PRECURSORS OF ADRENAL EPINEPHRINE AND NOREPINEPHRINE *IN VIVO*

by

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It is generally accepted that epinephrine is derived from phenylalanine. Evidence for this was obtained by GURIN AND DELLUVA[1] who demonstrated that either deuterium or tritium-labelled phenylalanine gave rise to labelled adrenal epinephrine in rats. A previous report from this laboratory[2] has indicated that both ^{14}C-labelled phenylalanine and tyrosine are precursors of adrenal epinephrine. However, little is known about the steps leading to norepinephrine and epinephrine. Some textbooks suggest that tyramine is an intermediate in this process[3], others consider dihydroxyphenylalanine (DOPA) as a more likely precursor[4].

The present communication describes experiments showing that phenylalanine, tyrosine and DOPA act as precursors of adrenal epinephrine and norepinephrine in the rat whereas phenylethylamine and tyramine do not. Evidence is also presented showing that in the adrenal gland the turnover rates of norepinephrine as well as epinephrine[2] are relatively slow.

MATERIALS

L-Epinephrine bitartrate and L-norepinephrine bitartrate were donated by Winthrop Stearns.

D-L Tyrosine-2-^{14}C and D-L phenylalanine-3-^{14}C were obtained from Tracerlab, Inc. D-L DOPA-3-^{14}C and D-L epinephrine-2-^{14}C were obtained from Nuclear Chemical and Instrument Co. Tyramine-1-^{14}C and phenylethylamine-2-^{14}C were prepared by decarboxylation of the corresponding amino acids as previously described[5].

METHODS

Epinephrine and norepinephrine were assayed by modifications of the fluorimetric procedures of LUND[6] using I_2 instead of MnO_2** for the initial oxidation. Excess I_2 was destroyed by adding an equivalent amount of thiosulfate after the reaction. In this procedure epinephrine and norepinephrine fluoresce to approximately the same extent at pH 5 whereas at pH 3 epinephrine fluoresces twenty times as much as does norepinephrine. If measurements of unknowns and standards are made at both pH values the amounts of each catecholamine in a mixture of the two can be determined. Epinephrine was determined at pH 3 with no correction for norepinephrine since its fluorescence contribution at this pH is very low, and in rat adrenals only about 20% of the total catecholamines is norepinephrine.

The ^{14}C-labelled compounds were administered intraperitoneally, in divided daily doses,

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** The rates of oxidation of the epinephrines were found to be more reproducible with I_2 than with MnO_2.

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for several days. Details of dosage are presented in the appropriate tables. One to two days after the last dose the animals were sacrificed, and the adrenals were removed and homogenized in 2 ml of 0.1 *N* HCl in a glass homogenizer. One of two techniques was then used for isolation of the catecholamines; the direct carrier method for epinephrine alone and paper chromatography for both epinephrine and norepinephrine.

Isolation of epinephrine by direct carrier method

Two ml of 15% trichloracetic acid was added to the homogenate and the proteins were removed by centrifugation. The supernatant solution was transferred to a glass-stoppered centrifuge tube, extracted several times with 10 ml portions of ether to remove the trichloracetic acid and the aqueous layer was then warmed to evaporate dissolved ether. An aliquot containing about 1 μg of epinephrine was taken for fluorimetric assay. In another aliquot, comprising almost all the remainder of the adrenal extract, 20–25 mg of L-epinephrine bitartrate was dissolved as carrier and the solution was evaporated to about 1 ml under a stream of nitrogen. Several crystals of sodium bisulfite* were added and the solution was chilled to 0°. An equal volume of ice-cold concentrated NH_3 saturated with sodium bisulfite was added and the solution was left standing in the cold until crystallization of epinephrine was complete. The tube was centrifuged and the supernatant solution was decanted. The crystals were washed twice with absolute alcohol, twice with anhydrous ether, suspended in 0.2–0.3 ml of ether, and transferred to a planchette. After the crystals had sedimented the ether was removed by evaporation under an infrared lamp. Radioactivity was measured in a gas flow proportional counter having a background of about 2 c.p.m.[7]. All samples were recrystallized to constant specific activity.

Recrystallization controls

Purification by recrystallization alone may give misleading results since solid solution may occur with closely related compounds and give the impression of isotopic homogeneity[8]. The control experiments shown in Table I indicate that the catechol compounds under investigation readily separate from one another during crystallization and that it is valid to use the attainment of

TABLE I

CONTROL EXPERIMENTS DEMONSTRATING THE SEPARABILITY OF VARIOUS CATECHOL COMPOUNDS DURING CRYSTALLIZATION

About 10 mg of either epinephrine or norepinephrine was used as carrier. To these were added μg quantities of various ^{14}C labelled compounds. The carriers were then crystallized several times (see text) and specific activity was determined after each recrystallization.

^{14}C Compound added	*Number of times recrystallized*	*Carrier epinephrine c.p.m./mg*	*Carrier norepinephrine c.p.m./mg*
Epinephrine	0	—	200
	2	—	53
Epinephrine	2	289	—
	3	302	—
	4	286	—
Norepinephrine §	2	2420	—
	3	1814	—
	4	1050	—
Norepinephrine §	2	—	112
	3	—	110
	4	—	112
Dopamine	0	2442	—
	1	180	—
	2	16	—
DOPA	0	1637	—
	1	2	—
	2	0	—

§ ^{14}C-norepinephrine was obtained enzymically from ^{14}C-DOPA by incubation with homogenized pheochromocytoma tumor.

* Sodium bisulfite is added to prevent oxidation of catecholamines during crystallization from ammonia.

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constant specific activity as a criterion of isotopic homogeneity. In addition, tyrosine, phenylalanine, tyramine and phenylethylamine are removed almost completely from carrier epinephrine and norepinephrine after one crystallization. Furthermore, because of their slow rates of turnover (see below) the epinephrines could be isolated from animals sacrificed when the specific activity of the precursors in the tissues had fallen to low levels.

Isolation of epinephrine and norepinephrine by paper chromatography

Adrenals were homogenized in 0.5 ml of 0.15 *N* HCl, 10 ml of ethanol was added and the mixture rehomogenized and allowed to stand 15 minutes. After centrifugation, the clear supernant solution was evaporated to dryness at 40° under a stream of nitrogen. The residue was extracted by trituration with 0.5 ml and then with 0.25 ml of 0.1 *N* HCl in 95% ethanol, and the extract was applied to Whatman No. 1 paper as a single small spot which was then blown dry with nitrogen. The following chromatographic methods are modifications of those of CRAWFORD AND OUTSCHOORN[9]. As markers, 25 μg each of epinephrine, norepinephrine, DOPA and dihydroxyphenylethylamine (DOPamine) were applied as adjacent spots at the origin. Descending chromatograms were developed overnight with water-saturated phenol through which SO_2 had been bubbled for 10 minutes at room temperature. Air-tight chromatogram jars in which the atmosphere was replaced by SO_2 were used. Following development of a chromatogram the paper was washed in benzene and dried at room temperature for 30–60 minutes. The control area was then cut out and sprayed with ferricyanide reagent[10]. Adrenal epinephrine and norepinephrine were assumed to be opposite their control spots and these areas were cut out and eluted with 1–2 ml of 0.01 *N* HCl by descending chromatography in an air-tight glass bell jar. The eluates were then washed three times with 2 ml portions of benzene to remove traces of phenol. Aliquots were then analysed for epinephrine or norepinephrine* and other aliquots were dried on planchets and counted directly. In some experiments the final aliquot was divided and counted both directly and after recrystallization with added carrier; the specific activity values obtained by both methods were in excellent agreement for both compounds.

RESULTS

The results of typical experiments in which various compounds were tested as possible precursors of adrenal epinephrine and norepinephrine are summarized in Table II.

TABLE II

COMPOUNDS TESTED AS PRECURSORS OF ADRENAL EPINEPHRINE

^{14}C Compound administered	*Total counts administered* $\times 10^6$	*Specific activity of adrenal epinephrine c.p.m./μmole*	*Specific activity of adrenal norepinephrine c.p.m./μmole*
D-L-Phenylalanine-3-^{14}C	9.4	180	—
D-L-Phenylalanine-3-^{14}C	18.8	600	—
D-L-Tyrosine-2-^{14}C	6.6	280	—
D-L-Tyrosine-2-^{14}C	6.6	310	—
D-L-Tyrosine-2-^{14}C	8.6	834	492
D-L-Tyrosine-2-^{14}C	8.6	648	994
Tyramine-1-^{14}C	4.1	0	—
Tyramine-1-^{14}C	4.1	0	—
Phenylethylamine-2-^{14}C	12.0	0	—
Phenylethylamine-2-^{14}C	12.0	0	—
D-L-Dihydroxyphenylalanine-3-^{14}C	6.0	2510	—
D-L-Dihydroxyphenylalanine-3-^{14}C	6.0	1760	—
D-L-Dihydroxyphenylalanine-3-^{14}C	6.0	1910	1250
D-L-Epinephrine-1-^{14}C	7.0	174	—

* Overall recoveries of epinephrine and norepinephrine carried through the entire chromatographic procedure were about 60–70%.

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^{14}C-labelled phenylalanine, tyrosine and DOPA served as precursors. The highest labelling was obtained with DOPA. ^{14}C-tyramine and phenylethylamine produced no labelling whatever. It is of interest that the administration of ^{14}C-D-L-epinephrine also yielded labelling of adrenal epinephrine, which though slight was significant since no other tissue examined contained ^{14}C-epinephrine.

Because of the slow turnover of adrenal epinephrine and norepinephrine it was necessary to administer precursors over several days to get significant labelling. In Table III are shown the specific activities of rat adrenal epinephrine and those of circulating tyrosine which were obtained at the time of sacrifice. Initially, tyrosine was more highly labelled than epinephrine but after several days the reverse was true, thus satisfying the criteria of a precursor end-product relationship. It is obvious that the activity of adrenal epinephrine, in the later periods, cannot be due to relabelling from tyrosine.

In Table IV are shown the specific activities of epinephrine and norepinephrine isolated chromatographically from rat adrenals 1–8 days following the final injection of labelled tyrosine. Although individual variation is too great to permit exact calculation of turnover rates it is evident that the half-lives of both catecholamines in the adrenal gland are of the order of several days.

TABLE III

COMPARISON OF ACTIVITIES OF ADRENAL EPINEPHRINE AND PLASMA TYROSINE

Expt.	*Days after last dose*	*Specific activity (c.p.m./μmole)*		*Epinephrine*
		Free plasma tyrosine	*Adrenal epinephrine*	*Tyrosine*
1	1	700	280	0.4
2	1	930	180	0.2
3	1	600	310	0.3
4	7	258	1088	4.0
5	7	148	1604	10.8
6	12	150	450	3.0
7	12	178	836	4.8

D-L-Phenylalanine-3-^{14}C ($2.6 \cdot 10^5$ c.p.m. per μmole) or D-L-Tyrosine-2-^{14}C ($2.0 \cdot 10^5$ c.p.m. per μmole) were administered in doses of about 1 mg per day for several days. The animals were sacrificed at specified times after the last dose.

TABLE IV

TURNOVER STUDIES ON ADRENAL EPINEPHRINE AND NOREPINEPHRINE IN THE RAT

Time lapse	*c.p.m./μmole*	
days	*Epinephrine*	*Norepinephrine*
1	1835	1350
1	1226	2100
1	1320	1945
5	618	1358
5	648	994
5	834	726
8	425	804
8	438	778

D-L Tyrosine-2-^{14}C ($2.0 \cdot 10^5$ c.p.m./μmole) were given i.p., 2 mg/day × 4 days, and the rats were sacrificed after the indicated time lapse.

DISCUSSION

The findings that phenylalanine, tyrosine and DOPA can serve as precursors of adrenal epinephrine and norepinephrine are in agreement with many currently accepted theories concerning the biogenesis of these hormonal agents. However, they present the first direct evidence of such roles for tyrosine and DOPA. They do not support the possibility that tyramine[11] and phenylethylamine[12] are intermediates.

Further problems remain concerning formation of these hormones. The intermediate between DOPA and norepinephrine may be either DOPamine or 3–4 dihydroxyphenylserine. Since the enzyme DOPA decarboxylase is present in adrenal medulla and in other tissues and since DOPA and DOPamine have been found in

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urine[13, 14], DOPamine is considered to be the more likely intermediate. This is supported by preliminary studies in this laboratory indicating that homogenates of pheochromocytoma tumors can convert DOPA and DOPamine to norepinephrine*. *In vitro* conversion of ^{14}C-DOPamine to norepinephrine has also been reported by HAGEN[15].

The finding that circulating epinephrine can be taken up by the adrenal medulla is of interest since it may demonstrate an inherent property of this tissue to bind epinephrine and norepinephrine. However, the small extent of the labelling obtained in this way indicates that the bulk of adrenal epinephrine is synthesized from some precursor within the gland. If one assumes that all the epinephrine and norepinephrine synthesized by the adrenal gland must first mix with material already stored there, before being secreted, then the slow turnover requires a chemical explanation. This would suggest that at least one step in their synthesis in the adrenal gland is slow. Since the turnover rates of both catecholamines in the rat adrenal gland are of approximately equal rates the methylation of norepinephrine cannot be limiting with respect to epinephrine formation. It may be that the slow step in this pathway is the conversion of tyrosine to DOPA.

An alternative explanation is that the slow turnover of adrenal catecholamines is only apparent. This would assume that their synthesis takes place at a rapid rate but that most of the newly synthesized material is continually secreted into the blood, only small amounts being taken up for storage in the gland. The stored supply of catecholamines in the gland must then be relatively inert to normal stimuli.

SUMMARY

Isotopically labelled phenylalanine, tyrosine, and dihydroxyphenyalanine, in the intact rat, serve as precursors of adrenal epinephrine and norepinephrine; tyramine and phenylethylamine do not. The turnover rates of adrenal epinephrine and norepinephrine are relatively low, both having half-lives of about one week.

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