Bovine Lactoperoxidase. Partial Characterization of the Further Purified Protein*

W. A. Rombauts,† W. A. Schroeder,‡ and Martin Morrison

ABSTRACT: Lactoperoxidase has been prepared from bovine milk by a modification of the procedure of Morrison and Hultquist (Morrison, M., and Hultquist, D. E. (1963), *J. Biol. Chem. 238*, 2847) and partially characterized. Examination of the enzyme by chromatography, reversed salting-out procedure with ammonium sulfate, polyacrylamide gel disc electrophoresis, and sedimentation equilibrium has given evidence of only minimal contamination. The molecular weight is about 77,500, and the iron content is 0.0729%. A quantitative determination shows that all the common amino acids are present. Glucosamine, galactosamine, and neutral carbohydrates are constituents of this glycoprotein, but acetylneuraminic acid is absent. Acetyl groups were also detected. Leucine (0.5 residue/mole) was the only significant N-terminal amino acid that was detected by the dinitrophenyl, phenylthiohydantoin, and cyanate methods. Peptide maps of the soluble tryptic peptides suggest that lactoperoxidase may consist of two nearly identical subunits.

Although peroxidatic activity was demonstrated in bovine milk by Arnold (1881), the protein responsible for the activity was isolated by Theorell and Åkeson (1943) by precipitation methods and termed lactoperoxidase. Although several criteria of purity were applied, the ratio of absorbances at 412 to 280 mμ ($A_{412}:A_{280}$) has been the most common criterion for comparing the purity of various preparations. A preparation of Theorell and Pedersen (1944) had an $A_{412}:A_{280}$ ratio of about 0.8. A significantly higher ratio of 0.9 was achieved by Polis and Shmukler (1953) who employed a chromatographic procedure in part. A simplified procedure of Morrison and co-workers (Morrison *et al.*, 1957; Morrison and Hultquist, 1963) yielded a product with a ratio of 0.91–0.95; it behaved as a homogeneous substance in immunological tests (Allen and Morrison, 1963).

Although several peroxidases have been characterized as to their amino acid composition, terminal groups, carbohydrate content, and molecular weight (for a review, see Paul, 1963), the only chemical investigation of lactoperoxidase has been the study of its heme component by Hultquist and Morrison (1963). Because the procedure of Morrison and Hultquist (1963) provides purified lactoperoxidase in good yield and quantity, we began the further characterization of this protein.

When initial experiments were made to determine the N-terminal residues of lactoperoxidase, several types of residues were detected in a fractional number of moles per mole of protein. These results were not anticipated because the immunological analyses of lactoperoxidase by Allen and Morrison (1963) had provided excellent evidence of homogeneity. This evidence from the N-terminal determination prompted extensive investigation into the method of purification and the removal of possible contaminants as well as the characterization of the homogeneity by other than immunological methods. A further modified method of purification has resulted that eliminates the need for passage through a column of Sephadex (Morrison and Hultquist, 1963). Despite this effort, it has not been possible to obtain entirely satisfactory results from the N-terminal data that agree with other criteria of purity.

Experimental Section

Purification of Lactoperoxidase. In the initial stages of this work, lactoperoxidase was purified according to the method of Morrison and Hultquist (1963). Later the following modified chromatographic procedure was used for the purification. All operations were performed in a cold room at 2–3°. Crude lactoperoxidase, which contained about one-third of the total protein as lactoperoxidase ($A_{412}:A_{280}$ about 0.3), was isolated by the batch procedure of Morrison and Hultquist (1963). After overnight dialysis against 0.02 M sodium acetate adjusted to pH 7.35 with glacial acetic acid,[1] the solution was centrifuged at 20,000*g*

* Contribution No. 3534 from the Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91109 (W. A. S.), and the Medical Research Institute, City of Hope Medical Center, Duarte, California 91010. *Received June 21, 1967.* This investigation was supported in part by Grants HE 02558 and GM-08964 from the National Institutes of Health, U. S. Public Health Service. W. A. R. held an "Aspirant" Fellowship from the National Fund for Scientific Research, Brussels, Belgium, and a C. R. B. Fellowship from the Belgian–American Educational Foundation, New York, N. Y., during part of this investigation.

† Present address: Virus Laboratory, University of California, Berkeley, Calif. 94720.

‡ To whom requests for reprints should be sent.

[1] All determinations of pH were made at room temperature.

to remove a small amount of insoluble material. The protein solution was then chromatographed on a cation-exchange resin. For analytical purposes, a 1 × 40 cm column easily accommodated 120 mg of crude preparation which contained about 40 mg of lactoperoxidase. The procedure below is described for this column. For preparative purposes, a 2.5 × 40 cm column and six times the amount of protein were used. All measures except the length of the column were increased sixfold.

After the resin (Bio-Rex 70, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) had been freed from fines by repeated decantation, it was suspended in 0.02 M sodium acetate at pH 7.35 and the pH was readjusted to 7.35 with stirring by means of glacial acetic acid. The suspension was allowed to settle, and the process was repeated until the pH of the supernatant remained constant overnight. Until used for chromatography, the resin was refrigerated in this solution with a drop of toluene to prevent mold formation.

After pouring in three sections under gravity, the column was equilibrated with 600 ml of the starting buffer (0.02 M sodium acetate, pH 7.35) at a flow rate of 30 ml/hr with a Milton Roy constant-volume Minipump. Shortly before completion of the equilibration, the top of the resin was stirred and permitted to settle under pressure to ensure a flat top. The dark green-brown lactoperoxidase solution (in 2–5 ml) was either layered on top of the resin under the buffer or applied on top of the resin after withdrawal of the supernatant buffer. The protein was adsorbed in the upper 6–7 cm as an olive-green zone. After a 5-cm layer of starting buffer had been placed on top of the resin, the column was connected to a system for generating linear gradients (Bock and Ling, 1954). The mixing reservoir contained 116.5 ml of the starting buffer, and the second reservoir contained 100 ml of 0.35 M sodium acetate solution at pH 7.90. Because of the large density difference, careful adjustment is necessary to attain hydrostatic equilibrium. Elution was controlled with a Milton Roy Minipump at a flow rate of 12 ml/hr. Collection of 3-ml fractions was started when the protein solution was being applied to the column. The absorbancy of the fractions was measured at 280 and 412 mμ. At the end of the first gradient, the green lactoperoxidase zone was located between 7 and 16.5 cm from the top. A more gradual gradient was then started with 120 ml of 0.35 M sodium acetate (pH 7.90) in the mixing reservoir and with 120 ml of 0.50 M sodium acetate (pH 7.90) in the other. This gradient completely eluted the lactoperoxidase. However, there remained a pink zone[2] in the upper 10 cm of the column and a faint yellow zone about a centimeter wide immediately below. These zones can be essentially separated by a gradient from 0.50 to 1.50 M acetate (240 ml of each, pH 7.90). Routinely, however, they were eluted together by pumping through 1 M sodium acetate, until the absorbancy of the eluent at 280 mμ was less than 0.01. After reequilibration with 600 ml of starting buffer, the column could be used again; reproducible patterns were obtained in successive runs.

Those fractions of the lactoperoxidase peak with an $A_{412}:A_{280}$ ratio above 0.9 were combined and concentrated in Schleicher & Schuell collodion membranes by ultrafiltration. Other fractions with a ratio $A_{412}:A_{280}$ above 0.5 were combined and rechromatographed.

Reverse Ammonium Sulfate Gradient Chromatography. The procedure is essentially that of Zahn and Stahl (1953). Hyflo Supercel (Johns-Manville) was settled ten times from water until all fine particles were removed. A 20-ml portion of settled material was placed in a beaker and as much water as possible was decanted from the settled material. An aqueous solution (27 mg in 2 ml) of lactoperoxidase (purified as above) was then added to the magnetically stirred suspension. A solution of 95% saturated ammonium sulfate which was buffered at pH 5.0 with 5 ml of 2 M acetate buffer/100 ml was then added at 1 ml/min until 80% saturation of ammonium sulfate was reached. The entire suspension was poured into a chromatographic tube (1-cm diameter) to form a column about 20 cm high. The effluent was discarded because there was no residual absorbance at 280 mμ. A linear gradient system was set up with 162 ml of 80% saturated ammonium sulfate (with 5 ml of 2 M acetate buffer of pH 5.0/100 ml) in the mixing chamber and 198 ml of water in the reservoir. Flow rate through the column was 18 ml/hr and 3-ml fractions were collected.[3] Finally, the column was washed with 100 ml of water. The absorbancy at 412 and 280 mμ was measured.

Analyses for Peroxidase Activity. Peroxidase activity was measured with guaiacol as the hydrogen donor (Chance and Maehly, 1954; Morrison *et al.*, 1957). The rate of oxidation of guaiacol was followed spectrophotometrically on a Gilford recording spectrophotometer at 470 mμ. The cuvet with a 1.0-cm light path contained 3 ml of a solution 33 mM with respect to guaiacol in 0.1 M phosphate buffer, pH 7.4, and 0.3 mM with respect to hydrogen peroxide. The assay was performed at room temperature (22–23°).

Test for peroxidatic activity of zones in acrylamide gel was made with the benzidine reagent (Allen and Morrison, 1963).

Polyacrylamide Gel Disc Electrophoresis. The system for basic proteins with "spacer" and "sample" gels according to Reisfeld *et al.* (1962) was used because the isoionic point of lactoperoxidase is near pH 9 (Polis and Shmukler, 1953). The pH of the tray buffer in this system is 4.5. The actual pH of the gel is about 4.0.

Because of unsatisfactory results (see Results and Discussion), modified conditions were used. In some experiments, the sample gel was omitted, and the

[2] The protein in this pink zone has been variously referred to as "red protein" and "lactotransferrin" (Groves, 1960; Johanson, 1960; Blanc and Islike, 1961; Gordon *et al.*, 1962). We shall refer to this protein as "red protein."

[3] These columns have little back pressure. The flow is easily controlled with a metering or a peristaltic pump.

sample in 20% sucrose solution was layered on the spacer gel. In other experiments, the 7% spacer gel was increased to the concentration of the "running" gel. The best results were obtained in a system similar to that of Broome (1963) in which sample and spacer gel were omitted and reagents were removed from the running gel by 3 hr of electrophoresis before the sample was applied. The 15% running gels proved to be more satisfactory than 10% gels. Electrophoresis was carried out at 35° with 3 ma/tube for 1 hr. The 15% gels gave the sharpest patterns and were routinely used. The gels were fixed and stained for 30 min in a 1% Naphthol Blue Black solution in 7% acetic acid and destained overnight by washing in 7% acetic acid. Normally, 100 μg of lactoperoxidase was used but in some tests as much as 80-fold this amount was applied. The resulting patterns were photographed on Polaroid transparency film. The transparencies were examined with a Joyce Loebl microdensitometer, and the protein concentration of each peak was determined from planimetry of the peak areas and from the known concentration ratio between the runs. Tests were made to ensure that the blackening of the film by the lactoperoxidase peak in experiments with normal concentration and the small impurity peaks at very high concentration both fell into the logarithmic sensitivity region of the photographic emulsion. It was assumed that all protein bands had the same affinity for the dye.

Iron Determinations. After dry ashing of the protein, colorimetric determination was made with the bathophenanthroline reagent. Lactoperoxidase solutions were thoroughly dialyzed, and aliquots were pipetted into weighed porcelain crucibles. The solutions were first evaporated to dryness in an oven at 90° to avoid spattering, and the residue was subsequently dried to constant weight at 110°. The dried protein was ashed in an electric oven at 520° for 24 hr.

The iron oxide was dissolved in 0.1 ml of 4 N HCl on a steam bath, transferred to a volumetric flask with several 0.2-ml portions of iron-free water, the procedure was repeated, and the solution was made to volume. The iron in appropriate aliquot portions was determined colorimetrically with bathophenanthroline according to Diehl and Smith (1960).

Amino Acid Analysis. Prior to analysis, solutions of lactoperoxidase were thoroughly dialyzed against water or in some cases consecutively against a dilute HCl solution of pH 3, distilled water, a NH_3 solution of pH 12, water, a 1 M NaCl solution, and finally exhaustively against water in order to remove, if present, any bound amino acids and/or peptides. A volume containing about 5 mg of purified lactoperoxidase was pipetted into weighed tubes, the water was evaporated at 90°, and the residue was dried to constant weight at 110°.

Hydrolysis was carried out as described by Schroeder *et al.* (1963). Automatic amino acid analysis was made on 0.6 × 9 and 1 × 60 cm columns according to Spackman (1963) and Spackman *et al.* (1958).

Methionine and the sum of cysteine and cystine were determined after oxidation with performic acid according to Moore (1963). His recovery factors of 94 ± 2% for cysteic acid and 100 ± 2% for methionine sulfone were used.

Tryptophan was determined spectrophotometrically by method II of Bencze and Schmid (1957). No correction factor for residual absorption in the region of 280–290 mμ could be extrapolated because of the presence of the absorption maximum at 412 mμ. Tryptophan was also determined after 50 and 70 hr of alkaline hydrolysis in 4 N $Ba(OH)_2$ as described by Noltmann *et al.* (1962) and modified by Henschen and Blömback (1964). The tryptophan was directly determined on the 0.6 × 9 cm column of the amino acid analyzer (Spackman, 1963). It was well separated from lysine, glucosamine, and galactosamine. The recovery of tryptophan and the other amino acids was examined with a purified lysozyme sample (three-times crystallized, Sigma Co.). Noltmann *et al.* (1962) used a tryptophan recovery factor equal to that of leucine which was 85 ± 5%. Under our conditions, the recovery of leucine from lysozyme was 83.7%, and the average from proline, glycine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine was 86.2 ± 3%. However, the recovery of tryptophan was 93.5% and this was used as the basis for calculating the quantity in lactoperoxidase.

Hexosamine Determinations. Glucosamine and galactosamine were identified in acid hydrolysates on the basis of the chromatographic behavior of authentic samples on the 0.6 × 9 cm column (Spackman, 1963). In this system, the two peaks were not completely separated, but integration of the glucosamine peak by the usual height–width method was possible because of the high glucosamine to galactosamine ratio. Then, the total quantity in both peaks was determined by summation of absorbances (Spackman *et al.*, 1958), and the amount of galactosamine was arrived at by difference. Alternatively, these amino sugars were also determined on the 1 × 60 cm column (Spackman, 1963), where glucosamine and galactosamine are eluted after phenylalanine. Because glucosamine and galactosamine are known to be destroyed to a large extent during 24-hr hydrolysis at 110° in 6 N HCl and because this destruction is aggravated by the presence of protein and is greater when less concentrated solutions are used (Boas, 1953; Fisher and Nebel, 1955), some hydrolyses were made under milder conditions (0.5 ml of 3 N HCl for 4.5 and 8 hr at 100° instead of 2 ml of 6 N HCl at 110° for 24 hr), and the results were extrapolated to zero time.

Neutral Carbohydrates. The procedure of Ota *et al.* (1964) was used to isolate neutral sugars. Colorimetric determination with anthrone reagent by the method of Scott and Melvin (1953) followed. Solutions of glucose (Nutritional Biochemicals Corp., reagent grade, anhydrous) were used to determine a standard curve.

Acetylneuraminic Acid. Purified samples of lactoperoxidase, containing about 0.15 μmole, were hydrolyzed in 0.1 M H_2SO_4 for 1 hr at 80–90°. The hy-

drolysates were then analyzed by the method of Svennerholm (1958) and Warren (1959) for acetylneuraminic acid. No evidence for this compound was obtained with either method, although the Svennerholm procedure did show the presence of other carbohydrates.

Studies of N and C Termini. The end groups were examined by several methods in the manner described below.

DINITROPHENYLATION METHOD. About 220 mg of lactoperoxidase was exhaustively dialyzed against water and dinitrophenylated in aqueous solution at pH 8.0 and 40° under nitrogen with 0.1 ml of FDNB.[4] The procedure was essentially that of Rhinesmith *et al.* (1957). The pH was controlled with a pH-Stat (Radiometer, Copenhagen) during 5 hr of reaction. Excess reagent was extracted with 3 × 25 ml of peroxide-free ether (Mallinckrodt). The last traces of ether were removed by aspiration. The dissolved DNP-lactoperoxidase was precipitated by adjusting the pH to 2 with 1 N HCl. The finely dispersed greenish-yellow precipitate was centrifuged at 20,000*g* for 30 min at 4°, washed with 7 × 20 ml of 0.01 N HCl, and finally dried in an air stream. The yield of DNP-lactoperoxidase was 125 mg. Samples (50 mg) were hydrolyzed by refluxing in 10 ml of 6 N glass-distilled HCl for various periods of time. The hydrolysates were extracted with 4 × 25 ml of peroxide-free ether. The ether extracts were washed with 4 × 5 ml of water to which 1 drop 6 N HCl had been added, and the ether phase was evaporated at 40° under reduced pressure. The original hydrolysate was further extracted with 4 × 25 ml ethyl acetate (reagent grade) in order to detect DNP-histidine, and the organic phase was washed as above. DNP-amino acids in both extracts were identified by chromatography on silicic acid–Celite columns according to Green and Kay (1952). Their quantity was calculated from their absorbance in glacial acetic acid and corrected for recovery with the correction factors of Rhinesmith *et al.* (1957).

PHENYLTHIOHYDANTOIN METHOD. About 40 mg (0.5 μmole) of lactoperoxidase in water was applied to six 1 × 7 cm paper strips and were carried through the degradation procedure as described by Schroeder *et al.* (1963). Semiquantitative estimation was made by serial dilution. In experiments in which the cyclization time was doubled or in which the coupling with phenyl isothiocyanate was repeated with intervening benzene washing as recommended by Sjöquist (1959), no significant difference in results was observed.

CYANATE METHOD. The procedure was exactly that described by Stark and Smyth (1963). The special procedures for tryptophan, cystine, or cysteine were omitted. Determinations used 40 mg (0.5 μmole) of lactoperoxidase, and a similar amount was carried through the procedure as a control without urea and cyanate. (When cyanate only is omitted, the urea produces enough cyanate during the period of reaction to effect nearly as complete a carbamylation as when cyanate is added.) Carbamylation was essentially complete as estimated from the recovery of free lysine and homocitrulline in a hydrolysate of the carbamylated protein (Stark and Smyth, 1963). The fractions called "C_1" and "C_2" (Stark and Smyth, 1963), which should only contain the hydantoins of histidine and arginine, respectively, were both found to contain large amounts of lysine, histidine, and arginine. When these fractions were heated at 100° for 30 min in 3 N HCl in order to open pyrrolidone carboxylyl residues (G. R. Stark, personal communication), histidine, lysine, and arginine were absent from fraction C_1 and histidine and arginine from fraction C_2 but the lysine amount in fraction C_2 was merely decreased.

HYDRAZINOLYSIS. The procedure of Funatsu *et al.* (1964) was applied to 0.5 μmole of lactoperoxidase.

CARBOXYPEPTIDASE DIGESTION. Carboxypeptidase A (10 μl) (Worthington two-times crystallized, aqueous suspension, 50 mg/ml) was added to 20 mg of lactoperoxidase in 4 ml of 0.2 M ammonium acetate at pH 8.0. Samples (700 μl) were taken at zero time (before adding the enzyme), at 15 and 45 min, and at 2 and 18 hr. They were acidified to pH 2.5 with 2 ml of 2 N acetic acid and directly analyzed on the 1 × 60 cm column of the amino acid analyzer.

Acyl Determinations. Acylated amino groups were determined by the method of Bartley (1953). Approximately 40 mg (0.5 μmole) of lactoperoxidase was used for each determination. As a measure of the reliability of the procedure, a determination was made of the acetyl content of a known amount of acetylglycine in the presence of 40 mg of gelatin. Control runs were made on reagent and on lactoperoxidase that had not been hydrolyzed in order to correct for the possible presence of small amounts of acetate ion.

Peptide Maps. About 40 mg of lactoperoxidase was denatured by precipitation in 12.5% trichloroacetic acid. After the precipitate had been centrifuged and washed with water until the supernatant was neutral, it was suspended in 5 ml of H_2O, the pH was adjusted to 8.0, and 0.6 mg of trypsin (trypsin, two-times crystallized, salt free, Worthington) in 200 μl of 0.001 N HCl was added. The digestion was carried out at room temperature under N_2 with pH controlled by a Radiometer pH-Stat. After 30 min, the suspension had dissolved to produce a clear red-brown solution. After 2 and 5 hr, an equal amount of trypsin was added. After 11.5 hr when the uptake of base had essentially stopped, the pH was adjusted to 6.5 and a small amount of a fine brown precipitate was centrifuged. The slightly yellow supernatant fluid was lyophilized. The peptide mapping procedure was essentially that of Ingram (1958). Chromatographic developers were 1-butanol–pyridine–acetic acid–water (30:20:6:24) (Waley and Watson, 1953) or pyridine–isoamyl alcohol–water (35:35:30) (Wittman and Bräunitzer, 1959). Peptides from 4 mg of protein were used for each map. Tests for specific residues in the peptides were made as follows: tyrosine (Acher and Crocker, 1952), histidine (Baldridge and Lewis, 1953), and tryptophan

[4] Abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; PTH, phenylthiohydantoin.

with a spray of a 0.4% solution of 4-dimethylaminocinnamaldehyde in ethanol–6 N HCl (9:1) (K. N. F. Shaw, personal communication). After drying, tryptophan-containing spots are purple against a fading carmine background.

Analytical Ultracentrifugations. Equilibrium sedimentation experiments were made in a Spinco Model E ultracentrifuge with an An-D rotor at 20.0°. The protein solution was prepared by dialysis for 2 days against 0.2 M sodium acetate at pH 7.35. The protein concentration was 0.8%. Short-column solutions (3 mm) were used in double-sector cells (Van Holde and Baldwin, 1958) with a 12-mm thick Kel F centerpiece. The average speeds (7926 and 9340 rpm) over the entire run were calculated from the odometer readings. Centrifugations lasted between 15 and 24 hr. The concentration distribution in the cell was measured with Rayleigh interference optics. Interference patterns were recorded on Kodak spectrographic plates, emulsion type II G with a Kodak 77 A filter at exposures between 80 and 120 sec. The plates were measured in a Nikon two-dimensional microcomparator. The apparent weight-average molecular weight was calculated from the relation (Svedberg and Pederson, 1940) $\bar{M}_{w,app} = 2RT(\mathrm{d} \ln c/\mathrm{d}r^2)/(w^2(1 - \bar{v}\rho))$. The value of $\mathrm{d} \ln c/\mathrm{d}r^2$ was obtained by determining the "hinge point" and equating the corresponding fractional fringe number to the fractional fringe number for c_0 as determined in a synthetic boundary run (J. Fessler, personal communication). All the observed fringe numbers were corrected accordingly. Finally, these corrected fringe numbers, which now correspond with the actual concentration values across the cell, are plotted against r^2, and from the slope of this curve the value for $\mathrm{d} \ln c/\mathrm{d}r^2$ was determined.

The density of the solvent was taken from the International Critical Tables (1926). The partial specific volume was calculated from the amino acid composition as described by Cohn and Edsall (1943). Because the difference in partial specific volume between glutamic and aspartic acids and their corresponding amides is small, the value for the partial specific volume of the acids was used. The partial specific volume for glucose which is 0.65 ml/g (Lansing and Kraemer, 1935) was used for all carbohydrate residues. The heme group was not included in the calculation.

Results and Discussion

Determination of the terminal groups of a purified protein offers a test of its homogeneity because an integral number of terminal residues is to be expected. As mentioned above, lactoperoxidase did not meet this criterion when a study of its N-terminal residues was undertaken. As a result, other criteria of purity were applied, and a modified method of purification was devised. For ease of discussion in the following pages, we shall refer to the methods of purification as "original" and "modified." The "original" method is that of Morrison and Hultquist (1963) in which lactoperoxidase is adsorbed batchwise on ion-exchange resin from milk, removed from the ion-exchange resin, concentrated by ammonium sulfate precipitation, chromatographed on ion-exchange resin, and finally passed through a column of Sephadex to separate traces of proteins that the ion-exchange chromatography did not remove. The "modified" procedure is identical through the precipitation with ammonium sulfate and as the sole remaining step employs chromatography on an ion-exchange resin under modified conditions. We shall describe and discuss first the experiments on the determination of the N-terminal sequence of lactoperoxidase that had been prepared by both procedures, and then consider other criteria of purity and further characterization of the molecule.

Terminal Group Determinations. The data of many experiments are summarized in Table I. When a quantitative dinitrophenyl (DNP) procedure was applied to lactoperoxidase from the original method, DNP-leucine was isolated in a yield of about 0.5 residue/mole of lactoperoxidase. Threonine and glycine were present at about 0.1 and 0.2 residue, respectively. A semiquantitative determination on the same preparation by the PTH method confirmed these values but also showed about 0.1 residue of alanine. The amount of alanine varied greatly from one preparation to the other and, as shown in Table I, became the major derivative in some batches. Red protein, which constitutes a major portion of the protein material in the crude lactoperoxidase preparations, has an N-terminal alanyl residue (Groves, 1960). However, the red protein is an unlikely source of the N-terminal alanyl residue in these lactoperoxidase preparations. Because its molecular weight is similar to that of lactoperoxidase (Groves, 1960) it would have to have been a gross contaminant to yield the observed amount of PTH-alanine. This possibility must be further discounted on the basis of the spectral and immunological evidence for homogeneity for these preparations (Allen and Morrison, 1963). When lactoperoxidase from the modified method was subjected to the PTH procedure, 0.25 residue of leucine was found and the alanine amount decreased to the level of the other trace contaminants (Table I). In this semiquantitative estimation of the PTH derivatives, no correction factors were applied.

When the quantitative cyanate procedure was applied to this same material (expt III in Table I), 0.4 residue/mole of leucine was found. In addition to traces of various amino acids, rather large recoveries of serine and glutamic acid were found. However, all these values are corrected for losses. Because the loss of serine is about 70%, the corrected value has limited meaning. The glutamic acid is evident because pyrrolidone carboxylyl peptides (Stark and Smyth, 1963) were not removed in this expt I. However, the recoveries of leucine by the PTH and cyanate methods agree rather well, if we consider that the PTH recovery is uncorrected. Only trace amounts of amino acids were detected in a blank determination with the cyanate method (expt II). Experiment III gives the result of a cyanate determination on another preparation of

TABLE I: Results of the N-Terminal Determinations on Lactoperoxidase.[a]

	Sample Designation[b]	Derivatives										
		Asp	Thr	Ser	Glu	Pro	Gly	Ala	Leu	Lys	His	Arg
					DNP Method							
1-hr hydrolysis	LP_{II}		0.10				0.10		0.24			
2.5-hr hydrolysis			0.14				0.24		0.66			
24-hr hydrolysis			0.10				0.20		0.44			
					PTH Method							
	LP_{II}		0.1				0.1	0.1	0.3–0.5			
	LP_{III}	Trace[d]	0.1	0.1			Trace	0.5	0.3			
	LP_{IV}[c]						0.2	0.3	0.4			
	LP_{V}		Trace	Trace			0.2	0.5	0.2		Trace	
	"Red protein"		0.2	0.2			0.2	0.5–0.7	0.2			
	LP_{VIII}	Trace	Trace	Trace	Trace		Trace	0.3	0.1			
	LP_{XII}	Trace	Trace	Trace	Trace		Trace	Trace	0.25			
					Cyanate Method							
Expt I[e]	LP_{XI}	0.05	0.03	0.34	0.26		0.15	0.06	0.40			
Expt II[f]	LP_{XII}	0.01		0.01	0.10	0.01	0.03	0.03	0.01	0.03	0.01	0.01
Expt III[g]	LP_{XII}	0.02	0.02	0.23	(−0.16)	0.01	0.01	0.10	0.42	1.0	0.08	0.06

[a] The results are expressed in residues per mole of lactoperoxidase (77,500) after correction for destruction and/or losses, except in the PTH method. [b] Samples LP_{II} to LP_{VIII} from the original method and LP_{XI} and LP_{XII} from the modified method. [c] After exhaustive dialysis against acid, base, and sodium chloride (see Experimental Section). [d] "Trace" is $\leqslant 0.05$. [e] Uncorrected for blank. Pyrrolidone carboxyl peptides were not removed. [f] Blank without urea or cyanate. [g] Corrected for blank in expt II. Pyrrolidone carboxylyl peptides were removed.

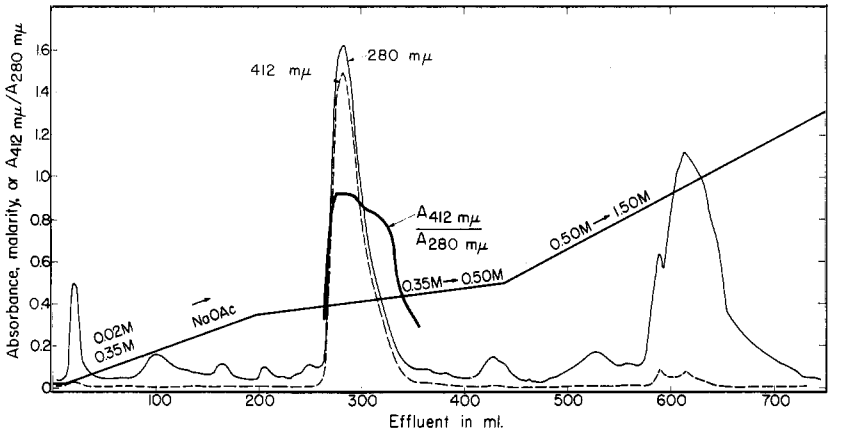

FIGURE 1: Chromatographic separation of lactoperoxidase from 150 mg of a crude preparation on a 0.9 × 40 cm column of Bio-Rex 70 resin at 4° with the indicated gradients.

lactoperoxidase according to the modified method. In this experiment, all fractions were treated to remove pyrrolidone carboxylyl peptides, and the values were corrected for the blank. Again, leucine appears in a yield approaching 0.5 residue/mole. The serine is without significance as discussed above and glutamic acid is absent. Lysine, which is very evident here even after the removal of pyrrolidone carboxylyl peptides was never detected with the PTH and DNP methods. Gaetjens *et al.* (1964) note that a high residual amount of lysine was detected in myosin with the cyanate procedure, and W. R. Holmquist (personal communication) reports the same for human hemoglobin in which lysine is not N terminal. If the pyrrolidone carboxylyl peptides were not removed, the amount of lysine approached ten residues per mole in both C_1 and C_2 fractions (see Experimental Section). The residual value is therefore rather low and should probably be disregarded (G. R. Stark, personal communication).

The small amounts of other amino acids have not been consistent by the three methods. Contamination at such a low level is often reported in N-terminal studies of highly purified enzymes (for example, Kanarek *et al.*, 1964), and in some cases their relative amounts decreased only very gradually over additional purification cycles (Dopheide and Trikojus, 1964). They are usually disregarded when one or more residues are near an integral value. Our results on lactoperoxidase do not permit such a clear-cut distinction. We believe, however, that leucine is a real N-terminal residue. The possibility must be considered that none of the N-terminal methods yields a satisfactory result with some proteins despite the fact that numerous excellent results with the DNP method might be cited. On the other hand, when the PTH method as used by us was tested with human hemoglobin,[5] the yield was only 50% of the theoretical. Laver (1961) recovered only 30% of the theroetical yield from horse hemoglobin with a PTH method using ^{35}S. Groves (1960) found 0.5 mole of alanine/mole of red protein with the DNP method after correction, and assumed this to be 1 mole/mole. Rombauts (1966) has reported a corrected recovery of 0.7 mole of proline/mole in PTH studies of the coat protein of the green tomato atypical mosaic virus. However, even if the results of the PTH method appear to be low for lactoperoxidase, they are in accord with the data from the DNP and cyanate methods.

The evidence from C-terminal analyses is inconclusive. Hydrazinolysis gave negative results as would be expected if the C-terminal residue is asparagine. The latter may be a C-terminal amino acid because the peptide Glu-Asn was present in 30% yield in a tryptic hydrolysate (unpublished data). Carboxypeptidase A after 18 hr of reaction gave $threonine_{0.1}$, serine, glutamine or $asparagine_{0.4}$ (which were not distinguished on the amino acid analyzer), $alanine_{0.1}$, and $leucine_{0.3}$ residues per mole.

Purification. A typical chromatographic separation of the components in "crude" lactoperoxidase by means of the modified method is illustrated in Figure 1. Four colorless zones emerged during the first gradient and were followed by lactoperoxidase itself at a molarity of about 0.4 during the second gradient. The bulk of the protein in the crude material was comprised of a yellow and a red protein which were removed by the final gradient. Although Polis and Shmukler (1953) thought that the red protein might be a degradation product of lactoperoxidase, it is not immunologically related to lactoperoxidase (Allen and Morrison, 1963).

The only heme-containing protein in the crude preparation is lactoperoxidase. In the several preparations of crude material that were chromatographed in this way, the content of lactoperoxidase was about one-third of the total protein and the other proteins were in the same approximate proportions as evident from Figure 1.

About 80% of the lactoperoxidase was in the center

[5] The authors are indebted to Mrs. J. B. Shelton for this determination.

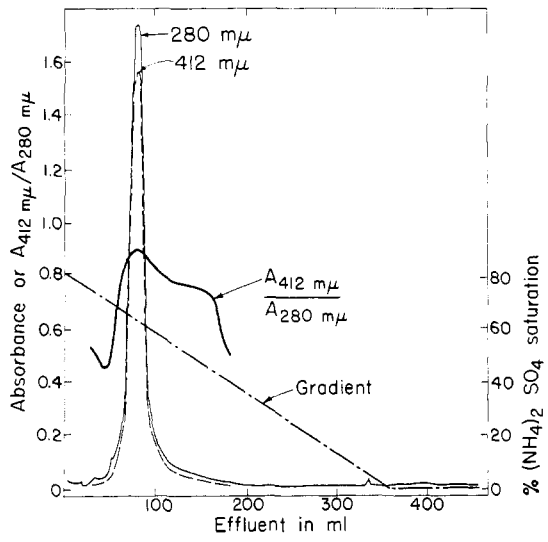

FIGURE 2: Reversed "salting-out" chromatography of lactoperoxidase.

part of the peak where the $A_{412}:A_{280}$ ratio was 0.93. This material was used for further characterization. Total recovery of lactoperoxidase, when front and tail sections were included, was essentially 100%. When those portions in which the ratio was <0.9 were rechromatographed, more material with a ratio >0.9 could be isolated.

Although a "second" lactoperoxidase has been reported by several authors (Polis and Shmukler, 1953; Morrison *et al.*, 1957; Burec *et al.*, 1963) and especially by Theorell and Paul (1944) in preparations from milk collected in the summer, no evidence for this second lactoperoxidase was observed in any chromatogram. It has been suggested by Morrison *et al.* (1957) that the "second" lactoperoxidase was an artifact of extraneous proteolytic action during preparative procedures. The subject is discussed in detail in that paper as well as by Morrison and Hultquist (1963).

Carlstrom (1965, 1966) and Carlstrom and Vesterberg (1967) have recently claimed that as many as six subcomponents are present in lactoperoxidase independently of whether or not rennet was used in the preparation or whether or not pooled milk or milk from a single animal was used. This extensive heterogeneity was apparent only after repeated chromatography (1965) or by means of isoelectric focusing (1967). It is not evident from his work whether the heterogeneity is present in the native enzyme or arises from the method of isolation.

Criteria of Purity. SPECTRAL DATA. As noted above, 80% of the lactoperoxidase in the chromatographic peak had an $A_{412}:A_{280}$ ratio of 0.93. This ratio compares well with that of 0.91–0.95 observed by Morrison and Hultquist (1963) and of 0.9 by Polis and Shmukler (1953). It is significantly different from that of 0.77 observed for the preparations of Theorell and Åkeson (1943). Carlstrom (1965) has reported a maximum ratio of 0.99. The modified method, therefore, produces a lactoperoxidase that is not significantly different in this absorbancy ratio from other recent preparations.

REVERSE AMMONIUM SULFATE GRADIENT CHROMATOGRAPHY. As an additional test of homogeneity, the central portion of the lactoperoxidase peak (absorbancy ratio >0.90) was examined by a reverse "salting-out" technique. The results are depicted in Figure 2. The lactoperoxidase emerged as a sharp peak at an ammonium sulfate saturation of 63%. Theorell and Paul (1944) reported that their preparation of lactoperoxidase salted out between 62 and 68% saturation of ammonium sulfate. When the center part of this peak was subjected to the same procedure, it behaved identically. Its absorbancy ratio of 0.96 indicated a possible slight increase in purity. We have not observed the effect of ammonium sulfate in decreasing this ratio as reported by Carlstrom (1965). The recovery was 80% of the amount applied.

DISC ELECTROPHORESIS. When disc electrophoresis with "spacer" and "sample" gels as described in the Experimental Section was used to examine the purified protein, there were frequently observed five sharp green bands which gave positive reactions with the benzidine reagent for peroxidase activity. The most rapidly moving band was in greatest amount and the other four varied in relative concentration. The distances between the bands did not decrease toward the top of the gel, as they would if aggregates of increasing degree of polymerization were present (Marinis and Ott, 1964).

Experiments were made to exclude the possibility that the multiple bands were caused by different oxidation states or altered ligands of the hemoprotein. However, when the protein solution in the buffer was made 20% in sucrose and layered directly on the "spacer" gel, the five bands still appeared. This eliminated the possibility that, during polymerization of the "sample" gel by ultraviolet light in the presence of protein, the ultraviolet light might have degraded the heme and generated differently charged molecules. Furthermore, the electrophoresis was made in 0.01 M cyanide or fluoride as well as with enzyme that had been reduced with dithionite or oxidized with ferricyanide. Although the mobility of the protein varied, it separated in all cases into the same number of components on electrophoresis.

In order to investigate the possibility that the multiple zones were artifacts, a concentrated solution of the enzyme was subjected to electrophoresis and separated into zones. The major zone could readily be distinguished without staining. When this part was cut out and placed on a small pore gel and the electrophoresis was repeated, all five zones were apparent. Thus, it was established that the multiple components were artifacts of the electrophoretic procedure. The procedure was repeated with the second zone. The major portion of this zone migrated as it had originally but did give traces of a slower moving zone. The major zone apparently is convertible to the other zones,

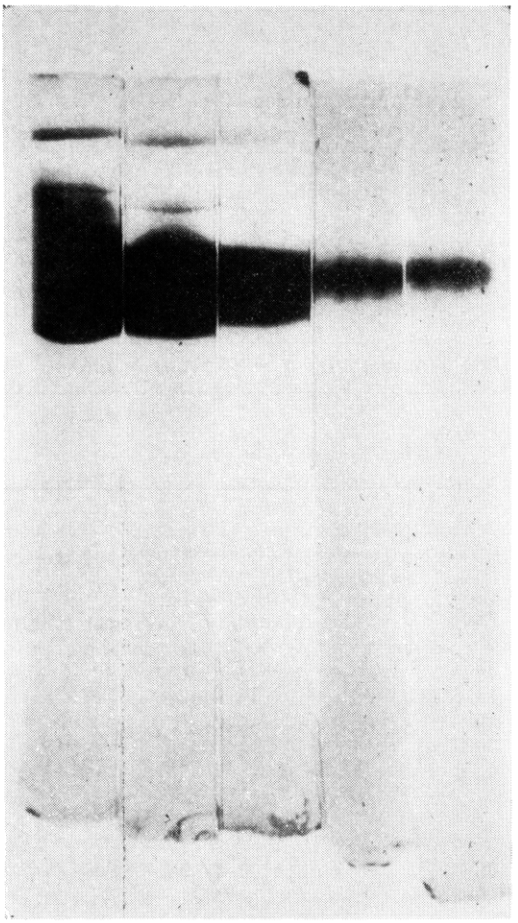

FIGURE 3: Disc electrophoreses with increasing amounts of purified lactoperoxidase. The 100 μg of lactoperoxidase for the right gel was increased successively to 2, 20, 40, and 80 times this amount. The pH was 4.5, and spacer and sample gels were omitted.

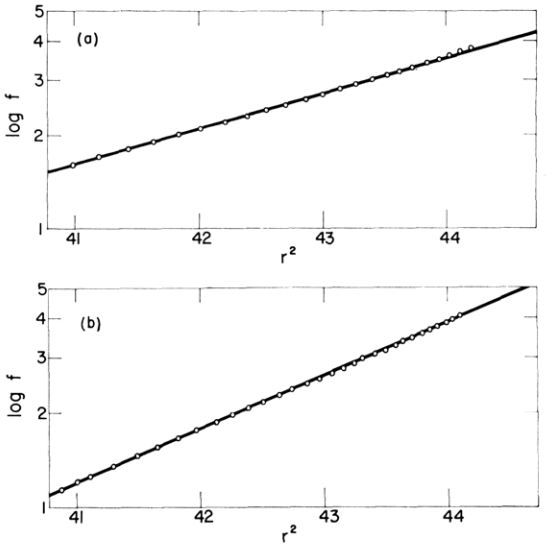

FIGURE 4: Concentration distribution of lactoperoxidase throughout the cell expressed as the logarithm of the fringe numbers *vs.* the square of the radius for an equilibrium sedimentation experiment after approximately 24 hr at 7926 rpm in a and at 9430 rpm in b.

but the conversion is not reversible. These irreversible conversions are comparable to the phenomena reported by Carlstrom (1966).

The various bands are also tested for immunological identity. Antisera to crude and purified lactoperoxidase were prepared in rabbits (Allen and Morrison, 1963). After separation by electrophoresis, the individual bands were placed onto a 1% agar plate. After the agar had solidified, a well was made parallel to and 5 mm from the gel. Antisera to lactoperoxidase were placed in the well, and the plate was kept at room temperature in a water-saturated atmosphere for as long as 2 weeks. The precipitin formed could be readily detected in the diffusion plate by the positive benzidine stain. All electrophoretic fractions gave bands of immunological identity.

On the other hand, when the concentration of the "spacer" gel, which normally is 7% and lower than that of the running gel was made the same (15%), only one sharp band appeared. It was thus concluded that the four more slowly moving bands were artifacts rather than isozymes. Swope *et al.* (1966) likewise have detected no heterogeneity by starch gel electrophoresis.

In the final experiments the system for disc electrophoresis was similar to that of Broome (1963) in which sample and spacer gels are omitted, excess reagents are removed by electrophoresis, and the sample is directly layered on top of the gel. Figure 3 shows the results of disc electrophoresis of increasingly larger amounts of lactoperoxidase that are derived from the center part of a peak as in Figure 1. As the amount was increased, two small bands became apparent. By planimetry of densitometric tracings of this photograph, it could be shown that the two small bands constituted only about 1% of the material. The major dark band is diffuse in some of these experiments because of overloading.

It would appear that the heterogeneity that Carlstrom (1966) observed in disc electrophoresis is the result of modification of lactoperoxidase by the procedure itself. Brewer (1967) has discussed the role of persulfate in this problem.

IRON DETERMINATIONS. Iron determinations were done in the early stages of this work on lactoperoxidase from the original method. The result of two determinations on separate samples was 0.0729 ± 0.0015%. From earlier studies (Theorell and Pedersen, 1944), it is known that only one iron atom per mole is present. The minimum molecular weight from the iron content is 76,500 ± 1600. Carlstrom (1965) lists iron contents of 0.062 and 0.061% from two fractions; this is equivalent to a molecular weight of about 91,000.

SEDIMENTATION EQUILIBRIUM. In Figure 4, the data from the sedimentation equilibrium determinations

TABLE II: A Comparison of Molecular Weights and Related Data.

	Theorell and Pedersen (1944)	Polis and Shmukler (1953)	This Investigation
Absorbance ratio	0.77	0.9	0.93-0.96
Fe content (%)	0.070 ± 0.002	0.069	0.0729 ± 0.0015
Molecular weight from Fe content	80,000 ± 2,300	81,000	76,500 ± 1,600
Molecular weight by other methods	93,000[a] (sedimentation velocity)	82,000 ± 2,000 (light scattering)	77,500 ± 3,000[b] (sedimentation equilibrium)

[a] $D_{20} = 5.95 \times 10^{-7}$ cm sec^{-1} (measured); $s_{20} = 5.37$ S; $\bar{V} = 0.764$ ml/g (measured). [b] $\bar{V} = 0.75$ ml/g (calcd).

TABLE III: Composition of Lactoperoxidase.[a]

	3 N HCl, 100°		6 N HCl, 110°			Av Values[b] (mol wt 77,500)
	4.5 hr	8 hr	24 hr	48 hr	106 hr	
Lys			32.1	33.2	32.9	33
His			13.8	14.1	14.0	14
Arg			38.5	38.9	39.0	39
$CySO_3H$						16
Asp			70.5	70.6	71.3	71
$MetSO_2$						12
Thr			27.3	27.0	24.2	28[c]
Ser			28.4	26.2	21.7	30[c]
Glu			59.4	60.3	60.9	60
Pro			40.1	42.3	42.6	42
Gly			40.1	41.4	41.0	41
Ala			38.9	40.7	39.6	40
Val			27.0	28.7	29.4	29[d]
Ile			24.5	27.1	28.0	28[d]
Leu			66.3	68.1	68.4	68[d]
Tyr			14.6	15.0	15.1	15
Phe			30.7	30.8	31.5	31
Trp				15.8[e]	15.8[e]	16[e]
Glucosamine	15.9	16.1	9.9	7.1	3.9	16[f]
Galactosamine	9.7	9.5	5.5	3.6	1.0	10[f]
Neutral carbohydrates (%)						1.5
Acetylneuraminic acid						Negative
Acetyl groups						10 ± 2[g]

[a] Data are expressed as residues per mole of protein. [b] Average values of the determinations unless noted otherwise. [c] Corrected to zero time. [d] The maximum value determined. [e] Alkaline hydrolysis for 50 and 70 hr. [f] Based on the data from hydrolysis in 3 N HCl. [g] Average of two determinations.

with purified lactoperoxidase are plotted as the logarithm of the protein concentration throughout the cell in corrected fringe numbers *vs.* r^2 at 7926 and 9340 rpm, respectively. At the bottom of the cell, the fringes were too closely spaced to be completely resolved by the photographic plate and are omitted from the drawings. The straight line in Figure 4a represents the sedimentation behavior for a homogeneous substance in ideal solution with an apparent molecu'ar weight of 75,000 ± 3000 and that in Figure 4b for 80,000 ± 3000 with the calculated partial specific volume of 0.75 for lactoperoxidase. The measured concentration values throughout the cell fit the straight lines rather well, except toward the bottom of the cell, where a slight upward curvature is present. This indicates a small degree of heterogeneity and may be

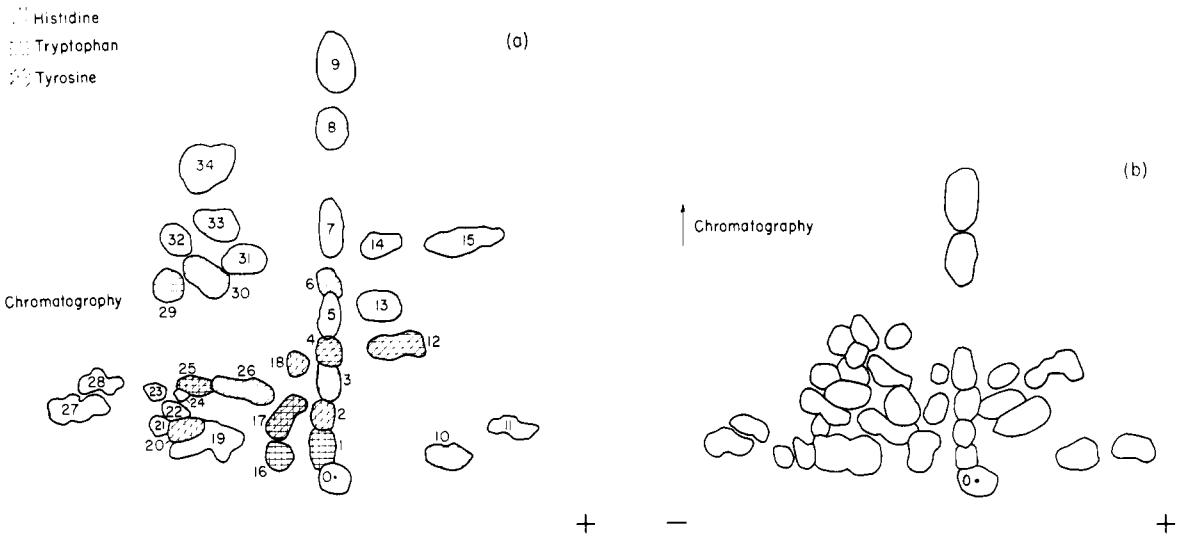

FIGURE 5: Tracings of peptide maps of the soluble tryptic peptides of lactoperoxidase. The point of application is marked o. (a) Developed with 1-butanol–pyridine–acetic acid–water and (b) with pyridine–isoamyl alcohol–water. The spots with positive reaction to specific stains are noted.

representative of the traces of impurity that appeared in disc electrophoresis.

The two values for the molecular weight 75,000 ± 3000 and 80,000 ± 3000 agree within the accuracy of the experiments. It should be noted that they are apparent molecular weights at 0.8% concentration. No extrapolation to zero concentration was made as our principal aim was the demonstration of homogeneity with respect to molecular weight.

In Table II, the available data about the molecular weight of lactoperoxidase are compared. The data of Polis and Shmukler and of this investigation are in essential agreement both on the basis of molecular weight from the iron percentage and from the physical measurements. Theorell and Pedersen's data apparently are in error; the $A_{412}:A_{280}$ ratio and the iron content suggest contamination with the red protein which according to Groves (1960) has a molecular weight of 93,000 and 86,000 as determined by iron content and sedimentation, respectively. Carlstrom's (1965) values of 0.061 and 0.062% iron are grossly different.

Amino Acid, Carbohydrate, and Acyl Analyses. The results of the several analyses of lactoperoxidase are presented in Table III. Only a few comments need be made about the amino acid analyses. An unidentified peak equivalent to about one residue per molecule eluted between proline and glycine. Spectrophotometric determination of tryptophan gave a value of 23 residues/molecule as compared to 16 by alkaline hydrolysis. The latter is judged to be the more exact because the usual corrections to the spectrophotometric data are invalidated because of absorption by the heme group. The values for the carbohydrates are probably minimal although there is reasonable agreement from the two types of hydrolysis if the results from hydrolysis in 6 N HCl are extrapolated to zero time as a pseudo-first-order reaction (14 residues of glucosamine and 10 of galactosamine). In addition to the hexosamines, about 1.5% neutral carbohydrates are present. These carbohydrate moieties probably are covalently linked to the peptide chain(s) because hexosamines can be detected in the amino acid analysis of carbamylated lactoperoxidase which has been exposed to 8 M urea for 15 hr at 100° and exhaustively dialyzed. Hexosamine residues were also detected in several peptides from a tryptic digest after chromatographic separation; in addition these and several other peptides showed a positive test with tetrazolium spray on paper (unpublished results). Thus, bovine lactoperoxidase belongs to the class of glycoproteins. Although many glycoproteins possess carbohydrate moieties that terminate in acetylneuraminic acid (Gottschalk and Graham, 1966), none were detected in lactoperoxidase. Nevertheless, 10 ± 2 acetyl residues/mole were detected. These could easily be accounted for by the presence of acetylated hexosamines with a possible contribution from acetylated N-terminal groups. The sum of the various components in Table III plus one heme group accounts for 98.2% of the weight of lactoperoxidase; this is well within the experimental error of the analysis.

The general presence of a carbohydrate moiety in many peroxidases (Morrison *et al.*, 1966; Paul, 1963) as well as lactoperoxidase suggests that it possesses a functional or structural necessity. It could conceivably be stabilizing the hemoprotein structure, or serve as internal hydrogen donor for the peroxidative function. Recently, however, Eylar (1966) noted that glycoproteins were far more frequent among extracellular than among intracellular enzymes, and suggested that the carbohydrate moiety was either added after synthesis of the protein in order to carry out a specialized function during the process of excretion through the cell wall,

or, alternatively, originated from parts of the cell wall being irreversibly linked to the protein during its passage.

Peptide Mapping. After tryptic digestion, about 2% of the material precipitated at pH 6.5. This precipitate which contained the heme group had the composition Lys_1,His_1,Arg_1,Asp_2,$Thr_{1.5}$,Ser_2,Glu_3,Pro_1,Gly_2,Ala_2,half-$Cys_{0.3}$,Val_1,$Met_{0.5}$,Ile_2,Leu_3,$Tyr_{0.5}$,$Phe_{1.5}$. When the precipitate was dissolved in 0.1 N ammonia and chromatographed on paper with 1-butanol–pyridine–acetic acid–water, a major spot and four minor spots appeared.

Figure 5 presents drawings of peptide maps from the soluble tryptic peptides of lactoperoxidase. From the amino acid analysis, 33 lysyl and 39 arginyl residues are present in the molecule of lactoperoxidase (77,500 mol wt). If these are all in unique positions and if asparagine is the C-terminal residue, tryptic hydrolysis should produce a maximum of 73 peptides or the same number of spots on the peptide map. Actually, 34 significant spots may be observed. The amino acid analysis of the protein shows 14 histidyl, 16 tryptophyl, and 15 tyrosyl residues in the molecule; with specific staining, seven to eight spots were positive for histidine, about seven for tryptophan, and about ten for tyrosine as one of the drawings of Figure 5 indicates. We need not belabor the uncertainties that arise in using such numbers as a measure of the subunit structure of a protein. However, the total number of spots as well as those with specific staining is about equal to the number that would be expected if lactoperoxidase has two identical subunits. It seems reasonable to conclude that lactoperoxidase is not composed of a single polypeptide chain or of two or more dissimilar chains because then the number of spots should be much larger. This conclusion is supported by preliminary sedimentation equilibrium studies in 8 M urea and 6 M guanidinium chloride which suggest a molecular weight of the order of 40,000 (W. A. Rombauts and A. J. Bailey, unpublished data). Certain data suggest that the proposed subunits, although probably very similar, may not be entirely identical. The determination of the N-terminal residues has not yielded enough derivative to account for even a single end group per molecule of molecular weight 77,500 let alone two. If two similar subunits are present, the N-terminal residue of one may be blocked. If lactoperoxidase, indeed, has two subunits, the single covalently bonded heme (Hultquist and Morrison, 1963) must either be bound to one of the two or it must bridge both. The distribution of the carbohydrate residues in the subunits also remains to be determined.

Conclusions

The properties of lactoperoxidase as prepared by the modified method do not differ in large measure from those of other preparations; *i.e.*, the ratio of absorbancies at 412 and 280 mμ is not higher. Immunochemical studies as previously reported (Allen and Morrison, 1963) suggested homogeneity. To this have been added now the data from reverse "salting-out" chromatography, from sedimentation equilibrium, and from electrophoresis. The latter shows only about 1% of any impurity and the centrifugation no evidence of any significantly greater amount. Reverse "salting-out" chromatography likewise points to essentially complete homogeneity. The minimum molecular weight from the iron content is in good agreement with the molecular weight by sedimentation equilibrium and with the value from light scattering by Polis and Shmukler (1953).

On the other hand, although traces of extraneous "N-terminal" amino acids are largely eliminated in preparations by the modified procedure, no amino acid is increased in amount to an integral number of N-terminal residues. Additional evidence is needed to distinguish between the possibilities that some yet undetected contamination is responsible for the fractional residue of leucine per molecule or that leucine is the real N-terminal residue and gives abnormally low yields in all procedures. Until other data are forthcoming, it would appear that the N-terminal determination is not a good measure of the purity of lactoperoxidase.

The results of the amino acid analyses and the carbohydrate determination when taken together with the known heme content account essentially quantitatively for the weight of the sample. This is, of course, in itself no real indicator of homogeneity. However, these data in conjunction with the molecular weight and the peptide maps lead to the conclusion that lactoperoxidase may be composed of two identical or nearly identical subunits.

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