A technique for the cannulation and perfusion of isolated rat epididymal fat pad

R. J. HO and H. C. MENG

Department of Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee

SUMMARY A method for study of the rat epididymal fat pad in vitro is described, in which perfusion via the internal spermatic artery is employed. The time interval between the stoppage of the blood flow and the establishment of the perfusion is less than 30 sec. Observations of perfusion pressure, flow rate, tissue edema, and oxygen consumption indicate that this system can be used to study the metabolism of adipose tissue. The sensitivity of the method with respect to free fatty acid release stimulated by epinephrine and ACTH is greater than that of incubated tissue, probably because of the increase in surface area of the fat cells exposed to the medium

THE ISOLATED rat epididymal fat pad has been used extensively for the study of lipid metabolism (1–3). In the methods employed to date, either the whole fat pad or pieces thereof have been incubated in vitro in suitable media. Under these conditions, however, only the surface of the tissue is in contact with the medium and it would appear possible that the metabolic function of deeper lying cells may be limited and modified by a relatively poor transfer of substrates, products, and hormones. With this in mind, we have devised a perfusion system in order to facilitate transfer of substances between medium and cells throughout the tissue. The system has been tested with regard to perfusion pressure, flow rate, tissue edema, and oxygen consumption. The release of free fatty acids (FFA) under the influence of epinephrine and ACTH has been compared with that obtained by the conventional incubation method.

We have presented a preliminary report of this work (4). A perfusion system for parametrial fat tissue has recently been reported by Robert and Scow (5).

METHODS AND MATERIALS

The epididymal fat pad is usually supplied by the epididymal branch of the internal spermatic artery (6). The internal spermatic arteries leave the aorta a short distance below the left renal artery, although occasionally they branch off from the renal arteries (Fig. 1). On the basis of this anatomical relationship, it is possible to perfuse the fat pad as described below.

Technique of Cannulation

Albino rats of the Sprague-Dawley strain weighing 150–200 g were used. All animals were fed Purina rat chow ad lib. The animal was anesthetized with Nembutal (50 mg/kg of body weight intraperitoneally), and heparin (5 mg/kg) was administered intracardially or intravenously to prevent coagulation of the blood. In later experiments heparin administration was found to be unnecessary.

An incision was made along the linea alba. After ligation of the right spermatic artery (ligation I in Fig. 1), the left perirenal fat was separated and the left kidney lifted up and pushed to the right so as to expose the underlying aorta. The aorta was cleared of enveloping tissues and ligated above and as close as possible to the lower lumbar arteries (ligation II in Fig. 1). A loose loop was made around the aorta just below the origin of the left renal and upper lumbar arteries. The aorta was clamped above the celiac artery. A small incision was made, and a No. 18 or 20 needle was inserted as cannula and tied (ligation III in Fig. 1). At this point, the left spermatic artery is the only remaining outflow from the tied aortic segment. Thus, it is functionally a cannulation of the left internal spermatic artery. It is important to note that occasionally the lower lumbar arteries leave the aorta at the same level as the spermatic artery. If this is the case, they can usually be tied off by placing the ligature II at an oblique angle.

Perfusion was then begun with a buffered medium at a pressure of about 100 mm Hg in order to wash the blood out of the intravascular compartment. A uniform

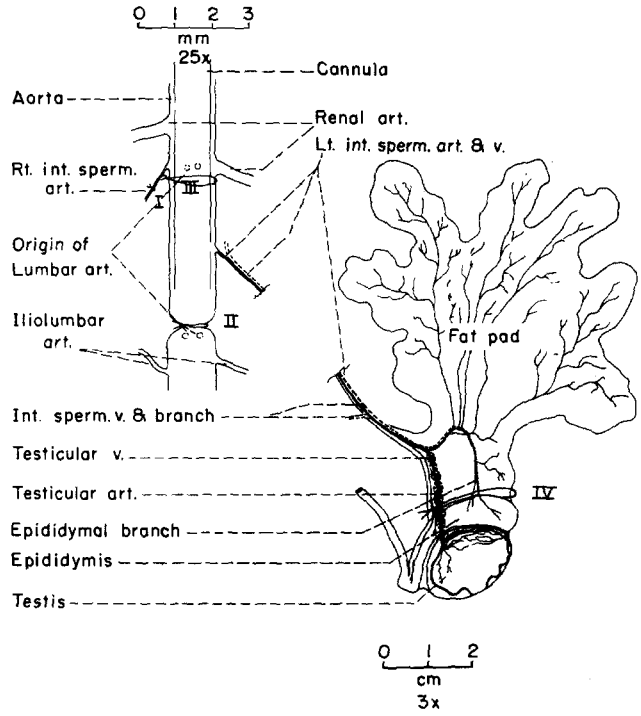

FIG. 1. Diagram showing the anatomical relationships in the cannulation of the aorta for perfusion of the rat epididymal fat pad. The order of ligations is indicated by roman numbers.

disappearance of color in the fat pad and the testis was taken as an indication of a satisfactory preparation. The time required from the clamping of the aorta to the beginning of perfusion was about 30 sec. The whole tissue mass, including testis, epididymis, left internal spermatic artery, and the segment of aorta, was removed from the rat by lifting up the various parts and cutting the underlying connective tissue attachments. The testicular artery was then ligated between the epididymis and testis, and the latter removed. In later experiments, it was found more desirable to place the ligation above the epididymis (ligation IV in Fig. 1) and to remove this tissue as well. The preparation was then placed in a beaker containing saline at room temperature to wash off any external blood. The fat pad was then ready for use.

In some cases, the left internal spermatic artery branches off directly from the left renal artery. When this anatomical relationship was encountered the right fat pad was used for perfusion.

In some experiments both the artery to and the vein from a fat pad were cannulated. The principle for the functional cannulation of the internal spermatic vein was the same as that of the artery; this was to make the left internal spermatic vein the only inflow to the inferior vena cava which had been cannulated. In this case, double ligations were made at the rectal level of the intestine about $^1/_2$ inch from the anus and the intestine was severed between the ligatures. The cephalic end of the intestine was separated from the mesentery up to the superior mesenteric artery in order to expose the veins in the interrenal region. The aorta and inferior vena cava were carefully separated and two loose loops were applied for further cannulations: one loop was to secure the aortic cannula (ligation III in Fig. 1), and the other (not shown in Fig. 1) was an oblique loop around the vena cava and left renal vein with which the venous cannula was ligated. For stopping the venous inflow other than via the left internal spermatic vein, the right internal spermatic vein was ligated with its accompanying artery (ligation I) and the vena cava was tied with the aorta (ligation II) as shown in Fig. 1. The left renal vein was also ligated close to the kidney, and the branch of the left internal spermatic vein was also tied. After clamping the vena cava at the right renal level, an incision was made below it. Heparinized polyethylene tubing was inserted until the tip reached the junction of the left renal vein and the vena cava; the loop was then secured. After both vein and artery had been cannulated, the ligation IV was made.

The oxygen consumption of the fat pad was determined by the arteriovenous difference in the oxygen tension from a doubly cannalated preparation. The oxygen tension was measured by the method of Sproule et al. (7).

Perfusion of the Epididymal Fat Pad

The washed fat pad was then transferred to the perfusion apparatus. Two variants were used. The first was employed when recirculation of a small volume of medium through the tissue was desired. In this system, the cannula was connected to an apparatus modified from that described by Morgan et al. (8) for perfusion of the rat heart. The medium was pumped through the aortic cannula into the fat pad, which rested on a sintered glass filter disk in the thermostatic tissue chamber. Medium left the tissue through the cut branches of the internal spermatic vein and returned to the tissue chamber (Fig. 2). The perfusate then passed through the filter and was recirculated. The flow rate was estimated by counting the drops falling into the bubble trap (Fig. 2).

The second system was used for perfusion without recirculation, the so-called open system. The medium was contained in a thermostatic reservoir consisting of a water-jacketed condenser of about 60 ml capacity. It was equilibrated with 95% O_2–5% CO_2 by gentle continuous bubbling. The outflowing medium passed through the peristaltic pump, the bubble trap, and the fat pad located in the tissue chamber as shown in Fig. 2 and was collected in 15-ml graduated conical centrifuge tubes. The flow rate was estimated by counting either

the drops falling from the top of the bubble trap or those from the tissue chamber into the collecting tube.

In both types of perfusion system the flow rate was maintained at about 0.5 ml/min by adjusting the pressure developed by the peristaltic pump. The first 5 ml of perfusate containing red blood cells were discarded and the perfusate collected thereafter was clear.

A three-way stopcock was inserted between the bubble trap and the tissue chamber just above the cannula. This provided a convenient route for the addition of hormones in the recirculation experiments, and for changing from one type of medium to another in the open system.

The perfusion medium was a Krebs-Ringer phosphate or bicarbonate buffer containing 5% bovine serum albumin as FFA acceptor (9).

Determinations of FFA and Tissue Protein Nitrogen

Free fatty acid content of the medium was determined by the method of Dole (10), modified to use Nile Blue A as an indicator in the titration mixture. Tissue FFA was determined by the same procedure except that the tissue was homogenized in the extraction mixture.

A segment of the tissue, weighing about 100 mg, was homogenized in 2 ml of saline and centrifuged. The underlying clear layer was used for the soluble protein nitrogen determination according to Lowry (11).

Experiments with Nonperfused Fat Pads

Whole fat pads or segments of them were quickly weighed on a torsion balance and then incubated in medium containing epinephrine in a Dubnoff metabolic shaker at about 100 cycle/min. The incubation time, volume, and other conditions are indicated under individual experiments. The FFA content in medium and tissue was determined after incubation according to methods described above.

Materials

Adrenalin chloride, 1 mg/ml in physiological saline, was supplied by Parke, Davis and Co., Detroit, Mich. Lyophilized ACTH, lot No. 116283, was supplied by Wilson Laboratories, Chicago, Ill.; 1 mg is equivalent to 100 units. Crystallized bovine serum albumin (albumin bovine crystalline 2×) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. In some experiments, bovine serum albumin was extracted according to the method of Goodman (12) with slight modification; this preparation has been designated as "FFA-free" serum albumin.

RESULTS

In a hydrodynamic system, the flow rate depends on the perfusion pressure, the tissue resistance, and the viscosity of the circulating medium. If the flow rate and the viscosity of the medium are constant, the pressure change is proportional to the tissue resistance. Perfusion of the rat epididymal fat pad was carried out on the basis of this relationship. At the beginning of the preliminary washing by perfusion, the pressure was about 100 mm Hg. During the first 5 min the pressure declined slowly and became relatively steady at between 25 and 75 mm Hg (varying from one pad to another). The pressure in 36 experiments was maintained at an average of 52 ±0.13 mm Hg during a 60 min perfusion period. The flow rate during perfusion was kept constant at about 0.4–0.5 ml/min. Fig. 3 represents the average of two experiments illustrating the time course of perfusion pressure and flow rate. Steady states in perfusion pressure and flow rate were also maintained during the 50 min experimental period when epinephrine (0.03 μg/ml) was employed.

Oxygen Consumption

The oxygen tension of the medium was determined before and after perfusion. That of the arterial sample taken from the medium reservoir during perfusion was

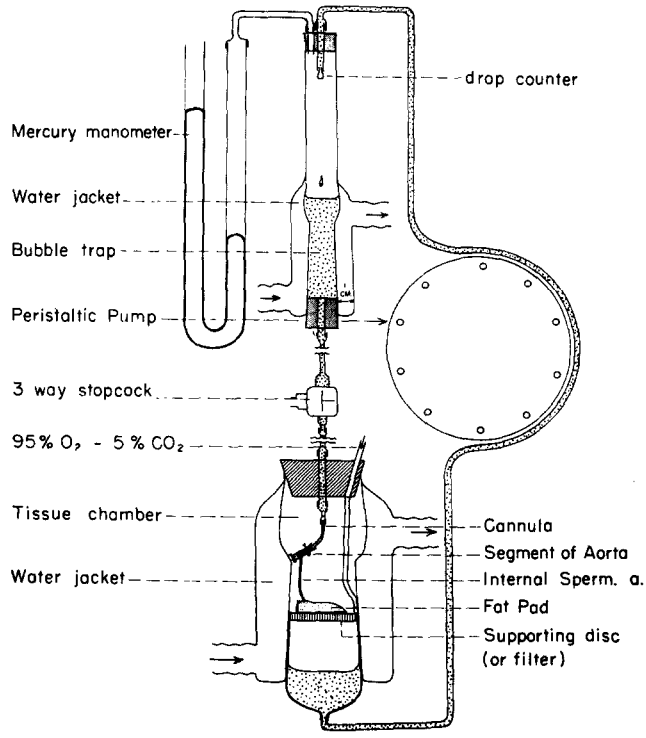

FIG. 2. Diagrammatic representation of the apparatus for perfusion of the fat epididymal fat pad. The diagram shows the recirculation of medium during perfusion. A small amount of medium usually collects above the filter disk and the gas inlet dips into this fluid; the continuous bubbling facilitates gas exchange. When an open circuit (non-recirculation) perfusion is carried out the filter disk is replaced by a coarsely perforated teflon plate.

about 505 mm Hg throughout the experiment. The venous sample was obtained directly from the venous cannula and collected into a gas-sealed syringe. It averaged 213 mm Hg at the basal state, with a range from 180 to 262 mm Hg. In the presence of epinephrine (0.01 μg/ml) plus glucose (100 mg/100 ml) the oxygen tension decreased to an average of 100 mm Hg; the lowest finding was 85 mm Hg. The mean tissue oxygen consumption was 386 μl/g per hr under the basal state conditions, and 561 μl/g/ per hr when epinephrine and glucose were added (Table 1).

Weight Gain of Epididymal Fat Pad During Perfusion

In the early recirculation experiments, a pressure of 100 mm Hg or higher was maintained. Following prolonged perfusion at this pressure gross edema was usually observed. The edema was less pronounced in fat pads perfused at low pressure (40–50 mm Hg). Table 2 shows the average weight gain of perfused fat pads with pressure maintained at about 50 mm Hg and flow rate at 0.4–0.5 ml/min.

Protein Nitrogen Content of the Perfused Fat Pads

The protein nitrogen content of the epididymal fat pads varied with the nutritional state of the animal and the amount of the plasma protein nitrogen trapped in the tissue. Table 3 shows the protein nitrogen contents of epididymal fat pads from fed and fasted rats both before and after washing by perfusion with bicarbonate buffer.

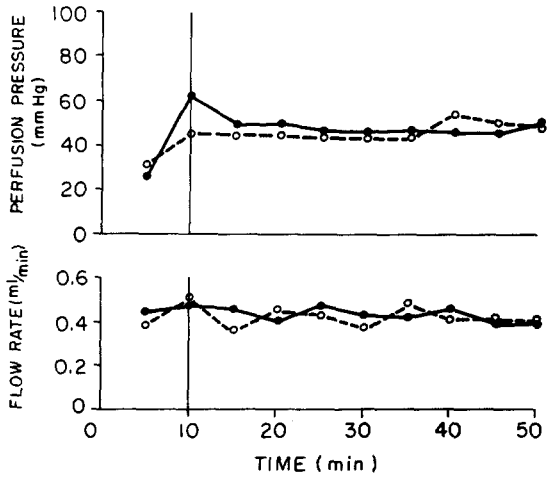

FIG. 3. The time course of perfusion pressure and flow rate during a 50 minute perfusion period. Fat pads from fed rats were perfused with a Krebs-Ringer bicarbonate buffer pH 7.4 containing 5% "FFA-free" bovine serum albumin. Perfusion was carried out without recirculation for 10 min, after which a medium containing epinephrine (0.03 μg/ml) was used. The results are averages of two representative experiments. ●—● basal medium without epinephrine; O---O basal medium with the addition of epinephrine (0.03 μg/ml).

Comparison of FFA Release from the Perfused and Incubated Fat Pads

Figure 4 shows that the perfused fat pads were more sensitive than the incubated tissues in respect to FFA release. The average net production of FFA from the perfused fat pads was 2.5 times as much and the FFA release was 11 times as much as that of the incubated tissues. The average tissue:medium FFA ratio of the incubated fat pads was 9, while that of the perfused fat pads was about 1.5.

Effect of Prior Injection of Heparin on the FFA Release Induced by Epinephrine

Although prior administration of heparin was not necessary, it did facilitate considerably the washing by perfusion of the fat pad. Since heparin is known to induce the release of the clearing factor lipase, the

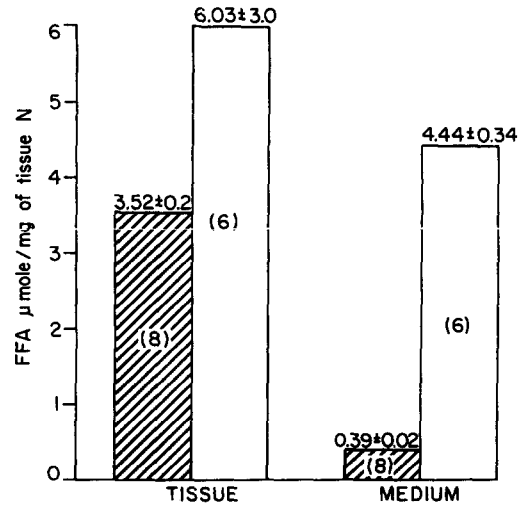

FIG. 4. Comparison of the increases in the FFA content of tissue and medium of the perfused and incubated epididymal fat pads of fed rats. The perfusion or incubation period was 30 min. A Krebs-Ringer phosphate buffer pH 7.4, containing 5μg of epinephrine per ml and 5% bovine serum albumin was used as medium for both incubation and perfusion experiments. The recirculation perfusion system was used. The fat pad of incubation was dissected, weighed and incubated in an Erylenmeyer flask containing 3.0 ml of buffer. ▨ incubation; □ perfusion. Figures in parentheses represent the number of fat pads. The average FFA content of the fat pads from 23 fed rats before incubation was 0.44 ± 0.004 μmole/mg of N. FFA content and tissue nitrogen before incubation were determined on the other fat pad of the same rat and the latter value was used to express the units of FFA in the tissue and medium. In these units average total FFA produced by the incubated and by the perfused fat pads was 3.91 and 10.47 μmole/mg of N reprectively. The total FFA produced thus calculated is actually the net FFA produced under the described experimental conditions since the quantities of FFA released by lipolysis and utilized for reesterification during incubation or perfusion were not known.

TABLE 1 OXYGEN CONSUMPTION OF THE ISOLATED PERFUSED EPIDIDYMAL FAT PADS*

Tissue Wt	AO_2† Before Perfusion	After Perfusion	Basal Medium Perfusion VO_2‡	$AO_2 - VO_2$	Flow Rate	O_2§ Consumption		Epinephrine Plus Glucose Perfusion VO_2	$AO_2 - VO_2$	Flow Rate	O_2 Consumption	
mg	*mm Hg*	*mm Hg*	*mm Hg*	*mm Hg*	*ml*	*μl/pad/hr*	*μl/g/hr*	*mm Hg*	*mm Hg*	*ml*	*μl/pad/hr*	*μl/g/hr*
660	504	501	180	325	0.4	251	379	85	420	0.4	325	490
626	506		184	321	0.4	248	394	108	397	0.5	384	610
700			206	299	0.5	288	412	100	405	0.5	391	559
650			262	243	0.5	234	358	108	397	0.5	383	585

* Epididymal fat pads from fed rats were doubly cannulated and perfused with a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% "FFA-free" bovine serum albumin first without and then with epinephrine (0.03 μg/ml) and glucose (100 mg/100 ml) for 15 min each. The arterial perfusate was taken from the medium reservoir and the venous perfusate was collected in a gas-sealed syringe for pO_2 assay.

† Oxygen tension of perfusate from the reservoir.

‡ Oxygen tension of venous perfusate.

§ The tissue oxygen consumption was calculated by multiplying the A−V oxygen difference(based on the oxygen solubility in water) by the flow rate and dividing by tissue weight. Means ± SE: 386 ± 12 μg/g/hr (basal), 561 ± 26 μg/g/hr (epinephrine).

TABLE 2 WEIGHT GAIN OF EPIDIDYMAL FAT PADS DURING PERFUSION*

No. of Expts.	Perfusion Time	Observed Weight Gain	Average Water Content
	min	%	%
4	0	0	16.3 ± 3.7†
6	30	15.9 ± 6.6	27.7‡
6	45	16.3 ± 4.0	27.8‡
6	60	18.0 ± 3.0	28.8‡

* Fat pads from fed rats were perfused with a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% "FFA-free" bovine serum albumin. Wet weight of each fat pad was obtained by weighing in a torsion balance before and after perfusion. The assumption was made that the weight of cannula and the segment of aorta were constant throughout the perfusion period.

† The average water content of fat pads before perfusion was estimated by difference between the weights of wet and dry tissue.

‡ Water content (%) of the fat pad after perfusion was calculated according to the following formula:

$$\frac{\text{Gain in wet wt during perfusion (mg)} + \text{Water content before perfusion (mg)}}{\text{Wet wt after perfusion (mg)}} \times 100$$

TABLE 3 SOLUBLE PROTEIN NITROGEN CONTENT OF THE PERFUSED AND THE NONPERFUSED FAT PAD

No. of Expts.	Condition	Before Perfusion	After Perfusion	Decrease
		mg N/g	*mg N/g*	%
5	Fed	2.68 ± 0.08	1.65 ± 0.04	39
10	Fasted 36 hr	3.25 ± 0.40	2.24 ± 0.70	31

Fat pads were perfused with a Krebs-Ringer bicarbonate buffer, pH 7.4 (no albumin) for 10–20 min until the perfusate was clear and free of blood cells.

TABLE 4 EFFECT OF PRIOR INJECTION OF HEPARIN ON THE FFA RELEASE INDUCED BY EPINEPHRINE

No. of Expts.	Pretreatment with Heparin	Time of Perfusion with Epinephrine	FFA Release
		min	*μmole/mg of N*
13	+	30	4.89 ± 0.27*
5	−	30	4.25 ± 0.84

Fat pads of fed rats were used in all experiments; epinephrine concentration was 0.03 μg/ml. Heparin (5 mg/kg) was injected intracardially or intravenously before surgical procedures. Fat pads were perfused with a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% "FFA-free" bovine serum albumin in a nonrecirculation system.

* Mean ± SE.

possibility of an effect of heparin on the epinephrine-induced FFA release was tested. Results are shown in Table 4. It can be seen that no significant difference in FFA release between the two groups was observed.

Time Course of FFA Release Induced by Epinephrine and ACTH

The time courses of FFA release induced by continuous perfusion with epinephrine and ACTH are shown in Figs. 5A and 5B respectively. Epinephrine (0.03 μg/ml) and ACTH (100 μU/ml) both stimulated the FFA release from fat pads.

The Ability of the Perfused Fat Pad to Respond to Repeated Stimulation

In this study, two equal stimulations of short duration were applied; the second stimulation was given after the tissue had recovered from its reponse to the first. The results of double stimulation by epinephrine are shown in Fig. 6. The amounts of FFA released following both stimulations were identical.

DISCUSSION

In studies in which organs are perfused the oxygen supply, perfusion pressure, flow rate, and tissue edema should always be considered. The oxygen supply of the perfused fat pad in the present work seems adequate since the oxygen tension of the arterial perfusate in the medium reservoir was maintained at a high and constant level throughout the experimental period. Furthermore, although the oxygen tension of the perfusate collected

after perfusion of the tissue was 55% lower than that of the arterial perfusate, it was higher than that of the venous or even arterial blood of a normal animal (13); the results suggest that there is an adequate oxygen supply for tissue consumption. This is true even in the presence in the perfusion medium of epinephrine, which increased the oxygen consumption by 46% above the basal state. The oxygen consumption of the perfused fat pads determined in the present study was higher than that reported by Hagen et al. using incubation techniques (14).

The fall in pressure during the preliminary washing by perfusion might have been due to the change of viscosity from blood to the buffer medium. Differences in tissue resistance might have caused the variation of pressure between individual fat pads during perfusion. However, in the steady state, even when the blood was completely replaced by the perfusion medium, occasional minor adjustments of the pump were necessary in order to maintain the flow rate constant at about 0.5 ml/min. The reason for using 0.5 ml/min rather than a higher rate was that less pressure was required to maintain this rate and, therefore, less edema of the tissue developed. The presence of some degree of edema did not apparently interfere with the FFA release from the fat pad. Furthermore, the water content remained fairly constant after the initial rise. This perfusion flow rate of the relatively nonviscous medium is about 5 times higher than that of the blood estimated in situ.[1]

The decrease in tissue protein nitrogen after washing by perfusion is believed to be due to the loss of plasma protein from the vascular compartment of the tissue, and indicates the importance of this preliminary step in the study of FFA mobilization, since plasma FFA is known to be bound to serum albumin.

The increase in sensitivity in FFA release from the perfused fat pad is probably due to the fact that the medium was supplied by a physiological route which increases the functional surface between the medium and fat cells. According to Winegrad every fat cell of the epididymal fat pad is surrounded by capillaries and every capillary is surrounded by fat cells (15). The

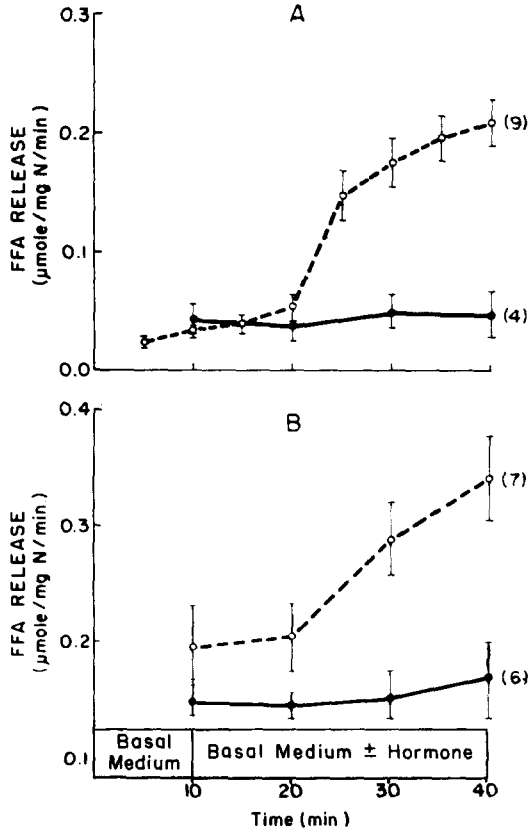

FIG. 5. The time course of FFA release from fat pads induced by epinephrine (*A*) and by ACTH (*B*). Fat pads were perfusion-washed with Krebs-Ringer bicarbonate buffer pH 7.4 containing 5% "FFA-free" bovine serum albumin until the venous perfusate was free of red blood cells. Samples of perfusate were collected at 5- to 10-min intervals. After collecting the control samples the medium containing epinephrine (0.03 μg) or ACTH (100 μU/ml) was introdced. The control groups were perfused with the same basal medium without the hormone throughout the experiment. Fed rats were used for investigating epinephrine effects (*A*) and fasted (36–48 hr) rats for examining the effect of ACTH (*B*). O-----O FFA release induced by epinephrine (*A*) or by ACTH (*B*). ●——● FFA release of the control group.

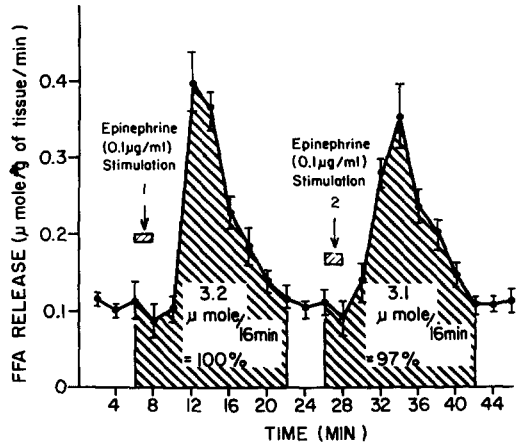

FIG. 6. The response of the perfused fat pad to repeated stimulation by epinephrine. Fat pads from fed rat were first perfused with the basal medium, a Krebs-Ringer bicarbonate buffer containing 5% "FFA-free" bovine serum albumin, pH 7.4, for 6 min to collect 3 samples at 2-min intervals. After this the perfusion was carried out with medium containing epinephrine (0.1 μg/ml) for 2 min by switching the 3 way stopcock located between the tissue chamber and the bubble trap; at the end of the 2 min, epinephrine-free basal medium was again used. Samples of perfusate were collected at 2-min intervals. The second stimulation was given after the FFA level had fallen to the initial level. Nonrecirculation perfusion was used. Vertical lines represent the standard errors. Each point represents the average of 4 pads.

[1] R. J. Ho and H. C. Meng, unpublished data.

volume of the vascular compartment of adipose tissue is comparable with that of muscle (16). Thus, the surface area of fat cells exposed to the perfusate is much larger than that exposed to the incubation medium. Furthermore, the mixing and the entering into cells of the substrates and hormones in the medium and the removing of the products from cells are probably facilitated by the perfusion process.

It is evident that the isolated perfused adipose tissue remains in a functional state and responds continuously to the stimulation of epinephrine; it also responds equally to the repeated stimulation. In fact, perfused fat pads have been maintained in good functional condition for as long as 100 min.[1]

Prior administration of heparin as an anticoagulant to rats seems to have no effect on the FFA mobilization induced by epinephrine. In fact, even the addition of heparin to the perfusion medium did not modify the FFA release induced by epinephrine from the epididymal fat pad.[2]

It is concluded that the methods of cannulation and perfusion of rat epididymal fat pad meet the basic requirements used as criteria in perfusion systems. Furthermore, this technique would be useful in many aspects in which the current incubation method is not satisfactory.

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[2] R. J. Ho, S. J. Ho, and H. C. Meng, unpublished data.

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