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Effects of L-Phenylalanine and Sodium Phenylpyruvate on the Formation of Adrenaline from L-Tyrosine in Adrenal Medulla *in vitro*

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While investigating the metabolism of L-tyrosine in rat-liver slices, Bickis, Kennedy & Quastel (1957) found that both L-phenylalanine and sodium phenylpyruvate inhibit the breakdown of this amino acid to acetoacetate and its incorporation into liver proteins. They suggested that these aromatic acids may affect other aspects of L-tyrosine metabolism, as, for example, the formation of hormones such as adrenaline, noradrenaline or thyroxine.

The biosynthetic pathway in bovine adrenal medulla has been established as: L-tyrosine $\rightarrow$ L-3:4-dihydroxyphenylalanine (dopa) $\rightarrow$ 3-hydroxytyramine $\rightarrow$ noradrenaline $\rightarrow$ adrenaline (Goodall & Kirshner, 1957). It is known that phenylpyruvate inhibits L-dopa decarboxylase in extracts of pig-kidney (Hartman, Akawie & Clark, 1955) and of guinea-pig adrenal medulla (Fellman, 1956), L-phenylalanine, however, having no effect. It has been suggested (Fellman, 1956) that interference with adrenaline formation by phenylalanine metabolites occurs by inhibition of L-dopa decarboxylase in adrenal medulla.

One of the phenomena associated with the inherited metabolic disorder, phenylketonuria, is a decreased concentration of plasma adrenaline. Testing 13 phenylketonuria patients and 117 normal humans, Weil-Malherbe (1955) found average basal adrenaline values of 0.62 and 1.52 $\mu\text{g./l.}$ respectively. Bickel, Gerrard & Hickmans (1954) reported that the blood concentration of tyrosine in phenylketonuria patients is normal or only slightly lower, but L-phenylalanine accumulates in the blood, reaching concentrations as high as 41 mg./100 ml. (2.5 mM) (Jervis, Block, Bolling & Kanze,

1940). This value is relatively constant in individual patients even after the ingestion of phenylalanine (Borek, Brecher, Jervis & Waelisch, 1950). Phenylpyruvic acid also accumulates in the blood; the concentration in fasting patients is usually not more than 1.8 mg./100 ml. (0.1 mM), but it may increase to four times this value after the ingestion of phenylalanine (Jervis, 1952). In normal human blood, the concentration of both L-tyrosine and L-phenylalanine is approx. 10 mg./100 ml. (0.06 mM) (Johnson & Bergeim, 1951) and phenylpyruvate is not detectable.

The present paper is concerned with the formation of adrenaline by adrenal medulla *in vitro* and the effects thereon of L-phenylalanine and phenylpyruvate under conditions approximating to the physiological. Although adrenal medulla contains ascorbic acid, additional quantities of this compound were added to the media in which the tissue was incubated in order to promote the conversion of tyrosine into dopa, considered by Holtz (1959) to be the rate-limiting reaction in the synthesis of catecholamines.

EXPERIMENTAL

Materials. Adult guinea pigs (800–1200 g.) were obtained locally. DL-[2- ^{14}C]Tyrosine (0.2 mCi; 8.54 mg.) came from Atomic Energy of Canada Ltd. DL-[^{14}C]Adrenaline D-bitartrate (0.1 mCi; 30.3 mg.) was obtained from Tracerlab Inc. L-Tyrosine, L-phenylalanine, sodium phenylpyruvate, DL-dopa, 3-hydroxytyramine-HCl, DL-noradrenaline and adrenaline (U.S.P.) were obtained from Nutritional Biochemicals Corp.

Preparation of biological material. Two guinea pigs were killed by decapitation and the adrenals were removed to chilled Ringer solution of the following composition: 0.14M-NaCl, 6 mM-KCl, 1 mM- CaCl_2 , 1 mM- MgSO_4 and 1 mM- KH_2PO_4 . The medullary tissue was prepared by the method of Hokin, Benfey & Hokin (1958). The bulk of the

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cortex (yellow) was removed by taking one slice from each side of an adrenal with a Stadie & Riggs (1944) slicer. The two slices were discarded and the remaining frill of cortex around the medulla (red) was trimmed off with scissors. The isolated medulla was sliced and the slices were divided evenly between two chilled Warburg vessels: a control and a test. Each vessel contained an average wet weight of 150 mg. of tissue.

Incubation technique. The reaction mixture consisted of Ringer solution, 0.01 M-phosphate buffer, pH 7.4 [0.01 M- Na_2HPO_4 -0.01 M- KH_2PO_4 (81:19, v/v)], 1 mM-sodium ascorbate, 5 mM-glucose and 0.05 mM-L-[2- ^{14}C]tyrosine in a total volume of 3 ml. The radioactive tyrosine was prepared by diluting DL-[2- ^{14}C]tyrosine with non-radioactive L-tyrosine so that the final solution contained 81% of the L-form. The initial radioactivity of L-[2- ^{14}C]tyrosine in each vessel was 43 000 counts/min. The total amount was 0.15 μmole and the specific activity was 287 000 counts/min./ μmole . The tyrosine was in the side arm and the other constituents were in the main compartment. The centre well contained a paper soaked in KOH to absorb CO_2 . In addition, the test vessel contained sodium phenylpyruvate or L-phenylalanine at concentrations ranging from 0.05 to 2.5 mM. The vessels were gassed with O_2 and equilibrated in a water bath at 38°. At zero time the tyrosine was tipped in and the vessels were incubated for 150 min.

Extraction of catechols. After incubation, the vessels were removed quickly to a tray of crushed ice and the reaction was stopped by the addition of 0.2 ml. of 2 N-HCl. Throughout the following procedures care was taken to keep the mixtures either cold and acidic or under N_2 , in order to protect the catechols against auto-oxidation. Extractions were performed with acidic ethanol (0.1 ml. of conc. HCl/100 ml. of 95% ethanol). The medium was decanted into chilled 15 ml. plastic centrifuge tubes, and the vessels were washed with 3 ml. of acidic ethanol, the wash being added to the medium.

A stock solution of 'carrier' catechols (adrenaline U.S.P., DL-noradrenaline, 3-hydroxytyramine-HCl and DL-dopa) had been previously prepared, containing 20 μg . of each catechol/20 μl . of 0.01 N-HCl; 20 μl . of this solution was added to the medium. Thus any radioactive catechols that might have been formed were further protected against degradation by the addition of non-radioactive 'carriers'.

The medium was centrifuged at 5000g for 20 min. at 4°. The precipitate was washed with 3 ml. of acidic ethanol and spun in the same way for 10 min., and the combined supernatants were evaporated to dryness at low pressure (water pump) under N_2 at 30-35°. The evaporation took just over 1 hr. The residue was taken up in three portions of 0.5 ml. of acidic ethanol, transferred to a 12 ml. glass

centrifuge tube, stoppered tightly and stored in the deep freeze overnight.

On the following day, the ethanol was evaporated to 0.1 ml. under a stream of N_2 , the contents of the tube were centrifuged at 4°, and the supernatant was applied from a micropipette as successive 5 μl . amounts to the origin of a paper chromatogram. Between applications, the spots were dried in a stream of N_2 . The residue was washed three times with 0.1 ml. of acidic ethanol and centrifuged, and the supernatant was spotted on the origin as before.

Separation of catechols. Two-dimensional paper chromatography on a sheet of Whatman no. 1 filter paper, 17 in. $\times$ 17 in. was carried out by the ascending method, the solvent systems being butan-1-ol-N-HCl-water (4:1:5, by vol.) (Hamberg & Euler, 1950) and phenol-0.1 N-HCl (85:15, w/v) (Vogt, 1952). 'Carrier' catechols (20 μl . of the stock solution) and the test solution were applied to the origin in the manner already described. The chromatogram was allowed to develop in each solvent in turn at room temperature for 36-40 hr. in an atmosphere of N_2 .

Identification of catechols. The dried chromatogram was dipped in 0.44% $\text{K}_3\text{Fe}(\text{CN})_6$ in 0.1 M-phosphate buffer, pH 7.8 (James, 1948). The catechols were immediately oxidized and appeared as coloured spots on a yellow background. The paper was dried and dipped in 0.4% ninhydrin in water-saturated butan-1-ol to locate the tyrosine spot. The test compounds were identified by their R_F values in two solvent systems and by their colours (Table 1). A total of 40 μg . of each 'carrier' catechol had been processed: 20 μg . added immediately after incubation and 20 μg . applied to the origin of the chromatogram. The ensuing spots thus gave easy identification and the method provided effective separation, as well as protection of newly synthesized radioactive catechols against loss.

It is known that the R_F values of substances may be affected by the presence of other compounds. However, in all experiments the R_F values were found to be the same as those of control compounds applied to a chromatogram as solutions in 0.1 N-HCl. This finding is in agreement with that of Euler & Hökfelt (1953), who found that there was no displacement of catecholamine spots if extractions were carried out with acidic ethanol.

Measurements of catechols. The radioactivity of the isolated spots was measured with an end-window Geiger-Müller tube (Tracerlab) which had 6% efficiency. Corrections were made for background and for absorption on the paper, and the results were finally expressed as counts/min./150 mg. wet wt. of adrenal medulla. The amount of each newly synthesized radioactive catechol was calculated from the specific activity of the experimental L-[2- ^{14}C]tyrosine and expressed as μm -moles.

Table 1. *Identification of catechols and tyrosine by paper chromatography*

For details see Experimental section.

	R_F		Colour	
	Butanol-HCl-water	Phenol-HCl	$\text{K}_3\text{Fe}(\text{CN})_6$	Ninhydrin
Adrenaline	0.17	0.61	Pink	Violet
Noradrenaline	0.11	0.30	Pink	Brown
3-Hydroxytyramine	0.21	0.53	Blue	Brown
Dopa	0.18	0.28	Blue	Dark blue
Tyrosine	0.51	0.60	—	Purple

Table 2. *Effects of sodium phenylpyruvate and L-phenylalanine on the synthesis of radioactive catechols from L-[2-¹⁴C]tyrosine in the presence of slices of guinea-pig adrenal medulla*

The incubation mixture contained 0.05 mM-L-[2-¹⁴C]tyrosine, 1 mM-sodium ascorbate, 5 mM-glucose, 0.01 M-phosphate buffer, pH 7.4 (see text), Ringer solution and various concentrations of sodium phenylpyruvate and L-phenylalanine. Total volume, 3 ml.; average wet wt. of adrenal medulla, 150 mg.; incubation time, 150 min.; atmosphere, O₂; temp., 38°. Catechols were extracted from the medium with acidic ethanol and separated by two-dimensional paper chromatography. Recoveries were calculated from radioactivity of isolated spots and from specific activity of [2-¹⁴C]tyrosine and expressed as μ m-moles.

Sodium phenylpyruvate (mM)	L-Phenylalanine (mM)	No. of observations	Dopa (μ m-moles)		3-Hydroxytyramine (μ m-moles)		Adrenaline (μ m-moles)		Inhibition of adrenaline synthesis (%)
			Mean	Range	Mean	Range	Mean	Range	
—	—	16	0	—	7.5	3.6–12.5	2.1	1.5–2.5	—
0.05	—	3	1.3	0.5–1.8	9.4	2.7–13.6	1.3	1.1–1.6	33
0.10	—	5	2.1	0.9–3.2	6.6	3.0–12.4	1.0	0.7–1.1	50
0.25	—	3	2.3	1.6–3.2	4.6	1.7–8.4	0.2	0.04–0.4	90
0.50	—	1	2.6	—	3.2	—	0.1	—	97
—	0.10	1	0	—	5.7	—	2.3	—	—
—	0.25	1	0	—	7.1	—	1.8	—	—
—	0.50	1	0	—	9.1	—	2.0	—	—
—	2.5	1	0	—	9.5	—	2.3	—	—

RESULTS

Recovery of adrenaline

To test for loss of adrenaline during the foregoing experimental procedures, 1 μ g. of DL-[¹⁴C]adrenaline D-bitartrate (2.5 μ m-moles of DL-[¹⁴C]adrenaline) was used in place of L-[¹⁴C]tyrosine. Incubation with adrenal-medulla slices, extraction, identification by paper chromatography and measurement of radioactivity were performed as before. The recovery corresponded to 100 % of the original amount of [¹⁴C]adrenaline, indicating that there had been no loss during either incubation or isolation.

Synthesis of catechols from L-[2-¹⁴C]tyrosine

Controls. The average amount of [¹⁴C]adrenaline formed in 16 control flasks was 2.1 μ m-moles (Table 2).

Radioactive 3-hydroxytyramine was also found. The average amount recovered was 7.5 μ m-moles. However, the results showed a much wider range than with adrenaline, indicating that there might have been considerable losses. Neither [¹⁴C]noradrenaline nor [¹⁴C]dopa could be recovered from control flasks.

Effect of sodium phenylpyruvate. There was a marked inhibitory effect on the formation of [¹⁴C]adrenaline. Phenylpyruvate concentrations of 0.05, 0.10, 0.25 and 0.50 mM caused inhibitions of 33, 50, 90 and 97 % respectively.

The effect on 3-hydroxy[¹⁴C]tyramine was not as clear. The conflicting results indicate that this compound was less stable than adrenaline under our experimental conditions. No [¹⁴C]noradrenaline could be detected. However, [¹⁴C]dopa was re-

covered from all the test flasks, although there was no well-defined effect of various concentrations of phenylpyruvate.

The recovery of labelled dopa suggested that phenylpyruvate had inhibited L-dopa decarboxylase, thereby causing both an accumulation of dopa and a decreased synthesis of adrenaline.

However, there remained the possibility that the reduced recovery of [¹⁴C]adrenaline might have been partly due to its degradation after synthesis. To test this possibility, 2.5 μ m-moles of DL-[¹⁴C]adrenaline were incubated with adrenal-medulla slices, both in the presence and in the absence of 0.50 mM-sodium phenylpyruvate. After extraction, identification and estimation of radioactivity, the recoveries of [¹⁴C]adrenaline from the control and test samples amounted to 94 and 105 % respectively. This result eliminated the possibility that phenylpyruvate had any effect on adrenaline after the latter's synthesis from L-tyrosine.

L-Phenylalanine. Addition of this substance had no effect on the recovery of radioactive adrenaline or 3-hydroxytyramine even when the concentration of L-phenylalanine was as high as 2.5 mM. As with the controls, radioactive dopa could not be detected.

DISCUSSION

Synthesis of catecholamines from L-tyrosine

In the present study, L-[2-¹⁴C]tyrosine was converted into labelled catecholamines in the presence of slices of guinea-pig adrenal medulla. Under experimental conditions, the rate of adrenaline synthesis was 2.1 μ m-moles/150 mg. wet wt. of adrenal medulla/150 min. A low rate of adrenaline synthesis from [¹⁴C]tyrosine was also found by

Goodall & Kirshner (1957), who used ox-adrenal-medulla slices, and by Rosenfeld, Leeper & Udenfriend (1958), who used isolated, perfused calf adrenals.

No [^{14}C]noradrenaline could be detected. This observation was in accord with that of Hökfelt (1951), who found virtually no noradrenaline in the adrenal glands of adult guinea pigs, and with that of Hokin *et al.* (1958), who found that adrenal-medulla slices released adrenaline but no noradrenaline into the medium during an incubation period of 2 hr. These observations suggest that the *N*-methylation of noradrenaline is very efficient in the adrenal medulla of adult guinea pigs. The present results indicate that this reaction is much faster than the conversion of 3-hydroxytyramine into noradrenaline.

However, 3-hydroxy[^{14}C]tyramine was recovered. Its rate of production from L-[2- ^{14}C]tyrosine was calculated to be 7.5 $\mu\text{m-moles/150 mg. wet wt. of adrenal medulla/150 min.}$ This rate was 3.5 times that of adrenaline. However, the presence of monoamine oxidase in adrenal medulla (Lange-mann, 1951) might have caused a decrease in the yield of 3-hydroxytyramine. Pellerin & D'Iorio (1957) found equal amounts of 3:4-dihydroxy-phenylacetic acid and 3-hydroxytyramine after the incubation of ox-adrenal-medulla homogenate with radioactive dopa. Goodall & Kirshner (1957) also reported a poor recovery of 3-hydroxytyramine after the incubation of ox-adrenal-medulla slices with radioactive tyrosine.

No [^{14}C]dopa could be recovered from control flasks. This finding is consistent with that of Demis, Blaschko & Welch (1955, 1956), who observed 94% decarboxylation of L-dopa by ox-adrenal-medulla homogenate during an incubation period of 2 hr. Kirshner (1959) reported that this reaction is apparently the fastest of the entire sequence in the conversion of L-tyrosine into adrenaline. Moreover, dopa is rapidly oxidized at a pH above 7. Thus it would be very unstable during an incubation period of 150 min. at pH 7.4.

Stability of adrenaline during incubation

The average amount of [^{14}C]adrenaline recovered was 2.1 $\mu\text{m-moles}$. When a similar amount of DL-[^{14}C]adrenaline (2.5 $\mu\text{m-moles}$) was incubated with adrenal-medulla slices, there was 100% recovery. Other workers have also reported on the stability of adrenaline under similar conditions. Euler & Floding (1958) incubated adrenaline with ox-adrenal-medulla homogenate for 2 hr. and observed its 'remarkable stability'. Hokin *et al.* (1958) incubated 20 $\mu\text{g.}$ of adrenaline bitartrate with slices of guinea-pig adrenal medulla for 90 min. and also found that there had been no loss.

In the present experiments, adrenaline may

have been protected against chemical oxidation by the presence of ascorbic acid in the medium. Heard & Welch (1935), Blaschko, Richter & Schlossmann (1937) and Euler (1956) are among those who have observed that ascorbic acid inhibits the auto-oxidation of adrenaline. Moreover, 3-hydroxytyramine may have protected adrenaline against deamination by monoamine oxidase, as suggested by Goldstein, Friedhoff & Simmons (1959). Adrenaline is a poor substrate for this enzyme, whereas 3-hydroxytyramine is an excellent one (Blaschko, 1952).

Effect of phenylpyruvate on adrenaline formation

The concentrations of aromatic acids used in the present experiments were those found in phenylketonuric blood. L-Phenylalanine, at concentrations as high as 2.5 mM, had no effect on the conversion of L-tyrosine into adrenaline by slices of guinea-pig adrenal medulla. However, 0.1 mM-sodium phenylpyruvate caused 50% inhibition of adrenaline biosynthesis. This result may be compared with that of Weil-Malherbe (1955), who found 62% reduction of plasma adrenaline in phenylketonuria.

Moreover, decrease of adrenaline synthesis in the present experiments was accompanied by an accumulation of dopa, suggesting that the decarboxylation of L-dopa was inhibited. Sodium phenylpyruvate inhibits this reaction, but L-phenylalanine has no effect. Thus, if observations made with slices of guinea-pig adrenal medulla *in vitro* can be applied to phenylketonuria, a possible explanation is found for the low plasma-adrenaline concentrations of these patients. It would seem that the accumulation of blood phenylpyruvate causes a decrease in adrenaline formation by inhibiting L-dopa decarboxylase on the biosynthetic pathway from L-tyrosine to adrenaline in the adrenal medulla.

Recent evidence indicates that dopa decarboxylase and 5-hydroxytryptophan decarboxylase are apparently the same enzyme (Westermann, Balzer & Knell, 1958; Holtz, 1959; Yuwiler, Geller & Eiduson, 1959; Werle & Aures, 1959; Fellman, 1959; Hagen, Weiner, Ono & Lee, 1960; Udenfriend, Lovenberg & Weissbach, 1960). The product of 5-hydroxytryptophan decarboxylation is serotonin, which occurs in mammalian brain and blood. Not only have low blood concentrations of serotonin been reported in phenylketonuria (Pare, Sandler & Stacey, 1957, 1958), but phenylpyruvate inhibits 5-hydroxytryptophan decarboxylase *in vitro* (Davison & Sandler, 1958). The possibility that phenylpyruvate inhibits the formation of cerebral serotonin and catecholamines might therefore be accountable in some measure for the mental abnormalities associated with phenylketonuria.

SUMMARY

1. Incubation of slices of guinea-pig adrenal medulla with L-[2-¹⁴C]tyrosine in well-oxygenated modified Ringer solution results in the formation of labelled 3-hydroxytyramine and adrenaline.

2. The addition of 0.1 mM-sodium phenylpyruvate *in vitro* causes a 50% decrease in the recovery of labelled adrenaline. This decrease is caused by inhibition of the synthesis of adrenaline, rather than by its degradation after synthesis.

3. Whereas, usually, no labelled dihydroxyphenylalanine (dopa) is observed during the incubation of slices of guinea-pig adrenal medulla in the presence of labelled tyrosine, the addition of sodium phenylpyruvate results in the appearance of labelled dopa. This effect indicates that dopa decarboxylation is inhibited by sodium phenylpyruvate. It is concluded that inhibition of adrenaline biosynthesis by sodium phenylpyruvate is largely due to inhibition of dopa decarboxylase.

4. The addition of L-phenylalanine *in vitro* has no effect on the synthesis of adrenaline.

5. The bearing of these results on the mental abnormalities associated with phenylketonuria are discussed.

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