Purification of Myeloperoxidases from the Bone Marrow of the Guinea Pig*

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ABSTRACT: Myeloperoxidase, an enzyme that appears in the leukocyte during maturation, constitutes a good model system for the study of enzyme differentiation because of its favorable physical and biological properties. A relatively simple method is described for the preparation of this enzyme from white blood cells of guinea pig bone marrow. Electrophoretic and immunological examination of the product indicate that it is suitable for the preparation of immunological reagents to be used in studies of differentiation. The purification procedure also resolves and purifies a second protein with peroxidase activity from this source which differs immunologically, spectrally, and in solubility from the previously described "myeloperoxidases."

One basic element in the differentiation of mammalian cells is the appearance of enzyme activities in the mature form of the cell which were absent from its progenitors. An example is the appearance of myeloperoxidase activity in the neutrophilic granulocyte (Thorell, 1947). Many properties of myeloperoxidase make it a favorable subject for a detailed investigation of enzyme differentiation. These include its color, its abundance, its localization to cells of the granulocytic series, its localization to specific granules within the cell, and the ease with which its activity can be determined. Furthermore, recent technical advances permit the preparation of useful quantities of marrow granulocytes in various stages of differentiation (Peterson and Evans, 1967).

Although several laboratories have published methods for the purification of this enzyme (Agner, 1958; Maehly, 1955; Newton *et al.*, 1965; Schultz and Shmukler, 1964; Rohrer *et al.*, 1966), none has used a source suitable for experimental study of normal differentiation. Since the cell separation procedures have been standardized on cells from guinea pig bone marrow, we have investigated the purification of myeloperoxidase from this source with a view to obtaining immunological reagents useful in the study of its maturation. The present communication describes a method which provides two distinct proteins with peroxidase activity from guinea pig bone marrow, both of which are free of detectable contaminants as judged by polyacrylamide gel electrophoresis and immunodiffusion. One of these corresponds closely to the enzyme isolated by others from human white cells in its absorption spectrum and other physical properties (Rohrer *et al.*, 1966). The second, apparently unrecognized previously, is characterized by different immunological properties, heme spectrum, and stability.

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Methods

Analytical. Cell counts were done with a Sanborn–Frommer counter. Protein determinations were by the method of Lowry *et al.* (1951) using bovine serum albumin (Armour Pharmaceutical Co.) as a reference standard. Absorption spectra were obtained with a Cary Model 14 MS recording spectrophotometer calibrated with didymium glass.

Myeloperoxidase activity was measured using guaiacol (Matheson Coleman and Bell) as a substrate. The 3-ml reaction mixture contained 0.013 M guaiacol, 0.00033 M H_2O_2, and 0.01 M phosphate buffer (pH 7.0). After initiation by the addition of enzyme, the reaction was followed at 470 mμ in the recording spectrophotometer. The rate was constant for the first minute at 25°. An enzyme unit was defined as the amount of enzyme required to produce an increase of one absorbance unit per minute under these conditions.

A convenient spot test for enzyme activity in chromatographic fractions was provided by the addition of 1 drop of the fraction to 2 drops of the reaction mixture. Appearance of an orange color (within 10 sec to 1 min) indicated the presence of enzyme.

Preparation of Homogenates. All cells were obtained from NIH strain guinea pigs weighing from 500 to 700 g. The methods for obtaining casein-stimulated peritoneal exudate (Stähelin *et al.*, 1956) and marrow cells (Peterson and Evans, 1967) have been described. The following presents a typical procedure in preparing myeloperoxidase (*cf.* Figure 1).

Twenty male guinea pigs were killed and the femurs and tibias were removed. All subsequent operations were carried out on an ice bath. The marrow obtained was pooled in groups, each containing the material from five animals. Each pool was suspended in 50 ml of a Krebs–Ringer–phosphate solution prepared without calcium or magnesium (henceforth referred to as NaKP) (Peterson and Evans, 1967). The suspension was centrifuged for 5 min at 1500 rpm in a refrigerated centrifuge (International, Model PR–1). All centrifuga-

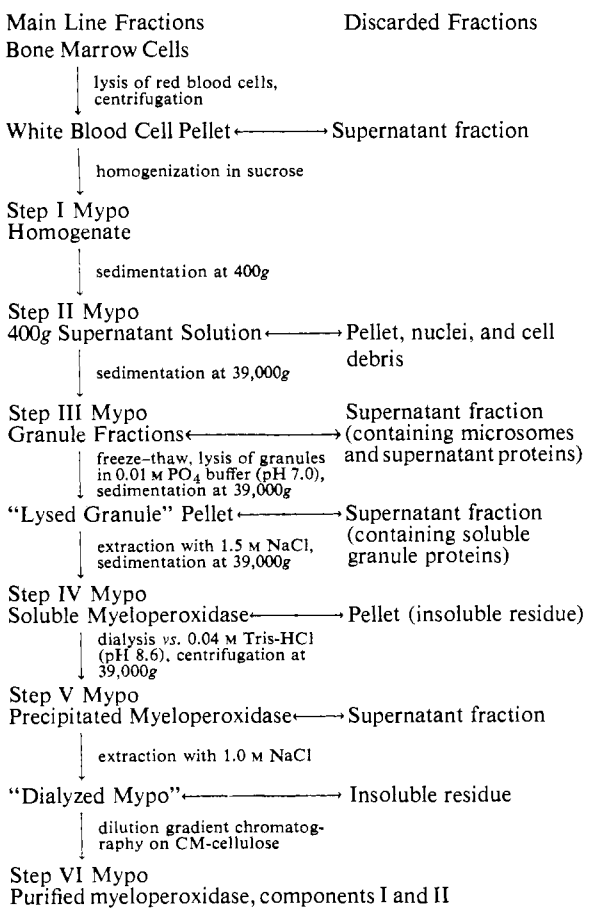

FIGURE 1: Purification of myeloperoxidase from bone marrow.

tion was done at 4°. The supernatant was discarded and each pellet was suspended in an additional 50 ml of NaKP and recentrifuged in the same manner. The supernatant solution was again discarded.

To each pellet was added 4 ml of 0.9% NaCl, and the cells were resuspended. Then 12 ml of distilled water was added to each tube and mixed for 30 sec. The solution was brought back to physiological tonicity by adding 4 ml of 3.6% NaCl and mixing. The solutions were then filtered through a single layer of silk, diluted each to 50 ml with NaKP, and centrifuged for 5 min at 1500 rpm. The supernatant solutions were discarded and the pellets were washed with an additional 50 ml each of NaKP in an identical manner.

The pellets were transferred to a single 17-ml Potter-Elvehjem homogenizer with 13 ml of 0.25 M sucrose and homogenized by 50 passes with a motor driven (1300 rpm) Teflon pestle (clearance 0.106 mm). The degree of cell breakage was determined by phase microscopy. Cell breakage was usually in excess of 90%. The homogenate (step I Mypo) was transferred to a centrifuge tube with sufficient sucrose to bring the final volume to 20 ml.

Differential Centrifugation. All operations were carried out at 4°. The whole homogenate was centrifuged at 1300 rpm (400*g*) for 10 min. The supernatant solution (400*g* supernatant fraction, step II Mypo) was saved. The pellet, containing nuclei, tissue debris, and some trapped granules, was washed with 7 ml of 0.25 M sucrose, centrifuged at 1300 rpm for 10 min, and the supernatant solution was added to the 400*g* supernatant fraction. This pooled preparation was then centrifuged at 39,000*g* for 10 min. The supernatant solution from this centrifugation was discarded and the pellet (39,000*g* pellet, step III Mypo) was frozen in an acetone–Dry-Ice bath and stored at −80° Freezing and thawing were necessary for maximal extraction of the enzyme.

Extraction of Myeloperoxidase from Granules. The 39,000*g* pellet was thawed and transferred to a 17-ml homogenizer with 10 ml of 0.01 M sodium phosphate buffer (pH 7.0). The sample was homogenized by 25 passes with a Teflon pestle. It was then centrifuged at 39,000*g* for 10 min and the supernatant liquid was discarded. The pellet was homogenized in 4.5 ml of 1.5 M NaCl by 25 passes in a 7-ml homogenizer. The homogenate was centrifuged for 10 min at 39,000*g* and the clear green supernatant solution was saved. The pellet was extracted once more with a 4.5-ml portion of 1.5 M NaCl in an identical manner and the supernatant solution from this extraction was pooled with the original extract. The combined extracts represent soluble myeloperoxidase (step IV Mypo).

Soluble myeloperoxidase was dialyzed for 18 hr against two 20-volume portions of 0.04 M Tris-HCl buffer (pH 8.4) on a rotating dialyzer. The copious precipitate was collected by centrifuging the dialyzed material for 10 min at 39,000*g*. The pellet was extracted with two 2.0-ml aliquots of 1.0 M NaCl, and the suspension was centrifuged at 39,000*g* after each extraction. The supernatant solutions were pooled to make dialyzed myeloperoxidase (step V Mypo).

Chromatographic Procedures. Whatman CM-cellulose (CM-23, no lot number given, nominally 1.0 mequiv/dry g) was used in all ion-exchange chromatographic experiments. The methods for preparing the adsorbent for use and the packing procedures employed have been described elsewhere (Peterson and Sober, 1962). The details of each chromatographic experiment are given in the captions to the figures. Experiments were performed with the aid of a Sigmamotor T-8 pump and a time-indexed fraction collector. Effluent solutions were monitored for absorbance at 280 mμ, 260 mμ, pH, conductivity, and enzyme activity. Gradients were produced with a five-chambered Varigrad (Peterson and Sober, 1959).

Sephadex G-200 (lot no. TO 43) was prepared for use by allowing it to swell in buffer for 2 weeks and then packing it in such a manner that the forming bed was exposed to a constant hydrostatic head of 10 cm of buffer. During experiments a constant head of 6 cm of buffer was maintained with the aid of a Mariotte bottle. Fractions were collected in polyethylene tubes of constant tare and the effluent volume was then determined by weighing each fraction on a Mettler top-loading balance accurate to ±0.01 g. Monitoring procedures were identical with those used in ion-exchange chromatographic experiments.

Polyacrylamide Gel Electrophoresis. Samples were

TABLE I: Distribution of Myeloperoxidase Activity in Subcellular Fractions of Bone Marrow and Exudate Cells from a Guinea Pig Injected with Casein.

Fraction	Marrow		Exudate	
	Enzyme Units/10^8 cells	% Whole Cell Act.[a]	Enzyme Units/10^8 cells	% Whole Cell Act.[a]
Whole homogenate	59	100	20	100
39,000*g* supernatant fraction	9	16[b]	0.2	1
Granule fraction	45	76	15	75
Nuclear fraction	15	25	6	30

[a] Slightly more peroxidase activity is invariably recovered in the fractions than is found in the whole homogenate because myeloperoxidase is inhibited by factors present in the nuclei but removed in the first centrifugation. [b] Of this activity, 84% sedimented to the bottom of the centrifuge tube when centrifuged at 105,000*g* for 1.5 hr.

examined in the standard system of Davis (1964) as modified by Felberg and Schultz (1968). Because of the problems encountered (see Results), various modifications were tested to find conditions under which myeloperoxidase would migrate rapidly. The best of the systems tested was the following. A 7% gel was prepared as described by Davis (1964) except that the buffer in the gel and the electrode chambers was 0.05 M sodium acetate buffer (pH 4.7)–0.05 M NaCl. Since residual ammonium persulfate gives a false positive histochemical test for myeloperoxidase, the gels were electrophoresed for 1 hr at 15 mA/gel tube before application of the sample. No spacer gel was used. Samples, mixed 1:1 with 40% sucrose, were applied by layering them over the upper gel surface under a layer of buffer. Electrophoresis was carried out for 1 hr at a constant current of 15 mA/tube (about 70 V). Gels were stained with Amido-Schwarz for protein or for peroxidase by the method of Graham and Karnovsky (1966).

Immunological Methods. Antibodies to myeloperoxidase were found in the serum of a sheep (NIH sheep 371) that had been hyperimmunized with red blood cell lysed (Fallon *et al.*, 1962) guinea pig bone marrow (2.5×10^8 cells/dose) in complete Freund's adjuvant intramuscularly, followed by periodic boosting (at 2 month intervals) with the same antigen preparation in incomplete Freund's adjuvant. Serum from another sheep (NIH sheep 612) that had been similarly immunized with red blood cell lysed guinea pig exudate was also found to contain antibodies to myeloperoxidase.

Precipitin analyses were done by double diffusion in gel (Ouchterlony, 1958). Gels were 1% agarose (*Seakem* Brand, Bausch & Lomb, Rochester, N. Y.) in 1 M NaCl with 0.01 M phosphate buffer (pH 7.4). The high salt concentration was used because one of the peroxidase components did not diffuse in 1% agarose–0.15 M NaCl plates.

Results

Subcellular Distribution of Peroxidase Activity. The distribution of peroxidase activity among the subcellular fractions of bone marrow and exudate cells obtained from the same animal is given in Table I. Both cell populations are rich in this enzyme activity, but the marrow contains 59 units/10^8 nucleated cells compared with only 20 units/10^8 nucleated cells found in the exudate. This discrepancy is surprising because the granulocytic cell line which contains the myeloperoxidase represents 80% of the exudate but is diluted about twofold by members of the red blood cell series in the marrow. It indicates that some cell type in bone marrow has a much higher activity of myeloperoxidase than the exudate leukocyte.

In keeping with earlier reports (Schultz and Kaminker, 1962), about 75% of the total peroxidase activity of both cell types is found in the granule fraction. Although substantial activity is present in the nuclear fraction of both, it is likely that this represents contamination with active granules, which is obvious on microscopic examination. A striking difference between the two cell lines lies in the amount of activity found in the 39,000*g* supernatant solution; 1% of the total cellular peroxidase activity (0.2 unit/10^8 cells) is present in this fraction of the exudate cells, confirming earlier findings (Evans and Rechcigl, 1967). This amount of activity is no more than would be expected from the degree of contamination of this fraction with granules. On the other hand, 16% (9 units/10^8 cells) of the total cellular peroxidase content of marrow cells is found in the 39,000*g* supernatant solution, an amount of activity too great to be explained by granule contamination of the degree observed. In absolute terms, the supernatant activity per cell in marrow is almost 50 times that found in the corresponding fraction of exudate cells. Ultracentrifugation at 105,000*g* for 1.5 hr caused 84% of this activity to sediment, indicating that it is bound to the microsomal components of the cell.

The proportion of the total peroxidase activity which is found in the 39,000*g* supernatant solution seems to be influenced by the state of stimulation of the marrow. When marrow from animals that have not been stimulated to form an exudate is examined, 5–7% of the total activity is found in the 39,000*g* supernatant solution, in contrast to the 16% found in this stimulated

TABLE II: Purification of Myeloperoxidase from the Bone Marrow of 20 Guinea Pigs.

Step	Fraction	Protein/Fraction (mg)	Enzyme Units/Fraction[a]	Sp Act. (units/mg)	Recov (%)
I	Homogenate	520	3280	6	
II	400*g* supernatant solution	420	5920	14	
III	39,000*g* pellet	184	4200	23	
IV	Soluble myeloperoxidase	27	3440	127	100[b]
V	Dialyzed myeloperoxidase	6.2	2100	339	68
VIa	CM-fraction I	1.4	805	575	30
VIb	CM-fraction II	0.4	226	565	

[a] For the definition of enzyme units see Methods. [b] This fraction was chosen to represent 100% activity because it constitutes the first clarified sample on which assays could be performed in a completely standardized manner.

animal; 5% of the total marrow enzyme activity is, however, still more than 20 times the absolute quantity found in the exudate on a per cellular basis.

Preparation of Purified Myeloperoxidase Components. A variety of alternative methods of purification were tested. That judged best in terms of convenience and purity of products is outlined in detail in Figure 1. Although each step in the procedure is listed for reference in repeating the method, it can be conveniently considered to consist of four major parts, namely, (1) preparation of a granule fraction, (2) extraction of the enzyme from the granule preparation with 1.5 M NaCl, (3) precipitation of the enzyme by dialysis against buffers of high pH and low ionic strength, followed by extraction with 1.0 M NaCl, and (4) ion-exchange chromatography on columns of CM-cellulose in a gradient of NaCl. As will be discussed below, this procedure yields enzyme that is homogeneous by disk electrophoresis but contains an enzymatically inactive contaminant detectable immunologically that can be removed by chromatography on Sephadex G-200. Although for many purposes this final step may not be required, it is useful if the product is to be used in producing monospecific antisera.

The results of a typical preparation according to Figure 1 are shown in Table II. The steps are labeled identically in the two charts to facilitate cross reference. From a cell homogenate which contained 520 mg of protein and 3280 units of peroxidase activity, two purified peroxidase components were obtained which together contained 1.9 mg of protein and 1030 units of enzyme activity. Thus, an over-all purification of 94-fold was achieved with a yield of about 30%. Completion of the procedure required approximately 4 days.

The apparent quantity of enzyme activity in the whole homogenate was always substantially less than that in the 400*g* supernatant solution or in the 39,000*g* pellet. This phenomenon, resulting from the presence of inhibitory substances in the nuclear fraction, so complicated the interpretation of analyses done on early fractions that the apparent enzyme content of soluble myeloperoxidase fraction (step IV Mypo) was arbitrarily chosen to represent 100% for recovery calculations. As will be discussed later, the losses incurred were largely from the component more tightly bound to CM-cellulose.

Step V (dialysis against dilute Tris-HCl buffer, and extraction of the resulting precipitate with 1.0 M NaCl) proved to be important in obtaining myeloperoxidase of the highest purity, even though it had drawbacks. Upon dialysis of the clear green salt extract of the granules under these conditions, a copious precipitate formed that contained all of the myeloperoxidase activity still measurable. Some protein, which was not separated from the enzyme by the remaining purification steps if dialysis were omitted, remained in the supernatant solution. Further purification of the enzyme was achieved when this precipitate was extracted with 1.0 M NaCl, for although the major part of the enzyme went into solution, a good deal of other material remained insoluble. An over-all purification of 2.7-fold was achieved by this step.

The results of ion-exchange chromatography of the enzyme on CM-cellulose (step VI) are depicted in Figure 2. Two distinct activity peaks were largely resolved and will be designated CM-1 and CM-2 in the order of their emergence from the column. As will be discussed below, this method of purification does not give a meaningful measure of the relative quantities of the two fractions in the original homogenate, since unequal losses occur earlier in the purification. The specific activities of the two components are, however, almost equal.

Direct Chromatographic Evaluation of Undialyzed Salt Extract of Granule Fraction. Studies of the stability of purified myeloperoxidase components CM-1 and CM-2 indicated that CM-2 (but not CM-1) was precipitated and rendered inactive by exposure to solutions of low ionic strength. This suggested that the dialysis of salt extracts of granules against dilute Tris-HCl buffer, used in step IV of the standard purification procedure, might cause a selective inactivation of this component. Therefore, step IV Mypo from marrow was examined by dilution to 0.2 M NaCl and direct application to the CM-cellulose columns, omitting dialysis. The results of such an experiment are shown in Figure 3. Two peaks of enzyme activity emerged at the same conductivities as the components found with

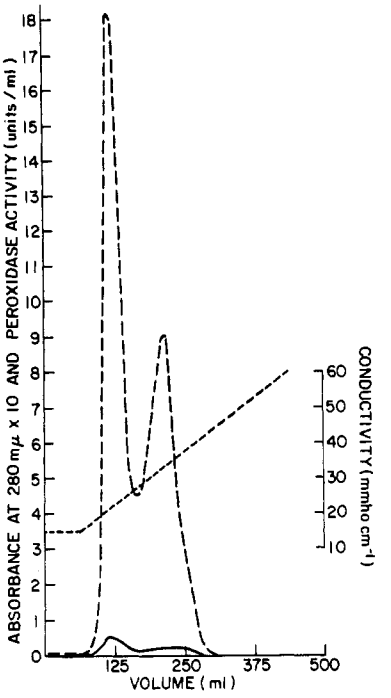

FIGURE 2: Ion-exchange chromatography of partially purified myeloperoxidase on CM-cellulose. Step V Mypo (see Table II) from 20 animals, containing 6.2 mg of protein and 2100 enzyme units, was chromatographed on a 1.3 × 23 cm column of CM-cellulose. Flow rate was 90 ml/hr. The linear gradient was of 500-ml volume from 0.025 M sodium acetate–0.025 M acetic acid (pH 4.7)–0.2 M NaCl to the same buffer 1.5 M in NaCl. Enzyme activity: long dashes; A_{280}, solid line, conductivity: short dashes.

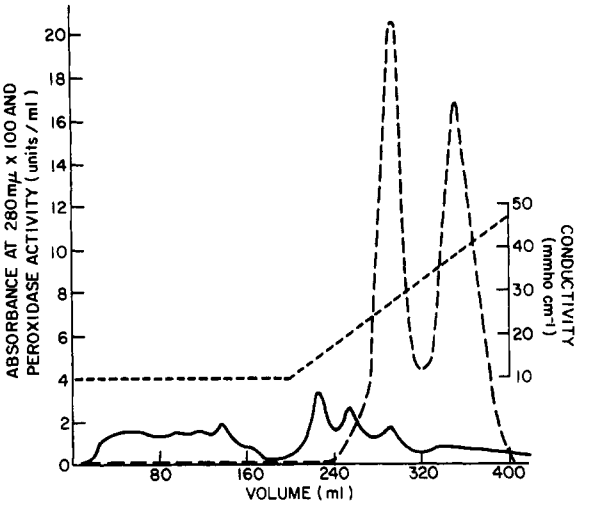

FIGURE 3: Ion-exchange chromatography of partially purified myeloperoxidase on CM-cellulose. Step IV Mypo (see Table II) from 20 animals, containing 38 mg of protein and 2260 enzyme units, was chromatographed directly (without dialysis against Tris-Hcl buffer) upon dilution on a 1.3 × 23 cm column of CM-cellulose. Flow rate was 100 ml/hr. The linear gradient was of 500-ml volume from 0.025 M sodium acetate–0.025 M acetic acid (pH 4.7)–0.2 M NaCl to the same buffer 1.5 M in NaCl. Symbols as in Figure 2.

dialyzed extracts. However, the quantities of CM-1 and CM-2 were nearly equal, in contrast to the preponderance of CM-1 found in the earlier experiments. This indicates that the dialysis step results in substantial selective losses of CM-2. Unfortunately, the specific activities of the components obtained from direct chromatography are only half as great as those obtained by chromatography after dialysis, and the contaminating material proved difficult to remove with later purification steps. Thus dialysis is included in the standard form of the purification procedure in spite of losses which its use entails.

Examination and Further Purification of CM-1 on Sephadex G-200. Because an enzymatically inactive contaminant was detected in CM-1 by immunodiffusion (see below), further purification of this component on Sephadex G-200 was attempted. The results are shown in Figure 4. In 0.05 M sodium acetate buffer (pH 4.7)–0.05 M NaCl, CM-1 was resolved into two components, one representing approximately 85% of the total applied protein, the other representing approximately 15%. Neither of these components contained an immunologically detectable inactive contaminant as judged by immunodiffusion.

The pooled fractions of the major Sephadex peak had a specific activity of 580 units/mg of protein. The minor Sephadex peak had a pooled specific activity of 50 units/mg of protein. No protein peak was found that corresponded to the enzymatically inactive component detected in the starting material by immunodiffusion, nor was this contaminant demonstrated in concentrates of the apparently protein-free portions of the Sephadex effluent.

Electrophoretic Examination of the Purified Enzyme Components. Examination of CM-1 in the electrophoretic system described by Felberg and Schlutz (1968) revealed a single band of protein, just beyond the origin of the cathodal running gel, which gave a positive histochemical test for peroxidase. On prolongation of the time of electrophoresis, the band remained in this location. No other bands of protein were detected in either the anodal or cathodal gels when quantities as high as 100 μg of CM-1 were applied. Examination of these preparations in polyacrylamide gel in 0.05 M sodium acetate buffer (pH 4.7)–0.05 M NaCl demonstrated a single protein band, which was enzymatically active, moving toward the cathode. No components moving toward the anode were detected. A small band of enzymatic activity, without demonstrable protein, remained at the origin. This was identified, as will be discussed below, as a small amount of CM-2. The poor stability of CM-2 in solutions of low salt concentration prevented us from evaluating this component by any of these electrophoretic systems.

Immunological Examination of Purified Myeloperoxidase Components. When CM-1 prepared by the standard method (Figure 1) was examined by double diffusion with an antiserum capable of detecting six components in the starting material (step IV Mypo), two bands were found, one active by histochemical stain, and the other inactive. When this material was further purified by chromatography on Sephadex

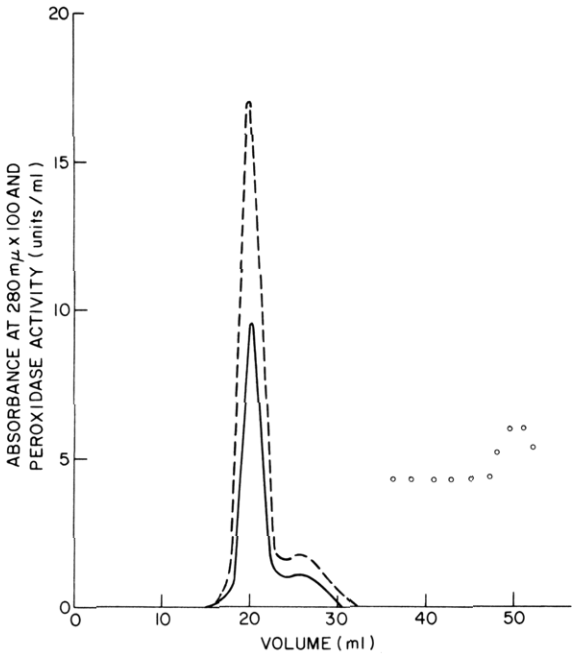

FIGURE 4: Chromatography of CM-1 on Sephadex G-200. CM-1 (0.75 ml) in a concentration of 1.2 mg/ml was chromatographed on a 38-ml bed (0.9 × 50 cm) of Sephadex G-200 in 0.05 M sodium acetate buffer (pH 4.7)–0.05 M NaCl. Flow rate was maintained at 3 ml/hr with a Mariotte flask and approximately 1-ml fractions were collected. Activity, dashed line; absorbance (280 mμ), solid line; conductivity, points.

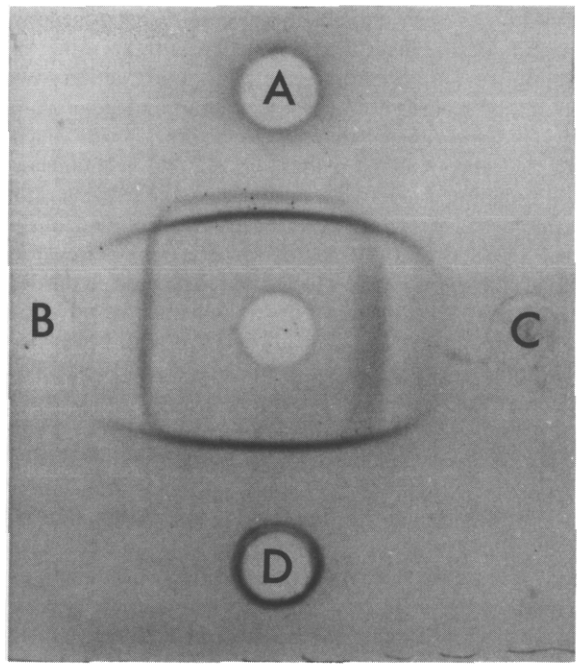

FIGURE 5: Immunologic comparison of two guinea pig myeloperoxidases. The central well contains antibody to red blood cell lysed guinea pig bone marrow (S 371). The peripheral wells contain: A, CM-2; B and C, CM-1, D, step IV Mypo. The precipitin lines from the two peroxidase components in step IV Mypo (well D) are superimposed. With other antisera, they appear distinct from each other. The plate is stained for myeloperoxidase activity.

G-200, the inactive band was removed, and only one, active, component was immunologically detectable. Since the contaminant was only detectable when the antigen concentration was increased to the point where immune precipitation of the enzyme occurred next to the antibody well, and since the specific activity of the myeloperoxidase preparation did not rise after this additional purification, it is judged that this contaminant, if protein, was present in small quantity. CM-2 contained only two components, both enzymatically active, when examined with this antiserum. Figure 5 illustrates the comparison of preparations of CM-1 and CM-2, each slightly contaminated with the other component. The minor component of each of these preparations was usually not detectable as a visible precipitin line before staining. The crossing of the precipitin lines from the main components of CM-1 and CM-2 (wells B and C *vs.* well A, Figure 5) indicates that they are antigenically distinct proteins.

Oxidized Absorption Spectra of the Purified Myeloperoxidase Components. The visible absorption spectra of the two myeloperoxidase components are shown in Figure 6. A distinct difference in the position of the Soret absorption band was found. CM-1 possessed a spectrum very similar to that reported for the human enzyme with a Soret maximum at 433 mμ and a ratio of absorbance at 430 mμ to absorbance at 280 mμ of 0.55 (*cf.* Schultz and Shmukler, 1964). CM-2, on the other hand, showed a Soret maximum at about 415 mμ.

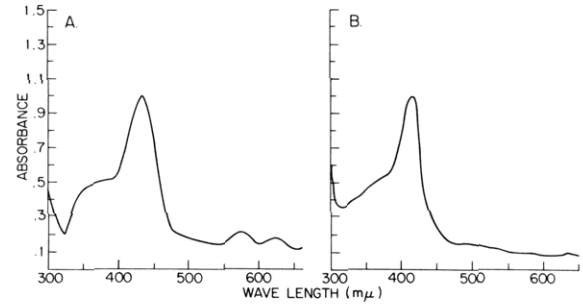

FIGURE 6: Visible absorption spectra of myeloperoxidase components. The absorption spectra of CM-1 (A) and CM-2 (B) from 300 to 650 mμ normalized so that both Soret maxima are equal to 1.0. The Λ_{max} of CM-1 is at 433 mμ and of CM-2 at 415 mμ.

Discussion

Since myeloperoxidase is difficult to extract in soluble form from leukocytes, it has become customary to trypsinize cell homogenates to prepare starting extracts (Maehly, 1955). Although evidence had been accumulated indicating that this approach does not degrade the enzyme, recent investigations by Felberg and Schultz (1968) have demonstrated the presence of at least ten components as judged by disc electrophoresis. Although no explanation for this multiplicity of com-

ponents has been offered, this degree of heterogeneity suggests partial digestion of the enzyme by trypsin. An alternative and preferable method of extraction was offered by Rohrer *et al.* (1968) who found that exposure of cell homogenates to high concentrations of NaCl released the myeloperoxidase in soluble form. They additionally discovered, however, that the enzyme thus released was bound to DNA which had simultaneously been released from the cell nuclei. Since the net charge of the enzyme molecule was found to be reversed during the course of purification, it was felt that the adventitiously bound nucleic acid had been removed. Nevertheless, unnecessary exposure of the enzyme to substances to which it binds tightly would be better avoided from the outset.

For these reasons, the extraction procedure of Rohrer *et al.* was chosen, in preference to the trypsinization used by earlier workers, but it was applied to a granule fraction of the marrow cells from which the ribosomes and nuclei had been largely removed. The data presented in Table I show that the granule fraction in marrow contains the major part of the total cellular myeloperoxidase activity, making this procedure feasible. It shows, additionally, however, that the subcellular distribution of the enzyme is not identical in marrow and exudate cells, the former containing a significant quantity of the enzyme in the 39,000*g* supernatant fraction. Since the bone marrow contains the undifferentiated members of the granulocytic cell series, this fraction may represent newly formed enzyme. The present data, however, do not allow exclusion of alternative explanations, including the existence of a lighter granule fraction in the marrow cells, or the possibility that the granules of marrow cells are more fragile than those of exudate and therefore leak peroxidase during homogenization which then becomes bound to microsomes because of its high positive charge.

The behavior of the enzyme on ion-exchange chromatography when extracted after removal of nuclei and ribosomes (Figure 2) is comparable with that observed by Rohrer *et al.* (1966) after their preliminary purification on DEAE-Sephadex. This corroborates their assumption that removal of adventitiously bound nucleic acid was responsible for the reversal of charge which myeloperoxidase underwent during their purification procedure. It also offers a simpler and more direct solution to the problem.

The resolution of two myeloperoxidase components by ion-exchange chromatography on CM-cellulose (Figures 2 and 3) is substantiated by the immunological and spectral differences found between the two fractions. They also differ in their stability and solubility properties. The present data do not, in themselves, enable a judgment concerning the significance of these components. Two major possibilities exist: the first that the two enzymes originate in different cell lines in the marrow, and the second that the neutrophilic granulocytes are the source of both components. In favor of the first alternative is the known existence of a peroxidase in eosinophils which shares some of the spectral properties of the present CM-2 (Archer *et al.*, 1965), but the low eosinophil content of guinea pig marrow renders the relationship dubious. In fact, the differential count of the starting material strongly suggests that granulocytes or granulocyte precursors are the only cells present in sufficient quantity to account for the large amount of CM-2 found. Definitive answers to these questions should become available when immunological reagents prepared with the purified myeloperoxidase components are applied to the analysis of fractionated cell populations. Such studies are now in progress. They should additionally identify the cell type which is responsible for the anomalously high marrow content of peroxidase as compared with peritoneal exudate (*cf.* Results).

The data presented here show that the purification scheme illustrated in Figure 1 is a convenient and rapid method of obtaining two myeloperoxidases of high purity. Although direct comparison with previous methods is rendered difficult by the difference in starting material, the data suggest that the present fractionation compares favorably in terms of yield, convenience, and purity of product. Direct assessment by acrylamide gel electrophoresis and immunological analysis indicates that both CM-1 and CM-2 are quite homogeneous by these criteria. In view of the strongly basic nature of these molecules and their mode of attachment to the granules, however, physical and immunological homogeneity does not necessarily imply that the simplest active peroxidase forms have been obtained. It is possible that the present preparations contain some portion of the granule matrix which is not essential for activity. This may be suggested by the fact that the ratio of A_{430} to A_{280} in the present preparation was 0.55 compared with ratios of 0.8 obtained by other workers using cells from other species (Schultz and Shmukler, 1964), although species differences cannot be excluded as an explanation. The possibility is presently under investigation, since, if true, it would provide a means of studying the nature of the attachment of the enzyme to the granule.

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Self-Associating Systems. II. Multinomial Theory for Nonideal Systems*

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ABSTRACT: A general expression for the calculation of equilibrium constants and virial coefficients of self-associating nonideal systems has been established. The derivations are an extension of the multinomial theory for ideal systems (Derechin, M. (1968), *Biochemistry 7*, 3253).

This general expression is applicable to association reactions of any degree of polymerization and is valid irrespective of whether the apparent weight-average, M_{app}, or number-average, $M_{n\ app}$, molecular weight is used in the calculations. Using this expression, equations to establish the equilibrium constants K_1, K_2, K_3, and K_4 have been derived. Directions for the calculation of virial coefficients are given.

In a previous paper (Derechin, 1969) self-association reactions of the type

$$nP_1 \leftrightarrows a_2P_2 + a_3P_3 + \cdots + a_mP_m$$

with

$$\sum_{i=2}^{m} ia_i = n \qquad (1)$$

for ideal systems have been examined. Many systems encountered in practice depart of course from ideality. It is, therefore, desirable to extend this theory to cover nonideal behavior.

Theoretical

It is assumed here that all species participating in the self-associating reaction have the same partial specific volume ($\bar{v}_1 = \bar{v}_2 = \cdots = \bar{v}$) and the same refractive index increment $(dn/dc_1)_{T,P} = (dn/dc_2)_{T,P} = \cdots = (dn/dc)_{T,P}$, and that for dilute systems the activity coefficient of each associating species can be represented by a series expansion as $\ln y_i = iB_iM_1c +$ higher terms in c, where B_i is the second virial coefficient of the species i, M_1 is the molecular weight of the monomer, and c is the total solute concentration in g/100 ml. Since we shall deal only with systems in dilute solution, higher powers in c are neglected. Also it is assumed that $B_1 = B_2 = \cdots = B$. In the particular case $i = 1$

$$\ln y_1 = BM_1c \qquad (2)$$

Then

$$\ln y_i = iBM_1c = \ln y_1{}^i \qquad (3a)$$

or

$$y_i = y_1{}^i \qquad (3)$$

The condition for chemical equilibrium can be stated in terms of the equilibrium constants, K_i, as

$$y_ic_i = K_iy_1{}^ic_1{}^i \qquad i = 1, 2, \ldots \qquad (4a)$$

or using eq 3,

$$c_i = K_ic_1{}^i \qquad i = 1, 2, \ldots \qquad (4)$$

Equation 4 is valid for ideal and nonideal systems. In the particular case $i = 1$, $K_i = 1$. Also since

$$c = \sum_{i=1}^{m} c_i \qquad i = 1, 2, \ldots, m \qquad (5)$$

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