Increase in Hepatic Catalase and Glycerol Phosphate Dehydrogenase Activities on Administration of Clofibrate and Clofenapate to the Rat

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1. Clofenapate (methyl 2-[4-(*p*-chlorophenyl)phenoxy]-2-methylpropionate) fed to the rat in the diet increased the content of mitochondrial protein in the liver by 50–60%. In this respect it resembled the related compound clofibrate (ethyl α -*p*-chlorophenoxyisobutyrate), which is widely used as an antihypercholesterolaemic drug. 2. Both compounds when fed to the rat enhanced the activity of α -glycerol phosphate dehydrogenase in the liver mitochondria manifold, but were without effect on the enzyme in the soluble fraction. 3. On the other hand, the catalase activity in the supernatant fraction increased twofold after administration of the drugs. The mitochondrial catalase activity showed a consistent decrease. 4. It was unlikely that under the influence of the drug the increase in catalase activity took place in the mitochondrial particles and was leached into the cytosol during isolation. 5. The increase in catalase activity in the cytosol under the influence of the drug is best explained on the assumption that peroxisomes which contain this enzyme, and which are known to increase on administration of the drug, were broken during the process of cellular fractionation and released the enzyme into the cytosol. 6. All the above effects shown by both drugs were fully reversed when drugs were withdrawn from the diet. 7. Clofenapate was effective in bringing about the above changes when administered to the animal at one-hundredth the concentration of clofibrate.

Administration of the antihypercholesterolaemic drug clofibrate (ethyl α -*p*-chlorophenoxyisobutyrate, also called Atromid-S) to experimental animals has been shown to increase the hepatic glycerol phosphate dehydrogenase [L-glycerol phosphate-(acceptor) oxidoreductase, EC 1.1.99.5] and catalase (hydrogen peroxide-hydrogen peroxide oxidoreductase, EC 1.11.1.6) activities (Hess *et al.*, 1965). Both catalase and glycerol phosphate dehydrogenase are present in the cytosol as well as in the mitochondria of liver. In studies reported so far on the drug-induced increase in the activities of these two enzymes, attention was confined to the total enzyme activity in liver homogenates without considering its intracellular distribution (Ruegamer *et al.*, 1969; Reddy *et al.*, 1971). It was reported from our laboratory that administration of clofibrate to the rat increased the content of hepatic mitochondria by 50–100% (Kurup *et al.*, 1970). In the light of this observation it was decided to examine whether the increase in the activities of glycerol phosphate dehydrogenase and catalase observed in whole liver under the influence of the drug is the result of an increase of the two proteins in one or both of the subcellular fractions. The results of such an investigation reported in the present paper indicate that the activities of the two enzymes in the mitochondrial fraction and cytosol respond differently to the drug. The data also show that the analogous hypolipidaemic compound clofenapate (methyl 2-[4-(*p*-chlorophenyl)phenoxy]-2-methyl-

propionate) (Thorpe, 1969) resembles clofibrate in eliciting the above enzymic responses in the liver.

Materials and Methods

Animals and administration of the drug

Male albino rats weighing 140–160 g from the stock colony of this Institute were used in all the experiments. The composition of the diet and the feeding schedule were the same as described by Kurup *et al.* (1970). After being kept for 7–10 days on the normal diet, the experimental animals were given 0.5% (w/w) clofibrate or 0.005% (w/w) clofenapate mixed with the diet for the period indicated, at the end of which period the animals were killed. Six animals were used for each treatment and six animals kept on the normal diet were also killed as controls. An equal number of control and experimental animals were killed at any one time with a view to minimizing variations. In the withdrawal experiments, the animals were supplied with the diet containing the drug at the above concentrations for the time-period indicated and were then replaced on a normal diet and kept on it for an equal period of time.

Subcellular fractionation

The animals were killed by stunning and decapitation. The liver was removed, weighed, chilled and

homogenized in 0.25M-sucrose. The nuclear and mitochondrial fractions were sedimented and washed as described by Kurup *et al.* (1970). The microsomal fraction was sedimented by centrifugation at 109000g for 60min in a Beckman model L3-50 refrigerated ultracentrifuge. The sediment was resuspended in 0.25M-sucrose and used without washing.

All homogenizations were carried out in an ice bath. The centrifugations for the sedimentation of nuclei and mitochondria were made in a Sorvall RC₂-B refrigerated centrifuge at 0–4°C. The sucrose medium was prepared fresh daily, in water previously boiled and cooled. The fractions were suspended in 0.25M-sucrose unless otherwise stated. The protein content of the fractions was measured by Folin's reagent after appropriate dilution (Lowry *et al.*, 1951).

Enzyme assays

The activity of catalase was determined spectrophotometrically as described by Lück (1963). The reaction system contained 198 μ mol of sodium phosphate-potassium phosphate buffer, pH7.0, and 37.5 μ mol of hydrogen peroxide, in a total volume of 3ml. The reaction was initiated by the addition of 20–50 μ g of protein from the subcellular fraction and the rate of decomposition of hydrogen peroxide was followed by measuring the decrease in E_{240} in a Cary model 14 recording spectrophotometer. Under the above conditions the reaction rate was linear for at least 1 min. The concentration of hydrogen peroxide was calculated by using the extinction coefficient of 0.036 μ mol⁻¹·ml⁻¹ (Lück, 1963).

The glycerol phosphate dehydrogenase activity in particulate fractions was measured manometrically as described by Ruegamer *et al.* (1964) in an Aminco Warburg apparatus at 30°C. The main compartment of the Warburg flask contained 250 μ mol of potassium phosphate buffer, pH7.4, 100 μ mol of KCl, 3 μ mol of KCN, 10 μ mol of MgCl₂, 1.8mg of bovine serum albumin, 1.8mg of phenazine methosulphate and 20–50mg of protein from the subcellular fraction. The KCN solution was always prepared fresh and neutralized before use. Alkali was present in the central well. After 10–15min of equilibration the reaction was started by tipping in 120 μ mol of disodium DL- α -glycerophosphate from the side arm. The total volume was 3ml. The uptake of oxygen was noted every 5min and the increase over blanks lacking substrate was used for the calculation of the enzyme activity.

The soluble fraction did not show any glycerophosphate-dependent oxygen uptake when assayed by the above method. Therefore the glycerol phosphate dehydrogenase activity in this fraction was determined by adapting the method of Hohorst (1963) for the enzymic determination of glycerophosphate. The reaction system contained hydrazine (400 μ mol)–

glycine (1000 μ mol) buffer, pH9.5, NAD⁺ (5 μ mol) and about 500 μ g of protein from the supernatant fraction, in a total volume of 3ml. The reaction was initiated by the addition of α -glycerophosphate (120 μ mol) and the reduction of NAD⁺ was followed by measuring the increase in E_{340} in a Cary model 14 recording spectrophotometer. Under the above conditions the reaction rate was proportional to protein concentration and was linear at least up to 2min. The NADH formed was calculated by using a molar extinction coefficient of 6.22cm⁻¹·mol⁻¹.

The activity of urate oxidase (urate-oxygen oxidoreductase EC 1.7.3.3) was determined as described by London & Hudson (1956). The reaction system contained 300 μ mol of sodium borate buffer, pH9.2, 90nmol of lithium urate and about 1–2mg of protein from the subcellular fraction. The disappearance of uric acid was followed by measuring the decrease in E_{293} in a Cary model 14 recording spectrophotometer. The concentration of uric acid was calculated by using the extinction coefficient of 12.61cm⁻¹· μ mol⁻¹ (Praetorius, 1948).

All the reagents used were of analytical grade and were dissolved in water double-distilled in an all-glass apparatus. All solutions were adjusted to pH7.4 before use. The samples of clofibrate and clofenapate were a generous gift from Dr. J. M. Thorp, Pharmaceuticals Division, Imperial Chemical Industries Ltd, Alderley Park, Macclesfield, Cheshire, U.K.

Results

Subcellular composition of liver

It was reported (Kurup *et al.*, 1970) that administration of clofibrate to the rat increased the mitochondrial protein content of the liver. The results in Table 1 confirm this observation. Dietary administration of clofibrate increased the content of mitochondrial protein by 56% in 20 days and 62% in 30 days. An increase in mitochondrial protein of the same order was obtained with clofenapate at one-hundredth the concentration of clofibrate. The former drug, when given in the diet (0.005%, w/w), increased the mitochondrial protein by 42% in 20 days and 65% in 30 days. This is consistent with the observation of Thorp (1969) that about 300 μ g of the compound/day per rat was enough for optimum hypolipidaemic effects, because under our conditions rats on an average consumed about 10g of food/day. (This would correspond to 500 μ g of the drug/day per rat.)

When the drugs were administered as the sodium salts of the free acids instead of in the ester form, the mitochondrial increase obtained was less. Thus in one experiment the free acids produced a mitochondrial increase of 30% when fed at the above dosages for 20 days. This may be due to the fact that the esters

Table 1. *Effect of feeding rats with clofibrate (0.5%) or clofenapate (0.005%) on the protein content of the subcellular fractions of liver*

The animals were given the drug mixed with the diet for 20 days in Expt. 1 and for 30 days in Expt. 2. In the set marked 'Withdrawn' the animals were replaced on the drug-free diet and kept on it for 20 and 30 days in Expts. 1 and 2 respectively. The values are the averages $\pm$ S.D. of six independent determinations (animals). The values represent mg of protein/g of fresh liver. The percentage of protein in any fraction, taking the total liver protein to be 100, is given in parenthesis. * Significant value: $P < 0.005$.

	Subcellular fraction	Fed			Withdrawn		
		Normal	Clofibrate	Clofenapate	Normal	Clofibrate	Clofenapate
Expt. 1	Nuclear	27 $\pm$ 3 (14 $\pm$ 1)	35 $\pm$ 6 (17 $\pm$ 2)	34 $\pm$ 5 (16 $\pm$ 3)	32 $\pm$ 6 (15 $\pm$ 3)	29 $\pm$ 3 (15 $\pm$ 1)	32 $\pm$ 8 (17 $\pm$ 3)
	Mitochondrial	50 $\pm$ 4 (26 $\pm$ 2)	78 $\pm$ 5* (34 $\pm$ 2)	71 $\pm$ 6* (32 $\pm$ 1)	58 $\pm$ 6 (27 $\pm$ 3)	58 $\pm$ 5 (27 $\pm$ 1)	60 $\pm$ 3 (27 $\pm$ 3)
	Microsomal	50 $\pm$ 4 (26 $\pm$ 1)	47 $\pm$ 5 (21 $\pm$ 1)	50 $\pm$ 5 (22 $\pm$ 1)	51 $\pm$ 7 (25 $\pm$ 3)	56 $\pm$ 7 (28 $\pm$ 3)	53 $\pm$ 5 (24 $\pm$ 2)
	Supernatant	66 $\pm$ 8 (34 $\pm$ 2)	66 $\pm$ 6 (29 $\pm$ 1)	71 $\pm$ 7 (31 $\pm$ 2)	64 $\pm$ 3 (33 $\pm$ 1)	63 $\pm$ 7 (31 $\pm$ 2)	65 $\pm$ 6 (32 $\pm$ 1)
Expt. 2	Nuclear	38 $\pm$ 4 (14 $\pm$ 1)	44 $\pm$ 5 (14 $\pm$ 2)	46 $\pm$ 4 (14 $\pm$ 2)	33 $\pm$ 7 (13 $\pm$ 2)	33 $\pm$ 3 (13 $\pm$ 1)	42 $\pm$ 10 (15 $\pm$ 2)
	Mitochondrial	62 $\pm$ 4 (25 $\pm$ 2)	100 $\pm$ 6* (31 $\pm$ 2)	103 $\pm$ 10* (32 $\pm$ 2)	62 $\pm$ 7 (24 $\pm$ 2)	64 $\pm$ 8 (24 $\pm$ 2)	57 $\pm$ 8 (23 $\pm$ 5)
	Microsomal	64 $\pm$ 13 (25 $\pm$ 3)	66 $\pm$ 9 (22 $\pm$ 2)	67 $\pm$ 12 (22 $\pm$ 2)	66 $\pm$ 7 (25 $\pm$ 2)	63 $\pm$ 6 (24 $\pm$ 1)	64 $\pm$ 5 (23 $\pm$ 2)
	Supernatant	94 $\pm$ 11 (36 $\pm$ 3)	106 $\pm$ 11 (33 $\pm$ 2)	106 $\pm$ 12 (33 $\pm$ 4)	98 $\pm$ 8 (38 $\pm$ 3)	100 $\pm$ 7 (39 $\pm$ 1)	105 $\pm$ 19 (39 $\pm$ 4)

are more uniformly absorbed than the free acids (Thorp, 1969).

The procedure for cell fractionation adopted in our laboratory yields a mitochondrial fraction relatively free of contaminating proteins from the nuclear and microsomal fractions (Kurup *et al.*, 1970). Our method, however, does not yield good nuclear preparations (Schneider & Kuff, 1969). This may account for the larger variations in the protein content of the nuclear fractions in Table 1.

When animals were returned to a normal diet after having been fed on a diet containing either drug, the mitochondrial protein content returned to normal values. The hypolipidaemic and hepatomegalic effects of clofibrate have also been shown to be reversible (Hess *et al.*, 1965).

Glycerol phosphate dehydrogenase

Glycerol phosphate dehydrogenase activity is present both in the particulate and in the soluble fractions. Although the activity in the particulate fraction is not coenzyme-dependent (Dewan & Green, 1937), that in the supernatant fraction is dependent on NAD⁺ (Baranowski, 1949). It has been reported that administration of clofibrate increased the particulate glycerol phosphate dehydrogenase activity of liver homogenates about six-fold (Hess *et al.*,

1965; Westerfeld *et al.*, 1968; Ruegamer *et al.*, 1969). The results in Table 2 indicate that both clofibrate and clofenapate increased the specific activity of glycerol phosphate dehydrogenase in the homogenate and the total activity in the liver almost to the same extent. The results further show that the specific activity of the enzyme in mitochondria also increased about fourfold on treatment with the drugs. In most cases 70–90% of the glycerol phosphate dehydrogenase activity in the liver was accounted for by the mitochondrial fraction. This is in agreement with the mitochondrial localization of the enzyme (Klingenberg, 1970).

On the withdrawal of the drug from the diet, the glycerol phosphate dehydrogenase activity returned to the basal value (Table 2). The specific activity of this mitochondrial enzyme has been shown to increase manyfold in rats kept on a hyperthyroidic diet and to revert to the basal value when the animals were returned to the normal diet (Lee *et al.*, 1959).

The NAD⁺-dependent glycerol phosphate dehydrogenase activity in the soluble fraction did not show any significant increase after administration of the drugs (Table 3). The values reported in Table 3 are of the same order as those reported by Lee *et al.* (1959), who used the same method of assay. Hyperthyroidic conditions also did not alter the activity of the soluble enzyme (Lee *et al.*, 1959).

Table 2. *Effect of feeding rats with clofibrate or clofenapate on the activity of particulate glycerol phosphate dehydrogenase in liver*

The concentration of the drugs used and the conditions of the experiment were the same as given in Table 1. The period of withdrawal was the same as the period of feeding. The enzyme assay has been described in the Materials and Methods section. The activity is expressed as ng-atoms of O/min. The values are the means of six independent determinations $\pm$ s.d.

Period of feeding (days)	Treatment	Fed			Withdrawn		
		Activity (per mg of protein)		Activity (per g of fresh liver)	Activity (per mg of protein)		Activity (per g of fresh liver)
		Homo-genate	Mito-chondria		Homo-genate	Mito-chondria	
20	Normal	1.6 $\pm$ 0.1	3.8 $\pm$ 0.8	305 $\pm$ 27	1.6 $\pm$ 0.1	4.1 $\pm$ 0.7	333 $\pm$ 49
	Clofibrate	7.0 $\pm$ 0.7	12.0 $\pm$ 2.5	1393 $\pm$ 131	2.1 $\pm$ 0.4	4.1 $\pm$ 1.0	425 $\pm$ 87
	Clofenapate	6.2 $\pm$ 0.9	14.1 $\pm$ 0.9	1312 $\pm$ 135	3.8 $\pm$ 0.4	6.6 $\pm$ 1.1	732 $\pm$ 15
30	Normal	2.4 $\pm$ 0.9	5.7 $\pm$ 0.2	504 $\pm$ 100	2.5 $\pm$ 0.6	5.7 $\pm$ 0.7	632 $\pm$ 120
	Clofibrate	15.9 $\pm$ 1.1	22.4 $\pm$ 1.0	4940 $\pm$ 135	2.9 $\pm$ 0.2	3.9 $\pm$ 0.4	760 $\pm$ 91
	Clofenapate	16.7 $\pm$ 1.2	23.8 $\pm$ 0.7	5250 $\pm$ 138	3.5 $\pm$ 0.7	3.7 $\pm$ 0.1	972 $\pm$ 70

Table 3. *Effect of feeding rats with clofibrate or clofenapate on the activity of soluble glycerol phosphate dehydrogenase in liver*

The conditions of the experiment are the same as described in Table 2. The enzyme activity was assayed spectrophotometrically as described in the Materials and Methods section. The activity is expressed as μ mol of NAD⁺ reduced/min. The values are the means of four independent determinations (animals) $\pm$ s.d.

Period of feeding (days)	Treatment	Fed		Withdrawn	
		Activity (per mg of protein)		Activity (per mg of protein)	
		Activity (per g of fresh liver)		Activity (per g of fresh liver)	
20	Normal	0.148 $\pm$ 0.043	12.8 $\pm$ 3.0	0.103 $\pm$ 0.028	9.1 $\pm$ 3.2
	Clofibrate	0.178 $\pm$ 0.025	17.2 $\pm$ 1.5	0.160 $\pm$ 0.051	13.6 $\pm$ 1.6
	Clofenapate	0.189 $\pm$ 0.028	18.9 $\pm$ 2.8	0.147 $\pm$ 0.024	13.5 $\pm$ 2.6
30	Normal	—	—	0.139 $\pm$ 0.050	15.8 $\pm$ 2.4
	Clofibrate	0.166 $\pm$ 0.048	21.5 $\pm$ 2.8	0.136 $\pm$ 0.025	13.9 $\pm$ 2.7
	Clofenapate	0.128 $\pm$ 0.032	14.0 $\pm$ 2.6	0.140 $\pm$ 0.014	13.3 $\pm$ 0.1

Catalase

The results in Table 4 indicate that the activity of catalase in the liver increased about twofold on administration of clofibrate to the rat. This is in agreement with previous reports (Hess *et al.*, 1965; Reddy *et al.*, 1971). Clofenapate was as effective as clofibrate in increasing the hepatic catalase activity. Since catalase, like glycerol phosphate dehydrogenase, is present in both the mitochondrial and supernatant fractions of rat liver it was decided to see how the activity of the enzyme in these two fractions responded to the drug. The results in Table 4 show that the specific activity of the enzyme in the mitochondrial fraction did not increase on administration of the drugs. In fact, the activity showed a 30–40% decrease when compared with controls. However, the total enzymic activity accounted for by the mitochondrial fraction showed a marginal increase (15–20%). This was because the increase in mitochondrial content more than compensated for the decrease in specific activity. The specific activity of the enzyme in the cytosol, however, increased about threefold on administration of the drugs. The total activity of the enzyme in the cytosol also showed a similar increase (Table 4). These changes are in striking contrast to the effect of the drugs on the activity of glycerol phosphate dehydrogenase in the two subcellular fractions. Further, catalase activity of the liver has been shown to decrease under hyperthyroidic conditions (Girkin & Kampschmidt, 1960).

The hepatic catalase activity as well as the activity changes in the mitochondrial and supernatant fractions reverted to the normal values when the animals were returned to the drug-free normal diet.

Mitochondrial catalase

It has been reported that the catalase activity associated with the mitochondrial fraction of rat

liver was largely leached out of the particles on washing with 0.25M-sucrose (Greenfield & Price, 1956; Radhakrishnan & Sarma, 1964). This raised the possibility that in the drug-fed animals the enzyme had increased in the mitochondria but had been leached into the supernatant fraction during fractionation. To test this possibility mitochondria were isolated from the livers of both normal and drug-fed animals by using 0.25M- and 0.88M-sucrose for homogenization. The speed and time of centrifugation for the sedimentation of the mitochondrial fraction were the same with both homogenizing media (Kurup *et al.*, 1970). Hyperosmotic sucrose, it was presumed, would prevent the leaching out of catalase from the particles with the result that mitochondria prepared in this medium would show a higher specific activity of the enzyme. On washing such particles with 0.25M-sucrose the enzyme was expected to be lost to the medium. The results presented in Table 5 show that, contrary to expectation, the specific activity of catalase in the mitochondrial fraction sedimented in 0.88M-sucrose was only about 20% of that in the corresponding fraction sedimented in 0.25M-sucrose. The pattern was the same in both normal and drug-fed animals. When this fraction was washed with 0.25M-sucrose only about 15% of the activity was leached out.

To understand the reasons for the relatively low specific activity of catalase in the mitochondrial fraction sedimented in 0.88M-sucrose, the subcellular distribution of the catalase under both conditions was studied. When the fractionation of the subcellular components was carried out in 0.25M-sucrose about 40% of the catalase activity was recovered in the mitochondrial fraction and about 50% in the cytosol. This is in good agreement with the values in the literature (de Duve & Berthet, 1954; Holmes & Masters, 1972). However, when the fractionation was done in 0.88M-sucrose, only 15% of the activity

Table 4. *Effect of feeding rats with clofibrate or clofenapate on the activity of catalase in liver*

The conditions of the experiment were the same as described in Table 3. The enzyme activity was assayed spectrophotometrically as described in the Materials and Methods section. The particulate fractions were assayed after the addition of deoxycholate and the soluble fraction without deoxycholate. The activity is expressed as mmol of H₂O₂ decomposed/min. The values are the means of six independent determinations $\pm$ S.D.

Period of feeding (days)	Treatment	Catalase activity (per mg of protein)			Catalase activity (per g of fresh liver)		
		Homo- genate	Mito- chondria	Cytosol	Homo- genate	Mito- chondria	Cytosol
20	Normal	—	0.68 $\pm$ 0.05	0.26 $\pm$ 0.08	—	30 $\pm$ 3	24 $\pm$ 6
	Clofibrate	—	0.42 $\pm$ 0.1	0.63 $\pm$ 0.19	—	34 $\pm$ 7	64 $\pm$ 12
	Clofenapate	—	0.45 $\pm$ 0.03	0.84 $\pm$ 0.12	—	35 $\pm$ 2	83 $\pm$ 4
30	Normal	0.27 $\pm$ 0.06	0.78 $\pm$ 0.18	0.26 $\pm$ 0.05	67 $\pm$ 11	49 $\pm$ 9	25 $\pm$ 8
	Clofibrate	0.40 $\pm$ 0.05	0.57 $\pm$ 0.14	0.76 $\pm$ 0.18	125 $\pm$ 17	57 $\pm$ 11	80 $\pm$ 16
	Clofenapate	0.53 $\pm$ 0.21	0.57 $\pm$ 0.15	0.84 $\pm$ 0.28	124 $\pm$ 19	54 $\pm$ 9	87 $\pm$ 31

Table 5. *Effect of the osmoticity of the homogenizing medium on mitochondrial catalase activity*

The animals were given the drug mixed with the diet, at the concentrations indicated in Table 1, for 20 days. One portion of the liver (2.5g) was homogenized in 0.25M-sucrose and another (2.5g) in 0.88M-sucrose. The mitochondria were sedimented as described in the Materials and Methods section and washed once and resuspended in the respective medium. Enough water was added to the suspension in 0.88M-sucrose (100mg of protein) to make the concentration of sucrose 0.25M. The diluted suspension was homogenized and the mitochondria were sedimented. Catalase activity is expressed as mmol of H_2O_2 decomposed/min per mg of protein. The values are the means of four independent determinations.

Mitochondrial preparation	Fed			Withdrawn		
	Normal	Clofibrate	Clofenapate	Normal	Clofibrate	Clofenapate
In 0.25M-sucrose	0.68	0.43	0.45	1.20	1.04	1.14
In 0.88M-sucrose	0.16	0.16	0.17	0.20	0.19	0.16
In 0.88M-sucrose, washed with 0.25M-sucrose	0.12	0.12	0.13	0.17	0.15	0.14
Activity (%) leached into the medium	12.3	9.1	5.7	24.3	25.6	25.0

was recovered in the mitochondrial fraction. The microsomal and supernatant fractions together, in this case, accounted for about 80% of the total catalase activity. There was no appreciable change in the protein content of the subcellular fractions or in the total catalase activity/g of fresh liver with the different osmoticities of sucrose used for homogenization (Table 6).

Peroxisomes

The above observation that in hyperosmotic sucrose more catalase activity was recovered in the microsomal fraction would lend partial support to the concept developed by de Duve (1966) that the catalase activity is confined to peroxisomes, which are organelles smaller in size than mitochondria. Recently Lazarow & de Duve (1971) have reported that the final steps in the synthesis of this enzyme may take place in peroxisomes. It is known that the content of peroxisomes in the liver increases about tenfold on administration of clofibrate to the rat (Svoboda & Azarnoff, 1966). If peroxisomes are the carriers of the majority of the catalase in the liver cell, then the increase in the activity of the enzyme in the supernatant fraction on the administration of the drug is best explained by the assumption that during the process of cell fractionation the peroxisomes break and release the enzyme into the cytosol, as has been suggested by Hess *et al.* (1965). Some support for this assumption is provided by the results presented in Table 7. Catalase activity in the mitochondrial fraction was increased by freezing and thawing or on treatment with deoxycholate (Table 7). There was some release of activity into the medium when mitochondria were frozen and thawed re-

peatedly. However, about 40% of the original activity was still retained with the particulate fraction even after repeated freezing and thawing.

Uricase

In addition to catalase, peroxisomes contain two other enzymes, namely uricase and D-amino acid oxidase [D-amino acid-oxygen oxidoreductase (deaminating), EC 1.4.3.3]. Of these, D-amino acid oxidase and catalase are localized in the sap whereas uricase is membrane-associated. D-Amino acid oxidase is also present in mitochondria. Therefore the intracellular distribution of uricase was studied with a view to finding the pattern of distribution of peroxisomes under our experimental conditions. No uricase activity could be detected in the cytosol, which is in accordance with the membranous association of the enzyme. The greatest activity was found in the microsomal fraction (49%) and more or less equal amounts in the other two fractions (Table 8). It may be mentioned that, in the cell, peroxisomes seem to exist in close association with the endoplasmic reticulum (Hruban & Swift, 1964; Svoboda & Azarnoff, 1966). When the fractionation was done in hyperosmotic sucrose more of the activity was associated with the mitochondrial and microsomal fractions. The enzyme activity showed no change on freezing the mitochondria. Also, addition of deoxycholate consistently inhibited the activity. This would indicate that under our experimental conditions the majority of peroxisomes would settle with the microsomal fraction. The possibility of some contamination of the mitochondrial fraction with peroxisomes is also indicated.

Table 6. Subcellular distribution of catalase in rat liver

Portions (2.5g) of the liver were homogenized in 0.25M- and 0.88M-sucrose. Subcellular fractions were prepared and catalase was assayed as described in the Materials and Methods section. The activity of catalase is expressed as mmol of H₂O₂ decomposed/min. The distribution of catalase activity in the various fractions is calculated by taking the total activity in the homogenate to be 100. The values are the means of two independent determinations.

Subcellular fraction	0.25M-Sucrose				0.88M-Sucrose			
	Catalase activity (per mg of protein)	Protein (mg/g of fresh liver)	Catalase distribution (%)		Catalase activity (per mg of protein)	Protein (mg/g of fresh liver)	Catalase distribution (%)	
Homogenate	0.42	227	100		0.40	210	100	
Nuclear	0.19	33	6		0.10	30	4	
Mitochondrial	0.90	44	38		0.25	48	15	
Microsomal	0.16	40	6		0.31	47	18	
Cytosol	0.55	90	47		0.61	80	59	

Discussion

The results presented in the present paper show that both clofibrate and clofenapate share the property of causing proliferation of mitochondria in the liver when administered in the diet (Table 1). These results lend biochemical support to the report of Paget (1963) that electron micrographs of the livers of rodents fed on clofibrate indicated an increase in the number of mitochondria. Pereira & Holland (1969) have observed that the protein recovered in the mitochondrial fraction of rat liver increased by 50% in 8 h and 90% in about 40 h after a single intraperitoneal dose of clofibrate (250 mg/kg body wt.). However, Khnen *et al.* (1971) observed no increase in the hepatic mitochondrial content on intramuscular administration of clofibrate (60 mg/100 g body wt.) for 9 successive days. However, the content of mitochondria in their control animals was abnormally high (98 mg of protein/g fresh wt. of liver) and was of the same order as obtained by us in drug-fed animals.

The results presented in the present paper confirm the previous reports that administration of clofibrate increases the glycerol phosphate dehydrogenase and catalase activities in liver and show that clofenapate also has this property. However, although the glycerol phosphate dehydrogenase activity increases manyfold in the mitochondrial fraction and remains unchanged in the cytosol, the increase in the catalase activity is detected only in the cytosol. In fact, on administration of the drug, the mitochondrial catalase activity shows a consistent decrease (Table 4). The increase in catalase on administration of the drug would seem to agree with the reported increase in peroxisomes (micro-bodies), which according to de Duve (1966) account for more than 70% of this enzyme in the liver. However, the increase in catalase activity is never more than twofold whereas the increase in peroxisomes under the influence of clofibrate is about tenfold as revealed by histochemical analysis (Svoboda & Azarnoff, 1966). This would indicate that either the majority of catalase in the cell is extra-peroxisomal or the peroxisomes produced under the influence of the drug have a low concentration of catalase in them. Holmes & Masters (1972) have reported the presence of multiple forms of catalase in rat liver cytosol with electrophoretic mobilities different from the peroxisomal enzyme. Uricase, which is also a peroxisomal enzyme, does not increase on feeding the animal with clofibrate. In fact, Hess *et al.* (1965) have reported that, under the influence of the drug, the uricase activity decreases by half. The results with catalase and uricase therefore indicate that the peroxisomes produced under the influence of the drug are dissimilar in composition to the normal ones. Hess *et al.* (1967) observed that the micro-bodies from normal and treated livers showed different sedimentation characteristics on density gradient centrifugation.

Table 7. *Effect of freezing and thawing on the activity of catalase in the mitochondrial fraction*

In treatment no. 2 the sample of freshly isolated mitochondria (74 mg of protein) was frozen and thawed five times (being kept frozen each time for 5 min) and the catalase activity determined. The particles were then sedimented by centrifugation at 100000g for 60 min. The supernatant fraction, which contained 5.25 mg of protein, was assayed for catalase. In treatment no. 3, a portion (37.8 mg) of the frozen-thawed and sedimented mitochondria (treatment no. 2) was kept frozen for 48 h, thawed, assayed for activity and sedimented as described above. The supernatant fraction containing 6.2 mg of protein was assayed for catalase activity. In treatment no. 4, the freshly isolated sample (74 mg) was kept frozen for 48 h and treated as in treatment no. 3. The supernatant fraction contained 22 mg of protein. In all experiments there was good agreement between the enzyme activity before centrifugations and the total activities recovered in the particulate and supernatant fractions. The supernatant fractions were assayed without the addition of deoxycholate. The enzyme activity is expressed as mmol of H_2O_2 decomposed/min per mg of protein. Other details are given in the Materials and Methods section.

Treatment no.	Mitochondria		Medium	
	Without deoxycholate	With deoxycholate	Without deoxycholate	Activity released (%)
1. Freshly isolated	0.34	1.10	—	—
2. Frozen and thawed five times	0.51*	1.02*	5.25	32
3. Sediment from no. 2 frozen for 48 h	0.37*	0.74*	1.07	24
4. Fresh mitochondria frozen for 48 h	0.68*	0.92*	1.87	61

* Activity before centrifugation.

Table 8. *Intracellular distribution of uricase in rat liver*

The experimental details are as given in Table 6. The activity is expressed as nmol of uric acid oxidized/min per mg of protein. The distribution of the enzyme activity in the various fractions is calculated by taking the total activity in the homogenate to be 100. The values are the means of two independent determinations. No activity could be detected in the cytosol.

Subcellular fraction	0.25 M-Sucrose			0.88 M-Sucrose		
	Uricase activity	Protein (mg/g of liver)	Uricase distribution (%)	Uricase activity	Protein (mg/g of fresh liver)	Uricase distribution (%)
Homogenate	7.2	211	100	8.5	210	100
Nuclear	11.5	32	23	10.2	30	18
Mitochondrial	10.7	42	28	15.1	46	40
Microsomal	18.3	42	49	16.7	45	42

The differences in the subcellular distribution of catalase and uricase observed by us support the assumption (Hess *et al.*, 1965) that during fractionation the micro-bodies break and release their catalase into the cytosol. The uricase, which remains associated with the membranes, gets distributed among the various particulate fractions. Most of the whole peroxisomes sediment with the mitochondria (catalase is taken as the marker). The ratio of peroxisomes to mitochondria in the normal liver cell is approx. 1:8 (Svoboda & Azarnoff, 1966), so that not more than 10% of the protein in the mitochondrial fraction will

be of peroxisomal origin (this assumes that the peroxisomes are recovered intact). On the other hand, in the drug-fed animals, in the livers of which the peroxisomes have presumably increased tenfold, considerable contamination of the mitochondrial fraction is possible. However, the catalase specific activity in these samples showed a consistent decrease of about 50% when compared with normal samples and the total catalase activity recovered in the mitochondrial fraction in both normal and drug-fed animals was of the same order. This, together with the observation that the specific activity of the enzymes

connected with oxidative phosphorylation did not alter in the mitochondria from drug-fed animals (Kurup *et al.*, 1970), would rule out extraneous proteins contaminating the mitochondrial fraction in drug-treated livers. The suggestion has been made that peroxisomes represent a stage in the biosynthesis of mitochondria (Rouiller & Bernhard, 1956). In our opinion the induction of both peroxisomal and mitochondrial biogenesis by substituted chlorophenoxy-isobutyrate offers a good system to verify this hypothesis.

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