Acute Hypervitaminosis A in Guinea Pigs. I. Effects on Acid Hydrolases.* (28017)

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Experiments by Dingle(1) indicate that the addition *in vitro* of an excess of Vit. A alcohol to lysosome-rich fractions of rabbit liver releases acid hydrolases. Since phagocytic cells contain abundant lysosomes (2), a similar release of lysosomal enzymes occurring *in vivo* might have profound effects on immunological mechanisms.

Accordingly, the effects of acute hypervitaminosis A in guinea pigs, were studied with attention to: 1. distribution of lysosomal hydrolases in subcellular fractions of guinea pig liver. 2. stability of granular fractions *in vitro* to incubation and U-V irradiation and 3. serum levels of one of these enzymes (beta-glucuronidase).

The effect of this treatment upon delayed-type hypersensitivity is reported in the following paper (28018).

Materials and methods. Vitamin A acid was generously provided by the Hoffmann-La Roche Co., Nutley, N. J. The yellow crystals were suspended in corn oil (80 mg/ml) and the animals fed one or 2 ml by mouth, as indicated below. In most experiments, animals were given 80 mg on 2 successive days and killed 24 hours after the last dose. Control animals were given the same volume of corn oil. Since Vit. A acid is not stored in the liver, these experiments should not be complicated by *in vitro* effects of the stored vitamin.

Fractionation of guinea pig liver. Animals were killed by a blow on the head and livers were removed and promptly minced in chilled 0.25M sucrose until the washing fluid was free of gross blood. All subsequent procedures were performed at 4°C. The wet tissue was weighed, and a 1:10 w/v homogenate in sucrose was made in a glass homogenizer with a motor-driven Teflon pestle at 900 r.p.m. (Tri-R Stir-R, Tri R Co., Jamaica, N. Y.). Nuclei, debris and unbroken cells were separated by centrifugation at 800 *g* for 10 minutes in a Servall "Superspeed" centrifuge. The pellet was resuspended as a 1:10 w/v suspension which was called the "nuclear" fraction. The supernatant solution was centrifuged at 15,000 *g* for 20 minutes; the pellet was resuspended in sucrose 1:10 w/v, washed a second time at 15,000 *g*, and resuspended in a glass homogenizer by hand. As a 1:10 suspension this fraction was used for studying distribution of enzymes; as a 1:5 suspension this fraction, called the "large granule" fraction was used in experiments detailed below. The supernatant of the first, 15,000 *g* centrifugation was spun at 100,000 *g* for 2 hours in a Spinco model "L" preparative ultracentrifuge. The pellet was resuspended 1:10 in sucrose and the resultant suspension termed the "small granule" fraction; the "supernatant" was directly assayed. All fractions were mechanically disrupted in a Waring blendor (micro attachment) for 3 minutes before enzyme activities were determined.

Incubation and irradiation. These procedures have been described(3). Briefly: Aliquots of the twice-washed large granule fraction were placed in 600 ml beakers, and fixed in a water bath at 37°C. To test the relative stability of lysosomes, granular fractions were subjected to 2 types of incubation: with and without irradiation(4). Fractions to be irradiated were placed 32 cm beneath a Hanovia 100 watt high-pressure mercury arc lamp, whose rated output of energy below 3130 A is 250 microwatts/cm^2 as measured at 50 cm in free air. Duplicate samples were incubated in the same water bath, but shielded from radiation by means of aluminum foil. Another portion of each large granule fraction was homogenized in a Waring blendor for 5 minutes and then incu-

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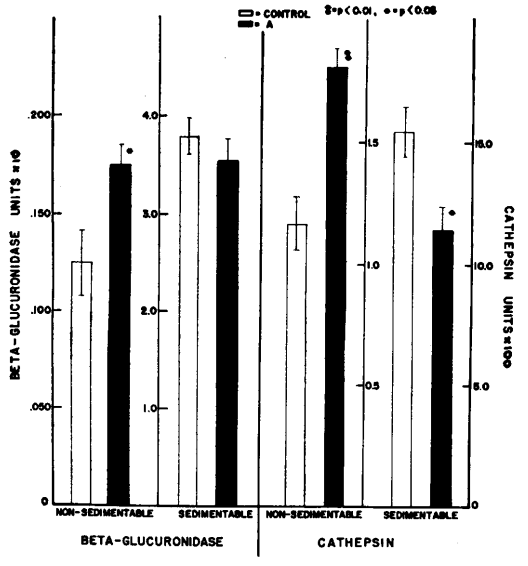

FIG. 1. Homogenates of control (open bars) and vitamin A acid-treated guinea pig liver (closed bars) have been made in 0.25M sucrose. Activities of cathepsins and beta-glucuronidase were measured in the fraction nonsedimentable at 15,000 × *g*, and in the fraction sedimentable at 15,000 × *g* (after disintegration of the granules in a Waring blendor).

bated to liberate the "total" activity of enzymes. Following incubation, these suspensions were again centrifuged at 15,000 *g* and the supernatants were analyzed for enzyme activity. "Unsedimentable" activity was determined by directly assaying that portion of the original homogenate which was not sedimentable at 15,000 *g*.

Cathepsin was determined by the method of Anson, using acid hemoglobin as substrate, as previously described(5).

Beta-glucuronidase of tissue extracts was measured by the method of Fishman *et al.* (6) using phenolphthalein glucuronide as the substrate. Serum beta-glucuronidase was determined as 0.2 ml samples of serum (obtained by retro-orbital bleeding) which were incubated with buffer and substrate for 18 hours.

Acid phosphatase was measured by the method of Huggins and Talalay(7) using phenolphthalein diphosphate (Sigma) as the substrate.

Protein was determined by the method of Lowry *et al.*(8) using crystalline egg white lysozyme as a standard.

Tabulation of results. All experiments with the large granule fraction were done simultaneously with control samples; "t" tests for differences between paired samples were used to test significance. Enzyme activity was expresed as units (μg of substrate hydrolyzed/μg protein/hr) or as % of total activity of the sample.

Results. Effect of acute hypervitaminosis A on distribution of acid hydrolases in liver. Sedimentation properties of acid protease(s) or cathepsin, and of beta-glucuronidase changed after 2 doses of Vit. A acid (80 mg) (Fig. 1). A decrease in activity of each enzyme contained within the large granule fraction (sedimenting between 800-15,000 × *g*) was accompanied by an increase in activity remaining in the supernatant (15,000 × *g*). The latter effect was more noticeable with beta-glucuronidase. To determine whether "unsedimentable" enzyme activity was entirely free in cell sap, or still remained bound to organelles or their breakdown products, a fractionation procedure described above was employed, and 4 fractions: nuclear, large granule, small granule and supernatant, were prepared. As seen in Table I, activities of 3 acid hydrolases were decreased in the large granule fraction prepared from vitamin A-treated animals, with comparable increases in the activity recovered in the supernatants and the small granule fractions (Table I). Enzymes recovered in this latter fraction could represent enzymes still attached to fragmented lysosomes, or non-specifically bound to microsomes, or enzymes associated with smaller lysosomes newly formed in the endoplasmic reticulum.

Lysosomal enzymes released from the large granules of liver might enter into the circulation. Serum beta-glucuronidase determinations summarized in Fig. 2 show that levels of this enzyme were significantly elevated in the serum of Vit. A-treated animals. The source of elevated serum beta glucuronidase is not necessarily the liver, therefore, an increased synthesis of enzyme at other sites could also result in these levels, as could a decrease in the removal of beta-glucuronidase from the circulation.

Release of acid hydrolases from the large

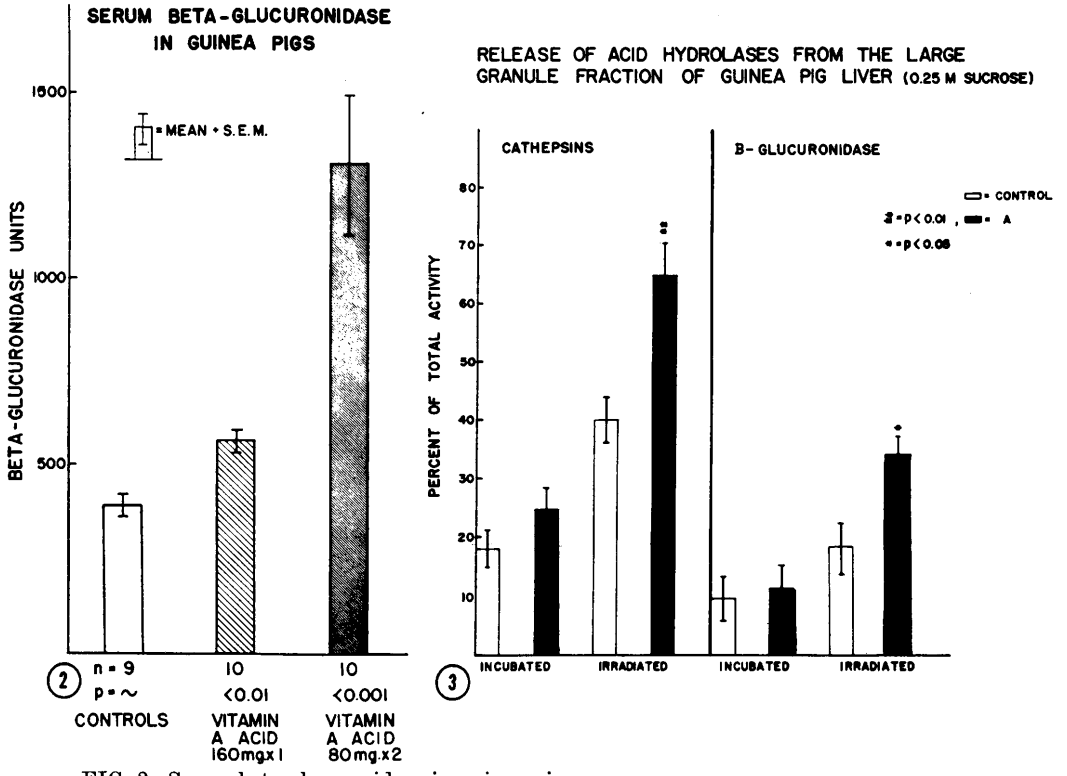

FIG. 2. Serum beta-glucuronidase in guinea pigs.

FIG. 3. Release of acid hydrolases from large granule fraction of guinea pig liver (0.25M sucrose). Large granule fractions sedimenting between 800 and 15,000 × *g* were isolated from normal (open bars) and vitamin A acid-treated guinea pig liver (closed bars). They were incubated with or without mercury arc irradiation for 40′ (see text). Following this treatment, suspensions were again centrifuged at 15,000 × *g* and the enzyme activity (expressed as % of total activity of the fraction) determined in the supernatant.

granule fraction of guinea pig liver. When the large granule fractions of guinea pig liver were incubated, particularly in the presence of mercury-arc irradiation, lysosomal hydrolases were more readily released from fractions which had been prepared from the livers of Vit. A-treated animals (Fig. 3). In view of similar findings in the rabbit and rat after endotoxin or traumatic shock(3,9) this finding could indicate an increased "fragility" of hepatic lysosomes. In accord with previous studies in rabbits of lysosomal damage induced by several different agents,(10,3,9) the release of acid protease(s) exceeds that of beta-glucuronidase from lysosome-rich fractions. This observation may indicate selective release of particular enzymes.

Discussion. These experiments suggest that Vit. A can cause release of lysosomal en-

TABLE I. Distribution of Acid Hydrolases in Subcellular Fractions of Guinea Pig Liver (0.25M Sucrose).

Animals	Fraction	Acid phosphatase	Beta glucuronidase	Cathepsin
Controls	Nuclear	16.1*	18.0*	27.5*
	Large granule	49.5	66.8	46.0
	Small "	19.8	9.9	22.0
	Supernatant	14.6	5.3	.5
	Total activity recovered (in units)	6.95†	2.76†	26.7‡
Vit A	Nuclear	18.7*	14.6*	28.8*
	Large granule	37.8	58.2	32.4
	Small "	22.0	16.9	18.3
	Supernatant	21.5	10.3	20.5
	Total activity recovered (in units)	5.53†	2.85†	29.5‡

* % of recovered activity.

† μg/phenolphthalein liberated/10.0 μg protein/hr.

‡ μg/acid-soluble tyrosine liberated/100 μg protein/hr.

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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