Phosphatidate Phosphohydrolase and Palmitoyl-Coenzyme A Hydrolase in Cardiac Subcellular Fractions of Hyperthyroid Rabbits and Cardiomyopathic Hamsters

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Activities of phosphatidate phosphohydrolase and palmitoyl-CoA hydrolase were determined in cardiac subcellular fractions prepared from rabbits which had received tri-iodothyronine and from hamsters with hereditary cardiomyopathy (strain BIO 14.6). 1. Both mitochondrial and microsomal fractions of hyperthyroid rabbit hearts produced 4–5 times as much diacylglycerol 3-phosphate from glycerol 3-phosphate and palmitate as did those of euthyroid hearts. 2. Phosphatidate phosphohydrolase, measured with phosphatidate emulsion, was activated by 1 mM-Mg²⁺ in all but the mitochondrial fraction of euthyroid rabbit hearts. The activation was more pronounced in subcellular fractions isolated from hyperthyroid hearts, so that the measured activities were significantly increased above those of the controls. The highest activity was found in the microsomal and lysosomal fractions. 3. In the absence of Mg²⁺ during incubation, the difference in phosphohydrolase activities between eu- and hyper-thyroid states was not significant. 4. The phosphohydrolase of subcellular fractions of control hamsters did not respond to addition of 0.5–8.0 mM-Mg²⁺. The enzyme from cardiomyopathic hearts was slightly inhibited by this bivalent cation and therefore significant increases in activity were observed only in the absence of Mg²⁺ from the assay system. 5. The rate of reaction by soluble phosphatidate phosphohydrolase was similar regardless of the nature of the substrate. Both when microsomal-bound phosphatidate was used as the substrate and when phosphatidate suspension was used, the activity of soluble enzyme was lower than that of the microsomal and lysosomal enzymes measured with phosphatidate suspension; this was especially so when the assay was carried out in the absence of Mg²⁺. Neither tri-iodothyronine nor cardiomyopathy influenced the soluble phosphohydrolase activity in the two species. 6. Neither tri-iodothyronine nor cardiomyopathy significantly changed palmitoyl-CoA hydrolase activities in subcellular fractions. 7. Microsomal diacylglycerol acyltransferase and myocardial triacylglycerol content were also unchanged in the hyperthyroid state.

There have been a number of studies on the control mechanisms regulating fatty acid oxidation in the myocardium (Bing *et al.*, 1954; Bing, 1965; Opie, 1968, 1969*a,b*). Since the membrane transport of fatty acids can be viewed as more or less a physico-chemical process (Opie, 1969*a,b*; Neely & Morgan, 1974), the intracellular availability of fatty acids and fatty acyl-CoA esters plays a dominant role in governing the rate of fatty acid oxidation in the ventricle (Randle *et al.*, 1966; Oram *et al.*, 1973; Neely & Morgan, 1974).

Acyl-CoA esters also serve as one of the precursors for complex lipid formation. Although the metabolic pathways of glycerol 3-phosphate acylation and phosphatidate dephosphorylation have been extensively investigated in a variety of tissues (Kennedy, 1961; Hübscher, 1970), relatively little work has been done on the heart enzymes. Many of the

studies involving heart tissue dealt with the increased rate of fatty acid esterification which accompanies a decreased rate of fatty acid oxidation in the hypoxic or ischaemic myocardium (Bing *et al.*, 1958; Opie, 1969*a,b*). However, pathological fat infiltration takes place in the ventricle under some other conditions, such as various forms of cardiomyopathies, heart failure and hyperthyroidism (Opie, 1969*a,b*; Kikuchi & Kako, 1970).

It is generally assumed that the rate of esterification of fatty acids into neutral lipids increases because of an increased supply of the precursors, fatty acyl-CoA esters, glycerol 3-phosphate or both. Such a control may be exerted indirectly through the regulation of the acyl-CoA availability by the carnitine palmitoyltransferase (EC 2.3.1.21) system (Fritz, 1968), carnitine (Bressler & Wittels, 1966), or carnitine acetyltransferase (EC 2.3.1.7) (Oram *et al.*,

1973). One of the difficulties inherent in this hypothesis is that the rate of microsomal diacylglycerol 3-phosphate formation in the heart is 3–10-fold higher than that of the biosynthesis in mitochondria (Liu & Kako, 1974). Similarly, phosphatidate phosphohydrolase (EC 3.1.3.4) activity in heart microsomal fractions is severalfold greater than that in heart mitochondrial fractions (Liu & Kako, 1975; see also below), thereby making less important the role played by the mitochondria in the acylation process. In liver and intestine, particulate-bound and non-bound phosphatidate are dephosphorylated to different extents by the soluble phosphohydrolase (Hübscher *et al.*, 1967; Johnston *et al.*, 1967; Smith *et al.*, 1967). Thus all the evidence obtained by using subcellular fractions suggests that the factors controlling the rate of neutral lipid biosynthesis are more complex than those implied by the above hypothesis. Accordingly, we have studied the properties of cardiac glycerol 3-phosphate-acylating enzymes (Liu & Kako, 1974; Kako & Liu, 1974; Liu *et al.*, 1974), as well as phosphatidate phosphohydrolase and palmitoyl-CoA hydrolase (EC 3.1.2.2) (Liu & Kako, 1975). The present paper describes our attempt to examine the alterations in enzyme activities that occur under two pathological conditions.

Experimental

Materials

ATP, CoA, palmitoyl-CoA, palmitic acid, 3,3',5-tri-iodo-L-thyronine (sodium salt), bovine serum albumin (fraction V, fatty acid-poor) and Triton X-100 (octyl phenoxypolyethoxyethanol) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. [$1\text{-}^{14}\text{C}$]Palmitic acid, [$1\text{-}^{14}\text{C}$]palmitoyl-CoA and *sn*-[$\text{U-}^{14}\text{C}$]glycerol 3-phosphate were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The purity of palmitoyl-CoA and glycerol 3-phosphate was determined by t.l.c. in the solvent systems butan-1-ol–acetic acid–water (5:2:3, by vol.) (Pullman, 1973) and phenol–water (5:2, w/v) (Benms & Proulx, 1971) respectively. Suppliers of other chemicals were: *sn*-glycerol 3-phosphate [C.F. Boehringer (Mannheim) Corp., New York, N.Y., U.S.A.]; *rac*-glycerol 3-phosphate (Sigma); Tween 20 (polyoxyethylene sorbitan monolaurate) (J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.); *sn*-1-stearoyl-2-oleoylglycerol, 1,2-dipalmitoylglycerol, 1,2-dioleoylglycerol and 1,2-diacylglycerol prepared from pig liver phosphatidylcholine (all from Serdary Research Laboratories, London, Ont., Canada); t.l.c. plates precoated with silica gel G (Brinkmann Instruments, Rexdale, Ont., Canada). Phosphatidic acid (diacylglycerol 3-phosphate) was purchased from either Pierce Chemical Co.

(Rockford, Ill., U.S.A.) or Serdary. It was prepared from egg phosphatidylcholine and its fatty acid composition was, by weight, 36% palmitic acid, 37% oleic acid, 15% stearic acid and 12% linoleic acid. Palmitic acid was combined with KOH by heating it in an ethanol–water mixture, and by evaporating off the ethanol. All other reagents were obtained from Fisher Scientific Co. (Montreal, Que., Canada) or J. T. Baker Chemical Co., and were of the highest purity available.

Animals and subcellular preparation

Male albino rabbits with body weights ranging from 1.8 to 2.2 kg were kept in individual cages. The animals received a daily injection of 125 μg of tri-iodothyronine/kg body wt. for 6–8 days. This dose induced the typical hyperthyroid state as judged from the loss of body weight (20–30%) and measured heart rate (162% of the control) (Kako & Liu, 1974). A pair of eu- and hyper-thyroid rabbits were anaesthetized by an intraperitoneal injection of 2.5 ml of 2% (w/v) α -chloralose in 20% (w/v) urethane/kg body wt.

Healthy hamsters were of the Syrian golden strain, and hamsters with hereditary muscular dystrophy were of the BIO 14.6 strain. They were housed individually in cages with wire-mesh bottoms and fed on laboratory chow and water *ad libitum*. They were killed at ages 3–6 months by decapitation (Kako *et al.*, 1974).

The heart of each animal was excised and immediately chilled in ice-cold homogenizing medium. The heart was trimmed to remove visible fat, weighed and then finely chopped with scissors. The tissue was homogenized with 4 vol. of 0.25 M-sucrose containing 0.02 M-Tris-HCl, pH 7.4, in a Potter–Elvehjem-type homogenizer with a Teflon pestle, as described previously (Liu & Kako, 1974). The homogenate was centrifuged at 800g (r_{av} 7.5 cm) for 15 min to remove nuclei, myofibrils and cell debris, and the supernatant was carefully decanted and centrifuged at 10000g (r_{av} 7.5 cm) for 15 min. The lysosomal fraction was isolated by collecting a fraction precipitated between 10000g (15 min) and 25000g (15 min, r_{av} 7.5 cm). The resulting supernatant was removed and further centrifuged at 100000g (r_{av} 5.74 cm) for 60 min. The pellet obtained was used as the microsomal fraction after being suspended in the homogenizing medium to give approx. 10 mg of protein/ml. The 10000g pellet was resuspended and centrifuged at 8000g for 15 min, yielding the mitochondrial pellet, which was suspended in the homogenizing medium to give approx. 7 mg of protein/ml. Hepatic microsomal fractions were isolated in some experiments and used as the source of the enzyme to synthesize labelled

Table 1. *Distribution of enzyme markers and protein in subcellular fractions of rabbit heart*

The subcellular fractions were isolated by differential centrifugation, as described in the Experimental section. Activities of cytochrome *c* oxidase and NADPH-cytochrome *c* reductase were assayed spectrophotometrically. Acid phosphatase activity in the fractions was determined by using β -glycerophosphate as substrate at pH 5.0, and ATPase activity by release of P_i from ATP in the presence and in the absence of Na^+ and K^+ . The values shown are means $\pm$ S.E.M. of four to five independent experiments. Protein concentration of crude tissue homogenate was 128 mg/g, 66.8% of which appeared in the precipitate of the low-speed (800g for 15 min) centrifugation; the distribution of remaining protein is shown in the row marked *.

Marker	Unit	Mitochondrial fraction	Lysosomal fraction	Microsomal fraction	Soluble fraction
Protein	mg/g wet wt.	10.1 $\pm$ 1.0	2.8 $\pm$ 0.3	3.6 $\pm$ 0.5	28.0 $\pm$ 2.3
Protein	% in homogenate*	6.7	2.1	2.3	24.5
Cytochrome <i>c</i> oxidase	μ M/min per mg of protein	584.6 $\pm$ 105.0	134.6 $\pm$ 20.5	111.8 $\pm$ 9.9	—
NADPH-cytochrome <i>c</i> reductase	μ M/min per mg of protein	1.26 $\pm$ 0.1	7.74 $\pm$ 1.0	12.52 $\pm$ 1.5	—
Acid phosphatase	nmol/min per mg of protein	5.60 $\pm$ 0.45	12.60 $\pm$ 1.20	7.45 $\pm$ 1.25	1.05 $\pm$ 0.16
(Na^+ + K^+ + Mg^{2+})-dependent ATPase	—	0.84 $\pm$ 0.06	0.86 $\pm$ 0.02	0.79 $\pm$ 0.05	—
Mg^{2+} -dependent ATPase	—	—	—	—	—

mono- and di-acylglycerol 3-phosphate. The protein content of each fraction was determined by the method of Lowry *et al.* (1951).

Cross-contamination among subcellular fractions was examined by measuring 'marker' enzyme activities. Activities of cytochrome *c* oxidase (EC 1.9.3.1) (Smith, 1955; Sottocasa *et al.*, 1967) and NADPH-cytochrome *c* reductase (EC 1.6.2.4) (Sottocasa *et al.*, 1967) were measured at 25°C, and the activities of acid phosphatase (EC 3.1.3.2) (Berthet & DeDuve, 1951) and (Na^+ + K^+)-dependent ATPase* (EC 3.6.1.3) (Stam *et al.*, 1969) were assayed at 37°C. The results (Table 1) indicate that both the mitochondrial and microsomal fractions were contaminated by each other to an extent of less than 20%, but that the lysosomal fraction was considerably contaminated by the microsomal fraction. The plasma-membrane fraction was randomly distributed among the three fractions. However, it is not known how exclusively these marker enzymes are localized in a single subcellular fraction in the myocardium; for instance, acid phosphatase may exist in fractions other than in the lysosomal fraction, and NADPH-cytochrome *c* reductase may be found in the outer mitochondrial membrane.

Assay of acyl-CoA-sn-glycerol 3-phosphate O-acyl-transferase (EC 2.3.1.15)

The optimum conditions for this enzyme assay have been reported previously (Liu & Kako, 1974; Kako & Liu, 1974). The reaction mixture contained; 2.0 μ mol of potassium palmitate, 0.8 μ mol of CoA, 12.0 μ mol of ATP, 6.0 μ mol of $MgCl_2$, 20 mg of fatty acid-poor serum albumin, 6.0 μ mol of [^{14}C]glycerol

3-phosphate (360000 d.p.m./ μ mol), 100 μ mol of Tris-phosphate buffer, pH 7.4, and fresh mitochondrial fraction (approx. 2.5 mg of protein) or microsomal fraction (approx. 0.8 mg of protein). The final volume was 2.0 ml. The incubation was carried out in a metabolic shaker at 37°C in air for 10 min (mitochondrial) or 5 min (microsomal fraction). Lipids were extracted with butan-1-ol and chromatographed on t.l.c. plates (Liu & Kako, 1974; Brindley, 1973). The plates were developed with chloroform-methanol-acetic acid-water (65:25:8:4, by vol.) (Skipski *et al.*, 1962). The lipids were detected by iodine vapour, compared with authentic standards and, in addition, identified by mild alkaline hydrolysis and chromatography (Marshall & Kates, 1972). Areas corresponding to mono- and di-acylglycerol 3-phosphate were scraped from the plate and suspended in a Ready-Solv solution (Beckman Instruments, Fullerton, Calif., U.S.A.) for radioactivity determination.

Assay of phosphatidate phosphohydrolase

This activity was in most cases determined by measuring the release of P_i from diacylglycerol 3-phosphate (phosphatidic acid) (Liu & Kako, 1975). The incubation mixture was prepared immediately before each experiment and contained 3.0 μ mol of sodium phosphatidate, 160 μ mol of Tris-acetate buffer, pH 7.0, and an enzyme preparation in a final volume of 2.0 ml. Phosphatidate was sonicated at 20 kHz and 150 W for 45 s at 4°C (Biosonik B 10-II; Bronwill Scientific, Rochester, N.Y., U.S.A.) to produce a homogeneous suspension. Some assays were performed in the absence of 1 mM- $MgCl_2$ and other assays in its presence (Jamdar & Fallon, 1973). In addition, the effects of an addition of 0.5–8.0 mM-

* Abbreviation: ATPase, adenosine triphosphatase.

MgCl₂, 1 mM-CaCl₂, 1 mM-MnSO₄ or 1 mM-EDTA were also examined. Incubation was in air for 45 min at 37°C. The enzyme assays in which phosphatidate was dissolved in diethyl ether and incubated as the substrate of the enzyme (Okuyama & Wakil, 1973) gave results which were identical with those obtained by the above procedure. The reaction rates were constant up to 60 min (Liu & Kako, 1975). The reaction was stopped by the addition of 2.0 ml of 12% (w/v) trichloroacetic acid. After removal of the precipitated protein by centrifugation, the P_i in the reaction mixture was determined by the method of Berenblum & Chain (1938).

Particulate-bound phosphatidic acid (Smith *et al.*, 1967; Lamb & Fallon, 1974) was prepared by incubating the hepatic microsomal preparation in the presence of [U-¹⁴C]glycerol 3-phosphate for 10 min at 37°C in the manner described above. The incubation mixture contained 50 mM-Tris-phosphate buffer, pH 7.4, 1 mM-potassium palmitate, 0.4 mM-CoA, 6 mM-ATP, 3 mM-MgCl₂, 20 mg of bovine serum albumin, 3 mM-glycerol 3-phosphate (approx. 1 µCi), and the microsomal fraction (approx. 2 mg of protein) in a final volume of 2.0 ml. As reported previously (Liu & Kako, 1974), 91–96% of the acylation products were mono- and di-acylglycerol 3-phosphate under these conditions. After incubation, the contents of ten tubes were pooled, chilled, layered over a cold 0.3 M-sucrose solution and centrifuged at 100 000 g (*r*_{av}, 5.74 cm) for 60 min at 4°C. The microsomal pellet was resuspended and used as particle-bound phosphatidic acid. This method of preparation of substrate resulted in a slightly larger quantity of mono- and di-acylglycerol 3-phosphate (see the Results section) than the method which involves heating the resuspended microsomal fraction (Jamdar & Fallon, 1973), but the measured activity of phosphohydrolase was uninfluenced.

The activity of phosphatidate phosphohydrolase was measured by incubating the membrane-bound phosphatidic acid (approx. 0.3–0.7 µmol in 5–8 mg of microsomal protein) in the presence and in the absence of the supernatant fraction in a final volume of 3.0 ml. Conditions for the assay were tested, and it was found that the reaction rate was constant up to 20 min. The above quantity of bound substrate apparently saturated the enzyme, so that the rate of reaction was proportional to the amount of soluble fraction (2.5–17.0 mg of protein). Water-saturated butan-1-ol was added to the zero-time sample and assay tubes, and the lipids were extracted by procedures previously published (Daae & Bremer, 1970; Sánchez *et al.*, 1973; Liu & Kako, 1974). The products were analysed by t.l.c. in the solvent system light petroleum (b.p. 30–60°C)–diethyl ether–acetic acid (40:10:1, by vol.) and their radioactivity was determined. From these data, the activity of the supernatant enzyme was estimated.

Assay of palmitoyl-CoA hydrolase

The subcellular fraction was incubated with 0.2 µmol of [¹⁴C]palmitoyl-CoA with a radioactivity of approx. 40 000 d.p.m., 80 µmol of Tris-phosphate buffer, pH 7.4, and 20 mg of bovine serum albumin in a final volume of 2.0 ml. The incubation was carried out at 37°C in a metabolic shaker (Liu & Kako, 1975). After 45 min the reaction was stopped by the addition of 10 ml of propan-2-ol–heptane–1 M-H₂SO₄ (20:5:1, by vol.), followed by addition of 6.0 ml of heptane and 4.0 ml of water. The mixture was then transferred to a separatory funnel, shaken vigorously, and was left for 5–10 min (Goss & Lein, 1967). After the separation of two phases, the heptane phase was rewashed with heptane-saturated water, and the aqueous phase with water-saturated heptane. A portion of the combined heptane phases was dissolved in a naphthalene–1,4-dioxan-based scintillating solution (Bray, 1960) for the determination of radioactivity. The combined heptane extracts contained 93–96% of the radioactivity when [¹⁴C]-palmitic acid was tested alone. By contrast, a negligible amount of [1-¹⁴C]palmitoyl-CoA was extracted by the heptane, indicating a satisfactory isolation of the radioactive product from its precursor. For the determination of radioactivity, a Beckman liquid-scintillation spectrometer, LS-150, was used. The counting efficiency was more than 90%.

Assay of acyl-CoA-1,2-diacylglycerol O-acyltransferase (EC 2.3.1.20) system

The activity of this enzyme was assayed with a modification of the procedure used by de Kruyff *et al.* (1970). The optimum assay conditions were 8.0 µmol of GSH, 12.5 µmol of ATP, 0.25 µmol of CoA, 9.0 µmol of MgCl₂, 0.4 mg of Tween 20, 0.7 µmol of 1,2-*sn*-diacylglycerol and 1.5 µmol of [¹⁴C]palmitic acid (0.1 µCi) in a total volume of 2.0 ml. A mixture of the last three chemicals was sonicated to produce a uniform suspension before each experiment. The subcellular fractions (up to 2 mg of protein) were added and the incubation was carried out for 10 min at 37°C. Larger amounts of enzyme sources or longer incubation times invariably decreased the incorporation of ¹⁴C into tri-acylglycerol. Addition of the supernatant fraction to microsomal fraction had no effect on the rate of the acyltransferase reaction. The replacement of Tween 20 with Triton X-100, catcum or gum arabic, and the use of synthetic 1-stearoyl-2-oleoylglycerol, 1,2-dipalmitoylglycerol or 1,2-dioleoylglycerol all decreased measured enzyme activities. Since the activity of palmitoyl-CoA synthetase (EC 6.2.1.3) of both mitochondrial and microsomal fractions of the heart is comparably high (De Jong & Hülsmann, 1970; Aas, 1971), the synthetase reaction

does not limit diacylglycerol formation in this assay. The products of the reaction were verified by t.l.c. in the solvent system light petroleum (b.p. 30–60°C)–diethyl ether–acetic acid (40:10:1, by vol.) in some experiments. The incubation was stopped by adding 5 ml of ethanol, and neutral lipids were extracted with carbon tetrachloride (Weiss *et al.*, 1960). Under these conditions, the radioactivity at zero time was generally one-tenth that of the incubated samples.

Triacylglycerol determination

Amounts of myocardial neutral glycerides were determined by a method based on chromotropic acid formation (Van Handel & Zilversmit, 1957). The heart tissue was frozen with liquid N₂ and pulverized. Lipids were extracted with chloroform-methanol (1:1, v/v) (Bligh & Dyer, 1959). After phospholipids were adsorbed with Zeolite (W. A. Taylor Co., Baltimore, Md., U.S.A.), neutral lipids were extracted, hydrolysed and their glycerol content was determined (Van Handel & Zilversmit, 1957).

Results

Thyroid hormone has been found to influence to different degrees the fatty acid-esterifying enzymes existing in the various subcellular compartments. The formation of diacylglycerol 3-phosphate by isolated mitochondrial and microsomal fractions from radioactive glycerol 3-phosphate and palmitic acid was increased four- and five-fold respectively by the tri-iodothyronine treatment. The rate of accumulation of microsomal monoacylglycerol 3-phosphate was not enhanced under the same experimental conditions and that of mitochondrial monoacylglycerol 3-phosphate was increased only twofold. Thus the

results suggest that, although the monoacylglycerol 3-phosphate formation was enhanced by the thyroid hormone, its products were rapidly removed as it formed. In other words, tri-iodothyronine stimulated the acyl-CoA–monoacylglycerol 3-phosphate acyltransferase reaction to a greater extent than the acyl-CoA–glycerol 3-phosphate acyltransferase reaction in these experiments.

Whether or not the third step of the esterification process in the heart was altered under the influence of thyroid hormone was therefore examined. Phosphatidate phosphohydrolase possesses characteristics which indicate the existence of different enzymes in different subcellular fractions (Sedgwick & Hübscher, 1965; Smith *et al.*, 1967; Jamdar & Fallon, 1973; Liu & Kako, 1975). The activity was therefore measured by using four subcellular fractions isolated from rabbit hearts and with phosphatidate suspension as the substrate (Table 2). Table 2 also shows the effect of Mg²⁺ on the enzyme activities. In euthyroid animals, the mitochondrial enzyme was uninfluenced by the presence of 1 mM-Mg²⁺ in the assay system, whereas activities in all other fractions were potentiated by Mg²⁺. This effect was observed with the concentration of Mg²⁺ up to 5.0 mM.

In hyperthyroid rabbits, highly significant increases in activities were observed when the mitochondrial, lysosomal and microsomal enzymes were assayed in the presence of Mg²⁺ (Table 2). On the other hand, the difference caused by the thyroid state was not as marked as when the assays were performed in the absence of Mg²⁺, and only the lysosomal enzyme showed an increase of borderline significance ($P < 0.05$). This is in part due to the greater effect of Mg²⁺ on the enzymes in hyperthyroid rabbits than in euthyroid rabbits. The activity of the soluble enzyme appeared to increase, but the change was not significant ($P < 0.1$).

Table 2. *Effect of tri-iodothyronine treatment on phosphatidate phosphohydrolase activity in subcellular fractions of rabbit hearts*

The preparation of subcellular fractions and the procedure for enzyme assays are described in the Experimental section. An aqueous suspension of phosphatidic acid was used as the substrate for the enzyme. Eu- and hyper-thyroid rabbits were fed in parallel, but the latter received a daily injection of 125 µg of tri-iodothyronine/kg body wt. for at least 5 days. The assays in which 1 mM-MgCl₂ was added to the reaction mixture are indicated as (+Mg²⁺) in the Table. Eight pairs of the animals were used for experiments. Statistical significance between the values obtained from eu- and hyper-thyroid animals was computed by Student's *t* test; * indicates $P < 0.01$; † $P < 0.025$; ‡ $P < 0.05$; § $P < 0.1$. Values are means ± S.E.M.

Conditions	Activity (nmol of substrate/min per mg of protein)			
	Mitochondrial fraction	Lysosomal fraction	Microsomal fraction	Soluble fraction
Euthyroid (no Mg ²⁺)	1.16 ± 0.17	2.97 ± 0.25	3.14 ± 0.22	0.21 ± 0.07
Hyperthyroid (no Mg ²⁺)	1.39 ± 0.14	3.86 ± 0.41†	3.09 ± 0.37	0.45 ± 0.12§
Euthyroid (+Mg ²⁺)	1.42 ± 0.11	5.24 ± 0.44	8.65 ± 0.67	0.85 ± 0.23
Hyperthyroid (+Mg ²⁺)	2.37 ± 0.24*	9.18 ± 1.17*	13.57 ± 1.80†	1.08 ± 0.21

Table 3. *Phosphatidate phosphohydrolase activity in subcellular fractions of hamsters with hereditary cardiomyopathy*

The subcellular fractionation and the assay methods are described in the Experimental section. An aqueous dispersion of phosphatidic acid was used as the substrate of the enzyme. The assays were carried out in the absence (–) and in the presence (+) of 1 mM-MgCl₂ and the number of animals used was seven in each case; results are means ± S.E.M. Hamsters with hereditary muscular dystrophy were of BIO 14.6 strain. The statistical significance between healthy and diseased hearts was tested by Student's *t* test; * indicates $P < 0.025$; † $P < 0.05$; ‡ $P < 0.01$.

Conditions	Activity (nmol of substrate transformed/min per mg of protein)			
	Mitochondrial fraction	Lysosomal fraction	Microsomal fraction	Soluble fraction
Control (no Mg ²⁺)	1.92 ± 0.32	3.87 ± 0.58	4.22 ± 0.48	0.51 ± 0.20
Cardiomyopathy (no Mg ²⁺)	2.02 ± 0.26	6.01 ± 1.11†	7.50 ± 1.35*	1.04 ± 0.21†
Control (+Mg ²⁺)	1.45 ± 0.29	3.51 ± 0.62	4.24 ± 0.56	0.54 ± 0.18
Cardiomyopathy (+Mg ²⁺)	1.67 ± 0.22	4.27 ± 0.77	6.08 ± 1.02‡	0.58 ± 0.19

The effect of Mg²⁺ on phosphatidate phosphohydrolase was found to be not a universal property of this enzyme. Previous investigators found a very high phosphohydrolase activity in the lysosomal fraction (Wilgram & Kennedy, 1963; Sedgwick & Hübscher, 1965). Therefore it was thought that its activity may alter under conditions where lysosomal changes may take place, such as in cardiomyopathy (Nadkarni *et al.*, 1972; Kako *et al.*, 1974). Consequently, the activity was measured with subcellular fractions prepared from hamsters with hereditary cardiomyopathy. The results obtained were quite different from those of eu- and hyper-thyroid rabbits, revealing a species difference in the effect of Mg²⁺ on the phosphohydrolase. In subcellular fractions of hamster hearts, 1 mM-Mg²⁺ did not influence the activities of this enzyme (Table 3). However, higher concentrations (3–8 mM) of Mg²⁺ were inhibitory. Moreover, in cardiomyopathic hamsters, the addition of 1 mM-Mg²⁺ suppressed to some extent the enzyme activity. Thus significant increases in activities in lysosomal, microsomal and soluble fractions prepared from diseased hamster hearts were detectable only when the assay medium did not contain Mg²⁺. The increase in microsomal activity, measured in the presence of Mg²⁺, was not statistically significant ($P < 0.1$). An addition of 1 mM-CaCl₂ decreased the activity of the enzyme by half and that of Mn²⁺ decreased the activity to one-fifth, in both rabbits and hamsters. On the other hand, 1 mM-EDTA did not influence the rate of phosphatidate hydrolysis by subcellular fractions.

There is evidence that the optimum substrate for the soluble phosphohydrolase is particulate-bound phosphatidic acid (Brindley *et al.*, 1967; Hübscher *et al.*, 1967; Mitchell *et al.*, 1971). These studies dealt with organs other than the heart. We investigated the difference between a phosphatidate suspension and membrane-bound phosphatidate as the substrate for cardiac phosphohydrolase in the rabbit and hamster. Our results showed that the activities of the

soluble enzyme were similar regardless of the nature of the substrate, i.e. either with phosphatidate emulsion or with microsomal-bound phosphatidate as the substrate (Tables 4 and 5). Since the preparation of the membrane-bound substrate required the use of Mg²⁺ ions (see the Experimental section), the effect of adding EDTA on the soluble phosphohydrolase activity was examined. Additions of 1 mM-EDTA decreased the activity to a very low value (one-third to one-tenth). Therefore in the absence of Mg²⁺, a phosphatidate suspension was a preferred substrate over membrane-bound substrate even for the soluble enzyme. This agrees with the results of Jamdar & Fallon (1973). Our results further indicate that the phosphohydrolase is activated by Mg²⁺, which is adsorbed on the microsomal fraction during the acylation of glycerol 3-phosphate.

Tables 2 and 4 also demonstrate that, in the rabbit heart, the highest specific activity is found in the microsomal and lysosomal fractions both in the presence and in the absence of Mg²⁺. Nevertheless, these data confirm that the particulate-bound substrate is susceptible to the action of the soluble phosphatidate phosphohydrolase. Consequently, an addition of the soluble fraction to the microsomal suspension augments neutral lipid formation (Tables 4 and 5). In the hamster heart also, the activities measured by using optimum conditions were considerably higher in the microsomal and lysosomal fractions than were those in the soluble fraction (Tables 3 and 5). Further, Tables 4 and 5 show that the activities of the soluble enzymes are not influenced by treatment with tri-iodothyronine in the rabbit or by the presence of cardiomyopathy in the hamster.

Palmitoyl-CoA hydrolase activity in subcellular fractions of the hearts of rabbits and hamsters was uninfluenced by tri-iodothyronine administration and by hereditary muscular dystrophy respectively (Table 6). High-energy bonds are wasted by the hydrolysis of acyl-CoA by this enzyme; consequently,

Table 4. *Neutral lipid formation from microsomal-bound phosphatidates catalysed by the soluble enzyme from the hearts of eu- and hyper-thyroid rabbits*

Phosphatidates were synthesized by incubating rabbit liver microsomal fractions under the conditions described in the Experimental section. The incubation was stopped by chilling the reaction mixture and the latter was centrifuged at 100000g for 1 h, layered on a cold 0.3 M-sucrose solution. Approx. 10 nmol of mono- and di-acylglycerol 3-phosphate/min per mg of protein was synthesized under these conditions. The microsomal-bound phosphatidate (approx. 0.39 μ mol) was incubated in the absence and in the presence of the 100000g-supernatant fraction of eu- or hyper-thyroid rabbit heart. After a 15 min incubation period, the reaction was stopped and the lipids were extracted with butan-1-ol and chromatographed on t.l.c. plates to separate phosphatidate and neutral lipids (see the Experimental section). The first row shows that 4.4% of the total lipids formed were neutral lipids before the second incubation; the incubation of the microsomal fraction for 15 min increased the proportion of neutral lipids to 9.1% of the total, which was further raised to 30–32% by the addition of the soluble fraction. The microsomal protein content in the reaction mixture was 5.43 ± 0.57 mg. The values are means $\pm$ S.E.M.

Experimental conditions	No. of animals	Ratio neutral lipid/total lipid (%)	Average soluble protein content (mg)	Rate of neutral lipid formation (nmol/min per mg of soluble protein)
Microsomal fraction before the 2nd incubation	9	4.4 ± 0.6	—	—
Microsomal fraction after the 2nd incubation	9	9.1 ± 0.8	—	0.244 ± 0.024
Microsomal fraction plus the euthyroid heart soluble fraction	8	31.8 ± 2.9	16.67 ± 1.53	0.350 ± 0.053
Microsomal fraction plus the hyperthyroid heart soluble fraction	8	30.4 ± 3.6	17.02 ± 1.61	0.321 ± 0.085

Table 5. *Effect of the soluble fraction on the neutral lipid formation by microsomal-bound phosphatidate in hamsters*

For details of these experiments, see the legend to Table 3. The rate of formation of mono- and di-acylglycerol 3-phosphate was approx. 12 nmol/min per mg of protein during the first incubation. For the assay of phosphatidate phosphohydrolase activities (second incubation), approx. 0.7 μ mol of the membrane-bound substrate was incubated in the presence or in the absence of the cardiac soluble fraction. The microsomal protein content was 8.50 ± 0.78 mg, and the number of animals used was six in each experiment. The values are means $\pm$ S.E.M.

Experimental conditions	Neutral lipid present in total lipid (%)	Protein content of soluble fraction (mg)	Rate of neutral lipid formation (nmol/min per mg of soluble protein)
Microsomal fraction before the 2nd incubation	9.0 ± 1.9	—	—
Microsomal fraction after the 2nd incubation	16.4 ± 1.2	—	0.456 ± 0.054
Microsomal fraction plus control hamster soluble fraction	26.2 ± 2.4	9.00 ± 0.70	0.552 ± 0.062
Microsomal fraction plus cardiomyopathic hamster soluble fraction	27.0 ± 3.0	9.99 ± 0.77	0.522 ± 0.055

Table 6. *Palmitoyl-CoA hydrolase activities in cardiac subcellular fractions of hyperthyroid rabbits and cardiomyopathic hamsters*

Subcellular fractions were prepared by differential centrifugation. The enzyme activities were calculated from the extent of hydrolysis of [1- 14 C]palmitoyl-CoA during a 45 min incubation period. Details of the experimental techniques are described in the Experimental section. * indicates $P < 0.1$. Values are means $\pm$ S.E.M.

Conditions	No. of animals	Activity (nmol of substrate hydrolysed/min per mg of protein)			
		Mitochondrial fraction	Lysosomal fraction	Microsomal fraction	Soluble fraction
Euthyroid rabbits	6	0.106 ± 0.012	0.170 ± 0.043	0.166 ± 0.022	0.076 ± 0.011
Hyperthyroid rabbits	6	0.122 ± 0.031	0.231 ± 0.048	0.209 ± 0.034	0.095 ± 0.017
Control hamsters	5	0.106 ± 0.017	0.229 ± 0.033	0.240 ± 0.057	0.083 ± 0.017
Myopathic hamsters	5	0.111 ± 0.012	0.227 ± 0.058	0.234 ± 0.052	$0.122 \pm 0.026^*$

it was thought that the activity might be increased in hypermetabolic states such as hyperthyroidism. Table 6 demonstrates at the same time that palmitoyl-CoA hydrolase showed the highest activity in the lysosomal fraction in homogenates of hamster hearts.

Activity measurements of acyl-CoA:diacylglycerol acyltransferase were less reproducible than other assays reported here, as has been noted previously (Mangiapane *et al.*, 1973). There was no change in activity of this enzyme in subcellular fractions of hyperthyroid rabbit hearts. The measured activity was low ($0.07\text{--}0.2\text{ nmol/min per mg}$ of microsomal protein), compared with that of hepatic microsomal fractions ($5.0\text{--}15.0\text{ nmol/min per mg}$ of protein). The work published recently indicated that, in the rat liver subcellular fraction, an aqueous dispersion of diacylglycerol was not as suitable a substrate for the enzyme as microsomal-bound diacylglycerol (Fallon *et al.*, 1975). The myocardial triacylglycerol content was also unchanged in hyperthyroid heart muscle ($5.88 \pm 0.48\text{ }\mu\text{mol/g}$, $n = 9$, in euthyroid compared with $5.82 \pm 0.63\text{ }\mu\text{mol/g}$, $n = 10$, in hyperthyroid). Whether or not cardiac lipase (diacyl- and triacyl-glycerol hydrolase) activity is increased under these conditions remains to be investigated.

Discussion

The optimum assay conditions, basic properties of enzymes, enzyme kinetics and pH optima have been previously investigated and separately reported (Liu & Kako, 1974, 1975). On the basis of these findings we further investigated the effect on the enzyme activities of tri-iodothyronine and hereditary cardiomyopathy in the two species of animals in this study.

Thyroid hormones act on a variety of biochemical processes in tissue (Tata, 1964; Fath & Kako, 1973). In particular, they induce lipolysis (Gold *et al.*, 1967; Silverman *et al.*, 1972) and increase the rate of protein synthesis (Lee *et al.*, 1959). Bressler & Wittels (1966) observed a more than twofold increase in the concentration of myocardial triacylglycerol of guinea pigs treated with L-thyroxine. This increase was probably a consequence of the increased mobilization of plasma free fatty acids and triacylglycerol, since the myocardial uptake of fatty acids under these conditions is increased (Gold *et al.*, 1967). In some cases, a thyrotoxic myocardium was accompanied by fat infiltration, myofibrillar degeneration and fibrosis, but in others, no specific lesion appeared (Sandler & Wilson, 1959; Hudson, 1970). Thus in the present study we examined the possible change of activities of fatty acid-esterifying enzymes in the heart. Further, enzyme activities were measured by using subcellular fractions, because the distribution of

these enzymes in myocardial tissue is non-homogeneous (Liu & Kako, 1974, 1975). Although the activities of some enzymes were dramatically altered (Table 2; see also Kako & Liu, 1974), no further attempt was made at this time to explore whether these changes are due to the potentiation of the enzymes by the thyroid hormone or by the adenylate cyclase system, or are due to enzyme protein biosynthesis.

The formation of diacylglycerol 3-phosphate from glycerol 3-phosphate and fatty acids is greatly increased in the hyperthyroid state (Kako & Liu, 1974). Such a dramatic change in glycerol 3-phosphate acylation has never been reported. The results suggested not only that acyl-CoA:glycerol 3-phosphate and acyl-CoA:monoacylglycerol 3-phosphate acyltransferase activity increases, but also that further conversion of diacylglycerol 3-phosphate into neutral and phosphoglycerides may be influenced by the hormone. Therefore it became necessary to assay the activities of phosphatidate phosphohydrolase, palmitoyl-CoA hydrolase and diacylglycerol acyltransferase in the hyperthyroid rabbits.

The properties of phosphatidate phosphohydrolase have been studied in some detail in the liver, brain, kidney and intestine of ox, pig, rabbit, guinea pig and rat (Coleman & Hübscher, 1962; Hübscher, 1970). In addition, the soluble enzyme has been partially purified by Coleman & Hübscher (1962) and Sedgwick & Hübscher (1967). The properties of the enzyme in adipose tissue and erythrocytes, as well as in organs of hamsters and cats, have also been documented (Hokin & Hokin, 1961; Johnston *et al.*, 1967; Jamdar & Fallon, 1973), but so far the heart enzymes have attracted little attention. Its study is essential for, at least, the following three reasons: (i) the enzyme, which is localized in subcellular compartments, shows somewhat different properties (Hübscher, 1970); (ii) the enzyme has been proposed to be rate-limiting in the synthesis of lipids *de novo* under some experimental conditions (Vavrecka *et al.*, 1969; Mangiapane *et al.*, 1973; Lamb & Fallon, 1974); (iii) the enzyme exists at a crucial branch point in biosynthetic pathways, namely between neutral-lipid formation and the CDP-linked phospholipid synthesis, thereby the activity of the enzyme could serve as a cellular regulator (McMurray & Magee, 1972; Krag *et al.*, 1974).

The effect of Mg^{2+} on phosphatidate phosphohydrolase varied greatly among the various subcellular fractions, as well as between the two species studied (Tables 2 and 3). Since cross-contamination between mitochondrial and microsomal fractions was shown not to be great (Table 1), it can be concluded that the Mg^{2+} sensitivity (Tables 2 and 3) of the mitochondrial enzyme does differ from that of the microsomal enzyme. Previously, Hokin & Hokin (1961) observed an activation of the erythrocyte enzyme when Mg^{2+} was added, whereas Coleman &

Hübscher (1962), Sedgwick & Hübscher (1965), Smith *et al.* (1967) and Krag *et al.* (1974) all observed a Mg^{2+} inhibition in various tissues. The Mg^{2+} effect was re-investigated by Jamdar & Fallon (1973), using rat adipose tissue. They claimed that it is not the nature of the substrate (i.e. phosphatidate suspension or membrane-bound phosphatidate) but the presence of Mg^{2+} which determines the apparent activities of the subcellular enzymes. In agreement with the latter view, the addition of Mg^{2+} to the microsomal and lysosomal fractions of the rabbit heart greatly increased the activity in our experiments. The activities of the soluble enzyme of the rabbit heart assayed in the presence of Mg^{2+} and with membrane-bound substrate were much lower than those obtained by the assay with phosphatidate suspension in the presence of Mg^{2+} (Tables 2 and 4). Previous results from our laboratory (Liu & Kako, 1975) contained an error in calculation of activities of the soluble enzyme. Recalculation revealed that the activities were erroneously overestimated in that paper. Thus our data obtained by using rabbit hearts are not in agreement with the view that the soluble enzyme exhibits the highest reaction rate among enzymes of subcellular fractions. In rat liver and hamster and cat intestine the enzyme in the supernatant fraction was most active when membrane-bound phosphatidate was used, whereas with phosphatidate suspension as the substrate, particulate enzyme activities amounted to 90% of the total, according to the reports published previously (Smith *et al.*, 1967; Hübscher *et al.*, 1967). The discrepancy is probably due to the organ-specificity of the phosphohydrolase, so that the characteristics of this enzyme in the heart and adipose tissue may differ from those in the liver and intestine. A similar organ-specificity of fatty acid esterification, namely that of acyl-CoA-glycerol 3-phosphate acyltransferase reaction, has been postulated before (Liu & Kako, 1974).

Both Wilgram & Kennedy (1963) and Sedgwick & Hübscher (1965) observed the highest specific activity of phosphatidate phosphohydrolase to be in the lysosomal fraction of the liver. Our work confirms this finding. Further, the activity was significantly increased on administration of tri-iodothyronine (Table 2). The lysosomal fraction also contained a very high activity of palmitoyl-CoA hydrolase (Table 6). However, the results of our experiments with heart homogenates prepared from hamsters with hereditary muscular dystrophy did not show significant increases in the activities. This could be explained by the facts (i) that the lysosomal fraction prepared by differential centrifugation is contaminated by other subcellular organelles (Table 1), (ii) that consistent changes in activities of enzymes, even among so-called 'lysosomal' enzymes, are infrequent in cardiomyopathies (Kako *et al.*, 1974)

and other heart lesions (Tolnai & Beznak, 1971), and (iii) that phosphohydrolase may not be a typical lysosomal enzyme because its responses to freeze-thawing, to the low ionic milieu and to detergents differ from those of 'lysosomal' enzymes (Sedgwick & Hübscher, 1965).

The palmitoyl-CoA hydrolase activities in lysosomal and microsomal fractions in the hyperthyroid state did not significantly deviate from those of the controls (Table 6). In contrast with phosphohydrolase, no definite metabolic significance has been assigned to palmitoyl-CoA hydrolase (McMurray & Magee, 1972), although its properties have been described in some detail (Barden & Cleland, 1969; Jezyk & Hughes, 1971; Barber & Lands, 1971; Jansen & Hülsmann, 1973). Smith & Hübscher (1966) and Johnston *et al.* (1967), using rat liver and hamster intestine, demonstrated that fatty acids, rather than fatty acyl-CoA esters, are more suitable substrates for glyceride biosynthesis. This may be related to a relatively high activity of the hydrolase as well as micelle-forming characteristics of acyl-CoA esters in the intracellular spaces.

A number of controversial results for the acyl-CoA-glycerol 3-phosphate and acyl-CoA-monoacylglycerol 3-phosphate acyltransferase reactions have previously been reported, partly because of unsuitable assay methods. In our assay with [^{14}C]glycerol 3-phosphate, monoacylglycerol 3-phosphate and diacylglycerol 3-phosphate accumulate linearly with time for a period of, but not beyond, 10 min (Liu & Kako, 1974). The rates of the reactions determined by our assay system could still be somewhat underestimated, since phosphatidate phosphohydrolase activity in cardiac particulate fractions is relatively high (Tables 2 and 3). This might result in the removal of the reaction products of acyl-CoA-glycerol 3-phosphate and acyl-CoA-monoacylglycerol 3-phosphate acyltransferase reactions. Indeed, a prolonged incubation under conditions similar to those for the acyltransferase assays results in an increased neutral lipid formation, which is facilitated by the presence of the soluble fraction (Tables 4 and 5). The latter point was more unequivocally demonstrated in the liver (Hübscher, 1970). However, to what extent synthesis of cardiac lipids *de novo* is facilitated by the cell-sap enzyme in more physiological situations remains to be investigated. In the perfused rabbit heart, over 80% of palmitate incorporation into tissue lipids was found in triacylglycerol (Kikuchi & Kako, 1970). Similar results were obtained with the perfused rat heart, in which almost a negligible quantity of the label was found in phosphatidic acid even after 10 min of perfusion, despite the formation of a large amount of [^{14}C]triacylglycerol and some diacylglycerol (S. C. Vasdev & K. J. Kako, unpublished work), suggesting that either the bulk of fatty acid incorporation may

occur through acyl-exchange reactions, or phosphatidate phosphohydrolase may not be rate-limiting under these conditions. With respect to the latter point, although the specific activity of soluble phosphohydrolase was not high (Tables 2–5), an amount of the soluble fraction in tissue is tenfold larger than the amount of the microsomal fraction (Table 1), and hence the total activity of the soluble phosphohydrolase in the heart is considerable. Recent evidence indicates that lipid enzymes react with particulate-bound (endogenous) substrate differently from aqueous suspension of the substrate (Hübscher, 1970; Fallon *et al.*, 1975; Kanoh & Ohno, 1975). Diacylglycerol may be synthesized in an intracellular organelle discovered in bovine heart (Christiansen, 1975). Lastly, cardiac enzymes of glyceride synthesis *de novo* exhibit little fatty acid specificity (Liu & Kako, 1974; Liu *et al.*, 1974; G. Zaror-Behrens & K. J. Kako, unpublished work), so that the stereospecific fatty acid distribution of tissue lipids in the rabbit heart must largely be caused by selective transacylation reactions.

Our data thus demonstrate that tri-iodothyronine controls the activities of acyl-CoA-glycerol 3-phosphate and acyl-CoA-monoacylglycerol 3-phosphate acyltransferase, and Mg^{2+} -activated phosphatidate phosphohydrolase in subcellular fractions of rabbit hearts. Its selective activation strongly suggests that these enzymes in subcellular fractions are different, or at least possess different properties. The lack of effect of Mg^{2+} on phosphohydrolase in hamster hearts, together with the finding that there is a significant change in Mg^{2+} -insensitive phosphohydrolase of cardiac microsomal fraction in myopathic hamsters, implies the existence of species specificity, as well as complicated intracellular compartmentalization of fatty acid-esterifying enzymes and their substrates. Membrane-bound phosphatidate was not a preferred substrate for the cardiac soluble phosphohydrolase, suggesting that the properties of the cardiac enzyme are dissimilar to those of the liver enzyme.

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