LYSOSOMAL ACID HYDROLASES IN MICE INFECTED WITH BCG*

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Infection of laboratory animals with BCG, an attenuated strain of tubercle bacilli, results in functional changes of the RES[1], especially enhanced activity. The heightened phagocytic capacity may well contribute toward resistance of such animals to homologous and heterologous virulent infection (2). One of the consequences of altered activity of the RES induced by infection with BCG is the well documented inhibitory effect of MP against phagocytized tubercle bacilli observed *in vivo* and *in vitro* (3, 4). At present it is best to assume that this inhibitory action may be the result of specific reactions based on the immune response of the animals, *i.e.* cellular or delayed hypersensitivity, combined with non-specific cellular responses to the immunizing stimulus (5). Such a reaction, *e.g.* increase of cellular acid phosphatase, has been observed histochemically in MP of granulomatous lesions (6) or in Kupffer's cells of the liver (7), and quantitatively in cultured peritoneal MP from BCG-infected rabbits (8); some workers, however, reported contradictory results (9, 10).

Lysosomes which were described by de Duve (11, 12) as a new group of cytoplasmic particles containing a variety of acid hydrolases are regarded to play an essential role in intracellular digestion of phagocytized and pinocytosed materials. Indeed, Cohn and Hirsch (13) and others (14, 15) clearly demonstrated the transfer of acid hydrolases from lysosomal granules to the phagocytic vacuoles in polymorphonuclear leucocytes and in MP. In this connection, the present investigation was undertaken to detect any changes in content of acid hydrolases in BCG-infected animals and to determine the distribution of

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[1] The following abbreviations are used in this paper: RES, reticuloendothelial system; MP, macrophages, histiocytes, and monocytes; saline, physiologic sodium chloride solution; BSS, balanced salt solution.

these enzymes in various isolated subcellular fractions of mechanically disrupted liver homogenates. The results obtained indicate that BCG infection causes a general elevation of the level of some acid hydrolases, and that the increase of these enzymes in the liver is mainly confined to a "large granular" fraction which consists of mitochondria and lysosomes.

Materials and Methods

Animals.—Female Swiss white mice of the ICR strain were purchased from a commercial breeder. The animals were kept in air-conditioned quarters, maintained at 25°C, and fed pellets and water *ad libitum*.

Infection with BCG.—Mice were injected intravenously with 0.2 ml of a 10- to 14-day-old culture of BCG grown in liquid medium containing tween 80 and albumin. The mice were used 10 to 14 days after the injection. In all experiments non-infected mice of the same batch served as controls.

Preparation of Cells and Tissues for Determination of Acid Hydrolases.—

Peritoneal macrophages: Suspensions of peritoneal MP were obtained without prior stimulation by washing the peritoneal cavity with 2.5 to 3.0 ml of Hanks' BSS containing 1:20,000 of heparin. The MP suspension was centrifuged at 1000 RPM (International refrigerated centrifuge, model PR-2) for 10 minutes at 4°C. The pellet of the cells was then washed once and finally suspended in saline[1] to an appropriate concentration. The number of cells was determined in a hemocytometer. The MP suspension thus prepared was treated with sonic oscillation at 10 kc/second (Raytheon sonic oscillator) for 10 minutes, and the suspension of the sonicated MP was used for determination of the enzymes. For experiments with cultured MP, monolayers of MP were prepared. Aliquots of 1 ml of a suspension in BSS with 10 per cent calf serum containing 2.5 to 3.5 $\times 10^6$ MP were placed into a number of Leighton tubes immediately after collection of the cells from the mice. The tubes were then kept at 37°C for 1.5 to 2 hours to allow the cells to settle on the flat surface of the tubes. After decanting the supernatant fluid, 1 ml of BSS containing 20 per cent of heat-inactivated calf serum was added to each tube, and the monolayer of MP was kept in a stationary state at 37°C under 2 per cent CO_2 in air. Phenol red was omitted from Hanks' BSS, because the color of the dye interfered with the colorimetric determinations of the enzymes. The pH of the culture medium was checked using a pH meter. Heat-inactivation of calf serum at 56°C for 60 minutes was necessary since calf serum contains rather high levels of acid hydrolases. For the determination of the enzymes after 3 washings with saline, the MP were harvested from the walls of the tubes with a rubber policeman into 0.1 M acetate buffer, pH 5.2. Cell suspensions from 3 to 5 tubes of the same experimental group were pooled and were treated with sonic oscillation as described.

Liver: Liver was removed from the mouse after perfusion with 5 ml of cold saline through the portal vein system for the purpose of expelling the blood. After washing with cold saline, the livers were homogenized in a glass homogenizer with a motor-driven teflon pestle for 2 minutes to make a 10 per cent w/v homogenate in saline. Coarse tissue fragments were removed by centrifugation at 300 RPM for 3 minutes and the supernatant was frozen and thawed six times in an ethanol dry ice bath. The homogenate was centrifuged at 2500 RPM for 15 minutes and the supernatant was used for enzymatic assays.

Blood plasma: Blood was collected by heart puncture using syringe and needle which were previously wetted with 16 per cent sodium citrate solution. The plasma was separated by centrifugation at 1000 RPM (International refrigerated centrifuge, model PR-2) for 10 minutes. During the preparation of plasma, precautions were taken to avoid disruption of blood cells.

Separation of subcellular fractions from liver homogenate: The livers collected as described above were washed in cold 0.25 M sucrose and were homogenized in a glass homogenizer

with a motor-driven teflon pestle for 2 minutes to make a 10 per cent w/v homogenate in 0.25 M sucrose solution. Then the homogenate was separated into various fractions by centrifugation (Servall refrigerated-automatic centrifuge) according to the method of Weissmann and Thomas (16). Thus, nuclei and unbroken cells were separated by centrifugation at 800 *g* for 10 minutes. The pellet was washed once with 0.25 M sucrose by centrifugation at 800 *g* for 10 minutes and the pellet was resuspended as a 1:5 w/v suspension in 0.25 M sucrose and termed the "nuclear" fraction. The supernatant of the first 800 *g* centrifugation was centrifuged at 15,000 *g* for 20 minutes, and this pellet was washed once with sucrose by centrifugation at 15,000 *g* for 20 minutes. The washed pellet, which contained the bulk of mitochondria, lysosomes, and microsomal elements, was resuspended in 0.25 M sucrose to make a 1:5 w/v suspension, and used as the "large granular" fraction. The supernatant of the first 15,000 *g* centrifugation (which contained smaller granules, ribosomes, and the cell sap) was used as the "supernatant" fraction. Plate 55 shows electron micrographs of the large granular fraction which was fixed with osmium oxide and then centrifuged at top speed for several minutes in an International clinical centrifuge to separate two layers of particles. The lower phase (Fig. 1) contained mainly structures characteristic for mitochondria and lysosomes, whereas the upper phase (Fig. 2) consisted of microsomes. All the fractions, except supernatant fraction, were frozen and thawed six times in an ethanol dry ice bath and were centrifuged at 15,000 *g* for 20 minutes. The clear or faintly clouded supernatants were used for enzymatic determinations.

For each enzymatic determination materials from at least 3 similarly treated mice were pooled. The temperature was kept between 0° and 5°C during the preparation of all specimens.

Enzymatic and Chemical Determinations.—As representatives for lysosomal acid hydrolases, acid phosphatase, β-glucuronidase, and cathepsin were examined.

Acid phosphatase was determined by the method of Lowry *et al.* (17). As substrate 2 mM of *p*-nitrophenylphosphate disodium 4 H_2O (California Corporation for Biochemical Research, Inc., Los Angeles) were used in 1.0 ml of reaction mixture, and the reaction was carried out in 0.1 M acetate buffer, pH 5.2, containing 1 mM $MgSO_4$. After incubation for 40 minutes in a 37°C water bath, the reaction was stopped by the addition of trichloroacetic acid, and the reaction product, *p*-nitrophenol, in the clear supernatant, was determined in a Beckman DU spectrophotometer at 410 mμ under alkaline condition.

Beta glucuronidase was determined according to the method of Talalay, Fishman, and Huggins (18). As substrate 0.625 mM of phenolphthalein glucuronidate (Sigma Chemical Company, St. Louis, Missouri) was used in 1.6 ml reaction mixture. The reaction was carried out in 0.1 M acetate buffer at pH 4.5. After incubation for 1 hour in a 37°C water bath, the reaction was stopped by the addition of 95 per cent ethanol. The liberated phenolphthalein was determined at 540 mμ after the addition of 0.4 M glycine buffer of pH 10.5. The final pH was checked and it was between 10.20 and 10.40. Undiluted preparations of blood plasma sometimes had a faint color or opalescence after the addition of ethanol. If this was the case, phenolphthalein was decolorized after the first colorimetric reading by the addition of a small amount of concentrated HCl. A second reading was then made for blank correction.

Cathepsin was determined as cathepsin D according to the method of Press, Porter, and Cebra (19). A 2.5 per cent solution of hemoglobin (Sigma Chemical Company) was used as substrate, and the reaction was carried out in 0.4 M citrate buffer at pH 3.0. After incubation at 37°C for 20 minutes trichloroacetic acid was added to the reaction mixture. The precipitate was removed by filtration. The absorption of the clear filtrate at 280 mμ was measured against a reagent blank which was carried out by adding trichloroacetic acid before the period of incubation. Tyrosine was used as a standard, and the results were expressed as tyrosine-equivalent.

Protein contents in MP sonicates, liver homogenates, and subcellular fractions isolated from liver homogenates were determined by the method of Lowry *et al.* (20) using crystallized bovine serum albumin (Pentex Inc., Kankakee, Illinois) as standard.

RESULTS

Acid Hydrolases in BCG-Infected Mice.—Activities of acid phosphatase, β-glucuronidase, and cathepsin in MP, liver, and blood plasma were determined 10 to 14 days after BCG inoculation and in normal animals. The results are summarized in Table I.

MP from BCG-infected mice exhibited about 1.9, 1.6, and 1.5 times as much activities per mg cell protein of acid phosphatase, β-glucuronidase, and cathep-

TABLE I

Effect of BCG Infection in Acid Hydrolases in Peritoneal Macrophages (MP), Liver, and Blood Plasma

Enzymes*	Peritoneal MP		Liver		Plasma	
	Control	BCG	Control	BCG	Control	BCG
Acid phosphatase						
(a)	0.365 ± 0.099 (11)‡	0.687 ± 0.133 (11)	0.838 ± 0.116 (4)	1.164 ± 0.128 (4)	—	—
(b)	0.030 ± 0.009 (11)	0.069 ± 0.016 (11)	—	—	—	—
(c)	—	—	—	—	1.047 ± 0.431 (5)	2.300 ± 0.804 (5)
β-glucuronidase						
(a)	73.0 ± 11.1 (11)	117.9 ± 15.8 (11)	25.5 ± 2.4 (4)	45.3 ± 7.6 (4)	—	—
(b)	5.7 ± 1.1 (11)	11.0 ± 2.4 (11)	—	—	—	
(c)	—	—	—	—	0.9 ± 1.3 (10)	16.6 ± 9.7 (10)
Cathepsin						
(a)	160.1 ± 19.0 (3)	239.0 ± 17.7 (3)	39.2 ± 11.7 (4)	66.8 ± 11.7 (4)	—	—
(b)	11.7 ± 1.0 (3)	25.3 ± 4.6 (3)	—	—	—	—

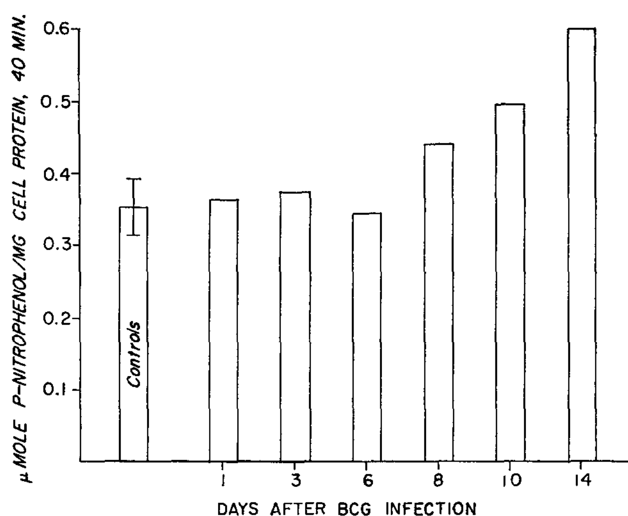
* Activities of enzymes are expressed as follows: Acid phosphatase, μmole of *p*-nitrophenol produced in 40 minutes; β-glucuronidase, μg of phenolphthalein produced in one hour; cathepsin, μg of tyrosine-equivalent produced in 20 minutes. (*a*) per 1 mg protein, (*b*) per 1 × 10^6 cells, (*c*) per 1 ml plasma.

‡ Number of determinations.

sin, respectively, as those from normal mice. When based on cell count rather than on protein the differences in the three enzymes between normal and BCG-infected mice became greater. This indicates that cells derived from BCG-infected mice contained approximately twenty per cent more protein than normal cells. The liver from BCG-infected mice exhibited about 1.4, 1.8, and 1.7 times the activities of acid phosphatase, β-glucuronidase, and cathepsin, respectively. In blood plasma also, BCG-infected mice showed variably higher activities of acid phosphatase and β-glucuronidase. It is noteworthy that in all individual experiments cells from BCG-infected mice exhibited invariably higher enzymatic activities than those from control mice.

The rate of increase of acid phosphatase in peritoneal macrophages was examined following BCG infection. A number of mice of one batch was divided into two groups; the one group was injected intravenously with BCG and the other served as uninfected control. Three mice of each group were killed on subsequent days after BCG injection and the acid phosphatase content of their MP was determined.

The results are illustrated in Text-fig. 1 in which the mean values for all control mice are combined. As can be seen, acid phosphatase levels began to increase significantly in BCG-infected mice by about twenty-five per cent after 8 days and increased further until 14 days after inoculation, when the experi-



TEXT-FIG. 1. Acid phosphatase in MP after BCG infection.

ment was terminated. As in the previous experiment the difference in enzyme activity between the cells from normal and BCG-infected mice was greater when based on cell number instead of on cell protein. Determinations done on the 21st day after BCG inoculation yielded similar values as on the 14th day. Apparently no further increase of enzyme occurred after the 14th day.

Thus, the results indicate elevated levels of activity of lysosomal acid hydrolases in BCG-infected mice. This elevation of enzyme activity could be due either to actual increase of enzyme levels or to inactivation of inhibitors of these enzymes present in normal cells. To examine this latter possibility, suspensions of disrupted MP from normal and BCG-infected mice were mixed in varying proportions, and the activities of acid phosphatase and β-glucuronidase were determined in these mixtures.

As shown in Table II the determined activities of both enzymes in the mixtures approximated those calculated from the relative proportions of either

TABLE II

Activities of Acid Hydrolases in Mixtures of MP Sonicates from Normal and BCG-Infected Mice

Normal and BCG	Acid phosphatase*		β-Glucuronidase‡	
Proportions of MP sonicates	Experimental values	Calculated values	Experimental values	Calculated values
ml				
2.0 + 0	0.400	—	40.0	—
1.5 + 0.5	0.570	0.575	78.0	66.0
1.0 + 1.0	0.740	0.750	102.0	92.0
0.5 + 1.5	0.930	0.925	128.0	118.0
0 + 2.0	1.100	—	144.0	—

* Activity of acid phosphatase was expressed as μmole *p*-nitrophenol produced in 40 minutes per 2 ml of MP sonicate.

‡ Activity of β-glucuronidase was expressed as μg phenolphthalein produced in 1 hour per 2 ml of MP sonicate.

TABLE III

*Distribution of Acid Hydrolases in Subcellular Fractions of Liver Homogenates of Normal and BCG-Infected Mice**

Fractions	Enzymes‡		
	Acid phosphatase	β-glucuronidase	Cathepsin
Normal mice			
Homogenate	1.140 ± .354	29.8 ± 1.3	60.1 ± 1.7
Nuclear	1.073 ± .698 (22.3%)‖	54.5 ± 10.3 (23.1%)	131.2§ (28.7%)
Large granular	2.600 ± .462 (54.0%)	171.2 ± 15.0 (72.7%)	310.6 ± 53.7 (68.0%)
Supernatant	1.140 ± .488 (23.7%)	9.9 ± 1.3 (4.2%)	14.9 ± 8.1 (3.3%)
BCG-infected mice			
Homogenate	1.303 ± .367	53.1 ± 10.8	93.3 ± 4.2
Nuclear	1.266 ± .615 (17.7%)	114.5 ± 23.7 (24.5%)	181.5 ± 83.3 (22.0%)
Large granular	4.863 ± .771 (67.9%)	338.5 ± 46.9 (72.6%)	618.8 ± 131.8 (74.9%)
Supernatant	1.031 ± .171 (14.4%)	13.4 ± 2.5 (2.9%)	25.6 ± 9.3 (3.1%)

* Results of 3 experiments.

‡ Activities of enzymes per 1 mg of protein were expressed as follows: Acid phosphatase, μmole of *p*-nitrophenol produced in 40 minutes; β-glucuronidase, μg of phenolphthalein produced in one hour; Cathepsin, μg of tyrosine-equivalent produced in 20 minutes.

§ Mean of the results of 2 experiments because the sample in one experiment was lost by accident.

‖ Percentage of the sum of the activities in the three fractions.

kind of MP sonicates. This finding argues against the existence of inhibitors for acid hydrolases in MP of normal mice.

Acid Hydrolasess in Various Fractions of Liver Homogenates from Normal and BCG-Infected Mice.—The results in the preceding section indicate elevated levels of lysosomal acid hydrolases in BCG-infected mice. To examine whether this increase of acid hydrolases actually reflects an increase of the enzymes in lysosomes, activities of the enzymes were determined in various subcellular fractions separated from liver homogenate of normal and BCG-infected mice. The results are shown in Table III.

By the infection with BCG the specific activity of acid phosphatase in the "supernatant" fraction did not change significantly, while that in the "nuclear" fraction appeared to increase slightly. On the other hand the "large granular" fraction from livers of BCG-infected mice showed a substantial increase of acid phosphatase (about 1.9 times as much activity as that of normal mice). The specific activities of β-glucuronidase and cathepsin in BCG-infected mice increased to variable extent in all fractions of the liver. The greatest increase of both enzymes was found to occur in the large granular fraction, and the activities of both almost doubled in BCG-infected mice.

In terms of percentage of total activity of all three fractions about 54 and 68 per cent of specific activity of acid phosphatase present in the three fractions were concentrated in large granular fractions of the livers from normal and BCG-infected mice, respectively. Specific activities of the other two enzymes, β-glucuronidase and cathepsin, in the large granular fraction amounted to about 70 per cent in both normal and BCG-infected mice. It is noteworthy that the original homogenate yielded less activity per milligram protein than did the fractions.

These results indicate that acid phosphatase, β-glucuronidase, and cathepsin are associated with the large granular fraction which consists of the bulk of mitochondria, lysosomes, and microsomal elements, and that infection with BCG causes increase of all the three acid hydrolases in this fraction. Furthermore, it seems interesting that the distribution of acid phosphatase in various fractions showed some difference from that of the other two enzymes.

Acid Hydrolases in Cultured Peritoneal MP.—Weiss and Fawcett (21) observed by histochemical techniques a marked increase of acid phosphatase in blood MP of chickens after cultivation *in vitro*. We undertook to examine this phenomenon quantitatively on MP from normal and BCG-infected mice. Activities of acid phosphatase and β-glucuronidase in MP were determined immediately after preparation of the cell-sheets and 24 hours after cultivation.

Table IV shows that *in vitro* cultivation for 24 hours of MP, either derived from normal or BCG-infected mice, caused a significant increase (1.5- to 3-fold) of cellular acid phosphatase activity. This suggests non-specific stimulation of this enzyme by physical means. The rate of increase was slightly greater in MP from BCG-infected mice than in those from normal mice. The rather

large standard deviation can be attributed to the manipulations necessary to obtain the cell preparation. In the culture medium an increase of acid phosphatase was also found after 24 hours of cultivation. This suggests net synthesis of acid phosphatase by the MP and release of the enzyme into the culture medium. By contrast, β-glucuronidase activity was found decreased in MP after 24 hours of cultivation.

In one experiment acid phosphatase and β-glucuronidase were determined up to 6 days of cultivation of MP from normal and BCG-infected mice. The peak of activity of acid phosphatase in MP either from normal or BCG-inefcted mice occurred after 48 hours of cultivation. Then activity decreased gradually, but even 6 days after the cultivation it still remained at a significantly higher level than that found immediately after preparation of the cell-sheets. By contrast, activity of β-glucuronidase in MP exhibited little change during cultivation. This different behavior of the two acid hydrolases in MP upon *in vitro* cultivation is quite interesting.

TABLE IV

Effect of Cultivation of MP on Their Acid Hydrolases

Time after cultivation	Acid phosphatase*		β-Glucuronidase‡	
	Normal	BCG	Normal	BCG
0	0.245 ± 0.099 (9)§	0.369 ± 0.090 (8)	91.4 ± 24.7 (5)	117.4 ± 33.0 (4)
24 hrs.	0.529 ± 0.179 (9)	0.829 ± 0.162 (8)	58.3 ± 20.1 (5)	97.7 ± 32.4 (4)
2 days	0.797	1.305	69.8	94.6
4 days	0.259	0.955	—	73.2
6 days	0.529	0.906	62.5	60.6

* Activity of acid phosphatase was expressed as μmole of *p*-nitrophenol produced in 40 minutes per mg of cell protein.

‡ Activity of β-glucuronidase was expressed as μg of phenolphthalein produced in 1 hour per mg of cell protein.

§ Number of determinations. Enzyme activities after 2, 4, 6 days of cultivation were determined in only one experiment.

DISCUSSION

The results presented in this paper deal with the general adaptability of the RES. There are specific and non-specific reactions involved, and their relative contributions have to be assessed.

Increase of acid hydrolases in cells of the RES after stimulation with various agents has been observed by many investigators. Thus infection with tubercle bacilli induces an increase of acid phosphatase demonstrable by histochemical techniques in MP of granulomatous lesions, (6) in Kupffer's cells, and peri-

toneal MP (7). Simultaneously, the liver content of this enzyme is enhanced. Cultured peritoneal MP from BCG-infected rabbits had a higher content in acid phosphatase than had normal cells (8), but this is apparently not always the case (9), presumably because of ineffective infection or poor timing. Furthermore, in most previous experiments, cells of induced exudates were used rather than cells normally present in the peritoneal cavity (10).

The results of the present study confirm earlier observations by the quantitative demonstration of significantly higher levels of acid hydrolases in BCG-infected mice compared with normal mice. It is unlikely that this increase of activity is due to the neutralization of some inhibitor of acid hydrolases, since the determination of the enzymes in mixtures of samples prepared from normal and BCG-infected mice corresponded to the calculated values. A parallel increase of levels of three kinds of acid hydrolases, *i.e.* acid phosphatase, β-glucuronidase, and cathepsin, is suggestive for an increased number of lysosomes or a higher content in enzymes per lysosome. Actually the determinations of acid hydrolases in various subcellular fractions of liver homogenates clearly indicated that the acid hydrolases were associated mainly with the "large granular" fractions, which consist of the bulk of mitochondria and lysosomes, and that infection with BCG causes an increase of the enzymes found in this fraction. The increased level of acid hydrolases in the plasma is possibly a consequence of the increase of enzymes within the cells. It is quite likely that this elevation of enzymes by infection with BCG represents a non-specific reaction, since similar alterations are induced by a variety of agents such as polyvinylpyrrolidone and periston (22).

The difference between acid phosphatase and the other two acid hydrolases, *i.e.* β-glucuronidase and cathepsin, observed in their distribution in the various fractions of liver homogenate could indicate the difference between each enzyme in the degree of release from lysosome membrane under physiological conditions, or in intrinsic genetic control on its intracellular location. Sawant *et al.* (23) described a difference in the degree of binding to the lysosome membrane of various enzymes, and Paigen (24) demonstrated in the mouse the genetic control of the intracellular location of β-glucuronidase.

The appearance of RES cells with quantitatively different properties at sites distant from the eliciting stimulus demands attention. This can be explained either by selection of a population with these new distinguishing properties at the expense of the original cells, or the already existing cells respond to a generalized stimulus. The latter could be provided by antigenic material derived from the immunizing infection or could be provided by an endogenous factor produced in response to infection with BCG and distributed throughout. The cytophilic antibodies of Boyden and Sorkin (25) and the 19S antibody found on RES cells after immunization with an attenuated strain of *Salmonella typhimurium* (26) could provide such a stimulus. The fact that MP are capable

of increasing their acid phosphatase content *in vitro* is an indication that these cells have adaptive capacities as far as their lysosomal enzymes are concerned.

SUMMARY

Experiments are reported dealing with the increase of lysosomal acid hydrolases induced by BCG infection. Acid hydrolases were determined quantitatively in peritoneal MP, liver homogenate, and plasma of normal and BCG-infected mice. A significant increase of acid phosphatase, β-glucuronidase, and cathepsin was found in MP and liver homogenate of BCG-infected mice. In plasma also a significant increase of acid phosphatase and β-glucuronidase was noticed. The results of the determination of the enzymes in centrifugally separated subcellular fractions of liver homogenate indicated clearly that the acid hydrolases associated mainly with the "large granular" fraction, which consists of mitochondria, lysosomes, and microsomes and that infection with BCG caused significant increase of the enzymes specifically in this fraction. Differences in the pattern of location among centrifugally separated fraction of liver homogenate were observed between acid phosphatase and the other two acid hydrolases. MP cultured *in vitro* doubled their acid phosphatases content within 24 hours, whereas β-glucuronidase rather decreased in the same cells.

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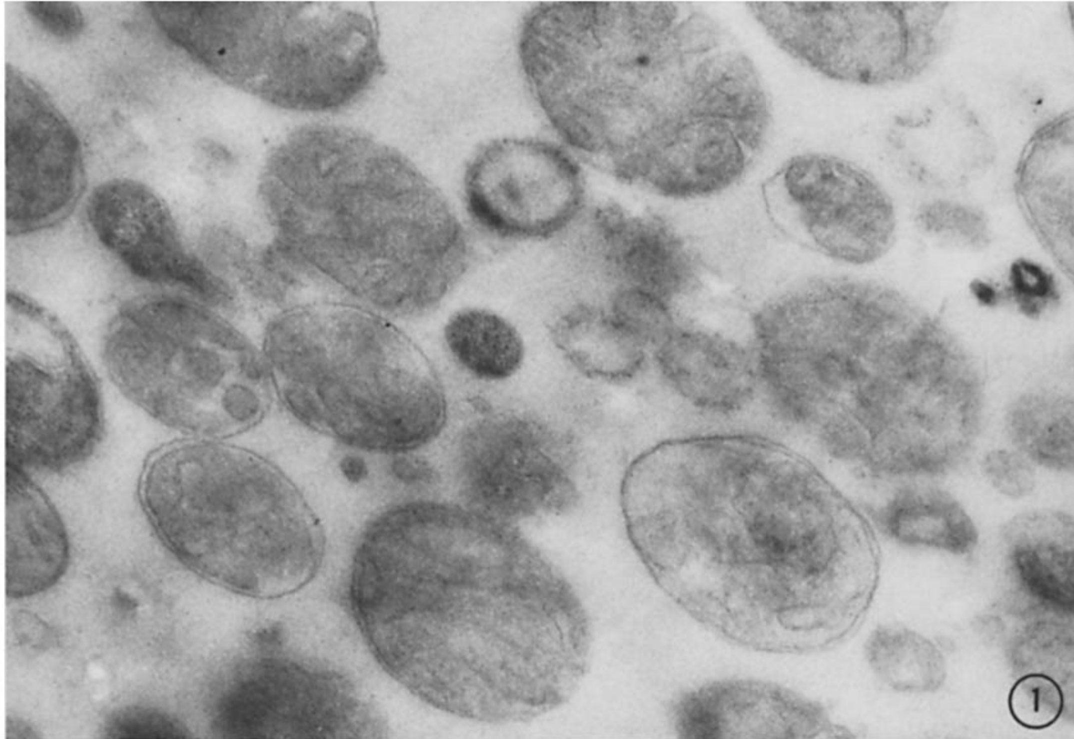
EXPLANATION OF PLATE 55

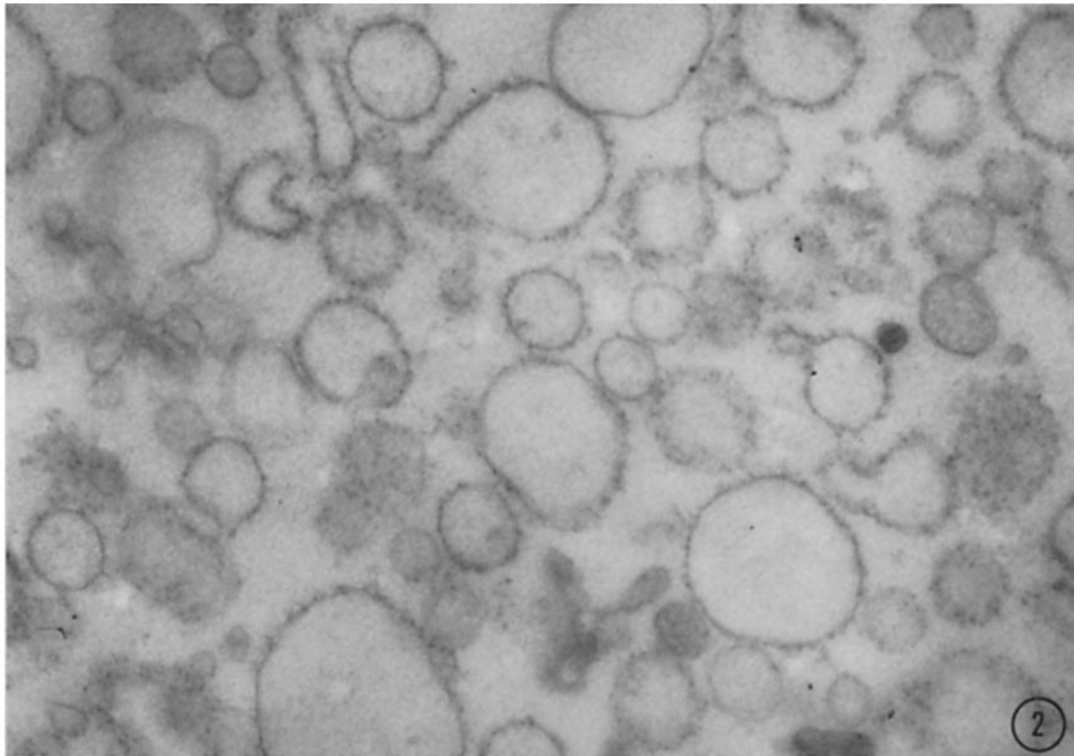
We are grateful to Dr. Joseph Shands of this department for the preparation of the electron micrographs.

FIGS. 1 and 2. Electron micrographs of the "large granular" fraction prepared from liver homogenate by differential centrifugation. This fraction was fixed with osmic acid and centrifuged at low speed to obtain two layers. The layers were embedded separately and sectioned.

FIG. 1. Lower layer of the "large granular" fraction with structures resembling lysosomes, autophagic vacuoles, and mitochondria. $\times$ 40,000.

FIG. 2. Upper layer of "large granular" fraction containing microsomes and lysosome-like structures. $\times$ 40,000.





(Saito and Suter: Lysosomal enzymes and BCG infection)