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SUBCELLULAR DISTRIBUTION OF ASCORBATE IN BOVINE ADRENAL MEDULLA

EVIDENCE FOR ACCUMULATION IN CHROMAFFIN GRANULES AGAINST A CONCENTRATION GRADIENT

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Summary

The subcellular distribution of ascorbate and catecholamines has been studied in homogenates of bovine adrenal medulla and cortex.

1. The recovery of the vitamin was found to be 4.10 ± 0.22 and 9.57 ± 1.37 μmol/g wet weight for the medulla and cortex, respectively. A major fraction (34.4%) of the vitamin was recovered in the particulate fraction of the medulla as compared to about 8% in the corresponding fraction of the cortex. In comparison, 78.9% of the catecholamines were found in the particulate fraction of the medulla.

2. Analytical differential centrifugation of medulla homogenates revealed a sedimentation profile of ascorbate which was identical to that obtained for noradrenalin and adrenalin. The co-sedimentation of these compounds indicates that ascorbate is an essential component of the heavy as well as the light population of chromaffin granules. The stoichiometry of catecholamines to ascorbate was approx. 25:1 in both subpopulations.

3. Based on an estimated volume fraction of ~13% for the chromaffin granules, as determined morphometrically (Kryvi, H., Flatmark, T. and Terland, O. (1979) Eur. J. Cell Biol. 20, 76—82), a concentration gradient (chromaffin granules:cytosol) of approx. 4 was estimated for ascorbate in the cells of adrenal medulla.

4. No ascorbate 2-sulfate was detected in any of the subcellular fractions isolated, and the content of dehydroascorbate in isolated chromaffin granules was <1% of the total ascorbate value.

Introduction

Ascorbate is present in all animal tissues (for review, see Ref. 1), but the significance of this vitamin in the cellular metabolism as well as its mechanism of action, is still a matter for discussion [2,3]. However, the finding in this laboratory that the catecholamine storage granules (chromaffin granules) isolated from the bovine adrenal medulla contain high concentrations of unmodified ascorbate in its fully reduced form [4] points to one of the most clearly defined biochemical roles of the vitamin. Thus, the hydroxylating enzyme dopamine β-monooxygenase, which is a membrane-enclosed enzyme confined to the chromaffin granules (for review, see Ref. 5), utilizes very efficiently ascorbate as electron donor [6]. On the basis of our estimates on the ascorbate content of the chromaffin granule matrix [4], which appears to be well above the K_m value of dopamine β-monooxygenase in an in vitro assay system [6,7], it is likely that ascorbate indeed functions as the immediate electron donor also in vivo. Furthermore, we have not been able to detect any alternative electron donor for this monooxygenase reaction in vivo (Terland, O., unpublished data).

In addition to these basic observations [4—7] it is important to know (1) the subcellular concentration gradients of ascorbate in the cells of adrenal medulla, (2) the mechanism by which ascorbate is taken up by the chromaffin granules, and (3) whether or not the chromaffin granules have a biochemical system to regenerate ascorbate when utilized as electron donor in the dopamine β-monooxygenase reaction. The purpose of the present study is to present data on the subcellular distribution of ascorbate in the bovine adrenal medulla from which an estimate of the concentration gradient (granule matrix vs. cytosol) has been obtained. In addition, the sedimentation profile of particle-bound ascorbate has been compared with that of the catecholamines.

Materials and Methods

Preparation of subcellular fractions of bovine adrenal cortex and medulla

Bovine adrenal glands were removed within a few minutes after death and kept on ice for approximately one hour prior to the start of the preparation procedures. The animals were predominantly Norwegian NRF dairy cattle. The cortex and medulla (approx. 4 g of tissue) were dissected apart and the medulla was first dispersed with a small manually operated stainless steel tissue press (Climpex Ltd.) before homogenization in 40 ml of ice-cold 0.25 M sucrose and 1.0 mM EDTA at pH 6.50, with a Teflon-glass Potter-Elvehjem homogenizer at a pestle speed of 1250 rev./min with only two passes. The cortex was homogenized directly at the same conditions. Even this gentle homogenization procedure gave a recovery of approx. 95% ascorbate (cortex) and 80% catecholamines (medulla) in the postnuclear fraction, which is in accordance with previously published values (catecholamines) [8].

The homogenates were centrifuged (in Sorvall RC-5 refrigereated centrifuge, HB-4 rotor with R_{min} = 6.2 cm and R_{max} = 14.4 cm) at a centrifugal effect of $\int_0^t (\text{rev./min})^2\, dt = 4 \cdot 10^7\ \text{min}^{-1}$ (equivalent to 2000 rev./min, $650 \times g_{max}$ for 10 min) in order to sediment any tissues which might have escaped disintegra-

tion, blood cells, incompletely broken cells and cell nuclei [9]. The supernatant fluid (post nuclear fraction) was centrifuged at a centrifugal effect of $\int_0^t (\text{rev./min})^2\ dt = 4 \cdot 10^9\ \text{min}^{-1}$ (equivalent to 12 600 rev./min, 25 780 $\times g_{max}$, 25 min) to give the particulate fraction (pellet) and an almost clear supernatant [9]. All procedures were carried out at 0—4°C.

Sedimentation profiles

The sedimentation profile of chromaffin granules was determined by analytical differential centrifugation in a homogeneous medium (0.25 M sucrose containing 1.0 mM EDTA) by parallel sedimentation essentially as described by Slinde and Flatmark [10] using a Sorvall RC-5 centrifuge with the swinging-bucket HB-4 rotor (R_{min} = 6.2 cm and R_{max} = 14.4 cm).

Preparation of tissue fractions for analysis of ascorbate and catecholamines

Particulate and non-particulate fractions were assayed for their ascorbate and catecholamine content. The particulate fractions were initially resuspended and gently homogenized in the centrifugation medium (0.25 M sucrose, 1 mM EDTA) and diluted by adding an equal volume of 50 mM potassium citrate buffer (pH 4) containing 1 mM EDTA, to obtain efficient lysis. Protein was then precipitated by adding an equal volume of absolute ethanol, and after 1 h at 0°C the precipitated protein was removed by centrifugation [9]. In some experiments the particulate material was lysed by resuspension in dilute acetic acid (pH 4) before addition of acid ethanol (pH 4). The supernatant fractions were initially diluted with citrate buffer, and protein was then precipitated by adding an equal volume of ethanol (see above). Care was taken to avoid contamination by the lipid layer on the top when the supernatants obtained following protein precipitation were removed for further analysis [9].

Estimation of catecholamines and ascorbate by high-performance liquid chromatography

Catecholamines and ascorbate were estimated by high-performance liquid chromatography using a Constametric II pump and variable wavelength UV detector (SpectroMonitor 1200 from Laboratory Data Control, FL, U.S.A.). The chromatographic separation of ascorbate was achieved at 20°C on a microparticulate bonded strong anion exchanger (Partisil-10 SAX, pre-packed from Whatman). The mobile phase, consisting of 20 mM ammonium phosphate buffer (pH 5.0), was pumped at a flow-rate of 2 ml/min (1400 lb/inch^2). Adrenalin and noradrenalin were separated at 20°C on a microparticulate bonded strong cation exchanger (Partisil-10 SCX, pre-packed from Whatman). The mobile phase, consisting of 50 mM acetate buffer (pH 4.3), was pumped at a flow-rate of 2 ml/min (1400 lb/inch^2).

Ascorbate 2-sulfate was assayed as recently described [11]. Dehydroascorbate was estimated by measuring the difference in ascorbate content after and before reduction by H_2S for 15 min in 50 mM potassium citrate buffer, pH 3.5 at 25°C [12].

The concentrations of adrenalin, noradrenalin and ascorbate standards were determined spectrophotometrically, using the extinction coefficient 2.6 ($mM^{-1} \cdot cm^{-1}$) at 280 nm for adrenalin and noradrenalin [4], and 12.6 ($mM^{-1} \cdot cm^{-1}$) at 265 nm and pH 5 for ascorbate [13].

Chemicals

L-Noradrenalin bitartrate was obtained from Hoechst AG (F.R.G.), L-adrenalin was the product of Merck AG (F.R.G.), and ascorbate 2-sulfate was a gift from Hoffman La Roche (Switzerland). All other chemicals were of reagent grade.

Results

Chromatographic systems for ascorbate and catecholamines

Two isocratic systems were developed for the assay of ascorbate (on Partisil 10-SAX) and adrenalin and noradrenalin (on Partisil 10-SCX), and typical chromatograms are shown in Fig. 1A—C. The retention times were 3.6 min (ascorbate), 4.2 min (noradrenalin) and 5.6 min (adrenalin), and a linear relationship was obtained between peak height and concentration for all these components within the actual measuring range (data not shown).

Estimation of ascorbate and catecholamines in cell subfractions of the bovine adrenal medulla and cortex

In order to determine the distribution of ascorbate between the particulate and non-particulate fraction of medulla and cortex, the post-nuclear fraction was centrifuged at a centrifugal effect which was sufficiently high to sediment 100% of all particles with $s > 3800$ S, including the total population of chromaffin granules [9,10]. As seen from the sedimentation profile (Fig. 2), this centrifugal effect is indeed sufficiently high to sediment all particle-bound

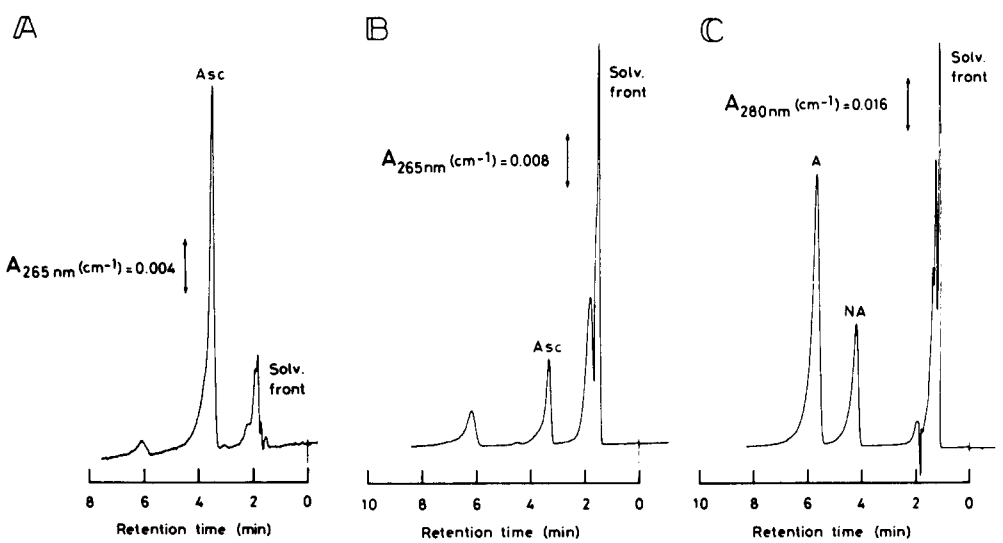

Fig. 1. HPLC elution pattern of ascorbate (A and B) and catecholamines (C). (A) 20 µl of an extract of the soluble fraction of the adrenal cortex homogenate was injected; ascorbate (Asc) was eluted with t_R = 3.6 min. (B) 20 µl of an extract of the particulate fraction of adrenal medulla homogenate. (C) 20 µl of the same material as in Fig. 1, B; noradrenalin (NA) and adrenalin (A) were eluted with t_R = 4.2 min and t_R = 5.6 min, respectively.

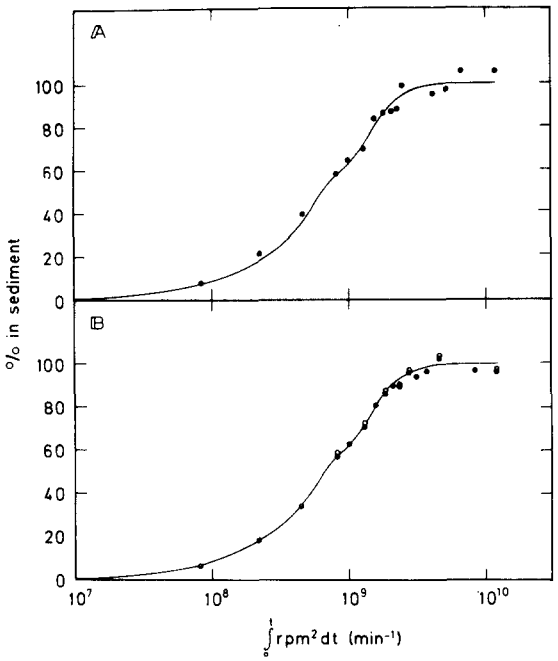

Fig. 2. Sedimentation of ascorbate (A) and catecholamines (B) as a function of $\int_0^t$ (rev./min)^2 dt when R_{min} = 6.2 cm and R_{max} = 14.4 cm; •, noradrenalin and ○, adrenalin (in B). The solid lines in A and B represent the best fit to the experimental values in B; the convergence value (100% in sediment) represents the mean of the sediment value (100% in sediment) obtained at $\int_0^t$ rev./min)^2 d$t \gtrsim 3 \cdot 10^9$ min^{-1}.

ascorbate. Using this centrifugal effect, it is seen from Table I that 34.4% of the ascorbate in the preparations of medulla was sedimented, whereas in the cortex only 8% was recovered in the sediment. From Table I it is also seen that 21% of the catecholamines in the medulla preparations were found in the non-particulate fraction, indicating that the degree of lysis was less than 21%. In the cortex, 55% of the catecholamines were found to sediment at the selected centrifugal effect. However, the total amount of catecholamines in the cortex is only 1.46 μmol/g wet weight as compared to 45.5 μmol/g wet weight in the medulla, indicating that the cortex is cross-contaminated by medulla to an extent of only 3%. The numbers given in Table I indicate that in spite of a certain degree of lysis of the chromaffin granules, as much as 34.4% of the ascorbate in the medulla homogenate is particle bound, whereas only a minor fraction of the ascorbate in the cortex homogenate is recovered in the particulate fraction. This unique distribution of ascorbate in the medulla is in accordance with our previous finding that the isolated chromaffin granules contain a high concentration of ascorbate [4].

Analytical differential centrifugation of particle bound ascorbate

Our particulate fraction contains mitochondria, lysosomes and microsomal elements in addition to chromaffin granules, and in order to show that ascorbate is present predominantly in the chromaffin granules, the particulate frac-

TABLE I

THE CONTENT OF ASCORBATE AND CATECHOLAMINES IN THE BOVINE ADRENAL MEDULLA AND CORTEX

The numbers given are expressed in $\mu mol \cdot g^{-1}$ wet weight ± S.D.

	Ascorbate		Catecholamines *	
	Post-nuclear fraction	Particulate fraction	Post-nuclear fraction	Particulate fraction
Medulla	4.10 ± 0.22 (100%) (n = 8)	1.41 ± 0.17 (34.4%) (n = 8)	45.5 ± 5.7 (100%) (n = 11)	35.9 ± 3.6 (78.9%) (n = 11)
Cortex	9.57 ± 1.37 (100%) (n = 6)	0.77 ± 0.17 (8%) (n = 6)	1.46 ± 0.54 (100%) (n = 12)	0.81 ± 0.43 (55.4%) (n = 12)

* Adrenalin + noradrenalin.

tion was further studied by analytical differential centrifugation. From Fig. 2A it is seen that maximum recovery of sedimented ascorbate was obtained at a time integral of about $3 \cdot 10^9$ min^{-1}. Any further increase in the centrifugal effect did not increase the yield of ascorbate in the pellet, indicating that this value represents the convergence value for the sedimentation of particle bound ascorbate essentially as found for adrenalin and noradrenalin (Fig. 2B).

Analyses of ascorbate 2-sulfate and dehydroascorbate

We were unable to detect ascorbate 2-sulfate in any of the fractions studied, using high-performance liquid chromatography as the analytical tool [11]. All fractions were also analyzed for dehydroascorbate by measuring ascorbate before and after reduction by H_2S. At most 1—2% increase in the amount of ascorbate was seen after H_2S reduction, indicating that essentially all the ascorbate present in the various fractions are in the reduced state.

Discussion

The standard procedures for the assay of ascorbate have been based on the reducing properties of ascorbate e.g. the reduction of 2,6-dichlorophenolindophenol [4,13]. These methods are not easily applicable to the analyses of subcellular fractions of the adrenal medulla with the high content of catecholamines which are also oxidized by 2,6-dichlorophenolindophenol [4]. Thus, the assay of ascorbate, e.g. in the chromaffin granules, by such methods makes it necessary to remove catecholamines prior to the reaction with the oxidizing agent [4]. On the other hand, by using high-performance liquid chromatography for the assay no interference from the catecholamines was observed (Fig. 1A and B).

The most marked differences between our measurements and those previously published by Hagen [14] was the subcellular distribution of ascorbate in the bovine medulla and cortex. Whereas Hagen [14] found almost no ascorbate in the sediment from high-speed centrifugation of cortex and medulla homogenates, a substantial fraction, i.e. 34.4% (medulla) was measured in the particulate fraction in the present study, while only about 8% was recovered in the

particulate fraction from the cortex. It is not clear why Hagen [14] did not detect ascorbate in the particulate fraction of medulla, but it may be related to the instability of ascorbate in chromaffin granule lysates if acid conditions and heavy metal chelating agents are not used. The finding that as much as 34.4% of the ascorbate present in the medulla is confined to the particulate fraction is in good agreement with our previous finding of a high concentration (~13 mM) of ascorbate in the chromaffin granules [4]. The catecholamine/ascorbate ratio in the particulate fraction (Table I) was estimated to about 25:1, whereas the corresponding ratio in chromaffin granules isolated by differential and density gradient centrifugation was about 40:1 [4]. This discrepancy is best explained by the difference in time required for the isolation of chromaffin granules (2 h in the present work and 6—8 h in a previous work [4]) since on storage of chromaffin granules in 1.6 M sucrose at 0—4°C a gradual increase in the catecholamine/ascorbate ratio was observed (Terland, O., unpublished data). The more rapid decrease in ascorbate content versus the catecholamine content is probably due to a more rapid leakage of ascorbate and/or a gradual oxidation of this compound. The fact that about 21% of the catecholamines are found in the non-particulate fraction (Table I), and that ascorbate is gradually lost from the granules, indicates that the amount of particle-bound ascorbate in the medulla (i.e. 34.4% of the total) represents a minimum value. Assuming the commonly accepted value of 550 mM for the intragranular concentration of the catecholamines (for review, see Ref. 16) and a catecholamine/ascorbate ratio of 25:1 (Table I), a matrix concentration of about 22 mM can be estimated for the total population of chromaffin granules. A similar value is arrived at by calculation based on the analytic values of the ascorbate content per g of wet weight, water content and the volume fraction of chromaffin granules. Our recent morphometric measurements [17] have revealed that the chromaffin granules occupy ~13% of the cytoplasmic volume, and a concentration gradient (chromaffin granules:cytosol) of 4 can then be estimated for ascorbate in the adrenal medullary cells using the values given in Table I, i.e. 1.41 μmol ascorbate in a volume fraction of ~13% (chromaffin granules) and 2.69 μmol ascorbate in a volume fraction of 87% (cytosol). This value must, however, be considered to be a minimum value, since the degree of lysis of chromaffin granules is not accounted for. Recent studies have revealed that dopamine is taken up by chromaffin granules in an energy-dependent process (for review, see Ref. 18) and kinetically linked to the function of the (H^+)-ATPase, i.e. the proton pump, of the granule membrane [19,20]. Since it has not been possible so far to demonstrate an ATP-driven uptake of ascorbate in chromaffin granules [21], an energy-independent mechanism of ascorbate uptake seems likely. The finding in the present study of a considerable concentration gradient of ascorbate between the granule matrix and the cytosol, therefore, indicates that the vitamin is included in the dynamic storage complex demonstrated for protein, catecholamines and adenine nucleotides by ^{1}H-NMR spectroscopy [22].

Since no ascorbate 2-sulfate was found in any fractions of the medulla, a regulatory function of ascorbate 2-sulfate in noradrenalin biosynthesis [23] can be ruled out. In agreement with previous studies [14], no dehydroascorbate was found when 1.0 mM EDTA was included in the homogenization and

extraction medium, indicating the presence of a powerful reducing mechanism for ascorbate in the chromaffin cells. At EDTA concentrations below 0.1 mM, however, a significant amount of dehydroascorbate was found in the extracts which can be attributed to metal catalyzed oxidation of ascorbate. Since no NADH or NADPH dependent dehydroascorbate reductase activity has been detected in the chromaffin granules (O. Terland, unpublished data), alternative mechanisms for the reduction of dehydroascorbate should be searched for.

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