# HYDROLYTIC ENZYMES OF RABBIT MONONUCLEAR EXUDATE CELLS

## I. Quantitative Assay and Properties of Certain Proteases, Non-Specific Esterases, and Lipases of Mononuclear and Polymorphonuclear Cells and Erythrocytes

ARTHUR M. DANNENBERG, Jr., M.D., and
WILLIAM E. BENNETT, Ph.D.

From The Henry Phipps Institute, Department of Public Health and Preventive Medicine, University of Pennsylvania School of Medicine, Philadelphia



ABSTRACT

Oil-induced mononuclear phagocytes (MN) were quantitatively assayed for various hydrolases as unfractionated suspensions of frozen and thawed cells. They apparently contain two proteases. The first, measured with urea- or acid-denatured hemoglobin, was similar to purified Proteinase I of lung with respect to pH optimum (pH 4), stability, hydrolytic and polymerizing activities, and reactions to various inhibitors. The second protease resembled chymotrypsin in its hydrolysis of glycyl-L-phenylalanine amide, acetyl-L-tyrosine ethyl ester and *N*-benzoyl-DL-phenylalanine-β-naphthol ester (BPN). With the latter, its pH optimum was between 5.0 and 5.8, and its action was inhibited by diisopropylphosphorofluoridate (DFP) and *p*-chloromercuribenzoate. When assayed under the above conditions, polymorphonuclear exudate cells (PMN) and red blood corpuscles (RBC) manifested little or no hydrolysis of either hemoglobin or BPN. MN also contained esterases that split methyl butyrate and β-naphthyl acetate. The pH optimum with the latter was 7.4, and its hydrolysis was partially inhibited by DFP, fluoride, taurocholate, and eserine. PMN had low esterase activity; RBC had little or none. MN, but not PMN or RBC, contained a stable lipase with a pH optimum of 6.1 in maleate buffer. Protamine, NaCl, heat, *p*-chloromercuribenzoate, ethylenediamine tetraacetate, taurocholate, and DFP were inhibitory, but no appreciable activation occurred in the presence of heparin or serum. Thus it possessed some of the characteristics of Korn's lipoprotein lipase, but not others.

## INTRODUCTION

The mononuclear phagocyte (MN)[1] is the functional element of the reticuloendothelial system, which is the body's major defense against microbial agents and their toxic products. The present

[1] Abbreviations: MN, mononuclear(s) or mononuclear exudate cells (used as a frozen and thawed suspension for enzyme studies); PMN, polymorphonuclear(s) or polymorphonuclear exudate cells; RBC and WBC, red and white blood corpuscles; urea-Hb and acid-Hb, urea- and acid-denatured hemoglobin; EDTA, ethylenediamine tetraacetate; DFP, (diisopropylphosphorofluoridate); BPN, *N*-benzoyl-DL-phenylalanine-β-naphthol ester; NDBB, Naphthanil Diazo Blue B (tetrazotized diorthoanisidine); GPA, glycyl-L-phenylalanine amide; ATEE, acetyl-L-tyrosine ethyl ester; Naph. Ac., β-naphthyl acetate.

investigation was undertaken to assay and characterize MN enzymes that hydrolyze proteins, lipids, polysaccharides, and nucleic acids, which are the major components of the many exogenous and endogenous substances (*e.g.*, bacteria and effete blood corpuscles) that are digested by these cells. MN proteases, esterases, and lipase are evaluated herein; MN lysozyme is evaluated in a subsequent report (1); and studies on MN nucleases are just beginning. Similar enzymes in PMN and RBC were also evaluated.

CELL COUNTS: The MN exudates contained from 10 million to 1 billion cells with an average of about 80 million. The RBC present averaged 15 per cent of the WBC, varying from 0 to 100 per cent. The differential cell counts usually showed 90 to 100 per cent MN (including 2 to 5 per cent lymphocytes) and 0 to 10 per cent PMN. For our quantitative enzyme studies allowance was made, when necessary, for both the RBC and the PMN present in the MN exudates.

COLLECTION OF PMN EXUDATES AND RBC: PMN exudates were induced by the intraperitoneal injection of 0.1 per cent of glycogen in saline (2) and harvested after 18 hours. RBC were collected from blood by repeated centrifugation with removal of the buffy coat. The PMN exudates averaged 85 per cent PMN (15 per cent MN), and the RBC specimens averaged 0.3 per cent WBC.

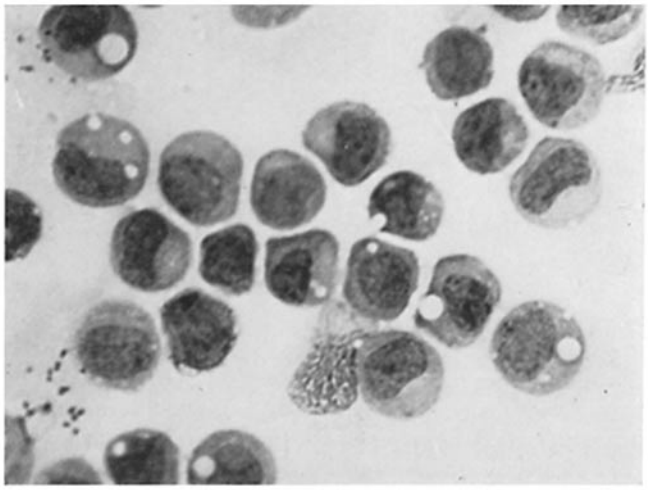

FIGURE 1 Typical oil-induced mononuclear exudate cells from the peritoneal cavity of the rabbit. Wright's stain. × 1000.

## EXPERIMENTAL PROCEDURES AND RESULTS

### *Preparation of Exudates for Quantitative Enzyme Assay*

COLLECTION OF MONONUCLEAR CELLS: Thirty-five to 40 ml of white medicinal mineral oil (U.S.P.) were injected intraperitoneally into each rabbit by means of a blunt 18-gauge needle. Five or 6 days later, the animal was killed by exsanguination, and its peritoneal cavity, including the omental pocket, was rinsed several times with 50 ml of citrate-saline solution (0.4 per cent sodium citrate in 0.85 per cent NaCl).

The peritoneal rinses were pooled, filtered through gauze, and centrifuged at about 900 *g* for 10 minutes. The sediment containing the exudate cells was suspended in 2 to 5 ml of citrate-saline. Total white and red cell counts were made, and smears were prepared for differential cell counts. The suspensions were frozen and stored at −25°C for 3 or more days, after which they were thawed and diluted for enzyme assay. Fig. 1 depicts representative MN.

DESOXYRIBONUCLEIC ACID (DNA) AND PROTEIN DETERMINATIONS: The amount of DNA should provide a fairly accurate check on the cell count, for all non-dividing cells of a given host contain the same amount of DNA (3); and the amount of protein should give some indication of cytoplasmic mass. These parameters were, therefore, assayed by the indole (4–6) and bromsulphalein (7) methods as modified by Bonting and Jones (8). Eight million exudate cells were sufficient for duplicate assays when 10 times the volumes of the reagents listed by Bonting and Jones were employed.

PERMEABILITY STUDIES ON MONONUCLEAR EXUDATE CELLS: Since enzymes may diffuse from these cells during their collection, it seemed advisable to test their permeability by means of trypan blue (9, 10) and eosin (11, 12). In 15 exudates, about 92 per cent of the MN population was

impermeable to trypan blue and 85 per cent to eosin. PMN averaged 6 per cent lower. The use of Hanks balanced salt solution (13) containing 1:10,000 heparin in place of citrate-saline did not increase the proportion of impermeable cells, nor did it increase their assayable lipase or proteinase.

PREPARATION OF CELL HOMOGENATES: The cell preparations were frozen for several days and subsequently thawed. Cohn (14) found that this procedure effectively ruptured MN lysosomes (15). In confirmation, we found that Potter-Elvehjem homogenization (16) or Triton X-100[2] (0.1 per cent) did not increase the proteinase activity of MN preparations. Since this enzyme resides in the lysosomes (14), one would also expect the activity of other lysosomal hydrolases to be unaffected by these additional procedures. Uncentrifuged suspensions of frozen and thawed cells were employed for the enzyme studies described in this report, since clearing the preparations by centrifugation at 900 *g* resulted in a loss of 25 to 40 per cent of MN proteinase and lipase activities to the sediment.

## *Proteinase Hydrolyzing Urea-Denatured Hemoglobin*

TECHNIQUE: The method of Anson (17) and Kunitz (18) as modified by Dannenberg and Smith (19) was employed. 5.2 million frozen and thawed MN in 1.3 ml of water were incubated at 38°C with 1.3 ml of 2 per cent urea-denatured hemoglobin (urea-Hb)[3] buffered with 0.1 M citrate to pH 3.9. At 0-hour and 1-hour, 1.0 ml samples were taken and mixed with 1.0 ml of 5 per cent trichloroacetic acid. The tubes were then kept at 38°C for ½ to 1 hour, and cleared by centrifugation. To 1.0 ml of each supernate, 2.0 ml of water were added, and the optical densities read at 280 mμ in a Beckman DU spectrophotometer. The amount of proteinase activity (see Table I) was expressed as the difference in optical density between the 0- and 1-hour samples, after allowance was made for changes in the control tubes that were free of enzyme.

PROPERTIES OF THIS PROTEINASE: Its activity was a linear function of cell concentration up to an optical density of 0.350, and then there was a gradual decrease in slope. Its pH optimum fell between 3.7 and 4.3 in citrate buffer (Fig. 2). The following compounds did not affect the activity of this enzyme: cysteine (0.01 M), *p*-chloromercuribenzoate (0.0002 M), iodoacetate (0.002 M), $Fe(NH_4)_2(SO_4)_2$ (0.004 M), $MnCl_2$ (0.004 M), $MgCl_2$ (0.004 M), ethylenediamine tetraacetate (EDTA) (0.01 M), diisopropylphosphorofluoridate (DFP) (0.0003 M), and crystalline soybean trypsin inhibitor[3] (250 μg per ml). This proteinase lost no activity when stored at −20°C for 5 years, but lost 90 per cent of its activity when it was heated at 58°C for 30 minutes.

Neither PMN nor RBC appreciably hydrolyzed urea-Hb at pH 3.9 when allowance was made for the MN that contaminated the former (Table I).

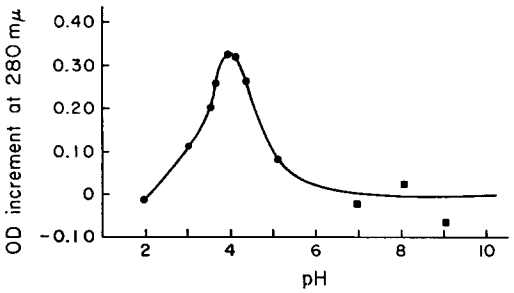


FIGURE 2 Proteinase activity of suspensions of frozen and thawed rabbit monouclear exudate cells as a function of pH with 1 per cent urea-denatured hemoglobin as substrate and 0.05 M citrate (●) and 0.05 M Tris (■) as buffers. The concentration of MN was 3.0 million per ml, and 0.004 M cysteine was employed as a possible activator.

## *Proteinase Hydrolyzing Acid-Denatured Hemoglobin*

TECHNIQUE: Since urea may be inhibitory for certain enzymes, hemoglobin was prepared without urea (17, 20). For this purpose, 1.25 gm of hemoglobin powder was ground in a mortar in a small amount of water and made up to 50 ml with additional water. It was cleared by centrifugation. Since less Hb went into solution than when urea was employed, 2.5 ml of Hb (buffered with acetate) and 0.5 ml of frozen and thawed MN (6 million cells) were incubated together, instead of equal quantities of each. The final protein concentration of the substrate now approximated that of the urea-Hb, when determined by the Bücher method (21). Because acid-Hb occasionally precipitated in citrate buffer, acetate buffer was employed in a final ionic strength of 0.1. Except for this, the assay technique with acid-Hb resembled that just described for urea-Hb.

[2] Rohm and Haas Co., Philadelphia (lot No. 7330).
[3] Bovine Hemoglobin Substrate Powder and Crystalline Soy Bean Trypsin Inhibitor were obtained from Worthington Biochemical Corp., Freehold, New Jersey.

TABLE I

*Proteinase Activity of MN, PMN, and RBC with Urea-Denatured Hemoglobin, Acid-Denatured Hemoglobin and N-Benzoyl-DL-Phenylalanine-β-Naphthol Ester as Substrates*

| Predominant cell type | No. of rabbits | Differential cell count MN* (per cent) | Differential cell count PMN (per cent) | Urea-hemoglobin hydrolysis: optical density units at 280 mμ. Cells per ml in parentheses. Uncorr. | Urea-hemoglobin hydrolysis: optical density units at 280 mμ. Cells per ml in parentheses. Corrected‡ | Urea-hemoglobin hydrolysis: optical density units at 280 mμ. Cells per ml in parentheses. Corrected‡ | Acid-hemoglobin hydrolysis: optical density units at 280 mμ. Cells per ml in parentheses. Uncorr. | Acid-hemoglobin hydrolysis: optical density units at 280 mμ. Cells per ml in parentheses. Corrected‡ | Acid-hemoglobin hydrolysis: optical density units at 280 mμ. Cells per ml in parentheses. Corrected‡ | Benzoyl-phenylalanine-naphthol ester hydrolysis: optical density units at 540 mμ§. No. of cells in parentheses. Uncorr. | Benzoyl-phenylalanine-naphthol ester hydrolysis: optical density units at 540 mμ§. No. of cells in parentheses. Corrected‡ | Benzoyl-phenylalanine-naphthol ester hydrolysis: optical density units at 540 mμ§. No. of cells in parentheses. Corrected‡ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MN | 10 | 95 | 5 | $(2 \times 10^6)$ | $(2 \times 10^6)$ | $(1 \times 10^6)$ | $(2 \times 10^6)$ | $(2 \times 10^6)$ | $(1 \times 10^6)$ | $(2 \times 10^6)$ | $(2 \times 10^6)$ | $(1 \times 10^6)$ |
| Average | | ±2 | ±2 | 0.346 | 0.363 | 0.181 | 0.187 | 0.196 | 0.098 | 0.262 | 0.276 | 0.138 |
| ‖ | | | | | ±0.026 | ±0.013 | | ±0.018 | ±0.009 | | ±0.015 | ±0.007 |
| PMN | 7 | 15 | 85 | $(10 \times 10^6)$ | $(10 \times 10^6)$ | $(1 \times 10^6)$ | $(2 \times 10^6)$ | $(2 \times 10^6)$ | $(1 \times 10^6)$ | $(10 \times 10^6)$ | $(10 \times 10^6)$ | $(1 \times 10^6)$ |
| Average | | ±1 | ±1 | 0.332 | 0.072 | 0.007 | 0.054 | 0.029 | 0.014 | 0.161 | 0.000 | 0.000 |
| ‖ | | | | | ±0.032 | ±0.003 | | ±0.007 | ±0.004 | | | |
| RBC | 8 | 0.3 per cent WBC | | $(10 \times 10^6)$ | | | $(2 \times 10^6)$ | | | $(10 \times 10^6)$ | | |
| Average | | | | 0.000 | | | 0.007 | | | 0.009 | | |
| ‖ | | | | | | | ±0.001 | | | ±0.002 | | |

* Includes 3 to 5 per cent lymphocytes.

‡ Corrected: MN for contaminating PMN; PMN for contaminating MN.

§ An optical density of 0.100 corresponds to the liberation of about 20 μg of β-naphthol from BPN.

‖ The average and its standard error $\left(\sqrt{\frac{\Sigma(x - \bar{x})^2}{n(n-1)}}\right)$ are listed.

PROPERTIES OF THIS PROTEINASE: MN hydrolyze both urea- and acid-Hb in similar ranges of pH and cell concentration (Table I and Fig. 3). The slight differences observed in the hydrolysis of these two substrates were also observed with purified Proteinase I of lung (see below). These findings suggest that both substrates may be hydrolyzed by the same MN enzyme, but this hypothesis can not be proven without purifying the enzymes involved. Additional evidence for it is provided by the MN exudates used for Table I, as well as a series of 16 exudates used to study the effect of tuberculosis on MN proteinases (22). In all of these the ratio of activities with urea-Hb and acid-Hb as substrates were constant within the range of experimental error.

PMN and RBC did not hydrolyze acid-Hb to a significant degree (Table I).

### *Comparison of the Proteinases from MN and Lung*

Proteinase I of beef lung (19) and rabbit MN proteinase had identical pH optima with either urea-Hb or acid-Hb as substrate (Fig. 3). Both enzymes appeared to be partially inhibited by urea in acetate buffer ($r/2 = 0.1$) and both hydrolyzed urea-Hb better in citrate buffer than in acetate buffer. Neither of them hydrolyzed the synthetic peptide substrates for pepsin and trypsin (see below), and neither was substantially affected by cysteine, $Fe^{++}$, $Mn^{++}$, EDTA, or DFP. A solution of purified Proteinase I (250 $\mu$g of protein N per ml), like MN proteinase, lost no activity in 5 years of storage at −20°C. These properties, as well as their ability to polymerize certain amino acid esters (23), suggest that Proteinase I of lung has much in common with MN proteinase and possibly arises from the MN-like cells found in lung. Purified Proteinase I, however, was somewhat less affected by heat (58°C for 30 minutes) and somewhat more responsive to SH inhibitors (19). This may have been due to differences in the purification of each preparation or in the species of origin.

### *Studies Employing Synthetic Substrates for Proteases*

The three main groups of tissue proteases, with hydrolytic activities resembling pepsin, trypsin, and chymotrypsin, respectively, may be assayed at pH 5 with the following compounds: (*a*) carbobenzoxy-L-glutamyl-L-tyrosine, (*b*) benzoyl-L-arginine amide and tosyl-L-arginine methyl ester, (*c*) glycyl-L-phenylalanine amide (GPA) and acetyl-L-tyrosine ethyl ester (ATEE) (24, 25). These compounds[4] were therefore employed as substrates for MN in the presence of 0.005 M cysteine, and the Grassmann-Heyde technique (19, 26) was used to titrate the degree of hydrolysis.

Both GPA and ATEE (0.05 M) were hydrolyzed 80 and 50 per cent, respectively, in 22 hours by

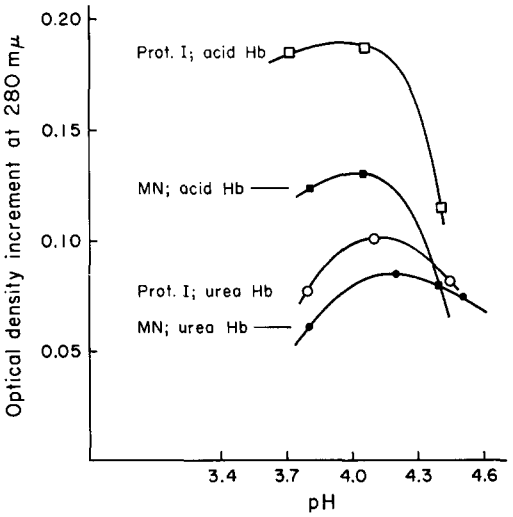


FIGURE 3 Comparison of purified Proteinase I of beef lung (6 $\mu$g protein N per ml) with the proteinase of rabbit MN (2.4 million cells per ml), employing one per cent urea- and acid-denatured hemolgobin as substrates at pH 3.7 to 4.5 in acetate buffer ($r/2 = 0.1$). The proteinase content of the MN employed in this experiment was slightly lower than usual (see Table I).

25 to 50 million disrupted MN in 2.5 ml of 0.04 M acetate buffer and 0.004 M cysteine at 38°C. The other substrates listed were not appreciably hydrolyzed during this time. The hydrolysis of ATEE may have been less than the hydrolysis of GPA, because the splitting of the ester bond, in contrast to the amide bond, results in a slight drop in pH when this technique is employed. These results suggest that MN contain an enzyme which is similar to chymotrypsin in its substrate specificity.

In order to obtain an assay method for this

[4] Obtained from Mann Research Laboratories, Inc., New York.

MN enzyme which did not require excessive cells and incubation time, the more sensitive colorimetric procedure of Ravin, Bernstein, and Seligman (27) for chymotrypsin-like enzymes was employed. Their substrate was *N*-benzoyl-DL-phenylalanine-β-naphthol ester (BPN)[5], and the color was developed by coupling the released β-naphthol with Naphthanil Diazo Blue B (NDBB)[5].

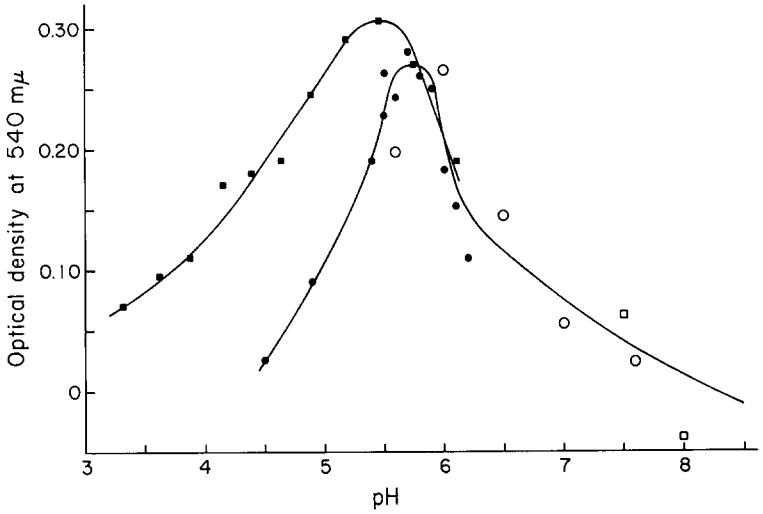


FIGURE 4 Hydrolysis of *N*-benzoyl-DL-phenylalanine-β-naphthol ester by suspensions of frozen and thawed rabbit mononuclear exudate cells as a function of pH. The buffers employed were acetate (■ and ●), phosphate (○), and Tris (□) in 0.08 M concentrations. The points in the alkaline range represent differences between samples run with and without enzyme, for the autohydrolysis of this substrate increased markedly as the pH rose from 7.0 to 9.0. The lower curve indicates the pH optimum with our standard technique. The upper curve indicates this optimum when a modification of our revised technique was employed, namely, the diazo coupling of the released β-naphthol at pH 7.4 in 0.03 M veronal buffer. The scale of each curve was adjusted to match at pH 5.7 the average value listed in Table I.

TECHNIQUE FOR BPN HYDROLYSIS BY MN: Two million frozen and thawed MN in 1.0 ml of water were added to 5.0 ml of dilute BPN substrate,[6] buffered at pH 5.7. The mixture was then incubated at 38°C for 1 hour with frequent shaking. During this hour 1.0 ml was removed from each tube in order to determine the pH. At the end of the incubation time, the remaining 5.0 ml were chilled in an ice bath for 5 minutes and 1.0 ml of cold, freshly prepared NDBB reagent[7] was added. After 3 minutes the reaction was stopped with 1.0 ml of 40 per cent trichloroacetic acid and the color extracted with 15 ml of ethyl acetate. The latter was cleared by a brief centrifugation, and its optical density (see Table I) was read immediately thereafter at 540 mμ. Controls added to measure the amount of non-enzymatic hydrolysis were essentially negative.

The diazo coupling reaction of β-naphthol and NDBB deserves comment. It was optimal between pH 7.0 and 8.2. Below 7.0 it was progressively inhibited, reaching zero at pH 4.3. At pH 5.7, the pH employed in the BPN technique, 20 μg of β-naphthol produced an optical density of 0.100, whereas at pH 7.4 only 4 μg were required. Because of this, it is recommended that future work with this enzyme be performed with 0.5 million MN incubated for 30 minutes at 38°C and pH 5.4 to 5.5 (see Fig. 4). The reaction should be stopped first by chilling to 3°C and then by adding 2.5 ml of 0.05 M Veronal buffer (pH 8.5)

[5] Dajac Laboratories, Borden Chemical Co., Philadelphia.

[6] Dilute BPN substrate was prepared as follows: 4.0 ml of concentrated substrate (2 mg per ml of acetone) were added just before use to 76.0 ml of 0.1 M acetate buffer (pH 5.7) at room temperature with a submerged pipette and constant shaking.

[7] The NDBB reagent was prepared just before use by adding 80 mg of NDBB to 20 ml of cold water.

to bring the pH to approximately 7.4 for diazo coupling

PROPERTIES OF BPN HYDROLASE: It had a pH optimum from 5.0 to 5.8 (Fig. 4), and there was a straight line relationship between the number of cells and the naphthol released, up to an optical density of 1.100. The enzyme was completely inhibited by DFP (0.00045 M), $HgCl_2$ (0.012 M), *p*-chloromercuribenzoate (0.0002 M) and by heating at 58°C for 30 minutes. It was also inhibited by 0.05 M GPA and 0.05 M ATEE about 30 and 60 per cent, respectively. $MnCl_2$, $MgCl_2$, and $CaCl_2$, (all 0.012 M), EDTA (0.01 M), iodoacetate (0.002 M) and soybean trypsin inhibitor (250 μg per ml) had no consistent effect on its activity. Since cysteine (0.01 M) and $NaN_3$ (0.02 M) interfered with the development of color from β-naphthol and NDBB, their effect on this enzyme (and the one hydrolyzing β-naphthyl acetate) could not be evaluated. MN samples collected 5 years ago and stored at −20°C possessed about two-thirds the BPNase activity of recently collected samples.

RBC and PMN did not hydrolyze this substrate to any appreciable degree (Table I).

The complete inhibition of the BPN hydrolase of MN by DFP and *p*-chloromercuribenzoate strongly suggests that this enzyme is different from the enzyme hydrolyzing urea-Hb, whose activity was not affected by either. In addition, there was no correlation between the activities of these two enzymes in the samples assayed for Table I and in 36 other samples (22). As further support for the individuality of these enzymes, it is of interest that BPN was not hydrolyzed by purified Proteinase I of lung, an enzyme whose hydrolyzing and polymerizing activities resemble MN proteinase (see above).

MN did not split *N*-α-benzoyl-DL-arginine-β-naphthyl amide[5] under the conditions used for BPN assay, whereas crystalline trypsin (0.1 mg) did.

### *Esterase Hydrolyzing Methyl Butyrate*

TECHNIQUE: The hydrolysis of methyl butyrate by MN was measured manometrically (28, 29). In the main chamber of a Warburg flask were placed 0.05 ml of redistilled methyl butyrate, 0.90 ml of 0.15 M $NaHCO_3$, and 1.15 ml of water. In each side arm were 0.5 ml of frozen and thawed MN (10 million per ml) and 0.1 ml of 0.15 M $NaHCO_3$. The chambers were then flushed for 3 minutes with 5 per cent $CO_2$ in nitrogen and the apparatus sealed. Although base line readings were taken, no true equilibrium was reached at 30°C, because methyl butyrate seemed to distill into the compartments containing the enzyme.

PROPERTIES OF THIS ESTERASE: The above technique results in a final pH of about 7.0, and there was a straight line relationship between the number of MN and the μl of $CO_2$ released from $NaHCO_3$, up to about 300 μl per hour. Boiled MN were inactive. Because of the relatively large number of MN required for assay, no other properties of this MN esterase were studied.

PMN hydrolyzed methyl butyrate about one-seventh as rapidly as MN; RBC did not hydrolyze it appreciably (see Table II).

### *Hydrolysis of β-Naphthyl Acetate*

TECHNIQUE: The following is essentially the technique of Nachlas and Seligman (30), as modified by Hardin *et al.* (31). One million frozen and thawed MN, in 3.0 ml of water, were chilled and then "incubated" at 3°C in an ice bath with 4.0 ml of cold, dilute β-naphthyl acetate (Naph. Ac.) substrate.[8] After 30 minutes the amount of released β-naphthol (see Table II) was determined by coupling to NDBB as described above for the hydrolysis of BPN.

PROPERTIES OF THE ESTERASE(S) HYDROLYZING β-NAPHTHYL ACETATE: The optimal pH was from 7.0 to 8.0 (Fig. 5). A straight line relationship existed between the naphthol liberated and the number of MN, up to an optical density of 1.00. The enzymatic activity proved rather stable, for it was reduced only to half when stored at −20°C for 4 years. It was inhibited about 85 per cent by DFP (0.0003 M), 75 per cent by NaF (0.02 M), 30 per cent by taurocholate (0.04 per cent), and by eserine (4 μM), 25 per cent by heat (58°C for 30 minutes), and 20 per cent by iodoacetate (0.002 M) and by *p*-chloromercuribenzoate (0.0002 M). EDTA (0.002 M) and $MnCl_2$, $CaCl_2$, and $MgCl_2$ (all 0.001 M) had no consistent effect. At the concentrations employed, none of these compounds interfered with the diazo coupling reaction.

PMN hydrolyzed Naph. Ac. about one-twen-

[8] Dilute Naph. Ac. substrate: 2 ml Naph. Ac. (Dajac) (5 mg per ml of acetone) were added with continuous shaking to 78 ml of 0.05 M Tris buffer at pH 7.4.

TABLE II

*Esterase and Lipase Activities of MN, PMN, and RBC*

| Predominant cell type | No. of rabbits | Differential cell count MN* (per cent) | Differential cell count PMN (per cent) | Methyl butyrate hydrolysis: $CO_2$ released at 30°C from $NaHCO_3$ in Warburg apparatus (μl/hr) 10 million cells Uncorr. | 10 million cells Corr.‡ | 50 million cells Uncorr. | 50 million cells Corr.‡ | Naphthyl acetate hydrolysis: optical density units at 540 mμ§ 1 million cells Uncorr. | 1 million cells Corr.‡ | 5 million cells Uncorr. | 5 million cells Corr.‡ | Coconut oil hydrolysis: optical density units at 570 mμ§ 5 million cells Uncorr. | 5 million cells Corr.‡ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MN Average ‖ | 10 | 95 ±2 | 5 ±2 | 146 | 153 ±28 (6.8 μM) | | 765 ±140 | 0.263 | 0.276 ±0.032 (7.7 μM) | | 1.380 ±0.160 | 0.212 | 0.223 ±0.045 |
| PMN Average ‖ | 5 | 14 ±1 | 86 ±1 | | | 208 | 117 ±108 | 0.028 | 0.000 | 0.252 | 0.072 ±0.032 | 0.018 | 0.000 |
| RBC Average ‖ | 8 | 0.3 per cent WBC | | | | 4 ±1 | | 0.000 | | 0.004 ±0.002 | | 0.000 | |

* Includes 3 to 5 per cent lymphocytes.

‡ Corrected: MN for contaminating PMN; PMN for contaminating MN; RBC for contaminating WBC.

§ An optical density of 0.100 corresponds to the liberation of about 4 μg of β-naphthol from β-naphthyl acetate, and to the liberation of about 1.0 μg of glycerol from coconut oil.

‖ The average and its standard error $\left(\sqrt{\frac{\Sigma(x - \bar{x})^2}{n(n-1)}}\right)$ are listed.

tieth as rapidly as MN; RBC showed no appreciable activity (Table II).

There was no correlation between the esterase activities with methyl butyrate and naphthyl acetate as substrates in either the samples assayed for Table II or those employed for other purposes (22). This finding suggests that more than one type of esterase may be present in MN.

### *Lipase Hydrolyzing Coconut Oil*

TECHNIQUE: One to 5 million frozen and thawed MN exudate cells in 0.5 ml of water were incubated at 37°C with 0.3 ml of buffered coconut oil substrate (Ediol)[9] with frequent shaking. At

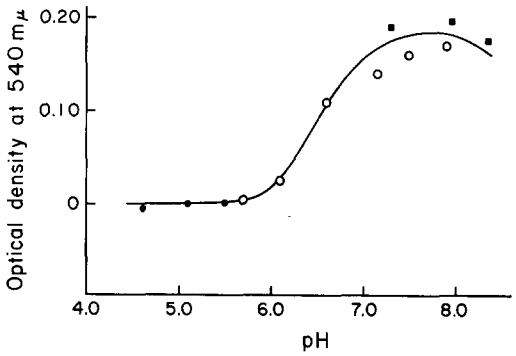


FIGURE 5 Hydrolysis of β-naphthyl acetate by suspensions of one million frozen and thawed rabbit mononuclear exudate cells as a function of pH. The buffers employed were acetate (●), phosphate (○), and Tris (■) in 0.025 M concentrations. The points above pH 7.8 represent differences between samples run with and without enzyme, for spontaneous hydrolysis began at this pH and increased with alkalinity.

0 hour and at 2 hours duplicate 0.100 ml aliquots were placed into 150 x 13 mm colorimeter tubes along with 0.10 ml of 1 N $H_2SO_4$. The samples were then frozen until assayed for glycerol by the following method. At room temperature 0.10 ml of 0.05 M sodium periodate (32–34) was added to each tube. After exactly 5 minutes 0.10 ml of 0.5 M sodium arsenite (32–34) was added, and then after 10 more minutes 4.5 ml of chromotropic acid reagent[10] (33, 34). Following this, the tubes were inverted several times and placed *in the dark* in a boiling water bath for 30 minutes. The optical density of the resulting purple color was read at 570 mμ (see Table II). An optical density increment of 0.100 corresponds to the liberation of approximately 1.0 μg of glycerol from coconut oil.

Since Ediol contains 12.5 per cent sucrose, in addition to 50 per cent coconut oil, it is important to ascertain that MN do not possess a sucrase, for fructose reacts with the glycerol reagent.[11] MN

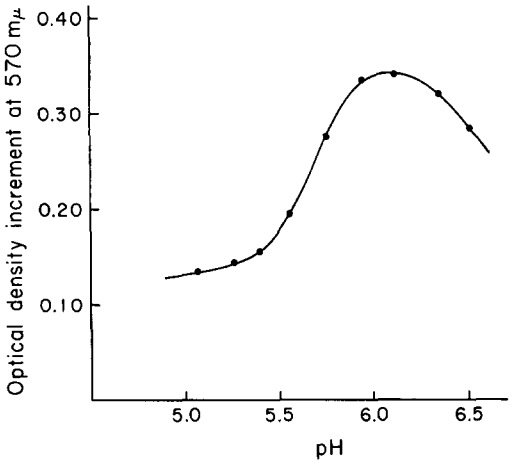


FIGURE 6 Hydrolysis of coconut oil by suspensions of 4.5 million frozen and thawed rabbit mononuclear exudate cells as a function of pH in maleate buffer.

were, therefore, incubated with sucrose of approximately the same concentration that was present in Ediol. No increase in the color produced by the glycerol reagent was found. This suggests that MN do not contain a sucrase.

PROPERTIES OF THIS LIPASE: Its pH optimum was between 5.8 and 6.3 in maleate buffer (Fig. 6). The $NH_4Cl$-$NH_3$ buffer system (employed by Korn (32, 33)), phosphate buffer, and acetate buffer also indicated that the 5.0 to 6.5 range of pH was optimal for this enzyme, but these buffers had poor capacity in this range. Up

[9] Buffered coconut oil substrate, prepared just before use, consisted of 1.4 ml of 0.25 N maleate buffer (pH 6.1), 0.5 ml of 3 per cent Ediol, 0.1 ml of 1 M $CaCl_2$, and 1.0 ml of water. Ediol, which is 50 per cent coconut oil, was obtained from Schenlabs Pharmaceuticals, Inc., New York.

[10] Chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid disodium salt) (practical) was obtained from Distillation Products Industries, Eastman Kodak Co., Rochester, New York.

[11] With this reagent the ratio of color produced from glucose, sucrose, fructose, and glycerol was 1:2:6:30 on a weight for weight basis.

to an optical density increment of 0.320 mμ, there was a straight line relationship between the MN concentration and MN lipase activity. Then there was a gradual decrease in slope. The amount of MN lipase was rather variable from one rabbit to another, and doubling the number of cells did not result in proportionate increases in every case. MN samples frozen for 18 months had 25 to 50 per cent of the lipase activity of fresh samples.

Various activators and inhibitors were tested on this enzyme, in order to delineate its properties

TABLE III

*Effect of Activators and Inhibitors on MN Lipase*

Frozen and thawed MN were incubated with the activator or inhibitor for 30 to 60 min. at room temperature before assay at pH 6.1. None of these substances appreciably affected the color produced from glycerol standards.

| Substance or procedure | Per cent of control* |
|---|---|
| $CaCl_2$ (0.012 M) | 180‡ |
| Heat (58°C for 15 min.) | 9 |
| Protamine ($5 \times 10^{-5}$ M) | 55 |
| NaCl (1 M) | 41 |
| NaCl (0.5 M) | 33 |
| Diisopropylphosphorofluoridate (0.0003 M) | 31 |
| *p*-Chloromercuribenzoate (0.0002 M) | 40 |
| Taurocholate (0.1 per cent) | 21 |
| EDTA (0.01 M) | 57§ |

* Average of three or four experiments.

‡ The control for $CaCl_2$ contained no $Ca^{++}$; the controls for the others had $Ca^{++}$ present (see text).

§ EDTA had no effect on MN lipase activity in the absence of added $Ca^{++}$.

and enable it to be compared with Korn's lipoprotein lipase (32, 33). The following showed no consistent effect: heparin (0.001 per cent), serum (0.5 per cent), albumin[12] (2 per cent), cysteine (0.005 M), iodoacetate (0.002 M), $CoCl_2$ (0.002 M), $MnCl_2$ (0.002 M) and oleic acid (0.05 per cent). Others, listed in Table III, showed varying degrees of activation or inhibition. It was estimated that rabbit MN had about one-fourth of the lipase activity of rat heart (32) on a wet weight basis; but, unlike heart and adipose tissue (35), intact MN did not release lipase when they were incubated *in vitro* in the presence of heparin (30 μg per ml).

PMN and RBC do not hydrolyze coconut oil under these experimental conditions (Table II).

[12] Crystallized Bovine Plasma Albumin, Armour and Co., Chicago. The effect of this substance was determined in the absence of $Ca^{++}$.

## DISCUSSION

This report characterizes two proteases of rabbit mononuclear exudate cells. The first is the proteinase described by Opie in 1906 (36) and subsequently by Weiss *et al.* (37, 38). It hydrolyzes hemoglobin at pH 4.0 and will be termed MN proteinase in this discussion. The second, termed BPNase, hydrolyzes the synthetic substrate *N*-benzoyl-DL-phenylalanine-β-naphthol ester.

Because of analogies with the Proteinase I of beef lung, which was partially purified by Dannenberg and Smith (19), we feel that MN proteinase is a pepsin-like enzyme. Proteinase I and MN proteinase both polymerize aromatic and dicarboxylic amino acid esters at pH 7 (23), and Proteinase I like pepsin splits the B chain of insulin adjacent to these same amino acids (23). Other analogies between these enzymes are listed in the preceding section. A proteinase which acted optimally at acid pH was partially purified from spleen (39–42), an organ which contains many MN. It, therefore, would be of interest to determine whether this splenic proteinase could polymerize amino acid esters.

Lymphocytes also possess an acid-acting proteinase (43, 44), but the literature on PMN is confusing. Some investigators (37, 38, 43, 45–47) found an acid-acting proteinase in PMN, but made no allowance for the MN and lymphocytes that contaminated their preparations. Mounter and Atiyeh (44), like ourselves, after making this allowance, found no such enzyme. However, Štefanovič, Webb and Lapresle (48) employed human serum albumin as the substrate and found that *extracts* of rabbit PMN had higher proteinase activity than *extracts* of MN both at pH 3.5 and 2.0, which were optimal for their cathepsins D and E, respectively (39). Acid-acting proteinases have been demonstrated in human (49) and rabbit (50) red blood corpuscles. In each case, the number of RBC employed for assay was apparently far greater than the number we employed.

The second protease of MN, BPNase, has not, to our knowledge, been previously described. It resembles chymotrypsin in that it splits glycylphenylalanine amide, acetyltyrosine ethyl ester,

and benzoylphenylalanine naphthol ester. One MN enzyme probably hydrolyzes all three substrates, because each of the first two inhibited the hydrolysis of the latter. However, much more characterization, including some steps towards the purification of this enzyme, would be necessary to establish that only one exists (*cf.* 51). Evidence for its ability to hydrolyze proteins is lacking, for any action on urea-denatured hemoglobin at pH 5 (52) was obscured by the Opie proteinase, and no activity was found with this substrate in the neutral or alkaline ranges (see Weiss *et al.* (38) for similar results with other protein substrates). MN BPNase may have been partially purified from spleen as cathepsin C (52, 53), identified in lung tissue (19), and demonstrated in tissue phagocytes by means of the histochemical substrate, *O*-acetyl-5-bromoindoxyl (54), but these suggestions still need to be investigated.

In several laboratories the esterases of MN, PMN, or RBC have been studied. Day and Harris (55) and Rossiter and Wong (56), using rabbit exudates, and Hardin *et al.* (31) and Frei *et al.* (57), using human blood, obtained results rather similar to those reported here. Rabbit exudate MN, however, had 6 times the esterase activity of human circulating MN (57). The histochemistry of the esterases of MN, PMN, and alveolar macrophages has been recently reviewed by Dannenberg *et al.* (58).

Day and Harris (55) identified MN lipase, but presented little data on either its characterization or quantitation. They employed relatively large numbers of MN at a single pH (7.3), rat chyle as a substrate, and titrimetric procedures. The method presented in this report seems preferable, in that it employed relatively few MN, a stable and commercially available substrate, and colorimetric procedures. Elsbach and Rizack (59), in contrast to ourselves, have found a lipase in rabbit PMN exudates. This disagreement may be due to the different assay methods employed or to the fact that they did not make allowance for the MN in their preparations.

Of interest is a comparison of MN lipase with the lipoprotein lipase of Korn (32, 33). They are similar in that both of them were inhibited by NaCl, protamine, heat, DFP, taurocholate, and *p*-chloromercuribenzoate (Table III), and both hydrolyzed chyle triglycerides (55). They are different in that MN lipase did not require serum (*i.e.* $\alpha$-lipoproteins) or heparin for optimal activity and functioned best at pH 6.1, rather than 8.5. One might postulate that both $\alpha$-lipoproteins and heparin are present in adequate concentrations in mononuclear cells, and that similar enzymes may have different pH optima in different tissues. Since these postulates remain to be investigated, all that can be stated at present is that MN lipase has some of the properties of lipoprotein lipase, but not others.

### *Biological Implications*

MN proteinases seem to be involved in many diverse biological phenomena, *e.g.* delayed hypersensitivity (60), the breakdown of antigens (61), the removal of effete red blood corpuscles, and the intracellular digestion of bacteria, viruses, and protozoa. Basic polypeptides, probably derived from the histones of injured mammalian tissues, have been found to have antimicrobial properties (62, 63). In this connection it is of interest that MN, which dispose of necrotic tissue, did not contain proteinases with a specificity for basic amino acids (19, 23, and this report) and did not hydrolyze protamine (38). They, therefore, should not alter the antimicrobial effect of the basic polypeptides of tissues.

MN lipases are important because MN seem to be among the body's main scavengers for many types of lipid material: immunological adjuvants (64), mycobacteria, and necrotic tissue contain lipids that are ingested by MN. The characterization of MN lipase herein presented may lead to a better understanding of how MN dispose of this material.

The effect of tuberculosis on MN proteases, esterases, and lipase is evaluated in another report (22).

We wish to thank Mrs. Margaret E. Carson and Mrs. Mildred L. Pennick for their valuable assistance during part of this work, and Dr. Lewis I. Pizer of the Department of Microbiology for his critical review of the manuscript. This investigation was supported by Contract Nonr-551(24) with the Office of Naval Research, and Grant AI 02048 from the National Institutes of Health, United States Public Health Service.

*Received for publication, April 25, 1963.*

## REFERENCES


1. CARSON, M. E., and DANNENBERG, A. M., JR., in preparation.
2. HIRSCH, J. G., *J. Exp. Med.*, 1956, **103**, 589.
3. VENDRELY, R., *in* The Nucleic Acids, **2**, (E. Chargaff and J. N. Davidson, editors), New York, Academic Press, Inc., 1955, 155.
4. DISCHE, Z., *Biochem. Z.*, 1929, **204**, 431.
5. CERIOTTI, G., *J. Biol. Chem.*, 1952, **198**, 297.
6. CERIOTTI, G., *J. Biol. Chem.*, 1955, **214**, 59.
7. NAYYAR, S. N., and GLICK, D., *J. Histochem. and Cytochem.*, 1954, **2**, 282.
8. BONTING, S. L., and JONES, M., *Arch. Biochem. and Biophys.*, 1957, **66**, 340.
9. ROUS, P., and JONES, F. S., *J. Exp. Med.*, 1916, **23**, 601.
10. STÄHELIN, H., SUTER, E., and KARNOVSKY, M. L., *J. Exp. Med.*, 1956, **104**, 121.
11. SCHREK, R., *Am. J. Cancer*, 1936, **28**, 389.
12. HANKS, J. H., and WALLACE, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1958, **98**, 188.
13. HANKS, J. H., and WALLACE, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 196.
14. COHN, Z. A., and WIENER, E., personal communication; and *J. Exp. Med.*, 1963, **118**, 991 and 1009.
15. DE DUVE, C., *in* Subcellular Particles, (T. Hayashi, editor), New York, Ronald Press Co., 1959, 128.
16. POTTER, V. R., *in* Methods of Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Academic Press, Inc., 1955, **1**, 10.
17. ANSON, M. L., *J. Gen. Physiol.*, 1938, **22**, 79.
18. KUNITZ, M., *J. Gen. Physiol.*, 1947, **30**, 291.
19. DANNENBERG, A. M., JR., and SMITH, E. L., *J. Biol. Chem.*, 1955, **215**, 45.
20. LENNEY, J. F., *J. Biol. Chem.*, 1956, **221**, 919.
21. BÜCHER, T., *Biochim. et Biophysica Acta*, 1947, **1**, 292.
22. DANNENBERG, A. M., JR., and BENNETT, W. E., *Arch. Path.*, 1963, **76**, 581.
23. DANNENBERG, A. M., JR., and SMITH, E. L., *J. Biol. Chem.*, 1955, **215**, 55.
24. BERGMANN, M., and FRUTON, J. S., *in* Advances in Enzymology, (F. F. Nord and C. H. Werkman, editors), New York, Interscience Publishers, Inc., 1941, **1**, 63.
25. FRUTON, J. S., *in* The Enzymes, (P. D. Boyer, H. Lardy, and K. Myrbäck, editors), New York, Academic Press, Inc., 1960, **4**, 233.
26. GRASSMANN, W., and HEYDE, W., *Z. physiol. Chem.*, 1929, **183**, 32.
27. RAVIN, H. A., BERNSTEIN, P., AND SELIGMAN, A. M., *J. Biol. Chem.*, 1954, **208**, 1.
28. UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F., Manometric Techniques, Minneapolis, Burgess Publishing Co., 1957.
29. CONNORS, W. M., PIHL, A., DOUNCE, A. L., and STOTZ, E., *J. Biol. Chem.*, 1950, **184**, 29.
30. NACHLAS, M. M., and SELIGMAN, A. M., *J. Biol. Chem.*, 1949, **181**, 343.
31. HARDIN, E. B., VALENTINE, W. N., FOLLETTE, J. H., and LAWRENCE, J. S., *Am. J. Med. Sc.*, 1955, **229**, 397.
32. KORN, E. D., *J. Biol. Chem.*, 1955, **215**, 1 and 15.
33. KORN, E. D., *in* Methods of Biochemical Analysis, (D. Glick, editor), New York, Interscience Publishers, Inc., 1959, **7**, 145.
34. LAMBERT, M., and NEISH, A. C., *Canad. J. Research*, 1950, **28B**, 83.
35. CHERKES, A., and GORDON, R. S., JR., *J. Lipid Research*, 1959, **1**, 97.
36. OPIE, E. L., *J. Exp. Med.*, 1906, **8**, 410.
37. WEISS, C., and CZARNETZKY, E. J., *Arch. Path.*, 1935, **20**, 233.
38. WEISS, C., KAPLAN, A., and LARSON, C. E., *J. Biol. Chem.*, 1938, **125**, 247.
39. LAPRESLE, C., and WEBB, T., *Biochem. J.*, 1962, **84**, 455.
40. ANSON, M. L., *J. Gen. Physiol.*, 1940, **23**, 695.
41. PRESS, E. M., PORTER, R. R., and CEBRA, J., *Biochem. J.*, 1960, **74**, 501.
42. LAPRESLE, C., and WEBB, T., *Biochem. J.*, 1960, **76**, 538.
43. BARNES, J. M., *Brit. J. Exp. Path.*, 1940, **21**, 264.
44. MOUNTER, L. A., and ATIYEH, W., *Blood*, 1960, **15**, 52.
45. TABACHNICK, J., and WEISS, C., *Arch. Path.*, 1956, **61**, 76.
46. PANTLITSCHKO, M., and STATTMANN, K., *Biochem. Z.*, 1955, **326**, 252.
47. DERNBY, K. G., *J. Biol. Chem.*, 1918, **35**, 179.
48. ŠTEFANOVIČ, J., WEBB, T., and LAPRESLE, C., *Ann. Inst. Pasteur*, 1962, **103**, 276.
49. MORRISON, W. L., and NEURATH, H., *J. Biol. Chem.*, 1953, **200**, 39.
50. GOETZE, E., and RAPOPORT, S., *Biochem. Z.*, 1954, **326**, 53.
51. PATTERSON, E. K., KEPPEL, A., and HSIAO, S-H., *J. Histochem. and Cytochem.*, 1961, **9**, 609; *J. Biol. Chem.*, 1963, **238**, 3611.
52. TALLAN, H. H., JONES, M. E., and FRUTON, J. S., *J. Biol. Chem.*, 1952, **194**, 793.
53. DE LA HABA, G., CAMMARATA, P. S., and TIMASHEFF, S. N., *J. Biol. Chem.*, 1959, **234**, 316.
54. HESS, R., and PEARSE, A. G. E., *Brit. J. Exp. Path.*, 1958, **39**, 292.
55. DAY, A. J., and HARRIS, P. M., *Quart. J. Exp. Physiol.*, 1960, **45**, 213.

56. Rossiter, R. J., and Wong, E., *J. Biol. Chem.*, 1949, **180,** 933.
57. Frei, J., Borel, C., Horvath, G., Cullity, B., and Vannotti, A., *Blood*, 1961, **18,** 317.
58. Dannenberg, A. M., Jr., Burstone, M. S., Walter, P. C., and Kinsley, J. W., *J. Cell Biol.*, 1963, **17,** 465.
59. Elsbach, P., and Rizack, M. A., *Fed. Proc.*, 1963, **22,** 428; *Am. J. Physiol.*, 1963, **205,** 1154.
60. Johanovský, J., and Škvařil, F., *Immunol.*, 1962, **5,** 469.
61. Sorkin, E., and Boyden, S. V., *J. Immunol.*, 1959, **82,** 332.
62. Skarnes, R. C., and Watson, D. W., *Bact. Rev.*, 1957, **21,** 273.
63. Hirsch, J. G., *Bact. Rev.*, 1960, **24,** 133.
64. Freund, J., *Advances Tuberc. Research*, 1956, **7,** 130.