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Purification and some properties of myeloperoxidase and eosinophil peroxidase from human blood

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Myeloperoxidase and eosinophil peroxidase have been isolated from outdated human blood. Peroxidase activity was extracted from washed leucocytes using 0.5 M-$CaCl_2$ and the extract further purified by chromatography on concanavalin A–Sepharose, phenyl-Sepharose and finally by gel filtration. The final enzyme preparations were highly purified according to spectral and gel-electrophoretic criteria. Under reducing and denaturing conditions on polyacrylamide-gel electrophoresis myeloperoxidase gave rise to bands of M_r 57000, 39000 and 15500, whereas the eosinophil enzyme yielded bands of M_r 50000 and 15500. Both enzymes were very resistant to denaturation either by the chaotropic agents urea and guanidinium chloride or by elevated temperatures. Spectral properties of the native and reduced forms of the enzymes are reported.

The presence of two haem peroxidases in leucocytes has been long established. The physiological role of myeloperoxidase, present in large amounts in neutrophils, and eosinophil peroxidase in eosinophil granulocytes, is uncertain. Both have been proposed to have anti-microbial and anti-parasitic functions (Klebanoff *et al.*, 1980; Clark & Klebanoff, 1979; Diamond *et al.*, 1980). Myeloperoxidase has been obtained in a stable, soluble and highly purified state from several sources, including human (Agner, 1958; Schultz & Shmukler, 1964; Zgliczyński *et al.*, 1968; Desser *et al.*, 1972; Bakkenist *et al.*, 1978; Andersen *et al.*, 1982). Attempts to purify the eosinophil peroxidase have, however, been far less successful. To our knowledge, eosinophil peroxidase has been only partially purified from guinea pig (Desser *et al.*, 1972) and rat (Archer *et al.*, 1965), although very recently a highly purified product has been isolated from horse blood (Jörg *et al.*, 1982). Eosinophil peroxidase has also been partially purified from human blood (Wever *et al.*, 1981). The purification and characterization of eosinophil peroxidase have been hampered by the tendency of the enzyme to aggregate and also to bind to dialysis tubing and gel-chromatography media. We now report a scheme for the isolation of homogeneous, unaggregated eosinophil peroxidase from human blood. Highly purified myeloperoxidase is obtained as a by-product from this process. In addition, we report briefly on some properties of these enzymes.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Sepharose-immobilized concanavalin A and phenyl-Sepharose were obtained from Pharmacia (Uppsala, Sweden) and cetyltrimethylammonium bromide and guaiacol from Sigma (St. Louis, MO, U.S.A.). Ultrogel AcA 44 was from LKB (Bromma, Sweden). H_2O_2 was obtained as a 30% (w/v) solution from the local chemist. Other reagents used were of analytical-reagent-grade purity.

Peroxidase activity was determined by using the method of Himmelhoch *et al.* (1969) as described by Olsen & Little (1981). One unit of enzyme activity was defined as giving an initial rate of 1 absorbance unit/min at 470nm and 23–24°C in a 3ml reaction mixture.

Protein was determined by the method of Bradford (1976), with pure myeloperoxidase as standard. The concentration of myeloperoxidase was calculated from A_{430} ($\varepsilon = 178\,mM^{-1}\cdot cm^{-1}$) (Agner, 1958). Spectra were recorded in a Hitachi 557 double-beam recording spectrophotometer.

Analytical polyacrylamide-slab-gel electrophoresis was carried out by using gel system 7 of Maurer (1971) with the following modifications: 10% gels were used, and cetyltrimethylammonium bromide (0.05%, w/v) and glycerol (25%, w/v) were present in the gel and in the electrode buffer. Riboflavin was used as catalyst both in the separating and stacking gel. The electrophoresis was carried out with gel slabs of dimensions 0.25cm × 8.0cm × 8cm and at a constant 100 V/slab. Detection of

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peroxidase activity and staining of protein were performed as described previously (Olsen & Little, 1979).

SDS/polyacrylamide-slab-gel electrophoresis was carried out at a constant 100 V by using the system of Laemmli (1970). Gels of 10% acrylamide were used, and before application, samples were boiled for 15 min in the presence of 5% (w/v) mercaptoethanol. Molecular weights were determined with the low-molecular-weight calibration kit from Pharmacia. After the run, the SDS was removed from the gel with propan-2-ol (25%, v/v) and acetic acid (10%, v/v) in gel destainer GD-4II (Pharmacia). In the same apparatus the gel was stained with Coomassie Brilliant Blue and subsequently destained.

Unless otherwise stated, all procedures were carried out at room temperature (22–24°C). Solutions of enzyme were concentrated by ultrafiltration in Diaflo equipment (Amicon Corp., Lexington, KY, U.S.A.) with a PM-10 membrane.

Results and discussion

Purification

Initial extract. Buffy coats, collected from 90 litres of outdated human blood, were obtained by centrifugation of the blood at 1000 ***g*** for 15 min and subsequently washed twice in a hypo-osmotic solution (0.155 M-NH_4Cl/0.01 M-$KHCO_3$/0.1 mM-EDTA) to lyse contaminating erythrocytes. The white cells were sedimented by centrifugation at 800 ***g*** for 15 min between the washings. The packed white cells were resuspended in 0.1 M-potassium phosphate buffer, pH 7.3, and homogenized in a Waring Blendor for 1 min at top speed. The homogenate was centrifuged at 20000 ***g*** for 20 min, the supernatant discarded and the pellet homogenized once more in the phosphate buffer. After a total of three such homogenizations, the pellet was resuspended in 1.5 litres of 0.5 M-$CaCl_2$ and homogenized (Waring Blendor, 1 min, top speed) and centrifuged (20000 ***g*** for 20 min). The pellet was rehomogenized in 1.5 litres of 0.5 M-$CaCl_2$ and centrifuged as above and the supernatants combined. To this pool was then added 0.1 vol. of 1 M-sodium acetate buffer, pH 5.6, containing cetyltrimethylammonium bromide (0.5%, w/v), 0.5 M-$CaCl_2$, 0.05 M-$MnCl_2$ and 0.05 M-$MgCl_2$.

Affinity chromatography. The initial extract was mixed with 150 ml (packed volume) of concanavalin A–Sepharose and the mixture (approx. 3 litres), in a 5-litre plastic bottle, was rolled on a Rollacell (New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.) for 1 h (approx. 30 rotations/min) before being centrifuged (800 ***g*** for 15 min). The pellet was washed three times by resuspension in 0.1 M-sodium acetate, pH 5.6, containing 0.05% (w/v) cetyltrimethylammonium bromide, 0.5 M-$CaCl_2$, 5 mM-$MnCl_2$ and 5 mM-$MgCl_2$, followed by centrifugation as described above. Finally the pellet was resuspended in a small volume of the above buffer and poured into a glass column (5·5 cm × 25 cm) and washed with two bed volumes of this buffer. The gel was then washed with two bed volumes of 0.1 M-sodium acetate (pH 5.6)/0.05% (w/v) cetyltrimethylammonium bromide/0.5 M-$CaCl_2$/5 mM-$MnCl_2$/5 mM-$MgCl_2$/25% (w/v) glycerol.

The peroxidase activity was eluted from the column with this same glycerol-containing buffer, but including 0.2 M-1-*O*-methyl α-D-glycopyranoside. Fractions containing peroxidase activity were pooled and concentrated to about 100 ml by ultrafiltration.

Hydrophobic adsorption chromatography. The enzyme solution was then loaded on to a column of phenyl-Sepharose (150 ml packed volume). After washing with two bed volumes of Buffer A [0.1 M-sodium acetate (pH 5.6)/0.5 M-$CaCl_2$/0.05% (w/v) cetyltrimethylammonium bromide/25% glycerol] enzyme activity was eluted with Buffer A containing 70% (w/v) (rather than 25%) glycerol. Fractions containing peroxidase activity were pooled, diluted with two volumes of Buffer A without glycerol and then concentrated by ultrafiltration to 3.7 ml.

Gel filtration. The highly concentrated enzyme solution was applied to a column (1.6 cm × 100 cm) of Ultrogel AcA 44 and eluted with Buffer A and fractions collected. The elution profile from this column is shown in Fig. 1. Two well-separated peaks of peroxidase activity can be seen. The first fractions of the less-retarded peak (fractions 25–27) were pooled, concentrated and rechromatographed and

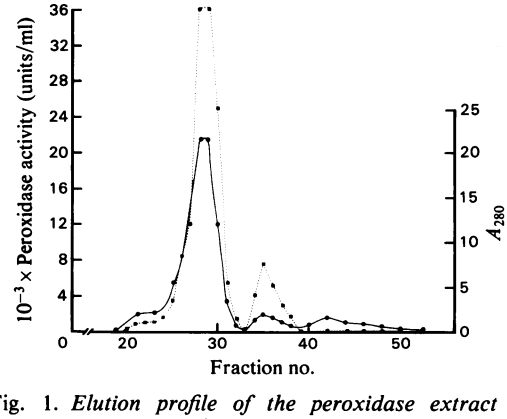

Fig. 1. *Elution profile of the peroxidase extract on Ultrogel AcA 44*

The peroxidase activity obtained after hydrophobic adsorption chromatography was concentrated and loaded on to an Ultrogel AcA 44 column (1.6 cm × 100 cm). Fractions (about 3.2 ml) were collected and tested for protein [A_{280} (——)] and peroxidase activity (— — — —).

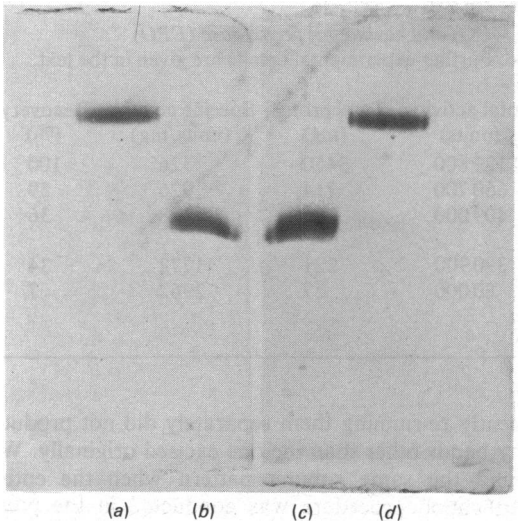

Fig. 2. *Analytical polyacrylamide-gel electrophoresis of the final products*

The electrophoresis was carried out as described in the Materials and methods section. After the run the gel slab was cut in two parts: one (*a*, *b*) was stained for protein and the other (*c*, *d*) was stained for peroxidase activity. Myeloperoxidase (15 μg) was applied to lanes (*a*) and (*d*), and eosinophil peroxidase (15 μg) to lanes (*b*) and (*c*).

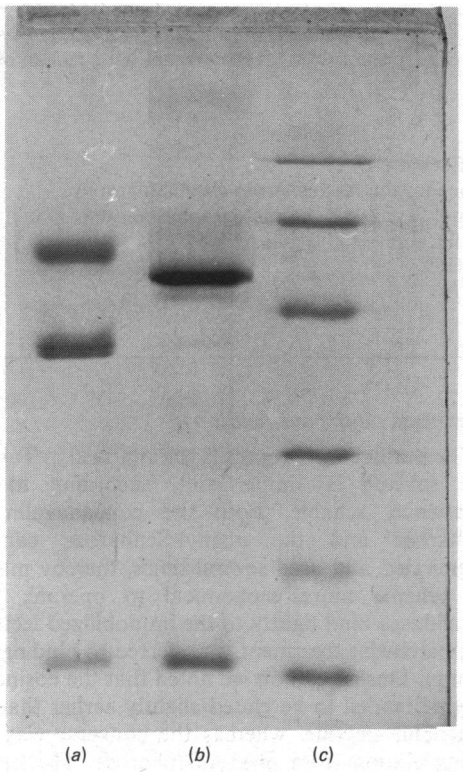

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of the purified enzymes*

Electrophoresis was performed on boiled reduced samples of the purified enzymes as described in the text. (*a*) Myeloperoxidase (25 μg), (*b*) eosinophil peroxidase (25 μg) and (*c*) standard proteins: phosphorylase *b* (mol.wt. 94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soya-bean trypsin inhibitor (20000) and lactalbumin (14400).

then the main peroxidase activity peak fractions combined with fractions 28–31 from the first gel filtration. Spectral analysis showed that the less retarded activity in Fig. 1 had a Soret absorption maximum at 434nm, which is characteristic for myeloperoxidase (Schultz & Shmukler, 1964; Himmelhoch *et al.*, 1969; Odajima, 1980), whereas the more retarded peroxidase (fractions 34–37 pooled) had a Soret absorption maximum at 415nm, as expected for the eosinophil peroxidase (Archer *et al.*, 1965; Wever *et al.*, 1981).

Purity of the final enzyme preparations

Both myeloperoxidase and eosinophil peroxidase were subjected to analytical polyacrylamide-gel electrophoresis and stained for both peroxidase activity and protein (Fig. 2). Each enzyme gave a single protein band with strong corresponding peroxidase activity. In addition, the myeloperoxidase sample gave, when stained for peroxidase activity, a second band just discernible and corresponding to the eosinophil enzyme. No corresponding protein band was detectable.

Both enzymes were also subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 3). Myeloperoxidase gave rise to three major bands with mol.wts. 57000, 39000 and 15500. The 39000-mol.wt. band consisted of two very similar sized polypeptides. The eosinophil peroxidase consisted of two major bands, one with mol.wt. 50000 and the other 15500. Faint traces of other polypeptides could also be detected.

The ratio between the absorption at the Soret peak and that at 280 nm (the *RZ* value) is commonly used as a criterion of purity of haem peroxidases. The final myeloperoxidase preparation had an *RZ* of 0.81, a value similar to that expected for the pure enzyme (Schultz & Shmukler, 1964; Matheson *et al.*, 1981; Andersen *et al.*, 1982). The *RZ* for eosinophil peroxidase was 1.05, a value significantly higher than that obtained by Wever *et al.* (1981) in their isolation procedure ($RZ = 0.7$). Later an *RZ* value of 1.0 has been reported for the human enzyme obtained by this isolation procedure (Buys *et al.*, 1981).

Table 1. *Purification of human myeloperoxidase (MPO) and eosinophil peroxidase (EPO)*
Buffy coats from 90 litres of outdated human blood were used. Further experimental details are given in the text.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)
Buffy-coat extract	3000	1127800	3450	326	100
Concanavalin A–Sepharose chromatography	102	660700	714	926	59
Phenyl-Sepharose chromatography	3.7	409000	287	1428	36
Gel filtration on Ultrogel AcA 44					
MPO	28.5	390900	221	1772	34
EPO	17.5	80000	27	2962	7

Comments on the purification

The purification process is summarized in Table 1. The scheme is simple and, according to our experience, reliable. Both the concanavalin A–Sepharose and the phenyl-Sepharose can be regenerated and used several times, thereby making the scheme more economical to operate. Both peroxidases bind tightly to the immobilized lectin so that batchwise treatment is preferred to binding in a column. During elution we noted that the eosinophil enzyme tended to be eluted slightly earlier than the neutrophil enzyme, whereas the converse was true during elution from phenyl-Sepharose. The myeloperoxidase peak from gel filtration was found to overlap partly with some higher-molecular-weight material with an absorption peak around 415nm. This material, which is probably an aggregated form of the eosinophil enzyme, was mostly removed on rechromatography of the relevant fractions. During rechromatography, this aggregated material mostly disaggregated and eluted at the same position as the non-aggregated eosinophil enzyme had done. The trace amounts of eosinophil peroxidase activity noted in the analytical disc-gel electrophoretogram of myeloperoxidase probably arise from residual traces of this aggregated enzyme. With the present buffers containing both glycerol and detergent, the eosinophil enzyme showed none of the tendencies to bind firmly to the stationary-phase material in gel filtration and to dialysis tubing that were previously reported (Wever *et al.*, 1981; Jörg *et al.*, 1982). The elution volume of the major peak of eosinophil peroxidase in the gel filtration used in the isolation procedure corresponds to that of a globular protein of approx. mol.wt. 50000. On SDS/polyacrylamide-gel electrophoresis a polypeptide with mol.wt. 50000 is found. In addition a polypeptide of approx. mol.wt. 15500 is present. Interestingly this subunit is exactly similar in size to the small subunit [denoted β-subunit by Andersen *et al.* (1982)] in myeloperoxidase. This small subunit must either be associated with the larger subunit or form an aggregate of approx. mol.wt. 50000. Slicing out these two subunits from the gel slab and subsequently re-running them separately did not produce any bands other than the one excised originally. We found the same subunit pattern when the entire purification procedure was conducted in the presence of the proteinase inhibitor phenylmethanesulphonyl fluoride.

The subunit pattern of myeloperoxidase is still a matter of dispute. Andersen *et al.* (1982) reported that myeloperoxidase from normal human neutrophils consists of two subunits with mol.wts. 55000 and 15000. A polypeptide with a mol.wt. of 39000 was also reported present. Olsson *et al.* (1972) found four subunits (62000, 54000, 38000 and 14000) in the enzyme isolated from leukaemic myeloid cells. In myeloperoxidase from human promyelocytic leukemia HL-60 cells, Yamada *et al.* (1981) reported the presence of two subunits (mol.wts. 59300 and 10150). Harrison *et al.* (1977) found a similar composition for canine myeloperoxidase. Other workers report a somewhat different subunit pattern. Thus Bakkenist *et al.* (1978) found that, after reduction, human myeloperoxidase on SDS/polyacrylamide-gel electrophoresis yielded an intense band (mol.wt. 63000) and a weaker band (mol.wt. 81000), whereas Matheson *et al.* (1981) found, under similar conditions, only one subunit (mol.wt. 59000).

In the present work the ratio between the amounts of the two peroxidases varied somewhat from purification to purification. Typically, however, the yield from 90litres of blood was 200–250mg of myeloperoxidase and 20–30mg of eosinophil peroxidase, with an overall yield of peroxidase activity of around 40%. These amounts of material are sufficient to permit detailed characterization and structural studies on both enzymes.

Stability properties of the peroxidases

When dissolved in Buffer A, both enzymes could be stored at −20°C for many months and even thawed and refrozen several times without detectable loss of activity. The myeloperoxidase seemed equally stable in 0.1M-phosphate buffer, pH 7·5, whereas, because of its tendency to aggre-

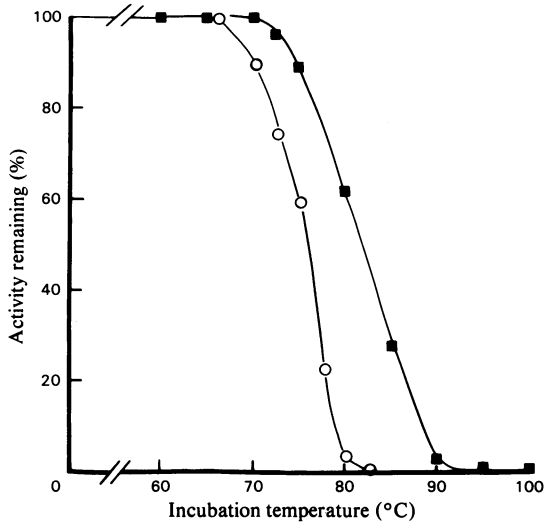

Fig. 4. *Thermal inactivation of myeloperoxidase and eosinophil peroxidase*

Myeloperoxidase (56 μg/ml) in Buffer A and eosinophil peroxidase (34 μg/ml) in Buffer A were exposed to different temperatures for 5 min and then cooled and assayed. ■, Myeloperoxidase; O, eosinophil peroxidase.

gate, especially in the absence of glycerol and detergent, the eosinophil enzyme was normally stored in Buffer A.

Some limited stability studies were also carried out on these two enzymes. Thermal-denaturation studies showed both enzymes could tolerate heating for 5 min at temperatures up to 65°C without being inactivated (Fig. 4). Indeed the myeloperoxidase suffered a mere 10% inactivation after 5 min at 75°C. Exposure of the peroxidases to chaotropic agents also produced interesting results. Myeloperoxidase was activated some 30–35% in solutions of guanidinium chloride of 1.8–3.0 M, with higher concentrations causing inactivation. Approx. 3.3 M-denaturant caused 50% inactivation (Fig. 5*a*). The eosinophil enzyme showed full activity after exposure to guanidinium chloride solutions up to 2.5 M, with approx. 3.1 M-guanidinium chloride causing 50% inactivation. Equivalent studies using urea showed that the eosinophil enzyme was not inactivated by concentrations up to 8 M, and only 18% inactivation occurred at 10 M. The myeloperoxidase was unaffected by urea concentrations up to 6.5 M, but retained only 15% activity after incubation in 10 M-urea (Fig. 5*b*).

Measurements of protein fluorescence, which are commonly used as a criterion for protein unfolding, provided no evidence for marked unfolding of either enzyme in urea solutions up to 10 M or in guanidinium chloride solutions up to 5 M. Clearly, therefore, both these enzymes have an extremely stable structure. For myeloperoxidase, this may result from

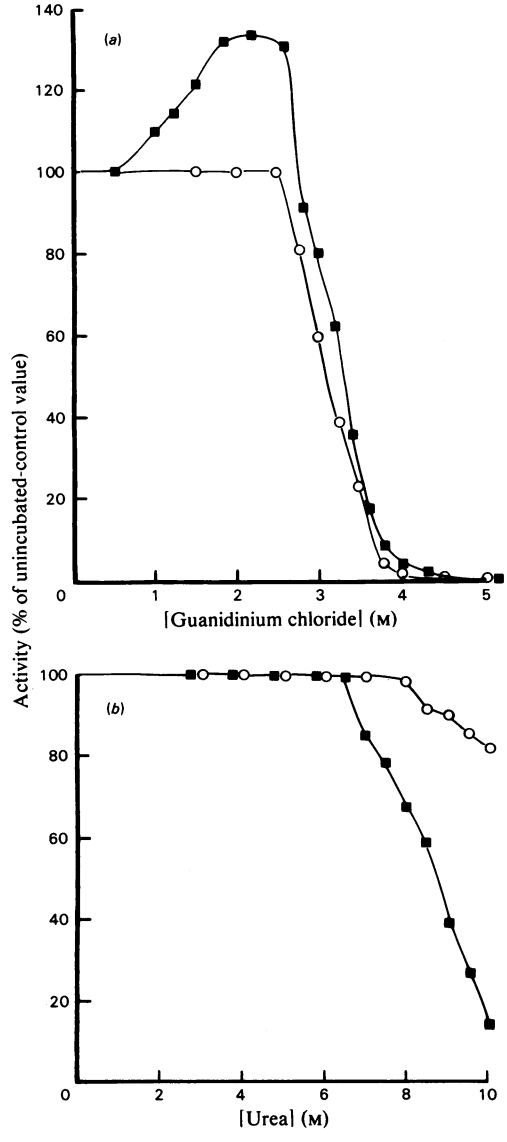

Fig. 5. *Effect of exposure to solutions of (a) guanidinium chloride or (b) urea on the catalytic activities of myeloperoxidase and eosinophil peroxidase*

Myeloperoxidase (40 μg/ml) and eosinophil peroxidase (34 μg/ml), dissolved in 0.1 M-potassium phosphate, pH 7.5, were mixed with solutions of the denaturants in the same buffer. The activities of myeloperoxidase (■) and eosinophil peroxidase (O) were measured after 6 h equilibration at room temperature and expressed as a percentage of the activities of the unincubated controls.

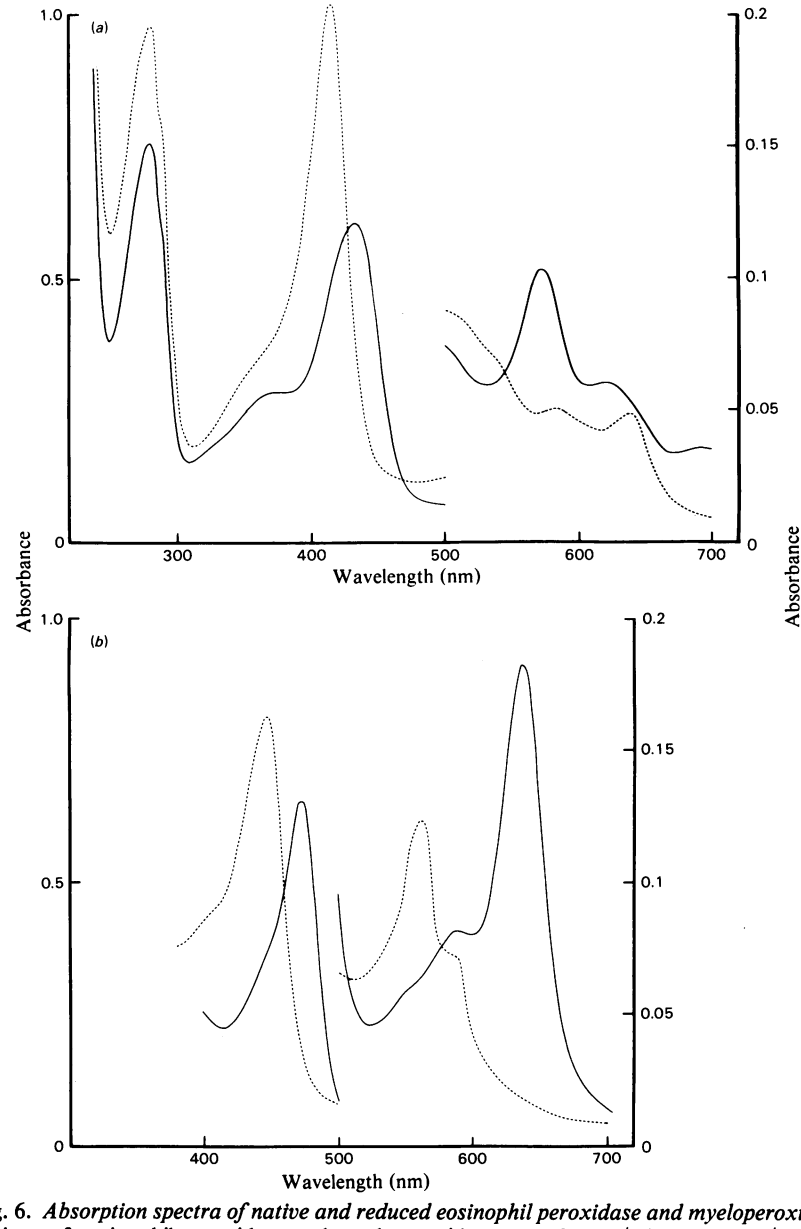

Fig. 6. *Absorption spectra of native and reduced eosinophil peroxidase and myeloperoxidase*
The concentrations of eosinophil peroxidase and myeloperoxidase were 0.6 mg/ml and 0.5 mg/ml respectively; (*a*) native enzymes; (*b*) reduced enzymes: ——, myeloperoxidase, – – –, eosinophil peroxidase. The left ordinates refer to the Soret and u.v. region, whereas the right ordinates refer to the other traces (above 500 nm).

its high content of cysteine, as shown in amino acid analysis (Matheson *et al.*, 1981), so that extensive intramolecular disulphide bridging may stabilize the structure. The reason for the extreme stability of the eosinophil enzyme is not known.

Spectral properties of the peroxidases

The final myeloperoxidase preparation in Buffer A had, in the native (oxidized) state, a major absorption peak at 434 nm with two smaller peaks centred at 573 and 620 nm; for the dithionite-reduced form, the peaks occurred at 473, 589 and 637 nm (Figs. 6*a* and 6*b*). These results agree well with the known visible absorption spectrum of this enzyme. The native eosinophil enzyme in Buffer A had a major absorption maximum at 415 nm with minor peaks at 500, 582 and 637 nm; for the dithionite-reduced form the peaks were at 447 nm

(major), 564 and 594nm, with the latter being more of a shoulder than a resolved peak (see Figs. 6*a* and 6*b*). These values are in general agreement with those reported by Wever *et al.* (1981) for the partially purified human enzyme. The fact that the two studies were carried out in quite diffent buffer systems may explain at least some of the minor differences. It should also be noted that these spectra are very similar indeed to those we have published recently for uterine peroxidase II from rat (Olsen *et al.*, 1982), an enzyme with which rat eosinophil peroxidase has immunological similarities (Olsen & Little, 1982).

In the present work, myeloperoxidase concentrations were based on $\varepsilon_{430} = 178\,mM^{-1} \cdot cm^{-1}$. This value was calculated from the data of Agner (1958) and is consistent with the spectral data for this enzyme published by Stelmaszyńska & Zgliczyński (1974). Certain workers (Bakkenist *et al.*, 1978; Wever & Plat, 1981) have quoted an $\varepsilon_{430} = 89\,mM^{-1} \cdot cm^{-1}$ for myeloperoxidase. As a further check, protein concentrations using the method of Bradford (1976) were also calculated with bovine serum albumin as standard, and the results obtained for the peroxidase concentrations were consistent with the higher absorption coefficient for myeloperoxidase and not the lower.

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References

Agner, K. (1958) *Acta Chem. Scand.* **58**, 89–94

Andersen, M. R., Atkin, C. L. & Eyre, H. J. (1982) *Arch. Biochem. Biophys.* **214**, 273–283

Archer, G. T., Air, G., Matoula, J. & Morell, D. B. (1965) *Biochim. Biophys. Acta* **99**, 96–101

Bakkenist, A. R. J., Wever, R., Vulsma, T., Plat, H. & Van Gelder, B. F. (1978) *Biochim. Biophys. Acta* **521**, 45–54

Bradford, M. M. (1976) *Anal. Biochem.* **78**, 248–254

Buys, J., Wever, R., van Stigt, R. & Ruitenberg, E. J. (1981) *Eur. J. Immunol.* **11**, 843–845

Clark, R. A. & Klebanoff, S. J. (1979) *J. Clin. Invest.* **64**, 913–920

Desser, R. K., Himmelhoch, S. R., Evans, W. R., Januska, M., Mage, M. & Shelton, E. (1972) *Arch. Biochem. Biophys.* **148**, 452–465

Diamond, R. D., Clark, R. A. & Haudenschild, C. C. (1980) *J. Clin. Invest.* **66**, 908–917

Harrison, J. E., Pablan, S. & Schultz, J. (1977) *Biochim. Biophys. Acta* **493**, 247–259

Himmelhoch, S. R., Evans, W. H., Mage, M. G. & Peterson, E. A. (1969) *Biochemistry* **8**, 914–921

Jörg, A., Pasquier, J.-M. & Klebanoff, S. J. (1982) *Biochim. Biophys. Acta* **701**, 185–191

Klebanoff, S. J., Jong, E. C. & Henderson, W. R., Jr. (1980) in *Eosinophils in Health and Disease* (Klebanoff, S. J., Jong, E. C. & Henderson, W. R., Jr., eds.), pp. 99–114, Mahmond, New York

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Matheson, N. R., Wong, P. S. & Travis, J. (1981) *Biochemistry* **20**, 325–330

Maurer, H. R. (1971) *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis*, De Gruyter, Berlin and New York

Odajima, T. (1980) *J. Biochem. (Tokyo)* **87**, 379–391

Olsen, R. L. & Little, C. (1979) *Eur. J. Biochem.* **101**, 303–339

Olsen, R. L. & Little, C. (1981) *Acta Chem. Scand.* **B35**, 1–4

Olsen, R. L. & Little, C. (1982) *Biochem. J.* **207**, 613–616

Olsen, R. L., Flatmark, T. & Little, C. (1982) *Biochem. J.* (1982) **201**, 91–94

Olsson, I., Olofsson, T. & Odeberg, H. (1972) *Scand. J. Haematol.* **9**, 483–491.

Schultz, J. & Shmukler, H. W. (1964) *Biochemistry* **3**, 1234–1238

Stelmaszyńska, T. & Zgliczyński, J. M. (1974) *Eur. J. Biochem.* **45**, 305–312

Wever, R. & Plat, H. (1981) *Biochim. Biophys. Acta* **661**, 235–239

Wever, R., Plat, H. & Hamers, M. N. (1981) *FEBS Lett.* **123**, 327–331

Yamada, M., Mori, M. & Suimura, T. (1981) *Biochemistry* **20**, 766–771

Zgliczyński, J. M., Stelmaszyńska, T., Ostrowski, W., Naskalski, J. & Sznajd, J. (1968) *Eur. J. Biochem.* **4**, 540–547