Biochem. J. (1971) **122**, 487–493
Printed in Great Britain

Enzymic Formation of *p*-Hydroxybenzoate from *p*-Hydroxycinnamate

By S. RANGANATHAN and T. RAMASARMA
Department of Biochemistry, Indian Institute of Science, Bangalore 12, *India*

(*Received* 9 *November* 1970)

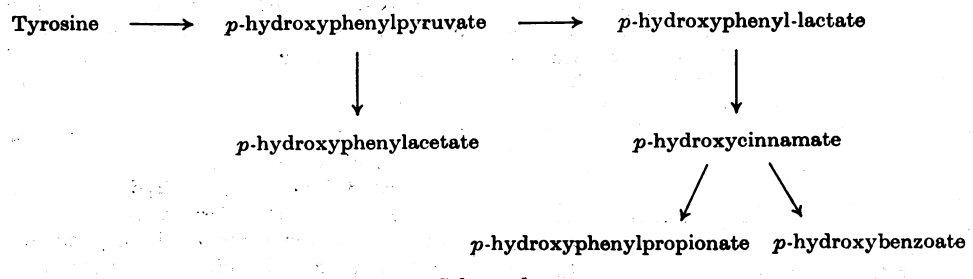
An enzyme that converts *p*-hydroxycinnamate into *p*-hydroxybenzoate was found in rat liver. It is localized in the mitochondria and requires ATP. The activity is lost when the mitochondria are stored frozen overnight. Addition of magnesium chloride, cytochrome *c*, GSH, coenzyme A or potassium cyanide did not have any effect on the activity. When the rats were fed with α-*p*-chlorophenoxyisobutyrate, the rate of formation of *p*-hydroxybenzoate increased twofold. The reaction has some similar properties to fatty acid oxidation, but appears to be different in many respects.

It was shown that [U-^{14}C]tyrosine and [U-^{14}C]-phenylalanine were incorporated into ubiquinone, although to a small extent, in the rat (Olson, Dialameh & Bentley, 1960) and the labelling was localized in the benzoquinone nucleus of ubiquinone (Bentley, Ramsey, Springer, Dialameh & Olson, 1961). Rudney & Parson (1963) and Olson *et al.* (1963) have further shown that *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate were incorporated more efficiently than tyrosine into ubiquinone. In a system incorporating [U-^{14}C]phenylalanine into ubiquinone with rat liver slices, addition of *p*-hydroxybenzoate as well as *p*-hydroxyphenylpyruvate, *p*-hydroxyphenyl-lactate, *p*-hydroxycinnamate, *p*-hydroxyphenylpropionate, *p*-hydroxybenzaldehyde, *p*-hydroxybenzyl alcohol and benzoate, *in vitro*, was found to produce a 'dilution effect,' suggesting that these compounds or others derived therefrom are intermediates in the tyrosine–*p*-hydroxybenzoate pathway (Olson, 1965). Scheme 1 shows the sequence of reactions from tyrosine to *p*-hydroxybenzoate proposed by Booth, *et al.* (1960), based on the excretion pattern of phenolic acids in the urine of rats dosed with tyrosine and other metabolites. In Scheme 1 the conversion of tyrosine into *p*-hydroxyphenylpyruvate by the transaminase and the following reaction to *p*-hydroxyphenyl-lactate by an enzyme similar to lactate dehydrogenase (Weber & Zannoni, 1966) are the two steps known so far to be catalysed by enzymes. The work presented in the present paper provides the first evidence for the enzymic conversion of *p*-hydroxycinnamate into *p*-hydroxybenzoate in rat liver mitochondria.

MATERIALS AND METHODS

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 catalyst (Booth *et al.* 1960). All other chemicals were of AnalaR grade.

Animals and preparation of tissues. Albino rats, weighing about 200 g, from the stock colony were used. The animals were killed by stunning and decapitation, the



Scheme 1.

livers quickly removed and kept in ice. For the experiments with minces, the livers were sliced with a pair of scissors until a fine mince was obtained. The livers were homogenized in a Potter–Elvehjem-type glass homogenizer in 0.25M-sucrose (10ml/g of liver). Mitochondria were obtained by the conventional differential-centrifugation procedure of Schneider & Hogeboom (1950) and were washed once with the homogenization medium and suspended in 0.25M-sucrose.

Incubation conditions and enzyme assay. The reaction mixture consisted of 25μmol of potassium phosphate buffer (pH7.4), or 50μmol of tris–HCl buffer (pH7.4), where mentioned, 5μmol of ATP and 0.2ml of mitochondrial suspension (about 5–8mg of protein) in a total volume of 0.5ml. Incubation was carried out for 60min, or a specified time, at 37°C in a water bath, with reciprocal shaking of the tubes. At the end of the incubation 0.2ml of 10M-$HClO_4$ was added, the mixture centrifuged and the clear supernatant was extracted three times with 2ml portions of diethyl ether. The pooled ether extract was evaporated to dryness and the residue was dissolved in a small volume of ethyl acetate for paper chromatography. *p*-Hydroxybenzoate was separated and determined as described below. The enzyme activity is expressed as nmol of *p*-hydroxybenzoate formed/h per mg of mitochondrial protein or per g of liver.

All the assays were run in duplicates for each sample. Duplicates showed good agreement and the mean values are given. No *p*-hydroxybenzoate was formed when *p*-hydroxycinnamate was incubated with boiled or $HClO_4$-treated enzyme.

Separation and determination of p-*hydroxybenzoate.* The ether extracts were chromatographed on Whatman no. 1 filter paper, with benzene–acetic acid–water (10:7:3, by vol.; organic phase) in one direction and aq. 2% (v/v) formic acid in the other. The phenolic compounds were detected under u.v. light (Mineral light UVS-12). They were further identified by spraying the paper with diazotized *p*-nitroaniline followed by aq. 5% (w/v) Na_2CO_3. Two blue spots corresponding to *cis*- and *trans*-forms of *p*-hydroxycinnamate (isomerized during chromatography in the first solvent) and a red spot corresponding to *p*-hydroxybenzoate were obtained on the chromatograms (Fig. 1).

The method of Bray, Humphris, Thorpe, White & Wood (1952) for phenolic compounds was used to determine *p*-hydroxybenzoate. The spot corresponding to it was cut out of the chromatogram and eluted with 2ml of ethanol. To this 0.2ml of diazotized *p*-nitroaniline reagent [freshly made by mixing 5ml of 0.3% (w/v) *p*-nitroaniline in 0.8M-HCl and 0.3ml of aq. 5% (w/v) $NaNO_2$ solution] and after 2min 0.8ml of aq. 5% (w/v) Na_2CO_3 solution were added. The volume was made up to 5ml with water and the colour intensity was read in a Klett–Summerson photoelectric colorimeter with green filter (500–570nm). The readings were proportional to the concentration of *p*-hydroxybenzoate in the range of 10–150nmol.

The recovery of added *p*-hydroxybenzoate after incubation, extraction and chromatography was about 95% over the range specified above. It was also found that the colour obtained was stable and the method could be used even after the paper had been sprayed with the reagents for visual detection.

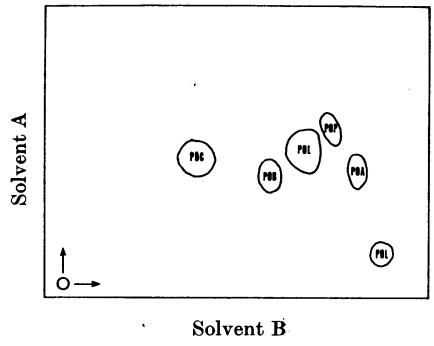


Fig. 1. Two-dimensional paper chromatogram of some phenolic acids. Solvent A: Benzene–acetic acid–water (10:7:3, by vol.; upper layer) (run: 18cm). Solvent B: 2% formic acid (run: 24cm). Abbreviations: POC, *p*-hydroxycinnamate; POB, *p*-hydroxybenzoate; POA, *p*-hydroxyphenylacetate; POP, *p*-hydroxyphenylpropionate; POL, *p*-hydroxyphenyl-lactate; O, origin.

p-Hydroxycinnamate was determined spectrophotometrically in alcoholic solution at 310nm ($E^{1\%}_{1cm}$ 678). *p*-Hydroxybenzoate did not show any extinction at this wavelength and *p*-hydroxycinnamate could be determined in the presence of *p*-hydroxybenzoate.

Measurement of oxidation of octanoate. The oxidation of octanoate by mitochondria was measured by the oxygen uptake in a Gilson KM Oxygraph. The medium consisted of 40μmol of potassium phosphate buffer (pH7.4), 3μmol of ATP, 10μmol of $MgCl_2$, 100μmol of KCl and 0.06μmol of cytochrome *c* (Sigma, type II), 2–4mg of mitochondrial protein and 1μmol of potassium octanoate in a total volume of 2ml made up with water. The rate of oxygen uptake was corrected for the endogenous rate.

RESULTS

Experiments with minces. In the first set of experiments liver minces were incubated in 0.05M-potassium phosphate buffer (pH7.4) with tyrosine, *p*-hydroxyphenyl-lactate and *p*-hydroxycinnamate. *p*-Hydroxybenzoate was formed only when *p*-hydroxycinnamate was used. It was found that shaking the reaction mixture in a Dubnoff metabolic shaker increased considerably the amount of *p*-hydroxybenzoate formed. No phenolic compound could be detected in the ether extract when tyrosine was used as substrate. Under similar experimental conditions kidney, brain and heart did not show formation of *p*-hydroxybenzoate from *p*-hydroxycinnamate. However, when 0.25M-sucrose was used as the medium for incubation, kidney and heart also showed formation of *p*-hydroxybenzoate.

Experiments with liver homogenates. In the second set of experiments, homogenates of liver were tested for their ability to convert *p*-hydroxycinnamate to *p*-hydroxybenzoate. The activity

could be detected only with homogenates made in 0.25M-sucrose and not in 1.1%(w/v) potassium chloride or 0.05M-potassium phosphate buffer (pH 7.4). Addition of nicotinamide, GSH, ferrous sulphate or sodium-hydrogensulphite did not show any effect. The activity could be enhanced by adding ATP (Table 1). The lack of activity in some cases was not due to destruction of *p*-hydroxybenzoate, because added *p*-hydroxybenzoate under the incubation conditions with homogenates was quantitatively recovered. The activity was found in the pH range 5.5–8.0 and was maximal around neutral pH values. When the homogenates in sucrose were stored frozen overnight, the activity was completely lost.

Experiments with mitochondria. On fractionation of the homogenates in sucrose into cell components and testing them, the activity was localized in the mitochondrial fraction (Table 2). Therefore further experiments were carried out with mitochondria.

Mitochondrial suspension (about 120mg of protein) was incubated with 10μmol of *p*-hydroxycinnamate, 100μmol of ATP and 1mmol of tris–HCl buffer, pH 7.4, for 2h in a Dubnoff metabolic shaker at 37°C. *p*-Hydroxybenzoate was purified from the ether extract by the procedure described above. The product was identified as *p*-hydroxybenzoate from its position in the two-dimensional paper chromatogram and u.v. spectrum in alcohol. It showed maximum absorption at 254nm and the peak shifted to 275nm when the solution was made alkaline (0.05ml of 1M-potassium hydroxide added to 3ml of *p*-hydroxybenzoate solution). These spectral characteristics are identical with those of the authentic *p*-hydroxybenzoate (Fig. 2).

Table 1. *Effect of adding nicotinamide, ATP, glutathione,* $NaHSO_3$ *and* $FeSO_4$ *on the formation of* p-*hydroxybenzoate from* p-*hydroxycinnamate by rat liver homogenate*

The incubation system consisted of 0.5μmol of *p*-hydroxycinnamate, 25μmol of KH_2PO_4 buffer (pH 7.0), 1ml of rat liver homogenate in 0.25M-sucrose (10ml/g of liver) and the other compounds in a total volume of 1.2ml. Incubation was carried out for 30min at 37°C with shaking.

Compound added	*p*-Hydroxybenzoate formed (nmol/h per g of liver)
None	280
Nicotinamide (1mg)	320
ATP (5μmol)	560
GSH (5μmol)	280
$FeSO_4$ (0.5μmol)	280
$NaHSO_3$ (1.0μmol)	280

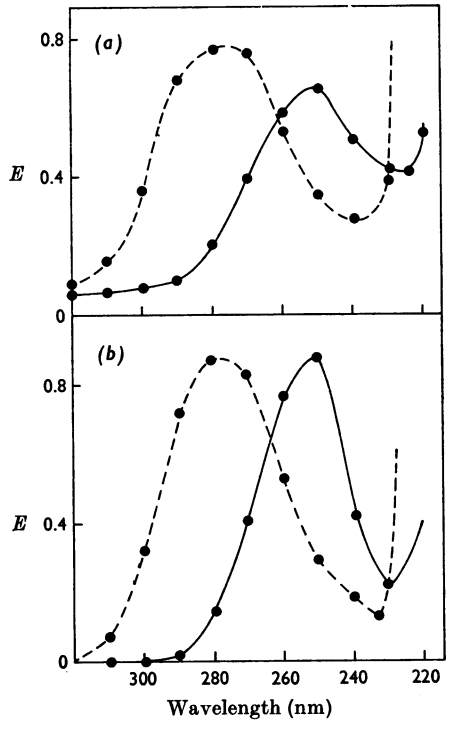


Fig. 2. Ultraviolet spectra of (*a*) *p*-hydroxybenzoate obtained from *p*-hydroxycinnamate by enzymic reaction, and (*b*) authentic *p*-hydroxybenzoate in neutral (——) and alkaline (----), solutions in ethanol.

Table 2. *Distribution of the enzyme activity in subcellular fractions of the liver*

The incubation system conisted of 1μmol of *p*-hydroxycinnamate, 10μmol of ATP, 100μmol of tris–HCl buffer (pH 7.4) and the subcellular fraction in a total volume of 1.3ml. Incubation was carried out for 90min at 37°C with shaking.

Cell fraction	*p*-Hydroxybenzoate formed (nmol/h per g of liver)	*p*-Hydroxybenzoate formed (nmol/h per mg of protein)	% of total activity in the fraction
Homogenate	258	0.55	100
Nuclei	25	0.38	9
Mitochondria	221	6.55	89
Postmitochondrial supernatant	0	0	0

Table 3. *Effect of adding various compounds on the formation of* p-*hydroxybenzoate from* p-*hydroxycinnamate by rat liver mitochondria*

The incubation system consisted of 0.5 μmol of *p*-hydroxycinnamate, 50 μmol of tris–HCl buffer (pH 7.4), freshly prepared mitochondria (4–6 mg of protein) and the respective compounds in a total volume of 0.5 ml. Incubation was carried out for 60 min at 37°C with shaking.

Expt. no.	Additions	ATP added (μmol)	*p*-Hydroxybenzoate formed (nmol/h per mg of protein)
1	None	0	0
	None	2	6.9
	None	5	8.5
	None	10	6.8
	Cytochrome *c* (0.1 mM)	0	0
	Cytochrome *c* (0.1 mM)	5	7.2
2	None	10	4.3
	Succinate (0.1 M) + ADP (10 mM)	0	1.4
	Succinate (0.1 M)	0	0
	NADP (0.5 mg) + isocitrate (4 mM)	0	0
	KCN (3 μmol)	10	4.1
	$MgCl_2$ (5 μmol)	10	4.5
3	None	10	6.2
	Coenzyme A (0.2 mg)	10	6.5

The enzyme activity in the mitochondria was lost on freezing and storage for 1 day. Even when the mitochondria were kept at 0–5°C without freezing for 24 h more than 90% of the activity was lost. The activity of the enzyme could not be restored by the addition of GSH, β-mercaptoethanol, fumarate, ferrous sulphate or cytochrome *c*.

With mitochondria addition of ATP was an absolute requirement for activity, and this could be replaced partly by succinate plus ADP, but not by cytochrome *c*, succinate alone or NADP plus isocitrate (Table 3). Maximal activity was obtained with 10 mM-ATP. This activity was not significantly affected by added magnesium chloride, cytochrome *c*, $\alpha\alpha$-bipyridyl, *o*-phenanthroline or coenzyme A.

The activity was maximal around neutral pH and decreased very sharply at pH values below 7.0 and above 7.5 (Fig. 3). The activity increased with increase in substrate concentration and the K_m for *p*-hydroxycinnamate calculated from the Lineweaver–Burk plot was 3.1×10^{-4} M (Fig. 4). Under the experimental conditions the activity was linear up to 90 min of incubation and the amount of *p*-hydroxybenzoate in the reaction mixture remained constant thereafter. This may be due either to the inactivation of the enzyme or to non-availability of ATP, which may have been hydrolysed by this time. Addition of ATP at 1 h intervals of time was therefore tested. The results in Fig. 5 show that the reaction rate was maintained beyond 90 min with extra ATP added.

It is not yet possible to decide whether the reaction is oxidative in nature. The rate of the reaction is too slow to measure the uptake of oxygen either in the Oxygraph or in a Warburg manometer. However, carrying out the reaction with reciprocal shaking of the tubes gave nearly a twofold increase in the activity. Also, carrying out the reaction in a nitrogen atmosphere decreased the activity to 50%. But no conclusion could be drawn since the required amount of oxygen, if any, being so small, may be available as dissolved oxygen.

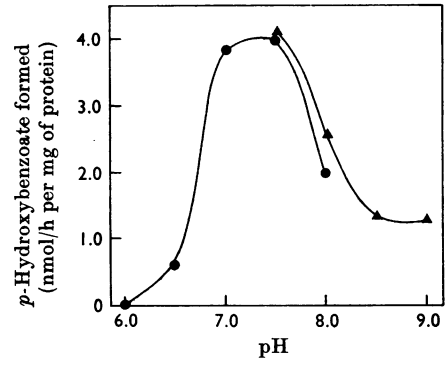


Fig. 3. Influence of pH on the formation of *p*-hydroxybenzoate from *p*-hydroxycinnamate in rat liver mitochondria. ●, Phosphate buffer; ▲, tris–HCl buffer.

Stoicheiometry. In order to study the stoicheiometry of the reaction, *p*-hydroxycinnamate was incubated under standard conditions for 90 min to obtain sufficient conversion. The phenolic compounds were extracted and separated by paper chromatography. The recovery of *p*-hydroxybenzoate and *p*-hydroxycinnamate, under con-

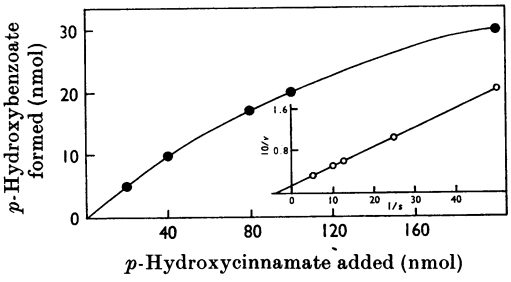


Fig. 4. Effect of concentration of *p*-hydroxycinnamate on the formation of *p*-hydroxybenzoate. Experimental details are given in the text. Double-reciprocal plot of the same results is given in the inset. The units for $1/s$ and $1/v$ are μmol^{-1} and (nmol of *p*-hydroxybenzoate/h)$^{-1}$, respectively.

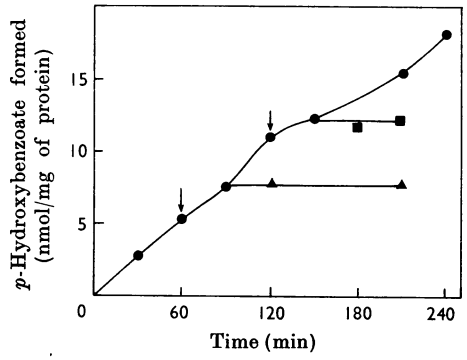


Fig. 5. Time-course of the formation of *p*-hydroxybenzoate and the effect of adding more ATP during incubation. The incubation system consisted of 0.5μmol *p*-hydroxycinnamate, 50μmol of tris–HCl buffer (pH 7.6), 4.4mg of mitochondrial protein and 5μmol of ATP in a total volume of 0.5ml. A further 5μmol of ATP was added at 60min and 120min (shown by arrows). The tubes that did not receive additional ATP at 60min and 120min are shown by ▲ and ■ respectively.

ditions when perchloric acid was added before mitochondria, was greater than 95%. The results in Table 4 show that all the *p*-hydroxycinnamate that disappeared was not accounted for as *p*-hydroxybenzoate. The *p*-hydroxycinnamate not accounted for was not in the form of any ether-extractable phenolic compound, since no other spot was detected in the chromatograms. The difference therefore must be in the bound form in the mitochondria, and its nature remains to be investigated.

Specificity. The formation of *p*-hydroxybenzoate was found under the standard experimental conditions only with *p*-hydroxycinnamate and *p*-hydroxyphenylpropionate as substrates, but not with *p*-hydroxyphenylacetate, *p*-hydroxyphenyllactate and tyrosine. The rate of formation of hydroxybenzoate was the same with both *p*-hydroxycinnamate and *p*-hydroxyphenylpropionate. *p*-Hydroxycinnamate was detected in the ether extracts when *p*-hydroxyphenylpropionate was incubated with mitochondria.

Effect of feeding with α-p-Chlorophenoxyisobutyrate ethyl ester. It was found in this laboratory that feeding rats with this drug, which is antihypercholesterolemic (Thorp & Waring, 1962), increased the hepatic concentration of ubiquinone (Krishnaiah, Inamdar & Ramasarma, 1967) and its synthesis from [2-^{14}C]mevalonate (Krishnaiah & Ramasarma, 1969). It was therefore decided to see whether the formation of *p*-hydroxybenzoate increases after feeding with this drug. The results in Table 5 show that formation of *p*-hydroxybenzoate increased twofold without affecting fatty acid oxidation in mitochondria, obtained from livers of rats fed for 15 days with 0.5% (w/w) of the drug in the diet. This increase was also observed with α-*p*-chlorophenoxyisobutyric acid (sodium salt) feeding, along with the lowering of serum sterol (Kritchevsky, Tepper, Sallata, Kabakjeen & Cristofalo, 1969) and elevation of hepatic ubiquinone (T. P. Krishnakanta & C. K. Ramakrishna Kurup, unpublished results).

DISCUSSION

The postulated conversion of *p*-hydroxycinnamate into *p*-hydroxybenzoate (Booth *et al.* 1960) is supported by the demonstration of such an enzymic conversion in the mitochondria. Although it is apparent from some of the properties that this is probably similar to the β-oxidation type of reactions there are some distinctive features. Knoop (1904) has shown that phenyl-substituted fatty acids undergo β-oxidation in intact animals. It is therefore appropriate to compare our system with fatty acid oxidation systems.

The requirement for fresh mitochondria and ATP in the media used is shared by the two systems. *p*-Hydroxybenzoate formation was irreversibly lost in aged mitochondria, whereas octanoate oxidation could be partially restored by the addition of fumarate. It does not resemble the GTP-dependent system for activation of fatty acids (Galzigna, Rossi, Sartorelli & Gibson, 1967) in being sensitive to dinitrophenol and not to P_i. Cyanide did not inhibit formation of *p*-hydroxybenzoate, but completely inhibited oxidation of octanoate.

Addition of octanoate inhibited formation of *p*-hydroxybenzoate from *p*-hydroxycinnamate. But addition of *p*-hydroxycinnamate, even at 10mM as used in the assay, did not inhibit oxidation of octanoate or uncouple oxidative phosphorylation in mitochondria. The most striking distinction

Table 4. *Stoicheiometry of disappearance of* p-*hydroxycinnamate and formation of* p-*hydroxybenzoate*

The incubation system consisted of *p*-hydroxycinnamate, 5 μmol of ATP, 50 μmol of tris–HCl buffer (pH 7.4) and the mitochondrial suspension, in a total volume of 0.5 ml. Incubation was carried out for 90 min at 37°C with shaking. The ether extract was dissolved in ethanol and *p*-hydroxycinnamate was determined spectrophotometrically. *p*-Hydroxybenzoate was determined after separation from *p*-hydroxycinnamate by paper chromatography.

Expt. no.	Mitochondrial protein added (mg)	*p*-Hydroxycinnamate added (nmol)	*p*-Hydroxycinnamate disappeared (nmol)	*p*-Hydroxybenzoate formed (nmol)	Phenolic compound not accounted for (nmol)
1	5.6	200	61	49	12
		200	60	54	6
		200	61	51	10
2	8.0	100	54	27	27
		50	26	13	13

Table 5. *Effect of feeding with* α-p-*chlorophenoxyisobutyrate* (*CPIB*) *on mitochondrial oxidation of octanoate and formation of* p-*hydroxybenzoate from* p-*hydroxycinnamate*

Experimental details are described in the text. Values are given ±S.D. with the numbers of animals used in parentheses.

	Normal	CPIB-fed	*P*-value
Octanoate oxidized (n-atoms of oxygen/min per mg of protein)	14.8±5.9 (5)	16.0+7.7 (6)	>0.1
p-Hydroxybenzoate formed (nmol/h mg of protein)	3.9±0.3 (5)	8.1+1.1 (6)	<0.001

between the two systems is the increase in the rate of conversion of *p*-hydroxycinnamate into *p*-hydroxybenzoate without altering the rate of octanoate oxidation in α-*p*-chlorophenoxyisobutyrate-treated animals.

It would be of value to know the fate of the C_2 fragment, the second product of the reaction. If the reaction were similar to the β-oxidation type of reactions, the two carbon atoms will be converted into CO_2 via acetyl-CoA. An alternative possibility exists, that the second product may be oxalate, since it was reported that oxalate was formed from the side chain of tyrosine and excreted in the urine in the rat (Cook & Henderson, 1969). This requires further investigation.

Many other questions also arise. There may be some intermediates between *p*-hydroxycinnamate and *p*-hydroxybenzoate. The part of the *p*-hydroxycinnamate not accounted for in stoicheiometry studies may be in the form of bound intermediates. The product *p*-hydroxybenzoate could be in the form of a CoA derivative in equilibrium with the free substance, or it may be a direct product. The requirement for the large amount of ATP remains to be explained. Addition of oligomycin did not give any change in the rate of formation of *p*-hydroxybenzoate, implying the absence of involvement of high-energy intermediates. The possibility of the activation of the carboxyl group, the energy being provided by ATP, is still not ruled out. ATP may also be serving to protect the mitochondria from swelling.

The specific increase in the rate of formation of *p*-hydroxybenzoate in the chlorophenoxyisobutyrate-treated animals deserves special mention. Ubiquinone content increases during this treatment, and it is logical that the formation of *p*-hydroxybenzoate, the precursor of the ring of ubiquinone, also increased which may be responsible for the observed increase in ubiquinone. This adds indirect support for the idea that this system is responsible for the physiological formation of *p*-hydroxybenzoate.

REFERENCES

Bentley, R., Ramsey, V. G., Springer, C. M., Dialameh, G. H. & Olson, R. E. (1961). *Biochem. biophys. Res. Commun.* **5**, 443.

Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O. H., Jones, F. T. & DeEds, F. (1960). *J. biol. Chem.*, **235**, 2649.

Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952). *Biochem. J.* **52**, 416.

Cook, D. A. & Henderson, L. M. (1969). *Biochim. biophys. Acta*, **184**, 404.

Galzigna, L., Rossi, C. R., Sartorelli, L. & Gibson, D. M. (1967). *J. biol. Chem.* **242**, 2111.

Knoop, F. (1904). *Beitr. chem. Physiol. Path.* **6**, 150.

Krishnaiah, K. V., Inamdar, A. R. & Ramasarma, T. (1967). *Biochem. biophys. Res. Commun.* **27**, 474.

Krishnaiah, K. V. & Ramasarma, T. (1969). *Biochem. J.* **116**, 321.

Kritchevsky, D., Tepper, A. S., Sallata, P., Kabakjeen, J. R. & Cristofalo, V. J. (1969). *Proc. Soc. exp. Biol. Med.* **132**, 76.

Olson, R. E. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.* **24**, 85.

Olson, R. E., Bentley, R., Aiyar, A. S., Dialameh, G. H., Gold, P. H., Ramsey, V. G. & Springer, C. M. (1963). *J. biol. Chem.* **238**, PC 3146.

Olson, R. E., Dialameh, G. H. & Bentley, R. (1960). *Fedn Proc. Fedn Am. Socs exp. Biol.* **19**, 220.

Rudney, H. & Parson, W. W. (1963). *J. biol. Chem.* **238**, PC 3137.

Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.

Thorp, J. M. & Waring, W. S. (1962). *Nature, Lond.*, **194**, 948.

Weber, W. & Zannoni, V. G. (1966). *J. biol. Chem.* **241**, 1340.