*Biochem. J.* (1973) **132**, 833–836
*Printed in Great Britain*



# The Effect of Cycloheximide on Adipose-Tissue Clearing-Factor Lipase

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(*Received* 31 *January* 1973)

The progressive increase in clearing-factor lipase activity that occurs during the incubation of adipose tissue from starved rats in an appropriate medium at 25°C is shown to occur in two stages. The first of these is not inhibited by cycloheximide, whereas the second is.

There is now considerable evidence that the main function of the enzyme clearing-factor lipase (or lipoprotein lipase) is to hydrolyse chylomicron and very-low-density-lipoprotein triglycerides and thereby facilitate the removal from the blood of their constituent fatty acids. In performing this function the enzyme probably acts at the luminal surface of the capillary endothelial cells of the extrahepatic tissues (Robinson, 1963).

Special consideration has been given in recent years to the exercise of this function of the enzyme in adipose tissue, and, in particular, to the changes in its activity in this tissue that occur in a variety of situations and that can be correlated with corresponding changes in triglyceride fatty acid uptake by the tissue (Robinson, 1970). Our particular interest in this respect relates to the marked alterations both in triglyceride fatty acid uptake and in enzyme activity that occur in the tissue with variations in nutritional status. In attempts to discover the factors controlling such changes in activity of the enzyme, we have devised systems where epididymal fat-bodies from starved rats, which have a low clearing-factor lipase activity, are incubated *in vitro* and in which the total enzyme activity increases progressively over several hours towards that characteristic of the tissue in the fed animal.

Such increases in enzyme activity have been shown to be inhibited at 37°C in the presence of an inhibitor of protein synthesis such as puromycin (Wing *et al.*, 1966; Wing & Robinson, 1968*a*), and this has led to the view that they involve a stimulation of enzyme synthesis. This was also the conclusion of Patten (1970), who observed in similar work with rat adipocyte incubation systems a close correlation in a variety of situations between changes in the rate of protein synthesis and changes in clearing-factor lipase activity.

There is, on the other hand, indirect evidence to suggest that such increases in enzyme activity are due rather to activation brought about by the conversion of one form of the enzyme into another and that this may result from a fall in the concentration of adipocyte cyclic AMP (Wing & Robinson, 1968*b*; Cunningham & Robinson 1969). Recently further important observations consistent with this view have been described. Thus Stewart & Schotz (1971) have reported increases in clearing-factor lipase activity in rat adipocyte incubation systems at 23°C that are not inhibited in the presence of cycloheximide, and defatted rat adipose-tissue preparations have been shown to contain two forms of the enzyme, the proportions of which vary when the activity of the enzyme in the tissue is altered as a result of a change in nutritional status (Garfinkel & Schotz, 1972; Schotz & Garfinkel, 1972).

In the present study we report experiments that may help to reconcile these apparently disparate concepts. The procedures used were similar to those described in our earlier work and involved the incubation of groups of epididymal fat-bodies from 24h-starved rats in a defined medium (2ml/fat-body) at pH7.4 under $O_2+CO_2$ (95:5) and the measurement of clearing-factor lipase activity in the incubation medium and in the fat-bodies at intervals thereafter (Wing & Robinson, 1968*a*,*b*). The incubation medium was a modification of that of Wing & Robinson (1968*a*), with the following composition: 1.2vol. of 0.85% NaCl containing chylomicrons to give a final concentration in the medium of triglyceride esterified fatty acids of 20$\mu$equiv./ml, 0.125vol. of 3.77% (w/v) $NaHCO_3$, 0.15vol. of a salt solution (see below) and 1.025vol. of water containing glucose (2.4mg/ml), casein hydrolysate (1.2mg/ml), insulin (12munits/ml) and heparin (2.4 units/ml) to give the final concentrations shown in parentheses. The salt solution was modified slightly from that of Salaman & Robinson (1966) to give a medium with the following final inorganic ion composition (mg/100ml): $Na^+$, 256; $K^+$, 14.2; $Ca^{2+}$, 8.7; $Mg^{2+}$, 2.4: $Cl^-$, 308; $PO_4^{3-}$, 3.2; $SO_4^{2-}$, 6.4; $HCO_3^-$, 137.

All the incubations were carried out at 25°C. Increases in clearing-factor lipase activity occur *in vitro* in such systems at both 25° and 37°C, but higher final activities are reached at 25°C, probably because the enzyme is more stable at this temperature (Robinson & Wing, 1970, 1971). Whereas rat serum was a component of several of our earlier incubation

Table 1. *Effect of cycloheximide on increases in adipose-tissue clearing-factor lipase activity during incubations at 25°C*

A number of incubations were carried out and in each epididymal fat-bodies from 24h-starved rats of the Wistar strain (body weight in fed state, 170–190g) were incubated in the medium described in the text, either in the absence or in the presence of cycloheximide at a final concentration of 10$\mu$g/ml. Up to 40 fat-bodies were included in each incubation. At the times shown, groups of fat-bodies were removed and acetone–ether-dried powders were prepared from these for assay of the tissue clearing-factor lipase activity as described by Wing & Robinson (1968*a*) and Robinson & Wing (1971). At least two fat-bodies were taken for each powder. Portions of the incubation medium were removed at the same times so as to keep the medium-volume/fat-body ratio constant, and the enzyme activity of a sample of this was also assayed (Wing & Robinson 1968*a*). The total activities recorded were obtained by adding the medium and the tissue activities. The values given are means ± S.D. and the numbers in parentheses are the numbers of measurements made.

| | | Clearing-factor lipase activity ($\mu$mol of free fatty acid/h per fat-body) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Expt. 1 | | Expt. 2 | | Expt. 3 | |
| Incubation time (h) | Cycloheximide ... | Absent | | Absent | Present | Present | |
| | | Total | Medium | Total | Total | Total | Medium |
| 0 | | 8.7 ± 2.5 (25) | — | 9.8 ± 2.6 (10) | 9.6 ± 4.9 (10) | 8.7 ± 2.1 (5) | — |
| 1 | | 15.1 ± 4.6 (9) | 0.6 ± 0.4 (3) | 15.6 ± 5.1 (7) | 14.5 ± 5.8 (7) | 14.0 ± 3.4 (5) | 0.7 (2) |
| 2 | | 21.2 ± 5.6 (24) | 1.3 ± 0.6 (10) | 22.3 ± 5.1 (10) | 21.9 ± 6.0 (10) | 20.8 ± 1.4 (5) | 1.6 (2) |
| 4 | | 28.6 ± 8.1 (16) | 4.8 ± 0.4 (6) | | | 16.6 ± 2.1 (5) | 4.7 (2) |
| 6 | | 39.5 ± 7.6 (11) | 10.2 ± 1.3 (5) | | | 21.4 ± 2.1 (5) | 11.1 (2) |

media, it is not necessary in incubations at 25°C (Robinson & Wing, 1970, 1971). It has been replaced by a chylomicron preparation in the present experiments, since these form part of a more extensive investigation in which use is to be made of the complexing of clearing-factor lipase in solution with its chylomicron triglyceride substrate (Robinson, 1970). Such complexing will also serve to stabilize further the enzyme in solution (Cunningham, 1970). The chylomicrons were obtained by thoracic-duct cannulation of rats fed with olive oil and were exposed to serum at 37°C before recovery by flotation and addition to the incubation medium (Cunningham, 1970). Although clearing-factor lipase appears in the medium during the incubations, little hydrolysis of the chylomicron triglyceride occurs because of the absence of any free (non-esterified) fatty acid acceptor. Thus, even after incubations lasting 6h, less than 5% of the triglyceride in the medium is hydrolysed.

The progressive increase in total (fat-body + medium) clearing-factor lipase activity when fat-bodies from starved rats are incubated at 25°C in the above medium is shown in Expt. 1 (Table 1). It is noteworthy that the increases during each of the 2h intervals are all highly significant, $P$, as determined by Student's $t$ test, being $<0.01$ in every case. Expt. 2 (Table 1) shows that the inclusion of cycloheximide [Sigma (London) Chemical Co., London S.W.6, U.K.] in the medium at a concentration of 10$\mu$g/ml does not affect this increase in total activity during the first 2h. On the other hand, Expt. 3 shows that it prevents completely the further rise in total activity that normally occurs in its absence (Expt. 3 versus Expt. 1, Table 1).

The failure of cycloheximide to inhibit the increase in total enzyme activity that occurs during the first 2h could be accounted for if such a period were necessary for the added cycloheximide to penetrate to the site of its action on the enzyme within the tissue. That this is not the explanation, however, is suggested by the results obtained in a number of separate incubations at 25°C in the presence and in the absence of cycloheximide (10$\mu$g/ml). In these the incorporation of L-[1-$^{14}$C]leucine (59–66mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) into the total tissue protein was measured by the method of Herrera & Renold (1960), and was found to be inhibited by more than 90% after incubation for

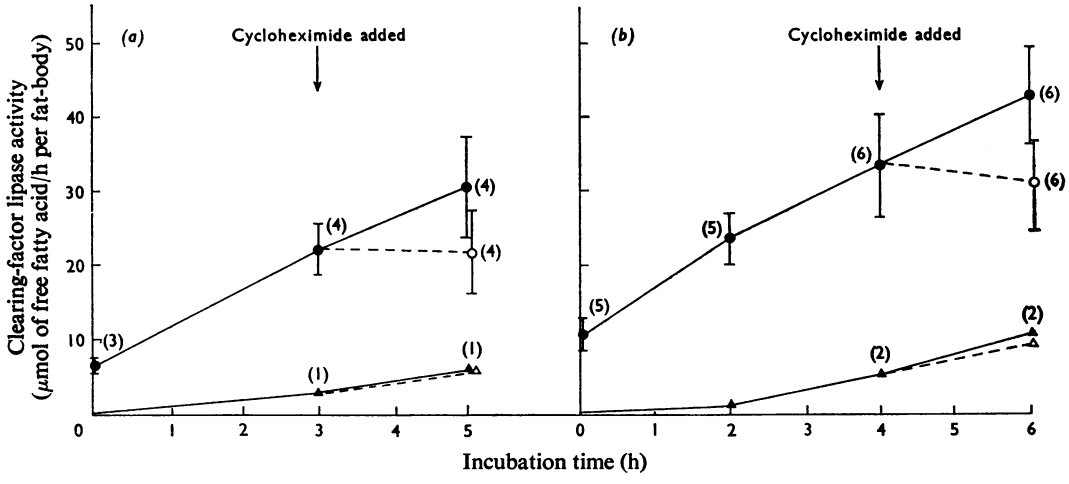


Fig. 1. *Effect of cycloheximide addition on increases in adipose-tissue clearing-factor lipase activity during incubations at 25°C*

Epididymal fat-bodies from 24h-starved rats of the Wistar strain (body weight in fed state, 170–190g) were incubated at 25°C in the medium described in the text. Cycloheximide was added in some cases (----), but not in others (——), at either 3h or 4h. Total (● and ○) and medium (▲ and △) clearing-factor lipase activities were determined as described in the legend to Table 1. The values shown at each time are the means and the bars indicate ±S.D. The numbers in parentheses are the numbers of measurements made.

1h in the presence of cycloheximide, as well as after incubation for 6h or for intermediate times. Further evidence for this view is provided, moreover, by the results in Fig. 1, which show that, under appropriate conditions, cycloheximide can inhibit enzyme activity increases that occur within 2h of its addition to the incubation medium. In these experiments separate groups of fat-bodies were incubated for either 3h or 4h in the absence of cycloheximide and then cycloheximide was added to some of the incubation media but not to others. The enzyme activity increase that occurred during the next 2h in the absence of cycloheximide was completely prevented in its presence.

These experiments suggest that the initial increases in enzyme activity occur by a process that is independent of protein synthesis, but that this is not the case with respect to the later increases. It is noteworthy that, although cycloheximide is without influence on the appearance of enzyme activity in the medium throughout the incubations (Table 1 and Fig. 1), its inhibitory effect first appears at the time when such activity begins to be detectable in the medium at a significant level. Appearance of enzyme in the medium is dependent on its release from the fat-bodies by the heparin present (Robinson & Wing, 1970). Further, in the absence of heparin, when enzyme is not extracted into the medium, the increases in activity during incubations at 25°C occur only during the first 2h (Robinson & Wing, 1971). In its presence, on the other hand, they continue for up to 10h (A. Cryer, B. Foster & D. S. Robinson, unpublished work). If therefore seems that the inhibition by cycloheximide of the increases that occur after 2h could be related to the extraction of enzyme from the tissue. Moreover, since the enzyme is more rapidly extracted into the incubation medium at 37°C than at 25°C (Wing, 1972), such a concept would reconcile the present findings with our earlier observation (Wing *et al.*, 1966; Wing & Robinson, 1968*a*) that puromycin inhibited the rise in clearing-factor lipase activity that occurred when fat-bodies were incubated in the presence of heparin at 37°C.

When considered in more general terms, the present findings clearly allow two distinct stages in the enzyme activity increases to be identified. Moreover, the view that only the first of these takes place by a process that is independent of protein synthesis seems consistent with the proposal that increases in clearing-factor lipase activity may occur initially through the conversion of one form of the enzyme into another of higher specific activity, and that this conversion may then be followed by the synthesis of more of the precursor (Robinson & Wing, 1970, 1971).

This work was supported in part by a grant from the Medical Research Council. D. R. W. was a member of the external staff of the Medical Research Council when the work was carried out.

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