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3,4-DIHYDROXYPHENYLALANINE (DOPA), DOPAMINE AND NOREPINEPHRINE STORAGE IN THE RAT HEART AFTER L-DOPA—FURTHER EVIDENCE FOR NOREPINEPHRINE RELEASE

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Abstract—The storage of 3,4-dihydroxyphenylalanine (dopa), dopamine (DA) and norepinephrine (NE) in the rat heart was studied after the administration of a single dose of L-dopa (100 mg/kg). Endogenous NE concentration was significantly reduced 45 min after L-dopa despite high levels of dopa and DA. Synthesis blockade with disulfiram did not prevent the decline in [^{3}H]NE or endogenous NE induced by DA. Therefore, acceleration of NE synthesis by precursor substrate loading distal to the rate-limiting step in the biosynthetic pathway cannot explain the increased cardiac NE turnover that occurs after L-dopa. Administration of L-dopa resulted in a significant decrease in the retention of tracer [^{3}H]NE in both the particulate and soluble cell fractions without changing the subcellular distribution of [^{3}H]NE. Dopa and DA were recovered from only the soluble fraction of tissue homogenates. The storage of dopa and DA in the heart was unaffected by reserpine pretreatment. The results are consistent with dopa-induced NE release of the type that occurs with indirect acting sympathomimetic amines. The relationship of these findings to autonomic dysfunction in humans after L-dopa is discussed.

THE USE of L-3,4-dihydroxyphenylalanine (L-dopa) in the symptomatic treatment of Parkinson's disease is associated with significant alterations in autonomic function. Orthostatic hypotension has been the most frequent autonomic side effect in patients given L-dopa,[1–3] but sympathomimetic effects, such as cardiac arrhythmias,[1–4] hypertensive reactions,[1,3] increased cardiac contractility (shortened pre-ejection period),[5–7] and pupillary dilatation,[8,9] have also been noted. In experimental animals acute administration of L-dopa produces increased cardiac contractile force[6,7] and elevated blood pressure.[10,11] The cardiovascular effects of L-dopa are blocked both in patients[4] and experimental animals[6,7,10,11] by decarboxylase inhibition which prevents the conversion of dopa to dopamine. Although much of the influence of L-dopa on the cardiovascular system has been attributed to the direct effect of dopamine on adrenergic receptors,[6,7] substantial evidence suggests that an interaction between L-dopa and the peripheral adrenergic nerve endings may be involved. Recent studies have clearly demonstrated that pharmacologic doses of L-dopa increase the turnover of cardiac NE in rats.[12–14] The effect of L-dopa on NE turnover is dose related up to 100 mg/kg, dependent on conversion *in vivo* to dopamine, and independent of central sympathetic

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activity.[14] It has been suggested that precursor substrate loading with L-dopa by passes the rate-limiting step in catecholamine biosynthesis and, by stimulating synthesis, results in increased turnover of NE.[13] Other evidence, however, supported by the findings presented here, indicates that L-dopa releases NE from the sympathetic nerve endings,[14–16] and that NE release is the basis for the increased cardiac NE turnover.

METHODS

Materials. Female 180 g Sprague–Dawley rats were used in these studies. DL-7-[^{3}H]NE, 10 Ci/m-mole, or L-7-[^{3}H]NE, 10·4 Ci/m-mole, L-3,4-dihydroxyphenylalanine-[^{3}H]G, 6–9 Ci/m-mole, and 3,4-dihydroxyphenylethylamine-[^{3}H]G, 15 Ci/m-mole, were purchased from New England Nuclear Corp. All radioactive compounds were purified prior to use by column chromatography on Alumina as described below. L-Dopa (base), dopamine hydrochloride and NE bitartrate were purchased from CalBiochem. The L-dopa administered to rats was kindly provided by Roche; it was dissolved in 0·45% NaCl and 0·15 N HCl and injected i.p. in a dose of 100 mg/kg (base) in a volume of 1·0 ml. Control animals received diluent alone. Disulfiram, kindly provided by Ayerst, was suspended in 1% carboxymethylcellulose in 0·9% NaCl and injected i.p. in a dose of 400 mg/kg in a volume of 1·0 ml. Control animals received diluent alone. Reserpine (Sandril, Lilly) was administered i.p. in a dose of 5 mg/kg; control animals received 0·9% NaCl.

[^{3}H]NE was administered to unanesthetized animals via the tail vein after dilution with 0·9% NaCl in a dose of 25 μCi/kg. L-[^{3}H]NE was used in the subcellular distribution studies. Animals were killed by a blow at the base of the skull or, when plasma was collected, by guillotine. The hearts were rapidly removed, homogenized in iced 0·4 N $HClO_4$, and the perchloric acid extracts applied to an Alumina column as described below. Blood was collected in 100 units (100 μl) of Na heparin, 100 μl of 2% EDTA, and 0·5 mg of Na metabisulfite. Plasma was separated in a refrigerated centrifuge (Sorvall) and 0·1 vol. of $HClO_4$ was added followed by sufficient 0·4 N $HClO_4$ to give a final volume of 10·0 ml. After vigorous shaking and centrifugation, the perchloric extract was applied to an Alumina column and eluted with acid. Tritium was counted in a Packard Tri-Carb liquid scintillation spectrometer at an efficiency of 18 per cent. Fluorescence was determined in an Aminco–Bowman spectrophotofluorometer.

Statistical significance was determined by the Student's *t*-test.

Isolation of catechols on Alumina. The isolation of catechols on Alumina was performed as previously described.[17] The perchloric acid extract was applied to a column of prepared Alumina at pH 8·6[18] and the catechols were eluted with 0·2 N acetic acid. NE was assayed on the Alumina eluate by the trihydroxyindole fluorescent technique of von Euler and Lishajko,[19] with values corrected for a recovery of 80–90 per cent as determined in each experiment. As previously noted,[18] the presence of very high levels of dopa interfered with determinations of NE. In order, therefore, to assess more accurately the changes in NE concentration and to determine dopa and dopamine levels, an additional column separation was made.

Separation of dopa, NE and dopamine on Dowex. Dowex-50WX8, 200–400 mesh (BioRad), was prepared according to the method of Hirs *et al.*,[20] which entails thorough washing with glass-distilled water, cycling through the sodium form by

washing in 40% NaOH, and converting to the H^+ form by washing in 3 N HCl. The column was 5·0 cm long by 0·5 cm wide. Before the sample was added, the column was washed with 20 ml of 2 N HCl, 5 ml of glass-distilled water, 10 ml of 1·0 N sodium acetate (pH 6) and 5 ml H_2O.[21] The sample (Alumina eluate) was poured over the column after the addition of 10 mg sodium metabisulfite and 0·5 ml EDTA and after the pH was adjusted to 2·0. The column was then washed with 15 ml of glass-distilled water. Dopa was eluted with 8·0 ml of 0·5 M potassium acetate (pH 6·5); NE was eluted with 11·0 ml of 1 N HCl; and dopamine was eluted with 8·0 ml of 2 N HCl. The average recovery after both columns (as determined by both radioisotope and fluorescent assays) was about 60 per cent for dopa, 80 per cent for NE and 50 per cent for dopamine, and results were corrected for recovery as determined in each experiment. Studies with labeled dopa, dopamine and NE revealed small degrees of cross contamination in the different fractions (< 5 per cent). The fluorescent assays described below, however, are sufficiently selective so that no interference was noted in assays of the different fractions.

Fluorometric assay of dopa, NE and dopamine. Assay of these compounds was based on the findings of Laverty and Taylor.[22] Prior to oxidation, the pH of the eluate was adjusted to 5·7 for dopa with citric acid–phosphate buffer, to 6·5 for NE, and to 7·0 for DA with Na_2HPO_4 and NaOH. Iodine was used as the oxidant and a sodium sulfite–sodium hydroxide mixture was the anti-oxidant.[22] Acidification of the mixtures with glacial acetic acid and heating of the dopa and DA samples at 100° for 40 min was carried out in order to develop fluorescence fully.[22] The most reproducible blanks were obtained by heating the eluate at 100° for 30 min followed by complete reversal of the acidification, alkalinization and oxidation procedures. Internal standards and blanks were run with each sample which were assayed in duplicate. The excitation and emission peaks were 330 and 380 nm for dopa, 380 and 480 nm for NE and 320 and 375 nm for DA.[22] Under these conditions, fluorescence was linear between 20 and 1000 ng for each of these compounds. The amount of substrate required to give readings twice blank was 22 ng for dopa, 11 ng for NE and 62 ng for DA.

Subcellular distribution studies. Differential centrifugation was performed as previously described.[23] The tissues were homogenized in iced 0·25 M sucrose and spun at 12,000 *g* in a Sorvall refrigerated centrifuge to remove the nuclei, unbroken cells and mitochondria. The supernatant was decanted and spun at 100,000 *g* in a Spinco model L preparative ultracentrifuge for 1 hr to separate the particulate or microsomal fraction from the supernatant or soluble fraction. The high speed pellet was resuspended and homogenized in 0·4 N $HClO_4$; the high speed supernatant was shaken with 0·1 vol. of 4·0 N $HClO_4$ and the perchloric acid extracts were applied to Alumina and Dowex as described above.

RESULTS

Effect of disulfiram pretreatment on [^{3}H]NE retention and endogenous NE levels in dopamine-treated rats

Previous studies have shown that inhibition of dopa decarboxylase prevents the increased cardiac NE turnover that occurs after administration of dopa but not after dopamine,[14] thus indicating that conversion *in vivo* of dopa to dopamine is essential

for this effect. As shown in Fig. 1, pretreatment with disulfiram, an inhibitor of dopamine-β-hydroxylase, did not prevent the increase in cardiac NE turnover after dopamine. Cardiac [^{3}H]NE concentration was significantly decreased in dopamine-treated animals with or without disulfiram pretreatment, thus indicating that dopamine is the active agent and that conversion to NE is not essential.

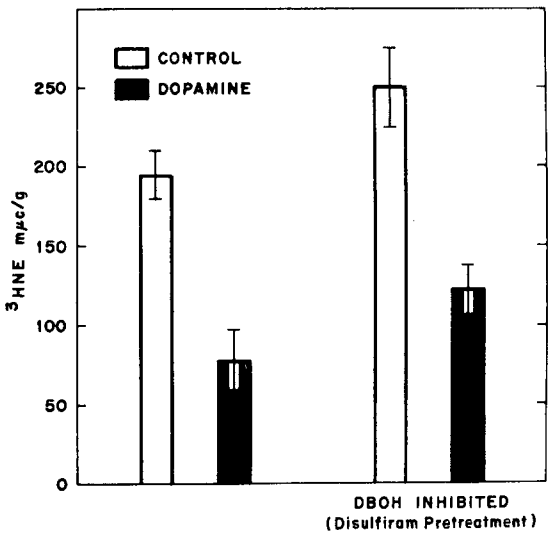

FIG. 1. Effect of DA on [^{3}H]NE retention in disulfiram-pretreated rats. Each group contained eight to nine animals. Bars represent means ±S.E.M. All animals were injected with [^{3}H]NE to label the cardiac NE stores. Three hr later, one-half the animals received disulfiram (400 mg/kg); the other half received diluent. One hr later, one-half of each 'pretreatment' group received 50 mg/kg of dopamine (black bar) and all animals were killed 2·5 hr later and the hearts assayed for endogenous and [^{3}H]NE as described in Methods. [^{3}H]NE concentration was significantly reduced in animals receiving DA when compared with un-pretreated controls ($P < 0·0002$) or controls pretreated with disulfiram ($P < 0·0001$). Animals pretreated with disulfiram did not differ significantly from untreated controls, but did differ significantly from those animals receiving disulfiram pretreatment and DA ($P < 0·0006$). Note that inhibition of dopamine-β-hydroxylase did not prevent [^{3}H]NE release by DA.

TABLE 1. EFFECT OF DISULFIRAM PRETREATMENT OF ENDOGENOUS NE CONCENTRATION AFTER DOPAMINE*

	Endogenous NE (μg/g)		
Treatment	Diluent pretreatment	Disulfiram pretreatment	P
Control	1·05 ± 0·05	0·82 ± 0·06	≦0·002
Dopamine	0·66 ± 0·05	0·63 ± 0·06	NS
P	≦0·0001	≦0·02	

* Each group contained eight to nine rats. Details of drug administration are given in the legend of Fig. 1. DA caused a significant fall in the concentration of cardiac NE after synthesis inhibition by disulfiram. The effectiveness of disulfiram in inhibiting dopamine-β-hydroxylase is shown by the significant fall in endogenous NE concentration in rats not receiving DA. NS = not significant.

As shown in Table 1, disulfiram pretreatment did reduce endogenous NE levels significantly, thus indicating effective blockade of NE synthesis. In disulfiram-pretreated rats, DA caused a further significant reduction in NE levels (Table 1). The data in Fig. 1 and Table 1 indicate that increased NE turnover occurs despite blockade of NE synthesis at the level of dopamine-β-hydroxylase.

Cardiac levels of dopa, DA and NE after L-*dopa*

Forty-five min after treatment with L-dopa, there was a significant reduction in cardiac NE levels (Fig. 2). This occurred despite concomitant high levels of NE precursor (dopa + DA), a finding not explicable in terms of increased NE synthesis secondary to precursor loading. The corresponding plasma levels of dopa and DA shown in Fig. 2 suggest that most of the cardiac DA is generated locally in the heart from dopa rather than from circulating DA.

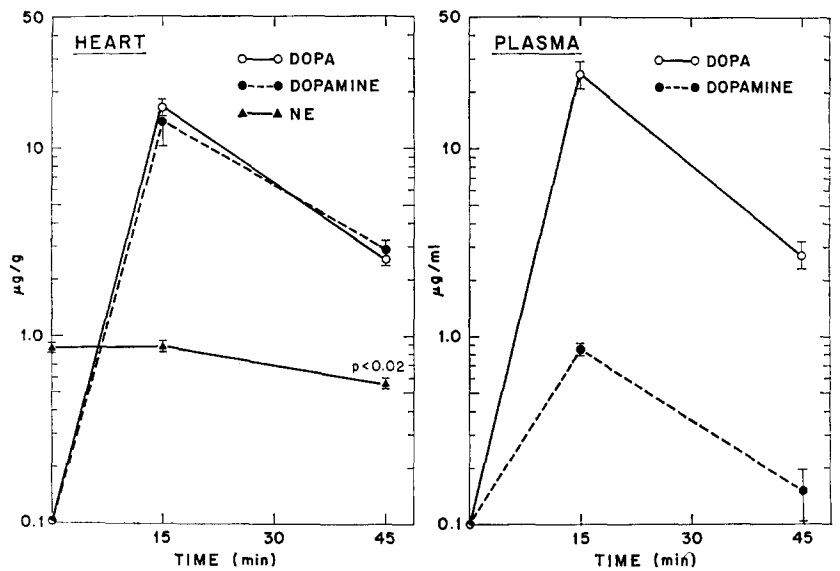

FIG. 2. Cardiac and plasma levels of dopa, DA and NE after L-dopa. Each group contained six to eight animals. L-Dopa-treated rats received 100 mg/kg i.p. and were killed 15 and 45 min later. Dopa, dopamine and NE were determined in heart and plasma as described in Methods. Tracer [^{3}H]NE was added to heart homogenates and individual recoveries were calculated on each column. Values for cardiac NE: controls, 0·85 μg/g ± 0·07; 15 min, 0·88 μg/g ± 0·5; 45 min, 0·57 μg/g ± 0·07. NE was not detectable in plasma. Despite high levels of dopa and DA in the heart (note semi-log scale), NE levels decreased significantly (30 per cent; $P < 0·02$) at 45 min.

Subcellular distribution of [^{3}*H*]*NE, dopa and DA after* L-*dopa*

The administration of L-dopa caused a significant decrease in the retention of [^{3}H]NE in both the particulate and soluble cell fractions (Fig. 3); the subcellular distribution of [^{3}H]NE was not altered. Both dopa and DA were located in the soluble fraction of cell homogenates (Table 2), but because of the relative insensitivity of the fluorescent DA assay these results do not exclude the possibility that small amounts of DA may be stored in the particulate fraction.

TABLE 2. SUBCELLULAR DISTRIBUTION OF DOPA AND DOPAMINE IN THE RAT HEART AFTER THE ADMINISTRATION OF L-DOPA*

Fraction	Dopa (μg/g heart)	Dopamine (μg/g heart)
Microsomes	0·11 ± 0·04	Not detectable
Supernatant	7·64 ± 1·4	2·58 ± 0·44

* Six animals were treated with L-dopa (100 mg/kg i.p.) and killed 15 min later. The homogenate fractions were separated by differential centrifugation as described in Methods. Both dopa and DA were present in the supernatant by fluorescent assay. The small amount of dopa in the microsomes (1·4 per cent of the supernatant) probably represents nonspecific contamination of the pellet.

Effect of reserpine on cardiac dopa and DA levels after L-*dopa*

The concentration of dopa and DA in the rat heart was unaffected by prior treatment with reserpine (Table 3). This is consistent with the presence of these compounds in the soluble fraction of tissue homogenates.

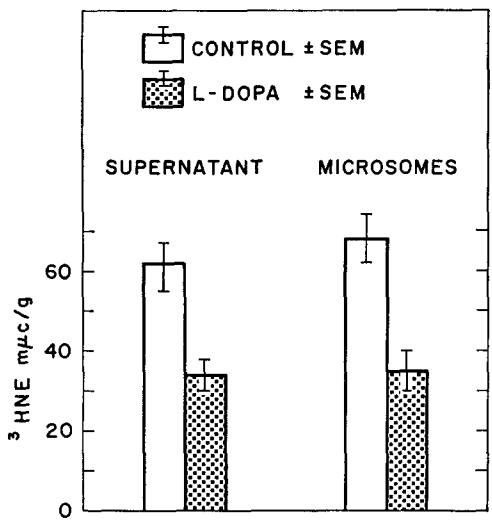

FIG. 3. Subcellular distribution of [^{3}H]NE after L-dopa. Each group contained six animals. All rats received 25 μCi/kg of L-[^{3}H]NE. One hr later, one-half the rats received L-dopa, the other half diluent. All animals were killed 1 hr later. The hearts were homogenized in iced sucrose and differential centrifugation was performed as described in Methods. [^{3}H]NE concentration was significantly reduced in the supernatant and microsomal fractions of L-dopa-treated rats ($P < 0·005$). Dopa was present in a concentration of 1·19 μg/g ± 0·23 in the supernatant. Dopa was not detectable in the pellet and no DA was detectable in either fraction at this time (1 hr).

DISCUSSION

Studies with decarboxylase inhibitors have clearly established that the cardiovascular effects of L-dopa depend upon conversion *in vivo* of dopa to dopamine.[4,6,7,10,11] The low or undetectable levels of dopamine compared with the high levels of dopa in the

TABLE 3. EFFECT OF RESERPINE ON DOPA AND DOPAMINE CONCENTRATION IN THE RAT HEART AFTER L-DOPA*

Pretreatment	Dopa (μg/g heart)	Dopamine (μg/g heart)
None	6·81 ± 0·77	4·07 ± 2·06
Reserpine	8·47 ± 1·78	3·22 ± 0·58

* Each group contained seven to eight animals. Reserpine (5 mg/kg i.p.) was administered 18 hr before the experiment to one-half the animals; the remainder received saline injections. L-Dopa, 100 mg/kg i.p., was administered to all the rats 0·5 hr before they were killed. Pretreatment with reserpine did not affect the concentration of dopa and dopamine. Reserpine-treated rat hearts contained less than 0·05 μg/g of NE, indicating the efficacy of reserpine pretreatment in depleting NE.

plasma of patients[21,24] and rats (Fig. 2) suggest that the conversion of dopa to DA occurs in peripheral tissues. The cardiovascular effects of dopamine formed in this manner have usually been attributed to direct stimulation of adrenergic receptors.[6,7] Substantial evidence now indicates, however, that NE release from the sympathetic nerve endings by DA contributes to these sympathomimetic effects. Since 1963 it has been recognized that DA resembles sympathomimetic amines in its capacity to decrease [^{3}H]NE[25] and endogenous NE[26] levels in the heart. Recently pharmacologic and biochemical evidence[14,16] has been advanced in support of NE release by L-dopa. The decline in endogenous NE after L-dopa administration noted both here (Fig. 2) and in previous works,[10,12] as well as the effect on subcellular distribution of [^{3}H]NE (Fig. 3), is similar to the effect of tyramine,[27] the prototype of indirect acting sympathomimetic amines. Hypertensive reactions after administration of L-dopa to patients on monoamine oxidase inhibitors provide further evidence for NE release.[28] According to this formulation, the increased turnover of cardiac NE in the rat subsequent to L-dopa injection[12–14] results from NE release followed by resynthesis from DA. An alternative possibility, that increased NE synthesis secondary to substrate precursor loading distal to the rate-limiting biosynthetic step explains the increased NE turnover, is not supported by the evidence presented here. Thus, NE release was demonstrable after synthesis blockade (Fig. 1, Table 1); furthermore, the transient fall in NE concentration after L-dopa (Fig. 2) is inconsistent with a primary effect on synthesis. Since DA is an NE precursor, the prolonged NE depletion that occurs with other indirect acting sympathomimetic amines does not occur; NE stores are regenerated within a few hours of a single dose of L-dopa.[12,16] That increased NE levels do not occur after precursor loading lends weight to the hypothesis that when substrate levels of dopamine accumulate in the nerve ending, vesicular uptake of DA becomes rate limiting in NE biosynthesis.[29,30]

NE release may thus be an important part of the pharmacology of L-dopa. The relative importance of direct receptor stimulation by DA as compared with NE release in the genesis of sympathomimetic effects is not known. Recent evidence, however, supports the view that a significant proportion of the cardiovascular effects of DA depends on NE release.[31] The effect of L-dopa on the subcellular distribution of [^{3}H]NE (Fig. 3), as well as the association of L-dopa with sympathomimetic

effects,[5–11] is consistent with the release of active NE rather than increased intraneuronal metabolism.

Studies on patients indicate that the effect of L-dopa on the pre-ejection period may not persist during therapy.[5–7] To determine whether or not NE release occurs chronically with prolonged L-dopa administration, studies on the adrenergic nerve endings after long-term treatment with L-dopa would be required.

The explanation for the postural hypotension which occurs in Parkinsonian patients treated with L-dopa remains obscure. NE depletion by L-dopa has been suggested,[32,33] but prolonged NE depletion has not been demonstrated, presumably because of NE synthesis from DA. Vasodilatation induced by circulating DA has also been considered,[24] but is unlikely, since circulating DA levels are very low[21,24] and DA is not known to be a potent vasodepressor. The possibility that DA accumulates in the sympathetic nerve endings and functions as a false neurotransmitter has also been raised.[32] For a substance to act as a false neurotransmitter, it must be stored in the same granular sites as NE and be released by sympathteic stimulation.[34] Although conflicting data have been reported as to the subcellular localization of tracer DA,[35,36] the evidence that tracer amounts of DA may be stored in the NE storage granules[36] and released by sympathetic stimulation appears convincing.[37] The results obtained with pharmacologic doses of L-dopa in these studies indicate that dopa and DA are localized in the supernatant fraction and not in the NE storage granule (Table 2). Because of the relative insensitivity of the fluorometric dopamine assay, however (see Methods), an amount of DA equal to the microsomal pellet NE concentration (0·1–0·2 μg/g) would not be detectable. Therefore, although most of the dopa and DA is in the supernatant subcellular fraction after pharmacologic doses of L-dopa, these studies do not exclude the possibility of physiologically significant DA storage, and hence are not inconsistent with the false neurotransmitter hypothesis. The same considerations apply to the data presented in Table 3; the failure of reserpine to prevent dopamine storage does not rule out retention of a small fraction of the endogenously generated DA in the NE storage granule. The most compelling evidence against the false neurotransmitter hypothesis is the well substantiated observation that hypotension subsequent to L-dopa administration is accentuated or unmasked after decarboxylation of dopa is inhibited in the peripheral tissues but not in the CNS.[10,11] The fact that hypotension is prevented after CNS decarboxylation is blocked implicates a central, DA-sensitive mechanism in the production of orthostatic hypotension.

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