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INHIBITION OF HEPATIC GLUCONEOGENESIS BY PHENETHYLHYDRAZINE (PHENELZINE)

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Abstract—The hepatic effects of phenethylhydrazine (phenelzine), an antidepressive drug occasionally causing hypoglycemia, were examined. Phenelzine (1 mM) inhibits gluconeogenesis from L-lactate, pyruvate, and propionate, but not from fructose in experiments with isolated perfused rat livers. Ketogenesis from endogenous sources as well as from hexanoate or fructose is likewise inhibited. Gluconeogenesis, urea formation, and net formation of L-lactate + pyruvate from L-alanine are inhibited in experiments with isolated hepatocytes. Phenelzine leads to a reduction of the cytoplasmic, but not of the mitochondrial $NAD^+/NADH$ system.

Cross-over plots of intrahepatic metabolites revealed forward cross-overs between L-malate and phosphoenolpyruvate (PEP) and fructose-1,6-bisphosphate (FDP) and fructose-6-phosphate (F-6-P) in the presence of phenelzine, when L-lactate was glucogenic precursor. With pyruvate as substrate forward cross-over points were between pyruvate and L-malate and between FDP and F-6-P. The concentrations of L-glutamate, L-aspartate, and α-oxoglutarate changed in the presence of phenelzine in a way compatible with an inhibition of the aspartate aminotransferase reaction. The overall concentrations of acetyl-CoA decreased in the presence of phenelzine, when pyruvate was substrate.

PEP-carboxykinase was inhibited *in vitro* by phenelzine, due to trapping of oxaloacetate by phenethylhydrazone formation. The same mechanism was found for aspartate aminotransferase when tested in the direction of L-aspartate formation. In the direction of oxaloacetate formation a competitive inhibition was observed (K_i app $= 7.2 \cdot 10^{-4}$ M), probably due to an interaction of phenelzine with the enzyme linked pyridoxal-5′-phosphate (PLP) as indicated by aldimine formation of phenelzine with PLP *in vitro*. Phenelzine (1 mM) inhibited significantly the incorporation of carbon from the C-1 and from the C-2 position of the lactate–pyruvate pool into CO_2, glucose, and (C-2 only) fatty acids, whereas the incorporation into the glyceride–glycerol fraction increased. The incorporation of hydrogen from ^3H_2O into total lipids and glyceride–glycerol was strongly, that into fatty acids completely, inhibited under the same conditions. Phenelzine did not inhibit acetoacetate reduction by isolated rat liver mitochondria with succinate, citrate or isocitrate as substrate, but it inhibited strongly when pyruvate, and slightly when L-malate, was the substrate.

The ability of phenelzine to form hydrazones with 2-keto acids increased in the sequence α-oxoglutarate < pyruvate < oxaloacetate. No inhibition of aspartate aminotransferase was observed in the presence of 2-phenethylhydrazonopentanoate or 2-phenethylhydrazonopropionate. Gluconeogenesis from L-lactate, but not from pyruvate, was inhibited by 0.1 mM 2-phenethylhydrazonopropionate.

It is concluded that phenelzine, if at all, affects gluconeogenesis only partly via its hydrazone derivatives. It acts mainly by restricting oxalacetate formation in the cytosol due to an inhibition of aspartate aminotransferase. In addition, phenelzine inhibits pyruvate oxidation. This effect is mainly responsible for the observed inhibition of fatty acid synthesis from carbohydrates. The mechanism of action precludes the use of this or similar drugs in the treatment of diabetes.

Phenethylhydrazine (phenelzine), an inhibitor of monoamine oxidase activity used for treatment of depression, is known to occasionally induce hypoglycemia as a side effect [1–3]. This is observed especially in those patients who have already been treated with sulfonylureas or insulin because of diabetes [4, 5]. Triner *et al.* [6] showed in experiments with isolated perfused rat livers that phenelzine inhibited gluconeogenesis from L-alanine. They explained this effect with an inhibition of hepatic L-glutamate pyruvate aminotransferase activity. A similar explanation had already been offered for the inhibitory effect of hydrazine on gluconeogenesis [7].

Recently, the inhibitory effects of phenelzine and hydrazine on gluconeogenesis have been attributed to an action of their corresponding hydrazones and not to direct effects of the original compounds [8].

The present studies were conducted in order to get more information on the mechanism of inhibition of gluconeogenesis by phenelzine.

MATERIALS AND METHODS

Male Wistar rats (weight 200–250 g) were obtained from Winkelmann, Kirchborchen, Germany. They were kept on standard food (Altromin-R pellets) *ad libitum*. Liver perfusion studies were performed in a recirculating system as described earlier [9]. The experiments were started after a recovery phase of 20 min and lasted for another 60 min. Phenelzine was added 10 min before addition of the substrates. Liver tissue was obtained by freeze-clamping [10] at the end of the perfusion. Liver cells were prepared by a modification of the method of Berry and Friend [11] with the main difference that hyaluronidase was omitted. Cells corresponding to about 15–20 mg dry wt. were incubated in 2 ml Krebs–Ringer bicarbonate buffer containing 2 g/100 ml dialyzed bovine serum albumin under the conditions mentioned in the legends to Tables 2 to 5.

Total lipids were extracted from liver cells after homogenization in methanol/chloroform (1/2, v/v)

according to Folch *et al.* [12]. They were saponified with 5% KOH in methanol (w/v). Unsaponifiable lipids were removed by extraction with heptane. After addition of water and acidification, the free fatty acids were extracted with heptane. The remaining aqueous phase and the combined heptane phases were used for the determination of radioactivity in glycerol and fatty acids, respectively.

Experiments with isolated rat liver mitochondria. Mitochondria were isolated from rat liver according to Myers and Slater [13]. The oxygen uptake and respiratory control were measured polarographically [14].

The reduction of acetoacetate in the presence of ATP and various metabolites and inhibitors was measured in the system described by Ernster and Lee [15].

Mitochondrial activities of ATPase and NADH oxidase were measured according to Pullman and Penefsky [16] and according to Mackler [17], respectively and the ATP-^{32}P exchange according to Pullman [18].

Analysis of metabolites. The freeze clamped tissue was powdered in a mortar under liquid nitrogen, weighted and deproteinized with a five-fold vol. (v/w) of ice-cold 1 N $HClO_4$. After centrifugation at 15.000 *g* for 10 min (4°) the supernatant was decanted and carefully brought to pH 6.5 with KOH · $KClO_4$ was sedimented by centrifugation (15.000 *g*, 10 min). An aliquot of the supernatant was treated with magnesium silicate (Florisil) (50 mg/ml), shaken for 2 min and filtered through a Millipore filter (pore size 100 mμ). The filtrate was used for the fluorometric analysis of G-6-P, F-6-P, FDP, G-1-P, DAP, 3-PG, 2-PG * and pyruvate. The untreated supernatant was used for analysis of all other metabolites.

The metabolites were either measured enzymic-optically using standard methods [19] or fluorometrically as described by Williamson and Corkey [20]. Fluorometry was performed with an Eppendorf fluorometer with a registration adapter.

Spectral data were obtained using either a Beckman model 25 recording spectrophotometer or an Amino DW-2 spectrophotometer.

Determination of enzyme activities. As a source for soluble enzymes, a high speed supernatant from rat liver was prepared, as described earlier [21].

The activity of soluble aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC-2.6.1.1) (AAT), was determined either in the direction of oxaloacetate formation (I), or of L-aspartate formation (II) using the following assay system (final concentrations):

(I) Sodium phosphate pH 7.6, 0.1 M; NADH 0.2 mM; α-oxoglutarate 8 mM; L-aspartate varied between 0.4 and 50 mM, as given in the respective figures; malate dehydrogenase 20μg/ml; lactate dehydrogenase 16 μg/ml.

(II) Sodium phosphate pH 7.6, 0.1 M; coenzyme A, 0.52 mM; cysteine 3.3 mM; NAD^+, 0.33 mM; L-glutamate, 25 mM; oxaloacetate varied between 0.05 and 0.3 mM; α-oxoglutarate dehydrogenase, 160 μg/ml. The activity of phosphoenolpyruvate carboxykinase (GTP:oxaloacetate carboxylase, EC4.1.1.32) was determined in the direction of PEP formation according to Henning *et al.* [22], the activity of pyruvate carboxylase (pyruvate:carbon dioxide ligase (ATP) EC 6.4.1.1) according to Seufert *et al.* [23]. α-Oxoglutarate dehydrogenase activity (2-oxoglutarate:lipoate oxidoreductase, EC 1.2.4.2) was assayed according to Sanadi [24]. Unless otherwise mentioned, phenelzine was added 10 min before initiating the enzymatic reactions. The reaction temperature for all assay systems was 30°.

Determination of equilibrium constants for 2-phenethyl-hydrazone formation between phenelzine and α oxoglutarate, pyruvate or oxaloacetate. The equilibrium constants were calculated for four different concentrations of phenelzine (0.5, 1, 1.5 and 2 mM) at an initial concentration of the respective 2-keto acid of 0.15 to 0.25 mM according to the equation:

$$K_{eq} = \frac{|\text{2-keto acid}| \times |\text{phenelzine}|}{|\text{acid-2-phenethylhydrazone}|}.$$

The 2-keto acids were incubated at room temperature and at pH 7.0 in the presence of phenelzine for increasing time intervals up to 240 min prior to the enzymatic determination of the free keto acid. Under these conditions unspecific loss of oxaloacetate was less than 5 per cent of the initial concentration. No significant loss of pyruvate or α-oxoglutarate occurred. The concentrations of free keto acids and of phenethylhydrazone at infinite time were calculated by extrapolation.

Preparative methods. α-Oxoglutarate dehydrogenase was prepared from pig heart as described by Sanadi [24]. After additional chromatography on Sephadex G-150 the enzyme was essentially free of aspartate aminotransferase activity.

2-Phenethylhydrazonopropionate (PEH-propionate) and 2-phenethylhydrazonopentanoate (PEH-pentanoate) were prepared from the respective 2-ketoacids as follows: to 200 ml of a 50 mM aqueous solution of phenelzine sulfate, pH 1.8, 30 ml of a 300 mM ethanolic solution of either pyruvate or α-oxoglutarate were added. After stirring for 2 hr at room temperature, the precipitating hydrazones were collected, extensively washed, and recrystallized at least three times. They were chemically homogenous as judged by spectroscopic analysis.

Materials. Phenelzine (phenethylhydrazine) was a kind gift of Dr. A. Knecht, Gödecke A.G., Freiburg/Brg., Germany.

All enzymes, coenzymes, oxaloacetate and α oxoglutarate were purchased from the Boehringer Mannheim Corp., Mannheim, Germany. Bovine serum albumin (fraction V) from Serva, Heidelberg, Germany, was extensively dialyzed and freeze-dried prior to use. Phenylacetate was obtained from Riedel de Haen, Hannover, Germany; all other chemicals (analytical grade) came from Merck A.G., Darmstadt, Germany.

Purified rat liver pyruvate carboxylase was a kind gift of Dr. C. D. Seufert, Göttingen.

[U-^{14}C]Pyruvate, [1-^{14}C]pyruvate, [2-^{14}C]pyruvate, and ^3H_2O came from the Amersham Buchler Company, Braunschweig, Germany.

* Abbreviations used: G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; G-1-P, α-glycerophosphate; DAP, dihydroxyacetone phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate.

Table 1. Effects of phenelzine on net changes of various metabolites in the perfusion medium in the presence of various glucogenic precursors

Precursor	No. of experiments (n)	Concentration of phenelzine (mM)	Net changes in the perfusion medium (nmole · min^{-1} · 100 g body weight^{-1}) Glucose	L-Lactate	Pyruvate	Total ketone bodies
L-Lactate	(13)	0	+2974 ± 277	−6070 ± 475	+1238 ± 149	+645 ± 147
L-Lactate	(14)	1	−60 + 49*	+1100 ± 165*	−134 + 22*	+18 ± 20*
L-Lactate	(3)	0.20	+564 ± 192*	−1167 ± 728*	+737 ± 176	
L-Lactate	(4)	0.05	+2318 ± 212	−5131 + 1055	+521 ± 282	
Pyruvate	(8)	0	+3009 ± 164	+4013 ± 348	−13703 + 1755	+523 ± 116
Pyruvate	(13)	1	+1401 ± 100*	+2639 ± 136*	−7902 ± 368*	+293 ± 64
L-Lactate + hexanoate	(9)	0	+3925 ± 243	−7675 ± 483	+421 + 93	+3263 ± 369
L-Lactate + hexanoate	(11)	1	−104 ± 36*	+640 ± 252*	+124 ± 35*	+1514 + 207*
Propionate	(8)	0	+1710 ± 118	n.m.	n.m.	n.m.
Propionate	(4)	1	+521 ± 74*	n.m.	n.m.	n.m.
Fructose	(5)	0	+9249 + 1065	+6058 ± 559	+3645 + 912	+2903 ± 646
Fructose	(5)	1	+8624 ± 1011	+5023 ± 367	+2541 ± 479	+1171 ± 296*

The initial precursor concentration was 20 mM except for propionate which was 10 mM. Where indicated hexanoate was added at the beginning of the experiment to give a final concentration of 1 mM. Moreover, 0.8 mmole/hr was continuously infused intraportally throughout the experiment. Net uptake or net release is indicated by − or + resp. (Mean values ± S.E.M.)

* $P < 0.05$ or smaller.

RESULTS

Equilibrium constants for 2-phenethylhydrazone formation between phenelzine and various 2-keto acids. The ability of phenelzine to form hydrazones with 2-keto acids increased in the sequence α-oxoglutarate < pyruvate < oxaloacetate. The equilibrium constants were 6.34 ± 0.15, 2.38 ± 0.15, 1.64 ± 0.05 moles · 10^{-4} for α-oxoglutarate, pyruvate and oxaloacetate, respectively. The initial velocities of the hydrazine formation obey first order kinetics with 'half lives' of 47.2, 75.1, 205 min, and rate constants of 14.7, 9.23, 3.38 · 10^{-3} · min^{-1} for oxaloacetate, pyruvate and α-oxoglutarate respectively.

Perfusion experiments. Phenelzine at a concentration of 1 mM completely abolished gluconeogenesis from 20 mM L-lactate (Table 1) whereas gluconeogenesis from 20 mM pyruvate was only inhibited by about 50 per cent and that from 10 mM propionate only by about 70 per cent. Gluconeogenesis from 20 mM fructose was not significantly affected by 1 mM phenelzine.

When gluconeogenesis from L-lactate was further stimulated by simultaneous infusion of hexanoate, phenelzine (1 mM) again completely abolished gluconeogenesis.

When L-lactate was the glucogenic precursor, phenelzine led to a net release of L-lactate in spite of the high concentration of L-lactate in the medium. This was independent of the presence or absence of hexanoate.

When pyruvate was the glucogenic precursor phenelzine diminished the net uptake of pyruvate as well as the release of L-lactate. The formation of L-lactate and pyruvate from fructose was only slightly diminished by phenelzine.

Gluconeogenesis from L-lactate (20 mM) and lactate uptake were still inhibited by concentrations of phenelzine as low as 0.5 mM (Table 1). Since phenelzine is metabolized in the liver to phenylacetate [25] the inhibitory effect of the latter compound was likewise examined. Phenylacetate at a concentration of 0.5 mM led to a small decrease of the net release of glucose and the uptake of L-lactate. However, these changes were not significant.

Effects of phenelzine on the metabolism of L-*alanine and pyruvate in isolated rat liver cells*

Effect on gluconeogenesis and ureogenesis from L-*alanine.* Formation of both glucose and urea was significantly reduced by 1 mM phenelzine (Table 2). This inhibition could still be seen at a phenelzine concentration of 0.5 mM. The conversion of L-alanine to L-lactate was also inhibited by phenelzine.

Effect of phenelzine on ketogenesis. Phenelzine (1 mM) led to an almost complete inhibition of ketogenesis in the presence of L-lactate (Table 1). Hexanoate in the presence of L-lactate stimulated the rate of ketogenesis about fivefold and this increased rate of ketogenesis was inhibited by more than 50 per cent in the presence of 1 mM phenelzine. The increased rate of ketone body formation in the presence of 20 mM fructose was likewise inhibited by more than 60 per cent in the presence of 1 mM phenelzine.

Effects of PEH-propionate on gluconeogenesis. Recently, the inhibitory effect of phenelzine on gluconeogenesis has been related to an action of its hydrazone derivatives, especially to PEH-propionate [18]. Therefore, the effects of this compound were examined.

Gluconeogenesis from 20 mM L-lactate was indeed significantly reduced in the presence of 0.1 mM PEH-propionate (Fig. 1). In contrast to the effects seen in the presence of phenelzine, gluconeogenesis from 20 mM pyruvate remained completely unaffected by PEH-propionate (0.74 ± 0.06 ($n = 5$) vs 0.70 ± 0.06 ($n = 5$) μmole glucose formed · min^{-1} · g^{-1} in the absence and presence of 0.1 mM PEH-propionate, respectively) during perfusion of isolated livers from 48 hr-starved rats.

Table 2. Effects of decreasing concentrations of phenelzine on gluconeogenesis, production of L-lactate and urea and utilisation of L-alanine in experiments with isolated liver cells from 48 hr starved rats

Experimental condition	Urea	Net production of glucose	L-Lactate	Net utilisation of L-alanine
	(μmole · g liver^{-1} · min^{-1})			(μmole · g liver^{-1} · min^{-1})
Control	0.19 ± 0.004	0.31 ± 0.003	< 0.003	
L-Alanine	1.15 ± 0.070	0.61 ± 0.014	0.34 ± 0.005	2.21 ± 0.04
L-Alanine + $1 \cdot 10^{-3}$ M Phenelzine	0.13 ± 0.005*	0.27 ± 0.02*	0.13 ± 0.010*	0.60 ± 0.01*
L-Alanine + $5 \cdot 10^{-4}$ M Phenelzine	0.45 ± 0.060*	0.31 ± 0.07*	0.21 ± 0.008*	1.73 ± 0.09*
L-Alanine + $2 \cdot 10^{-4}$ M Phenelzine	1.02 ± 0.060	0.48 ± 0.005*	0.31 ± 0.008*	1.92 ± 0.09*

The net production of pyruvate was of the order of 0.01 μmole · g wet weight^{-1} · min^{-1} in all groups. Mean values ± S.E.M. ($n = 4$).
* $P < 0.05$ or smaller.

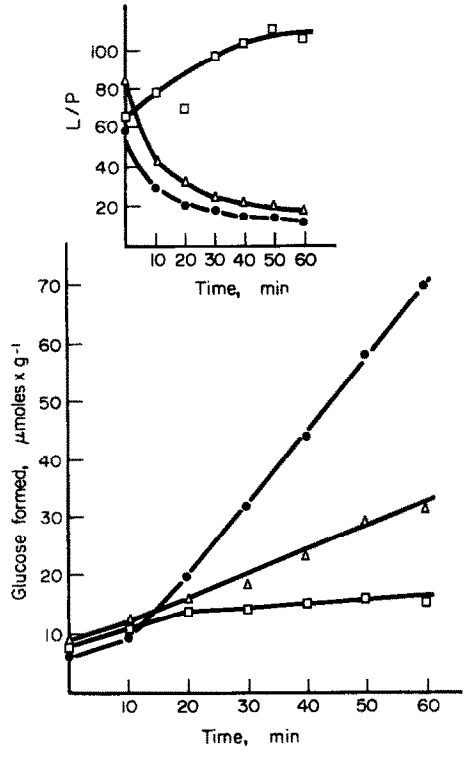

Fig. 1. Effect of PEH-propionate or phenelzine on gluconeogenesis with 20 mM L-lactate as glucogenic precursor in isolated perfused livers from 48 hr starved rats.

Experimental conditions were as given in the methods section, except that a hemoglobinfree perfusion medium was used. O——O 20 mM L-lactate; Δ——Δ 20 mM L-lactate plus 0.1 mM PEH-propionate; □——□ 20 mM L-lactate-plus 1 mM phenelzine. The L-lactate/pyruvate ratio (L/P) in the perfusate is given in the insert.

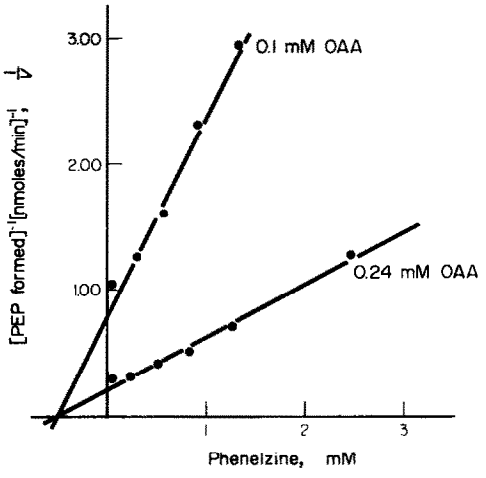

Fig. 2. 'Apparent' non-competitive inhibition of PEP carboxykinase from rat liver by phenelzine.

PEP carboxykinase activity was measured in direction of PEP formation. The initial concentration of oxaloacetate was 0.24 or 0.1 mM respectively. The reciprocals of the enzyme velocities are plotted against the concentration of phenelzine (Dixon plot). For further details see Materials and Methods.

Inhibition of specific enzymatic steps by phenelzine — Effects on PEP-carboxykinase and pyruvate carboxylase

An examination of the effect of phenelzine on the activity of phosphoenolpyruvate carboxykinase in rat liver 100,000 *g* supernatant revealed a non-competitive inhibition when the reaction was measured in the direction of PEP formation (Fig. 2). To test whether phenelzine had a direct effect on this enzyme or whether phenelzine acted mainly by reacting with its substrate oxaloacetate, the enzyme activity was followed as a function of the "free" oxaloacetate concentration at varying oxaloacetate/phenelzine ratios (Fig. 3). Immediately before addition of the enzyme, aliquots were taken from the incubation mixture for timed analysis of oxaloacetate. The substrate dependency of phosphoenolpyruvate carboxykinase, when related to the measured instead of the calculated concentrations of oxaloacetate, was now exactly identical in the presence or absence of phenelzine. This indicates that phenelzine does not directly act on the enzyme itself, but inhibits the reaction by decreasing the actual substrate concentration, probably via hydrazone formation.

The rate of pyruvate carboxylation *in vitro* was not significantly altered by phenelzine up to a concentration of 5 mM when using purified rat liver pyruvate carboxylase (results not shown).

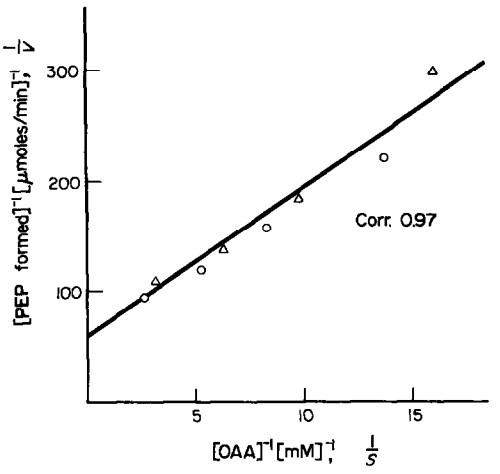

Fig. 3. Dependency of the reaction velocity of PEP carboxykinase from the actual concentration of free oxaloacetate.

PEP carboxykinase activity was followed for 5 min in the direction of PEP formation in the absence (circles) or presence (triangles) of 1 mM phenelzine. Immediately before addition of the enzyme, aliquots were taken from the reaction mixture for analysis of oxaloacetate.

The initial concentrations of oxaloacetate varied between 0.1 and 0.5 mM. Reciprocals of the enzyme velocity are plotted against the reciprocals of the measured oxaloacetate concentrations (Lineweaver–Burk plot).

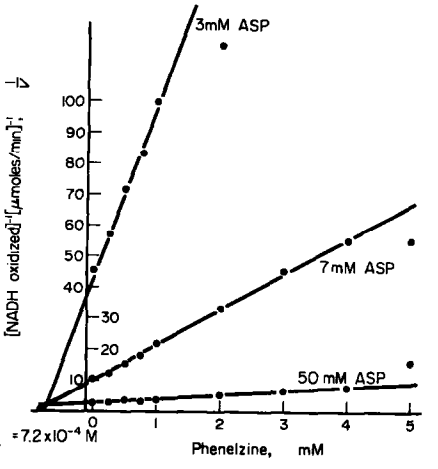

Fig. 4. Competitive inhibition by phenelzine of cytosolic aspartate aminotransferase from rat liver.

Aspartate aminotransferase activity was tested in the direction of oxaloacetate formation (assay I). L-Aspartate and phenelzine were varied as indicated in the figure. The reciprocals of the enzyme velocities are plotted against the phenelzine concentration (Dixon plot). Further details as given in Methods.

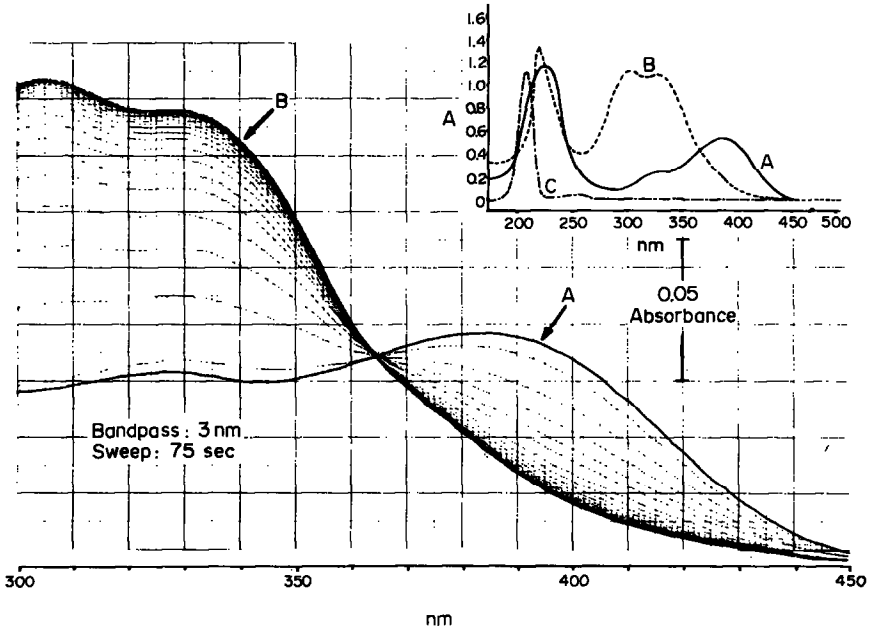

Fig. 5. U.v. spectral changes accompanying the reaction between pyridoxal-5′-phosphate and phenelzine. The sample cuvette contained (final concentrations): 0.1 M phosphate buffer, pH 7.6; pyridoxal-5′-phosphate (PLP) 0.01 mM; phenelzine 0.1 mM. The reference cuvette contained only the phosphate buffer. Spectrum A was recorded before ($t = 0$ min), spectrum B one hour after the addition of phenelzine. Time difference between each spectral curve is 2.5 min. Note that the reaction has almost completed after 30 min. Insert: u.v. absorption spectra of pyridoxal-5′-phosphate (A), pyridoxal-5′-phosphate plus phenelzine (B), or phenelzine (C). 20 mM phosphate buffer (pH 7.2) was used as a solvent. The reference cuvette contained either phosphate buffer (A and C), or 0.5 mM phenelzine in phosphate buffer (B). The difference spectrum (B) was recorded 20 min after the addition of the reactants. The absorption coefficient at $\lambda = 330$ nm was found to be $\varepsilon = 12.5 \cdot \text{mM}^{-1} \cdot \text{cm}^{-1}$. All spectra were recorded at room temperature.

Effects on aspartate aminotransferase

Aspartate aminotransferase (AAT) was inhibited competitively by phenelzine (K_i app = $0.72 \cdot 10^{-3}$ M) when tested in the direction of oxaloacetate formation (Fig. 4). To evaluate whether this inhibition was an 'apparent' effect, due to hydrazone formation with the intermediary product oxaloacetate generated in this coupled enzyme reaction, a system was chosen to mimick such a situation: into cuvettes containing malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) and NADH at concentrations as used in the AAT assay system, plus varying amounts of phenelzine, oxaloacetate was infused at rates computed for maximal AAT velocities (0.25, 0.12, and 0.025 μmoles/min/ml, as given by the three intercepts with the ordinate in Fig. 4). Under these conditions no 'apparent' inhibition due to substrate trapping could be observed even at molar ratios phenelzine/oxaloacetate as high as 80/1. (Results not shown.)

The identified type of inhibition, namely competitive with L-aspartate, could indicate an interaction with the coenzyme pyridoxal-5′-phosphate (PLP).

Phenelzine reacted *in vitro* with PLP, as revealed by spectral analysis (Fig. 5). The absorption maximum at 330 nm is characteristic for an aldimine linkage (Schiff base formation), as it occurs normally between amino acid and PLP during transamination [26].

The aldimine between PLP and phenelzine is readily formed at room temperature. The initial velocity of the reaction obeys first order kinetics with a 'halflife' of 6.2 min and a rate constant of $111.8 \cdot 10^{-3} \cdot \text{min}^{-1}$. Hydrazone formation with 2-ketoacids in comparison occurs relatively slowly, e.g. with pyruvate $t_{\frac{1}{2}} = 72$ min.

In contrast to the effect seen in the presence of phenelzine, the hydrazones themselves exhibited comparably weak effects on AAT (Fig. 6), a small activation of the enzyme was observed in the presence of PEH-propionate, a slight inhibition in the presence of PEH-pentanoate (+ 13% and − 17% at 5 mM hydrazone resp.).

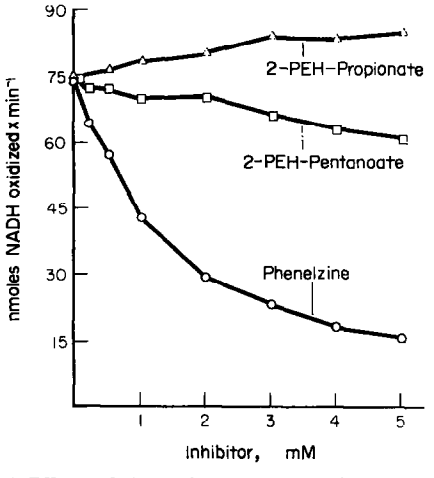

Fig. 6. Effects of phenelzine, 2-PEH-propionate and 2-PEH-pentanoate on aspartate aminotransferase activity.

Aspartate aminotransferase activity was determined in the direction of oxaloacetate formation as given in Methods (assay I), except that L-aspartate was 0.4 mM (final concentration). Phenelzine or its derivatives were added to the reaction cuvette at concentrations indicated in the figure. After standing for 10 min at 30° the reaction was initiated by addition of α-oxoglutarate.

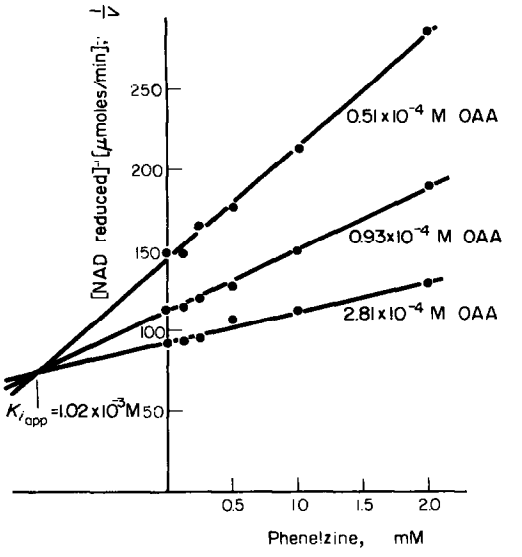

Fig. 7. 'Apparent' competitive inhibition by phenelzine of cytosolic aspartate aminotransferase from rat liver.

Aspartate aminotransferase was tested in the direction of L-aspartate formation (assay II). Oxaloacetate and phenelzine were varied as indicated in the figure. The reciprocals of the enzyme velocities are plotted against the concentrations of phenelzine (Dixon plot). Further details are given in Methods.

An "apparent" inhibition of AAT by phenelzine was likewise observed when testing this enzyme in the (forward) direction of L-aspartate formation (Fig. 7). However this inhibition is clearly related to substrate trapping by phenelzine, as can be shown by the good correlation between enzyme activity and the actual oxaloacetate concentration (Fig. 8).

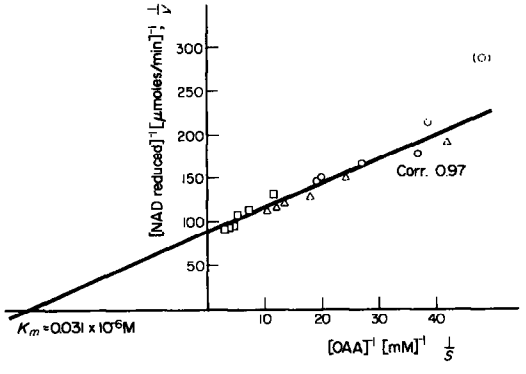

Fig. 8. Dependency of the aspartate aminotransferase activity from the concentration of free oxaloacetate.

Data are taken from Fig. 7. The reciprocals of the enzyme velocity are plotted against the reciprocals of the corresponding concentration of free oxaloacetate (Lineweaver–Burk plot). The true oxaloacetate concentrations in the presence of varying amounts of phenelzine were measured immediately before addition of L-glutamate (start of reaction). The initial oxaloacetate concentrations were as follows: ⋯—⋯, $2.81 \cdot 10^{-4}$ M; Δ——Δ, $0.93 \cdot 10^{-4}$ M; and O——O, $0.51 \cdot 10^{-4}$ M oxaloacetate.

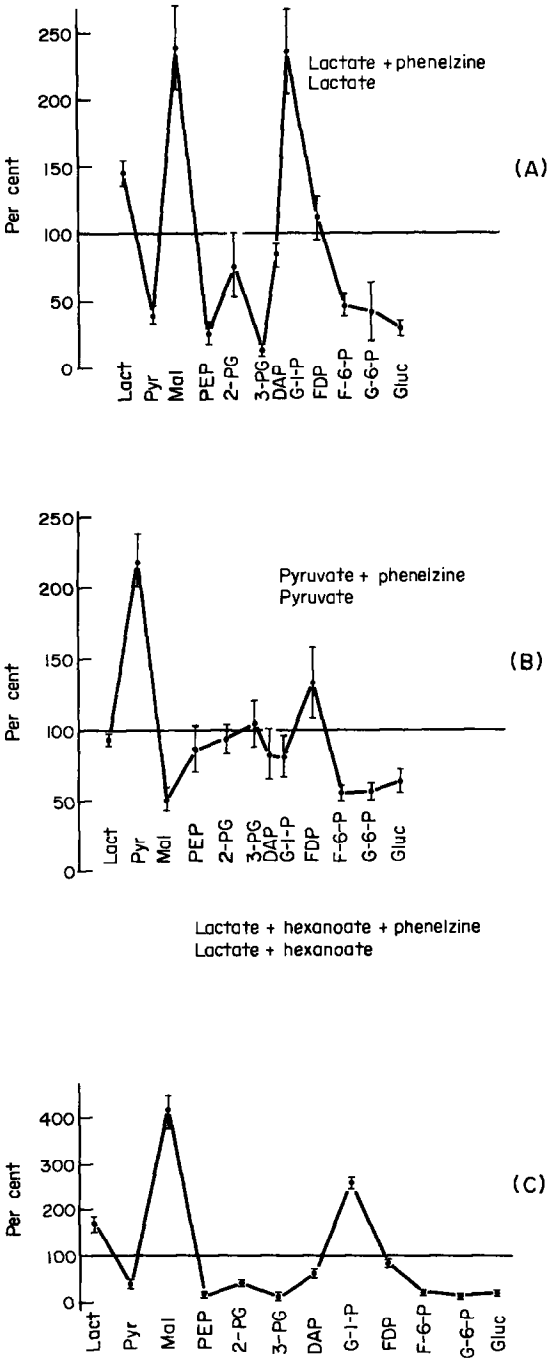

Fig. 9. Effects of phenelzine (1 mM) on the cross-over plot of metabolite concentrations of glycolytic intermediates in isolated perfused rat livers.

The glucogenic substrates were L-lactate (A), pyruvate (B) and L-lactate plus hexanoate (C). L-Lactate and pyruvate were given at an initial concentration of 20 mM. Hexanoate was added at $t = 0$ min (1 mM initial concentration) and 0.8 mmole/min of hexanoate was infused intraportally throughout the experiment.

The livers were freeze-clamped after 60 min. The metabolite concentrations measured in the absence of phenelzine were taken as 100%. Absolute values are given in Table 3.

The points represent means, the bars ± S.E.M.

Intrahepatic metabolite concentrations and cross-over plots

The results of these measurements are summarized in Table 3 and in Fig. 9.

With L-lactate as substrate a forward cross-over occurred between L-malate and PEP and again between FDP and F-6-P (Fig. 9A).

The cross-over between L-malate and PEP became even more pronounced when phenelzine was added in the presence of hexanoate (Fig. 9C).

With pyruvate as substrate, phenelzine caused a forward crossover between pyruvate and L-malate, and between FDP and F-6-P (Fig. 9B).

The concentrations of citrate, α-oxoglutarate, and L-aspartate under the same conditions are summarized in Table 3. With L-lactate as glucogenic precursor, the concentrations of citrate and α-oxoglutarate did not significantly change in the presence of phenelzine whereas the concentration of L-glutamate decreased and the concentration of L-aspartate increased significantly.

Hexanoate caused a significant increase in the concentration of citrate, a small increase in the concentration of α-oxoglutarate but no measurable changes in the concentrations of L-glutamate and L-aspartate.

Phenelzine led in the presence of hexanoate + L-lactate to a strong increase of the concentrations of citrate and L-aspartate, while the concentrations of L-glutamate and α-oxoglutarate did not significantly change.

In the presence of pyruvate, phenelzine caused a strong decrease in the overall concentrations of citrate and L-glutamate, and a smaller decrease of the α-oxoglutarate concentration, while the concentration of L-aspartate increased.

Changes in the overall concentrations of acetyl-*S*-CoA are given in Table 3.

Phenelzine provoked under all conditions a significant fall of the overall concentration of acetyl-*S*-CoA. The lowest absolute values were found when phenelzine was given in the presence of pyruvate.

Phenelzine did not significantly affect the adenine nucleotide levels or the ATP/ADP ratios in the various experimental groups except in the presence of L-lactate + hexanoate where phenelzine was accompanied by an increase of the ATP concentration and of the ATP/ADP ratio (Table 3).

Redox-state of NAD⁺/NADH linked redox couples. The results are summarized in Table 4. In the presence of L-lactate as precursor, phenelzine led to a significant reduction of the cytosolic free NAD⁺/NADH system as indicated by the increased L-lactate/pyruvate and G-1-P/DAP ratios, whereas the redox state of the mitochondrial NAD⁺/NADH system as reflected by the 3-hydroxybutyrate/acetoacetate ratio did not significantly change.

When pyruvate was the glucogenic precursor the L-lactate/pyruvate ratio was more oxidized in the presence of phenelzine, but this difference was not statistically significant. The G-1-P/DAP ratio was about the same in the presence and absence of phenelzine (Table 4). The 3-hydroxybutyrate/acetoacetate ratio showed only a small and insignificant increase in the presence of phenelzine.

Table 3. Effects of phenelzine on the steady state concentrations of various intermediates in isolated perfused livers from 48 hours starved rats

	L-Lactate control ($n = 6$)	L-Lactate + phenelzine ($n = 12$)	Pyruvate control ($n = 6$)	Pyruvate + phenelzine ($n = 8$)	L-Lactate + hexanoate control ($n = 8$)	L-Lactate + hexanoate + phenelzine ($n = 9$)
			(nmole · g liver^{-1})			
Lactate	8212 ± 1049	11932 ± 756*	2672 ± 321	2483 ± 114	5445 ± 207	9206 ± 855*
Pyruvate	403 ± 76	155 ± 19*	2240 ± 297	4886 ± 406*	217 ± 10	82 ± 10
Malate	235 ± 69	562 ± 78*	372 ± 42	188 ± 25*	902 ± 94	3721 ± 320*
PEP	106 ± 26	25 ± 4*	943 ± 163	811 ± 151	157 ± 9	23 ± 3*
2-PG	21 ± 2	16 ± 5	181 ± 33	171 ± 18	45 ± 5	13 ± 3*
3-PG	152 ± 54	17 ± 4*	1528 ± 305	1582 ± 245	287 ± 19	33 ± 6*
DAP	15 ± 3	13 ± 1	10 ± 2	8 ± 1	21 ± 9	13 ± 2
G-1-P	168 ± 36	395 ± 52*	95 ± 23	77 ± 14	393 ± 44	1033 ± 40*
FDP	4 ± 1	4 ± 1	2 ± 1	3 ± 0	7 ± 1	6 ± 1
F-6-P	11 ± 3	4 ± 1*	8 ± 1	4 ± 0*	9 ± 1	2 ± 1*
G-6-P	44 ± 6	19 ± 4*	44 ± 9	25 ± 3*	42 ± 5	6 ± 1*
Glucose	4682 ± 813	1324 ± 254*	3431 ± 502	2183 ± 266*	6056 ± 272	1157 ± 201*
Citrate	796 ± 44	699 ± 44	1509 ± 256	552 ± 26*	2281 ± 225	5415 ± 191*
L-Glutamate	3133 ± 594	1896 ± 233*	3265 ± 613	1601 ± 282*	3030 ± 419	2681 ± 202
L-Aspartate	370 ± 56	809 ± 128*	201 ± 43	347 ± 34*	423 ± 20	1290 ± 77*
α-Oxoglutarate	357 ± 27	350 ± 32	669 ± 141	436 ± 111	539 ± 44	614 ± 44
Acetyl-S-CoA	24.8 ± 1.9	15.2 ± 1.0*	22.4 ± 1.8	6.0 ± 0.8*	106.8 ± 7.1	72.6 ± 4.3*
ATP	1956 ± 110	2170 ± 95	1725 ± 148	2044 ± 70	1453 ± 10	1939 ± 54*
ADP	836 ± 60	874 ± 65	581 ± 44	674 ± 31	707 ± 31	652 ± 10
AMP	479 ± 83	413 ± 71	382 ± 77	356 ± 54	614 ± 77	360 ± 46*
			(ratio)			
ATP/ADP	2.38	2.48	3.09	3.09	2.11	2.99

The initial concentration of L-lactate and pyruvate was always 20 mM, that of phenelzine 1 mM. Hexanoate was added to give an initial concentration of 1 mM followed by a continuous intraportal infusion of 0.8 mmole · hr^{-1}. Liver tissue was obtained in all experiments by freeze-clamping after 60 minutes of perfusion. (Mean values ± S.E.M.)

* $P < 0.05$ or smaller.

When L-lactate was given together with hexanoate the L-lactate/pyruvate ratio, the G-1-P/DAP ratio, and the 3-hydroxybutyrate/acetoacetate ratio were significantly elevated in the presence of phenelzine.

The strong reduction of the cytosolic $NAD^+/NADH$ system in the presence of phenelzine with L-lactate as glucogenic precursor (Table 4), as reflected by a high L-lactate/pyruvate ratio in the perfusate, was not observed in the presence of PEH-propionate (Fig. 1).

Experiments with [1-^{14}C]*pyruvate and* ^3H_2O. In the presence of 1 mM phenelzine the oxidation of pyruvate as judged from the formation of $^{14}CO_2$, decreased significantly (Table 5). The incorporation of ^{14}C-radioactivity into glucose decreased likewise. The incorporation of ^{14}C-radioactivity into total lipids, however, increased significantly. At the same time the incorporation of radioactivity from ^3H_2O into total lipids decreased significantly.

The incorporation of ^{14}C-radioactivity into the glycerol moiety of lipids increased significantly, while the incorporation of ^{3}H-radioactivity from ^3H_2O into this fraction decreased by about 50%. The incorporation of ^{3}H-radioactivity into the fatty acids fraction of total lipids was nearly completely abolished by phenelzine.

Table 4. Effect of 1 mM phenelzine on the intrahepatic redox state of various NAD-linked substrate couples in the presence of different glucogenic precursors

Precursor	Absence(−) or presence (+) of phenelzine	Lactate/pyruvate (ratio)	α-Glycerophosphate/dihydroxyacetonephosphate (ratio)	3-Hydroxybutyrate/acetoacetate (ratio)
L-Lactate	(−)	18.9 ± 4.0	14.8 ± 2.1	0.67 ± 0.17
L-Lactate	(+)	80.9 ± 8.7*	24.9 ± 3.2*	0.70 ± 0.09
Pyruvate	(−)	1.5 ± 0.4	13.1 ± 3.5	0.35 ± 0.07
Pyruvate	(+)	0.6 ± 0.1*	9.2 ± 2.1	0.44 ± 0.12
L-Lactate + hexanoate	(−)	26.7 ± 3.0	19.3 ± 2.6	1.02 ± 0.07
L-Lactate + hexanoate	(+)	133.0 ± 23.4*	92.0 ± 13.3*	4.27 ± 0.34*

The experiments are identical with those of Table 1.

* $P < 0.05$ or smaller.

Table 5. Effects of phenelzine on the incorporation of carbon from the C-1 or C-2 position of the lactate–pyruvate pool and on the incorporation of hydrogen from ^3H_2O into various metabolites by isolated liver cells from fed rats

Labeled precursor	Experimental condition (n)	Incorporation of carbon from the C-1 or C-2 position of the lactate–pyruvate pool or from ^3H_2O into				
		CO_2	Glucose	Total lipids	Fatty acids	Glyceride glycerol
		(ng atom carbon · g liver^{-1} · min^{-1})				
[1-^{14}C]Pyruvate	Control (4)	557 ± 31	95.3 ± 5.7	5.39 ± 0.40	—	5.25 ± 0.20
[1-^{14}C]Pyruvate	Phenelzine (4)	215 ± 12*	45.8 ± 13.9*	17.76 ± 1.20	—	14.51 ± 0.98*
[1-^{14}C]Pyruvate	Control (5)	49.9 ± 7.7	212 ± 20	7.75 ± 0.11	1.68 ± 0.06	5.14 ± 0.05
[2-^{14}C]Pyruvate	Phenelzine (5)	18.1 ± 1.1*	53 ± 4*	13.28 ± 1.10*	0.56 ± 0.06*	11.79 ± 1.07*
		(ng atom H · g liver^{-1} · min^{-1})				
^3H_2O	Control (8)	—	—	34.0 ± 2.4	7.4 ± 0.1	24.8 ± 2.2
^3H_2O	Phenelzine (8)	—	—	10.4 ± 0.6*	0+	10.5 ± 0.6*

The medium contained L-lactate + pyruvate (L:P = 9:1 (mol/mol)) at an initial concentration of 5 mM together with 1 μCi of either [1-^{14}C]- or [2-^{14}C]pyruvate/incubation flask or tritiated water (initial specific activity 3.6 μCi/mole H_2O) resp. The concentration of phenelzine was always 1 mM. Further experimental details are given under Materials and Methods. (The numbers are mean values ± S.E.M.).

* $P < 0.05$ or smaller.

+ Counting rate in the liquid scintillation spectrometer not distinguishable from background rate.

Experiments with [2-^{14}C]pyruvate and ^3H_2O. Phenelzine inhibited significantly the formation of $^{14}CO_2$ and the incorporation of ^{14}C-radioactivity into glucose (Table 5). The incorporation of ^{14}C-radioactivity into total lipids increased by about 50 per cent in the presence of 1 mM phenelzine.

This resulted from a strong increase of ^{14}C-incorporation into the glycerol moiety of total lipids while the incorporation into the fatty acid fraction of total liver lipids was significantly reduced. Measurements of the incorporation of ^{3}H-radioactivity from ^3H_2O into the various fractions revealed values similar to those obtained in the experiments with [1-^{14}C]pyruvate and ^3H_2O.

Experiments with isolated rat liver mitochondria. Phenelzine in concentrations up to 5 mM did not significantly alter respiration and respiratory control ratio with the following substrates: succinate, 3-hydroxybutyrate, and L-malate. Phenelzine had only a very weak inhibitory effect on the activity of mitochondrial NADH oxidase and ATPase (12% and 5% inhibition resp. at 1 mM phenelzine). No significant effect on the ATP-^{32}P exchange reaction could be observed with phenelzine concentrations up to 5 mM. Reversed electron flow as measured by the reduction of acetoacetate in the presence of succinate + ATP was not significantly affected by 1 mM phenelzine, whereas rotenone and antimycin exerted the well known inhibitory effect (not shown). The ATP independent reduction of acetoacetate by pyruvate, on the other hand, was significantly inhibited by 1 mM phenelzine (7.42 ± 0.02 versus 3.10 ± 0.02 nmoles · mg^{-1} · min^{-1} acetoacetate reduced in the absence or presence of phenelzine resp. at an endogenous reduction of 2.97 ± 0.16 and 0.51 ± 0.07 nmoles · mg^{-1} · min^{-1} resp.).

DISCUSSION

Phenelzine inhibited gluconeogenesis from all precursors entering the gluconeogenic pathway below the triosephosphates, but did not significantly affect gluconeogenesis from fructose. Therefore, it is unlikely that the inhibition resulted from a lack of energy-rich phosphates. Moreover, phenelzine did not alter the levels of the adenine nucleotides or the ATP/ADP ratio. The cross-over at the fructose-1,6-bisphosphatase/phosphofructokinase step. The phenomenon did not correlate with changes in the overall levels of citrate, an inhibitor of liver phosphofructokinase. The citrate level decreased in the presence of pyruvate, remained unchanged in the presence of L-lactate, and increased in the presence of L-lactate + hexanoate. Changes of the levels of the adenine nucleotides were too small to offer an explanation (e.g. inhibition of fructose-1,6-bisphosphatase or stimulation of phosphofructokinase by 5′-AMP). However, one has to state that the overall measurements do not exactly reflect the concentrations in the cytosol. The fact that gluconeogenesis from fructose was not affected by phenelzine shows that the cross-over at the fructose-1,6-bisphosphatase-phosphofructokinase step rather reflects a new steady state at this step, than a site of flux inhibition by phenelzine.

In the presence of L-lactate (plus or minus hexanoate) a forward cross-over was also observed between L-malate and phosphoenolpyruvate, demonstrating that phosphoenolpyruvate carboxykinase was also a site of interaction in the presence of phenelzine. With pyruvate as precursor the second cross-over occurred between pyruvate and L-malate.

The cross-over at the phosphoenolpyruvate carboxykinase step most probably indicates one site of inhibition of gluconeogenesis since gluconeogenesis from propionate which does not involve the pyruvate carbox-

ylase reaction was also inhibited by phenelzine. The cross-over occurring at the pyruvate carboxylase step with pyruvate as precursor could indicate that in this case pyruvate carboxylation is also inhibited which would be explained by the extreme drop in the level of acetyl-CoA, a positive effector of pyruvate carboxylation [27]. Our results do not support the idea of a direct inhibition of phosphoenolpyruvate carboxykinase by phenelzine as was proposed by Ray *et al.* [28] for the effect of hydrazine on hepatic phosphoenolpyruvate carboxykinase. It is much more likely that the flux through the phosphoenolpyruvate carboxykinase step is limited by the concentration of oxaloacetate. A direct trapping of oxaloacetate by phenelzine seems inconceivable, since the affinity of oxaloacetate generated by an enzymic reaction (e.g. AAT) is much higher for oxaloacetate utilizing enzymes than for phenelzine (see Results: Effects on aspartate aminotransferase). It is more likely that the PEP-carboxykinase reaction appears inhibited due to a lack of free oxaloacetate resulting from an inhibition of cytosolic aspartate aminotransferase. This is consistent with the observed inhibition of transamination in the direction of oxaloacetate generation.

The identified type of inhibition points to a competition between L-aspartate and phenelzine for enzyme-linked pyridoxal-5′-phosphate (PLP). Hydrazone formation between PLP and hydrazides has been described by others [29]. According to our results phenelzine too reacts rapidly and at room temperature with PLP as indicated by the appearance of an absorption maximum at 330 nm which is characteristic for an aldimine linkage. A similar inhibitory mechanism has been described for isoniacid, a tuberculostastic hydrazide (K_i app = 14 mM, competitively with L-aspartate on AAT) [30], or recently for the neurotoxin gabaculine, which inhibits γ-aminobutyrate transaminase via aldimine formation with enzyme bound PLP [31]. The evidence for an inhibition of transamination by phenelzine *in vitro* makes it likely that the same inhibitory mechanism is also operative *in vivo*.

The view that AAT is the main site of interaction is supported by the observed changes in the tissue levels of L-aspartate, L-glutamate and α-oxoglutarate as well as the different degree of inhibition of gluconeogenesis with L-lactate, propionate or pyruvate as glucogenic precursor. Gluconeogenesis from L-lactate but not from pyruvate involves an intra- and extramitochondrial transamination step [32], since intramitochondrially formed L-aspartate is exported out of the mitochondria to be converted into oxaloacetate in the cytosol. Gluconeogenesis from propionate would be inhibited to a minor degree, since in this case carbon and reducing equivalents are leaving the mitochondrial matrix mainly as L-malate and only to a minor extent as L-aspartate. When pyruvate is glucogenic precursor no transamination step is involved. Carbon and reducing equivalents are leaving the mitochondrial compartment mainly as L-malate.

An inhibition of transamination reactions by phenelzine is further supported by the following results: (1) The inhibition of formation of glucose, lactate and urea from L-alanine in experiments with isolated rat liver cells; and (2) the decreased incorporation of ^3H-radioactivity from ^3H_2O into the glycerol moiety of liver lipids concomitantly with an increased incorporation of ^{14}C-radioactivity from [1-^{14}C]- and [2-^{14}C]pyruvate. Radioactivity from ^3H_2O is incorporated into the methyl group of L-lactate and pyruvate during transamination due to hydrogen exchange [33]. Inhibition of transamination inhibits this exchange.

The inhibitory effect of phenelzine on gluconeogenesis has been recently attributed to the action of PEH propionate [8]. The authors observed a complete inhibition of gluconogenesis with 0.05 mM PEH-propionate in isolated perfused guinea pig liver using either L-lactate or pyruvate as glucogenic precursor. However, in rat liver PEH-propionate and phenelzine act by different mechanisms. In our experiments with isolated perfused rat livers PEH-propionate failed to inhibit gluconeogenesis from pyruvate, but inhibited gluconeogenesis from L-lactate (Fig. 1). With L-lactate as glucogenic precursor, phenelzine but not PEH-propionate caused a rapid reduction of cytoplasmic $NAD^+/NADH$ system as reflected by an increase in the L-lactate/pyruvate ratio of the perfusate (Fig. 1), suggesting a strong inhibition of the L-aspartate shuttle in the presence of phenelzine. This further supports the view of a direct effect of phenelzine on AAT, especially since no interference with the mitochondrial L-aspartate carrier system could be detected (unpublished results).

Whether the reported effects in the guinea pig liver are a consequence of the more reduced cytoplasmic redox state in this species [34] or whether they are the result of other species differences [35], remains to be investigated.

Under conditions where pyruvate is generated and subsequently utilized by the liver (L-lactate as glucogenic precursor), hydrazone formation with pyruvate may be too slow to become a rate limiting factor in the overall reaction. Since AAT activity is inhibited significantly by phenelzine but not by its hydrazone *in vitro*, the inhibition of transamination must have resulted from a direct action of phenelzine.

The increased incorporation of ^{14}C-radioactivity from [1-^{14}C]- and [2-^{14}C]pyruvate into the glyceride glycerol fraction in the presence of phenelzine seems to be in contrast with the decreased carbon flux from pyruvate or L-lactate to glucose. However, this result is explained by the increased intracellular level of α-glycerophosphate (see Table 3) which favours esterification of fatty acids [2]. This can occur without any alteration or even during a decrease of the carbon flux from pyruvate to the triose phosphates.

An inhibition of pyruvate oxidation by phenelzine is indicated by the following findings: (1) decreased formation of $^{14}CO_2$ from [1-^{14}C]pyruvate in experiments with isolated liver cells; (2) decreased incorporation of ^{14}C-radioactivity from [2-^{14}C]pyruvate into fatty acids; (3) decreased reduction of acetoacetate with pyruvate as substrate in experiments with isolated liver mitochondria; and (4) a decreased formation of ketone bodies in experiments with fructose. It has been described earlier [36] that under these special conditions the carbon of the ketone bodies is mainly derived from fructose via pyruvate oxidation. The inhibition of ketogenesis from fructose is therefore indicating an inhibition of this step.

The effects of phenelzine on pyruvate metabolism are certainly independent from the effect on the aminotransferases.

As we have observed *in vitro* an inhibition of

the α-oxoglutarate dehydrogenase reaction (K_i app. = $2.2 \cdot 10^{-3}$ M, results not shown), it seems conceivable that phenelzine affects the thiamine pyrophosphate dependent step of the α-oxoglutarate and pyruvate decarboxylation reactions. In addition, at low concentrations of pyruvate, effects of PEH-propionate could contribute to the inhibition of pyruvate utilisation.

According to our studies hypoglycemia in patients treated with phenelzine most likely results from an inhibition of hepatic gluconeogenesis. This effect is based mainly on the inhibition of pyridoxalphosphate-depending transamination reactions. In addition, phenelzine inhibits pyruvate oxidation and thus also hepatic fatty acid synthesis. This combination of effects, including the inhibitory effects on monoamine oxidase, prohibits the use of phenelzine or similar hydrazine derivatives in the treatment of diabetes.

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