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SUBCELLULAR LOCALIZATION OF ETHYLENEDIAMINETETRAACETATE IN THE PROXIMAL TUBULAR CELL OF THE RAT KIDNEY*

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Abstract—Ca-EDTA-^{14}C was administered to rats at three different dose levels, only one of which was capable of inducing vacuolization in proximal convoluted tubular cells within 24 hr. Differential centrifugation indicated that the percentage uptake with all three doses was similar over a period of 30 min to 24 hr. The behaviour of the particles relative to time as well as on sucrose:water density gradients suggested that the EDTA-^{14}C eventually shares particle space with lysosomal enzymes.

THE NEPHROTOXICITY ascribed to EDTA and calcium EDTA (Ca-EDTA) is primarily based on a number of observations that these agents will induce vacuolization of the renal tubular cells in rats[1–3] and humans[1, 4–8] when administered in large doses. The nature and course of EDTA- and Ca-EDTA-induced vacuolization is unknown. A previous study[2] has indicated that doses of Ca-EDTA capable of producing changes in the cells of the proximal convoluted tubules (PCT) were also associated with secondary changes in the subcellular distribution of the lysosomal enzymes, acid phosphatase, aryl sulfatase, and acid ribonuclease. In order to define the relationship of the vacuoles to lysosomes in greater detail, information regarding the properties of the vacuoles is required. It is especially important to know if the vacuoles at any time contain the chelate. The present investigation was carried out to learn if the Ca-EDTA enters the PCT cells and, if so, the subcellular localization of the chelate.

METHODS

Male, albino, Sprague–Dawley rats, 210–260 g, were used. Solutions of disodium Ca-EDTA-^{14}C, labeled in the carboxyl positions, were prepared so that for all dose levels each animal received 5·0 μc of activity and a total injected volume of 1·0 ml/200 g body wt. Doses of 10 and 100 mg/kg of the chelate were administered as a single injection via the jugular vein. Doses of 1000 mg/kg were administered in four divided doses over 30 min via a polyethylene cannula in the femoral vein. This was done to avoid death of the animal from the acute toxic effects of the chelate. Kidneys were removed at 30 min, 1, 2, 3, 6, and 24 hr after injection. When the rats were kept for more than 3 hr, they were allowed food (standard laboratory chow) and water *ad libitum*.

* From the Bureau of Medicine and Surgery, Navy Department, Research Task No. MR005.20-0031. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large. The experiments reported herein were conducted according to the principles enunciated in *Guide for Laboratory Animal Facilities and Care*, NAS-NRC.

At the designated time after drug administration, the rats were anesthetized with ether, the kidneys perfused with 0·25 M sucrose solution, and the cortical homogenate prepared in 0·9 M sucrose solution (1:10 wet w/v) by using previously described procedures.[2]

Red cells, whole cells, connective tissue, and other debris were removed by centrifugation at 100 *g* for 2 min. Differential centrifugation was carried out on the supernatant and successive supernatants as follows: 650 *g* for 20 min, 5000 *g* for 20 min, 15,000 *g* for 20 min, and 150,000 *g* for 60 min. For purposes of calculating distribution of activity in the subfractions, the 100 *g* supernatant was considered the whole homogenate. Total cortical activity is based on the activity of the whole homogenate plus that of the 100 *g* sediment. To correct for surface adsorption and the trapping of soluble activity, aliquots of Ca-EDTA-^{14}C were added to cold homogenates which were centrifuged and sampled concurrently with homogenates from treated animals. All values presented are corrected for the per cent of activity adsorbed and trapped. The per cent of activity trapped (±S.E.M.) as observed in 11 control runs is as follows: 650-*g*, 4·96 ± 0·30; 5000-*g*, 3·98 ± 0·24; 15,000-*g*, 3·17 ± 0·20; 150,000-*g*, 2·32 ± 0·18; total cortical sediment, 14·41 ± 0·71.

The medullary tissues from both kidneys were also combined and homogenized in distilled water (1:10 wet w/v).

Aliquots of the whole cortical homogenate, sediment, 150,000 *g* supernatant, and medullary homogenate were digested in Hyamine 10-X* (1 M methanolic solution) and counted in a liquid scintillation system modified after Bray.[9] The values presented are the means of 2–3 experiments performed for each dose at each time interval.

The distribution of the more dense cortical particles containing the bulk of radioactivity and lysosomal enzymes was measured across a sucrose gradient. These studies were carried out for all three dose levels at 1, 3, and 24 hr after injection. In this case, homogenization of the cortex was carried out in 0·7 M sucrose adjusted to pH 7. Centrifugation was performed at 100 *g* for 1 min and the resulting supernatant spun at 15,000 *g* for 10 min. The 15,000 *g* sediment contained a dark bottom layer and a loosely packed top layer of lighter colored material. The top layer was easily removed and the bottom layer was resuspended in 0·7 M sucrose solution, pH 7, containing 2% dextran (average mol. wt. 73,000). An aliquot of this suspension was layered above a continuous gradient of sucrose, 0·70 to 2·0 M (d = 1·09 to 1·25) containing 2% dextran. This was centrifuged at 144,000 *g* for 6 hr in a Beckman model L-2 ultracentrifuge with an SW 39 rotor. Sixteen fractions of 18 drops each were collected from the bottom of the tube. Radioactivity, acid phosphatase activity, and cytochrome oxidase activity were determined in each fraction.

Radioactivity was measured by the liquid scintillation methods described above. Acid phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.2), aryl sulfatase (aryl-sulfate sulfohydrolase; EC 3.1.6.1), and acid ribonuclease (polyribonucleotide 2-oligotransferase, cyclyzing; EC 2.7.3.16) were assayed according to previously published procedures.[2] Cytochrome oxidase (cytochrome *C*:O_2 oxidoreductase, EC 1.9.3.1) activity was assayed according to the method of Smith and Stotz.[10] Protein was determined by the method of Lowry *et al.*[11] with bovine plasma albumin as the reference standard.

* Registered trade mark of Rohm and Haas for *p*-(diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium hydroxide.

RESULTS

Differential centrifugation experiments

The data in Table 1 show that some activity from Ca-EDTA-^{14}C remained in the renal cortex between 30 min and 24 hr after dosing. This amount gradually decreased

TABLE 1. RADIOACTIVITY APPEARING IN THE KIDNEYS OF RATS TREATED WITH Ca-EDTA-^{14}C

	Percent of injected dose per tissue fraction (both kidneys) at designated time (hr) after administration of the chelate					
	0·5	1	2	3	6	24
Dose: 10 mg/kg						
Total cortex	0·340	0·230	0·180	0·220	0·180	0·110
Cortical sediment*	0·065	0·062	0·066	0·087	0·074	0·034
Cortical supernatant†	0·275	0·168	0·114	0·133	0·106	0·076
Total medulla	0·103	0·134	0·043	0·040	0·046	0·018
Dose: 100 mg/kg						
Total cortex	0·500	0·190	0·180	0·200	0·130	0·150
Cortical sediment*	0·087	0·058	0·066	0·083	0·055	0·059
Cortical supernatant†	0·413	0·132	0·114	0·117	0·070	0·091
Total medulla	0·230	0·120	0·070	0·026	0·029	0·061
Dose: 1000 mg/kg						
Total cortex	0·260	0·240	0.210	0·100	0·120	0·100
Cortical sediment*	0·049	0·057	0·069	0·040	0·041	0·027
Cortical supernatant†	0·211	0·183	0·136	0·060	0·079	0·073
Total medulla	0·290	0·120	0·070	0·030	0·017	0·018

* 650 *g* through 150,000 *g* fractions combined.

† 150,000 *g* supernatant.

with time. The per cent of the injected dose occurring in the cortical sediment (650 *g* through 150,000 *g* combined) was small but persistent. The peak concentration appeared within 30 min with all three doses. It will be recalled that the 1000 mg/kg dose was administered over a 30-min period. Considering the rapid elimination of Ca-EDTA,[12] the 30-min level of activity in the cortex for that dose is probably less than it would have been had the dose been given all at once.

The temporal behavior of the activity occurring in the cortical sediment and that occurring in the cortical supernatant were considerably different. In the supernatant, there was a sharp decrease in activity over the first 2–3 hr, whereas the sedimentable activity showed little, if any, tendency for a decrease until after 6 hr.

Table 2 expands the cortical activity data. Of the total injected activity that *appeared in the cortex*, the fraction that appears in the sediment increased over the first 3- to 6-hr period for the 3 dose levels tested. By 24 hr, a decrease from the peak levels in the per cent cortical activity in the sediment occurred. A similar pattern was seen with the 650 *g* fraction which comprised the major activity component of the sediment.

Table 1 shows that the per cent of injected activity in the medulla decreased rapidly during the first 3 hr after administration. This decrease is somewhat similar to that seen for the cortical supernatant.

Isopycnic density gradient experiments

When a lysosome-rich fraction from an untreated animal was allowed to equilibrate across the sucrose gradient, a distribution pattern as displayed in Fig. 1 was observed. The method of presentation of the density equilibrium studies follows that described by Beaufay *et al.*[13] The relative concentration (ordinate) is the ratio of the observed

TABLE 2. RADIOACTIVITY APPEARING IN RENAL CORTICAL SUBFRACTIONS OF RATS TREATED WITH Ca-EDTA-^{14}C

	Per cent total cortical activity per subfraction at designated time (hr) after administration of the chelate					
Fraction (*g*)	0·5	1	2	3	6	24
Dose: 10 mg/kg						
650	9·17	16·45	21·24	25·07	28·59	27·53
5000	6·15	6·77	10·42	10·97	10·59	4·78
15,000	2·25	1·26	0·97	1·29	1·92	0·37
150,000	2·21	3·26	0·29	1·23	1·00	2·07
Total sediment*	19·78	27·74	32·92	38·56	42·10	34·75
Supernatant	80·22	72·26	67·08	61·44	57·90	65·25
Dose: 100 mg/kg						
650	8·86	16·83	29·12	27·96	34·53	25·60
5000	4·74	5·50	6·46	8·23	7·56	9·41
15,000	2·08	0·84	1·00	1·27	1·48	3·20
150,000	1·85	2·61	1·21	0·36	0·66	0·92
Total sediment*	17·53	25·78	37·79	37·82	44·23	39·13
Supernatant	82·47	74·22	62·21	62·18	55·77	60·87
Dose: 1000 mg/kg						
650	7·56	12·16	25·76	31·62	27·01	20·40
5000	5·73	5·72	6·69	6·56	4·90	4·80
15,000	2·69	1·10	0·49	2·04	0·70	0·88
150,000	2·65	1·62	0·76	0·18	1·30	0·67
Total sediment*	18·63	20·60	33·70	40·40	33·91	26·75
Supernatant	81·37	79·40	66·30	59·60	66·09	73·25

* Sum of values for 650 *g* through 150,000 *g* fractions.

activity (C) to that which would have been found if the activity had been homogenously distributed throughout the whole gradient (Ci). With fractions from normal animals, the peaks for the three lysosomal enzymes studied—acid phosphatase, aryl sulfatase, and acid ribonuclease—were all in a position indicating a somewhat greater density than that of mitochondria, indicated by the location of the cytochrome oxidase peak. Acid phosphatase was the only lysosomal enzyme determined when the gradients were run on fractions from Ca-EDTA-^{14}C-treated animals. Fig. 2 shows the results obtained 1 hr after a dose of 1000 mg/kg of the labeled chelate. The peak ^{14}C activity was located in the same density zone (d = 1·22) as the peak acid phosphtase activity, while the cytochrome oxidase equilibrated in a zone of a somewhat lesser density. The major difference between the carbon-14 activity and the acid phosphatase activity is that the latter is more generally distributed than the former. The same results were obtained at 3 and 24 hr after the 1000 mg/kg dose and 1, 3 and 24 hr after the 10 mg/kg and 100 mg/kg doses. Protein distributed with a single major peak corresponding to the cytochrome oxidase activity. When cold homogenates

with added activity were treated in a similar manner, no radioactivity was observed on the gradient. Though the control data from saline-injected animals are not presented here, Fig. 1 shows the typical acid phosphatase pattern obtained with the controls. It was evident that no change in density in acid phosphatase particles occurred between control and treated animals.

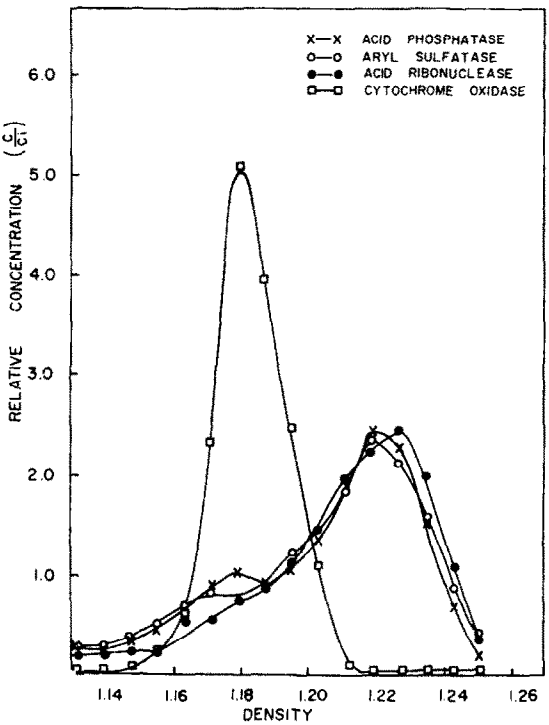

FIG. 1. Distribution of mitochondrial and lysosomal enzymes across a sucrose:water density gradient. Renal cortex from a normal rat.

DISCUSSION

Over a dose range of 10–1000 mg/kg of disodium Ca-EDTA, the amount of chelate appearing in the renal cortex at any one time is small. It is probable that the activity observed in the cortex is a reflection of unchanged EDTA. Foreman *et al.*[12] administered Ca-EDTA-C^{14}, labeled on the α-carbons, and noted that less than 0·15 per cent of the activity appeared as $C^{14}O_2$. The urinary and serum radioactivity represented unchanged EDTA, as indicated by radiochromatography. That urinary activity represents unchanged EDTA has been confirmed in this laboratory by radiochromatography. Considering that only one labeled compound, EDTA has been detected in serum and urine, any oxidation that occurs must occur on all four acetate groups. Since the compound used here was labeled on the carboxyl carbons, any oxidation occurring would most certainly deprive the compound of its label. It is probable, therefore, that the activity observed in the cortex is a reflection of unchanged EDTA. It is not certain, however, whether the EDTA enters the cell with or without the metal ion attached.

The mechanism by which the chelate enters the cell is unclear. Contrary to previous reports,[12, 14] it is now probable that the chelated EDTA is cleared in the kidney by glomerular filtration with no accompanying contribution by tubular secretion.[15, 16] Thus, the chelate is most likely presented to the tubular cells on the luminal side. The fact that a fairly constant fraction of the dose enters the cell irrespective of a two-log

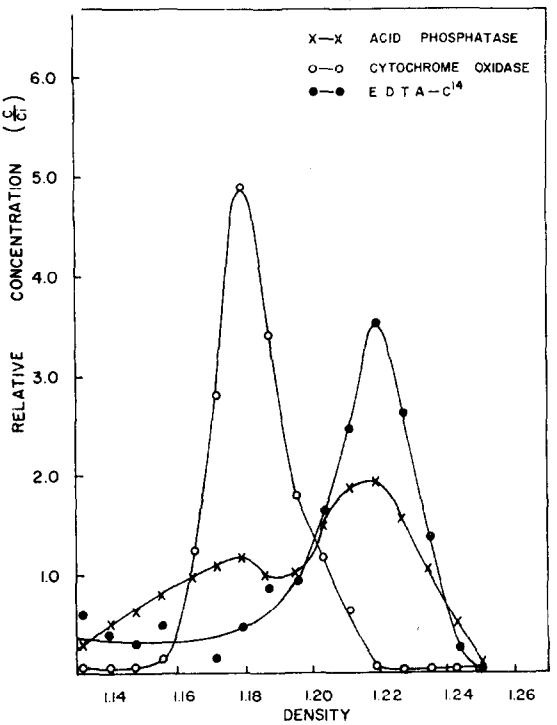

FIG. 2. Distribution of cytochrome oxidase, acid phosphatase, and EDTA-^{14}C across a sucrose:water density gradient. Renal cortex from a rat 1 hr after the administration of 1000 mg/kg of disodium Ca-EDTA-^{14}C.

unit dose range may suggest a passive movement of the compound from the glomerular filtrate into the cytoplasm. It is likely that a part of the supernatant activity observed is a result of urinary contamination. The early rapid decrease in supernatant activity is similar to that seen in the urine after the administration of Ca-EDTA.[12] Considering the constant residual activity that remains in the supernatant after the initial drop, it is also likely that not all of the activity in that fraction is of urinary origin.

How the activity becomes sedimentable after entering the cell is a matter of speculation. Some of the chelate may enter already segregated, possibly by a process akin to pinocytosis. Over the first 3 hr, the 650 *g* fraction reflects an increasing fraction of the total cortical activity. It is this fraction which has been previously shown to contain the bulk of the sedimentable lysosomal enzymes, acid phosphatase, aryl sulfatase and acid ribonuclease.[2] (The 650 *g* fraction described here is a combination of the 300 *g* and 650 *g* fraction described in the previous report.[2]) Further studies utilizing density gradients indicate that the chelate and acid phosphatase share the

same density zone and perhaps the same particle space. Thus, the coupled pinocytosis–lysosome system or "vacuolar apparatus"[17] may be involved.

The appearance of EDTA in a sedimentable fraction of the proximal tubular cell may be just coincident to the vacuolization process. It should be mentioned that, as seen by light microscopy, obvious vacuolization is induced with the 1000 mg/kg dose, whereas at the 10 and 100 mg/kg levels it has not been possible to say whether the number of vacuoles seen is significantly different from that observed in the kidneys of untreated animals.

The possibility has been considered that Ca-EDTA manifests its vacuologenic activity via the binding of metals in the cortex. Studies along this line have revealed that the chelate does not alter the renal cortical content of zinc, iron, manganese, copper, magnesium, cobalt, or calcium by 24 hr after the administration of vacuologenic doses.[3] Thus, any binding of metals by the intracellular EDTA would most likely have to occur in the absence of a subsequent removal of the metals.

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