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INACTIVATION OF LIPOPROTEIN LIPASE IN BUFFERED SALINE SOLUTIONS

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SUMMARY

1 The rate of inactivation of lipoprotein lipase (glycerol-ester hydrolase, EC 3.1 1 3) in dilute buffered saline solutions has been investigated, and found to be dependent on the ionic strength of the incubation medium and not upon its molar concentration

2 Unesterified long-chain fatty acids protect the enzyme from the effects of ions by formation of stabilised enzyme–free fatty acid complexes.

3. Lipoprotein lipase purified from post-heparin plasma, or adipose, heart, lung or diaphragm tissues shows the same first-order kinetics of inactivation in each case, and appears to represent a single enzyme species.

4 Crude enzyme preparations from these tissues are partially stabilised in comparison with the purified enzyme.

INTRODUCTION

Lipoprotein lipase (clearing factor lipase) (glycerol-ester hydrolase, EC 3.1 1.3) catalyses the hydrolysis of the triglyceride moiety of chylomicrons and low-density lipoproteins, and is probably involved in the uptake of triglyceride fatty acids from the blood[1,2] It is released into the bloodstream following the intravenous injection of heparin[3] The enzyme has also been studied using acetone-dried powders of several tissues including heart and adipose tissues[4–6] Although the enzyme activity from these sources is identical in many properties a difference in the 'activation energies' of heart and adipose tissue enzyme preparations has recently been reported[7]. In the present study the different rates of inactivation of several enzyme preparations in buffered saline solution have been investigated. Evidence is presented that the differences observed may be the result of the binding of different amounts of a stabilising factor to a single enzyme species.

MATERIALS AND METHODS

Fully fed, male, Wistar strain albino rats were used throughout Whole blood from rats weighing 300–400 g was collected from the dorsal aorta into 0.05 vol of

7 5%, w/v, sodium citrate in plastic tubes After centrifugation at 2500 × *g* for 20 min the plasma was recalcified with 0 025 vol of 1 M $CaCl_2$, and after removal of the fibrin clot the serum was dialysed overnight against 100 vol of 0.145 M NaCl at 4°. Triglyceride emulsion (Intralipid 20%, Vitrum, Stockholm, Sweden) was incubated with 19 vol. of serum for 1 h at 37° to produce lipoprotein substrate[6] In some experiments further purification of this substrate was carried out by centrifugation of the lipid–serum mixture at 75 000 × *g* for 1 h The upper lipid layer was resuspended in the same volume of 0 145 M NaCl The centrifugation and resuspension procedure was repeated twice, and the final lipid supernatant suspended in 0 145 M NaCl and used within 24 h

All manipulations involving enzyme solutions were carried out at 4°. Epididymal adipose tissue obtained from rats weighing 120–140 g was homogenised in 0.145 M NaCl (1 ml saline/epididymal fat pad) for 10 sec at the top speed of a Silverson blender. The homogenate was extracted with 40 vol of acetone and 20 vol. of diethyl ether, and the precipitate dried under vacuum[8]. The dried matter was shaken gently with 0.05 M Tris–HCl buffer (pH 8 1) containing heparin (Evans Medical Co , Liverpool, Great Britain) at 1 0 I.U /ml, for 1 h (15 mg dried residue per ml of buffer). Heparin increased the amount of enzyme liberated into solution but was without effect on its properties. The homogenate obtained was centrifuged at 5000 × *g* for 30 min and the supernatant, which contained 3–4 mg protein/ml (by the method of LOWRY *et al* [9]) and 10–15 enzyme units/ml* was used as a source of adipose tissue lipoprotein lipase Three further extractions with fresh portions of buffer removed over 90% of the homogenate enzyme activity into solution In most experiments the supernatant from a single extraction was used. This contained about 60% of the homogenate activity. Supernatant preparations of heart, lung and diaphragm tissue enzyme were prepared in the same way

Post-heparin plasma was taken from rats which had been injected 10 min previously with heparin (100 I U./kg) *via* the femoral vein The plasma after centrifugation was extracted with 40 vol of acetone and 20 vol of diethyl ether. The dried powder was dissolved in Tris buffer–heparin (15 mg/ml) This solution had an activity of 10–15 units/ml. Bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Great Britain) was added as carrier protein in some experiments

Preliminary experiments to purify the enzyme from plasma and tissue preparations showed that absorption on $Ca_3(PO_4)_2$ gel, which gave good purifications with human post-heparin plasma enzyme[10,11] was unsuitable for use with the enzyme from rat plasma or tissue Following the technique of NIKKILA[10] no more than 20% of the absorbed enzyme could be recovered in the citrate eluate following oxalate treatment Two other methods giving almost complete recovery have been used in the purification of the enzyme from the rat When post-heparin plasma was diluted with 15 vol of distilled water, and the pH adjusted to 5.7 with 0 1 M HCl, the whole lipoprotein lipase activity was precipitated[12] After centrifugation at 2500 × *g* for 20 min this precipitate was dissolved in Tris buffer–heparin and the solution extracted with acetone and ether as before. The dried powder was finally re-dissolved in the same buffer An 8–10-fold purification was obtained

Lipoprotein lipase forms a stable complex with its substrate and has been purified several 100-fold from human plasma by separation of the upper lipid layer after centrifugation of the plasma–substrate mixture[13] Post-heparin plasma or tissue

* One enzyme unit catalyses the release of 1 μmole of free fatty acid per h

enzyme solution from the rat was mixed with an equal volume of purified substrate prepared as described, and the mixture stood in ice for 1 h It was then centrifuged at 75 000 × *g* for 1 h and the upper layer separated and resuspended as described The process was repeated 4 times Purification from plasma was 300–500-fold, from adipose tissue 40–50-fold. However, the stability of the enzyme obtained after a single treatment is not changed by further purification After addition of carrier protein the mixture of lipid and bound protein was extracted with acetone and ether and the dried powder made up in Tris buffer–heparin

Bovine serum albumin was freed of bound long-chain fatty acid[14] and a 5% solution (pH 7 4), made up in 0 145 M NaCl Solutions of the potassium salts of long-chain fatty acids (oleic, stearic, palmitic, linoeic, linolenic acids, Calbiochem, Calif., more than 99% pure, confirmed in the laboratory by gas–liquid chromatography of the methyl esters) were prepared in twice the calculated quantity of 0.1 M KOH at 50°, diluted as required with 0 145 M NaCl. Fatty acid at known concentrations was bound to the defatted albumin preparation[15]

An assay system based on that suggested by ROBINSON[2] has been used. 1 ml of enzyme solution in Tris buffer–heparin was mixed with the same volume of 0 145 M NaCl, and added to 3 ml of the following assay mixture: 24 ml of 15%, w/v, bovine serum albumin in Krebs–Ringer solution (pH 8 1) + 4.3 ml of Tris–HCl buffer, 1 35 M (pH 8 1) + 14 ml of plasma–triglyceride mixture pre-incubated as described + 0.7 ml of heparin solution in 0.145 M NaCl (40 I.U /ml) Two initial samples of 1 ml of reaction mixture were taken for measurement of the free fatty acid content[16] The reaction flask was incubated in a shaking water bath at 37° for periods of up to 2 h, after which two further 1-ml samples were taken for assay of free fatty acid as before Control flasks containing buffer without enzyme were incubated routinely The increase in the free fatty acid of the flask containing enzyme, corrected for any increase in the control flask, gave the enzyme activity of the original enzyme solution in the units defined Duplicate assays were reproducible to within 5%. Under the conditions described the production of free fatty acid was zero order and linear.

In the absence of added substrate no free fatty acid was produced from the acetone–ether extracted enzyme preparations Intralipid triglyceride emulsion in the absence of plasma was not hydrolysed by the enzyme (*cf* ref 4).

RESULTS

Effects of buffer concentration and ionic strength on adipose tissue enzyme

The acetone- and ether-extracted powder from adipose tissue enzyme–substrate complex was dissolved in either Tris–HCl buffer, 0.088 M, I = 0 04 (pH 8 1) or in Na_2HPO_4–NaH_2PO_4 buffer, 0 0137 M, I = 0 04 (pH 8 1), 15 mg powder per ml of buffer in each case. Each buffer contained heparin (0.5 I U /ml) By dilution with distilled water or with either buffer at higher concentration (I = 0.20) solutions of the same concentration of enzyme but made up in buffer of I 0 01–0 10 were prepared The proportion of initial enzyme activity remaining after an incubation of 1 h at 37° was measured In other experiments part of the ionic strength contribution of the buffer was replaced with NaCl or $MgCl_2$ In no case did these changes affect the enzyme assay The differences found were therefore due to loss of enzyme activity during incubation, not to inhibition of the assay The results of such an experiment are shown in Fig. 1.

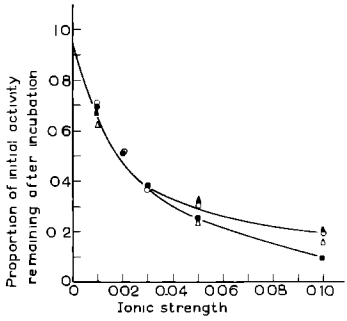

Fig 1 The effect of increased ionic strength on the stability of purified adipose tissue enzyme Two 2-ml samples of adipose tissue enzyme purified from enzyme–substrate complex (6 4 enzyme units/ml) in Na_2HPO_4–NaH_2PO_4 buffer (0 0137 M) or Tris–HCl buffer (0 088 M), pH 8 1 in each case, and diluted to I 0 01–0 1, in one case assayed directly after addition of 3 ml of assay mixture, in the other incubated 1 h at 37° before assay in the same way The results are expressed as the proportion of activity assayed directly recovered after the incubation period ○—○, Tris–HCl buffer, ●—●, Na_2HPO_4–NaH_2PO_4 buffer, ▲—▲, Tris buffer, I = 0 01 + NaCl, △—△, phosphate buffer, I = 0 01 + NaCl

At low ionic strength, the same rate of loss of activity was obtained with both buffers Above I = 0 05 phosphate showed a greater effect Substitution of NaCl for Tris–HCl buffer did not change the proportion of enzyme activity recovered, provided that the ionic strength was unchanged Substitution of NaCl for phosphate relieved in proportion the extra inhibitory effect of this buffer In the presence of Tris–HCl buffer, $MgCl_2$ inhibited to a greater extent than predicted on grounds of ionic strength alone. Addition of $MgCl_2$ to phosphate buffer, with the formation of relatively insoluble magnesium phosphates, gave a reduction in the inhibitory effect of the buffer

Kinetics of inactivation of lipoprotein lipase

In further experiments on ionic strength effects, a standard buffered saline solution was used, prepared by mixing equal volumes of 0 145 M NaCl and 0 05 M Tris–HCl buffer (pH 8 1) The ionic strength of the mixture was 0 085 Loss of enzyme activity during incubation in this solution could therefore be considered in terms of ionic strength effects

Plasma and adipose tissue enzyme solutions were prepared in Tris buffer–heparin, and mixed with an equal volume of 0 145 M NaCl held at 37°. Samples were withdrawn at zero time of incubation and thereafter at intervals, and these were assayed in the usual way so that the time course of the loss of enzyme activity could be followed

For first-order kinetics, the constant $k_1 = 2\ 303/(t_x - t_0) \log c_0/c_x$ for enzyme concentrations c_0 and c_x after incubation periods t_0 and t_x The plot of log c_0/c_x will be linear against time t. Adipose tissue enzyme accurately followed first-order kinetics (Fig 2) In some experiments the reaction was followed until 10% of the initial enzyme activity remained, the kinetics of the reaction remained unchanged over the whole time course The first-order constant was 0.016 min^{-1} (three experiments, range 0.0156–0 0174) With post-heparin plasma a similar initial rate of inactivation decreased as the reaction proceeded (Fig 2) Solutions of lipoprotein lipase from

acetone- and ether-extracted lung, heart and diaphragm tissues were prepared in Tris buffer–heparin These were incubated with an equal volume of 0.145 M NaCl as described above The first-order constant was the same as that of adipose tissue enzyme

Post-heparin plasma enzyme purified by precipitation at acid pH and low ionic strength[12] was dissolved in buffered saline solution and the time course of loss of enzyme activity at 37° obtained The reaction followed first-order kinetics, $k_1 = 0\ 039\ min^{-1}$ (three experiments, range 0 0360–0 0411) Enzyme from plasma and from the tissues given above was also purified by absorption to substrate[13], subsequently extracted with acetone and ether. These preparations also followed first-order kinetics, with a rate constant identical with that of plasma enzyme purified by the previous technique

Effects of plasma on the rate of inactivation of lipoprotein lipase

The difference observed in the stabilities of unpurified plasma and tissue enzymes might result from the influence of a factor present in plasma To investigate this possibility the NaCl component of the buffered saline incubation medium was replaced with an equal volume of normal plasma dialysed against the same NaCl solution The course of inactivation of the enzyme during incubation at 37° was followed as before

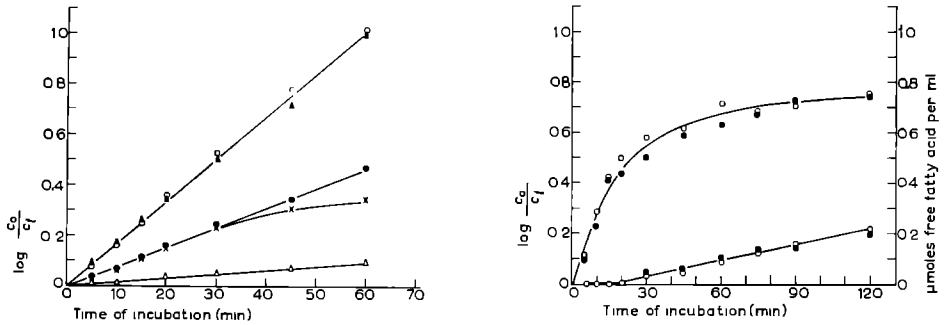

Fig 2 The rate of loss of activity of several enzyme preparations at 37° Five ml of the enzyme prepared as described under METHODS in Tris buffer–heparin was incubated at 37° with an equal volume of 0 145 M NaCl At intervals, 0 5-ml samples were withdrawn, mixed with 1 5 ml of a mixture of equal parts of Tris buffer–heparin and with 3 ml of assay medium, and assayed in the usual way Where the enzyme has been incubated in potassium oleate solution this is made up in 0 145 M NaCl, the concentration given is the final concentration in the incubation medium The proportion of initial enzyme activity remaining after a given period of incubation is expressed as the log of the initial enzyme concentration in enzyme units (c_0) to concentration (c_x) after an incubation period of t_x min ○—○, purified post-heparin plasma, ▲—▲, purified adipose tissue enzyme, ●—●, crude adipose tissue enzyme, ×—×, crude post-heparin plasma; △—△, purified adipose tissue enzyme + 0 10 µM potassium oleate

Fig 3 The rate of loss of enzyme activity from adipose tissue and plasma preparations in the presence of normal serum Six ml of unpurified plasma or tissue enzyme solution, prepared as described under METHODS, incubated at 37° with the same volume of whole normal plasma dialysed against 0 145 M NaCl Assay procedure and expression of results as in Fig 2 At the same times that portions were withdrawn for enzyme assay, 0 5-ml portions were assayed directly for free fatty acid content (method of DOLE[16]) Results expressed as free fatty acid concentration in the incubation medium ○—○, crude post-heparin plasma enzyme, ●—●, crude adipose tissue enzyme Upper curve free fatty acid in incubation flask. Lower curve. rate of loss of enzyme activity, calculated as described in the text

At the same time the free fatty acid concentration of the incubation medium was recorded In this way the hydrolysis of plasma lipoprotein could be monitored

Identical results were obtained from adipose tissue and post-heparin plasma preparations (Fig. 3). In a first phase lasting about 30 min there was no loss of enzyme activity, and simultaneously a rapid increase in the free fatty acid concentration of the incubation medium. Subsequently, the production of free fatty acid markedly declined, and at the same time there occurred a slow loss of enzyme activity which followed first-order kinetics, $k_1 = 0.004$ min^{-1} (three experiments, range 0.0038–0.0046) In two further experiments the normal plasma used in the incubation medium had been previously extracted with acetone and ether, as described previously for post-heparin plasma; the precipitated protein was re-dissolved in the original volume in 0.145 M NaCl After mixing with enzyme solution in Tris buffer–heparin the rate of loss of enzyme activity was followed as before The first phase was absent The slow loss of enzyme activity began immediately, with the same first-order constant as before

Lipoprotein lipase may be distinguished from other mammalian triglyceride lipases in its inhibition by strong NaCl solutions[4]. When tissue or plasma enzyme was incubated with normal plasma in the presence of 1 M NaCl the increase in medium free fatty acid concentration found in the previous experiments (Fig 3) was abolished. Purified substrate, prepared as described under METHODS, was diluted with 0.145 M NaCl to a lipid concentration of 0.3%, w/v, and incubated with tissue enzyme as before. The enzyme was completely stabilised. Intralipid artificial triglyceride emulsion, in the absence of plasma, was without effect on the stability of the enzyme

Effects of long-chain fatty acids on the rate of loss of enzyme activity

The above experiments suggested that the free fatty acid present in the incubation medium might be involved in the stabilisation of lipoprotein lipase by plasma. Solutions of the potassium salts of several long-chain fatty acids were prepared in 0.145 M NaCl as described under METHODS. These were incubated with an equal volume of adipose tissue enzyme in Tris buffer–heparin The rate of loss of enzyme activity was followed as before At free fatty acid concentrations above 0.02 μM a progressive reduction of the rate of inactivation was obtained with increasing concentrations of free fatty acid such that at 0.15 μM complete stability to incubation at 37° was attained. Examples of such experiments are shown in Fig 2 The partially stabilised preparations lost activity according to first-order kinetics Although most experiments were carried out using potassium oleate, similar results were obtained with the salts of linoleic and linoenic acids. Potassium stearate and palmitate were completely inactive at concentrations of up to 2.0 μM Adipose tissue or plasma enzyme stabilised by pre-incubation with potassium oleate (0.20 μM/ml) was extracted with acetone and ether as under METHODS, and the dried powder made up in the original volume of Tris buffer–heparin, and the rate of inactivation of the preparation during incubation was found. Extraction did not remove the stabilisation achieved by prior incubation with fatty acid. A second extraction into the same volumes of acetone and ether removed no further measurable fatty acid from the precipitated material, and had no effects on stability. More than 99.5% of serum unesterified fatty acid is bound to albumin at physiological concentrations (approx 0.5 μM). Only 0.01% is in free solution; the remainder is bound to the lipoprotein fraction[17,18] The effect of albumin-bound free fatty acid on the rate of

TABLE I

EFFECTS OF FREE AND ALBUMIN-BOUND FATTY ACIDS ON ADIPOSE TISSUE ENZYME ACTIVITY

The results of two separate experiments are recorded In each, 1 ml of lipoprotein lipase solution from epididymal adipose tissue (3 4 mg protein/ml, 11 1 enzyme units/ml) was added to 1 ml of 0 145 M NaCl containing fatty acid as the potassium salt in free solution or bound to albumin Three samples were prepared in each case One was assayed directly after addition of 3 ml of assay mixture as described under METHODS The other two were first incubated at 37° for 1 h, and then assay mixture added as before The results are given as the percentage of the enzyme activity as assayed directly, retained after 1 h incubation

Fatty acid solution	*Protein (μM)*	*Fatty acid (μM)*	*Initial activity remaining after incubation (%)*	
			Expt 1	*Expt 2*
Potassium oleate	—	0 21	84	84
Potassium stearate	—	0 25	48	41
Defatted albumin	0 72	—	38	41
Defatted albumin + oleate	0 80	0 23	42	36
Defatted albumin + stearate	0 77	0 29	44	39
0 145 M NaCl only	—	—	37	42

loss of enzyme activity was investigated Adipose tissue enzyme solution in Tris buffer–heparin was incubated with a solution of albumin which contained a known concentration of bound stearic or oleic acid (Table I) The proportion of initial enzyme activity recovered after an incubation of 1 h at 37° was determined. Albumin-bound free fatty acid was completely inactive in stabilising the enzyme Unpurified post-heparin plasma, or tissue enzyme in the presence of albumin (7 5 mg/ml) added as carrier protein, was not stabilised by free fatty acid concentrations of up to 2 0 μM

Effects of a second extraction with acetone and ether

Samples of each of the enzyme preparations already described were prepared

TABLE II

EFFECTS OF A SECOND ACETONE AND ETHER EXTRACTION ON LIPOPROTEIN LIPASE PREPARATIONS

10 ml of each preparation obtained in solution as described under METHODS was assayed (1 0 ml) and the remainder after the addition of albumin as carrier protein (7 5 mg/ml) except in the case of unpurified post-heparin plasma, precipitated with acetone and ether The powder, after drying in vacuum, was made up to the same volume with Tris buffer–heparin and 1-ml samples added to 1 ml of 0 145 M NaCl and 3 ml of assay mixture for assay as usual The results are given as the percentage of the enzyme activity assayed initially, retained after extraction with acetone and ether and subsequent re-assay The results of two separate experiments are recorded

Enzyme preparation	*Initial activity remaining after extraction (%)*	
	Expt 1	*Expt 2*
Unpurified post-heparin plasma	77	82
Post-heparin plasma purified by the method of HOLLETT AND MENG[12]	25	30
Post-heparin plasma purified by the method of ANFINSEN AND QUIGLEY[13]	28	34
Substrate–adipose tissue enzyme complex[13]	30	33
Unpurified adipose tissue enzyme	39	44
Adipose tissue pre-incubated with potassium oleate (0 10 μM)	82	71

in Tris buffer–heparin, a portion assayed, and the remainder then precipitated a second time with acetone and ether The precipitate was dried under vacuum and the powder re-dissolved to the same volume in this buffer and immediately re-assayed. The proportion of initial enzyme activity recovered was determined Purified plasma and tissue enzyme preparations and purified enzyme–substrate complex, were unstable to this treatment Approx. 0 3 of the initial activity was recovered Unpurified tissue enzyme was more stable (Table II) Preparations stabilised to incubation with plasma or free fatty acid were by the same process made stable to extraction with acetone and ether.

DISCUSSION

The lipoprotein lipase preparations used had each been extracted with acetone and ether. Incubation of these in assay medium without added substrate gave no formation of free fatty acid The foregoing experiments on the stability of the enzyme to incubation were therefore carried out in the absence of significant amounts of lipoprotein substrate

The stability of such enzyme preparations to incubation in dilute buffers and buffered saline solutions is dependent on the ionic strength of the incubation medium and not upon its molar concentration There is a rapid loss of activity at 37° even at low ionic strengths At $I = 0\ 05$, 70% of activity is lost in 1 h (Fig 1) Considerable evidence is available that heparin is bound to the enzyme released into post-heparin plasma[19,20] Removal of heparin from post-heparin plasma greatly reduces the stability of this enzyme preparation to incubation[21], this is restored by the addition of heparin to the incubation medium The identical kinetic constants obtained for purified plasma and tissue enzymes in a heparin-containing incubation medium (Fig 2) provide indirect evidence that tissue enzyme under these conditions may also bind heparin. Increased ionic strength may therefore affect the stability of lipoprotein by reducing interaction with the heparin polyanion, by formation of buffer anion–enzyme complexes Alternatively increased ionic strength might reduce the solubility of the lipophilic enzyme molecule in buffer solutions by reducing the binding of water molecules[22] In view of the low ionic strengths which are effective in reducing enzyme stability the former explanation is more likely The sensitivity of the enzyme to low concentrations of phosphate as reported by KORN[4] is a particular example of inactivation due to ionic strength effects

Clear differences have been found in the rates of inactivation of different enzyme preparations (Fig 2). Adipose tissue enzyme follows first-order kinetics, but plasma enzyme activity shows a more irregular decline. Comparison of the rate of inactivation of plasma enzyme in normal and post-heparin plasma (Figs 2, 3) shows that a change in the properties of the plasma must have occurred following the injection of heparin, since plasma enzyme in normal plasma is inactivated according to first-order kinetics (Fig 3). No evidence is available on the nature of this change. Nevertheless, purification from post-heparin plasma by either of two methods[12,13] produces a preparation with the same stability to incubation, and to acetone and ether extraction, as that obtained from purified tissue enzyme (Fig 2, Table II) The purified preparations therefore appear to represent a single species

The presence in the incubation medium of increasing concentrations of long-

chain fatty acids is associated with a progressive reduction in the rate of inactivation of the enzyme Precipitation of the stabilised enzyme with acetone and ether, followed by solution in the same buffer as before, does not reduce the rate constant of the inactivation process. The effect of fatty acids therefore appears to be by formation of a complex stable to extraction with acetone and ether Whole plasma is also effective in stabilising the enzyme (Fig 3) Albumin-bound free fatty acid, and free fatty acid in the presence of serum albumin, is without effect Lipoprotein lipase therefore competes unsuccessfully with albumin for free fatty acid when the concentrations of each reactant are similar to those in plasma Extraction of plasma with acetone and ether does not reduce its ability to stabilise the enzyme Experiments in progress have shown that these solvents are unable to remove free fatty acid bound to highly purified enzyme under the extraction conditions described under METHODS Free fatty acid bound to protein other than albumin, such as α- and β-lipoprotein[18], may be involved in stabilisation by plasma The very similar properties of plasma-stabilised and free fatty acid-stabilised enzyme preparations make it unlikely that different mechanisms are involved.

Unpurified tissue enzyme has a stability intermediate between that of purified and fully-stabilised preparations, both to incubation and to extraction with acetone and ether. In these properties it is indistinguishable from purified enzyme incubated with low concentrations of fatty acid. The formation of stabilised enzyme-free fatty acid complexes may play a part in the regulation of enzyme level in the tissues.

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REFERENCES

1 E D KORN, *Methods Biochem Anal*, 7 (1959) 145
2 D S ROBINSON, *Advan. Lipid Res*, 1 (1963) 133
3 D S ROBINSON AND J E FRENCH, *Quart J Exptl Physiol*, 38 (1953) 233
4 E D KORN, *J Biol Chem*, 215 (1955) 1
5 E D KORN AND T W QUIGLEY, *Biochim Biophys. Acta*, 18 (1955) 143
6 E D KORN AND T W QUIGLEY, *J Biol Chem*, 226 (1958) 833
7 M BRADY AND J A HIGGINS, *Biochim Biophys Acta*, 137 (1967) 140
8 M R SALAMAN AND D S ROBINSON, *Biochem. J*, 99 (1966) 648
9 O H LOWRY, N J ROSEBROUGH, A L FARR AND R J RANDALL, *J Biol Chem*, 193 (1951) 265
10 E A NIKKILA, *Biochim Biophys Acta*, 27 (1958) 612
11 A N PAYZA, H B EIBER AND I DANISHEVSKY, *Biochim Biophys Acta*, 111 (1965) 159
12 C. HOLLETT AND H C. MENG, *Biochim. Biophys. Acta*, 20 (1956) 421
13 C. B. ANFINSEN AND T W QUIGLEY, *Circulation*, 8 (1953) 435
14 R F CHEN, *J Biol Chem.*, 242 (1967) 173
15 P B GARLAND, E A NEWSHOLME AND P J RANDLE, *Biochem J*, 93 (1964) 665
16 V P. DOLE, *J Clin Invest*, 35 (1956) 150
17 D S. GOODMAN, *J Am Chem Soc*, 80 (1958) 3892
18 D S GOODMAN AND E SHAFRIR, *J Am Chem Soc*, 81 (1958) 612
19 K REBER AND A STUDER, *Experientia*, 14 (1958) 462
20 B BASKYS, E KLEIN AND W F LEVER, *Arch Biochem Biophys*, 99 (1962) 25
21 D S ROBINSON, *Quart J Exptl Physiol.*, 41 (1956) 195
22 K J LAIDLER, *The Chemical Kinetics of Enzyme Action*, Clarendon Press, Oxford, 1958, p 391.