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Clearing-Factor Lipase in Adipose Tissue

STUDIES WITH PUROMYCIN AND ACTINOMYCIN

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1. When adipose tissue from starved rats is incubated in a medium containing glucose, insulin, heparin and actinomycin (5 μg./ml.) the total clearing-factor lipase activity of the system increases at least tenfold over a period of 9 hr. In the absence of actinomycin, enzyme activity also increases, but to a lesser extent and for only about 3 hr. Some enzyme activity appears in the incubation medium in both the presence and the absence of actinomycin. 2. When the glucose and insulin of the incubation medium are replaced by pyruvate and heparin is omitted, an increase in the total clearing-factor lipase activity in the presence of actinomycin still occurs, but only after a lag of several hours. When only heparin is omitted from the medium, the rise in enzyme activity begins immediately, but there is a shoulder in the time-course curve after a few hours. In the absence of heparin, little enzyme activity appears in the incubation medium. 3. The increases in enzyme activity in the presence of actinomycin are prevented if puromycin (0·5 mg./ml.) is present in the incubation medium. 4. Catecholamines and corticotrophin inhibit the increase in enzyme activity caused by actinomycin. 5. The clearing-factor lipase activity of adipose tissue from fed animals declines with a half-life of between 1 and 1·5 hr. when the tissue is incubated in the presence of puromycin. The clearing-factor lipase activity of adipose tissue from starved animals is stable under similar circumstances, as is the raised activity found after such tissue has been incubated in the presence of actinomycin. 6. Clearing-factor lipase extracted from adipose tissue of fed animals is less stable in solution than that extracted from the tissue of starved animals after this has been incubated in the presence of actinomycin.

The enzyme clearing-factor lipase or lipoprotein lipase is thought to play an important physiological function in determining the pattern of uptake of triglyceride fatty acids from the bloodstream by the extrahepatic tissues (see Robinson, 1963). Consistent with such a role is the finding that in rat adipose tissue the activity of the enzyme is high in the fed state but falls to a low value on starvation (Hollenberg, 1959; Cherkes & Gordon, 1959; Páv & Wenkeová, 1960; Robinson, 1960; Salaman & Robinson, 1961). In the starved animal, in contrast with the fed, triglyceride fatty acids are not removed from the blood by this tissue (Bragdon & Gordon, 1958).

In recent studies (Salaman & Robinson, 1966; Wing, Salaman & Robinson, 1966) it was shown that the low enzyme activity characteristic of adipose tissue from starved animals rises progressively to a higher plateau value when such tissue is incubated *in vitro* in a suitable medium. The increase in activity is prevented by puromycin and, since puromycin was also shown to lower the high activity of clearing-factor lipase in adipose tissue from fed animals *in vivo*, it was suggested that the enzyme in this tissue is inducible, its activity in various physiological states being regulated by alterations in the rates of synthesis and breakdown.

In a preliminary report (Eagle & Robinson, 1964) it was shown that there is also a progressive rise in the low clearing-factor lipase activity of adipose tissue from starved rats after the injection of actinomycin D *in vivo* and when the tissue is incubated *in vitro* in suitable media containing this substance. The increase in activity *in vitro* is greater than that occurring in the absence of actinomycin, and is also prevented by puromycin. Since actinomycin D inhibits DNA-dependent RNA synthesis (Reich & Goldberg, 1964) these observations were unexpected and appeared to merit further investigation. In the present work

various factors influencing the increase in clearing-factor lipase activity brought about by actinomycin D and the stability of the enzyme have been studied.

MATERIALS AND METHODS

Animals. Epididymal fat bodies were taken from male albino rats of the Wistar strain. These had been either starved for 48hr. or given their normal laboratory diet before being killed. When fed rats were used, the experiments were started between 8 a.m. and 9 a.m. The animals weighed 120–145g. before starvation and 100–130g. afterwards.

The clearing-factor lipase activities of samples of epididymal adipose tissue from fed and from starved rats have been found to be somewhat lower in the present experiments than in those reported by Salaman & Robinson (1966) and Wing *et al.* (1966). The rats were of the Wistar strain throughout, but their diet was Oxoid modified 41B in the earlier studies whereas it was an Oxoid pasteurized breeding diet in this work. Moreover, the rats were housed in a different animal house in the present study and the air temperature was 23° instead of 21°. Any of these factors could be responsible for the differences in enzyme activity.

Techniques of incubation of fat bodies and assay of clearing-factor lipase. Most of the experiments that are reported involved the incubation of groups of epididymal fat bodies in particular media and the subsequent assay of the total clearing-factor lipase activity either of the whole incubation system or of a proportion of the system.

When heparin was a component of the incubation medium, enzyme was extracted from the fat bodies during the course of the incubation (Salaman & Robinson, 1966). Assay of the total clearing-factor lipase activity therefore required the measurement of enzyme activity in the medium as well as in the tissue. This was done by making acetone–ether-dried preparations from fat bodies and medium combined and determining the enzyme activity in samples of a homogenate of such preparations (Salaman & Robinson, 1966). When heparin was not a component of the incubation medium, enzyme was not extracted from the tissue (Wing *et al.* 1966) and total clearing-factor lipase activities could be measured by assaying the enzyme in acetone–ether-dried preparations made from the fat bodies alone. Nevertheless, it was convenient to include the medium when making the acetone–ether-dried preparations because, in this way, the weight of the preparations was increased and their handling and sampling for assay were thereby facilitated.

To follow the time-course of changes in total clearing-factor lipase activity two procedures were used. In the first, a large number of fat bodies were incubated in a relatively large volume of medium and proportions of the fat bodies and medium were removed at various times, leaving the ratio of the number of fat bodies to the volume of the medium in the remainder of the system unchanged. An acetone–ether-dried preparation for total clearing-factor lipase assay was then made from each sample of fat bodies plus incubation medium. In the second procedure, smaller numbers of fat bodies were incubated separately for different periods and an acetone–ether-dried preparation of each system was made and assayed for enzyme activity.

In some experiments, the activity of clearing-factor lipase in an incubation medium was measured. In such cases samples of the medium were taken for direct assay of the enzyme (Salaman & Robinson, 1966); an allowance was made in the way described by Wing *et al.* (1966) for the effect of enzyme removed in the samples of incubation medium on the total enzyme activity, when this was to be determined at a later stage.

The incubation media used are described as modifications of that (CRM*) described by Salaman & Robinson (1966); this medium had the 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 solution. The salt solution contained NaCl, KCl, $CaCl_2$, NaH_2PO_4 and $MgSO_4$ in the amounts given in Table 1 of Salaman & Robinson (1961). Penicillin and streptomycin were present in all the incubation media at concentrations of approximately 0·1mg./ml.

Incubations were carried out in stoppered flasks at 37° and at pH7·3–7·5 under $O_2 + CO_2$ (95:5). In some experiments comparisons of enzyme activity were made between systems containing groups of paired fat bodies by the technique described by Wing *et al.* (1966). In this technique, fat bodies, taken alternately from the right and left sides of the animals, were distributed in equal numbers to each incubation system. In other experiments, fat bodies from a large group of rats were distributed at random into different incubation media. When large numbers of fat bodies had to be collected before the incubations were started, they were stored at room temperature in gassed CRM from which the heparin, glucose and insulin had been omitted. This storage period never exceeded 15min. The fat bodies were then rinsed thoroughly in 0·9% NaCl and put into their respective incubation media.

When fat bodies were transferred from one incubation medium to another, they were thoroughly rinsed at least three times in large volumes of 0·9% NaCl and blotted on filter paper before being put into the fresh medium.

In the experiment to determine the effect of starvation on the clearing-factor lipase activity of epididymal adipose tissue, the fat bodies were put into a solution of albumin (5%, w/v) and heparin (2·4units/ml.) in the salt solution of CRM, pH7·4, 2·5ml. of medium for each fat body. Acetone–ether-dried preparations were then made as described by Salaman & Robinson (1966) and Wing *et al.* (1966). Assays of acetone–ether-dried preparations were carried out after these had been stored overnight *in vacuo* at 4°. Less than 5% of the activity of the preparations was lost under these conditions. Clearing-factor lipase was assayed in incubation media immediately after the samples were taken. All clearing-factor lipase activities were expressed, as in the study of Salaman & Robinson (1966), as μmoles of FFA released from chylomicron triglycerides/fat body/hr. of assay incubation. The modification of Dole's method described by Salaman & Robinson (1961) was used to determine FFA, but with 2N-H_2SO_4 to lower the pH sufficiently to extract the FFA quantitatively (Dole & Meinertz, 1960).

Chemicals. Sources of most of the constituents of the incubation media and assay media have been given by

* Abbreviations: CRM, complete reconstituted medium; FFA, free fatty acid(s).

Salaman & Robinson (1961) and Wing *et al.* (1966). Puromycin hydrochloride was obtained from American Cyanamid Co., Pearl River, N.Y., U.S.A., and the actinomycin D was a gift from Merck, Sharp and Dohme, Rahway, N.J., U.S.A. Neither puromycin nor actinomycin affected the assay of clearing-factor lipase, whether added before the acetone–ether-dried preparations were made or directly to the assay media.

RESULTS

Increase in clearing-factor lipase activity when epididymal fat bodies from starved rats are incubated in the presence of actinomycin D

Time-course of increase in enzyme activity. When Eagle & Robinson (1964) studied the increase in clearing-factor lipase activity that occurs when fat bodies from starved rats are incubated in the presence of actinomycin, they made use of the fact that, since the incubation media contained heparin, a proportion of the enzyme was extracted from the tissue (see Robinson, 1960; Salaman & Robinson, 1961; Wing *et al.* 1966). Thus in most experiments they followed only the rise that occurred in the enzyme activity of the medium. In the present study it was necessary to determine the precise relationship between the increase in the medium and the increase in the total enzyme activity (see the Materials and Methods section) of the incubation system, and this was done in the experiment shown in Fig. 1(*a*). In the presence of actinomycin, total enzyme activity rises from that of the tissue in the starved animal at an approximately linear rate for at least 9hr., when it reaches a value similar to that found in tissue from fed animals (see Fig. 8). Activity in the medium also rises progressively as the incubation proceeds, but at a lower rate.

The medium used in this experiment was that (CRM) described by Salaman & Robinson (1966) and Wing *et al.* (1966) and, as they showed, there is a progressive increase in total clearing-factor lipase activity when the fat bodies from starved rats are incubated in this medium containing no actinomycin. However, the time-course and the

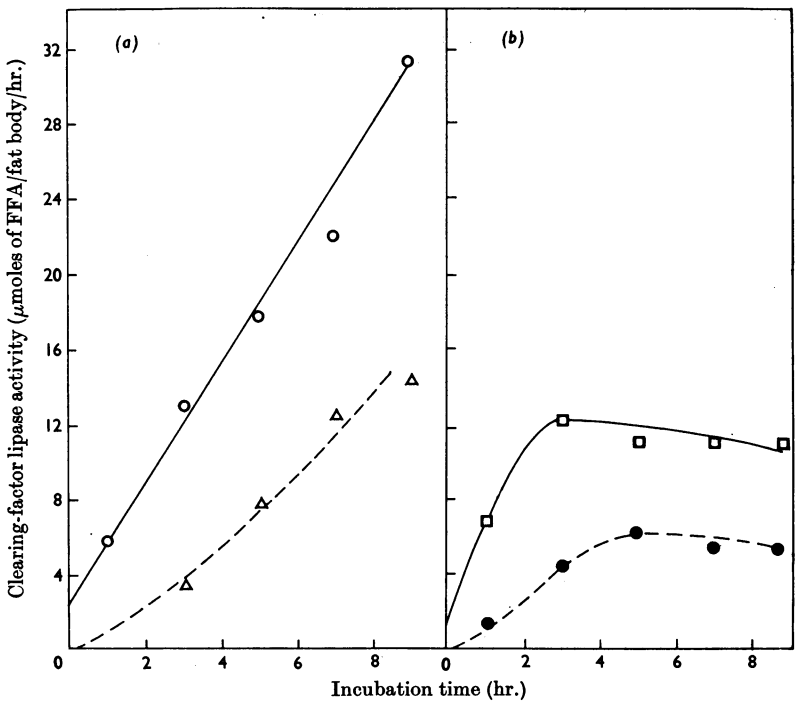

Fig. 1. Rise in clearing-factor lipase activity when adipose tissue from starved rats is incubated in the presence and absence of actinomycin. In Expt. (*a*), five groups of ten fat bodies, each from a separate starved rat, were each incubated at 37° in 25ml. of CRM that contained actinomycin (5μg./ml.); in Expt. (*b*), similar incubation systems with the paired fat bodies were set up in CRM alone. In each group, the clearing-factor lipase activity of a sample (1ml.) of the incubation medium (△, ●) and of an acetone–ether-dried preparation made from the fat bodies and the remainder of the medium (○, □) was determined after a known incubation period.

extent of this increase in activity, as shown in Fig. 1(*b*), are quite different from those found in the presence of actinomycin.

Effects of heparin and of glucose and insulin. The increase in total clearing-factor lipase activity that occurs in CRM in the absence of actinomycin is markedly decreased when heparin is omitted from the incubation medium or when the glucose and insulin components are replaced by pyruvate (Salaman & Robinson, 1966; Wing *et al.* 1966). The effect of omitting heparin from the medium and replacing the glucose and insulin by pyruvate on the increase in total clearing-factor lipase activity brought about by actinomycin is shown in Fig. 2. Under these conditions, a progressive rise in the enzyme activity of the system still occurs, but the rate of increase is initially slow, and only after several hours does it approach that observed in the presence of heparin, glucose and insulin. Very little enzyme activity appears in the incubation medium, since heparin is absent. Thus in the experiments shown in Fig. 2 the enzyme activity of the medium, even after incubation for 10hr., was only 10–15% of the total.

When fat bodies are incubated in the absence of actinomycin, the omission of heparin alone from CRM lowers, but does not completely abolish, the rise in total clearing-factor lipase activity that occurs during the first few hours (Wing *et al.* 1966).

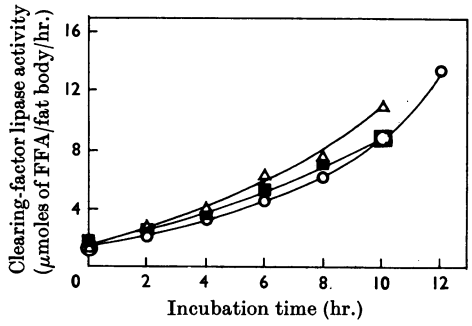

Fig. 2. Rise in clearing-factor lipase activity when adipose tissue from starved rats is incubated in a medium containing actinomycin but without heparin, glucose and insulin. The results of three experiments are shown (○, △, ■). In each, 30 fat bodies (one from each of 30 rats) were incubated in 75ml. of a medium containing actinomycin (5μg./ml.) and all the constituents of CRM except heparin, glucose and insulin. The glucose and insulin were replaced by pyruvate (2·4mg./ml.). At intervals, acetone–ether-dried preparations were made from five fat bodies and 11·5ml. of medium taken from the incubation systems, and clearing-factor lipase was assayed in these. Samples of the media (1ml.) were also taken at intervals for measurement of the enzyme activity of the medium; the activities of the medium were low and are not shown in the Figure (see the text).

Under similar conditions with actinomycin present the time-course is as shown in Fig. 3. The shoulder that is evident could indicate that two processes are occurring in such a system; namely, an initial increase in clearing-factor lipase activity due to the presence in the medium of the CRM components, glucose and insulin, and the later actinomycin-induced increase in enzyme activity shown in Fig. 2 (see the Discussion section).

Effect of puromycin. Eagle & Robinson (1964) showed that puromycin prevents the increase in total clearing-factor lipase activity that occurs when fat bodies from starved rats are incubated in CRM in the presence of actinomycin. It has a similar effect when the incubations are carried out in the presence of actinomycin but in CRM from which the heparin has been omitted and in which the glucose and insulin have been replaced by pyruvate. In an experiment with the paired fat bodies of eight rats, the total activities (μmoles of FFA released/fat body/hr.) after an incubation period of 7hr. were 0·8 in the presence of puromycin (0·5mg./ml.) and 9·6 in its absence. Puromycin added at a later stage of the incubation also stops the increase of enzyme activity in the presence of actinomycin. Thus in a similar experiment in which the total incubation period was 10hr. and puromycin (0·5mg./ml.) was added to one incubation system after 7hr., the final total activities were 5·5 in the presence of puromycin and 11·0 in its absence.

Effects of catecholamines and corticotrophin. The increase in total clearing-factor lipase activity that

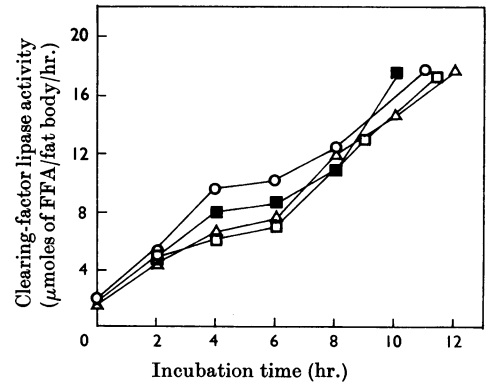

Fig. 3. Rise in clearing-factor lipase activity when adipose tissue from starved rats is incubated in the presence of actinomycin: effect of omission of heparin from the medium. The results of four experiments are shown (○, △, □, ■). Each was carried out essentially as described in the legend to Fig. 2, except that the media contained glucose (2·4mg./ml.) and insulin (12milliunits/ml.) but no pyruvate.

Table 1. *Effect of catecholamines and of corticotrophin on the increase caused by actinomycin in the clearing-factor lipase activity of fat bodies from starved rats*

The design of the experiments is given in the text. In Expts. 1 and 2, each incubation system consisted of three fat bodies in 7·5ml. of a medium consisting of CRM from which heparin had been omitted, but containing actinomycin (5μg./ml.). Adrenaline bitartrate (0·05μmole/ml.), noradrenaline bitartrate (0·05μmole/ml.) or corticotrophin (1·35i.u./ml.) were present as indicated. Incubation was for 5hr. at 37°. In Expt. 3, similar conditions were used but the media contained pyruvate (2·4mg./ml.) instead of glucose and insulin and incubation was for 9hr. A second addition of adrenaline bitartrate (0·05μmole/ml.) was made to the medium containing it after 5hr. Besides the total clearing-factor lipase activities of the systems at the end of the incubation (in μmoles of FFA released/fat body/hr. of incubation), the table also gives in parentheses the final FFA concentration in the medium (μmoles/ml.) and the change in this concentration during the incubation.

	Total clearing-factor lipase activity					
Addition to medium.....	None	Adrenaline	None	Noradrenaline	None	Corticotrophin
Expt. 1	11·9 (0·41;−0·14)	4·1 (1·29;+0·74)	11·7 (0·60;+0·05)	7·2 (0·97;+0·42)	11·2 (0·72;+0·17)	7·1 (1·04;+0·49)
Expt. 2	9·1 (0·66;+0·11)	4·8 (1·03;+0·48)	9·1 (0·68;+0·13)	5·4 (0·90;+0·35)	10·3 (0·62;+0·07)	5·8 (0·97;+0·42)
Expt. 3	11·1 (0·78;+0·14)	1·4 (1·85;+1·21)				
	9·2 (0·78;+0·14)	1·0 (1·64;+1·00)				
	11·1 (0·71;+0·07)	1·2 (1·90;+1·26)				

occurs when fat bodies from starved rats are incubated in CRM is inhibited by adrenaline, noradrenaline and corticotrophin (Wing *et al.* 1966).

To study the effects of these hormones on the actinomycin-induced increase in clearing-factor lipase activity, experiments were carried out by a paired-fat-body technique (see the Materials and Methods section). Groups of fat bodies were incubated in media similar to CRM but containing actinomycin, and the paired fat bodies were incubated in media that contained, in addition to actinomycin, adrenaline, noradrenaline or corticotrophin. Heparin was omitted from all the media and, in some of the experiments, glucose and insulin were replaced by pyruvate. To check that the hormones were active in mobilizing fatty acids from the tissue under the experimental conditions (see Rudman, 1963), samples of each medium were taken before the fat bodies were added and at the end of the incubation for measurement of the concentration of FFA. At the end of the incubations, total clearing-factor lipase activity was measured in acetone–ether-dried preparations made from the fat bodies and incubation medium combined. The results in Table 1 show that all three hormones inhibit the increase in enzyme activity brought about by actinomycin.

Effect of puromycin on the clearing-factor lipase activity of adipose tissue

Puromycin, both *in vivo* and *in vitro*, causes a rapid decline in the high clearing-factor lipase activity of adipose tissue from fed rats (Wing *et al.* 1966). This action of puromycin is compared below with its effect on the low clearing-factor lipase activity of adipose tissue from starved rats and on the increased enzyme activities when such tissue has been incubated in CRM in the absence of actinomycin and in CRM or similar media in the presence of actinomycin.

Effect when tissue from fed animals is used. In the presence of puromycin, the total clearing-factor lipase activity of adipose tissue from fed rats falls at an exponential rate *in vitro* for at least 2·5hr., the half-life of the enzyme being between 1 and 1·5hr. (Fig. 4). (Enzyme was not extracted into the incubation medium in these experiments since they were carried out without added heparin.)

Effect when tissue from starved animals is used. In contrast with its effect on the enzyme in adipose tissue from fed rats, puromycin does not cause a decline in the low clearing-factor lipase activity of tissue from starved animals. Three experiments were carried out, in each of which three epididymal fat bodies from starved rats were incubated for 10min. in 4ml. of CRM from which the heparin had been omitted and in the presence of puromycin (0·5mg./ml.), and their paired fat bodies were incubated for 1·5hr. under the same conditions. The total enzyme activities of acetone–ether-dried preparations made from the fat bodies and medium combined (μmoles of FFA released/fat body/hr.) were 3·7, 3·2 and 3·3 at 10min. and 4·3, 3·6 and 4·7 at 1·5hr. In a similar experiment, clearing-factor

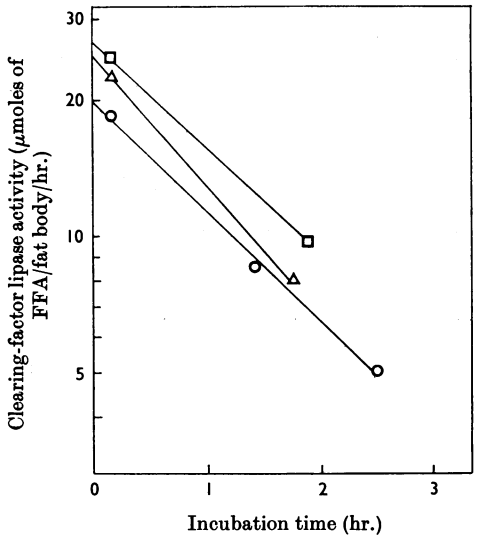

Fig. 4. Effect of puromycin on the clearing-factor lipase activity of adipose tissue from fed rats. Epididymal fat bodies were incubated at 37° in CRM from which the heparin had been omitted but which contained puromycin hydrochloride (0·5mg./ml.). In one experiment (○), 24 fat bodies from 12 rats were incubated in 30ml. of medium, and clearing-factor lipase was measured in acetone–ether-dried preparations made from eight of the fat bodies and 10ml. of the medium after 10min., 1·5hr. and 2·5hr. In the others (△, □), fat bodies from three rats were incubated in 4ml. of medium with the paired-fat-body technique (see the Materials and Methods section), and clearing-factor lipase was measured in acetone–ether-dried preparations made after 10min. or 1·75hr.

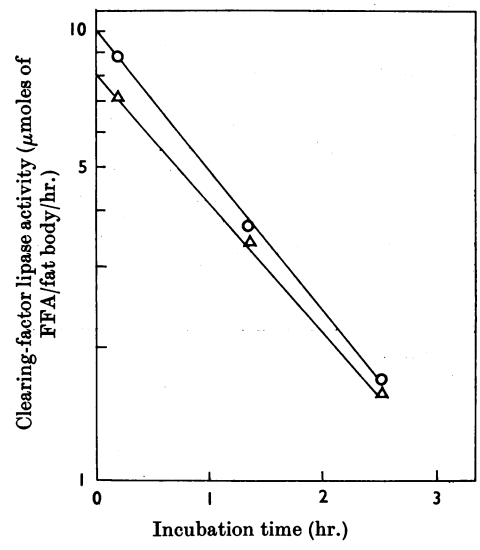

Fig. 5. Effect of puromycin on the clearing-factor lipase activity of adipose tissue from starved rats after incubation of the tissue in CRM. Two experiments were carried out (○, △). In each, epididymal fat bodies from 12 starved rats were incubated at 37° in 60ml. of CRM for 2·5hr. At this time, the fat bodies were removed from the CRM, rinsed, and reincubated in 30ml. of CRM from which the heparin had been omitted but which contained puromycin hydrochloride (0·5 mg./ml.). Clearing-factor lipase was measured in acetone–ether-dried preparations made from eight of the fat bodies and 10ml. of the medium after 10min., 1·3hr. and 2·5hr.

lipase was measured in groups of at least four fat bodies that were incubated for 10min., 1·5hr., 4hr. or 6hr. in the presence of puromycin. Total activities of 2·8, 3·6, 2·4 and 2·2 μmoles of FFA released/fat body/hr. respectively were found.

Effect when tissue from starved animals is incubated in CRM. To study the stability towards puromycin of the raised clearing-factor lipase activity when adipose tissue from starved rats is incubated in CRM, incubation of fat bodies in this medium was first carried out for 2·5hr. Though some enzyme appeared in the medium during this time, the enzyme activity of the tissue also increased severalfold (see Fig. 1*b*). At this stage, the fat bodies were removed from the initial incubation medium and transferred to fresh CRM containing puromycin but from which heparin had been omitted. As shown in Fig. 5, total enzyme activity declines exponentially at a similar rate ($t_{\frac{1}{2}}$ 1hr.) to that found with tissue from fed animals.

Effect when tissue from starved animals is incubated in the presence of actinomycin. The effect of puromycin on the raised enzyme activity that is observed when fat bodies from starved animals are incubated with actinomycin was studied in two types of experiments.

In the first type, fat bodies were incubated for 10hr. with actinomycin in CRM from which only heparin had been omitted (see Fig. 3). After being rinsed, the fat bodies were then incubated again in the presence of puromycin in fresh CRM from which heparin was absent, clearing-factor lipase activity in the systems being measured at intervals thereafter. The results of three such experiments are shown in Fig. 6. The rate of decline in the total enzyme activity in the presence of puromycin is much less than that observed with adipose tissue from fed animals (Fig. 4) or adipose tissue from starved animals that has been incubated in CRM (Fig. 5).

Greater stability in the presence of puromycin was also observed in the second type of experiment, when the initial incubation in actinomycin was in a medium that lacked heparin, glucose and insulin,

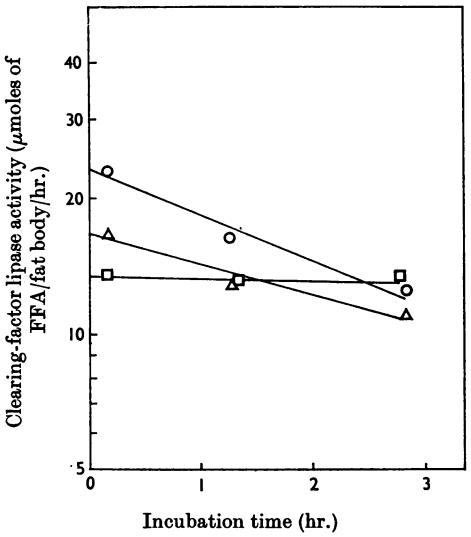

Fig. 6. Effect of puromycin on the clearing-factor lipase activity that is induced in adipose tissue of starved rats by actinomycin. Three experiments were carried out (○, □, △). In each, 12 fat bodies from starved rats were incubated at 37° in 30ml. of medium containing actinomycin (5μg./ml.) for 10hr. The fat bodies were then removed from the medium, rinsed, and incubated in 15ml. of medium containing puromycin hydrochloride (0·5mg./ml.). Clearing-factor lipase was measured in acetone–ether-dried preparations made from four of the fat bodies and 5ml. of the fresh medium after approximately 10min., 1·25hr. and 2·75hr. of this second incubation. The incubation medium to which the actinomycin or puromycin hydrochloride was added was CRM from which heparin had been omitted.

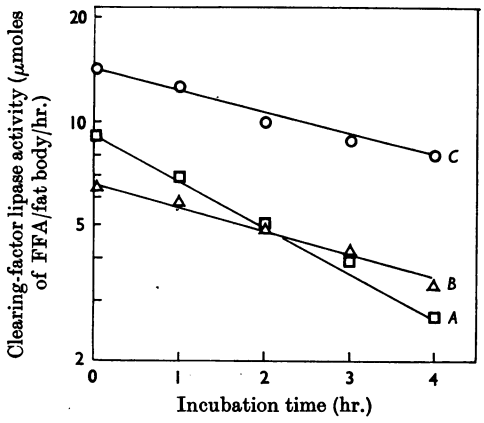

Fig. 7. Stability of clearing-factor lipase in solution. Clearing-factor lipase was extracted from: *A*, fat bodies from fed rats, by incubating them for 4hr. at 37° in CRM from which glucose and insulin had been omitted; *B*, fat bodies from starved rats, that had been incubated at 37° for 11hr. in CRM containing actinomycin (5μg./ml.) but no heparin. The fat bodies were then rinsed and enzyme was extracted from them, by incubation at 37° for 4hr. in the medium used in *A*; *C*, fat bodies from starved rats, by incubating them for 6hr. at 37° in CRM containing actinomycin (5μg./ml.) but no glucose or insulin. After extraction of the enzyme, the media containing it were incubated at 37° for 4hr., samples (1ml.) being removed at intervals for clearing-factor lipase assay. Two or three experiments were carried out with each system and mean curves for the decline in enzyme activity in solution are shown.

the glucose and insulin being replaced by pyruvate (see Fig. 2). The experiments were carried out in a similar way to that described in the legend to Fig. 6; in one experiment, the initial period of incubation with actinomycin was 12hr. and the total clearing-factor lipase activities of groups of four fat bodies after subsequent incubation for 10min., 1·5hr. and 2·5hr. in the presence of puromycin were 8·1, 8·8 and 9·6μmoles of FFA released/fat body/hr. respectively. In a second experiment in which the initial incubation period was 9hr., the total activities of groups of 12 paired fat bodies that were then incubated in the presence of puromycin for either 0·25hr. or 2·25hr. were 9·9 and 7·9μmoles of FFA released/fat body/hr. respectively.

Stability of clearing-factor lipase in solution at 37°

To study the stability of clearing-factor lipase in solution, enzyme was extracted from adipose tissue from fed animals and from tissue from starved animals after this had been incubated for several hours with actinomycin. In the latter case enzyme in solution was obtained in two ways. (1) A preliminary incubation in the presence of actinomycin but in the absence of heparin was carried out and then, when the tissue activity was raised, enzyme was extracted into fresh medium that contained heparin. (2) Alternatively, it was extracted as the activity increased in the presence of actinomycin by including heparin in the incubation medium. The results in Fig. 7 show that, whereas the activity of the enzyme extracted from tissue from fed animals declines at 37° with a half-life of about 2hr., that of the enzyme extracted from tissue that has been incubated in the presence of actinomycin has a half-life at 37° of at least 4hr. Actinomycin added to the medium was shown not to affect the rate of loss of the activity of the enzyme extracted from tissue from fed animals. The pH of the medium in all cases was between 7·1 and 7·25 throughout.

Rate of decline of adipose-tissue clearing-factor lipase activity on starvation

Although it is well established that the clearing-factor lipase activity of adipose tissue falls to a low

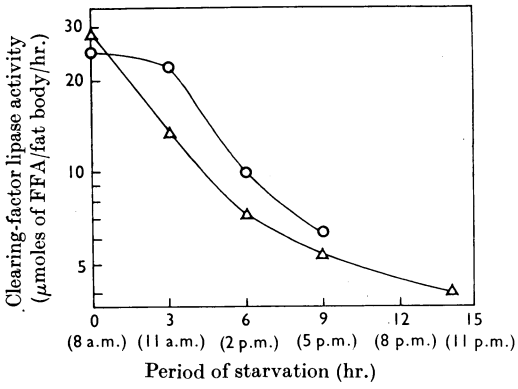

Fig. 8. Effect of starvation on adipose-tissue clearing-factor lipase activity. Two experiments were carried out (○, △). Food was taken from cages each containing four rats, at 8 a.m.; at various times thereafter, clearing-factor lipase was measured in acetone–ether-dried preparations made from the grouped fat bodies of the rats in each cage.

value on starvation, the rate of decline of the activity is not known. To provide this information, rats were starved from 8 a.m. and the activity of the enzyme was measured at various times thereafter in adipose tissue taken from groups of animals. The results of two such experiments are shown in Fig. 8. In both, there was a period of about 6hr. during which the clearing-factor lipase activity of the tissue fell extremely rapidly. Since the rat is a nocturnal feeder, it seems possible that food continued to be absorbed for some time after the diet was removed from the cages in the experiment in which the rapid decline in enzyme activity was delayed.

DISCUSSION

The lipase activity of all the preparations used in this study has been shown to be directed towards triglycerides in chylomicra or in artificial lipid emulsions that have been appropriately activated, but not towards triglycerides in unactivated emulsions, to have a pH optimum between 8·0 and 8·5, and to be completely inhibited in 0·5M-sodium chloride (D. R. Wing & D. S. Robinson, unpublished work). On the basis of these three properties, which distinguish it from other lipases in adipose tissue (Robinson, 1963), the enzyme has been referred to throughout as 'clearing-factor lipase'. However, in the absence of adequate methods for its purification, it is recognized that this identification must be provisional and that it does not exclude the possibility, discussed below, that the enzyme may exist in more than one form in the different preparations.

It has not yet been shown unequivocally that the increases in the total clearing-factor lipase activity of adipose tissue that are associated either with the change from the starved to the fed state or with the incubation of adipose tissue from starved rats in CRM *in vitro* are due to increases in the enzyme content of the tissue. In an earlier study (Wing *et al.* 1966) increase in activity *in vitro* was provisionally interpreted as being due to an increase in enzyme content because it is inhibited by puromycin, depends on aerobic conditions and takes place over a period of several hours. These findings were thought to be inconsistent with a process of activation of a pre-existing enzyme or enzyme precursor. However, Appleman & Kemp (1966) showed that puromycin, at concentrations similar to those used in our earlier study and in the present work, as well as acting as an inhibitor of protein synthesis (Greengard, 1963), also increases the concentration of adenosine cyclic 3′,5′-monophosphate (cyclic AMP) in the rat diaphragm. Cyclic AMP is believed to mediate the activating and inhibiting actions of a number of hormones on the enzymes concerned in glycogen synthesis and breakdown (Sutherland, Øye & Butcher, 1965). It may have a similar action on the lipase concerned in the mobilization of triglyceride fatty acids from adipose tissue (Rizack, 1964; Butcher, Ho, Meng & Sutherland, 1965; Butcher, Sneyd, Park & Sutherland, 1966). Catecholamines and corticotrophin are known to raise the concentration of cyclic AMP in adipose tissue (Butcher *et al.* 1965). In these circumstances, the finding (Wing *et al.* 1966) that these hormones, as well as puromycin, inhibit the increase in clearing-factor lipase activity that occurs when adipose tissue from starved rats is incubated in CRM, could indicate a role for cyclic AMP in the control of clearing-factor lipase activity. Thus it is possible that changes in the relative proportions of active and inactive forms of the enzyme in adipose tissue, rather than changes in the enzyme content of the tissue, explain the increase in enzyme activity *in vitro* and also the increase that occurs during the transformation from the starved into the fed state *in vivo*.

'Stable' and 'unstable' forms of clearing-factor lipase. The clearing-factor lipase activity of adipose tissue from starved rats is 'stable' for several hours at 37° in the presence of puromycin *in vitro*, whereas under similar conditions the enzyme activity of tissue from fed animals has a half-life of 1–1·5hr. These findings suggest that the change from the starved to the fed state involves the appearance in the tissue of an 'unstable' form of clearing-factor lipase. The increased enzyme activity that is found when adipose tissue from starved animals is incubated in CRM *in vitro* is also lost rapidly in the presence of puromycin. It seems therefore that the enzyme is also in an 'unstable' form under these conditions.

The foregoing conclusions are based on the results of studies *in vitro*, but they are supported by a limited series of experiments *in vivo*. Thus the low enzyme activity in adipose tissue from starved rats does not decline after the injection of puromycin (D. R. Wing & D. S. Robinson, unpublished work), whereas the high enzyme activity in the tissue in fed rats falls rapidly after puromycin or cycloheximide injection (Wing *et al.* 1966; Wing, Fielding & Robinson, 1967). The rapid loss of enzyme activity from adipose tissue that occurs in starvation (Fig. 8) is also consistent with the presence of 'unstable' enzyme in the tissue in the fed animal.

Instability of clearing-factor lipase in adipose tissue from fed animals could be due to a variety of causes. It could result from (*a*) the conversion of an active form of the enzyme into an inactive form, as discussed above, (*b*) the absence of some stabilizing cofactor such as heparin (Robinson, 1963) or (*c*) the action of proteolytic enzymes in the tissue. The finding that differences in stability can be shown with clearing-factor lipase that has been extracted from the tissue and incubated in solution (Fig. 7) does not favour the last possibility.

Effect of actinomycin on clearing-factor lipase activity. The time-course of the increase in total clearing-factor lipase activity when adipose tissue from starved rats is incubated *in vitro* in the presence of actinomycin is markedly influenced by heparin and by glucose and insulin. Thus in the absence of heparin and when glucose and insulin are replaced by pyruvate, increase in enzyme activity at an appreciable rate begins only after a lag of several hours (Fig. 2). In the presence of glucose and insulin, but in the absence of heparin, the increase in activity begins immediately, but there is a shoulder in the time-course curve after 4–6hr. (Fig. 3). When glucose, insulin and heparin are all present, enzyme activity increases from the beginning of the incubation and continues to do so at a steady rate for several hours (Fig. 1*a*).

The increased enzyme activity in adipose tissue from starved rats, several hours after this has been incubated in a medium containing actinomycin, resembles that originally present in the tissue in being 'stable' in the presence of puromycin (Fig. 6). In this respect it differs from the raised activity found on incubation of the tissue in CRM in the absence of actinomycin (Fig. 5) and from that present in tissue from fed animals (Fig. 4). The observed variations in the time-course in the presence of actinomycin might be explained if, when glucose, insulin and heparin were present in the medium, an increase in the activity of the 'unstable' form of the enzyme occurred during the lag, before the increase in the activity of the 'stable' form was brought about by actinomycin. Omission of heparin from the incubation medium would diminish the increase in activity of the 'unstable' form (Wing *et al.* 1966), and hence a shoulder might be expected to appear in the time-course curve when the increase in the activity of the 'unstable' enzyme had ceased and that of the 'stable' enzyme had not started. Some support for this explanation is provided by experiments in which fat bodies were incubated with actinomycin in the presence of glucose and insulin but in the absence of heparin, as described in Fig. 6, but for 4hr., instead of 10hr. In these circumstances, when the enzyme activity of the tissue would be expected to have risen only to the shoulder of the time-course curve shown in Fig. 3, the half-life of the enzyme when the tissue was subsequently incubated in the presence of puromycin was only 1–2hr. The enzyme activity showed therefore the stability characteristics of that found in fat bodies from starved rats that have been incubated in CRM.

Eagle & Robinson (1964) suggested that the rise in clearing-factor lipase activity brought about by actinomycin might be due to an inhibitory effect of this substance on the formation of enzymes that are normally active in degrading clearing-factor lipase. The present finding that clearing-factor lipase activity in adipose tissue from starved animals is in a 'stable' form, whether or not the tissue has been incubated in the presence of actinomycin, does not support this view. Neither are the experiments shown in Fig. 7 readily explained on this basis. In these experiments, clearing-factor lipase was extracted from the adipose tissue of starved animals, after this had been incubated in a medium containing actinomycin, and also from the adipose tissue of fed animals. These two soluble enzyme preparations showed differing stabilities when they were incubated at 37° under identical conditions.

Mechanism of actinomycin action. Increases in the activity of certain inducible enzymes by actinomycin have been reported (see e.g., Rosen, Raina, Milholland & Nichol, 1964; Garren, Howell, Tomkins & Crocco, 1964). However, the mechanism of action of the actinomycin was not definitely established and the changes in activity were not as rapid or as marked as those described in the present paper.

The effects of actinomycin on clearing-factor lipase activity are difficult to interpret because of the uncertainty whether the changes in the enzyme activity of the tissue that occur in its absence are due to alterations in enzyme content or in the proportions of two enzyme forms of different specific activity. If the latter explanation is correct, the two forms of clearing-factor lipase could differ in their stability and also be interconvertible by enzyme action, as are the enzymes concerned in glycogen synthesis and in glycogen breakdown,

glycogen phosphorylases *a* and *b* (EC 2.4.1.1) and UDP-glucose–glycogen glucosyltransferases I and D (EC 2.4.1.11) (Caputto, Barra & Cumar, 1967). If synthesis of the interconverting enzyme were under the control of a short-lived messenger RNA, this could be the point of action of actinomycin, which would thereby prevent the interconversion. In these circumstances, an increase in the amount of a 'stable' form of the enzyme could take place if the synthesis of this form occurred first in the tissue from a messenger RNA of relatively long half-life.

Alternatively, if the 'stable' and 'unstable' forms of clearing-factor lipase were non-interconvertible isoenzymes, their synthesis could be determined by different messenger RNA molecules. If only the messenger RNA for the 'unstable' form of the enzyme had a short half-life, actinomycin would presumably inhibit the formation of this enzyme form, but not that of the 'stable' enzyme. Under such conditions, the consequent freeing of some common enzyme constituent, such as heparin, which was in short supply in the tissue, might then result in increased formation of 'stable' enzyme.

Other mechanisms for the action of actinomycin cannot be excluded. It might, for instance, act exclusively through an effect on the formation or destruction of a cofactor such as heparin, which may stabilize the enzyme (Robinson, 1963); or it might interfere with the formation of RNA molecules that, either directly or through the synthesis of repressor proteins, normally prevent formation of the enzyme. There is evidence for the operation of such mechanisms in certain inducible enzyme systems (Garren *et al.* 1964; Garren, Ney & Davis, 1965; Drysdale & Munro, 1965; Kenney & Albritton, 1965). Finally, Kenney (1967) has suggested that, since processes of enzyme synthesis and degradation may show a differential sensitivity to actinomycin, accumulation of inducible enzymes may occur in its presence because degradation is more effectively blocked than synthesis.

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