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# Iron-Binding Fragments from the *N*-Terminal and *C*-Terminal Regions of Human Lactoferrin

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Digestion of lactoferrin with pepsin at pH 3.0 gave an iron-binding half-molecule that represents the *C*-terminal part of the native protein. Tryptic or chymotryptic digestion of 30%-iron-saturated lactoferrin yielded the *N*- and *C*-terminal half molecules, which could be separated by DEAE-Sephadex chromatography. The *N*- and *C*-terminal fragments did not show any immunological cross-reaction. The carbohydrate of lactoferrin was distributed equally between the two fragments.

Lactoferrin, a glycoprotein present in the external secretions and neutrophilic leucocytes of mammals, resembles transferrin in its ability to bind two atoms of $Fe^{3+}$ with the incorporation of two molecules of bicarbonate. Both proteins have a single-chain structure of mol. wt. 76000 and are clearly related to one another (Johansson, 1960; Montreuil *et al.*, 1960; Blanc & Isliker, 1961; Masson & Heremans, 1968; Querinjean *et al.*, 1971; Bluard-Deconinck *et al.*, 1974). However, lactoferrin and transferrin differ in several ways. They show no immunological cross-reaction unless denatured (P. L. Masson, B. Teuwissen, J. Van Snick, T. K. Roberts, J. F. Heremans & R. C. Topp, unpublished work). Further, the affinity of lactoferrin for iron is largely retained at pH values below 4.0, whereas transferrin completely releases its iron under such conditions (Johansson, 1960; Montreuil *et al.*, 1960; Blanc & Isliker, 1961). Receptors for lactoferrin and transferrin reside on different cells, since transferrin combines with reticulocyte membranes (Fielding & Speyer, 1974) and lactoferrin combines with the membranes of macrophages and lymphocytes (Van Snick & Masson, 1976).

Williams (1974, 1975) has shown that iron-binding fragments representing the *N*-terminal and *C*-terminal halves of hen ovotransferrin can be prepared by limited proteolysis. Brock *et al.* (1976) have also obtained two iron-binding fragments from bovine transferrin by trypsin digestion. Line *et al.* (1976) reported that an iron-containing fragment of mol.wt. 39500 can be obtained by peptic digestion of human lactoferrin.

These observations are of interest in various fields. Comparison of fragments from transferrin and lactoferrin might provide useful information on the evolution of these iron-binding proteins, and cleavage into two fragments is clearly a useful step in the determination of the amino acid sequence of the proteins. The availability of the separate fragments should also facilitate physical studies of the iron-binding sites and biological investigations of the interaction of these proteins with cellular membranes.

In the present work we show that the peptic fragment of human lactoferrin represents the *C*-terminal half of the protein, and that two iron-binding fragments can be obtained from lactoferrin by proteolysis with trypsin or chymotrypsin.

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## Materials and Methods

### *Materials*

Pepsin, trypsin (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated), chymotrypsin and carboxypeptidases A and B were obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Lactoferrin was isolated from human milk as described previously (Querinjean *et al.*, 1971).

### *Methods*

Iron was removed from lactoferrin or its proteolytic fragments by treatment with 0.1M-citrate and 4M-urea at room temperature (20°C) for 30min. The Fe–citrate complex was then retained on a column (5.0cm×2.0cm) of Dowex 1 (X2; 200–400 mesh; Bio-Rad, Richmond, CA, U.S.A.). The protein was dialysed against water and freeze-dried.

The antiserum used for the immunological study was raised in a goat by intradermal injections of intact Fe-saturated lactoferrin with complete Freund's

adjuvant. The specificity of the antiserum was tested by immunoelectrophoresis of human milk and plasma. The antiserum was devoid of precipitating activities against milk or serum proteins other than lactoferrin.

*Reduction and alkylation.* Proteins were reduced by 1% 2-mercaptoethanol in 3M-Tris/HCl buffer, pH8.6, containing 8M-urea and 0.01% EDTA at room temperature, under $N_2$, and alkylated after 4h with an excess of iodoacetic acid.

*Molecular weights.* The molecular weights of lactoferrin and its proteolytic fragments were measured by electrophoresis in 6% polyacrylamide gel containing 1% sodium dodecyl sulphate by the procedure of Shapiro *et al.* (1967), with bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), pepsin (mol.wt. 36000), trypsin (mol.wt. 23000) and lysozyme (mol.wt. 14000) as standards. The electrophoreses were run in the presence or absence of 1% 2-mercaptoethanol.

Sedimentation equilibrium was carried out in the 12mm synthetic-boundary cell of the Spinco model E ultracentrifuge, by using Rayleigh interferometric optics. Equilibrium was checked before termination of the run by comparison of two plates exposed at intervals of 12h.

Lactoferrin was studied in a high-speed equilibrium run (Chervenka, 1970) by using 50$\mu$l of protein solution (1.9mg/ml) plus 15$\mu$l of FC 43 oil (Beckman, Palo Alto, CA, U.S.A.) in one sector, and 440$\mu$l of 0.067M-potassium phosphate buffer (pH7.4) plus 10$\mu$l of FC 43 oil in the second sector. The protein was centrifuged at 15315rev./min at 20°C.

The peptic fragment of lactoferrin was studied in a low-speed equilibrium run, by using 100$\mu$l of the protein solution (3.5mg/ml) plus 15$\mu$l of FC 43 oil in one sector and 115$\mu$l of the above phosphate buffer plus 10$\mu$l of FC 43 oil in the second sector. The runs were performed at 7883rev./min at 24°C.

The partial specific volumes of lactoferrin and its proteolytic fragments were determined with an Anton Paar digital densitometer (Kratky *et al.*, 1969).

*Neuraminidase treatment.* Protein samples were incubated with neuraminidase (10 units/100$\mu$g of protein where 1 unit of enzyme converts 1$\mu$mol of substrate/min) (Behringwerke A.G., Marburg/Lahn, Germany) for 24–48h in 0.05M-sodium acetate buffer, pH5.5, containing 0.15M-NaCl and 0.009M-$CaCl_2$.

*Amino acid analysis.* Protein samples were hydrolysed with 5.7M-HCl at 105°C in sealed evacuated tubes for periods of 24, 48 and 72h. Cysteine was determined as cysteic acid after performic acid oxidation (Hirs, 1967) and tryptophan was determined after hydrolysis in the presence of toluene-*p*-sulphonic acid and tryptamine (Liu, 1972). Analyses were performed on an LKB 3201 amino acid analyser. Each determination was performed twice.

*Carbohydrate analysis.* The analysis of carbohydrate in lactoferrin and in the proteolytic fragments was carried out by Professor J. Clamp, Department of Medicine, University of Bristol, by g.l.c. (Clamp, 1974).

*End-group analyses.* *N*-Terminal residues of protein samples were identified by dansylation in the presence of sodium dodecyl sulphate (Gray, 1972). Performic acid-oxidized protein (0.5mg) was dissolved in 100$\mu$l of 1% (w/v) sodium dodecyl sulphate solution. To this was added 200$\mu$l of *N*-ethylmorpholine and 300$\mu$l of dansyl chloride (25mg/ml of acetone). After reaction for 1h at 37°C the protein was dried and hydrolysed with 5.6M-HCl for 4h at 110°C. Dansyl-amino acids were identified by t.l.c. *C*-Terminal residues were identified by digestion with carboxypeptidases A and B in 0.1M-*N*-ethylmorpholine acetate buffer, pH8.5. An enzyme/substrate ratio of 1:100 (w/w) was used. Samples were removed at different times and applied to the amino acid analyser.

*Peptide 'maps'.* Samples dissolved in 5% (v/v) formic acid were digested by pepsin and analysed by high-voltage paper electrophoresis at pH6.5 for 30min as described by Williams (1974).

## Results

### *Preparation of peptic fragment of lactoferrin*

Fe-saturated lactoferrin was incubated with pepsin for 3h at 37°C in 0.9% NaCl maintained at pH3.0, 3.5, or 4.0 in an autotitrator (Radiometer, Copenhagen, Denmark) at enzyme/substrate ratios of 1:10 or 1:50 (w/w). After neutralization with $NH_4HCO_3$, the digest was concentrated and passed through a Sephadex G-100 column (80cm×3cm) in 0.1M-$NH_4HCO_3$. Four major fractions were distinguished (Fig. 1*a*) by $A_{280}$ and the orcinol reaction for carbohydrate (Winzler, 1955). Carbohydrate was found in the first three fractions. Fractions I and II had a pink colour, indicating that they contained iron. After concentration, they were analysed by electrophoresis in starch gel at pH7.6 (Poulik, 1957) and agarose gel at pH8.6 (Bodman, 1960). In both gels fraction I had the same mobility and the same diffuse pattern as intact lactoferrin, whereas fraction II was resolved into two major bands migrating slightly faster than native lactoferrin (Fig. 2). Fractions III and IV, which represent low-molecular-weight glycopeptides and non-carbohydrate peptides respectively, were not studied further.

After treatment of fraction II with neuraminidase, the faster-running bands diminished and the slowest band increased (Fig. 2), which suggests that different sialic acid contents were responsible for the electrophoretic heterogeneity of fraction II.

As shown below, the material constituting fraction II represented the *C*-terminal fragment of lactoferrin.

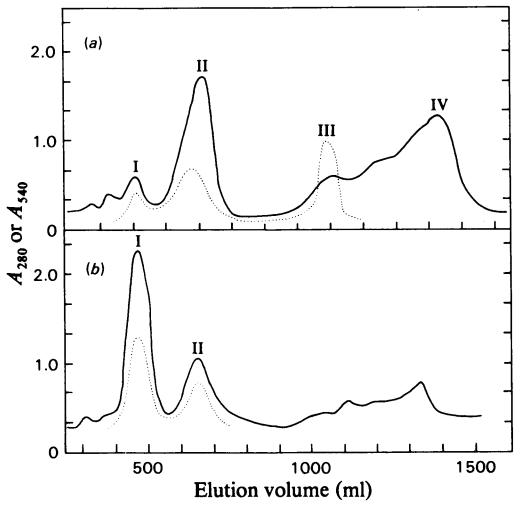


Fig. 1. *Gel filtration of (a) pepsin digest of iron-saturated lactoferrin and (b) chymotrypsin digest of* 30%*-iron-saturated lactoferrin, on Sephadex G-100*
Column effluent was monitored by $A_{280}$ (——) and by the orcinol reaction for carbohydrate by using $A_{540}$ (· · · ·). I, II, III and IV indicate the fractions referred to in the text.

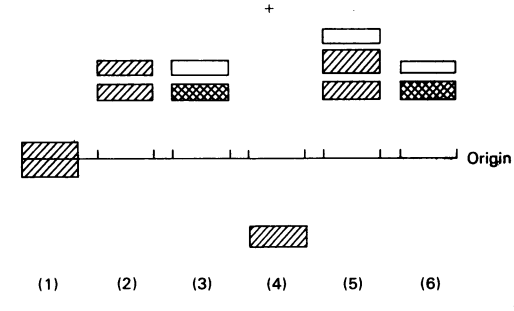


Fig. 2. *Diagram of agarose-gel electrophoresis at pH* 8.6
The numbered samples are as follows. (1) Lactoferrin. (2) Fraction II from pepsin digest of iron-saturated lactoferrin. (3) The same material after incubation with neuraminidase. (4) The basic component from fraction II from trypsin digest of partially iron-saturated lactoferrin. The basic component was separated from acidic material by chromatography on DEAE-Sephadex. (5) The acidic components from the same fraction. (6) The acidic components after incubation with neuraminidase. The stained protein bands are represented by rectangles, the intensity of staining being represented by the amount of cross-hatching.

The greatest yield of peptic fragment was obtained when the protein was digested at pH 3.0 at an enzyme/substrate ratio of 1:10. As judged by $A_{280}$, 30% of the starting material was recovered as the fragment, 55% as Fe-free peptides and 15% as undigested Fe-saturated lactoferrin with an $A_{280}/A_{470}$ ratio of 22.

*Preparation of chymotryptic and tryptic fragments of lactoferrin*

Digestion of partially iron-saturated lactoferrin with chymotrypsin and trypsin was performed because this method was earlier found to yield the *N*-terminal fragment of hen ovotransferrin (Williams, 1974). Samples of lactoferrin 30% saturated with ferric nitrilotriacetate were incubated at 37°C in 0.1M-$NaHCO_3$ with chymotrypsin or trypsin at an enzyme/substrate ratio of 1:30. After 3h, the quantity of enzyme was doubled, and the incubation was continued for another 6h, but no digestion was observed, even when the experiment was repeated in the presence of 2M-urea. On the basis of a previous study on the denaturation of lactoferrin (Teuwissen *et al.*, 1974), solid urea was then added to 30%-iron-saturated lactoferrin to give a urea concentration of 5.4M, a concentration that does not cause significant release of iron. To avoid denaturation of the enzyme, the lactoferrin solution in 5.4M-urea was added dropwise, with constant stirring, to chymotrypsin or trypsin in 3vol. of 0.1M-$NaHCO_3$. The digestion conditions described above were then applied.

Partial digestion was obtained as indicated by the resolution of the material into two main fractions by chromatography on Sephadex G-100 in 0.01M-$NH_4HCO_3$ (Fig. 1*b*). Fraction I contained undigested lactoferrin, as shown by agarose-gel electrophoresis at pH 8.6. Under these electrophoretic conditions, fraction II, which like fraction I was pink and contained carbohydrate, was resolved into one basic band migrating towards the cathode and three acidic bands migrating towards the anode (Fig. 2). As shown below, the acidic material corresponded to the *C*-terminal fragment of lactoferrin, whereas the basic band represented the *N*-terminal fragment. After treatment with neuraminidase, the heterogeneity of the *C*-terminal fragment was decreased, with an increase in the concentration of the slower-moving components (as shown in Fig. 2).

The yield of fragments after chymotrypsin digestion was 4% of the starting material. Undigested Fe-saturated lactoferrin represented 27%, and the residual 69% corresponded to small iron-free peptides. Trypsin digestion gave similar results.

The *C*-terminal fragments were separated by chromatography on a DEAE-Sephadex A-50 column (10cm×2cm) equilibrated with 0.02M-Tris/HCl buffer, pH 8.5. The *N*-terminal fragments were not

adsorbed under these conditions, whereas the *C*-terminal fragments were adsorbed by the ion-exchanger and were then eluted with 0.1M-Tris/HCl buffer, pH8.5, containing 0.13M-NaCl. In general, as judged by $A_{280}$, fraction II from chymotrypsin or trypsin digestions contained about 25% of *N*-terminal and 75% of *C*-terminal fragment.

*Characterization of the N- and C-terminal fragments of lactoferrin*

*Molecular weight.* The molecular weights of the various fragments were estimated by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate and by analytical ultracentrifugation (Table 1). A value of about 40000 was obtained for the molecular weight of most fragments. When electrophoresis was performed in the presence of 1% 2-mercaptoethanol, no change in molecular size was observed. Reduced and alkylated peptic fragment was eluted from Sephadex G-100 in the same position as the non-reduced fragment.

*N- and C-Terminal amino acids.* The *N*-terminal residue of the peptic fragments was alanine. The *N*-terminus of the basic fragment from chymotryptic and tryptic digests was glycine, as also is that of lactoferrin itself (Bluard-Deconinck *et al.*, 1974; Bezkorovainy & Grohlich, 1974).

Carboxypeptidase A, acting alone, yielded no free amino acids from lactoferrin or from the peptic fragment. A mixture of carboxypeptidases A and B released arginine, leucine, lysine and phenylalanine from both the intact protein and the peptic fragment. Although the data do not allow the *C*-terminal amino acid sequence to be deduced, it is likely from the failure of carboxypeptidase A to release free amino acids that in both cases arginine is *C*-terminal and that the peptic fragment represents the *C*-terminal half of the protein.

*Peptide maps.* High-voltage electrophoreses at pH 6.5 of the peptides obtained by pepsin digestion of the peptic fragment and the acidic chymotryptic fragments gave similar patterns, whereas the basic chymotryptic fragment gave a different pattern.

*Amino acid and carbohydrate compositions.* The peptic and acidic chymotryptic fragments had similar amino acid compositions (Table 2), but significant differences were observed between the basic chymotryptic fragment and the *C*-terminal fragments, notably in the values for glutamic acid, proline, isoleucine, phenylalanine, arginine and tryptophan. The sum of the compositions of the *C*-terminal and *N*-terminal fragments agreed reasonably with the composition of lactoferrin, except for threonine, serine and histidine. The sum of the carbohydrate compositions of the *C*-terminal peptic and *N*-terminal chymotryptic fragments was similar to that of lactoferrin (Table 2).

*Iron-binding capacity.* All the fragments were pink, suggesting the presence of iron complexes. The peptic fragment was treated with citric acid and urea to remove iron as described under 'Methods' and then reloaded with increasing amounts of ferric nitrilotriacetate (Fig. 3). The progressive saturation of the protein was monitored at 245nm (Teuwissen *et al.*, 1972), and maximal absorbance was obtained with 0.8g-atom of iron/mol of fragment.

To test the stability of the iron complexes at low pH, *N*-terminal and *C*-terminal fragments and whole lactoferrin were dissolved in 0.1M-$NaHCO_3$ at a concentration of 5mg/ml. The pH of the solutions and the $A_{470}$ were measured 5min after additions of HCl. In the three preparations, the absorbances decreased in similar fashion as the pH was lowered from 3.5 to 2.3 (Fig. 4).

*Immunological studies.* The fragments were studied by double diffusion in agarose gel with a goat antiserum raised against whole lactoferrin (Fig. 5). The *C*-terminal chymotryptic fragment was contaminated by the *N*-terminal chymotryptic fragment and, by radial immunodiffusion (Mancini *et al.*, 1965), it was estimated that the degree of contamination was about

Table 1. *Molecular weights of lactoferrin and its fragments*

Abbreviation: ND, not determined.

| Material | Mol.wt. By sodium dodecyl sulphate/polyacrylamide-gel electrophoresis | Mol.wt. By ultracentrifugation* |
|---|---|---|
| Peptic fragments | 40750 | 41950 |
| Acidic tryptic fragments | 36100 | ND |
| Acidic chymotryptic fragments | 40000 | ND |
| Basic tryptic fragment | 39800 | ND |
| Basic chymotryptic fragment | 46250 | ND |
| Intact lactoferrin | 79430 | 75500 |
| Sum of acidic and basic tryptic fragments | 75900† | |
| Sum of acidic and basic chymotryptic fragments | 86250† | |

* A partial specific volume of 0.715 was obtained for intact lactoferrin, and 0.709 for the peptic fragment.

† Calculated by addition of the values obtained for each fragment by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Table 2. *Number of amino acid and carbohydrate residues in lactoferrin and in the peptic and chymotryptic fragments*
The values for the fragments are averages of two determinations. The values for lactoferrin are taken from Querinjean *et al.* (1971). A molecular weight of 41350 was used for the peptic fragment. Values of 40000 and 46250, respectively, were used for the acidic and basic chymotryptic fragments. Abbreviation: ND, not determined.

| | Peptic fragment | Acidic chymotryptic fragment | Basic chymotryptic fragment | Sum of acidic and basic chymotryptic fragments | Sum of peptic fragment and basic chymotryptic fragments | Lactoferrin |
|---|---|---|---|---|---|---|
| Cysteic acid | 14 | 12 | 14 | 26 | 28 | 26 |
| Aspartic acid | 37 | 28 | 28 | 56 | 65 | 65 |
| Threonine | 15 | 14 | 12 | 26 | 27 | 33 |
| Serine | 24 | 21 | 30 | 51 | 55 | 46 |
| Glutamic acid | 27 | 24 | 36 | 60 | 63 | 69 |
| Proline | 14 | 11 | 23 | 33 | 36 | 33 |
| Glycine | 26 | 21 | 28 | 49 | 54 | 49 |
| Alanine | 28 | 26 | 30 | 56 | 59 | 56 |
| Valine | 24 | 21 | 24 | 45 | 48 | 34 |
| Methionine | 2 | 4 | 2 | 6 | 4 | 6 |
| Isoleucine | 4 | 5 | 12 | 17 | 16 | 16 |
| Leucine | 22 | 31 | 24 | 55 | 46 | 54 |
| Tyrosine | 9 | 10 | 10 | 20 | 19 | 20 |
| Phenylalanine | 9 | 12 | 20 | 31 | 29 | 28 |
| Lysine | 19 | 20 | 22 | 42 | 41 | 39 |
| Histidine | 4 | 4 | 4 | 8 | 8 | 10 |
| Arginine | 13 | 19 | 27 | 46 | 40 | 38 |
| Tryptophan | 3 | 4 | 9 | 13 | 12 | 11 |
| Mannose | 2 | ND | 2 | | 4 | 6 |
| Fucose | 1 | ND | 1 | | 2 | 2 |
| Galactose | 2 | ND | 2 | | 4 | 4 |
| *N*-Acetylglucosamine | 3 | ND | 2 | | 5 | 8 |
| Neuraminic acid | 1 | ND | 2 | | 3 | 3 |

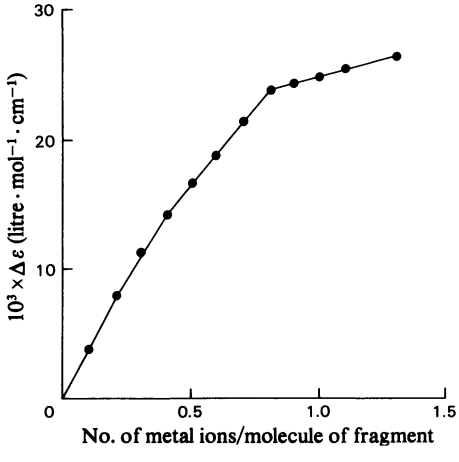


Fig. 3. *Titration of the peptic fragment of lactoferrin with ferric nitrilotriacetate followed by difference spectrometry at 245 nm*
For details see Results section of the text.

5%. The peptic, tryptic and chymotryptic *C*-terminal fragments were immunologically identical, as were the tryptic and chymotryptic *N*-terminal fragments, but no common antigenic determinants were detected between the *N*- and *C*-fragments. With some preparations no spur was observed between the precipitin lines given by the mixture of *C*- and *N*-fragments and whole lactoferrin, suggesting that no antigenic determinant had been lost during the digestion, but in other cases a small spur was seen; and in these cases loss of one or more antigenic determinants is likely.

## Discussion

In the present work, we have shown that proteolytic digestion of lactoferrin yields fragments capable of binding one atom of iron and representing about half the lactoferrin molecule. The fragment obtained by peptic digestion is likely to represent the *C*-terminal half of lactoferrin, because of the results of carboxypeptidase digestion. From the manner of preparation and the results of *N*-terminal end-group and amino acid analysis, this fragment appears to be very similar to that described by Line *et al.* (1976).

Two fragments that were separable on the basis of their charges were obtained by tryptic or chymotryptic digestion. The *C*-terminal fragments provided by these digestions were identified by comparison of their peptide 'maps' and their immunological proper-

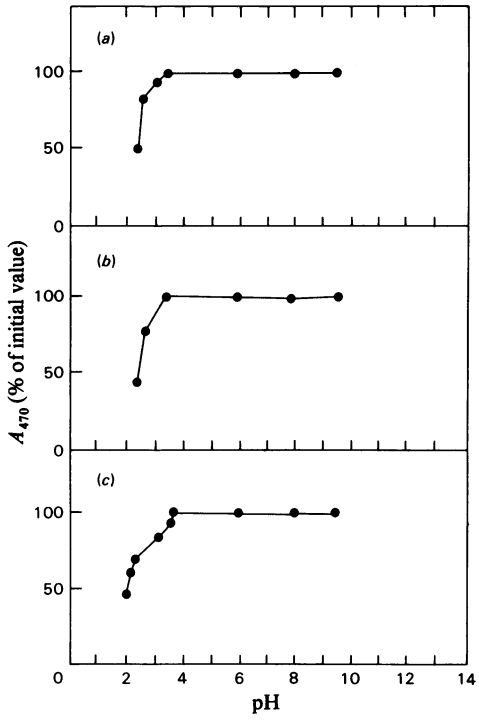


Fig. 4. *Effect of lowering pH on the stability of the iron complexes of lactoferrin and its half-molecules*
Dissociation of the complex was monitored by the decrease in $A_{470}$. The ordinate represents $A_{470}$ as a percentage of the initial value. (*a*) *N*-Terminal tryptic fragment. (*b*) *C*-Terminal tryptic fragment. (*c*) Intact lactoferrin.

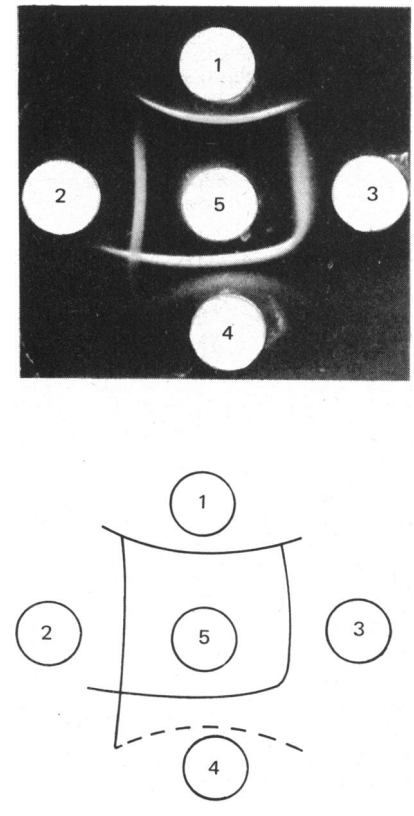


Fig. 5. *Immunological precipitin reactions between anti-lactoferrin serum and the proteolytic fragments of lactoferrin*
Well 1 contained intact lactoferrin, well 2 the *N*-terminal chymotryptic fragment, well 3 the peptic. fragment, well 4 the *C*-terminal chymotryptic fragment and well 5 goat anti-lactoferrin antiserum. A line drawing and a photograph of the reactions are shown.

ties with those of the peptic fragment. The other fragments obtained by tryptic or chymotryptic digestions were assigned to the *N*-terminal part of lactoferrin because of the identity of their *N*-terminal amino acids with that of whole lactoferrin. These *N*-terminal fragments are basic and are immunologically identical, but they show no immunological cross-reaction with the *C*-terminal fragments whatever the mode of preparation of the latter. The *N*-terminal and *C*-terminal fragments of hen ovotransferrin have also been found to be immunologically distinct (Williams, 1975).

The protein–metal complexes of lactoferrin and transferrin dissociate at low pH. There is no gross difference between the *N*-terminal and *C*-terminal half-molecules of lactoferrin in the rate of iron loss. This is in contrast with the results obtained with hen ovotransferrin, where the *N*-terminal half-molecule loses iron much faster than the *C*-terminal half-molecule (Williams, 1975).

It is likely that the *N*-terminal half of iron-saturated lactoferrin is more susceptible than the *C*-terminal half to digestion by pepsin. A similar differential susceptibility has been reported for hen ovotransferrin, where subtilisin preferentially attacks the *N*-terminal half of the molecule (Williams, 1975). It is not yet known whether the *N*- and *C*-terminal fragments produced by digestion of 30%-iron-saturated lactoferrin with trypsin or chymotrypsin arise from the digestion of the iron-free halves of monoferric lactoferrin or from cleavage of diferric lactoferrin complexes, and further work on this is required.

As mentioned in the introduction, iron-binding half-molecules have now been isolated from a variety

of transferrins. This is consistent with the idea that gene doubling leading to internal homology has occurred in the evolutionary history of these proteins, with subsequent differentiation between the two half-molecules.

Compelling evidence in favour of this is provided by the studies by MacGillivray & Brew (1975) and MacGillivray *et al.* (1977), who have shown a large number of amino acid identities in the sequences of the *N*-terminal and *C*-terminal halves of human transferrin. Since similarities of amino acid sequence between lactoferrin and transferrin have been found (Bluard-Deconinck *et al.*, 1974; Jolles *et al.*, 1976), it is likely that internal homology will prove to be a general characteristic of this class of protein.

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