Effect of total exclusion of the exocrine pancreas in the rat upon in vitro esterification of C^{14}-labeled cholesterol by the intestine and upon lymphatic absorption of C^{14}-labeled cholesterol

W. J. LOSSOW, R. H. MIGLIORINI,* NATHAN BROT, and I. L. CHAIKOFF

Department of Physiology, University of California, Berkeley, California

SUMMARY The esterification of free cholesterol-4-C^{14} by extracts of acetone powders prepared from the small intestines was measured in normal rats and in rats in which all of the pancreas, except for a small portion along the splenic vessels, was removed. The acinar cells of the pancreatic remnant degenerated, thus providing a nondiabetic rat containing no functioning exocrine pancreatic tissue. Depriving the rat of its external secretions of the pancreas in this manner resulted in a reduction—but not in complete loss—of the intestine's capacity to esterify free cholesterol. The absorption of tube-fed cholesterol-4-C^{14} into thoracic duct lymph of the rats totally deprived of functioning exocrine pancreatic tissue was depressed, but the proportion of sterol-C^{14} recovered in the esterified form in the thoracic duct lymph of the operated rats was normal. It is concluded that an enzyme capable of esterifying free cholesterol, not derived from the pancreas, is present in the small intestines of the rat. The fact that the pancreatic juice enzyme that esterifies cholesterol was absent from the intestines of our operated rats could account for the reduction in esterification of the sterol and in its absorption. However, other mechanisms that could just as well explain these observations have also been considered.

MUELLER, IN 1916, observed that ligation of the pancreatic ducts in the dog greatly diminished the increase in cholesterol that appears in thoracic duct lymph after the animal is fed a meal containing cholesterol (1). He concluded, from other experiments, that a good part of the free cholesterol in the intestines was esterified just before its absorption into lymph, and, in addition, he found that ground pancreas esterified free cholesterol. On the basis of these findings, Mueller postulated that, for cholesterol absorption, a substance in pancreatic juice capable of accelerating cholesterol esterification was required. Two studies in the rat—in one 95% of the pancreas had been excised (2), in the other both pancreatic juice and bile were diverted from the intestines and only bile returned (3)—have shown that esterification of cholesterol is considerably reduced in the absence of a normal flow of pancreatic juice. These studies suggested the possibility that the pancreas is the exclusive source in the rat of the enzyme in the intestines that esterifies free cholesterol. Recently, however, Murthy and Ganguly (4) conducted studies in which the specificity of cholesterol esterases extracted from acetone powders of rat pancreas and small intestines to various inhibitors was determined, and concluded that the enzymes esterifying cholesterol in these two tissues are of independent origin. It seemed necessary, therefore, to determine whether or not the small intestine of the rat totally deprived of its external secretions of the pancreas for an extended period of time would lose completely its capacity to esterify free cholesterol and whether the absorption of cholesterol into the thoracic duct lymph by such rats could occur.

The anatomical peculiarities of the rat pancreas make total excision exceedingly difficult. In the 95%-pancreatectomized rat, which has been widely used for metabolic studies, all of the pancreas except the portion between the bile duct and the duodenum is removed. This preparation has its limitation for studying the

* This work was carried out during the tenure of a Fellowship from The Rockefeller Foundation. Permanent address: Departamento de Fisiologia, Faculdade de Medicina, Ribeirão Preto, Estado de São Paulo, Brazil.

effects of exclusion of the exocrine secretions of the pancreas from the intestines because pancreatic juice from the pancreatic lobules not removed can be delivered to the bile duct. Attempts to exclude pancreatic juice permanently from the intestines by implanting the upper segment of the common bile duct (above the point of entrance of the pancreatic ducts) into the intestines in order to maintain an uninterrupted flow of bile to the intestines, and ligating and cutting the remaining portion (that part into which the pancreatic secretions enter) has one serious drawback : rechanneling between the pancreas and intestines may occur. Clowes and Macpherson (5) observed acinar degeneration in only 58% of rats operated on in this manner; the rest appeared normal in all respects. A satisfactory procedure for total pancreatectomy in the rat has recently been developed by Scow (6). Since the ideal animal for studying the effects of excluding pancreatic juice from the intestines for an extended period of time would be one with no functional exocrine pancreatic tissue but with sufficient islet tissue to remain nondiabetic, we adapted Scow's procedure to prepare such an animal. All pancreatic tissue was excised, except for a portion (about 20% of the gland) alongside the splenic vessels, at a considerable distance from the common bile duct. After this operation the exocrine tissue in the pancreatic remnant degenerates. The esterification of free cholesterol-4-C^{14} by extracts of acetone powders prepared from the small intestines of normal rats and rats operated on in this manner was compared. The absorption of tube-fed cholesterol-4-C^{14} into the thoracic duct lymph was also studied.

EXPERIMENTAL METHODS

Labeled Cholesterol

Cholesterol-4-C^{14} with a specific activity of 16.83 mc/mmole was purchased from the Nuclear-Chicago Corporation and purified, before use, on silicic acid columns (7).

Treatment of Animals

Male, Long-Evans rats that had been maintained on an adequate stock diet (Diablo Labration), and weighing 136–162 g, were used. Pancreatectomy was performed as described by Scow (6) except that a small portion of the pancreas (about 20%) alongside the splenic vessels was not removed. After the operation a synthetic diet, supplemented with pancreatin, was substituted for the stock diet. The composition of the synthetic diet was the same as that used by Clowes and Macpherson (5) for their series IV rats, with the following exceptions: fat-soluble vitamins A, D, and E (15,000 U of A, 3000 U of D, and 50 mg of E per kg of diet) were added, dissolved in corn oil; the B vitamin mixture was that described in reference 8, and the salt mixture was U.S.P. XIV (Nutritional Biochemicals Corporation, Cleveland, Ohio). Pancreatin (Viokase powder, 4 × U.S.P. potency, VioBin Corporation, Monticello, N.Y.) was added (1.5 g/kg of diet). Serial sections of the remnant of pancreatic tissue excised 1 week after the operation showed complete degeneration of acinar cells, with ducts dominating the field. The islet tissue appeared normal. The experiments described here were performed 2–5 weeks after the operation. At that time, in a number of the rats, the blood glucose levels, as measured by the Mendel-Hoogland method (9), were found to be normal. Pancreatin was eliminated from the diet not less than 1 week before the enzyme or absorption studies were carried out. The control rats were not operated on, but were fed the same diet as that of the operated rats, for the same length of time.

Preparation of Acetone Powders of Small Intestines

Rats were exsanguinated by withdrawal of blood from the heart (usually 8–10 ml) under ether anesthesia. Each small intestine, from a point just below the entry of the bile duct to approximately 6 cm above the cecum, was removed and divided into two sections to facilitate washing. Each section was washed twice with 30 ml of 0.9% NaCl, and placed in ice-cold 0.9% NaCl. The procedure used to prepare the acetone powders is based on that described by Morton (10). In a 4° cold room, the intestines were minced with a razor blade and homogenized in a Potter-Elvehjem type of homogenizer provided with an electrically driven Teflon pestle, in 3 volumes of 0.005 M pyrophosphate buffer of pH 6.5. After 1 hr the homogenates were centrifuged for 30 min at 1450 × *g*. The supernatant fractions were added dropwise, with stirring, to 10 volumes of acetone that had been cooled to −15°. The mixtures were allowed to stand for at least 10 min and were then centrifuged at 1560 × *g*. The precipitates were washed with 15 volumes of acetone at −15°. The mixtures were again centrifuged, and the precipitates were collected on Buchner filter funnels and washed with small amounts of acetone. The powders were dried overnight in vacuo at 4° in a desiccator containing $CaCl_2$. They were stored at −15° until used.

Collection of Pancreatic Juice

A polyethylene cannula was inserted into the bile duct as close as possible to the liver so as to drain off the bile secretions. Pancreatic juice was then collected from a second cannula inserted into the bile duct as close as possible to the duodenum. The juice was collected in ice-cold tubes and stored up to 3 days at −15°. It was determined beforehand that the esterification of free cholesterol by pancreatic juice that had been frozen for a

TABLE 1 ESTERIFICATION OF FREE CHOLESTEROL-4-C^{14} BY ACETONE POWDER EXTRACTS PREPARED FROM SMALL INTESTINES OF NORMAL RATS AND RATS DEPRIVED OF EXOCRINE PANCREATIC TISSUE

Incubation Mixture*		% Sterol-C^{14} in Esterified Form					
		Intestinal Powder Extracts of:				No Intestinal Powder Extracts (Control Flasks)	
		Pancreatectomized Rats		Normal Rats			
Cholesterol	Pancreatic Juice	Range†	Average	Range†	Average	Range†	Average
mg	*ml*						
1.0	None	0.7–5.4 (7)	3.6	18.5–21.1 (6)	20.0	0.1–0.4 (7)	0.2
0.1	None	1.6–10.5 (4)	6.0	21.2–22.0 (4)	21.6	0.1–0.2 (3)	0.1
0.01	None	1.6–10.2 (5)	5.3	19.1–25.9 (5)	22.7	0.1–0.4 (5)	0.2
1.0	0.2 heated	1.0–13.9 (7)	6.3	61.1–71.4 (5)	60.7	0.1–0.2 (4)	0.2
1.0	0.2 unheated	48.9–81.2 (6)	69.3	48.9–87.5 (5)	72.5	69.7–81.1 (4)	75.4

* See text for other constituents in the incubation mixture.
† The figures in parentheses give the number of samples analyzed.

few days and then thawed was the same as that by freshly collected juice.

Measurement of Esterification of Free Cholesterol-4-C^{14} by Extracts of Acetone Powders of Small Intestine

The acetone powders were used 1–3 days after their preparation. The powders were extracted in a cold room (4°) with 15 volumes of 0.05 M phosphate buffer of pH 6.5, with stirring, for 1 hr. The extract was centrifuged for 1 hr at 4° at 19,850 × *g* in a Spinco ultracentrifuge, and the clear supernatant solution thus obtained was used as the enzyme preparation. In preliminary experiments it was determined that the optimum pH for cholesterol esterification in our extracts was between 6.0 and 6.5. This agrees well with pH optimum curves reported by Murthy and Ganguly (4) for esterification of cholesterol by extracts of rat intestinal acetone powder. Murthy and Ganguly (4) and Swell and co-workers (2) added sodium oleate to their systems. The former investigators observed a stimulating effect with oleic acid. Swell et al. (2) have reported that sodium taurocholate is required for cholesterol esterification in extracts of rat intestines. In preliminary experiments we found that the addition of ATP, CoA, and $MgCl_2$ did not affect the esterification. Neither Swell and co-workers nor Murthy and Ganguly supplemented their systems with ATP and CoA. In the present experiments the incubations were carried out as follows: 1 ml of the acetone powder extract was incubated at 37° for 3 hr, with shaking, in 0.1 ml of a 0.1 M, phosphate buffer of pH 6.4 to which had been added 0.5 ml of a suspension of cholesterol-4-C^{14} (11) containing either 1.0, 0.1, or 0.01 mg of cholesterol, 10 mg of sodium taurocholate and 1.5 mg of sodium oleate. Mixtures containing 0.2 ml of pancreatic juice, either unheated or heated for 1 hr at 60°, were also incubated. (Heating pancreatic juice for 1 hr at 60° inactivates it.) Control mixtures containing no intestinal powder extract were also incubated. The total volume of the incubation mixture was 5 ml in all cases. At the end of the incubation period ethanol–ethyl ether 3 : 1 (v/v) was added to each flask.

Collection of Thoracic Duct Lymph after Feeding Labeled Cholesterol

Thoracic ducts of rats were cannulated as described in reference 12, and on the following day 1.5 μc cholesterol-4-C^{14} dissolved in 0.4 ml of olive oil was administered by stomach tube. Lymph was collected in ice-cold centrifuge tubes at various intervals up to 24 hr and, after removal of the fibrin clot, each lymph sample was transferred to ethanol–ethyl ether 3 : 1.

Analytical Procedures

Lipids were extracted as described in part C of Methods in reference 13, and the esterified and free sterol fractions were separated on silicic acid columns (7). Dried lipid samples were dissolved in 15 ml of toluene containing 45 mg of 2,5-diphenyloxazole and 1.5 mg 1,4-*bis*-2-(5-phenyloxazolyl)benzene, and assayed for C^{14} in a Tri-Carb liquid scintillation counter (Packard Instrument Co., La Grange, Ill.). The protein content of the acetone powder extracts was determined by the Folin-Ciocalteu method as modified by Lowry et al. (14).

RESULTS

Acetone Powder Experiments

Average percentages of cholesterol esterified in four separate experiments are presented in Table 1. In each experiment acetone powders were prepared from the pooled intestines of pancreatectomized rats, killed 3–5 weeks after surgery, that had been deprived of pancreatin supplements for 9–14 days. The rats ranged in weight from 184 to 305 g, and in each experiment powders were also prepared from the intestines of control rats of comparable weight. In the first three experiments the intes-

tines from 3 pancreatectomized and 3 control rats were used. In the fourth the powders were prepared from one pancreatectomized and one control rat. In the first experiment 1.0 mg of cholesterol was added. In the second and third, three different concentrations (Table 1) were used. In the fourth, the highest and lowest concentrations were used. The protein contents of the extracts prepared from the intestines of pancreatectomized and control rats were almost identical, ranging from 19.1 to 34.0 mg/ml for the pancreatectomized rats and from 24.3 to 30.0 mg/ml for the controls.

The esterification of cholesterol by extracts prepared from intestines of rats deprived of their exocrine pancreatic secretions was lower than that by extracts prepared from the control rats at all three cholesterol concentrations. At the two lower concentrations (0.01 and 0.1 mg per flask) the difference between the two groups was 3.5-4 fold. At the highest concentration (1.0 mg per flask) the difference was about 5.5-fold. The esterification was proportional to the concentration in the extracts of the control rats at all three concentrations, but was no longer proportional at the highest concentration in the extracts of the pancreatectomized rats. Further increases in cholesterol concentration, therefore, would be expected to increase the difference between the two groups until enzyme saturation was reached with the controls. Addition of enzymatically inactive (heated at 60° for 1 hr) pancreatic juice did not restore to normal the esterification of cholesterol by the intestinal preparation of pancreatectomized rats. It did, however, bring about a 3-fold increase in esterification of cholesterol by the intestinal powder extracts of the control rats and a little less than a 2-fold increase in the experiments with extracts of the pancreatectomized rats.

Absorption Experiments

The cumulative percentages of tube-fed cholesterol-C^{14} recovered in thoracic duct lymph at various intervals up to 24 hr and the proportion of the lymph sterol-C^{14} esterified at each time interval are recorded for three pancreatectomized and three control rats in Table 2. The labeled cholesterol was fed to the pancreatectomized rats from 19 to 25 days after surgery, and these rats had been deprived of pancreatin supplements from 1 to 3 weeks. Elimination of all exocrine pancreatic tissue decreased the absorption of the administered labeled cholesterol, but did not affect the proportion of labeled sterol in the esterified form in the thoracic duct lymph.

TABLE 2 Lymphatic Absorption and Esterification of Cholesterol-4-C^{14} by Normal Rats and Rats Deprived of Exocrine Pancreatic Tissue

Condition of Rat	% of Fed Cholesterol-4-C^{14} Recovered in Thoracic Duct Lymph		% of Lymph Sterol-C^{14} in Esterified Form	
	Interval	Recovered	Interval	Esterified
	hr		*hr*	
Pancreatectomized	2	0.06	0–2	51.5
	6	0.31	2–6	70.7
	12	0.50	6–12	71.9
	24	0.81	12–24	68.8
Pancreatectomized	2	0.04	0–2	48.1
	6	0.14	2–6	71.6
	12	0.18	6–12	70.1
Pancreatectomized	24	0.51	0–24	65.4
Normal	2	0.38	0–2	38.6
	6	1.74	2–6	62.1
	12	5.91	6–12	69.1
	24	9.92	12–24	74.8
Normal	2	0.16	0–2	55.1
	6	1.42	2–6	68.1
	12	3.17	6–12	70.8
Normal	24	10.21	0–24	69.8

DISCUSSION

Our finding that extracts prepared from the small intestines of rats that had been totally deprived of external secretions of the pancreas for weeks retained some capacity for esterifying free cholesterol is compatible with the view of Murthy and Ganguly (4) that the enzymes esterifying cholesterol in the small intestine and pancreas of the rat have different origins. Murthy et al. (15) raised the question of the relative importance of the two enzymes for cholesterol absorption, and concluded from their studies that the intestinal enzyme was the more important. This conclusion was based on the observation that the specificity of fatty acids for the esterification of cholesterol increased progressively with the unsaturation of the fatty acid in enzyme preparations of rat intestines (but not in those of the pancreas), and that this corresponded to the preponderance of polyunsaturated fatty acids in cholesterol esters in blood. It should be noted, however, that the fatty acid composition of the cholesterol esters of thoracic duct lymph is not identical with that of the esters of plasma. The esters of plasma (16–22) are composed principally of polyunsaturated fatty acids, but those of lymph (16) are not. No conclusion concerning the relative importance of the pancreatic and intestinal cholesterol esterifying enzymes can be drawn, therefore, from the fatty acid specificity data. Our own studies show that the capacity of intestinal extracts of rats deprived of functional exocrine pancreatic tissue to esterify cholesterol was severely reduced. This could have been a consequence of depriving the intestines of the pancreatic cholesterol-esterifying enzyme. If this is the case, our findings suggest that the pancreatic enzyme is the more important one. But other mechanisms can just as well account for the reduction in cholesterol esterification. The absence of pancreatic juice may

have limited the supply of cofactors or other substances required for the esterification in the intestines. Our results indicate that pancreatic juice contains a heat-stable substance(s) (identity not known) that enhances cholesterol esterification. The heated juice did not restore the esterification to normal, but pancreatic juice might also contain nonenzyme, heat-labile substances capable of enhancing cholesterol esterification. It is conceivable that a normal flow of pancreatic juice in some manner creates conditions, in vivo, essential for maximum esterification of cholesterol in the intestines in vitro.

The observations that cholesterol absorption was not completely eliminated in our pancreatectomized rats and that the proportion of sterol-C^{14} recovered in the esterified form in the thoracic duct lymph was similar in the pancreatectomized and control rats again show that the small intestine of the rat contains an enzyme, not derived from the pancreas, capable of esterifying free cholesterol. The reduction in the absorption of the cholesterol into the thoracic duct lymph in our rats as well as in Mueller's duct-ligated dogs (1) might, in accordance with Mueller's original postulation, be explained by the reduction in the esterification of cholesterol by the intestines of these animals. The effect of depriving an animal of its external pancreatic secretions upon absorption of cholesterol may, on the other hand, be independent of the effect on the esterification of cholesterol in the intestines. The absence of pancreatic lipase and other digestive enzymes in the juice might create unfavorable conditions for cholesterol absorption. The action of pancreatic juice might be important for the formation of complex micellar structures in the intestines. Any interference with the formation of these structures, which are probably involved in fat absorption, would be very likely to restrict the absorption of cholesterol.

This work was aided by grants from the U.S. Public Health Service and the Life Insurance Medical Research Fund.

Manuscript received June 24, 1963; accepted November 18, 1963.

References

1. Mueller, J. H. *J. Biol. Chem.* **27:** 463, 1916.
2. Swell, L., J. E. Byron, and C. R. Treadwell. *J. Biol. Chem.* **186:** 543, 1950.
3. Hernandez, H. H., I. L. Chaikoff, and J. Y. Kiyasu. *Am. J. Physiol.* **181:** 523, 1955.
4. Murthy, S. K., and J. Ganguly. *Biochem. J.* **83:** 460, 1962.
5. Clowes, G. H. A., Jr., and L. B. Macpherson. *Am. J. Physiol.* **165:** 628, 1951.
6. Scow, R. O. *Endocrinol.* **60:** 359, 1957.
7. Creech, B. G., and B. W. Sewell. *Anal. Biochem.* **3:** 119, 1962.
8. Hill, R., N. Baker, and I. L. Chaikoff. *J. Biol. Chem.* **209:** 705, 1954.
9. Mendel, B., and P. L. Hoogland. *Lancet* **259:** 16, 1950.
10. Morton, R. K. *Methods Enzymol.* **1:** 34, 1955.
11. Nieft, M. L., and H. J. Deuel, Jr. *J. Biol. Chem.* **177:** 143, 1949.
12. Bloom, B., I. L. Chaikoff, W. O. Reinhardt, C. Entenman, and W. G. Dauben. *J. Biol. Chem.* **184:** 1, 1950.
13. Lossow, W. J., N. Brot, and I. L. Chaikoff. *J. Lipid Res.* **3:** 207, 1962.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. *J. Biol. Chem.* **193:** 265, 1951.
15. Murthy, S. K., S. Mahadevan, P. S. Sastry, and J. Ganguly. *Nature* **189:** 482, 1961.
16. Swell, L., M. D. Law, H. Field, Jr., and C. R. Treadwell. *J. Biol. Chem.* **235:** 1960, 1960.
17. Lis, E. W., and R. Okey. *J. Nutr.* **73:** 117, 1961.
18. Okey, R., R. Ostwald, A. Shannon, and J. Tinoco. *J. Nutr.* **76:** 353, 1962.
19. Arvidson, G., and T. Olivecrona. *Acta Physiol. Scand.* **55:** 303, 1962.
20. Monsen, F. R., R. Okey, and R. L. Lyman. *Metab. Clin. Exp.* **11:** 1113, 1962.
21. Albers, H. J., and S. Gordon. *Proc. Soc. Exptl. Biol. Med.* **109:** 860, 1962.
22. Glomset, J. A. *Biochim. Biophys. Acta*, **65:** 128, 1962.