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Clearing-Factor Lipase in Adipose Tissue

A MEDIUM IN WHICH THE ENZYME ACTIVITY OF TISSUE FROM STARVED RATS INCREASES *IN VITRO*

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1. When epididymal fat bodies from starved rats are incubated for 3·5hr. at 37° in a defined medium *in vitro* the total clearing-factor lipase activity rises to approximately twice its initial value. 2. During the incubation period part of the tissue clearing-factor lipase activity appears in the medium. 3. Heparin, glucose, insulin, and HCO_3^- and K^+ ions are shown to be important medium constituents.

The removal of chylomicron and low-density-lipoprotein triglycerides from the circulating blood by the extrahepatic tissues is known to occur at a rapid rate and is thought to involve hydrolysis of the triglycerides by an enzyme, clearing-factor lipase or lipoprotein lipase, which is widely distributed in the extrahepatic tissues and which may be associated with the endothelial cells lining the blood capillaries (Robinson, 1963*a*; Bezman-Tarcher, Otway & Robinson, 1965). FFA‡ released at this site as a result of triglyceride hydrolysis would be expected to leave the blood and enter the tissue cells extremely rapidly.

If the uptake of triglyceride fatty acids from the blood in the extrahepatic tissues is dependent on their hydrolysis by clearing-factor lipase, then this enzyme presumably regulates the distribution of triglyceride fatty acids to the tissues. That is, the activity of clearing-factor lipase in a particular tissue will determine the capacity of that tissue to remove triglyceride fatty acids from the circulation.

Recent observations on the clearing-factor lipase activity of adipose tissue and the mammary gland are consistent with the above view. Triglyceride fatty acids are taken up from the blood by adipose tissue of fed animals, but not by that of starved animals (Bragdon & Gordon, 1958; Havel, Felts & van Duyne, 1962), and the activity of clearing-factor lipase in adipose tissue is high in the fed animal but falls to a low level on starvation (Hollenberg, 1959; Cherkes & Gordon, 1959; Robinson, 1960; Páv & Wenkeová, 1960; Salaman & Robinson, 1961). In the mammary gland, triglyceride fatty acids of chylomicra and very low-density lipoproteins are taken up by the gland from the blood during lactation in amounts sufficient for them to be a major source of the milk fatty acids (Barry, Bartley, Linzell & Robinson, 1963); and the clearing-factor lipase activity of the gland rises markedly at parturition when this uptake begins and persists at a high level throughout lactation (McBride & Korn, 1963; Robinson, 1963*b*).

The mechanism whereby these changes in clearing-factor lipase activity are achieved is evidently of interest. In an attempt to throw light on this mechanism, experiments have been carried out to determine conditions in which the clearing-factor lipase activity of epididymal adipose tissue from starved rats is increased *in vitro*.

MATERIALS AND METHODS

Adipose tissue. Epididymal adipose tissue was obtained from male albino rats of the Wistar strain that had been starved for 48hr. The animals weighed 120–145g. before starvation and 100–130g. afterwards.

Serum. (*a*) For incubation media. Blood was obtained by the technique described by French, Robinson & Florey (1953) from male albino rats of the Wistar strain. Unless specified otherwise, these were on their normal diet and weighed 200–350g. Blood samples were allowed to clot for 30min. at 37° and were then centrifuged at 2500*g* for 15min. at room temperature. The serum was recovered and usually dialysed at 4° against a large volume of 0·85% NaCl soln., pH7·7, on a rocking dialyser for 16–24hr.

(*b*) For assay media. Serum prepared by the clotting of whole blood has been reported to contain inhibitors of clearing-factor lipase (Mitchell, 1959; Hollett & Nestel, 1960). Although no evidence for the action of such inhibitors was ever observed under the conditions of our experiments, serum used in the assay of the enzyme was nevertheless

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‡ Abbreviation: FFA, free fatty acids.

obtained from blood collected in 7·6% (w/v) trisodium citrate soln. (final proportions, 19ml. of blood to 1ml. of citrate). After centrifuging, the plasma was separated and M-$CaCl_2$ soln. was added to give a final concn. of 25mM. Clotting was allowed to occur for 30min. at 37° before the serum was separated from the fibrin clot. Dialysis was then carried out as described above.

Serum samples were stored at 4° and no sample was kept for longer than 5 days after its collection. Before use, the pH, which varied slightly from sample to sample, was adjusted to 7·4 with dilute HCl or NaOH.

Serum-lipoprotein fractions. Low-density ($d < 1·063$) and high-density (d 1·063–1·21) serum-lipoprotein fractions were separated from each other and from the serum proteins of $d > 1·21$ by the ultracentrifugal technique described by Robinson & Harris (1961). The serum used was collected as described above for incubation media. Lipoprotein fractions were dialysed in a rocking dialyser against several changes of 0·85% NaCl soln., pH 7·7, at 4° until the concn. of the KBr added in the centrifugal fractionation was calculated to be less than 1·25mM. They were used immediately after dialysis.

Miscellaneous materials and chemicals. Chyle was obtained by cannulation of the thoracic ducts of rats that had been fed with olive oil (French *et al.* 1953). Serum albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex (bovine albumin, fraction V). Casein hydrolysate (Bacto-Casamino Acids B230) was obtained from Difco Laboratories, Detroit, Mich., U.S.A. Insulin (Wellcome) was obtained from Burroughs, Wellcome and Co., London, N.W. 1. Heparin (Pularin) was obtained from Evans Medical Ltd., Liverpool. Diethyl ether (anaesthetic grade) and acetone were obtained from Harrington Bros. Ltd., London, E.C. 1. Other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, and, except for casein, were of AnalaR grade.

Method of incubation of epididymal fat bodies in vitro. A standard method used in studies on the metabolism of intact adipose tissue *in vitro* is that in which the two epididymal fat bodies of a rat are used in conjunction, the activity of one serving as a control for that of the other (see, e.g., Winegrad & Renold, 1958). This 'paired fat-body technique' eliminates the effect of variation in tissue metabolic activity from one animal to another and was employed in the experiments described in this study. Results presented in the accompanying paper (Wing, Salaman & Robinson, 1966) show that the differences in clearing-factor lipase activity between paired fat bodies are small and that there is no consistent difference between the activities of right and left fat bodies. In the experiments reported, right and left fat bodies were used alternately in test and control systems.

Such a paired fat-body technique has allowed two types of experiment to be carried out. Either paired fat bodies were incubated in different incubation media for a given time and then the clearing-factor lipase activities compared, or the clearing-factor lipase activities of paired fat bodies were compared before and after their incubation in a particular medium.

The standard experimental procedure was as follows. With the rat under ether anaesthesia, the epididymal fat bodies were exposed through an incision in the abdominal wall. Each fat body was grasped at its tip with forceps and removed by cutting across the tissue at right angles to the long axis and tangentially to the prominent vein lying at its base. After being rinsed in 0·85% NaCl soln. and blotted on filter paper, the fat bodies were weighed, put into stoppered test tubes (40ml. capacity) containing 2·5ml. of incubation medium and incubated for either 5min. (0hr.) or 3·5hr. in a shaking water bath at 37°.

Although various incubation media were used in the present study most can readily be related to one, which is referred to below as complete reconstituted medium (CRM), with the following composition: 1·2vol. of dialysed serum, 0·0125vol. of heparin (2·4units/ml.), 0·0125vol. of D-glucose (2·4mg./ml.), 0·0125vol. of insulin (12milliunits/ml.), 0·25vol. of $NaHCO_3$ (1·5mg./ml.), 0·0125vol. of casein hydrolysate (1·2mg./ml.) and 1·0vol. of salt soln. The concentrations given in parentheses are final ones. The salt soln. component contained NaCl, KCl, $CaCl_2$, NaH_2PO_4 and $MgSO_4$. The final inorganic ion composition of the medium is shown in Table 1. Values for Krebs–Ringer bicarbonate soln. and rat plasma are given for comparison.

All the incubation media that contained bicarbonate were gassed, with occasional shaking of the vessels, with $O_2 + CO_2$ (95:5) for 5–10min. before the insulin, and then the fat bodies, were introduced. When bicarbonate was absent (Expt. 1 in Table 5) O_2 was the gas phase. The

Table 1. *Ionic composition of CRM*

Data for the ionic composition of Krebs–Ringer bicarbonate soln. and of rat plasma are taken from Long (1961). Values for rat plasma are means ±S.D. with the numbers of animals in parentheses. Phosphate is expressed as PO_4^{3-}.

	Concentration (mg./100ml.)		
Ion	CRM	Krebs–Ringer bicarbonate	Rat plasma
Na^+	357	330	327 ±9 (20)
K^+	13·3	23·2	15·6±1·6 (20)
Ca^{2+}	5·8	10·2	12·1±1·0 (18)
Mg^{2+}	1·7	2·9	3·2±0·5 (4)
Cl^-	508	455	371 ±14 (20)
PO_4^{3-}	6·4	11·4	—
SO_4^{2-}	6·5	11·5	6·5±0·4 (28)
HCO_3^-	109	152	171 ±21 (20)

initial pH of the incubation media that were gassed with O_2+CO_2 (95:5) was 7·3–7·5 and this decreased only slightly during incubation, never falling below 7·1.

Methods of assay of clearing-factor lipase. (*a*) In acetone–ether-dried preparations. At least two lipases are now thought to be of major physiological importance in adipose tissue (see Renold & Cahill, 1965). One of these is responsible for the hydrolysis of the adipose-tissue triglycerides during conditions of fatty acid mobilization and the other is clearing-factor lipase. Both lipases are present in aqueous homogenates of adipose tissue but only clearing-factor lipase is stable to treatment of the tissue with acetone and ether (Korn, 1959; Robinson, 1963*a*).

In the present study such acetone–ether-dried preparations were made from epididymal fat bodies and their incubation media and the activity of the enzyme in homogenates of these preparations was used as a measure of the total clearing-factor lipase activity, i.e. that present in the tissue and medium combined.

The lipase present in such homogenates cannot hydrolyse artificial triglyceride emulsions in the absence of added serum proteins [in one experiment the rate of FFA release from Lipomul (Upjohn Co., Kalamazoo, Mich., U.S.A.) was raised from 0·6 to 8·6 μmoles of FFA/fat-body equivalent/hr. when serum was added to the assay mixture] and is more than 90% inhibited by 0·6 M-NaCl. It therefore shows two of the characteristic properties of clearing-factor lipase (Korn, 1959). The preparation procedure is described below.

Immediately after the incubation each fat body was removed from its medium, rinsed in 0·85% NaCl soln. and cut with scissors into a coarse mince that was homogenized in a glass homogenizer with a Teflon plunger in acetone at 4° (20 ml./fat body). The incubation medium was added at this stage and the mixture was rehomogenized and filtered through Whatman no. 1 filter paper on a Buchner funnel. The residue on the filter paper was not allowed to dry but was washed successively with 150 ml. of acetone at 4°, 250 ml. of acetone at room temperature and finally with 250 ml. of ether at room temperature. Excess of ether was removed by suction on the water pump for 1 min. and then, while still moist, the residue was transferred with a spatula from the filter paper to a 10 ml. centrifuge tube. In most of the experiments described the moist preparation was put *in vacuo* at room temperature for 20 min. at this stage, and a homogenate was prepared immediately afterwards. In some, however, the preparations were stored *in vacuo* overnight at 4° before being homogenized. Less than 5% of the activity of the preparation was lost on storage for this time.

Homogenates were prepared by adding cold 25 mN-NH_3, adjusted to pH 8·1 with N-HCl, to the preparation (6 ml./fat-body equivalent) and then stoppering the centrifuge tube and rotating it at 4° on a mechanical turntable for 5–10 min. During this time most of the serum protein went into solution without frothing. The coarse suspension of particles remaining was next homogenized at 4° as above, giving a satisfactory mixture for representative samples to be removed for assay. The enzyme activity was stable for at least 2 hr. at 4° and assays were always started within this time.

Assays were carried out by measuring the amount of FFA produced from chyle triglycerides. The assay system, which is based on one described by Robinson (1963*a*) for the assay of the enzyme in defatted tissue preparations, contained 1·45 ml. of albumin in 0·85% NaCl soln., pH 8·1 (60 mg./ml.), 0·5 ml. of tris buffer, pH 8·1 (0·15 M), 1 ml. of chyle–serum mixture (10 μequiv. of chyle triglyceride fatty acid/ml.), 0·05 ml. of heparin (0·5 unit/ml.) and 1·5 ml. of enzyme preparation; the final concentrations are shown in parentheses. The amount of heparin present gives maximum activity under these assay conditions (Salaman, 1964). A chyle–serum mixture was used to provide the triglyceride substrate on the basis of experiments showing that the rate of FFA release from chylomicron triglycerides is increased in the presence of serum (Salaman, 1964). The chyle and serum were incubated together for 30 min. at 37° at a 1:3 volume ratio before being added to the assay system. The amount of triglyceride present was such that less than 20% was hydrolysed during the assay. Neither it, nor the albumin, limited the rate of hydrolysis, which was linear throughout the assay period.

(*b*) In incubation media. Clearing-factor lipase is extracted from intact adipose tissue into media that contain heparin, and the level of enzyme activity in the medium has been shown to reflect the tissue activity (Robinson, 1960; Salaman & Robinson, 1961). Moreover, in the accompanying paper (Wing *et al.* 1966) it is shown that, when epididymal fat bodies from starved rats are incubated in a medium in which the total (tissue plus medium) clearing-factor lipase activity increases with time of incubation, this increase is accompanied by a progressive rise in the enzyme activity in the medium. Although the precise time-course of the increase in activity in the medium varies in different experiments it seems legitimate to assume that, where paired fat-body systems are being compared, the relationships of the activities of the media will give a valid measure of the relationships of the total activities, at least at the incubation time of 3·5 hr. that was used in the present study. Assays of incubation media are more conveniently performed than the ones described above involving the making of defatted preparations and were used in some of the present experiments. The assay system used for the homogenates of acetone–ether-dried preparations was employed but samples of incubation medium replaced the 1·5 ml. of homogenate. If necessary, the total volume was made up to 4·5 ml. with 0·85% NaCl soln.

When such measurements of the enzyme activity of incubation media were made, the samples of media used frequently contained substances that were already present as constituents of the assay system. However, where these were known to affect the activity of the enzyme, e.g. serum proteins and heparin, it had been shown that the amounts in the assay system were sufficient to produce maximum activation (Salaman, 1964). Glucose, insulin, amino acids and HCO_3^- ions, which were also present in the incubation media, were shown not to affect the assay. Since Korn & Quigley (1955, 1957) have reported an activating effect of Ca^{2+} and Mg^{2+} ions and of NH_4^+ ions on the assay of adipose-tissue clearing-factor lipase, experiments were carried out to determine whether differences in ionic composition that existed between some of the media (see Table 5) would influence the assay. No effects were found. Moreover, when enzyme in the incubation medium was assayed in the presence and absence of NH_4^+ ions no differences in activity were observed. It is possible that, under the present assay conditions, trishydroxymethylmethylammonium ions, from the tris buffer, may be able

to substitute for NH_4^+ ions and produce maximum activation.

All assays were carried out at 37° in a shaking water bath for 1–3 hr., depending on the expected enzyme activity. The pH, which was initially 8·1, did not fall below 8·0 during the incubation period. Duplicate samples (1 ml.) of the test assays, and of control assays without enzyme, were taken at zero time and at the end of the assay for the measurement of FFA by the modification of Dole's method described by Salaman & Robinson (1961) but with 2N-H_2SO_4 to lower the pH sufficiently to extract the FFA (Dole & Meinertz, 1960).

Lipase activities are expressed as μmoles of FFA produced/fat body/hr. of assay incubation. Differences in activity observed with paired fat bodies in the replicate tests of an experiment were subjected to statistical analysis where they numbered four or more. The procedure used was to determine by the *t* test whether the mean of the differences between the paired fat bodies differed significantly from zero, values of $P < 0.05$ being accepted as showing a significant effect.

RESULTS

Demonstration of an increase in clearing-factor lipase activity when fat bodies from starved rats are incubated in vitro

Hollenberg (1959) found that, when epididymal fat bodies from starved rats were incubated at 37° in a medium containing serum from starved animals, heparin and a triglyceride emulsion, the rate of appearance of FFA in the medium was very low, but that if glucose and insulin were added the rate increased. Although this finding may be interpreted as indicating a rise in the activity of clearing-factor lipase in the system during the incubation period in the presence of glucose and insulin, it is clearly necessary to demonstrate directly that an increase in total clearing-factor lipase activity occurs *in vitro* to establish this point. A series of replicate tests were therefore carried out with the paired epididymal fat bodies of five starved rats. In each test one fat body was incubated in a medium containing 2·4 ml. of serum, 0·01 ml. of insulin (3 units/ml.) and 0·1 ml. of heparin (60 units/ml.) for 5 min. and its pair was incubated under the same conditions for 3·5 hr. Total clearing-factor lipase activity was determined in acetone–ether-dried preparations made from each fat body and its medium combined after incubation. The mean activities (μmoles of FFA/fat body/hr.) at 5 min. and 3·5 hr. were 5·4 (range 3·0–8·3) and 8·1 (range 6·4–11·0) respectively. The mean of the differences between paired fat bodies (±S.E.M.) is 2·7 (±0·28), representing a significant difference at the 0·1% level.

When this experiment was repeated with dialysed serum, no increase in clearing-factor lipase activity occurred unless the serum diffusate was also present, suggesting that the requirements in the medium for an increase in enzyme activity were complex. Further studies were therefore carried out that led to the development of a medium containing dialysed serum and heparin, and in which the diffusate was replaced by glucose, insulin, amino acids, sodium hydrogen carbonate and other salts. This medium, with the composition described in the Materials and Methods section, has been called CRM and in Table 2 is shown the increase in clearing-factor lipase activity that occurs when fat bodies from starved rats are incubated in CRM. This increase is at least as great as that observed in the experiment with undialysed serum.

Table 2. *Clearing-factor lipase activity of fat bodies from starved rats incubated* in vitro *in CRM*

Each pair of values refers to a test with the paired fat bodies of one rat. In each test one fat body was incubated at 37° for 5 min. (0 hr.) in 2·5 ml. of CRM, and the paired fat body was incubated under the same conditions for 3·5 hr. After incubation the clearing-factor lipase activity of acetone–ether-dried preparations made from each fat body and medium combined was determined. The differences between the 0 hr. and 3·5 hr. values represent a significant difference at the 0·1% level.

	Total clearing-factor lipase activity (μmoles of FFA/fat body/hr.)	
Incubation time.....	0 hr.	3·5 hr.
	6·8	9·5
	5·6	11·8
	3·4	6·2
	3·9	8·8
	3·0	9·8
	6·1	13·6
	4·4	7·9
	5·7	9·2
	3·0	10·0
Mean	4·7	9·6

Evidence for participation of individual CRM components

Heparin. When fat bodies from starved rats are incubated for 3·5 hr. in CRM from which the heparin has been omitted, no significant increase in the total clearing-factor lipase activity of the incubated system occurs (Table 3).

Glucose, insulin and amino acids. When fat bodies from starved rats are incubated for 3·5 hr. in CRM from which amino acids, glucose and insulin have been omitted, the amount of clearing-factor lipase activity that appears in the medium and the total clearing-factor lipase activity of the fat body and medium combined is less than when they are present (Expts. 1 and 2 in Table 4).

Experiments to differentiate between the effects

Table 3. *Clearing-factor lipase activity of fat bodies incubated* in vitro: *effect of omission of heparin from CRM*

Each experiment consisted of a series of replicate tests with the paired fat bodies of starved rats. The conditions were as described in Table 2 with the incubation periods shown. Where indicated, heparin was absent from CRM during the incubation. In such cases it was added to the medium immediately before the acetone–ether-dried preparations were made.

Expt. no.	Omission from CRM during incubation	Time of incubation (hr.)	Mean total clearing-factor lipase activity (μmoles of FFA/fat body/hr.) (no. of observations in parentheses)	Mean of differences between paired fat bodies ± S.E.M. (*P* value in parentheses)
1	Heparin	0	6·2 (4)	0·1 ± 0·5 (*P* > 0·05)
	Heparin	3·5	6·3 (4)	
2	None	3·5	12·1 (5)	5·4 ± 0·28 (*P* < 0·001)
	Heparin	3·5	6·7 (5)	

Table 4. *Clearing-factor lipase activity of fat bodies incubated* in vitro: *effect of omission of glucose, insulin and amino acids from CRM*

Each experiment consisted of a series of replicate tests with the paired fat bodies of starved rats. The tests were carried out under the conditions described in Table 2, but with omissions from the incubation media as indicated. Incubations were for 3·5 hr. and clearing-factor lipase was assayed either in the incubation medium alone or in acetone—ether-dried preparations made from each fat body and its incubation medium combined (total activities).

Expt. no.	Omission from CRM	Mean clearing-factor lipase activity (μmoles of FFA/fat body/hr.) (no. of observations in parentheses) Medium	Total	Mean of differences between paired fat bodies ± S.E.M. (*P* value in parentheses)
1	None	4·9 (5)	—	3·8 ± 0·80 (*P* < 0·01)
	Glucose, insulin, amino acids	1·1 (5)	—	
2	None	—	9·6 (5)	5·6 ± 0·49 (*P* < 0·001)
	Glucose, insulin, amino acids	—	4·0 (5)	
3	None	—	10·3 (6)	4·2 ± 0·99 (*P* < 0·01)
	Glucose, insulin	—	6·1 (6)	
4	None	—	12·0 (4)	0·8 ± 0·7 (*P* > 0·05)
	Amino acids	—	11·2 (4)	
5	Amino acids	5·3 (6)	—	4·1 ± 0·73 (*P* < 0·01)
	Amino acids, glucose	1·2 (6)	—	
6	Amino acids	4·2 (6)	—	1·6 ± 0·39 (*P* < 0·01)
	Amino acids, insulin	2·6 (6)	—	
7*	Amino acids	4·6 (6)	—	2·6 ± 0·59 (*P* < 0·01)
	Amino acids, insulin	2·0 (6)	—	

* In Expt. 7 the dialysed serum component of the media was obtained from rats that had been starved for 48 hr.

of amino acids on the one hand and glucose and insulin on the other show that, in the absence of glucose and insulin, the total clearing-factor lipase activity after 3·5 hr. is markedly decreased, but that the omission of amino acids produces no significant effect (Expts. 3 and 4 in Table 4). Similar experiments were carried out in which a mixture of amino acids, at their reported concentrations in the serum of fed rats, replaced the casein hydrolysates of CRM and again no significant effect of the omission of amino acids was observed.

The effect of the omission from the incubation medium of glucose and of insulin separately was studied by using the technique of direct assay of the incubation medium. When either glucose alone or insulin alone was omitted from CRM the clearing-factor lipase activity in the medium after incubation for 3·5 hr. was markedly lowered (Expts. 5 and 6 in Table 4), the effect of insulin omission being similar whether the dialysed serum component of the medium was obtained from fed or from starved rats (Expt. 7 in Table 4). Amino acids were not present in the media used in these experiments.

Bicarbonate and other salt ions. When fat bodies

were incubated in CRM from which sodium hydrogen carbonate was omitted, the clearing-factor lipase activity in the medium after 3·5hr. was much less than when it was present (Table 5). These results suggest that HCO_3^- ions play an important role in CRM. However, the possibility cannot be entirely excluded that small differences in the pH of the incubation media were responsible for the observed effects. In the experiment shown in Table 5 the pH fell from 7·4 to 7·0 in the absence of bicarbonate, instead of from 7·4 to 7·2 as in CRM.

Attempts have been made to maintain the pH of the medium in the absence of bicarbonate by the use of other buffers. When imidazole–hydrochloric acid (0·05M) or sodium phosphate (0·1M) buffer solutions replaced bicarbonate in CRM (initial pH 7·6, final pH 7·4) no activity at all was detected in the medium after 3·5hr., and in one experiment in which a glycylglycine–sodium hydroxide buffer solution (0·05M) was substituted for bicarbonate (initial pH 7·4, final pH 7·2) the activity in glycylglycine was 2·0 μmoles of FFA produced/fat body/hr. as compared with a value of 7·7 for the paired fat body incubated in CRM.

Experiments were carried out to determine whether other salt ions in CRM participated in the increase in clearing-factor lipase activity. All the incubation media contained Na^+ and Cl^- ions at the concentrations in which they are present in CRM, but K^+, Ca^{2+}, PO_4^{3-}, Mg^{2+} and SO_4^{2-} ions were variously present or absent. Casein hydrolysate was omitted from all the media since it might have contributed small quantities of the ions under consideration: its effect on the increase in clearing-factor lipase activity is negligible (see Table 4). The results suggest that, in addition to HCO_3^- ions, K^+ ions also play an important part in the increase in clearing-factor lipase activity that occurs on incubation in CRM (Table 5).

Dialysed serum. Experiments were carried out to investigate whether the protein constituents of dialysed serum in CRM participated in the increase in clearing-factor lipase activity. Fat bodies from starved rats were incubated in CRM, or in CRM in which the dialysed serum component was replaced by 0·85% sodium chloride, by albumin, or by casein. In all cases the activity in the medium after 3·5hr. was lower in the absence of serum.

In further experiments in which the dialysed serum of CRM was replaced by casein, serum-lipoprotein fractions were also added. When both high-density (d 1·063–1·21) and low-density (d < 1·063) lipoproteins were present, the clearing-factor lipase activity in the medium after incubation for 3·5hr. was significantly higher than in their absence (Expt. 1 in Table 6). No evidence was obtained for an effect of the high-density fraction alone. The results obtained with the low-density lipoproteins (Expts. 2 and 4 in Table 6) suggest that this fraction may be active alone but further studies are necessary to establish this.

Table 5. *Clearing-factor lipase activity of fat bodies incubated* in vitro: *effect of salt composition of medium*

Each experiment consisted of a series of replicate tests with the paired fat bodies of starved rats. In each test one fat body was incubated at 37° for 3·5hr. in 2·5ml. of a medium with the salt composition of CRM, and the paired fat body was incubated in a medium of modified salt composition. The incubation media were: Expt. 1, CRM or CRM from which the $NaHCO_3$ was omitted and replaced by water; Expts. 2–6, CRM or CRM that lacked various inorganic ions. Amino acids were also lacking from all the media in Expts. 2–6. Media containing $NaHCO_3$ were gassed with O_2+CO_2 (95:5); those without were gassed with O_2. After incubation samples of the media were assayed for clearing-factor lipase.

Expt. no.	Salt composition of incubation medium	Mean clearing-factor lipase activity (μmoles of FFA/fat body/hr.) of incubation medium (no. of observations in parentheses)	Mean of differences between paired fat bodies ± S.E.M. (P value in parentheses)
1	CRM	3·7 (7)	2·6 ± 0·32 (P < 0·001)
	CRM − $NaHCO_3$	1·1 (7)	
2	CRM	5·7 (3)	0·5 ± 0·7
	CRM − NaH_2PO_4	5·2 (3)	
3	CRM	6·2 (3)	0·3 ± 0·8
	CRM − $MgSO_4$ − $CaCl_2$	6·5 (3)	
4	CRM	3·8 (5)	1·8 ± 0·19 (P < 0·001)
	CRM − KCl	2·0 (5)	
5	CRM	6·9 (5)	3·4 ± 0·52 (P < 0·01)
	NaCl, $NaHCO_3$	3·5 (5)	
6	CRM	5·1 (4)	0·4 ± 0·6 (P > 0·05)
	NaCl, KCl, $NaHCO_3$	5·5 (4)	

Table 6. *Clearing-factor lipase activity of fat bodies incubated* in vitro: *effect of serum lipoproteins in medium*

Each experiment consisted of a series of replicate tests, with the paired fat bodies of starved rats, carried out under conditions similar to those described in Table 5. The incubation media were modifications of CRM containing 0·5ml. of 2·8% (w/v) casein in 0·85% NaCl in place of the dialysed serum and, in some cases, a serum low-density-lipoprotein fraction (LDL) or a serum high-density-lipoprotein fraction (HDL) or both in addition. The final concentrations of lipoproteins were calculated to be the same as in CRM. Insulin was present at twice its concentration in CRM and the volume of the medium in each test was made up to 2·5ml. with 0·85% NaCl. After incubation for 3·5hr. samples of the media were assayed for clearing-factor lipase.

Expt. no.	Addition to incubation medium	Mean clearing-factor lipase activity (μmoles of FFA/fat body/hr.) of incubation medium (no. of observations in parentheses)	Mean of differences between paired fat bodies ± S.E.M. (P value in parentheses)
1	None	3·4 (6)	1·7 ± 0·48 ($P < 0·02$)
	LDL, HDL	5·1 (6)	
2	None	3·4 (6)	0·7 ± 0·4 ($P > 0·05$)
	LDL	4·1 (6)	
3	None	3·6 (3)	0·1 ± 0·4
	HDL	3·5 (3)	
4	HDL	3·6 (3)	0·8 ± 0·3
	HDL, LDL	4·4 (3)	
5	LDL	3·6 (3)	0·5 ± 0·1
	LDL, HDL	3·1 (3)	

DISCUSSION

The experiments reported in this paper show that the clearing-factor lipase activity of adipose tissue from starved rats is increased when the tissue is incubated in a defined medium *in vitro*. A full discussion of the possible significance of the results, and in particular of the role in the incubation medium of heparin, glucose and insulin, is given by Wing *et al.* (1966), who also present the results of further studies with the same experimental system. The requirements for HCO_3^- ions and for K^+ ions have not been investigated further, however, and the possible functions of these components in the incubation medium may be considered briefly at this time.

A prominent reaction in adipose tissue requiring HCO_3^- ions is that concerned with the formation of malonyl-CoA from acetyl-CoA during the synthesis of fatty acids (Martin & Vagelos, 1965). Though there is no evidence at present to link fatty acid synthesis directly with clearing-factor lipase action, except insofar as both are increased in tissue from fed animals, it is noteworthy that K^+ ions have also been shown to increase the synthesis of fatty acids from acetate in the liver (Hastings & Longmore, 1965). Their effect on fatty acid synthesis in adipose tissue has not been studied to our knowledge.

A link between the requirement for HCO_3^- and K^+ ions in the incubation medium is also provided by the evidence that both may facilitate glucose utilization, possibly at the stage of glucose 6-phosphate formation. Evidence for such a function has been obtained in studies with both liver and adipose tissue (Hastings & Buchanan, 1942; Zahnd, Dagenais & Thorn, 1960; Hagen, Ball & Cooper, 1959; Minard & Davis, 1961; Hastings & Longmore, 1965), and the possibility that the presence of these components in the incubation medium is necessary for this purpose cannot be excluded at present.

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REFERENCES

Barry, J. M., Bartley, W., Linzell, J. L. & Robinson, D. S. (1963). *Biochem. J.* **89**, 6.

Bezman-Tarcher, A., Otway, S. & Robinson, D. S. (1965). *Proc. Roy. Soc.* B, **162**, 411.

Bragdon, J. H. & Gordon, R. S. (1958). *J. clin. Invest.* **37**, 574.

Cherkes, A. & Gordon, R. S. (1959). *J. Lipid Res.* **1**, 97.

Dole, V. P. & Meinertz, H. (1960). *J. biol. Chem.* **235**, 2595.

French, J. E., Robinson, D. S. & Florey, H. W. (1953). *Quart. J. exp. Physiol.* **38**, 101.

Hagen, J. M., Ball, E. G. & Cooper, O. (1959). *J. biol. Chem.* **234**, 781.

Hastings, A. B. & Buchanan, J. M. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 478.

Hastings, A. B. & Longmore, W. J. (1965). *Advanc. Enzyme Regulation*, **3**, 147.

Havel, R. J., Felts, J. M. & van Duyne, C. M. (1962). *J. Lipid Res.* **3**, 297.

Hollenberg, C. H. (1959). *Amer. J. Physiol.* **197**, 667.
Hollett, C. & Nestel, P. J. (1960). *Amer. J. Physiol.* **199**, 803.
Korn, E. D. (1959). *Meth. biochem. Anal.* **7**, 145.
Korn, E. D. & Quigley, T. W. (1955). *Biochim. biophys. Acta*, **18**, 143.
Korn, E. D. & Quigley, T. W. (1957). *J. biol. Chem.* **226**, 833.
Long, C. (1961). *Biochemists' Handbook*, pp. 58, 873. London: E. and F. N. Spon Ltd.
McBride, O. W. & Korn, E. D. (1963). *J. Lipid Res.* **4**, 17.
Martin, D. B. & Vagelos, P. R. (1965). In *Handbook of Physiology, Section* 5: *Adipose Tissue*, p. 211. Ed. by Renold, A. E. & Cahill, G. F. Washington, D.C.: American Physiological Society.
Minard, F. N. & Davis, R. V. (1961). *J. Lipid Res.* **2**, 369.
Mitchell, J. R. A. (1959). *Lancet*, i, 169.
Páv, J. & Wenkeová, J. (1960). *Nature, Lond.*, **185**, 926.
Renold, A. E. & Cahill, G. F. (1965). *Handbook of Physiology, Section* 5: *Adipose Tissue*. Washington, D.C.: American Physiological Society.
Robinson, D. S. (1960). *J. Lipid Res.* **1**, 332.
Robinson, D. S. (1963*a*). *Advanc. Lipid Res.* **1**, 133.
Robinson, D. S. (1963*b*). *J. Lipid Res.* **4**, 21.
Robinson, D. S. & Harris, P. M. (1961). *Biochem. J.* **80**, 361.
Salaman, M. R. (1964). D.Phil. Thesis: University of Oxford.
Salaman, M. R. & Robinson, D. S. (1961). In *Enzymes of Lipid Metabolism*, p. 218. Ed. by Desnuelle, P. Oxford: Pergamon Press Ltd.
Winegrad, A. I. & Renold, A. E. (1958). *J. biol. Chem.* **233**, 267.
Wing, D. R., Salaman, M. R. & Robinson, D. S. (1966). *Biochem. J.* **99**, 648.
Zahnd, G. R., Dagenais, Y. M. & Thorn, G. W. (1960). *Acta endocr., Copenhagen*, **35** (Suppl. 51), 947.