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Factors Affecting the Activity and Stability of the Palmitoyl-Coenzyme A Hydrolase of Rat Brain

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Palmitoyl-CoA hydrolase (EC 3.1.2.2) catalyses the irreversible hydrolysis of long-chain acyl-CoA thioesters. This enzyme is found primarily in the postmicrosomal supernatant fraction prepared from homogenates of rat brain. Either of two forms of the hydrolase, a lower-molecular-weight species of approx. 70000 or a higher-molecular-weight species of approx. 130000 can be isolated by gel filtration. The higher-molecular-weight form is obtained from columns of Sephadex G-200 eluted with buffer containing 10μM-palmitoyl-CoA or 20% (v/v) glycerol, whereas the lower-molecular-weight form is obtained when the eluting buffer does not contain palmitoyl-CoA or glycerol. The two forms of the hydrolase have the same pH optimum of 7.5, are equally sensitive to the thiol-blocking reagents *p*-hydroxymercuribenzoate, $HgCl_2$, and 5,5′-dithiobis-(2-nitrobenzoic acid), and exhibit the same K_m (1.8μM) with palmitoyl-CoA as substrate. The two forms differ in the availability or reactivity of certain external thiol groups, as determined by covalent chromatography with activated thiol Sepharose. Dilute solutions of the lower-molecular-weight form of the hydrolase rapidly lose activity (50% in 60min at 0°C), but there is no change in the K_m with palmitoyl-CoA as substrate during this progressive inactivation. Dilutions of the hydrolase in buffer containing 10μM-palmitoyl-CoA retain full activity. However, addition of palmitoyl-CoA to solutions of the lower-molecular-weight form will not restore previously lost hydrolase activity. The evidence supports the conclusion that the substrate palmitoyl-CoA promotes the formation of a relatively stable dimer from two unstable subunits. This process may not be reversible, since the removal of palmitoyl-CoA or glycerol from solutions of the higher-molecular-weight form does not result in the appearance of the lower-molecular-weight form of the hydrolase.

The possibility that long-chain acyl-CoA thioesters play a physiologically significant role in the regulation of intermediary metabolism, particularly lipid metabolism, has received increasing attention (Nordlie *et al.*, 1967; Lerner *et al.*, 1972; Goodridge, 1973; Hsu & Powell, 1975; Kawaguchi & Bloch, 1976). Although general non-specific effects of long-chain acyl-CoA esters are provoked by virtue of their potent detergent properties (Taketa & Pogell, 1966), relatively low concentrations specifically and reversibly alter a number of transport and enzyme systems *in vitro*. Although extrapolation of the significance of studies *in vitro* to the situation *in vivo* has often been questioned (Taketa & Pogell, 1966; Shafrir & Ruderman, 1974; Srere, 1965), several groups of investigators have defended the notion that long-chain acyl-CoA esters may play a regulatory role *in vivo* under certain circumstances. Aberrations of lipid metabolism, as in the liver in diabetes, may in part result from an increased intracellular concentration of long-chain acyl-CoA esters (Lerner *et al.*, 1972; Cheema-Dhadli & Halperin, 1973; Goodridge, 1975; Tubbs & Garland, 1964; Seitz *et al.*, 1977).

Palmitoyl-CoA hydrolase (EC 3.1.2.2) is a ubiquitous enzyme to which no definite physiological role has been ascribed [unlike the thioesterase activity of the fatty acid synthetase complex, which functions in the release of newly synthesized fatty acids from the acyl-carrier protein (Jacob *et al.*, 1968; Phillips *et al.*, 1970)]. Since this enzyme utilizes long-chain acyl-CoA esters as substrates, it seemed possible that it might have a profound influence on the intracellular concentration of these compounds. The present paper reports the results of a study of the factors affecting the activity *in vitro* of the palmitoyl-CoA hydrolase of brain, which is the richest source of the enzyme from the rat (Kurooka *et al.*, 1972).

Experimental

Palmitoyl-CoA was purchased from P-L Biochemicals Inc., Milwaukee, WI, U.S.A. [1-^{14}C]-Palmitoyl-CoA (sp. radioactivity 60mCi/mmol) and glycerol tri[^{14}C]oleate (sp. radioactivity 83mCi/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Determinations of the purity and quantification of palmitoyl-CoA were

accomplished spectrophotometrically (Lamb & Fallon, 1970). The purities of radioactively labelled glycerol trioleate and palmitoyl-CoA were determined by chromatography on thin layers of silica gel G with n-hexane/diethyl ether/acetic acid (70:30:1, by vol.) and butan-1-ol/acetic acid/water (5:2:3, by vol.) respectively as developing solvents.

Other chemicals and supplies and their sources were: palmitic acid and glycerol trioleate from Applied Science Laboratories, State College, PA, U.S.A.; dithiothreitol, *p*-hydroxymercuribenzoate, bovine serum albumin, L-cysteine hydrochloride, 5,5′-dithiobis-(2-nitrobenzoic acid), and coenzyme A from Sigma Chemical Co., St. Louis, MO, U.S.A.; fatty acid-poor bovine serum albumin from Pentex Inc., Kankakee, IL, U.S.A.; thin-layer plates of silica gel G, Brinkman Instruments, Westbury, NY, U.S.A.; Sephadex G-25 and G-200, activated thiol Sepharose, and column apparatus from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. For calibration of Sephadex G-200 columns, a Combithek was purchased from Boehringer Mannheim, Indianapolis, IN, U.S.A. All other chemicals and reagents were of the highest quality available. Male Sprague–Dawley rats (250–300g) were purchased from Flow Laboratories, Dublin, VA, U.S.A., and were fed Purina Laboratory Chow.

Brains were quickly removed from rats after decapitation, chilled in ice-cold 0.9% NaCl, blotted, and weighed. Homogenates (10 or 20%, w/v) were prepared in 0.25M-sucrose and 0.10M-potassium phosphate buffer, pH7.4, by using a Polytron ST 10 (Brinkman Instruments) at low speed. Whole cells, cellular debris, and nuclei were removed from the homogenate by centrifugation for 5min at 700*g* ($r_{av.}$ 4.13cm) in a refrigerated centrifuge. Particulate and supernatant fractions were obtained by centrifuging the 700*g* supernatant for 60min at 105000*g* ($r_{av.}$ 5.95cm) in a refrigerated ultracentrifuge. The particulate fraction was suspended in a volume of homogenization medium equal to the original volume of the 700*g* supernatant. Except as noted, all operations were conducted at 0–4°C.

Enzyme assays

Palmitoyl-CoA hydrolase was measured *in vitro* by using either a radioassay or a spectrophotometric assay. The standard radioassay mixture consisted of 0.5–10μg of protein and 88nmol of palmitoyl-CoA (containing 0.05μCi of [1-^{14}C]palmitoyl-CoA) in a total volume of 1.0ml of 0.10M-potassium phosphate, pH7.4, containing 1mM-EDTA. Experiments to determine the optimal assay conditions revealed that, in addition to the pH of the incubation, the nature and the ionic strength of the buffer were important. Hydrolase preparations in Tris buffer were much less active than in potassium phosphate buffer. Hydrolase activity increased with increasing ionic strength of phosphate buffer until a plateau was reached at 0.075M.

After 5–60min at 37°C in a metabolic-shaker bath, the incubations were stopped by the addition of chloroform/methanol/acetic acid (50:50:1, by vol.). The chloroform phase was removed after centrifugation and the aqueous phase extracted again with chloroform. The combined chloroform extracts were washed with water, dried under N_2, and the residue taken up in n-hexane. Portions of the hexane were taken for radioassay or for analysis by chromatography on thin layers of silica gel G with n-hexane/diethyl ether/acetic acid (70:30:1, by vol.) as the developing solvent. Lipid standards were applied with the radioactive samples. The chromatograms were visualized by exposure to I_2 vapour or by spraying with Rhodamine 6G, and selected areas of the chromatograms were transferred directly into scintillation vials for radioassay.

The spectrophotometric assay of palmitoyl-CoA hydrolase was conducted by the method of Barnes (1975) except that bovine serum albumin was deleted and the temperature was 37°C. An incubation mixture consisted of 0.10mM-5,5′-dithiobis-(2-nitrobenzoic acid), 0.5–2.5 units of enzyme activity (1 unit hydrolyses 1nmol of palmitoyl-CoA/min at 37°C and at substrate saturation), and 1–100μM-palmitoyl-CoA in a final volume of 2.0ml of 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA. A Beckman Acta III recording spectrophotometer was used to measure the increase in A_{412}.

The activity of triacylglycerol lipase in fractions eluted from Sephadex columns was determined by a modification of the method described by Baginsky & Brown (1977). An assay mixture consisted of 10mg of fatty acid-poor bovine serum albumin, 2.0μmol of glycerol trioleate containing 0.08μCi of glycerol tri[^{14}C]oleate, and 20–200μl of enzyme solution in a final volume of 1.0ml of 0.10M-Tris/HCl buffer, pH7.4. The substrate was emulsified by sonication with 5% gum arabic in 0.10M-Tris/HCl buffer, pH7.4. The final concentration of gum arabic in the incubations was 7.5mg/ml. The incubations at 30°C were stopped after 10–60min, extracted (Pittman *et al.*, 1975), and the liberation of [^{14}C]oleate determined by liquid-scintillation spectrometry.

Gel filtration

All column-chromatographic procedures were performed at 2°C. Columns of Sephadex G-200 were calibrated by using the following pure proteins: aldolase (mol.wt. 158000), bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), chymotrypsinogen A (mol.wt. 25000), and cytochrome *c* (mol.wt. 12500). For desalting protein samples, a column of Sephadex G-25 was calibrated by its

ability to separate tryptophan and bovine serum albumin from a mixture of the two.

Covalent chromatography

The principles and techniques of covalent chromatography of proteins containing thiol groups by using columns of Sepharose–(glutathione–2-pyridyl disulphide) conjugate have been discussed by Brocklehurst *et al.* (1973). Except for a substitution of eluting buffer (potassium phosphate, pH 7.4, for Tris/HCl, pH 8.0), their procedures were followed closely. The hydrolase samples were reduced with 5mM-dithiothreitol, desalted, applied to the column, and elution begun with 0.10M-potassium phosphate, pH 7.4, containing 1mM-EDTA. After collecting five fractions (approx. 20–25ml), elution was continued with buffer containing 20mM-L-cysteine. The collected fractions were analysed for a sharp rise in A_{343} indicative of cysteine displacement of 2-thiopyridone and protein covalently bound by a disulphide linkage to the glutathione immobilized on the Sepharose matrix. After dialysis overnight against 0.10M-potassium phosphate, pH 7.4, containing 1mM-EDTA to remove the 2-thiopyridone and cysteine, the fractions were analysed for A_{280} and for palmitoyl-CoA hydrolase activity.

Radioactivity was determined by liquid-scintillation spectrometry with external standardization to determine counting efficiencies. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results and Discussion

Assay of palmitoyl-CoA hydrolase

When portions of cell-free homogenates of brain were incubated for the standard radioassay described above, the appearance of radioactivity in the chloroform extracts of the incubations was found to occur linearly with respect to protein concentration and time up to 40% hydrolysis of the palmitoyl-CoA substrate. The same specific activity was obtained whether protein concentration or incubation time was varied. T.l.c. of portions of the chloroform extracts revealed that approx. 95% of the radioactivity co-migrated with palmitic acid standard.

The hydrolase activities of subcellular fractions prepared from cell-free homogenates of brain were measured by the standard radioassay. Over 85% of the enzyme activity was found in the 105000*g*-supernatant fraction. Separation of the homogenates into particulate and soluble components did not result in the appearance or disappearance of hydrolase activity. For all subsequent work, the 105000*g*-supernatant fraction was used as the source of palmitoyl-CoA hydrolase. From the 105000*g* supernatants of 19 homogenates (10%, w/v) of fresh brain, a mean (±S.D.) specific activity of 325±53 units/mg of protein and a mean (±S.D.) total activity of 815±115 units/ml were calculated. Although the yield of total activity was decreased substantially (30–50%), the subcellular distribution of hydrolase activity was the same for frozen (−50°C for up to 2 months) and fresh brain.

Gel-filtration studies

Representative profiles of the elution of palmitoyl-CoA hydrolase from columns of Sephadex G-200 are shown in Fig. 1. When the eluting buffer was 0.10M-potassium phosphate, pH 7.4, containing 1mM-EDTA, two peaks of activity were observed (Fig. 1*a*). The smaller peak coincides with the void volume and probably represents the hydrolase activity associated with the fatty acid synthetase complex (Kumar, 1975; Lornitzo *et al.*, 1975). The larger peak of hydrolase activity eluted in a region consistent with a molecular weight of approx. 70000.

In an effort to increase the recovery of hydrolase activity from Sephadex columns, glycerol (20%, v/v) was included in the eluting buffer (Fig. 1*b*). Although the recovery of activity was not substantially increased, a new peak of hydrolase activity emerged from the column earlier than in the absence of glycerol (Fig. 1*a*). Based on its elution from calibrated columns of Sephadex G-200 (glycerol did not alter the elution of the standard calibration proteins), this higher-molecular-weight form of the hydrolase had an approx. mol.wt. of 130000.

When a sample of 105000*g* supernatant was applied to a Sephadex column equilibrated and eluted with buffer containing 10μM-palmitoyl-CoA (Fig. 1*c*), a broad peak of hydrolase activity was found to elute earlier than in the absence of palmitoyl-CoA. However, the elution profile with palmitoyl-CoA was similar to that with glycerol in the eluting buffer. In addition, the inclusion of palmitoyl-CoA in the buffer nearly doubled the recovery of hydrolase activity from the columns (80–90% against 40–50%).

A portion of the pooled peak fractions from the column depicted in Fig. 1(*c*) was desalted to remove non-protein-bound palmitoyl-CoA, concentrated, applied to a Sephadex G-200 column, and eluted with potassium phosphate buffer containing EDTA (Fig. 2*a*). A single peak of hydrolase activity eluted in a region consistent with a mol.wt. of approx. 130000. In a similar set of experiments, 60nmol of [^{14}C]palmitoyl-CoA with and without 10mg of protein from the lower-molecular-weight-hydrolase peak from a Sephadex G-200 column (Fig. 1*a*) was applied to Sephadex G-25 desalting columns. In the absence of protein all the radioactivity eluted in the

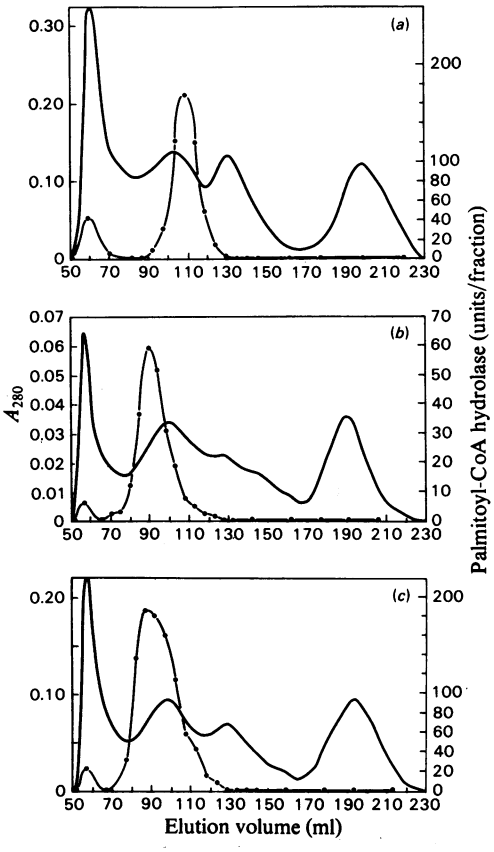

Fig. 1. *Gel filtration of rat brain* 105000*g supernatant* (*a*) A portion (5ml) of 105000*g* supernatant was applied to a Sephadex G-200 column (2.6cm×36cm) equilibrated and eluted by ascending flow (9ml/h) with 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA. Fractions (5.3ml) were collected and assayed for palmitoyl-CoA hydrolase activity by the standard radioassay. Recovery of hydrolase activity was 46% of the applied activity. The specific activity of the major peak was 415nmol of palmitoyl-CoA hydrolysed/min per mg of protein, whereas that of the applied 105000*g* supernatant was 159nmol/min per mg of protein. (*b*) A portion (1ml) of 105000*g* supernatant was adjusted with glycerol to 20% (v/v) and applied to a Sephadex G-200 column (2.6cm×36cm) equilibrated and eluted by ascending flow (8ml/h) with 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA and 20% (v/v) glycerol. Fractions (5.3ml) were collected and assayed for palmitoyl-CoA hydrolase activity by the standard radioassay. Recovery of hydrolase activity was 47% of the applied activity. The specific activity of the major peak was 530nmol of palmitoyl-CoA hydrolysed/min per mg of protein, whereas that of the applied 105000*g* supernatant was 161nmol/min per mg of protein. (*c*) A portion (3ml) of 105000*g* supernatant was applied to a Sephadex G-200 column (2.6cm×36cm) equilibrated and eluted by ascending flow (9ml/h) with 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA and 10μM-palmitoyl-CoA. Fractions (5.3ml) were collected and assayed for palmitoyl-CoA hydrolase activity by the standard radioassay. Recovery of hydrolase activity was 84% of the applied activity. The specific activity of the leading portion of the major peak was 818nmol of palmitoyl-CoA hydrolysed/min per mg of protein, whereas that of the applied 105000*g* supernatant was 143nmol/min per mg of protein. In each Figure palmitoyl-CoA hydrolase activity (●) and A_{280} (——) are shown.

salt region from the Sephadex G-25 columns. When the palmitoyl-CoA was mixed with protein from the Sephadex G-200 column, two distinct peaks of radioactivity eluted from the desalting column. Approx. 70% of the radioactivity eluted in the salt region, whereas the remaining 30% eluted with the protein. After extracting the protein fraction with chloroform/methanol/acetic acid (50:50:1, by vol.), approx. 75% of the radioactivity was recovered in the chloroform extract, 5% in the aqueous phase, and the rest remained bound to the coagulated protein pellet removed from the solvent interface.

When a portion of the pooled peak fractions from a glycerol column (Fig. 1*b*) was desalted, applied to the Sephadex G-200 column, and eluted with buffer containing no glycerol or palmitoyl-CoA, only 5% of the applied hydrolase activity was recovered, all as the higher-molecular-weight form (mol.wt. 130000). Thus the removal of glycerol from solutions of the brain hydrolase results in a nearly complete loss of activity, whereas a substantial portion of the original activity remains after excess non-protein-bound palmitoyl-CoA is removed by gel filtration. Two plausible explanations for these findings are: first, the high-molecular-weight form of palmitoyl-CoA hydrolase produced by glycerol is fundamentally different from that produced by palmitoyl-CoA; or secondly, there is only one high-molecular-weight form, but the mechanisms by which glycerol and palmitoyl-CoA affect the hydrolase are different. The latter explanation is favoured because no differences in their properties (pH optimum, K_m, susceptibility to thiol-blocking reagents etc.) have been observed. Unlike the case with glycerol, sufficient palmitoyl-CoA to stabilize the hydrolase may remain bound to the protein during gel filtration. Accordingly, the concentration of palmitoyl-CoA used throughout these studies (10μM) may be well in excess of that required for subunit aggregation.

Although attempts to generate the lower-molecular-weight form of the hydrolase from the higher-molecular-weight form failed, the reverse process could be accomplished. The higher-molecular-

weight form can be generated from the lower-molecular-weight form by exposure to palmitoyl-CoA. A portion of the pooled peak fractions from a Sephadex column eluted without palmitoyl-CoA or glycerol in the buffer (Fig. 1*a*) was adjusted to 10μM-palmitoyl-CoA and eluted from a Sephadex column with buffer containing palmitoyl-CoA (Fig. 2*b*). The hydrolase activity eluted in the higher-molecular-weight region.

Some properties of the long-chain acyl-CoA hydrolase of pig (Srere *et al.*, 1959) and bovine (Anderson & Erwin, 1971) brain have been reported. Anderson & Erwin (1971) showed that the bovine brain hydrolase had a pH optimum of approx. 7.7, was inactivated by exposure to *p*-chloromercuribenzoate, and had a K_m of approx. 5μM with palmitoyl-CoA. Their gel-filtration studies revealed that two forms of the hydrolase could be eluted from a column of Sephadex G-100 run at 2°C. They further demonstrated that the higher-molecular-weight species (mol.wt. 96000) was partially converted into the lower-molecular-weight species (mol.wt. 46000) after 5h at 25°C. Thus, there are similarities between the hydrolases of bovine and rat brain, including subcellular distribution, pH optimum, susceptibility to thiol-blocking reagents, and the occurrence of two forms of the enzyme differing in molecular weight. There is, however, a substantial species difference with regard to the molecular weights of and the relationship between the two forms of hydrolase.

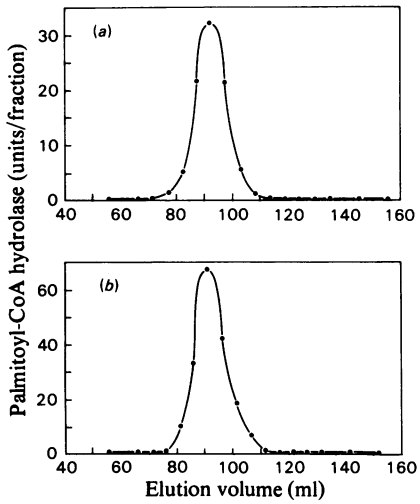

Fig. 2. *Gel filtration of rat brain palmitoyl-CoA hydrolase*
(*a*) Portions (5ml) of the pooled peak fractions from the higher-molecular-weight region of a Sephadex G-200 column (Fig. 1*c*) were desalted to remove excess palmitoyl-CoA, concentrated, and applied to a Sephadex G-200 column (2.6cm×36cm) equilibrated and eluted by ascending flow (9ml/h) with 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA. Fractions (5.3ml) were collected and assayed for palmitoyl-CoA hydrolase activity by the standard radioassay. Recovery of hydrolase activity was 75% of the applied activity. (*b*) Portions (5ml) of the pooled peak fractions from the lower-molecular-weight region of a Sephadex G-200 column (Fig. 1*a*) were adjusted to 10μM-palmitoyl-CoA and applied to a Sephadex G-200 column (2.6cm×36cm) equilibrated and eluted by ascending flow (9ml/h) with 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA and 10μM-palmitoyl-CoA. Fractions (5.3ml) were collected and assayed for palmitoyl-CoA hydrolase activity by the standard radioassay. Recovery of hydrolase activity was 60% of the applied activity. The results are representative of three independent experiments with different 105000*g* supernatants as starting material.

Characteristics of the two forms of palmitoyl-CoA hydrolase

pH optima. Hydrolase activity against pH was determined in triplicate for the lower- and the higher-molecular-weight forms (results not shown). Both have a pH optimum of approx. 7.5, and 1pH unit either side of this optimum, hydrolase activity was decreased by 50%.

Activated thiol Sepharose covalent chromatography. A difference in the availability of reactive thiol groups on the higher- and the lower-molecular-weight forms of palmitoyl-CoA hydrolase is demonstrated by covalent chromatography on columns of activated thiol Sepharose (Fig. 3). The higher-molecular-weight form was not retained by the column, indicating the lack of availability or reactivity of certain thiol groups. On the other hand, when portions of the lower-molecular-weight species from Sephadex columns were applied to an activated thiol Sepharose column, the bulk of the activity was retained until the cysteine wash (Fig. 3*b*). Some of the applied enzyme activity was not retained, which may be due to spontaneous formation of the non-reactive higher-molecular-weight form.

Sensitivity to thiol-blocking reagents. In view of their difference in retention on columns of activated thiol Sepharose, the sensitivities of the lower- and higher-molecular-weight forms to thiol-blocking reagents were determined (Table 1). The two forms were found to be equally sensitive to the three reagents used. The decrease in activity was particularly dramatic with the two mercuric compounds. 5,5′-Dithiobis-(2-nitrobenzoic acid), which is commonly employed in the spectrophotometric assay of palmitoyl-CoA hydrolase (Barnes, 1975), decreased the activity of the brain enzyme, although it was several orders of magnitude less effective than mercurials.

Triacylglycerol hydrolase activity. The list of glycerol ester hydrolases or lipases that have associated palmitoyl-CoA hydrolase activity is extensive (Barber & Lands, 1971; Jansen & Hülsmann, 1973; Serrero *et al.*, 1975; Baginsky & Brown, 1977). A radioassay with a stabilized emulsion of glycerol trioleate was employed to determine if either the lower-molecular-weight form of rat brain palmitoyl-CoA hydrolase or the higher-molecular-weight form generated from it has lipase activity. In incubations with up to 400 units of palmitoyl-CoA hydrolase activity, no lipase activity was detected. To validate the method, cell-free homogenates and postmitochondrial supernatant fractions of rat liver were assayed under identical conditions and found to rapidly hydrolyse the glycerol trioleate substrate.

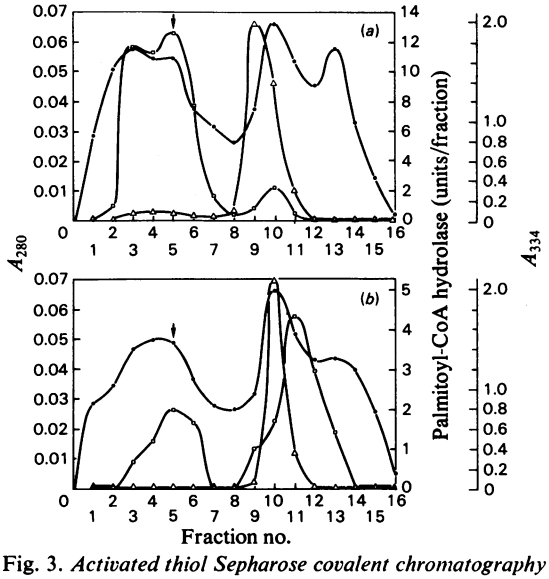

Fig. 3. *Activated thiol Sepharose covalent chromatography of rat brain palmitoyl-CoA hydrolase*

(*a*) A 5.0ml portion of the pooled peak fractions from the higher-molecular-weight region of a Sephadex G-200 column (Fig. 1*c*) was reduced with dithiothreitol, desalted, and applied to an activated thiol Sepharose column (0.9cm×15cm) equilibrated and eluted (5ml/h) with 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA and 10μM-palmitoyl-CoA. At the point indicated by the arrow, elution was continued with buffer containing 20mM-cysteine. Fractions (4.5ml) were collected and assayed for A_{343} (△), dialysed, and assayed for A_{280} (●) and for palmitoyl-CoA hydrolase activity (□) by the standard radioassay. Overall recovery of hydrolase activity was 72% of the original activity. Distribution of hydrolase from this column was 92% in the non-bound-activity peak and 8% in the bound-activity peak. (*b*) A 4.0ml portion of the pooled peak fractions from the lower-molecular-weight region of a Sephadex G-200 column (Fig. 1*a*) was reduced with dithiothreitol, desalted, and applied to an activated thiol Sepharose column (0.9cm×15cm) equilibrated and eluted (5ml/h) with 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA. At the point indicated by the arrow, elution was continued with buffer containing 20mM-cysteine. Fractions (4.5ml) were collected and assayed for A_{343} (△), dialysed, and assayed for A_{280} (●) and for palmitoyl-CoA hydrolase activity (□) by the standard radioassay. Overall recovery of hydrolase activity was 20% of the original activity. The distribution of hydrolase from this column was 33% in the non-bound-activity peak and 67% in the bound-activity peak. The results are representative of three independent experiments with different 105000*g* supernatants as starting material.

Substrate stabilization of palmitoyl-CoA hydrolase

Although concentrated (>100 enzyme units/ml) solutions of the lower-molecular-weight form of hydrolase from Sephadex columns are stable for several weeks when frozen at −50°C, a progressive loss of activity of diluted solutions prepared for assay was noted. As depicted in Fig. 4(*a*), the activity of dilute solutions of the lower-molecular-weight form of the hydrolase decreased rapidly even at 0°C. When the same preparation was diluted in buffer containing 10μM-palmitoyl-CoA, full activity was retained. However, palmitoyl-CoA was ineffective in regenerating the hydrolase activity of a dilute solution of the lower-molecular-weight form lost during 60min at 0°C. Neither 10μM-palmitic acid (rapidly injected into the protein solution in a trace amount of ethanol), 10μM-CoA, nor a combination of the two stabilized the hydrolase activity as did palmitoyl-CoA. It is possible that the lower-molecular-weight form itself is comprised of subunits, and inactivation results from dissociation of these subunits. However, gel-filtration studies provide no evidence of a catalytically active subunit smaller than mol.wt. 70000. Furthermore, the rate of loss of hydrolase activity is zero-order, not first-order, as might be expected for a simple unimolecular-dissociation process.

At 20min after dilution of the lower-molecular-weight preparation in buffer with and without palmitoyl-CoA, portions were assayed for linearity with time and protein concentration (Figs. 4*b* and 4*c*). The difference in activity between the two dilutions was manifested at each protein concentration and time point during the enzyme assay. This suggests that further loss of activity in the dilution without palmitoyl-CoA was arrested by exposure to the substrate in the incubation.

Spectrophotometric assay of palmitoyl-CoA hydrolase

The kinetic parameters of the two forms of brain hydrolase were determined by spectrophotometric assay. Fig. 5 shows Eadee–Hofstee plots of the data

Table 1. *Sensitivity to thiol-blocking reagents of the lower- and the higher-molecular-weight forms of palmitoyl-CoA hydrolase*
Portions (2.5 enzyme units) of either the lower- or the higher-molecular-weight form of palmitoyl-CoA hydrolase were added to solutions of *p*-hydroxymercuribenzoate, $HgCl_2$, or 5,5′-dithiobis-(2-nitrobenzoic acid). After 5min at 37°C, the hydrolase activities were measured by the standard radioassay. Results are expressed as means±S.E. for three independent experiments.

Reagent	Final concentration (μM)	Hydrolase activity (% of control) Lower-molecular-weight form	Higher-molecular-weight form
p-Hydroxymercuribenzoate	1	47±4	54±5
	10	8±2	11±3
	100	3±1	7±2
$HgCl_2$	0.5	32±6	26±4
	1	19±3	19±4
	10	4±1	5±3
5,5′-Dithiobis-(2-nitrobenzoic acid)	10	94±8	92±9
	100	71±2	69±5
	1000	40±5	45±4

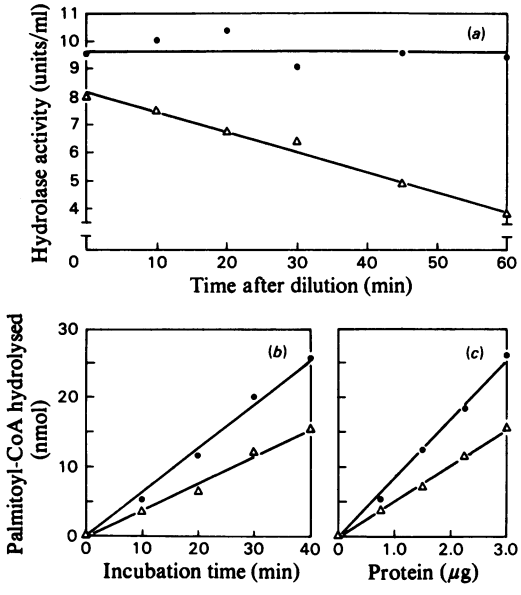

Fig. 4. *Substrate stabilization of palmitoyl-CoA hydrolase*
Equal portions of the lower-molecular-weight form of rat brain palmitoyl-CoA hydrolase were diluted 1:15 in either 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA (△), or the same buffer containing 10μM-palmitoyl-CoA (●). The hydrolase activities of the two solutions were determined by the standard radioassay at various times after the dilution (*a*). At 20min after dilution, both solutions were assayed for linearity with respect to incubation time (*b*) and protein concentration (*c*).

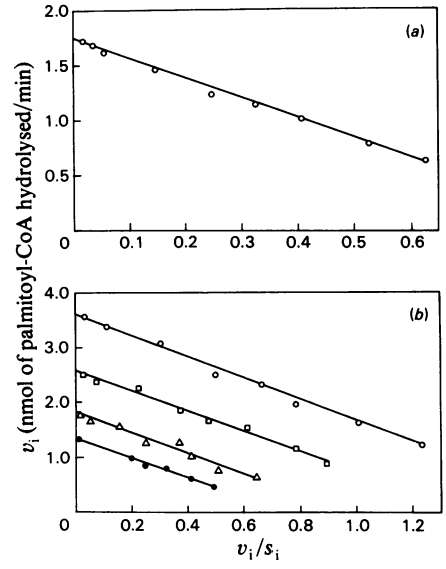

Fig. 5. *Eadee–Hofstee plots of kinetic data from the spectrophotometric assay of palmitoyl-CoA hydrolase*
Each incubation (at 37°C) consisted of 0.10mM-5,5′-dithiobis-(2-nitrobenzoic acid), 1–100μM-palmitoyl-CoA, and enzyme in a final volume of 2.0ml of 0.10M-potassium phosphate buffer, pH7.4, containing 1mM-EDTA. (*a*) Each point is the mean for three or four assays of the higher-molecular-weight form of rat brain hydrolase prepared by gel filtration. (*b*) Each point represents a single assay of the lower-molecular-weight form of rat brain hydrolase prepared by gel filtration. The second set of assays (□) was completed 35min after the first (○), the third set (△) 25min after the second, and the last set (●) 20min after the third. s_i is the concentration of palmitoyl-CoA (μM).

drawn according to least-squares linear-regression analyses. The higher-molecular-weight form had a K_m of approx. 1.8μM for palmitoyl-CoA. The $V_{max.}$

obtained by the spectrophotometric assay was approx. 75% of the rate measured by the standard radioassay with a considerable excess of substrate (88 μM). This difference is probably due to the presence of 100 μM-5,5'-dithiobis-(2-nitrobenzoic acid) in the spectrophotometric assay (see Table 1). There was no substantial difference (±5%) in the hydrolase velocities observed in four successive sets of nine substrate concentrations (approx. 30 min between completion of each set), again reflecting the stability of the higher-molecular-weight form.

With the lower-molecular-weight form, there was a progressive decrease in hydrolase velocity at each substrate concentration from the first set of assays to the last (Fig. 6*b*). The average K_m for the four sets of assays was approx. 1.8 μM, and there was no significant difference in K_m between any of the sets. These data suggest that there is no alteration in the substrate-binding affinity of the catalytic site of the enzyme during the progressive inactivation in dilute solutions.

It would be of considerable interest to ascertain whether the lower-molecular-weight form *per se* has hydrolase activity. The activity may be latent and expressed only on creation of the dimer by the palmitoyl-CoA substrate used in the enzyme assay. Although there was no apparent initial lag during the spectrophotometric assay of the lower-molecular-weight form, more sophisticated analyses may reveal a rapid activation of the hydrolase after addition of substrate.

Palmitoyl-CoA is capable of altering both the activity and the physical structure of several multi-subunit enzymes (Kawaguchi & Bloch, 1976; Wititsuwannakul & Kim, 1977). Palmitoyl-CoA has always been found to inhibit or inactivate enzyme activities, and whenever the quaternary structure of a protein is altered, dissociation of a higher-molecular-weight species to lower-molecular-weight subunits has been demonstrated. The palmitoyl-CoA hydrolase of rat brain is an apparent exception to the paradigm. The evidence presented here supports the concept that palmitoyl-CoA promotes the aggregation of subunits to form a more stable higher-molecular-weight form of the enzyme. High concentrations of glycerol can substitute for palmitoyl-CoA, suggesting that this phenomenon is a physical process of aggregation. Apparently, this process internalizes certain thiol groups on the protein, since the higher-molecular-weight form does not covalently exchange with the 2-pyridyl disulphide group on the Sepharose matrix during activated thiol Sepharose chromatography.

Since the gel-filtration columns eluted with buffer containing palmitoyl-CoA (Fig. 1*c*) were run at 2°C, hydrolysis of palmitoyl-CoA during the run would occur slowly (approx. 5% of the rate at 37°C). It is possible that at the conclusion of the run all of the palmitoyl-CoA in the eluting buffer would have been hydrolysed, suggesting the possibility that palmitate or CoA is responsible for the subunit aggregation of the hydrolase. However, the enzyme would be constantly exposed to fresh palmitoyl-CoA as it traversed the length of the column. Furthermore, the data of Fig. 2(*a*) suggest that after initial exposure the continued presence of 10 μM-palmitoyl-CoA is unnecessary for stabilization of the higher-molecular-weight form of the hydrolase. The data in Fig. 4 suggest that the effect of palmitoyl-CoA is immediate and not apparently dependent on the slow hydrolysis of palmitoyl-CoA at 0°C. Finally, palmitate, CoA, or a combination of both, at the appropriate concentrations, will not duplicate the effect of palmitoyl-CoA. Thus it seems unlikely that palmitate or CoA is responsible for the subunit aggregation and stabilization of the hydrolase.

The question of the physiological significance of the alteration *in vitro* of enzyme activities by long-chain acyl-CoA remains unsettled. Previously, all results in support of this hypothesis were considered in terms of negative feedback inhibition of certain enzymes of lipid metabolism (Kawaguchi & Bloch, 1976). The results reported in the present paper demonstrate for the first time a positive effect of a long-chain acyl-CoA on an enzyme activity. The activity of palmitoyl-CoA hydrolase may be altered *in vivo* by fluctuations in the intracellular concentration of long-chain acyl-CoA thioesters, either by substrate activation of an inactive precursor or by stabilization of an active form of the enzyme. The lowest concentration of palmitoyl-CoA used in these studies was 10 μM, which is probably well above the intracellular concentration of free long-chain acyl-CoA (that is, not bound to protein). The total concentration of long-chain acyl-CoA in brain is approx. 11 nmol/g wet wt. of tissue (Veloso & Veech, 1974). Either small fluctuations in the low concentration of long-chain acyl-CoA are required to alter palmitoyl-CoA hydrolase activity, or this phenomenon has little significance in a tissue like brain. However, the concentration of long-chain acyl-CoA in liver is much higher than in brain, from 15–50 nmol/g wet wt. of tissue (Bortz & Lynen, 1963; Tubbs & Garland, 1964). Furthermore, since the liver is an important homoeostatic regulator of the body's lipid metabolism, the hepatic concentration of long-chain acyl-CoA fluctuates to a much greater extent than in brain. Indeed, in starvation, which does not alter the concentration of long-chain acyl-CoA in brain (Veloso & Veech, 1974), these concentrations in liver may rise to 110 nmol/g wet wt. of tissue (Tubbs & Garland, 1964). Conditions such as diabetes also markedly increase the intrahepatic concentration of long-chain acyl-CoA (Tubbs & Garland, 1964). The possibility that the observed increase of hepatic palmitoyl-CoA hydrolase activity in the rat after induction of diabetes by alloxan

(Kurooka *et al.*, 1971) or streptozotocin (T. E. Knauer & G. R. Knauer, unpublished work) occurs in response to the increased concentrations of long-chain acyl-CoA in the liver requires investigation.

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