zymes *in vivo*. A large dose of Vit. A acid in the guinea pig not only caused a change in subcellular distribution of lysosomal enzymes, but also rendered the granules more "fragile" to *in vitro* incubation and irradiation. It is, therefore, suggested that along with other effects(11), Vit. A acts upon the surface of lysosomes to cause release of acid hydrolases. The demonstration of increased serum levels of beta-glucuronidase after administration of Vit. A acid indicates that the enzymes not only leave granules within the cell, but also enter the circulation. The consequences of this regimen of Vit. A on immune responses in these animals are discussed in the following paper.

Summary. Acute hypervitaminosis A was induced in guinea pigs by oral administration of Vit. A. This treatment caused much of the activity of acid phosphatase, beta-glucuronidase and cathepsin to become unsedimentable at 15,000 *g* in homogenates prepared in 0.25M sucrose. Lysosome-rich fractions of hypervitaminotic livers released beta-glucuronidase and cathepsin more readily into the incubation medium *in vitro* than did control fractions. These events were accompanied by increases of serum beta-glucuronidase, and are compatible with a direct effect of excess Vit. A on lysosomes.

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Acute Hypervitaminosis A in Guinea Pigs. II. Effects on Delayed-Type Hypersensitivity.* (28018)

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Evidence is accumulating that specialized subcellular particles, the lysosomes, may play an important role in the phagocytosis or pinocytosis of colloidal materials(1,2,3) and in the mediation of certain types of tissue damage(4,5,6). It was, therefore, of interest to study the effects upon immune mechanisms of an agent such as Vit. A, which has been shown to release lysosomal enzymes *in vitro* and *in vivo*(7,6).

The studies described below have shown that induction of acute hypervitaminosis A in the guinea pig can substantially suppress the expression of delayed-type hypersensitivity without a detectable effect on the phagocytic function of the reticuloendothelial system or on antibody formation. This action(8) was associated with the release of lysosomal hydrolases of liver from granules into the cell sap and circulation.

Materials and methods. Antigens and antibodies. Ovalbumin (Ea) 2 × recrystallized, was obtained from Pentex; crystalline bovine plasma albumin (BPA) from Armour, Inc; and diphtheria toxoid KP59a (To) containing 50 Lf/ml from Massachusetts Dept. of Health. All were diluted in physiological

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saline. A preparation of bacteriophage ΦX 174 containing 4.5×10^{11} plaque-forming particles per ml was prepared and purified as previously described(9). Rabbit anti-BPA containing 820 μg antibody protein/ml, rabbit anti-Ea containing 5.4 mg/ml and rabbit antitoxin containing 90 units/ml were used. The antibody content of these sera was determined by quantitative precipitation according to the method of Gitlin(10), except for rabbit antitoxin which was measured by toxin neutralization in rabbit skin(11).

Immunization with bacteriophage φX 174. Dilutions of phage in saline were injected intravenously into the hindleg of guinea pigs. For tracing phage in the circulation, quantitative bleedings of 0.1 ml or 0.5 ml were performed from the retro-orbital space using a capillary pipette rinsed with heparin. The blood was immediately placed in 0.9 ml chilled heparinized saline or for low concentrations of phage was not diluted. The number of plaque-forming particles in whole blood was determined by the pour plate method of Adams(12) using strain C of *E. coli.* For serum antibody determinations 1 ml of blood was obtained and allowed to clot. The standard phage neutralization assay was employed (13). Results are expressed as K, the first order inactivation constant for the neutralization of phage by specific antibody.

Sensitization. a) Delayed-type hypersensitivity. For all experiments except one, animals were injected with 3 μg of To in complete Freund's adjuvant contained in a volume of 0.5 ml and distributed into all 4 footpads. In one experiment, the To was in the form of a washed specific precipitate with excess rabbit antitoxin(14). Skin testing was usually performed on the 6th day with 3 μg To and the diameter of erythema measured at 24 hours. The area of erythema corresponded to the area of induration in control, sensitized animals. b) Passive Arthus. Animals were injected intravenously with either 820 μg of rabbit anti-BPA or 5400 μg of rabbit anti-Ea immediately before skin testing with 100 μg of the specific antigen. c) Passive cutaneous anaphylaxis. Animals received an intradermal injection of 0.1 ml containing 1 μg of rabbit anti-Ea. Five hours later they were injected intravenously with 3 mg of Ea in 1 ml of 0.25% Evans blue dye(15).

Administration of Vitamin A. Vit. A acid, (Hoffmann-La Roche) suspended in corn oil at a concentration of 40-80 mg/ml, was administered by a plastic dropper inserted into the mouth of guinea pigs until the sucking reflex was elicited. By expelling the oil during active sucking, aspiration was minimized. Vit. A palmitate (Nutritional Biochemicals) in corn oil containing 10^6 I.U./ml was injected intramuscularly.

Results. General effects of acute hypervitaminosis A. Within 24 hours after oral administration of 80 mg of A acid or its equivalent/500 g body weight, guinea pigs displayed weakness, weight loss, hair loss and decreased muscle tonus. Aspiration of small amounts of the Vit. A in oil probably occurred routinely since all of 3 animals sacrificed 48 hours after a single oral dose of Vit. A acid in oil showed scattered areas of atelectasis and pneumonitis and white blood cell counts showed a moderate granulocytosis.

Delayed-type hypersensitivity. Guinea pigs were sensitized by footpad injection of 3 μg of diphtheria toxoid in complete Freund's adjuvant. At various times, ranging from 30 hours before to 20 hours after skin testing, groups of 3-8 animals received either Vit. A acid or corn oil by mouth or were injected with Vit. A palmitate intramuscularly. For skin testing, 3 μg of toxoid was injected intradermally 6 days after sensitization and the reactions were measured 24 hours later.

Table I summarizes several individual experiments. As can be seen, Vit. A given at times ranging from 30 hours to 5 hours before skin testing inhibited development of skin reactions of the expected size and induration. Thus, 2 doses of either Vit. A acid or Vit. A palmitate resulted in skin reactions whose average area in mm^2 was 145 and 90 respectively and average induration ±, compared to 920 and 580 mm^2 for 2 control groups with average induration 1-2+. Suppression was greatest with the larger doses of Vit. A administered for 2 days. Vit. A given 5 or 20 hours after skin testing did not alter the expected reaction.

A second experiment was carried out to

TABLE I. Effect of Acute Hypervitaminosis A on Delayed-Type Skin Reactivity.

Group*	Treatment: Dose/day	Treatment: No. of days	No. of animals	Area of skin reactions at 24 hr (mm²): Avg	Area of skin reactions at 24 hr (mm²): Range	Avg induration†
	a) 1 ml oil	1	3	920	(380-1575)	1 to 2+
	b) 1 ml oil	2	8	580	(290-1200)	1 to 2+
Vit A	a) 80 mg A acid‡	1	4	285	(120- 420)	1+
	b) 160 mg A acid	1	4	195	(100- 360)	± to 1+
	c) 80 mg A acid	2	4	145	(90- 225)	±
	d) 10^6 units A palmitate§	1	4	225	(100- 380)	± to 1+
	e) *Idem*	2	4	90	(30- 170)	±
	f) 160 mg A acid 8 hr after skin testing	1	4	450	(335- 550)	1 to 2+
	g) 160 mg A acid 20 hr after skin testing	1	4	650	(400-1050)	1 to 2+

* Guinea pigs were sensitized with 3 μg of To in complete Freund's adjuvant injected into the footpads. Skin testing was performed 6 days later with 3 μg To, and, unless otherwise stated, 5 hr after the last administration of corn oil or Vit. A.

† ± doubtful, 1+ slight, 2+ moderate.

‡ Administered by mouth in 0.5-1 ml oil.

§ Injected intramuscularly.

test the effect of acute hypervitaminosis A on the skin reactivity of more highly sensitized guinea pigs. Ten guinea pigs were sensitized with 3 μg of To in the form of a To-rabbit antitoxin precipitate. On both the 13th and 14th day after sensitization 5 animals received 80 mg of Vit. A and 5 received 1 ml of corn oil. Five hours after the last dose all were skin tested with 3 μg of toxoid. The average area in mm² of the skin reactions was 740 in controls (range 156-1225) and 970 in Vit. A-treated animals (range 625-1480). Almost all the reactions in both groups were considerably more indurated (3+) than those of the animals in the previous experiment. Thus, there was no evidence that administration of the doses of Vit. A employed in this study suppressed delayed-type hypersensitivity skin reactions in highly sensitized animals.

Other inflammatory responses. Animals in these experiments were either pretreated with 80 mg of Vit. A acid or 1 ml of corn oil 24 hours and 1 hour before sensitization (5 hours before diphtheria toxin testing). As shown in Table II acute hypervitaminosis A caused suppression of a moderate, but not a severe Arthus reaction observed 6 hours after skin testing, and possibly a slight decrease in amount of dye extravasated in the PCA reaction. Although the area of inflammatory reaction to diphtheria toxin did not appear to be affected by hypervitaminosis A, the induration and intensity of erythema of the Vit. A-treated group was considerably diminished. Thus, administration of large doses of Vit. A can diminish several types of skin inflammation in the guinea pig.

Removal of antigen from the circulation. It has been previously shown that bacteriophage ΦX 174 is removed from the circulation in 2 exponential phases: the first or non-immune phase is unaffected by prior x-irradiation(9) and is due to removal of phage by reticuloendothelial cells. The second or accelerated phase is due to production and release in the circulation of specific antibody. These functions were studied in guinea pigs with acute hypervitaminosis A.

Five animals received by mouth 80 mg of Vit. A acid and 3 control animals an equal volume of corn oil on 3 successive days. 10^9 ΦX was injected intravenously 5 hours after the second dose. The animals were bled at intervals and phage content of the blood determined. The hypervitaminotic A animals died between 5-8 days after the beginning of the experiment.

There was no detectable difference between the 2 groups in rate of non-immune elimination, time of onset of detectable immune elimination and time in which less than 0.0001% of the original level of phage particles was left in the circulation (Table III). This experiment, therefore, failed to detect an effect of acute hypervitaminosis A on either

TABLE II. Effect of Acute Hypervitaminosis A on Several Types of Inflammatory Skin Reaction.

			Area of skin reaction in individual animals (mm^2)	Avg induration‡
Passive Arthus*	820 μg antibody	Hypervitaminosis A†	0, 0	0
		Control	700, 575	2+
	5400 μg "	Hypervitaminosis A†	1400, 945	3+
		Control	875, 575	3+
Passive cutaneous anaphylaxis§		Hypervitaminosis A†	170, 145, 150, 550	
		Control	272, 180, 1125, 875	
Inflammatory reaction to 1/400 mld diphtheria toxin‖		Hypervitaminosis A†	143, 165, 320, 272, 81, 225, 216, 224	±
		Control	121, 240, 256, 182	1+

* 100 μg specific antigen injected intradermally immediately after intravenous injection of antibody. Reaction measured at 6 hr.

† 80 mg vitamin A acid by mouth 24 and 1-5 hr before sensitization.

‡ ± doubtful, 1+ slight, 2+ moderate, 3+ severe.

§ Sensitized with 1 μg anti-Ea. 5 hr later, challenged with Ea, in Evans blue dye and reactions measured 20 min later.

‖ Injected intradermally. Reactions measured at 24 hr.

removal of the phage by the reticuloendothelial system or upon antibody formation to the phage. It was not possible, however, to measure quantitatively the amount of antibody produced to ΦX since all hypervitaminotic A animals in the preceding experiment died within 3 days after immune elimination. A second experiment was, therefore, performed in which 5 guinea pigs received 80 mg of Vit. A acid and 5 control animals 1 ml of corn oil on only 2 successive days. 5×10^9 ΦX was injected intravenously 5 hours after the second dose. All the animals were alive 7 days later and were bled for antibody determinations at that time. The mean K of the "A" group was 0.24 (range 0.12-0.42) and the control group 0.49 (range 0-1.4). This difference is not statistically significant.

Discussion. The experiments described above indicate that induction of acute hypervitaminosis A before skin testing of animals with delayed hypersensitivity to diphtheria toxoid substantially suppresses the size and intensity of the resultant delayed-type skin reactions. For example, in animals sensitized 6 days previously, the skin reactions were approximately 25% of the area of and considerably less indurated than reactions observed in control animals. In guinea pigs sensitized 14 days previously, in which the intensity of

TABLE III. Effect of Acute Hypervitaminosis A on Elimination of Bacteriophage ΦX 174 from the Circulation of Guinea Pigs.

Group	No. of animal	Rate* of non-immune elimination	Time of onset of immune elimination (hr)	Time at which less than 0.0001% of phage left in circulation (hr)
Hypervitaminosis A†	1	.56	44	72
	2	.56	44	68
	3	.99	44	64
	4	.32	48	77
	5	.32	45	73
Control	1	.67	44	60
	2	.99	44	64
	3	.37	48	80

* Rate $= \frac{\text{1 log decline in phage}}{\text{Time in hr}}$.

† 80 mg vitamin A acid was administered by mouth on 3 successive mornings. 10^9 phage was injected 5 hr after second dose.

sensitization was considerably greater, acute hypervitaminosis A did not detectably diminish delayed-type skin reactions. Similarly, in the passive Arthus reaction, acute hypervitaminosis A diminished the 6 hour skin response of mild, but not of severe reactions. It was also shown that Vit. A decreased the skin reactions to diphtheria toxin, and possibly the amount of dye extravasation in the PCA reaction. No effect of Vit. A was noted on the removal of bacteriophage ΦX 174 from the circulation or on amount of antibody produced to the phage 1 week after immunization.

These observations suggest that acute hypervitaminosis A can effect the expression of immunologic and non-specific inflammatory processes associated with cellular infiltration in the skin. This effect was accompanied by alterations in the subcellular distribution of 3 acid hydrolases in homogenates of liver, an increase in the serum levels of one of these enzymes (beta-glucuronidase) and an increased "fragility" of the large granule fraction of liver *in vitro*(8). Thus, there is correlative evidence implicating lysosomes as necessary for the expression of several kinds of inflammatory processes in the skin.

Several other recent studies are also consistent with this hypothesis. Leukocytes reportedly contain specific granules which in many ways resemble lysosomes(3). Moreover, induction of hypervitaminosis A in the guinea pig depletes significantly the acid phosphatase content of peritoneal leukocytes (16). Cells lacking their normal complement of lysosomal enzymes might be unable to participate normally in the inflammatory response to injury. Of particular pertinence are recent unpublished experiments of Janoff and Kaley which indicate that subcutaneous injection of lysosomal enzymes can initiate inflammation and also that hypervitaminosis A can depress tissue reactions to endotoxin, epinephrine, xylene, and vasoactive amines.

The effects of excessive Vit. A on other functions essential for inflammation in the skin are not known. It is possible, therefore, that the change in skin reactivity induced by Vit. A is a consequence of alterations in such functions as skin circulation, capillary permeability and leukocyte sticking, etc. rather than from a direct action on lysosomes.

Summary. Induction of acute hypervitaminosis A in the guinea pig can substantially suppress delayed-type hypersensitivity and Arthus reactivity of moderate intensity and the inflammatory response to intradermal injection of diphtheria toxin. No effect of Vit. A was demonstrated on the clearance of bacteriophage ΦX 174 from the circulation or on antibody formation to the phage. The results suggest that acute hypervitaminosis A can suppress inflammatory responses of moderate intensity in the skin of guinea pigs, possibly as a consequence of an action on lysosomes.

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