ASCORBATE AS A NATURAL CONSTITUENT OF CHROMAFFIN GRANULES FROM THE BOVINE ADRENAL MEDULLA

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1. Introduction

The copper enzyme dopamine β-hydroxylase (dopamine β-mono-oxygenase: EC 1.14.17.1), which catalyzes the hydroxylation of dopamine to noradrenaline has been located within the limiting membrane of the chromaffin granules of adrenal medulla [1,2]. In this typical mono-oxygenase reaction ascorbate supports high rates of hydroxylation catalyzed by the solubilized or the purified enzyme [3], but whether or not this vitamin functions as the electron donor for the enzyme in vivo, is still uncertain. Thus, while ascorbate is an effective donor when tested on highly purified enzyme, one cannot be certain if this is also the case during hydroxylation in vivo since no decrease in the tissue levels of catecholamines has been detected in the severely scorbutic guinea pig [4,5]. Furthermore, in his estimations of the ascorbate content of the subcellular fractions of bovine adrenal medulla Hagen [6] found little if any ascorbate in the cytoplasmic particles. Although indirect evidence has been presented for the presence of reducing equivalents in the intact chromaffin granules [1,7], the function of ascorbate has not yet been demonstrated.

In support of the function of ascorbate in the hydroxylation of dopamine in the adrenal medulla we report that the chromaffin granules contain a high concentration of this vitamin in its fully reduced form.

Abbreviations: DCPI, 2,6-dichlorophenolindophenol.

2. Methods

2.1. *Preparation of protein-free lysate of chromaffin granules*

Chromaffin granules were prepared from bovine adrenal medulla by sedimentation in 1.6 M sucrose as described [8]. The granules were then subjected to hypo-osmotic lysis and soluble proteins were precipitated by 50% (v/v) ethanol in 10 mM acetic acid containing 1 mM EDTA [9]. The pH was adjusted to 3.0 by 0.5 M HCl, and following an incubation period of 1 h at 0°C the precipitated proteins were removed by centrifugation.

2.2. *Chromatographic procedures*

Catecholamines of the protein-free lysate (pH adjusted to 5.3) were quantitatively removed by adsorption onto a column of the strong cation-exchange resin Dowex 50W-X8 (Na^+ form) [10] equilibrated with 10 mM Na-acetate buffer, pH 5.3 containing 1 mM EDTA.

Ascorbate [11] as well as adenine nucleotides of the protein-free lysate (pH adjusted to 5.3) were quantitatively removed by adsorption onto a column of the moderately strong anion-exchange resin Dowex 2 (acetate form) equilibrated with 10 mM Na-acetate buffer, pH 5.3 containing 1 mM EDTA.

The low-molecular weight compounds of the protein-free lysate were separated on a column (1.9 cm × 46 cm) of Sephadex G-10 (Pharmacia Fine Chemicals, Sweden) equilibrated and eluted (10 ml/h) with 10 mM Na-acetate buffer, pH 5.3 or pH 3.3, containing 1 mM EDTA and 0.3 M NaCl. was added to the buffer in order to increase the

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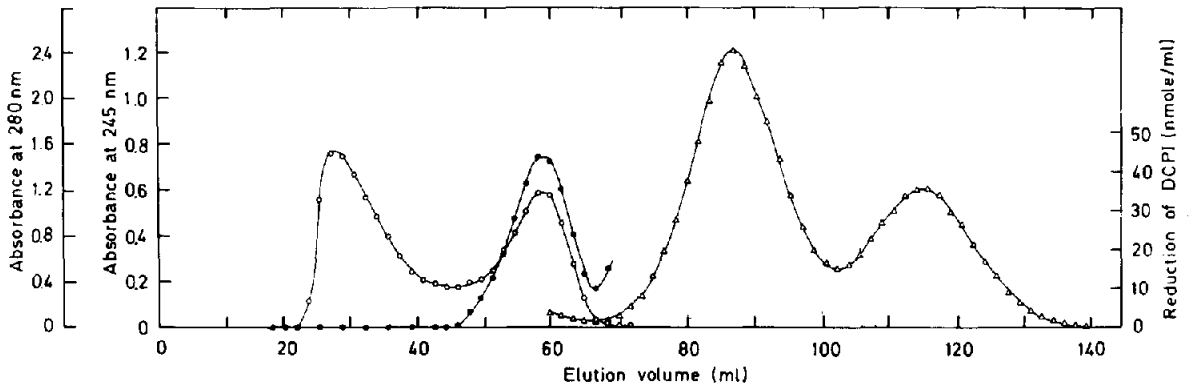

Fig.1. Absorbance at 245 nm (o) and 280 nm (△) and 2,6-dichlorophenolindophenol (DCPI) reducing ability (•) of the fractions (1.6 ml) of the acid ethanol-soluble portion of the chromaffin granule lysate, after separation on Sephadex G-10. The column (1.9 × 46 cm) was equilibrated and eluted with 10 mM Na-acetate buffer, pH 3.3 containing 0.3 M NaCl and 1 mM EDTA.

retardation of the catecholamines typical of aromatic compounds [12] and thus achieve a separation from e.g. ascorbate in spite of nearly identical molecular weights, as well as a separation of adrenaline from noradrenaline (see fig.1). Aliquots were assayed for absorbance at 245 nm and 280 nm and titrated with 2,6-dichlorophenolindophenol to locate ascorbic acid/ascorbate containing fractions (see below).

EDTA (1 mM) was included in all buffers used in order to protect the ascorbate from oxidation since metal ions catalyze the autoxidation of this vitamin [13].

2.3. *Analytical methods*

Ascorbate was assayed by its reduction of 2,6-dichlorophenolindophenol (from the Sigma Chemical Co., U.S.A.) at pH 7.0 (0.1 M K-phosphate buffer) as followed spectrophotometrically (Shimadzu model MPS-50L recording spectrophotometer) by the decrease in absorbance at 600 nm, using the extinction coefficient 16.1 mM^{-1} cm^{-1} [14].

Adrenaline and noradrenaline were identified by a high-speed amino-acid analyzer (Bio-Cal, type BC-200) [9] and assayed spectrophotometrically using the extinction coefficient 2.6 mM^{-1} cm^{-1} at 280 nm for both components (O. Terland, unpublished results).

L-Ascorbic acid was the product of Baker Chemicals, The Netherlands. Quartz distilled water was used throughout.

3. Results

3.1. *Demonstration and assay of DCPI-reducing substance(s) of the chromaffin granule lysate*

The chromaffin granule lysate, from which proteins had been removed by acid-ethanol precipitation, was assayed for substances able to reduce 2,6-dichlorophenolindophenol (DCPI) as well as for catecholamines (table 1). Quite unexpected was the finding that the catecholamine-free lysate contained high concentrations of DCPI-reducing substance(s). Furthermore, it is seen from table 1 that there is an almost constant molar ratio between the catecholamines and the DCPI reduced when determined on different preparations of adrenal medulla (the molar ratio was approx. 40:1 as an average of 6 determinations). This result indicates that the naturally occurring reducing substance(s) is present in the chromaffin granule matrix at a concentration of about 13 mM assuming a two-electron donor and a concentration of 0.55 M for the catecholamines [15].

3.2. *Identification of the major DCPI-reducing substance*

Molecular-sieve chromatography on Sephadex G-10 of the acid ethanol-soluble portion of the granule lysate gave four distinct UV-absorbing peaks in the elution diagram (fig.1). The first peak (at 28 ml) was identified spectroscopically as adenine nucleotides whereas the third peak (at 86 ml) and

Table 1
The molar ratio of catecholamines to DCPI reducing substance(s) in the lysate of chromaffin granules

Expt. No.	Catecholamines (μmol)	DCPI reduced (μmol)	Molar ratio catecholamines / DCPI reduced
1[a]	9.2	0.227	40.5
2[a]	8.2	0.213	38.5
3[a]	8.9	0.212	42.0
4[a]	8.7	0.215	40.5
5[b]	25.0	0.653	38.3
6[b]	25.7	0.627	41.0

[a]Derived from 1.66 g of medulla (wet wt)
[b]Derived from 1.4 g of medulla (wet wt)
The amount of DCPI reduced was determined after the catecholamines had been removed as described in Methods. The amount of catecholamines (adrenaline plus noradrenaline) was determined after DCPI-reducing substance(s) and adenine nucleotides had been removed as described in Methods. The data in Expt. No. 5 and 6 were obtained from experiments identical to that given in fig.1.

fourth peak (at 114 ml) were found to represent adrenaline and noradrenaline, respectively. The second peak (at 54 ml), which was found to contain material which rapidly reduced DCPI, was well separated from adenine nucleotides and catecholamines at pH 3.3, and co-chromatographed with L-ascorbic acid/ascorbate at this pH as well as at pH 5.3 (table 2). The identity of this DCPI-reducing substance with ascorbic acid/ascorbate was confirmed by a comparison of the u.v. absorption spectrum of the peak

Table 2
The elution volumes of ascorbic acid/ascorbate, the DCPI-reducing substance, adenine nucleotides, adrenaline and noradrenaline at two different pH values on molecular sieve chromatography (Sephadex G-10)

Compound	Elution volume (ml)	
	pH 3.3	pH 5.3
Ascorbic acid/ascorbate	55	42
DCPI-reducing substance	54	43
Adenine nucleotides[a]	28	28
Adrenaline	86	86
Noradrenaline	114	114

[a]Mainly ATP
Experimental conditions as in fig.1. The volumes are the mean of two determinations.

fractions (equivalent to 56 μM DCPI) with an authentic sample of L-ascorbic acid/ascorbate at strongly acidic pH (fig.2A) and pH 5.3 (fig.2B).

Since the molar ratio between the catecholamines and the ascorbate thus determined was also approx. 40:1 (table 1) it may be concluded that the major DCPI reducing component of the chromaffin granules is ascorbate.

Although we do not know why ascorbic acid/ascorbate revealed a strongly pH-dependent elution profile on the Sephadex G-10 column (table 2), our data may indicate that the vitamin exists as a dimer in its dissociated form.

4. Discussion

Previous observations [1,7] that preparations of crude chromaffin granules of adrenal medula convert dopamine into noradrenaline even in the absence of added ascorbate, indicated the presence of reducing equivalents in the granules although the conclusion of a more direct study was negative [6]. From the data presented here, however, it is apparent that the chromaffin granule matrix contains appreciable amounts of a DCPI-reducing substance. This substance was identified as ascorbic acid/ascorbate

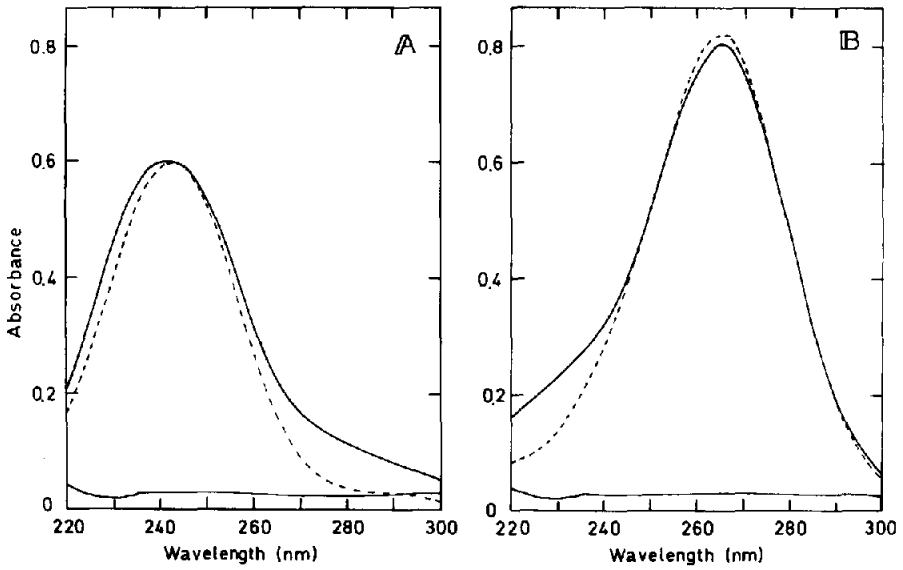

Fig.2. The solid lines represent the u.v. absorption spectra of the DCPI-reducing substance eluted from the Sephadex G-10 column at 54 ml (see fig.1) recorded at pH 1.0 (A) and pH 5.3 (B). The amount of material was equivalent to 56 μmol of DCPI. The broken lines represent the spectra of 55 μmol of an L-ascorbic acid/ascorbate standard solution. The common baselines are indicated in each figure.

by its chromatographic behavior on Sephadex G-10 and its spectral comparison with an authentic sample of L-ascorbic acid/ascorbate.

On the basis of our discovery and estimates on the average ascorbate content of the chromaffin granule matrix (approx. 13 mM), which is well above the K_M value of dopamine β-hydroxylase in an in vitro assay system [16,17], as well as the localization of this enzyme within the limiting membrane of the granules [1,2], it is likely that ascorbate indeed functions as the immediate electron donor also in vivo.

Since dehydroascorbate is formed when the oxidation of ascorbate is coupled to the β-hydroxylation of dopamine [3] and it has been reported that a conjugated form of ascorbate (ascorbate 2-sulfate) is present in the adrenal gland [18] a more detailed analysis of the content of these compounds in the chromaffin granules is now in progress.

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References

[1] Kirshner, N. (1962) J. Biol. Chem. 237, 2311–2317.
[2] Belpaire, F. and Laduron, P. (1968) Biochem. Pharmacol. 17, 411–421.
[3] Friedman, S. and Kaufman, S. (1965) J. Biol. Chem. 240, 4763–4773.
[4] Jonsson, G., Lohmander, S. and Sachs, C. (1974) Biochem. Pharmacol. 23, 2585–2593.
[5] Lokoshko, O. A. and Lyesnykh, L. D. (1971) Ukr. Biokhim. Zh. 43, 536–539.
[6] Hagen, P. (1954) Biochem. J. 56, 44–46.
[7] Lishajko, F. (1969) Acta Physiol. Scand. 76, 159–171.
[8] Helle, K. B., Flatmark, T., Serck-Hanssen, G. and Lønning, S. (1971) Biochim. Biophys. Acta 226, 1–8.
[9] Flatmark, T., Bødtker-Naess, V. and Slinde, E. (1975) Abstr. Commun. Meet. Fed. Eur. Biochem. Soc., No. 1311.
[10] Bertler, Å., Carlsson, A. and Rosengren, E. (1958) Acta Physiol. Scand. 44, 273–292.

[11] Williams R. C., Baker, D. R. and Schmit, J. A. (1973) J. Chromat. Sci. 11, 618–624.
[12] Bretthauer, R. K. and Golichowski, A. M. (1957) Biochim. Biophys. Acta 155, 549–557.
[13] Hanaki, A. (1969) Chem. Pharm. Bull. 17, 1839–1846.
[14] Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M. (1969) Data for Biochemical Research, 2nd edn. pp. 436–437.
[15] Kirshner, N. (1974) Life Sci. 14, 1153–1167.
[16] Foldes, A., Jeffrey, P. L., Preston, B. N. and Austin, L. (1973) J. Neurochem. 20, 1431–1442.
[17] Aunis, D., Miras-Portugal, M.-T. and Mandel, P. (1973) Biochim. Biophys. Acta 327, 313–327.
[18] Mumma, R. O. and Verlangieri, A. J. (1972) Biochim. Biophys. Acta, 273, 249–253.