THE EFFECT OF LIGATION OF THE COMMON BILE DUCT ON CHOLESTEROL SYNTHESIS IN THE RAT*

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Obstruction of the biliary tract has long been known to be accompanied by hypercholesterolemia (1). This phenomenon has been studied in the rat by Chanutin and Ludewig (2) and, more extensively, by Byers, Friedman, and their colleagues (3–7). In a recent review the latter authors have mentioned possible mechanisms involved, including altered absorption, synthesis, excretion, and destruction of cholesterol (8).

The purpose of the present study was to investigate by isotopic methods the effect of bile duct ligation on cholesterol synthesis in the rat. As indices of the rate of cholesterol synthesis we used the incorporation of carboxyl carbon from 1-C^{14}-acetate, and hydrogen from tritium-labelled water, into cholesterol in the intact animal, in liver slices, or in cell-free homogenates.

In all the systems which we employed, the rate of incorporation of these isotopes into cholesterol was increased following ligation of the common bile duct.

EXPERIMENTAL

Rats utilized in these studies were of the Sprague-Dawley and Wistar strains, both males and females, weighing between 140 and 320 gm., and unless otherwise specified, were maintained on a steroid-free, fat-free diet of the following composition: casein (vitamin-free) 125 gm., sucrose 355 gm., salt mixture "W" (9) 20 gm., choline chloride 500 mg., thiamine hydrochloride 10 mg., riboflavine 10 mg., pyridoxine hydrochloride 10 mg., nicotinic acid 10 mg., calcium-*d*-pantothenate 20 mg., and linoleic acid 0.47 gm. All surgical procedures were performed under ether anesthesia. Ligations of the common bile duct were made with double silk ligatures. Maintenance of obstruction was determined at autopsy by the presence of a dilated, bile-filled duct proximal to the ligatures with no evidence of leakage. The peritoneal cavities of all control animals were opened and the intestines manipulated.

Isolation and Assay of Cholesterol.—Cholesterol was isolated from liver in the following manner: 0.5 gm. of liver was hydrolyzed in 2.5 ml. of a 15 per cent solution of KOH in 95 per cent ethanol for 16 hours at 100°C. An equal volume of water was then added, followed by extraction with four 10 ml. portions of petroleum ether (b.p. 30–60°). The extracts were combined and taken to dryness on the steam bath, the residue was dissolved in 4 ml. of 1:1 acetone-

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ethanol and filtered, and cholesterol was precipitated by addition of 2 ml. of a 0.5 per cent solution of digitonin in 95 per cent ethanol. When larger amounts of liver were extracted, the proportions were varied accordingly. After at least 3 hours, the precipitates were filtered on Whatman No. 50 filter paper and counted with a thin window counter, as previously described for $BaCO_3$ (10). After being counted the samples were dried over P_2O_5, scraped from the filter paper, and weighed. The results were corrected for self-absorption to a thickness of 4 mg. spread over the 3.7 $cm.^2$ area (approximately the amount of cholesterol digitonide usually obtained from 0.5 gm. of normal rat liver).

Tritium Assays.—Tritium assays were made by counting of tritiated methane obtained from labeled cholesterol and body water. Tritiated water from the combustion of 15 to 25 mg. of vacuum-desiccated cholesterol digitonide was collected in a low pressure trap, as described by Glascock (11). The water was measured as vapor in a gas burette. It was then introduced into an evacuated reaction vessel and converted to methane by reaction with methyl magnesium bromide in butyl ether. Its radioactivity was measured by introduction of the methane directly into a proportional type gas counter. This method (12) of counting of tritiated water is essentially that of Robinson (13) with the significant modification that a 2- to 3-fold excess of Grignard reagent is used, instead of a 2-fold excess of sample water. Making the water the limiting reactant increases the over-all counting efficiency, and is of advantage when limited quantities of water are available. It also decreases the possibility of isotopic effects, since almost all the water is used up in the reaction. Because half of the hydrogen in the water is lost to MgBrOH in the Grignard reaction, and not all of the methane produced ultimately reaches the sensitive volume of the counter, the efficiency even of this method is limited to little more than 25 per cent.

Radioactive Materials.—1-C^{14}-sodium acetate was prepared by the reaction of $C^{14}O_2$ with methyl magnesium bromide (14) in a vacuum system designed for efficient handling of small amounts of carbon dioxide (15).

Tritium-labelled water was prepared by equilibrating tritium gas with water in the presence of a platinum oxide catalyst (16).

Purification of Cholesterol.—Cholesterol was purified by two passages through the dibromide according to the method of Schoenheimer (17) as modified by Schwenk and Werthessen (18). Schoenheimer's method of recovery of cholesterol from the dibromide was retained.

Serum cholesterol and bile cholesterol were extracted and determined quantitatively by the Schoenheimer-Sperry method (19). When necessary, aliquots of the acetone-ethanol filtrate were set aside, sufficient carrier cholesterol was added to bring the total sample weight to 4 mg., and the cholesterol was precipitated and counted in the same manner utilized for liver cholesterol.

Quantitative determinations of liver cholestrol were carried out according to the Schoenheimer-Sperry method (19).

Total nitrogen of liver tissue was determined by the method of Ma and Zuazaga (20).

RESULTS

A. The Effect of Bile Duct Ligation on Incorporation of Acetate into Cholesterol Single Animal Experiments (Animal Acting as Its Own Control).—

In each of two Sprague-Dawley rats weighing approximately 290 gm., polyethylene catheters of internal diameter 0.011 and 0.023 inch respectively were placed in the common bile duct and the femoral vein, secured by ligatures, and passed through the body wall. The animals were then placed in restraining cages and the venous catheter connected to a constant infusion of 5 per cent dextrose, 2.5 per cent sodium citrate

solution. Blood samples could thus be obtained and acetate injected without further trauma. Bile was collected from the common bile duct catheter for analysis of cholesterol content and radioactivity.

3 to 7 hours after the operative procedure, when the rat was observed to have resumed feeding, 100 μc. of 1-C^{14}-sodium acetate in physiological saline was administered intravenously in a single injection. 3½ hours later blood was obtained. Approximately 20 hours after the initial acetate injection, the biliary drainage tube was obstructed with a clamp, and 24 hours later, a second blood sample was taken, followed by a second injection of C^{14}-acetate. 3½ hours later a final blood sample was taken.

TABLE I

Appearance of C^{14} from Acetate in Plasma Cholesterol before and after Bile Duct Obstruction in the Same Rat

Time	Plasma cholesterol			Bile cholesterol
	Concentration	Radioactivity		Radioactivity Total excreted*
	mg./100 ml.	*C.P.M./ml.*	*C.P.M./mg.*	*C.P.M.*
Experiment 1				
3½ hrs. after tracer 1‡	62	115	186	
24 hrs. after obstruction	120	173	144	943
3½ hrs. after tracer 2‡	120	2250	1875	
Experiment 2				
3½ hrs. after tracer 1‡	70	344	491	
24 hrs. after obstruction	209	426	204	1934
3½ hrs. after tracer 2‡	243	7184	2956	

* total C.P.M. excreted as cholesterol during 13 to 16 hours from injection of tracer 1 to time duct was obstructed.

‡ 100 μc. 1-C^{14}-sodium acetate in saline intravenously.

The results are shown in Table I. It should be noted that both total radioactivity and specific activity of cholesterol were greatly increased after biliary obstruction. Assuming the plasma volume of these rats to be approximately 7 ml., it is possible to estimate the total radioactivity in the plasma cholesterol following the second tracer. This figure can be compared with the total C^{14} excreted in the bile as cholesterol over the entire 13 to 16 hour period after the first acetate tracer. It can then be seen that, were bile duct obstruction to cause regurgitation into the plasma of the entire radioactivity derived from C^{14}-acetate and excreted as biliary cholesterol, it would be possible to account for only a small fraction of the observed rise in radioactivity in plasma cholesterol after biliary obstruction. Hence, cessation of normal biliary cholesterol excretion alone will not explain the results obtained in these experiments.

Since the initial control tracer in these animals was administered rather early

in the postoperative period, a time during which a depression of normal cholesterol synthesis could conceivably exist, it was felt that a different type of control was necessary. Therefore the experiments discussed below were undertaken.

In these animals, and in all the subsequent experiments, the blood cholesterol was found to rise significantly following biliary obstruction.

Incorporation into Liver Cholesterol of C^{14}-Acetate Injected Intraperitoneally.—

Ligations of the bile duct and sham operations were performed on two groups of eight Sprague-Dawley rats each. The animals were placed in cages with food and water for 48 hours. A single injection of 50 μc. of C^{14}-acetate was then given intraperitoneally to each. 1 hour later, the rats were decapitated, and the livers were perfused with distilled water and removed for extraction of cholesterol. Blood for cholesterol determination was taken from the tail just prior to operation and at the time of sacrifice.

As demonstrated in Table II, the mean specific activity of the cholesterol from the livers of the rats subjected to bile duct ligation was higher than that of the controls by a factor of 19 to 1.

Incorporation of C^{14}-Acetate into Cholesterol by Liver Slices.—

Two groups of Wistar female rats were subjected to ligation of the bile duct or to sham operation as in the previous experiment, and either: (*a*) placed in cages with food and water for 48 hours; or, since rats with ligated bile ducts were observed to eat little food after operation compared with the controls, (*b*) placed in cages with water, but no food, for the 48 hour period. The animals were then decapitated, the livers removed, and slices of approximately 0.5 mm. thickness were prepared with a Stadie-Riggs slicer. 0.5 gm. of slices was placed in each of 44 vessels containing 3.5 ml. of Krebs-Ringer phosphate buffer at pH 7.4 and 2.5 mg. of sodium acetate having a total radioactivity of 2.72×10^4 C.P.M. The vessels were incubated for 2 hours at 37° in an oxygen atmosphere in the Dubnoff shaker. The slices were then transferred to alcoholic KOH and hydrolyzed, and the cholesterol extracted.

As indicated in Table III, the incorporation of acetate into cholesterol by liver slices was again significantly higher in the ligated animals. Of greater interest are the results in the animals from which food was withheld after operation. In this experiment, the livers from bile duct–ligated rats showed a rate of incorporation roughly comparable to that obtained when food was offered the animals. However, the rate in the fasted control animals was markedly reduced, and the difference between ligated and control animals exceeded 20-fold.

Incorporation of C^{14} from Acetate into Cholesterol by Cell-Free Liver Homogenates.—

Cell-free homogenates were prepared from liver from rats in the previous slice experiments according to the method of Bucher (21). The incubation vessels contained,

in final concentration, potassium phosphate buffer 0.03 M, nicotinamide 0.019 M, $MgCl_2$ 0.003 M, sucrose 0.087 M, DPN 0.016 M, AMP 0.008 M, and total nitrogen from liver tissue ranging from 1.2 to 2.1 mg./ml. The vessels were incubated in an oxygen atmosphere at 37°C for 2 hours in the Dubnoff shaker. Cholesterol was then extracted and the radioactivity determined as before. Since the total nitrogen was determined

TABLE II

Cholesterol Synthesis in Vivo in Rats with Ligated Bile Ducts 1 Hour after Injection of 50 μc. of C^{14}-Acetate

Rat	Body weight	Operation	Plasma cholesterol mg./100 ml		Specific activity of liver cholesterol
			0°	48°	
	gm.				*C.P.M./mg.*
1	170	Sham	55	58	79
2	179	"	70	52	51
3	163	"	58	81	83
4	146	"	47	51	45
5	259	"	54	71	92
6	240	"	58	73	115
7	228	"	51	59	71
8	274	"	48	68	76
Mean.....	199				77*
9	183	Ligation	65	202	587
10	176	"	63	302	2788
11	138	"	86	318	1558
12	153	"	59	250	2373
13	241	"	49	200	890
14	274	"	48	159	554
15	223	"	49	256	780
16	233	"	45	174	2244
Mean.....	210				1472*

* $p < 0.001$.

on a separate aliquot of the homogenate, the radioactivity of the digitonide was expressed in terms of the quantity of tissue nitrogen in each vessel.

Again, utilizing the cell-free system, we found that cholesterol synthesized by liver from the bile duct—ligated animals had a higher specific activity (Table III).

Effect of Ligation on Total Liver Cholesterol.—

Cholesterol was extracted from the livers of ligated and sham animals by the Schoenheimer-Sperry method (19). Total nitrogen was determined on aliquots of the same

TABLE III

Cholesterol Synthesis from C^{14}-Acetate by Liver Slices and Cell-Free Homogenates after Ligation or Sham Operation

Rat	Operation	Dietary intake	Plasma cholesterol		Specific activity in liver slices	Specific activity in homogenates
			0°	48°		
			mg./100 ml.	*mg./100 ml.*	*C.P.M./mg.**	*C.P.M./mg. of nitrogen**
1	Ligation	Fed	56	204	704	371
2	"	"	77	181	885	216
3	"	"	60	175	968	270
4	"	"	79	166	400	103
5	"	"	76	225	637	
6	"	"	59	315	597	
Mean.....					699‡	240
7	Sham	"	71	89	197	20
8	"	"	89	106	230	17
9	"	"	70	80	163	16
10	"	"	69	91	110	2
11	"	"	80	92	113	
12	"	"	56	68	228	
Mean.....					174‡	14
13	Ligation	Fasting	81	246	926	200
14	"	"	89	224	806	27
15	"	"	55	208	396	
16	"	"	68	240	353	
17	"	"	69	120	133	
Mean.....					533§	
18	Sham	"	89	99	4	<1
19	"	"	68	75	66	<1
20	"	"	46	48	13	
21	"	"	69	75	12	
22	"	"	61	69	21	
Mean.....					23§	

* All values represent the average of incubation in duplicate.

‡ $p < 0.001$.

§ $p < 0.005$.

liver homogenate and the cholesterol to nitrogen ratio was established. The results are shown in Table IV. No significant change in total liver cholesterol was observed after ligation, in agreement with the findings of Byers and Friedman (5).

B. Turnover Studies with Tritium-Labelled Body Water

Six Sprague-Dawley male rats (230 to 320 gm.) were divided into two groups. The bile ducts of three were ligated, and the three other rats were submitted to a sham operation. All were permitted food and water *ad libitum*. 24 hours postoperatively, each rat received intraperitoneally approximately 0.25 ml. of water containing 4 mc./ml. of tritium. Ordinary drinking water was then replaced with water containing tritium at a specific activity of 0.008 mc./ml., in order to maintain the specific activity of the body water approximately constant throughout the experimental period. At intervals of 1, 16, and 24 hours after the initial injection of tritium, blood samples were withdrawn from the tail for determination of the specific activity in the body water hydrogen. After the 24-hour blood sample, the animals were killed by decapitation, the livers removed, and the cholesterol isolated as the digitonide for analysis of its tritium content.

TABLE IV

Liver Cholesterol Content in Bile Duct-Ligated Animals and Controls

Rat	Operation	Liver homogenates, 4 gm. of wet tissue plus 4 ml. of water		
		Cholesterol	Nitrogen	$\frac{\text{Mg. cholesterol}}{\text{Mg. nitrogen}}$
		mg./ml.	*mg./ml.*	
5	Sham	1.16	15.7	0.074
6	"	1.07	15.3	0.070
7	"	1.11	16.4	0.068
8	"	1.16	17.0	0.068
13	Ligation	1.23	16.8	0.073
14	"	1.28	16.4	0.078
15	"	1.39	17.0	0.082
16	"	1.32	16.7	0.079

The results are found in Table V. An average of the values for body water hydrogen for each rat was used in the calculations. The half-time of liver cholesterol is found to be reduced from an average of 4.1 days in the sham-operated rats to an average of 1.4 days in the bile duct–ligated group.

Purification of Cholesterol through the Dibromide.—

The common bile duct of a rat was ligated. 48 hours later 100 μc. of C^{14}-acetate was administered intraperitoneally. After 1 hour the liver was removed and its cholesterol was extracted, precipitated with digitonin, and assayed for radioactivity. The dibromide of the cholesterol was prepared, and the cholesterol was regenerated from the dibromide and again counted. A second passage through the dibromide was carried out in the same way. Duplicate values of the specific activity of the cholesterol digitonide, in C.P.M./mg., were as follows: initial, 35.3, 34.7; after one passage through the dibromide, 34.5, 34.5; after two passages through the dibromide, 33.6, 33.8.

TABLE V

Turnover of Liver Cholesterol as Measured with Tritium-Labelled Body Water in Rats with Ligated Bile Ducts

Rat	Operation	Body weight	Specific activity of body water hydrogen			Specific activity of liver cholesterol hydrogen	Half-life of liver cholesterol
			1 hr.	16 hrs.	24 hrs.		
		gm.	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*	*days*
1	Sham	319	60,000	58,900	57,700	4140	4.6
2	"	310	74,000	76,000	65,000	5130	4.5
3	"	254		87,000	89,000	8550	3.2
Mean..							4.1*
4	Ligation	321	45,000	51,500	47,300	13,650	0.8‡
5	"	253		85,000	83,000	13,580	1.8
6	"	233		98,000	98,000	17,130	1.6
Mean..							1.4*

* $p < 0.01$.

‡ These estimates of the half-life of liver cholesterol in the rats with ligated bile ducts do not take into account the fact that a net transfer of cholesterol from liver to serum is occurring.

A comparison of the rate of synthesis of serum-liver cholesterol in the two groups of animals may be made, based on the assumptions that the amount and specific activity of body water and the size of the cholesterol pool are constant, that cholesterol is freely exchangeable between serum and liver, that no exchange with other compartments occurs, and that half of the hydrogen atoms of cholesterol are derived from water (22). Under these conditions, $C^* = W^*\left(1 - e^{\frac{-k_1 W}{C} t}\right)$, in which C = weight of cholesterol hydrogen/2, W = weight of hydrogen in body water, C^* = specific activity of cholesterol hydrogen × 2, W^* = specific activity of water hydrogen, t = time, and k_1 is the rate constant for cholesterol synthesis. These assumptions appear fairly justifiable in the normal rat. After bile duct ligation, however, the size of the cholesterol pool is not constant. Nevertheless, although the serum cholesterol rises considerably, the fractional change in the total pool during a 24 hour period is not great, because of the high and virtually constant cholesterol content of the liver.

One may attempt to take into account the change in the size of the cholesterol pool by assuming that cholesterol destruction and excretion are a first order process (having a rate proportional to the total serum and liver cholesterol) and calculating rate constants which will lead to the observed rate of change in amount and specific activity of cholesterol. The following equations apply:—

$$C = \frac{k_1 W}{k_2} + \left(C_0 - \frac{k_1 W}{k_2}\right) e^{-k_2 t},$$

$$C' = \frac{k_1 W'}{k_2} - \frac{k_1 W'}{k_2} e^{-k_2 t}$$

in which the primed letters represent total radioactivity rather than specific activity, k_2 is the rate constant for cholesterol elimination, and C_0 is the size of the cholesterol pool at the time of injection of the tritiated water. These assumptions lead to the conclusion that after bile duct ligation the rate of synthesis from water of liver plus serum cholesterol rises by a factor of about 4.0, as compared with the value 3.7 obtained from the simpler expression.

Both of these calculations were based on the same typical values of the various parameters, as follows:—

For the sham rat, t = 1 day, W = 18 gm., $C_0 = 1.55 \times 10^{-3}$ gm., W^* = 100,000 C.P.M./mg., and C^* = 15,940 C.P.M./mg. 1 day after administration of the isotope; for the rat after ligation of the bile duct, $C_0 = 1.83 \times 10^{-3}$ gm., $C = 2.11 \times 10^{-3}$ gm. 1 day after administration of the isotope, C^* = 41,600 C.P.M./mg., all other values the same as for the sham rat.

This reduction in specific activity by passage through the dibromide is well within the range observed with biosynthetic cholesterol from normal animals. Therefore, this experiment provides no evidence that radioactive "cholesterol" synthesized by a rat with biliary obstruction is less pure than that synthesized by normal rats.

DISCUSSION

The indices employed in these experiments appear to demonstrate that an increased rate of synthesis of cholesterol in the liver follows ligation of the bile duct in the rat. Not only is the incorporation of carbon from acetate into cholesterol increased, but also that of hydrogen from body water.

The effect of bile duct ligation is especially striking when all animals are fasted. The effect of fasting upon cholesterologenesis, as noted by others (22, 23), is apparent in the controls. We have observed that the ligated animal eats less and loses more weight during the 48-hour postoperative period; and, in these experiments, we feel that a better comparison between ligated and sham animals is offered in the fasting series. Our results do not provide adequate information about the relationship between caloric intake and cholesterol synthesis in the animals with biliary obstruction.

The half-time of liver cholesterol as obtained with tritium is somewhat shorter than that reported by Pihl, Bloch, and Anker (24), who obtained values of approximately 6 days in the normal rat by a different experimental approach. All animals used in our series, however, were in the postoperative state, and we feel that the series was so small that its use should be limited to comparison with the ligated animals.

The effect of ligation upon plasma cholesterol concentration was in complete agreement with that reported by Chanutin and Ludewig (2), and by Byers, Friedman, and Michaelis (3). The latter authors have proposed the biliary excretion of cholesterol as an index of cholesterol synthesis by the liver (7). They found, however, no change in the excretion of biliary cholesterol immediately following the release of biliary obstruction (5). On the basis of this index it would be reasonable to conclude that synthesis by the obstructed liver is not increased. Our isotopic data, on the other hand, appear to be in disagreement with this conclusion.

Isotopic studies of metabolic syntheses are subject to the limitation that exchange reactions are difficult to distinguish from true synthesis. Rittenberg and Schoenheimer were led to the conclusion that the occurrence of exchange was unlikely in the case of their studies of cholesterol synthesis from D_2O (25).

Our experiments with C^{14} do not contain information concerning possible variations in the size of the acetate pool. This deficiency is perhaps of less importance in the slice experiments, in which large amounts of carrier acetate were added. That considerations of this nature are of more than academic inter-

est is emphasized by the lack of agreement in the degree of increase in cholesterol synthesis after ligation of the bile duct, as judged from the two labelled precursors of cholesterol. All conditions which we employed, however, point to a substantial increase. The relative importance of this phenomenon in the development of postobstructive hypercholesterolemia cannot yet be assessed, since at least one other factor, an altered liver-plasma cholesterol partition, appears to be involved.

SUMMARY

The effect on cholesterol synthesis of ligation of the common bile duct was studied in the rat. The bile ducts of rats were ligated; 24 to 48 hours later, estimates of the rate of cholesterol synthesis were made, either by injection of labelled water or acetate into the intact animal, or by incubation of slices or homogenates of the liver in the presence of 1-C^{14}-acetate. These various criteria all indicated that cholesterol synthesis was increased following ligation of the bile duct. The average ratios of the rate of synthesis in the experimental animals to that in the controls were as follows:

1. Synthesis from C^{14}-carboxyl-labelled acetate: (*a*) in the intact rat fed *ad libitum*, 19; (*b*) in liver slices from the fasted rat, 23; (*c*) in liver slices from the rat fed *ad libitum*, 4; (*d*) in cell-free homogenates from the fasted rat, >27; (*e*) in cell-free homogenates from the rat fed *ad libitum*, 17.
2. Synthesis from tritium-labelled water in the intact rat fed *ad libitum*, 4.

BIBLIOGRAPHY

1. Flint, A., Jr., *Am. J. Med. Sc.*, 1862, **44,** 305.
2. Chanutin, A., and Ludewig, S., *J. Biol. Chem.*, 1936, **115,** 1.
3. Byers, S. O., Friedman, and Michaelis, F., *J. Biol. Chem.*, 1950, **184,** 71.
4. Byers, S. O., Friedman, M., and Michaelis, F., *J. Biol. Chem.*, 1951, **188,** 637.
5. Byers, S. O., and Friedman, M., *J. Exp. Med.*, 1952, **95,** 19.
6. Friedman, M., and Byers, S. O., *Am. J. Physiol.*, 1952, **168,** 292.
7. Byers, S. O., and Friedman, M., *Am. J. Physiol.*, 1952, **168,** 297.
8. Byers, S. O., Friedman, M., and Rosenman, R. H., *Metabolism*, 1952, **1,** 479.
9. Wesson, L. G., *Science*, 1932, **75,** 339.
10. Zamecnik, P. C., Frantz, I. D., Jr., Loftfield, R. B., and Stephenson, M. L., *J. Biol. Chem.*, 1948, **175,** 299.
11. Glascock, R. F., *Biochem. J.*, 1952, **52,** 699.
12. Loud, A. V., unpublished data.
13. Robinson, C. V., *Rev. Scient. Instr.*, 1951, **22,** 353.
14. Houben, J., and Kesselkaul, L., *Ber. chem. Ges.*, 1902, **35,** 2519.
15. Loftfield, R. B., unpublished data.
16. Fischer, R. B., Potter, R. A., and Voskuyl, R. J., *Anal. Chem.*, 1948, **20,** 571.
17. Schoenheimer, R., *Z. Physiol. Chem.*, 1930, **192,** 86.
18. Schwenk, E., and Werthessen, N. T., *Arch. Biochem. and Biophysic.*, 1952, **40,** 334.

19. Sperry, W. M., and Webb, M., *J. Biol. Chem.*, 1950, **187,** 97.
20. Ma, T. S., and Zuazaga, G., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14,** 280.
21. Bucher, N. L. R., *J. Am. Chem. Soc.*, 1953, **75,** 498.
22. Tomkins, G. M., and Chaikoff, I. L., *J. Biol. Chem.*, 1952, **196,** 569.
23. Van Bruggen, J. T., Hutchens, T. T., Claycomb, C. K., Cathey, J. W., and West, E. S., *J. Biol. Chem.*, 1952, **196,** 389.
24. Pihl, A., Bloch, K., and Anker, H. S., *J. Biol. Chem.*, 1950, **183,** 441.
25. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, 1937, **121,** 235.