Biochemical Pharmacology, Vol. 20, pp. 1811–1819. Pergamon Press, 1971. Printed in Great Britain

EFFECT OF PHENELZINE ON THE METABOLISM AND MEMBRANAL TRANSPORT OF GLUCOSE IN BRAIN

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(*Received* 14 *October* 1970; *accepted* 28 *November* 1970)

Abstract—A colorimetric method for the estimation of phenelzine (β-phenylethylhydrazine) based on its reaction with *p*-dimethylaminobenzaldehyde is presented. Using this method it was shown that *in vivo* phenelzine rapidly penetrated all regions of the brain. *In vitro* with glucose as substrate the drug inhibited the respiration of tissue slices from all regions of the brain; with pyruvate as substrate the inhibition was much weaker and slower to develop. It was concluded that the inhibition of respiration was due to the drug's reducing the permeability of the cell membrane to glucose, since the respiration of brain homogenates was not inhibited and that of slices was more resistant to the action of the drug when the glucose concentration in the medium was increased. It was also shown that phenelzine did not inhibit any of the enzymes of glycolysis nor did it react to any significant extent with any of the glycolytic intermediates.

THE CLINICALLY important anti-depressant drugs fall broadly into two main classes: the tricyclics, e.g. imipramine [*N*-(γ-dimethylaminopropyl)-dibenzazepine] and the hydrazine derivates, e.g. phenelzine (β-phenylethylhydrazine) or their isosteres, e.g. tranylcypromine (2-phenylcyclopropylamine). The tricyclics are believed to act by inhibiting the post stimulatory re-uptake of transmitter at the adrenergic presynaptic element, while the hydrazine derivatives are thought to exert their effect by inhibition of monoamine oxidase [Monoamine: 0_2 oxidoreductase (deaminating) EC 1.4.3.4].

However, hydrazine or its derivatives, some of which are also monoamine oxidase inhibitors (MAOI), are known to inhibit a number of other enzymes. They will, for example, interfere with the enzymic decarboxylation of dihydroxyphenylalanine (DOPA),[1] 5-hydroxytryptophan[2] and glutamate,[3] the oxidation of diamines, amphetamine and choline,[4] the phosphorylation of pyridoxal[5] and the hydrolysis of acetylcholine.[4] In addition there is evidence, albeit conflicting, that the MAOI have an effect on the respiration and carbohydrate metabolism of various tissues,[6–8] and that they have an insulin-like effect on the blood sugar level[9] which correlates with their efficacy as anti-depressants.[10] These considerations led us to investigate the possibility of an interaction between carbohydrate metabolism, respiration and phenelzine in the brain.

We found that phenelzine inhibited the *in vitro* respiration of tissue slices from all regions of the brain with glucose as the substrate but that the effect was much less pronounced with pyruvate as substrate. We were able to show that the effect was not mediated by the inhibition of any of the glycolytic enzymes nor by interaction with any of the carbonyl intermediates of glycolysis. However, the respiration of brain

homogenates was not affected by phenelzine and the effect of the drug on the respiration of brain slices was reduced by increasing the glucose concentration of the medium. It is therefore concluded that phenelzine interferes with the passage of glucose into the neurons.

METHODS

In those experiments concerned with the rate of entry, exit and distribution of phenelzine in the brain, female New Zealand white rabbits (3–4 kg) were used. Phenelzine sulphate brought to pH 7·4 was injected intraperitoneally (40 mg/kg) and the animals killed at various times thereafter by injection of air into the ear vein. The brain was rapidly removed and dissected.

Approximately 0·5 g of tissue was transferred immediately after excision to 4 ml ice cold zinc sulphate solution (6% w/v $ZnSO_4.7H_2O$) contained in a tared all-glass homogenizer, homogenized without delay and weighed. An aliquot of the homogenate (2 ml) was spun down, 1·5 ml of alkali (4% w/v NaOH) was added, allowed to stand for 5 min and spun down. The supernatant was decanted, saturated with sodium chloride and extracted with 10 ml benzene. [The benzene (AR) had been previously washed with N HCl followed by a water wash and dried over anhydrous sodium sulphate. It was found that some samples of benzene gave high blanks if this procedure was omitted.] An aliquot of the benzene layer (9 ml) was re-extracted with 1 ml N HCl and 0·5 ml of the acid layer added to 0·5 ml of a 4% (w/v) solution of *p*-dimethylamino-benzaldehyde in ethanol–HCl, 2N(4:1, v/v). After mixing and standing 5 min the colour was read at 470 nm.

A blank and two phenelzine standards were run with each estimation.

Recoveries of added phenelzine were from brain 87 per cent, heart 64 per cent, liver and kidney 50 per cent and blood 40 per cent.

Respiration of brain slices

New Zealand white rabbits were also used for the *in vitro* regional respiratory studies. They were killed by air injection. The brain was quickly removed and dissected. Tissues were held in ice-cold Krebs–Ringer solution[11] buffered to pH 7·4 at 2° with tris (50 mM final concentration) until cut into 0·33 mm slices using the apparatus previously described.[12] The effect of phenelzine on the *in vitro* respiration of brain slices was measured using the standard constant volume Warburg technique. The incubation medium was Krebs–Ringer solution buffered to pH 7·4 at 37° with tris (50 mM final concentration). The drug, dissolved in the incubation medium and adjusted to pH 7·4, was added from the side arm to give a final concentration of 10 mM. The substrate was either glucose (11 mM) or pyruvate (11 mM); the gas phase was oxygen.

To investigate the effect of increasing the glucose concentration in the medium on the phenelzine-induced inhibition of respiration of brain slices adult Wistar rats were used. The rats were killed by decapitation and the excised brain divided down the mid-sagittal line. Starting from the optic chiasma, frontal slices 0·33 mm thick were cut caudad,[12] groups of six contiguous slices being placed in the same Warburg flask. Groups of slices from one side of the brain were used as controls for the corresponding slices from the other side which were treated with phenelzine. The other experimental details were as above except the final glucose concentrations in the pair of flasks were increased to 40, 80 or 100 mM.

Respiration of brain homogenates

In these experiments, adult Wistar rats were killed by decapitation, the brain rapidly excised and a 10% (w/v) homogenate prepared in 0·4 M phosphate buffer pH 7·4. The standard Warburg constant volume technique was used. The incubation medium was that devised by Larner, Jandorf and Summerson.[13] To test the effect of phenelzine on the respiration of the homogenate the drug was added to the incubation medium to give a final concentration of 10 mM. The incubation medium was placed in the main well of the Warburg flask and the homogenate added from the side arm after thermal equilibration. The gas phase was oxygen.

Assay of activities of glycolytic enzymes

In these experiments, adult Wistar rats were killed by decapitation. The brains were rapidly removed, weighed and homogenized in teflon-in-glass homogenizers at 0° using the buffer in which the assay was to be carried out. Where that buffer was tris, which has a large pH–temperature coefficient, the homogenate was made in 0·9% (w/v) sodium chloride. To obtain optically clear solutions of the enzymes for the assays the homogenates were centrifuged at 70,000 *g* for 30 min. All steps in the preparation of the supernatants were carried out at 0–4°.

The assays used were modifications of standard enzymatic methods devised to measure substrate concentrations by linking a reaction between the substrate and an enzyme directly or indirectly to the oxidation or reduction of NAD or NADP and measuring the change of absorbance at 340 nm.[14] In these methods the amount of substrate is limiting. In the present methods sufficient substrate, cofactors and linking enzymes were employed to ensure that the limiting factor was the activity of the enzyme under investigation and this was estimated in terms of the rate of change of absorbance at 340 nm. Care was taken to ensure that the rate of change of absorbance was linear with time.

To measure the effect of the drug on enzymic activity an aliquot of the supernatant from the high speed centrifugation was incubated with buffered (pH 7·4) phenelzine (10 mM, final concentration) at 37°. Samples were removed from the incubation mixture at 10 min intervals for 1 hr and assayed immediately. Control aliquots of the supernatant appropriately buffered and diluted were run in parallel with the tests. Endogenous rates of activity were also measured and corrected for when present.

The Unicam SP800, controlled at 30 ± 0·25° was used throughout.

Formation of β-phenylethylhydrazones

Each of the carbonyl intermediates of glycolysis was separately incubated at 37° with phenelzine in tris buffer (pH 7·4 at 37°) and the absorption spectra examined after various periods. Control aliquots of phenelzine and of the intermediates were similarly treated.

RESULTS AND DISCUSSION

After intraperitoneal injection the blood level of phenelzine rises rapidly to a maximum at about 9 min and thereafter falls with a "half life" of about 20 min. The drug rapidly enters the brain reaching a maximum, depending on the brain region, at between 12 and 20 min. The highest levels and earliest maxima are attained in the caudate nucleus and the diencephalon. In these structures the concentration of the

drug is not markedly different from the level in the blood at any time. The cerebral cortex and the hippocampus reach about 70 per cent of the blood level and the cerebellum and brain stem about 50 per cent with correspondingly later maxima (Fig. 1). There is no evidence of any marked retention of the drug in any brain region but our techniques were almost certainly too gross to detect the probably small amount of the drug required to exert its pharmacological effect at specific and highly circumscribed receptor sites.

Effect of phenelzine on the respiration of tissue slices

The addition of phenelzine to a final concentration of 10 mM in the medium in which brain slices were respiring with glucose as substrate caused a progressive decline in oxygen uptake. As Table 1 indicates, the effect was a general one. In most regions the inhibition was reproducible, the sole exception being the caudate nucleus which in three experiments out of seven showed no inhibition. In general, the time taken to reach 50 per cent inhibition (T_{50}) was about 40 min and there was no significant difference (5 per cent) between the brain regions.

With pyruvate as substrate inhibition occurred less frequently in all regions except the diencephalon and the T_{50} was greater, approaching the time at which respiration

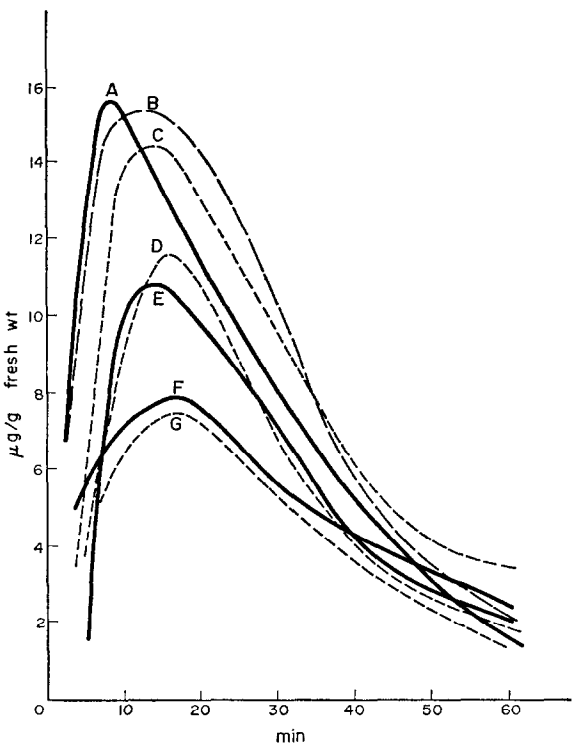

FIG. 1. The concentrations of phenelzine in blood and in various brain regions of the rabbit after intraperitoneal injection. The drug was injected at zero time and samples taken at 3, 5, 10, 20, 30, 45 and 60 min after injection. Curve A, blood; B, caudate nucleus; C, diencephalon; D, cerebral cortex; E, hippocampus; F, cerebellum; G, brain stem.

TABLE 1. THE EFFECT OF PHENELZINE (10 mM) ON THE *in vitro* RESPIRATION OF SLICES FROM VARIOUS BRAIN REGIONS

Brain region	Oxidizable substrate			
	Glucose (11 mM)		Pyruvate (11 mM)	
	Frequency of inhibition	T_{50}(min)	Frequency of inhibition	T_{50}(min)
Cerebral cortex	7/7	50·8 ± 8·1	2/8	150
Caudate N.	4/7	42·0	0/5	—
Hippocampus	7/7	36·1 ± 6·7	5/8	108
Diencephalon	3/3	34·0	3/3	118
Cerebellar cortex	7/7	45·1 ± 9·8	4/8	56
Corpus callosum	7/7	37·7 ± 5·8	0/5	—

The oxidizable substrates were as indicated. The time to 50 per cent inhibition (T_{50}) is expressed as the mean ± S.E.M. The values for the diencephalon are the weighted means of a series of frontal slices from A3 to P4.[12] There was no significant variation along the rostrocaudal axis in the diencephalon.

of brain slices *in vitro* begins normally to decline. The persistent sensitivity of the diencephalon is perhaps significant in view of the concentration of biogenic amines in this region.

These results establish the effect as primarily one on glycolysis although there is some evidence of an effect operating on or peripheral to the citric acid cycle. The most likely peripheral site is the γ-aminobutyrate shunt,[15] a pyridoxal phosphate dependent, alternative pathway between α-oxoglutarate and succinate which operates in brain and is sensitive to the presence of hydrazides, e.g. thiosemicarbazide. However, addition of pyridoxal phosphate up to 0·5 mM did not relieve the inhibition. The addition of γ-aminobutyrate (final concentration 10^{-3} M) produced a result whose possible significance was not clear until after some of the results reported below became available: with glucose (11 mM) as substrate and in the presence of phenelzine (10 mM) and γ-aminobutyrate (10 mM) the respiratory rates of slices from all brain regions began their usual decline but after about 30 min the decline ceased and the respiratory rates became linear with time at about 50–60 per cent of the initial values. This effect was quite reproducible.

Effect of phenelzine on the activity of the glycolytic enzymes

Every enzyme in the glycolytic sequence between glucose and lactate was investigated. In every case full activity of the enzyme was observed even after 1 hr incubation with the drug which is long enough completely to inhibit monoamine oxidase and to reduce the *in vitro* respiration to a very low level.

Formation of hydrazones

The possibility that phenelzine was involved in hydrazone formation with one or more of the carbonyl intermediates of glycolysis thereby reducing substrate availability and so reducing the glycolytic flux was next considered. Only pyruvate and glyceraldehyde-3-phosphate showed significant spectral changes on incubation with phenelzine.

Phenelzine showed a high peak of absorption at 217 nm and a smaller one at 257 nm.

Pyruvate had a single peak at 230 nm. On incubating phenelzine and pyruvate together a new peak immediately appeared at 227 nm which increased slowly with time and shifted to a slightly longer wavelength. A second peak appeared at 260 nm and continued to increase for several hours (Fig. 2).

Glyceraldehyde-3-phosphate showed a broad assymetrical absorption band between 215 and 325 nm. The reaction between glyceraldehyde-3-phosphate and phenelzine produced a new broad band with a maximum at 236 nm which increased in intensity with time eventually swamping a minor peak at 217 nm which also increased (less rapidly) with time and shifted to a slightly longer wavelength (Fig. 3).

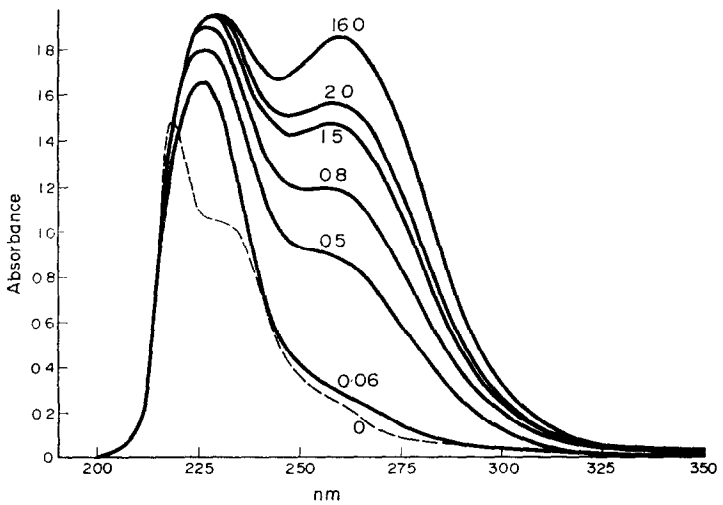

FIG. 2. Absorption spectra of the products of the reaction between phenelzine and pyruvate at pH 7·4 and 37°. The values on the curves are the times in hours after mixing. The dotted curve is the sum of the absorption spectra of pyruvate (5·5 mM) and phenelzine (1 mM) read separately. The concentrations of the reactants in the mixture were also 5·5 and 1 mM respectively.

The hypothesis that hydrazone formation was reducing the availability of glyceraldehyde-3-phosphate and/or pyruvate and so reducing the glycolytic flux was examined directly using the triose phosphate isomerase and lactate dehydrogenase assay systems. In the control assays the amounts of the substrates were adjusted so that they became the rate limiting factors. In the test assays samples of the respective substrates at the adjusted concentrations were incubated with phenelzine before being added to the assay reaction mixture. That a reaction had occurred between the substrates and phenelzine was checked spectrophotometrically. No significant difference between the rates of reaction with the untreated substrates and those with the phenelzine-treated substrates could be detected from which it was concluded that either the hydrazones were adequate substrates for the respective enzymes—a highly improbable proposition —or that the amount of hydrazone formed was insignificantly small. In this connection it should be noted that some hydrazones absorb strongly in the u.v. with extinction coefficients of the order of 1·5 to 2×10^4.

The failure of phenelzine to inhibit any of the glycolytic enzymes or to "lock-up" significant amounts of any of the glycolytic intermediates suggested that the site of

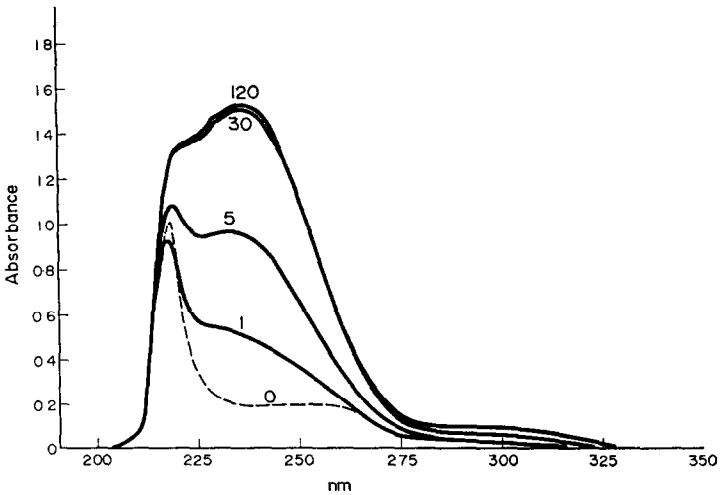

FIG. 3. The absorption spectra of the products of the reaction between phenelzine and glyceraldehyde-3-phosphate at pH 7·4 and 37°. The values on the curves are the times in minutes after mixing. The dotted curve is the sum of the absorption spectra of phenelzine (1 mM) and glyceraldehyde-3-phosphate (4·1 mM) read separately. The concentrations of the reactants in the mixture were also 1 and 4·1 mm respectively.

the phenelzine induced inhibition of respiration was not intracellular. This conclusion was confirmed by the observation that phenelzine failed to inhibit the respiration of brain tissue homogenates with glucose as substrate.

Respiratory studies with rat brain homogenates

The incubation medium of Larner *et al.*[13] contains both pyruvate and glucose. Using this complete medium the control aliquots of the homogenate respired at the same rate as aliquots incubated in a medium containing phenelzine (Table 2). Since the possibility existed that the respiration was occurring at the expense of pyruvate, a second series of experiments was performed in which the pyruvate was omitted. This resulted, predictably, in lower respiratory rates but there was still no difference between the tests and the controls (Table 2).

TABLE 2. THE EFFECT OF PHENELZINE (FINAL CONCENTRATION 10 mM) ON THE RESPIRATION OF WHOLE RAT BRAIN HOMOGENATES

	Incubation medium			
	Complete		Pyruvate omitted	
	Control	With phenelzine	Control	With phenelzine
Mean	6·2	6·0	3·6	3·2
Range	5·4–7·0	5·5–6·6	3·2–4·0	2·6–3·6

The complete incubation mixture (Larner *et al.*[15]) contained, *inter alia*, glucose and pyruvate. Respiratory rates expressed as $\mu 10_2$/mg tissue, fresh wt./hr. There were four experiments in each group.

The cumulative indication of all these results is that phenelzine inhibits respiration by an action at the cell membrane since it is inhibitory towards preparations containing intact cells but not towards homogenates or individual enzymes or substrates. It was not considered likely that phenelzine interacted with enzymic cofactors since for the most part these are common to the steps which precede and follow pyruvate in glucose catabolism. Specifically, it was postulated that phenelzine reduced the uptake of glucose by the cells. Such a reduction could be brought about either by reducing the binding of glucose to the cell membrane (by direct competition or allosteric intervention) or by interference with the mechanism which carries glucose across the membrane. The latter is as yet a theoretical and untestable concept. On the other hand interference at the glucose binding site can be investigated by changing the relative

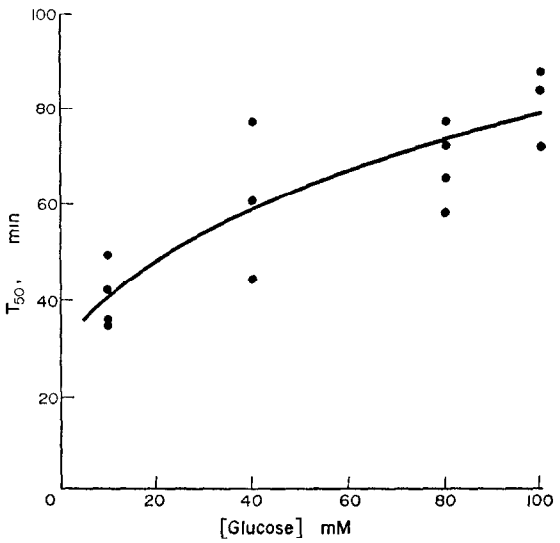

FIG. 4. The effect of increasing the glucose concentration in the medium on the phenelzine induced inhibition of respiration of brain slices *in vitro*. T_{50} is the time taken to reduce the respiration to 50 per cent of its uninhibited value. In every case the concentration of phenelzine was 10 mM.

concentrations of glucose and phenelzine in the incubation medium. In the case of direct competition between glucose and phenelzine for a common binding site increasing the concentration of glucose should favour its inward transport but if the intervention is of the allosteric type it should not be affected by the glucose concentration.

The results of such an experiment are shown in Fig. 4. It is seen that increasing the glucose concentration in the medium from 10 mM to 100 mM increased the T_{50} from 40 to 79 min. This suggests that glucose and phenelzine compete for common, or at least overlapping, binding sites but it does not exclude some form of competition involving the transmembranal carrier system.

It will be recalled that γ-aminobutyrate was able to exert some measure of protection against the inhibition of respiration by phenelzine. The nature of this protection was initially obscure on an intracellular basis but it could now find an explanation in terms of competition with phenelzine for its binding site. Consideration of the chemical constitutions of the two compounds suggests a considerable degree of steric similarity

that could allow γ-aminobutyrate to substitute for phenelzine at the binding site, and the steady respiration at a subnormal level in the presence of both phenelzine and γ-aminobutyrate might reflect the attainment of an equilibrium between the two at a common binding site.

The passage of glucose into mammalian cells occurs by facilitated diffusion, a process which is not as specific nor as endergonic as active transport but which is more selective than concentration-directed diffusion across a passive membrane. A carrier molecule in the membrane is almost certainly involved in facilitated diffusion and the inward movement of glucose appears to be associated with the outward transport of Na^+. The present results suggest that phenelzine interferes with this system but whether the interference is with the inward transport of glucose or the outward transport of Na^+ is not clear.

It has already been shown that anti-depressants can influence membrane transport mechanisms. For example, imipramine blocks the uptake by the adrenergic presynaptic membrane of biogenic amines[16] and also the passage of noradrenaline across the platelet membrane,[17] desipramine acts as a competitive inhibitor of the intracellular mechanism for the transport of the biogenic amines into their storage granules[18] and the MAOI, nialamide reduces the rate at which K^+ traverses membranes.[19] In the absence of a precise definition of any of these transport systems it is not possible to suggest reaction mechanisms. It is, however, interesting to note that two groups of compounds with very different chemical structures but a common therapeutic function also apparently show a common participation in membrane transport phenomena.

Acknowledgements—The phenelzine was generously supplied by W. R. Warner and Co. Ltd., Eastleigh, Hampshire, England.

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