

## Induction of carnitine acetyltransferase by clofibrate in rat liver

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Administration of the anti-hypercholesterolaemic drug clofibrate to the rat increases the activity of carnitine acetyltransferase (acetyl-CoA–carnitine *O*-acetyltransferase, EC 2.3.1.7) in liver and kidney. The drug-mediated increase in enzyme activity in hepatic mitochondria shows a time lag during which the activity increases in the microsomal and peroxisomal fractions. The enzyme induced in the particulate fractions is identical with one normally present in mitochondria. The increase in enzyme activity is prevented by inhibitors of RNA and general protein synthesis. Mitochondrial protein-synthetic machinery does not appear to be involved in the process. Immunoprecipitation shows increased concentration of the enzyme protein in hepatic mitochondria isolated from drug-treated animals. In these animals, the rate of synthesis of the enzyme is increased 7-fold.

Administration of the anti-hypercholesterolaemic drug clofibrate [2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester] to the rat has been shown to increase the activities of fatty acyltransferases in liver (Solberg *et al.*, 1972; Daae & Aas, 1973; Kahonen & Ylikahri, 1974). Among these transferases, the short-chain fatty-acid-transferring enzyme carnitine acetyltransferase (acetyl-CoA–carnitine *O*-acetyltransferase, EC 2.3.1.7), which is at a barely detectable concentration in normal rat liver, is activated most by the drug. The drug-induced increase in the activity of the enzyme has been shown to occur in microsomal, peroxisomal and mitochondrial fractions. However, the increase is highest in the mitochondrial fraction (Markwell *et al.*, 1977). We have recently purified to homogeneity the enzyme from hepatic mitochondria of drug-treated rats (Mittal & Kurup, 1980*a*). By using antibody raised against the purified enzyme, we have attempted to study the mode of drug-induced increase in enzyme activity. The results presented in the present paper show that the increase in enzyme activity is due to increase in content of the enzyme protein because of its enhanced rate of synthesis.

### Materials and methods

#### *Animals and administration of drug*

Male albino rats, 3 months old, weighing about 150 g and obtained from the Central Animal Facility of the Institute, were fed with a vitamin-supplemented casein diet (Kurup *et al.*, 1970), and used in the experiments reported here. Clofibrate was

administered either orally as a water emulsion (50 mg in 0.2 ml/rat per day) or mixed with the diet (0.5%, w/w).

#### *Subcellular fractionation*

Subcellular fractionation of liver was carried out essentially as described previously (Kurup *et al.*, 1970). Mitochondria were sedimented by centrifugation at 8250 *g* for 10 min and a 'peroxisome-rich' fraction was isolated by centrifugation at 17000 *g* for 20 min in a refrigerated RC 2-B Sorvall centrifuge. The microsomal fraction was obtained by centrifugation at 100000 *g* for 60 min in a Beckman model L5-50 ultracentrifuge. The mitochondrial fraction was washed once with 0.25 M-sucrose. Mitoplasts were prepared by digitonin treatment as described by Greenwalt (1974).

Pure preparations of peroxisomes were obtained from rat liver by using isopycnic sucrose-density-gradient centrifugation, essentially as described by Baudhuin (1974). The animals had received an intraperitoneal injection of Triton WR-1339 (10 mg/100 g body wt.) 5 days before killing. The purity of the catalase-rich peroxisomes was checked by the assay of marker enzymes for mitochondria, microsomal fraction ('microsomes') and lysosomes. Protein was measured by the biuret method (Gornall *et al.*, 1949) or by the method of Lowry *et al.* (1951).

#### *Enzyme assays*

Carnitine acetyltransferase was assayed spectrophotometrically by measuring the rate of release of

coenzyme A, using 5,5'-dithiobis-(2-nitrobenzoic acid) (Bieber *et al.*, 1972; Mittal & Kurup 1980a). One unit of the enzyme activity releases 1  $\mu$ mol of coenzyme A under the assay conditions.  $\alpha$ -Glycerophosphate dehydrogenase [L-glycerol 3-phosphate-(acceptor) oxidoreductase, EC 1.1.99.5] was assayed manometrically as described by Krishnakantha & Kurup (1972). Catalase (hydrogen peroxide-hydrogen peroxide oxidoreductase, EC 1.11.1.6) was determined spectrophotometrically as described by Luck (1963). Other marker enzymes were assayed by standard procedures as described previously (Mittal & Kurup, 1980b).

#### Preparation of enzyme and antibody

The procedure for the purification of carnitine acetyltransferase from mitochondria isolated from the livers of clofibrate-fed rats has been described previously (Mittal & Kurup, 1980a). The purified enzyme was emulsified with complete Freund's adjuvant (1:1, v/v) and injected subcutaneously into a rabbit on three separate occasions at 10 day intervals (300  $\mu$ g of protein for the first time and 100  $\mu$ g subsequently). Fourth injection (100  $\mu$ g) was given intramuscularly. One week later, a booster injection (100  $\mu$ g, no adjuvant) was given intravenously and the animal was bled 6 days later. Serum was prepared and a  $\gamma$ -globulin fraction was purified as described by Campbell *et al.* (1970). The purified preparation was stored in 1 ml lots at  $-15^{\circ}\text{C}$  until use.

#### Rate of enzyme synthesis

*Chlorella* protein hydrolysate (100  $\mu$ Ci of  $^{14}\text{C}$ ; 0.2 ml/rat) was injected intraperitoneally into animals that were given clofibrate (50 mg/rat per day orally) for 4 days. At 1 h after injection, the animals were killed and a hepatic microsomal fraction was prepared. The preparation (35 mg of protein) from control and drug-fed animals was treated with

excess antibody (4 mg). The immunoprecipitate was washed, dissolved in formic acid (0.2 ml), spotted on Whatman no. 1 filter paper strip and the radioactivity was determined, after addition of 5 ml of toluene containing 2,5-diphenyloxazole (0.5%, w/v), in a Beckman LS-100 Counter. The counting efficiency was 30% and the error was  $<2.5\%$ .

#### Reagents

All biochemicals, dextran, ethidium bromide, chloramphenicol and actinomycin D were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Cycloheximide was obtained from Upjohn Co., Kalamazoo, MI, U.S.A. Triton WR-1339 was obtained from Rohm and Haas, Philadelphia, PA, U.S.A. Thiamphenicol glycinate hydrochloride was generously given by Dr. D. D. Bella, Zambon Research Laboratories, Milan, Italy. Clofibrate was generously given by Dr. Vasant S. Palkar, Nivedita Chemicals, Pvt., Bombay, India. All common reagents were of analytical grade. All solutions were prepared with triple-quartz-distilled water.

#### Results

##### *Carnitine acetyltransferase activity in rat tissues*

The effect of prolonged feeding with clofibrate on the activity of the enzyme in different tissues of the animal is shown in Table 1. The enzyme activity, which is very low (at the barely detectable level) in the livers of normal animals, increases tremendously on administration of clofibrate. In hepatic mitochondria the specific activity of the enzyme increases almost 30-fold (Table 1). In kidney, the enzyme increases about 5-fold. In the heart, which shows a fairly high activity of the enzyme, the increase in activity on administration of the drug is marginal. No increase is detected in the brain or in testes. The latter tissue shows very high

Table 1. *Effect of clofibrate administration on the activity of carnitine acetyltransferase in tissues of the rat*  
Animals were fed with clofibrate (0.5%, w/w) in the diet for 45 days. The activity of the enzyme was determined in the tissue homogenate and mitochondria. The values are means for three independent determinations (animals). Means  $\pm$  s.d. are given.

Tissue	Enzyme activity (m-units/mg of protein)			
	Control		Clofibrate-fed	
	Homogenate	Mitochondria	Homogenate	Mitochondria
Liver	—	1.5 $\pm$ 0.2	23.0 $\pm$ 2.7*	44.4 $\pm$ 2.1*
Kidney	2.6 $\pm$ 1.0	5.0 $\pm$ 0.9	15.0 $\pm$ 1.4*	28.4 $\pm$ 2.7*
Heart	8.0 $\pm$ 0.9	11.4 $\pm$ 2.9	9.2 $\pm$ 0.8	13.5 $\pm$ 2.8
Testes	23.8 $\pm$ 3.3	—	22.2 $\pm$ 0.4	—
Brain	2.0 $\pm$ 0.1	—	2.0 $\pm$ 0.1	—

\*  $P < 0.01$  (drug-fed versus control).

enzyme activity in normal animals. No increase was detected in smooth muscle or skeletal muscle as well (results not shown).

#### Pattern of increase in liver

The effect of progressive feeding of rats with clofibrate on the specific activity of carnitine acetyltransferase in hepatic mitochondria is illustrated in Fig. 1. The enzyme activity increases rapidly and becomes about 10-fold higher in 4 days. In contrast with this, the increase in the activity of mitochondrial glycerophosphate dehydrogenase, which is also induced by the drug (Krishnakantha & Kurup, 1972), takes place at a much slower rate, the activity increasing only 2-fold in 4 days (Fig. 1).

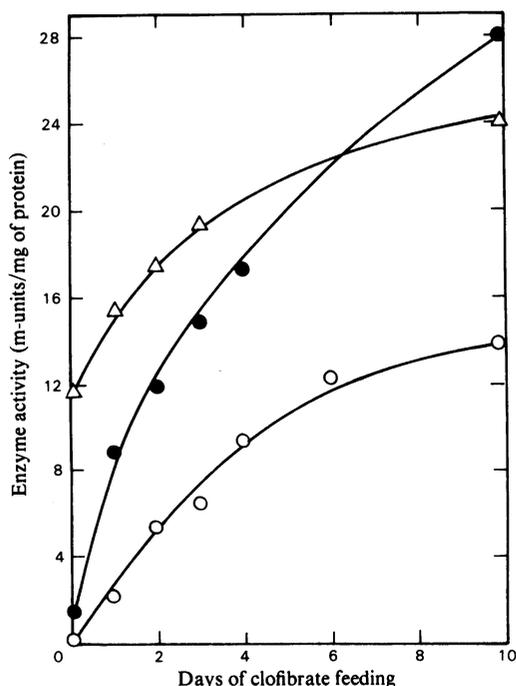


Fig. 1. Increase in carnitine acetyltransferase and glycerophosphate dehydrogenase activities in rat liver on progressive feeding with clofibrate

The drug was given mixed with the diet (0.5%, w/w) for the time period indicated. The activities of transferase in hepatic mitochondria (●) and post-mitochondrial supernatant fraction (○) are shown. The activity of mitochondrial glycerophosphate dehydrogenase is also shown (Δ). The values are means for two independent determinations. The variation between determinations was less than 10%. One unit of enzyme activity is defined as the enzyme required for the release of  $1\ \mu\text{mol}$  of CoA/min (transferase) or uptake of  $1\ \mu\text{g}$ -atom of O/min (dehydrogenase). Other details are given in the Materials and methods section.

With continued feeding, mitochondrial transferase activity increases enormously, reaching values as high as 360 m-units/mg of mitochondrial protein in about 90 days. In contrast, the dehydrogenase activity rarely increases beyond 5–6-fold.

Clofibrate-induced increase in transferase activity is not confined to the mitochondria (Kahonen, 1976; Markwell *et al.*, 1977). The enzyme activity increases, though to a lesser extent, in the peroxisomal and microsomal fractions also. Under our experimental conditions, in animals that are fed with the drug for 3 months, 85% of the total activity in liver is accounted for by the mitochondria, 9–10% by the 'peroxisomal' fraction and about 5% by the microsomal fraction. The activity in cytosol (100000 g) is at the limits of detection.

We have reported previously (Mittal & Kurup, 1980c) that even in regenerating liver, the drug is able to bring about increase in liver size and mitochondrial population. It was therefore of interest to see whether the drug would induce these enzymes when administered after partial hepatectomy. The results presented in Fig. 2 show that in regenerating conditions, the enzyme activity increases progressively with feeding of the drug. In the first 24 h, the increase in activity is relatively small (Fig. 2). It is noteworthy that during this period, there is very little change in the content of mitochondrial protein in the liver (Mittal & Kurup, 1980c). It may also be seen from the Figure that the glycerophosphate dehydrogenase activity increases at a much slower rate than does the transferase activity.

Considerable increases in the activity of the enzyme in liver mitochondria can be observed even after a single oral dose of the drug. The pattern of increase is shown in Fig. 3. The specific activity of the enzyme in the mitochondrial fraction remains unchanged for about 6 h after a single dose of clofibrate. Thereafter it increases progressively with time and appears to reach a maximum in 30 h. The specific activity decreases thereafter. The activity of the enzyme in the 'peroxisomal' fraction appears to increase earlier than in the mitochondrial fraction, the activity increasing 2-fold between 3 and 6 h ( $P < 0.01$ ). Thereafter, the increase is progressive with time and follows the same pattern as shown by the mitochondrial fraction (Fig. 3). The specific activity of the enzyme in the microsomes doubles within 3 h after the administration of the drug ( $P < 0.01$ ). The subsequent increase follows the same course as the 'peroxisomal' and mitochondrial fractions. In normal animals, the specific activity of the enzyme in the 'peroxisomal' fraction is higher than that in the mitochondrial fraction. The activity of microsomes is less than half that of mitochondria. However, the maximum activity attained by the particulate fractions is more or less of the

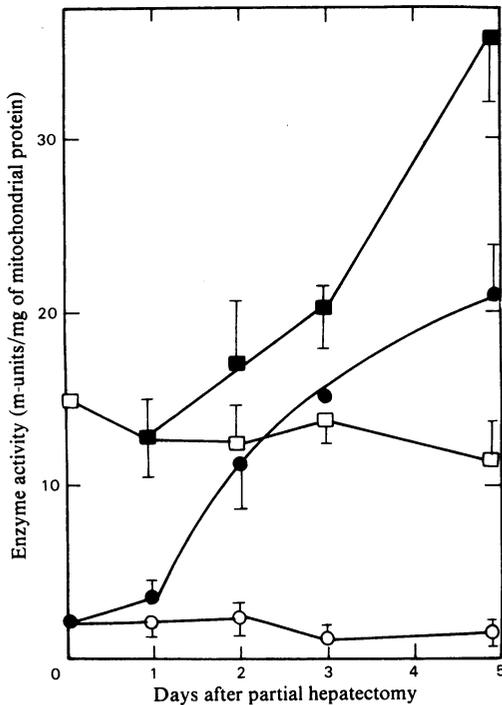


Fig. 2. Increase in carnitine acetyltransferase and glycerophosphate dehydrogenase activities in rat liver mitochondria on progressive feeding with clofibrate after partial hepatectomy

Rats were partially hepatectomized as described by Bresnick (1971). Wherever indicated, animals were fed with the drug orally (50 mg/rat in 0.2 ml of water as an emulsion) on the day of surgery and in the diet (0.5%, w/w) from the next day. Hepatic mitochondrial transferase activities from control (○) and drug-fed (●) animals are given. The dehydrogenase activities of liver mitochondria from control (□) and drug-fed (■) animals are also given. The values are means  $\pm$  s.d. for three to four independent estimations (animals). Other details are given in the legend for Fig. 3.

same order (Fig. 3). It may also be mentioned that at no time could the carnitine acetyltransferase activity be detected in the cytosol during these experiments.

As may be seen from Fig. 3, the specific activity of the enzyme in all the particulate fractions decreases from 30h after a single dose of the drug. The decrease, though rapid in the initial stages, slows down after 24h, taking about 1 week for the activity to return to the normal values. It may be mentioned that the concentration of the drug reaches a maximum in serum (1.3 mM) and liver (200 nmol/g of liver) 2h after ingestion, decreases

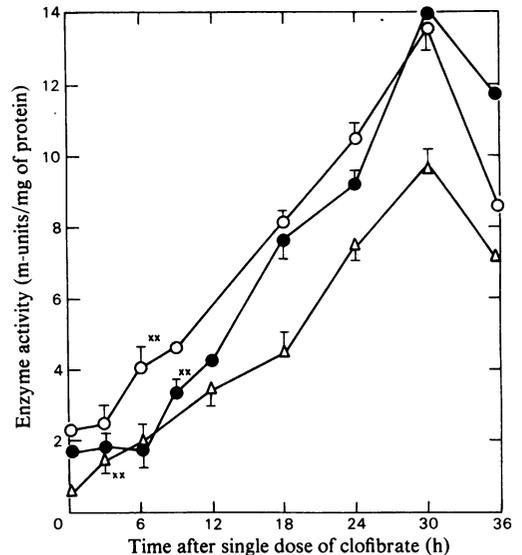


Fig. 3. Pattern of increase of carnitine acetyltransferase activity in rat liver subcellular fractions after a single oral dose of clofibrate

Rats were given clofibrate (50 mg/rat) orally as an emulsion in water (0.2 ml) at zero time. The animals were killed at the time period indicated, the hepatic subcellular fractions were isolated and the enzyme activity was assayed. The specific activities of the enzyme in the mitochondrial (●), 'peroxisomal' (○) and microsomal (△) fractions are given. Significance ( $P < 0.01$ , versus zero time) is indicated by xx. The values are means  $\pm$  s.d. for six to ten independent determinations. Other details are given in the Materials and methods section.

thereafter and practically disappears from the system in 24h (Kurup & Chhabra, 1977).

In animals that have been fed with the drug for 90 days, the mitochondrial carnitine acetyltransferase activity did not show any significant decrease in activity for the first 5 days after withdrawal of the drug from the diet. In 10 days the activity decreased by 75%, but was still about 5-fold higher than the value in control mitochondria. In contrast, the glycerophosphate dehydrogenase activity decreased by 50% in the first 5 days after withdrawal of the drug (results not shown).

#### Inhibition of drug-induced carnitine acetyltransferase

To see whether the drug-induced increase in enzyme activity involves synthesis of RNA and protein *de novo*, the effect of actinomycin D (200  $\mu$ g/rat) and cycloheximide (300  $\mu$ g/rat) was tested. One injection at 30 min before the administration of clofibrate totally prevented any

increase in the activity of the enzyme in all the particulate fractions for 18h. Treatment of the animal with either antibiotic (6, 9 or 12h) subsequent to the administration of clofibrate effectively arrested further increase in the activity of the enzyme in mitochondria up to 18h. The same results were obtained when multiple injections of the antibiotics were given (results not shown).

The effect of inhibitors of mitochondrial protein synthesis on clofibrate-induced carnitine acetyltransferase activity in mitochondria was also tested. Repeated injections of chloramphenicol (100 mg/rat, every 6h) or of ethidium bromide (2 mg/rat) failed to inhibit clofibrate-mediated increases in enzyme activity in mitochondria in normal as well as partially hepatectomized animals. However, in these experiments we failed to observe any decrease in the specific activity of cytochrome oxidase, the synthesis of which is known to be inhibited by these compounds (Kroon & De Vries, 1971). When partially hepatectomized animals were injected with thiamphenicol (45 mg/rat) twice daily for 3 days, the cytochrome oxidase activity of hepatic mitochondria decreased by 40–45%. Administration of clofibrate (single dose) to such animals resulted in an uninhibited increase in the activity of carnitine acetyltransferase. It may be mentioned that thiamphenicol is more slowly metabolized in the body than chloramphenicol (Della Bella *et al.*, 1968). Conditions that are characterized by severe mitochondrial degeneration, like acute starvation (Chhabra *et al.*, 1979) or cuprizone feeding (Subramanian *et al.*, 1975), also did not interfere with the induction of carnitine acetyltransferase activity by clofibrate.

#### Increase in enzyme protein

The results presented in Fig. 3 give a clear indication that the clofibrate-mediated increase in carnitine acetyltransferase activity in rat liver is due to stimulation of the synthesis of the enzyme by the drug. To confirm this, antibody against the drug-induced enzyme was prepared. The purified immunoglobulins cross-reacted with microsomal, peroxisomal and mitochondrial fractions, giving in each case a single precipitin line (Fig. 4), indicating the immunological identity of the enzyme in the three particulate fractions.

To ascertain that the enzyme activity in the 'peroxisomal' fraction does not arise from contamination by other subcellular fractions, particularly mitochondria, pure preparations of hepatic peroxisomes from drug-fed (15 days) animals were obtained by isopycnic sucrose-density-gradient centrifugation. These preparations showed high catalase activity (17.7 mmol of  $H_2O_2$  decomposed/min per mg of protein) and practically no cytochrome oxidase activity. The specific activity of

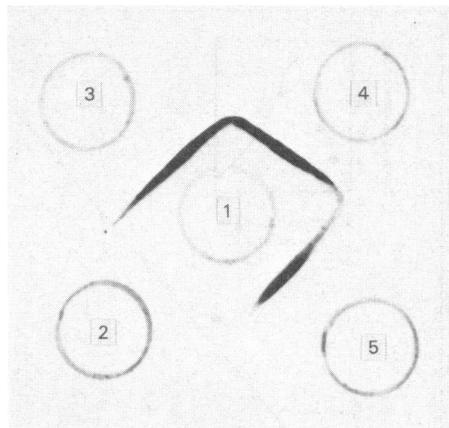


Fig. 4. Immunoprecipitin reaction between carnitine acetyltransferase antibody and subcellular fractions. Purified antibody (250  $\mu$ g) was placed in the central well (1). Phosphate buffer, pH 7.4 (2), solubilized mitochondria (2 mg of protein) (3), solubilized peroxisomes (2 mg of protein) (4) and solubilized microsomes (2 mg of protein) (5) were placed in the other wells as indicated. The subcellular fractions were isolated from the livers of animals fed with clofibrate for 90 days. The plates were incubated for 4 days at room temperature. The precipitin bands were stained with Coomassie Blue.

carnitine acetyltransferase was 52.1 m-units/mg of protein. The mitochondrial preparation (cytochrome oxidase activity 545 ng-atom of O/min per mg of protein; catalase activity 0.24 mmol of  $H_2O_2$ /min per mg of protein) showed a transferase activity of 76.1 m-units/mg of protein. The transferase activity in both preparations (peroxisomes and mitochondria) were completely precipitated by the antibody raised against the mitochondrial enzyme. The peroxisomal preparation did not show any contamination by lysosomal or microsomal fractions also. The enzyme activity in microsomes was not due to peroxisomal contamination. The existence of the enzyme in microsomes has already been demonstrated (Kahonen, 1976; Markwell *et al.*, 1977).

The pattern of precipitation of mitochondrial carnitine acetyltransferase activity by its antibody, depicted in Fig. 5, reveals that the organelles isolated from the livers of clofibrate-fed animals contain more of the enzyme (protein) per mg of particulate protein. With both mitochondrial preparations, 50  $\mu$ g of the antibody was equivalent to about 22 m-units of carnitine acetyltransferase activity. From regression analysis the amount was calculated as 23.8 and 21.0 m-units for mitochondria from normal and drug-fed animals respectively. This

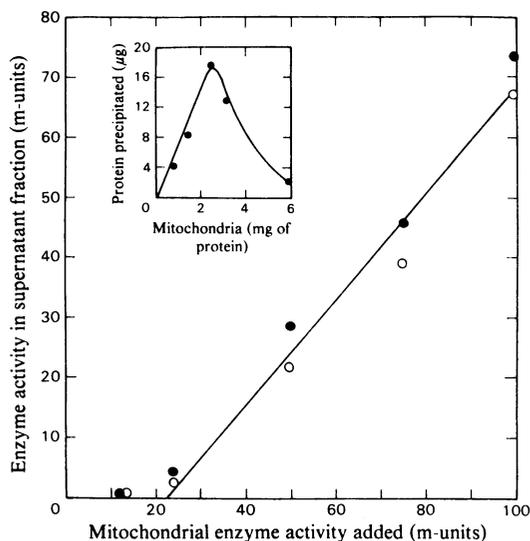


Fig. 5. Immunoprecipitation of mitochondrial carnitine acetyltransferase activity in control (○) and clofibrate-fed (●) animals

Mitochondria, with carnitine acetyltransferase activity as indicated, were solubilized with Triton X-100 (1%, w/v) and mixed with 50 µg of purified  $\gamma$ -globulin preparation containing antibody for the enzyme and left for 24 h at 4°C. The total volume was 0.5 ml. The precipitate was removed by centrifugation and the enzyme activity in the supernatant fraction was estimated. To obtain 50 m-units of activity, the amount of mitochondrial protein required was 0.8 mg in the case of clofibrate-fed animals and 9.6 mg in the case of control animals. Regression lines were fitted by least-squares method for immunoprecipitation of carnitine acetyltransferase in mitochondria from control ( $y = 0.854x - 20.3$ ;  $r = 0.99$ ) and drug-fed animals ( $y = 0.913x - 19.2$ ;  $r = 0.99$ ). The regression line for both values analysed together ( $y = 0.883x - 19.73$ ;  $r = 0.98$ ) is indicated. The inset shows the titration of clofibrate-induced enzyme with the antibody. Various concentrations of the mitochondrial preparation were treated as above with 150 µg of the antibody preparation. The immunoprecipitate was washed three times with 20 mM-phosphate buffer, pH 7.4, containing 0.3 M-NaCl and the protein content was estimated.

amount of enzyme is present in 0.33 mg of mitochondrial protein from clofibrate-fed animals or 4.2 mg of mitochondrial protein from control animals. Addition of increasing concentrations of the enzyme to a fixed concentration of antibody yields a typical immunotitration curve (inset in Fig. 5).

#### Rate of enzyme synthesis

The rate of carnitine acetyltransferase synthesis in

the livers of normal and drug-fed animals was compared by pulse labelling the proteins with radioactive amino acids. The radioactivity precipitated by the antibody from 35 mg of microsomal protein was 110 d.p.m. and 700 d.p.m. respectively from normal and drug-fed animals. This would indicate that in clofibrate-fed animals the enzyme is synthesized at a rate 7-fold higher than in normal animals.

#### Transport of the enzyme

With a view to understanding the mode of transport of the enzyme from the site of synthesis to peroxisomes and mitochondria, the exchange of enzyme activity from one type of organelle to another, on mixing, was studied. When enzyme-rich microsomes (specific activity 24.2 m-units/mg of protein) or peroxisomes (21.6 m-units/mg of protein) were incubated with hepatic mitochondria (specific activity 1.3 m-units/mg of protein) freshly isolated from control animals, no apparent transport of the enzyme took place. Mitoplasts prepared by digitonin treatment (Greenwalt, 1974) of the incubated mitochondria showed no significant increase in specific activity of the enzyme (about 3 m-units/mg of protein). Additions of succinate or ATP also had no effect. During incubation, the enzyme did not accumulate in the cytosol either.

To see whether mitochondria take up the enzyme from solution, the organelles freshly isolated from normal livers were incubated with the enzyme purified from hepatic mitochondria of drug-fed animals. The activity imbibed by mitochondria on mixing with the purified enzyme was readily destroyed by proteolytic enzymes, indicating that it was adsorbed on the outer membrane. Mitoplasts prepared from such mitochondria did not show enhanced enzyme activity.

#### Discussion

The results presented in the present paper report convincing evidence that the increase in activity of carnitine acetyltransferase in the livers of clofibrate-fed rats results from a stimulation of the rate of synthesis of the enzyme under the influence of the drug. The enzyme protein that increases in the particulate fractions is immunologically identical with that present in normal rat liver. In this context, it may be pertinent to point out that the fatty acid oxidase system induced by the drug in peroxisomes is different from that present in mitochondria (Lazarow & de Duve, 1976; Lazarow, 1978). Even though the mitochondrial fraction accounts for about 80% of the total enzyme activity in drug-fed animals, the enzyme (which has two subunits of unequal size; Mittal & Kurup, 1980a) is synthesized outside the mitochondrion. Protein-synthetic ma-

chinery in the organelle appears to have no role to play. In this respect, the enzyme differs from other mitochondrial enzymes, like ATPase and cytochrome oxidase, in the synthesis of which both mitochondrial and cytoplasmic ribosomes are involved (Tzagoloff *et al.*, 1979).

The mode of transport of the enzyme from the endoplasmic reticulum into the peroxisomes and mitochondria is intriguing. Our experiments do not appear to support the intermediate passage of proteins through the cytosol as proposed by Hallermayer *et al.* (1977) and Schatz (1979). Under no condition could we detect reasonable enzyme activity in the cytosol. Moreover, inhibition of 'cytoplasmic' protein synthesis resulted in an immediate cessation of the accumulation of activity in mitochondria. Also, the purified enzyme was not taken up by mitochondria when the two were mixed. In this respect, this enzyme resembles malate dehydrogenase, which, though of mitochondrial origin, is not taken up by the intact organelle from solution (Strasberg *et al.*, 1979). In contrast, mitochondrial aspartate aminotransferase is readily and specifically taken up by mitochondria from solution (Marra *et al.*, 1978).

Direct transfer of the enzyme synthesized on endoplasmic reticulum to peroxisomes and mitochondria is conceivable according to the 'direct insertion' hypothesis of protein transfer advanced by Kellems & Butow (1974). It may be pertinent to mention that clofibrate proliferates not only mitochondria but peroxisomes as well (Svoboda & Azarnoff, 1966). Electron-micrographic evidence for the intimate association and continuity between endoplasmic reticulum and peroxisomes in liver has been presented (Reddy & Svoboda, 1973). Our experiments, however, failed to show any significant movement of the enzyme from microsomes or peroxisomes into mitochondria. The suggestion (Rothman & Fine, 1980) that vesicles that bud-off from one membrane and fuse with another may be the key intermediates in the intracellular transport of proteins remains to be tested.

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