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The Clearing-Factor Lipase Activity of Isolated Fat-Cells

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1. When fat-cells are isolated from the epididymal adipose tissue of 24h-starved rats and incubated at 25°C in the presence of dialysed serum, glucose, insulin, amino acids and heparin, the total clearing-factor lipase activity of the incubation system increases progressively over a period of several hours. 2. All of the increase in activity is accounted for by the appearance of enzyme in the incubation medium and the fat-cell activity does not change significantly. Cycloheximide, at a concentration that prevents protein synthesis, does not affect the appearance of enzyme in the incubation medium, but the fat-cell enzyme activity is decreased in its presence. 3. The magnitude of the increase in total clearing-factor lipase activity is unaffected by the omission of heparin from the medium. However, less enzyme is extracted in its absence and the fat-cell activity increases. Cycloheximide again only affects the rise in cell activity and does not alter the activity in the incubation medium. 4. When serum in the incubation medium is replaced by casein, the distribution of enzyme between the cells and the medium is changed, but the magnitudes of the increases in total enzyme activity are similar. 5. These characteristics of the clearing-factor lipase response of isolated fat-cells differ in several respects from those observed earlier with intact adipose tissue from 24h-starved rats (Robinson & Wing, 1971; Cryer *et al.*, 1973). The differences could be due, in part, to changes in the relative amounts of two different molecular forms of the enzyme that occur during the isolation of the fat-cells.

In adipose tissue the main function of the enzyme clearing-factor lipase (lipoprotein lipase) is to hydrolyse the plasma triglycerides and thereby facilitate the uptake of their constituent fatty acids by the tissue. Important supporting evidence for this role is provided by the finding that the activity of the enzyme falls on starvation and rises again on re-feeding, in parallel with corresponding alterations in the uptake of plasma triglyceride fatty acids (see Robinson, 1970).

Though some of the enzyme in adipose tissue is present in the fat-cells (Rodbell, 1964), this fraction is not believed to be active in the hydrolysis of the plasma triglycerides. In fact, there is now strong evidence that such hydrolysis occurs through the action of enzyme that is present at the luminal surfaces of the capillary endothelial cells of the tissue (see Robinson, 1970). The finding that, when the activity of the whole tissue falls on starvation, the activity associated with the fat-cell shows only little change (Cunningham & Robinson, 1969), is consistent with this view and it has been proposed on the basis of this and other evidence that the fat-cell enzyme is the precursor of the functional enzyme at the endothelial cell surface (see Robinson & Wing, 1970, 1971).

When intact adipose tissue from starved rats is incubated in the presence of glucose, insulin and heparin, the clearing-factor lipase activity of the incubation system increases progressively over several hours towards the value characteristic of tissue from animals in the fed state (see Robinson & Wing, 1970, 1971). Such rises in activity occur during incubations at either 25° or 37°C, and we have described certain characteristic features of the increases at each of these temperatures. For example, during incubations at 37°C in the presence of heparin, much of the rise in activity is due to enzyme that appears in the incubation medium and the tissue activity shows little change. The increases in activity at this temperature are, moreover, completely prevented in the presence of inhibitors of protein synthesis (Wing *et al.*, 1966). However, during similar incubations at 25°C, the rise in activity that occurs during the first 2h is almost wholly within the tissue and it is only the increase after this time that takes place in the incubation medium. Further, the rise in activity over the first 2h is not affected when protein synthesis in the tissue is blocked, although the increase that occurs at later times is completely prevented (Cryer *et al.*, 1973).

During incubations of the intact tissue at 25°C in the absence of heparin, increases in activity occur during the first 2h that are again wholly within the tissue and unaffected by inhibition of protein synthesis. However, in this situation there is neither any further increase in activity during incubations for longer periods nor any significant appearance of

enzyme activity in the incubation medium (Robinson & Wing, 1971; A. Cryer, E. R. Williams & D. S. Robinson, unpublished work).

In attempting to relate these findings to the control of the activity of the enzyme in adipose tissue *in vivo*, we have assumed that, through carrying out incubations at 25°C, it has been possible to separate two distinct processes leading to increases in enzyme activity which, although they presumably also occur at 37°C, are not readily distinguished at this temperature. Thus, we have suggested: (*a*) that increases in enzyme activity occur initially in the fat-cell fraction of the tissue through the conversion of one enzyme form into another of higher specific activity by processes independent of protein synthesis, (*b*) that the subsequent release of this enzyme form from the fat-cell and its transport, either into the medium during incubations *in vitro* or to the functional site of action of the enzyme in the tissue *in vivo*, induces the synthesis of more of the precursor form and a further rise in activity (Robinson & Wing, 1970, 1971; Cryer *et al.*, 1973). Similar concepts have been advanced by Schotz and his co-workers in a related series of studies (Stewart & Schotz, 1971; Schotz & Garfinkel, 1972; Garfinkel & Schotz, 1973; Stewart & Schotz, 1974), which have led to the separation of two molecular species of the enzyme from intact adipose-tissue preparations.

We have already reported briefly that progressive increases in clearing-factor lipase activity also occur when fat-cells, which have been isolated from the adipose tissue of starved rats, are incubated at 25°C in a medium similar to that used for the incubation of the intact tissue (Robinson & Wing, 1971). In the present study we have investigated the effects of an inhibitor of protein synthesis, cycloheximide, and of heparin on these increases in enzyme activity, with particular reference to the effects of these substances on the distribution of the enzyme between the cells and the incubation medium. We also provide direct evidence for the existence in the fat-cells of two molecular species of clearing-factor lipase.

Materials and Methods

Animals and tissue preparations

Male albino rats of the Wistar strain were used throughout. They were maintained on Oxoid pasteurized diet 41B (H. Styles Ltd., Bewdley, Worcs., U.K.) and weighed 170–190g in the fed state. Epididymal fat-pads were removed from the animals while they were under light ether anaesthesia and after they had been starved for 24h from between 09.00 and 10.00h the previous day. Fat-cells were isolated from the fat-pads as described by Cunningham & Robinson (1969), except that an additional step was introduced after the incubation with collagenase by filtering the disrupted tissue through nylon mesh (0.3mm diam.) to remove any undispersed tissue elements. After the cells had been washed twice as described by Cunningham & Robinson (1969), they were washed a third time in 20vol. of the medium in which they were eventually to be incubated, but modified by the omission of the glucose, insulin and heparin components.

Fat-pads from several rats were used in each isolation and the fat-cells were pooled, so that in appropriate experiments, samples representative of the pool could be incubated under different conditions and the results compared directly with each other. For the distribution of the samples, the cells were taken in suspension with a silicone-treated pipette, the tip aperture of which was such (2mm) as to minimize cell damage.

Incubation media

The incubation media used were variations of the 'complete reconstituted medium' described by Salaman & Robinson (1966) and Wing & Robinson (1968).

A serum-based medium was used in many of the experiments, and this had the following composition: 1.2vol. of serum previously dialysed against 0.85% NaCl as described by Salaman & Robinson (1966), 0.375vol. of an amino acid solution, 0.25vol. of a salts solution, 0.125vol. of D-glucose (48mg/ml) 0.125vol. of insulin (0.24unit/ml), 0.125vol. of heparin (48 units/ml), 0.125vol. of $NaHCO_3$ (37.7mg/ml) and 0.175vol. of water. The amino acid solution contained a mixture of L-amino acids such that their final concentration in the medium was equal to twice their concentration in rat plasma (Miller & Biegelman, 1967; East *et al.*, 1973). The salts solution contained a mixture of salts such that the final concentrations of individual ions in the medium (expressed as mg/100ml) were as follows: 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.

In some experiments a casein-based medium was used. It differed from the serum-based medium described above solely by the replacement of the serum by an equal volume of a 4% (w/v) casein solution that had also been dialysed against 0.85% NaCl.

The heparin solution was omitted from the media in some experiments and the volume of water added was then increased accordingly. In other experiments, a solution of cycloheximide was added and, in this case, it replaced an equivalent volume of water. All the media were gassed with O_2+CO_2 (95:5) to give a final medium pH of between 7.3 and 7.5 (Salaman & Robinson, 1966).

Most of the constituents of the media were obtained from the sources described by Salaman & Robinson (1966). The casein (soluble, light white) and the amino acids were purchased from British Drug Houses Ltd., Poole, Dorset, U.K.

Assay of clearing-factor lipase

Clearing-factor lipase activity was determined throughout the study by measuring the release of free fatty acids from a suitable triglyceride substrate at 37°C. The method of assay was essentially that described by Salaman & Robinson (1966). However, instead of chylomicrons, which were used as the triglyceride substrate in their study, the artificial triglyceride emulsion Intralipid (Vitrum, Stockholm, Sweden) was used. Morever, the composition of the assay medium was slightly modified as described by Cunningham & Robinson (1969). Recent studies with a variety of rat adipose-tissue preparations have shown that, with this assay system, the rates of free fatty acid release are the same with chylomicron and Intralipid triglycerides as substrate (Riley & Robinson, 1974). Other authors have reached a similar conclusion (Boberg & Carlson, 1964).

Assays of the enzyme were carried out on aqueous homogenates of acetone–ether-dried preparations made from intact fat-pads, from the fat-cells isolated from the fat-pads, or from the fat-cells together with the media in which they had been incubated. They were also carried out in some experiments directly on samples of the incubation media alone.

The methods used to make the acetone–ether-dried preparations and homogenates thereof were essentially those described by Salaman & Robinson (1966) and Cunningham & Robinson (1969). Usually the preparations were made from single fat-pads or from a quantity of cells (or cells and incubation medium combined) equivalent to that isolated from a single fat-pad. To facilitate the subsequent handling of the preparations, it was necessary to increase their bulk by the addition of extra protein. For preparations made from fat-pads, this was done by homogenizing the tissue in 1.5ml of a solution of 5% (w/v) casein that had been dialysed against 0.85% NaCl and then dispersing the homogenate in acetone at 4°C. For preparations made from fat-cells, or from fat-cells and incubation medium combined, a homogeneous sample of the cells, or cells and medium, was mixed with 1.5ml of the 5% (w/v) casein solution and this was then pipetted directly into the acetone. No heparin was added (cf. Salaman & Robinson, 1966).

Separation of the fat-cells from the incubation medium was carried out by centrifugation for 1min at 1000*g*. Under these conditions the cells formed a layer at the surface, and this was recovered by removing the subnatant medium with a fine-tipped silicone-treated Pasteur pipette. When the enzyme was to be assayed in the incubation medium directly, the same procedure provided the medium samples.

Assays of the enzyme in incubation media were carried out immediately after the medium samples were obtained. The acetone–ether-dried preparations were, however, stored at −20°C overnight and then assayed on the next day. To assess the likely loss of enzyme activity under such conditions, acetone–ether-dried preparations were made from samples of the incubation medium which were also assayed directly. The activity of the acetone–ether-dried preparations was 90–95% of that measured by direct assay.

In previous work, the activity of clearing-factor lipase in rat epididymal fat-pads has been expressed as units of enzyme/fat-pad, where 1 unit is equal to the release of 1μmol of free fatty acid/h at 37°C (Salaman & Robinson, 1966). In this study, however, most of the assays were carried out on preparations derived from fat-cells, rather than from fat-pads. In the course of the investigation we made 35 measurements of the clearing-factor lipase activity of single intact fat-pads, immediately after their removal from 24h-starved rats, and 39 measurements of the activity of the total yield of fat-cells isolated from such single fat-pads. The respective mean (±S.D.) activities were 12.2±4.5 and 12.5±3.6 units. Thus in such starved animals there is no loss of enzyme activity during the isolation procedure (see also Cunningham & Robinson, 1969). We therefore expressed the fat-cell clearing-factor lipase activities as units of enzyme/fat-pad equivalent, whether the activities were measured in the fat-cells or in the media in which the cells were incubated. We should emphasize, however, that we did not assess the fat-cell yield in the present study. If this was significantly less than 100% it would imply an activation of clearing-factor lipase during the isolation procedure. This possibility certainly cannot be excluded in view of the evidence that a change in the relative proportions of different molecular species of the enzyme occurs during the isolation procedure (see the Results section).

Where at least four clearing-factor lipase activities are measured they are expressed as means±S.E.M. unless otherwise stated. The significance of differences of activity has been tested where appropriate by applying Behren's modification of Student's *t* test (Fisher & Yates, 1957), values of $P<0.05$ being accepted as significant.

The relatively high S.D. of the above mean values indicates that the activities of individual fat-pads from 24h-starved rats vary considerably between experiments. We have evidence that some of this variation may be accounted for by seasonal factors (A. Cryer, P. Davies & D. S. Robinson, unpublished work).

Amino acid incorporation studies

The incorporation of amino acids into fat-cell protein was studied with L-[1-^{14}C]leucine of specific radioactivity 60mCi/mmol (The Radiochemical Centre, Amersham, Bucks., U.K.) by using the modification of the method of Mans & Novelli (1961) as applied to fat-cells by Miller & Biegelman (1967). Radioactivity was determined by using a Beckman

LS-230 counter. The liquid-scintillation solvent contained 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene.

Gel-filtration chromatography

This was carried out on extracts of acetone–ether-dried preparations of fat-pads and of fat-cells by using columns of Sepharose 6B essentially as described by Davies *et al.* (1974). The acetone–ether-dried preparations were in this case prepared by homogenizing either 24 fat-pads, or the fat-cells isolated from 28 fat-pads, directly in acetone; that is, without the addition of any carrier protein. Extracts of the preparations were prepared in 50mM-NH_3–NH_4Cl buffer, pH 8.1 and were concentrated to 5% of their original volume in an Amicon ultrafiltration cell (model 202) with a PM30 filter before application to the column. The extracts contained approx. 50% of the clearing-factor lipase activity of the original preparations. Suitable portions were applied to columns (80cm×2cm) of Sepharose 6B (Pharmacia Ltd., Uppsala, Sweden) that had been pre-equilibrated with the extractant buffer. Elution of the fractions was carried out with a constant head (25cm) of the buffer at a flow rate of 14.5ml/h.

Results

Fat-cell incubations in the presence of heparin

Fig. 1 shows the time-course of the rise in total clearing-factor lipase activity that occurs when fat-cells, isolated from the epididymal adipose tissue of rats starved for 24h, are incubated at 25°C and pH 7.4 in media that contain glucose, insulin, amino acids, heparin and either serum or casein.

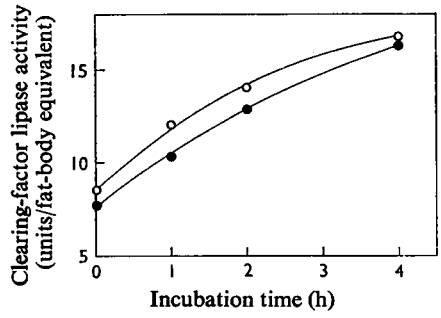

Fig. 1. *Increase in total clearing-factor lipase activity when isolated fat-cells are incubated at 25°C*

Fat-cells were isolated from 16 epididymal fat-pads taken from rats that had been starved for 24h. After washing, the cells were pooled and then equal portions were suspended at 25°C in 24ml of either a serum-based (○) or a casein-based (●) medium (see the Materials and Methods section). At intervals, acetone–ether-dried preparations were made from samples of the cells and media combined and the clearing-factor lipase activity of homogenates of these was determined. Duplicate preparations were made at each interval and the values shown are the means of the measured enzyme activities.

We have carried out six separate experiments of this kind with the medium that we have designated 'serum-based medium' (see the Materials and Methods section), and in these the total enzyme activity at 3h was 194±10% of the initial value of 10.3±1.3 units/fat-pad equivalent. In these same experiments, the distribution of the increase in enzyme activity between the fat-cells and the medium was followed. A small, but significant, amount of clearing-factor lipase activity, amounting to 13±2% of the total activity of the system, was already present in the medium at the beginning of the incubation. This increased progressively during the incubation, as shown in Fig. 2(*a*), so that after 3h it accounted for essentially all of the increase in the total activity. On the other hand, the final cell activity (9.3±1.3 units/fat-pad equivalent) was not significantly different from the initial cell activity (8.7±1.3 units/fat-pad equivalent) and, in further experiments in which the cell activity was measured at 1h intervals during a 3h incubation, no significant change was observed at any time.

In the presence of cycloheximide at a concentration of 10μg/ml, protein synthesis in intact epididymal adipose tissue is inhibited during incubations of the tissue at 25°C by at least 90% (Cryer *et al.*, 1973). In the present study, the same concentration was shown to inhibit protein synthesis to a similar extent during incubations of isolated fat-cells at 25°C (Fig. 3). Despite this Fig. 2(*b*) shows that the rise in clearing-factor lipase activity in the medium which occurs during such incubations is not affected in the presence of this concentration of cycloheximide. The fat-cell activity does fall significantly under such circumstances, however. Thus in the experiments of Fig. 2(*b*), the fat-cell activity after 3h was 6.4±1.2 units/fat-pad equivalent, a fall to 68±4% of the initial cell activity of 9.4±1.6 units/fat-pad equivalent, which was statistically significant ($P<0.05$). Because of this fall in fat-cell activity, the increase in total clearing-factor lipase activity was also decreased in the presence of cycloheximide from 9.1±1.3 to 5.5±1.2 units/fat-pad equivalent.

Three experiments similar to those described above have been carried out with a casein-based medium (see the Materials and Methods section). The rise in total enzyme activity that occurs when fat-cells are incubated in this medium is also wholly due to the increase in activity in the medium and this is unaffected in the presence of cycloheximide. Again, however, cycloheximide decreases the rise in total activity because of a decline in the cell activity that occurs in its presence.

Fat-cell incubations in the absence of heparin

The omission of heparin from the incubation medium does not significantly alter the increase in

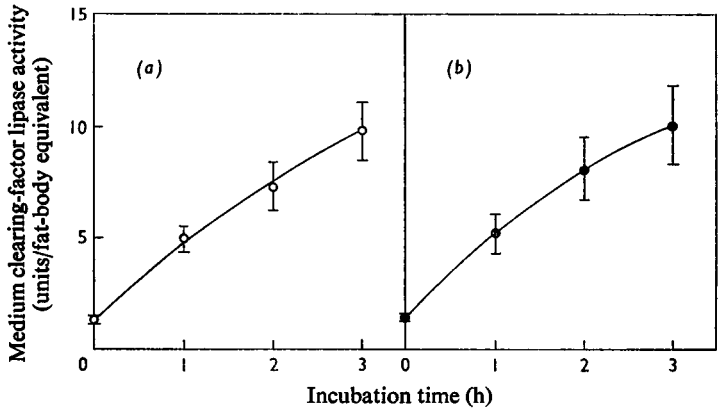

Fig. 2. *Increase in clearing-factor lipase activity in the medium when isolated fat-cells are incubated at 25°C in a serum-based medium in the absence and the presence of cycloheximide*

Fat-cells were isolated from the epididymal fat-pads of rats that had been starved for 24h. After washing, the cells were pooled and then portions were suspended in either the serum-based medium described in the Materials and Methods section (*a*) or in the same medium containing cycloheximide (Sigma Chemical Co., London S.W.6, U.K.) at a concentration of 10μg/ml (*b*). In each incubation, fat-cells derived from at least eight fat-pads were used and the volume of the medium was 3ml/fat-pad equivalent of cells. Portions of the incubation mixture were removed at intervals and the clearing-factor lipase activity of the incubation medium was measured in these after removal of the fat-cells by flotation in the centrifuge. The values shown are the means of duplicate assays in six completely independent experiments carried out without cycloheximide and in four parallel experiments carried out with cycloheximide. The bars represent S.E.M.

total clearing-factor lipase activity that occurs when isolated fat-cells are incubated in a serum-based medium at 37°C (Robinson & Wing, 1971). In the present study, we have shown that this is also true for the increases that occur during incubations in the same medium at 25°C. Thus in four experiments the total clearing-factor lipase activities after incubation for 3h in the presence and absence of heparin were increased respectively to 170±10 and 166±4% of the initial values. As Fig. 4(*a*) shows, however, the rise in the activity of enzyme in the incubation medium was less in the absence of heparin than in its presence (cf. Fig. 2*a*) and the similarity of the increase in total activity in the two situations is therefore accounted for by the increase in the cell activity that occurred during incubations in the absence of heparin. In the presence of cycloheximide, the rise in the enzyme activity in the medium was unaffected, as it was during the incubations carried out in the presence of heparin, but the increase in the fat-cell activity was less (Fig. 4*b*). As a result, the extent of the rise in total enzyme activity during the 3h incubation was also less in the presence of cycloheximide than in its absence, decreasing from 11.9±0.7 to 5.9±0.9 units/fat-pad equivalent ($P<0.001$).

Fat-cell incubations in the absence of serum

Stewart & Schotz (1973) have reported that the release of clearing-factor lipase from isolated fat-cells incubated in the presence of albumin, glucose and salts, but no heparin or insulin, is enhanced sixfold when serum is added to the medium. It is not clear from their paper whether any increase in total clearing-factor lipase activity occurred under their conditions of incubation. However, besides incubating fat-cells in serum-based medium from which the heparin component had been omitted, we have also carried out incubations using casein-based medium (see the Materials and Methods section), again with the heparin component omitted.

In six such experiments, the total clearing-factor lipase activity of the system increased after incubation for 3h at 25°C to 167±11% of the starting activity, which is very similar to the rise found in the studies using the equivalent serum-based medium. However, in the same experiments, the activity present in the medium at 3h was 6.8±0.4 units/fat-pad equivalent, which is higher than that found with the serum-based medium (cf. Fig. 4*a*). Consistent with the evidence for increased extraction of the enzyme was the finding that there was no increase in the fat-cell activity during incubations in the casein-based medium comparable with that found during incubations in the serum-based medium. In fact, in four separate experiments, the fat-cell activity after 3h was 101±4% of the initial value.

As in the studies with serum-based medium, cycloheximide did not decrease the enzyme activity in the medium in these experiments. Thus in five experiments in which cycloheximide was present, the acti-

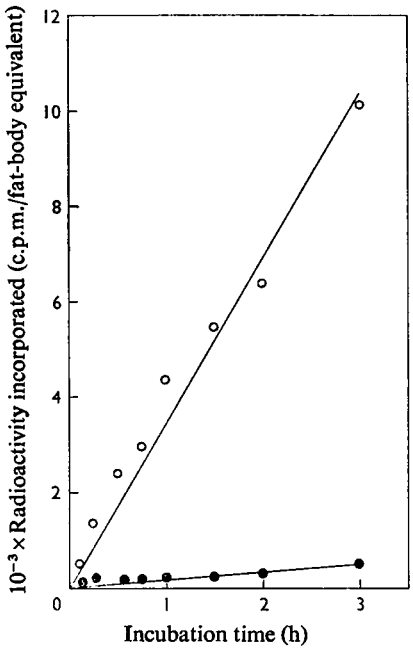

Fig. 3. *Inhibition by cycloheximide of protein synthesis in isolated fat-cell incubations*

Fat-cells were isolated from six epididymal fat-pads taken from rats that had been starved for 24h. After washing, the cells were pooled and then equal portions were incubated as described in the legend to Fig. 2 in the presence (●) and absence (○) of cycloheximide (10 μg/ml). The media also contained 1 μCi of L-[1-^{14}C]leucine/ml. Portions (0.1 ml) of the incubation mixture were removed in triplicate at intervals and the extent of incorporation of amino acids into the fat-cell protein was measured as described in the Materials and Methods section. Mean values for the incorporation at each time-interval are shown.

vity in the medium at 3h was 7.2±0.4 units/fat-pad equivalent. Cycloheximide did, however, significantly lower the cell activity and, in four experiments in which this was measured directly after a 3h incubation at 25°C, it was only 50±2% of the initial value. As a result, the rise in total enzyme activity was also less in the presence of cycloheximide and, in five experiments, it increased to only 116±10% of the initial value during a 3h incubation.

Molecular forms of fat-cell clearing-factor lipase

Schotz & Garfinkel (1972) have reported the existence in intact rat adipose tissue of two molecular species of clearing-factor lipase that are separable by gel-filtration chromatography. A form of high molecular weight (form 'a'), which is eluted at the void volume, predominates in extracts of tissue taken from rats in the fed state, whereas a form of much lower molecular weight (form 'b') predominates in tissue extracts of starved rats (Garfinkel & Schotz, 1973). We have confirmed and extended these findings (Davies *et al.*, 1974). Since the fat-cell contains virtually all of the clearing-factor lipase activity of adipose tissue in starved rats (see the Materials and Methods section), we expected that the distribution of the two enzyme forms in fat-cells would be similar to that in the intact tissue. However, as the results in Fig. 5 show, this is not the case. In fact, the pattern resembles, at least superficially, that in adipose tissue taken from rats in the fed state (cf. Fig. 1 of Davies *et al.*, 1974). The changes do not appear to be due to the use of collagenase in the isolation of the cells, since similar alterations occur when the intact tissue is incubated at 37°C, though not at 25°C, in the absence of collagenase (Davies *et al.*,

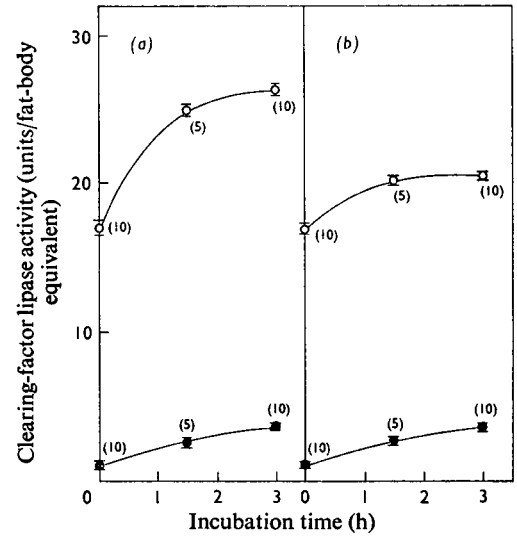

Fig. 4. *Effect of cycloheximide on the increase in fat-cell and medium clearing-factor lipase activity when isolated fat-cells are incubated at 25°C in the absence of heparin*

Fat-cells were isolated from the epididymal fat-pads of rats that had been starved for 24h. After washing, the cells were pooled and then portions were suspended either in the serum-based medium described in the Materials and Methods section from which the heparin component had been omitted (*a*) or in the same medium containing cycloheximide at a concentration of 10 μg/ml (*b*). In each incubation, cells isolated from six fat-pads were used and the volume of the medium was 3 ml/fat-pad equivalent of cells. Portions of the incubation mixture were removed at intervals and the clearing-factor lipase activity of the fat-cells (○) and of the medium (●) was measured after separation of the fat-cells by flotation in the centrifuge. The values shown are the means of duplicate assays and the bars represent S.E.M. The number of observations at each time-interval is shown in parentheses.

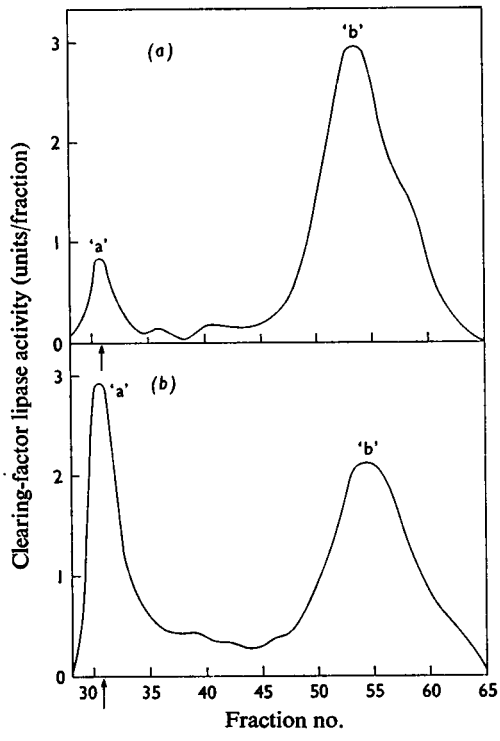

Fig. 5. *Elution profiles of clearing-factor lipase in epididymal fat-pads from 24h-starved rats and in fat-cells isolated therefrom*

Extracts of acetone–ether-dried preparations were made from fat-pads (*a*) and from fat-cells (*b*) as described in the Materials and Methods section. Samples of each extract were applied to Sepharose 6B columns and eluted at 4°C. Alternate fractions (1 ml) of the eluates were assayed for clearing-factor lipase. The column void volume is indicated by the arrow. The recovery of enzyme applied to the column, calculated from the areas under the curves, was 75% (*a*) and 81% (*b*). Two peaks of enzyme activity, referred to in the text as 'a' and 'b', are shown.

1974; A. Cryer, P. Davies & D. S. Robinson, unpublished work).

Discussion

The results of the present study show that the increase in clearing-factor lipase activity that occurs during incubations of isolated fat-cells in appropriate media at 25°C differs in several respects from that which takes place during similar incubations of intact adipose tissue. Thus the magnitude of the increase is considerably less than is observed during incubations of the intact tissue carried out immediately after its removal from the animal (Robinson & Wing, 1971), and it appears that in the presence of heparin it is the capacity for sustained increases in the enzyme activity, which depends on continuing protein synthesis, that is most markedly decreased (Cryer *et al.*, 1973). Again, whereas the increases during incubations of the intact tissue in the absence of heparin, or during the first 2h of incubations in the presence of heparin, take place wholly within the tissue (Robinson & Wing, 1971; Cryer *et al.*, 1973), those that occur during incubations of isolated fat-cells under similar conditions are accounted for, either wholly or in part, by rises in the enzyme activity in the incubation medium. Because of these differences, it is difficult to relate the present findings directly to the problem of the control of the activity of the enzyme in the intact tissue. However, they do not appear to conflict with the essential features of the hypothesis concerning that control which has been advanced in the introduction and elsewhere (Cryer *et al.*, 1975). Thus increases in activity that are at least partially independent of protein synthesis do occur during the incubation of fat-cells at 25°C and much of this increased activity is due to enzyme that is released from the cells.

Any explanations that are offered for the differences between the isolated fat-cell and the intact tissue incubation systems must clearly be tentative at the present time. However, there are obvious differences in the structural organization of the two systems which could influence the accessibility of heparin and other components of the incubation media to the sites of control and release of the enzyme. The changes that occur in the relative proportions of different molecular forms of the enzyme during the isolation of the fat-cells (see Fig. 5) could also be important. Moreover, we have obtained evidence that the enzyme of peak 'b' in Fig. 5 itself exists in two further forms ('b' and 'b'') and, during the increase in clearing-factor lipase activity, which occurs when the intact tissue is incubated in an appropriate medium at 25°C, there is a change in the relative proportions of these which suggests the conversion of one into the other by a process that is independent of protein synthesis (Davies *et al.*, 1974; Cryer *et al.*, 1975). Clearly, not only could the relative amounts of these two forms also alter during the isolation of the fat-cells, but the increases in activity that occur during the incubation of the isolated fat-cells could involve a further change in their proportions.

Stewart & Schotz (1971, 1974) have suggested that the release of clearing-factor lipase from isolated fat-cells is accompanied by activation of the enzyme. Some of the observations in the present study are consistent with this view. Thus, during incubations of fat-cells in the presence of heparin and cycloheximide, the mean rise in the enzyme activity in the medium was 8.7 units/fat-pad equivalent (Fig. 2*b*) and this was accompanied by a fall in the mean cell activity of only 3.0 units. However, the results of the corresponding experiments carried out in the absence of heparin are not easily interpreted in this way. It seems therefore

that either the release process in the presence of heparin is different from that which occurs in its absence, or the changes in the cell and medium enzyme activities are not directly related. In this connexion it is noteworthy that although cycloheximide does not affect the increases in medium enzyme activity it has variable effects on the cell activity. Moreover, increases in total activity that occur in the absence of cycloheximide are very similar in the presence and absence of heparin, despite the differences in the enzyme activity that is released into the medium in the two situations.

The failure of cycloheximide to affect the extent of the release of clearing-factor lipase into the medium during the incubation of fat-cells at 25°C agrees with the observations of Stewart & Schotz (1971, 1974) using a somewhat different experimental system. However, our findings differ from those of these authors in that the fat-cell enzyme activities that they reported were much lower than those obtained in the present study and did not change during incubations in the presence of cycloheximide. In our experiments the cell activity either fell in the presence of cycloheximide, or did not increase as much as in its absence. Stewart & Schotz (1971, 1974) isolated fat-cells from the adipose tissue of rats that were in the fed state and assayed the enzyme in aqueous homogenates of such cells by a method which we have shown gives much lower enzyme activities than the one used in the present study (Riley & Robinson, 1974). The differences in the results may therefore be due to these differences of technique. It is also noteworthy that the assay system used by Stewart & Schotz (1971, 1974) did not contain heparin as a routine, as did that used here (see Salaman & Robinson, 1966). Thus the increased activity of clearing-factor lipase in the fat-cell which they observed during incubations in the presence of heparin, but which we have not found, may be due to an action of heparin on the assay of the enzyme and be exerted, not during the incubation of the fat-cells, but after their disruption for enzyme assay.

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