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The Metabolism of Phenolic Acids in the Rat

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Some of the enzyme systems in the formation of *p*-hydroxybenzoate from tyrosine have been studied in the rat liver *in vitro*. The conversion of *p*-hydroxycinnamate into *p*-hydroxybenzoate, which was found in rat liver mitochondria showed a number of differences when compared with the β-oxidation of fatty acids. Studies with *p*-hydroxy-[U-^{14}C]cinnamate indicated that $^{14}CO_2$ was released during the formation of *p*-hydroxybenzoate. The formation of *p*-hydroxycinnamate from tyrosine or *p*-hydroxyphenyl-lactate could not be demonstrated *in vitro*. The interconversion of *p*-hydroxycinnamate and *p*-hydroxyphenylpropionate was demonstrated in rat liver mitochondria.

Several phenolic acids are excreted in the urine of animals. The number of these can be decreased considerably by feeding on a purified diet. Rats excrete a number of phenolic acids even during starvation showing that the catabolism of endogenous aromatic amino acids is involved in the formation of these phenolic acids. On the basis of their studies on the excretion of phenolic acids after administration of tyrosine and related compounds to the rat, Booth *et al.* (1960) have proposed a pathway for the formation of these phenolic acids from tyrosine (Scheme 1). The following four phenolic acids have been identified as intermediates in the formation of *p*-hydroxybenzoate from tyrosine: *p*-hydroxyphenylpyruvate, *p*-hydroxyphenyl-lactate, *p*-hydroxyphenylpropionate and *p*-hydroxycinnamate.

The conversion of tyrosine into these phenolic acids appears to be independent of the intestinal micro-organisms, since the feeding of antibiotics (streptomycin) and sulpha drugs (sulphathiazole) to the rats did not decrease the excretion of phenolic acids, with or without extra dietary tyrosine. Bernhart & Zilliken (1959) reported that increased excretion of phenolic acids in tyrosine-fed rats was not altered by the administration of aureomycin.

In Scheme 1, the conversion of tyrosine into *p*-hydroxyphenylpyruvate is catalysed by tyrosine aminotransferase, and conversion of *p*-hydroxyphenylpyruvate into *p*-hydroxyphenyl-lactate has been shown to be catalysed by an aromatic 2-oxo acid reductase (Weber & Zannoni, 1966). This enzyme is widely distributed in animal tissues. The formation of *p*-hydroxycinnamate or *p*-hydroxyphenylpropionate from *p*-hydroxyphenyl-lactate has not been shown *in vitro*. Ranganathan & Ramasarma (1971) reported the enzymic conversion of *p*-hydroxycinnamate into *p*-hydroxybenzoate. Additional properties of this enzymic conversion and also the interconversion between *p*-hydroxycinnamate and *p*-hydroxyphenylpropionate are presented in the present paper.

Materials and Methods

Chemicals

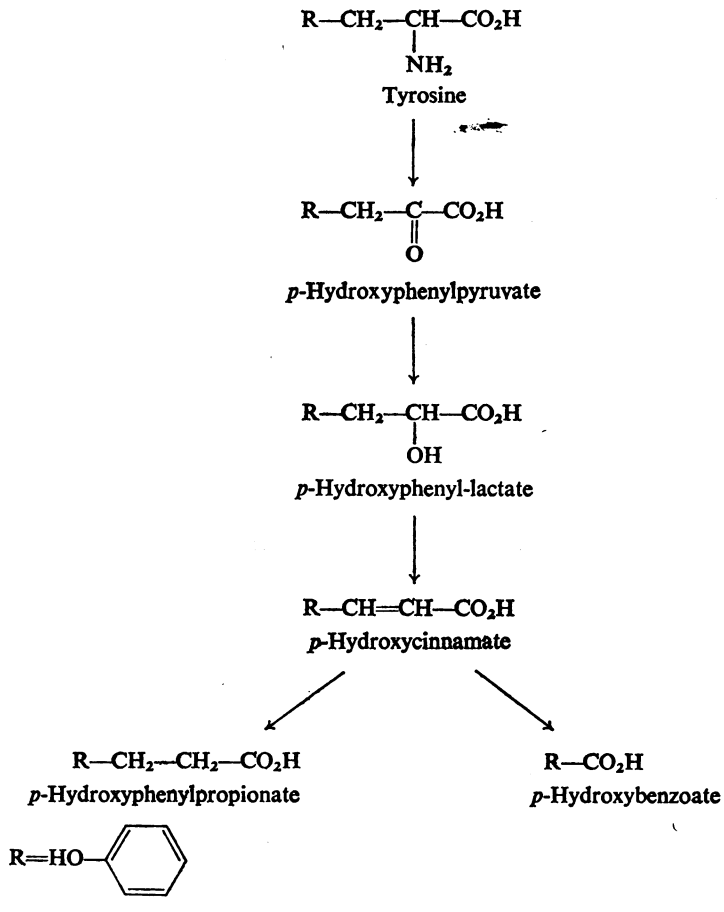
p-Hydroxycinnamate was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. *p*-Hydroxybenzoate, ATP, cytochrome *c* and octanoate were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. *p*-Hydroxyphenylpropionate was prepared by hydrogenation of *p*-hydroxycinnamate with Pd on charcoal as the catalyst. When examined by paper chromatography the phenolic acids showed as single spots. *p*-Hydroxy[U-^{14}C]-cinnamate (10Ci/mol) was prepared enzymically from [U-^{14}C]tyrosine by using tyrosine ammonia lyase from *Rhizoctonia solani* and was purified twice by paper chromatography (Ranganathan & Ramasarma, 1971). All other chemicals were of AnalaR grade obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Animals and preparation of tissues

Male albino rats (about 150g) from the Institute colony were used for all the experiments. They were fed with pellet diet obtained from Hindustan Lever Ltd., Bombay, India. The rats were killed by stunning and decapitation and the livers were quickly removed and kept on ice. Homogenates were made in 0.25M-sucrose (10ml/g of liver) by using a Potter–Elvehjem-type glass homogenizer. Mitochondria were prepared by the conventional differential-centrifugation procedure of Schneider & Hogeboom (1950), washed once with the homogenization medium and suspended in the same medium.

Methods of assay

The enzyme assays, separation of phenolic acids by paper chromatography and their determination, and the measurement of the rate of oxidation of octanoate were as described by Ranganathan &



Scheme 1. *Formation of phenolic acids from tyrosine*

Ramasarma (1971). To measure *p*-hydroxyphenylpropionate, 2ml of water was used for elution instead of ethanol.

To study the release of $^{14}CO_2$ from labelled *p*-hydroxycinnamate the reaction mixture containing *p*-hydroxy[U-^{14}C]cinnamate (48000c.p.m.), 5μmol of ATP, 50μmol of potassium phosphate buffer, pH7.4, and freshly prepared rat liver mitochondria (3mg of protein) was incubated at 37°C in a closed Warburg flask with 0.1ml of 2M-KOH in the central well and 0.5ml of 1M-H_2SO_4 in the side arm. At the end of the incubation for 2h the reaction was stopped by tipping H_2SO_4 from the side arm into the central well and after 30min the contents of the central well were transferred quantitatively into vials containing 5ml of scintillation fluid (see below) and the radioactivity was measured.

The liver was homogenized in water, the proteins were precipitated with $HClO_4$ and the protein-free supernatant was extracted with diethyl ether. The ether extract was chromatographed on paper in a two-dimensional system to detect the phenolic acids (Ranganathan & Ramasarma, 1971).

Measurement of radioactivity

Radioactivity measurements were carried out by using a Beckman model LS-100 liquid-scintillation spectrometer. A solution (5ml) containing 0.2% PPO (2,5-diphenyloxazole) and 0.002% POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] in a toluene–methanol mixture (1:1, v/v) was used as scintillation fluid (Yardley, 1964). The addition of small amounts of alkali to this scintillation fluid did not significantly alter the counting efficiency.

Results

Comparative study of p-hydroxybenzoate formation from p-hydroxycinnamate and fatty acid oxidation

Further studies were done on the comparison of the properties of the conversion of *p*-hydroxycinnamate into *p*-hydroxybenzoate, and fatty acid oxida-

Table 1. *Effect of fatty acids on the formation of p-hydroxybenzoate from p-hydroxycinnamate*

The reaction mixture consisted of 25 μmol of potassium phosphate buffer (pH 7.4), 5 μmol of ATP, 0.5 μmol of *p*-hydroxycinnamate and 0.2 ml of rat hepatic mitochondrial suspension (6 mg of protein) in a total volume of 0.5 ml. The incubation was carried out with shaking for 60 min at 37°C. Other details of the experiments are given in the Materials and Methods section.

Additions	Concentration of fatty acids (mM)	*p*-Hydroxybenzoate formed (nmol/h per mg of protein)
None	—	3.9
Octanoate	1.0	0.4
Laurate	0.8	0.2
Palmitate	0.4	0.3

Table 2. *Effect of cinnamate and benzoate on the formation of p-hydroxybenzoate from p-hydroxycinnamate*

The experimental conditions were the same as in Table 1. The substrate concentration (*p*-hydroxycinnamate) was 1 mM. Cinnamate and benzoate were added as Na^+ salts.

	p-Hydroxybenzoate formed (nmol/h per mg of protein)	
Concentration of (μM)	Cinnamate added	Benzoate added
Nil	9.0	9.0
20	8.1	6.7
40	7.4	6.4
80	4.8	3.6
120	3.6	2.4

tion by the rat liver mitochondria. Table 1 shows that the addition of fatty acids to the reaction mixture inhibited the formation of *p*-hydroxybenzoate. Addition of *p*-hydroxycinnamate to the reaction mixture in the Oxygraph did not, however, effect the rate of oxidation of octanoate. The ADP/O ratio, respiratory control ratio and the rate of O_2 uptake, with succinate as the substrate were not affected by the addition of 1 mM-*p*-hydroxycinnamate.

Addition of small concentrations of cinnamate and benzoate to the reaction mixture inhibited the formation of *p*-hydroxybenzoate (Table 2). The inhibitory effect could be due to the competition of these compounds with *p*-hydroxycinnamate for the active site of the enzyme. This was clarified by studying the inhibitory effect at different concentrations of the substrate and drawing Lineweaver–Burk plots. The inhibition was found to be neither competitive nor non-competitive, both the K_m and $V_{max.}$ values having changed.

Table 3. *Effect of various compounds on the formation of p-hydroxybenzoate from p-hydroxycinnamate and on fatty acid oxidation*

The experimental techniques for *p*-hydroxybenzoate formation and fatty acid (octanoate) oxidation are described in the Materials and Methods section. The concentrations of *p*-hydroxycinnamate and octanoate used were 1 and 0.5 mM respectively.

Addition (mM)	*p*-Hydroxybenzoate formed (nmol/h per of protein)	Fatty acid (octanoate) oxidation (ng-atoms of O/min per mg of protein)
None	4.3	14
Hydroxylamine (100)	4.6	0
Cyanide (6)	4.1	0
Benzoate (8)	0.8	12
Diethyldithiocarbamate (7)	2.0	16
p-Chlorophenoxyisobutyrate (Na^+ salt) (2)	2.5	20
o-Phenanthroline (10)	0.7	14
Carnitine (3)	6.6	16
Arsenite (0.25)	1.4	8
p-Chloromercuribenzoate (0.25)	0	0
Deoxycholate (2.5)	0	0

Table 3 shows the effects of various other compounds on the formation of *p*-hydroxybenzoate from *p*-hydroxycinnamate and the oxidation of octanoate. Hydroxylamine and cyanide specifically inhibited the oxidation of octanoate without affecting the formation of *p*-hydroxybenzoate. Benzoate, diethyldithiocarbamate, α-*p*-chlorophenoxyisobutyrate Na^+ salt and *o*-phenanthroline only inhibited the formation of *p*-hydroxybenzoate from *p*-hydroxycinnamate.

Nature of the second product

If the reaction were to follow the β-oxidation type of reactions, the two carbon atoms would be converted into acetyl-CoA and then into CO_2. Experiments with *p*-hydroxy[U-^{14}C]cinnamate indicated that $^{14}CO_2$ was liberated during the formation of *p*-hydroxybenzoate. If *p*-hydroxybenzoate (7 carbons) were to be formed from *p*-hydroxy[U-^{14}C]cinnamate (9 carbons) by β-oxidation and the acetyl-CoA formed in the reaction oxidized completely to CO_2, the radioactivity in the CO_2 released should be 28.5% of that in the *p*-hydroxybenzoate formed. However, the results showed that the radioactivity in the CO_2 was only about 10% of that in *p*-hydroxybenzoate. This could be due to the incorporation of a part of the acetyl-CoA formed into lipids, tricarboxylic acid cycle intermediates and other metabolites.

Table 4. *Effect of shaking on the formation of p-hydroxyphenylpropionate and p-hydroxybenzoate from p-hydroxycinnamate*

The incubation mixture contained 50μmol of phosphate buffer (pH7.4), 0.5μmol of *p*-hydroxycinnamate and rat hepatic mitochondria (5mg of protein) in a total volume of 0.5ml. ATP (5μmol) was added where indicated. The incubation was carried out for 1h at 37°C, with or without shaking of the tubes containing the reaction mixture, as indicated. (N_2) indicates flushing the reaction mixture with N_2 before the incubation. *p*-Hydroxybenzoate and *p*-hydroxyphenylpropionate were isolated from the reaction mixture and determined as given in the text.

Shaking	ATP	*p*-Hydroxyphenylpropionate (nmol/h per mg of protein)	*p*-Hydroxybenzoate (nmol/h per mg of protein)
−	−	0.6	1.1
−	+	6.6	3.3
−(N_2)	+	8.2	2.2
+	−	0.5	1.2
+	+	1.3	5.6

Table 5. *Effect of various compounds on the formation of p-hydroxyphenylpropionate from p-hydroxycinnamate*

The incubation mixture contained 50μmol of phosphate buffer (pH7.4,) 0.5μmol of *p*-hydroxycinnamate, 5μmol of ATP and rat liver mitochondria (6mg of protein) in a total volume of 0.5ml. The incubation was carried out at 37°C for 1h without shaking.

Additions	*p*-Hydroxyphenylpropionate (nmol/h per mg of protein)
None	3.6
Cyanide (3mM)	4.3
Antimycin A (1μg)	0
Succinate (50mM)	7.1
α-Glycerophosphate (60mM)	6.1
α-Glycerophosphate (60mM) +cyanide (3mM)	5.3
α-Glycerophosphate (60mM) +cyanide (3mM) +cytochrome *c* (0.1mM)	5.8
α-Glycerophosphate (60mM) +antimycin A (1μg)	1.1
Glucose 6-phosphate (0.1mg) +NADP (0.1mg) +rat liver supernatant (0.1ml)	4.4
NADH (1.0mM)	1.6
p-Chloromercuribenzoate (0.4mM)	0
CoA (0.2mg)	5.3
Hydroxylamine (80mM)	0
Octanoate (0.2mM)	0

Formation of p-hydroxyphenylpropionate from p-hydroxycinnamate

Preliminary experiments showed that when rat liver homogenates were incubated with *p*-hydroxycinnamate, traces of *p*-hydroxyphenylpropionate could be detected in the reaction mixture, in addition to *p*-hydroxybenzoate. When the reaction was carried out without shaking, it was found that the amount of *p*-hydroxyphenylpropionate formed was more than that of *p*-hydroxybenzoate. Experiments with different fractions of the rat liver homogenate showed that the conversion occurred only with the mitochondrial fraction.

Table 4 shows that the formation of *p*-hydroxyphenylpropionate is maximum when the incubation was carried out without shaking and in the absence of O_2. Very little *p*-hydroxybenzoate was formed under these conditions. Since the reaction is reductive in nature, anaerobic conditions are presumably favourable.

It was decided to study the nature of the reducing equivalents required for the formation of *p*-hydroxyphenylpropionate. The conversion is not stimulated by the addition of glucose 6-phosphate, $NADP^+$ and the supernatant fraction of rat liver, indicating that NADPH is probably not involved in the reaction, but addition of NADH significantly inhibited this conversion. Addition of α-glycerophosphate or succinate to the reaction mixture enhanced the formation of *p*-hydroxyphenylpropionate (Table 5). Mitochondrial α-glycerophosphate dehydrogenase and succinate dehydrogenase are well-known flavoproteins and these substrates apparently can provide the reducing equivalents through a common intermediate. Addition of cytochrome *c* did not have any effect. The conversion was inhibited by antimycin A but not by cyanide. Antimycin A is known to inhibit the re-oxidation of ubiquinol and cyanide inhibits cytochrome oxidase. These results indicate that a reduced component associated with respiratory chain at the level of ubiquinol or before is required for the reduction of *p*-hydroxycinnamate to *p*-hydroxyphenylpropionate.

The formation of *p*-hydroxyphenylpropionate was inhibited by the addition of small concentrations of octanoate, as in the case of formation of *p*-hydroxybenzoate (Table 5). *p*-Chloromercuribenzoate was inhibitory at the concentration of 0.4mM, hydroxylamine was also inhibitory, but the addition of CoA considerably stimulated the reaction.

Incubation of *p*-hydroxyphenylpropionate with rat liver mitochondria resulted in the formation of *p*-hydroxybenzoate. Traces of *p*-hydroxycinnamate were also detected in the reaction mixture. This indicates that *p*-hydroxycinnamate may be the intermediate in the formation of *p*-hydroxybenzoate

from *p*-hydroxyphenylpropionate. The interconversion of *p*-hydroxycinnamate and *p*-hydroxyphenylpropionate may be a reversible reaction. However, it was not possible to study the rate of formation of *p*-hydroxycinnamate, because measurable amounts of *p*-hydroxycinnamate could not be obtained in the reaction mixture. The rates of formation of *p*-hydroxybenzoate from *p*-hydroxycinnamate and *p*-hydroxyphenylpropionate were nearly the same (3.5 and 3.8nmol/h per mg of protein respectively). Hence the rate of formation of *p*-hydroxycinnamate from *p*-hydroxyphenylpropionate must be the same or greater.

These reactions could not be obtained with mitochondria stored frozen overnight. The activity in such preparations could not be restored by any agents tested such as GSH, 2-mercaptoethanol, fumarate or cytochrome *c*. Storage in the presence of substrate or ATP could not stabilize the enzyme activity.

Formation of p-hydroxycinnamate

Attempts were made to show the formation of *p*-hydroxycinnamate from tyrosine and other suggested intermediates in the rat *in vitro*. Considerable amounts of radioactivity could be obtained in the ether extract when [^{14}C]tyrosine was incubated with rat liver homogenate or liver slices in the presence of α-oxoglutarate and pyridoxal phosphate. The ether extract was chromatographed on paper for the separation of phenolic acids in the two-dimensional system described by Ranganathan & Ramasarma (1971) after the addition of 50μg of each of the following unlabelled compounds: *p*-hydroxyphenyl-lactate, *p*-hydroxycinnamate, *p*-hydroxyphenylpropionate and *p*-hydroxybenzoate. Considerable radioactivity was associated with *p*-hydroxyphenyl-lactate, whereas no significant radioactivity could be detected in the other phenolic acids.

According to the pathway suggested by Booth *et al.* (1960), *p*-hydroxycinnamate is presumably formed from *p*-hydroxyphenyl-lactate. This suggestion was based only on the excretion pattern of phenolic acids after administration of *p*-hydroxyphenyl-lactate to the rat. Several experiments were carried out to demonstrate this conversion in the rat tissues *in vitro*. Incubation of *p*-hydroxyphenyl-lactate with slices and homogenates of rat liver and kidney in the presence of ATP, NAD^+ and other cofactors at different pH conditions, did not result in the formation of *p*-hydroxycinnamate. In another set of experiments *p*-hydroxyphenyl-lactate was injected intraperitoneally into the rats and the livers were analysed for the presence of *p*-hydroxycinnamate and other phenolic compounds at various time-intervals. No other phenolic metabolites could be detected in the liver.

Discussion

Comparison of the formation of *p*-hydroxybenzoate from *p*-hydroxycinnamate with fatty oxidation showed that there were a number of distinctive features (Table 6). Studies with *p*-hydroxy[^{14}C]-cinnamate showed that $^{14}CO_2$ was formed during the reaction, indicating that the reaction probably followed the β-oxidation type of reactions, the two carbons being first removed as acetyl-CoA, and then oxidized to CO_2. If the conversion were to follow the β-oxidation type of reactions, *p*-hydroxycinnamate has to be activated to the acyl-CoA form and ATP may be providing the energy. Derivatives of CoA were shown to be involved in the formation of benzoate from cinnamate in plants (Alibert & Ranjeva, 1971). Walton & Butt (1971) demonstrated cinnamyl-CoA synthetase activity in leaf extracts. Also Vollmer *et al.* (1965) showed that acetate was formed during the formation of *p*-hydroxybenzoate from *p*-hydroxycinnamate in wheat shoots. Cell-free extracts of the wood destroying fungus, *Sporobolomyces roseus* are also known to catalyse the conversion of *p*-hydroxycinnamate into *p*-hydroxybenzoate (Moore *et al.*, 1968).

p-Hydroxyphenylpropionate, one of the phenolic acids excreted in urine, has been shown here to be formed from *p*-hydroxycinnamate. This enzyme

Table 6. *Comparison between p-hydroxybenzoate formation from p-hydroxycinnamate and fatty acid oxidation*

+, Inhibited; −, not inhibited. The data are compiled from the present work and that of Ranganathan & Ramasarma (1971) Both systems required fresh mitochondria, ATP and O_2.

	p-Hydroxybenzoate formation from *p*-hydroxycinnamate	Fatty acid (octanoate) oxidation
Activity		
With aged mitochondria	Lost	Partly lost
With aged mitochondria +fumarate	Not restored	Restored
Inhibition by:		
Cyanide	−	+
Hydroxylamine	−	+
Benzoate	+	−
Cinnamate	+	−
α-*p*-Chlorophenoxy-isobutyrate	+	−
o-Phenanthroline	+	−
Diethyldithiocarbamate	+	−
Feeding on α-*p*-chlorophenyoxy-isobutyrate	Increased	No effect

activity is associated with the mitochondrial fraction of rat liver. During the present studies it was also found that *p*-hydroxyphenylpropionate can be converted into *p*-hydroxycinnamate. It has not been possible to decide whether these reactions are catalysed by specific enzymes or whether they are simply a part of the general *β*-oxidation system of fatty acids. Inhibition by octanoate and enhancement of the activity by CoA give some support to the latter possibility.

The only reaction which has not been understood in the tyrosine → *p*-hydroxybenzoate pathway, is the formation of *p*-hydroxycinnamate. In plants and micro-organisms *p*-hydroxycinnamate is formed directly from tyrosine, the reaction being accomplished by tyrosine ammonia lyase (Neish, 1961; Power *et al.*, 1965; Young *et al.*, 1966). However, this enzyme has not so far been found in the animal tissues. Booth *et al.* (1960) showed that feeding *p*-hydroxyphenyl-lactate to rats resulted in the excretion of large amounts of *p*-hydroxycinnamate. As this conversion has not at present been demonstrated *in vitro* it is likely that intestinal micro-organisms may be responsible. Tanaka (1968) reported that *Proteus vulgaris* can convert *p*-hydroxyphenyl-lactate added to the medium into *p*-hydroxycinnamate. Thus the elusive link between *p*-hydroxyphenyl-lactate and *p*-hydroxycinnamate or *p*-hydroxyphenyl-propionate has yet to be resolved to explain the origin of *p*-hydroxyphenylpropionic acid.

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