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Clearing-Factor Lipase in Adipose Tissue

FACTORS INFLUENCING THE INCREASE IN ENZYME ACTIVITY PRODUCED ON INCUBATION OF TISSUE FROM STARVED RATS *IN VITRO*

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1. Evidence is presented that the increase in clearing-factor lipase activity that occurs when adipose tissue from starved rats is incubated in a defined medium *in vitro* is due to an increase in the total enzyme content of the system. It is shown that the clearing-factor lipase activity rises to reach a plateau level where, it is suggested, rates of enzyme synthesis and of enzyme destruction become balanced. 2. The presence of heparin in the incubation medium results in the extraction of part of the clearing-factor lipase originally present in the adipose tissue and this could provide the stimulus for the increase in total enzyme content. 3. Glucose is required in the incubation medium at a very low concentration. It can be replaced by fructose, but not by pyruvic acid, lactic acid, glyceric acid or dihydroxyacetone. 4. Adrenaline and corticotrophin inhibit the increase in enzyme activity when they are present in the incubation medium. 5. The high clearing-factor lipase activity associated with adipose tissue of fed rats is decreased by 50% within 3hr. of the injection of puromycin.

In the preceding paper (Salaman & Robinson, 1966) it was shown that the clearing-factor lipase activity of epididymal fat bodies from starved rats increased when they were incubated *in vitro* in a defined medium that was designated complete reconstituted medium or CRM. In the present paper, additional studies are reported with this incubation system and the nature of the increase in enzyme activity that occurs is investigated.

MATERIALS AND METHODS

Epididymal fat bodies were obtained from male albino rats of the Wistar strain weighing 120–140g. that had been either fed on their normal diet or starved for 48hr. before being killed. The methods used for the incubation of fat bodies *in vitro*, and for the assay of clearing-factor lipase in incubation media and in acetone–ether-dried preparations made from incubation media and fat bodies combined (total activities), were essentially as described by Salaman & Robinson (1966). When heparin was absent from a medium during the incubation period it was added immediately before the acetone–ether-dried preparations were made. Clearing-factor lipase activities are expressed, as by Salaman & Robinson (1966), as μmoles of FFA† liberated from chylomicron triglycerides/fat body/hr. of assay incubation.

Salaman & Robinson (1966) compared the clearing-factor lipase activities of paired fat bodies from individual rats. In an experiment in which right and left fat bodies from five starved rats were incubated separately in CRM for 5min. under the conditions described by Salaman & Robinson (1966), the mean of the arithmetic differences in total activity (±S.D.) between the paired fat-body systems was +0·1 (±0·4) μmoles of FFA/fat body/hr., the mean total activities being 3·9 (right) and 3·8 (left). The corresponding mean difference measured in a similar experiment after incubation for 3·5hr. was −0·5 (±2·3), the mean total activities being 11·0 (right) and 11·5 (left). On the basis of these findings it seemed that the number of replicate tests performed could be reduced by comparing clearing-factor lipase activities of groups of paired fat bodies. This was the usual procedure in the present study, at least four fat bodies, taken alternately from the right and left sides of the animals, being used in each group. Only in Tables 1 and 2 are the activities of unpaired groups of fat bodies compared.

In experiments in which the time-course of the increase in enzyme activity in the incubation medium was followed, serial samples of the medium were assayed for clearing-factor lipase, the volume being maintained by the addition of fresh medium. Allowance has been made for enzyme removed with the samples of medium by adding to the activity (μmoles of FFA/fat body/hr.) determined at each time-interval a correction for the measured activities of samples removed at earlier times. The volumes of the samples of media that were assayed were 0·5–1ml. depending on the enzyme activity present. Though it is recognized that the correction can only be an approximate one, no

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† Abbreviation: FFA, free fatty acids.

appreciable error is likely to be involved, in view of the total volumes of medium used, except in the experiment described in Fig. 1, and in this the form of the time-course curve will not be affected. In experiments in which enzyme activities were measured both in the medium and in acetone–ether-dried preparations made from the medium and tissue combined (total activities), a similar correction was applied in calculating the total enzyme activity.

Post-heparin plasma. Plasma that contained clearing-factor lipase was prepared by centrifuging for 10min. at 2500*g* blood obtained from rats that had been injected intravenously 10min. previously with 50 units of heparin/kg. body wt.

Chemicals. Sources of several of the constituents of the incubation media and of the assay media have been given by Salaman & Robinson (1966). D-Fructose, D-galactose, D-mannose, sodium palmitate, sodium oleate, EDTA, cysteine hydrochloride and L-ascorbic acid were obtained from British Drug Houses Ltd., Poole, Dorset. DL-Glyceric acid and 2-mercaptoethanol were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Adrenaline hydrochloride and adrenaline bitartrate were obtained from Burroughs Wellcome and Co., London, and corticotrophin (Acthar) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. 1,3-Dihydroxyacetone was obtained from Wallerstein Co., Staten Island, N.Y., U.S.A., sodium pyruvate was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, West Germany, and puromycin hydrochloride was from American Cyanamid Co., Pearl River, N.Y., U.S.A. L-Lactate was kindly provided by Mr T. Gascoyne of the Department of Biochemistry, University of Oxford. When included in incubation media these substances were dissolved in the salt solution of the medium before the latter was made up. Injections of puromycin hydrochloride suspension in 0·04M-KH_2PO_4 soln., adjusted to pH7·4 with 2N-NaOH, were made subcutaneously.

RESULTS

Increase in clearing-factor lipase activity on incubation of fat bodies from starved rats in CRM

Time-course. When fat bodies from starved rats are incubated in media such as CRM that contain heparin, part of the clearing-factor lipase of the tissue appears in the medium (Salaman & Robinson, 1966) and, at a medium volume (ml.)/fat body ratio 2·5:1, the proportion of the total enzyme activity that is extracted rises to a maximum of 40–50% after incubation for 3·5hr. (Table 1).

The results in Figs. 1 and 3 show that, under the same conditions, the absolute level of activity in the medium rises, after a short lag period, to a plateau level that is reached after 5–8hr. When considered in relation to the presence of 40–50% of the total enzyme activity in the incubation medium after 5–8hr., these observations suggest that the total clearing-factor lipase activity of the incubation system also rises to a plateau level. This conclusion is supported by the results shown in Table 2, which are taken from experiments carried out at various times during this study. The time-course of the increase in total activity cannot be determined more precisely, because, in a single experiment, only one determination of total activity can be made and because the extent of the increase in activity varies markedly from one experiment to another.

Table 1. *Extraction of clearing-factor lipase when fat bodies are incubated in CRM*

Groups of five fat bodies, each from a starved rat, were incubated in 12·5ml. of CRM for different periods at 37°. Clearing-factor lipase was measured in a sample of the medium and in acetone–ether-dried preparations made from the fat bodies and the rest of the medium combined at the end of the incubation period.

Time of incubation (hr.)	Percentage extraction of enzyme activity	
	Individual values	Mean
1·0	12, 13	12·5
2·5	16, 24	20
3·0	25, 30, 40	32
3·5	38, 40, 44, 50	43
5·0	44, 44, 57	48
6·0	35, 41, 48, 62	46·5
12·0	45, 46, 65	52

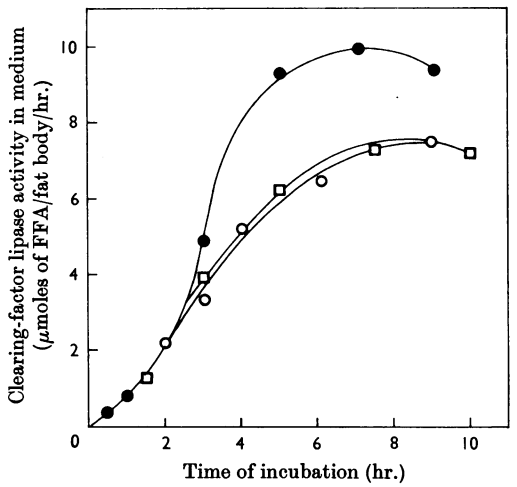

Fig. 1. Time-course of the increase in clearing-factor lipase activity in the incubation medium. In each experiment (○, ●, □) groups of six fat bodies, each from a starved rat, were incubated at 37° in 15ml. of CRM. Samples of the incubation medium were removed at intervals for assay of clearing-factor lipase.

Effect of volume of medium. When fat bodies from starved rats are incubated in 8ml. of CRM/fat body, the total clearing-factor lipase activity reaches a higher level than when 2ml. of medium/fat body

Table 2. *Time-course of increase in total clearing-factor lipase activity when fat bodies are incubated in CRM*

Groups of four or five fat bodies, each from a starved rat, were incubated in CRM (2·5ml. of medium/fat body) at 37°. Clearing-factor lipase was measured in acetone–ether-dried preparations made from the fat bodies and the medium combined at the end of the incubation period. Values are expressed ± S.D. with the ranges in parentheses.

Time of incubation (hr.)	No. of groups	Total clearing-factor lipase activity (μmoles of FFA/fat body/hr.)
0·1	21	5·7 ± 1·6 (3·3–9·8)
3·0	6	11·1 ± 0·9 (9·6–12·0)
3·5	21	13·0 ± 3·3 (7·5–19·7)
5·0	7	10·9 ± 3·0 (7·8–15·8)
6·0	6	11·3 ± 3·3 (7·3–16·3)
10·0	6	14·0 ± 1·8 (10·8–16·1)

Table 3. *Effect of volume of medium on the total clearing-factor lipase activity of fat bodies incubated in CRM*

In each experiment five fat bodies, each from a starved rat, were incubated at 37° in 10ml. of CRM and the five paired fat bodies were incubated in 40ml. of CRM. Clearing-factor lipase activity was measured in acetone–ether-dried preparations made from the fat bodies and the medium combined at the end of the incubation period.

Expt. no.	Time of incubation (hr.)	Clearing-factor lipase activity (μmoles of FFA/fat body/hr.)	
	Vol. of medium ...	10ml.	40ml.
1	3·5	11·9	11·1
2	6	12·9	14·9
3	9	11·1	18·2

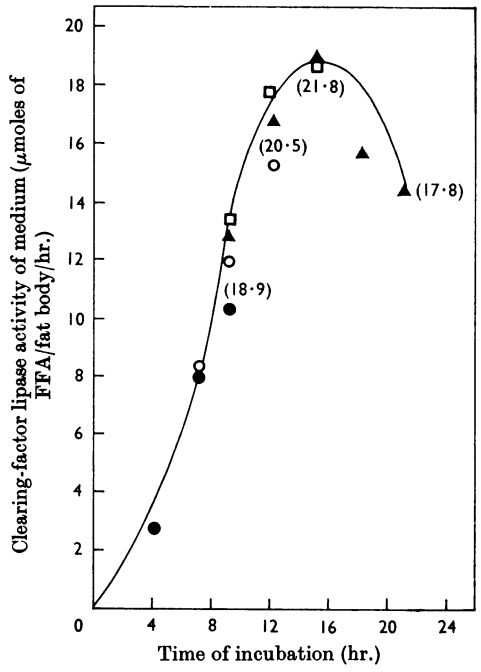

Fig. 2. Increase in clearing-factor lipase activity with a medium volume (ml.)/fat body ratio 8:1. Two experiments were carried out. In each, groups of five fat bodies, each from a starved rat, were incubated at 37° in 40ml. of CRM and a duplicate incubation system was set up with the five paired fat bodies. Small amounts of streptomycin and penicillin were added to the incubation media. The clearing-factor lipase activities of samples of the incubation media were measured at intervals and are shown in the Figure as follows: Expt. 1, ● and ○; Expt. 2, □ and ▲. At the end of each incubation the enzyme activity of an acetone–ether-dried preparation made from the fat bodies and the rest of the medium combined was measured and these values are given in parentheses.

is used, and with the high-volume system maximum activity is reached in the medium only after incubation for 12–18hr. (Table 3 and Fig. 2). This may be compared with the earlier maximum observed when 2·5ml. of medium/fat body is used. With the high-volume system, the percentage of the total activity that is extracted is also increased (Fig. 2).

The total activity of 21·8μmoles of FFA/fat body/hr. achieved in Expt. 2 of Fig. 2 is about 50% of the activity of fat bodies from fed rats (see Table 8).

Effect of gas phase. Replacement of the CRM gas phase, namely oxygen + carbon dioxide (95:5), by air + carbon dioxide (95:5) did not significantly alter the increase in total clearing-factor lipase activity that occurred during a 3·5hr. incubation period at 37°. When the gas phase was nitrogen + carbon dioxide (95:5), however, the final total clearing-factor lipase activity was markedly decreased. In one experiment with groups of paired fat bodies, in which the incubation time was 10hr., the final total activities (μmoles of FFA/fat body/hr.) were 3·6 in nitrogen + carbon dioxide (95:5) and 10·4 in air + carbon dioxide (95:5).

Effect of replacement of glucose in the medium. Salaman & Robinson (1966) showed that the increase in clearing-factor lipase activity when fat bodies from starved rats were incubated in CRM was markedly lowered when glucose was omitted from the medium. Their finding is substantiated and extended by the results of the experiments shown in Table 4. In the absence of glucose from the incubation medium, or when glucose is replaced by pyruvate, dihydroxyacetone or glyceric acid,

there is only a slight rise in the total clearing-factor lipase activity of the system. Enzyme activity still appears in the medium under these conditions. Fructose appears to be an adequate replacement for glucose, but with galactose and mannose the increase in activity is substantially less than with glucose.

When the glucose in CRM was replaced by L-lactate (2·4mg./ml.) the total clearing-factor lipase activity fell from an initial value (μmoles of FFA/fat body/hr.) of 5·5 to a final value of 0·4 after incubation for 3·5 hr. at 37°.

Effect of concentration of glucose in the medium. In Table 5 are shown the results of experiments in which the glucose concentration in CRM was varied over a wide range. Although the uncertainty as to what may be taken as a significant difference between paired fat-body systems makes it impossible to decide at what precise concentration glucose becomes limiting, decreased activities were only regularly observed at concentrations of 0·06mg./ml. and below.

Effect of omission of heparin. Although it was evident from the results of the preceding paper (Salaman & Robinson, 1966) that heparin was an important constituent of CRM it was not clear whether, when fat bodies from starved rats were incubated in its absence, the clearing-factor lipase activity remained constant or increased more slowly than in CRM. These possibilities were therefore investigated further. In a series of experiments, in each of which five fat bodies from starved rats were incubated in 12·5ml. of CRM from which the heparin component had been omitted, the following total clearing-factor lipase activities (μmoles of FFA/fat body/hr.) were recorded at the incubation times shown in parentheses: 8·7, 10·0 and 6·2 (3hr.), 8·3 (5hr.), 9·1 (6hr.), 9·1 (7hr.), 8·9 (9hr.), 6·5 (13hr.) and 4·2 (18hr.). In view of the mean initial activity of 21 groups of unpaired fat bodies of 5·7 μmoles of FFA/fat body/hr. (see Table 2), it seems that, in the absence of heparin, clearing-factor lipase activity may increase slightly to a maximum. No clearing-factor lipase activity could be detected in the medium in these experiments.

Effect of adrenaline, corticotrophin and free fatty acids. Both adrenaline and corticotrophin inhibited the increase in clearing-factor lipase activities of fat

Table 4. *Effect of replacement of glucose in the medium on the clearing-factor lipase activity of fat bodies incubated* in vitro

The incubation media were modifications of CRM in which the glucose was replaced by the substances shown, each at a final concn. of 2·4mg./ml. Four fat bodies, each from a starved rat, were incubated in 10ml. of a medium for 5min. (0hr.) at 37° and the four paired fat bodies were incubated in the same medium for 3·5hr. Clearing-factor lipase was measured in acetone–ether-dried preparations made from the fat bodies and medium combined at the end of the incubation period.

	Total clearing-factor lipase activity (μmoles of FFA/fat body/hr.)													
Glucose replaced by.....	Absent		Pyruvate		Pyruvate+ dihydroxyacetone		Pyruvate+ glyceric acid		Fructose		Mannose		Galactose	
Time of incubation (hr.).....	0	3·5	0	3·5	0	3·5	0	3·5	0	3·5	0	3·5	0	3·5
	5·9	6·2	6·4	8·2	4·5	6·5	3·5	5·7	5·3	14·4	4·2	6·7	3·3	6·1
	6·3	7·6	5·8	6·4	4·5	7·8	4·9	7·3	3·5	8·3				
			5·6	6·9					7·5	14·2				

Table 5. *Effect of the concentration of glucose in the medium on the clearing-factor lipase activity of fat bodies incubated* in vitro

Comparisons of enzyme activity are made between paired groups, each containing five fat bodies from starved rats. The incubation media were modifications of CRM containing glucose at the concentrations shown. The volume of medium was 12·5ml. and incubation was for 3·5hr. at 37°. At the end of the incubation, clearing-factor lipase was measured in acetone–ether-dried preparations made from the fat bodies and medium combined.

	Total clearing-factor lipase activity (μmoles of FFA/fat body/hr.)													
Concn. of glucose (mg./ml.).....	1·6	0·75	1·6	0·5	1·6	0·25	1·6	0·06	1·6	0·02	1·6	0·01	2·4	0
	13·6	15·2	14·7	13·3	9·8	12·4	9·6	7·0	12·9	9·7	13·2	9·5	11·5	8·2
							9·2	8·9	9·5	8·4	17·0	12·4	13·1	7·4
									12·4	11·4	10·6	9·8	12·4	8·9
													10·3	6·7

Table 6. *Effect of adrenaline, corticotrophin and free fatty acids on the clearing-factor lipase activity of fat bodies incubated in CRM*

Four fat bodies, each from a starved rat, were incubated in 10ml. of CRM for 3·5hr. at 37° and the four paired fat bodies were incubated under the same conditions but in CRM containing added adrenaline hydrochloride or bitartrate (0·1μmole/ml.), corticotrophin (1·35 i.u./ml.), sodium oleate (1·4μmoles/ml.) or sodium palmitate (1·4μmoles/ml.). When oleate and palmitate were added, Ca^{2+} and Mg^{2+} were omitted from the salt soln. included in CRM. After incubation, the clearing-factor lipase activity of acetone–ether-dried preparations made from the fat bodies and the medium combined was determined. Samples of each incubation medium were taken before adding the fat bodies and at the end of the incubation for the determination of FFA. The initial FFA concentration in the medium and the change in concentration during the incubation (μmoles/ml.) are given in parentheses.

Incubation medium	Total clearing-factor lipase activity (μmoles of FFA/fat body/hr.)							
	CRM	CRM + adrenaline	CRM	CRM + corticotrophin	CRM	CRM + oleate	CRM	CRM + palmitate
	12·3	5·9*	10·1	6·5	14·9	12·7	10·7	10·0
	(0·45; +0·24)	(0·45; +0·78)	(0·34; +0·18)	(0·34; +0·45)	(0·34; +0·38)	(1·37; −0·53)	(0·34; +0·14)	(1·34; −0·23)
	10·2	5·6*	17·6	11·6	17·3	16·0		
	(0·34; +0·34)	(0·34; +0·49)	(0·45; +0·39)	(0·45; +0·53)	(0·34; +0·28)	(1·37; −0·46)		
	10·6	5·4†						

* Adrenaline bitartrate. † Adrenaline hydrochloride.

bodies from starved rats incubated in CRM (Table 6). The concentrations of hormones used were sufficient to raise the FFA concentration of the medium during the incubation period, presumably through their activation of the lipase that acts on the adipose tissue triglycerides *in situ* (Rizack, 1965). However, when sodium oleate or sodium palmitate was added to CRM in amounts sufficient to maintain an FFA concentration in the medium at least as great as that finally achieved in the presence of the hormones, no inhibition of the increase in clearing-factor lipase activity was observed. It is noteworthy that there is an increase in the FFA concentration of the medium when fat bodies from starved rats are incubated in CRM.

Evidence that the increase in clearing-factor lipase activity when fat bodies from starved rats are incubated in CRM is due to a rise in the enzyme content of the system

Effects of puromycin. Puromycin is known to be a general inhibitor of protein synthesis (Greengard, 1963) and its effect on the increase in clearing-factor lipase activity observed when fat bodies from starved rats are incubated *in vitro* was studied. When puromycin was present from the beginning of the incubation in CRM the total enzyme activity did not rise but instead fell to a low level (Table 7).

The addition of puromycin when fat bodies had already been incubated in CRM for several hours also caused a decline in enzyme activity, both in the tissue and in the incubation medium. Thus when a group of five fat bodies from starved rats was incubated in 12·5ml. of CRM for 8hr. before puromycin (0·5mg./ml.) was added, the clearing-factor

Table 7. *Effect of puromycin on the clearing-factor lipase activity of fat bodies incubated in CRM*

In each experiment comparisons of enzyme activity are made between paired groups, each containing five fat bodies from starved rats. The incubation media were CRM or CRM containing puromycin (0·5mg./ml.). The volume of medium was 12·5ml. and incubation was for the periods shown at 37°. At the end of the incubation, clearing-factor lipase was measured in acetone–ether-dried preparations made from the fat bodies and medium combined.

Expt. no.	Time of incubation (hr.)	Total clearing-factor lipase activity (μmoles of FFA/fat body/hr.)	
		Puromycin present	Puromycin absent
1	0·1	6·4	
	3·5	2·3	
2	6·5	1·8	10·5
3	6·5	1·2	11·7

lipase activities (μmoles of FFA/fat body/hr.) of the tissue and medium assayed after incubation for a further 4hr. were 0·3 and 2·8, by comparison with activities of 7·6 and 6·3 in the tissue and medium of the paired fat-body system that was incubated in the absence of puromycin for the whole 12hr. period. Puromycin (0·5mg./ml.) was shown to have no effect on the assay of clearing-factor lipase.

Significance of the plateau level of clearing-factor lipase activity. The foregoing observations with puromycin suggest that the rise in total clearing-factor lipase activity in CRM arises from an increase in the enzyme content of the system, and that the plateau level of enzyme activity that is eventually reached is due to a balance between enzyme formation and destruction. Evidence consistent with this view is provided by the experiments shown in Fig. 3. In Expt. 1 (Fig. 3*a*) two groups of paired fat bodies from starved rats were incubated separately in CRM (systems *A* and *B*). After 6hr., one group of bodies was transferred to fresh CRM (*A'*) and the other was left in its original medium. Both systems (fat bodies *A* and medium *A'*, fat bodies *B* and medium *B*), as well as medium *A*, were then incubated for a further 6hr. During the second incubation period the clearing-factor lipase activity in medium *B* remained high, that in medium *A* fell and that in medium *A'* rose to a level at least as great as that in medium *B*.

The proportion of the rise in activity in medium *A'* that was due to the extraction of enzyme present in the fat bodies at the time of their transference could not be determined in Expt. 1 (Fig. 3*a*). In Expt. 2 (Fig. 3*b*) therefore both groups of fat bodies (*A* and *B*) were transferred after incubation for 6hr. in CRM, one into fresh CRM (*A'*) and the other into CRM in which the glucose component had been replaced by pyruvate (*B'*). In medium *B'* enzyme activity is maintained but not increased appreciably (see Table 4). The clearing-factor lipase activities (μmoles of FFA/fat body/hr.) of the medium and of the fat body and medium combined at the end of the incubation were 8·4 and 11·1 respectively in system *A'* and 4·7 and 4·7 respectively in system *B'*. It therefore appears that an increase in total activity of about 6μmoles of FFA/fat body/hr. occurred in medium *A'* during the second incubation, a similar increase to that normally observed when fat bodies from starved rats are first incubated in CRM. It is noteworthy that in medium *B'* all the enzyme activity at the end of the second incubation was in the medium.

Direct evidence that the total clearing-factor lipase activity increases during the second incubation is provided by the results of experiments in which groups of six paired fat bodies from starved rats were first incubated in 15ml. of CRM for 5hr. The activity of one group of fat bodies was determined at this time and the paired group was transferred to fresh CRM and incubated for a further 3hr.

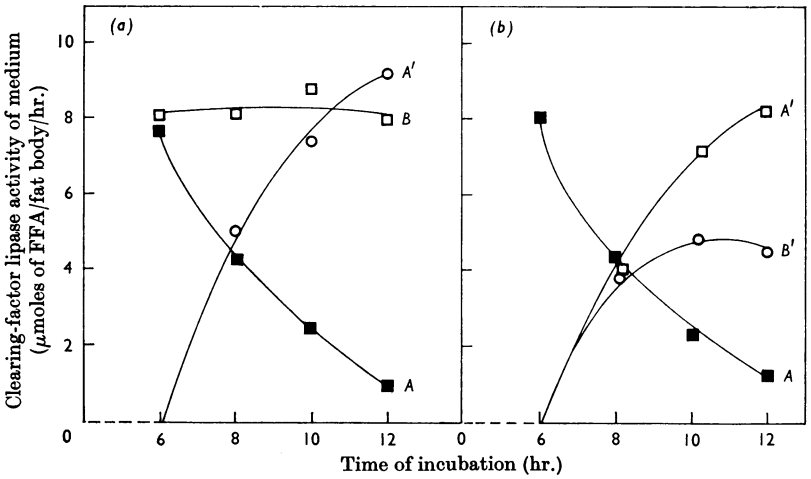

Fig. 3. Incubation of fat bodies in CRM: effect of replacement of medium. (*a*) Expt. 1. Six fat bodies, each from a starved rat, were incubated in 15ml. of CRM (medium *A*) at 37° for 6hr. and then transferred, after being rinsed in 0·85% NaCl soln., to 15ml. of fresh CRM (medium *A'*) and incubated for a further 6hr. Medium *A* alone was also incubated for a further 6hr. The six paired fat bodies were incubated in 15ml. of CRM (medium *B*) for 12hr. (*b*) Expt. 2. A similar procedure was followed except that, at 6hr., both groups of fat bodies were transferred to fresh media and incubated for a further 6hr. The fresh media were 15ml. of CRM (medium *A'*) or 15ml. of a medium (medium *B'*) in which the glucose of CRM was replaced by sodium pyruvate (2·4mg./ml.). Samples were taken from the incubation media at intervals for assay of clearing-factor lipase.

In two such experiments, the activities (μmoles of FFA/fat body/hr.) of the fat bodies at 5hr. were 4·8 and 3·8, whereas the activities of the paired fat bodies and the incubation medium combined at 8hr. were 8·3 and 14. At 8hr., 51 and 44% of the total activity was in the medium.

If our interpretation of Expts. 1 and 2 in Fig. 3 is correct, the rate of formation of clearing-factor lipase between 6 and 12hr. in system *B* of Expt. 1 (Fig. 3*a*) must be at least as great as the initial rate of decline in enzyme activity in medium *A*. And if enzyme activity is being destroyed within the fat bodies, as well as in the medium (and we have no evidence on this point at present), it will be greater than this.

Experiments were carried out to determine whether any of the constituents of the medium are exhausted after incubation for several hours at 37°. Fat bodies from starved rats were incubated for 6hr. in CRM and then more glucose and insulin were added to the medium in amounts equal to those originally present. The total clearing-factor lipase activity after incubation for a further 6hr. was not significantly different from that of a similar system containing the paired fat bodies incubated for 12hr. but without the additional glucose and insulin. When more heparin was added after 6hr., this also was found to have no significant effect on the total enzyme activity at 12hr.

Decline of clearing-factor lipase activity at 37°. The addition of EDTA (1mM), 2-mercaptoethanol (10mM), cysteine (10mM) or ascorbic acid (0·2mM) to samples of CRM in which fat bodies from starved rats had been incubated for several hours did not alter the rate of decline of clearing-factor lipase activity when the medium was subsequently incubated alone at 37°. Nor did replacement of the oxygen + carbon dioxide (95:5) gas phase by nitrogen + carbon dioxide (95:5) have any effect. When chyle was added, however, the rate of loss of enzyme activity was decreased, in one experiment by 50% and in another by 100%.

The clearing-factor lipase activity of post-heparin plasma and of extracts of acetone–ether-dried preparations made from fat bodies of fed rats has been found to fall exponentially at 37° at a rate similar to that shown in Fig. 3. It seems reasonable on present evidence to assume that this loss of activity is due to the instability of the enzyme under these conditions, rather than to postulate a specific enzyme that destroys clearing-factor lipase and that is present in all the above enzyme preparations.

Studies on epididymal fat bodies from fed rats

Fat bodies from rats fed on their normal diet have a much higher clearing-factor lipase activity than those from starved animals (Robinson, 1963). The results in Table 8 show that the activity falls when the fat bodies are incubated in CRM at 37°. Decline of activity also occurs in the absence of heparin, when little enzyme is extracted from the tissue. In the presence of puromycin both the percentage decline and the absolute loss of activity are increased.

Puromycin also causes a marked and rapid decline in the clearing-factor lipase activity of the epididymal fat bodies of fed rats *in vivo*. When puromycin hydrochloride in phosphate buffer was injected in three doses, each of 10mg., at 1·25hr. intervals to four rats, the activity of an acetone–ether-dried preparation made from the grouped fat bodies 3hr. after the initial injections was 16μmoles of FFA/fat body/hr. In a control group of animals, injected with the buffer solution only, the corresponding activity at 3hr. was 30μmoles of FFA/fat body/hr.

DISCUSSION

A rise in the activity of an enzyme in a tissue preparation *in vitro* need not necessarily be the result of an increase in the amount of enzyme present (Weber, 1963). There are several reasons,

Table 8. *Effect of incubation* in vitro *on the clearing-factor lipase activity of fat bodies from fed rats*

Groups of five fat bodies, each from fed rats, were incubated in 12·5ml. of medium at 37° for 5min. (0hr.) and the five paired fat bodies were incubated under the same conditions for 3·5hr. The incubation medium was CRM or a modification thereof in which heparin was omitted or puromycin (final concn. 0·5mg./ml.) was added. After incubation the clearing-factor lipase activity of acetone–ether-dried preparations made from the fat bodies and medium combined was determined.

	Total clearing-factor lipase activity (μmoles of FFA/fat body/hr.)							
Incubation medium	CRM		CRM − heparin		CRM + puromycin		CRM − heparin + puromycin	
Time of incubation (hr.)	0	3·5	0	3·5	0	3·5	0	3·5
	61	38	46	22	58	16	64	21
	41	34	26	13	52	18	34	3

however, for assuming that the rise in clearing-factor lipase activity described above is associated with the synthesis of new enzyme protein. Thus it does not occur under anaerobic conditions, it takes place over a period of several hours and, most significantly, it is prevented by puromycin, a general inhibitor of protein synthesis (Greengard, 1963). It is therefore provisionally assumed in the following discussion that the increase in clearing-factor lipase activity when fat bodies from starved rats are incubated in CRM *in vitro* is due to a corresponding rise in the amount of enzyme present in the system.

The falls in the clearing-factor lipase activity of epididymal fat bodies that have been found *in vivo* after the injection of puromycin into fed rats, and *in vitro* when fat bodies from starved rats are incubated in the presence of puromycin, suggest that the amount of the enzyme in this tissue is normally determined by a balance between enzyme formation and inactivation. Alterations in amount may therefore occur by a change in the rate of either of these processes. Whether the rise in total clearing-factor lipase activity when fat bodies from starved animals are incubated in CRM is the result of an increase in the rate of enzyme synthesis or a decrease in the rate of its inactivation cannot, however, be determined on present evidence. Although the pattern of increase in activity, namely a rise to a plateau level, is similar to that which Schimke, Sweeney & Berlin (1965), in their studies on the tryptophan pyrrolase of rat liver, have interpreted on kinetic grounds as indicating an increased rate of enzyme synthesis, the situation in the fat body–CRM system is complicated by the appearance of part of the enzyme in the incubation medium and its destruction there.

Role of enzyme extraction in the rise of clearing-factor lipase activity in CRM

The extent of the increase in clearing-factor lipase activity when fat bodies from starved rats are incubated in CRM is diminished when heparin is omitted from the medium. Heparin is necessary for extraction of the enzyme from the tissue and it could be argued that the extraction process results in a lowering of the amount of clearing-factor lipase in the tissue and that this provides the stimulus for the formation of more enzyme. Consistent with this view would be the observations that, though the total enzyme activity rises when fat bodies are incubated in CRM, the tissue activity shows little change, and that, when the medium volume/fat body ratio is raised, the higher level of total enzyme activity that is reached is largely accounted for by the rise in activity in the medium. On the other hand, in related studies (D. R. Wing, M. R. Salaman & D. S. Robinson, unpublished work), in which a rise of clearing-factor lipase activity was observed when fat bodies from starved rats were incubated *in vitro* in the presence of actinomycin (Eagle & Robinson, 1964), the increase in enzyme activity was not dependent on the presence of heparin in the medium.

Role of components of the incubation medium in the rise of clearing-factor lipase activity

If the rises in the clearing-factor lipase content of adipose tissue from starved rats that occur *in vivo* on re-feeding (Robinson, 1963), and *in vitro* on incubation of the tissue in CRM, are each due to changes in the rates of enzyme formation or destruction, the particular components of the medium found to be important in the system *in vitro* could also play a role *in vivo*. For this reason the possible modes of action of certain of the individual components of CRM are examined below in some detail.

Heparin. There is some evidence that heparin, or a heparin-like substance, may form an integral part of clearing-factor lipase (Korn, 1957). If heparin enters the adipose-tissue cells from the incubation medium in the present experiments (and there is no evidence on this point at present) it could provide an essential component of the active enzyme at its site of synthesis. Alternatively, since heparin has been shown to stabilize the clearing-factor lipase of post-heparin plasma (Robinson, 1956), its action might be that of stabilizing newly formed enzyme. The possibility that endogenous heparin in the fat bodies, possibly derived from mast cells that are present in the tissue (Sheldon, 1965), could be a physiological regulator of the amount of clearing-factor lipase may require further consideration (Jennings, Florey, Robinson & Salaman, 1963).

Serum lipoproteins. The extent of the rise in clearing-factor lipase activity in CRM is decreased when serum lipoproteins are omitted from the medium (Salaman & Robinson, 1966). In view of the protection against enzyme inactivation now shown to be afforded by chylomicra, and the evidence of previous studies showing that the enzyme in post-heparin plasma is protected against loss of activity under certain conditions in the presence of chylomicra (Robinson, 1956), it seems possible that this lipoprotein effect may be due to substrate stabilization of enzyme that appears in the medium.

Glucose. Two characteristics of the requirement for glucose in the incubation medium are noteworthy. One is the low concentration in which it is required and the other is the inability of pyruvic acid, lactic acid, dihydroxyacetone or glyceric acid

to replace it. If glucose is not functioning primarily as an energy source it may be providing some specific component needed for enzyme synthesis, and if heparin cannot enter the tissue cells (i.e. its major role in CRM is that of causing enzyme extraction) but is nevertheless an integral part of the enzyme, this component could be the glucuronic acid or glucosamine constituents of the heparin moiety.

Changes in the concentration of glucose in the plasma seem unlikely to be concerned in regulating the amount of clearing-factor lipase in adipose tissue *in vivo*. The plasma concentration of glucose in the starved rat is about 0·75mg./ml. (D. R. Wing, M. R. Salaman & D. S. Robinson, unpublished work), and, though lower than in the fed animal (about 1·5mg./ml.), is much higher than that which limits the increase in enzyme activity in CRM.

Insulin, adrenaline and corticotrophin. Salaman & Robinson (1966) showed that, when fat bodies from starved rats were incubated in CRM from which the insulin component had been omitted, the rise in the clearing-factor lipase activity of the incubation medium was markedly lowered. This observation has been confirmed in studies in which total enzyme activities were measured (D. R. Wing, M. R. Salaman & D. S. Robinson, unpublished work). Both the concentration of insulin in the plasma and the activity of clearing-factor lipase in adipose tissue fall in the starved animal (Randle & Taylor, 1961; Robinson, 1963) and in the alloxan-diabetic animal (Páv & Wenkeová, 1960; Schnatz & Williams, 1963; Kessler, 1963). The plasma insulin concentration could therefore be a factor controlling clearing-factor lipase activities in adipose tissue *in vivo*. However, Salaman & Robinson (1966) showed that the insulin requirement in CRM could be demonstrated whether the serum component of the incubation medium was obtained from fed or from starved rats. It is possible that the insulin activity of the serum was decreased on its dialysis, since insulin is known to be readily inactivated (Randle & Taylor, 1961), and that an insulin requirement was displayed regardless of the serum source for this reason. However, further studies are evidently required before the possibility of a regulating action of the plasma insulin concentration *in vivo* can be accepted. Such a function would need to be restricted to the enzyme in adipose tissue since in other tissues in the rat, e.g. in heart muscle, clearing-factor lipase activity is not appreciably decreased after starvation for 48hr. (Bjorntorp & Furman, 1962; Robinson & Jennings, 1965).

Recent evidence has suggested that insulin, on the one hand, and adrenaline and corticotrophin, on the other, may respectively inhibit and promote the mobilization of fatty acids from adipose tissue, possibly through their action on the lipase that is concerned with the hydrolysis of the adipose-tissue triglycerides *in situ* (Jungas & Ball, 1963; Rizack, 1965). The present findings that insulin augments, whereas adrenaline and corticotrophin inhibit, the rise in clearing-factor lipase activity in the fat body–CRM system raise the further possibility that these hormones may also play a role in controlling the deposition of triglycerides in adipose tissue through their regulation of the tissue clearing-factor lipase activity.

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