DIFFERENCES IN ENZYME CONTENT OF AZUROPHIL AND SPECIFIC GRANULES OF POLYMORPHONUCLEAR LEUKOCYTES

I. Histochemical Staining of Bone Marrow Smears

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ABSTRACT

Histochemical procedures for PMN granule enzymes were carried out on smears prepared from normal rabbit bone marrow, and the smears were examined by light microscopy. For each of the enzymes tested, azo dye and heavy metal techniques were utilized when possible. The distribution and intensity of each reaction were compared to the distribution of azurophil and specific granules in developing PMN. The distribution of peroxidase and six lysosomal enzymes (acid phosphatase, arylsulfatase, β-galactosidase, β-glucuronidase, esterase, and 5′-nucleotidase) corresponded to that of azurophil granules. Progranulocytes contained numerous reactive granules, and later stages contained only a few. The distribution of one enzyme, alkaline phosphatase, corresponded to that of specific granules. Reaction product first appeared in myelocytes, and later stages contained numerous reactive granules. The results of tests for lipase and thiolacetic acid esterase were negative at all developmental stages. Both types of granules stained for basic protein and arginine. It is concluded that azurophil and specific granules differ in their enzyme content. Moreover, a given enzyme appears to be restricted to one of the granules. The findings further indicate that azurophil granules are primary lysosomes, since they contain numerous lysosomal, hydrolytic enzymes, but the nature of specific granules is uncertain since, except for alkaline phosphatase, their contents remain unknown.

INTRODUCTION

In a previous paper (1) we demonstrated that rabbit polymorphonuclear leukocytes (PMN) contain two types of granules which can be distinguished by differences in size and density and in their time and mode of origin during PMN maturation in the bone marrow. Azurophil granules are formed early in PMN development and are larger and denser than specific granules which appear later. The presence of two morphologically distinct types of granules with separate origins suggested that differences may exist in the contents of the two granule types. Granule fractions prepared from exudate of rabbit PMN have been shown to contain numerous lysosomal enzymes (2), peroxidase (3), alkaline phosphatase (2), lipase (4), antibacterial basic proteins (2, 5), and acid mucopolysaccharides (6). However, except for histochemical findings on acid and alkaline phosphatases (7, 8) and on acid mucopolysaccharides (9), there is no information avail-

able to indicate the distribution of these substances in the two types of granules.

Initially, we undertook the investigation of this problem by carrying out cytochemical tests on cells which had been fixed and incubated in suspension and by trying to localize enzyme reaction product at the fine structural level, an approach similar to that used by Wetzel et al. (7, 8). However, as reported elsewhere (10, 11), we found that with lead salt techniques, enzyme activity is latent in mature granules and only the immature granules are reactive. In the present study, we circumvented the latency problem by using bone marrow smears which are well known (12) to be reactive for many of the enzymes present in PMN granules. This approach also enabled us to use the more numerous azo dye methods in conjunction with metal salt techniques. We carried out histochemical procedures for granule enzymes on bone marrow smears and, for each enzyme tested, we compared the distribution of the reaction product with that of azurophil and specific granules. The findings, briefly reported earlier (13), show that differences exist in the enzyme content of the two types of granules.

MATERIALS AND METHODS

Preparation of Smears

Femoral marrow was obtained from 30 normal adult New Zealand rabbits which either were under deep ether anesthesia or had been sacrificed by intravenous injection of sodium pentobarbital. Cover glass smears (14) were prepared by squashing or imprinting and were dried for 30 min at room temperature. Some were refrigerated at 4° C and subsequently processed unfixed; others were fixed as described below.

Staining Techniques

FIXATION: With the exception of the routine Wright's stain, a number of methods of fixation were tried including heat fixation (1 hr at 120° C), fixation for 30 min in ethyl alcohol or for 1 min in methyl alcohol or cold acetone. The preferred method is given below.

STAINING: (*a*) *Wright's stain.* Unfixed smears were stained for 5 min, after which an equal volume of phosphate buffer (pH 6.4) was added for an additional 25 min. (*b*) *Azure A.* Acetone-fixed smears were stained for 30 min with 0.02% azure A, pH 5.0 (9). (*c*) *Biebrich scarlet.* Methanol- or heat-fixed smears were stained for 30 min with 0.05% Biebrich scarlet in glycine buffer (pH 9.9) (15). (*d*) *Fast green FCF.* Heat-fixed smears were stained for 30 min in 0.1% aqueous fast green FCF freshly adjusted to pH 8.1 (16, 17). (*e*) *Modified Sakaguchi reaction.* Methanol- or heat-fixed smears were stained for 10 min in reagent containing 2,4-dichloro-α-naphthol (18).

After staining with all except the last method, smears were washed thoroughly in 0.05 M acetate-Veronal buffer (pH 7.4), air-dried, and mounted in Permount.

Enzyme Histochemistry

FIXATION: The following procedures were used routinely: (*a*) cold acetone, 1 min; (*b*) formol-calcium, pH 7.4 (19) (freshly prepared from paraformaldehyde; see reference 20), 15 min, followed by washing in distilled water with 7% sucrose; (*c*) 1.5% distilled (21) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (22), with 1% sucrose, 15 min, followed by washing in the same buffer with 7% sucrose. Other fixatives were used when recommended in the original procedure (see Table I).

Fixation was carried out at 4° C. Specimens were then briefly washed three times and stored for short periods (from 15 min to 2 hr) at 4° C.

The preferred fixative is indicated in the third column of Table I. In general, acetone and formol-calcium fixations were preferred because they provided the best preservation of both morphology and enzyme activity. With two enzymes, arylsulfatase and 5′-nucleotidase, glutaraldehyde fixation was preferable because the reaction was more intense. However, glutaraldehyde fixation was not satisfactory for most azo dye methods because the cells became diffusely yellow, a phenomenon previously noted by Janigan (43).

HISTOCHEMICAL METHODS: Tests were carried out for 11 enzymes. Most of the tests performed were for enzymes which are known constituents of rabbit PMN granules (see Introduction for references). The remaining tests (β-galactosidase, indoxyl esterase, and thiolacetic acid esterase) are for enzyme activities recognized as lysosomal in other tissues.[1] For each of these enzymes, several methods, primarily metal salt and azo dye methods, were utilized when available. All these procedures are summarized in Table I. With the azo dye and

[1] β-Galactosidase is present in lysosomal fractions of liver (44) and many other tissues. Activity with Holt's indoxyl esterase method has been demonstrated in lysosomes of kidney and liver by histochemical staining (45). Similarly, staining has been demonstrated in kidney and liver lysosomes with the thiolacetic acid method and is usually attributed to a cathepsin (41, 42).

TABLE I

Enzyme Tests: Histochemical Methods

Enzyme	Method	Preferred fixative*	Incubation medium					
			Substrate	Capture or postcoupling agent	pH	Temp.	Recommended time	Inhibitor
						°C		
Acid phosphatase	*a*, Burstone (23)	A, F	Naphthol AS-BI phosphate	Fast red violet LB	5.2	25	60 min	0.01 M NaF
	b, Barka and Anderson (24)		Naphthol AS-BI phosphate	Hexazonium pararosanilin	5.0	37	60 min	0.01 M NaF
	c, Gomori (modified, see reference 24)		β-Glycerophosphate	Lead	5.0	37	90 min	0.01 M NaF
Alkaline phosphatase	*a*, Burstone (23)	A, F	Naphthol AS-MX phosphate	Fast red violet LB	8.3	25	30 min	0.001 M cysteine
	b, Gomori (25)		β-Glycerophosphate	Calcium	9.2	37	60 min	0.001 M cysteine
	c, Wetzel et al. (7)		β-Glycerophosphate	Calcium	9.2	37	60 min	0.001 M cysteine
	d, Hugon and Borgers (26)		β-Glycerophosphate	Lead	9.0	37	60 min	0.001 M cysteine
Arylsulfatase	*a*, Austin and Bischel (27)	U	6-benzoyl-2-naphthyl sulfate	Fast blue RR	6.1	37	48 hr	
	b, Goldfischer (28)	G	*p*-Nitrocatechol sulfate	Lead	5.5	37	90 min	
	c, Hopsu-Havu et al. (29)	G	*p*-Nitrocatechol sulfate	Lead	5.5	37	90 min	
β-Galactosidase	Rutenburg et al. (30)	U, A	6-bromo-2-naphthyl-β-D-Galactopyranoside	Fast blue B	5.0	37	120 min	

β-Glucuronidase	*a*, Hayashi et al. (31)	A, F	Naphthol-AS-BI-β-D-glucuronic acid	Hexozonium pararosanilin	5.2	37	60 min	
	b, Fishman and DeLellis (32)		Naphthol-AS-BI-β-D-glucuronic acid	Fast dark blue R	4.5	25	120 min	
DNase	*a*, Aronson et al. (33)	G	DNA	Lead	5.0	37	16 hr	0.01 M NaF
	b, Vorbrodt (34)		DNA	Lead	5.9	37	16 hr	
Esterase	Holt (35)	F	*o*-Acetyl-5-bromoindoxyl		6.0	37	60 min	
Lipase	Gomori (25)		Tween 80	Calcium	7.2	37	12 hr	
5′-nucleotidase	Wachstein and Meisel (36)	G	Adenosine-5′-monophosphate	Lead	7.2	37	90 min	0.001 M cysteine
					4.0	37	90 min	0.01 M NaF
Peroxidase	*a*, Goodpasture (37)	A, F, G	H_2O_2	Benzidine	—	25	5 min	0.01 M KCN
	b, Wachstein and Meisel (38)		H_2O_2	Benzidine	—	25	30 min	0.01 M KCN
	c, Karnovsky (39, 40)		H_2O_2	3-3′-diaminobenzidine	7.6	25	30 min	0.01 M KCN
Thiolacetic acid esterase	*a*, Wachstein et al. (41)		Thiolacetic acid	Lead	7.0	37	120 min	E-600
	b, Bell and Barrnett (42)		Thiolacetic acid	Lead	5.0	37	120 min	

* Abbreviations for fixatives: G, glutaraldehyde; A, acetone; U, unfixed; F, formol-calcium.

benzidine methods, some smears were blotted dry of incubation media and were examined unmounted. Others were briefly washed in distilled water, lightly counterstained with 1% methyl green in 0.1 M phosphate-citrate buffer (pH 4.0) or Harris' hematoxylin, and mounted in glycerogel. Specimens incubated in metal salt media were treated with dilute $(NH_4)_2S$ and then washed in distilled water, counterstained briefly in Harris' hematoxylin, differentiated in tap water, dehydrated in alcohol, cleared in heptane, and mounted in Piccolyte. When calcium was the reaction product, it was converted to the corresponding lead or cobalt salt prior to sulfide treatment, as outlined in the original methods.

For each test, control procedures were as follows: (*a*) omission of the substrate or the capture agent; (*b*) dry heat inactivation at 120° C for 1 hr; or (*c*) addition of an inhibitor to the incubation media, as indicated in Table I. In addition, with the peroxidase and thiolacetic acid esterase tests, some specimens were preincubated for 1 hr in the appropriate inhibitor.

Microscopy

Mounted smears were examined by bright-field and phase contrast microscopy. Since morphological preservation of cells in the bone marrow smear is quite variable, in evaluating the distribution of enzyme reaction products, we avoided areas in which cells were shrunken or condensed as well as those with broken or distorted cells which were frequently unreactive (46). Selected fields were photographed with a Zeiss photomicroscope at original magnifications of 400 or 1000 with a 100 X planapochromat objective.

Materials

Stains were obtained from Allied Chemical Corp., New York. The following materials were obtained from Sigma Chemical Co., St. Louis, Mo.: acid phosphatase (type I), adenosine-5′-monophosphate (type II), 6-benzoyl-2-naphthyl sulfate, β-glycerophosphate (type I), 6-bromo-2-naphthyl-β-D-galactopyranoside, 3-3′-diaminobenzidine tetrahydrochloride, DNase (type I), fast blue B, fast blue RR, fast dark blue R, fast red violet LB, naphthol AS-BI-β-D-glucuronic acid, *p*-nitrocatechol sulfate, *o*-acetyl-5-bromoindoxyl, and sodium salts of naphthol AS-BI and AS-MX phosphoric acid. Benzidine was obtained from Merck & Co., Inc., Rahway, N. J.; E-600 from L. Light and Co., Ltd., Colnbrook, England; pararosaniline HCl from Allied Chemical Corp.; thiolacetic acid and 2,4-dichloro-α-naphthol from Eastman Organic Chemicals, Rochester, N. Y.; and Tween 80 from Nutritional Biochemicals Corporation, Cleveland, Ohio.

RESULTS

Distribution of Azurophil and Specific Granules in Developing PMN

Nomenclature and identification of the stages in PMN maturation were clarified in an earlier paper (1), where it was shown that developing PMN granulocytes produce two types of granules in two different generations: azurophil granules, formed during the progranulocyte stage, and specific granules, formed during the myelocyte stage.

The distribution of azurophil granules as seen in smears is illustrated in Fig. 1. Azurophil granules are present at all stages of maturation after the myeloblast. In the progranulocyte, their number varies since they are produced throughout this stage; the large, fully granulated progranulocyte contains numerous granules, as do early myelocytes. All stages thereafter, including the mature PMN, contain relatively few azurophil granules because no new azurophil granules are made after the progranulocyte stage, and their number is progressively reduced by several mitotic divisions which occur during the myelocyte stage.[2]

The distribution of specific granules is illustrated in Fig. 2. None are seen in the progranulocyte. They first appear in the early myelocyte and, since they are formed continuously during this stage, their number increases progressively, so that they become the predominant granule of the late myelocyte and of all later stages.

From the foregoing, it follows that the key stages for assessing the enzyme content of these two granule populations are the progranulocyte, which contains azurophil granules exclusively, and the mature PMN, which contains mainly (80–90%) specific granules. If a given enzyme is present exclusively in azurophil granules, the progranulocytes should contain numerous reactive granules and mature cells should contain fewer; on the other hand, if a given enzyme is present exclusively

[2] Special precautions must be taken to demonstrate azurophil granules, for they are poorly preserved by the routine Wright's staining procedure (compare Figs. 1 and 2). If smears are heat-fixed and subsequently stained with Wright's stain diluted with an equal volume of phosphate buffer prior to its application to the slide, many more azurophil granules can be visualized at all stages than can be when the undiluted staining mixture is applied directly to the air-dried smear and diluted thereupon.

in specific granules, the mature cells should contain numerous reactive granules and the progranulocytes none. If an enzyme is present in both granule populations, all developmental stages should contain numerous reactive granules.

Distribution of Enzymes in Developing PMN

The results of tests for 11 enzymes on smears of bone marrow cells are shown in Table II. Results with a given enzyme were the same regardless of the method used. Tests were repeated at least five times with each method and, except for DNase (see below), were consistent and reproducible.

With seven of the enzymes tested, acid phosphatase (Figs. 4 and 7), peroxidase (Fig. 8), 5′-nucleotidase, pH 4.0 (Fig. 9), indoxyl esterase (Fig. 10), β-galactosidase (Fig. 11), arylsulfatase, pH 5.5 (Fig. 12), and β-glucuronidase (Fig. 13), reaction product was found primarily in progranulocytes which contained many reactive granules; the later stages contained only a few. Hence these enzymes correspond in distribution to azurophil granules.

With two other enzymes, alkaline phosphatase (Figs. 3, 5, and 6) and DNase, progranulocytes were nonreactive; reaction product first appeared in myelocytes, and later stages contained numerous reactive granules. The distribution of these two enzymes corresponds, therefore, to that of specific granules. The results for DNase are open to question, however, because the preparations were reactive in only two out of 12 tests, and further work is necessary to establish the localization of this enzyme.

Finally, with two of the tests, the Tween method for lipase and the thiolacetic acid esterase method (with E-600), all stages were negative at the pH's tested.

CONTROLS: No reaction was seen in PMN of controls except in the case of aldehyde-fixed specimens reacted for peroxidase in which activity was only partially inhibited by KCN.

Effect of Varying pH

Tests for several enzymes were carried out at various pH's. The effect of pH on the distribution of reaction product and intensity of the enzyme reaction is summarized in Table III. Of the substrates investigated, only with adenosine-5′-phosphate and with β-glycerophosphate was the distribution of reaction product altered as a function of pH.

With adenosine-5′-phosphate, when the reaction was carried out at pH 4.0, the optimal pH for human leukocyte 5′-nucleotidase (47), the distribution of reaction product corresponded to that of azurophil granules (Fig. 9); progranulocytes contained numerous reactive granules, and mature cells contained fewer granules. As reported earlier (13), however, when the reaction was carried out at pH 7.2, the distribution of reaction product corresponded to that of specific granules; progranulocytes were unreactive, and mature cells contained numerous reactive granules. The reactivity of specific granules at pH 7.2 could be due to their high alkaline phosphatase activity (see reference 49) or to the presence of a second nucleotidase active at neutral pH (see reference 50).

With β-glycerophosphate at pH 5.0, distribution of reaction product corresponded to that of azurophil granules; the reaction was inhibited by NaF and hence was assumed to be due to the presence of acid phosphatase. At pH 7.2 and pH 9.0, however, the distribution pattern corresponded to that of specific granules; since the reaction was inhibited by cysteine, it was assumed to be due to alkaline phosphatase.

Several other findings were also of interest. The *p*-nitrocatechol sulfate reaction was positive only at pH 5.5 and was negative at pH 4.2, indicating the presence of arylsulfatase activity with a pH optimum corresponding to that of arylsulfatase "B" rather than that of "A" (see reference 28). The diaminobenzidine reaction for peroxidase was positive at neutral and alkaline pH but not at pH 6.0. Holt's indoxyl esterase method was reactive at all pH's tested.

Distribution of Basic Protein

Several tests were carried out for basic proteins and arginine, since PMN granule fractions are known to contain strongly basic proteins rich in arginine. With the Biebrich scarlet (pH 9.9) and fast green FCF (pH 8.1) methods, after heat or methanol fixation, all stages (progranulocytes, myelocytes, and mature forms) stained intensely orthochromatic (Figs. 14 and 15). Similarly, all stages were stained intensely with the Sakaguchi method for arginine. These results indicate that

The following abbreviations are used for figs. 1–15: *P*, progranulocyte; *M*, mature PMN, band cell, or metamyelocyte; *Y*, myelocyte.

All figures are light photomicrographs of smears prepared from normal rabbit bone marrow. Fig. 1 shows the distribution of azurophil granules. Fig. 2 shows the distribution of specific granules. Figs. 3–13 show the results of tests for various enzymes in the granules. The distribution of reaction product in Figs. 4 and 7–13 corresponds to that of the azurophil granules in Fig. 1. The distribution of reaction product in Figs. 3, 5, and 6 is similar to that of the specific granules in Fig. 2. Figs. 14 and 15 show the results of stains for basic proteins in which both azurophil and specific granules appear stained. All figures, × 1000.

FIGURE 1 Azure A stain (pH 5.0), acetone fixation. Azurophil granules are stained red to purple. The progranulocyte (*P*) contains many more granules than do the more mature cells (*M*).

FIGURE 2 Wright's stain, air-dried. Specific granules are stained pink. None are present in the progranulocyte (*P*), but many are present in the myelocyte (*Y*) and in the mature cells (*M*). Some purple-staining azurophil granules are seen in the progranulocyte and a few in the myelocyte, but none are visible in mature cells (see footnote 2).

FIGURE 3 Alkaline phosphatase, fixed in formol-calcium, incubated in Burstone's medium (pH 8.3), and counterstained with hematoxylin. A large unstained progranulocyte (*P*) is surrounded by several mature cells (*M*) which are stained deep red.

FIGURE 4 Acid phosphatase, fixed in acetone, incubated in a modified Gomori medium and counterstained with hematoxylin. More granules stain in the progranulocyte (*P*) than in the adjacent mature cells (*M*).

FIGURE 5 Alkaline phosphatase, fixed in formol-calcium, processed by Gomori's calcium-cobalt sulfide method (pH 9.2), and counterstained with hematoxylin. A large progranulocyte (*P*) which is unreactive is encircled by several intensely-stained mature cells.

FIGURE 6 Alkaline phosphatase, fixed in formol-calcium, incubated in Gomori's medium (pH 9.2), followed by treatment with lead nitrate. Several mature cells (*M*) contain many reactive granules. A large progranulocyte (*P*) is unstained.

FIGURES 7–13 In all these figures progranulocytes (*P*) contain more reactive granules than do mature cells (*M*). Fig. 7, acid phosphatase, fixed in acetone and incubated in Burstone's medium (pH 5.2); Fig. 8, peroxidase, fixed in acetone, and incubated in Karnovsky's medium (pH 7.6); Fig. 9, 5′-nucleotidase, fixed in glutaraldehyde, incubated in Wachstein and Meisel's medium (pH 4.0), and counterstained with hematoxylin (the cell on the upper left with large reactive granules is an eosinophil); Fig. 10, indoxyl esterase, fixed in formol-calcium, and incubated in Holt's medium (pH 6.0); Fig. 11, β-galactosidase, fixed in formol-calcium, and incubated at pH 5.0 in the medium of Rutenburg et al; Fig. 12, arylsulfatase, fixed in glutaraldehyde, incubated in Goldfischer's medium (pH 5.5), and counterstained with hematoxylin; Fig. 13, β-glucuronidase fixed in acetone, and incubated in Fishman and De Lellis' medium (pH 4.5).

FIGURE 14 Biebrich scarlet (pH 9.9), methyl alcohol fixation. All stages after the myeloblast, i.e. progranulocytes (*P*), myelocytes (*Y*), and mature cells (*M*), contain numerous stained granules.

FIGURE 15 Fast green FCF (pH 8.1), fixed in methyl alcohol. As in the case of Fig. 14, all stages after the myeloblast contain numerous stained granules.

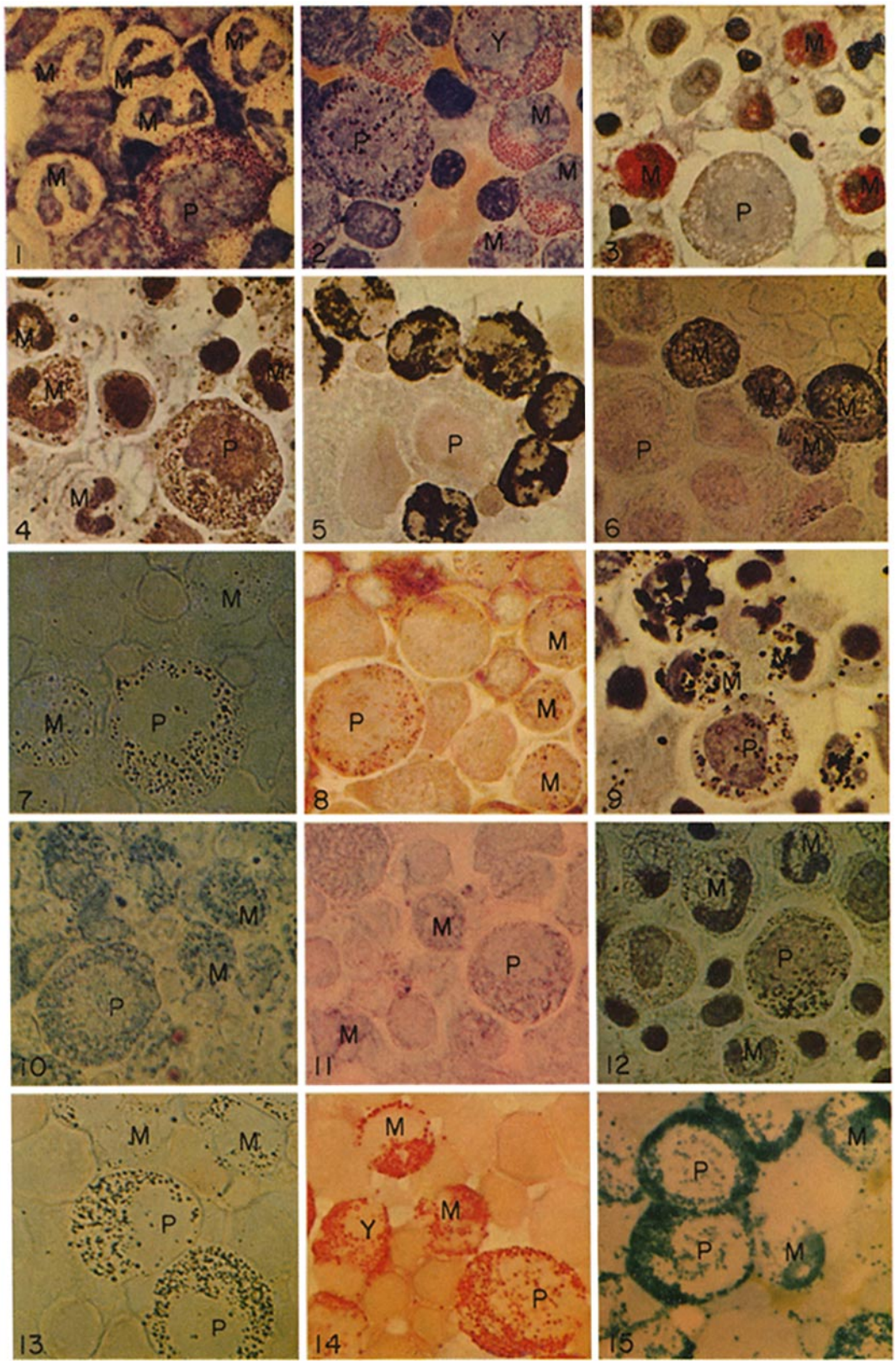
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TABLE II

Distribution of Reactive Granules in Progranulocytes and Mature PMN

Enzyme	pH	Number of reactive granules*	
		Progranulocyte	Mature PMN
Acid phosphatase	5.0	many	few
Arylsulfatase	5.5	many	few
β-Galactosidase	5.0	many	few
β-Glucuronidase	4.5	many	few
Indoxyl esterase	6.0	many	few
5′-nucleotidase	4.0	many	few
Peroxidase	7.6	many	few
Alkaline phosphatase	9.0	none	many
DNase	5.0, 5.9	none	many
Lipase	7.0, 9.0	none	none
Thiolacetic acid esterase	5.0, 7.0	none	none

* Few = few to moderate; many = numerous reactive granules.

basic protein (17, 51) and arginine (18) are present in both azurophil and specific granules.[3]

DISCUSSION

Our findings provide information on the localization of a number of enzymes in azurophil and specific granules of PMN leukocytes. Based on the similarity in their distribution to that of azurophil granules in developing PMN, peroxidase and six lysosomal enzymes (acid phosphatase, arylsulfatase, β-galactosidase, β-glucuronidase, 5′-nucleotidase, and an esterase) were localized within azurophil granules. On a similar basis, alkaline phosphatase was localized within specific granules. Taken together, our findings indicate that azurophil and specific granules differ in their enzyme composition, i.e. that there is enzyme heterogeneity among PMN granules. Furthermore, they also suggest that a given enzyme (the clearest case is alkaline phosphatase) is restricted to a single granule population and is not found in both. Only the tests for basic protein and arginine gave a positive reaction in both types of granules.

The present results support in general the data obtained on rabbit PMN by cell fractionation procedures which have clearly established the presence of seven lysosomal enzymes (acid phosphatase, 5′-nucleotidase, RNase, DNase, β-glucuronidase, cathepsin, and arylsulfatase), as well as alkaline phosphatase, peroxidase, lysozyme, and antibacterial proteins in the granules (see references 2, 3, 5, and 52). Similar enzymes have also been found in PMN granule fractions from the guinea pig (53) and man (54, 55). Of the present results, only those with lipase, which were negative at all developmental stages, are at variance with cell fractionation data. Lipase activity has been ascribed to rabbit PMN granules by Elsbach and Rizack (4); however, these results have been questioned by Dannenberg and Bennett (56). In addition to confirming data obtained by cell fractionation on the enzyme content of PMN granules, our results have introduced another element, namely the existence of heterogeneity in the enzyme content of PMN granules. This element escaped detection by cell fractionation procedures because all the work cited dealt with a common fraction, presumably containing both granule types.

Our results also confirm previous histochemical studies on the time of appearance of various enzymes during PMN maturation, most of which were carried out on human bone marrow. The most comprehensive study of this type was that of Ackerman (12), who noted that peroxidase, acid phosphatase, and arylsulfatase appear early in PMN development, and alkaline phosphatase appears late. Similar findings had been reported earlier by other investigators (57–59).[4] However, with the exception of the work by Wetzel et al. (7, 8) on developing PMN, these and other histochemical studies (see references 12, 62, 63) describing the distribution of various enzymes in PMN did not indicate whether enzyme hetero-

[3] However, when smears were fixed in 95% ethanol before staining with Biebrich scarlet, we found, like Horn and Spicer (9), that staining was restricted to azurophil granules. Apparently, some basic protein constituent of specific granules is extracted or masked by ethanol treatment.

[4] It is of interest to note that the peroxidase test has been used diagnostically to establish the lineage of undifferentiated leukemias (46) ever since Sabin et al. (60) and Richter (61) demonstrated that peroxidase appeared very early in PMN maturation and marked the development of myeloblasts to more differentiated cells.

TABLE III

Effect of pH on Distribution and Intensity of Enzyme Reaction Product

Substrate	Method (reference)	pH optimum (determined by biochemical assay)	(reference)	Species‡	pH of media	Number of reactive granules*	
						Progranulocyte	Mature PMN
Adenosine-5′-phosphate	(36)	4.0	(47)	H	4.0	many	few
					7.2	none	many
					9.0	none	many
β-Glycerophosphate	(24)	5.0	(48)	R	5.0	many	few
	(25, 26)	10.0	(48)	R	7.2	none	many§
					9.0	none	many
p-Nitrocatechol sulfate	(28)	—	—		4.2	none	none
					5.5	many	few
3-3′-diaminobenzidine	(39, 40)	8.6	‖	H	5.0	none	none
					6.0	none	none
					7.0	many	few
					9.0	many§	few
o-Acetyl-5-bromoindoxyl	(35)	—	—		6.0	many	few
					7.0	many	few
					8.0	many§	few

* Few = few to moderate; many = numerous reactive granules.

‡ *H* = human; *R* = rabbit.

§ Reaction positive, but less intense.

‖ Schultz, J. 1967. Personal communication.

geneity exists among the granules, primarily because at the time they were carried out there was no clear understanding of the relationship between the two granule types.

Finally, our findings confirm and extend those of Wetzel et al. (7, 8) whose work by electron microscopy and cytochemistry first suggested the existence of enzyme heterogeneity among PMN granules. The new findings in our work are the localization of peroxidase or, more precisely, myeloperoxidase (55), as well as a number of lysosomal enzymes in azurophil granules, which indicates that enzyme heterogeneity is more extensive than had been previously appreciated and suggests that lysosomal enzymes are largely restricted to azurophil granules.[5]

As to the contents of specific granules, the only substance so far detected therein is alkaline phosphatase which is not a typical lysosomal enzyme. The remaining constituents of specific granules, which constitute the majority (~85%) of the total granules of normal, mature PMN (1), remain to be established. Do specific granules contain lysosomal enzymes? Our present findings suggest that they do not, and, except for acid phosphatase which was found around immature (forming) granules, our electron microscopic studies reported in the accompanying paper (11) support this conclusion.

Of the substances known from cell fractionation studies to be present in PMN granules, the main ones not accounted for by our experiments are lysozyme and the antibacterial proteins. Recently, antibacterial activity of rabbit (5, 64) and guinea pig (53, 65) PMN granules has been separated from lysosomal enzymes and lysozyme and has been found to consist of a mixture of arginine-rich cationic proteins with isoelectric points above 11.0 and which stain at high pH with acidic dyes such as fast green (16, 66). Do specific granules contain antibacterial cationic proteins?[6] The results of our

[5] Sulfated acid mucopolysaccharide has also been localized within azurophil granules by histochemical staining and radioautography (9).

[6] In addition to their antibacterial activities, the cationic proteins from rabbit PMN granules may have other properties such as pyrogenicity, anticoagulant activity, and promotion of tissue damage and adhesion and emigration of leukocytes (see reference 64).

tests for arginine and for basic protein suggest that they may, for, with appropriate fixation procedures, large numbers of stained granules were found in mature cells where specific granules predominate. However, interpretation of these observations is complicated by the fact that these methods are not specific for antibacterial protein. Staining could be due to the presence of any one of several substances (antibacterial cationic proteins, lysozyme, DNase, or RNase), all of which are basic proteins (53) and are constituents of PMN granules. Since no specific histochemical tests are available for the antibacterial proteins, their precise localization remains to be determined by future cell fractionation work when separation of the two types of granules can be achieved and their contents established.

It is of interest, however, that the available evidence suggests that specific granules may not be lysosomes; they may represent instead a special type of secretion granule containing the antibacterial agents and/or lysozyme. The segregation of lysosomal enzymes into one type of granule and nonlysosomal secretory products into the other would allow separate synthesis, packaging, and storage of these substances and individual control over these processes, since we have previously shown that specific and azurophil granules have separate origins (1). It would also have the advantage of allowing the two types of granules to function independently, discharging their contents at different times. In this connection it should be mentioned that after phagocytosis, bacterial cell death occurs much more rapidly than digestion (67). The membrane-damaging action of the cationic proteins and resultant nonviability of the bacterial cell are apparently prerequisites for degradation of bacteria by lysosomal hydrolytic enzymes in the phagocytic vacuole (65).

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