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# Metabolism of 2-Phenylethylamine to Phenylacetic Acid, *Via* the Intermediate Phenylacetaldehyde, by Freshly Prepared and Cryopreserved Guinea Pig Liver Slices

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**Abstract.** *Background: 2-Phenylethylamine is an endogenous amine, which acts as a neuromodulator of dopaminergic responses. Exogenous 2-phenylethylamine is found in certain foodstuffs and may cause toxic side-effects in susceptible individuals. Materials and Methods: The present investigation examined the metabolism of 2-phenylethylamine to phenylacetic acid, via phenylacetaldehyde, in freshly prepared and cryopreserved liver slices. Additionally, it compared the relative contribution of aldehyde oxidase, xanthine oxidase and aldehyde dehydrogenase by using specific inhibitors for each oxidizing enzyme. Results: In freshly prepared and cryopreserved liver slices, phenylacetic acid was the main metabolite of 2-phenylethalamine. In freshly prepared liver slices, phenylacetic acid was completely inhibited by disulfiram (inhibitor of aldehyde dehydrogenase), whereas isovanillin (inhibitor of aldehyde oxidase) inhibited acid formation to a lesser extent and allopurinol (inhibitor of xanthine oxidase) had no effect. In cryopreserved liver slices, isovanillin inhibited phenylacetic acid by 85 %, whereas disulfiram inhibited acid formation to a lesser extent and allopurinol had no effect. Conclusion: In liver slices, 2-phenylethylamine is rapidly oxidized to phenylacetic acid, via phenylacetaldehyde, by aldehyde dehydrogenase and aldehyde oxidase with no contribution from xanthine oxidase.*

2-Phenylethylamine is a naturally occurring endogenous amine, which is distributed in trace amounts throughout the mammalian brain (1, 2). It is synthesized by decarboxylation of the amino acid L-phenylalanine in dopaminergic neurons of the nigrostiatal system (3), and can act as a neuromodulator of catecholamine neurotransmission in the brain (4, 5). It has been suggested that 2-phenylethylamine may exert its effects by potentiating the post-synaptic effects of dopamine (2, 6) and sufficiently high doses of 2-phenylethylamine can produce effects comparable to those of cocaine or methamphetamine (7).

Exogenous 2-phenylethylamine is found in certain foodstuffs and has been known to trigger migraine attacks in susceptible individuals (8, 9). 2-Phenylethylamine, an ingredient in chocolate, may initiate a headache by alteration of cerebral blood flow and release of norepinephrine from sympathetic nerve cells (8, 9). From other common dietary precipitants, a large number of cheeses contain 2-phenylethylamine (10, 11), as do some red wines (12, 13).

2-Phenylethylamine is metabolized to phenylacetaldehyde by a monoamine oxidase B catalyzed oxidative deamination (14, 15), which is then converted to phenylacetic acid by aldehyde dehydrogenase (EC 1.2.1.3, aldehyde-NAD(P)$^+$ oxidoreductase) (16) and possibly by xanthine oxidase (EC 1.2.3.2, xanthine-oxygen oxidoreductase) and aldehyde oxidase (EC 1.2.3.1, aldehyde-oxygen oxidoreductase) (17).

Precision-cut liver slices are used extensively as a suitable *in vitro* tool to study xenobiotic metabolism (17-19). They are easily prepared from a number of animal species, including man, by using a similar procedure. Because no proteolytic treatment is needed for their preparation, the normal tissue architecture, the cell heterogeneity and cell-to-cell interactions are maintained in slices (20, 21). Cryopreservation of liver slices would greatly facilitate their storage for long periods of time and thus reduce the number of laboratory animals used. The application of a rapid freezing technique, by direct immersion into liquid nitrogen, allows the maintenance of urea synthesis, sulfoconjugation and CYP-dependent oxidation of ethoxycoumarin, testosterone hydroxylation and N-deethylation of lidocaine at the same level as non-cryopreserved rat or human precision-cut liver slices after 2 or 3 hours of incubation (22,

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*Key Words:* Aldehyde dehydrogenase, aldehyde oxidase, allopurinol, biogenic amines, liver slices, isovanillin, phenylacetaldehyde, 2-phenylethylamine, xanthine oxidase, disulfiram.

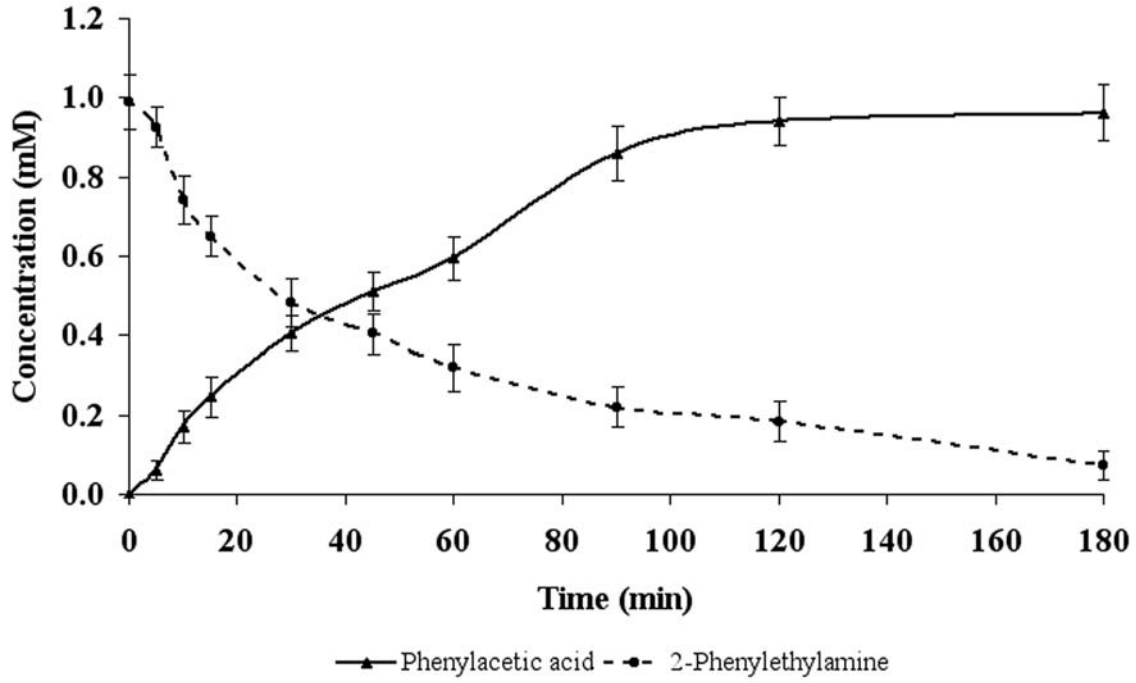


Figure 1. *Metabolism of 2-phenylethylamine by freshly prepared guinea pig liver slices. 2-Phenylethylamine ($1x10^{-3}$M) was incubated with four freshly prepared guinea pig liver slices (120 mg) in 3 ml of oxygenated Krebs-Henseleit buffer pH 7.4 containing $2.4x10^{-2}$M bicarbonate at 37°C. Each point is the mean of seven determinations. Values are expressed as means±SE.*

23) and with no significant loss of both phase I- and phase II-mediated drug metabolism (24, 25).

In the present study, the enzymatic oxidation of 2-phenylethylamine to phenylacetaldehyde and its subsequent conversion to phenylacetic acid was examined in guinea pig freshly prepared and cryopreserved liver slices. Incubations were also performed in the presence of specific inhibitors for several oxidizing enzymes, in order to determine which of these enzymes are involved in the formation of phenylacetic acid from phenylacetaldehyde and thus from 2-phenylethylamine. The specific inhibitors used were isovanillin for aldehyde oxidase (26, 27), allopurinol for xanthine oxidase (28) and disulfiram for aldehyde dehydrogenase activity (29, 30). Finally, it was determined if the enzymes monoamine oxidase, aldehyde oxidase, xanthine oxidase and aldehyde dehydrogenase were active in cryopreserved liver slices compared to freshly prepared liver slices.

## Materials and Methods

*Animals.* Dunkin-Hartley guinea pigs, weighing 450-950 g, were used in this study. The animals had free access to food and tap water and were maintained in a strictly controlled temperature (18±1°C), humidity (50-55%) and lighting cycle (07.00-19.00 h light, 19.00-07.00 h dark). The animals were fed with FD1 pellets supplemented with ascorbic acid and received hay three times weekly. They were handled with humane care in accordance with the National Institutes of Health guidelines.

*Chemicals.* Phenylacetaldehyde, 2-phenylethanol, 2-phenylethylamine hydrochloride, tetraethylthiuram disulfide (disulfiram), allopurinol, diethylamine, DL-mandelic acid and isovanillin were supplied from Sigma-Aldrich Chemical Company Ltd. (Poole, Dorset, UK), phenylacetic acid and perchloric acid from BDH Chemicals Ltd. (Poole, Dorset, UK), and acetonitrile HPLC grade from Rathburn Chemicals Ltd. (Walkeburn, Scotland).

*Preparation of freshly prepared and cryopreserved guinea pig liver slices.* Guinea pigs were killed by cervical dislocation and their livers were placed in ice-cold Krebs-Henseleit solution pH 7.4 containing $2.4x10^{-2}$M bicarbonate, which was continuously oxygenated with 95 % $O_2$/5% $CO_2$. Freshly prepared liver slices were obtained according to the method of Panoutsopoulos *et al.* (17) and stored in oxygenated Krebs-Henseleit buffer pH 7.4 containing $2.4x10^{-2}$M bicarbonate at 4°C until required. Cryopreserved liver slices were then prepared by direct immersion of the freshly prepared liver slices in liquid nitrogen and kept there until required.

*Incubation conditions with freshly prepared and cryopreserved guinea pig liver slices.* 2-Phenylethylamine ($1x10^{-3}$M) was incubated with four

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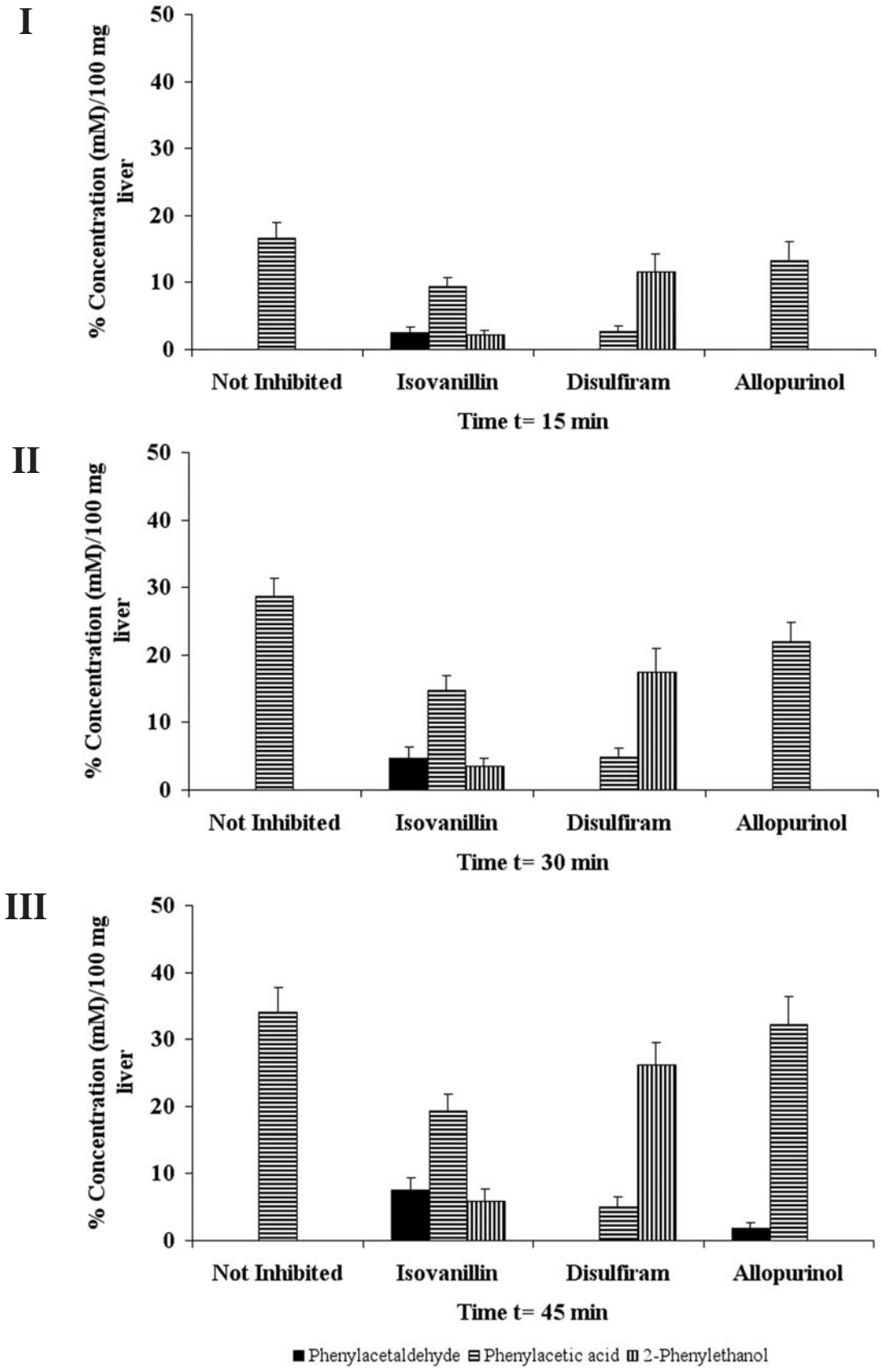
*Figure 2. Effect of inhibitors on the oxidation of 2-phenylethylamine by freshly prepared guinea pig liver slices. 2-Phenylethylamine ($1x10^{-3}$M) was incubated with freshly prepared guinea pig liver slices in Krebs-Henseleit buffer pH 7.4 containing $2.4x10^{-2}$M bicarbonate in the absence and presence of the inhibitors isovanillin ($1x10^{-3}$M), disulfiram ($1x10^{-4}$M) and allopurinol ($1x10^{-4}$M) at: (i) t=15 min, (ii) t=30 min, and (iii) t=45 min. Each point is the mean of seven determinations. Values are expressed as means±SE.*


I
% Concentration (mM)/100 mg liver
50
40
30
20
10
0
Not Inhibited
Isovanillin
Disulfiram
Allopurinol
Time t= 15 min
II
% Concentration (mM)/100 mg liver
50
40
30
20
10
0
Not Inhibited
Isovanillin
Disulfiram
Allopurinol
Time t= 30 min
III
% Concentration (mM)/100 mg liver
50
40
30
20
10
0
Not Inhibited
Isovanillin
Disulfiram
Allopurinol
Time t= 45 min
Phenylacetaldehyde
Phenylacetic acid
2-Phenylethanol

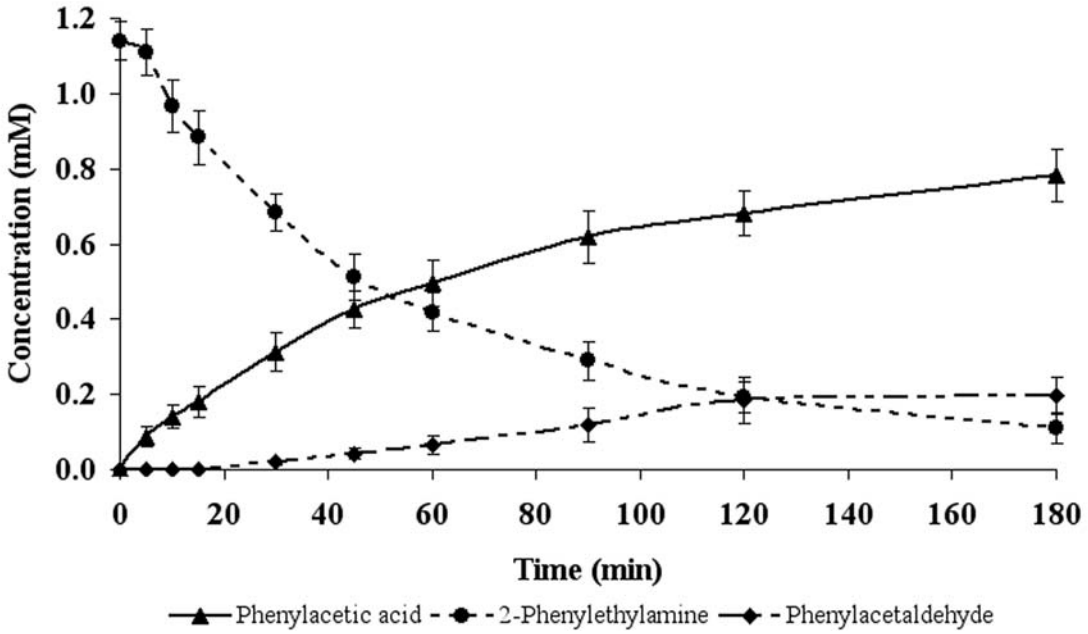



Figure 3. *Metabolism of 2-phenylethylamine with cryopreserved guinea pig liver slices. 2-Phenylethylamine ($1x10^{-3}$M) was incubated with four cryopreserved guinea pig liver slices (122 mg) in 3 ml of oxygenated Krebs-Henseleit buffer pH 7.4 containing $2.4x10^{-2}$M bicarbonate at 37˚C. Each point is the mean of six determinations. Values are expressed as means±SE.*

freshly prepared or cryopreserved liver slices in a total volume of 3 ml Krebs-Henseleit buffer pH 7.4. The addition of the liver slices indicated the beginning of the experiment. The incubations were carried out at 37˚C in a shaking water bath and the medium was oxygenated with 95 % $O_2$/5 % $CO_2$ initially and every subsequent hour for 5 min. Preliminary experiments determined that this amount of oxygenation was sufficient to maintain optimum metabolite production.

Samples (0.2 ml) were removed at t=0 min and at various time intervals, added to 0.1 ml of 3.6 % perchloric acid and centrifuged for 2.5 min at maximum speed on a Beckman microfuge B. The supernatant was then analyzed by HPLC. Control incubations, without the addition of liver slices, were also performed. Standard solutions of the amine and its possible metabolites were also analyzed by HPLC. After analysis, the slices were blotted dry and weighed to determine the total weight of liver used in each incubation.

Incubations with freshly prepared or cryopreserved liver slices were also performed in the presence of inhibitors. For comparison reasons, the results on the effect of inhibitors in freshly prepared and cryopreserved liver slices were normalized per 100 mg of liver.

*HPLC analysis of 2-phenylethylamine and its metabolites.* Reverse-phase HPLC analysis was performed with a Waters Associates (Northwich, Cheshire, UK) system comprising a single piston reciprocating 501 pump, a WISP 710B auto-injector, a Lambda-Max 481 LC variable wavelength detector and a Data Module 740. Samples from all incubations were analyzed on a 25 cm x 4.6 mm (i.d.) 5 μm stainless steel Hypersil ODS column with a Waters Guard Pak pre-column and a C18 insert. The reactions were monitored at 250 nm and 20 μl samples were injected for each analysis. HPLC separation was achieved with a mobile phase containing 30% of HPLC grade acetonitrile and 70 % $2.2x10^{-1}$M orthophosphate buffer, pH 2.9, containing $1.1x10^{-1}$M diethylamine at a flow rate of 1.5 ml/min. The mobile phase was filtered and degassed for 10 min under vacuum before use.

## Results

*Metabolism of 2-phenylethylamine with freshly prepared guinea pig liver slices.* A progressive decrease in the concentration of 2-phenylethylamine (Rt= 2.9±0.12 min, n=15) was observed when it was incubated with freshly prepared guinea pig liver slices (Figure 1). Simultaneously, a single metabolite appeared, which increased with time and accounted for 100 % conversion of 2-phenylethylamine within 180 min (Figure 1). This metabolite was co-chromatographed with phenylacetic acid (Rt=7.7±0.4 min, n=15). Thus, 2-phenylethylamine was quantitatively converted to phenylacetic acid by freshly prepared guinea pig liver slices.

*Inhibition of 2-phenylethylamine metabolism in freshly prepared guinea pig liver slices.* The presence of isovanillin ($1x10^{-3}$M) reduced the production of phenylacetic acid by 48±5% (n=7, t=30 min) and the intermediate phenylacetaldehyde (12.2±0.45 min, n=15) was also apparent in the chromatograms. In addition, small amounts of 2-phenylethanol (7.5±0.3 min, n=15) were also observed in the chromatograms, which could

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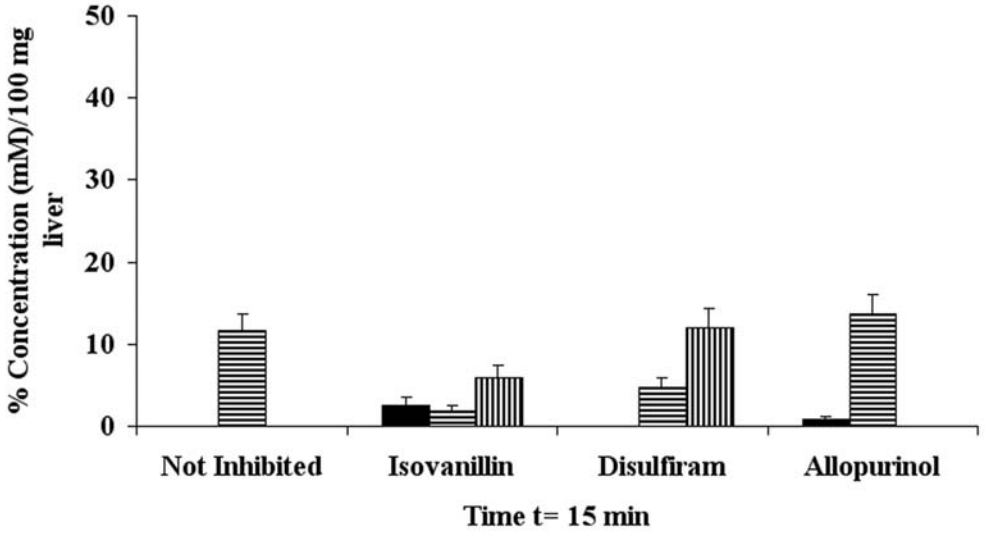
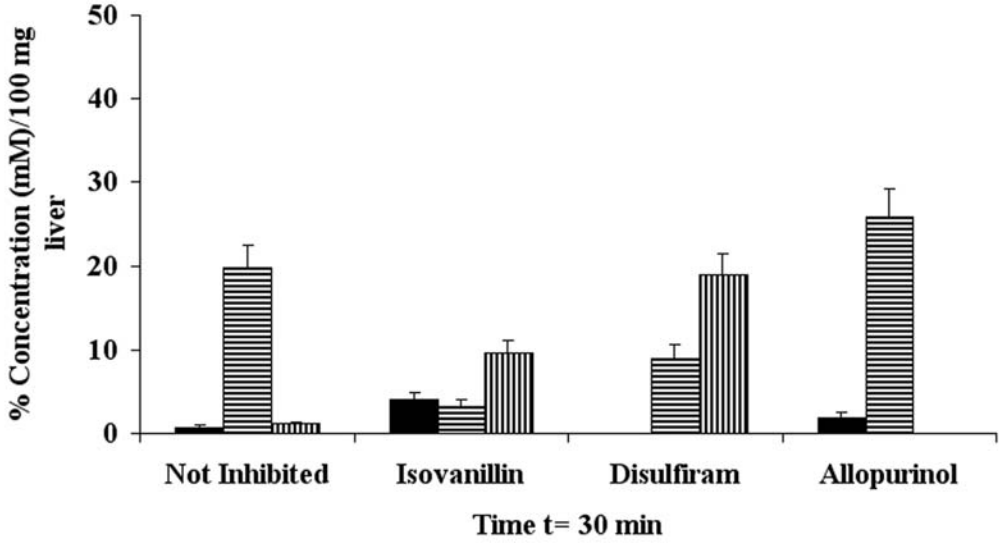
Figure 4. *Effect of inhibitors on the oxidation of 2-phenylethylamine by cryopreserved guinea pig liver slices. 2-Phenylethylamine ($1x10^{-3}$M) was incubated with cryopreserved guinea pig liver slices in Krebs-Henseleit buffer pH 7.4 containing $2.4x10^{-2}$M bicarbonate in the absence and presence of the inhibitors isovanillin ($1x10^{-3}$M), disulfiram ($1x10^{-4}$M) and allopurinol ($1x10^{-4}$M) at: (i) t=15 min, (ii) t=30 min, and (iii) t=45 min. Each point is the mean of six determinations. Values are expressed as means±SE.*

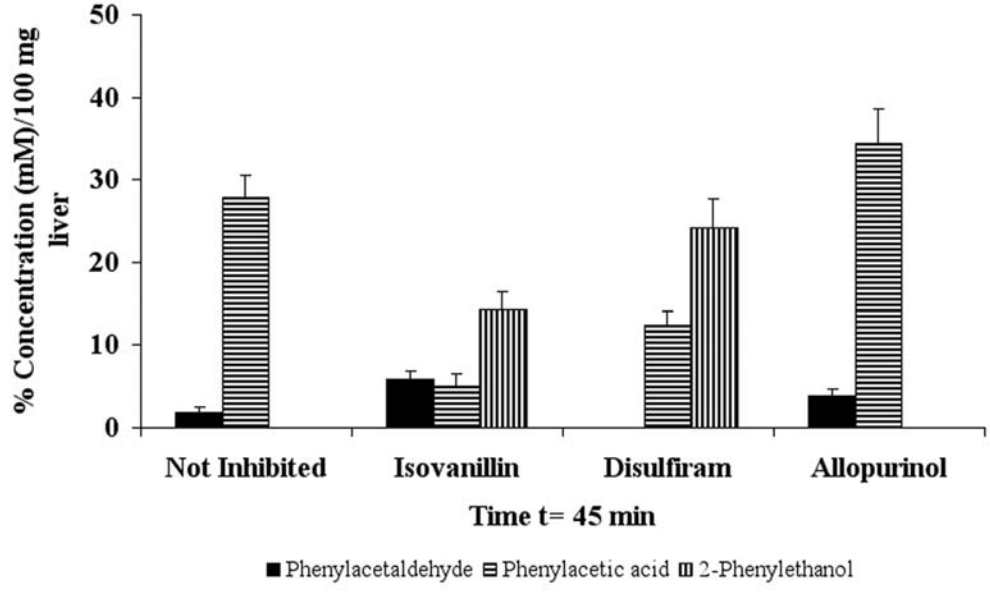
I


% Concentration (mM)/100 mg liver
50
40
30
20
10
0
Not Inhibited
Isovanillin
Disulfiram
Allopurinol
Time t= 15 min

II


% Concentration (mM)/100 mg liver
50
40
30
20
10
0
Not Inhibited
Isovanillin
Disulfiram
Allopurinol
Time t= 30 min

III


% Concentration (mM)/100 mg liver
50
40
30
20
10
0
Not Inhibited
Isovanillin
Disulfiram
Allopurinol
Time t= 45 min
Phenylacetaldehyde
Phenylacetic acid
2-Phenylethanol

be formed by reduction of phenylacetaldehyde in liver slices (Figure 2(i), (ii) and (iii)).

In the presence of allopurinol ($1x10^{-4}$M), there was a small decrease in the production of phenylacetic acid (Figure 2(i), (ii) and (iii)). However, small amounts of phenylacetaldehyde were also observed at 45 min (Figure 2(iii)), which increased with time up to 90 min and then gradually decreased (data not shown).

Disulfiram ($1x10^{-4}$M) caused an 83±6 % (n=7, t=30 min) inhibition in the production of phenylacetic acid over the incubation time period. However, there was a significant increase in the formation of 2-phenylethanol (Figure 2(i), (ii) and (iii)).

*Metabolism of 2-phenylethylamine with cryopreserved guinea pig liver slices.* In cryopreserved guinea pig liver slices, 2-phenylethylamine progressively disappeared with time and two metabolites were formed. The major metabolite was phenylacetic acid, whereas the minor metabolite was 2-phenylethanol (Figure 3). The concentrations of both metabolites accounted for all of the 2-phenylethylamine transformed.

*Inhibition of 2-phenylethylamine metabolism in cryopreserved guinea pig liver slices.* Isovanillin ($1x10^{-3}$M) caused an 84±7% (n=6, t=30 min) inhibition of phenylacetic acid formation in cryopreserved liver slices (Figure 4(i), (ii) and (iii)). The two other minor metabolites, phenylacetaldehyde and 2-phenylethanol, were slightly increased with time.

The presence of allopurinol ($1x10^{-4}$M) caused a slight enhancement in both phenylacetic acid and phenylacetaldehyde production (Figure 4(i), (ii) and (iii)).

Disulfiram ($1x10^{-4}$M) resulted in 55±8 % (n=6, t=30 min) inhibition in the production of phenylacetic acid (Figure 4(ii)). In addition, it was shown that, upon inhibition with disulfiram, there was a significant increase of 2-phenylethanol, which was the major metabolite (Figure 4(i), (ii) and (iii)).

## Discussion

The results showed that, in freshly prepared liver slices, 2-phenylethylamine is converted into a single metabolite, which is phenylacetic acid. The effects of the inhibitors tested showed that disulfiram, a specific inhibitor of aldehyde dehydrogenase activity (29, 30), caused an 85 % inhibition in phenylacetic acid formation and simultaneously 2-phenylethanol was also observed, which increased with time. 2-Phenylethanol could be formed by reduction of the intermediate phenylacetaldehyde in liver slices. Incubations, under similar conditions, with homovanillamine and 5-hydroxytryptamine, also resulted in their corresponding alcohol derivatives (31). Isovanillin, a specific inhibitor of aldehyde oxidase (26, 27), also inhibited phenylacetic acid production, but to a lesser extent than that of disulfiram. In addition, the intermediate phenylacetaldehyde and 2-phenylethanol were also apparent in the chromatograms, which both increased with time. Finally, allopurinol, a specific inhibitor of xanthine oxidase (28, 32), caused a slight inhibition in the production of phenylacetic acid.

Therefore, the results with freshly prepared liver slices indicate that the enzymes involved in the oxidation of 2-phenylethylamine, *via* the intermediate phenylacetaldehyde, are thought to be aldehyde oxidase and aldehyde dehydrogenase, whereas xanthine oxidase does not appear to contribute in the phenylacetaldehyde metabolism.

In cryopreserved liver slices, the major metabolite of 2-phenylethylamine is phenylacetic acid, whereas the minor metabolite is the intermediate phenylacetaldehyde, which increased with time. The enzymes involved in the oxidation of the intermediate phenylacetaldehyde are thought to be aldehyde oxidase and aldehyde dehydrogenase, since phenylacetic acid production is 85% inhibited by isovanillin and 55% inhibited by disulfiram, respectively. The presence of allopurinol on phenylethylamine oxidation, *via* phenylacetaldehyde, caused a small enhancement in phenylacetic acid production, which was not significant and therefore does not contribute to the metabolism of the intermediate phenylacetaldehyde. A similar enhancement in acid production was seen when phenylacetaldehyde (17) or vanillin (33) was incubated with guinea pig fresh liver slices in the presence of allopurinol. This enhancement may be due to xanthine dehydrogenase, which is present in liver slices. Xanthine oxidase differs from xanthine dehydrogenase in the presence of a sulphydryl group (34). Conversion of xanthine oxidase from dehydrogenase (D form) into an oxidase (O form) occurs upon oxidation or binding of sulphydryl groups by several oxidizing agents or ligands (35, 36). This may increase the formation of phenylacetic acid.

Phenylacetaldehyde is a substrate of ALDH-1 (Km=$1.5x10^{-6}$M) (37) and ALDH-2 with Km values of $0.6x10^{-6}$M (37) and $2.9x10^{-8}$M (38). The reaction of phenylacetaldehyde with rat brain mitochondrial aldehyde dehydrogenase has been reported by Weiner and Ardelt (39), who found a Km value of $1.3x10^{-5}$M. Oxidation of phenylacetaldehyde to phenylacetic acid, *via* guinea pig liver aldehyde oxidase, has been previously reported ($K_m$=$5.3x10^{-5}$M, $V_{max}$=0.44 μmol/min/mg protein and $K_s$=5.0 ml/min/mg protein) using ferricyanide as an electron acceptor (40).

Precision-cut liver slices are a simple preparation system where both the cells and the integrity of the organ remain intact. Although biochemical parameters of viability, such as measurement of the intracellular $K^+$ and ATP content (20, 41), which reflect on alterations in cell membrane integrity, were not tested here, the freshly prepared and cryopreserved

liver slices were viable in respect of the oxidation of compounds tested in this study. This may, in part, be due to the short incubation time periods used during experimentation.

The fact that 2-phenylethylamine is transformed to phenylacetic acid shows that monoamine oxidase activity (14, 15, 42) in both freshly prepared and cryopreserved liver slices is also maintained. As monoamine oxidase is a mitochondrial enzyme, this suggests that there is no difficulty in the diffusion of substrate into the liver slices. Incubations of 4-hydroxy-3-methoxy-2-phenylethylamine with freshly prepared liver slices also transformed into their acid derivatives under the same conditions (31). However, in order to determine the relative contribution of each form of monoamine oxidase in the deamination of the amines, further studies could involve a specific monoamine oxidase A substrate, such as noradrenaline (14, 42) and 5-hydroxytryptamine (14), or the use of selective monoamine oxidase inhibitors. Such selective inhibitors are widely used where monoamine oxidase A is preferentially inhibited by clorgyline (43, 44) and monoamine oxidase B is selectively inhibited by pargyline (45) and L-deprenyl (44, 45).

This study showed that several enzyme systems were maintained within freshly prepared and cryopreserved liver slices when incubated in Krebs-Henseleit buffer pH 7.0. These include the oxidizing enzymes, aldehyde oxidase, xanthine oxidase and aldehyde dehydrogenase, as was shown by using potent selective inhibitors for each enzyme tested. In addition, the formation of alcohol metabolites from aldehydes indicates that alcohol dehydrogenase was also active in guinea pig liver slices.

Although it has been demonstrated that aldehyde oxidase activity is maintained in cryopreserved liver slices where aldehyde dehydrogenase activity seems to be low when a simple, rapid freezing technique is used, the optimum conditions for preservation have still to be determined. Previous work has shown that cryopreserved pig and human liver slices retain between 80-85% and 54-89% viability, respectively, compared to freshly prepared liver slices. In these studies, slices were gradually frozen at 12˚C/min in fetal calf serum and DMSO and the intracellular $K^+$ content and protein synthesis was monitored (46).

However, in order to positively establish the relative contribution of each of these enzymes and, in particular, the role of aldehyde oxidase in guinea pig liver, other ways of inhibiting the aldehyde oxidase activity should also be tried. Therefore, it would be of interest to decrease aldehyde oxidase levels *in vivo* by treatment of the animals with tungsten (47) and/or the selective inhibitor hydralazine (48).

In freshly prepared and cryopreserved liver slices, the results confirmed the involvement of aldehyde oxidase in the metabolism of the aldehydes derived from biogenic amines. Therefore, the enzymes involved in the oxidation reaction of 2-phenylethylamine are both aldehyde oxidase and aldehyde dehydrogenase, whereas xanthine oxidase did not appear to contribute to their metabolism.

In conclusion, it would appear that aldehyde oxidase is not only important in the metabolism of aldehydes derived from drugs, xenobiotics, foods and flavourings, but also in the metabolism of the intermediate aldehyde derivatives from biogenic amines, such as 2-phenylethylamine.

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