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6-HYDROXYDOPAMINE INDUCED DEGENERATION OF NORADRENALINE NEURONS IN THE SCORBUTIC GUINEA-PIG

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Abstract—The effect of the neurotoxic compound 6-hydroxydopamine and its immediate precursor 6-hydroxy-DOPA on noradrenaline uptake and storage in central and peripheral catecholamine neurons of scorbutic and normal guinea-pigs has been investigated. Endogenous noradrenaline in heart and brain as well as the *in vitro* uptake-accumulation of ^{3}H-noradrenaline in iris and slices of heart and brain were not significantly changed in scorbutic animals. The *in vitro* formation of ^{3}H-noradrenaline from ^{3}H-dopamine was markedly reduced in heart slices of scorbutic guinea-pigs, due to ascorbic acid being a co-factor for dopamine-β-hydroxylase. There was an increased depletion of brain noradrenaline following tyrosine hydroxylase inhibition produced by α-methyl-*p*-tyrosine methylester in scorbutic animals, indicating an increased NA turnover. Administration of 6-hydroxydopamine or 6-hydroxy-DOPA resulted in a similar reduction of endogenous NA in brain and heart as well as of the *in vitro* uptake of ^{3}H-noradrenaline in iris, and slices from heart, cerebral cortex and hypothalamus in scorbutic and control guinea-pigs. These results are discussed in view of current hypotheses on mechanisms involved in the neurotoxic action of 6-hydroxydopamine on catecholamine neurons.

6-HYDROXYDOPAMINE (6-OH-DA) is known to produce an acute and selective degeneration of both central and peripheral catecholamine containing neurons (see symposium volume edited by Malmfors and Thoenen).[1] The molecular mechanism involved in the accomplishment of this chemically induced denervation is not fully understood. There are, however, two factors which seem to be of fundamental importance in this process; (1) 6-OH-DA has to be taken up and accumulated in high concentrations in the catecholamine nerve terminals, which is brought about by a specific uptake mechanism;[2–4] (2) 6-OH-DA is very susceptible to oxidation, especially at physiological pH.[5–9] Two theories have been put forward attempting to explain the molecular mechanisms related to the 6-OH-DA induced degeneration process. In one of these theories it has been suggested that quinonoid products formed from the oxidation of 6-OH-DA bind covalently with nucleophilic groups present in macromolecules of the neuron leading to degeneration.[5] The other theory proposes that the effective agent is hydrogen peroxide (H_2O_2) formed during autooxidation of 6-OH-DA.[6,7] These latter authors have shown that ascorbate markedly potentiates the formation of hydrogen peroxide from 6-OH-DA *in vitro*. In view of these findings it was thought of interest to investigate the degenerative action of 6-OH-DA on catecholamine neurons in scorbutic animals.

MATERIALS AND METHODS

Male albino Duncin–Hartley guinea-pigs (250–300 g body wt) have been used in the experiments. In each experiment the animals were divided into two groups, a con-

trol and a scorbutic group. Both groups were fed *ad libitum* a purified scorbutogenic diet containing no more than 0·2 μg vitamin C per g.[10] The animals had free access to water. The controls received 0·1% L-ascorbic acid in the drinking water. The diet produced a rapid development of scurvy in the experimental animals which started to lose weight after 13–15 days. The weight curves were essentially as described by Friberg and Lohmander.[10] The animals were taken for the experiments after 15–20 days at which time they showed moderate signs of scurvy.

Ascorbic acid assay. As a control of the state of ascorbic acid deficiency in the animals the ascorbic acid content of the adrenal glands of the guinea-pigs was determined using a slight modification of the method of Roe and Kuether.[11] In one experiment ascorbic acid was assayed in the crude synaptosomal fraction of guinea-pig brain, containing pinched-off nerve endings. This fraction was obtained by homogenizing the brain tissue in 10 volumes (w/v) of 0·25 M sucrose, using a homogenizer with a teflon pestle running at a speed of 1000 rev/min (10 strokes). The original homogenate was centrifuged at 3000 *g* for 10 min and after decantation, the supernatant was centrifuged at 10,000 *g* for 15 min to obtain a pellet, the so called crude synaptosomal fraction which was used for ascorbic acid determination as described above.

NA assay. Endogenous NA was determined in brain and heart according to the method of Bertler *et al.*[12] NA was extracted with 10 vol. 0·4 N perchloric acid and isolated by ion-exchange chromatography on Dowex 50W-X4 columns. After oxidation NA was determined fluorimetrically.

In vitro *incubations and determination of radioactivity.* Irides and approximately 0·5 mm thick slices from heart and brain (standardized size; diameter 3 mm) were incubated *in vitro* in a modified Krebs–Ringer bicarbonate buffer, pH 7·4, saturated with 93·5% O_2 and 6·5% CO_2.[13,14] Ascorbic acid was omitted in all experiments. The incubation medium contained 0·1 μM ^{3}H-NA and its volume was 2 ml per four slices or irides. All incubations were, unless otherwise stated, performed at 37° using a metabolic shaker. After termination of the incubation, the tissue pieces were dissolved in 0·5 ml Soluene® and after addition of 10 ml of toluene phosphor, the radioactivity was determined by liquid scintillation spectrometry. The irides, after the *in vitro* incubation, were prepared as whole mounts, dried and exposed to formaldehyde gas for the fluorescence histochemical demonstration of catecholamines according to Falck and Hillarp.[15–17] After fluorescence microscopy, the irides were scraped off the slides and dissolved in 0·5 ml Soluene® and the radioactivity was determined as described above. Since previous studies have shown that the main part (80 per cent or more) of radioactivity taken up in slices under these conditions represents unchanged ^{3}H-NA[13,14,18,19] the radioactivity values obtained after incubation in ^{3}H-NA have been expressed as unchanged ^{3}H-NA. In heart slices and irides the ^{3}H-NA content was expressed as dpm/mg wet weight of the tissue, whereas in brain slices as dpm/slice. ^{3}H-NA uptake at 0° has for all tissues been subtracted from that at 37° in order to correct for extraneuronal uptake (see refs. 13 and 14).

In one set of experiments heart slices were incubated *in vitro* in 0·1 μM ^{3}H-DA in order to investigate the formation of ^{3}H-NA from ^{3}H-DA. In this case, the slices, after the incubation, were weighed and extracted with 3 ml 0·4 N perchloric acid. ^{3}H-NA and ^{3}H-DA were isolated by ion-exchange chromatography (Dowex 50W-X4)

and determined as described by Jonsson and Sachs.[20] The radioactivity values were expressed as dpm/mg wet weight of the slices.

Drugs and substances used: DL-NA-7-^{3}H-HCl (5–10 Ci/m-mole), DA-7-^{3}H-acetate (2·4 Ci/m-mole, New England Nucl. Corp., Boston, Mass., U.S.A.); 6-OH-DA-HCl, 6-OH-DOPA; α-methyl-*p*-tyrosine methylester, H44/68 (AB Hässle, Göteborg, Sweden); nialamide (Niamid®, Pfizer, Stockholm, Sweden).

RESULTS

Endogenous NA. There was no significant difference between the endogenous NA in heart and brain of control and scorbutic guinea-pigs (Table 1). The disappearance of NA following tyrosine hydroxylase inhibition produced by α-methyl-*p*-tyrosine methylester (H44/68) was similar in the heart of control and scorbutic guinea-pigs, whereas H44/68 caused a somewhat greater depletion ($P < 0.001$) in the brain of scorbutic animals compared with control.

TABLE 1. EFFECT OF TYROSINE HYDROXYLASE INHIBITION ON ENDOGENOUS NA IN HEART AND BRAIN OF CONTROL AND SCORBUTIC GUINEA-PIG

	Heart (μg/g)†	(%)‡	Brain (μg/g)†	(%)‡
Control	1·88 ± 0·112	100	0·24 ± 0·007	100
+H44/68*	0·98 ± 0·169	52	0·12 ± 0·002	50
Scorbutic	1·72 ± 0·110	92	0·24 ± 0·005	100
+H44/68*	1·06 ± 0·115	56	0·10 ± 0·002	42

* α-Methyl-*p*-tyrosine methylester (H44/68), 250 mg/kg i.p., 4 hr.
† μg NA/g wet weight of the tissue.
‡ Expressed as a percentage of untreated control. n = 8–12.

The concentration of ascorbic acid in adrenal glands of scorbutic animals was 2–6 per cent of that in controls. The crude synaptosomal fraction from brains of scorbutic guinea-pigs had an ascorbic acid concentration of about 10 per cent (10·8 ± 3·1 per cent; n = 5) of controls.

Intracardial injection of 6-OH-DA led to a marked dose-dependent reduction of endogenous NA in heart, with approximately similar effects in both scorbutic and control guinea-pigs (Table 2). Administration of 6-OH-DOPA (i.p.) to nialamide pretreated animals also resulted in a marked depletion of heart NA with a somewhat more marked effect in scorbutic animals (Table 2). This treatment caused a small reduction of brain NA which was quantitatively similar in both groups of animals investigated.

^{3}H-NA synthesis, uptake and retention. In order to examine the diffusion conditions *in vitro* and the volume of the extracellular space in heart slices from control and scorbutic guinea-pigs, incubations with the extracellular marker ^{3}H-sorbital were carried out.[13] Heart slices were incubated in Krebs–Ringer buffer containing 2·5 μCi/ml of ^{3}H-sorbitol (sp. act. 1·4 Ci/m-mole) for 2–10 min at 37° after which the radioactivity in the slices was determined. Equilibrium was reached already after 2 min with similar results in both control and scorbutic animals. The sorbitol space, calculated as

$$\frac{^3\text{H-sorbitol/mg tissue}}{^3\text{H-sorbitol/}\mu\text{l medium}} \times 100,$$

TABLE 2. EFFECT OF 6-OH-DA OR NIALAMIDE + 6-OH-DOPA ON THE ENDOGENOUS NA IN HEART AND BRAIN OF CONTROL AND SCORBUTIC GUINEA-PIG

	Dose (mg/kg)	Heart (μg/g)‡	Heart (%)§	Brain (μg/g)‡	Brain (%)§
Control	—	1·88 ± 0·112	100	0·24 ± 0·007	100
6-OH-DA*	20	0·80 ± 0·132	43	—	—
6-OH-DA*	50	0·21 ± 0·009	11	—	—
Nialamide†	500				
+6-OH-DOPA	100	0·63 ± 0·113	34	0·17 ± 0·009	70
Scorbutic	—	1·72 ± 0·110	91	0·24 ± 0·005	100
6-OH-DA*	20	0·54 ± 0·169	29	—	—
6-OH-DA*	50	0·42 ± 0·053	22	—	—
Nialamide†	500				
+6-OH-DOPA	100	0·28 ± 0·019	15	0·18 ± 0·009	75

* 6-OH-DA (20 or 50 mg/kg) was injected intracardially 4 hr before sacrifice.

† Nialamide (500 mg/kg) was injected i.p. 30 min before 6-OH-DOPA (200 mg/kg i.p.) and the animals were sacrificed 4 hr thereafter.

‡ μg NA/g wet weight of the tissue.

§ Expressed as a percentage of untreated control. n = 6–12.

was similar in slices both from controls (29 per cent) and scorbutic animals (30 per cent). These results point to similar diffusion conditions in slices from both groups studied.

The formation of ^{3}H-NA in slices from heart incubated *in vitro* in 0·1 μM ^{3}H-DA for 30 min and washed in fresh buffer for 10 min was reduced by 40 per cent in slices from scorbutic animals, whereas the ^{3}H-DA recovered was markedly increased (Fig. 1).

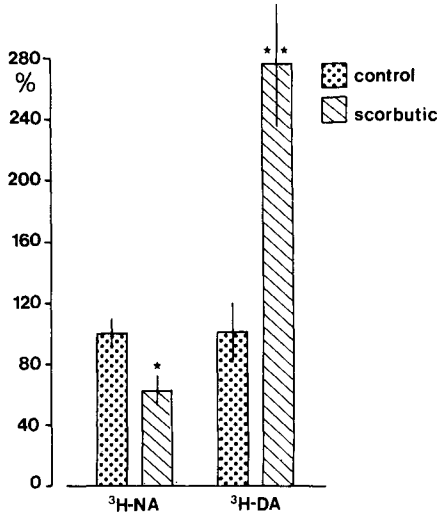

FIG. 1. Formation of ^{3}H-NA from ^{3}H-DA in heart slices of control and scorbutic guinea-pigs. The slices were incubated *in vitro* in 0·1 μM ^{3}H-DA for 30 min and washed in fresh buffer for 10 min. Each column represents the mean ± S.E.M. of 6–8 determinations and the results are expressed as a percentage of ^{3}H-NA and ^{3}H-DA recovered from control slices.

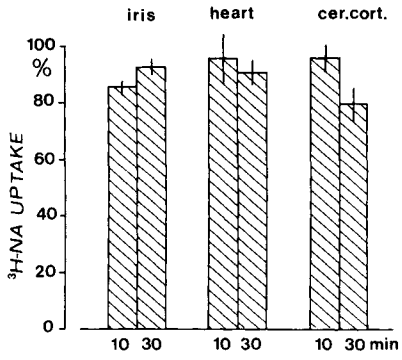

FIG. 2. *In vitro* uptake and retention of ^{3}H-NA (0·1 μM, 10 or 30 min) in iris and slices from heart and cerebral cortex (cer. cort.) of scorbutic guinea-pigs. After incubation in ^{3}H-NA for 10 min, the tissue pieces were taken directly for radioactivity determination, whereas after incubation for 30 min an additional incubation in fresh buffer for 10 min was carried out. Each column represents the mean ± S.E.M. of 6–8 determinations and is expressed as a percentage of ^{3}H-NA uptake-accumulation in respective control tissue.

The *in vitro* uptake and retention of ^{3}H-NA in iris and slices from heart and brain from scorbutic animals did not differ significantly from that of controls (Fig. 2), although there was a tendency for a slight reduction of both uptake and retention.

6-OH-DA treatment caused a marked dose-dependent reduction of the *in vitro* uptake of ^{3}H-NA in slices from heart and iris, with similar effects in control and scorbutic guinea-pigs (Fig. 3). There was also a reduced ^{3}H-NA uptake *in vitro* in iris and in slices from heart and brain after nialamide + 6-OH-DOPA treatment (Fig. 4). Again in no case was there any significant difference between tissues from control and scorbutic guinea-pigs.

Fluorescence histochemistry. The fluorescence morphology of the adrenergic nerves in iris demonstrated with the formaldehyde-fluorescence method of Falck and Hillarp was not notably affected in scorbutic animals compared to control. 6-OH-DA treatment as in Fig. 3 or nialamide + 6-OH-DOPA treatment as in Fig. 4 resulted in a marked reduction in the nerve density with similar effects in both groups of animals studied.

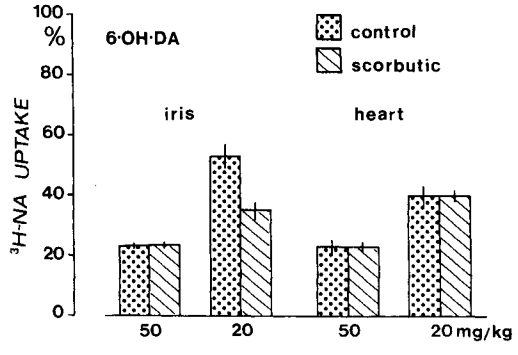

FIG. 3. Effect of 6-OH-DA (50 or 20 mg/kg intracardially, 4 hr) on the *in vitro* uptake of ^{3}H-NA (0·1 μM, 10 min) in iris and slices from heart of control and scorbutic guinea-pigs. Each column represents the mean ± S.E.M. of 6–8 determinations and is expressed as a percentage of the ^{3}H-NA uptake in respective control tissue from animals not treated with 6-OH-DA.

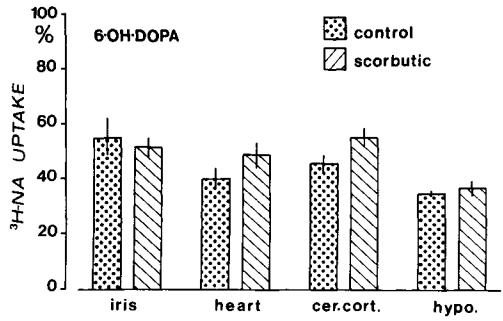

FIG. 4. Effect of 6-OH-DOPA (200 mg/kg i.p., 4 hr) in nialamide pretreated (500 mg/kg i.p., 30 min before 6-OH-DOPA) control and scorbutic guinea-pigs on the *in vitro* uptake of ^{3}H-NA (0·1 μM, 10 min) in irides and slices from heart, cerebral cortex and hypothalamus. Each column represents the mean ± S.E.M. of 6–8 determinations and the results are expressed as percentages of respective control tissue not treated with nialamide + 6-OH-DOPA.

Ascorbic acid treatment. Treatment of control guinea-pigs with a large dose of ascorbic acid resulted in a small reduction of ^{3}H-NA uptake in both iris and heart slices (Table 3). Pretreatment of the animals with ascorbic acid 15 min before the administration of 6-OH-DA caused a potentiation of the 6-OH-DA induced reduction of the ^{3}H-NA uptake in both tissues investigated. Considering the somewhat reduced uptake after ascorbic acid alone, the potentiation was only clear-cut in the iris.

DISCUSSION

Previous studies have shown that the endogenous NA concentration and the ^{3}H-NA uptake-accumulation is somewhat decreased in hearts of scorbutic guinea-pigs.[21] Consistent with these findings Sears[22] found a marked reduction of endogenous NA in the iris following vitamin C deficiency. The results of the present study revealed, however, no clear-cut or very small changes in endogenous NA in both brain and heart of scorbutic animals, in agreement of the findings of Lokoshko and

TABLE 3. EFFECT OF ASCORBIC ACID TREATMENT ON THE 6-OH-DA INDUCED REDUCTION OF THE *in vitro* UPTAKE OF ^{3}H-NA IN IRIS AND HEART SLICES OF CONTROL GUINEA-PIG

	^{3}H-NA uptake (%)§	
	Iris	Heart
Untreated	100 ± 6	100 ± 6
Ascorbic acid*	83 ± 2	74 ± 5
6-OH-DA†	44 ± 3	47 ± 4
Ascorbic acid‡ +6-OH-DA	25 ± 1	36 ± 1

* 1000 mg/kg i.p. 4 hr 15 min.

† 20 mg/kg intracardially 4 hr.

‡ Ascorbic acid (1000 mg/kg i.p.) was given 15 min before 6-OH-DA (20 mg/kg, intracardially, 4 hr).

§ The results are expressed as a percentage of the ^{3}H-NA uptake in untreated control. n = 6.

Lesnykh.[23] The ability of iris, heart and brain slices from scorbutic guinea-pigs to take up and accumulate ^{3}H-NA *in vitro* was also largely unaffected. In addition, there were no changes in the fluorescence morphology of the adrenergic nerves in iris following a scorbutogenic diet. The present data would thus point to almost unaffected NA uptake and storage in both central and peripheral NA neurons of scorbutic guinea-pigs and indicate that ascorbic acid is not primarily involved in maintaining the NA uptake and storage functions. However, it could always be argued that in spite of a very marked ascorbic acid reduction (90 per cent in synaptosomes from brain tissue) in the scorbutic animals, the amounts left are enough to leave NA uptake and storage practically unaffected. Previous studies have also shown that there seems to be a considerable sparing of brain ascorbate in states of general depletion.[24,25]

The discrepancy between the results of Thoa *et al.*,[21] and Sears[22] on one hand and the present data on the other might be related to these former investigators using animals with more advanced scurvy. During such conditions it is possible that the changes in NA uptake-storage are more indirect and associated with a more or less general damage of the neurons, since it has been observed that vitamin C deficiency can induce degenerative signs of autonomic ganglion cells.[26] These authors therefore suggested that ascorbic acid is necessary to maintain the integrity of the autonomic neuron.

The reduction of heart and brain NA in scorbutic animals following tyrosine hydroxylase inhibition produced by α-methyl-*p*-tyrosine was also similar to that of controls, although there was a tendency for an increased depletion of brain NA. This latter observation may indicate a more rapid turnover of brain NA (see Ref. 27), in scorbutic animals. A shorter turnover time for NA has also been observed in iris of scorbutic guinea-pigs as revealed by tyrosine hydroxylase inhibition, although the turnover rate was observed to be largely unaffected.[22] The reason for the increased brain NA turnover in scorbutic animals may be related to a stress-effect, since it has been shown that other stress conditions increase NA turnover (see Ref. 28).

It is known that the enzyme catalyzing the conversion of DA to NA, DA-β-hydroxylase, requires oxygen and ascorbate as cofactors.[29] In agreement it was observed in the present study that the *in vitro* formation of ^{3}H-NA from ^{3}H-DA was markedly reduced in heart slices of scorbutic guinea-pigs.

As regards the neurotoxic effect of 6-OH-DA and 6-OH-DOPA on catecholamine neurons, they were similarly potent in scorbutic and control animals. It has been shown in *in vitro* experiments by Heikkila and Cohen[6,7] that 6-OH-DA generates H_2O_2 upon autooxidation which led these authors to suggest that H_2O_2 may partly contribute to the neurotoxicity *in vivo* of 6-OH-DA. Furthermore, it was shown that ascorbic acid potentiates the formation of H_2O_2 from 6-OH-DA. If the theory of Heikkila and Cohen is correct one would expect a reduced effect of 6-OH-DA and 6-OH-DOPA on catecholamine neurons after lowering the ascorbic acid content, as in scorbutic animals. Although the present study did not show this, it does not mean that the H_2O_2 hypothesis can be ruled out. The negative results may only reflect that the model used to test the hypothesis is not adequate. This is obvious for a number of reasons, e.g. the catecholamine neurons in scorbutic animals may have other changes in addition to a reduced ascorbic acid content and therefore be more vulnerable; the remaining amount of ascorbic acid intraneuronally may be enough for the potentiation of H_2O_2 formation.

The present experiments with exogenous administration of a large dose of ascorbic acid showing a potentiation of the neurotoxic action of 6-OH-DA can be taken as a support for the H_2O_2 hypothesis, but it can always be argued that this effect is related to an antioxidant effect on 6-OH-DA. Ascorbic acid would then prevent the extraneuronal oxidation of 6-OH-DA leading to more 6-OH-DA being able to reach the neurons. The results after ascorbic acid administration are complicated also by the fact that ascorbic acid itself seems to affect ^{3}H-NA uptake. The reason for this is unknown but may be related to the observation that ascorbic acid can decrease the endogenous NA in the heart[30] which has been suggested to be an indirect effect of ascorbic acid acting on the sodium potassium pump. More recent studies by Cohen and Heikkila[31] have revealed that in addition to H_2O_2, superoxide and hydroxyl radicals are formed during the autooxidation of 6-OH-DA with a complex interaction of these radicals with H_2O_2, 6-OH-DA and catecholamines, and may explain why effects of lowering and increasing tissue concentrations of ascorbic acid are not easy to interpret. In any case, further investigations have to be carried out to get more precise information as to the causal agent(s) producing nerve degeneration after 6-OH-DA.

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