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DESTABILIZATION OF MOUSE LIVER LYSOSOMES BY VITAMIN A COMPOUNDS AND ANALOGUES*

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Abstract—In an investigation of the basis for toxicity of vitamin A, 13 vitamin A compounds and analogues were evaluated for destabilization of lysosomes. Those most effective for the release of liver acid phosphatase were, in decreasing order, retinol, *N,N*-diethylretinamide, *N*-ethylretinamide, retinyl acetate, 9-*cis*-retinal, retinoic acid, retinal, 13-*cis*-retinal, 13-*cis*-retinoic acid. For the release of liver β-glucuronidase, deoxyribonuclease and *N*-acetyl-β-D-glucosaminidase, these compounds were about equally active. The differential release of acid phosphatase, compared to the other enzymes, and a differential stabilization by chloroquine and 3′,5′-cyclic AMP of the retinol-induced release of the four enzymes were consistent with the idea that a heterogeneous population of lysosomes exists within liver cells. Chloroquine, which prevented the release of some lysosomal enzymes, did not reverse the toxicity of retinyl acetate to mice; and the release of β-glucuronidase form mouse L-929 fibroblast lysosomes correlated poorly with toxicity of the vitamin A compounds to these cells in culture. The results indicate that the toxicity of vitamin A compounds is not due solely to destabilization of lysosomes.

Although there is a mandatory requirement for vitamin A in the diet, little is known about the biochemical functions of vitamin A, with the exception of its activity in the visual process [1]. By reversing squamous, metaplastic alterations [2] and/or by inhibition of carcinogen activation [3], vitamin A compounds exhibit an anticarcinogenic effect in several animal test systems [2, 4].However, the potency of these compounds is limited by toxicity to the host, for large doses can cause weight loss, dermatitis and death of experimental animals and man [5].

Due to the toxic action of released hydrolytic enzymes from destabilized lysosomes, the tissue matrix of chick embryo cartilage in culture is degraded, and carbohydrate is liberated into the medium when excess vitamin A is present [6, 7]. Destabilization of lysosomal membranes also occurs in intact animals, for rats fed a diet containing large amounts of retinyl acetate have increased levels of free acid protease activity in their livers and kidneys [8]. This information is consistent with the hypothesis that the changes seen in hypervitaminosis A in living animals are associated with the release of hydrolytic enzymes from lysosomes [9, 10].

The availability to us of a series of vitamin A compounds and analogues allowed an evaluation of the toxicity of these agents and their capacity to destabilize mouse lysosomes.

MATERIALS AND METHODS

Materials. Retinyl acetate, retinoic acid, methyl retinoate, ethyl retinoate, 13-*cis*-retinoic acid, *N*-ethylretinamide, *N*, *N*-diethylretinamide, and Ro 8-7699† were prepared by the Hoffmann-LaRoche Co., Nutley, N.J., and supplied to us by the Lung Cancer Branch of the National Cancer Institute. Retinol, retinal, 9-*cis*-retinal, 13-*cis*-retinal, retinyl palmitate, retinyl acetate in corn oil, *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate), vitamin K_1, α-tocopherol, chloroquine diphosphate, hydrocortisone, calf thymus DNA (type I), 3′,5′-cyclic AMP, *p*-nitrophenyl-*N*-acetyl β-D-gluscosaminide, and *p*-nitrophenyl-β-D -glucuronide were purchased from Sigma Chemical Co., St. Louis, Mo.

Crude preparations of liver lysosomes from female mice were obtained essentially according to the procedure of Fukuzawa *et al.* [11]. The mice were killed by cervical dislocation, and livers were excised and placed on ice. All subsequent steps of the preparation were accomplished at 4°. The livers were then washed and homogenized in 9 vol. of 0·44 M sucrose. The preparation was centrifuged at 2000 *g* for 5 min and the resulting supernatant centrifuged again at 13,000 *g* for 10 min to give a pellet containing lysosomes. The pellet was suspended and washed once with 0·44 M sucrose. The lysosomal preparation was resuspended in 0·44 M sucrose–0·177 M KCl at a concentration of 0·1 g liver/ml.

L-929 cells were obtained from Microbiological Associates and grown in monolayer culture in SRI-14 medium [12] supplemented with 10% calf serum. Log-phase cells were harvested by trypsinization and homogenized in 10 vol. of 0·25 M sucrose in a Dounce homogenizer with a loose-fitting pestle until about 95 per cent of the cells were broken, which required about 100 up-and-down strokes. The homogenates were spun at 800 *g* for 10 min to precipitate nuclei. The supernatant was further spun at 13,300 *g* for 10 min to yield a crude lysosomal fraction. To prepare the microsomal fraction, the post-lysosomal supernatant was spun at 105,000 *g* for 60 min. For

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† Ro 8-7699: 9-(2-acetyl-5,5-dimethyl-1-cyclopen-1-yl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid.

destabilization studies, the lysosomal pellets were suspended in 0·25 M sucrose in a volume ratio of 1:6.

Analytical methods. For release of lysosomal enzymes by vitamin A compounds, each standard system contained 0·85 ml of the lysosomal preparation, 0·1 ml of 0·1 M Tris-chloride buffer (pH 7·1) and 0·05 ml of 35 mM test compound. Acidic vitamin A compounds were dissolved in 90% ethanol containing an equivalent amount of NaOH, and others were dissolved in ethanol. An appropriate control was run for each test compound. The preparations were incubated at 37° for 15 min, placed in ice and centrifuged at 13,000 *g* for 10 min at 4°. The supernatants were removed and assayed for lysosomal enzymes.

The assay for acid phosphatase was similar to that used by Fukuzawa *et al.* [11]. To a reaction mixture containing 6·25 mM *p*-nitrophenyl phosphate and 50 mM sodium acetate buffer (pH 5·0), 0·05 ml of the supernatant was added to start the enzymatic reaction. The final volume was 1 ml. After incubation of the tubes at 37° for 10 min, the reactions were stopped by addition of 0·15 ml of 10% NaOH; the absorbance of *p*-nitrophenol was measured spectrophotometrically at 420 nm. *N*-acetyl-*β*-D-glucosaminidase was assayed in the same manner as acid phosphatase, except that 3·1 mM *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosoaminide was used as the substrate.

The procedure for assay of *β*-glucuronidase was identical to that for acid phosphatase, except that the substrate was *p*-nitrophenyl-*β*-D-glucuronide and that 0·2 ml supernatant was used. The reaction was stopped with 1 ml stopping solution, which consisted of 0·133 M glycine, 0·067 M NaCl and 0·083 M Na_2CO_3, pH 10·0 [13]; the samples were centrifuged before measurement of the released *p*-nitrophenol.

The method of Bernardi and Griffe [14] was used to assay for deoxyribonuclease. Calf thymus DNA (0·4 mg/ml) was dissolved in 0·15 M sodium acetate (pH 5·0) containing 10 mM EDTA and 10 mM cysteine. To 0·7 ml of the DNA solution, 0·15 ml supernatant was added to start the reaction. After incubation of the tubes at 37° for 10 min, the reaction was stopped by the addition of 0·25 ml of 12% $HClO_4$. The preparations were placed in ice for 10 min and centrifuged to precipitate DNA. The absorbance of the supernatant was measured at 260 nm.

The procedure for stabilization of retinol-treated lysosomes was the same as that for the release of lysosomal enzymes, except that 0·75 ml of the lysosomal preparation and 0·1 ml of stabilizing agent in H_2O or 10% ethanol were used. The enzymatic assays were also the same as described above, except that 0·1 ml of the supernatant was used in the assay for acid phosphatase and *N*-acetyl-*β*-D-glucosaminidase and that the incubation time was 30 min for the assay of *β*-glucuronidase and deoxyribonuclease.

In all the experiments, a control containing the potential destabilizing compound and, if necessary, the stabilizing agent being tested and all the assay components except the enzyme substrate was used to determine the absorbance due to the test compound. The absorbance was subtracted from values obtained with the complete system. The amount of enzyme released was measured by the difference between the absorbance values for systems with and without destabilizing agents. Each assay for enzymatic activity was performed in duplicate.

Toxicity studies. To test for possible reversal of the toxicity of retinyl acetate by lysosomal stabilizing agents, four groups (A, B, C and D) of 10 female BDF_1 mice weighing 20–22 g were utilized. On days 1, 2 and 3 of the experiment, groups A and C were injected with the stabilizer, while groups B and D received only saline. On day 2, groups C and D received 160 mg retinyl acetate in corn oil; groups A and B received only corn oil. The weights and the deaths of animals were recorded daily for a period of 16 days.

Toxicity studies with mammalian cells in culture were accomplished with L929 and KB cells. Mouse L-929 fibroblasts were propagated in Eagle's MEM medium [15] supplemented with 10% fetal calf serum. To a sterile, screwcap tube, 5×10^4 L-929 cells in 2 ml medium was added, and the tubes were placed at an angle of 5° from horizontal in an incubator set at 37°. After a 24-hr establishment period, protein was determined by the procedure of Oyama and Eagle [16]. The average value was 30 *μ*g/tube. Fresh medium, to which vitamin A compounds had been added at concentrations of 0, 1, 10 or 100 *μ*g/ml, was placed in the remaining cultures; the cultures were incubated further for 72 hr, after which control tubes contained an average of 330 *μ*g protein. The amount of test compound required to reduce growth to 50 per cent of control (ED_{50}) was calculated by use of a computer program based on that of Drug Research and Development, National Cancer Institute, NIH.

Propagation and inhibition of growth of human KB cells were accomplished by an identical procedure, except that Eagle's basal medium [17] containing 10% calf serum was used.

RESULTS

Conditions for release of lysosomal enzymes. The extent of release of the four lysosomal enzymes, acid phosphatase, *β*-glucuronidase, deoxyribonuclease and *N*-acetyl-*β*-D-glucosaminidase, was dependent on several factors. The release of deoxyribonuclease by retinol was essentially complete as early as 7·5 min after adding the destabilizer (Fig. 1). However, a 15-min incubation with destabilizing agents was routinely used for release of the enzyme prior to assay and was convenient in that it allowed more samples to be included in each experiment. An increase in the concentration of the test compound above 1·75 mM did not produce greater release of enzymes (Fig. 1). Curves similar to these were obtained for each test compound with each of the four enzymes. The release of the lysosomal enzymes was also dependent upon the pH of the suspending buffer. The profiles for acid phosphatase are shown in Fig. 2. Similar results were observed for the other enzymes. The lysosomes were most stable at pH 6 and below.

When the lysosomal preparation was sonicated prior to exposure to vitamin A compounds, the enzyme activity released was greater than for any of the destabilizers, and there was no further release of either of the tested enzymes and no indication of direct inhibition of the enzymes by the vitamin A compounds or stabilizers being tested (results not shown).

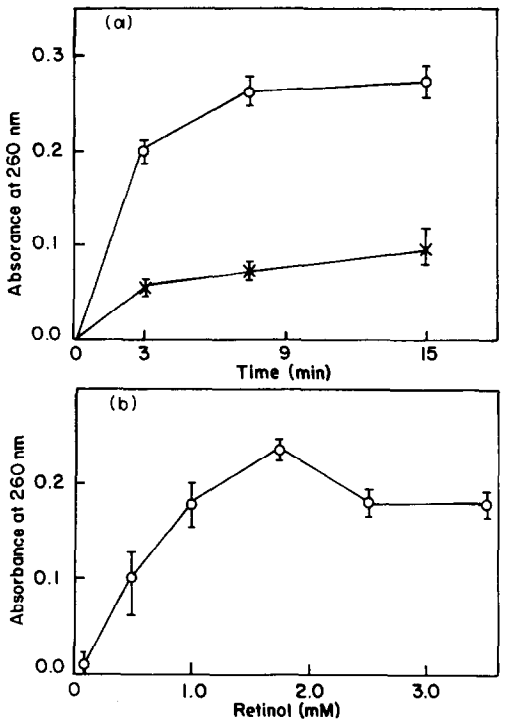

Fig. 1. Relationship between deoxyribonuclease release and time of incubation (a) and retinol concentration (b). The upper (○) and lower points (×) in Fig. 1a represent the release of enzyme in the presence and absence of retinol respectively. The values in Fig. 1b have been corrected for the values obtained in the absence of retinol. Each value represents mean ± S. D.

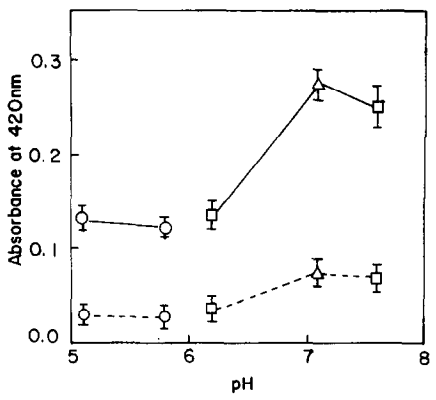

Fig. 2. Effect of pH on the release of acid phosphatase by retinol (1·75 mM). The buffer concentration is 0·01 M in each case for sodium acetate (○), potassium phosphate (□) and Tris–Cl (△). The dashed lines represent the release of enzyme in ethanol control.

Effect of vitamin A and analogues on the release of lysosomal enzymes. Table 1 presents the relative values for the release of the four marker enzymes by vitamin A compounds and analogues. The release of the enzymes as the result of action by retinol was arbitrarily set at 100; all other values are relative to that. The most effective destabilizers were retinol, *N,N*-diethylretinamide, *N*-ethylretinamide, retinyl acetate, 9-*cis*-retinal, retinoic acid, retinal, 13-*cis*-retinal and 13-*cis*-retinoic acid. Less active were Ro 8-7699, retinyl palmitate, methyl retinoate and ethyl retinoate.

Table 1. Release of lysosomal enzymes by vitamin A compounds and analogues and toxicity of these agents to cultured cells

	%Release of lysosomal enzymes compared with that from retinol-treated lysosomes*					Toxicity (ED_{50})†	
	Liver acid phosphatase	Liver β-glucuronidase	Liver deoxyribonuclease	Liver *N*-acetyl-β-D-glucosaminidase	L-929 β-glucuronidase	L-929	KB
Retinol	100	100	100	100	100	2·3	22
N,N-diethylretinamide	87 ± 17	97 ± 25	82 ± 7	79 ± 10	91	2·8	2·2
N-ethylretinamide	76 ± 11	82 ± 12	76 ± 2	69 ± 14	37	2·4	13
Retinyl acetate	74 ± 17	82 ± 3	98 ± 5	95 ± 12	8	2·8	27
9-*cis*-Retinal	64 ± 4	94 ± 13	85 ± 11	95 ± 21	103	1·2	3·1
Retinoic acid	58 ± 3	123 ± 28	78 ± 5	110 ± 2	125	5·6	24
Retinal	55 ± 4	79 ± 3	79 ± 5	78 ± 7	83	ND§	3·2
13-*cis*-Retinal	54 ± 15	83 ± 7	73 ± 11	82 ± 15	104‖	1·2	2·7
13-*cis*-Retinoic acid	39 ± 6	136 ± 11	74 ± 2	107 ± 5	170	2·0	15
Ro 8-7699	34 ± 4	89 ± 1	46 ± 5	60 ± 14	27	33	65
Retinyl palmitate	16 ± 8	7 ± 3	2 ± 2	−2 ± 3‡	19	>200	96
Methyl retinoate	7 ± 5	10 ± 3	14 ± 4	−5 ± 4‡	21‖	7·3	18
Ethyl retinoate	4 ± 3	5 ± 1	2 ± 2	−14 ± 3‡	20‖	18	18

* The amount of liver acid phosphatase, β-glucuronidase, deoxyribonuclease, *N*-acetyl-β-D-glucosaminidase and L-929 cells β-glucuronidase released from retinol-treated lysosomes was 22 ± 4, 37 ± 13, 61 ± 10, 50 ± 12 and 49 per cent respectively, of the "total" acid hydrolase activities, determined by incubation of lysosomal fractions in buffer containing 0·1% Triton X-1000. Values are mean ± S.D.

† ED_{50} is defined as that concentration of compound in μg/ml which inhibits cell growth to 50 per cent of control cell growth. The values are averages of two separate determinations.

‡ Average relative value for two separate determinations.

§ Not determined.

‖ Single determination.

Table 2. Effect of chloroquine, hydrocortisone, α-tocopherol, vitamin K_1 and 3′,5′-cyclic AMP on the release of enzymes from retinol-treated lysosomes*,†

Compound added	% Release of lysosomal enzymes compared with that from retinol-treated lysosomes			
	Acid phosphatase	β-Glucuronidase	Deoxyribonuclease	*N*-acetyl-β-D-glucosaminidase
None	100	100	100	100
Chloroquine (1 mM)	45 ± 17	66 ± 1	110 ± 16	24 ± 4
Hydrocortisone (0·3 mM)	87 ± 16	91 ± 9	78 ± 12	81 ± 3
α-Tocopherol (0·3 mM)	103 ± 9	89 ± 8	84 ± 20	87 ± 16
Vitamin K_1 (0·3 mM)	96 ± 12	95 ± 17	86 ± 18	83 ± 10
3′,5′-Cyclic AMP (1 mM)	47 ± 31	72 ± 29	57 ± 8	8 ± 11

* Relative values are the average of three separate determinations ± S.D.

† The amount of acid phosphatase, β-glucuronidase, deoxyribonuclease and *N*-acetyl-β-D-glucosaminidase released from retinol-treated lysosomes was 35 ± 14, 54 ± 3, 46 ± 5 and 30 ± 17 per cent respectively, of the "total" acid hydrolase activities, determined by incubation of lysosomal fractions in buffer containing 0·1% Triton X-100.

Some of the analogues (e.g. retinoic acid) release β-glucuronidase, deoxyribonuclease and *N*-acetyl-β-D-glucosaminidase to a greater extent than acid phosphatase.

Evaluation of potential lysosomal stabilizers. Several compounds, reported to be lysosomal stabilizers and therefore potentially capable of reversing the action of the vitamin A compounds, were evaluated for prevention of destabilization by retinol (Table 2). As detected by assays for acid phosphatase and *N*-acetyl-β-D-glucosaminidase, chloroquine and 3′,5′-cyclic AMP were stabilizing agents. That is, they prevented, to some extent, the retinol-induced release of these two enzymes. On the other hand, the release of β glucuronidase and deoxyribonuclease was stabilized only by chloroquine and 3′,5′-cyclic AMP respectively. Neither hydrocortisone, α-tocopherol nor vitamin K_1 at maximum solubility reduced the release of the four lysosomal enzymes by retinol.

Lack of effect of chloroquine and hydrocortisone on the toxicity of retinyl acetate. Experiments designed to test for reversal of the toxicity of retinyl acetate to mice were accomplished. Weight loss and the median day of death of mice given the lowest lethal dose (160 mg) of retinyl acetate were the same whether or not the highest non-toxic dose of chloroquine (20 mg/kg) was given. No reversal of toxicity was noted, and similar results were observed when the highest non-toxic dose of hydrocortisone (50 mg/kg) was used as a potential reversing agent.

Toxicity of vitamin A compounds to human KB and mouse L-929 cells in culture. The toxicity of the vitamin A compounds and analogues was determined for mammalian KB and L-929 cells in culture (Table 1). The KB cells were generally less sensitive to the compounds. For retinol and retinyl acetate, there was about a 10-fold difference in toxicity to the two cell lines.

Correlation between the release of β-glucuronidase and the toxicity of vitamin A compounds to L-929 cells. In the subcellular fractionation studies with L-929 cells, β-glucuronidase, *N*-acetyl-β-D-glucosaminidase, acid phosphatase and deoxyribonuclease were found to have similar distribution patterns (not shown). The enrichment of the specific activity of the lysosomal fraction, compared to the specific activity of the homogenate, was 3·3, 1·9, 1·5, and 1·6 for the four enzymes respectively. Since β-glucuronidase was most enriched in this fraction, it was used as a marker enzyme in the study of lysosomal labilization with vitamin A compounds and analogues (Table 1). Although some of the vitamin A compounds and analogues released L-929 β-glucuronidase to extents similar to those for liver β-glucuronidase, retinyl acetate and *N*-ethylretinamide were notable exceptions.

DISCUSSION

One of the best known effects of vitamin A compounds is that they labilize lysosomes and allow release of the lysosomal enzymes [6–10]. Our studies extend considerably the number of vitamin A compounds and analogues known to have such an effect. A previous investigation involving the release of a protease from rat liver lysosomes by vitamin A compounds shows that retinol is very effective but that neither retinyl acetate, retinoic acid nor retinyl palmitate is very active [7]. We find, however, from studies on acid phosphatase, β-glucuronidase, deoxyribonuclease and *N*-acetyl-β-D-glucosaminidase of mouse liver that retinyl acetate and retinoic acid are almost as active as retinol (Table 1). The fact that different species of animal was used and that different enzymes were assayed in the two experiments may account for this difference. The effects of pH on lysosome stability (Fig. 2) are not greatly different from those reported [9].

In our experiments, the release of each of the lysosomal enzymes is essentially complete within 7·5 min. After that time, further release of enzymes can be attributed to lysosomal autolysis (see Fig. 1). These results are similar to those involving retinol-induced release of arylsulfatase A from liver lysosomes of vitamin A-deficient rats (after subtraction, in both cases, of values for appropriate controls) [18]. In contrast, the retinol-induced release of cathepsin from rat liver lysosomes is apparently linear with time for 30 min [9], and the release of acid phosphatase and malic dehydrogenase from rabbit liver lysosomes is linear for 40 min [19]. Perhaps the extent of linearity of release of enzymes from destabilized lysosomes varies with different enzymes and with the type of animals used.

Compared to β-glucuronidase, deoxyribonuclease and *N*-acetyl-β-D-glucosaminidase, there is a differential release of acid phosphatase from liver lysosomes by some of the vitamin A compounds and analogues (Table 1). The relative values for release of acid phosphatase are graded, whereas those for deoxyribonuclease, β-glucuronidase and *N*-acetyl-β-D-glucosaminidase are polarized into high and low categories. This fact is consistent with the presence of a heterogeneous population of lysosomes in mouse liver. Such heterogeneity was noted by Fukuzawa *et al.*[11] in studies of acid phosphatase and β-glucuronidase of mouse liver lysosomes and by Futai *et al.*[20] in rat liver lysosomes. The heterogeneous distribution of acid hydrolases in Chinese-hamster ovary fibroblasts was also suggested [21].

In contrast to vitamin A, two other fat-soluble vitamins have been implicated as lysosome stabilizers. α-Tocopherol reportedly inhibits, to a small extent, the release of acid phosphatase and β-glucuronidase by mouse liver lysosomes [11], and dietary α-tocopherol stabilizes lysosomes from the livers of vitamin A-deficient rats [18, 21]. Vitamin K_1 inhibits the vitamin A-induced release of arylsulfatase from isolated rat colon lysosomes and the release *in vitro*, during hypervitaminosis A, of arylsulfatase, acid phosphatase and β-glucuronidase from these structures [23]. Other compounds which have been reported to have a stabilizing effect on isolated liver lysosomes include 3′,5′-cyclic AMP [24, 25], chloroquine [26] and hydrocortisone [27, 28]. Hydrocortisone and other steroids prevent the release of acid phosphatase more than the release of β-glucuronidase [28].

The results shown in Table 2 confirm that chloroquine and 3′,5′-cyclic AMP act as stabilizers in the prevention of the retinol-induced release of acid phosphatase and *N*-acetyl-β-D-glucosaminidase from mouse liver lysosomes. Little or no such stabilization is noted for hydrocortisone, α-tocopherol or vitamin K_1. The release of β-glucuronidase is reduced by chloroquine, whereas the release of deoxyribonuclease is inhibited by 3′,5′-cyclic AMP. These results also tend to support the idea that lysosomes are a heterogeneous mixture of particles [11, 20].

The action of vitamin A compounds on lysosomes apparently contributes to the toxicity of the vitamin to organs maintained in culture [7, 29]. However, for the vitamin A compounds studied here, there is no correlation between release of the four lysosomal enzymes and toxicity to KB cells, an established human cell line. There are only poor correlations between release of β-glucuronidase, deoxyribonuclease and *N*-acetyl-β-D-glucosaminidase from liver lysosomes and toxicity to mouse L-929 fibroblasts and between release of β-glucuronidase from the lysosomes of L-929 cells and toxicity to L-929 cells (Table 1). The lack of correlation between toxicity and lysosome destabilization may be due to the alteration of membranes other than those of lysosomes by the test compounds.

It seemed possible that chloroquine and hydrocortisone might prevent the release of hydrolytic enzymes in intact mice and reverse some of the toxicity of vitamin A because hydrocortisone has some protective effect for rabbits to which excess amounts of retonic acid have been administered [27] and for organ culture explants of limb bone rudiments exposed to high levels of vitamin A [30]. However, in our experiments *in vivo*, no reversal of vitamin A toxicity was observed, and it is therefore not likely that the toxicity of retinyl acetate to intact mice is due entirely to its effect on liver lysosomes.

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