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STUDIES OF THE NEPHROTOXICITY OF ETHYLENEDIAMINETETRAACETIC ACID*

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Abstract—The renal cortices of rats were examined for changes in lysosomal enzymes 2 and 24 hr after the administration of 1·0 and 2·5 g of calcium disodium ethylenediaminetetraacetic acid per kg. The enzymes measured were aryl sulfatase, acid phosphatase, and acid ribonuclease. Though vacuolization occurred at both time intervals with both doses, enzymatic changes were observed only at the 24-hr interval. The changes were manifested as a decrease in the concentration of the three enzymes in the nuclear-weight fractions. It is suggested that part of the chelate enters the cell via pinocytosis, resulting in the formation of a vacuole analogous to the phagosome described by previous investigators. A subsequent combination of the vacuoles with lysosomes is proposed.

ONE of the first reports associating a nephrotoxic hazard with the use of ethylenediaminetetraacetic acid (EDTA) was made by Holland *et al.*[1] Autopsy findings from two patients who received large doses of disodium EDTA (175 mg/kg and 235 mg/kg), in the course of treatment of hypercalcemia associated with multiple myeloma and malignant melanoma, revealed vacuolization of renal tubular cells similar to that found in sucrose nephrosis. Other reports indicating similar changes following large doses of EDTA have since been made.[2–6] One such report[5] dealt with a male, suffering from subacute chronic lead poisoning, who was erroneously treated with about 600 mg calcium disodium EDTA ($CaNa_2$ EDTA)/kg/day for five days, at which time death occurred. Foreman *et al.*,[4] employing rats, carried out a series of experiments on the dose–effect relationship of EDTA-induced lesions in the kidney. They found that varying dosages (125–3000 mg/kg daily) over varying periods of time (2–16 days) caused pathologic changes ranging from fine droplet degeneration of the proximal convoluted tubules (PCT) to the formation of huge vacuoles in the PCT, severe hydropic degeneration of the PCT, and lesions of the distal convoluted tubules (DCT). Seven[7] was able to review the slides of Dudley *et al.*[2] and of Vogt and Cottier[5] and to compare them with autopsy material from the studies done in rats by Foreman *et al.*[4] He found "striking similarities" between the rat and human lesions.

The similarities between sucrose and EDTA vacuolization in the kidney and the

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implication that sucrose vacuoles are lysosomal in nature[8, 9] suggested that a relationship between EDTA-induced vacuolization and alteration in renal lysosomes might exist. To study this relationship, $CaNa_2$ EDTA was administered to rats in high doses; the concentration and distribution of lysosomal enzymes in renal cortical tissue were then examined at two time intervals after its administration.

METHODS

Male albino Sprague–Dawley rats, 175–225 g, were used. Both treated and control tissues were obtained, homogenized, centrifuged, and assayed at the same time. The data presented for each dose group were obtained from five separate experiments unless otherwise indicated. Calcium disodium ethylenediaminetetraacetic acid, as a commercially available solution (200 mg/ml), was administered i.p. in single doses of 1·0 or 2·5 g/kg. Control animals were given a corresponding volume of 0·9% saline by the same route. Kidneys were removed at either 2 or 24 hr after injection. When the rats were kept for 24 hr, they were allowed food (standard laboratory chow) and water *ad libitum*.

At the designated time after drug administration, the animal was anesthetized with ether. After the necessary surgery, the kidneys were perfused rapidly from the aorta with ice-cold 0·25 M sucrose solution until they became pale. This usually required from 10–15 ml of the sucrose solution. The kidneys were then excised, capsules removed, and cortices separated. The cortical tissue was then homogenized in ice-cold 0·9 M sucrose (1:10 wet wt./v) in a glass homogenizer with a motor-driven Teflon pestle. Red cells, whole cells, connective tissue, and other debris were removed by centrifugation at 300 *g* for 2 min. The sediment was discarded and the resultant supernatant considered as the whole homogenate. Differential centrifugation was carried out on the homogenate and the successive supernatants as follows: 300 *g* for 10 min; 650 *g* for 20 min; 5000 *g* for 20 min; 15,000 *g* for 20 min; and 150,000 *g* for 60 min. A Sorvall model RC-2 centrifuge was used for the first four fractions, and a Beckman model L-2 ultracentrifuge for the last fraction. The five sedimented fractions and the 150,000 *g* supernatant were diluted according to the needs of the enzyme assay and adjusted to contain a final concentration of 0·1% Triton X-100* in 0·05 M acetate buffer, pH 5.

Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was assayed by a modification of the method of Lowry *et al.*,[10] with *p*-nitrophenylphosphate (PNPP) as the substrate.

Preliminary observations indicated the presence of an unstable component capable of the enzymatic hydrolysis of PNPP. This unstable activity, probably from glucose-6-phosphatase,[11] was completely eliminated after incubation for 20 min at 37°. Preincubation studies of up to 3 hr at 37° were made. After the loss of the unstable activity, no significant further changes in activity were noted during the 3-hr period studied.

To ensure the stability of the activity being tested, all fractions were preincubated for 90 min at 37° before assay.

The PNPP was prepared as a 0·020 M solution in 0·05 M acetate buffer, pH 5·0. To 1·0 ml of the PNPP solution was added 1·0 ml of homogenate subfraction (about 300–900 μg of protein), diluted in the buffer as described above. Incubation was

* The Triton X-100 was a gift of the Rohm and Haas Co., Philadelphia, Pa., U.S.A.

carried out in a water bath at 37° for 10 min with agitation. At the end of this period, 1·0 ml of the incubation mixture was added to 4·0 ml of a 0·1 N sodium hydroxide solution. This resulted in both termination of the enzymatic activity and the development of the yellow color (*p*-nitrophenol) which was measured in a Beckman DU spectrophotometer at 410 mμ. A reagent blank was run concurrently. With the above procedure, a tissue blank was found to be unnecessary. Acid phosphatase activity is expressed as the number of millimicromoles of *p*-nitrophenol liberated per milligram protein per minute.

Aryl sulfatase (aryl-sulfate sulfohydrolase, EC 3.1.6.1) was determined by a modification of the method of Roy,[12] with 4-nitrocatechol sulfate (NCS) as the substrate. To 1·0 ml of a 0·010 M solution of NCS was added 1·0 ml of the homogenate subfraction (about 300–900 μg protein) prepared in buffer (see above). Incubation in a water bath at 37° was allowed to proceed for 15 min with agitation. At the end of this time 1·0 ml of the incubation mixture was pipetted into 5·0 ml of alkaline quinol reagent.[12] The resultant reddish solution (4-nitrocatechol) was measured at 520 mμ against a tissue and reagent blank.

Aryl sulfatase activity is expressed as the number of millimicromoles of 4-nitrocatechol liberated per milligram protein per minute.

Acid ribonuclease [polyribonucleotide 2-oligonucleotidotransferase (cyclizing), EC 2.7.3.16] activity was determined by a modification of the method of Duve *et al.*[13] The substrate was prepared as a solution (2·0 mg/ml) of yeast ribonucleate in 0·05 M acetate buffer, pH 5, containing 0·1% Triton X-100. A mixture of 0·5 ml of the substrate solution and 0·5 ml of the homogenate subfraction in adequate dilution (about 150–450 μg protein) prepared in buffer (see above) was incubated for 30 min at 37° in a water bath. The reaction was stopped by the addition of ice-cold 10% perchloric acid containing 0·25% uranyl acetate. The mixture was centrifuged, and to 1·0 ml of the clear supernatant was added 3·0 ml water. The optical density of this mixture at 260 mμ was determined against a tissue and reagent blank. The acid RNAase specific activity is expressed as the change in optical density observed after 30 min under the above conditions per mg protein.

Protein was determined by the method of Lowry *et al.*,[14] with bovine plasma albumin as the reference standard.

Tissues for microscopic examination were fixed in phosphate-buffered formalin for 24 hr. Paraffin-embedded tissues were sectioned at 5 μ and stained with hematoxylin-eosin.

All data are presented as the mean ± the standard deviation. Comparisons were made by the Student's *t*-test.[15]

RESULTS

Pathological changes

The pathological changes observed with light microscopy are seen in Fig. 1. In general, the site of vacuolization was predominantly the outer cortex of the kidney (the most proximal portions of the proximal convoluted tubules). Two hours after the administration of $CaNa_2EDTA$ (1·0 g/kg), very fine vacuolization focally located was seen (Fig. 1A). Though only a portion of the tubules is involved, generally all the proximal cells of the affected nephron showed changes. Twenty-four hours after the administration of the same dose of the chelate, vacuolization was much more diffuse

throughout the PCT cells, and the individual vacuoles were much larger (Fig. 1B). Similar in appearance to the latter section were the histologic changes observed 2 hr after the administration of 2·5 g chelate/kg (Fig. 1C). Again, there was a diffuse vacuolization with vacuoles a great deal larger than those seen 2 hr after the lower dose. When the kidney was examined 24 hr after the higher dose (Fig. 1D), it was noted that the vacuolization was greater than that seen at 2 hr. In general, the vacuolization noted 24 hr after both doses was about the same. However, the dose of 2·5 g/kg sometimes induced overt cell damage in 24 hr as can be noted by the disruption of the brush border and the occasional presence of free nuclei in the tubular lumena.

Enzymatic changes

The specific activities of the enzymes studied in each of the fractions are shown in Tables 1–4. When the kidneys were removed 2 hr after the administration of either 1·0 g or 2·5 g $CaNa_2EDTA$ per kg (Tables 1 and 2), there were no apparent changes in the enzyme content of any of the fractions or in the total specific activity (total recovered activity/total recovered protein).

In contrast to the 2-hr interval studies, when the cortices were assayed for lysosomal enzymes 24 hr after the administration of either 1·0 g or 2·5 g $CaNa_2EDTA$ per kg, significant changes in enzyme content of the subfractions were seen. With the dose of 1·0 g/kg (Table 3), there were significant decreases in the aryl sulfatase content of the 300-*g*, 650-*g*, and 5000-*g* fractions, along with a decrease in the total aryl sulfatase content. When acid phosphatase content was examined, significant decreases in specific activity were seen in all the fractions, with the 300-*g*, 650-*g*, and 5000-*g* fractions showing the most striking changes. The total acid phosphatase content was somewhat more decreased than the total aryl sulfatase content.

Acid ribonuclease activity shows a slightly different behavior. The specific activities of the 300- and 650-*g* fractions show no significant differences between control and treated groups. Though the mean specific activities of these two fractions show enough differences to suggest significant changes, the standard deviations are quite large.

In this case, however, a comparison of *relative* specific activity may be made. Relative specific activity is defined here as the ratio of the per cent of total recovered activity occurring in the fraction to the per cent of total recovered protein occurring in the fraction. The total recovered activity or protein is the summation of the activity or protein in the six fractions. Relative specific activity represents, therefore, the degree of enzyme concentration in the component fraction as compared to that in the combined fractions. Thus, if the total specific activity is essentially unchanged between two groups, then any two fractions compared between the groups, having the same relative specific activity, will also have the same actual enzyme concentration (specific activity). It can be noted from Table 3 that there is little difference between the total specific activities of the control and experimental groups. Thus, comparisons of relative specific activities may be used to reflect similarities or differences in actual enzyme concentration of the fractions. Table 5 shows the relative specific activity of acid RNAase 24 hr after both doses. It can be seen that in the case of the lower dose, significant decreases occur in the 300- and 650-*g* fractions. Between Tables 3 and 5 it can also be seen that there is a significant *increase* in the activities of the 150,000-*g* and supernatant fractions.

TABLE 1. SPECIFIC ACTIVITIES OF LYSOSOMAL ENZYMES IN PCT CELLS 2 HR AFTER ADMINISTRATION OF $CaNa_2EDTA$

Dose: 1·0 g/kg

Fraction	Aryl sulfatase (mμmoles 4-nitrocatechol liberated/ min/mg protein)			Acid phosphatase (mμmoles *p*-nitrophenol liberated/ min/mg protein)			Acid ribonuclease (change in OD_{260}/30 min/mg protein)		
(*g*)	Control	EDTA	P	Control	EDTA	P	Control	EDTA	P
300	33·87 ± 4·51	32·25 ± 7·66	>0·70	77·21 ± 18·75	71·56 ± 7·94	>0·50	1·75 ± 0·11	1·71 ± 0·24	>0·70
650	43·30 ± 8·95	33·30 ± 6·53	>0·10	92·85 ± 15·19	86·41 ± 11·79	>0·40	1·50 ± 0·10	1·55 ± 0·27	>0·60
5,000	22·58 ± 6·61	25·00 ± 3·70	>0·60	79·85 ± 14·32	72·72 ± 8·74	>0·30	0·96 ± 0·20	1·31 ± 0·34	>0·05
15,000	18·54 ± 2·90	27·65 ± 5·40	>0·05	64·77 ± 13·06	68·11 ± 7·25	>0·60	0·55 ± 0·26	0·70 ± 0·29	>0·40
150,000	9·43 ± 4·03	12·90 ± 6·04	>0·40	61·44 ± 11·39	65·92 ± 9·55	>0·60	0·53 ± 0·25	0·67 ± 0·32	>0·40
Supernatant	8·31 ± 1·29	10·72 ± 1·21	>0·05	46·94 ± 4·03	53·27 ± 10·58	>0·60	0·44 ± 0·13	0·49 ± 0·12	>0·50
Total	14·68 ± 5·32	15·81 ± 3·87	>0·70	62·94 ± 11·68	61·10 ± 4·77	>0·70	0·75 ± 0·14	0·78 ± 0·21	>0·70

TABLE 2. SPECIFIC ACTIVITIES OF LYSOSOMAL ENZYMES IN PCT CELLS 2 HR AFTER ADMINISTRATION OF $CaNa_2EDTA$

Dose: 2·5 g/kg

Fraction	Aryl sulfatase (mμmoles 4-nitrocatechol liberated/ min/mg protein)			Acid phosphatase (mμmoles *p*-nitrophenol liberated/ min/mg protein)			Acid ribonuclease (change in OD_{260}/30 min/mg protein)		
(*g*)	Control	EDTA	P	Control	EDTA	P	Control	EDTA	P
300	40·72 ± 4·92	37·09 ± 3·06	>0·10	76·75 ± 13·62	73·75 ± 11·70	>0·70	1·81 ± 0·12	1·57 ± 0·37	>0·20
650	36·85 ± 7·66	31·69 ± 7·58	>0·20	95·49 ± 17·01	79·74 ± 19·74	>0·20	1·40 ± 0·23	1·23 ± 0·10	>0·20
5,000	22·98 ± 6·21	24·25 ± 5·00	>0·40	83·53 ± 13·90	77·21 ± 9·55	>0·40	0·88 ± 0·22	1·03 ± 0·11	>0·20
15,000	21·13 ± 6·37	22·98 ± 2·50	>0·50	70·41 ± 11·28	68·12 ± 14·97	>0·70	0·42 ± 0·16	0·66 ± 0·17	>0·10
150,000	15·32 ± 5·08	15·89 ± 1·21	>0·80	69·27 ± 11·79	78·59 ± 14·03	>0·20	0·45 ± 0·08	0·67 ± 0·19	>0·20
Supernatant	6·21 ± 2·18	7·26 ± 1·45	>0·30	43·61 ± 7·31	53·96 ± 17·48	>0·20	0·34 ± 0·08	0·44 ± 0·12	>0·20
Total	15·72 ± 3·47	16·28 ± 1·45	>0·70	60·29 ± 7·36	64·20 ± 11·56	>0·50	0·62 ± 0·06	0·71 ± 0·12	>0·20

TABLE 3. SPECIFIC ACTIVITIES OF LYSOSOMAL ENZYMES IN PCT CELLS 24 HR AFTER ADMINISTRATION OF $CaNa_2EDTA$

Dose: 1·0 g/kg

Fraction	Aryl sulfatase (mμmoles 4-nitrocatechol liberated/min/mg protein)			Acid phosphatase (mμmoles *p*-nitrophenol liberated/min/mg protein)			Acid ribonuclease (change in OD_{260}/30 min/mg protein)		
(*g*)	Control	EDTA	P	Control	EDTA	P	Control	EDTA	P
300	37·74 ± 4·28	23·71 ± 6·77	<0·005	85·83 ± 10·07	45·68 ± 8·00	<0·001	2·01 ± 0·21	1·72 ± 0·19	>0·05
650	40·16 ± 6·13	18·87 ± 3·14	<0·005	98·26 ± 6·10	48·21 ± 6·50	<0·001	1·64 ± 0·18	1·41 ± 0·11	>0·05
5,000	27·25 ± 4·05	14·52 ± 3·63	<0·005	86·99 ± 5·93	49·82 ± 5·41	<0·001	1·22 ± 0·11	1·16 ± 0·22	>0·60
15,000	20·64 ± 4·33	18·71 ± 5·00	>0·50	67·54 ± 1·73	51·43 ± 3·91	<0·001	0·63 ± 0·11	0·73 ± 0·26	>0·50
150,000	11·29 ± 6·75	7·90 ± 7·10	>0·40	69·61 ± 4·77	61·44 ± 3·16	<0·02	0·89 ± 0·18	1·27 ± 0·25	>0·05
Supernatant	6·93 ± 0·72	6·45 ± 0·57	>0·40	51·89 ± 6·17	26·34 ± 12·48	<0·025	0·47 ± 0·10	0·64 ± 0·10	>0·05
Total	16·77 ± 1·53	11·29 ± 1·29	<0·001	67·31 ± 10·24	39·58 ± 7·13	<0·005	0·89 ± 0·11	0·95 ± 0·14	>0·50

TABLE 4. SPECIFIC ACTIVITIES OF LYSOSOMAL ENZYMES IN PCT CELLS 24 HR AFTER ADMINISTRATION OF $CaNa_2EDTA$

Dose: 2·5 g/kg

Fraction	Aryl sulfatase (mμmoles 4-nitrocatechol liberated/ min/mg protein)			Acid phosphatase (mμmoles *p*-nitrophenol liberated/ min/mg protein)			Acid ribonuclease (change in OD_{260}/30 min/mg protein)		
(*g*)	Control	EDTA	P	Control	EDTA	P	Control	EDTA	P
300	46·67 ± 6·21	30·97 ± 11·29	<0·05	95·84 ± 15·36	68·00 ± 23·64	=0·05	1·78 ± 0·38	1·35 ± 0·17	=0·05
650	42·90 ± 5·80	26·77 ± 8·31	<0·01	114·82 ± 12·08	72·37 ± 23·53	<0·01	1·67 ± 0·35	1·28 ± 0·98	⩾0·10
5,000	24·68 ± 1·69	23·06 ± 6·29	>0·50	89·40 ± 10·47	67·20 ± 21·80	<0·05	0·95 ± 0·22	0·98 ± 0·27	>0·80
15,000	22·26 ± 2·02	21·93 ± 3·71	>0·80	74·21 ± 12·60	66·27 ± 12·89	>0·30	0·66 ± 0·17	0·70 ± 0·12	>0·60
150,000	15·00 ± 1·45	15·32 ± 6·37	>0·80	74·90 ± 13·69	68·92 ± 9·20	>0·40	0·62 ± 0·32	1·02 ± 0·23	<0·05
Supernatant	8·87 ± 2·26	9·52 ± 1·53	>0·60	50·17 ± 10·99	46·14 ± 9·20	>0·50	0·50 ± 0·12	0·77 ± 0·20	<0·05
Total	18·38 ± 1·53	15·00 ± 2·09	<0·02	68·46 ± 10·36	56·95 ± 12·94	>0·10	0·81 ± 0·10	0·91 ± 0·14	>0·20

Table 4 shows the data obtained when the kidney cortices were assayed 24 hr after the administration of 2·5 g $CaNa_2EDTA$/kg. The changes in aryl sulfatase specific activity are in a direction similar to those seen 24 hr after the 1·0 g/kg dose. An exception is noted by the observation that there is no decrease in the activity of the 5000-*g* fraction, as was observed with the lower dose.

There also appears to be some difference in the activity of acid phosphatase. Whereas all the fractions showed a significant decrease 24 hr after the 1·0 g/kg dose, only the 300-, 650-, and 5000-*g* fractions showed statistically significant decreases. It is worth noting that there is no decrease in the total acid phosphatase content of the cell, as was seen with the lower dose.

With regard to acid RNAase, the changes are the same as those seen 24 hr after the administration of the lower dose except that a significant difference in specific activity can be demonstrated in the 300-*g* fraction. Table 5 shows the relative specific activity data and the significant decreases in both the 300- and 650-*g* fractions, with an accompanying increase in the 150,000-*g* and supernatant fractions.

It is necessary to mention that in all experiments described here, except those in Table 5, when total specific activity of any enzyme for both control and experimental groups was the same, changes in specific activity paralleled changes in relative specific activity.

TABLE 5. RELATIVE SPECIFIC ACTIVITIES OF ACID RIBONUCLEASE IN PCT CELLS 24 HR AFTER ADMINISTRATION OF $CaNa_2EDTA$

	(1·0 g/kg)			(2·5 g/kg)		
Fraction (*g*)	Control	EDTA	P	Control	EDTA	P
300	2·31 ± 0·33	1·85 ± 0·22	<0·05	2·23 ± 0·45	1·49 ± 0·11	<0·01
650	1·83 ± 0·06	1·49 ± 0·11	<0·001	2·08 ± 0·26	1·40 ± 0·29	<0·005
5,000	1·33 ± 0·12	1·19 ± 0·10	>0·05	1·19 ± 0·26	1·07 ± 0·28	>0.50
15,000	0·76 ± 0·17	0·76 ± 0·20	>0·80	0·85 ± 0·34	0·79 ± 0·20	>0·70
150,000	0·96 ± 0·11	1·32 ± 0·25	<0·02	0·77 ± 0·40	1·15 ± 0·38	>0·10
Supernatant	0·55 ± 0·12	0·69 ± 0·03	<0·05	0·63 ± 0·11	0·84 ± 0·13	<0·025

Separate experiments were performed to ascertain the effect, if any, of $CaNa_2EDTA$ on the enzyme systems described above. The same chelate solution that was administered to the rats was added to control homogenates to give final concentrations of 20–200 μmoles drug/ml homogenate. Enzymatic data revealed that in this concentration range, $CaNa_2EDTA$ had no effect on the assays of the three enzymes studied.

DISCUSSION

The nature of EDTA nephrotoxicity is unknown. In certain clinical situations its effects have been considered clearly inimical, and this may largely be true; but, unfortunately, the complicating factors that existed render a definitive judgment impossible. Nor have the experimental models been of any decisive aid, for these have lacked the data relating to renal function, and the histologic studies have largely ignored the evolution of the lesions. Certainty can be attached to only one point, which is *the association of vacuolar changes in the tubular epithelium with a large dose*

of the chelate. Similar changes are seen in association with large doses of sucrose,[8] mannitol,[9] low-molecular weight dextran,[9] and to a lesser degree with glucose,[9] inulin[16] and xylose.[16]

Are the vacuolar changes observed related to any biochemical changes within the cell? If so, a clue to the nature of the nephrotoxicity of EDTA may be found. Proposals have been made by Trump and Janigan[8] and by Maunsbach *et al.*[9] that sucrose-induced vacuoles and glucose-, mannitol-, and dextran-induced vacuoles are associated with lysosomes. On this basis, the biochemical changes searched for in the present study were those associated with the renal lysosomes. Acid phosphatase, aryl sulfatase, and acid ribonuclease were chosen as representative enzymes of the acid hydrolases occurring within lysosomes.

The initial biochemical basis for the presence of lysosomes in renal cortical cells and the involvement of the particles in protein absorption in the kidney is credited to Straus.[17–19] He was able to isolate "droplets" from the renal cortical cells of rats pretreated with egg white and to show that these droplets contained about 5–7 times the specific activity of acid phosphatase compared to that of the total homogenate.[17] Further studies[18] revealed that normal kidney cells contained similar droplets ranging in diameter from 0·1 to 5·0 μ and that these particles contained high concentrations of acid phosphatase, acid ribonuclease, acid deoxyribonuclease, β-glucuronidase and cathepsin relative to the specific activities of the homogenate and mitochondrial fraction. More recently, Shibko and Tappel[20] have reported on the isolation of rat kidney lysosomes in an even greater degree of purity.

Figure 2 represents a summation of the control data obtained in all four experiments, i.e. two dose levels at two time intervals. These data are displayed as relative specific activity vs. per cent of recovered protein. When the relative specific activity is plotted against per cent of recovered protein in the fraction, as in Fig. 2, the area of each block will be proportional to the per cent of total recovered activity occurring in the fraction the block represents.

Immediately evident from this presentation is that the lysosomal enzymes in renal cortical cells are distributed in favor of the heavier centrifugal fractions, with the bulk of activity associated with particles of nuclear-like density (300 *g* and 650 *g*), though there is a somewhat more generalized distribution of acid phosphatase. These findings are essentially in agreement with those of Straus[18] and compare favorably with those of Shibko and Tappel.[20] The latter authors did report, however, a greater activity in the mitochondrial fractions than was observed in these studies. Some differences may be expected, since the latter authors used considerably different suspending media and centrifugal parameters than those described here.

The effects of large doses of EDTA on the concentration of the lysosomal enzymes in the six fractions studied were, in part, time-related. It was seen that 2 hr after the administration of 1·0 or 2·5 g $CaNa_2EDTA$ per kg there were no biochemical changes. Vacuolization occurred with both doses but to a greater extent with the larger doses. However, after 24 hr there were definite biochemical changes at both dose levels. These changes were reflected mainly as a decrease in the aryl sulfatase and acid phosphatase activity of the heavier (300- and 650-*g*) fractions and a decrease in the total content of both enzymes, with an exception to the latter in the case of acid phosphatase in experiments involving the 2·5 g/kg dose after 24 hr. Acid ribonuclease activity showed some variation relative to the other two enzymes. In only one case

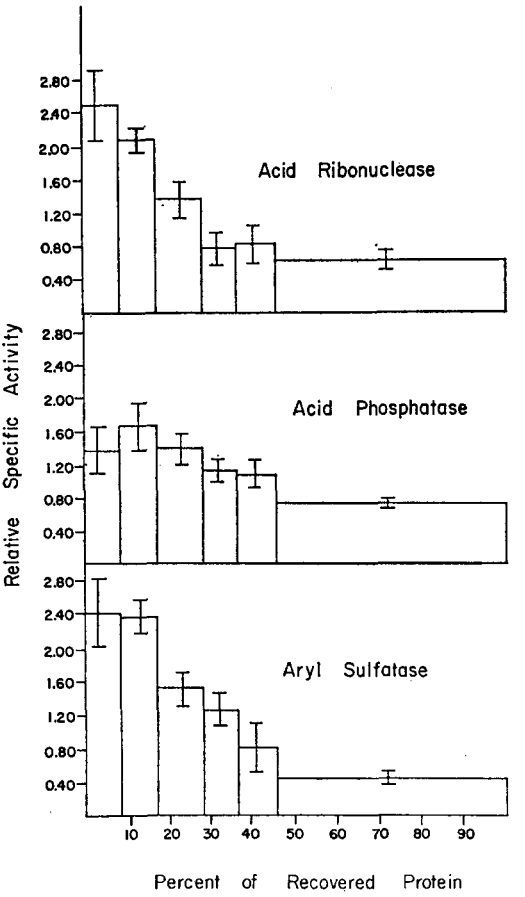

FIG. 2. Distribution patterns of aryl sulfatase, acid phosphatase, and acid ribonuclease in the renal cortex of the untreated rat. Ordinate: mean relative specific activity ± standard deviation calculated from combined control values of all experiments. Abscissa: mean relative protein content of fractions in the order of separation (left to right: 300 *g*; 650 *g*; 5000 *g*; 15,000 *g*; 150,000 *g*; supernatant) calculated from combined control values of all experiments.

was there a significant decrease in the acid RNAase specific activity of a heavy fraction. However, when *relative* specific activity was determined, a significant decrease in the activity of the heavier fractions was demonstrated. The basis for the presentation of the data in this manner was discussed under Results. Also noted in the case of acid RNAase was an increase in the enzyme content of the microsomal-weight fraction (150,000 *g*) and in the supernatant.

The underlying mechanisms in both the morphologic and enzyme changes can only be speculated on, but the data do imply that the two processes are related. However, the time lag between the formation of the vacuoles and the decrease in lysosomal marker-enzymes in the heavier centrifugal fractions indicate that the enzyme changes are secondary to the morphologic changes.

We have observed no significant decrease in the renal cortical content of zinc, iron, manganese, copper, magnesium, cobalt, or calcium at 24 hr after a dose of 2100 mg

$CaNa_2EDTA$/kg. There were, however, increases in calcium and zinc content, with the values exceeding those of the control by some 25%.* Also relevant is the evidence that within 6–24 hr, virtually all of an administered dose of this chelate has been excreted in the urine.[21] The histologic changes noted at 24 hr are therefore probably not due either to metal binding *in situ* or to depletion occasioned by the renal transit of the compound. One can further reason that the $CaNa_2EDTA$ had little if any direct effect on the enzymes studied, for no significant changes were noted in the 2-hr specimens, nor were the enzyme systems affected by addition of $CaNa_2EDTA$ *in vitro*. Speculation on the results of this study is therefore, in effect, predicated on an absence of any direct effect on the enzymes or of depletion of essential metals, and on the belief that the basic calcium-chelated EDTA molecule was excreted rapidly and intact.[21]

The most reasonable explanation for the morphologic changes is that the vacuoles form as a result of pinocytosis of the EDTA and that the vacuoles enlarge, owing to confluence with themselves and possibly other cytoplasmic particles. A similar process has been described for the vacuolization caused by sucrose[8, 9, 22] and dextran,[9] and to some extent for the tubular absorption of trypan blue.[23]

It has been reported that the vacuoles induced by the carbohydrates[8, 9, 22] and by trypan blue[23] at some point contain acid phosphatase. Ultimately, the EDTA vacuoles may also contain lysosomal enzymes. This may possibly occur by a combination of the vacuole and lysosome after the fashion of the phagosome–lysosome combination described by Straus[24] for horseradish peroxidase or by a less specific confluence of vacuoles with cytoplasmic particles than that mentioned above. Studies by Foreman *et al.*[21] suggest that by 24 hr all the EDTA has been cleared from the kidney .Thus, the loss of EDTA from the vacuoles and the cell may be accompanied by a loss of the lysosomal enzymes from the vacuoles and the cell. This would explain some of the enzyme changes observed in these studies. Some of the differences among the enzymes, especially in the case of RNAase, would be left unexplained, but the heterogeneity of content and the existence of various species of lysosomes should be considered.[13, 20]

The possibility that the lysosomal enzymes may be part of a large group of enzymes being nonspecifically altered should not be overlooked. Perhaps if additional enzymes had been studied, other types of responses might have been observed.

Whether the enzyme changes noted are due to a combination of lysosomes and vacuoles after the fashion of the formation of phago-lysosomes or are due to a less orderly, nonspecific engulfment of a variety of organelles, the demonstration of these changes is of interest in that it adds a functional dimension to changes heretofore known only in morphologic terms.

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