Fat transport and lymph and plasma lipoprotein biosynthesis by isolated intestine

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Abstract An apparatus and procedure are described for investigating fat transport and lipoprotein biosynthesis in isolated, lymph-cannulated rat intestine perfused with blood under physiological conditions. The small bowel, cecum, proximal half of the colon, and attached mesentery were removed into a tissue bath and perfused vascularly in a recycling system free of blood–air interfaces. Perfusion was continued for 5 hr. Lymph flow, glucose utilization, and oxygen consumption continued unchanged, as did intestinal motility and glucose and water transport from the lumen. No measurable lactate was produced. When 70 μmoles of soybean oil and 9 μmoles of lecithin were infused luminally, more than 50% of the fatty acids were recovered in the lymph, 90% as triglycerides of which 75% appeared in chylomicrons with average diameters estimated to be 100–200 nm, based on their phospholipid content. The preparation incorporated [^{3}H]-lysine into the protein moieties of lipoproteins of d $<$ 1.006 g/ml (chylomicrons plus very low density) which appeared in lymph and accounted for more than 30% of all labeled lymph protein. No labeled d $<$ 1.006 lipoproteins appeared in the perfusate. [^{3}H]Lysine was also incorporated into the d 1.006–1.21 lipoproteins of both lymph and perfusate, but the specific activity of the former was 500 times as high as the latter, indicating that d 1.006–1.21 as well as d $<$ 1.006 lipoproteins are produced by gut and reach the blood via mesenteric lymph. Most of the labeled d 1.006–1.21 protein appeared to be high density lipoprotein (d 1.063–1.21).

Supplementary key words vascularly perfused intestine · perfusion apparatus · intestinal metabolism

Although evidence from various sources suggests that intestine, as well as liver, may be a source of the protein moieties of some circulating lipoproteins, definitive identification has not been achieved, due largely to the lack of a suitable experimental preparation. Studies involving incorporation in vivo of labeled amino acids into mesenteric lymph or thoracic duct lymph lipoproteins (1–5) have implied synthesis by the gut. However, mesenteric lymph contains proteins synthesized by extraintestinal tissues and filtered from plasma, in addition to proteins derived from intestine. The whole spectrum of plasma lipoproteins is found in mesenteric lymph (5–8), but of the various apoproteins associated therewith, an intestinal origin has been determined with some certainty for only β-lipoprotein apoprotein (5). Evidence for some intestinal lipoprotein synthesis is also provided by studies in vitro with intestinal slices (9), mucosal cells (2, 4), and cell-free intestinal preparations (10). Vascular and lymphatic channels were disrupted in these preparations, so the lipoproteins synthesized and released into the incubation medium may not correspond to those released in vivo into the circulation. Furthermore, the quantities of protein synthesized were small. A preparation might be expected to yield more precise information if it provided for complete isolation of the intestine from the animal while keeping intact the vascular and lymphatic channels.

Techniques for extracorporeal vascular perfusion have been developed for studying successfully a variety of isolated organs, including liver, kidney, brain, heart, lungs, adipose tissue, spleen, pancreas, and thyroid (11, 12). Compared with other organs, however, vascular perfusion of intestine has proved difficult, and during early attempts it is unlikely that the morphology or function of the tissue were adequately preserved (13). Abnormal vascular resistance, intense spasmodic hypermotility, hypersecretion of fluids into the lumen, and sloughing of the mucosal epithelium limited the success of the procedure. Recent work from this labora-

Abbreviations: VLDL, very low density lipoproteins, d $<$ 1.006 g/ml; (LDL + HDL), low density lipoproteins plus high density lipoproteins, d = 1.006–1.21 g/ml.

tory suggested that these difficulties are related to the loss of central sympathetic innervation, and demonstrated that this loss can be adequately compensated by the continuous infusion of small amounts of norepinephrine into the recycling perfusate, provided it contained sufficient levels of a glucocorticoid (14). Thus, a technique was developed for extracorporeal vascular perfusion *in situ* of the small bowel, cecum, and part of the large bowel of the rat (14). During 5 hr of perfusion, this preparation produced lymph and transported glucose and water. Electron microscopy revealed that the mucosal epithelium remained essentially normal with brush borders intact.

The perfusion technique for intestine has undergone continuing development. This report describes the procedural refinements and the applicability of the preparation to investigations of intestinal fat transport. Modifications described provide for complete removal, without circulatory interruption, of the intact intestine from the animal into a constant-temperature, fluid-filled bath; for optional use of pulsatile blood flow; and for blood oxygenation and perfusion circuitry that avoid any direct blood–gas interface. The last may be particularly important during metabolic studies involving circulating lipoproteins because they may undergo irreversible structural alterations at liquid–air interfaces (15).

Quantitative data have been obtained regarding glucose and oxygen consumption, lactate and lymph production, and the production of lymph chylomicrons and other lipoproteins from lipid infused into the intestinal lumen. In addition, evidence is presented for intestinal biosynthesis of protein moieties of both lymph and perfusate lipoproteins.

EXPERIMENTAL

Rats and diets

Rats used as blood donors were mature males of the Osborne-Mendel or Sprague-Dawley strains. They had free access to Purina Chow and water until blood was drawn by aortic puncture under ether anesthesia. Rats used as intestine donors were 260–280-g males from the National Institutes of Health pathogen-free Osborne-Mendel colony. For 7–20 days before perfusion, these rats were fed ad lib. one of two semisynthetic diets. The basal diet (W-8) contained 68% corn starch, 20% casein, 5% corn oil, 0.3% DL-methionine, and 2% choline chloride, plus adequate amounts of vitamins and minerals (16). A fat-free diet (W-8FF) had the same composition as diet W-8, except the 5% corn oil was replaced with an equal weight of corn starch. Unless otherwise indicated, the diets, but not water, were withheld for 12 hr preceding perfusion.

Perfusion apparatus

The perfusion apparatus used previously (14) was extensively modified and is shown schematically in Fig. 1. The perfusate reservoir was a 14 × 16 cm flat silicone rubber envelope, which provided an expandable air-free compartment for the bulk of the perfusate. The reservoir was mounted on a metal platform and rocked mechanically at approximately 25 cycles/min to provide gentle mixing and to prevent settling of the blood cells. Pumps A and B (Holter Co., model RL-175) have dual silicone rubber pumping chambers. The arterial circuit was fed by one channel of pump A. In some experiments, the nearly nonpulsatile flow delivered by the pump was made pulsatile as shown in Fig. 1. The pulser was a modified rodent respirator (Harvard Apparatus Co., model 680) operated at 300 strokes/min and adjusted to provide a pulse height in the arterial cannula of approximately 40 mm Hg. The blood filter contained glass wool. The intestine was supported in a jacketed silicone rubber bath (gut bath) with a transparent plastic lid and a molded spout which fitted into the abdominal cavity of the rat during the surgical preparation. The gut bath temperature was maintained at 37°C by circulating water through the jack from a constant-temperature water bath.

The venous circuit was operated by the second channel of pump A and one channel of pump B, which was set to pump continuously at approximately 3 ml per min. The valve (Fig. 1) in the venous circuit, fabricated, like the reservoir, by cementing two opposing sheets of silicone rubber together at their edges, prevented the siphoning of venous blood from the intestine to the pumps. Venous pressure could be precisely and easily regulated by adjusting the height of the valve relative to the level of the intestine. Furthermore, by use of this valve, venous pressure, measured with a water manometer, was stable and independent of the rate of blood flow.

Venous blood from the intestine en route back to the reservoir was pumped through a membrane lung (17) (Mini-lung membrane oxygenator, Dow Corning Corp., Midland, Mich.) where it was equilibrated with a mixture of O_2 and CO_2. The gasses diffused across a 5 mil thick, nylon reinforced silicone rubber membrane with a surface area of 250 cm^2. At the start of perfusion, a gas mixture of 95% O_2 and 5% CO_2 was used. The CO_2 content of the mixture was gradually reduced to zero during the course of a 5-hr perfusion in order to counteract the pH-lowering effect of accumulating lactate. In this way the perfusate pH was maintained between 7.36 and 7.40 throughout an experiment.

A specially fabricated flow cell was interposed in the venous circuit (Fig. 1) to permit determination of the oxygen saturation of the venous blood. The flow cell, made of stainless steel and lined with silicone rubber,

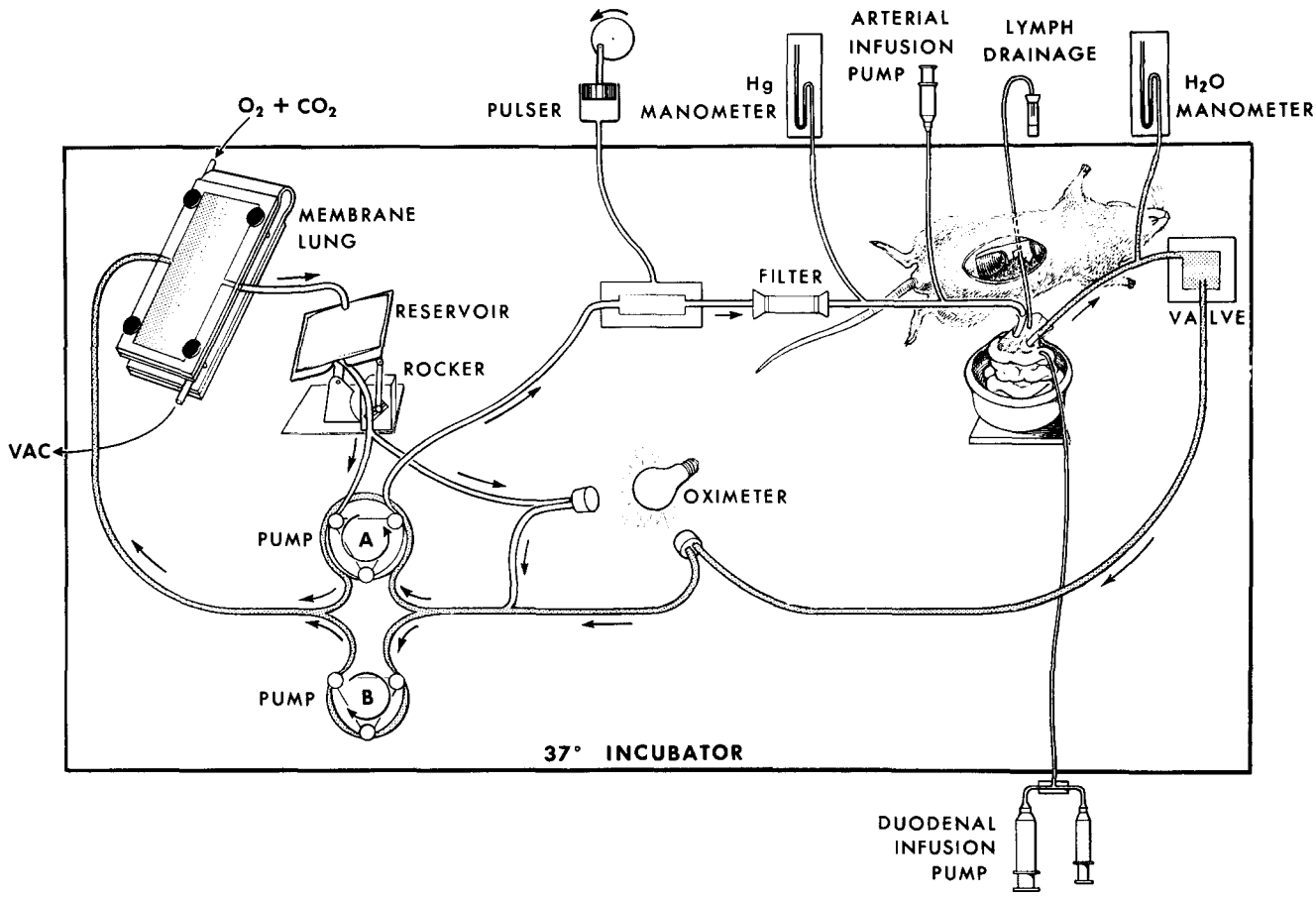

FIG. 1. Schematic diagram of apparatus for perfusing isolated rat intestine. In the perfusion circuit, indicated by the arrows, stippling indicates venous blood from the intestine and no stippling indicates fully oxygenated blood. *Vac*, vacuum.

had a round glass window (1.5-cm diameter) and was mounted on a modified reflection oximeter (American Optical Co., cat. no. 10841). A similar cell was interposed in the shunt between the reservoir and the venous circuit (Fig. 1). This cell was also mounted on the oximeter and determined the oxygen saturation of the arterial blood.

Silicone rubber tubing was used throughout the perfusion circuit. Between experiments it was rinsed with water, soaked in a solution of trypsin for several hours, rinsed thoroughly with water, and autoclaved. The volume of blood required to prime the arterial and venous circuits was 57 ml. All circuit components were contained within a portable enclosure maintained at 37°C.

Perfusion procedure

The gut-donor rat, anesthetized with a regulated mixture of ether and O_2, was supported on a sloping platform designed to pivot around one of its four corners. Through a midline incision, the small bowel, cecum, and proximal half of the large bowel were exteriorized and draped into the gut bath, the spout of which fitted into the abdominal cavity with its leading edge adjacent to the vena cava. The intestine was immediately immersed in the gut bath by filling it with fluid (37°C) (see below). Isolation of the intestine with ligatures, and cannulation of the duodenal lumen, mesenteric lymph channel, superior mesenteric artery, and superior mesenteric vein were then performed as previously described (14), with the exception that one additional ligature was placed across the mesentery between the lymph channel and the superior mesenteric vein (Fig. 2). Blood flow through the tissue was uninterrupted during the isolation procedure.

Once the extracorporeal circulation through the intestine was established and the rat killed, the perfused segment of intestine was completely excised and the body of the rat was pivoted away from the gut bath, as indicated in Fig. 1. At the same time, venous pressure was adjusted to 150 mm H_2O and average arterial pressure to 105 mm Hg. These values were maintained throughout the experiment. Venous pressure was set by positioning the height of the valve in the venous circuit. Arterial pressure was adjusted by controlling the rate of blood flow with pump A (Fig. 1) and the rate of norepi-

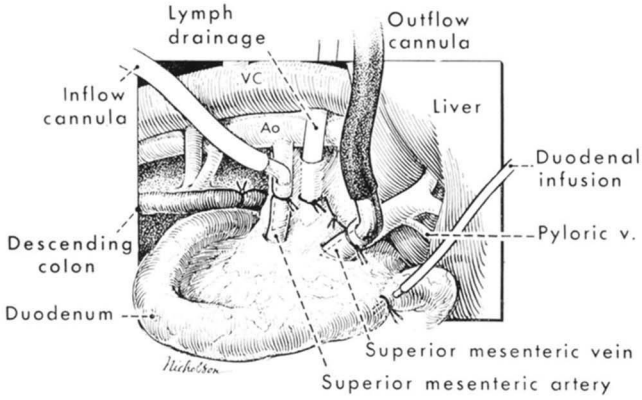

FIG. 2. Schematic diagram showing placement of cannulas and ligatures.

nephrine infusion from a syringe driven by a (continuously) variable-speed motor (arterial infusion pump, Fig. 1). Blood-flow rate varied from 9.8 to 13.1 ml/min and the rate of norepinephrine infusion varied from 0.05 to 0.5 μg/min (14). Typically, the blood-flow rate was adjusted to obtain 75–80% O_2 saturation of the hemoglobin in the venous circuit, since similar values were observed in portal vein blood of rats in vivo. In the arterial circuit, hemoglobin O_2 saturation was always 96–97%. In most experiments the rate of norepinephrine delivery was gradually reduced from an initial high value of 0.3–0.5 μg/min to a low plateau of 0.05 μg/min, reached after about 1 hr of perfusion, when the vascular resistance of the preparations typically stabilized. Total surgical time was about 45 min.

Solutions were delivered continuously into the intestinal lumen by a motor-driven duodenal infusion pump (Fig. 1) (Harvard Apparatus Co., model 975). The basic luminal infusate and the fat emulsion (see below) were delivered from separate syringes, as shown in Fig. 1. During the surgical preparations and until luminal infusion was started, a 5% glucose solution was infused continuously into the perfusate at a rate sufficient to maintain the plasma glucose concentration between 1 and 2 mg/ml. Lymph was collected in ice.

Solutions

The perfusate was 120–150 ml of freshly drawn rat blood plus 25 mg of sodium heparin, 100,000 units of penicillin G, 5 mg of streptomycin sulfate, 25 μg of dexamethasone (9α-fluoro-16α-methylprednisolone; Decadron; Merck, Sharp & Dohme), and 50 mg of glucose per 100 ml of blood. The hematocrit was 39–42%. Blood temperature was not allowed to fall below 35°C during preparation of the perfusate.

The solution bathing the intestine in the gut bath was Earle's balanced salt solution (18) with addition of 200 mg of glucose, 100,000 units of penicillin G, and 5 mg of streptomycin sulfate per 100 ml. Approximately 20 ml of solution was required to fill the bath and cover the intestine.

Norepinephrine was freshly prepared as a 0.2 mg/ml solution of L-arterenol-D-bitartrate (Mann) in 0.9% NaCl, and was delivered from a gas-tight syringe (Hamilton).

The basic luminal infusate was Earle's balanced salt solution with addition of 40 mg of glucose, 6 mg of sodium taurocholate, and 2.5 mg of fatty acid-free bovine serum albumin (19) per ml. Albumin was included to provide a continuing source of amino acids for the preparation. The sodium taurocholate (Pierce Chemical Co.) was free of deoxycholate (20). Unless otherwise indicated, beginning 15 min after the start of perfusion this solution was infused at 0.15 ml/min for 10 min (priming dose) and then at a rate of 2.4 ml/hr for the remainder of the experiment.

Fat was infused luminally as an aqueous triglyceride–phospholipid emulsion (Intralipid; Vitrum AB, Stockholm, Sweden) containing 243 μmoles of fatty acid and 17 mg of glycerol per ml. Approximately 92% of the fatty acid in the emulsion was supplied by fractionated soybean oil and 8% by fractionated egg yolk lecithin.

Isolation and counting of proteins

Aliquots of lymph and perfusate, sampled during experiments in which [^{3}H]lysine was added to the perfusate, were layered under 0.15 M NaCl and centrifuged for 16 hr at 40,000 rpm in a 40.3 Spinco rotor at 5°C. The top 2 ml (chylomicrons + VLDL) were recovered after slicing the tube and were washed twice by layering under 0.15 M NaCl and recentrifuging as before. The infranatant fractions after the initial centrifugation were adjusted to a density of 1.21 g/ml by adding a solution of NaCl and KBr and were centrifuged for 48 hr at 40,000 rpm in the same rotor. The top 2 ml (LDL + HDL) were recovered and washed once by layering under a solution of NaCl and KBr, d = 1.21 g/ml, and recentrifuging for 48 hr as before. All salt solutions used during isolation and washing of lipoproteins contained 3 mM EDTA, pH 7.4.

Proteins in portions of whole lymph, in perfusate (plasma), and in lipoprotein fractions were precipitated with 5% trichloroacetic acid after the addition of unlabeled L-lysine and, with all but the plasma samples, 1.3 mg of carrier bovine serum albumin. The precipitates were washed four times with 10 vol of 5% trichloroacetic acid and 0.1% L-lysine–HCl, then dissolved in 0.5 ml of NCS solubilizer (Amersham/Searle Corp.), and the radioactivity was determined in a liquid scintillation counter after the addition of 8.5 ml of Liquifluor (New England Nuclear Corp.). Specific radioactivity was calculated by relating the radioactivity, determined as

described above, to the amount of protein, determined directly on a separate aliquot of the sample. To determine the fraction of lipoprotein counts present in the lipid portion of the complex, trichloroacetic acid precipitates, prepared and washed as described above, were dissolved in 0.2 ml of 0.4 M potassium phosphate, pH 7.5. These solutions were treated with 6 ml of cold chloroform–methanol 2:1 (v/v), as described previously for albumin-containing solutions (21). The chloroform phase was recovered after the addition of water, and an aliquot was evaporated in a counting vial and dissolved in Liquifluor for counting.

Analytical procedures

The total hemoglobin and plasma hemoglobin concentrations of the perfusate were determined spectrophotometrically as cyanomethemoglobin (22). Microhematocrit determinations were also done routinely. The volume of fluid absorbed from the intestine into the perfusate was calculated from the reduction in hematocrit and total hemoglobin concentration.

Oxygen consumption by the perfused intestine was routinely calculated from the arterio-venous difference in hemoglobin O_2 saturation, as determined with the oximeter. O_2 consumption (ml/min) = blood flow rate through the intestine (ml/min) × hemoglobin concentration (g/ml blood) × 1.34 ml O_2 per g hemoglobin × (% O_2 saturation of arterial blood − % O_2 saturation of venous blood)/100. On two occasions, O_2 uptake was also measured by Van Slyke gas analysis of arterial and venous blood. Van Slyke analysis gave values for O_2 uptake which averaged 10% higher than oximetry.

Total lipid extracts of lymph and plasma were prepared by the method of Albrink (23). After removal of phospholipids with silicic acid, triglycerides were determined as described by Moore (24), except that excess periodate was reduced with sodium metabisulfite rather than sodium arsenite. Cholesterol in the lipid extracts was determined as described by Pearson, Stern, and McGavack (25), and phospholipid by the method of Bartlett (26). 1 mole of lipid phosphorus was assumed to be equivalent to 1 mole of phospholipid. Total fatty acids in the lipid extracts were assayed by titration after saponification, acidification, and extraction into *n*-hexane. Plasma unesterified fatty acids were determined by the double extraction method of Dole and Meinertz (27).

Glucose was determined in protein-free plasma filtrates (28) with glucose oxidase (29), and lactate in perchloric acid extracts of whole blood with lactic dehydrogenase (Lactate Stat-Pack, Calbiochem). Protein was measured according to Lowry et al. (30), with crystalline bovine serum albumin as standard. Plasma Na^+ and K^+ were measured by flame photometry, and the insulin content was determined by radioimmunoassay (31).

RESULTS

Gross features of intestine

The perfused tissue included almost the entire small bowel, the cecum, the proximal half of the large bowel, and the attached mesentery (14). The portion of pancreatic tissue, approximately 20% of the total, located in the duodenal mesentery was also included. Average wet weight of perfused tissue minus intestinal contents was 11.5 g, and dry weight was 2.8 g.

The color and gross appearance of the intestine remained normal throughout the 5-hr experiments. Intestinal tissue and mesentery remained free of hemorrhages, although in some experiments there was a small blood loss into the intestinal lumen. There was no blood loss into the gut bath. Peristaltic activity, both mixing and propulsive in character, was continuous and increased in vigor when solutions were infused into the lumen, particularly during the initial priming dose. There was no accumulation of fluid in the lumen despite infusion of more than 13 ml of material in a typical 5-hr experiment, demonstrating net transport of fluid. The cecum, in fact, appeared to shrink slightly and the contents became less fluid during perfusion. Net fluid absorption from the lumen was confirmed by the observed decrease in hematocrit and perfusate total hemoglobin concentration. Of the 13 ml of fluid infused into the lumen, one-third, on the average, was accounted for as lymph, and two-thirds appeared in the perfusate.

Lymph production

The rate of lymph production and lymph protein content remained fairly constant (Table 1). The volume collected was about 80% of the rate of mesenteric lymph flow in vivo observed under similar conditions of fluid input, and the protein concentration was also similar (5). In several early perfusion experiments conducted under conditions different from those in Table 1, lower rates of lymph production were observed. In each instance, the experimental conditions employed resulted in a net accumulation of fluid in the lumen, e.g., when (*a*) insufficient norepinephrine was administered, less than about 0.05 μg/min; (*b*) the lipid emulsion (Intralipid) was infused duodenally at an excessive rate, supplying more than 100 μmoles of fatty acid per hour; (c) an emulsion of oleic acid and monoolein was infused luminally. This last lipid mixture seemed to irritate the bowel, increasing motility and producing some hyperemia.

TABLE 1. Lymph production, glucose and oxygen consumption, and lactate accumulation during perfusion of isolated intestine

	Lymph Production		Glucose Utilization		Oxygen Consumption	Increase in Perfusate Lactate	
			By Perfusate[a]	By Intestine[b]		During Perfusion of Intestine[c]	During Recycling without Intestine
	μl/hr	*mg lymph protein/hr*	*μmoles/100 ml cells*	*μmoles*	*μl/min*	*μmoles/100 ml perfusate*	
No. of experiments	10	3	1	2	13[d]	5	1
Perfusion interval							
1st hr	870 ± 150	8.6	609	102	323 ± 15	265 ± 15	247
2nd hr	750 ± 100	5.1	667	110	334 ± 9	254 ± 69	254
3rd hr	640 ± 110	4.5	706	102	333 ± 9	196 ± 10	233
4th hr	840 ± 140	5.0	674	133	315 ± 11	208 ± 10	214
5th hr	1170 ± 180	7.1	769	125	314 ± 8	254 ± 14	200

For perfusion conditions see Experimental section. Unless otherwise indicated, the intestinal lumen was infused with the basic luminal infusate, and in some experiments with the lipid emulsion. Values are means of the indicated number of experiments ± SE.

[a] Determined by recycling perfusate through the apparatus in the absence of an intestine.

[b] Calculated as follows: glucose used by intestine per hr = glucose infused per hr minus the increase in glucose content of perfusate and gut bath during the hr minus the glucose used by cells in the perfusate per hr (see footnote *a*; an average value of 685 μmoles of glucose/100 ml of cells was used). In one experiment the glucose was infused continuously into the perfusate, and in the other experiment it was infused continuously into the duodenum of the perfused intestine.

[c] Values include lactate which diffused from the perfusate into the gut bath.

[d] Each value is the mean of 20–27 determinations made in 13 perfusion experiments.

Lymph collected from perfused intestines of rats fed the fat-free diet was colorless and only slightly lactescent. There was a noticeable increase in lactescence within 45 min after the start of the luminal fat infusion, and within 1.5 hr the lymph was milky white. All lymph samples contained some white cells, but only occasionally were a very few erythrocytes observed.

Metabolic activity

There was little change in O_2 uptake throughout a 5-hr perfusion (Table 1). The average rate observed (Q_{O_2}) was 7.0 ml of O_2/g of dry tissue/hr. No consistent difference in O_2 uptake was observed between preparations in which the lumen was infused with the basic infusate mixture plus lipids and preparations involving no luminal infusion. Therefore, all the data were pooled. The average respiratory quotient, determined by Van Slyke gas analysis of perfusate in two experiments, was 1.07.

The intestine continuously removed glucose from the perfusate at an average rate of 114 μmoles/hr (Table 1). Complete aerobic oxidation of this quantity of glucose theoretically would require 15.3 ml of O_2/hr. The average rate observed was 19.4 ml/hr. Throughout these experiments, perfusate glucose concentration was maintained between 1 and 2 mg/ml of plasma by glucose infusion either directly into the perfusate or into the duodenum. The necessary rate of infusion was similar by both routes, indicating virtually complete absorption of all luminally infused glucose.

The rate of lactate accumulation in the perfusate was not measurably greater during the perfusion of an intestine that was transporting glucose and fat than during recycling of perfusate through the apparatus in the absence of an intestine (Table 1). This indicates that the perfused gut did not effect an appreciable net production of lactate, even while it was transporting glucose. Lactate produced by the cells in the perfusate was apparently responsible for the gradual decline in perfusate pH. In order to maintain perfusate pH in the range of 7.36 to 7.40, the CO_2 content of the gas mixture delivered to the membrane oxygenator was reduced stepwise from 5% to 0% during the course of a 5-hr experiment. A similar adjustment was required during a control experiment when blood was recycled through the apparatus in the absence of an intestine.

There was no change in perfusate Na^+ concentration, a slight decline in the perfusate K^+ concentration, and a marked increase in the insulin concentration (Table 2), indicating, possibly, a response by pancreatic tissue to the increase in perfusate glucose concentration (32) observed in these particular experiments (Table 2).

Red cell hemolysis

Cumulative hemolysis of red cells in the perfusate after 5 hr of perfusion was approximately 1% (Table 3),

TABLE 2. Sodium, potassium, and insulin content of perfusate plasma

Duration of Perfusion	Perfusate Plasma Concentration			
	Na^+	K^+	Insulin	Glucose
hr	*meq/liter*		*μU/ml*	*mg/ml*
0	135	6.42	135	1.63
5	136	5.72	485	2.51

Perfusion conditions were similar to those in Table 1. A 5% glucose solution was infused continuously into the perfusate, and no luminal infusion was used. Each value is the mean of two experiments.

TABLE 3. Hemoglobin and unesterified fatty acid content of perfusate plasma

Duration of Perfusion	Perfusate Plasma Concentration		Red Cell Hemolysis
	Unesterified Fatty Acid	Hemoglobin (Free)	
hr	*μeq/ml*	*mg/ml*	%
0	0.29 (1)	0.6 (4)	0.4 (4)
5	0.61 (1)	1.4 (4)	1.0 (4)

Perfusion conditions were similar to those described in Table 1 and Fig. 3. Numbers in parentheses indicate number of experiments.

of which nearly half occurred before perfusion was begun, i.e., during preparation of the perfusate, priming of the apparatus, and removal of all air bubbles from the circuits.

Fat transport

The capacity of the perfused gut for fat transport is indicated by the data in Figs. 3–6.

Luminal infusion of 230 μmoles of long-chain fatty acid, 92% as soybean oil and 8% as lecithin, during the first 3 hr resulted in fatty acid recovery in lymph of greater than 50% by the end of 5 hr (Fig. 3). Of the fatty acid recovered in lymph, the proportion recovered as triglyceride increased from 83% during the first hour to 91% during the fourth hour, the period of greatest lipid recovery. The remaining fatty acids were nearly all recovered as phospholipids. The above values for fat transport capacity by this preparation are minimal estimates, since milky lymph was still flowing when the perfusions were terminated. Also, during several experiments, lymph collection was incomplete due to small accumulations of milky lymph within the mesentery.

Peak recovery of triglyceride in lymph was during the fourth hour (Fig. 4). When taurocholate was omitted from the luminal infusate in one experiment, there was partial inhibition of triglyceride transport. Only small amounts of triglyceride appeared in lymph when no fat was infused (Fig. 4).

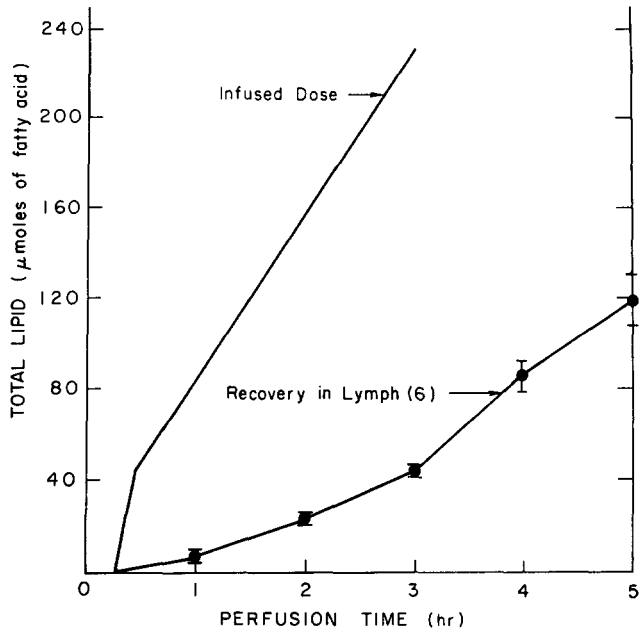

FIG. 3. Lymphatic recovery of duodenally infused lipid in isolated hemoperfused intestine. Intestine donors were fed the fat-free diet (W-8FF) and fasted for 12 hr prior to perfusion. Beginning at 15 min after the start of perfusion, the duodenum was infused continuously with the basic luminal infusate (see Experimental section). In addition, from 15 to 180 min, a triglyceride–phospholipid emulsion (Intralipid) was infused intraduodenally. An accelerated rate of infusion (priming dose) was used during the first 10 min, from 15 to 25 min after the start of perfusion. Recovery values were corrected for the endogenous fatty acid content of lymph, 2–4 μmoles/hr, determined from perfusion experiments in which no lipid was administered intraduodenally (see Figs. 4–6). Recovery data are mean values from six experiments ± SE.

Duodenal lipid infusion also increased the efflux of phospholipids and cholesterol in lymph (Figs. 5 and 6), and the rate of efflux changed little when taurocholate was omitted from the luminal infusate. During the first 3 hr of perfusion, the cholesterol and phospholipid contents of lymph were much lower than those found in mesenteric lymph of rats in vivo (zero-time points, Figs. 5 and 6), due to the absence of bile, which contributes phospholipids (33) and cholesterol (34) to intestinal contents. The observed increase in lymph phospholipid after fat infusion can be readily accounted for by phospholipid present in the infused mixture (35). However, no cholesterol was infused, so the increased lymph cholesterol must be of endogenous origin, representing synthesis by the gut (36) or increased filtration from the perfusate.

The lipoprotein distribution of the lymph lipids during the 4 hr of greatest fat transport is shown in Table 4. From earlier work in the rat it is known that chylomicrons (37) and VLDL of intestinal origin (38) are not discrete fractions but represent a continuum of lipoprotein particles with ultracentrifugal flotation rates

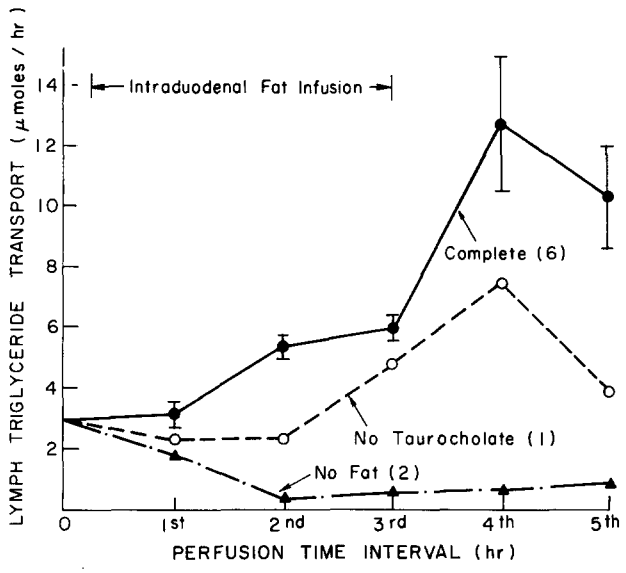

FIG. 4. Rate of lymphatic triglyceride transport by isolated hemoperfused intestine. ●——●, complete; these data are from the same experiments described in Fig. 3. ○- - -○, no taurocholate; conditions as described in Fig. 3, with the exception that sodium taurocholate was omitted from the luminal infusate. ▲—·—▲, no fat; conditions as described in Fig. 3, with the exception that the basic luminal infusate, but no lipid emulsion, was given intraduodenally. Data are mean values ± SE for the number of experiments indicated in parentheses. The zero-time transport rate is the average rate of triglyceride transport observed in mesenteric lymph of intact 280-g rats fed a fat-free diet (5).

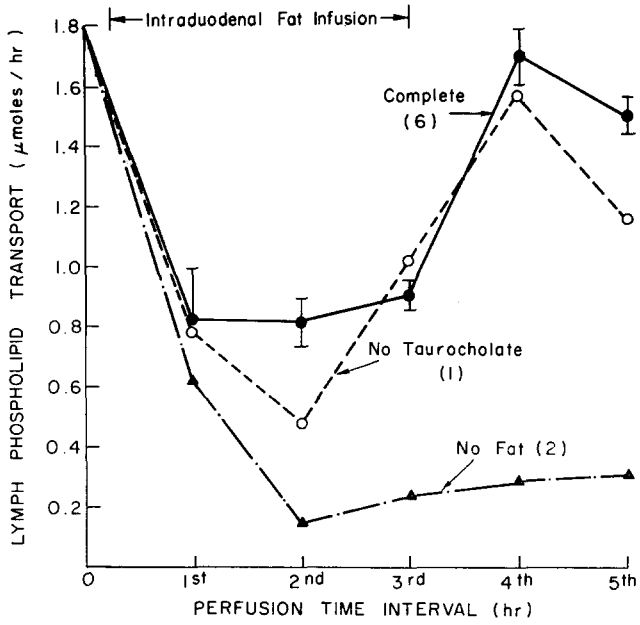

FIG. 5. Rate of lymphatic phospholipid transport by isolated hemoperfused intestine. Experiments are the same as described in Fig. 4.

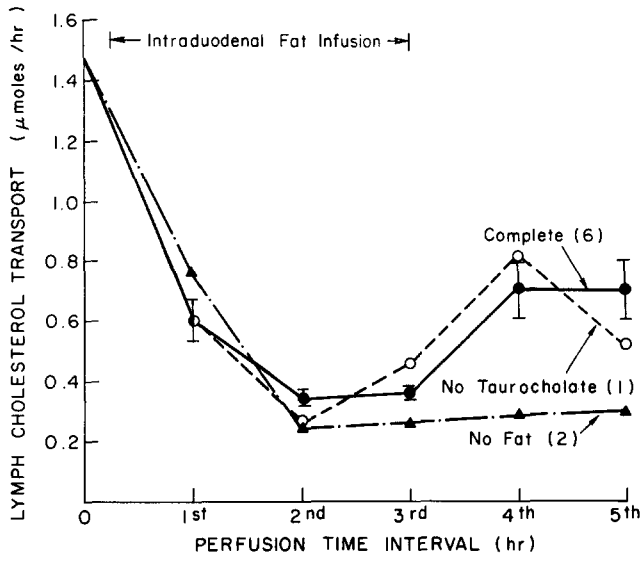

FIG. 6. Rate of lymphatic cholesterol transport by isolated hemoperfused intestine. Experiments are the same as described in Fig. 4.

ranging from a low of S_f 20 for the smallest VLDL complexes to S_f 10^5 for large chylomicrons. In the present study, chylomicrons were collected after centrifugation for 3×10^6 *g*-min (Table 4), conditions which would result in inclusion of all particles with $S_f > 250$ (39). This chylomicron fraction contained 75% of the triglyceride and nearly one-half of the phospholipid and cholesterol released into lymph by the perfused intestine. Average size of the chylomicrons can be estimated from their phospholipid content, which was 7.5% by weight of total chylomicron lipid (Table 4). This value is similar to the phospholipid content of the S_f 1100 to S_f 3200 chylomicron subfraction of rat intestinal lymph collected in vivo (38) and corresponds to particles with diameters of about 100–200 nm (40). Likewise, from analytical data on rat lymph chylomicrons by Fraser, Cliff, and Courtice (41) and on dog lymph chylomicrons by Yokoyama and Zilversmit (42), 7.5% phospholipid is expected for particles with mean diameters of 100–150 nm.

The remaining 25% of the lymph triglyceride not recovered in chylomicrons was recovered in VLDL, which also contained approximately 40% of lymph phospholipid and cholesterol. Approximately 14% of lymph phospholipid and lymph cholesterol and less than 1% of the triglyceride were found in the fraction with $d > 1.006$ g/ml.

No measurable change was observed in the perfusate concentration of phospholipids, total cholesterol, or total fatty acids during the 5-hr perfusions. There was a small loss of triglyceride, about 0.14 μmole/ml of plasma in 5 hr. This was accompanied by an increase of 0.3 μmole/ml in the perfusate concentration of unesterified fatty acids (Table 3) and probably reflects the action of a lipoprotein lipase. An analysis (43) of plasma following 5 hr of perfusion indicated the presence of a small amount of lipase activity which was inhibited by

TABLE 4. Lipid distribution in lymph lipoproteins from isolated intestine during fat transport

Lymph Fraction	Lipids in 4-hr Lymph Sample						Molar Ratio	
	Triglyceride (TG)		Phospholipids (PL)		Cholesterol (CHOL)		CHOL/PL	TG/PL
	μmoles	*% of total*	*μmoles*	*% of total*	*μmoles*	*% of total*		
Chylomicrons	19.00	74.6	1.77	44.4	0.78	44.6	0.44	10.73
VLDL	6.35	24.9	1.63	40.8	0.73	41.7	0.45	3.90
d > 1.006 g/ml	0.12	0.5	0.59	14.8	0.24	13.7	0.41	0.20
Total	25.47	100.0	3.99	100.0	1.75	100.0		

Perfusion conditions were as described in Fig. 3. Milky lymph (6.25 ml) from a single intestine was collected during the 1–5-hr interval after the start of perfusion. The lymph was layered under 0.15 M NaCl, 3 mM EDTA, pH 7.4, and centrifuged 60 min at 25,000 rpm in a Spinco 39-L swinging bucket rotor (avg 3.1 × 10^6 *g*-min). The thin floating chylomicron layer was recovered and washed once by layering under 0.15 M NaCl, 3 mM EDTA, and centrifuging as before. The infranatant fraction after the initial chylomicron isolation was layered under 0.15 M NaCl, 3 mM EDTA in a Spinco 40.3 rotor and spun for 16 hr at 40,000 rpm (avg 1.37 × 10^8 *g*-min). VLDL was recovered from the top 1.5 ml of the tubes. Sedimented proteins, with density > 1.006 g/ml, were also recovered and analyzed for lipids.

TABLE 5. Incorporation of [^{3}H]lysine into lymph and perfusate proteins by isolated intestine

Sample	Protein Fraction	Perfusion Time	Protein Content		Radioactivity of Protein		
		hr	*μg/hr*	*% of total*	*dpm/hr* (× 10^{-3})	*% of total*	*dpm/μg protein*
Lymph	Total	1	8760	100	326	100	37
		2	5750	100	1850	100	322
		3	4170	100	1900	100	455
		4	5100	100	2300	100	450
		5	6530	100	2000	100	306
	Chylomicrons + VLDL	1	75	0.9	95	29.1	1270
		2	73	1.3	545	29.5	7470
		3	73	1.8	694	36.5	9510
		4	90	1.8	647	28.1	7190
		5	70	1.1	530	26.5	7570
	LDL + HDL	1	106	1.2	49	15.0	462
		2	35	0.6	146	7.9	4170
		3	22	0.5	120	6.3	5460
		4	32	0.6	166	7.2	5190
		5	40	0.6	140	7.0	3500
			mg/ml		*dpm/ml* (× 10^{-3})		
Perfusate plasma	Total	0	58.0	100	0	100	0
		2	55.0	100	27.3	100	0.5
		5	51.0	100	62.3	100	1.2
	Chylomicrons + VLDL	0	0.04	0.1	0		0
		2	0.04	0.1	0	0	0
		5	0.03	0.1	0	0	0
	LDL + HDL	0	1.00	1.7	0		0
		2	0.98	1.8	5.7	20.8	5.8
		5	0.95	1.9	11.4	18.3	12.0

The intestine donor was fed diet W-8 until the time of perfusion. During perfusion, the duodenum was infused continuously with the basic luminal infusate. In addition, during the first 4 hr, a triglyceride–phospholipid emulsion (Intralipid), containing 243 μmoles of fatty acid/ml, was infused at 0.128 ml/hr. For more details, see Experimental section. 20 min after perfusion was begun, 0.9 mCi of L-[G-^{3}H]lysine (3.91 Ci/mmole, New England Nuclear) was added to the perfusate. Hourly lymph samples were collected for the next 5 hr. Total lymph volume was 3.5 ml. Perfusate was sampled immediately after the [^{3}H]lysine addition and after 2 and 5 hr of perfusion. Protein fractions were isolated as described in Experimental section.

1 M NaCl and which may have been released from the tissue by heparin in the perfusate (44).

Biosynthesis of lymph and plasma proteins

In several experiments, radioactive amino acids were added to the perfusate, and incorporation into lymph and perfusate proteins was measured. Table 5 shows the results of an experiment with L-[G-^{3}H]lysine. Nonfasted rat intestine was infused with a small amount of fat in order to ensure a continuous production of chylomicrons and VLDL. Lymph was milky throughout the experiment.

The specific radioactivity of lymph total protein and lipoproteins increased rapidly during the first 2 hr, after which there was little change. Approximately 0.4% of the [^{3}H]lysine appeared in lymph protein in 5 hr. Of this, approximately 30% was recovered in (chylomicrons + VLDL) and approximately 7% in (LDL + HDL) (Table 5). The remaining labeled proteins were not identified. The (LDL + HDL) content of lymph is very low (38), so these fractions were not further separated. The highest specific radioactivity was observed in the (chylomicron + VLDL) fraction.

No detectable radioactivity was incorporated into the perfusate (chylomicron + VLDL) fraction. There was, however, some incorporation into the perfusate (LDL + HDL) fraction. The specific radioactivity was very low compared with the (LDL + HDL) in the lymph, indicating that this latter group of proteins was delivered from the site of synthesis directly into the lymph and did not first equilibrate with similar lipoproteins in the perfusate. Although the specific activity of perfusate (LDL + HDL) was low, due to dilution by the large pool of unlabeled HDL in the perfusate, the total amount of [^{3}H]lysine incorporated into plasma (LDL + HDL) was approximately equivalent to the total incorporation into lymph (LDL + HDL).

In none of the lymph or perfusate lipoprotein fractions was more than 0.3% of the radioactivity from [^{3}H]lysine recovered in the lipid fraction of the complex.

Results qualitatively similar to those in Table 5 were obtained when L-[4,5-^{3}H]leucine was the radioactive precursor used in an experiment similar to that described in that table. However, with [^{3}H]leucine there was extensive incorporation into the lipid as well as the protein moieties of the lymph lipoprotein complexes.

Biosynthesis of tissue protein

Following an experiment similar to that described in Table 5, the intestine was divided into four portions, proximal small bowel, distal small bowel, cecum plus colon, and mesentery, and the extent of [^{3}H]lysine incorporation into soluble and insoluble tissue proteins was determined. After flushing out intestinal contents, each portion was homogenized in 0.25 M sucrose and centrifuged at 30,000 *g* for 30 min to separate the soluble from the insoluble protein. Protein in the sucrose supernatant fraction was precipitated with 5% trichloroacetic acid and counted as described for lipoproteins. The sucrose-insoluble residue was washed twice with 0.25 M sucrose, twice with absolute ethanol, and finally with diethyl ether. Following removal of residual ether in vacuo, weighed portions of the dried powder were dissolved in dilute KOH and counted.

A total of 6% of the [^{3}H]lysine added to the perfusate was recovered in soluble plus insoluble tissue protein at the end of the experiment. Soluble protein from all four portions of the preparation had approximately the same specific activity, 600–900 dpm/μg, the highest being distal small bowel. Thus, soluble tissue protein specific activity was approximately 10% of that of lymph VLDL (Table 5). The specific activity of insoluble tissue protein was also similar for the four portions of the gut and equaled about 0.7 times the value for soluble tissue proteins.

DISCUSSION

Development of a vascularly perfused gut preparation was undertaken primarily to facilitate definitive characterization of the lipoproteins synthesized by the intestine and released into the circulation. Thus, the perfusion conditions were chosen to approach the physiological norm as closely as possible. The perfusate was undiluted homologous blood, and arterial and venous pressure, perfusate pH, perfusate glucose concentration, and temperature were all maintained close to normal values in vivo. The preparation was bathed by a physiological salt solution similar in composition to peritoneal fluid (45), and establishment of the extracorporeal circulation was accomplished without interrupting the flow of oxygenated blood through the tissue. In addition, by use of a membrane oxygenator and a closed silicone rubber blood reservoir, an extracorporeal circuit was devised which eliminated blood–gas interfaces, a site of plasma protein denaturation and blood cell trauma (17). Hemolysis after 5 hr of perfusion was not more than 1% (Table 3). The blood pumping system was designed to mimic the rat heart with respect to frequency and amplitude of the pulse. It was observed, however, that, in relation to the gross and microscopic appearance of the intestine, experiments involving pulsatile flow could not be distinguished from experiments in which the pulsator was omitted and blood flow was nearly linear. Likewise, neither O_2 uptake, perfusate pH, fat and water transport, gut motility, nor vascular responsiveness to norepinephrine was noticeably influenced by the pulsatile flow. In the later experiments, therefore, the pulsator was not used. McLaughlin, Hammond, and Austen (46) have reported that pulsatile flow is required to preserve the viability of segments of anesthetized canine intestine perfused with blood. Crucial to the success of the rat intestine preparation is the use of norepinephrine and dexamethasone (14).

In addition to the normal gross and histological appearance (14) of the perfused intestine, a variety of metabolic and transport measurements now reinforce the conclusion that this is a viable, functioning preparation. For the duration of 5-hr experiments, O_2 consumption (Q_{O_2}) continued unabated at a rate of about 7 ml/g of

dry tissue/hr (Table 1). It is difficult to compare this value with others reported for preparations in vitro which typically do not include mesenteric tissue. For example, Wilson and Wiseman (47) reported an O_2 uptake of 12 ml/hr/g of lipid-free dry weight for rat everted small bowel sacs in vitro. The rate we observed during perfusion accounts for approximately 8.5% of the basal metabolic rate for a rat the size of the intestine donors (48). The perfused tissue represents, on the average, 4.6% by weight of the body. Lymph was produced continuously, the volume and protein content being similar to mesenteric lymph collected in vivo (Table 2). Furthermore, it is clear that the preparation transported water and glucose, and preliminary data indicate that during the course of perfusion the perfusate becomes considerably enriched in a variety of amino acids.[1] Glucose transport was not associated with an accumulation of lactate in the perfusate (Table 1). Thus, in this respect the perfused intestine resembles more nearly the intestine in vivo, where glucose transport also gives rise to little or no blood lactate (49), than it does the incubated everted sac preparation, which does produce lactate (50).

Demonstrating a capacity for fat transport was considered a necessary prerequisite to studying lipoprotein biosynthesis in this preparation. Evidence presented for chylomicron formation (Figs. 3–6, Table 4) indicates that, at least qualitatively, the isolated intestine is capable of performing all required steps in the luminal as well as mucosal phases of this process (51), including partial hydrolysis of triglycerides and phospholipids, absorption of the products of lipolysis into mucosal cells, reesterification, assembly of lipid and protein components into chylomicrons and related lipoprotein complexes, and release of these complexes into lymph. As in the intact rat (38, 52), the bulk of transported fat appeared in lymph in chylomicrons and a lesser amount in VLDL (Table 4). Based on phospholipid content, the average diameter of the chylomicrons was 100–200 nm. Chylomicrons in this size range are abundant in the lymph of fat-fed rats (1, 37). Furthermore, as in the intact rat (53), lymph became visibly enriched in lipid within 40 min after the start of fat infusion into the lumen.

Quantitatively, the gut perfused in vitro appeared to have a lower fat transport capacity than the intact rat. The highest hourly rate of transport observed in our experiments was 78 μmoles of fatty acid. The maximum transport capacity in vivo for long-chain fatty acids is of the order of 470 μmoles/hr, observed when the duodenum was being infused continuously with 750 μmoles/hr of oleic acid as triolein (54). The factors which limit fat transport in vitro or in vivo are not known. During perfusion, delivery of more than 90–100 μmoles of fatty acid per hour to the lumen resulted in an accumulation of luminal fluid and a decreased flow of lymph. Likewise, the luminal infusion of an alcoholic solution of unesterified long-chain fatty acids (14) or an emulsion of oleic acid and monoolein appeared to irritate the bowel, causing slight hyperemia, luminal fluid accumulation, and a low and delayed yield of lymph chylomicrons. Infusion of the triglyceride–phospholipid emulsion (Intralipid) produced the highest rates of fat transport.

There may be three sources for the lipase activity which affected luminal lipolysis: residual pancreatic lipase present in the lumen, lipase of intestinal origin (55), and lipase released by perfused pancreatic tissue. The presence of functional pancreatic tissue was indicated by the observed increase in perfusate insulin content (Table 2). The relatively high efficiency of fat transport when taurocholate was omitted from the luminal infusate (Figs. 4–6) may be related to residual bile in the lumen as well as the emulsified state of the fat infusion (56).

The intestine did not effect a measurable net change in the perfusate content of phospholipids, total cholesterol, or total fatty acid. A small net reduction in triglyceride was accompanied by a complementary increase in the perfusate unesterified fatty acid content (Table 3), the product, most likely, of the low levels of lipoprotein lipase detected in the perfusate. To test the possibility that the lipase activity and triglyceride loss were related to the presence of heparin in the perfusate, one intestinal preparation was perfused with defibrinated rather than heparinized rat blood. Perfusate triglyceride recovery was 96%, compared with an average of 87% in nine perfusions with heparin. Replacing heparinized blood with defibrinated blood did not significantly alter transport of luminal lipid into lymph. With defibrinated blood, vascular resistance was high and blood flow through the preparation was low during the first 10 min of perfusion. Otherwise, there was little to distinguish this experiment from those done with heparinized blood.

Johnston (57), using everted segments of hamster intestine, has demonstrated the transfer of tracer quantities of fatty acids from the mucosal solution into glycerides which appear in the serosal fluid. To our knowledge, however, the perfused rat gut is the first preparation in vitro which can effect net transport of large quantities of lipids.

In addition to transporting fat, the perfused intestine continuously incorporated radioactive lysine into lymph and plasma lipoproteins, which, in 5 hr, accounted for approximately 0.2% of the radioactive amino acid added to the perfusate (Table 5). An additional 6% of the radioactivity was incorporated into tissue protein. Nearly 40% of the label which appeared in lymph pro-

[1] Windmueller, H. G., and M. Slavik. Unpublished results.

tein was in lipoprotein, mostly in chylomicrons and VLDL (Table 5), indicating that lipoproteins constitute a major part of the newly synthesized protein which enters the circulation via the mesenteric lymph. Chylomicron and VLDL protein synthesized by the intestine appeared only in lymph, while newly synthesized (LDL + HDL) protein was divided approximately equally between lymph and perfusate. This may be a reflection of the difference in particle size between these two groups of lipoproteins. VLDL particles are probably too large to enter blood capillaries and therefore appear only in lymph, while some HDL can apparently cross the capillary endothelium (58). In the time course experiment shown in Table 5, LDL and HDL were not separated. In another similar experiment,[2] however, all lymph produced during a 5-hr perfusion was pooled, and lymph and perfusate lipoproteins were fractionated by ultracentrifugation. Of the total radioactivity in the (LDL + HDL) fraction, 50% in lymph and 86% in perfusate was recovered in a fraction with density between 1.063 and 1.21 g/ml, indicating clearly that much of this radioactivity is in HDL. Evidence for intestinal synthesis of HDL as well as VLDL protein may also be found in the data of Rodbell, Fredrickson, and Ono (2) and Roheim, Gidez, and Eder (3) with lymph-cannulated dogs.

Recent work (59–61) has shown that rat plasma VLDL and HDL each contain several different proteins which have been isolated (61) and partially characterized. Evidence for hepatic synthesis of most of these proteins has been obtained (62). It will be of interest to determine which of these apoproteins can also be synthesized by the intestine. Identification of [^{3}H]lysine-labeled lipoprotein apoproteins synthesized by a perfused gut preparation is presently underway.

Intestine, in the past, has been studied by a variety of techniques in vitro, the most popular in recent years being the everted gut sac preparation of Wilson and Wiseman (47). Lately, however, evidence has appeared that neither this preparation (53) nor any gut preparation without an intact blood supply (63) may be suitable for studies involving intestinal lipid absorption. Furthermore, the morphological integrity of this type of preparation is short-lived, 50–75% of the normal epithelium disappearing during a 30-min incubation in oxygenated buffer at 37°C (64). While vascular perfusion of intestine is certainly a more involved and intricate technique, it may offer an alternative procedure for the study of intestine in vitro with unique advantages in a variety of applications.

[2] Windmueller, H: G., P. N: Herbert, and R. I. Levy: Unpublished results.

Note Added In Proof. An analysis, recently completed, of the ^{3}H-labeled lipoprotein protein moieties, separated by polyacrylamide gel electrophoresis following delipidation, has shown that the isolated perfused rat intestine incorporates [^{3}H]lysine into all the major apoproteins of lymph VLDL and lymph and perfusate HDL, with the exception of the low molecular weight peptides (apparent mol wt of about 10,000) that are common to both VLDL and HDL. Although these peptides are found in the lymph lipoproteins, they do not appear to be synthesized by the gut and must therefore be acquired from other lipoproteins, presumably HDL, which filter into lymph from plasma. Isolated perfused rat liver, in contrast to gut, can synthesize all the major VLDL and HDL apoproteins, including the low molecular weight peptides (65).

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