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Lactoperoxidase

Identification of Multiple Molecular Forms and their Interrelationships

ANDERS CARLSTRÖM

Biokemiska avdelningen, Medicinska Nobelinstitutet, S-104 01 Stockholm 60, Sweden

The microheterogeneity of lactoperoxidase was investigated by disc electrophoresis and the heterogeneity pattern was found to be consistent with those obtained by ion-exchange chromatography, isoelectric focusing, and moving boundary electrophoresis. Lactoperoxidase A and B of Polis and Shmukler were shown to be composed of 4 and 6 subfractions, respectively. A convertibility of the subfractions within the B-group and between the B- and A-groups was studied and discussed. Mildly enriched peroxidase from individual cows was analysed and found to be heterogeneous. It was suggested that the heterogeneity is not due to preparative manipulations but to catabolic processes *in vivo*.

Since the first preparation of a highly purified peroxidase from cow's milk was reported by Theorell and Åkeson [1] several observations on the heterogeneity of this enzyme have been made.[2–7] By ion-exchange chromatography,[5] by the method of isoelectric focusing of ampholytes,[6] and by moving boundary electrophoresis,[6] the existence of multiple forms of LPO * was clearly established. These methods, however, are time consuming and require large amounts of enzyme. A rapid procedure applicable for the analyzis of small amounts of material is experimentally desirable. In a previous short communication the resolution of multiple forms of LPO by disc electrophoresis was briefly described.[7] The present paper details this electrophoretic system and the separation obtained. Disc electrophoresis was also used to correlate the heterogeneity patterns obtained by different methods, and to study the convertibility of the subfractions and the nature of mildly enriched peroxidase from individual cows. An artifactual heterogeneity produced by a disc electrophoretic system was also studied.

* LPO = lactoperoxidase.

MATERIALS AND METHODS

LPO from pooled milk was prepared as described earlier.[5] In the preparation of LPO from individual cows, an aliquot of the mixed morning milk yield was centrifuged at 3000 *g* for 20 min in an International Refrigerated Centrifuge which separated the cream as a firm floating cake. The skimmed milk thus obtained, was centrifuged in a Spinco Model L centrifuge at 19 000 rpm in the 21 rotor for 3 h within 90 min after the cow was milked. All operations were performed at +4°C. The transparent supernatant, 150 ml, freed from most of the casein particles, was applied to CM-cellulose in a 3×7 cm column, equilibrated with 0.05 M phosphate buffer, pH 7.0. The whey was allowed to flow through the column under a hydrostatic pressure of approximately 45 cm of water. After all of the whey had passed through the column, the CM-cellulose was washed with 0.05 M phosphate buffer, pH 7.0, until the absorbancy of the eluate was below 0.030 cm^{-1} at 280 mμ. The adsorbed peroxidase was then eluted with 0.1 M phosphate buffer, pH 7.0, containing 0.2 M sodium chloride. The fractions containing peroxidase activity were pooled (less than 50 ml) and concentrated by ultrafiltration through a collodion membrane until the volume was about 0.5 ml. The sample was then transferred to a 2×55 cm column containing Sephadex G-75, equilibrated with the 0.1 M phosphate buffer, pH 7.0, 0.2 M in sodium chloride. The preparation was subjected to gel filtration over-night and the fractions containing peroxidase activity were pooled and concentrated by ultrafiltration to give a volume of approximately 0.5 ml. A few ml of 0.06 M potassium acetate buffer, pH 6.7, was added and the peroxidase solution was again concentrated by ultrafiltration. This latter procedure was repeated several times in order to decrease the ionic strength of the solution. The preparation procedure was completed within 36 h after milking. The peroxidase was then immediately analyzed by disc electrophoresis.

CM-cellulose and DEAE-cellulose were prepared according to Peterson and Sober,[8] but omitting the last alcohol-drying step. Special care was taken to remove small particles by repeated decantation in order to obtain cellulose columns with high flow rates.

Sephadex G-75, Sephadex G-200, and DEAE-Sephadex A-50 were obtained from AB Pharmacia, Uppsala, Sweden. The materials were handled according to the manufacturer's description.

Chemicals. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine and 2,6-lutidine of analytical grade were obtained from Eastman Organic Chemicals, Rochester, New York.

Isoelectric focusing. Preparation of electrolysis column for isoelectric focusing was performed as described previously.[6] However, as the commercially available carrier ampholytes do not quite cover the pH region around pH 10, 150 mg of arginine (free base) and 70 mg of lysine (free base) were added to the ampholyte solution. Carrier ampholytes were purchased from LKB-Produkter AB, Stockholm, Sweden. After focusing, the zones were eluted, dialyzed against 0.06 M potassium acetate buffer, pH 6.7, and used for disc electrophoresis.

Chromatography on DEAE-Sephadex. The chromatography was performed essentially according to the previous description.[5] DEAE-Sephadex in a 2.2×22 cm column was equilibrated with 0.01 M Tris-HCl buffer, pH 9.04. The protein sample, extensively dialyzed against the same buffer, was applied on top of the column which subsequently was washed with a few ml of buffer. The elution was then effected by 0.01 M Tris-HCl buffer, pH 8.00, at a flow rate of 1.5 ml/cm^2/h. LPO B-4 and LPO B-5 were finally eluted with 0.1 M Tris-HCl buffer, pH 8.0. Five ml fractions were collected which were pooled according to Fig. 4 and analysed by disc electrophoresis without previous dialysis. The contents of those tubes containing homogeneous subfractions were concentrated by ultrafiltration and stored frozen at −15°C. Tubes containing non-homogeneous zones of LPO B-1 and LPO B-2_I were pooled and rechromatographed using 0.01 M Tris-HCl buffer, pH 8.5, as an eluent. However, complete separation was rarely achieved, although homogeneous subfractions were obtained from the leading and trailing edge, respectively.

Chromatography on Amberlite CG-50. Amberlite CG-50 was treated according to Paléus.[9] The ammoniacal form of the ion exchange material was transformed to the sodium form by repeated washings with 0.5 M disodium hydrogen phosphate. The Amberlite was then treated according to Morrison and Hultquist [4] and the column was packed to give the dimensions reported by these authors. Approximately 250 mg of

LPO was applied on top of the column and the chromatography was performed according to Morrison and Hultquist.[4] Fractions from the eluate were concentrated by ultrafiltration, dialyzed against 0.06 M potassium acetate buffer, pH 6.7, and subjected to disc electrophoresis.

Moving boundary electrophoresis. The experiments were performed at +0.8°C in the 11 ml cell of a Beckman/Spinco Model H electrophoresis-diffusion instrument. Before electrophoresis the samples were dialyzed against the appropriate buffers for 36 h at +4°C.

Peroxidase assays. In the preparation of LPO from individual cows the eluent fractions of peroxidase from the CM-cellulose and the Sephadex G-75 chromatography were continuously followed by the addition of 100-μl aliquots to a mixture containing 7×10^{-3} M guaiacol and 1.5×10^{-4} M hydrogen peroxide. If no colour was developed within 3 min, the fraction was regarded as containing no peroxidase and was discarded.

Disc electrophoresis. Disc electrophoresis in polyacrylamide gel was carried out in different buffer systems. The 2,6-lutidine-glycine/KOH-glycine buffer system (the "6.6-system") of Ornstein and Davis [10] was modified for the separation of LPO. Electrophoresis in this system was found to reflect the true heterogeneity in contrast to the fractionation obtained in both the β-alanine-acetic acid/KOH-acetic acid buffer system (the "4.3-system"), described by Reisfeld *et al.*,[11] and in a modification of this system.

Stock solutions for the modified "6.6-system". (A) KOH-glycine buffer (pH 8.3): KOH 1 M, 8 ml; glycine, 19 g; *N,N,N',N'*-tetramethylethylenediamine, 75 μl; water to make 100 ml. (B) KOH-glycine buffer (pH 10.3): KOH 1 M, 48 ml; glycine, 4.8 g; water to make 100 ml. (C) Monomer solution: Acrylamide, 30 g; *N,N'*-methylenebisacrylamide, 0.8 g; water to make 100 ml. (D) Ammonium persulfate solution: Ammonium persulfate, 0.4 g; water to make 100 ml. (E) Tray buffer (pH 8.3): Glycine, 13.7 g; 2,6-lutidine, 38.2 ml; water to make 1000 ml.

Preparation of lower gel (pH 8.3): Glass tubes (0.5×7 cm) were filled with 0.9—1.0 ml of a deaerated mixture of A, C, and D (6:1:1 v/v). Water was carefully layered on top of the monomer solution. The gel polymerized within 30 min.

Preparation of upper gel (pH 10.3): Buffer B and water were carefully mixed (1:7 v/v) and Sephadex G-200 powder was added to form a thick slurry.[12] A layer (approximately 0.2 cm) of the Sephadex slurry was placed on top of the polyacrylamide gel.

Preparation of sample gel (pH 10.3): Sephadex G-200 powder was added to a mixture (1:7 v/v) of buffer B and LPO, dialyzed against 0.06 M KOH-acetic acid buffer, pH 6.7. The thick slurry was carefully layered on top of the upper gel. The amount of sample gel applied was adjusted to fit the actual protein concentration.

Electrophoresis was performed at room temperature in an apparatus according to Ornstein and Davis.[10] The working tray buffer was obtained from buffer E and water (3:7 v/v). An unregulated power supply was used and the current was maintained at 2.5—3.0 mA. Only one gel was run at a time. The enzyme was allowed to migrate in the lower gel for 20—30 min.

The subfractions of lower mobility migrated as sharper zones if, before the experiment, the gels were "electrolyzed" in the electrophoresis apparatus for 1—2 h at 8—10 mA. KOH-glycine buffer, 1.9 M and pH 8.3 (lower gel buffer) was then used as a tray buffer. Probably this treatment gives a more even distribution of the buffer capacity in the gel and therefore the slower zones are not blurred in passing from the upper gel into the lower gel.

Electrophoresis in the β-alanine-acetic acid/KOH-acetic acid system ("4.3-system") was carried out in 7.5 % gel either according to Reisfeld *et al.*[11] with the modification of Broome [12] or according to the following empirical modification of this system.

Stock solution for the modified "4.3-system". (A′) KOH-acetic acid buffer (pH 5.4) KOH 1 M, 48 ml; glacial acetic acid, 5 ml; *N,N,N',N'*-tetramethylethylenediamine, 2 ml; water to make 100 ml. (B′) KOH-acetic acid buffer (pH 6.7): KOH 1 M, 48 ml; glacial acetic acid, 2.80 ml; water to make 100 ml. (C′) Monomer solution: Acrylamide, 30 g; *N,N'*-methylenebisacrylamide, 0.8. (D′) Ammonium persulfate solution: Ammonium persulfate, 0.07 g; water to make 100 ml. (E′) Tray buffer (pH 4.5): β-alanine, 31.2 g; glacial acetic acid, 8.0 ml; water to make 1000 ml.

Preparation of lower gel (pH 5.4). A′, C′, D′, and water (1:8:16:7 v/v) were carefully mixed and deaerated. The glass tubes were filled as described above for the "6.6-system".

Preparation of upper gel (pH 6.7). Buffer B′ and water mixed (1:7 v/v) and the upper gel was made by the addition of Sephadex G-200 powder as described above for the "6.6-system".

Preparation of sample gel (pH 6.7). Sephadex G-200 powder was added to a solution of LPO, dialyzed against buffer B′ and water (1:7 v/v). The sample gel was then handled as described above for the "6.6-system".

Electrophoresis. The equipment described for the "6.6-system" was used. Buffer E′ and water were mixed (1:9 v/v) to form the tray buffer. The runs were performed at room temperature at 4 mA and were finished in about 30 min. "Electrolyzed" gels were obtained as described above for the "6.6-system" with the exception that buffer A′ and water (1:31) was used as tray buffer. For subsequent introduction of catalyst(s) into the gel, the gel was run in the same buffer mixture containing the appropriate catalyst(s). The concentration of ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine was then the same as in the original lower gel.

Localization and identification of LPO zones in the polyacrylamide gel. The zones of LPO could in general be localized visually. Permanent staining was obtained by 1 % amidoschwartz in 7 % acetic acid according to Ornstein and Davis.[10] Staining for peroxidase activity by guaiacol and hydrogen peroxide was not useful as the coloured reaction product tends to spread. The subfractions were identified by cutting out the zones and analyzing them by disc electrophoresis in the presence of known fractions obtained by isoelectric focusing.

Quantitative determination of the LPO zones. Electrophoresis was carried out in glass tubes (0.9×7 cm) at a current of 8–10 mA. Before use, the gels were "electrolyzed" at 8–10 mA for 2 h as described above for the "6.6-system". The LPO zones, localized by eye, were cut out from the gel with a razor blade and the gel sections were placed in small test tubes and minced by means of a spatula. Phosphate buffer, 0.1 M and pH 7.0, was added to give a final volume of 1.0 ml. The gel was extracted in this way for 48 h and the supernatant was filtered through glass wool. The concentration of LPO in the supernatant was determined in a Beckman DU spectrophotometer at the Soret band, 412 mμ.

EXPERIMENTS AND RESULTS

Disc electrophoresis ("6.6-system"), isoelectric focusing, and moving boundary electrophoresis. Disc electrophoresis in the 2,6-lutidine-glycine/KOH-glycine system separates LPO into six subfractions corresponding to zones, LPO 1, LPO 2_I, LPO 2_{II}, LPO 3, LPO 4, and LPO 5, obtained by isoelectric focusing of the enzyme.[6] The electrophoretic pattern is very typical and resembles in this part the one that is seen in the focusing column. Earlier, LPO 2_I and LPO 2_{II} were not resolved by disc electrophoresis [7] but in this system they can now be separated as two close zones. In addition, disc electrophoresis reveals another four minor bands with peroxidase activity on the cathodic side of the group of six subfractions (Fig. 1). These four bands repeat the mutual positions of LPO 1, LPO 2_I, LPO 2_{II}, and LPO 3. By isoelectric focusing of the same preparation these four bands are not seen,[6] though they are present in the disc electrophoretic pattern. However, at an early stage of the focusing process a small amount of coloured precipitate is observed. The precipitate probably corresponds to the cathodial subfractions seen in disc electrophoresis. The relative amounts of the cathodial subfractions vary in different preparations and in one preparation none of them was observed. This inconsistency, however, could not be correlated to the time of the year. Moreover, the proportion of the four fractions can be increased, *e.g.* by dialyzing the peroxidase against water for several days at +4°C or by incubation of LPO in 0.08 M glycine buffer, pH 10.3, at room temperature for two days.

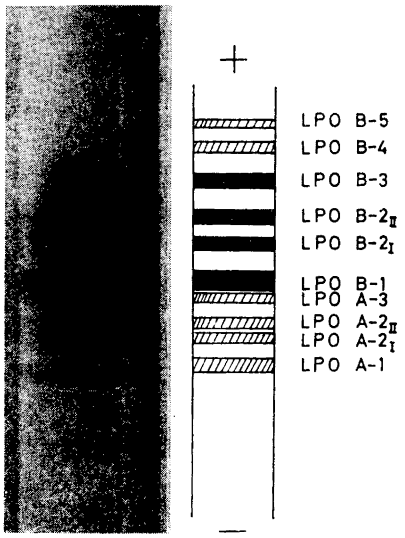


Fig. 1. The microheterogeneity of LPO, prepared from pooled milk, as revealed by disc electrophoresis in polyacrylamide. The pattern shown to the left is a photography of an unstained gel which is made more clear in the drawing to the right. Gels stained with amidoschwartz are not suitable for photographic reproduction as the stained zones tend to flow into each other at that high protein concentration necessary to visualize all zones.

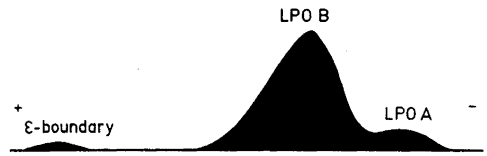


Fig. 2. Moving boundary electrophoresis of LPO, approximately 10 mg/ml; acetate buffer, pH (20°)=5.00, $I=0.1$, $t=435$ min. Descending boundary, cathode towards the right.

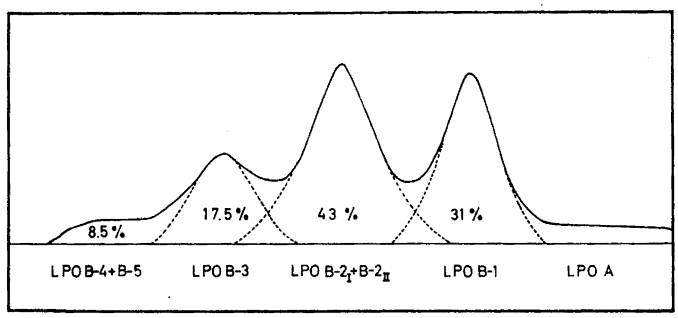


Fig. 3. Moving boundary electrophoresis of LPO, approximately 10 mg/ml; phosphate buffer, pH (20°)=7.75, $I=0.1$, $t=1075$ min. Descending boundary, cathode towards the right. The enzyme preparation was an aliquot of that used in the experiment illustrated in Fig. 2 and in the quantitation of the subfractions of the LPO B-group by disc electrophoresis (Table 1). Areas were analyzed by planimetry. The identities of the peaks were assumed to be those denoted in the figure.

The heterogeneity of LPO was originally demonstrated by Polis and Shmukler [2] by moving boundary electrophoresis in acetate buffer, $\mu=0.1$, pH 5.0. LPO was separated into two main components, LPO A and LPO B, with mobilities equal to 3.94 and 2.85 10^{-5} cm^2 V^{-1} sec^{-1}, respectively. In our hands, electrophoresis at the same conditions yielded a minor component, mobility=3.86, and a major component, mobility=2.70 10^{-5} cm^2 V^{-1} sec^{-1} (Fig. 2). From the mobility determinations it is obvious that these latter components represent LPO A and LPO B, respectively. Disc electrophoretic analysis of the boundary front in the ascending limb, *i.e.* the peak with a mobility=3.86 10^{-5} cm^2 V^{-1} sec^{-1}, showed that this front was mainly composed of the minor basic peroxidase bands described above. Thus the bulk of LPO A, as named by Polis and Shmukler,[2] is made up of the four cathodial subfractions, and LPO B of LPO 1—LPO 5. This is further substantiated by the heterogeneity pattern revealed by moving boundary electrophoresis in phosphate buffer, $\mu=0.1$, pH 7.75 (Fig. 3). In the electrophoresis pattern obtained in the phosphate buffer there is seen a flat tailing fraction on the cathodical side of the main peaks. With great probability, this tailing material constitutes the subfractions of the LPO A-group and the main peaks are the partially separated subfractions of the LPO B-group. However, by moving boundary electrophoresis it is not possible to separate completely the LPO A- and the LPO B-groups.

Convertibility of the LPO subfractions. For convenience, the subfractions of the LPO B-group are henceforth called LPO B-1, LPO B-2$_{\text{I}}$, LPO B-2$_{\text{II}}$, LPO B-3, LPO B-4, and LPO B-5 (previously named LPO 1—LPO 5) [6,7] and likewise the subfractions of the A-group are called LPO A-1, LPO A-2$_{\text{I}}$, LPO A-2$_{\text{II}}$, and LPO A-3. The use of suffixes for LPO A-2$_{\text{I}}$ and LPO A-2$_{\text{II}}$ in analogy with their use in the B-group is quite relevant, as each subfraction of the A-group could be formed from the B-group as shown by the following experiments. Homogeneous subfractions of the B-group were obtained by chromatography on DEAE-Sephadex (see below) and incubated in 0.1 M phosphate buffer, pH 7.0, for several days at room temperature. Disc electrophoretic analysis of the incubated subfractions demonstrated in each case a major band, with a mobility identical to that of the original subfraction, and a small zone with higher mobility. A mixture of the incubated LPO B-1—LPO B-3 showed by electrophoresis the typical pattern of the four main zones of the LPO B-group in addition to four minor zones moving ahead of the B-group. Heterogeneity analysis of this mixture, run together with an unresolved preparation of LPO known to contain the four fractions of the A-group, still did not reveal more than four zones in front of the LPO B-group. Thus, the subfractions formed during the incubation, most probably correspond to LPO A-1, LPO A-2$_{\text{I}}$, LPO A-2$_{\text{II}}$, and LPO A-3. Conversion between the LPO B-group and the LPO A-group was also obtained by dialysis against distilled water in the cold for several days or by incubation in 0.08 M glycine-KOH buffer, pH 10.3, for 48 h at room temperature.

In an earlier report [7] the conversion within the LPO B-group was described. This study has now been repeated and extended as the former LPO 2 now is known to be composed of two subfractions, LPO B-2$_{\text{I}}$ and LPO B-2$_{\text{II}}$. As in the previous study, disc electrophoresis in the "6.6-system" was used

for the heterogeneity analysis. Homogeneous LPO B-1—LPO B-3, obtained by chromatography, were dialysed against 0.01 M Tris—HCl buffer, pH 9.0. Glycine-KOH buffer, 0.64 M and pH 10.3, was added to each subfraction to make the protein solutions 0.08 M with respect to the glycine-KOH buffer. Solid Sephadex G-200 was added to give a slurry, part of which immediately was used as sample gel in the disc electrophoresis in order to check the homogeneity of each subfraction. The rest of the slurry was left for 48 h at room temperature and again analyzed by disc electrophoresis. The zones that were found were identified by electrophoresis in the presence of known homogeneous subfractions. Thus, LPO B-1 was the origin of two new zones with mobilities identical with LPO B-2_{I} and LPO B-2_{II}, LPO B-2_{I} of one new zone identical with LPO B-2_{II}, and LPO B-2_{II} of one new zone identical with LPO B-3. In all experiments minor zones of the LPO A-group were formed. The convertibility of LPO B-3 and LPO B-4 was not investigated because of the lack of homogeneous preparations of LPO B-4 and LPO B-5, and because of difficulties in getting sharp zones of these less basic subfractions. Neither the conversion of the LPO B-group, to yield the components of the LPO A-group, nor the conversion within the LPO B-group was found to be reversible at the conditions used.

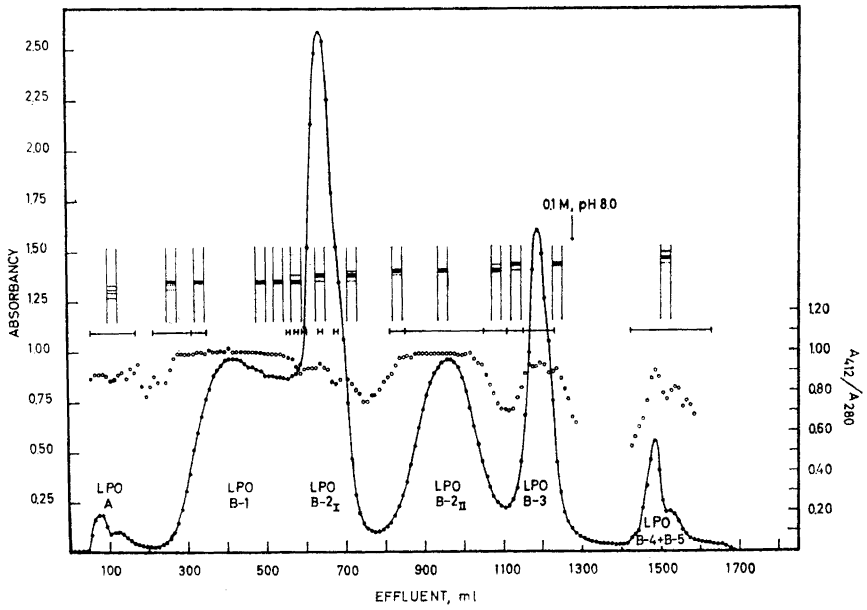


Fig. 4. Chromatography of LPO on a column of DEAE-Sephadex, 2.2 × 22 cm. Approximately 325 mg of enzyme, $A_{412}/A_{280}=0.87$, in 0.01 M Tris-HCl buffer, pH 9.04, was applied to the column, equilibrated with the same buffer. The elution was started by 0.01 M Tris-HCl buffer, pH 8.00. The arrow indicates the buffer change. Fractions of 5 ml were collected at a flow rate of 1.5 ml/cm²/h. The horizontal lines indicate fractions or pooled fractions which were subjected to disc electrophoresis. The results of the electrophoreses are shown in the figure. A_{280}: ●; A_{412}/A_{280}: ○.

Ion-exchange chromatography. The first demonstration of more than two molecular forms of LPO was made by Carlström [5] by means of ion-exchange chromatography on DEAE-Sephadex. As this method offered possibilities of getting homogeneous subfractions in preparative amounts, the fractions thus obtained were analyzed by disc electrophoresis. As seen from Fig. 4 the result is a confirmation of the heterogeneity patterns of LPO as obtained by other methods. The amount of subcomponents of the LPO A-group was comparatively small in this experiment. By rechromatography it was possible to obtain homogeneous subfractions of the LPO B-group. No effort was made to resolve chromatographically the LPO A-group and separate completely LPO B-4 and LPO B-5. The mean ratio, A_{412}/A_{280}, was 1.00 for LPO B-1.

Morrison and Hultquist [4] have described an inconsistent separation of LPO into two fractions by chromatography on Amberlite CG-50. However, the separation was not always seen. A major tailing fraction was eluted by 0.25 M ammonium acetate and a minor fraction by 0.5 M ammonium acetate. This chromatography was repeated according to the description of Morrison and Hultquist [4] in order to investigate the relationship between the fractionation thus obtained, and that reported in the present paper. The LPO preparation used was rich in the subfractions of the LPO A-group. By disc electrophoretic analysis it was found that the long tailing fraction eluted by 0.25 M ammonium acetate was composed of all the subfractions of both the LPO A and LPO B-groups. As expected the more acidic subfractions were quantitatively better represented in the first portion of this fraction. The second fraction, obtained by elution by 0.5 M ammonium acetate contained, however, only LPO A-1 and LPO A-2_I.

Heterogeneity analysis of LPO from individual cows. In order to investigate the heterogeneity of LPO in the native milk, enriched peroxidase from individual cows was analysed. Without losses of enzyme during the enrichment there were great difficulties in obtaining preparations of LPO that were useful for disc electrophoretic analysis. Disturbance of the process in disc electrophoresis which concentrates the enzyme into a narrow starting zone in the sample gel and upper gel was readily seen. This phenomenon was probably due to a relatively high quantity of acidic contaminating proteins, which force LPO, a more basic protein which normally moves anodically at the pH prevailing in the sample gel and upper gel, to continue its anodic migration into the upper tray buffer. Thus, group separation of whole milk on Sephadex G-100 or Sephadex G-200 and subsequent concentration of the peroxidase active fraction by ultrafiltration was not a successful way of preparing LPO for disc electrophoretic analysis. Attempts to combine this method with chromatography on DEAE-cellulose according to Groves [13] yielded a preparation of LPO which could be analyzed by disc electrophoresis. However, all of the LPO applied to the DEAE-cellulose column was not eluted by the 0.005 M phosphate buffer, pH 8.2, used by Groves, and the preparation could not be regarded as representing the true composition of the LPO subfractions in milk.

In the procedure finally adopted and described under "Materials and methods", the gel filtration step was essential in order to obtain a narrow

Table 1. The relative distribution of the subfractions of the LPO B-group in LPO prepared from pooled milk [5] (I) and in a preparation from a single cow (II). The analysis of the moving boundary electrophoresis pattern was performed according to Fig. 3. LPO B-4 in the "single cow preparation" was not determined due to difficulties in localizing this zone in the unstained polyacrylamide gel.

Subfraction	Percental composition of the LPO B-group		
	I analyzed by moving boundary electrophoresis	I analyzed by disc electrophoresis	II analyzed by disc electrophoresis
LPO B-1	31	33	50
LPO B-2_I	43	19	32
LPO B-2_{II}		24	11
LPO B-3	17.5	17	7
LPO B-4	8.5	5	not determined
LPO B-5		2	0

starting zone of the enzyme by disc electrophoresis. LPO from five milk samples from different cows was analyzed and in no case was there found a homogeneous peroxidase. The concentration of LPO in different milk samples varied widely. In those milk samples where the concentration of LPO was high and enough material could be applied to disc electrophoresis, at least four and probably five zones of the LPO B-groups were observed. These zones correspond to LPO B-1, LPO B-2_I, LPO B-2_{II}, LPO B-3, and (LPO B-4 ?). The fifth zone was seen in the beginning of the run but was later getting so blurred that it could not be localized and identified. Subfractions of the A-group were not seen in all samples. Table 1 shows the quantitative distributions of the subfractions of the B-group in LPO, as prepared by the ordinary procedure from pooled milk and as enriched from milk from a single cow.

Disc electrophoresis in the "4.3-system". The disc electrophoresis system of Reisfeld *et al.*,[11] β-alanine-acetic acid/KOH-acetic acid (the "4.3-system"), theoretically seemed to be a very suitable system for the fractionation of the LPO subfractions. By using the original system [11] with the modification of Broome,[12] a separation was obtained which was not quite reproducible and also resulted in blurred zones. In order to improve the separation, the system was empirically modified as described in the methodological part of this paper. Thus, four sharp zones were obtained, all containing peroxidase activity. However, no correlation was found between these zones and the subfractions isolated by chromatography on DEAE-Sephadex or by isoelectric focusing; different homogeneous subfractions gave the same pattern upon disc electrophoresis in this buffer system. The most cathodical zone from the polyacrylamide gel was cut out and re-run on disc electrophoresis and again four zones were obtained. By handling the second zone, number two from the cathode, in the same way three zones were seen including the original one. The third

and fourth zone gave analogous results. By other means, in this disc electrophoresis system there is a conversion from cathodic to anodic zones during the process of electrophoresis. Furthermore, there is no relation between these zones and the subfractions of LPO that were found by other methods. However, after "electrolytic" removal of *N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate, disc electrophoresis of LPO revealed no heterogeneity. Also by the addition of either *N,N,N',N'*-tetramethylethylenediamine or ammonium persulfate to the tray buffer used for electrolysis, a gel was obtained that upon electrophoresis showed only one zone of LPO. If both the polymerization catalysts were added simultaneously, however, disc electrophoresis again produced four zones. The apparent heterogeneity of a homogeneous subfractions (LPO B-1) was also observed in the original system of Reisfeld *et al.*[11]

DISCUSSION

The original demonstration of a heterogeneity of LPO was made by Polis and Shmukler.[2] These authors separated LPO into two peaks, LPO A and LPO B, by moving boundary electrophoresis. It is now shown that the six subfractions of LPO, earlier described by Carlström and Vesterberg,[6] have their counterparts in the zones obtained by disc electrophoresis in polyacrylamide, by ion-exchange chromatography on DEAE-Sephadex, and by moving boundary electrophoresis. However, in addition to these six subfractions another four minor peroxidases are seen in the polyacrylamide column. These latter zones together correspond to LPO A, and the former six subfractions to LPO B of Polis and Shmukler.[2] It was not possible to determine unequivocally by disc electrophoretic analysis if the slower of the four minor subfractions migrates in the LPO A or LPO B peak by moving boundary electrophoresis, employing the conditions of Polis and Shmukler.[2] However, these subfractions can be assigned to the LPO A-group since each of the four most cathodical subfractions can be formed by conversion from LPO B-1, LPO B-2_{I}, LPO B-2_{II}, and LPO B-3, respectively.

In addition to LPO A-1, LPO A-2_{I}, LPO A-2_{II}, and LPO A-3 there might exist another two fractions of the LPO A-group that correspond to LPO B-4 and LPO B-5, but then these hypothetical LPO A-4 and LPO A-5 probably occur in such a low concentration that they escape detection. Furthermore, as the heterogeneity pattern of the LPO A-group is a partial repetition of the LPO B-group pattern, somewhat displaced against the cathode, the supposed existence of LPO A-4 and LPO A-5 could be hidden by the LPO B zones.

The analysis of peroxidase from individual cows showed that the enzyme most likely exists in multiple forms in the native milk. The probability is very small that the heterogeneity within the LPO B-group is due to conversion of LPO B-1 during the procedure of enrichment. Pure LPO B-1 subjected to this procedure remained as a single component upon subsequent disc electrophoretic analysis. However, there is a discrepancy between the relative distributions of the subfractions in the B-group of LPO, as prepared by the ordinary procedure from pooled milk and as enriched from individual cows (Table 1). In the author's opinion this difference does not necessarily reflect

a conversion of LPO B-1 during the former procedure. The stage where a conversion would take place in the preparative procedure, is at the elution of LPO from the Amberlite CG-50 column by 1.0 M dipotassium hydrogen phosphate. However, incubation of LPO B-1 for 48 h in this phosphate solution in the cold does not cause any conversion within the B-group. It is more likely that the use of Amberlite CG-50 and CM-cellulose selects for the more acidic subfractions, as the last tailing fraction of LPO eluted from these ion-exchange materials are discarded because of a low ratio of peroxidase to impurities. The variation within the B-group might also be due to individual differences within different cows. Thus, the preparations from individual cows should reflect a true distribution of the subfractions in the native milk as there is no loss of peroxidase activity during the enrichment. However, the inconsistent presence of the LPO A-group in some milk samples could be due to an occasional conversion from the B-group during the course of enrichment. On the other hand, it does not seem to be likely that transformations of the subfractions of LPO occur during the preparative manipulations that are performed in a short time at +4°C and at mild conditions, but not in the udder of the cow, where the milk is stored for many hours above 35°C, and moreover, in the presence of hydrolytic enzymes of different kinds. Because of the convertibility and the multiplicity of the subfractions it is also not very probable that each subfraction is separately synthesized on the basis of genetic factors. Therefore, the extensive heterogeneity of LPO should probably be regarded as a reflection of the first stages of the catabolic breakdown *in vivo* of LPO B-1, which possibly originates from myeloperoxidase.[16]

The present results on the heterogeneity of LPO clearly demonstrates the necessity of the use of a rapid analytical technique, requiring small amounts of material, in order to follow the effect of preparative and other manipulations of the protein. Disc electrophoresis in the "6.6-system" has turned out to be an excellent method for this purpose. However, all methods do not reflect the true composition of LPO. Thus, it was found that disc electrophoresis in the modified "4.3-system" as well as the system of Reisfeld *et al.*,[11] which is the origin of the modified "4.3-system", separates LPO into zones that are artefacts formed during the procedure itself. This is in agreement with the results of Rombauts *et al.*[17] The production of artefacts in these systems was observed in 1964 by the present author and therefore they have not been used for the analysis of the heterogeneity of LPO.[6,7] Thus, the statement of Rombauts *et al.*[17] that "It would appear that the heterogeneity that Carlström (1966) observed in disc electrophoresis is the result of modification of lactoperoxidase by the procedure itself" is not justified. The formation of artefacts in the system described above, is a phenomenon that might be due to the peroxidase activity of the enzyme. By the enzymatic assay of LPO in the presence of hydrogen peroxide and guaiacol, the enzyme is destroyed during the course of the reaction, probably because of formation of free radicals.[6] An analogous process can possibly occur in the polyacrylamide gel, as one of the catalysts, sodium persulfate, is a potential source of hydrogen peroxide and *N,N,N',N'*-tetramethylethylenediamine is a hydrogen donor that would be able to act as a substrate for LPO. This explanation is supported by the fact that both catalysts had to be present simultaneously, otherwise

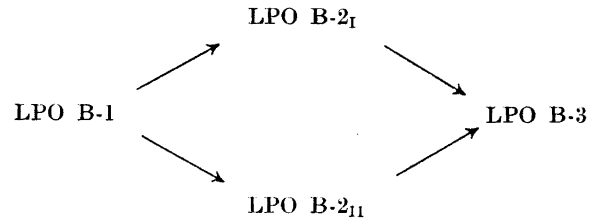
the artefacts were not seen. The role of persulfate in this problem has been discussed by Brewer [18] and Fantes and Furminger.[19]

Disc electrophoresis in the "6.6-system", isoelectric focusing, moving boundary electrophoresis, and chromatography on DEAE-Sephadex give somewhat different but consistent pictures of the heterogeneity of LPO. Disc electrophoresis shows the most complete pattern. Previously LPO B-2_{I} and LPO B-2_{II} were not resolved in the polyacrylamide gel.[7] The discrepancy with the present results is probably due to improved technical handling of the method. By comparing the relative distribution of the subfractions as obtained by disc electrophoresis with that obtained by moving boundary electrophoresis at pH 7.75 the relation of the two patterns is obvious (Table 1). The difference in mobility of LPO B-2_{I} and LPO B-2_{II} is apparently not big enough to resolve these subfractions in the moving boundary method, not even after 1075 min. Also the components of the LPO A-group are not resolved into discrete peaks at the conditions used. By isoelectric focusing these latter subfractions are not found as focused bands at a time when the zones of the LPO B-group have reached their equilibrium positions in the pH gradient. However, some proteins are known, *e.g.* horse liver alcohol dehydrogenase and catalase,[20] that precipitate during the procedure of isoelectric focusing. Therefore it is reasonable to believe that the coloured precipitate, which is observed in the focusing column, originates from the subfractions of the LPO A-group. In general this group has shown a tendency to precipitate much more easily than the B-group peroxidases. Thus the LPO A-group escaped detection by disc electrophoresis for a long time, probably because of too high ohmic heating during the runs.

The observation of multiple forms of peroxidase in milk from individual cows is in agreement with our earlier results [5] but contradictory to the conclusion made by Swope *et al.*[21] In their study the enzyme was analysed by starch gel electrophoresis. Probably, the power of resolution of this method, at the conditions that were used, was not sufficient to resolve the subfractions of LPO. Groves [13] found two fractions with peroxidase activity in milk from single cows. However, this author used the disc electrophoretic system of Reisfeld *et al.*,[11] the system that in our hands produces the artefactual heterogeneity of LPO. In 1957 Morrison *et al.*[3] confirmed the results of Polis and Shmukler [2] as they found that LPO was composed of two components. Several years later Morrison and Hultquist [4] reported that this heterogeneity was inconsistent in different preparations and recently Rombauts, Schroeder and Morrison [17] could not find any evidence of more than one component in LPO. The latter authors base their statement upon immunological studies, reverse "salting-out" chromatography, sedimentation equilibrium studies, and disc electrophoresis in the system of Reisfeld *et al.*[11] They summarily deal with previous reports by the present author on the heterogeneity of LPO [5–7] by the conclusion that "this extensive heterogeneity was apparent only after repeated chromatography or by means of isoelectric focusing". None of the homogeneity criteria used by Rombauts *et al.* excludes the existence of the heterogeneity of LPO as described in the present and previous [5–7] papers.

In previous publications [6,7] it was suggested that the conversion within the LPO B-group was due to hydrolysis of labile amide groups of glutamine

and/or asparagine residues in the protein. As LPO B-2_I and LPO B-2_{II} differ very little in isoelectric point compared to the difference between the other subfractions of the B-group it was also proposed that LPO B-2_I and LPO B-2_{II} were chemical isomers,[6] *i.e.* the conversion of LPO B-1 to LPO B-3 was following the route:



However, the results presented in this paper excludes this mechanism, and instead favours a consecutive process:

$$\text{LPO B-1} \longrightarrow \text{LPO B-2}_I \longrightarrow \text{LPO B-2}_{II} \longrightarrow \text{LPO B-3}$$

The conversion of the major subfractions of the LPO B-group to the corresponding subfractions of the LPO A-group makes the hypothesis very attractive that the same chemical change is responsible for each of the transformations from the B-group to the A-group. Recently it was found by the present author [14] that LPO B-1—LPO B-3 have identical amino acid and carbohydrate compositions. No significant difference in amino acid composition between the LPO B- and LPO A-groups was found, but there is a definite difference in the carbohydrate content. The LPO A-group contains less mannose, glucosamine, and galactosamine. This is a difference that very well could constitute one heterosaccharide residue. Very often the heterosaccharide in glycoproteins is bound to the protein moiety by a β-aspartylglycosylamine linkage which involves the amide group of asparagine.[15] If this is the case for LPO, there might be a close and interesting analogy between the deamidation theory for the conversion within the LPO B-group and the loss of carbohydrates in the conversion of the B-group into the A-group. Thus, hydrolysis of an amide group of an asparagine residue bound to a heterosaccharide would cause a conversion of the type: LPO B-1$\longrightarrow$LPO A-1, while the hydrolysis of an asparagine residue which is not linked to any carbohydrate moiety will result in a conversion within the LPO B-group.

From the present knowledge about the heterogeneity pattern of LPO and the routes of conversion between the subfractions, it is obvious that LPO B-1 can act as a parent peroxidase for all the other subfractions, probably also including LPO B-4 and LPO B-5. There is a possibility that LPO A-2_I, LPO A-2_{II}, and LPO A-3 can be formed from LPO B-1 by two alternative routes. Either, (1) LPO B-1 is first transformed to LPO B-2_I, LPO B-2_{II}, and LPO B-3, hypothetically by deamidation, and then these subfractions lose a heterosaccharide residue to give LPO A-2_I, LPO A-2_{II}, and LPO A-3, respectively, or, (2) LPO B-1 is primarily converted to LPO A-1 which then gives rise to LPO A-2_I, LPO A-2_{II}, and LPO A-3.

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