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CALCIUM ETHYLENEDIAMINETETRAACETATE (CaEDTA) TOXICITY—INTERACTION OF CaEDTA, CORTISOL AND VITAMIN A PALMITATE ON LYSOSOMES *IN VIVO* IN THE RAT*,†

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Abstract—Lysosomal stabilizers and labilizers attenuate and accentuate, respectively, the effect of CaEDTA in enhancing the degradation of collagen, as evidenced by urinary hydroxyproline excretion. The present study is concerned with evaluating directly the ability of CaEDTA to alter the integrity of rat liver and kidney lysosomes *in vivo*. The integrity of lysosomes was assessed by measuring the degree of redistribution of lysosomal marker enzymes from the sedimentable or bound fraction to the unsedimentable or soluble fraction and by measuring the activity of lysosomal enzymes in blood plasma. CaEDTA, compared to controls, enhanced the redistribution of cathepsin D and acid phosphatase to the soluble fraction. Total cathepsin D activity in the kidney was increased whereas total acid phosphatase activity in the liver and kidney was decreased. Cortisol (50 mg/kg/8 hr, i.m.) reduced the effect of CaEDTA on redistribution of lysosomal enzymes and abolished the effect of CaEDTA on total renal cathepsin D activity. Vitamin A palmitate (250,000 I.U./kg/12 hr, s.c.) itself produced effects similar to CaEDTA on renal lysosomes; when given concurrently with CaEDTA the increase in total renal cathepsin D activity was additive. The activity of lysosomal enzymes in blood plasma reflected changes in tissues, indicating clearly that release of lysosomal enzymes occurred *in vivo*. Thus, direct measurement of lysosomal enzyme activity, especially cathepsin D, revealed a close correlation between enzyme activity and the degradation of collagen associated with the administration of CaEDTA, cortisol and vitamin A palmitate. A marked reduction in the concentration of plasma proteins occurred in rats treated with CaEDTA and vitamin A palmitate. A rational basis for the use of cortisol in CaEDTA toxicity is provided by the fact that this drug abolished CaEDTA-induced depletion of plasma proteins.

We have previously shown that collagen degradation is markedly enhanced after the administration of CaEDTA to rats[1]. In the preceding companion paper, we showed that the concurrent administration of lysosomal stabilizers (cortisol, dexamethasone) attenuated and a lysosomal labilizer (vitamin A palmitate) accentuated CaEDTA-induced collagen degradation[2]. This suggested that lysosomes might be involved in CaEDTA-induced collagen degradation.

The objective of the present study is to determine directly lysosomal enzyme activity *in vivo* after the administration of CaEDTA, as well as with the concurrent administration of lysosomal stabilizers and labilizers. This was accomplished by measuring the distribution and total activities of lysosomal marker enzymes in liver and kidney, as well as their presence in blood plasma. It will be shown that CaEDTA activates lysosomes, as evidenced by a redistribution of acid hydrolases in tissues and their appearance in blood plasma in increased amounts. The total amount of cathepsin D in the kidney increased. These effects were prevented by cortisol and enhanced by vitamin A palmitate. In addition, it will be shown that the concentration of plasma protein was decreased by the administration of CaEDTA and vitamin A palmitate and that cortisol abolished this effect of CaEDTA.

MATERIALS AND METHODS

Details of animal preparation and the administration of drugs have been described in the preceding companion paper[2].

Distribution of acid hydrolases in liver and kidney. Tissues were prepared by a modification of commonly employed procedures[3, 4]. Rats were killed by decapitation; the livers and kidneys were removed immediately into ice-cold (4°) 0·25 M sucrose solution. The tissues were rinsed, blotted, and then weighed in tared beakers. Entire organs were minced finely with scissors prior to homogenization. Tissue homogenates were made with exactly three up–down strokes with a Potter–Elvehjem-type homogenizer (55-ml capacity) consisting of a smooth-walled glass tube and Teflon pestle (A. H. Thomas Co., Philadelphia). The pestle was driven by a motor at about 500 rev/min for liver and 200 rev/min for kidney. Homogenates were made up to a final volume of 10 ml/g of fresh tissue.

After homogenization, the tissue suspensions were centrifuged (0–4°) at 1000 *g* for 10 min

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(Beckman J-21 centrifuge) and the sediment was discarded. The supernatant liquid was divided into two fractions. One fraction was treated with 0·1% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis) to release total (soluble + latent) lysosomal enzyme activity. The second fraction was centrifuged at 50,000 *g* for 30 min to sediment lysosomes; this supernatant was treated with 0·1% Triton X-100 before assaying for soluble lysosomal enzyme activity. This centrifugal force was chosen over the more conventional 15,000 *g* for 20 min because it was determined that considerable latent enzyme activity remained in the supernatant liquid after the less rigorous centrifugation (see Table 1). Further increasing the *g* forces, or the time of centrifugation, did not result in an increase in sedimentable activity. Thus, 50,000 *g* for 30 min separated soluble from sedimentable (latent) lysosomal enzyme activity under the conditions of these experiments.

Acid phosphatase activity was determined at pH 5·0 by measuring the release of inorganic phosphate from beta-glycerophosphate. The method used was modified from Appelmans *et al.*[5]. Appropriate aliquots of tissue (0·1 to 0·5 ml) were added (Biopet disperser with plastic tips) to glass test tubes containing 0·4 ml acetate buffer (0·25 M), pH 5·0. The volume was made up to 1·6 ml with 0·25 M sucrose solution before starting the reaction with 0·2 ml of 0·5 M beta-glycerophosphate (Sigma Chemical Co., St. Louis, 98% D-isomer, freshly prepared, pH adjusted to 5·0). Incubation was carried out at 37°C for 20 min, while shaking at 100 c/min. The reaction was stopped by the addition of 0·2 ml of 50% (w/v) trichloroacetic acid. Blanks were prepared by adding trichloroacetic acid to enzyme preparations and substrate incubated separately. The release of inorganic phosphate was measured colorimetrically by an automated adaptation (Technicon Autoanalyzer II) of the procedure of Lowry and Lopez[6]. The enzymatic release of inorganic phosphate was taken as the difference in inorganic phosphate content between experimental and blank samples.

Cathepsin D activity was determined at pH 3·5 by measuring the release of tyrosine from denatured hemoglobin[7]. Appropriate aliquots of tissue (0·1 to 0·3 ml) were added (Biopet dispenser with plastic tips) to glass test tubes containing 0·2 ml acetate buffer (0·25 M), pH 3·5. The volume was made up to 0·7 ml with 0·25 M sucrose solution before starting the reaction with 0·3 ml denatured hemoglobin (Schwarz/Mann, Orangeburg, N. Y., freshly prepared, 4%, w/v, denatured with 6 M urea, pH adjusted to 3·5). Incubation was carried out at 37° for 30 min, while shaking at 100 c/min. The reaction was stopped by the addition of 1·0 ml of 10% (w/v) trichloroacetic acid. Blanks were prepared by adding trichloroacetic acid to enzyme preparations and substrate incubated separately. The release of tyrosine was measured colorimetrically by an automated adaptation (Technicon Autoanalyzer II) of the procedure of Anson[8]. The enzymatic release of tyrosine was taken as the difference in tyrosine content between the experimental and blank samples.

Measurement of protein and acid hydrolases in plasma. Blood (heparinized) was withdrawn from the abdominal aorta under light ether anesthesia. Plasma protein concentrations were determined with a Goldberg refractometer (American Optical). Analysis for acid phosphatase in 0·2 ml plasma was

Table 1. Comparison of per cent soluble lysosomal enzyme activities in liver and kidney of control and CaEDTA-treated rats from centrifugation at 15×10^3 *g* (20 min) vs 50×10^3 *g* (30 min)

	Per cent soluble enzyme activity†			
	Liver		Kidney	
Treatment*	15×10^3 *g* (20 min)	50×10^3 *g* (30 min)	15×10^3 *g* (20 min)	50×10^3 *g* (30 min)
Acid phosphatase				
Control (6)	16·8 ± 1·1	9·7 ± 0·6	44·4 ± 1·5	29·8 ± 0·9
CaEDTA (6)	26·1 ± 2·7	15·5 ± 1·7	51·0 ± 1·5	34·6 ± 0·7
% Increase over control	55	60	15	16
Cathepsin D				
Control (4)	17·8 ± 1·0	12·1 ± 0·9	30·2 ± 2·7	22·2 ± 2·5
CaEDTA (4)	24·4 ± 3·3	19·8 ± 1·8	47·5 ± 1·5	41·7 ± 1·3
% Increase over control	37	63	57	87

* All rats were infused i.v. with 0·9% NaCl solution for 24 hr. Treated rats then were infused i.v. with CaEDTA for the next 48 hr at a rate of 6 m-moles/kg/24 hr. Control rats continued to receive 0·9% NaCl solution during this time. Number of rats per group is stated in parentheses.

† Per cent soluble enzyme activity is defined as the amount of enzyme released into the supernatant of either the 15×10^3 *g* (20 min) or the 50×10^3 *g* (30 min) centrifugation expressed as per cent of total activity released by Triton X-100 (0·1%, v/v). Triton X-100 also was added to the supernatants containing soluble enzymes after centrifugation. Results are given as means ± S. E.

done similar to that described for liver and kidney except that the samples were incubated for 60 min. Analysis for cathepsin D in 0·2 ml plasma was carried out similar to that described for liver and kidney except that the acetate buffer was 1·0 M and the samples were incubated for 60 min. Beta-glucuronidase was determined by a modification of the procedure of Fishman *et al.*[9]. Plasma (0·5 ml) was incubated with 0·6 ml acetate buffer (0·2 M), pH 4·5, and 0·1 ml of 0·01 M phenolphthalein glucuronide (Sigma Chemical Co., St. Louis, Mo.) for 60 min at 37°. The reaction was stopped by the addition of glycine buffer (0·2 M), pH 10·4. The amount of phenolphthalein liberated was determined at 540 nm (Beckman DB spectrophotometer).

RESULTS

Initial experiments revealed the presence of insoluble enzyme activity in the supernatant after the 15,000 *g* (20 min) centrifugation, i.e. increasing the force and time of centrifugation resulted in a decrease in soluble enzyme activity for acid phosphatase and cathepsin D. This prompted a preliminary investigation into the influence of force of centrigugation on the apparent effect of CaEDTA on the latency of lysosomes (Table 1). It is evident that the increase in soluble acid phosphatase after CaEDTA treatment, compared to control, was similar using either centrifugal force. However, a marked difference occurred with cathepsin D activity. An increase of 37 per cent in soluble cathepsin D activity occurred in the liver of CaEDTA-treated rats over controls when 15,000 *g* (20 min) was used; in contrast, an increase of 63 per cent was evident at 50,000 *g* (30 min). Likewise, a 57 per cent increase in soluble renal cathepsin D activity occurred at 15,000 *g* (20 min), whereas an 87 per cent increase occurred at 50,000 *g* (30 min).

Effect of CaEDTA on hepatic and renal lysosomes in vivo. Significant redistribution of lysosomal enzymes occurred in the livers and kidneys of rats treated with CaEDTA. In the liver, redistribution of acid phosphatase and cathepsin D from the sedimentable to the soluble fraction increased (compared to controls) 63 and 88 per cent respectively (Fig. 1). In the kidney, CaEDTA effected increases of 17 per cent for acid phosphatase and 69 per cent for cathepsin D (Fig. 2).

Treatment with CaEDTA produced differing effects on the total enzyme content of liver and kidney. Total hepatic and renal acid phosphatase was decreased by 46 per cent (Fig. 3) and 60 per cent (Fig. 4) respectively. On the other hand, total catheptic activity in livers of CaEDTA-treated rats did not differ significantly from control rats (Fig. 3). However, CaEDTA treatment resulted in a marked increase in the total catheptic activity (58 per cent) in the kidney (Fig. 4). This result raised the possibility that CaEDTA remaining in the tissues might enhance the activity of cathepsin D. CaEDTA (0·1 M) was added to soluble preparations of cathepsin D and acid phosphatase from liver and kidney. Measurement of the activity of these enzymes before and after the addition of CaEDTA revealed no difference. Thus, CaEDTA *per se* does not affect the activity of the enzymes.

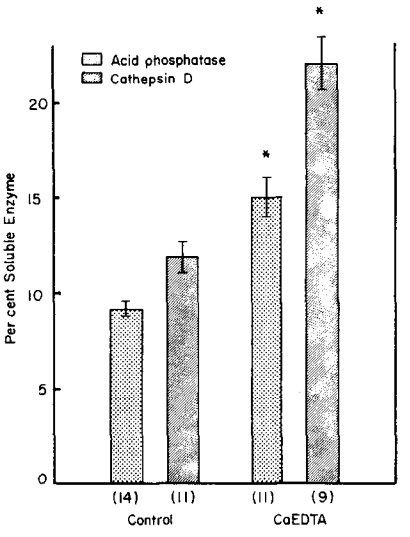

Fig. 1. Effect of CaEDTA *in vivo* on the release of acid phosphatase and cathepsin D from rat liver lysosomes. All rats were infused i.v. with 0·9% (w/v) NaCl solution for 12 hr. Treated rats were then infused i.v. with CaEDTA for 48 hr at a rate of 6 m-moles/kg/24 hr; control rats continued to receive 0·9% NaCl solution during this time. Results are expressed as per cent of the total enzyme activity released by treatment with Triton X-100. Number of experiments is stated in parentheses; bars represent means ± S. E. The asterisk indicates a significant difference from control, $P < 0.001$ (Student's *t*-test).

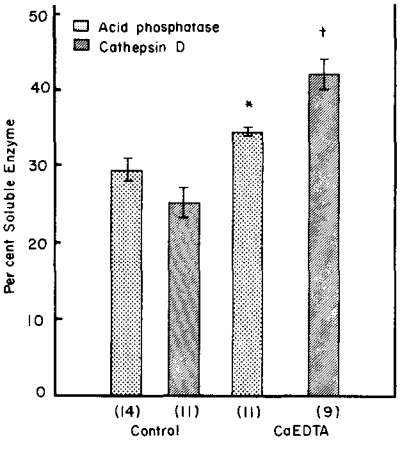

Fig. 2. Effect of CaEDTA *in vivo* on the release of acid phosphatase and cathepsin D from rat kidney lysosomes. All rats were infused i.v. with 0·9% (w/v) NaCl solution for 12 hr. Treated rats were then infused i.v. with CaEDTA for 48 hr at a rate of 6 m-moles/kg/24 hr; control rats continued to receive 0·9% NaCl solution during this time. Results are expressed as per cent of the total enzyme activity released by treatment with Triton X-100. Number of experiments is stated in parentheses; bars represent means ± S. E. Key: (*) significantly different from control, $P < 0.020$ (Student's *t*-test); and (†) significantly different from control, $P < 0.001$ (Student's *t*-test).

Interactions of cortisol and CaEDTA on lysosomes in vivo. Cortisol exerted a protective effect against the redistribution of lysosomal enzymes induced by CaEDTA (Table 2). Although the protective effect of cortisol against the labilizing effect of CaEDTA on soluble acid phosphatase in

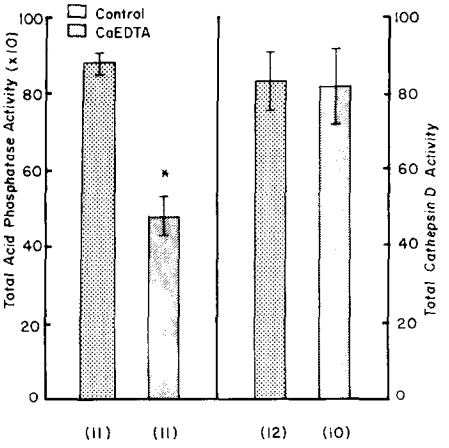

Fig. 3. Effect of CaEDTA *in vivo* on the total activities of acid phosphatase and cathepsin D of rat liver lysosomes. All rats were infused i.v. with 0·9% (w/v) NaCl solution for 12 hr. Treated rats were then infused i.v. with CaEDTA for 48 hr at a rate of 6 m-moles/kg-24 hr; control rats continued to receive 0·9% NaCl solution during this time. Total enzyme activity was released by treatment of homogenates of entire livers with Triton X-100. Number of experiments is stated in parentheses bars represent means ± S. E. Acid phosphatase activity is expressed as μmoles inorganic phosphate released/20 min/total liver, and cathepsin D activity as μmoles tyrosine released/30 min/total liver. The asterisk indicates a significant difference from control, $P < 0·001$ (Student's *t*-test).

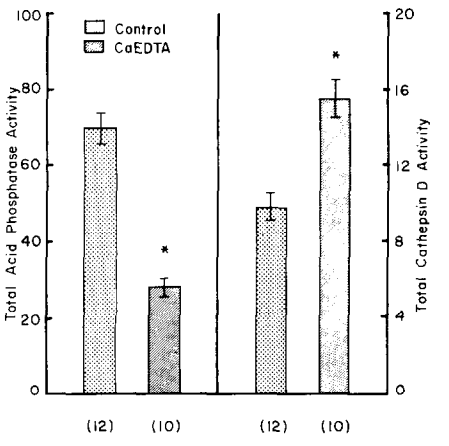

Fig. 4. Effect of CaEDTA *in vivo* on the total activities of acid phosphatase and cathepsin D of rat kidney lysosomes. All rats were infused i.v. with 0·9% (w/v) NaCl solution for 12 hr. Treated rats were then infused i.v. with CaEDTA for 48 hr at a rate of 6 m-moles/kg/24 hr; control rats continued to receive 0·9% NaCl solution during this time. Total enzyme activity was released by treatment of homogenates of entire kidneys with Triton X-100. Number of experiments is stated in parentheses; bars represent means ± S. E. Acid phosphatase activity is expressed as μmoles inorganic phosphate released/20 min/total kidney, and cathepsin D activity as μmoles tyrosine released/30 min/total kidney. The asterisk indicates a significant difference from control, $P < 0·001$ (Student's *t*-test).

Table 2. Effect of cortisol on the action of CaEDTA on lysosomes *in vivo*

Organ	Enzyme	Treatment* Control (11)	CaEDTA (9)	CaEDTA + cortisol (6)
		Per cent soluble enzyme (mean ± S. E.)		
Liver				
	Acid phosphatase	9·2 ± 0·4	15·0 ± 1·0†	13·1 ± 1·3†
	Cathepsin D	11·7 ± 0·8	22·0 ± 1·4†	18·7 ± 0·6†,‡
Kidney				
	Acid phosphatase	29·5 ± 1·7	34·4 ± 0·8†	31·6 ± 0·6‡
	Cathepsin D	24·9 ± 2·0	42·1 ± 2·1†	33·8 ± 2·1†,‡
		Total enzyme activity (mean ± S. E.) (μmoles tyrosine/30 min/kidney)		
Kidney	Cathepsin D	9·8 ± 0·7	15·5 ± 1·0†	10·8 ± 0·6‡

* All rats were infused with 0·9% NaCl solution for 12 hr. CaEDTA-treated rats then were infused i.v. (6 m-moles/kg/24 hr for 48 hr) alone or concurrently with cortisol (50 mg/kg/8 hr, i.m.). Control rats continued to receive 0·9% NaCl solution during this time. Number of rats per group is stated in parentheses.

† $P < 0·01$ compared to control (Student's *t*-test).

‡ $P < 0·025$ compared to CaEDTA (Student's *t*-test).

liver was of doubtful statistical significance, the reduction in soluble acid phosphatase in kidney and cathepsin D in liver and kidney clearly was significant. Cortisol abolished the CaEDTA-induced increase in total cathepsin D activity in kidney, but was without effect in antagonizing the marked decrease in total acid phosphatase activity. The administration of cortisol alone (50 mg/kg/8 hr, i.m., for 48 hr) did not alter the release or total amount of either acid phosphatase or cathepsin D except in the liver. Cortisol alone caused a significant decrease in total hepatic cathepsin D activity (from 85 to 55 μmoles tyrosine released/30 min/liver).

Interactions of vitamin A palmitate and CaEDTA on lysosomes in vivo. When vitamin A palmitate was administered alone (250,000 I.U./kg/12 hr, s.c., for 48 hr), increases occurred in the soluble and total cathepsin D activity in the kidney (Table 3). The increases were of the same

Table 3. Interaction of vitamin A with CaEDTA on lysosomes *in vivo*

Organ	Enzyme	Treatment* Control (11)	CaEDTA (9)	Vitamin A palmitate (5)	CaEDTA + vitamin A palmitate (5)
		Per cent soluble enzyme (mean ± S. E.)			
Kidney	Cathespsin D	24·9 ± 2·0	42·1 ± 2·1†	39·1 ± 1·0†	40·0 ± 2·4†
		Total enzyme activity (mean ± S. E.) (μmoles tyrosine/30 min/kidney)			
	Cathepsin D	9·8 ± 0·7	15·5 ± 1·0†	14·8 ± 0·6†	18·3 ± 0·7†‡

* All rats were infused with 0·9% NaCl solution for 12 hr. CaEDTA-treated rats then were infused i.v. (6 m-moles/kg/24 hr for 48 hr) alone or concurrently with vitamin A palmitate (250,000 I.U./kg/12 hr, s.c.). Control rats and rats treated with vitamin A palmitate alone continued to receive 0·9% NaCl solution during this time. Number of rats per group is stated in parentheses.

† $P < 0.001$ compared to control (Student's *t*-test).

‡ $P < 0.025$ compared to either CaEDTA or vitamin A palmitate (Student's *t*-test).

magnitude as those induced by CaEDTA. The vitamin had no effect on either the redistribution or total activity of acid phosphatase or cathepsin D in the liver. The inability of vitamin A palmitate to affect hepatic lysosomes is in agreement with data of Weissmann and Thomas[3], which showed the vitamin to cause cartilage damage in rabbits although it was without effect on hepatic lysosomes.

The effect of concurrent administration of vitamin A palmitate and CaEDTA on lysosomes was indistinguishable from that of CaEDTA alone with one exception; the effect on total renal cathepsin D activity was additive (Table 3).

Vitamin A alcohol (300,000 I.U. or 90 mg/kg/12 hr, p.o., for 48 hr) did not produce any measurable effect on hepatic or renal lysosomes. When combined with CaEDTA, the effects were the same as those described for CaEDTA alone. The ineffectiveness of vitamin A alcohol in this study may have been due to insufficient dosage. It was shown recently that this form of the vitamin caused lysosomal disruption at 175 and 350 mg/kg, p.o., to rats but was without effect at 50 or 100 mg/kg[10].

Presence of acid hydrolases in blood plasma. The presence of acid hydrolases in plasma generally reflected soluble enzyme activity measured in liver and kidney. Thus, the increased amounts of acid hydrolases in plasma resulting from CaEDTA administration were significantly reduced by cortisol (Fig. 5). Cortisol alone either

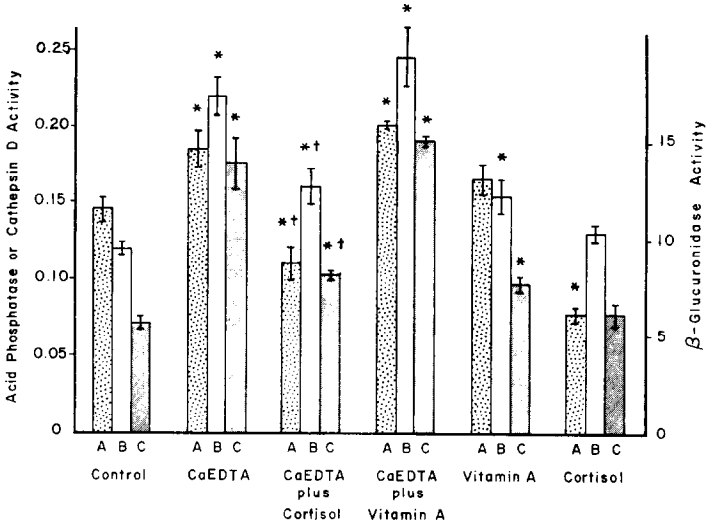

Fig. 5. Concentration of acid hydrolases in plasma after treatment with CaEDTA, vitamin A palmitate or cortisol. All rats were infused i.v. with 0·9% (w/v) NaCl solution for 12 hr. CaEDTA-treated rats then were infused i.v. (6 m-moles/kg/24 hr for 48 hr) alone or concurrently with either cortisol (50 ml/kg/8 hr for 48 hr, i.m.) or vitamin A palmitate (250,000 I.U./kg/8 hr for 48 hr, s.c.). Control rats, and rats treated with cortisol or vitamin A alone, continued to received 0·9% NaCl solution during this time. "A" signifies acid phosphatase activity, expressed as μmoles inorganic phosphate liberated/ml of plasma/hr; "B" signifies cathepsin D activity, expressed as μmoles tyrosine liberated/ml of plasma/hr; and "C" signifies β-glucuronidase activity expressed as μg phenolphthalein liberated/ml of plasma/hr. Bars represent means ± S. E. of four experiments. Key: (*) significantly different from control, $P < 0.025$ (Student's *t*-test); and (†) significantly different from CaEDTA, $P < 0.010$ (Student's *t*-test).

reduced (acid phosphatase) or did not materially alter their presence relative to control rats. Vitamin A palmitate alone effected increases in cathepsin D and beta-glucuronidase levels in plasma. When the vitamin was given simultaneously with CaEDTA, somewhat greater activities of the enzymes were measured than with CaEDTA alone.

Effect of CaEDTA, vitamin A palmitate and cortisol on the concentration of plasma proteins. The concentration of plasma proteins was found to be depressed in rats treated with CaEDTA and vitamin A palmitate (Fig. 6). This effect of CaEDTA was abolished by the simultaneous administration of cortisol. An increased concentration of plasma protein was present in rats treated with cortisol alone.

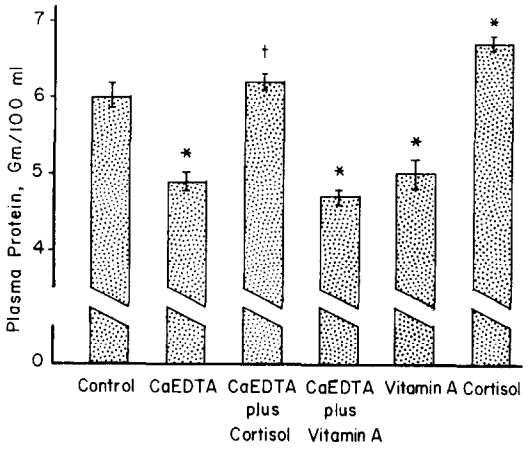

Fig. 6. Concentration of plasma proteins after treatment with CaEDTA, vitamin A palmitate or cortisol. All rats were infused i.v. with 0·9% NaCl solution for 12 hr. CaEDTA-treated rats then were infused i.v. (6 m-moles/kg/24 hr for 48 hr) alone or concurrently with either cortisol (50 mg/kg/8 hr for 48 hr, i.m.) or vitamin A palmitate (25,000 I.U./kg/8 hr for 48 hr, s.c.). Control rats, and rats treated with cortisol or vitamin A alone, continued to receive 0·9% NaCl solution during this time. Bars represent means ± S. E. of four experiments. Key: (*) significantly different from control, $P < 0.025$ (Student's *t*-test); and (†) significantly different from CaEDTA, $P < 0.0005$ (Student's *t*-test).

DISCUSSION

Data presented in this study indicated that CaEDTA labilized lysosomes of liver and kidney *in vivo* at doses that induced collagen degradation in the rat[1, 2]. The fact that plasma concentrations of acid hydrolases were elevated and reflected the release of lysosomal enzymes in tissues clearly indicates that CaEDTA labilized lysosomes *in vivo*. Thus, it is not likely that CaEDTA merely rendered the lysosomes more susceptible to rupture during the process of homogenizing tissues. A good correlation between plasma levels of lysosomal enzymes and changes in the integrity of lysosomes *in vivo* also has been demonstrated in traumatic and endotoxin shock in rats and rabbits[11] and in adjuvant-induced polyarthritis in rats[12].

It is of interest that CaEDTA exerted a more pronounced effect in increasing the per cent soluble cathepsin D activity relative to acid phosphatase activity after the 50,000 *g* (30 min) compared to the 15,000 *g* (20 min) centrifugation. This suggested either that CaEDTA enhanced the release of cathepsin D more effectively from lighter lysosomes or that the content of cathepsin D in lighter lysosomes is greater than that of the heavier lysosomes sedimented at 15,000 *g* (20 min). It has been shown by others that acid hydrolases are located in different subclasses of lysosomal particles[13] and that considerable heterogenity exists within lysosomal particles with respect to the lability of various acid hydrolases *in vivo* [3, 14–16].

It has been suggested that vitamin A induces labilization of lysosomes by penetration and expansion of the lysosomal membrane[17], probably by interacting with lipid components of the membrane[18]. The vitamin is selectively concentrated in lysosomes relative to other cellular structures[19]. It is inconceivable that CaEDTA, a highly water-soluble, negatively charged substance and limited in distribution to the extracellular space[20] could act by a similar mechanism. There is, however, some evidence indicating that CaEDTA may produce lysosomal labilization by its ability to exchange calcium for zinc *in vivo*. The ability of this drug to promote the urinary excretion of zinc is well established[21–23]. Recent evidence implicates zinc as an important factor in maintaining the stability of biological membranes, including lysosomal membranes[24], by suppressing lipid peroxidative changes[25]. In a previous study, we demonstrated that malondialdehyde production by the liver was significantly enhanced within 2 hr after the start of CaEDTA infusion[26]. At this time, the concentration of zinc in liver was significantly lower than controls while the concentration of iron remained unchanged. The addition of zinc to liver lysosomes *in vitro* has been shown to prevent their labilization when subjected to the fragility test[27, 28]. Thus, CaEDTA may produce lysosomal labilization by virtue of chelating zinc, thereby rendering the lysosomal membranes susceptible to lipid peroxidative deterioration. Labilization of lysosomes does not occur when ZnEDTA is administered to rats (unpublished observations).

Large doses of cortisol have been shown to stabilize lysosomes *in vivo* to labilizing agents including vitamin A[11, 29] and bacterial endotoxins[30], or the labilization resulting from traumatic shock[11] and applied mechanical and osmotic forces[31], or the labilization that develops during the progression of adjuvant polyarthritis in rats[12]. Anti-inflammatory steroids, such as cortisol, interact with lipid constituents of cellular membranes and possess structural features that tend to protect membranes from disrupting agents[32]. In the present study, it was shown that cortisol partially prevented the CaEDTA-induced release of lysosomal enzymes *in vivo* and completely prevented the effect of CaEDTA in enhancing total renal cathepsin D activity. Thus, cortisol antagonized the lysosomal labilizing effect of CaEDTA as well as CaEDTA-induced collagen degradation[2].

The additive effect on increased total renal cathepsin D activity resulting from the concurrent administration of CaEDTA and vitamin A palmitate is of interest in view of the synergistic effect of this

drug combination in enhancing urinary hydroxyproline excretion[2]. Although cathepsin D has been suggested as being responsible for collagen-degrading activity[33], recent studies with purified cathepsin D indicate that this enzyme has no action on collagen[34]. Nevertheless, the importance of cathepsin D in the degradation of proteoglycans seems established[35].

The interactions of CaEDTA, cortisol and vitamin A palmitate on the concentration of plasma proteins represents a preliminary study that fortuitously came about during the course of evaluating the effect of these drugs on plasma acid hydrolases. Although depletion of plasma proteins occurred after the administration of lysosomal labilizing agents, and this effect of CaEDTA was completely prevented by the administration of cortisol, it may be premature to suggest lysosomal mechanisms as an underlying cause. Appreciable quantities of plasma proteins can be lost from the body either by the kidney or intestine[36]. Even though albuminuria is not a consequence of CaEDTA administration[37], we have shown that intestinal permeability is enhanced between hr 24 and hr 36 of CaEDTA infusion[38], the same time that decreased plasma protein concentrations become apparent (unpublished observations). Thus, impaired intestinal function could constitute a means to account for the loss of plasma proteins. Nevertheless, whatever the mechanism, a rational basis for the use of cortisol in CaEDTA toxicity is provided by the fact that this drug abolishes CaEDTA-induced depletion of plasma proteins.

REFERENCES

1. A. L. Aronson and K. M. Rogerson, *Toxic. appl. Pharmac.* **21**, 440 (1972).
2. K. M. Rogerson and A. L. Aronson, *Biochem. Pharmac.* **00**, 000 (0000).
3. G. Weissmann and L. Thomas, *J. clin. Invest.* **42**, 661 (1963).
4. R. L. Deter and DeDuve, *J. Cell Biol.* **33**, 437 (1967).
5. F. Appelmans, R. Wattiaux and C. DeDuve, *Biochem. J.* **59**, 438 (1955).
6. O. H. Lowry and J. A. Lopez, *J. biol. Chem.* **162**, 421 (1946).
7. J. F. Woessner and T. H. Brewer, *Biochem. J.* **89**, 75 (1963).
8. M. L. Anson, *J. gen. Physiol.* **20**, 565 (1936).
9. W. H. Fishman, B. Springer and R. Brunetti, *J. biol. Chem.* **173**, 449 (1948).
10. L. J. Ignarro, *J. Pharmac. exp. Ther.* **182**, 179 (1972).
11. A. Janoff, G. Weissmann, B. W. Zweifach and L. Thomas, *J. exp. Med.* **116**, 451 (1962).
12. L. J. Ignarro and J. Slywka, *Biochem. Pharmac.* **21**, 875 (1972).
13. M. A. Verity, R. Caper and W. J. Brown, *Biochem. J.* **109**, 149 (1968).
14. K. Tanaka and T. Iizuka, *Biochem. Pharmac.* **17**, 2023 (1968).
15. J. J. Reynolds, in *Lysosomes in Biology and Pathology* (Eds. J. T. Dingle and H. B. Fell), Vol. 2, p. 163. American Elsevier, New York (1969).
16. L. J. Ignarro, *Biochem. Pharmac.* **20**, 2847 (1971).
17. J. T. Dingle, in *Lysosomes* (Eds. A. V. S. deReuck and M. P. Cameron), p. 384. CIBA Foundation Symposium, Little, Brown & Co., Boston (1963).
18. A. D. Bangham, J. T. Dingle and J. A. Lucy, *Biochem. J.* **90**, 133 (1964).
19. A. C. Allison and M. R. Young, *Life Sci.* **3**, 1407 (1964).
20. A. L. Aronson and F. A. Ahrens, *Toxic. appl. Pharmac.* **18**, 1 (1971).
21. H. M. Perry, Jr. and E. F. Perry, *J. clin. Invest.* **38**, 1452 (1959).
22. F. Havlicek, *Strahlentherapie* **134**, 296 (1967).
23. V. B. C. Braide, *Ph.D. Thesis*, Cornell University, Ithaca, New York (1973).
24. M. Chvapil, *Life Sci.* **13**, 1041 (1973).
25. A. A. Barber and F. Bernheim, *Adv. gerontol. Res.* **2**, 355 (1967).
26. M. Chvapil, A. L. Aronson and Y. M. Peng, *Expl molec. Path.* **20**, 216 (1974).
27. M. Chvapil, J. N. Ryan and C. F. Zukoski, *Proc. Soc. exp. Biol. Med.* **140**, 642 (1972).
28. M. Chvapil, J. N. Ryan and C. F. Zukoski, *Proc. Soc. exp. Biol. Med.* **141**, 150 (1972).
29. L. Thomas, R. T. McCluskey, J. Li and G. Weissmann, *Am. J. Path.* **42**, 271 (1963).
30. G. Weissmann and L. Thomas, *J. exp. Med.* **116**, 433 (1962).
31. L. J. Ignarro, *J. Pharmac. exp. Ther.* **182**, 179 (1972).
32. A. Allison, *Adv. Chemother.* **3**, 253 (1968).
33. J. A. Woessner, Jr., in *Tissue Proteinases* (Eds. A. J. Barrett and J. T. Dingle), p. 291. American Elsevier, New York (1971).
34. M. C. Burleigh, A. J. Barrett and G. S. Lazarus, *Biochem. J.* **137**, 387 (1974).
35. A. R. Poole, R. M. Hembry and J. T. Dingle, *J. Cell Sci.* **14**, 139 (1974).
36. R. Hoffenberg, in *Plasma Protein Metabolism. Regulation of Synthesis, Distribution and Degradation* (Eds. M. A. Rothschild and T. Waldmann), p. 239. Academic Press, New York (1970).
37. F. A. Ahrens and A. L. Aronson, *Toxic. appl. Pharmac.* **18**, 10 (1971).
38. V. B. C. Braide and A. L. Aronson, *Toxic. appl. Pharmac.* **30**, 52 (1974).