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# DOPAMINE-β-OXIDASE ACTIVITY IN MAN, USING HYDROXYAMPHETAMINE AS SUBSTRATE

BY

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Hydroxyamphetamine was administered orally to five human subjects in daily doses of 26 to 118 mg. Approximately half the dosage administered was recoverable in the urine as unchanged drug (free plus conjugated) and 3.7 to 9.1% was excreted as the β-hydroxylated metabolite, hydroxynorephedrine (free plus conjugated). Since conversion of hydroxyamphetamine to hydroxynorephedrine occurs *in vitro* by the action of dopamine-β-oxidase, a simple method is suggested for measuring the activity of this enzyme and the effect of its inhibitors in man. No impairment of β-hydroxylation was noted in an adrenalectomized subject. The β-hydroxylation of hydroxyamphetamine *in vivo* has not been described previously.

In recent years, useful therapeutic agents have been developed based on activities in altering the storage and metabolism of noradrenaline in tissues. It might be anticipated that an effective inhibitor of the biosynthesis of noradrenaline would also have interesting pharmacological properties. To date, attempts to block the synthesis of this amine have focused chiefly on the decarboxylation of dopa (3,4-dihydroxyphenylalanine) to dopamine (3,4-dihydroxyphenethylamine) by dopa decarboxylase. While several potent inhibitors of this enzyme have been developed, it has not been possible to inhibit it sufficiently *in vivo* to deplete tissue stores of noradrenaline (Hess, Connamacher, Ozaki & Udenfriend, 1961).

Considerable attention is now being directed to dopamine-β-oxidase, the enzyme responsible for the conversion of dopamine to noradrenaline. This enzyme catalyses the β-hydroxylation of many phenethylamine compounds (Bridgers & Kaufman, 1962 ; Goldstein & Contrera, 1962 ; Creveling, Daly, Witkop & Udenfriend, 1962a), and certain isosteres of phenethylamine have an affinity for the enzyme and serve either as substrates or inhibitors (Creveling *et al.*, 1962a ; Creveling, van der Schoot & Udenfriend, 1962b ; Kuntzman, Costa, Creveling, Hirsch & Brodie, 1962). Since it is likely that inhibitors of this type will become available for study in man, it seemed important to develop methods to assess dopamine-β-oxidase activity in the intact human.

One approach to this problem would be to measure the urinary excretion of naturally occurring β-hydroxylated compounds such as adrenaline, noradrenaline, the methoxy catechol amines, synephrine, norsynephrine, and their acid metabolites.

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Since the amounts of these compounds excreted normally are rather small, however, the effects of enzyme inhibition might be difficult to measure. Alternatively, one could increase the excretion of β-hydroxylated derivatives by administering either a natural substrate or a foreign compound which is hydroxylated by the same enzyme system; determination of the amount of the hydroxylated compound excreted in the urine would then afford an index of enzyme activity. We decided to use hydroxyamphetamine (*p*-hydroxy-α-methylphenethylamine; Paredrine, Smith, Kline & French Laboratories), since this compound is a substrate for dopamine-β-oxidase *in vitro* (Creveling *et al.*, 1962a) and the presence of a methyl group in the alpha position on the side-chain prevents metabolism by monoamine oxidase. As shown in Fig. 1, the compound formed would be hydroxynorephedrine (2-amino-1-*p*-


Hydroxyamphetamine Hydroxynorephedrine

Fig. 1. Structures of hydroxyamphetamine and its β-hydroxylated derivative, hydroxynorephedrine.

hydroxyphenylpropan-1-ol). The present studies include development of methods for assay of hydroxyamphetamine and hydroxynorephedrine in urine and demonstrate that β-hydroxylation is a significant pathway for the metabolism of hydroxyamphetamine in man.

## METHODS AND MATERIALS

Five hospital patients without significant renal or hepatic disease were the subjects of this study; three had mild labile hypertension, one had periodic angioneurotic oedema, and the fifth had phaeochromocytoma. All took the regular hospital diet. Hydroxyamphetamine (as hydrobromide) was administered orally in tablets or capsules for 2 to 5 days in a daily dose of 26 to 118 mg of the base. The drug was administered every 6 hr, except to the patient with phaeochromocytoma who received only single doses of 26 mg before removal of the tumour, since marked pressor responses were observed. With the other patients no significant changes in blood pressure or pulse rate were recorded. At the time of surgery in the patient with phaeochromocytoma, it was necessary to remove the left adrenal gland along with the tumour. Since she had undergone surgery with removal of a similar tumour in the right adrenal gland several months previously, she was totally adrenalectomized following the second operation. β-Hydroxylation studies were repeated 21 days after the second operation; at this time she was asymptomatic on maintenance steroid therapy. Urine specimens (24 hr) were collected into glass bottles containing 15 ml. of 6 N-HCl during 3 control days, during the period of administration of hydroxyamphetamine, and for 2 days thereafter.

*Measurement of hydroxyamphetamine in urine.* Free plus conjugated hydroxyamphetamine in acid-hydrolysed urine samples was measured using a modification of the method of Axelrod (1954). The major difference from the original method consisted of removal of excess reagent, in the final step, with an organic solvent.

Urine (2.5 ml.) was pipetted into a test-tube, 0.5 ml. of 12 N-HCl added and the tube placed in boiling water for 20 min. After cooling, the *p*H was adjusted to 9.0 to 10.0 by the addition of 0.4 ml. of 10 N-NaOH and solid $Na_2CO_3$. The liquid was transferred to a 60-ml. glass-stoppered bottle, 1 g of NaCl and 30 ml. of peroxide-free ether were added, and the bottle was shaken in a mechanical shaker for 10 min. The bottle was centrifuged and a 25 ml. aliquot of the ether layer was transferred to a glass-stoppered centrifuge tube with tapered

end, containing 4 ml. of 0.1 N-HCl. The tube was shaken for 10 min and then centrifuged. The ether layer was removed, and 3 ml. of the acid layer was transferred to a glass-stoppered centrifuge tube. Next, 1 ml. of nitric acid reagent (50 ml. of 2.5 N-$HNO_3$ with 1 ml. of a 2.5% aqueous solution of $NaNO_2$) and 1 ml. of a 0.1% solution of 1-nitroso-2-naphthol in ethanol were added, the reagents mixed and heated for 30 min in a water-bath at 55° C. After cooling, the liquid was shaken with 5 ml. of ethylene dichloride to remove the excess of reagent. The organic layer was discarded and the optical density measured at 470 mμ against a reagent blank in a Beckman DU Spectrophotometer. The colour was stable for at least 1 hr. Appropriate standards were carried through the procedure during each analysis. Hydroxyamphetamine (1 to 100 μg), added to 2.5 ml. urine samples, was recovered with adequate precision (95±5%).

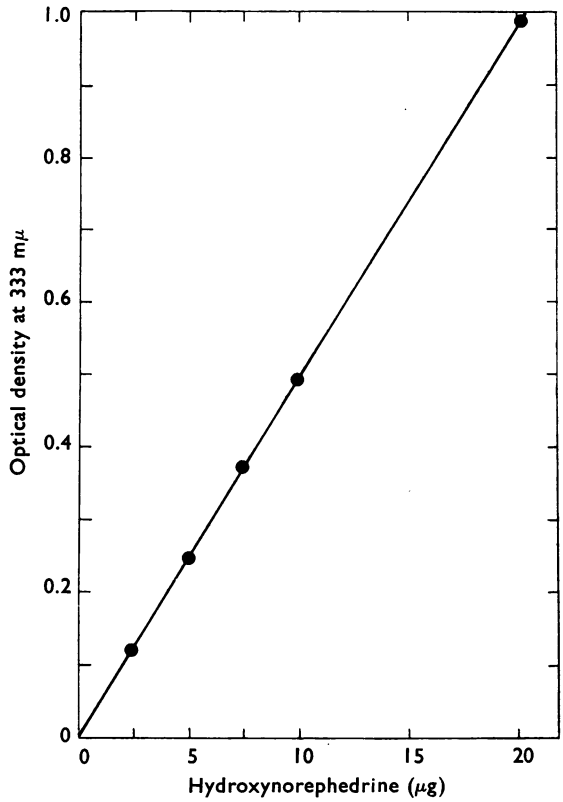


Fig. 2. Standard curve for hydroxynorephedrine following periodate oxidation. The ordinate indicates optical density at 333 mμ and the abscissa gives the amounts of hydroxynorephedrine (measured as *p*-hydroxybenzaldehyde) in a final reaction volume of 4.2 ml. (Pisano, 1960). Each value is the average of at least four determinations.

*Assay of hydroxynorephedrine in urine.* Preliminary studies showed that hydroxynorephedrine can be oxidized by means of periodate, essentially as described by Pisano, Oates, Karmen, Sjoerdsma & Udenfriend (1961) for the closely related compounds, synephrine [*p*-hydroxy-α-(methylaminomethyl)benzyl alcohol] and norsynephrine (α-aminomethyl-*p*-hydroxybenzyl alcohol). The oxidized product is *p*-hydroxybenzaldehyde, which gives maximum light absorption at 333 mμ. In a model experiment it could be shown that a linear relationship exists following periodate oxidation between the optical density at 333 mμ and the concentration of hydroxynorephedrine (Fig. 2). Thus, it was possible to apply the methods

described previously for the synephrine and norsynephrine (Pisano *et al.*, 1961) and the *m*-methoxy catechol amines (Pisano, 1960) to the assay of hydroxynorephedrine in urine. Before assay, 10 ml. samples of urine were acidified with 2 N-HCl to a *p*H less than 1, and hydrolysis was carried out at 100° C for 20 min. Appropriate hydroxynorephedrine standards were added to urine routinely to check the percentage recovery, which was consistently in the range 95 to 100%.

## RESULTS

*Chromatographic identification of the β-hydroxylated urinary metabolite of hydroxyamphetamine.* A great increase in the excretion of a basic compound which on treatment with periodate yielded a substance with a maximal optical density at 333 mμ was found in the urine of patients receiving hydroxyamphetamine. The following studies were performed to establish that this basic compound was hydroxynorephedrine.

Aliquots of urine (100 ml.) from two of the patients were applied to Amberlite CG-50 columns buffered at *p*H 6.0 to 6.5 (Pisano, 1960). Each column was washed with 300 ml. of water and then eluted with 160 ml. of concentrated $NH_4OH$. The eluate was evaporated to dryness under reduced pressure and the residue dissolved in a small amount of methanol. Extracts of control and experimental urines from the same subjects, as well as 5 to 10 μg of authentic hydroxyamphetamine and hydroxynorephedrine (both supplied as the hydrobromide salts by Smith, Kline & French Laboratories) were subjected to paper chromatography in several solvent systems. The chromatographic characteristics of authentic hydroxyamphetamine and hydroxynorephedrine are shown in Table 1. Comparison of control and experi-

TABLE 1

CHROMATOGRAPHIC CHARACTERISTICS OF HYDROXYAMPHETAMINE AND ITS β-HYDROXYLATED DERIVATIVE

The solvent systems (syst.) were: 1, *n*-butanol saturated with 1 N-HCl, descending; 2, isopropyl alcohol : ammonia solution : water (16:2:2), ascending; 3, *n*-butanol : acetic acid : water (12:3:5), descending. Whatman No. 3 paper was used

| | $R_F$ values | | | Colour reactions | | |
|---|---|---|---|---|---|---|
| Compound | Syst. 1 | Syst. 2 | Syst. 3 | Ninhydrin | *p*-Nitro-aniline | Gibb's reagent |
| 1. Hydroxyamphetamine | 0·69 | 0·84 | 0·81 | Yellow→brown | Violet | Light brown |
| 2. Hydroxynorephedrine | 0·48 | 0·71 | 0·76 | Purple | Pink | Blue |

mental urine extracts showed the appearance of four different spots coincident with ingestion of hydroxyamphetamine by the patient. Two of the spots had the same $R_F$ values and colour reactions as authentic hydroxyamphetamine and hydroxynorephedrine. The other two spots, which gave the same colour reactions as hydroxyamphetamine and hydroxynorephedrine, disappeared if the urine was first subjected to acid hydrolysis. It was concluded that hydroxyamphetamine and hydroxynorephedrine are excreted in the urine in both free and conjugated forms, the latter being susceptible to acid hydrolysis.

Further identification of hydroxyamphetamine and hydroxynorephedrine in urine extracts was obtained by means of gas chromatography. Aliquots of urine (25 ml.) were adjusted to *p*H 9.5 and shaken twice with two volumes of ether. The ether extracts were combined and evaporated to dryness; the residues were dissolved

in a small amount of methanol and applied to a Barber–Coleman model 15 gas chromatograph. A QF-1 column with 10% coating was used, prepared in the manner described by Fales & Pisano (1962). Temperature conditions were: column, 150° C; detector cell, 240° C; and flash heater, 245° C. Authentic samples of hydroxyamphetamine and hydroxynorephedrine exhibited retention times of 4.6 and 14.0 min respectively. The urine extracts from patients receiving hydroxyamphetamine showed peaks identical to those of the authentic compounds.

*Measurement of β-hydroxylation.* In control urines from these and other patients, no significant interference was encountered in the assays for hydroxyamphetamine. However, in the hydroxynorephedrine assays, natural occurrence of material absorbing at 333 mμ was always encountered, presumably due chiefly to the excretion of synephrine. Current studies are showing that the excretion of synephrine varies considerably with changes in the diet. Assay of control urines in the present

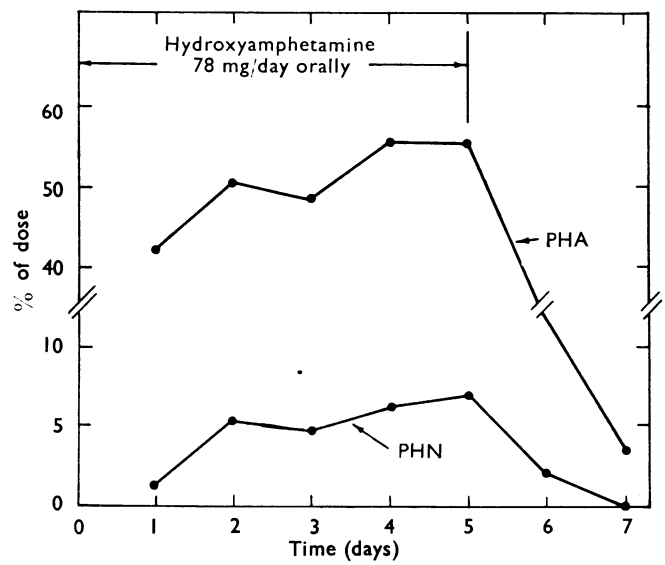


Fig. 3. Time course of urinary excretions of hydroxyamphetamine (PHA) and hydroxynorephedrine (PHN) in patient no. 3 (see Table 2).

investigation showed amounts of synephrine equivalent to 0.5 to 1.2 mg/day of hydroxynorephedrine; these values were subtracted from those obtained during the administration of hydroxyamphetamine to give the amounts actually due to hydroxynorephedrine.

The time course of excretion of hydroxyamphetamine and hydroxynorephedrine in a patient who received 78 mg of hydroxyamphetamine daily for 5 days is shown in Fig. 3. Maximum excretion of hydroxyamphetamine and hydroxynorephedrine occurred by the fourth day and small amounts of both compounds were still present in urine obtained during the second day after cessation of treatment. A summary of results for all five patients is given in Table 2. About half the total dosage of hydroxyamphetamine was excreted in each patient as unchanged drug (free plus conjugated). In addition, 3.7 to 9.1% of the hydroxyamphetamine administered

TABLE 2

URINARY EXCRETION OF HYDROXYAMPHETAMINE AND HYDROXYNOREPHEDRINE AFTER ORAL ADMINISTRATION OF HYDROXYAMPHETAMINE

Studies in patient no. 5 were done (A) before and (B) 21 days after removal of phaeochromocytoma and total adrenalectomy. Values for urinary hydroxyamphetamine (PHA) and hydroxynorephedrine (PHN) are expressed as the percentage of the total dose of hydroxyamphetamine administered

| Patient | Maximum dose (mg/day) | Total dose | Days of treatment | PHA (%) | PHN (%) |
|---|---|---|---|---|---|
| 1 | 104 | 520 | 5 | 52 | 4·7 |
| 2 | 78 | 390 | 5 | 51 | 3·7 |
| 3 | 78 | 390 | 5 | 55 | 5·8 |
| 4 | 118 | 470 | 4 | 53 | 6·5 |
| 5 (A) | 26 | 52 | 2 | 45 | 5·8 |
| 5 (B) | 78 | 150 | 2 | 48 | 9·1 |

was recovered in the urine as hydroxynorephedrine (free plus conjugated). The highest percentage of hydroxylation occurred in the patient with phaeochromocytoma, after the operation.

The possibility that β-hydroxylation of hydroxyamphetamine might be carried out by bacterial flora in the intestine was eliminated by the finding of similar results in patient no. 4 when the study was repeated concomitantly with the administration of neomycin (0.5 g every 6 hr for 10 days).

## DISCUSSION

The enzyme, dopamine β-oxidase, has been purified from beef adrenals, and many of its requirements have been determined (Levin, Levenberg & Kaufman, 1960). The enzyme has also been demonstrated in brain (Udenfriend & Creveling, 1959) and is presumably distributed throughout sympathetically innervated tissues. Because of its probable unique association with sympathetic neural elements, it seemed rather unlikely that a foreign substrate administered orally would be metabolized to a β-hydroxylated urinary product in sufficient amount to permit measurement by other than isotopic methods. However, as shown here, the β-hydroxylation of hydroxyamphetamine to hydroxynorephedrine could be easily measured by chemical assay of urine.

The lack of effect of administration of neomycin to one patient indicates that the hydroxylation occurs in body tissues. From what is known of the enzyme's distribution, one might assume that hydroxylation would occur mainly in adrenal glands. Similarly, a higher percentage of β-hydroxylation might be expected in patients with tumours highly active in synthesizing noradrenaline, such as phaeochromocytomata. Studies in one patient (no. 5, Table 2) with this tumour were revealing in both respects. In spite of a highly active β-hydroxylating system, as indicated by urinary noradrenaline excretion averaging 2.0 mg daily, the percentage of the hydroxyamphetamine dose excreted as hydroxynorephedrine was in the same range as that of the other subjects. A somewhat higher conversion to hydroxynorephedrine was noted after removal of the tumour, the patient also having been adrenalectomized. This indicates that a major portion of the β-hydroxylation of hydroxyamphetamine occurs in non-adrenal tissue. Unfortunately, at the present time one cannot be completely certain that the hydroxylation of hydroxyamphetamine *in vivo* is accomplished by the same enzyme which converts dopamine to noradrenaline.

The present studies form the basis of a potentially useful test for determining the effectiveness of inhibitors of dopamine-β-oxidase in man. One problem concerns the fact that the method of assay for hydroxynorephedrine also measures the excretion of synephrine, a compound known to be a normal urinary metabolite in man (Pisano *et al.*, 1961). Thus, values for the excretion of synephrine during control periods must be subtracted from the values obtained during administration of hydroxyamphetamine. If a constant diet is employed, rather constant levels of synephrine are excreted each day.

β-Hydroxylation was not previously known to be a pathway for the metabolism of hydroxyamphetamine. Axelrod (1954) showed that considerable amounts of dexamphetamine are metabolized to hydroxyamphetamine in dogs and rats. He suggested that the effects of amphetamine in these species could be mediated in part by the *p*-hydroxylated product. Apparently, hydroxylation of amphetamine has not been studied in man. In any event, it seems unlikely that hydroxynorephedrine could contribute significantly to the pharmacological effects of amphetamine or even hydroxyamphetamine in man. Less than 10% of a dose of hydroxyamphetamine is recovered as hydroxynorephedrine, and studies by Chen, Wu & Henriksen (1929) have shown that, while hydroxynorephedrine is slightly more potent than ephedrine as a pressor substance in animals, single oral doses of 50 mg in man have no significant effect.

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