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Two mechanisms produce tissue-specific inhibition of fatty acid oxidation by oxfenicine

Thomas W. STEPHENS,* Alan J. HIGGINS,† George A. COOK‡ and Robert A. HARRIS*
**Department of Biochemistry, Indiana University School of Medicine, Indianapolis, IN 46223, U.S.A.,*
†Department of Medicinal Biology, Pfizer Central Research, Sandwich, Kent DT13 9NJ, U.K., and
‡Department of Pharmacology, College of Medicine, University of Tennessee Center for the Health Sciences, Memphis, TN 38163, U.S.A.

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Oxfenicine [*S*-2-(4-hydroxyphenyl)glycine] is transaminated in heart and liver to 4-hydroxyphenylglyoxylate, an inhibitor of fatty acid oxidation shown in this study to act at the level of carnitine palmitoyltransferase I (EC 2.3.1.21). Oxfenicine was an effective inhibitor of fatty acid oxidation in heart, but not in liver. Tissue specificity of oxfenicine inhibition of fatty acid oxidation was due to greater oxfenicine transaminase activity in heart and to greater sensitivity of heart carnitine palmitoyltransferase I to inhibition by 4-hydroxyphenylglyoxylate [I_{50} (concentration giving 50% inhibition) of 11 and 510 μM for the enzymes of heart and liver mitochondria, respectively]. Branched-chain-amino-acid aminotransferase (isoenzyme I, EC 2.6.1.42) was responsible for the transamination of oxfenicine in heart. A positive correlation was found between the capacity of various tissues to transaminate oxfenicine and the known content of branched-chain-amino-acid aminotransferase in these tissues. Out of three observed liver oxfenicine aminotransferase activities, one may correspond to asparagine aminotransferase, but the major activity could not be identified by partial purification and characterization. As reported previously for malonyl-CoA inhibition of carnitine palmitoyltransferase I, 4-hydroxyphenylglyoxylate inhibition of this enzyme was found to be very pH-dependent. In striking contrast with the kinetics of malonyl-CoA inhibition, 4-hydroxyphenylglyoxylate inhibition was not affected by oleoyl-CoA concentration, but was partially reversed by increasing carnitine concentrations.

Oxfenicine [*S*-2-(4-hydroxyphenyl)glycine] inhibits fatty acid oxidation and stimulates carbohydrate oxidation (Higgins *et al.*, 1980). Transamination of oxfenicine to HPG is required for these effects, and HPG inhibits palmitoylcarnitine formation from palmitate plus carnitine more strongly with heart mitochondria than with liver mitochondria (Higgins *et al.*, 1981). Oxfenicine is of interest because of effectiveness in decreasing chest pain in patients with obstructive coronary-artery disease (Bergman *et al.*, 1980) and in protecting heart from necrotic tissue damage during ischaemia (Higgins *et al.*, 1980). In addition, the greater specificity of oxfenicine as an inhibitor of heart fatty acid oxidation compared with liver is characteristic of the naturally occurring modulator of fatty acid oxidation, malonyl-CoA (Saggerson & Carpenter, 1981*b*). Furthermore, the finding by Higgins *et al.* (1981) that HPG did not inhibit palmitoylcarnitine oxidation by heart mitochondria suggested that this compound might act at the same site as malonyl-CoA regulation of fatty acid oxidation, i.e. at the formation of long-chain acylcarnitine esters by CPT I.

The present study demonstrates that HPG does indeed inhibit CPT I and that two separate mechanisms are involved in the tissue-specific inhibition of fatty acid oxidation by oxfenicine. In addition, HPG is shown to inhibit CPT I in a pH-

Abbreviations used: CPT, carnitine palmitoyltransferase (EC 2.3.1.21); CPT I, CPT located on the outer aspect of the mitochondrial inner membrane; CPT II, CPT located on the inner aspect of the mitochondrial inner membrane; HPG, 4-hydroxyphenylglyoxylate; I_{50}, concentration giving 50% inhibition.

sensitive manner analogous to that of malonyl-CoA, but kinetically different in that HPG competes with carnitine rather than fatty acyl-CoA.

Experimental

Materials

Oxfenicine and HPG were given by Pfizer Central Research, Sandwich, Kent, U.K. Phenylglyoxylate, phenylpyruvate, 4-hydroxyphenylpyruvate, CoA, palmitic acid, palmitoyl-CoA, palmitoylcarnitine, ATP and ADP were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chromatofocusing gel (PBE94), Polybuffer 96 and Polybuffer 74 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Sources of other materials have been given previously (Stephens *et al.*, 1983). Wistar rats (chow-fed, 150–200g, male) were obtained from Harlan Industries (Indianapolis, IN, U.S.A.).

Preparation of mitochondria and hepatocytes

Rat heart and liver mitochondria were prepared essentially as described by Vercesi *et al.* (1978) and Johnson & Lardy (1967) respectively. Respiratory control ratios were always above 5 with 10mM-glutamate plus 0.5mM-malate as substrates. Rat hepatocytes were isolated by the method of Berry & Friend (1969) with modifications described previously (Harris, 1975). Rat myocytes, isolated as described by Farmer *et al.* (1982), were kindly given by Mrs. Barbara Farmer. CoA and CoA esters were assayed as described by Michal & Bergmeyer (1974).

Assay of CPT I

The radioisotope method was used as described previously (Stephens *et al.*, 1983). For the pH studies the complete system (1ml) contained 82–84mM-sucrose, 60–70mM-KCl, 35mM-imidazole, 35mM-Hepes, 1mM-EGTA, 5mM-reduced glutathione, 50μM-oleoyl-CoA, 4mg of bovine serum albumin/ml, 0.2mM-L-carnitine (5μCi of DL-[*methyl*-^{3}H]carnitine/μmol) and 1μg of antimycin A/ml. Other studies were carried out with the same conditions except with 50mM-imidazole (pH7.0), 60mM-KCl and 100mM-sucrose. CPT activity of heart mitochondria was corrected for that portion of the enzyme activity that was insensitive to HPG inhibition. This correction [originally used by Saggerson & Carpenter (1981*a*) and later by McGarry *et al.* (1983) for malonyl-CoA inhibition] is based on extrapolating the line obtained by plotting (% inhibition by HPG)$^{-1}$ versus [HPG]$^{-1}$ to infinite inhibitor concentration. This approach equates to plotting $v_0/(v_0-v_i)$ versus I^{-1}, where v_0 = control velocity, v_i = inhibited velocity and (% inhibition)$^{-1} = v_0/100(v_0-v_i)$. Plots of this type have been applied to enzymes exhibiting partial or hyperbolic inhibition (Webb, 1963). Application to a mixture of two enzymes, where only one enzyme is inhibited, is analogous. Derivations of equations describing this case for several different types of inhibition have been performed and are available on request (from R.A.H.). Plots with heart mitochondrial carnitine palmitoyltransferase were linear ($r = 0.989 \pm 0.009$, $n = 6$).

Binding of HPG with bovine serum albumin

Binding studies were modelled after the measurement of the association of branched-chain 2-oxo acids with albumin as described by Livesey & Lund (1982). Measurements of HPG were made in 0.5M-glycine buffer (pH10) to provide the maximum absorption coefficient and to avoid interference by phenylpyruvate and albumin. Individual assays were blanked by a similar solution made 1M in H_2O_2 and incubated for 20min to destroy the absorbance caused by HPG. Binding constants were determined by fitting the data by non-linear least squares (Marquardt, 1963):

$$\frac{[\text{P–HPG}]}{[\text{P}]_t} = \frac{n_1 K_1[\text{HPG}]}{1+K_1[\text{HPG}]} + \frac{n_2 K_2[\text{HPG}]}{1+K_2[\text{HPG}]}$$

where [P–HPG] refers to bound HPG, $[P]_t$ to total protein, [HPG] to free HPG, n_i to number of binding sites of type i (1, 2), and K_i to affinity constant for site i (1, 2).

Assay of aminotransferase activities

Oxfenicine aminotransferase assays were based on the A_{335} of HPG ($A_{mM} = 25$ litre·mmol^{-1}·cm^{-1}). A kinetic assay system (1ml) for oxfenicine aminotransferase activity contained 0.33M-triethanolamine (pH8.5), 50μM-pyridoxal 5-phosphate, 31mM-dithiothreitol, 1mM-EDTA and 0.5–10mM-oxfenicine. The reaction was monitored at 335nm and 30°C, and was initiated by addition of 2-oxoglutarate (20mM). An end-point assay system (0.325ml) contained 0.12M-triethanolamine (pH8.5), 62μM-pyridoxal 5-phosphate, 0.6mM-EDTA, 6.2mM-oxfenicine, 15mM-dithiothreitol and 30mM-2-oxoglutarate. Assays were conducted on an automated American Monitor Micro KDA analyser (Indianapolis, IN, U.S.A.) programmed for 340nm and 37°C. The reaction was terminated by addition of 1.8ml of 1M-NaOH.

Assays of the following enzyme activities were performed by previously published procedures: tyrosine aminotransferase (Granner & Tomkins, 1970); glutamine aminotransferase, type K (Cooper, 1978); asparagine aminotransferase (Cooper, 1977); glutamine aminotransferase, type

L (Cooper & Meister, 1973, 1974); branched-chain-amino-acid aminotransferase (Cappucino *et al.*, 1978); aspartate aminotransferase (Bergmeyer *et al.*, 1978); alanine aminotransferase (German Society of Clinical Chemistry Committee, 1972); lactate dehydrogenase (Bergmeyer & Bernt, 1974); citrate synthase (Shepard & Garland, 1969); and fatty acid oxidation by isolated hepatocytes and myocytes (McCune & Harris, 1979).

Distribution of oxfenicine aminotransferase between the soluble and mitochondrial fractions was determined by assaying total oxfenicine aminotransferase activity with the kinetic assay in post-mitochondrial supernatant versus total activity in mitochondria after homogenization with 0.5% Triton X-100. Distribution was corrected by the method of Akerboom *et al.* (1979), by using lactate dehydrogenase and citrate synthase as cytosolic and mitochondrial-matrix-space markers respectively.

Tissue homogenates for the determination of total oxfenicine aminotransferase activity were prepared with the Polytron PT-10 homogenizer (3 × 15s, maximum speed) in 5 vol. of ice-cold 25mM-Tris/HCl (pH8.3), containing 2mM-dithiothreitol, 1mM-EDTA, 10μM-pyridoxal 5-phosphate, 0.5μM-leupeptin and 0.5μM-pepstatin A.

Separation of aminotransferases by chromatofocusing chromatography

Homogenates prepared as described above were centrifuged at 12000*g* for 15min. The supernatant was decanted through cheesecloth and centrifuged at 165000*g* for 90min. The clear middle layer was concentrated in dialysis bags against poly(ethylene glycol) (M_r 20000). The concentrate was clarified by centrifugation (12000*g* for 5min) and the supernatant desalted on a Sephadex G-25 column equilibrated with 25mM-Tris acetate (pH8.0) containing 2mM-2-mercaptoethanol. The sample was placed on a PBE-94 chromatofocusing column (9mm × 60cm) equilibrated with the same buffer and eluted with 400ml of 5% (v/v) Polybuffer 96/5% (v/v) Polybuffer 74/2mM-2-mercaptoethanol (pH5 at 4°C). The pH of each fraction (4.4ml) was determined at 4°C and a stabilizing solution was added [0.63ml of 92% (v/v) glycerol containing 35mM-HCl and 91μM-pyridoxal 5-phosphate to fractions with pH > 7.0; same solution with 35mM-NaOH replacing HCl to fractions with pH < 7.0].

HPG concentration in tissues of oxfenicine-treated rats

Rats were injected subcutaneously with saline, oxfenicine (100mg/kg body wt.) or HPG (100mg/kg body wt.). Hearts and livers were rapidly removed 2h later, freeze-clamped at liquid-N_2 temperature (Wollenberger *et al.*, 1960), powdered under liquid N_2, and extracted with 4vol. of 1M-$HClO_4$. Samples of the extract were adjusted to pH6.8 or 9.5 with 0.67M-K_2HPO_4/0.33M-KOH or 1M-K_2CO_3 respectively. The solutions were clarified by centrifugation and the difference in A_{335} between the two solutions was determined. An absorption coefficient of 19.9 litre·mmol^{-1}·cm^{-1} (difference between absorption coefficients at pH6.8 and 9.5) was used to calculate HPG concentration.

Results

Site of inhibition of fatty acid oxidation by HPG

Higgins *et al.* (1981) located the site of action of HPG at either fatty acid activation or formation of the carnitine ester. HPG inhibited CPT I in both heart and liver mitochondria (Fig. 1). In experiments not shown, HPG did not prevent accumulation of palmitoyl-CoA when heart mitochondria were incubated with palmitate plus CoA, but did prevent loss of palmitoyl-CoA in incubations carried out with palmitoyl-CoA plus carnitine. Thus HPG appears to be a specific inhibitor of CPT I activity.

Effectiveness of HPG inhibition of heart and liver CPT I

Fatty acid oxidation by isolated myocytes was inhibited by 31 ± 4% ($n = 3$) by 0.5mM-oxfenicine, whereas fatty acid oxidation by isolated hepatocytes was not inhibited by oxfenicine at concentrations up to 5mM. Higgins *et al.* (1981) demonstrated greater HPG inhibition of palmitoylcarnitine formation with heart than with liver mitochondria. Heart mitochondrial CPT I was much more sensitive to HPG than was the liver enzyme, but a greater percentage of the CPT activity could be inhibited with liver mitochondria than with heart mitochondria (Fig. 1). Even at very high concentrations of HPG, the maximum inhibition of CPT of heart mitochondria (70–80%) was less than that for liver mitochondria (100%). These findings are analogous to the effects of malonyl-CoA as an inhibitor of CPT in mitochondria from these tissues and are consistent with HPG acting as a specific inhibitor of the outer surface enzyme (CPT I), with no inhibitory effect upon the inner surface enzyme (CPT II). An alternative explanation would invoke partial inhibition of heart CPT I and complete inhibition of liver CPT I. However, heart mitochondrial preparations contain more damaged mitochondria (Chao & Davis, 1972) and would be expected to express more malonyl-CoA-insensitive CPT activity than do liver mitochondrial preparations. As also known for malonyl-CoA inhibition of CPT (McGarry *et al.*, 1978), damage to liver mitochondria by hypo-osmotic

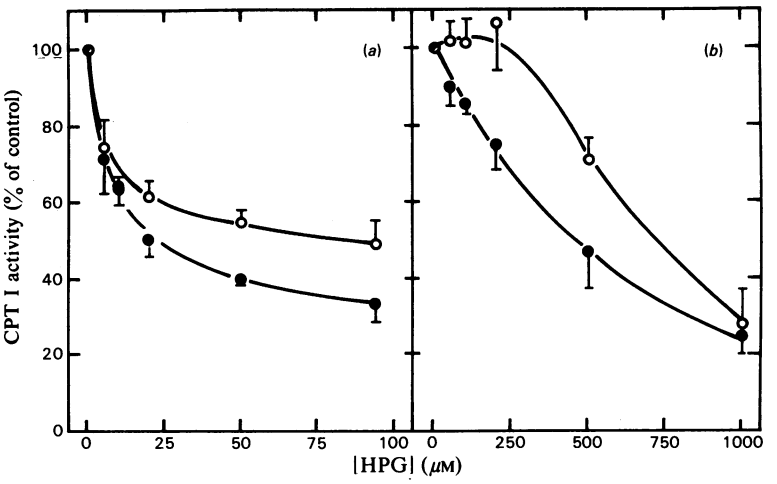

Fig. 1. *Sensitivity of heart (a) and liver (b) CPT I of mitochondria to inhibition by HPG*

Heart (*a*) or liver (*b*) mitochondria were isolated as described in the Experimental section. CPT was assayed as described in the absence (○) or the presence (●) of 0.5mM-phenylpyruvate. Control activities averaged 2.68 and 1.66nmol/min per mg in the absence of phenylpyruvate and 9.56 and 5.76 nmol/min per mg in the presence of phenylpyruvate for heart and liver respectively. Each point represents the mean ± S.D. for three mitochondrial preparations.

shock and freeze–thaw caused a decrease in the maximum HPG inhibition (from 100% to 50%), presumably because of loss of latency of CPT II activity. Thus inhibition of CPT by HPG appears very similar to that exerted by malonyl-CoA, in terms of both greater sensitivity of the heart than of the liver enzyme and specificity for CPT I rather than CPT II.

The inhibition pattern of HPG for CPT I of liver mitochondria (Fig. 1*b*) was distinctly different from that of heart mitochondria (Fig. 1*a*). The relative lack of effect at low HPG concentration with liver mitochondria was traced to a competition between oleoyl-CoA and HPG for binding sites on bovine serum albumin. Equilibrium binding studies to measure interaction of HPG with albumin yielded association constants of $K_1 = 6.1 \times 10^3$ and $K_2 = 0.13 \times 10^3$ litres/mol for two types ($n_1 = 1$ and $n_2 = 4$) of binding sites. Interaction of oleoyl-CoA and HPG at common sites on albumin complicated quantitative analysis of inhibition curves, because displacement of oleoyl-CoA increased free oleoyl-CoA concentration, resulting in greater CPT I activity at subsaturating concentrations of oleoyl-CoA. A method was found to minimize this effect of HPG. Phenylpyruvate apparently binds at the same sime on albumin as HPG and oleoyl-CoA, yet exerts no inhibitory effects on CPT I activity (results not shown). A constant amount (0.5mM) of phenylpyruvate was added to the assay system to minimize the effect of albumin and maximize the inhibition of CPT I by HPG. The presence of phenylpyruvate made HPG a more effective inhibitor of both the heart and liver enzymes (Figs. 1*a* and 1*b*). The heart mitochondrial enzyme remained much more sensitive to HPG inhibition ($I_{50} = 11 \pm 2\,\mu$M; mean ± S.E.M. for six mitochondrial preparations) than the liver enzyme ($I_{50} = 510 \pm 60\,\mu$M; mean ± S.E.M. for nine mitochondrial preparations). The I_{50} value for the heart mitochondrial enzyme was corrected for insensitive CPT activity (see the Experimental section).

Displacement of oleoyl-CoA by HPG had different effects on the pattern of inhibition with heart and liver mitochondria (Figs. 1*a* and 1*b*). These differences can be attributed to the relative affinities of HPG for CPT and albumin ($I_{50} = 11\,\mu$M for heart, $I_{50} = 510\,\mu$M for liver, and dissociation constant of $160\,\mu$M for binding of HPG to albumin) and the expression of an insensitive form of CPT (CPT II) with heart mitochondria. At low concentrations, HPG affinity for heart mitochondrial CPT is much greater than for albumin, and therefore inhibition is much more evident than displacement of oleoyl-CoA. At high HPG concentrations CPT I is totally inhibited, but CPT II activity is stimulated by the displacement of oleoyl-CoA from albumin. Therefore displacement effects are only important at high HPG concentrations with heart mitochondria. The situation is different with liver mitochondria, where the affinity of HPG is greater for albumin than for CPT I and no CPT II is

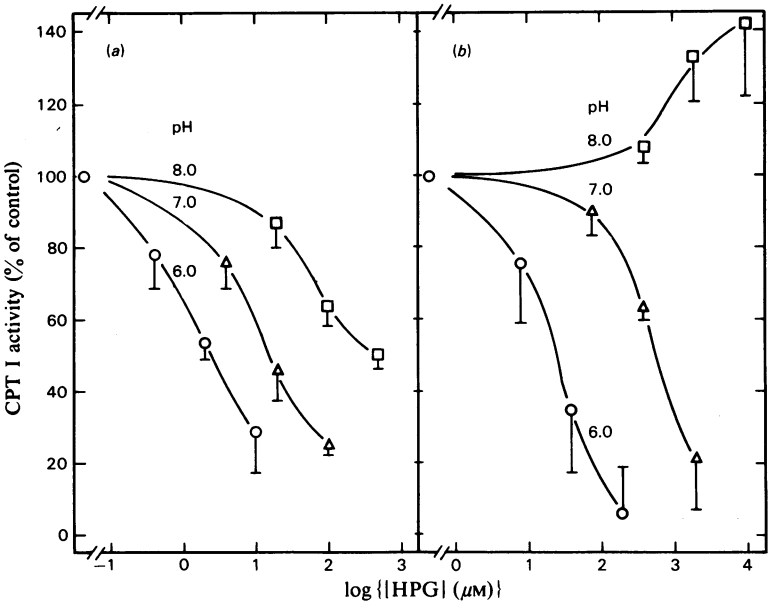

Fig. 2. *Effect of pH on inhibition of CPT I by HPG*
CPT I activity of heart (*a*) and liver (*b*) mitochondria was assayed as described in the Experimental section. Each point represents the mean ± S.E.M. for mitochondria prepared from three rats.

expressed. In this case displacement effects are more important at low HPG concentrations. All the CPT I activity is inhibited at high HPG concentrations, and displacement has no effect, because no CPT II is expressed.

pH, substrate concentrations and HPG inhibition of CPT I

As known for malonyl-CoA (Stephens *et al.*, 1983), the sensitivity of CPT I to inhibition by HPG is affected by pH (Fig. 2). Inhibition increased at low pH and decreased at high pH. Liver CPT I appears significantly more sensitive to this pH effect. Indeed, activation rather than inhibition was found at pH 8.0, perhaps reflecting HPG displacement of oleoyl-CoA from albumin. pH effects on the binding of HPG to albumin cannot explain these results. Bound HPG is relatively unaffected below pH 7.0, where inhibition of CPT changes significantly. Free HPG increases above pH 7.0, where CPT inhibition by HPG decreases (results not shown). Phenylglyoxylate, an analogue of HPG, was also found to inhibit liver mitochondrial CPT I in a pH-dependent manner (250 μM inhibited liver mitochondrial CPT I by 60% at pH 6.0, but caused no inhibition at pH 7.0).

Further studies on the type of inhibition of CPT I caused by HPG were performed with liver mitochondria incubated at pH 6.0. Dixon plots were constructed with reciprocal velocity plotted

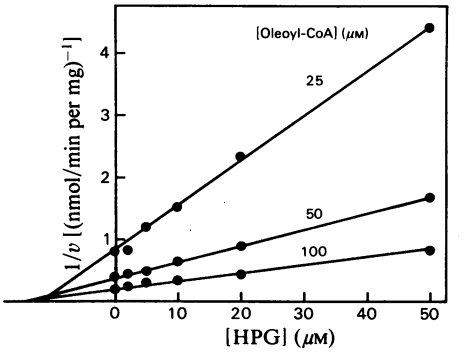

Fig. 3. *Dixon plot for inhibition of CPT I by HPG versus oleoyl-CoA concentration*
CPT I activity of liver mitochondria was assayed as described in the Experimental section, except that the final assay pH was 6.0 and the oleoyl-CoA concentration was varied as indicated. Each point represents the mean obtained with liver mitochondria prepared from three rats.

against inhibitor concentration at different oleoyl-CoA concentrations (Fig. 3). The intersection of these lines was close to the inhibitor-concentration axis, suggesting non-competitive inhibition by HPG with oleoyl-CoA as the varied substrate.

A similar study was made with carnitine as the varied substrate (Fig. 4). Upward curvature of the plot was found at the lower concentrations of

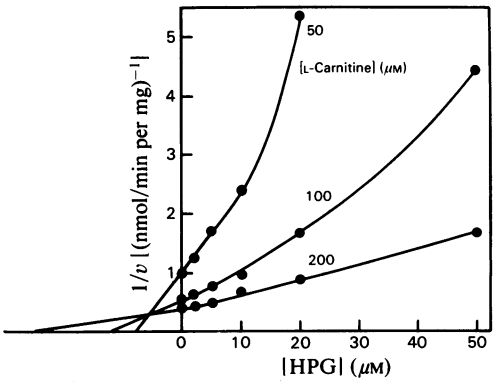

Fig. 4. *Dixon plot for inhibition of CPT I by HPG versus carnitine concentration*

Conditions were as in Fig. 3, except that carnitine was the varied substrate and oleoyl-CoA concentration was held constant at 50μM.

carnitine. Tangents drawn to these curves at zero HPG concentration intersect in the upper left quadrant above the HPG concentration axis. An intersection in this region is consistent with either simple or mixed competitive inhibition. The positive curvature indicates that the degree of inhibition increases at higher concentrations of HPG more than can be explained by simple linear competitive inhibition. Interpretation of kinetic data in a system this complex admittedly requires considerable caution. However, Fiol & Bieber (1984) have found positive co-operativity in the binding of carnitine and fatty acyl-CoA with CPT purified from bovine heart (Hill coefficient, *h*, 1.8). Furthermore, Zammit (1984) demonstrated an effect which can be explained by slow-acting positive co-operativity in the malonyl-CoA inhibition of CPT I, and Cook (1984) reported positive co-operative inhibition of CPT I by malonyl-CoA. Therefore the results may be explained by positive co-operative inhibition of CPT I by HPG. A Hill coefficient determined in the present studies for the HPG inhibition of CPT I was 1.3 ± 0.1 (mean ± S.E.M. for three mitochondrial preparations).

Tissue contents of HPG in oxfenicine-treated rats

Much higher contents of HPG were found in heart than in liver of oxfenicine-treated rats (102 ± 2 and 23 ± 5 nmol/g wet wt. for heart and liver respectively; mean ± S.E.M. for three animals), suggesting greater uptake and/or transamination activity by heart than liver. Neither tissue accumulated much HPG when an equivalent amount of this compound was injected (5 ± 1 and <2 nmol/g wet wt. for heart and liver respectively; mean ± S.E.M. for three animals), suggesting that transamination of oxfenicine in one tissue followed by transport in the blood and uptake by another tissue is unlikely. Rates of oxfenicine uptake and transamination to HPG must be important factors in determining the intracellular concentration of HPG.

Transamination of oxfenicine in various tissues

Oxfenicine aminotransferase activity was much higher in heart (451 ± 81 munits/g wet wt., $n = 3$) than in liver (37 ± 8 munits/g wet wt., $n = 3$). The intracellular distribution was 37% and 62% in heart, and 33% and 67% in liver, for the cytosolic and mitochondrial-matrix spaces respectively.

An effort was made to identify the enzymes responsible for oxfenicine transamination by chromatofocusing chromatography of tissue extracts (Fig. 5). Oxfenicine aminotransferase activity from heart tissue was resolved from mitochondrial and cytosolic aspartate aminotransferase and glutamine aminotransferase (type K) by this technique (Fig. 5). However, oxfenicine aminotransferase activity co-purified with branched-chain-amino-acid aminotransferase and alanine aminotransferase, being eluted at pH 5.2. Oxfenicine aminotransferase activity of fractions eluted from the column was significantly correlated by multivariate regression analysis with branched-chain-amino-acid aminotransferase activity ($r = 0.98$; $P < 0.001$), but not with alanine aminotransferase activity.

The active fractions from the chromatofocusing column were subsequently chromatographed on a phenyl-Sepharose column, which resolved oxfenicine aminotransferase activity from alanine aminotransferase but not from branched-chain-amino-acid aminotransferase. Multivariate regression analysis again yielded a correlation coefficient ($r = 0.999$) for oxfenicine aminotransferase versus branched-chain-amino-acid aminotransferase that was statistically significant ($P < 0.001$). Although branched-chain-amino-acid aminotransferase has been reported to have no activity with aromatic amino acids (Taylor & Jenkins, 1966), oxfenicine was not tested as substrate. Furthermore, a preparation of branched-chain-amino-acid aminotransferase from pig heart (given by Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.) was found to be rich in oxfenicine aminotransferase activity. In crude homogenates of heart, the pH optimum of oxfenicine aminotransferase was greater than 8, again consistent with branched-chain-amino-acid aminotransferase (Taylor & Jenkins, 1966). Finally, oxfenicine aminotransferase activity of heart extracts was strongly inhibited by branched-chain amino acids (e.g. 80% inhibited by 20mM-isoleucine). The

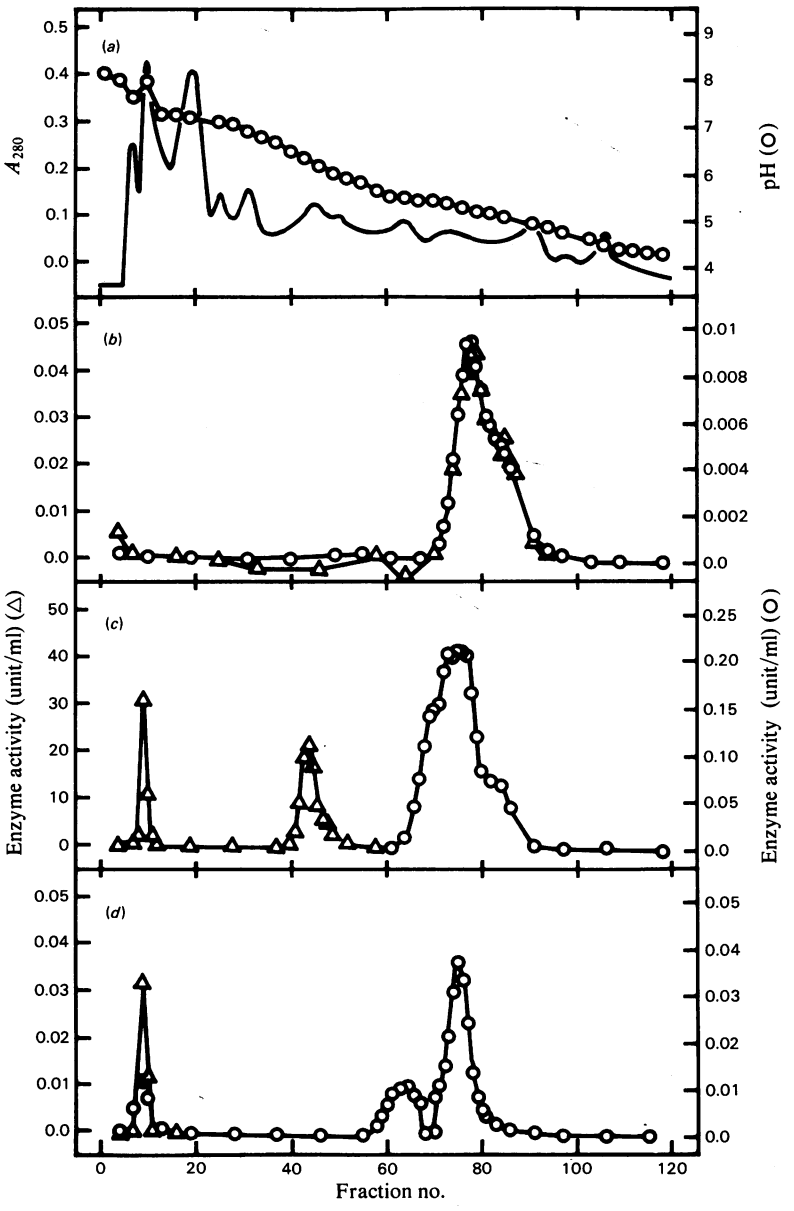

Fig. 5. *Separation of heart aminotransferase activities by chromatofocusing chromatography*
The aminotransferases of heart were separated by chromatofocusing chromatography and assayed as described in the Experimental section. (*a*) ○, pH; ——, A_{280}. (*b*) ○, Oxfenicine aminotransferase; △, branched-chain amino acid aminotransferase. (*c*) ○, Alanine aminotransferase; △, aspartate aminotransferase. (*d*) ○, Glutamine aminotransferase; △, tyrosine aminotransferase.

results therefore indicate that branched-chain-amino-acid aminotransferase (isoenzyme I) is responsible for transamination of oxfenicine in heart.

Ichihara (1975) and Ichihara *et al.* (1975) reported a marked variation in content of branched-chain-amino-acid aminotransferase in various tissues of the rat. Additional evidence that branched-chain-amino-acid aminotransferase is responsible for oxfenicine transamination was obtained by comparing reported activities of branched-chain-amino-acid aminotransferase in various tissues with the capacity of tissue extracts to transaminate oxfenicine (Fig. 6). The correla-

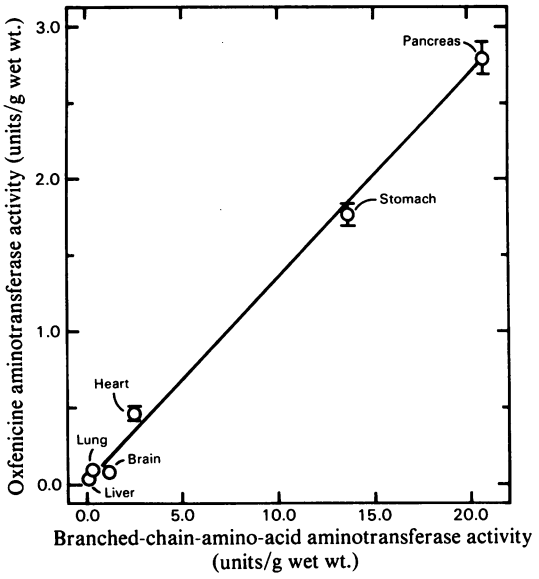

Fig. 6. *Correlation of oxfenicine aminotransferase activities versus reported branched-chain-amino-acid aminotransferase activities in various rat tissues*

Tissues were homogenized and oxfenicine aminotransferase activity was measured as described in the Experimental section. Values given are means ± S.E.M. for $n = 3$ (liver), $n = 7$ (lung), $n = 4$ (brain), $n = 3$ (heart), $n = 7$ (stomach) and $n = 6$ (pancreas). Overall correlation coefficient was $r = 0.999$ ($P < 0.001$). Values for branched-chain-amino-acid aminotransferase are from Ichihara (1975) and Ichihara *et al.* (1975).

tion was nearly perfect for all tissues investigated except the brain, which contains isoenzyme III rather than isoenzyme I of branched-chain-amino-acid aminotransferase (Ichihara, 1975).

To include liver on the line drawn in Fig. 6 is misleading, since liver has little capacity to transaminate either oxfenicine or branched-chain amino acids. Indeed, oxfenicine aminotransferase activity from whole liver homogenate was separated by chromatofocusing chromatography into three major peaks, eluted at pH 7.62, 7.35 and 6.98. The first peak corresponded to asparagine aminotransferase (pI reported to be 8.0; Noguchi *et al.*, 1979). None of the major peaks corresponded to mitochondrial or cytosolic aminotransferases for aspartate, glutamine (type K or L), leucine, tyrosine or alanine. Studies with whole liver homogenates had indicated that the pattern would be different from that of heart, since the pH optimum of oxfenicine-transaminating activity was between pH 7.0 and 7.5. Isoleucine had little effect on oxfenicine aminotransferase in liver, inhibiting by only 15% at 20mM. The oxfenicine aminotransferase activity in liver therefore appears to be the combined result of several aminotransferase activities.

Since the major peak (pH 7.35) of oxfenicine aminotransferase from rat liver did not correspond to any of the activities monitored, this enzyme was further purified (145-fold) by DEAE-Sephacryl, phenyl-Sepharose, chromatofocusing and Sephadex G-200 chromatography. 2-Oxoglutarate was the best amino-group acceptor and was equally effective at 20 and 2mM, whereas pyruvate was less effective at 20mM and a very poor amino-group acceptor at 2mM. The holoenzyme M_r was estimated to be 110000 by a sedimentation-equilibrium method (Bothwell *et al.*, 1978). The preference for 2-oxoglutarate as an amino-group acceptor eliminates asparagine aminotransferase and glutamine aminotransferases types K and L as possible enzymes. The holoenzyme M_r further eliminates branched-chain-amino-acid aminotransferase (isoenzymes I, II and III). The identity of the major oxfenicine aminotransferase activity in liver, however, was not determined.

Discussion

The organ specificity of oxfenicine inhibition of fatty acid oxidation has two underlying causes: (1) greater sensitivity of heart CPT I ($I_{50} = 11\,\mu M$) than of liver CPT I ($I_{50} = 510\,\mu M$) to HPG inhibition, and (2) greater oxfenicine aminotransferase activity in heart than in liver. The degree to which these separate mechanisms contribute to the tissue specificity is unknown, but may be put into perspective by the finding that fatty acid oxidation by isolated hepatocytes was not inhibited by oxfenicine at concentrations up to 5mM, but was inhibited by 35% at 5mM-HPG. These values can be compared with those for isolated myocytes, where 0.5mM-oxfenicine inhibited fatty acid oxidation by 31%. Oxfenicine and HPG have been shown to be equally effective in increasing pyruvate oxidation by isolated diaphragms (Higgins *et al.*, 1981). Therefore the lack of oxfenicine transamination capacity in liver appears to limit formation of HPG. However, the lack of sensitivity of liver CPT I to HPG inhibition makes oxfenicine a very poor inhibitor even if HPG could accumulate to high concentrations. The overall result is a very high degree of specificity for oxfenicine as an inhibitor of fatty acid oxidation in heart rather than in liver.

The requirement for albumin in the assay system for CPT I resulted in an underestimation of HPG inhibition. This appears to be caused by HPG binding to albumin, which displaces oleoyl-CoA, increasing free oleoyl-CoA concentration and decreasing free HPG concentration. Oleoyl-CoA

and phenylpyruvate were competitive with this binding. Addition of phenylpyruvate to the assay decreased HPG binding to albumin and thereby minimized an interaction that complicated kinetic analysis of the inhibition curves.

HPG inhibition of CPT I was independent of the oleoyl-CoA concentration, but was decreased by high carnitine concentrations. The results therefore suggest that HPG is competitive with carnitine and non-competitive with oleoyl-CoA. This is different from malonyl-CoA inhibition of CPT I, which is competitive with acyl-CoA substrate (McGarry *et al.*, 1978; Mills *et al.*, 1983).

HPG inhibition of CPT I is sensitive to pH, with inhibition increasing as the pH is lowered. Both liver and heart CPT I are affected in the same manner, but the liver enzyme was more sensitive. This pH effect, which is similar to that previously reported for malonyl-CoA inhibition of CPT I (Stephens *et al.*, 1983; Mills *et al.*, 1984), may be due to dissociation of a proton from an ionizable group of the enzyme, substrate (long-chain acyl-CoA or carnitine) or inhibitor (malonyl-CoA and HPG). However, phenylglyoxylate also inhibits CPT I in a pH-sensitive manner, and its pK_a is not in the appropriate pH range. Dissociation at an enzyme residue probably accounts, therefore, for the dramatic effect of pH on malonyl-CoA and HPG inhibition of the enzyme.

Oxfenicine aminotransferase activity was much higher in heart than in liver. This explains why HPG accumulated more in heart than in liver in rats treated with oxfenicine. Oxfenicine aminotransferase activity of heart corresponds to cytosolic and mitochondrial isoenzyme I of branched-chain-amino-acid aminotransferase. This is the first report of aromatic amino acid activity for this enzyme. Liver oxfenicine aminotransferase activity appears to result from several weak aminotransferase activities. One appears to be due to asparagine aminotransferase, whereas the remaining activities could not be attributed to any of the other aminotransferases monitored.

Apparent competition between HPG and carnitine suggests that the sensitivity of CPT I from various tissues to HPG inhibition should be related to the K_m for carnitine. This is indeed the case, i.e. heart CPT-I has a higher K_m for carnitine and a much lower I_{50} for HPG than the liver enzyme. This same relationship has previously been reported for malonyl-CoA inhibition of CPT I (McGarry *et al.*, 1983). The latter was unexpected, however, because malonyl-CoA inhibition of CPT I is competitive with palmitoyl-CoA rather than carnitine (McGarry *et al.*, 1978). Although competition between malonyl-CoA has been confirmed by binding studies (Mills *et al.*, 1983), the results remain difficult to rationalize. Analogous binding studies with HPG may prove informative as to the relationship involved.

In spite of strong similarities in the actions of HPG and malonyl-CoA as inhibitors of CPT I, striking differences appear to underlie their mechanisms of inhibition. Further studies of these inhibitors should improve our understanding of the regulation of CPT I and fatty acid oxidation.

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