stems from the fact that in this instance TG are contributed to the plasma by both liver and intestine, therefore making the post-Triton hypertriglyceridemia only a doubtful measure of hepatic TG secretion. One way to circumvent this difficulty would be to fast the animals prior to challenging them with Triton. Unfortunately, this approach is not useful in the case of animals with a CD fatty liver, since fasting has been shown to exert a lipotropic action (9). Thus, to obtain meaningful information, one must test hepatic TG secretion in CD rats while the animals are consuming the diet. To avoid the contribution of dietary fat to plasma TG, Recknagel and Goshal (9) performed a Triton test on rats fed a CD fat-free diet. In such animals evidence of an impaired secretion of hepatic TG was obtained, but only after several days of feeding. Another way in which a meaningful Triton test can be performed, even in animals ingesting a high fat diet, was suggested by some of the results obtained in the present studies. After intravenous injection of labeled palmitic acid, the amount of radioactivity contributed to plasma TG by TG of intestinal origin appeared to be very small relative

to that contributed by the liver. Indeed, the SA of intestinal mucosa TG was negligible in comparison to that of plasma and liver TG, and similar in both CS and CD rats (Fig. 1). Furthermore, less than 10% of plasma TG radioactivity was present in chylomicra TG (Table 2). Therefore, it appears that a reliable test can be performed in fed animals by injecting intravenously both Triton and a labeled fatty acid, and by measuring the increment with time in total plasma TG radioactivity, rather than the post-Triton hypertriglyceridemia. Such an increment would in fact be determined to a very large extent by newly synthesized hepatic TG released into plasma as a moiety of VLDL. When a test was so performed, 6.3% of the injected radioactivity was found to accumulate in 60 min in plasma TG of CS animals, compared with only 1.9% in CD rats (Fig. 3), indicating again a block in hepatic TG secretion in CD animals. This result also rules out the possibility that the lower radioactivities of plasma TG, observed in CD rats in the previous experiments, could have been due to a faster utilization of plasma TG by peripheral tissues. It is also evident from Fig. 3 that considerably less radioactivity accumulated in plasma PL of CD rats than of CS animals.[1]

It is known that even during active fat absorption, the liver, rather than the intestine, is the major source of plasma PL (42). Thus a simpler way of performing a Triton test in fed animals is to measure the post-Triton hyperphospholipidemia. Plasma PL, as well as TG, are secreted by the liver as a moiety of lipoproteins. Therefore, this modified test could provide indirect information on hepatic TG secretion. Again, in CD rats the post-Triton hyperphospholipidemia was less pronounced than in controls (Fig. 6). However, when performed in this way, the test is much less sensitive than when the response of plasma TG to Triton is measured. Indeed, after 3 hr there was less than a 3-fold increase in plasma PL, whereas up to 20-fold increases in plasma TG have been observed 90 min after Triton in fasted animals (8). The main reasons for such a low sensitivity are undoubtedly (*a*) the considerably slower turnover of plasma PL (28, 29, 39, and Fig. 3), and (*b*) the fact that among plasma lipoproteins, the VLDL fraction has the fastest turnover (43, 44), but the lowest content of PL (15, 36).

The significance of the results presented here lies in the fact that they were obtained during the very early stages of choline deficiency. Particularly relevant in this regard are the results of expt. 5, shown in Fig. 5, since they were obtained in animals which had been fed the diets for only 8–9 hr. It seems likely, therefore, that the fatty liver (Table 1) induced in rats by choline deficiency results from, rather than is the cause of, impaired release of hepatic TG into plasma, as a moiety of plasma lipoproteins.

As for the primary lesion responsible for such an impaired release during the early stages of choline deficiency, available evidence indicates that it involves the metabolism of phospholipids. Indeed, reduced levels of liver (6 and Table 1) and plasma (6, 7, 12, and Fig. 6) PL have been observed within 24 hr of choline deficiency, and the rate of PL, as well as TG, secretion into plasma is reduced (Figs. 3 and 6). Furthermore, a gradual decline in the concentration of plasma PL has been shown to accompany the early deposition of TG in the liver (6). Oral administration of choline to CD rats prevented completely any further deposition of TG in the liver, as well as any further decline in the level of plasma PL. These findings suggest that synthesis of PL is impaired in rats consuming a CD diet, and that the onset of fatty liver is related to this insufficiency. Alterations in the metabolism of PL, in particular of lecithins, have of course been known for a long time to occur in CD animals (45). However, their significance in terms of the lipotropic action of choline has been recently questioned or discounted (4, 46), mainly because of evidence, obtained from ^{32}P-incorporation studies, indicating that the turnover of liver lecithins is increased in CD animals (45, 47). Lecithins are a very heterogenous class of polar lipids, and several subclasses or species have been separated and identified on the basis of their fatty acid composition (48). The various pathways through which lecithins can be synthesized seem to be responsible for this heterogeneity (48). Of these pathways, two are of quantitative importance for net synthesis of lecithins in the liver (49): (*a*) incorporation of preformed, free choline via cytidine disphosphate choline and diglycerides; and (*b*) the stepwise methylation of phosphatidyl ethanolamines by S-adenosylmethionine. There is evidence that the first pathway is the most active in rats, especially males (50). Recently, it has been found in this laboratory that labeling of liver and plasma lecithins, after injection of 2-dimethylaminoethanol-1,2-^{14}C or methionine-CH_3-^{14}C, is several-fold faster and greater in CD than in CS animals. This finding could explain a reduced over-all synthesis of lecithins in CD rats, in spite of an increased turnover of some subclass or species of lecithins. Lecithins represent the major PL class in plasma (51, 52) and plasma lipoproteins (53), and there is evidence that PL play a role in the conjugation of the protein moiety to the nonpolar lipid moiety of plasma lipoproteins (54). It appears, therefore, likely that either an insufficient availability of lecithins, or a lack of specific lecithins, in CD animals may lead to failure in the conjugation of the

[1] In performing the test we have assumed that the vascular clearance and hepatic uptake of the injected label is similar in CS and CD rats injected with Triton. Therefore, our interpretation of these results depends upon the validity of this assumption.

moieties of plasma lipoproteins and, thereby, to impaired release of hepatic TG.

A second possibility was, however, suggested by results obtained in a previous study (12). Although protein synthesis was equal in livers of CD and CS rats, the release into plasma of nonlipoprotein proteins, as well as of lipoprotein proteins, was inhibited or delayed. It therefore seems possible that the alterations in PL synthesis that occur in CD rats may affect hepatic cellular membranes, plasma and(or) microsomal, in such a way that even if plasma lipoproteins are synthesized normally they cannot be secreted into the plasma. This possibility would also account for the impaired or delayed release by the liver of plasma proteins other than lipoproteins.

The valuable assistance of Miss Eileen LaRocca is gratefully acknowledged.

This work was supported in part by research grants from the United States Public Health Service: Grants AM 06334 from the National Institute of Arthritis and Metabolic Diseases, GM-10269 from the National Institute for General Medical Sciences, and Training Grant GM-00135 from the latter institute.

Manuscript received 5 December 1967; accepted 11 March 1968.

References

1. Recknagel, R. O. 1967. *Pharmacol. Rev.* **19:** 145.
2. Lombardi, B. 1966. *Lab. Invest.* **15:** 1.
3. Lombardi, B. 1965. *Federation Proc.* **24:** 1200.
4. Lucas, C. C., and J. H. Ridout. 1967. Progress in the chemistry of fats and other lipids. Pergamon Press, Oxford. **X** (Pt. 1): 1–150.
5. McHenry, E. W., and J. M. Patterson. 1944. *Physiol. Rev.* **24:** 128.
6. Lombardi, B., G. Ugazio, and A. N. Raick. 1966. *Am. J. Physiol.* **210:** 31.
7. Rosenfeld, B., and J. M. Lang. 1966. *J. Lipid Res.* **7:** 10.
8. Lombardi, B., and R. O. Recknagel. 1962. *Am. J. Pathol.* **40:** 571.
9. Recknagel, R. O., and A. K. Goshal. 1964. *Federation Proc.* **23:** 243.
10. Lombardi, B., and M. C. Schotz. 1963. *Proc. Soc. Exptl. Biol. Med.* **112:** 400.
11. Young, R. J., C. C. Lucas, J. M. Patterson, and C. H. Best. 1965. *Can. J. Biochem. Physiol.* **34:** 713.
12. Lombardi, B., and A. Oler. 1967. *Lab. Invest.* **17:** 308.
13. Friedberg, S. J., W. R. Harlan, Jr., D. L. Trout, and E. H. Estes. 1960. *J. Clin. Invest.* **39:** 215.
14. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. *J. Clin. Invest.* **34:** 1345.
15. Lombardi, B., and G. Ugazio. 1965. *J. Lipid Res.* **6:** 498.
16. Bligh, E. G., and W. J. Dyer. 1959. *Can. J. Biochem. Physiol.* **37:** 911.
17. Van Handel, E. 1961. *Clin. Chem.* **7:** 249.
18. Shin, Y. S. 1962. *Anal. Chem.* **34:** 1164.
19. Layne, E. 1957. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press, Inc., New York. **3:** 450.
20. Burton, K. A. 1956. *Biochem. J.* **62:** 315.
21. Laurell, S. 1959. *Acta Physiol. Scand.* **47:** 218.
22. Zilversmit, D. B., C. Entenman, and M. C. Fishler. 1943. *J. Gen. Physiol.* **26:** 325.
23. Schotz, M. C., N. Baker, and M. N. Chavez. 1964. *J. Lipid Res.* **5:** 569.
24. Baker, N., and M. C. Schotz. 1964. *J. Lipid Res.* **5:** 188.
25. Havel, R. J., J. M. Felts, and C. M. Van Duyne. 1962. *J. Lipid Res.* **3:** 297.
26. Recknagel, R. O., B. Lombardi, and M. C. Schotz. 1960. *Proc. Soc. Exptl. Biol. Med.* **104:** 608.
27. Schlunk, F. F., and B. Lombardi. 1967. *Lab. Invest.* **17:** 299.
28. Stein, Y., and B. Shapiro. 1959. *Am. J. Physiol.* **196:** 1238.
29. Borgström, B., and T. Olivecrona. 1961. *J. Lipid Res.* **2:** 263.
30. Havel, R. J., and A. Goldfien. 1961. *J. Lipid Res.* **2:** 389.
31. Olivecrona, T. 1962. *Acta Physiol. Scand.* **54:** 295.
32. Maling, H. M., A. Frank, and M. G. Horning. 1962. *Biochim. Biophys. Acta.* **64:** 540.
33. Mishkel, M. A., and B. Morris. 1964. *Quart. J. Exptl. Physiol.* **49:** 21.
34. Haines, D. S. M. 1966. *Can. J. Biochem.* **44:** 45.
35. Rosenfeld, B., and J. M. Lang. 1958. *Proc. Can. Fed. Biol. Soc.* **1:** 43.
36. Ugazio, G., and B. Lombardi. 1965. *Lab. Invest.* **14:** 711.
37. Otway, S., and D. S. Robinson. 1967. *J. Physiol.* **190:** 321.
38. Kellner, A. 1950. *Am. J. Pathol.*, **26:** 732.
39. Friedman, M., and S. O. Byers. 1953. *J. Exptl. Med.* **97:** 117.
40. Scanu, A., and P. Oriente. 1961. *J. Exptl. Med.* **113:** 735.
41. Byers, S. O., and M. Friedman. 1960. *Am. J. Physiol.* **198:** 629.
42. Fishler, M. C., C. Entenman, M. L. Montgomery, and I. L. Chaikoff. 1943. *J. Biol. Chem.* **150:** 47.
43. Haft, D. E., P. S. Roheim, A. White, and H. A. Eder. 1962. *J. Clin. Invest.* **41:** 842.
44. Gidez, L. I., P. S. Roheim, and H. A. Eder. 1967. *J. Lipid Res.* **8:** 7.
45. Zilversmit, D. B., and N. R. Diluzio. 1958. *Am. J. Clin. Nutr.* **6:** 235.
46. Mookerjea, S., D. Jeng, and J. Black. 1966. *Can. J. Biochem.* **45:** 825.
47. Mookerjea, S., and W. Thompson. 1966. *Proc. Can. Fed. Biol. Soc.* **9:** 71.
48. Lands, W. E. M. 1965. *Ann. Rev. Biochem.* **34:** 313.
49. Bjørnstad, P., and J. Bremer. 1966. *J. Lipid Res.* **7:** 38.
50. Artom, C. 1965. *Federation Proc.* **24:** 477.
51. Lyman, R. L., A. Shannon, R. Ostwald, and P. Miljanich. 1964. *Can. J. Biochem.* **42:** 365.
52. Newman, H. A. I., C. T. Liu, and D. B. Zilversmit. 1961. *J. Lipid Res.* **2:** 403.
53. Skipski, V. P., M. Barclay, R. K. Barclay, V. A. Fetzer, J. J. Good, and F. M. Archibald. 1967. *Biochem. J.* **104:** 340.
54. Sodhi, H. S., and R. Gordon Gould. 1967. *J. Biol. Chem.* **242:** 1205.