

STUDIES ON LYSOSOMES. II. THE EFFECT OF CORTISONE ON THE RELEASE OF ACID HYDROLASES FROM A LARGE GRANULE FRACTION OF RABBIT LIVER INDUCED BY AN EXCESS OF VITAMIN A *

BY GERALD WEISSMANN† AND LEWIS THOMAS

(From the Department of Medicine, New York University School of Medicine, New York, N. Y.)

(Submitted for publication October 24, 1962; accepted January 17, 1963)

Recent studies on the dissolution of cartilage matrix by vitamin A *in vivo* and *in vitro* have indicated that the vitamin acts, at least in part, by releasing endogenous acid proteases from cartilage cells (1-5). Dingle (6) found that vitamin A alcohol *in vitro* liberated acid protease(s) from a particulate fraction of rat liver homogenized in 0.25 M sucrose. This fraction of tissue homogenates contained the bulk of acid hydrolases intimately bound to lysosomes, cytoplasmic organelles described by de Duve (7).

Observations from this laboratory, based on earlier experiments by Dingle, suggested that the induction of hypervitaminosis A *in vivo* caused a similar release of acid protease from lysosomes (8, 9); the action of such enzymes was held responsible for the degradation of protein-polysaccharide complexes in connective tissue. Morphologic expressions of this phenomenon could be seen in rabbit ear and articular cartilage (8) and in the connective tissue (9) of amphibian tails, tentacles, and chondrocranium. These studies, and others carried out at the Strangeways Research Laboratory in Cambridge, England, have also indicated that cortisone and hydrocortisone antagonized the effects of excess vitamin A (10), perhaps by stabilizing the lysosomes of tissues against the vitamin and other injurious agents. Hydrocortisone retarded the release of acid hydrolases from a large granule fraction prepared from rat and rabbit liver after traumatic shock (11) or the injection of bacterial endotoxins (12), and diminished the release effected by mercury arc irradiation (13). It appeared possible, therefore, that

adrenal steroids might partially protect isolated lysosomes against an excess of vitamin A *in vivo* and *in vitro*.

Evidence presented in this paper suggests that an excess of vitamin A, when fed to young rabbits, caused the augmented release of acid hydrolases from a large granule fraction of liver homogenates *in vitro*; an appreciable increase in the non-sedimentable activity of these enzymes *in vivo* was also observed. Depletion of cartilage matrix in hypervitaminotic animals was associated with an increased liberation of acid hydrolases from cartilage. Both effects were diminished in animals given cortisone together with the vitamin. Hydrocortisone added to rabbit liver homogenates partially antagonized the effects of an excess of vitamin A administered during life.

MATERIALS AND METHODS

Animals. Young hybrid, male, albino rabbits were obtained from commercial sources and fed a stock laboratory diet. They weighed between 800 and 1,000 g.

Vitamin A. The yellow crystals of Vitamin A acid¹ were suspended in corn oil, 50 mg per ml, before feeding animals by stomach tube. Vitamin A alcohol² was dissolved in anhydrous ethanol before use. The vitamin A palmitate² was dissolved in cottonseed oil, 10⁶ international units per ml, and was also administered by stomach tube.

Cortisone acetate. Cortisone acetate in aqueous suspension³ was given intramuscularly, 50 mg per kg, for 3 days. On day 4, 50 mg of hydrocortisone hemisuccinate (Solu-Cortef)⁴ was given intravenously 30 minutes before death of animals by air embolus. For *in vitro* experiments, hydrocortisone was added to the whole homogenates 30 minutes before further fractionation.

Incubation and irradiation. These procedures are described in detail elsewhere (12). Briefly, a large granule

* Supported by grants A 5316 and RG 6367 (C2) from the National Institutes of Health, Bethesda, Md., and contract DA 49 007 MD 590 from the Surgeon General's Office, Dept. of the Army, Washington, D. C.

† Senior Investigator, Arthritis and Rheumatism Foundation.

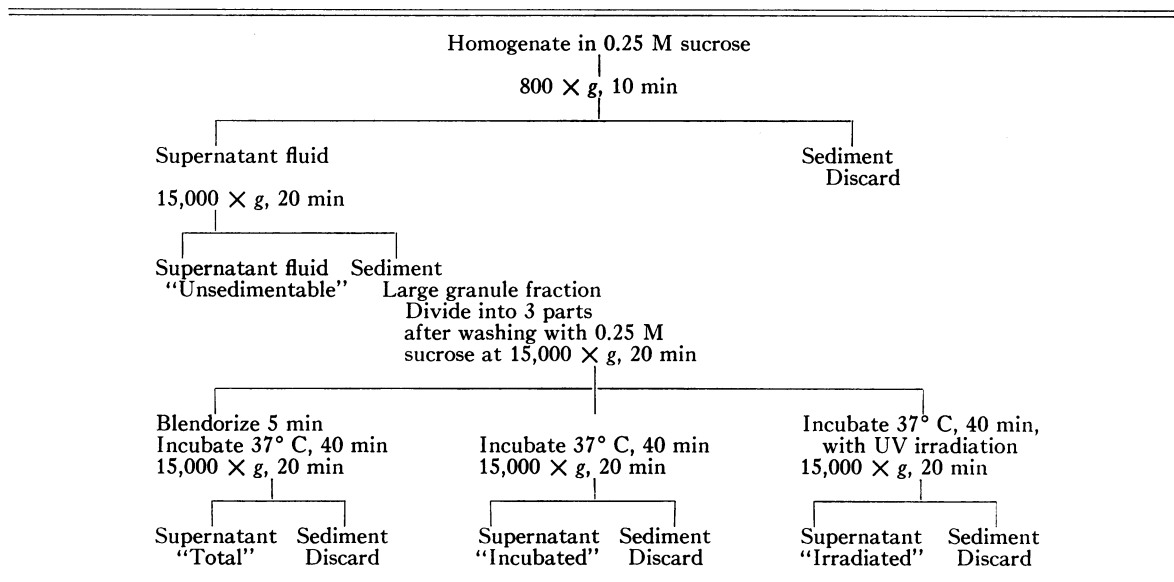
¹ Hoffman-LaRoche Co., Nutley, N. J.

² Nutritional Biochemicals, Inc., Cleveland, Ohio.

³ Philadelphia Ampoule Laboratories, Philadelphia, Pa.

⁴ The Upjohn Company, Kalamazoo, Mich.

TABLE I
*Fractionation scheme for preparation of rabbit liver granules**



* See text for details of this scheme and reference 12.

fraction of rabbit liver homogenized in 0.25 M sucrose was isolated between $800 \times g$ for 10 minutes and $15,000 \times g$ for 20 minutes in a Servall superspeed centrifuge. Samples of the large granule fraction were either incubated at 37°C for various times, or the fragility of lysosomes was tested by incubating them in the presence of mercury-arc irradiation, a procedure leading to the formation of lipid peroxides (14) and release of lysosomal enzymes (13). After incubation and irradiation, fractions were again centrifuged at $15,000 \times g$ for 20 minutes, and the enzyme activities of the supernatant fluids determined. These samples are termed "incubated" and "irradiated." "Total" activity represented activity released into the $15,000 \times g$ supernatant fluid of the large granule fraction after it had been mechanically disintegrated in a Waring Blendor for 5 minutes. "Unsedimentable" activity represented enzyme activity in the original homogenate which was unsedimentable after an initial $15,000 \times g$, 20-minute centrifugation. Our use of this term differs slightly from that of de Duve (7). These steps are summarized in Table I.

Enzyme assay. Cathepsin was assayed by the method of Anson (15), beta-glucuronidase by the method of Fishman, Springer, and Brunetti (16), and acid phosphatase by the method of Huggins and Talalay (17) with phenolphthalein diphosphate as the substrate. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall with crystalline egg-white lysozyme as a standard (18). Details of these procedures have been described before (8).

Tabulation of results. Paired samples were used in most experiments, and the appropriate *t* test performed. Results are expressed as percentage of total enzyme ac-

tivity of the large granule fraction, or in absolute units, micrograms substrate hydrolyzed per $100 \mu\text{g}$ protein per hour. It must be emphasized that changes in protein concentration could therefore alter these values, independent of any changes in enzyme activity. In cases where the effects of several agents were to be compared, all data have been transposed to percentages of control values.

Viscosity studies. Samples of a purified chondromucoprotein from bovine nasal septum (PP-L) in powdered form were generously provided by Dr. Maxwell Schubert. The preparation and properties of this material are described elsewhere (19). Solutions, 0.5% wt/vol, in 0.1 M acetate buffer at pH 4.5 were freshly prepared daily. Viscosities were determined in Ostwald viscosimeters having a water flow time of 30 to 40 seconds at 37°C . PP-L solutions, 2.5 ml, were placed together with 0.5-ml samples of the $15,000 \times g$ supernatant fluids of the irradiated large granule fractions prepared from the livers of normal and vitamin A-treated rabbits. Sucrose, 0.5 ml, was added to control samples; in some experiments, cysteine hydrochloride, 10 mmoles, or iodoacetamide, 10 mmoles, was also added to each viscosimeter.

Incubation of cartilage slices. Immediately after sacrifice, ears were removed from rabbits in a cold room at 4°C . Skin and perichondrial tissues containing soft tissue and blood vessels were dissected free, and the thin, avascular cartilaginous plates were sliced into squares ($0.5 \text{ cm} \times 0.5 \text{ cm}$). Slices obtained from both ears of each animal were incubated at 37°C for 18 hours in 40 ml of acetate buffer, pH 4.5, 0.1 M. The supernatant medium was decanted, filtered through Whatman no. 1 filter paper, and the slices washed three times in acetone

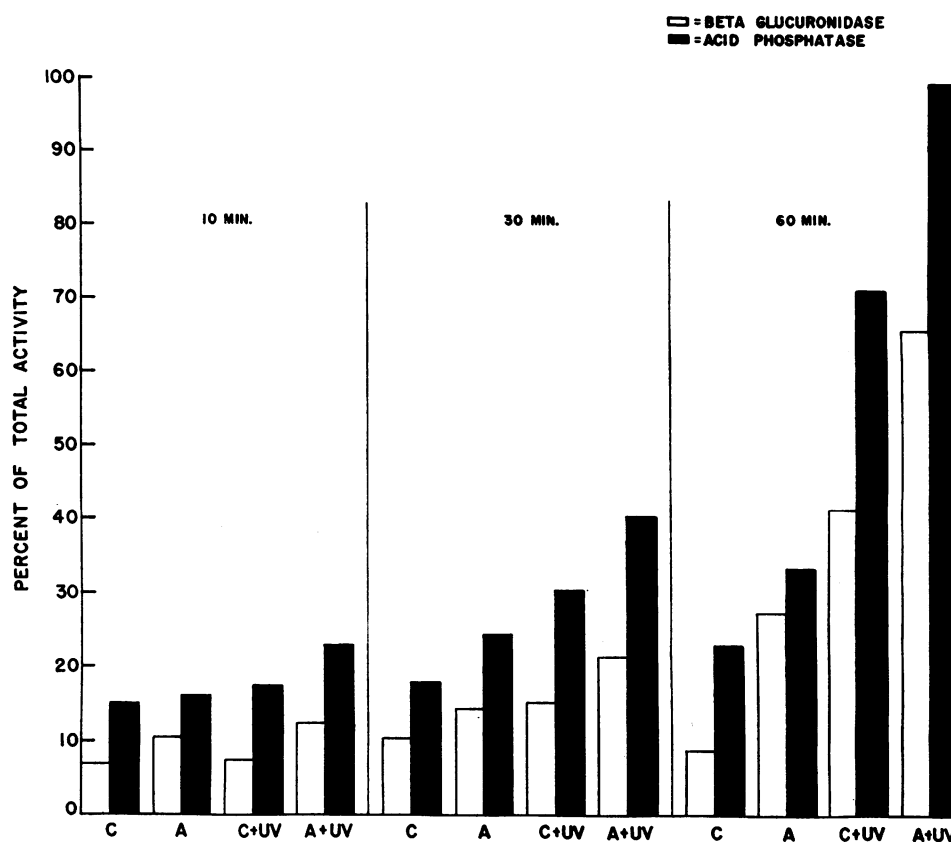


FIG. 1. VITAMIN A ALCOHOL *in vitro*. Vitamin A induced release of beta-glucuronidase and acid phosphatase from the large granule fraction of rabbit liver homogenized in 0.25 M sucrose. The fractions sedimenting between $800 \times g$ and $15,000 \times g$ were incubated and irradiated *in vitro* at 37°C for times shown. They were then centrifuged for 20 minutes at $15,000 \times g$ and the enzyme activity of the supernatant fluid determined. C = control liver fraction incubated at 37°C ; C + UV = control liver fraction, incubated and irradiated by a mercury arc lamp at 37°C ; A = liver fractions incubated with vitamin A alcohol, $3.5 \times 10^{-4} \text{ M}$ at 37°C ; and A + UV = liver fraction incubated with vitamin A alcohol 3.5×10^{-4} at 37°C , and irradiated.

and dried *in vacuo* over CaCl_2 to a constant weight. Enzyme activities were determined in samples of the buffer medium, and expressed as micrograms substrate hydrolyzed per milligram dry weight of the cartilage slices.

RESULTS

The effects of vitamin A alcohol in vitro on release of acid hydrolases from a large granule fraction of rabbit liver. Dingle (6) had found that $3.5 \times 10^{-4} \text{ M}$ vitamin A alcohol would release bound protease from the large granule fraction of rat liver. This amount of the vitamin was added in ethanol to the large granule fraction of rabbit liver; an equal volume of ethanol (0.25 ml per 50 ml) was added to control suspensions. Fractions,

20 ml, were incubated and irradiated for periods indicated in Figure 1, when 5-ml portions were withdrawn. It may be seen that the vitamin *in vitro* caused gradual release of acid phosphatase and beta-glucuronidase, more being released from those fractions which were exposed to the mercury beam. A period of 40 minutes' incubation was chosen for subsequent experiments. Data in Table II show that three acid hydrolases are released in roughly parallel fashion. Enzyme activities of unsedimentable and total fractions did not differ significantly from controls, since the vitamin was added *in vitro*. The results suggest that all three enzymes were released as a result of the addition of vitamin A, and that the vitamin

TABLE II
Activity of acid hydrolases in rabbit liver

Animals	Treatment of animals	Treatment of granules	Fraction*	Cathepsins		Beta-glucuronidase		Acid phosphatase	
				Enzyme activity	SD	Enzyme activity	SD	Enzyme activity	SD
no. 21	None (control)	None (control)	Incubated	11.7†	± 3.3	9.2†	± 2.3	16.1†	± 7.3
			Irradiated	27.0†	± 5.9	22.0†	± 3.9	32.9†	± 6.7
			Total	21.0†	± 3.8	6.1§	± 1.4	1.60§	± 0.14
			Unsedimentable	1.7†	± 0.2	0.025§	± 0.004	0.75§	± 0.12
				Percentage of control values		Percentage of control values		Percentage of control values	
6	None	Vitamin A alcohol, 3.5 × 10 ⁻⁴ M	Incubated	154.0¶	± 20.	151.0¶	± 19.3	179.0**	± 19.2
			Irradiated	200.2**	± 18.	165.0¶	± 21.9	141.5¶	± 17.6
			Total	112.8	± 20.1	100.3	± 14.3	107.8	± 16.1
			Unsedimentable	100.4	± 13.0	109.1	± 12.9	94.0	± 14.5
9	Vitamin A acid, 50 mg/kg × 4	None	Incubated	138.0¶	± 17.4	93.9	± 19.8	154.0¶	± 18.9
			Irradiated	152.2¶	± 20.2	100.8	± 23.2	119.0	± 20.2
			Total	95.2	± 24.3	69.0¶	± 12.6	110.2	± 17.3
			Unsedimentable	147.0**	± 12.4	130.0**	± 8.7	140.4**	± 12.1
4	Vitamin A acid, 50 mg/kg × 4, and cortisone	None	Incubated	53.0¶	± 21.7	75.4	± 26.2		
			Irradiated	49.0¶	± 20.7	79.6	± 22.2		
			Total	90.1	± 21.4	35.5**	± 18.3		
			Unsedimentable	15.5**	± 16.4	26.0**	± 12.7		
4	Cortisone, 50 mg/kg × 4	None	Incubated	87.2	± 20.2	67.0	± 19.2		
			Irradiated	83.0	± 23.2	83.6	± 30.0		
			Total	82.8	± 18.2	79.3	± 28.1		
			Unsedimentable	17.5**	± 12.3	54.0¶	± 17.4		
4	Vitamin A acid, 50 mg/kg × 4	Hydro-cortisone, 10 ⁻³ M	Incubated	39.5¶	± 20.6	70.8	± 26.2		
			Irradiated	69.6¶	± 19.8	82.5	± 28.3		
			Total	110.5	± 24.3	77.1	± 16.4		
			Unsedimentable	134.1¶	± 12.6	138.0¶	± 15.2		
4	None	Hydro-cortisone 10 ⁻³ M	Incubated	77.8	± 24.	72.4	± 20.3		
			Irradiated	77.2	± 19.	71.8	± 28.4		
			Total	106.8	± 17.3	101.2	± 21.3		
			Unsedimentable	101.3	± 10.8	96.4	± 12.8		
4	Vitamin A palm-itate, 10 ⁶ × 4	None	Incubated	81.2	± 19.4	93.2	± 21.2		
			Irradiated	106.7	± 32.1	90.7	± 19.8		
			Total	94.3	± 24.3	84.9	± 23.2		
			Unsedimentable	123.6	± 14.8	99.7	± 18.9		

* See Table I for preparation of these fractions.

† Activity found in the supernatant fluid of incubated or irradiated granules after centrifugation at 15,000 g for 20 minutes expressed as percentage of the value similarly obtained for the Blendor-treated preparation (total).

‡ Cathepsin unit: μg acid-soluble tyrosine per 100 μg protein per hour.

§ Beta-glucuronidase or acid phosphatase unit: μg phenolphthalein liberated per 100 μg protein per hour.

|| Control values arbitrarily assigned, 100%.

¶ $p < 0.05$.

** $p < 0.01$ vs. controls.

augmented the release effected by mercury arc irradiation. This is compatible with an effect upon the stability of lysosomes.

The effect of vitamin A acid in vivo on release of acid hydrolases from a large granule fraction of rabbit liver. To determine whether the *in vivo* effect of vitamin A in excess would be similar to the *in vitro* action, vitamin A acid was chosen. This form of the vitamin is not stored in the liver

to any appreciable degree (20), and therefore the amount still present in subsequent *in vitro* studies would be negligible. An earlier study (8) has indicated that significant cartilage lesions may be effected by 250 mg of vitamin A acid a day given orally. To determine whether a lesser amount, not associated with epilation, weight loss, or other toxicity, would affect liver lysosomes, vitamin A acid was given for four successive days

at a dose of 50 mg per kg in corn oil, while controls received corn oil alone. The last dose was given one hour before sacrifice. In Figure 2 the results of this treatment upon the subsequent release of acid protease *in vitro* may be seen. In each case, the vitamin A-treated fractions released more enzyme than did controls. This effect was magnified in fractions exposed to the mercury beam. In subsequent experiments, an incubation period of 40 minutes was used, and the vitamin was given at a dose of 50 mg per kg. In Table I the results of this treatment on release of acid hydrolases may be seen. A 30 to 40% increase in the unsedimentable activity of all three enzymes was found. This is compatible with a release of these enzymes from granules into the cytoplasm *in vivo*, before homogenization. A comparable decrease in the total, or sedimentable, activity was constant only in the case of beta-glucuronidase. A compensatory increase in synthesis of acid pro-

tease and acid phosphatase may have followed hypervitaminosis A. Such a finding has been reported by Dingle and Fell (21) for bone rudiments exposed to vitamin A *in vitro*. *In vitro* incubation and irradiation showed that this treatment schedule resulted in more active release of acid protease and acid phosphatase than of beta-glucuronidase from the particles *in vitro*. A preferential release of the protease has been noted previously in fractions prepared from animals injected with bacterial endotoxin (12).

The effect of cortisone and hydrocortisone upon the release of acid hydrolases from a large granule fraction of rabbit liver. When cortisone was administered intramuscularly in doses of 50 mg per kg together with the vitamin, release of enzymes into the unsedimentable fraction was significantly inhibited (Table II). Whereas no appreciable change in the total content of acid protease remaining sedimentable was noted, beta-glucuroni-

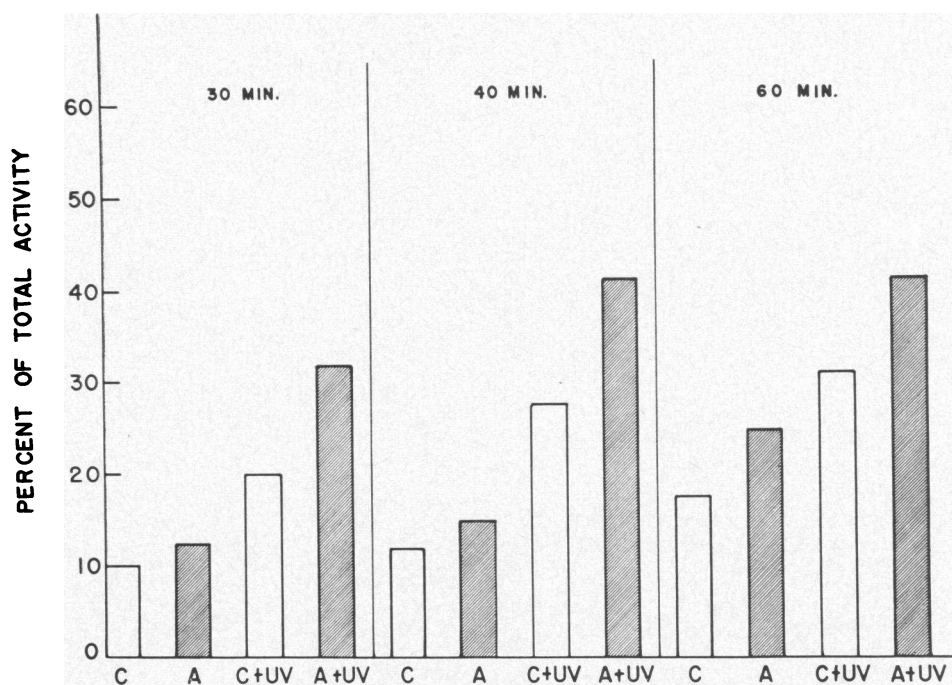


FIG. 2. VITAMIN A ACID *in vivo*. Vitamin A induced release of acid protease from the large granule fraction of rabbit liver homogenized in 0.25 M sucrose. The fractions sedimenting between 800 to 15,000 $\times g$ were incubated and irradiated *in vitro* at 37° C for times shown. They were then centrifuged for 20 minutes at 15,000 $\times g$ and the enzyme activity of the supernatant fluid determined. C = control liver fraction incubated at 37° C; C + UV = control liver fraction incubated and irradiated with a mercury arc lamp at 37° C; A = liver fraction prepared from a rabbit given 50 mg vitamin A acid for 4 days, incubated at 37° C; and A + UV = liver fraction prepared from a rabbit given 50 mg vitamin A acid for 4 days, incubated at 37° C.

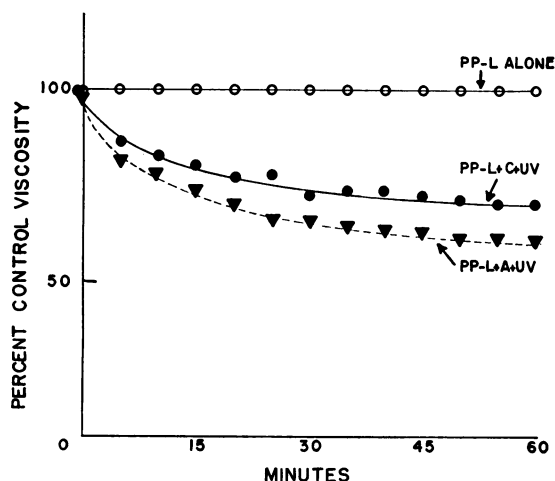


FIG. 3. VISCOSITIES OF A CHONDROMUCOPROTEIN FRACTION FROM COW NASAL SEPTUM (PP-L) IN 0.1 M ACETATE BUFFER, pH 4.5, 37° C. PP-L alone = 2.5 ml 0.5% PP-L plus 0.5 ml sucrose; PP-L + C + UV = 2.5 ml 0.5% PP-L plus 0.5 ml of C + UV fraction (see Figure 2); and PP-L + A + UV = 2.5 ml 0.5% PP-L plus 0.5 ml of A + UV fraction (see Figure 2).

dase was depressed even more than in animals given vitamin A alone. An additive effect of vitamin A and steroid may be seen in the data for animals given cortisone alone. Beta-glucuronidase is decreased significantly in these total fractions. *In vitro* release of acid protease from the large granule fraction prepared from animals given vitamin A and steroid together was significantly lower than from control fractions. Comparison of these data with those obtained from animals given cortisone alone (Table II) suggests that the stabilizing effect of adrenal corticoids is relatively independent of the presence of the vitamin.

When animals were made hypervitaminotic and hydrocortisone, 10^{-3} M, was added *in vitro*, release of hydrolases was retarded, both in incubated and irradiated fractions (Table II). This suggested that steroids given after the initial insult to the particles could still serve to stabilize the system(s) responsible for their integrity. This amount of hydrocortisone itself, in the absence of excess vitamin A, caused a 25 to 30% decrease of enzymes released from all fractions. This decrease was as marked for beta-glucuronidase as for acid protease.

The effect of vitamin A palmitate on release of acid hydrolases. Vitamin A palmitate given by

stomach tube in doses of 10^6 international units per day causes collapse of the ears of rabbits (4, 8). Yet this form of the vitamin, which is eventually stored as an ester in the liver, is unable to release acid protease from rat liver particles *in vitro* and is similarly inactive to causing damage to embryonic cartilage in organ culture (22). When the palmitate was administered to rabbits, the expected cartilage changes followed (8), but as may be seen in Table II, no effects on hepatic lysosomes could be demonstrated. A modest increase of unsedimentable activity was noted, but no other changes were seen. This would imply that the esterified vitamin is not active upon lysosomes of the tissues in which it is chiefly stored.

The effect of enzyme(s) from the large granules of rabbit liver on a chondromucoprotein. To study whether the protease(s) released from the large granule fraction of rabbit liver could be held responsible for degradation of connective tissue following hypervitaminosis A, viscosities of PP-L were determined in the presence of 1) sucrose alone, 2) the supernatant fluid of a large granule fraction prepared from a normal rabbit and subsequently exposed to a mercury beam, and 3) the supernatant fluid of a similar fraction from a rabbit given 50 mg per kg of vitamin A acid for four days. The results of such an experiment are shown in Figure 3. There was insufficient proteolytic activity in nonirradiated fractions to change the viscosity of PP-L. When similar incubations were carried out in the presence of 10 mmoles cysteine, no increase in the viscosity-reducing effects of the fractions could be detected, and 10 mmoles iodoacetamide did not cause any change in the opposite direction. Changes in viscosity of

TABLE III
Release of acid hydrolases from rabbit ear cartilage *in vitro* *

Animals	Acid phosphatase†	Beta-glucuronidase†	Acid protease‡
Control	1.00	2.66	6.36
Vitamin A§	1.15	3.90	11.60
Vitamin A + cortisone§	1.10	2.86	7.34
Cortisone§	1.02	1.53	2.94

* Cartilage slices were incubated in 0.1 M acetate buffer, pH 4.5, for 18 hours at 37°C, the medium was filtered, and enzyme activity determined.

† Micrograms phenolphthalein hydrolased per milligram cartilage per 18 hours.

‡ Micrograms acid-soluble tyrosine released per milligram cartilage per 18 hours.

§ See text for treatment schedule.

similar substrates brought about by papain (23, 24), trypsin (25), or plasmin (26) are generally regarded to result from the action of protease upon a protein-polysaccharide complex, with the formation of a protein-poor chondroitin sulfate of lesser molecular weight and lesser relative viscosity. Failure of sulfhydryl compounds to alter the curves would suggest absence of sulfhydryl-dependent proteases in the irradiated supernatant fluids.

The effect of hypervitaminosis A on release of hydrolases from cartilage slices in vitro. Cartilage slices from normal rabbits, from rabbits given vitamin A acid, 50 mg per kg, from rabbits given cortisone, 50 mg per kg, and from rabbits given both the vitamin and the steroid were incubated as described above. Results are listed in Table III. All three hydrolases were released to a greater degree from the cartilage of animals given only the vitamin than from the other three groups. This would suggest that vitamin A acted not only upon hepatic lysosomes, but also upon those of cartilage cells. It is of course impossible to determine whether increased synthesis also played a role in such a system.

DISCUSSION

The data presented above support the hypothesis that vitamin A *in vivo* as well as *in vitro* (6) causes release of acid hydrolases from lysosomes. This has been shown not only for particles isolated from the liver of rabbits, but also for cartilage slices. Adrenal glucocorticoids inhibited the release of lysosomal enzymes, both *in vitro* and *in vivo*. These findings also agree with the hypothesis that one action of cortisol and its analogues is the stabilization of lysosomes against the effects of vitamin A in excess.

The limitations inherent in this isolation procedure of hepatic granules with the presence of numerous mitochondria have been discussed at length elsewhere (8). Changes in protein concentration caused by various treatments would of course alter the interpretation of results; similar objections could be raised against use of dry weight, wet weight, or DNA measurements as a reference in treated animals.

During these experiments, and in experiments dealing with the effects of endotoxin, shock, and

mercury arc irradiation (11-13), it has become increasingly apparent that the particles within the large granule fraction may be quite heterogeneous. At low *in vivo* doses of vitamin A, *in vitro* studies indicate a fragility of the particles mainly with respect to acid protease, and very little with respect to beta-glucuronidase. Similarly, both hypervitaminosis A and cortisone pretreatment can reduce the total activity of beta-glucuronidase of the granules, without changing the activity of acid protease or acid phosphatase. Most *in vitro* experiments in which agents are added to the fractions after isolation indicate a parallel release of all lysosomal enzymes (27). This was the effect of vitamin A *in vitro*, and the actions of other *in vitro* agents on lysosomes such as mechanical disintegration (27), freezing and thawing (28), exposure to detergents (27, 28), and mercury-arc irradiation have been similar. The discrepancy between *in vivo* and *in vitro* effects suggests that lysosomes *in vivo* may respond selectively to various stimuli by release or storage of particular enzymes.

Although hypervitaminosis A may have more general membrane effects than upon the lysosomes alone (29), it would appear that damage to cartilage matrix and connective tissue (30) may be attributed to release of acid protease from lysosomes. Lysosomal protease has been studied in acute experiments at pH 4.5 (6). In longer experiments, however, Dingle has found such enzymes to be active on matrix at near-neutral pH (31). Since acid proteases are active upon chondromucoprotein *in vitro* and are released in increased amounts from cartilage removed from hypervitaminotic animals, the above hypothesis would seem warranted. Further evidence is afforded by the histologic similarities between the effects of vitamin A excess and the exogenous protease, papain *in vivo* (4) and *in vitro* (5). This plant protease can degrade chondromucoprotein (24), and upon intravenous injection, papain causes a loss of metachromasia from cartilage (32). These actions can be simulated by enzyme(s) released from lysosomes by hypervitaminosis A.

A protective effect of adrenal glucocorticoids on isolated granules of rat and rabbit liver has been previously demonstrated against damage caused by ultraviolet irradiation (13), injections of bac-

terial endotoxins (12), and simple incubation at acid pH (33). Indeed, several fat-soluble agents, such as vitamin D₂, act *in vitro* to labilize the granules; others, such as cholesterol, act to stabilize them (33). Thus, a coincidence between the *in vitro* and *in vivo* actions of vitamin A as a labilizer and cortisol as a stabilizer can conceivably be fortuitous. However, on morphologic grounds, too, protection of lysosomes by glucocorticoids has been shown against hypervitaminosis A *in vivo* (8, 9) and *in vitro* (10), and against ultraviolet irradiation *in vivo* (34) and *in vitro* (35). Desoxycorticosterone acetate was not effective in such systems. It seems reasonable, therefore, to assume that cortisone acts, at least in part, by stabilizing lysosomes in a variety of situations. Since recent experiments by Janoff and Kaley (36) have indicated that lysosomal enzymes are a potent inflammatory stimulus in the skin of small laboratory animals, the anti-inflammatory action of cortisol and its analogues may also be partially due to a stabilization of lysosomes.

SUMMARY

Vitamin A acid was fed to young rabbits and the activities of three lysosomal hydrolases determined in liver homogenates prepared in 0.25 M sucrose. An excess of the vitamin *in vivo* caused an increase in the activities of acid protease, beta-glucuronidase, and acid phosphatase unsedimentable at $15,000 \times g$. *In vitro* incubation and ultraviolet irradiation caused a greater release of acid protease and acid phosphatase from granular fractions prepared (between 800 and $15,000 \times g$) from hypervitaminotic animals than from control fractions. The simultaneous administration of cortisone largely prevented the change of sedimentability and retarded release of hydrolases from granules. Hydrocortisone *in vitro* also stabilized the granules against the effects of pretreatment with vitamin A. Cartilage slices prepared from the ears of hypervitaminotic rabbits released significantly more acid hydrolases into the surrounding medium; extracts of their liver granules caused a reduction in the viscosity of chondromucoprotein. These results are compatible with the hypothesis that excess vitamin A releases acid hydrolases from liver and cartilage lysosomes *in vivo* and *in vitro*, and that cortisone antagonizes this action.

ADDENDUM

Experiments initiated before the ones described in this paper and confirming an action of excess vitamin A on lysosomes *in vivo* have recently been reported (37).

ACKNOWLEDGMENT

We thank Mr. Ernest Bell and Miss Beverly Becher for their valuable technical assistance. We also thank Dr. Honor B. Fell and Mr. John Dingle for many stimulating suggestions and advice.

REFERENCES

1. Fell, H. B., and L. Thomas. Comparison of the effects of papain and vitamin A on cartilage. II. The effects on organ cultures of embryonic skeletal tissue. *J. exp. Med.* 1960, **111**, 719.
2. Dingle, J. T., J. A. Lucy, and H. B. Fell. Studies on the mode of action of excess of vitamin A. I. Effect of excess of vitamin A on the metabolism and composition of embryonic chick-limb cartilage grown in organ culture. *Biochem. J.* 1961, **79**, 497.
3. Lucy, J. A., J. T. Dingle, and H. B. Fell. Studies on the mode of action of excess of vitamin A. II. A possible role of intracellular proteases in the degradation of cartilage matrix. *Biochem. J.* 1961, **79**, 500.
4. Thomas, L., R. T. McCluskey, J. L. Potter, and G. Weissmann. Comparison of the effects of papain and vitamin A on cartilage. I. The effects in rabbits. *J. exp. Med.* 1960, **111**, 705.
5. Weissmann, G. Alterations in connective tissue and intestine produced by hypervitaminosis A in *Xenopus laevis*. *Nature (Lond.)* 1961, **192**, 235.
6. Dingle, J. T. Studies on the mode of action of excess of vitamin A. III. Release of a bound protease by the action of vitamin A. *Biochem. J.* 1961, **79**, 509.
7. De Duve, C. Lysosomes: a new group of cytoplasmic particles in *Subcellular Particles*, T. Hayashi, Ed. New York, Ronald, 1959, p. 128.
8. Thomas, L., R. T. McCluskey, J. Li, and G. Weissmann. Prevention by cortisone of the changes in cartilage induced by an excess of vitamin A in the rabbit. *Amer. J. Path.* 1963, **42**, 271.
9. Weissmann, G., E. Bell, and L. Thomas. Prevention by hydrocortisone of changes in connective tissue induced by an excess of vitamin A acid in amphibia. *Amer. J. Path.* In press.
10. Fell, H. B., and L. Thomas. The influence of hydrocortisone on the action of excess vitamin A on limb bone rudiments in culture. *J. exp. Med.* 1961, **114**, 343.
11. Janoff, A., G. Weissmann, B. Zweifach, and L. Thomas. Studies on the pathogenesis of irreversible shock. IV. Studies on lysosomes in normal and tolerant animals subjected to lethal trauma and endotoxemia. *J. exp. Med.* 1962, **116**, 451.

12. Weissmann, G., and L. Thomas. Studies on lysosomes. I. The effects of endotoxin, endotoxin tolerance, and cortisone on the release of acid hydrolases from a granular fraction of rabbit liver. *J. exp. Med.* 1962, **116**, 433.
13. Weissmann, G., and J. Dingle. Release of lysosomal protease by ultraviolet irradiation and inhibition by hydrocortisone. *Exp. Cell Res.* 1961, **25**, 207.
14. Barber, A. A., and A. Ottolenghi. Effect of EDTA on lipid peroxide formation and succinoxidase inactivation by ultraviolet light. *Proc. Soc. exp. Biol. (N. Y.)* 1957, **96**, 471.
15. Anson, M. L. The estimation of cathepsin with hemoglobin and the partial purification of cathepsin. *J. gen. Physiol.* 1936, **20**, 565.
16. Fishman, W. H., B. Springer, and R. Brunetti. Application of an improved glucuronidase assay method to the study of human blood β -glucuronidase. *J. biol. Chem.* 1948, **173**, 449.
17. Huggins, C., and P. Talalay. Sodium phenolphthalein phosphate as a substrate for phosphatase tests. *J. biol. Chem.* 1945, **159**, 399.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 1951, **193**, 265.
19. Gerber, B. R., E. C. Franklin, and M. Schubert. Ultracentrifugal fractionation of bovine nasal chondromucoprotein. *J. biol. Chem.* 1960, **235**, 2870.
20. Dowling, J. E., and G. Wald. The role of vitamin A acid. *Vitamins and Hormones* 1960, **18**, 515.
21. Dingle, J. T., and H. B. Fell. Unpublished observations.
22. Fell, H. B., J. T. Dingle, and M. Webb. Studies on the mode of action of excess of vitamin A. IV. The specificity of the effect on embryonic chick-limb cartilage in culture and on isolated rat-liver lysosomes. *Biochem. J.* 1962, **83**, 63.
23. Muir, H. The effect of papain on the viscosity of chondroitin sulfate. *Biochem. J.* 1956, **62**, 26 P.
24. Weissmann, G., J. L. Potter, R. T. McCluskey, and L. Thomas. The effects of papain on cartilage and chondromucoprotein. *Biochem. J.* 1961, **78**, 32 P.
25. Shatton, J., and M. Schubert. Isolation of a mucoprotein from cartilage. *J. biol. Chem.* 1954, **211**, 565.
26. Lack, C. H. Chondrolysis. *Ann. phys. Med.* 1961, **6**, 93.
27. De Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* 1955, **60**, 604.
28. Cohn, Z. A., and J. G. Hirsch. The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leukocytes. *J. exp. Med.* 1960, **112**, 983.
29. Lucy, J. A., and J. T. Dingle. The effect of vitamin A on the stability of the erythrocyte membrane. *Biochem. J.* 1962, **82**, 31 P.
30. Weissmann, G. Changes in connective tissue and intestine caused by vitamin A in amphibia, and their acceleration by hydrocortisone. *J. exp. Med.* 1961, **114**, 581.
31. Dingle, J. T. Unpublished observations.
32. Thomas, L. Reversible collapse of rabbit ears after intravenous papain, and prevention of recovery by cortisone. *J. exp. Med.* 1956, **104**, 245.
33. De Duve, C., R. Wattiaux, and M. Wibo. Effects of fat-soluble compounds on lysosomes *in vitro*. *Biochem. Pharmacol.* 1961, **9**, 97.
34. Järvinen, K. A. J. Effect of cortisone on reaction of skin to ultra-violet light. *Brit. Med. J.* 1951, **11**, 1377.
35. Weissmann, G., and H. B. Fell. The effect of hydrocortisone on the response of fetal rat skin in culture to ultraviolet irradiation. *J. exp. Med.* 1962, **116**, 365.
36. Janoff, A., and G. Kaley. To be published.
37. Dingle, J. T., I. M. Sharman, and T. Moore. The influence of vitamin A status on the proteolytic activity of lysosomes from the livers and kidneys of rats. *Proc. Nutr. Soc.* 1963, **10**, 22.