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Study of the Mechanism of Renal Vacuologenesis Induced in the Rat by Ethylenediaminetetraacetate

Comparison of the Cellular Activities of Calcium and Chromium Chelates

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SUMMARY

Ca-EDTA-^{14}C, ^{45}Ca-EDTA, Cr-EDTA-^{14}C, and ^{51}Cr-EDTA were administered individually to rats. Radioactivity was found in the renal cortical cells in all cases, and was partly sedimentable. The activities derived from the two chromium forms were equal in the compartments studied, whereas radioactivity from the two calcium forms followed separate courses. The chromium remained attached to the EDTA, whereas the calcium probably was removed or exchanged for endogenous metal ions. Both chromium chelates were distributed in the same manner as the radioactivity from Ca-EDTA-^{14}C in renal cortical cells, 1 and 24 hr after administration, except for the supernatant fraction at 24 hr, which contained higher levels derived from Ca-EDTA-^{14}C. On sucrose-water density gradients, radioactivity from administered Ca-EDTA-^{14}C and the two chromium forms formed peaks in the same density zone as lysosomal acid phosphatase, while the peak for radioactivity from administered ^{45}Ca-EDTA coincided with that for mitochondrial cytochrome oxidase. These findings, in conjunction with histological observations, suggest that EDTA-induced vacuologenesis is a reflection of the induction of pinocytosis by the chelate.

INTRODUCTION

We have found that a relationship exists between the vacuoles induced by ethylenediaminetetraacetate in renal proximal tubular cells and the lysosomal organelles of the kidney, and have suggested that the induction of vacuoles by EDTA involves the pinocytosis-lysosome system (1, 2). Evidence for this relationship was obtained by differential centrifugation of homogenates of renal cortical tissue from rats which had received Ca-EDTA-^{14}C (2). By this means, we found that the sedimentable radioactivity accumulated in fractions which had previously (1) been shown to contain the largest concentrations of the lysosomal enzymes: acid phosphatase, arylsulfatase, and acid ribonuclease. Isopycnic-density gradient studies also showed the radioactivity to be localized in the same density zone as the lysosomal enzymes (2).

Others have suggested that vacuolization may be initiated by chelation of an

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essential metal component of a metalloenzyme (3, 4). This postulate assumes that an administered metal-EDTA complex dissociates and that the chelating characteristics of the EDTA are essential to vacuologenesis. These assumptions and the further evaluation of the participation of pinocytosis in EDTA-induced vacuologenesis are the subjects of the present study. For this purpose the subcellular distribution of Ca-EDTA, in which the cation is easily displaced by other metal ions, was compared with the localization of Cr-EDTA, in which the cation is not readily displaced by other metals. To distinguish between distribution of the cations and the organic acids, the cellular radioactivity concentrations of ^{45}Ca-EDTA, Ca-EDTA-^{14}C, ^{51}Cr-EDTA, and Cr-EDTA-^{14}C were compared.

METHODS[3]

The materials used were Ca-EDTA-^{14}C, ^{45}Ca-EDTA, Cr-EDTA-^{14}C, and ^{51}Cr-EDTA. The ^{14}C-compounds were labeled in the carboxyl positions. The chromium chelate was prepared from EDTA by a modification of the method of Hamm (5), in which chromium(III) chloride was substituted for chromium(III) nitrate. All the isotopes were supplied by the New England Nuclear Corporation. Solutions of chelate were prepared to contain 0.53 mmole and 10 μCi of (Ca-EDTA-^{14}C and ^{45}Ca-EDTA), 3 μCi of Cr-EDTA-^{14}C, or 1 μCi of ^{51}Cr-EDTA per milliliter.

Male albino Sprague-Dawley rats weighing 200–265 g were used. Each animal received 2.67 mmoles/kg of the chelate (equivalent to 1000 mg/kg of disodium Ca-EDTA) by slow injection into the jugular vein. Either 1 or 24 hr after drug administration, the rats were anesthetized with ether and the kidneys were perfused *in situ* with cold 0.25 M sucrose solution, removed, and dissected. Cortical homogenates were prepared in 0.90 M sucrose solution (1 g, wet weight, per 10 ml) by procedures described previously (1).

[3] The experiments reported herein were conducted according to the principles enunciated in "Guide for Laboratory Animal Facilities and Care," issued by the National Research Council–National Academy of Sciences.

The homogenate was divided into two equal portions. An internal standard consisting of 10 μl of injection solution was added with complete mixing to one portion, and both portions were processed simultaneously through all subsequent steps. Red cells and other unbroken cells, connective tissue, and other debris were removed by centrifugation at 100 $\times$ g for 2 min. The supernatant fluid was then centrifuged at 150,000 $\times$ g for 60 min, and the resulting sediment and supernatant fraction were retained.

The fraction of *injected activity* appearing in the *total cortex* was calculated on the basis of the original homogenate before the whole cells were removed by centrifugation. To determine the fraction of cortical radioactivity in the sedimentable and soluble fractions, the 100 $\times$ g supernatant fraction, which contained no whole cells, was considered representative of the whole homogenate. These data were then combined to determine the fraction of injected activity occurring in the sedimentable and soluble fractions. The portion of the original homogenate containing the internal standard was used to correct for radioactivity adsorbed, trapped, and/or otherwise bound *in vitro* during centrifugation and other procedures.

When the mean correction factors (percentage of radioactivity adsorbed, trapped, etc.) were computed for each of the eight groups (i.e., four isotopic forms at two time intervals), six were not significantly different from one another. The grand mean correction factor ($\pm$standard error) for the six groups was 5.06 $\pm$ 0.26%. The exceptions were the two ^{45}Ca-EDTA groups, in which a much larger degree of sedimentability of the internal standard radioactivity was observed. This was found to be due to displacement of calcium from EDTA in homogenates *in vitro*, followed by uptake of these ions by mitochondria.

Previously described liquid scintillation methods were used for counting ^{14}C and ^{45}Ca (2). A well counter was utilized for counting ^{51}Cr activity.

For the density gradient experiments, a cortical homogenate prepared in 0.7 M sucrose, pH 7, was centrifuged at 100 $\times$ g for 1 min. The resulting supernatant fluid

was separated and centrifuged at 5000 × *g* for 10 min to produce a sediment containing a dark bottom layer and a lighter, loosely packed top layer. The latter was removed easily, and the bottom layer was resuspended in 0.7 M sucrose, pH 7, containing 2% dextran (average mol wt 73,000). An aliquot of this suspension was layered above a continuous gradient of sucrose and water, 0.70–2.0 M (density = 1.09–1.25), containing 2% dextran, and centrifuged at 144,000 × *g* for 6 hr in a Beckman model L2 ultracentrifuge with the 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 measured in each fraction.

Radioactivity was determined by the methods indicated above. Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) activity was assayed according to procedures described previously (1). Cytochrome oxidase (cytochrome c:O_2 oxidoreductase, EC 1.9.3.1) activity was assayed according to the method of Smith and Stotz (6).

In prior studies it had been assumed that the radioactivity observed in renal cortical tissue following the administration of Ca-EDTA-^{14}C represented the unchanged EDTA molecule (2). To test the validity of this assumption, a chemical assay procedure was developed to determine the quantity of EDTA in the tissue fractions. The assay is based on the quantitative binding of trivalent cobalt by the chelating agent and was performed as follows. Following the intravenous administration of Ca-EDTA-^{14}C, a renal cortical homogenate (1 g, wet weight, per 10 ml) was prepared in 0.7 M sucrose solution and centrifuged at 5000 × *g* for 10 min to yield a sediment composed of a light-colored upper layer and a dark bottom layer, as described above. The supernatant fluid and top layer of sediment were removed, and the bottom layer of sediment was set aside until a second yield was obtained by combining the top layer of sediment, the supernatant fractions, and the 100 × *g* sediment from the initial centrifugation and again centrifuging at 100 × *g* for 1 min and 5000 × *g* for 10 min. The two dark pellet fractions were combined and suspended in 0.5 ml of water to which were added 2 ml of acetone. After thorough mixing, the suspension was centrifuged and the acetone was removed by evaporation. To the remaining aqueous phase were added 0.5 ml of water, 1 drop of glacial acetic acid, 0.1 ml of 30% H_2O_2, and 0.025 ml of 1% $Co(OH)_3$. $Co(OH)_3$ was prepared prior to use by the reaction of $Co(NO_3)_2$ with NaOCl. The reaction mixture was applied to a 7 × 20 mm DEAE-cellulose column (Cellex D, Bio-Rad) moistened with 1% acetic acid.

The tube which had contained the reaction mixture was rinsed with 1.0 ml of 1% acetic acid, and the rinse was added to the column. The purple band, which contained the Co-EDTA, was eluted with 0.1 N sodium chloride solution. After the eluate was adjusted to a final volume of 1.0 ml with 0.1 N sodium chloride solution, its absorbance at 535 mμ was determined and the amount of EDTA was read from a standard curve. The standard used was prepared by the reaction of $CaNa_2EDTA$ solution with $Co(OH)_3$. An aliquot of the eluate was then used to determine radioactivity.

Histological studies. Single doses of Ca-EDTA or Cr-EDTA were administered to rats via the intraperitoneal route. The animals were exsanguinated 24 hr later. The kidneys were removed, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3- or 5-μ thickness, and stained with hematoxylin and eosin for microscopic examination. The extent of vacuolization in the tubular cells was graded on a 0–4 scale. An explanation of this grading has been presented in an earlier report (7).

Statistical analysis. The results are presented as means ± standard error. When the analysis was limited to two groups of measurements, Student's t-test was used, but when comparisons were made among more than two groups, an F value was computed. If the value of F indicated significant differences among the groups ($p < 0.05$), a multiple-range test utilizing the Newman-Keuls method (8) was done to determine which means were significantly different from the others.

TABLE 1

Occurrence of radioactivity in renal cortex of rats following administration of 2.67 mmoles/kg of chelate (n = 5 for each group)

Time after administration	Group	Chelate	Radioactivity found in		
			Cortex	Cortical sediment	Cortical supernatant
hr			*% injected dose ± SEM*		
1	1	Ca-EDTA-^{14}C	0.41 ± 0.020	0.062 ± 0.008	0.351 ± 0.020
	2	^{45}Ca-EDTA	0.12 ± 0.006[a]	0.041 ± 0.002[a]	0.081 ± 0.005[a]
	3	Cr-EDTA-^{14}C	0.43 ± 0.005	0.070 ± 0.003	0.356 ± 0.014
	4	^{51}Cr-EDTA	0.44 ± 0.015	0.080 ± 0.006	0.359 ± 0.011
24	5	Ca-EDTA-^{14}C	0.12 ± 0.002[b]	0.019 ± 0.001	0.103 ± 0.001[b]
	6	^{45}Ca-EDTA	0.0039 ± 0.0003[c]	0.0014 ± 0.0001[c]	0.0025 ± 0.0001[c]
	7	Cr-EDTA-^{14}C	0.062 ± 0.005	0.014 ± 0.0007	0.048 ± 0.006
	8	^{51}Cr-EDTA	0.060 ± 0.004	0.014 ± 0.002	0.046 ± 0.003

[a] Significantly different from groups 1, 3, and 4.
[b] Significantly different from groups 6–8.
[c] Significantly different from groups 5, 7, and 8.

RESULTS

Renal cortical radioactivity observed after the administration of the chelates is summarized in Table 1. The sedimentable radioactivity expressed as a fraction of the injected dose of Ca-EDTA-^{14}C was similar to that reported previously (2). The values for sedimentable radioactivity expressed as a fraction of the injected doses of Ca-EDTA-^{14}C, Cr-EDTA-^{14}C, and ^{51}Cr-EDTA were not statistically different either 1 or 24 hr following administration. The sedimentable radioactivity derived from ^{45}Ca-EDTA was significantly lower than these values at both time intervals.

In the supernatant fractions, the values for radioactivity derived from Ca-EDTA-^{14}C and the two chromium chelates were not different 1 hr after administration; by 24 hr, however, the level of radioactivity derived from Ca-EDTA-^{14}C was about twice that of the chromium forms. For Ca-EDTA-^{14}C the ratios of supernatant radioactivity to sedimentable radioactivity were 5.96 ± 0.76 and 5.63 ± 0.28 at 1 and 24 hr, respectively—not a significant difference. For Cr-EDTA-^{14}C the two ratios were 5.08 ± 0.29 and 3.56 ± 0.57, and for ^{51}Cr-EDTA they were 4.62 ± 0.31 and 3.33 ± 0.30, at 1 and 24 hr, respectively, after administration of the chelates. In both cases the reduction in ratio at 24 hr was significant. The activity in the supernatant fraction following the administration of ^{45}Ca-EDTA was many times lower than that of the other isotopic forms.

Figure 1 shows the results of density gradient experiments on tissues obtained 1 hr after the administration of the various isotopes. In addition to radioactivity, concurrent determinations of the distribution of cytochrome oxidase and acid phosphatase were made for each isotope studied. The curves shown for the two enzymes were obtained by combining the data from all isotope studies. The radioactivity derived from the administration of Ca-EDTA-^{14}C, Cr-EDTA-^{14}C, and ^{51}Cr-EDTA was distributed similarly to the acid phosphatase activity, i.e., in the zone of lysosomal enzymes (2). Radioactivity resulting from the administration of ^{45}Ca-EDTA was distributed similarly to cytochrome oxidase activity, i.e., along with mitochondria. The results of density gradient studies made 24 hr after the administration of the isotopes were essentially the same as those shown in Fig. 1.

In separate studies, the isotopes were added *in vitro* to nonradioactive cortical homogenates and subjected to density gradient centrifugation. The radioactivity from ^{45}Ca-EDTA appeared in the mito-

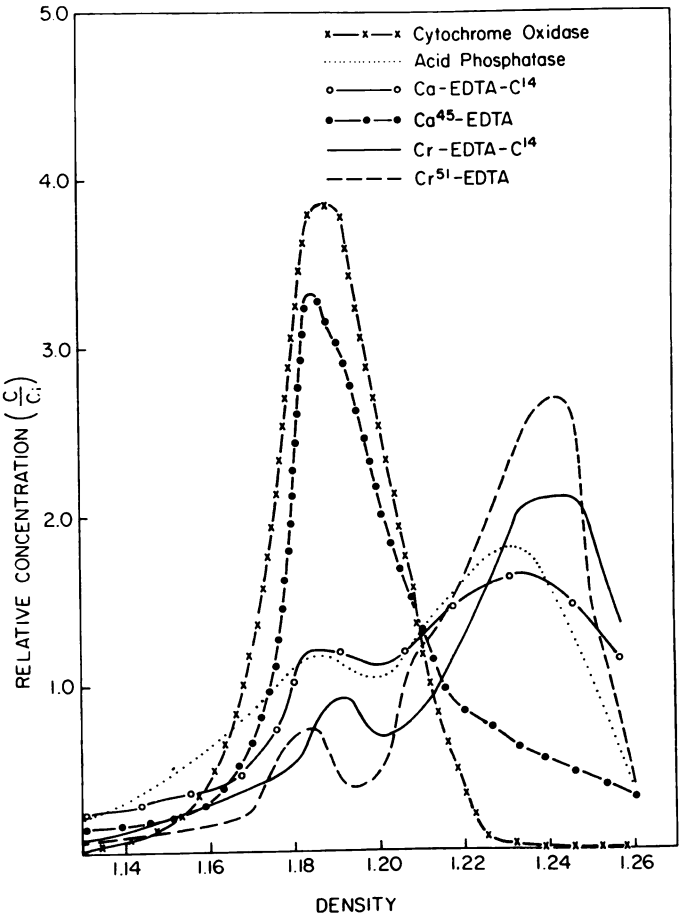

FIG. 1. *Distribution of cytochrome oxidase, acid phosphatase, and radioactivity in renal cortex of rats 1 hr after administration of 2.67 mmoles/kg of Ca-EDTA-^{14}C, ^{45}Ca-EDTA, Cr-EDTA-^{14}C, and ^{51}Cr-EDTA*

chondrial fraction, whereas none of the other three forms was distributed over the gradient.

To verify that the material measured as ^{14}C radioactivity was unmetabolized EDTA, we compared the radioassay results with those obtained by the photometric analysis for EDTA. Ca-EDTA-^{14}C was administered to three rats from which tissue samples were taken at 1 hr, and to three rats from which samples were taken at 24 hr. The EDTA content of the cortices was determined isotopically and photometrically, using the method described above. The resulting data were expressed as a ratio of EDTA content determined photometrically ($EDTA_p$) to the EDTA content determined isotopically ($EDTA_i$). One hour after the administration of Ca-EDTA-^{14}C to three rats, the $EDTA_p$:$EDTA_i$ ratios were 0.98, 1.07, and 1.07 (1.04 $\pm$ 0.03). After 24 hr the ratios were 1.11, 1.15, and 1.03 (1.06 $\pm$ 0.02). In neither group were the ratios significantly different from the value of 1.0 that would be expected if the isotopic material were chemically the same as EDTA determined photometrically.

Over 200 animals were examined in the comparison of Ca-EDTA- and Cr-EDTA-induced vacuologenesis. Both compounds were vacuologenic to the cells of the proximal tubule. However, the calcium chelate was more active in this respect than the chromium chelate. For example, the administration of 3.34 mmoles of Ca-EDTA per kilogram to five animals caused vacuolar changes

graded as 2 in each case, but similar changes were not seen with Cr-EDTA until after the administration of 9.35 mmoles/kg. These and other results of our comparative studies suggest that the calcium chelate was about 2.5 to 3 times more vacuologenic than the chromium chelate.

DISCUSSION

The results obtained in this and the previous study (2) suggest that EDTA-induced vacuoles are pinocytic. We noted previously that when various doses of Ca-EDTA-^{14}C are administered to rats, the amount of EDTA that enters cortical cells is concentration-dependent and that the kinetics of the entry process is compatible with both pinocytosis and passive diffusion (2). In the same study we showed that radioactivity derived from administered Ca-EDTA-^{14}C accumulates in the subcellular fractions of rat renal tubules, which are richest in lysosomal enzymes (2). We also noted that the prevailing concept of pinocytosis predicts the combination of pinosomes with lysosomal organelles (9), and that our data suggested that EDTA entered the cells by pinocytosis (2). These conclusions are confirmed and extended by the present data, which show that Ca-EDTA-^{14}C, Cr-EDTA-^{14}C, and ^{51}Cr-EDTA are localized in the same fractions as lysosomal enzymes.

Our observation (Table 1) that the radioactivity levels from ^{51}Cr-EDTA and Cr-EDTA-^{14}C were the same in both the sediment and supernatant fraction indicates that this complex does not dissociate *in vivo*. Consequently, the vacuologenic activity of Cr-EDTA could not be due to a chelation phenomenon, as has been suggested as a mechanism for the vacuologenic activity of EDTA chelates in general (3, 4). The difference between the vacuologenic activities of Ca-EDTA and Cr-EDTA may be based on the greater binding of the former to the cell membrane. Bennett (10) has proposed that pinocytosis begins with the attachment of molecules to the plasma membrane and is followed by membrane vesiculation and the pinching off of vesicles. However, little is known about the nature of the attachment of molecules to the plasma membrane or about the mechanism that "triggers" membrane vesiculation and pinosome interiorization. Cohn and Parks (11), in their studies of pinocytosis in mouse mononuclear phagocytes, concluded that negatively charged ions are the best inducers of pinocytosis in these cells. Among the materials we studied, it seems probable that, because of the hexadentate binding of calcium and chromium by EDTA, the less stable chelate has the greater tendency toward negativity, and that less Ca-EDTA than Cr-EDTA would be required for activation of the plasma membrane. On the other hand, since Cr-EDTA is less vacuologenic than Ca-EDTA although the two are equally sedimentable, our data suggest that there may be more Cr-EDTA per vacuole.

The source of the radioactivity in the supernatant fraction remains in question. In the animals held for 24 hr, urine samples were collected hourly for the first 6 hr. An exponential decrease in urinary concentration of radioactivity was observed. The half-lives for urine concentration were: Ca-EDTA-^{14}C, 42.6 min; Cr-EDTA-^{14}C, 42.8 min; ^{51}Cr-EDTA, 42.7 min. On this basis, the supernatant activity could not have been due entirely to contamination with tubular urine; otherwise the radioactivity in the 24-hr fraction would have been infinitesimal. The supernatant activity could also have resulted from rupture of the particles during sedimentation. No doubt this was involved to some extent, but it does not explain the differences between the activities contained in the supernatant fraction observed for Ca-EDTA-^{14}C and Cr-EDTA at 24 hr. As mentioned above, the kinetics observed previously (2) for the uptake of chelate by renal tubular cells is also characteristic of passive diffusion, and some chelate may enter the cell by such a route. If so, back-diffusion is not complete, since after 24 hr the concentration in urine decreased essentially 100% whereas the concentration in supernatant fluid decreased by only about 71% for Ca-EDTA-^{14}C and about 87% for the chromium chelate. If, therefore, chelate does exist in the soluble portion of the cell, a portion of it is bound in some manner, possibly as a ternary complex, in which the Cr-EDTA is less capable of participating.

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