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*

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Following the death of cells, a number of previously inactive intracellular enzymes apparently become activated and bring about the autolysis of tissues. de Duve and his collaborators have presented evidence that such activation is due to the release of acid hydrolases from a heterogeneous group of particles called lysosomes (1). Within these granules, which sediment with the mitochondrial fraction of tissue homogenates prepared in sucrose, are found such enzymes as beta glucuronidase, acid phosphatase, acid DNAase and RNAase, the cathepsins, and other hydrolases having an acid pH optimum. The full activities of these enzymes may be released from lysosome-rich subcellular fractions by treatment with non-ionic detergents, by exposure to hypoosmolar conditions, or by repeated freezing and thawing. Recently, more "physiologic" means of releasing these potentially harmful enzymes from particles both *in vitro* and *in vivo* have been described, such as their exposure to an excess of vitamin A (2–4), irradiation with the mercury spectrum (5), or simple incubation at 37°C at pH 5 (1).

The possibility that these subcellular particles might be a target for the effects of bacterial lipopolysaccharides was suggested by Martini, who found that the free catheptic activity in homogenates of rat liver and rat muscle was increased after the injection of endotoxin, while the total activity was relatively unaffected (6). It was impossible to determine, however, whether this effect, which was observed after 2 hours, was the consequence of a primary action of endotoxin on lysosomes, or simply a secondary reaction to tissue damage or fever. When techniques became available for measuring the graded release of enzymes from suspensions rich in lysosomes, it was possible to test whether an effect on the stability of lysosomes might be an early result of the injection of bacterial endotoxin. Underlying this hypothesis was the

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assumption that many of the diverse actions of endotoxin injected *in vivo* might result from the liberation of active hydrolases into cell sap or circulation, causing, in effect, "antemortem autolysis."

The experiments described in this report demonstrate that promptly after the intravenous injection of *A. aerogenes* endotoxin in young rabbits, there is a significant increase in the release of two lysosomal enzymes from the large granule fraction of liver homogenates prepared in sucrose. Granular fractions from the livers of animals made tolerant to endotoxin, and subsequently challenged with a single injection, no longer responded in this manner, nor did fractions prepared from animals pretreated with cortisone and hydrocortisone.

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 1000 gm.

Endotoxin.—Bacterial lipopolysaccharide prepared from *A. aerogenes* was obtained through the courtesy of the Charles Pfizer Co., Brooklyn, New York as "preparation LPS." 10 μg of this material was sufficient to raise the body temperature of 18/18 rabbits by at least 1.5°C 1 hour after intravenous injection. The generalized Shwartzman reaction (7) was induced in 3/6 rabbits with two injections of 10 μg each, spaced 18 hours apart. At intervals described below, rabbits were injected in a marginal ear vein with 40 μg of endotoxin and killed by cervical luxation, followed by rapid exsanguination in a cold room.

Preparation of a Granular Fraction Containing Lysosomes.—A method somewhat modified from those described previously was employed (2, 5); all procedures were carried out in a cold room at 4°C. Following death, livers were minced in ice cold 0.25 M sucrose until free of gross blood. Samples of the wet tissues were weighed and a 1:10 *w/v* homogenate in 0.25 M sucrose was made in a glass homogenizer with a motor-driven teflon pestle at 900 RPM (Tri-R Stir-R, Tri-R Instruments Jamaica, New York). Nuclei, debris, and unbroken cells were separated by centrifugation at 800 *g* for 10 minutes in a Servall Superspeed centrifuge. The pellet was again resuspended as a 1:5 *w/v* suspension in sucrose and used as the "nuclear" fraction in subsequent experiments. The supernatant was centrifuged at 15,000 *g* for 20 minutes, and this pellet (which in similar homogenates of rat liver contained the bulk of mitochondria and lysosomes (2, 8)) was resuspended 1:5 *w/v* in sucrose. After a second centrifugation at 15,000 *g* for 20 minutes, the pellet was resuspended by hand in a glass homogenizer to a final 1:5 concentration in sucrose. This fraction, termed the "large granule fraction," was subjected to the procedures detailed below. The supernatant of the first, 15,000 *g* centrifugation (which contained smaller granules, ribosomes, and the cell sap) was termed the "supernatant" fraction. This fraction was directly assayed for the "free" activities of lysosomal enzymes.

Incubation and Irradiation.—Aliquots (usually 20 ml) of the large granule fraction were placed in 600 ml beakers to assure a maximum surface area, and fixed in a water bath at 37°C. To test the relative stability of lysosomes, granular fractions were subjected to two types of incubations; with and without irradiation. Simple incubation serves to release a modest amount of enzyme from such suspensions, whereas the effect of mercury arc irradiation is to release into the supernatant a considerable percentage of total activity of the large granule fraction of liver (5). Fractions to be irradiated were 32 cm beneath a Hanovia 100 watt high pressure mercury arc lamp, whose rated output of radiations below 3130 A is 250 microwatts/cm^2 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. There was no appreciable change of

temperature during the irradiation (±0.05°C) and no significant change in pH (6.56 ± 0.15). To exclude any possible effects of evaporation and low wavelength radiation, some samples were irradiated while covered with a thin (1.0 mm) quartz plate, and no difference in release of lysosomal enzymes between these and uncovered samples was detected. Another portion (usually 10 ml) of each large granule fraction was homogenized in a Waring blendor (micro attachment) for 5 minutes (9) and then incubated at 37°C to liberate the total activity of the enzymes. In early experiments, 0.1 per cent *v/v* triton X-100 (Rohm and Haas, Philadelphia) was added to samples assayed for "total" activity. Enzyme activity *per milliliter of homogenate* was equivalent to that which was freed in the blendor, but when expressed as *activity per microgram of protein*, was less than in the mechanically disrupted fractions. This finding led to adoption of the blendor technique for releasing total activity. Preliminary experiments also indicated that the optimal incubation time for demonstrating differences between control and experimental large granule fractions was 40 minutes, and this incubation period was used in all experiments.

Following incubation, suspensions were centrifuged at 15,000 *g* for 20 minutes for the third and last time, and the slightly turbid supernatants were analyzed for enzyme activity.

Cathepsin.—Proteolytic activity of the samples was determined on a 4 per cent *w/v* hemoglobin substrate in 0.1 M acetate buffer at pH 4 by a modification of the method of Anson (10) as described by Dingle (2). With each set of determinations, tubes containing substrate with pepsin, and tyrosine alone, were incubated as controls. Care was taken to keep the reactants at 4°C except when actually incubated. Results are expressed as per cent of the total activity released by homogenization in a Waring blendor, or as micrograms of acid-soluble tyrosine/μg protein/hour released by 1 ml of suspension at 37°C.

Betaglucuronidase was determined by the method of Fishman *et al.* (11) using phenolphthalein glucuronide as the substrate. Readings were at 550 mμ rather than at 540 mμ, since the absorbance of the chromogen under these conditions was greatest at this wavelength. Tubes containing the entire reaction mixture without phenolphthalein glucuronide served as controls; activity was measured in 0.2 to 0.5 ml of homogenates incubated for 30 minutes at 37°C. After incubation all samples were centrifuged at 900 *g* for 5 minutes to insure a clear solution. Results are expressed as the per cent of the total activity released by homogenization in a Waring blendor, or as micrograms of phenolphthalein/μg of protein/hour released by 1 ml of suspension at 37°C.

Protein was determined by the method of Lowry *et al.* (12) using crystalline egg white lysozyme as a standard. The absorbance increment caused by the reaction of Folin reagent with the amount of sucrose present in each sample was subtracted from the total.

Tolerance to endotoxin was induced in rabbits by the daily intravenous injection of endotoxin in gradual increments for a total of 3 weeks until 20 μg/day had been given for 3 days. Animals were then rested for 48 hours and challenged.

Cortisone acetate in aqueous suspension (Philadelphia Ampoule Laboratories) was given (50 mg/kg) intramuscularly for 3 days. On the 4th day, 50 mg/kg of hydrocortisone 21-sodium hemisuccinate (solu-cortef, Upjohn) was given intravenously before challenge with endotoxin. Animals pretreated in this manner, and control animals, were killed 30 minutes after challenge. One injection of hydrocortisone alone was without detectable effect on release of lysosomal enzymes from granules.

DOCA (desoxycorticosterone acetate, Ciba) was given intramuscularly (20 mg/kg) for 4 days, the last injection 30 minutes prior to injection of the challenging dose of endotoxin.

Analysis of Results.—Since all experiments were performed simultaneously with controls, *t* tests for paired samples were done to test significance. Since in most cases, however, this led to the awkward expression "per cent of per cent control activity" the majority of data have been plotted as group means, ±SD (standard deviation) and SEM (standard error of

the mean). This made possible direct comparison of the release of beta glucuronidase and cathepsin on the same ordinates.

RESULTS

The Effects of 40 μg of Endotoxin on the Release of Hydrolases from the Large Granule Fraction of Rabbit Liver

When animals were killed 30 minutes after 40 μg of endotoxin, there was a statistically significant increase of both catheptic and beta glucuronidase

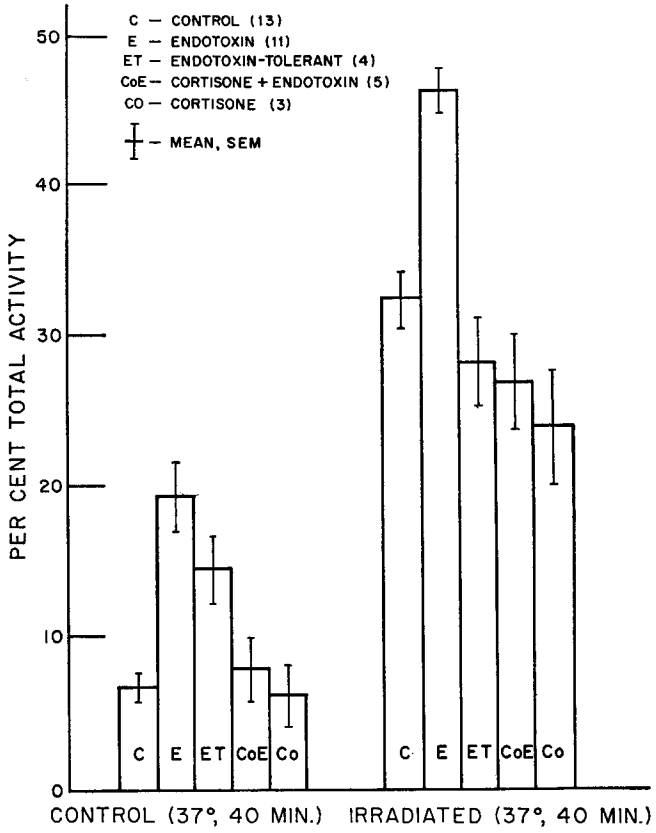

FIG. 1. Release of cathepsin into the supernatant (15,000 *g*) of a large granule fraction of rabbit liver homogenized in 0.25 M sucrose. Results are expressed as per cent of total activity (micrograms acid-soluble tyrosine/micrograms protein/hour) of a sample of the fraction exposed to a Waring blendor for 5 minutes. Denatured hemoglobin (4 per cent *w/v* in 0.1 M acetate buffer) was the substrate. Control fractions were incubated at 37°C for 40 minutes. Irradiated fractions were exposed to a mercury vapor lamp for 40 minutes at 37°C (SEM, standard error of the mean). See text for details of the pretreatment of animals before preparation of large granule fraction sedimenting between 800 *g* (10 minutes) and 15,000 *g* (20 minutes).

activity in the 15,000 *g* supernatants of both irradiated and non-irradiated large granule fractions incubated at 37°C. Similarly there was a trend towards a decrease in total catheptic activity of this fraction (see Figs. 1 and 2, Table I), while the depletion of beta glucuronidase was statistically significant. Ac-

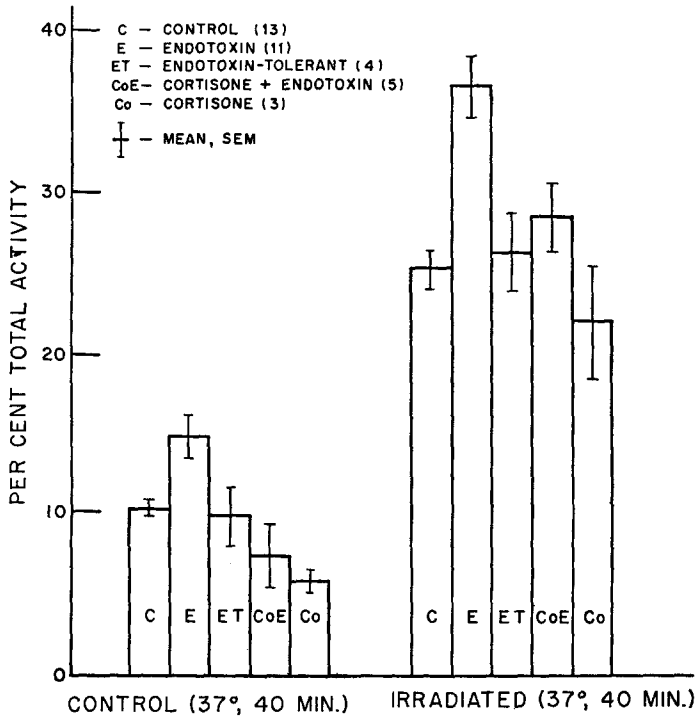

FIG. 2. Release of beta glucuronidase into the supernatant (15,000 *g*) of a large granule fraction of rabbit liver homogenized in 0.25 M sucrose. Results are expressed as per cent of total activity (micrograms phenolphthalein/micrograms protein/hour) of a sample of the fraction exposed to a Waring blendor for 5 minutes. Phenolphthalein glucuronide (0.001 M in acetate buffer pH 4.5, 0.1 M) was the substrate. Control fractions were incubated at 37°C for 40 minutes, irradiated fractions were exposed to a mercury vapor lamp for 40 minutes at 37°C (SEM, standard error of the mean). See text for details of the pretreatment of animals before preparation of large granule fraction sedimenting between 800 *g* (10 minutes) and 15,000 *g* (20 minutes).

companying a decrease in the total activity of these two hydrolases, was an increase in the free activities of 15,000 *g* supernatants of fresh homogenates, representing enzymes which were unsedimentable under these conditions (Fig. 3). This activity may have been present either in the cell supernatant, or in smaller granules, from which it could be released by further homogenization. Experiments clarifying this point will be presented below.

The Effect of Endotoxin Tolerance on Release of Hydrolases from the Large Granule Fraction of Rabbit Liver after Endotoxin

As may be seen in Table I and Figs. 1 and 2, the induction of tolerance to endotoxin largely abolished the increased liberation of large granule hydrolases, when compared to controls. However, there were significant decreases in the catheptic and beta glucuronidase activities of fractions prepared from the livers of tolerant animals. The free activities recovered in the 15,000 *g* supernatant did not differ from control values.

TABLE I A

Activities of Two Acid Hydrolases Released into the Supernatant of the Large Granule Fraction of Rabbit Liver Homogenized in 0.25 M Sucrose

Treatment* (No. of animals)	Treatment of fraction	Cathepsin				
		Per cent of total activity	±SD		Cathepsin units‡	±SD
Control (13)	Incubated	6.6	±3.2	Total§	25.5	±4.0
	Irradiated	32.4	±6.6	Free‖	1.5	±0.2
Endotoxin (11)	Incubated	19.4¶	±7.6	Total	21.2**	±3.4
	Irradiated	46.2¶	±9.9	Free	2.1	±0.6
Endotoxin - Tolerant (4)	Incubated	12.1	±4.0	Total	19.1**	±3.3
	Irradiated	28.5	±5.3	Free	0.8	±0.1
Cortisone + Endotoxin (5)	Incubated	7.9	±4.1	Total	22.3	±3.6
	Irradiated	27.0	±6.4	Free	0.2**	±0.1
Cortisone (3)	Incubated	6.1	±3.0	Total	22.0	±4.4
	Irradiated	24.0	±5.3	Free	0.0**	0.0

* See text for dosage schedule.

‡ Micrograms acid-soluble tyrosine/micrograms protein/hour.

§ Activity of fraction after 5 minutes in a Waring blendor.

‖ Activity of microsome and cell supernatant of whole homogenate.

¶ P of paired samples (*vs.* controls) < 0.01.

** P of paired samples (*vs.* controls) < 0.05.

The Effect of Pretreatment with Cortisone on Release of Hydrolases from the Large Granule Fraction of Rabbit Liver

Pretreatment of animals with cortisone alone caused a definite, although modest, decrease in the release of both enzymes, which was most readily seen in the samples both irradiated and incubated (Figs. 1 and 2, Table I). The most pronounced change effected was a decrease of measurable activity in the 15,000 *g* supernatants of fresh homogenates. These results are in agreement with those previously presented (5). When pretreated animals were challenged with endotoxin, no augmentation of enzyme release into the supernatants of incubated and irradiated large granule fractions was observed. Indeed most

activities were below control values. Free activities were also significantly lower than normal. The decrease in total beta glucuronidase activities of the large granule was found in both those animals treated with cortisone alone, and in those treated with cortisone and challenged with endotoxin. No ready explanation for the discrepancy between this finding and the maintenance of normal catheptic activity in both groups is apparent.

The Effects of Pretreatment with DOCA.—Animals were pretreated with

TABLE I B

Activities of Two Acid Hydrolases Released into the Supernatant of the Large Granule Fraction of Rabbit Liver Homogenized in 0.25 M Sucrose

Treatment* (No. of animals)	Treatment of fraction	Beta Glucuronidase: Per cent of total activity	±SD		Beta glucuronidase units‡	±SD
Control (13)	Incubated	10.3	±2.1	Total§	12.5	±1.6
	Irradiated	25.3	±4.1	Free‖	0.24	±0.04
Endotoxin (11)	Incubated	14.8¶	±4.1	Total	7.6¶	±1.1
	Irradiated	36.6¶	±5.9	Free	0.29	±0.05
Endotoxin - Tolerant (4)	Incubated	9.8	±3.2	Total	6.6¶	±1.0
	Irradiated	26.4	±4.3	Free	0.23	±0.04
Cortisone + Endotoxin (5)	Incubated	7.3	±3.8	Total	6.9**	±1.3
	Irradiated	28.5	±4.3	Free	0.15**	±0.02
Cortisone (3)	Incubated	7.4	±4.0	Total	7.7	±1.9
	Irradiated	22.1	±5.1	Free	0.14**	±0.02

* See text for dosage schedule.

‡ Micrograms phenolphthalein/micrograms protein/hour.

§ Activity of fraction after 5 minutes in a Waring blendor.

‖ Activity of microsome and cell supernatant of whole homogenate.

¶ P of paired samples (*vs.* controls) < 0.01.

** P of paired samples (*vs.* controls) < 0.05.

20 mg/kg of DOCA daily for 4 days (a dose pharmacologically more drastic than that of cortisone). The results are listed in Table II and represent the means of two experiments. No effects comparable to those of the glucocorticoids were evident. The enzyme activities released by all fractions after DOCA and endotoxin were comparable to those released by fractions prepared from control animals injected with endotoxin alone (compare Table I).

The Effect of Endotoxin on the Distribution of Hydrolase Activity in Rabbit Liver

Fractions were prepared from the livers of control animals and from rabbits injected with 40 μg of endotoxin 30 minutes before death. Nuclear, large granule, and supernatant fractions were prepared as described above. Portions of each fraction, and of the whole liver homogenates, were mixed in a Waring blendor for 5 minutes and then incubated at 37°C. To determine that amount of enzyme which might be passively released under physiologic conditions,

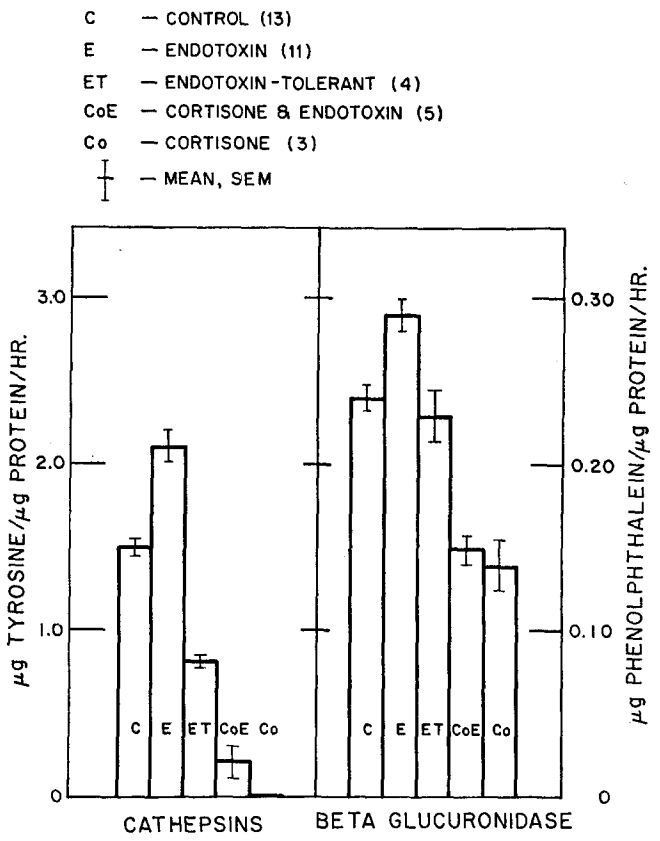

FIG. 3. Free activities of two acid hydrolases in the supernatant (15,000 *g*) of rabbit liver homogenates prepared in 0.25 M sucrose. Results expressed as indicated on ordinates. Aliquots of this fraction, representing small granules, ribosomes, and cell supernatant were directly assayed for enzyme activity (SEM, standard error of the mean). See text for details of pretreatment of animals before preparation of homogenate.

another portion was incubated in the unbuffered, near neutral sucrose for 40 minutes at 37°. All fractions were then centrifuged at 15,000 *g* for 20 minutes and the supernatants assayed. The results, expressed as the means of three experiments, are listed in Table III.

The per cent of enzyme activity released by simple incubation was greater in each endotoxin-treated fraction than in the controls, both as beta glucuronidase and as cathepsin. After endotoxin there appeared to be a modest diminution in the total activity (per microgram of protein) in all fractions: this was greatest for beta glucuronidase. When the activities of samples that had been exposed to the blendor were measured, no appreciable change in the distribution of enzyme activities after endotoxin was found; this was in sharp contrast to

TABLE II

The Effect of Pretreatment with DOCA on the Activities of Two Acid Hydrolases Released into the Supernatant (15,000 g) of the Large Granule Fraction of Rabbit Liver Homogenized in 0.25 M Sucrose

Treatment of animals*	Treatment of fraction	Per cent of total activity		Cathepsin units‡	Treatment of fraction	Per cent of total activity		Beta glucuronidase units§
DOCA + endotoxin	Incubated	18.7	Total	20.4	Incubated	15.1	Total	6.9
	Irradiated	47.3	Free	2.3	Irradiated	37.8	Free	0.28
DOCA alone	Incubated	7.2	Total	24.3	Incubated	11.0	Total	14.3
	Irradiated	31.8	Free	1.7	Irradiated	24.2	Free	0.20

* See text for dosage schedule. Results are means of two experiments.

‡ Micrograms acid-soluble tyrosine/micrograms protein/hour.

§ Micrograms phenolphthalein/micrograms protein/hour.

TABLE III

Effect of Intravenous Endotoxin on the Subcellular Distribution of Two Acid Hydrolases

Enzyme	Subcellular fraction	Treatment*	Total activity released in Waring blendor	Per cent of total recovered activity of homogenate	Per cent of total activity released by incubation (37°C, 40 min.)
Beta glucuronidase	Nuclei and unbroken cells (< 800 *g*)	Control	10.0‡	40.5	11.5
		Endotoxin	6.5	36.0	11.9
(Total recovery >180 per cent)	Large granules (800–15,000 *g*)	Control	12.5	50.6	8.4
		Endotoxin	10.4	57.8	11.3
	Supernatant and small granules (> 15,000 *g*)	Control	2.2	8.9	68.1
		Endotoxin	1.1	6.2	87.0
Cathepsin	Nuclei and unbroken cells (< 800 *g*)	Control	10.4§	26.8	23.3
		Endotoxin	10.6	28.0	43.6
(Total recovery >140 per cent)	Large granules (800–15,000 *g*)	Control	24.0	61.3	8.4
		Endotoxin	23.6	62.5	13.8
	Supernatant and small granules (> 15,000 *g*)	Control	4.8	11.9	69.2
		Endotoxin	3.6	9.5	119.0

* See text for dosage, etc. Results are means of three experiments.

‡ Beta glucuronidase, micrograms phenolphthalein/micrograms protein/hour.

§ Cathepsin, micrograms tyrosine/micrograms protein/hour.

findings when activities were determined *without* prior disintegration. Enzyme activity in the fractions non-sedimentable at 15,000 *g* was approximately 70 per cent for both enzymes (determined after simple incubation) indicating that most of the enzymes of this fraction had ready access to substrate. In contrast, only 8.4 per cent of the enzyme activity in the large granule fraction could be

detected without alteration of the suspension, either by pretreatment with endotoxin, or by mechanical disintegration. Nearly 100 per cent of enzyme activity was freely measurable after simple incubation of the supernatant fractions prepared from endotoxin-treated animals. This finding is in accord with the data presented in Fig. 3 which show the increased free activity of both hydrolases in these fractions. The relative inhomogeneity of the lysosomal suspensions may be appreciated: some of the smaller granules present in control samples appear to have remained intact in the 15,000 *g* supernatant, where their enzymes were made available to substrate by mechanical rupture.

TABLE IV

The Effect of Prior Freezing and Thawing on the Activities of Two Acid Hydrolases Released into the Supernatant (15,000 g) of the Large Granule Fraction of Rabbit Liver Homogenized in 0.25 M Sucrose

Treatment of animals*	Treatment of fractions‡	Cathepsin					Beta glucuronidase				
		Per cent of total activity§		Cathepsin units‖			Per cent of total activity§		Beta glucuronidase units¶		
Controls	Incubated	88.5	(6.6)	Total	22.4	(25.5)	66.8	(10.3)	Total	12.0	(12.4)
	Irradiated	55.0	(32.4)	Free	1.0	(1.5)	64.0	(25.3)	Free	0.39	(0.24)
Endotoxin	Incubated	70.0	(19.4)	Total	20.2	(21.2)	70.4	(14.8)	Total	6.6	(7.6)
	Irradiated	53.0	(46.2)	Free	0.5	(2.1)	70.2	(36.6)	Free	0.30	(0.29)

* See text for dosage schedule. Results are means of two experiments.

‡ All fractions frozen 18 hours at −20°C, thawed, and treated.

§ Figures in parentheses represent values for non-frozen and thawed samples.

‖ Micrograms of acid-soluble tyrosine/micrograms protein/hour.

¶ Micrograms of phenolphthalein/micrograms protein/hour.

The Effect of Freezing on the Release of Hydrolases from the Large Granule Fraction of Rabbit Liver

An increased liberation of enzymes from large granules might not be due to a direct effect of mercury arc irradiation or of endotoxin upon subcellular particles, but might result from an increase in the suspensions after endotoxin pretreatment or irradiation, of "activating" cofactors, metabolites, or hormones. Were such a mechanism to act *independently* of structured subcellular particles, the simple freezing and thawing of large granule fractions would abolish neither the effects of endotoxin, nor of mercury arc irradiation, since the hypothetical agent(s) would still be present. The participation of mitochondria cannot of course be excluded; de Duve (1) has established that freezing and thawing effect the release of enzymes from both lysosomes *and* mitochondria.

To test this possibility, large granule fractions and supernatants, prepared in the usual manner were frozen at 20°C for 18 hours, allowed to thaw at 4°C, and then incubated and irradiated as before. The results, expressed as the mean

of three experiments, are listed in Table IV. It is apparent that the increased liberation of enzymes due either to pretreatment with endotoxin or to mercury arc irradiation was essentially abolished. Indeed, the acid protease activity of irradiated samples was less than that of fractions which had been simply in-

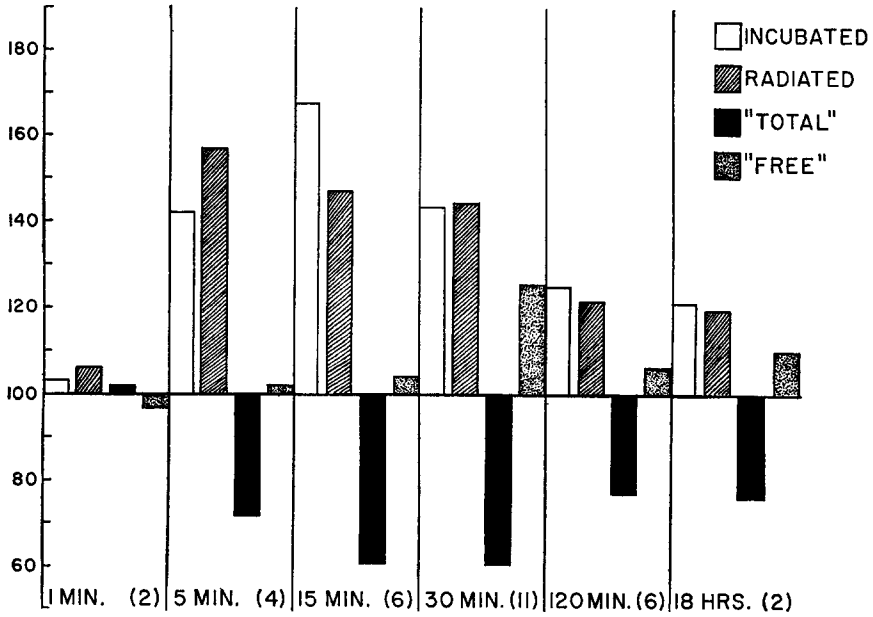

FIG. 4. Beta glucuronidase activities of four fractions prepared from rabbit liver homogenized in 0.25 M sucrose at varying times after animals had received 40 μg of *A. aerogenes* endotoxin. All results are expressed as per cent of control values which were obtained from litter mates not injected with endotoxin. Time indicated in minutes or hours, number of animals in parentheses. "Incubated" and "irradiated" samples represent supernatants of large granule fractions sedimenting between 800 *g* (10 minutes) and 15,000 *g* (20 minutes), which had either been simply incubated at 37°C for 40 minutes, or been exposed to a mercury vapor lamp for 40 minutes at 37°C. "Total" activities were determined by exposing samples of this fraction to a Waring blendor for 5 minutes, incubating, and determining the activities in a 15,000 *g* supernatant. Free activities represent enzyme which was not sedimentable at 15,000 *g* in the original homogenate.

cubated; this effect may be due to inactivation of the enzyme by the mixed beam in the absence of intact granules absorbing in the ultraviolet.

The Release of Hydrolases from the Large Granule Fraction of Rabbit Livers Removed at Varying Times after Endotoxin

Fractions were prepared from the livers of young rabbits at intervals of 1, 5, 15, 30, and 120 minutes, and 18 hours after 40 μg of endotoxin had been injected intravenously. Release of enzymes from incubated and irradiated large granule fractions, and free activities of 15,000 *g*

supernatants, and the total activity of the large granule fractions, are shown in Figs. 4 and 5. These have been expressed as per cent of control activities which have been assigned values of 100 per cent.

Beta Glucuronidase.—Five minutes after endotoxin, but not after 1 minute, there was an increase in the activity of this enzyme, released into the super-

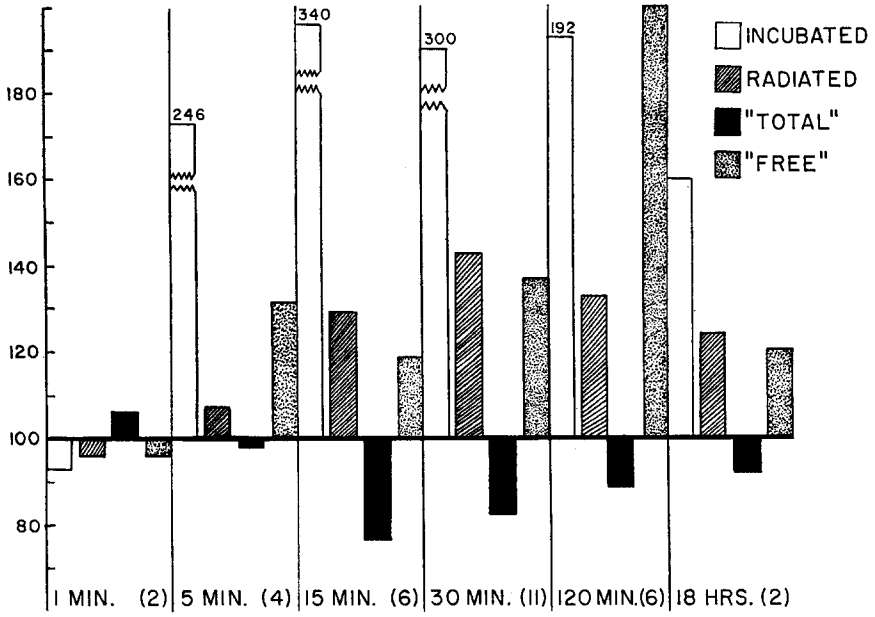

FIG. 5. Cathepsin activities of four fractions prepared from rabbit liver homogenized in 0.25 M sucrose at varying times after animals had received 40 μg of *A. aerogenes* endotoxin. All results are expressed as per cent of control values which were obtained from litter mates not injected with endotoxin. Time indicated in minutes or hours, number of animals is in parentheses. Incubated and irradiated samples represent supernatants of large granule fractions sedimenting between 800 *g* (10 minutes) and 15,000 *g* (20 minutes) which had either been simply incubated at 37°C for 40 minutes or been exposed to a mercury vapor lamp for 40 minutes at 37°C. Total activities were determined by exposing samples of this fraction to a Waring blendor for 5 minutes, incubating, and determining the activities in a 15,000 *g* supernatant. Free activities represent enzyme which was not sedimentable at 15,000 *g* in the original homogenate.

natants of both incubated and irradiated suspensions amounting to 140 to 150 per cent of the control values. This increase lasted for at least 30 minutes before dropping to 120 per cent of control values at 2 and 18 hours. The amount of enzyme activity remaining in the large granule fraction was decreased to 72 per cent of control values after 5 minutes, remained at 60 per cent of control values for at least 30 minutes, and then remained at approximately 80 per cent of controls for 2 and 18 hours after endotoxin. Not until 30 minutes after endo-

toxin was there a significant increase in the free activity of this enzyme. It must be noted, however, that the activities of this fraction were so low as to make interpretation of these data difficult (*cf.* Table I).

Cathepsin.—Five minutes after endotoxin, but not after 1 minute, there was a marked increase in the activity of acid protease in the incubated large granule fraction, which reached a peak somewhere between 15 and 30 minutes after endotoxin. Moderate increases, more in accord with comparable figures for beta glucuronidase, were found in fractions subjected to mercury arc irradiation. Here the increases were maximum at 15 to 30 minutes, when activities of 130 and 140 per cent of control values were found in supernatants. Depletion of catheptic activity of the large granule fraction reached its maximum at 15 to 30 minutes after endotoxin: 76 per cent of control values. Free activity was maximal at 2 hours after endotoxin; activity per microgram of protein in this fraction was quite low (see Table I).

There was rough general agreement between the results for beta glucuronidase and the acid protease suggesting that the effects of endotoxin were mediated through a mechanism common to both enzymes. Discrepancies were greatest in those fractions in which the enzyme activity/microgram protein was lowest (Table I).

DISCUSSION

The results presented above suggest that two acid hydrolases are released more readily from the large granule fractions of rabbit liver *in vitro*, when animals are injected with bacterial endotoxin. Within 5 to 30 minutes after endotoxin, there is partial depletion of the total beta glucuronidase and cathepsin activity of the large granule fractions, accompanied by an increase in activity not sedimentable with these particles. These data are consistent with the hypothesis that an early consequence of the injection of endotoxin in young rabbits is an altered stability of certain lysosomes, with subsequent release of their enzymes into cell sap or surrounding fluids.

Several earlier studies would suggest the validity of this hypothesis. Cohn and Hirsch (13) have demonstrated the similarity of the leukocyte granules of the rabbit to the lysosomes of rat liver described by de Duve *et al.* (14). Following the phagocytosis of bacteria or inert particles, leukocytes became degranulated and their enzymes were liberated into the non-sedimentable portion of cell homogenates in sucrose. Previously, Strauss and Stetson (15) had shown that endotoxin caused an increased oxygen uptake by leukocytes, an effect resembling that initiated by phagocytosis of inert particles. Evans and Karnofsky (16) postulated that this metabolic response to phagocytosis may be accompanied by the liberation of DPNH oxidase activity from granular form. Kerby (17) had demonstrated that endotoxin *in vitro* liberated lysozyme, a lysosomal enzyme, from leukocytes and this effect was held responsible for the

increases in plasma lysozyme levels after the intravenous injection of endotoxin in rabbits (18). These studies, however, involved a very specialized cytoplasmic granule, and it was not until the experiments of Martini (6) that involvement of the lysosomes of other tissues by bacterial endotoxins became a definite possibility. Finally, there is ample evidence that cortisone pretreatment will prevent the shock-producing and lethal effects of endotoxin (7).

Young rabbits, which are relatively resistant to the lethal effects of endotoxin, were used in the present experiments. This made it possible to compare early and late effects of injections of lipopolysaccharide without complications introduced by profound vascular collapse or the death of a number of animals. The large granule fraction of liver was employed only as a source of readily available lysosomes; any generalization to other tissues is unwarranted. Indeed, it is impossible to be certain whether any direct effect upon lysosomes has been demonstrated. The granular fraction employed in similar studies contained at least 80 to 90 per cent mitochondria when examined by electron microscopy (19). However, active mitochondrial metabolism would be minimal under the above conditions of incubation: without adequate aeration and without agitation. In fact, in the only vessels with adequate aeration, ultraviolet light of an intensity known to uncouple oxidative phosphorylation (20) served to exclude the major effects of mitochondrial respiration. In any experiments involving granular fractions not subjected to prolonged density gradient procedures, effects upon mitochondria and lysosomes cannot readily be distinguished from each other, and this would be as true of "mitochondrial swelling" experiments (21) as of the ones described above. We have been concerned exclusively with the liberation of lysosomal enzymes and have not studied the actions of endotoxin on the mitochondria. It is possible that lipopolysaccharides may have general effects upon other subcellular particles and/or their limiting membranes.

Mercury arc irradiation may not actually test the "stability" of the lysosomes; in fact the view of such particles as simply sacs of hydrolytic enzymes surrounded by a lipoprotein membrane, as postulated by de Duve (1), has been repeatedly challenged (22, 23). Experiments showing that the release of enzymes from lysosomes by an excess of vitamin A (2) and ultraviolet irradiation (5) is dependent upon pH and temperature, suggest, however, that systems analogous to those responsible for the integrity of the red cell membrane may be operative (24).

Freezing and thawing, as reported above, released most of the enzyme activity present in the granules and eliminated the augmented release that followed endotoxin pretreatment or mercury arc irradiation. Were either of these procedures to act by increasing the concentration of cofactors, activating a proenzyme, destroying an inhibitor, or increasing the available oxygen of the suspension, their effects should not be abolished by freezing and thawing. Thus,

the probability of effecting quantitatively similar changes in two separate enzymes through these means would seem slight. These experiments favor the interpretation that freezing and thawing, mercury arc irradiation, and endotoxin pretreatment all act upon the surface of a subcellular particle, to release bound enzyme. With each of the means chosen to injure the lysosomes, the activity of cathepsin in the supernatants was greater (when expressed as per cent of total) than that of beta glucuronidase. Preliminary experiments with Dr. John Ayvazian indicate that acid DNAase, while behaving in the same manner as the other two enzymes in response to mercury arc irradiation and endotoxin, is also released more slowly than cathepsin. This would be in accord with the suggestion of de Duve (1) that the initial step in autolysis may be the release of protease, which in turn digests the lysosomal membrane to liberate the other hydrolases.

It is possible that the effects described as due to endotoxin may have been secondary to vascular alterations induced *in vivo* by endotoxin. The prompt changes in stability of lysosomes to incubation and irradiation, which were observed 5 minutes after an injection of endotoxin, would argue against this view; ligature of a whole branch of the hepatic artery does not affect the distribution of lysosomal enzymes in the anoxic lobe until a later time (1). No histologic evidence of tissue necrosis is seen this soon after endotoxin (7) nor do macrophages which are rich in lysosomal enzymes (25) accumulate in reaction to cell damage.

The experiments are pertinent in a consideration of tolerance to endotoxin. Repeated injections of bacterial lipopolysaccharides may leave a relatively resistant population of lysosomes remaining in the tissues, which would react slowly to the challenging dose. Evidence has accumulated that exogenous (26, 27) and perhaps endogenous (28) proteases in human and rabbit serum are bound to alpha globulins which prevent their entry into tissue spaces. Thus endotoxin-resistant animals may release potentially harmful enzymes more slowly from more resistant lysosomes, at rates not exceeding the capacity of carrier proteins in serum. A similar phenomenon has been held responsible for the acquisition of tolerance to traumatic shock in rats, and may underlie the mechanism of tolerance to a variety of injurious agents (29).

A protective effect of hydrocortisone upon granules rich in lysosomes has been reported before (5). In organ culture studies of the rudimentary limb cartilages of chicks (30) and the fetal skin of rats (31), hydrocortisone *in vitro* has been found to be protective against two agents active on lysosomes: excess of vitamin A or irradiation with ultraviolet light below 3000 A. In the experiments with endotoxin, conditions were chosen which partially overcame the protection against irradiation afforded by cortisone pretreatment. But even at this level of radiation the steroid diminished the effect of endotoxin on the release of enzymes from granular form. It appears unlikely that such an action

is due to a non-specific absorption of radiant energy by any steroid configuration, since DOCA was ineffective. Cortisone also caused less lability of the granules to simple incubation, and decreased the non-sedimentable activity of the initial homogenates. These findings support the observations of de Duve *et al.* (32) who showed that hydrocortisone *in vitro* retarded the release of acid phosphatase from similar fractions of rat liver incubated at pH 5. The pharmacologic effects of glucocorticoids in inflammation may be mediated through their action upon the integrity of lysosomes. In cartilage, both *in vivo* and *in vitro*, hydrocortisone antagonizes the effects of an excess of vitamin A (30, 33). The vitamin causes release of a protease from lysosomes which is active upon the protein-polysaccharide complex of cartilage matrix (3); this effect is retarded in the presence of hydrocortisone. The presumed *in vivo* activation of lysosomal enzymes by vitamin A acid in the larvae of *Xenopus laevis* is also inhibited by the simultaneous administration of hydrocortisone (34). Similarly, pretreatment with hydrocortisone protects skin *in vivo* (35) and *in vitro* (31) against the vesication and digestion of intercellular connective tissue which follow ultraviolet irradiation. Thus glucocorticoids, in a variety of experimental situations, appear to decrease the liberation of potentially harmful enzymes from lysosomes, and may in fact function physiologically to stabilize the boundaries of these subcellular particles.

SUMMARY

Granular fractions sedimenting between 800 *g* and 15,000 *g* have been prepared in 0.25 M sucrose from the livers of normal young rabbits and from the livers of rabbits injected with *A. aerogenes* endotoxin. As early as 5 minutes after endotoxin, there was an augmented release of two enzymes, beta glucuronidase and cathepsin, into the supernatant of fractions which had been incubated or incubated and irradiated with a mercury vapor lamp at 37°C. These effects were maximum at 30 minutes after endotoxin and were associated with a depletion of the total activities of the two enzymes within the granules. Concurrently there was a rise in the activity of the two enzymes in fractions of the homogenate which were unsedimentable at 15,000 *g*. Fractions prepared from animals made tolerant to endotoxin no longer responded to incubation and irradiation by an augmented release of these two hydrolases, nor did fractions prepared from animals pretreated with glucocorticoids. Pretreatment with DOCA did not prevent release of enzymes after endotoxin.

The results have been interpreted to indicate that one consequence of the injection of endotoxin is an effect upon the stability of lysosomes, with subsequent release of acid hydrolases into the cell sap or surrounding tissue. Glucocorticoids may exert their pharmacologic effects through the protection of these subcellular particles against a variety of injurious agents.

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