

Enzymatic Oxidation of Vanillin, Isovanillin and Protocatechuic Aldehyde with Freshly Prepared Guinea Pig Liver Slices

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Key Words

Aldehyde dehydrogenase • Aldehyde oxidase • Allopurinol • Disulfiram • Isovanillin • Liver slices • Protocatechuic aldehyde • Vanillin • Xanthine oxidase

Abstract

Background/Aims: The oxidation of xenobiotic-derived aromatic aldehydes with freshly prepared liver slices has not been previously reported. The present investigation compares the relative contribution of aldehyde oxidase, xanthine oxidase and aldehyde dehydrogenase activities in the oxidation of vanillin, isovanillin and protocatechuic aldehyde with freshly prepared liver slices. **Methods:** Vanillin, isovanillin or protocatechuic aldehyde was incubated with liver slices in the presence/absence of specific inhibitors of each enzyme, followed by HPLC. **Results:** Vanillin was rapidly converted to vanillic acid. Vanillic acid formation was completely inhibited by isovanillin (aldehyde oxidase inhibitor), whereas disulfiram (aldehyde dehydrogenase inhibitor) inhibited acid formation by 16% and allopurinol (xanthine oxidase inhibitor) had no effect. Isovanillin was rapidly converted to isovanillic acid. The formation of isovanillic acid was not altered by allopurinol, but

considerably inhibited by disulfiram. Protocatechuic aldehyde was converted to protocatechuic acid at a lower rate than that of vanillin or isovanillin. Allopurinol only slightly inhibited protocatechuic aldehyde oxidation, isovanillin had little effect, whereas disulfiram inhibited protocatechuic acid formation by 50%. **Conclusions:** In freshly prepared liver slices, vanillin is rapidly oxidized by aldehyde oxidase with little contribution from xanthine oxidase or aldehyde dehydrogenase. Isovanillin is not a substrate for aldehyde oxidase and therefore it is metabolized to isovanillic acid predominantly by aldehyde dehydrogenase. All three enzymes contribute to the oxidation of protocatechuic aldehyde to its acid.

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Introduction

Precision-cut liver slices are described as valuable tools for in vitro drug metabolism of xenobiotics and toxicological studies [1-4]. They are easily prepared from different animal species, including man, using the same method and they closely resemble the organ from which

they are derived. Liver slices can retain their normal liver hepatocyte population and their physiological and biochemical functions for many hours [5, 6]. Successful experimentation depends on the maintenance of slice viability over the designated experimental period. The slices, however, must be thin enough to allow nutrients, gases and waste exchange to occur [7, 8].

Aldehydes are reactive organic compounds, which occur as natural constituents in a wide variety of foods and food additives [9, 10]. Many drugs, with an amino group, can be transformed into aldehydes via the action of monoamine oxidase. Hydroxylation of an aromatic methyl group to an alcohol, can be subsequently oxidized to form an aldehydic group [11]. Other exogenous aldehydes can also occur through the ingestion of metabolic precursors such as the primary alcohols. Endogenous aldehydes are produced via oxidative deamination of compounds such as biogenic amines catalyzed by monoamine oxidase [3, 12].

Biogenic and xenobiotic aldehydes can either be oxidized to the corresponding acids, which may be catalyzed by aldehyde dehydrogenase (EC 1.2.1.3, aldehyde-NAD(P)⁺ oxidoreductase), aldehyde oxidase (EC 1.2.3.1, aldehyde-oxygen oxidoreductase) and/or xanthine oxidase (EC 1.2.3.2, xanthine-oxygen oxidoreductase), or reduced by aldehyde reductase and/or alcohol dehydrogenase to the corresponding alcohols. However, the main metabolic route is conversion to acids.

4-Hydroxy-3-methoxybenzaldehyde (vanillin) has been shown to be an excellent substrate of aldehyde oxidase with a lower affinity for xanthine oxidase [13, 14]. In contrast, 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde) and 3-hydroxy-4-methoxybenzaldehyde (isovanillin) were shown to be reasonable substrates of xanthine oxidase but potent competitive inhibitors of aldehyde oxidase [13, 14].

Vanillin and isovanillin have been previously tested as substrates for aldehyde dehydrogenase, where both compounds were found as substrates for guinea pig liver mitochondrial aldehyde dehydrogenase [14] and for *Rhodospseudomonas acidophila* M402 aldehyde dehydrogenase [15]. Protocatechuic aldehyde was found as substrate of human [16] and guinea pig [14] liver mitochondrial aldehyde dehydrogenase.

Vanillin is found naturally in vanilla beans and is synthesized in the food industry for use as a flavoring agent in confectionery, beverages and foodstuffs [17]. It is also found in barrel-aged wine [18] and is a by-product of curcumin from turmeric [19].

Vanillin acts as an anticarcinogen by reducing the number of small intestinal tumors, which are induced from several carcinogens [20, 21]. It also acts as an antioxidant and pro-oxidant by decreasing the induced damage from H₂O₂ [22]. It exerts its antimutagenic activity in damaged cells by promoting recombination and rejoining of DNA at homologous sites [23] and/or by inhibiting DNA repair at the non-homologous end-joining due to DNA-dependant protein kinase activity inhibition from vanillin [24]. Therefore, vanillin might have beneficial antimutagenic effects and the ability to potentiate the effectiveness of anticancer drugs.

Protocatechuic aldehyde has been demonstrated to induce apoptotic cell death in cytotoxic T-cells (CTL-2) either by inhibiting the ornithine decarboxylase activity, which is induced by IL-2, or by interfering with MAPK signaling pathways [25, 26].

The present investigation examines the oxidation of the substituted benzaldehydes vanillin, isovanillin and protocatechuic aldehyde with freshly prepared precision-cut guinea pig liver slices and followed by HPLC. Incubations were also performed in the presence of specific inhibitors for several oxidizing enzymes in order to determine the relative contribution of each enzyme in the oxidation of these aldehydes. Guinea pig liver was chosen in this study as aldehyde oxidase has similar substrate specificity to the human liver enzyme [27, 28].

Materials and Methods

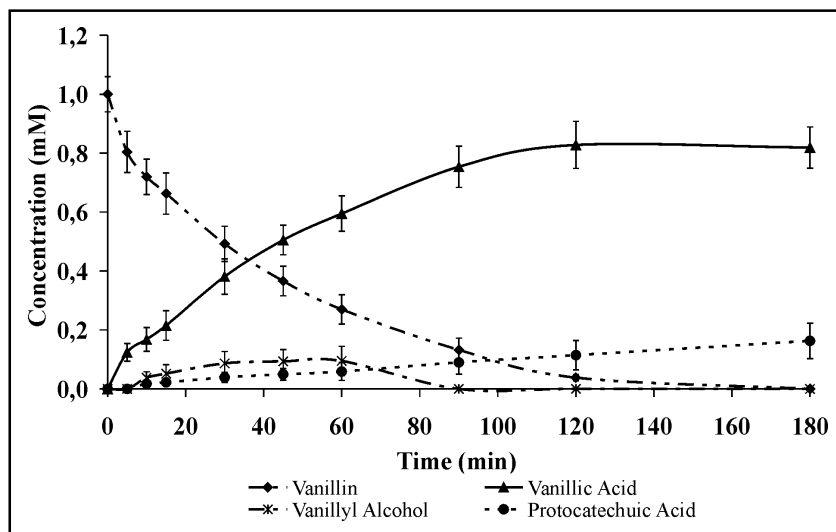
Animals

Dunkin-Hartley female guinea pigs (450-950 g) were used for the investigation. Animals were fed with FDI pellets supplemented with ascorbic acid and received hay three times weekly. All animals were allowed food and water ad libitum and maintained in a strictly controlled temperature (18±1°C), humidity (50 %) and lighting cycle (07.00-19.00 h light, 19.00-07.00 h dark). Animals were handled with humane care in accordance with the National Institutes of Health guidelines.

Chemicals

4-Hydroxy-3-methoxybenzaldehyde (vanillin), 4-hydroxy-3-methoxybenzylalcohol (vanillyl alcohol), 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde), 3,4-dihydroxybenzoic acid (protocatechuic acid), 3-hydroxy-4-methoxybenzaldehyde (isovanillin), 3-hydroxy-4-methoxybenzoic acid (isovanillic acid), tetraethylthiuram disulfide (disulfiram), allopurinol, diethylamine from Sigma Chemical Company Ltd, 3-hydroxy-4-methoxybenzylalcohol

Fig. 1. Metabolism of vanillin by freshly prepared guinea pig liver slices. Vanillin ($1 \times 10^{-3} \text{M}$) was incubated with four guinea pig liver slices (147 mg) in 3 ml of Krebs-Heinseleit buffer pH 7.4 at 37°C . Each point is the mean of five determinations. Values are expressed as means \pm SE.



(isovanillyl alcohol) from Aldrich Chemical Co Ltd, perchloric acid, 4-hydroxy-3-methoxybenzoic acid (vanillic acid) from BDH Chemicals Ltd, and acetonitrile HPLC grade from Rathburn Chemicals Ltd.

High performance liquid chromatography (HPLC)

Reverse-phase HPLC was performed using a system from Waters Associates (Northwich, Cheshire, UK), which consisted of a single piston reciprocating 501 pump, a WISP 710B auto-injector, a Lambda-Max 481 LC spectrophotometer and a Data Module 740. Compounds were separated with a stainless steel Hypersil ODS column $5 \mu\text{m}$ ($25 \text{ cm} \times 4.6 \text{ mm}$ internal diameter). A Waters Guard Pak pre-column with a C18 insert was fitted to the HPLC system for protection of the column from the biological materials. The optimum wavelength was 285 nm and $20 \mu\text{l}$ samples were injected for each analysis. HPLC separation was achieved with a mobile phase containing 15 % acetonitrile and 85 % orthophosphate buffer ($2.2 \times 10^{-1} \text{M}$) pH 2.9 containing $1.1 \times 10^{-1} \text{M}$ diethylamine at a flow rate of 1.5 ml/min. The mobile phase was filtered and degassed for 10 min under vacuum before use.

Preparation of freshly prepared guinea pig liver slices

Guinea pigs were killed by cervical dislocation and their livers were immediately excised. The livers were placed in ice-cold Krebs-Henseleit solution pH 7.4 containing $2.4 \times 10^{-2} \text{M}$ bicarbonate, which was continuously oxygenated with 95 % $\text{O}_2/5 \text{ \% CO}_2$. Liver slices were then obtained according to the method of Panoutsopoulos et al [3] and the freshly prepared liver slices were stored in oxygenated Krebs-Henseleit buffer pH 7.4 containing $2.4 \times 10^{-2} \text{M}$ bicarbonate at 4°C until required.

Incubation conditions with liver slices

Vanillin, isovanillin or protocatechuic aldehyde ($1 \times 10^{-3} \text{M}$) was incubated with 4 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. Incubation mixtures were

maintained at 37°C in a shaking water bath. The medium was oxygenated with 95 % $\text{O}_2/5 \text{ \% CO}_2$ initially and every subsequent hour for 5 min. Incubations with freshly prepared liver slices were also performed in the presence of inhibitors. The inhibitors used were isovanillin for aldehyde oxidase activity [13, 14], allopurinol for xanthine oxidase activity [29] and disulfiram for aldehyde dehydrogenase activity [30, 31].

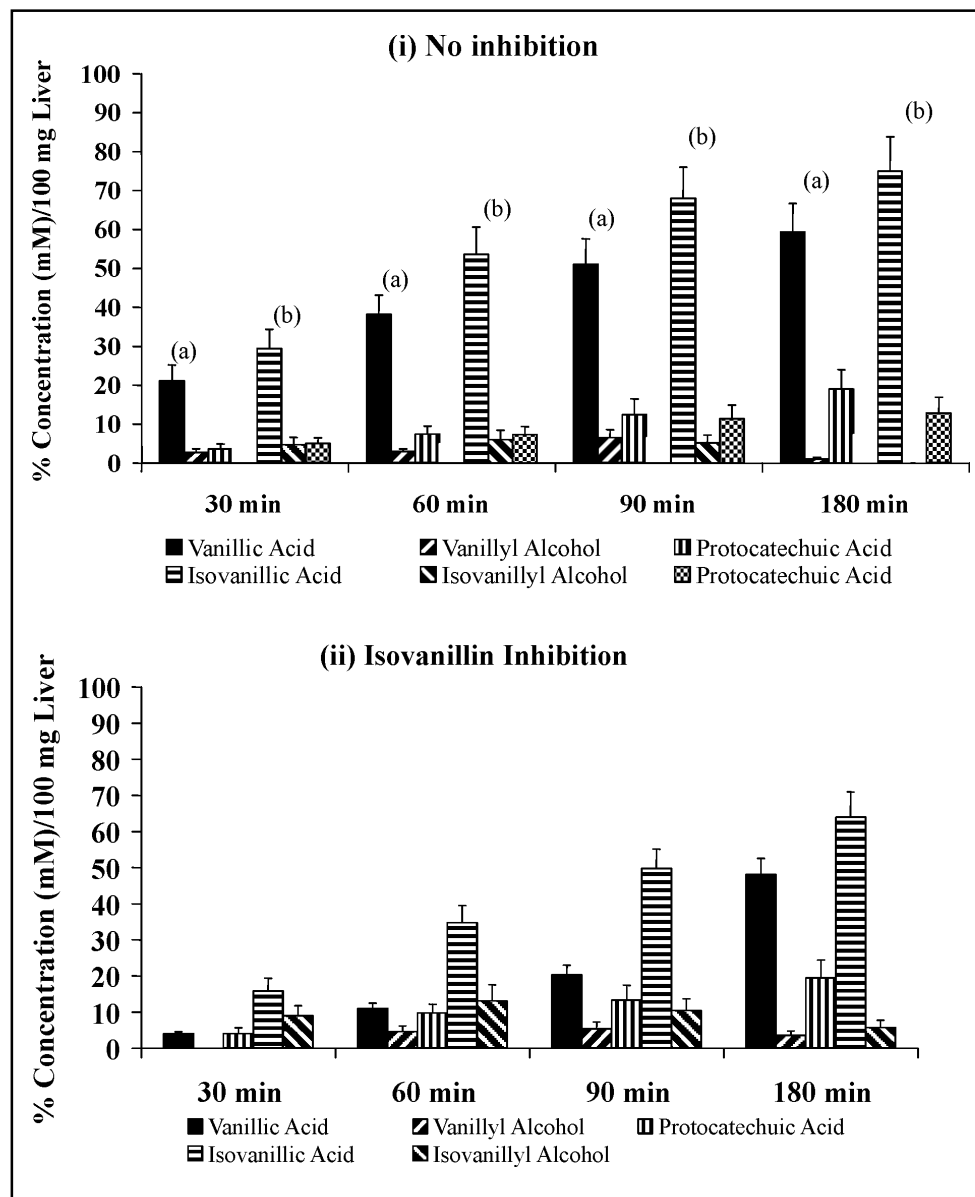
Aliquots (0.2 ml) were removed at $t = 0$ min and at various time intervals, added to 0.1 ml of 3.6 % perchloric acid and centrifuged for 2.5 min at maximum speed on a Beckman microfuge B. The supernatant was then analyzed by HPLC. Control incubations without the liver slices present were also performed. Standard solutions of the aldehydes and their possible metabolites were also analyzed by HPLC. After analysis, the slices were blotted dry and weighed to determine the total weight of liver used. For comparison reasons, the results on the effect of inhibitors in liver slices have been normalized per 100 mg of liver.

Results

Metabolism of vanillin with freshly prepared guinea pig liver slices

Vanillin ($R_t = 13.2 \pm 1.0 \text{ min}$, $n = 7$) concentration was progressively decreased with time, so that after 180 min (Fig. 1) it had been completely transformed into three metabolites. The major metabolite was vanillic acid ($R_t = 7.4 \pm 0.5 \text{ min}$, $n = 7$), which accounted for approximately 82 % conversion of the substrate. The other two minor metabolites were vanillyl alcohol and protocatechuic acid ($R_t = 4.3 \pm 0.5 \text{ min}$, $n = 7$). Vanillyl alcohol increased to its maximum concentrations at 90 min and thereafter gradually disappeared, whereas protocatechuic acid

Fig. 2. Effect of isovanillin on vanillin oxidation by freshly prepared guinea pig liver slices. (i) Vanillin ($1 \times 10^{-3} \text{M}$) (a) and isovanillin ($1 \times 10^{-3} \text{M}$) (b) were incubated individually with four guinea pig liver slices in 3 ml of Krebs-Heinseleit buffer pH 7.4 at 37°C . (ii) Vanillin ($1 \times 10^{-3} \text{M}$) and isovanillin ($1 \times 10^{-3} \text{M}$) were incubated together with four guinea pig liver slices in 3 ml of Krebs-Heinseleit buffer pH 7.4 at 37°C . Each point is the mean of five determinations. Values are expressed as means \pm SE.



accounted for approximately 16 % conversion of vanillin after 180 min and was thought to arise via an O-demethylation reaction. The total amount of all three metabolites accounted for all of the vanillin transformed. Control incubations of vanillin, in the absence of guinea pig liver slices, showed no such decrease in the concentration of vanillin and likewise, no metabolites were formed.

Protocatechuic acid formation in vanillin incubations could arise via O-demethylation of vanillic acid or from O-demethylation of vanillin to protocatechuic aldehyde and subsequent oxidation to the corresponding acid. Therefore, in order to elucidate which of these pathways was the most likely, vanillic acid ($1 \times 10^{-3} \text{M}$) was incubated

with freshly prepared guinea pig liver slices ($n=4$). A 13 % decrease of vanillic acid concentration was observed after 90 min and a 23 % decrease after 180 min (data not shown). One main metabolite and traces of a number of other polar metabolites were produced. However, the main metabolite was protocatechuic acid, which accounted for 8 % conversion of the vanillic acid after 90 min and 16 % after 180 min (data not shown). Thus, it would appear that microsomal O-demethylation of vanillic acid to protocatechuic acid occurred in liver slice incubations.

Effect of inhibitors on vanillin oxidation by freshly prepared guinea pig liver slices
In the presence of isovanillin ($1 \times 10^{-3} \text{M}$), vanillin

Fig. 3. Effect of inhibitors on vanillin oxidation by freshly prepared guinea pig liver slices. Vanillin ($1 \times 10^{-3} \text{M}$) was incubated in the absence of inhibitors and in the presence of (a) allopurinol ($1 \times 10^{-4} \text{M}$), (b) disulfiram ($1 \times 10^{-4} \text{M}$) and (c) disulfiram ($1 \times 10^{-4} \text{M}$) and allopurinol ($1 \times 10^{-4} \text{M}$), with four guinea pig liver slices in 3 ml of Krebs-Heinseleit buffer pH 7.4 at 37°C and $t=90 \text{ min}$. Each point is the mean of four determinations. Values are expressed as means \pm SE.

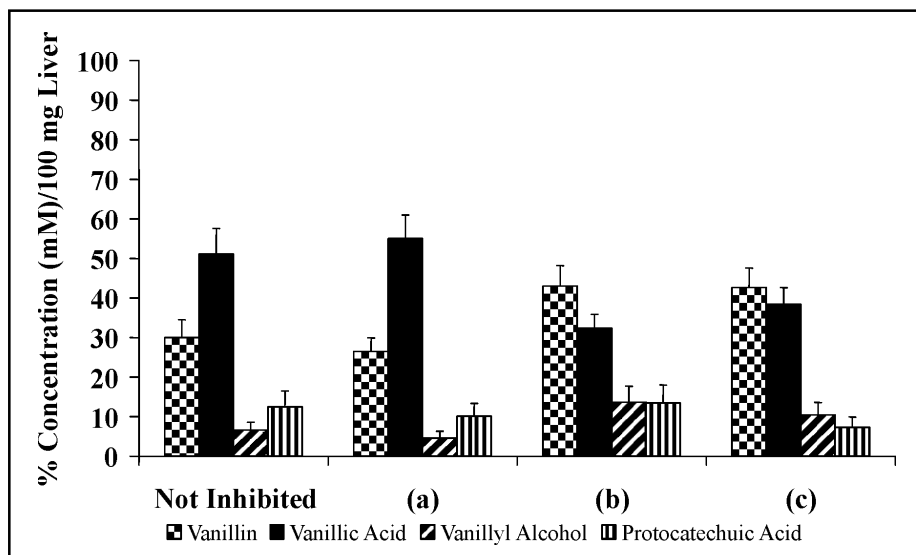
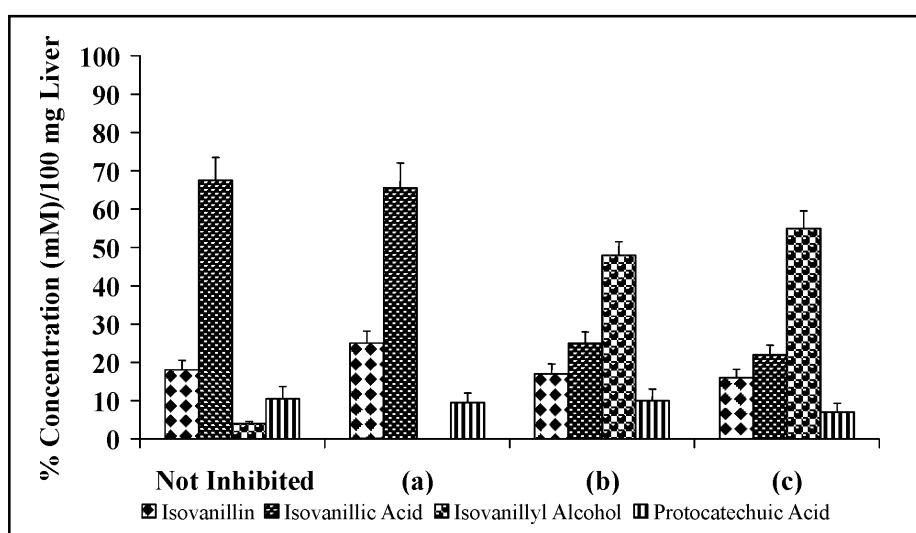


Fig. 4. Effect of inhibitors on isovanillin oxidation by freshly prepared guinea pig liver slices. Isovanillin ($1 \times 10^{-3} \text{M}$) was incubated in the absence of inhibitors and in the presence of (a) allopurinol ($1 \times 10^{-4} \text{M}$), (b) disulfiram ($1 \times 10^{-4} \text{M}$) and (c) disulfiram ($1 \times 10^{-4} \text{M}$) and allopurinol ($1 \times 10^{-4} \text{M}$), with four guinea pig liver slices in 3 ml of Krebs-Heinseleit buffer pH 7.4 at 37°C and $t=90 \text{ min}$. Each point is the mean of four determinations. Values are expressed as means \pm SE.



oxidation by liver slices was significantly reduced with only small amounts of vanillic acid produced in 30 min, and hence resulting in an 82 % inhibition ($n=5$). It was noted that during the first 90 min of incubation, isovanillin acted as a good inhibitor exhibiting 72 % and 58 % inhibition of vanillic acid formation after 60 min and 90 min, respectively (Fig. 2(i) and 2(ii)). However, after 90 min, most of the isovanillin had been metabolized into isovanillic acid and therefore, less inhibition was observed (16 % after 180 min) (Fig. 2(i) and 2(ii)).

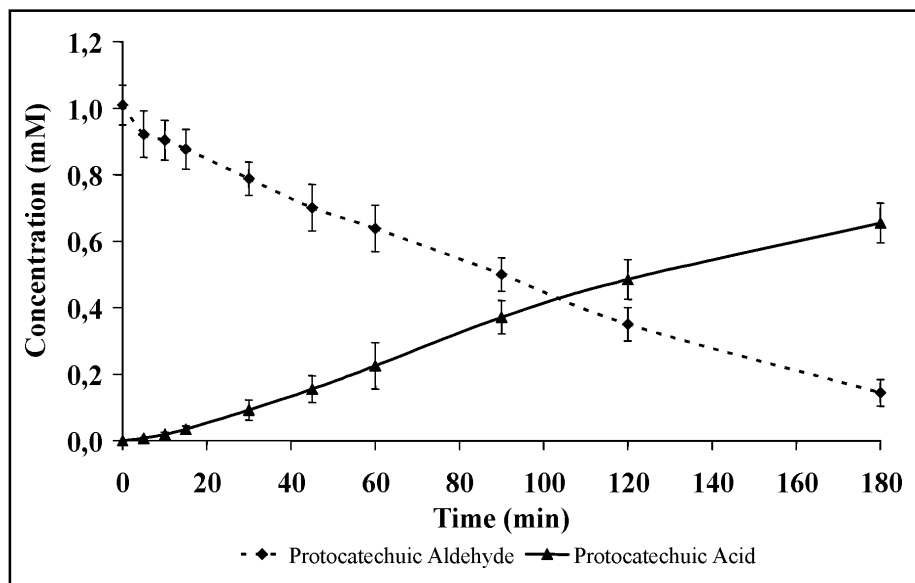
In the presence of allopurinol ($1 \times 10^{-4} \text{M}$) the rate of vanillin breakdown over 90 min was slightly enhanced (11 %) and the amount of the minor metabolite, protocatechuic acid was 19 % lower than in incubations with vanillin alone (Fig. 3). However, neither of these changes was significant and, in contrast, vanillic acid

production was slightly enhanced (by 14 %).

Disulfiram ($1 \times 10^{-4} \text{M}$) caused a 43 % ($n=4$) inhibition in vanillin breakdown and 33 % inhibition in vanillic acid formation. This degree of inhibition was about twice that observed with the oxidation of vanillin, by guinea pig liver aldehyde oxidase, when disulfiram was present [14]. However, disulfiram had little effect (8 % enhancement) on protocatechuic acid formation (Fig. 3), whereas there was a significant increase in the formation of vanillyl alcohol.

In incubations containing both allopurinol ($1 \times 10^{-4} \text{M}$) and disulfiram ($1 \times 10^{-4} \text{M}$) vanillin breakdown ($n=4$) was inhibited by 42 %, vanillic acid formation by 21 % and protocatechuic acid formation by 42 % (Fig. 3). Once again there was an increase (58 %) in the production of vanillyl alcohol.

Fig. 5. Metabolism of protocatechuic aldehyde with freshly prepared guinea pig liver slices. Protocatechuic aldehyde ($1 \times 10^{-3} \text{M}$) was incubated with four guinea pig liver slices (125 mg) in 3 ml of Krebs-Heinseleit buffer pH 7.4 at 37°C . Each point is the mean of four determinations. Values are expressed as means \pm SE.



Metabolism of isovanillin with freshly prepared guinea pig liver slices

The time profile of isovanillin metabolism ($R_t = 12.6 \pm 1.1$ min, $n=5$), with freshly prepared guinea pig liver slices, was similar to that observed in vanillin metabolism (Fig. 1). A progressive decrease in aldehyde concentration was observed, which continued until it had been completely transformed during the 180 min incubation time. Simultaneously, three metabolites appeared which increased with time and accounted for approximately 100 % conversion of the aldehyde after 180 min (data not shown). The major metabolite formed was isovanillic acid ($R_t = 8.2 \pm 0.6$ min), which accounted for 87 % conversion of isovanillin. The other two metabolites were protocatechuic acid (13 % conversion) and isovanillyl alcohol ($R_t = 5.7$ min), which achieved maximum concentrations between 45-60 min and then subsequently decreased with no alcohol remaining after 180 min. Control incubations of isovanillin without guinea pig liver slices, did not show any change in isovanillin concentration and no metabolites were formed.

Due to the unexpected formation of protocatechuic acid in isovanillin incubations with liver slices, incubations of isovanillic acid with guinea pig liver slices were also performed. A decrease in isovanillic acid concentration (12.5 %) was noted, at $t = 180$ min, but to a lesser extent than vanillic acid incubation under similar conditions (data not shown). The major metabolite observed was protocatechuic acid (4.5 %, $t = 180$ min), whereas the other two minor polar metabolites had retention values of 3.7 min and 4.0 min. Neither of these compounds co-

eluted with protocatechuic aldehyde (data not shown). Thus, it would appear that O-demethylation of isovanillic acid to protocatechuic acid occurred in liver slice incubations.

Effect of inhibitors on isovanillin oxidation with freshly prepared guinea pig liver slices

The presence of allopurinol ($1 \times 10^{-4} \text{M}$) appeared to enhance the turnover of isovanillin by 24 %, whereas the formation of isovanillic acid and protocatechuic acid were inhibited by 6 % and 23 % respectively (Fig. 4).

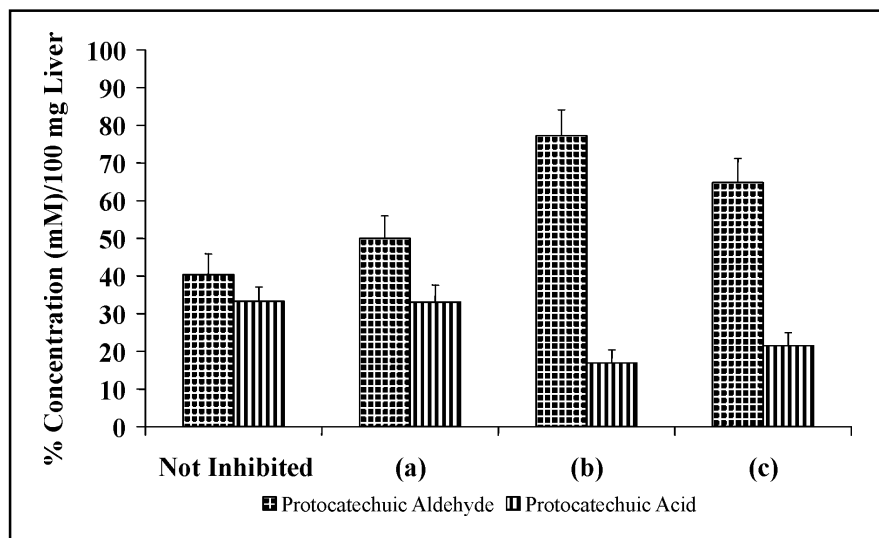
Disulfiram ($1 \times 10^{-4} \text{M}$) had little effect on isovanillin disappearance in guinea pig liver slices ($n=4$). However, there was a 61 % inhibition in the production of isovanillic acid, whereas protocatechuic acid production was unaffected. Moreover, there was a very significant increase in isovanillyl alcohol (Fig. 4). A higher concentration of disulfiram ($2 \times 10^{-4} \text{M}$) did not cause any more inhibition in isovanillic acid formation (data not shown).

The combined effect of disulfiram ($1 \times 10^{-4} \text{M}$) and allopurinol ($1 \times 10^{-4} \text{M}$) on isovanillin incubations with guinea pig liver slices ($n=4$) was similar to the results seen in the presence of disulfiram alone (Fig. 4).

Metabolism of protocatechuic aldehyde with freshly prepared guinea pig liver slices

When protocatechuic aldehyde was incubated with freshly prepared guinea pig liver slices ($n=4$), an 88 % decrease in protocatechuic aldehyde ($R_t = 6.4 \pm 0.8$ min) concentration was observed after 180 min (Fig. 5). Four

Fig. 6. Effect of inhibitors on protocatechuic aldehyde oxidation by freshly prepared guinea pig liver slices. Protocatechuic aldehyde ($1 \times 10^{-3} \text{M}$) was incubated in the absence of inhibitors and in the presence of (a) allopurinol ($1 \times 10^{-4} \text{M}$), (b) disulfiram ($1 \times 10^{-4} \text{M}$) and (c) disulfiram ($1 \times 10^{-4} \text{M}$) and allopurinol ($1 \times 10^{-4} \text{M}$), with four liver slices in 3 ml of Krebs-Heinseleit buffer pH 7.4 at 37°C and $t=90$ min. Each point is the mean of four determinations. Values are expressed as means \pm SE.



metabolites were formed, the major one being protocatechuic acid ($R_t = 4.3 \pm 0.5$ min), which accounted for 68 % of substrate conversion (Fig. 5). The other 3 metabolites were more polar and eluted at 2.7 min, 3.2 min and 3.6 min. These metabolites remained unidentified due to lack of relevant assay standards for possible metabolites. These metabolites accounted for the remaining 20 % of protocatechuic aldehyde breakdown. Protocatechuic aldehyde was stable in control incubations of protocatechuic aldehyde without liver slices.

In order to see if these minor metabolites are due to protocatechuic aldehyde breakdown or due to further metabolism of protocatechuic acid formation, incubations of protocatechuic acid with guinea pig liver slices were performed. An 8 % decrease in acid concentration after 180 min was observed (data not shown) and three metabolites were formed, which eluted at 1.9 min, 2.7 min and 3.2 min. Although metabolism of protocatechuic acid may account for some of the unaccounted metabolites in protocatechuic aldehyde incubations, it is likely that the aldehyde itself is also subject to further metabolic breakdown.

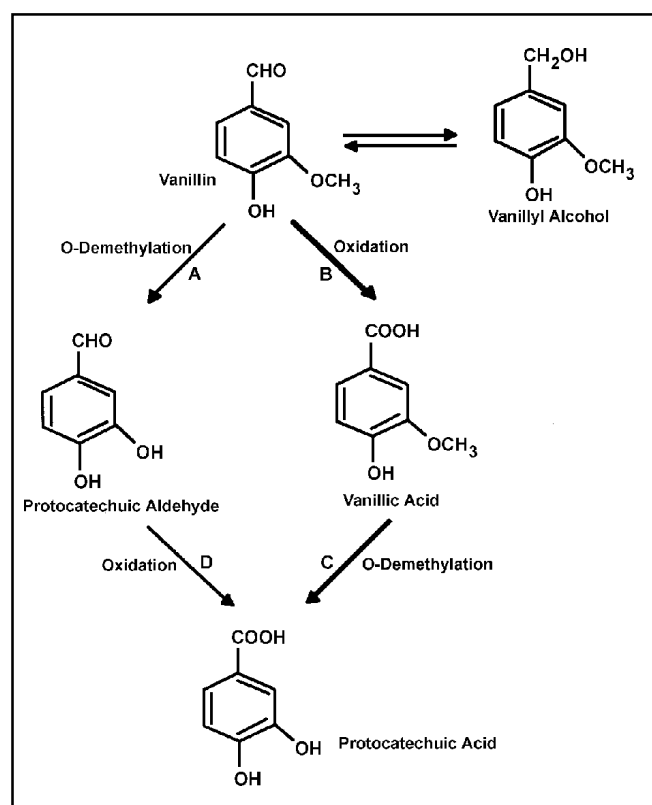


Fig. 7. Proposed metabolic pathways of vanillin by freshly prepared guinea pig liver slices.

Effect of inhibitors on protocatechuic aldehyde oxidation by freshly prepared guinea pig liver slices

The presence of isovanillin ($1 \times 10^{-3} \text{M}$) resulted in a 20 % inhibition of protocatechuic aldehyde breakdown although there was an apparent increase (7 %) in the formation of protocatechuic acid at 90 min ($n=4$) (data not shown). However, protocatechuic acid is also formed in isovanillin incubations with liver slices and thus some of the acid may be derived from isovanillin rather than

protocatechuic aldehyde. If the concentration of protocatechuic acid formed in isovanillin incubations is subtracted from that in the combined incubation then the residual amounts of acid are less than in protocatechuic aldehyde incubations with liver slices. This indicates that, in fact, isovanillin does inhibit protocatechuic

acid formation as well as protocatechuic aldehyde breakdown.

Allopurinol caused a 17% inhibition of protocatechuic aldehyde breakdown, but the formation of protocatechuic acid was not greatly affected (Fig. 6).

In the presence of disulfiram, the breakdown of protocatechuic aldehyde was inhibited by 62% with a concomitant 49% inhibition of protocatechuic acid formation (Fig. 6).

The addition of both the allopurinol and disulfiram in the incubation of the protocatechuic aldehyde with guinea pig liver slices resulted in a 40% inhibition in the breakdown of protocatechuic aldehyde and a 35% inhibition in the production of the acid (Fig. 6). This is a lower inhibition than that observed with disulfiram alone and suggests that allopurinol protects protocatechuic aldehyde from the effects of disulfiram.

Discussion

In freshly prepared liver slice incubations, vanillin was rapidly converted to vanillic acid, whereas only small amounts of vanillyl alcohol and protocatechuic acid were produced. The formation of protocatechuic acid in vanillic acid incubations is similar to the formation of protocatechuic acid in vanillin incubations. Thus, it is highly probable that O-demethylation of vanillic acid occurs in preference to O-demethylation of vanillin. Hence, routes B and C in Fig. 7 are more likely to occur than A and D. This is also supported by the results from isovanillic acid incubations with liver slices.

The effects of isovanillin and disulfiram on vanillin oxidation resulted in 82% and 16% inhibition respectively, which were both similar to those obtained with partially purified hepatic aldehyde oxidase under similar conditions [14]. Allopurinol caused an enhancement of 11% in vanillic acid production. A similar enhancement in acid production (17%) was seen when vanillin was incubated with aldehyde oxidase in the presence of allopurinol [14], which is attributed to the presence of xanthine dehydrogenase in liver slices [3, 14].

Thus, it is concluded that, in liver slices, vanillin is rapidly oxidized by aldehyde oxidase with little or no contribution from either xanthine oxidase or aldehyde dehydrogenase.

In isovanillin incubations, the aldehyde was rapidly converted to its acid with small amounts of protocatechuic acid and isovanillyl alcohol been formed. The formation

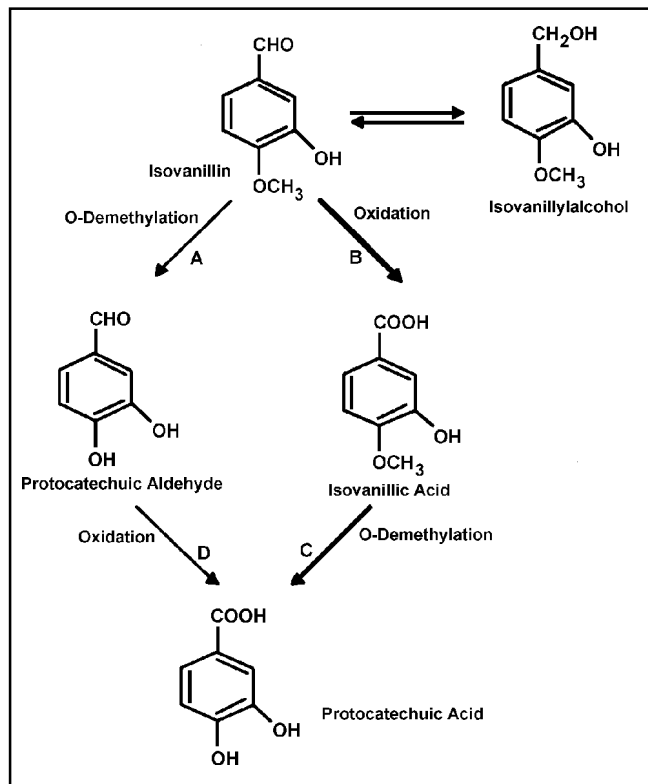


Fig. 8. Proposed metabolic pathways of isovanillin by freshly prepared guinea pig liver slices.

of isovanillic acid was not altered in the presence of allopurinol, but was considerably inhibited by disulfiram. Taken with the evidence that isovanillin is not a substrate for guinea pig liver aldehyde oxidase [13, 14] it is proposed that in liver slices, isovanillin is metabolized predominantly by aldehyde dehydrogenase activity. As protocatechuic aldehyde did not inhibit isovanillin metabolism, the slight enhancement in isovanillin oxidation by aldehyde dehydrogenase activity in liver slices (data not shown) may arise from displacement of isovanillin by protocatechuic aldehyde from less productive binding sites on other enzymes.

Therefore, it is likely that isovanillin is converted first to isovanillic acid and then O-demethylated to protocatechuic acid. Hence, routes B and C in Fig. 8 are the most likely. A similar sequence of events has also been postulated for the metabolism of vanillin to protocatechuic acid.

However, the inhibition of isovanillic acid formation by disulfiram in the liver slices seemed to be less than

that with guinea pig liver aldehyde dehydrogenase activity (almost 100 % inhibition) [14]. In both cases significant increases of isovanillyl alcohol were noted. The reason for the reduced inhibition might be because disulfiram, itself, is metabolized in liver slices and therefore the inhibition seen is less (inhibition is marked after 90 min compared to 15-30 min).

In protocatechuic aldehyde incubations, the aldehyde was converted to protocatechuic acid at a lower rate than that of vanillin or isovanillin. Allopurinol only slightly inhibited the aldehyde oxidation, whereas disulfiram had a greater effect (50 % inhibition) and isovanillin had little effect. Although menadione is often used as a specific inhibitor of aldehyde oxidase [32] and it would be of interest to try this inhibitor on protocatechuic aldehyde oxidation in liver slices, this was not performed because it was found to interfere with the HPLC assay and was unstable under the incubation conditions.

Thus, all three enzymes contribute to the oxidation of protocatechuic aldehyde to its acid in liver slices. However, this conclusion is not entirely consistent with the results obtained with the enzymes in separate preparations [14]. Protocatechuic aldehyde showed minimal transformation with aldehyde oxidase [14], although it is a competitive inhibitor of aldehyde oxidase [13]. Xanthine oxidase does catalyze protocatechuic aldehyde oxidation, although not as rapidly as isovanillin [14]. In contrast, incubations with mitochondrial aldehyde dehydrogenase activity gave only minimal formation of protocatechuic acid [14]. This suggests that mitochondrial aldehyde dehydrogenase activity does not contribute significantly to the oxidation of protocatechuic aldehyde in liver slices. Therefore, there must be another enzyme

present which is susceptible to disulfiram inhibition. One possibility is cytosolic aldehyde dehydrogenase, which can also be inhibited by disulfiram [33, 34].

Therefore it is suggested that protocatechuic aldehyde may be slowly oxidized by xanthine oxidase, cytosolic aldehyde dehydrogenase and perhaps aldehyde oxidase or another molybdenum hydroxylase isozyme.

It has been shown in this study that oxidation of aromatic aldehydes in guinea pig liver slices occurs very rapidly and that the activity is retained in a simple Krebs medium for up to 3 hours. Similarly, phenylacetaldehyde metabolism was examined in both guinea pig fresh liver slices [3] and in separate enzyme preparations [35]. The results showed that the aldehyde was rapidly converted to phenylacetic acid and 2-phenylethanol. Isovannillin inhibited phenylacetic acid by 14 %, whereas disulfiram caused a 70-80 % inhibition and allopurinol caused a slight enhancement. Therefore, the results obtained with phenylacetaldehyde are in accordance to those in the present investigation. However, high turnover rates in guinea pig liver slices are not restricted to aldehydes because phthalazine, a heterocyclic substrate of aldehyde oxidase [27], is also rapidly oxidized to 1-phthalazinone [36].

The results obtained with vanillin and isovanillin indicate that the activity of both aldehyde oxidase and mitochondrial aldehyde dehydrogenase in freshly prepared guinea pig liver slices is high and their oxidation is mainly inhibited by isovanillin and disulfiram respectively. Therefore, vanillin and isovanillin are predominantly metabolized by aldehyde oxidase and aldehyde dehydrogenase respectively, whereas protocatechuic aldehyde is metabolized by all three enzymes.

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