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Human Tear and Human Milk Lysozymes*

Jacqueline Jollès and Pierre Jollès

ABSTRACT: Lysozymes from human tears and human milk were obtained in a chromatographically pure state by ion-exchange chromatography on Amberlite CG-50. The amino acid compositions were determined and the heat stability of these enzymes was studied. The tryptic digests of human milk and hen egg white lysozymes were compared with the help of a peptide AutoAnalyzer.

The present data and others previously obtained indicate the probable identity of the lysozyme of all human tissues and secretions; it has approximately 3.5 times the activity of hen egg white lysozyme.

Many studies have been devoted since 1893 to the bactericidal action of tears. They were reviewed by Thompson (1941) and more recently at the 2nd International Symposium on Lysozyme (1961). One of the most important observations was due to Fleming (1922) who indicated that tears, in common with many other agents, have a remarkable lytic activity against many saprophytes. He obtained evidence that the substance(s) concerned in the lysis was an enzyme and he gave it the name lysozyme (EC 3.2.1.17). Though many experiments concerning the biological activity were performed with the lysozyme from tears (Fleming, 1929; Thompson, 1941), its complete purification has not been achieved and no studies have been made with the enzyme obtained in a chromatographically pure state. The milk of most species contains lysozyme and human milk is a comparatively rich source of the enzyme (Bordet and Bordet, 1924; Rosenthal and Lieberman, 1931). Human milk lysozyme, the purification of which was described by Jollès and Jollès (1961), was the first entirely purified human lysozyme. This paper deals with the chromatographic purification of human tear and milk lysozymes, their amino acid composition, and some of their properties. Both lysozymes will then be compared to previously purified human lysozymes (Jollès, 1964; Jollès *et al.*, 1965; Charlemagne and Jollès, 1966) and some of the peptides obtained by tryptic digestion to those of hen egg white lysozyme.

Materials and Methods

Tears were always obtained from the same person. Because a hypersecretion causes a diminution of the concentration of lysozyme, only the first tears (0.05 ml) were collected with a pipet and frozen until used, when a sample of 1 ml was available. *Milk samples*, obtained from several mothers (1 l.), were stored at 2° during 1 night and immediately used for the preparation of lysozyme. In a few cases, the samples were kept frozen for up to 1 week before use. *Hen egg white lysozyme* was obtained from Armour, Kankakee, Ill. (lot 638040).

The lytic activity was determined by observing spectrophotometrically the extent of lysis of a suspension of dead cells of *Micrococcus lysodeikticus* (Jollès *et al.*, 1965). *The protein* content was determined qualitatively at 280 mμ with a spectrophotometer Beckman DU or with the ninhydrin reagent of Hirs *et al.* (1956) after alkaline hydrolysis. *The quantitative amino acid compositions* were determined after total hydrolysis (6 N HCl, 110°, 18 and 48 hr, sealed tubes under vacuum) with a Technicon AutoAnalyzer. Cystine was estimated as cysteic acid after performic acid oxidation

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and tryptophan according to the procedure of Spies and Chambers (1949).

Tryptic Hydrolysis. Lysozymes were reduced by mercaptoethanol or thioglycolic acid and carboxymethylated under the conditions described by Jollès *et al.* (1963). They were then digested with 2% by weight of trypsin (EC 3.4.4.4) (Worthington) at 37°, pH 7.8 (trimethylamine), 5 hr. Trypsin was pretreated during 16 hr by 0.06 N HCl at 37°. The peptides obtained after tryptic hydrolysis were separated and characterized with a Technicon AutoAnalyzer. The sample (3 or 6 mg) was applied at pH 3.2 to a 90 × 0.6 cm jacketed column of cation-exchange resin (Chromo-Beads P®, Technicon) previously equilibrated with a 0.2 N sodium citrate buffer, pH 3.2. The column was then developed at 37° with a flow rate of 0.42 ml/min. For the elution gradient a nine-chambered Varigrad (90 ml/chamber) was used (0.2 (pH 3.2)–2.0 N (pH 6.8)).

The peptides obtained by tryptic digestion were also separated by thin layer electrophoresis chromatography on cellulose MN-300; electrophoresis: 2°, 2 hr, 55 v/cm, pH 6.2, pyridine–water–acetic acid (250:2225:25, v/v); chromatography: 6 hr, 1-butanol–pyridine–water–acetic acid (340:200:125:70, v/v).

Heat Stability. Heating was carried out for 0.5 or 1 min at 100° at a variety of pH values (0.05 M sodium acetate, sodium phosphate, or Tris·HCl buffers) and the lysozyme activity was calculated as a per cent of the initial activity before heating.

Chromatographic Purification Procedure. Jollès *et al.* (1962) described a purification procedure which included the following four main steps: (1) preparation of a lysozyme-rich material; (2) chromatography on CM-cellulose; (3) filtration on Sephadex G-25; and (4) ion-exchange chromatography on Amberlite CG-50.

Human tear lysozyme was directly submitted to the ion-exchange chromatography. For human milk lysozyme, the general method was used with an important modification. The lysozyme-rich extract (first step) was obtained by adjusting the pH of the milk to 4.6 with 30% acetic acid and by eliminating the precipitate (mainly casein) by centrifugation. The lysozyme in solution at pH 4.6 was adsorbed on Amberlite CG-50 previously buffered with a 0.2 N phosphate buffer of pH 6.5. The resin was washed with water and with the 0.2 N phosphate buffer of pH 6.5; the lysozyme was then eluted with a 0.8 M phosphate buffer of pH 6.5 and desalted on Sephadex G-25 with water as eluent. As no significant purification was observed during the chromatography on CM-cellulose (second step), a treatment with $(NH_4)_2SO_4$ was introduced. To the lysozyme-rich extract dissolved in water, $(NH_4)_2SO_4$ was added until the percentage of the salt in the final solution was 40%. After centrifugation, the supernatant contained the lysozyme and was desalted on Sephadex G-25 with water as eluent (third step). The fourth step was the ion-exchange chromatography on Amberlite CG-50; this latter was absolutely necessary as inactive proteins were still eluted in the void volume; it was verified by amino acid analyses that in these conditions the composition of the main active peak remained constant from one preparation to another. The active material obtained by omitting the ion-exchange chromatography (material after treatment with $(NH_4)_2SO_4$, followed eventually by a filtration on Sephadex G-75), had different amino acid compositions from one preparation to another. Finally, the ion-exchange chromatography was usually followed by a filtration on Sephadex G-75 with 0.1 N acetic acid as eluent.

Determination of the Molecular Weight by Filtration on Sephadex G-100. The gel filtration behavior of a series of proteins (ribonuclease, hen and duck egg white lysozymes, trypsin, and kallikrein) was compared on Sephadex G-100 (95 × 2 cm) with a 0.05 M Tris·HCl buffer of pH 8 plus 0.1 M KCl as eluent. As described by Whitaker (1963) for simple proteins, the effluent volume can be plotted *vs.* the log of the molecular weight of these proteins.

Results

Lysozyme Content. The lysozyme content of the tears used during these experiments was 7 mg (±20%)/ml; the content of the milk samples was more variable (100–500 mg/l.). All these quantities were calculated from the enzymic activities, assuming that the enzymes had the same activity as hen egg white lysozyme. The higher specific activity of the human enzymes was not taken here into account.

Purification, Chromatographic Behavior, and Specific Activity. The tears (1 ml) were chromatographed without further preparation with a 0.2 N phosphate buffer of pH 6.98 on a column of Amberlite CG-50 (21 × 0.9 cm) previously equilibrated with this same buffer. The proteins inactive against suspensions of *M. lysodeikticus* were recovered in the void volume. The second peak retained by Amberlite (see Figure 1) contained the whole lytic activity (yield 70%). Its specific activity was constant; if it is set to 1 for hen egg white lysozyme, it is, in the present case, 3 ± 0.5. The ion-exchange chromatography of human milk lysozyme on Amberlite CG-50 was done again at pH 6.98 with a 0.2 N phosphate buffer; a small active peak ($\leq$10% of the total lytic activity) was eluted before the main one, the specific activity of which remained constant (3 ± 0.5). The over-all yield starting from the milk reached here 25%.

Human tear and milk lysozymes as all previously studied human lysozymes (from saliva, placenta, normal serum and leucocytes, and serum and leucocytes of patients suffering from chronic myelogenous leukemia)[1] have the same chromatographic behavior during their ion-exchange chromatography on Amberlite CG-50 (Figure 2) and a very different one from the bird egg white lysozymes. Hen egg white lysozyme (Figure 2, upper right diagram) and duck egg white lysozyme (Jollès, 1964) are chromatographed, respectively, at pH 7.18 and 7.56; at pH 6.98, they remain adsorbed

[1] Abbreviation used: CML, chronic myelogenous leukemia.

on the resin. The slight displacement of the curves for normal leucocytes and leukemia (CML) leucocytes lysozymes is here not significant.

Quantitative Amino Acid Analyses and Molecular Weight. The amino acid compositions of human tear and milk lysozymes are quite comparable (see Table I). The results are expressed as g of residues/100 g of protein. In each case, the analyses of three different samples are in agreement and show no differences which cannot be accounted for by methodologic error. Also tabulated in Table I are the estimated minimal number of amino acid residues in both lysozymes calculated from the averaged values of the three analyses on the basis of one histidine. The minimal molecular weight calculated by this manner (14000–15000) is in fact the real one, as was also verified by the following experiments: (a) by dialysis with calibrated cellophane membranes; (b) by filtration on Sephadex G-100 (The two human lysozymes have the same behavior on Sephadex G-100 as hen egg white lysozyme (mol wt 14386)); and (c) by ultracentrifugal analyses; the sedimentation constant ($s_{20,w}$) of both, calculated to infinite dilution, was 1.9–2.0 S corresponding to a molecular weight of the order of 15.000. The ultra-

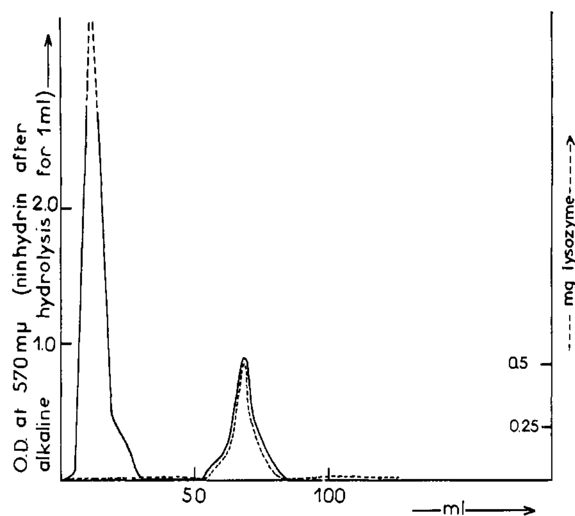

FIGURE 1: Ion-exchange chromatography on Amberlite CG-50 (21 × 0.9 cm) of human tears (1 ml), 0.2 M phosphate buffer of pH 6.98; —, optical density at 570 mμ (ninhydrin after alkaline hydrolysis); ——, milligrams of lysozyme calculated from the enzymic activity (see Results, lysozyme content).

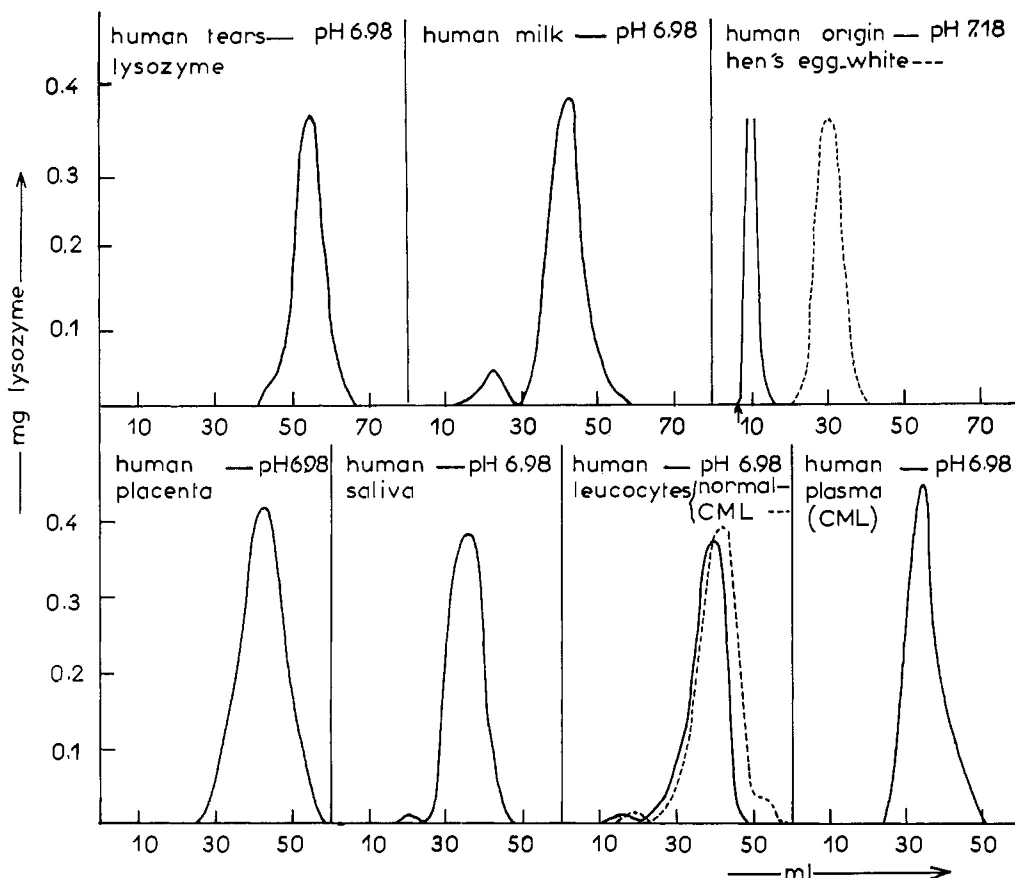

FIGURE 2: Ion-exchange chromatography on Amberlite CG-50 (15 × 1 cm, 0.2 M phosphate buffer) of seven human lysozymes (pH 6.98) and of hen egg white lysozyme (pH 7.18). Quantities calculated from the enzymic activities.

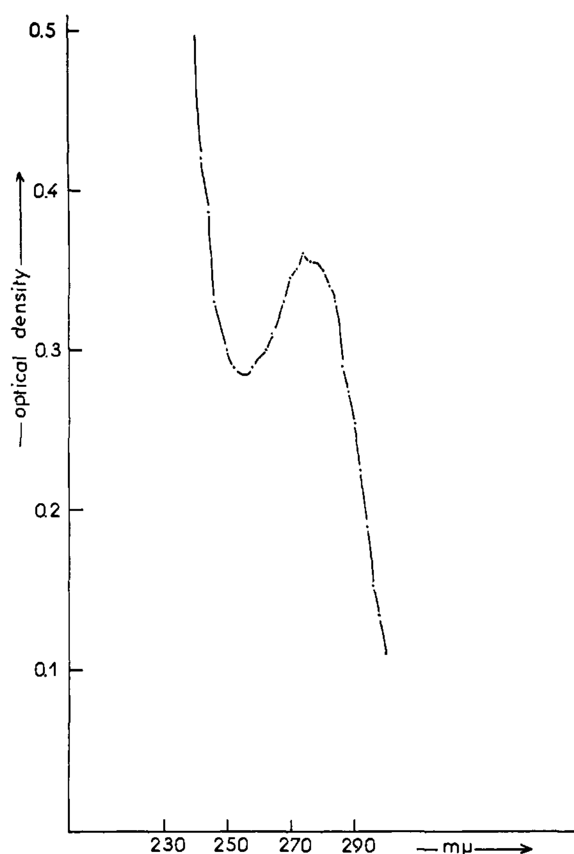

FIGURE 3: Ultraviolet spectrum of human milk lysozyme in water (1.16×10^{-5} M).

violet spectrum of human milk lysozyme is indicated in Figure 3.

Heat Stability. Figure 4 shows that the behavior of the two human lysozymes when heated during 0.5 or 1 min at 100° and at different pH is identical. Both are a little more heat sensitive after 1 min than hen egg white lysozyme.

Tryptic Hydrolysis. Like native hen egg white lysozyme native human milk lysozyme was only scarcely digested by trypsin. Therefore, the tryptic digest of the reduced carboxymethylated human milk lysozyme was analyzed on a peptide AutoAnalyzer and compared to the tryptic digest of reduced carboxymethylated egg white lysozyme. As indicated in Figure 5, the two tryptic digests seem different. This observation was confirmed by thin layer electrophoresis chromatography. In Figure 5 the tryptophan-containing peptides are missing. They were eluted from the resin with difficulty and a low yield only after 30 hr without giving real peaks. Similar observations were made during control experiments when purified tryptophan-containing peptides obtained by tryptic digestion of hen egg white lysozyme (Jollès *et al.*, 1963) were analyzed on the peptide AutoAnalyzer. Furthermore, the tryptic peptides of reduced carboxymethylated milk and saliva lysozymes were compared by thin layer electrophoresis chromatography and very similar and probably identical peptide maps were obtained.

Discussion

Amino acid analyses of all the human lysozymes

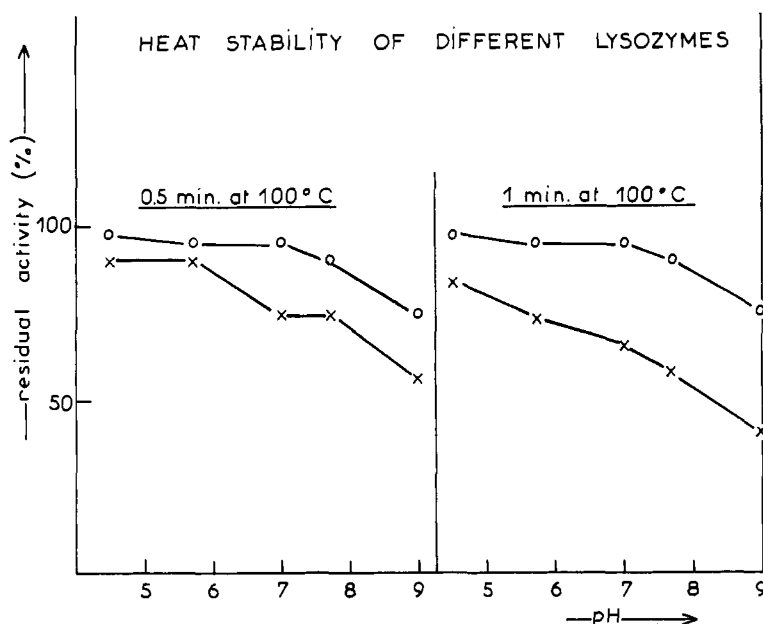

FIGURE 4: Heat stability of human tear and milk lysozymes (X—X) (different pH, 100°). Comparison with hen egg white lysozyme (O—O).

TABLE I: Amino Acid Compositions of Human Tear and Human Milk Lysozymes.[c]

Amino Acid	Human Tear Lysozyme									Human Milk Lysozyme									Data of Jollès *et al.* (1965) Estimated from Analyses of Human Salivary, Placental, and Leucocytic Lysozymes	Hen Egg White Lysozyme (Jollès *et al.*, 1963)	Duck Egg White Lysozyme (Jollès *et al.*, 1965)
	Lot 1				Lot 2		Lot 3			Lot 1				Lot 2		Lot 3					
	18-hr Hydrolysis		48-hr Hydrolysis		18-hr Hydrolysis		18-hr Hydrolysis		Nearest Integer	18-hr Hydrolysis		48-hr Hydrolysis		18-hr Hydrolysis		18-hr Hydrolysis		Nearest Integer			
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*b*	*b*	*b*	*b*
Asp	13.70	16.15	11.65	14.3	12.40	14.8	13.60	16.1	17	13.05	15.5	12.00	14.0	13.70	15.8	13.35	15.5	17	18 ± 1	21	19 ± 1
Thr	3.56	4.8	2.93	4.0	3.17	4.3	4.28	5.8	5–6	3.78	5.15	3.24	4.3	3.47	4.5	3.32	4.4	5–6	6 ± 1	7	7
Ser	3.50	5.55	2.73	4.4	4.29	6.8	4.26	6.7	6–7	3.85	6.1	3.06	4.7	3.70	5.7	3.29	5.0	6–7	6 ± 1	10	10–11
Glu	8.60	9.05	8.82	9.6	8.75	9.3	8.30	8.6	9–10	8.26	8.85	8.67	9.0	8.38	8.7	8.66	9.0	9	9 ± 1	5	5
Pro	1.98	2.75	2.29	3.3	1.50	2.35	1.69	2.35	3	1.67	2.35	1.95	2.7	1.75	2.4	1.22	1.7	3	2–3	2	2
Gly	4.93	11.7	4.86	12	4.93	11.9	4.97	11.9	12	4.68	11.3	4.95	11.6	4.33	10.2	4.17	11.0	12	12 ± 1	12	12
Ala	6.20	11.8	6.55	13	6.48	11.7	6.40	12.2	12–13	6.08	11.8	6.60	12.4	6.08	11.4	6.08	11.4	12–13	12 ± 1	12	11–12
Val	4.46	6.1	5.72	8.1	4.50	6.3	4.90	6.7	7–8	4.90	6.8	5.37	7.25	4.71	6.35	5.24	7.0	7–8	7	6	6
(Cys-)	3.92	5.2	3.04	4.2	4.28	5.7	3.82	4.95	6	4.54	6.1	3.40	4.45	4.46	5.8	4.56	6.0	6	6	8	8
Met	1.83	1.9	1.68	1.8	1.43	1.5	1.23	1.25	2	1.45	1.55	1.17	1.3	1.64	1.7	1.45	1.5	2	2	2	2
Ile	3.20	3.85	4.23	5.25	3.79	4.65	3.79	4.6	5	3.33	4.05	4.32	5.1	3.62	4.3	3.60	4.25	5	5	6	5–6
Leu	5.96	7.15	6.51	8.1	5.88	7.2	6.49	7.8	8	6.30	7.7	6.83	8.1	6.98	8.2	6.74	7.9	8	8	8	8
Tyr	5.98	5.0	5.47	4.4	5.93	5.0	5.05	4.2	5	6.42	5.45	5.23	4.3	6.00	4.9	6.49	5.3	5–6	5	3	4–5
Phe	1.50	1.4	1.79	1.7	2.06	1.95	2.35	2.15	2	2.05	1.95	1.86	1.7	1.81	1.7	1.90	1.7	2	2	3	1
Trp[d]	6.80	5.1	6.60	5.0	7.27	5.4	6.60	4.7	5	7.18	5.3	6.67	4.8	6.87	4.9	7.40	5.3	5–6	5 ± 1	6	5–6
Lys	4.11	4.35	4.66	5.1	4.10	4.45	4.66	4.9	5	3.81	4.1	4.58	4.8	4.47	4.6	4.13	4.1	5	5	6	6
His	1.01	1.0	0.98	1.0	1.01	1.0	1.02	1.0	1	0.99	1.0	1.02	1.0	1.03	1.0	1.04	1.0	1	1	1	0
Arg	13.05	11.3	12.30	11.1	12.70	11.2	13.40	11.4	11–12	13.32	11.7	13.38	11.5	13.43	11.4	13.30	11.3	11–12	11–12	11	13 ± 1
Total	94.87		91.81		94.47		96.81		124 ± 3	95.66		94.30		96.43		95.94		124 ± 3	122 ± 5	129	125 ± 5

[a] Residues (g)/100 g of protein. [b] Residues per mole calculated on the basis of one histidine (human and hen egg white) and one phenylalanine (duck egg white). [c] Comparison with other human and egg white lysozymes. [d] Characterized following the procedure of Spies and Chambers (1949).

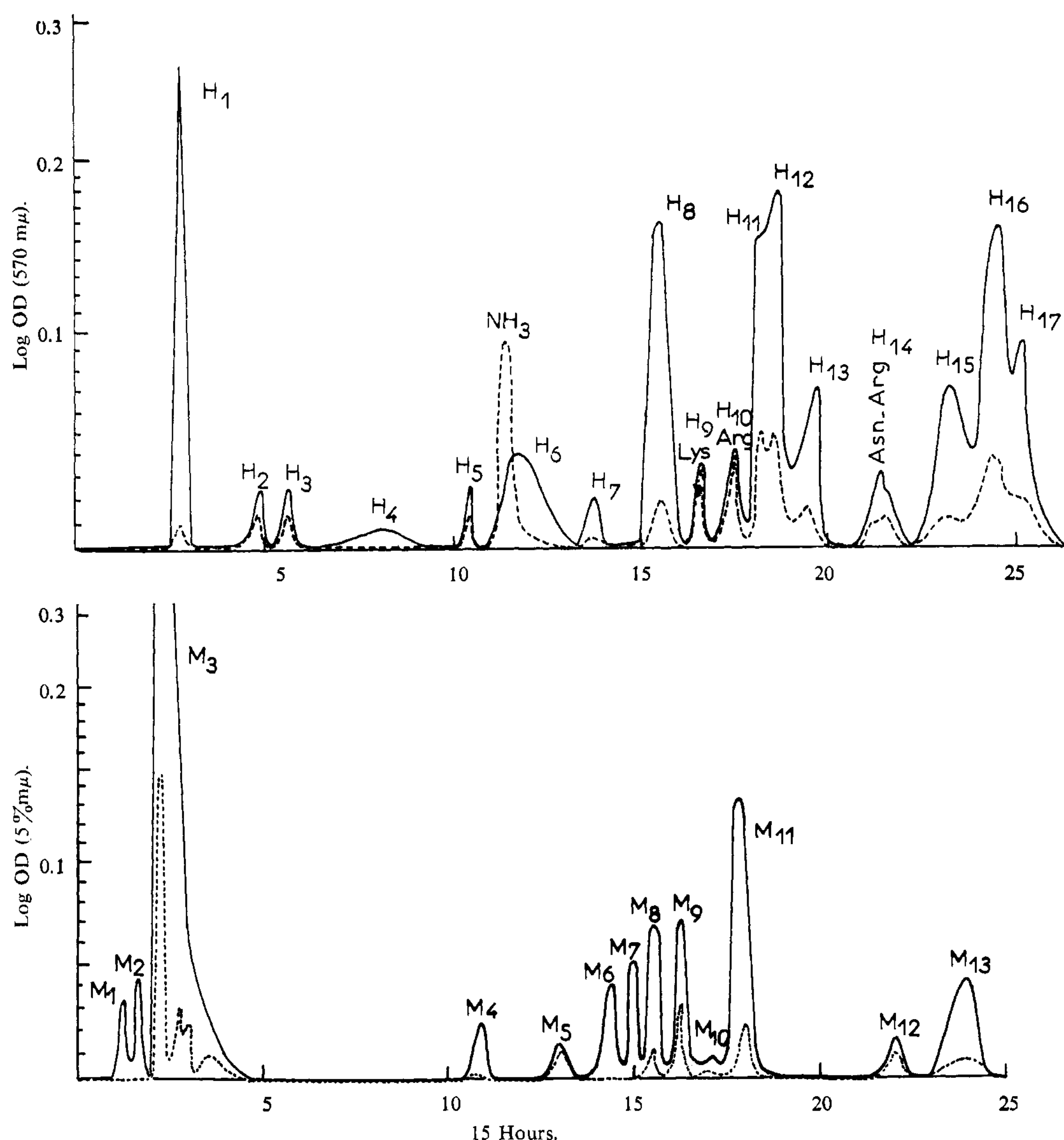

FIGURE 5: Tryptic peptides from reduced carboxymethylated human milk (bottom) and hen egg white lysozymes (top) separated with a Technicon peptide AutoAnalyzer. ——, ninhydrin after alkaline hydrolysis; – – –, ninhydrin without alkaline hydrolysis. Lys, Arg, and Asn-Arg were identified during a control experimental run only with these substances.

yielded comparable results. These data indicate the probable identity of the enzyme in all human tissues and secretions. Our analytical data are in agreement with recent results of Osserman and Lawlor (1966), who purified human serum and urinary lysozymes in monocytic and monomyelocytic leukemias and found a close correspondence with the values indicated in Table I. Furthermore, they reported some immunochemical data which suggest again the identity of the human lysozymes studied until now.

When compared with bird egg white lysozymes (Table I), these human proteins show several significant differences in their compositions and also in their structures which are under investigation. Dissimilarities were further substantiated by the failure of the antiserum to a human lysozyme to inhibit the activity of the egg enzyme (Osserman and Lawlor, 1966). Finally, if all the human lysozymes were found to have an identical chromatographic behavior, two hen lysozymes (egg white and lung) presented a different one (Jollès, 1964).

Human tear and saliva lysozymes are almost as stable as hen or duck lysozymes when heated for 1 min at 100°. They possess only three cystine residues per mole compared to four for the two bird enzymes. In a recent study on the relationship between cystine and tryptophan contents of five lysozymes and their heat stability, Jollès *et al.* (1966) indicated that a lysozyme with a cystine content lower than three residues per molecule, like goose egg white lysozyme (two residues of cystine and two residues of tryptophan per mole-

cule) was no more heat stable.

All our human lysozymes have approximately 3–3.5 times the activity of hen egg white lysozyme (at equivalent concentrations when titrated with suspensions of *M. lysodeikticus*). Comparable results were obtained by Osserman and Lawlor (1966) when similar methods were used; however, these authors found with another method (lysoplate method) that their human lysozyme has 8–12 times the activity of hen egg white lysozyme. Finally, recent results of Sharon *et al.* (1966) indicated that the mechanism of action of human tear lysozyme was very similar to that of hen egg white lysozyme.

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