Hydrocortisone inhibition of ascorbic acid transport by chromaffin cells

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Adrenal chromaffin cells have been found to accumulate ascorbic acid by a saturable high affinity mechanism that is inhibited by hydrocortisone. The rate of ascorbic acid transport into cells was linear for at least 1 h and had a K_m of 103 μM, a value approaching the reported concentration of ascorbic acid in the adrenal vein during stress. The uptake process itself, representing net accumulation rather than exchange, was inhibited by 0°C, lack of sodium, ouabain, and by dinitrophenol and iodoacetate. Hydrocortisone but not the inactive analogue hydrocortisone hemisuccinate was found to inhibit ascorbic acid uptake in a reversible manner, with an ID_{50} of 62 μM. This value was within the reported steroid concentration in the adrenal portal system during a significant stress. Both ascorbic acid and hydrocortisone are secreted from cortical cells during stress into the adrenal portal system and thus contact medullary chromaffin cells. We suggest that the control of ascorbic acid uptake by hydrocortisone indicates the existence of a heretofore unanticipated biochemical aspect of the adrenal stress response.

Ascorbic acid *Hydrocortisone* *Chromaffin cell* *Transport*

1. INTRODUCTION

During stress, ACTH activates the adrenal cortex to release stored ascorbic acid and subsequently to release newly synthesized corticosteroids [1,2]. Both substances enter the adrenal sinusoids, which serve as a portal system supplying the chromaffin cells of the medulla [3,4]. The medulla also contains a substantial amount of ascorbic acid [5,6], raising the questions of whether ascorbate in the portal system might be transported by chromaffin cells, and whether corticosteroids might regulate this process. An ascorbate transport system has been indicated in [7,8]. We now report that ascorbic acid is transported into chromaffin cells in a concentration dependent manner, and that the corticosteroid hydrocortisone reversibly inhibits ascorbic acid uptake. We suggest that ascorbic acid uptake into chromaffin cells and its regulation by corticosteroids may be an important and previously unrecognized biochemical aspect of the adrenal response to stress.

2. MATERIALS AND METHODS

2.1. *Cell preparation and culture*

Chromaffin cells were prepared by collagenase digestion using a modification of the method in [9], as described [10]. After preparation, the cells were incubated for 20 h in tissue culture flasks containing Basal Eagle Medium with Earle's Salts (Gibco), supplemented with glutamine 2.93 mM, gentamicin 5 μg/ml, cytosine arabinoside 10 μg/ml, streptomycin 100 μg/ml, and 5% fetal calf serum (Gibco). The cell concentration was 1×10^6/ml medium.

2.2. *Assay of transport*

Cells in suspension culture were washed twice at room temperature by centrifugation and resuspended in a standard medium containing 118 mM NaCl, 1.2 mM $MgSO_4$, 4.7 mM KCl, 10 mM glucose, 2.2 mM $CaCl_2$, and 25 mM HEPES-buffer (pH 7.4) with the addition of 0.1% bovine serum albumin (Calbiochem). The experiments

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were initiated by adding cells to reaction mixtures prewarmed to 37°C. The reaction mixtures consisted of cells at 1.0×10^6/mol in 10 ml containing (*R*)-[^{14}C]ascorbic acid (1 μCi/ml), 2.0 mM thiourea [11,12] standard medium and 0.1% final conc. bovine serum albumin.

Cell aliquots (400 μl) were withdrawn at different times with an EMI pipettor and blunt 16 gauge needles and pipetted into polypropylene tubes (Selectopette tips, Becton Dickson Co.) that had been heat sealed at the bottom. The tubes each contained 0.4 ml of a 1:2 mixture of Ficoll Hypaque and standard medium which was preshaken to the bottom of the tubes. The cell aliquots were pipetted 1–2 cm above this shelf, with an air space between the 2 layers, and the tubes cooled on ice until the end of the time course. The tubes were then spun in an RC-2 Sorvall Centrifuge at 2600 rev./min at 0°C for 5 min and frozen in an ethanol–dry ice mixture. The ends of the tubes containing the cell pellets and radioactive ascorbate were cut with an offset snips, dissolved in 0.5 ml protosol (New England Nuclear) and counted in a liquid scintillation counter.

2.3. *HPLC Analysis*

HPLC analysis of ascorbic acid was performed on a 5 μm radial compression C^{18} column using an ultraviolet detector at 254 nm (Waters). The mobile phase was 0.2% dicyclohexylamine phosphate at 3.0 ml/min and 1500 lb/in^2. (*R*)-Ascorbic acid eluted at 6.0 min.

Cells incubated with (*R*)-[^{14}C]ascorbic acid for 2 h were washed, lysed with water and centrifuged at 30000 $\times$ *g* for 10 min. Supernatant samples, which contained 99% of the total cell homogenate radioactivity, were directly injected and fractions collected at 1 min intervals for determination of radioactivity. More than 95% of the collected counts corresponded to the ascorbic acid peak.

2.4. *Chemicals and assays*

Protein was determined by Bradford Assays as described by this laboratory for application to chromaffin tissue [13]. Catecholamines were analyzed by the spectrofluorometric method [14]. Chemicals were reagent grade. *R*-[^{14}C]Ascorbic acid was purchased from New England Nuclear (spec. act. 7.6 mCi/mmol).

3. RESULTS

3.1. *Transport of ascorbic acid by chromaffin cells*

(*R*)-[^{14}C]Ascorbic acid was transported into chromaffin cells in a linear fashion for nearly 1 h at 37°C (fig. 1A). In addition the uptake of (*R*)-[^{14}C]ascorbic acid was found to be dependent on ascorbic acid concentration in a saturable manner, exhibiting a K_m of 103 μM (fig. 1B). To evaluate the stability of labelled ascorbic acid in cells, supernatants of water-lysed cell homogenates were analyzed by high-performance liquid chromatography. More than 95% of the counts collected corresponded to the ascorbic acid peak (not shown). Thus, we concluded that *R*-[^{14}C]-ascorbic acid was indeed transported by chromaffin cells, that transport was concentration dependent, and that the transported radiolabelled marker remained as ascorbic acid.

We then determined whether the transported ascorbic acid was free inside the cells, or bound. Cells that had been exposed to (*R*)-[^{14}C]ascorbic acid were lysed in water. Under these conditions, 96% of the label was released into the soluble fraction of the cell homogenate (table 1). Furthermore, when this soluble fraction was analyzed by ultrafiltration, we found that any possible site of ascorbate binding was $\leq$ 10 kDa. By contrast, Dounce homogenization of labelled cells in an isotonic medium revealed that 14% of the total label was contained in a particulate but osmotically labile fraction which also contained >90% of the catecholamines, presumably in chromaffin granules. Since chromaffin granules are known to contain ascorbic acid [15], the data indicated that under these incubation conditions ascorbic acid in the cytoplasm had access to the granule pool. However, most of the labelled newly transported ascorbic acid remained in a non-sedimenting cytoplasmic compartment.

3.2. *Ascorbate transport leads to net accumulation by cells*

The uptake of ascorbic acid in chromaffin cells appeared to involve net accumulation rather than simple exchange, as shown in a direct analysis by HPLC (table 2). Cells incubated in 200 μM (*R*)-ascorbic acid for 3 h contained 8-fold more ascorbic acid than cells without ascorbate in the

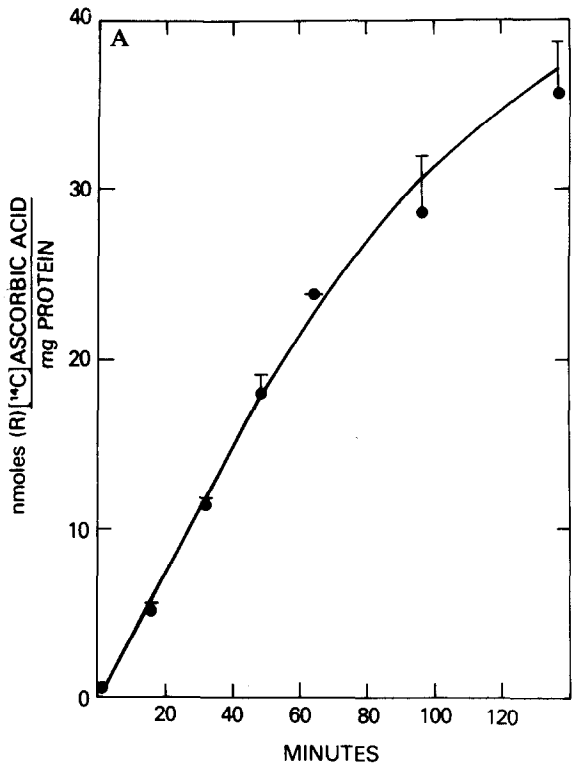


Fig. 1A. Uptake of (*R*)-[^{14}C]ascorbic acid into chromaffin cells as a function of time; ascorbic acid was 132 μM.

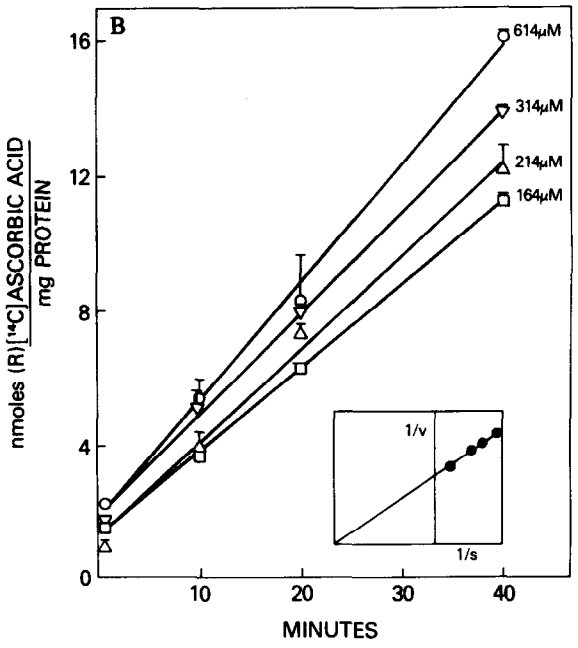


Fig. 1B. Uptake of (*R*)-[^{14}C]ascorbic acid into chromaffin cells as a function of varying concentrations of ascorbic acid: (□) 164 μM; (△) 214 μM; (▽) 314 μM; (○) 614 μM. Inset: Lineweaver–Burk plot of data in fig. 2; K_m was 103 μM.

medium. Furthermore, by estimating cell volume using a chromaffin cell diameter of 18 μm, we calculated a maximum spherical cell volume and a minimum intracellular ascorbate concentration. Ascorbate in the loaded cells was at least 1.8 mM, compared with 200 μM in the incubation medium (table 2). The data therefore suggested that the mechanism of ascorbic acid transport involved a concentrative mechanism.

3.3. *Energetics of ascorbic acid transport*

As anticipated from studies of ascorbic acid transport in other systems [11,12,16], the uptake process in chromaffin cells was blocked at 0°C, by metabolic inhibitors, and by lack of sodium. Ouabain inhibited transport by 50%, and replacement of NaCl by choline also resulted in a 50% reduction of transport. Dinitrophenol in combination with iodoacetate also inhibited uptake by 75% (not shown).

Table 1

Percent of (*R*)-[^{14}C]ascorbic acid in supernatants of disrupted cells

Disruption method	Centrifugation	Ultrafiltration
Water lysis	95.5 ± 3.1	91.4 ± 6.7
Dounce homogenization in isotonic medium	86.3 ± 9.5	80.3 ± 13.3

Chromaffin cells were incubated with (*R*)-[^{14}C]ascorbic acid for 2 h. They were then washed twice and were either lysed in 10 vol. water or passed in a Dounce homogenizer in isotonic standard medium. The lysate and homogenate were each subjected to centrifugation of 30000 × *g* for 30 min or ultrafiltration, using an M_r 10000 retention filter (Millipore). Percentages represent the percent of total cpm for each fraction remaining in the supernatant or filtrate; n = 3

Table 2

Ascorbic acid accumulation in chromaffin cells

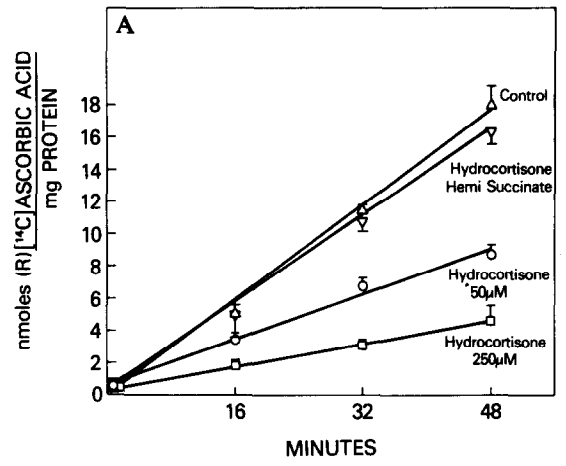
	Cells incubated with no ascorbic (N = 4)	Cells incubated with 200 μM ascorbic acid for 3 h (N = 6)
μg/10^6 cells	0.12 ± 0.02	0.97 ± 0.11
mM	0.22 ± 0.04	1.80 ± 0.20

Chromaffin cells were incubated with (R)-ascorbic acid as described and washed 3 times. Cells (1 vol.) were then extracted in chloroform: methanol 2:1, v/v) and the aqueous phase acidified to pH 3.5 prior to HPLC analysis. The amount of ascorbic acid accumulated by cells was identical whether ascorbic acid was radiolabelled or unlabelled (not shown). Cell volume was calculated using both a cell diameter of 18 μM and a spherical model, and thus ascorbate concentrations reflect minimum values

3.4. *Influence of corticosteroids on ascorbic acid transport*

Because of the sequential nature of ascorbic acid and corticosteroid secretion in the adrenal gland [17,18], we investigated the ability of corticosteroids to regulate ascorbic acid uptake. Hydrocortisone, the bovine physiological glucocorticoid, was found to inhibit ascorbic acid transport into chromaffin cells in a dose-dependent manner (fig. 2A). By contrast, hydrocortisone-21-sodium hemisuccinate, a water-soluble derivative which must be hydrolyzed to be active, had no activity over 10–500 μM. A detailed analysis of the hydrocortisone effect (fig. 2B) indicated that the steroid exhibited over 90% of its inhibitory potency over 2 log doses. In different experiments the ID_{50} ranged from 50–62 μM, which is in the physiological range for corticosteroids in the adrenal portal system under conditions of stress [17–19].

We further reasoned that if hydrocortisone inhibition of ascorbic acid uptake were of physiological significance, then the inhibition ought to be reversible. Indeed, when chromaffin cells were exposed to hydrocortisone for 1 h and washed twice in steroid-free medium, full ascorbic acid transport activity was restored. In addition, re-introduction of hydrocortisone to the washed cells resulted in prompt and complete inhibition of transport again (not shown). The ready reversibility of the process as well as the effective concentration range of the steroid indicated to us that we could be analyzing a physiologically relevant process.



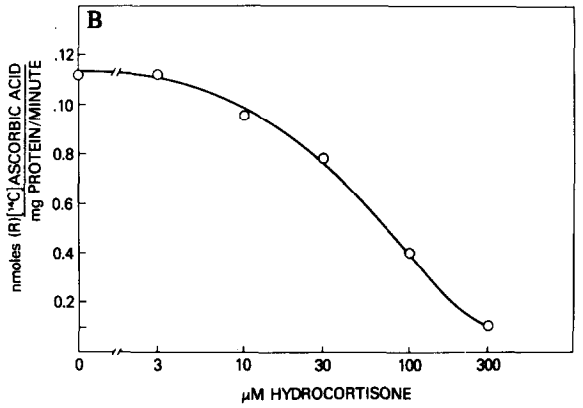


Fig. 2A. Corticosteroid inhibition of (*R*)-[^{14}C]ascorbic acid uptake in chromaffin cells: (○) hydrocortisone 50 μM; (□) hydrocortisone 250 μM; (▽) hydrocortisone 21-sodium hemi-succinate at 10 μM, 50 μM and 200 μM also show no inhibition of ascorbic acid uptake (not shown).

Fig. 2B. Hydrocortisone inhibition of (*R*)-[^{14}C]ascorbic acid uptake in chromaffin cells as a function of hydrocortisone concentration. Velocity data points, each representing 8 values, were determined by measuring (*R*)-[^{14}C]ascorbic acid uptake over 64 min at 16 min intervals, in the presence of the appropriate hydrocortisone concentration; ascorbate was 132 μM.

4. DISCUSSION

These data demonstrate that ascorbic acid is accumulated and concentrated in cultured bovine chromaffin cells by a saturable mechanism that is reversibly sensitive to physiological levels of hydrocortisone. It is therefore possible that this process is part of the adrenal stress response. However, the exact role of ascorbic acid in the adrenal stress response has remained an unsolved puzzle. Historically, it has been appreciated that the mammalian adrenal cortex and medulla contain the highest levels of ascorbic acid per mg protein of any tissue [6,20]. It has also been appreciated that when ACTH stimulates the cortex during stress, 50% of cortical ascorbic acid is released peripherally [1,2,17,18]. This means, of course, that the chromaffin cells are the first cells the newly released ascorbic acid reaches as it travels through the adrenal portal system. Thus, the chromaffin cells might initially accumulate ascorbate by the mechanism we have described. Subsequently, newly synthesized hydrocortisone secreted from the adrenal cortex would reach local concentrations in the medulla [17–19] which could then regulate ascorbic acid uptake. The ascorbic acid initially available for transport into chromaffin cells of the medulla would therefore become biochemically shunted to the general circulation.

The reasons underlying the proposed initial uptake and then redirection of ascorbic acid from chromaffin tissue to the periphery remain to be elucidated. It is possible, though, that such processes may occur if our results on cultured chromaffin cells reflect the in vivo condition. We anticipate that the system we have described will have general application for eventually understanding the exact role of ascorbic acid in adrenal physiology.

REFERENCES

[1] Sayers, G., Sayers, M.A., Liang, T.S. and Long, C. (1946) Endocrinology 38, 1.
[2] Sayers, M.A., Sayers, G. and Woodbury, L. (1948) Endocrinology 42, 349.
[3] Handbook of Physiology (1975) Section 7, Endocrinology, vol. 7, ch. 20.
[4] Bennett, H.S. and Lawrence, K. (1940) Anat. Rec. 77, 447.
[5] Ingebretsen, O.C., Terland, O. and Flatmark, T. (1980) Biochim. Biophys. Acta 628, 182.
[6] Glick, D. and Gerson, G.R. (1935) J. Biochem. 110, 1.
[7] Levine, M.A. and Pollard, H.B. (1982) Endocrinology, 110, suppl. 80 abstr.
[8] Daniels, A.J., Dean, G., Viveros, O.H. and Diliberto, E.J. jr (1982) Science 216, 737–739.
[9] Greenberg, A. and Zinder, O. (1983) Cell Tissue Res. in press.
[10] Pollard, H.B., Pazoles, C.J., Creutz, C.E., Scott, J. and Zinder, O. (1983) J. Biol. Chem. in press.
[11] Sharma, S.K., Johnstone, R.M. and Quastel, J.H. (1964) Biochem. J. 92, 564–573.
[12] Spector, R. and Greene, L.A. (1977) Brain Res. 136, 131–140.
[13] Pollard, H.B., Menard, R., Brandt, H.A., Pazoles, C.J., Creutz, C.E. and Ramu, A. (1978) Analyt. Biochem. 86, 761–763.
[14] Anton, A.H. and Sayre, D.F. (1962) J. Pharmacol. Expt. Ther. 138, 360–375.
[15] Terland, O. and Flatmark, T. (1975) FEBS Lett. 59, 52–55.
[16] Finn, F.M. and Johns, P.A. (1980) Endocrinology 106, 811–817.
[17] Slusher, M.A. and Roberts, S. (1951) Endocrinology 61, 98–105.
[18] Lipscomb, H.S. and Nelson, D.H. (1961) Endocrinology 66, 144–146.
[19] Wurtman, R.J., Pohorecky, L.A. and Baliga, B.S. (1972) Pharm. Rev. 411, 426.
[20] Hornig, D. (1975) Ann. NY Acad. Sci. 258, 103–118.