A HISTOCHEMICAL STUDY OF PHAGOCYTIC AND ENZYMATIC FUNCTIONS OF RABBIT MONONUCLEAR AND POLYMORPHONUCLEAR EXUDATE CELLS AND ALVEOLAR MACROPHAGES

I. Survey and Quantitation of Enzymes, and States of Cellular Activation

ARTHUR M. DANNENBERG, JR., M.D., MARVIN S. BURSTONE, D.D.S., PAUL C. WALTER, and JUNE W. KINSLEY

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

ABSTRACT

The cytochrome oxidase (CO), aminopeptidase (AMP), succinic dehydrogenase (SD), acid phosphatase, esterase, and alkaline phosphatase of rabbit mononuclear (MN) and polymorphonuclear (PMN) peritoneal exudate cells and pulmonary alveolar macrophages (AM)—air dried on Mylar strips—were characterized by histochemical techniques with respect to stability, activators, inhibitors, and pH optima. A granule count method was established for the quantitation of these enzymes. For the acid phosphatase of MN, in which the most precise results were obtained, time, pH, substrate, and inhibitor curves resembled those commonly obtained biochemically. Five of these enzymes were usually more active in AM than MN, whereas the sixth, alkaline phosphatase, was not present in either cell type. AM also tended to consume more oxygen than MN and to divide more frequently. Since the most active cells in the population would be first involved in the host's defense against microbial agents, a comparison was made of the 10 per cent of the AM and MN with the highest enzymatic activities. No differences were found in the granule counts that were not reflected by the means. However, within a given AM population, cells containing ingested dust particles seemed to have higher enzymatic activities than those without particles. MN had greater acid phosphatase and SD activities than PMN and consumed more oxygen, but the CO, AMP, and esterase activites of both types of cells were of similar magnitude. PMN showed high alkaline phosphatase activity; MN showed none. A survey of the histochemical literature indicates that a positive correlation between the enzymatic and phagocytic activities of both MN and PMN exists *in vivo*.

Mononuclear phagocytes (MN)[1] are essential components of the reticuloendothelial system—the chief defense of the host against many micro organisms and their products. Similar functions are served by polymorphonuclears (PMN) in the blood and in areas of inflammation, and by alveolar macrophages (AM) in the lung. The present report (*a*) characterizes six enzymes of rabbit MN

[1] The abbreviations used in this report are as follows: MN, mononuclear(s), or mononuclear peritoneal exudate cells; PMN, polymorphonuclear(s) or polymorphonuclear peritoneal exudate cells; AM, alveolar macrophage(s) of the lung; ip, intraperitoneal; CO, cytochrome oxidase; AMP, aminopeptidase; SD,succinic dehydrogenase; Tris, tris(hydroxymethyl)aminomethane.

and PMN peritoneal exudate cells and alveolar macrophages by utilizing *unfixed* Mylar strip preparations in aqueous histochemical reagents, (*b*) establishes the granule count method for the quantitation of such enzymes, and (*c*) utilizes this technique to compare the enzyme contents of MN and AM, and MN and PMN. The following paper (3) evaluates the role of these enzymes in the phagocytic process.

MATERIALS AND METHODS

Collection and Quantitation of MN, PMN, and AM

MN exudates were elicited in rabbits by the intraperitoneal (ip) injection of 35 ml of heavy mineral oil (U.S.P.). Five to 6 days later, the animals were killed by exsanguination, and their peritoneal cavities washed with citrate-saline (0.4 per cent sodium citrate in 0.85 per cent sodium chloride) or Hanks' solution (4) containing 0.01 per cent heparin. The exudate cells were filtered through gauze, collected by centrifugation, and either used as such or washed once with physiological saline (PSS) or Hanks' solution. Total counts ranged from 30 to 150 million cells per exudate, with a mean of about 80 million. Differential cell counts employing Wright's stain ranged from 80 to 99 per cent MN, with a mean of about 95 per cent. A high percentage of the mononuclear cells were of medium size with a fairly basophilic cytoplasm that sometimes contained mineral oil vacuoles. This type of cell proved most active in the phagocytosis of particles, and for this reason was selected for all the studies on MN exudates reported below. The other cells in these exudates were ignored; *e.g.*, larger, paler MN, PMN, and occasional lymphocytes and mesothelial cells.

PMN exudates were elicited by a method adapted from that of Hirsch (5), namely, the ip injection of 250 to 500 ml of 0.1 per cent glycogen (Nutritional Biochemicals Corporation, Cleveland) in PSS, and the collection of cells 4 (or 18) hours later in a manner similar to that used for MN. Total PMN counts averaged above 100 million cells. Differential cell counts averaged 85 to 95 per cent PMN.

Alveolar macrophages were obtained by a method adapted from that of Myrvik *et al.* (6). Specifically, about 35 ml of PSS, citrate-saline, or heparinized Hanks' solution were injected intratracheally after removal of the lungs from a recently sacrificed rabbit. The fluid was then allowed to drain out. This procedure was repeated and the two washings were pooled, centrifuged and counted in a manner similar to that used for the peritoneal exudates. Total counts averaged about 20 million cells; differential counts averaged 98 per cent AM and 2 per cent PMN.

The *viabilities* of the exudate cells were determined by the trypan blue and eosin methods. These methods, adapted from others (7–10), consisted of mixing, on a microscope slide, one drop of exudate, one drop of autologous serum, and a third of a drop of 1 per cent trypan blue or 0.5 per cent eosin Y in PSS, covering the mixture with a coverslip, and incubating it at 37°C for 10 minutes. Examination under the microscope revealed stained cells (dead) and unstained cells (alive). AM and MN averaged 93 to 99 per cent viability with trypan blue. PMN averaged about 6 per cent lower. With eosin the viabilities of all three types of cells appeared 5 to 10 per cent less than with trypan blue. High degrees of viability were also found in phagocytosis studies on AM, MN, and PMN (3), which have been shown by Tullis (11) to be a more rigorous criterion than dye-permeability tests.

Preparation of Mylar Strips

The cell suspensions were smeared on Mylar[2] strips (12, 13), employing the end of a microscope slide. The strips were air-dried at room temperature for about 20 minutes; no fixation was employed. They then were placed in the refrigerator (5°C) until samples were removed for the determination of succinic dehydrogenase (usually within 3 hours, though sometimes on the next day). After this, the strips were stored in the deep freezer (−25°C). Aminopeptidase and cytochrome oxidase were usually assayed within 3 days, but the activities of the other 3 enzymes described in this report (esterase, acid phosphatase, and alkaline phosphatase) seemed more stable. Although they were usually assayed within a week, they were readily demonstrable in the Mylar preparations after several weeks of storage.

In contrast with preparations on glass, unfixed exudate cells adhered rather well to the Mylar strips during their incubation face down on the surface of the reagent solutions, during their subsequent dips in distilled water, and during their counterstaining with half-strength Ehrlich's acid alum hematoxylin (frequently omitted). Occasionally, however, there was some loss of cells in certain areas. This loss can be decreased, if necessary, by the addition of serum to the exudate cells before they are smeared. After histochemical staining, the strips were air-dried, mounted face up in glycerol gelatin on glass slides, and covered by a coverslip.

Measurement of Oxygen Consumption

The oxygen consumption of cells was determined manometrically in standard Warburg flasks by the

[2] DuPont Mylar, type 50C, 0.25 mil thick (13), is available from Brownell Distributers, Inc., New York. It resembles cellophane or Saran wrap.

method described by Umbreit *et al.* (14). Although the experiments varied, the following amounts of materials are representative of those employed: In the main compartment were phagocytes in citrate-saline (0.5 ml), 1 per cent glucose in PSS (0.6 ml), autologous serum (0.3 ml), and serum from a rabbit immunized against yeast (3) and diluted 1:6 with PSS (0.8 ml). The pH was around 7.0. In each of the two side arms were 0.4 ml of boiled yeast suspension (3). In the center well was 0.20 ml of 20 per cent KOH. The yeast was employed in the phagocytosis studies presented in another report (3); the endogenous respiration of the various phagocytes is presented in this report. Controls made up similarly, but with no phagocytes in the flask, showed no oxygen uptake.

EXPERIMENTAL DATA AND RESULTS

Histochemical Studies on Six MN, PMN, and AM Enzymes Whose Activities May Reflect the Metabolic Potentialities of These Cells

Cytochrome Oxidase (CO)

This enzyme was demonstrated in MN, PMN, and AM by the histochemical method of Burstone (15, 16). Naphthol AS-L3G[3] or 8-amino-1,2,3,4-tetrahydroquinoline[4] (10 mg) and *p*-aminodiphenylamine[5] (10 mg) were dissolved in 0.5 ml of ethanol and made up to 50 ml with 0.1 M Tris buffer (tris(hydroxymethyl)aminomethane) at pH 7.4. The solution was then filtered into small beakers, and Mylar strips of exudate cells were floated on the surface for 1 to 2 hours of incubation at room temperature. A brown-black or blue-black color resulted, depending on the reagents used. The strips were then fixed for 1 hour in a solution containing 10 per cent of each of the following materials: cobalt acetate, formalin, and 0.2 M acetate buffer, (pH 5.2). They were then washed in water and mounted in glycerol gelatin. Figs. 1, 13, and 14 show representative results.

The color in MN and AM was considerably augmented by cytochrome C in a final concentration of 0.2 mg per ml (see Table I). This substance was used in all routine CO determinations; in fact, it enabled the demonstration of this oxidase after the Mylar preparations had been stored in the deep freezer for several days. Catalase had no apparent effect on the enzyme's activity, whereas 10 per cent neutral formalin destroyed it within 5 minutes (see Hannibal *et al.*, 17). Other inhibitors are listed in Table I. These observations suggest that cytochrome oxidase, rather than peroxidase (18), is demonstrated by this reaction in rabbit MN and AM. Our studies with PMN, however, were not extensive enough to favor one enzyme above the other.

No precise quantitation of this enzyme was possible, because of the variability in the amount of color developed in adjacent cells of the same exudate and in adjacent areas of the same Mylar strip.

Aminopeptidase (AMP)

This enzyme was demonstrated in MN, PMN, and AM by the histochemical method of Burstone and Folk (19) which had been modified from Gomori (20). DL-alanyl-β-naphthylamide[6] and fast garnet GBC[7, 8] (each 1 mg per ml) were used as substrate and coupler, respectively, in 0.1 M Tris buffer (pH 7.1). The solution was filtered and Mylar strips incubated on its surface for 1 to 2 hours at room temperature. Figs. 2 and 3 show representative, though rather heavily stained, results for MN and AM. The effects of certain inhibitors on this enzyme are presented in Table I.

Because of a certain amount of non-specific yellow staining, (the specific color is orange-brown), this method was not too satisfactory for quantitative studies. In addition, with storage the color occasionally fades and the dye reorganizes into a coarse precipitate.

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[3] Pfister Chemical Works, Inc., Ridgefield, New Jersey. (See reference 16 for chemical name).

[4] Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York, (practical grade).

[5] British Drug Houses, Ltd., Poole, England, available from the Earling Corporation, Cambridge, Massachusetts. (See reference 16 for chemical name and formula, synonyms, and alternate sources of supply.)

[6] Synthesized by Dr. M. S. Burstone (19); commercially available from Mann Research Lab., New York.

[7] Dyestuff Chemical and Pigments Division, General Aniline and Film Corporation, New York.

[8] The Azoic Diazo Number, Color Index Number, structural formula, chemical name, molecular weight and synonyms of this stable diazonium salt are listed by Lillie (21).

FIGURE 1

Cytochrome oxidase activity of rabbit polymorphonuclear (PMN) and mononuclear (MN) exudate cells. The densely stained cell is an MN; the rest are PMN. In other microscopic fields PMN stained as densely as MN. Glycogen exudate. × 1000.

FIGURE 2

Aminopeptidase activity of MN. One of the more densely stained groups of cells is depicted. Mineral oil exudate. × 1000.

FIGURE 3

Aminopeptidase activity of alveolar macrophages (AM) from the same rabbit that provided the MN of Fig. 2. Both the AM and MN were incubated simultaneously in the same histochemical reagents. One of the more densely stained groups of cells is depicted. × 1000.

FIGURE 4

Succinic dehydrogenase activity of MN and PMN. The two cells with the most dye granules are MN. The rest are PMN. All but a few of the PMN depicted are unstained. An MN can usually be differentiated from a PMN by observing its nucleus at different levels of focus. Glycogen exudate, counterstained with half-strength Ehrlich's acid alum hematoxylin. × 1000.

FIGURE 5

Succinic dehydrogenase activity of MN. One of the more densely stained groups of cells is depicted. Mineral oil exudate. × 1000.

FIGURE 6

Succinic dehydrogenase activity of AM from the same rabbit that provided the MN in Fig. 5. The cells represented in both of these figures were incubated simultaneously in the same histochemical reagents. One of the more densely stained groups of cells is depicted. × 1000.

FIGURE 7

Acid phosphatase activity of PMN and MN. In general, the MN are more darkly stained. Four are present (at 1, 2, 3, and 7 o'clock respectively). Glycogen exudate, counterstained with half-strength Ehrlich's acid alum hematoxylin. × 1000.

FIGURE 8

Esterase activity of MN. One of the more densely stained groups of cells is depicted. Mineral oil exudate. × 1000.

FIGURE 9

Esterase activity of AM from the same rabbit that provided the MN in Fig. 8. The cells represented in both of these figures were incubated simultaneously in the same histochemical reagents. One of the more densely stained groups of cells is depicted × 1000.

FIGURE 10

Alkaline phosphatase activity of PMN and MN. The two very pale cells to the right of the center are MN. The rest are PMN. Glycogen exudate, counterstained with half-strength Ehrlich's acid alum hematoxylin. × 1000.

FIGURE 11

Esterase activity of PMN and MN. The four largest cells are MN; the rest are PMN. In this example, the MN stained slightly darker than the PMN, but in other examples the two groups stained with equal intensities. Glycogen exudate. × 1000.

FIGURE 12

AM that have ingested autologous red blood corpuscles (rbc) *in vivo*. The center cell contains three rbc, the lower cell contains one. A few dust particles are also visible in the center cell. Wright's stain. × 1000.

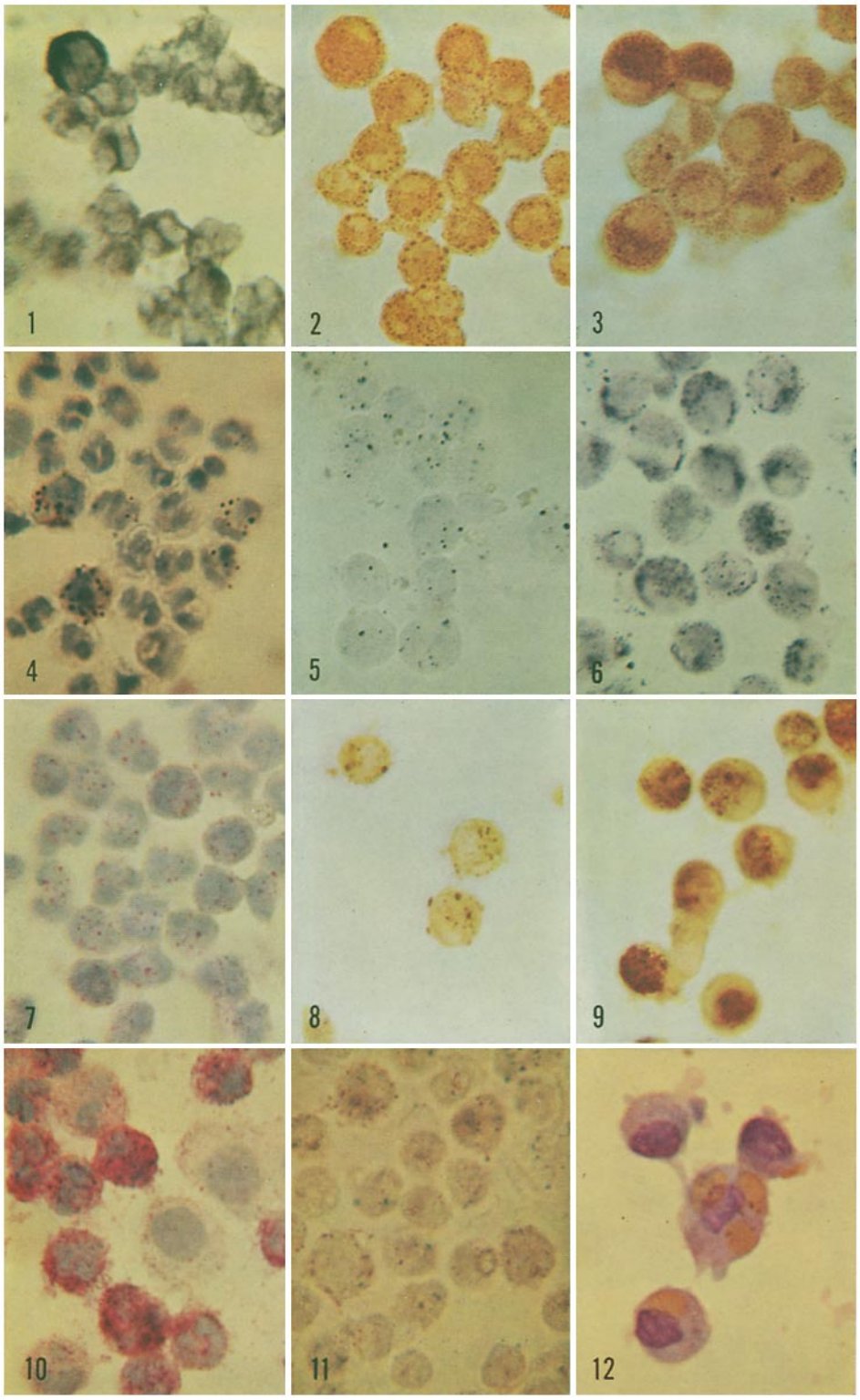
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TABLE I

The Effect of Inhibitors and Activators on Certain Histochemically Demonstrable Enzymes of MN, PMN, and AM

Enzyme	Inhibitor (I) or activator (A)	Amount of inhibition or activation* MN	PMN	AM
Cytochrome oxidase	A Cytochrome C‡ (0.2 mg per ml)	++++	++	++++
	I NaCN (0.001 M)	++	++	
	I Na_2S (0.01 M)	++	+++	
	I Sodium azide (0.01 M)	++	0	
	I Catalase (0.02 per cent)	0	0	0
Aminopeptidase	I KCN (0.01 M)	+	+	+
	I $CuSO_4$ (0.01 M)	++	++++	++
	I $CuSO_4$ (0.0015 M)	+++	+++	+++
Succinic dehydrogenase	I Sodium malonate (0.008 M)	++	+	+
	I *p*-chloromercuribenzoate (0.0002 M)	+++	++++	+++
Acid phosphatase	I Sodium molybdate (0.005 M)	++++	+++	++++
	I Sodium arsanilate (0.001 M)	0		0
	I NaF (0.01 M)	+++	+++	++
	A $MnCl_2$ (0.002 M)	±		
Alkaline phosphatase	I Sodium molybdate (0.005 M)		+	
	I Sodium arsanilate (0.001 M)		+	
	I NaF (0.01 M)		+	
	I Sodium iodoacetate (0.002 M)		+	
	A $MnCl_2$ (0.002 M)		0	
Esterase	I Eserine salicylate (0.01 M)	0	±	0
	I Sodium azide (0.01 M)	++++	++++	+++
	I Diisopropyl fluorophosphate (0.001 M)§	+++	++++	++
	I NaF (0.01 M)	++	++++	0

* 0, no inhibition (or activation); ++++, complete or almost complete inhibition (or activation); others (+, ++, +++) were in between these extremes. Each category represents the results of 2 to 5 experiments.

‡ Cytochrome C, Type II, from horse heart, Sigma Chemical Company, St. Louis.

§ Merck and Co., Inc., Chemical Division, Rahway, New Jersey.

Succinic Dehydrogenase (SD)

Dehydrogenase activity was demonstrated in MN, PMN, and AM, essentially by the method of Nachlas *et al.* (22). To 10 mg of Nitro-BT[9] (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride) dissolved in 0.5 ml of ethanol was added 500 mg of sodium succinate in 40 ml of 0.1 M Tris buffer at pH 7.4. The solution was then filtered into small beakers, and the Mylar strips were incubated on the surface for 0.5 to 2.5 hours at 37°C.

The dehydrogenase of these phagocytes seemed to be a rather unstable enzyme, and so it was generally assayed on the same day that the exudates were withdrawn from the animal. Figs. 4, 5, and 6 show representative results in PMN, MN, and AM. For both MN and PMN the pH optimum was around 7 to 8. At pH 5.7 and 8.9, the activity

[9] Dajac Laboratories, Borden Company Chemical Division, Philadelphia.

was somewhat reduced. The pH optimum for AM was not determined. The effects of certain inhibitors on this reaction are listed in Table I.

When sodium succinate was omitted from the Nitro-BT reagent solution, only a rare formazan granule appeared in MN and AM. With PMN, however, the number of formazan granules formed in the absence of substrate seemed to equal the number formed in its presence. Thus our studies on the SD activity of PMN probably do not concern this specific substrate, but rather an as yet undefined substrate within these cells.

The formazan granules formed by the reduction of Nitro-BT showed more variation in size than the dye granules produced by the other enzymes. An additional complication arose from the *instability* of this dehydrogenase. In phagocytes injured during their collection or during the preparation of the Mylar smear, SD activity was poorly preserved, whereas in morphologically intact MN and AM the dehydrogenase frequently showed high activity.[10] In fact, Wachstein and Meisel (23, 24) consider the activity of this enzyme a most sensitive indicator of cell damage.

Acid Phosphatase

This enzyme was demonstrated in MN, PMN, and AM by the method of Burstone (25, 26). Five mg of naphthol AS–BI phosphate[11] was dissolved in 0.5 ml of dimethylformamide. Fifty ml of 0.1 M acetate buffer (pH 5.2) and 10 mg of fast red violet LB[8, 12] were added and also two drops of 10 per cent $MnCl_2$ solution as a possible activator. The somewhat opalescent solution was then filtered, and the Mylar strips incubated on its surface at room temperature for 10 to 60 minutes. Figs. 7, 16, and 17 show representative results in PMN, MN, and AM.

The azo dye granules formed in MN usually were discrete, fairly uniform in size, and almost never small enough to create a diffuse appearance. This enzyme was rather stable, for Mylar strips of MN still showed activity after several months of storage in the deep freezer (−25°C). Because of these properties, MN acid phosphatase was used to establish the granule count method described in the next section, where time, pH, substrate, and inhibitor (NaF) relationships are presented.

The dye deposits in AM and PMN were both diffuse and granular and could not be quantitated as precisely as those in MN. Table I presents the effects of inhibitors on all three types of phagocyte.

The method for alkaline phosphatase (see below), with the addition of $MnCl_2$, was used to construct a pH curve for PMN phosphatase. It had two peaks: one at pH 4.7 to 5.2, and one, 1.5 times as large, at pH 8.3 to 8.9. Some activity was present, however, from pH 4.1 to 8.9 which was the entire pH range tested.

Alkaline Phosphatase

This enzyme does not seem to be present in rabbit MN or AM, but only in PMN. It was demonstrated by the method of Burstone (25) employing 5 mg of naphthol AS–MX phosphate,[11] 0.5 ml of dimethylformamide, 10 mg of fast red violet LB,[8, 12] and 50 ml of Tris buffer (0.1 M) at pH 8.3. The procedure was similar to that for acid phosphatase, described above. Incubation times varied from 2 to 15 minutes at room temperature. The type of granule produced was rather fine and somewhat diffuse (Fig. 10), so that *quantitative* studies on this enzyme were less precise.

At incubation times of 2 and 3 minutes, pH curves showed an optimal alkaline phosphatase activity above 8.0. Follette *et al.* (27) found biochemically an optimum between 8.3 and 8.9. The effects of certain inhibitors and activators are presented in Table I.

Esterase

The activity of this enzyme was demonstrated in MN, PMN, and AM by the method of Burstone (28). Naphthol AS–D acetate[13] (5 mg) was dissolved in 0.5 ml of dimethylformamide, and 50 ml of 0.1 M Tris buffer at pH 7.1 was added, followed by fast garnet GBC[7] (10 mg). The mixture was then filtered, and the cell preparations incubated

[10] Injured and morphologically intact phagocytes can be readily differentiated in Wright's stained-preparations. The injured cell appears larger (presumably because it has absorbed fluid), its nucleus is less basophilic, and its edge is less distinctly demarcated.

[11] Synthesized by Dr. M. S. Burstone (25, 26); commercially available from Sigma Chemical Company, St. Louis.

[12] Verona Dyestuffs, Verona Chemical Co., Union, New Jersey.

[13] Naphthol AS-D acetate was synthesized by Dr. M. S. Burstone (29) and used instead of naphthol AS–LC acetate. It is commercially available from Sigma Chemical Company, St. Louis.

on the surface of the filtrate for 3 to 40 minutes at room temperature. Figs. 8, 9, and 11 show representative results for MN, AM, and PMN.

The azo dye granules produced ranged in size. Some of them were so small that a diffuse appearance resulted, whereas some were as large as *Rickettsia* or even larger. Quantitative studies on this enzyme, therefore, were not very precise. The esterase of both intact and injured MN, PMN, and AM seemed rather stable in the deep freezer. Its pH optimum was around 7.0 to 7.6 for both MN and PMN; the optimum for AM was not evaluated.

Table I presents the effect of certain inhibitors on the esterase activity of these three cell types.

Histochemical Quantitation by the Granule Count Method

Studies with MN Acid Phosphatase

The acid phosphatase reaction of MN produced discrete dye granules of fairly uniform size and therefore seemed suitable for the development of a new quantitative procedure which we have termed the granule count method. Granule counts were made in the following manner: A unit granule approximately 0.3 μ in diameter (Fig. 21) was first selected, and granules of other sizes were designated as fractions or multiples of this unit. The total number of granule units per

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FIGURE 13

Cytochrome oxidase activity of MN. One of the more densely stained groups of cells is depicted. Mineral oil exudate. × 1000.

FIGURE 14

Cytochrome oxidase activity of AM from the same rabbit that provided the MN in Fig. 13. The cells represented in both of these figures were incubated simultaneously in the same histochemical reagents. One of the more densely stained groups of cells is depicted. × 1000.

FIGURE 15

Acid phosphatase activity of AM. The center cell contains the most "dust" as well as enzyme. However, some dust and other foreign particles are present in all of the cells shown. × 1000.

FIGURE 16

Acid phosphatase activity of MN. One of the more densely stained groups of cells is depicted. Mineral oil exudate. × 1000.

FIGURE 17

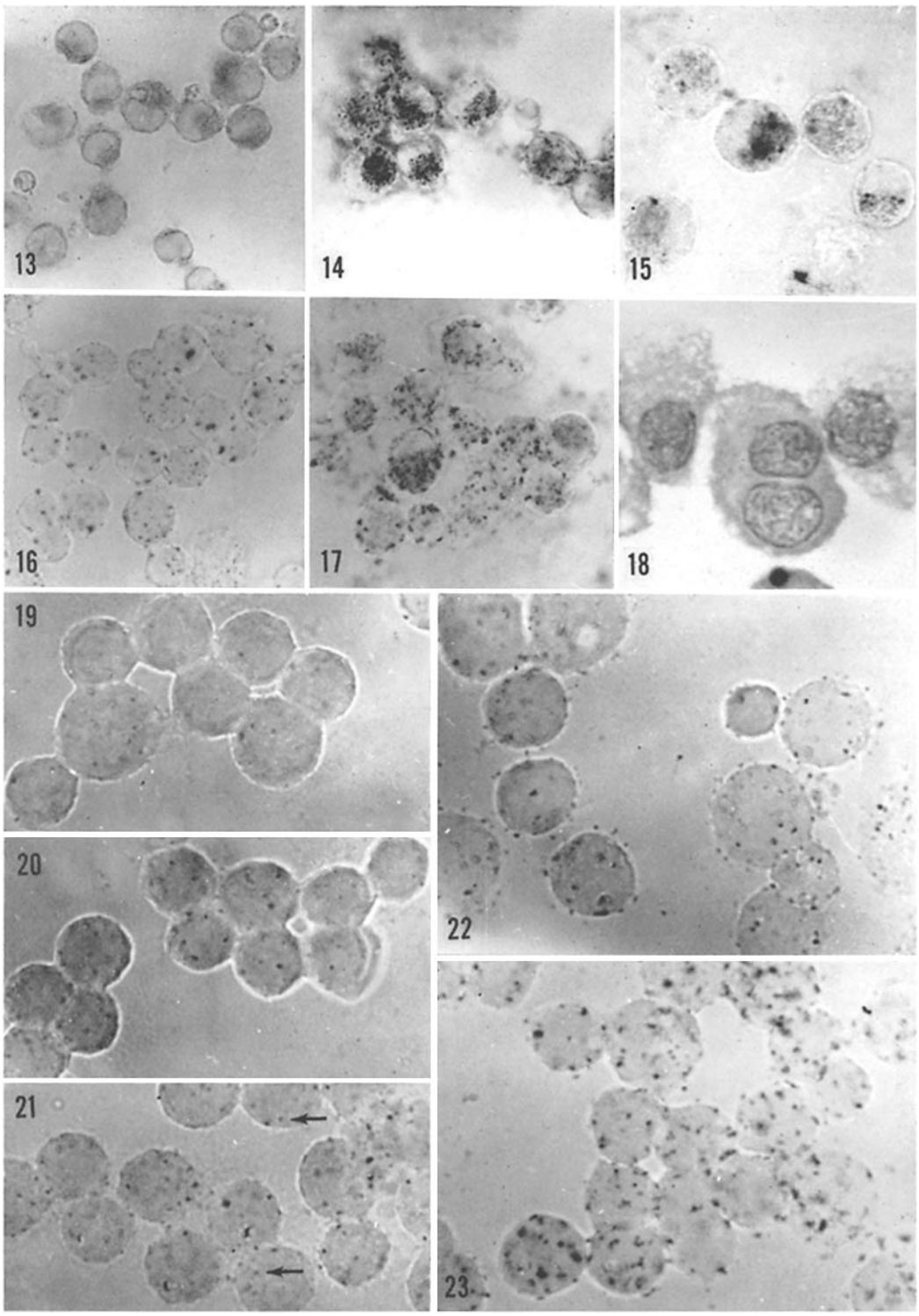
Acid phosphatase activity of AM from the same rabbit that provided the MN in Fig. 16. The cells represented in both of these figures were incubated simultaneously in the same histochemical reagents. One of the more densely stained groups of cells is depicted. × 1000.

FIGURE 18

An alveolar macrophage with 2 nuclei, indicative of cell division. Wright's stain. × 1000.

FIGURES 19 TO 23

The effect of incubation time on MN acid phosphatase activity. The MN represented in these figures were incubated 4, 8, 16, 20, and 45 minutes, respectively, at room temperature. They are the same cells that are represented by black dots in Fig. 24. The smaller granules in Fig. 21 were considered unity for purposes of quantitation. Two of these are marked with arrows. Not all the granules in a given cell are visible at a single level of focus. Mineral oil exudate. × 1500.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cell was then considered a measure of its enzyme content.

As a test of the reliability of this method, the acid phosphatase reaction of MN was run at varying time, pH, and substrate and inhibitor concentrations. The results for varying time are depicted in Figs. 19 to 23 and plotted in Fig. 24. The others are plotted in Figs. 25 to 27. These results resemble curves obtained for enzymes by biochemical techniques. The standard deviation of the mean for a given point on a given curve was usually less than 10 per cent of its numerical value, provided that a single investigator did all the counting.

It seems, therefore, that the granule count method is a valid quantitative procedure and can be used to compare *different* Mylar preparations. However, it is most suitable for the study of individual cells or of different types of cells in the *same* Mylar preparation, *e.g.* cells that have ingested particles and those that have not (see reference 3), or MN and PMN in the same exudate (see below).

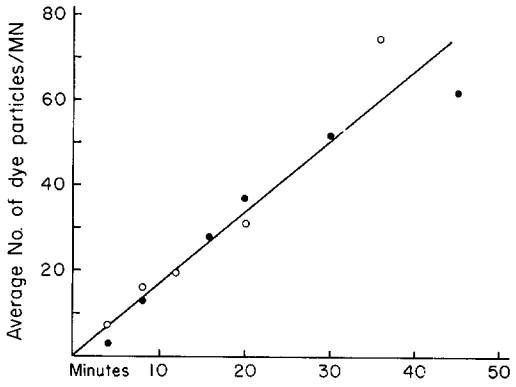

FIGURE 24

Effect of incubation time on acid phosphatase activity of MN. ● and ○ represent the results of two independent investigators and two separate experiments, adjusted to a common base line.

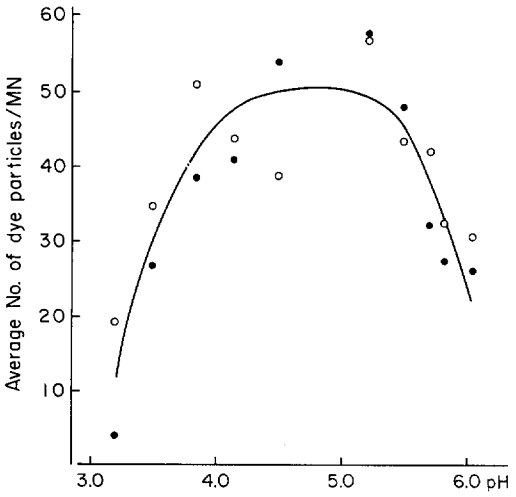

FIGURE 25

Effect of pH on acid phosphatase activity of MN. ● and ○ represent the results of two independent investigators, adjusted to a common base line.

STUDIES WITH OTHER ENZYMES

Although these studies could not be so precise as those just plotted for MN acid phosphatase, they

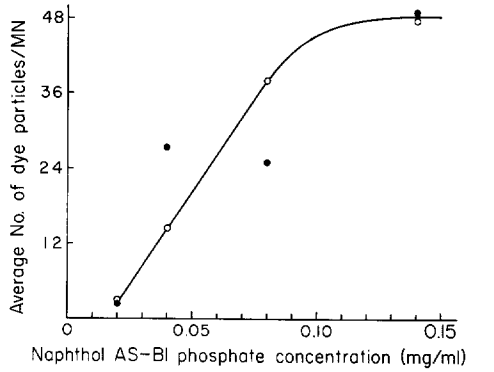

FIGURE 26

Effect of substrate concentration on acid phosphatase activity of MN. ● and ○ represent the results of two independent investigators, adjusted to a common base line.

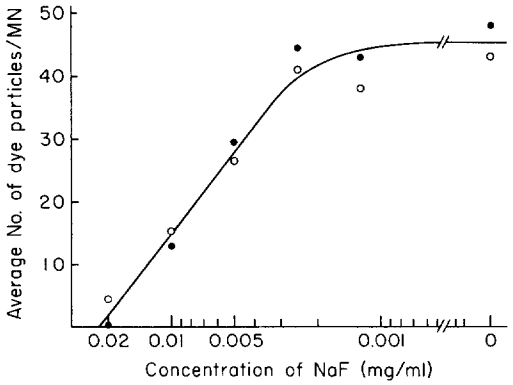

FIGURE 27

Effect of concentration of NaF inhibitor on acid phosphatase activity of MN. ● and ○ represent the results of two independent investigators, adjusted to a common base line.

did follow a similar pattern. There was a straight line relationship between time and the number of granule units resulting from the succinic dehydrogenase and esterase activities of MN and PMN and the acid and alkaline phosphatase activities of PMN. The effect of pH on these enzymes was reported in the previous section. No time and pH studies were made on AM.

A Comparison of MN to AM and PMN with Respect to Certain Histochemically Demonstrable Enzymes and Oxygen Consumption

Peritoneal and alveolar macrophages represent two varieties of mononuclear phagocytes. The former (MN) are mobilized into the peritoneal cavity by mineral oil, a stimulus that is present 5 to 6 days. The latter (AM) are *continuously* mobilized into the pulmonary air spaces in response to the inhalation of dust and bacteria, and possibly to the extravasation of occasional red corpuscles (Fig. 12). Although AM resemble MN in general morphology, they are slightly larger, occasionally have a somewhat eosinophilic cytoplasm,[14] and may contain dust particles. MN, on the other hand, may contain vacuoles filled with mineral oil.

MN and PMN represent the two major types of phagocyte in the body. The former clear the blood as a part of the reticuloendothelial system and are active in areas of *chronic* inflammation. The latter circulate in the blood, and emigrate to areas of *acute* inflammation.

Because of the differences in function of all three kinds of phagocyte, it is of interest to determine whether some of their enzymes are qualitatively or quantitatively different and to compare their oxygen consumption as a measure of their general activity.

Histochemical Studies on MN and AM

COMPARISON OF AM AND MN ENZYMES: AM and MN were removed from the same rabbit at the time of death, smeared on Mylar strips, incubated for *identical* periods in histochemical reagents, and mounted side by side on the same slide. The results were read by two methods. In the first, the paired strips were studied alternately until the amount of color of the cells in the AM strip relative to that of the cells in the MN strip could be determined (Table II). In the second, the amount of granular and diffuse dye material in each cell was assigned numerical values (Table III). A different series of rabbits was used with each method in order to extend the number of observations.

From these tables which present the results, it is apparent that AM showed more enzymatic activity than MN in over half of the cases. In the rest they were approximately equal, except for rare instances in which the reverse occurred. Figs. 2 and 3; 5 and 6; 8 and 9; 13 and 14; and

TABLE II

*A Histochemical Comparison of Various Enzyme Activities of AM and MN from the Same Rabbit—Survey Method**

Rabbit No. (Series I)	Enzyme	AM	MN	Ratio: AM/MN
1	Cytochrome	++++	1/2	8
2	oxidase	++	++	1
3		++++	1/2	8
4		++	++	1
8		++	+	2
12		++++	++	2
13		++	++	1
1	Aminopep-	++	+	2
4	tidase	++	+	2
5		+	+	1
8		++++	+	4
11		++++	+	4
12		++	++	1
2	Acid phos-	+++	+	3
8	phatase	++++	1/2	8
9		++	+	2
10		++	+	2
5	Esterase	++	+	2
6		++++	+	4
7		++++	+	4
10		++++	+	4
12		++	+	2
13		++	+	2
1	Succinic de-	++++	+	4
6	hydrogenase	++++	+	4
7		++	++	1
8		++	+	2

* See text

[14] Possibly from the previous ingestion of red blood corpuscles (30); they contain ferritin (31).

TABLE III

A Histochemical Comparison of Various Enzyme Activities of AM and MN from the Same Rabbit—Granule Count Method

Rabbit No. (Series II)	Enzyme	No. of cells evaluated		Average No. of histochemical dye granules per cell*						Ratio: AM/MN for		
				In the darkest staining 10 per cent of the cells		In the darkest staining 20 per cent of the cells		In 100 per cent of the cells				
		AM	MN	AM	MN	AM	MN	AM	MN	Top 10 per cent	Top 20 per cent	100 per cent
II	Cytochrome oxidase	50	55	711	640	693	606	543	386	1.1	1.1	1.4
III		53	52	650	671	616	616	384	376	1.0	1.0	1.0
V		50	50	692	474	670	456	527	301	1.5	1.5	1.8
I	Aminopeptidase	100	100	200	178	190	173	157	159	1.1	1.1	1.0
II		54	59	602	520	633	487	423	294	1.2	1.3	1.4
V		50	65	621	402	588	372	439	171	1.5	1.6	2.6
II	Succinic dehydrogenase	41	62	206	313	191	318	106	179	0.7	0.6	0.6
III		100	100	105	103	99	92	58	59	1.0	1.1	1.0
V		118	134	108	72	89	55	27	14	1.5	1.6	1.9
VI		50	50	356	133	318	119	184	67	2.7	2.7	2.8
I	Acid phosphatase	100	100	54	37	48	35	78	22	1.5	1.4	3.6
II		55	43	206	50	185	30	70	18	4.1	6.2	3.9
IV		100	100	113	90	114	80	184	48	1.3	1.4	3.8
V		61	61	321	373	229	349	115	171	0.9	0.7	0.7
I	Esterase	100	100	132	54	149	48	123	27	2.4	3.1	4.6
II		81	145	588	526	531	499	372	271	1.1	1.1	1.4
IV		100	80	532	493	495	448	351	304	1.1	1.1	1.2
V		66	55	495	683	468	640	232	269	0.7	0.7	0.9

* When diffuse dye material was present in a cell, its equivalent in dye-granules was estimated and added to the number of granules present. This was accomplished by assigning the diffuse staining to one of 5 intensities that ranged from 75 to 375 granules. Succinic dehydrogenase of AM and MN and acid phosphatase of MN rarely were manifest by diffuse staining, whereas the other enzymes commonly were. This explains why many of the granule counts of the latter group appear high.

16 and 17 show representative results from rabbits whose AM had distinctly higher enzymatic activity. With the exception of succinic dehydrogenase, all the enzymes studies in AM were represented by dye material that was partly diffuse and therefore were only amenable to semiquantitative estimation.

COMPARISON OF THE ENZYMATICALLY MOST ACTIVE CELLS IN AM AND MN PREPARATIONS: A group of AM or MN may be characterized by its most active members, for these are the cells that ingest the foreign particles in the alveolar air or the blood stream, as the case may be, not the sluggish cells. For a comparison of the enzymatically most active AM and MN, studies were made of the 5, 10, 15, and 20 per cent of the cell population with the highest enzyme content. The results at the top 10 and 20 per cent levels typify the others and are included in Table III. From this, it is apparent that most of the AM:MN ratios were not appreciably altered at higher enzyme levels.

TABLE IV

Correlations between the Number of Dust Particles in AM and Their Enzymatic Activities*

Rabbit No.	Enzyme	No. of cells evaluated	Type of cell evaluated	Average No. of histochemical dye granules per cell	Average No. of dust* particles per cell
14	Esterase	66	Top 10 per cent‡	240	33
			Top 20 per cent	220	34
			Bottom 80 per cent	75	13
15	Acid phosphatase	55	Top 10 per cent	78	30
			Top 20 per cent	65	12
			Bottom 80 per cent	15	6
16	Acid phosphatase§	100	Top 20 per cent	48	0.0
			Bottom 80 per cent	21	0.5

* Some of these "dust" particles may be hemosiderin granules[14] and other material.
‡ "Top 10 per cent" refers to the 10 per cent of the cells that contained the most *dye* granules.
§ In this experiment there seemed to be too few dust particles to make a reliable study.

TABLE V

Oxygen Consumption of PMN, MN, and AM

Rabbit No. and differential cell count (in parentheses)*			No. of cells per Warburg flask (in millions)			μl O_2 consumed per hour per million cells			Ratio:
PMN	MN	AM	PMN	MN	AM	PMN	MN	AM	MN:AM
1 (91)	6 (90)	6 (100)	34.0	6.0	6.0	0.47	2.2	3.2	1:1.5
2 (85)	7 (96)	7 (99)	21.3	7.2	7.2	0.56	2.2	2.2	1:1.0
3 (88)	8 (99)	8 (100)	23.6	18.0	18.0	0.25	1.8	2.4	1:1.3
2 (85)	9 (85)	9 (100)	213.	8.7	9.8	0.32	1.7	4.3	1:2.5
3 (88)	10 (93)	10 (99)	236.	3.8	3.8	0.21	7.4	6.8	1:0.92
4 (81)			180.			0.29			
5 (91)			200.			0.29			
Average						0.34	3.1	3.8	1:1.4
Median						0.29	2.2	3.2	1:1.3
Ratio: Average PMN:MN:AM						1 :	9.1 :	11.2	
Median PMN:MN:AM						1 :	7.6 :	11.0	

* For example, the cell preparations listed on the top line contained, respectively, 91 per cent PMN, 90 per cent MN, and 100 per cent AM. The values listed for oxygen consumption represent the cell preparation as a whole with no adjustment for its differential cell count.

Throughout the course of these studies, it was observed that AM with the highest enzyme activities frequently contained the most "dust" particles (Fig. 15). In the two experiments where these factors were adequately quantitated, this correlation was indeed found to be the case (Table IV). It is of interest that AM containing dust particles are among the first cells to ingest inhaled tubercle bacilli (32).

Oxygen Consumption of AM and MN

Not only do AM tend to have higher levels of certain enzymes than MN, but they also tend to

TABLE VI

A Histochemical Comparison of Various Enzyme Activities of PMN with Those of MN in the Same Glycogen Exudate

Enzyme	Rabbit No.	No. of MN studied	No. of PMN studied	Average No. of histochemical granules per cell		Ratio: MN/PMN
				MN	PMN	
Cytochrome oxidase	1	6	33	54	33	1.6
	2	25	150	43	43	1.0
	3	Numerous	Numerous	++	++	1.0
	4	Numerous	Numerous	+++	++	1.5
Aminopeptidase	1	26	105	73	89	0.8
	2	30	75	78	50	1.6
	5	35	138	60	104	0.6
	3	Numerous	Numerous	++	++	1.0
	4	Numerous	Numerous	++	++	1.0
Succinic dehydrogenase	1	20	84	95 + 100*	32	6.1
	2	51	255	94 + 100*	93	2.1
	4	50	50	47	13	3.6
	6	20	50	95 + 100*	28	7.0
	7	Numerous	Numerous	++++	+	4.0
	8	Numerous	Numerous	+++++	+	5.0
Acid phosphatase	8	19	50	47	6	7.8
	9	20	50	56	15	3.7
	10	20	50	61	20	3.1
	11	30	70	80	21	3.8
	12	35	72	125	31	4.0
Alkaline phosphatase	1	19	84	0	50	0.0
	2	30	187	4‡	46	0.1
	6	52	53	9‡	45	0.2
	8	25	60	8‡	32	0.3
	10	25	50	7‡	36	0.2
	11	25	50	6‡	36	0.2
Esterase	1	15	24	37	36	1.0
	8	25	45	11	12	0.9
	11	25	50	19 + 32*	15 + 38*	1.0
	12	30	40	12 + 18*	13 + 26*	0.8
	13	17	110	52	32	1.6

* Estimate in granules of the amount of diffuse dye material present in the cells. Such diffuse color was rare with succinic dehydrogenase. Its presence in three of the samples listed may have been due to the use of glycogen to produce these exudates.

‡ These values probably represent MN *acid* phosphatase acting suboptimally at this alkaline pH.

have higher rates of oxygen consumption (Table V). The respiration of MN, expressed in microliters of oxygen per hour per million cells, averaged 2 to 3, and that of AM, 3 to 4. Comparable figures of Myrvik (33) for rabbits were approximately 1.5 and 4 μl, respectively; and comparable figures from Karnovsky's laboratory (34, 35) for guinea pigs were 1.4 and 8.2 μl, respectively.

An additional indication of greater AM activity was their more frequent cell division (*cf.* Lurie, 36), which we observed in Wright's stained preparations, but did not quantitate (Fig. 18).

Histochemical Studies on MN and PMN

Since MN and PMN from different rabbits in response to different irritants are not strictly comparable (*cf.* Elberg, 37), a histochemical comparison was made of these cells when they were simultaneously present in the same 18-hour glycogen exudate. In this type of exudate, MN and PMN show high viabilities both by the dye tests (see Materials and Methods) and by their ability to phagocytize particles (3).

The results are presented in Table VI and Figs. 1, 4, 7, 10, and 11. From these it is apparent that MN had greater acid phosphatase and succinic dehydrogenase activities, and PMN greater alkaline phosphatase activity (MN showed none). Their cytochrome oxidase, aminopeptidase, and esterase activities were of similar magnitude.

Oxygen Consumption of MN and PMN

Table V indicates that rabbit MN exudates have a higher oxygen consumption than PMN. PMN consumed about 0.3 μl of oxygen per hour per million cells and MN consumed from 2 to 3 μl. Comparable figures of Stähelin, Suter, and Karnovsky (8, 34) for PMN and MN exudates of guinea pigs were 0.3 and 1.0 μl, respectively; and comparable figures of Frei *et al.* (38) for PMN and MN of human blood were 0.15 and 0.5 μl, respectively.

Exudate MN are slightly larger than PMN, but not enough to account for their greater enzymatic activities and oxygen consumption. Their motility as observed by time-lapse cinematography (39) was also greater. These three parameters indicate that MN are metabolically and functionally more active cells.

DISCUSSION

This paper reports our attempts to quantitate by the granule count technique several histochemically demonstrable enzymes in rabbit mononuclear and polymorphonuclear exudate cells (MN and PMN) and pulmonary alveolar macrophages (AM). We were successful with acid phosphatase in MN and partially successful with succinic dehydrogenase in all three types of phagocyte. With the others (cytochrome oxidase, aminopeptidase, and esterase) the method became semiquantitative in nature, because part of the color produced was diffuse and not amenable to granule counting.

The drying of the cells on the Mylar strips evidently caused rupture of such structures as lysosomes and mitochondria whose enzymes became more freely distributed throughout the cytoplasm. This redistribution enabled the quantitative procedures herein described, for the sites of enzymatic activities as evidenced by the dye granules became dependent on the enzyme concentration rather than on the number of organelles (*cf.* Doyle, 40). The granule count method therefore may not be applicable to cells fixed in formol calcium or other agents that seem to maintain organelle structure.

Other methods have been introduced for the histochemical quantitation of enzymes in individual cells. Benditt and Arase (41) studied the time, substrate, and inhibitor relationships of a chymotrypsin-like enzyme in mast cells by matching color intensities. Similar methods were employed by Hopsu and Glenner (42) to evaluate the trypsin-like enzymes of mast cells, and by Kaplow (43) and Quigley *et al.* (44) to evaluate the alkaline phosphatase activity of PMN in blood smears. The use of particle sizing and particle counting was described by Eränkö in his text on quantitative histochemical methods (45), but their application to enzymatic reactions was not discussed.

Survey of Literature

There have been few if any previous histochemical studies on the enzymes of rather pure populations of phagocytes, but there have been many such studies on these phagocytes in blood and bone marrow films and in tissue sections.

Baker and Klapper (46) demonstrated cytochrome oxidase in foreign body giant cells (which are closely related to MN). Hannibal *et al.* (17) found strongly positive reactions in human MN and PMN, which they believed were due to *both* the G-Nadi reaction for cytochrome oxidase and the M-Nadi reaction for peroxidase. Positive reactions in blood and bone marrow cells have also been described by others (47–49).

The aminopeptidase of human blood leukocytes has been evaluated by Ackerman (50), and identified in tissue MN (19, 51) and PMN (19).

Dehydrogenase activity specific for succinate has been described in PMN, MN, and lymphocytes of human blood and bone marrow (52–56). Ackerman (54) was able to show mitochondrial

localization of succinic dehydrogenase in viable blood cells by exposing them to a non-ionic, surface-active agent. Balogh and Cohen (56) studied this enzyme in *unfixed* human blood leukocytes by means of phase microscopy.

Acid phosphatase has been demonstrated by histochemical methods in the MN of man (57), rabbits, mice, and rats (58), and chickens (59), but has not been found in the MN of guinea pigs (58). This enzyme is found in the lysosomes, phagosomes, and pinocytotic vacuoles of cells (60–66).

Among the first histochemical reports on the alkaline phosphatase of PMN are those of Gomori (67), Wachstein (68), and Wislocki and Dempsey (69). Many other investigators have since confirmed the presence of alkaline phosphatase in the PMN of blood (see references 70 and 71). It is of interest that Grogg and Pearse (58) demonstrated alkaline phosphatase in PMN of rats, guinea pigs, and rabbits, but could not detect it in PMN of mice. It was absent in the MN of most species. A rather complete biochemical characterization of purified alkaline phosphatase from human leukocytes was made by Trubowitz *et al.* (72). This enzyme is low in chronic myeloid leukemia, and rather high in leukemoid states (73–76), a finding that may be of considerable diagnostic importance.

There are many reports that describe esterases in tissue phagocytes. Among these are the following: for PMN, (references 58, 77–81), for MN (58, 77, 80–91), and for AM (82, 87, 89, 90, 92). With few exceptions, the inhibitor studies reported in the literature (77, 82–88, 92) agree with our results. AM and MN appear to contain both the inhibitor-sensitive and inhibitor-resistant varieties of esterase described by Shnitka and Seligman (93). Increased MN esterase activity was found in the lesions of cutaneous tuberculosis (83, 85), BCG infection (91), leprosy (85, 91), and leishmaniasis (94). In infectious leukocytosis circulating PMN likewise showed increased esterase activity (80).

Our findings that AM contained higher concentrations of enzymes than MN are in agreement with other investigators. Myrvik *et al.* (95, 96) found that AM had 2 to 5 times more lysozyme, β-glucuronidase, and acid phosphatase. Cohn and Wiener (65) have confirmed these results and added an acid-acting proteinase and ribonuclease to the list. Using histochemical methods Nachlas and Seligman (89) and Chessick (90) observed higher esterase activity in AM than in Kupffer cells.

With respect to our observations on the enzymes of PMN and MN, there are both conflicting and confirmatory reports. The biochemical studies of Frei *et al.* (38) showed that human blood MN had over twice the esterase activity of PMN, whereas the histochemical studies of Ackerman (81) showed the reverse. In glycogen exudates from rabbits we found that MN and PMN contained equal amounts of esterase. Our studies on their phosphatases are in agreement with others (58, 67, 68). The peak we observed in the acid range of the pH curve for PMN phosphatase seems to be of special significance, because such a peak determined biochemically (97) might have arisen from contaminating MN.

In Vivo Activation of PMN

Ludwik Fleck has termed this phenomenon "leukergy" and found that circulating PMN were more active following certain stress situations, *i.e.* infections, trauma, and endotoxin injection (98, 99). With such PMN, the cells' "stickiness," motility, phagocytic ability, and activity in destroying staphylococci were increased, as were their contents of alkaline phosphatase and glycogen (100–106). Other investigators have undoubtedly studied similar phenomena by evaluating under various conditions PMN alkaline phosphatase (43, 68, 73, 107–110), "lipase" (esterase) (111), peroxidase (112), and glycogen contents (112). A stress-induced adrenal response may be at least partially responsible for PMN activation, for ACTH and 17-hydroxy-corticosteroids increased PMN alkaline phosphatase levels in man (109, 110).

It seems from the references just listed that PMN circulate in two states of activation: (*a*) a relatively inactive PMN with low alkaline phosphatase content, and (*b*) an activated, probably more mature (113) PMN with high alkaline phosphatase content. Evidently conditions of stress increase the proportion of activated PMN in the blood. Circulating PMN are only a small fraction of those stored in the bone marrow, spleen, and lung. A study of these reserves may be necessary to elucidate the mechanisms responsible for PMN activation (*cf.* reference 114).

In Vivo Activation of MN

Since the original work of Lurie (36), there have been many investigations of this nature

(115–120), for MN are the functional element of the reticuloendothelial system. Among the stimuli employed were live, attenuated, or dead bacteria, endotoxin, drum trauma, and thyroid hormone. Among the indices of MN activation were phagocytic ability and resistance to shock-producing and infectious agents.

In spite of the extensive work on activation of the reticuloendothelial system, there have been relatively few reports on accompanying changes in the enzyme content of MN. Lurie (36) has demonstrated that MN from tuberculous animals are more active phagocytically than controls. Such MN also have higher dehydrogenase (121) and acid phosphatase activities (58, 121–123). Connective tissue histiocytes of mice that were most phagocytic for several substances *in vivo* were highest in acid phosphatase, aminopeptidase, adenosine triphosphatase, and β-glucuronidase (124). Connective tissue MN of man that contained hemosiderin were highest in esterase activity (88), and those of guinea pigs that probably ingested particles of adjuvant were highest in lipase activity (125).

Most of the other studies concerned acid phosphatase whose activity was increased (*a*) in Kupffer cells most active in the phagocytosis of particles from the blood stream (63, 123, 126, 127), (*b*) in MN in contact with various foreign bodies placed subcutaneously in mice and rats (128), (*c*) in the reticular cells of the rabbit appendix that had ingested the cell debris caused by radiation damage (129), (*d*) in reticuloendothelial cells of rat thymus and lymph nodes following the intravenous injection of killed typhoid bacteria (63) and in those of the liver following the ip injection of *Salmonella typhimurium* (130), (*e*) in mouse macrophages that had ingested red corpuscles in the peritoneal cavity (62), and (*f*) in alveolar phagocytes containing the most dust particles (this report). In addition, both the acid phosphatase and phagocytic activities of Kupffer cells can be increased by endotoxins (123, 126, 127).

The following paper (3) will present studies on *in vitro* activation of phagocytes and suggest a theory that seems to unify current knowledge concerning their enzymatic and phagocytic functions.

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