Molecular and Cellular Biochemistry 63, 165–189 (1984).

REVIEW

What's new in lysozyme research?*

Always a model system, today as yesterday

Pierre Jollès and Jacqueline Jollès
Laboratory of Proteins (C.N.R.S. E.R. 102 and I.N.S.E.R.M. U-116), University of Paris V, 45 rue des Saints-Pères, F 75270 Paris Cedex 06, France

Summary

The present review is focused on the main achievements realized in the lysozyme research field since the meeting held in 1972 to commemorate the fiftieth anniversary of the discovery of this enzyme. Despite of extensive structural, physico-chemical, crystallographic, genetic, immunological and evolutionary studies devoted to lysozymes, their biological role is still not exactly known.

1. Introduction

Already more than 60 years slipped away since Fleming's discovery in 1922 of lysozyme (EC 3.2.1.17), a remarkable bacteriolytic element found in tissues and secretions (1). Lysozyme behaved as a model system for many studies; the first protein which was sequenced and contained together all the twenty usual amino acids was hen lysozyme (2, 3, 4); the first enzyme which was submitted to a complete X-ray crystallographic analysis was hen lysozyme (5); the first enzyme for which a detailed mechanism of action was proposed was again hen lysozyme (6). In the same way many evolutionary or immunological or physico-chemical studies were performed with lysozyme as a model compound. Our knowledge concerning this enzyme was summarized in a book entitled: 'Lysozyme' (7) reporting a series of papers presented at a meeting held in 1972 to commemorate the fiftieth anniversary of the discovery of this enzyme.

The present review will be focused on the main achievements realized in the lysozyme research field since ten years and will also contain developments which, at least in part, might be considered as model studies for other biochemical investigations. Despite of extensive studies devoted to lysozymes, their biological role is still not exactly known and this aspect will be discussed in the last part of the review.

* 121st communication on lysozymes.

2. Definition

Lysozymes are defined as 1,4-β-N-acetylmuramidases cleaving the glycosidic bond between the C-1 of N-acetylmuramic acid (Mur NAc) and the C-4 of N-acetylglucosamine (GlcNAc) in the bacterial peptidoglycan (6). Some lysozymes also display a more or less pronounced chitinase (EC 3.2.1.14) activity corresponding to a random hydrolysis of 1,4-β-N-acetylglucosamine linkages in chitin.

A slight esterase activity of hen lysozyme has been described (8): histidine was claimed to be involved in this activity (9) but in the authors' laboratory it was shown that histidine-less lysozymes possess also a weak and probably non-specific esterase activity (10).

3. Activity determination and purification

Despite a great number of efforts achieved in order to prepare some simple, well-defined substrates (11, 12), such as glycolchitin (13, 14) or

derivatives of N-acetyl-β-chitotetraoside (12), lysozyme activity continues to be determined turbidimetrically by measuring the decrease in absorbance of a suspension of *M. luteus* cells (15, for a review see Ref. 16). Highly sensitive radioimmunoassays (17, 18), an assay based on fluorescence polarization or fluorescence intensity utilizing a fluorescent peptidoglycan substrate (19) as well as some automated methods (20, 21, 22) have also been described. Most of the lysozymes have been prepared by classical, rather standardized purification procedures including a prepurification step followed by molecular sieving on Sephadex G-25 or G-75 and ion-exchange chromatography on a cationic resin (CM-cellulose 32). Quite recently some affinity chromatography procedures have also been described (23). Immunoadsorption affinity chromatography was used to isolate and purify human lysozyme: the immunoadsorbent was prepared by coupling sheep anti-(human leukemic lysozyme) IgG to epoxy-activated Sepharose 6B (24). Bacteriophage T4 lysozyme was purified by affinity chromatography on a C6 muropeptide-Affigel 202 column (C6 muropeptide: GlcNAc-MurNAc-L-Ala-D-Glu-meso A2pm-D-Ala) (25). The very high isoelectric pH value of lysozyme enabled the enzyme to be separated from many other proteins in a single chromatographic separation on heparin-Sepharose (26). Two other one-step chromatographic procedures using agarose columns (27) and exclusion chromatography based on isoelectric point (28) have also been reported.

4. Characterization and study of distinct types of lysozymes

Lysozymes are ubiquitous enzymes; the most intensively investigated are those from avian egg-white and there are two distinct types of enzyme from this source: hen egg-white lysozyme (chicken-type, c type) and goose egg-white lysozyme (g type). The complete primary structures are known for 18 lysozymes c: from hen (2, 3, 4), quail (4 different enzymes) (29, 30, 31), turkey (32), guinea fowl (33), duck (5 different enzymes from Kaki or Peking-duck) (34, 35, 36), pheasant (37) and chachalaca (38) egg-whites as well as from human milk (39) (or human urine, 40), baboon (41) rat (42) and cow stomach (43); 10 sequences have been established in the authors' laboratory (Fig. 1). Partial sequences are known for some more enzymes, from pigeon (44), mouse (45), tortoise (46), *Bombyx mori* (47), *Galleria mellonella* (47) and *Spodoptera littoralis* (47). Only 3 complete lysozyme g sequences have so far been determined, from black swan (48), ostrich (49) and Emden goose (50) egg-white. As our research proceeded, we demonstrated the existence of other distinct types of lysozyme which differ from the c and g types on the basis of structural, catalytic and immunological criteria (51).

4.1. *c type lysozymes for in-depth studies of molecular evolution*

The c type lysozymes have been characterized at high concentration only in two orders of birds, the Galliformes and the Anseriformes (52). We shall not come back here to the now classical studies which have been devoted to the sequence (2, 3, 4), the three-dimensional structure at 4 °C and 20 °C (5) and the mechanism of action of hen egg-white lysozyme (6). We want to restrict the discussion to recently studied c type lysozymes: these investigations were necessary for in-depth studies of molecular evolution and antigenic structure. Furthermore knowledge of the positions at which sequence variation occurs has enhanced understanding of the structural requirements for lysozyme function.

4.1.1. *Contrast between protein evolution and evolution at the organismal level: chachalaca egg-white lysozyme*

The amino acid sequence of lysozyme c from chachalaca egg-white was determined (38) (Fig. 1). Like other bird lysozymes c, that of the chachalaca has 129 amino acid residues. It differs from other avian lysozymes c by 27 to 31 amino acid substitutions as well as by being devoid of phenylalanine. Although the chachalaca is classified zoologically in the order Galliformes, which includes chickens and other pheasant-like birds, its lysozyme differs more from those of pheasant-like birds than do the lysozymes c of ducks. Phylogenetic analysis (Fig. 2) of the sequence comparisons confirms that the lineage leading to chachalaca lysozyme c separated from that leading to other galliform lysozymes c before the duck lysozyme c lineage did. This indicates a contrast between protein evolution and evolution at the organismal level. Immunological

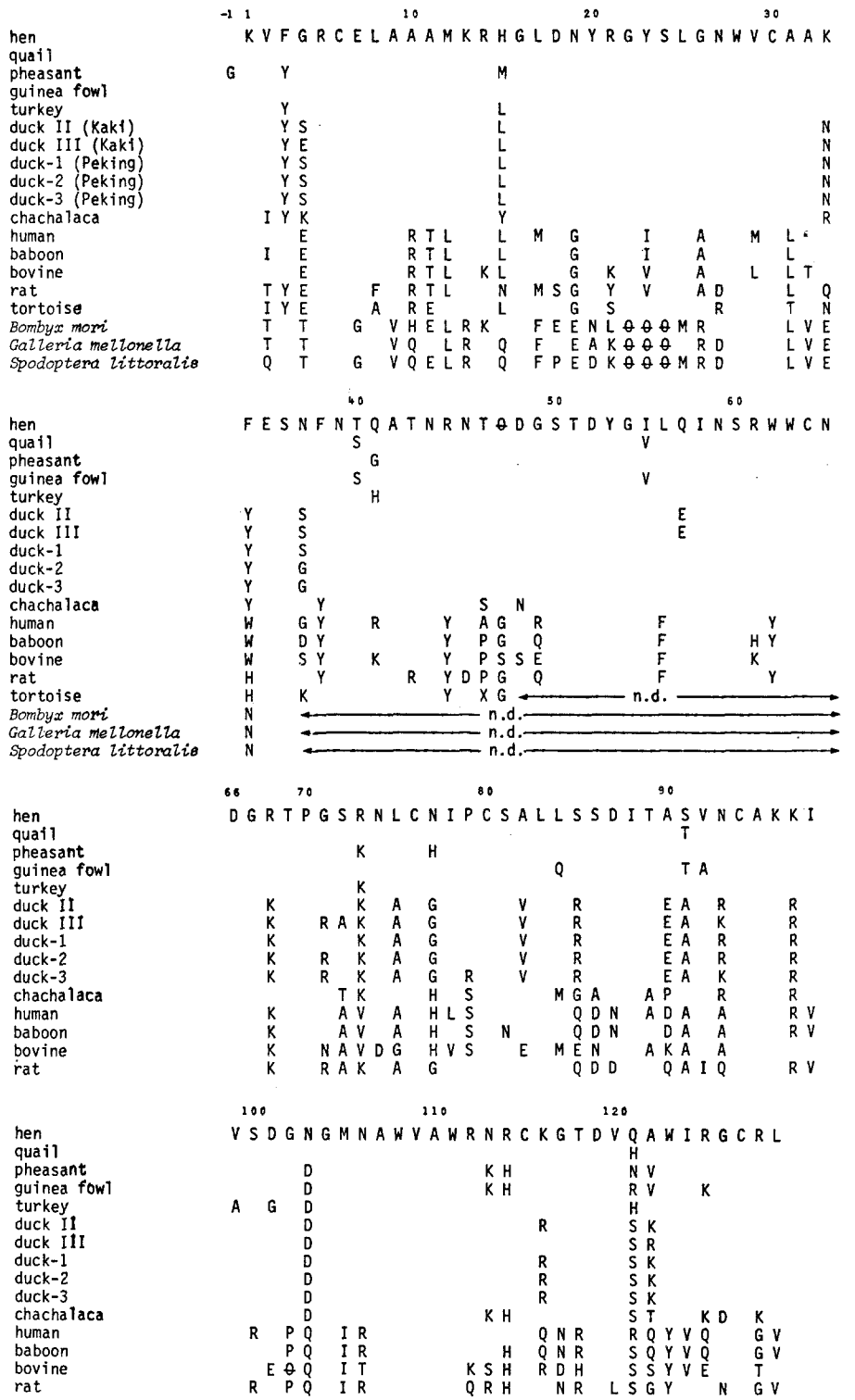

Fig. 1. Amino acid sequences of different c type lysozymes. The numbering refers to the hen lysozyme sequence. The one-letter amino acid abbreviation system was used. *θ*:deletion.

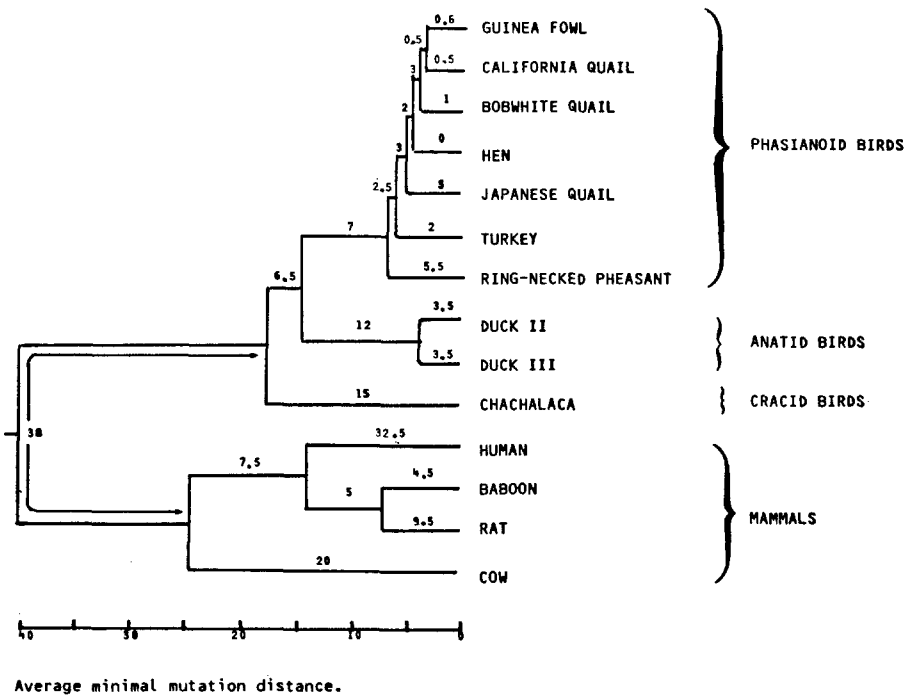

Fig. 2. Phylogenetic tree of animal (c type) lysozymes (37, 38, 43) constructed from minimal mutation distances according to the Farris method.

comparison of chachalaca lysozyme c with other lysozymes of known sequence provides further support for the proposal that immunological cross-reactivity is strongly dependent on degree of sequence resemblance among bird lysozymes (Table 1).

4.1.2. *Evolutionary change affecting processing of prelysozyme: pheasant lysozyme*

Hen lysozyme mRNA was translated in a reticulocyte lysate and the *in vitro* product was shown to be larger than lysozyme synthesized *in vivo* (53). The N-terminal sequence of prelyozyme is:

$$\overset{-18}{\text{Met}}\text{-Arg-Ser-}\overset{-15}{\text{Leu}}\text{-Leu-Ile-Leu-Val-}\overset{-10}{\text{Leu}}\text{-Cys-Phe-}$$

$$\overset{-5}{\text{Leu}}\text{-Pro-Leu-Ala-Ala-Leu-}\overset{-1}{\text{Gly}}\downarrow\overset{1}{\text{Lys}}\text{-Val-...}$$

where lysine is the N-terminus of lysozyme.

The amino acid sequence of ring-necked pheasant egg-white lysozyme c was determined (37): besides differing from the chicken enzyme by 9 amino acid substitutions, ring-necked lysozyme has an extra glycine at the amino terminus. Four other species of pheasant were shown to have lysozymes with a conventional amino terminus, beginning with lysine. The amino termini are now known for a total of 28 lysozymes c, and all except the ring-necked pheasant enzyme begin with lysine. To account for the extra residue in the ring-necked pheasant lysozyme, it is suggested that an evolutionary shift in the site of proteolytic cleavage of prelysozyme has occurred. Phylogenetic analysis (Fig. 2) of 10 avian and 4 mammalian lysozymes of known sequence placed the pheasant at a greater distance from the chicken that the turkey is from the chicken, in contrast to traditional taxonomic placement (54), but consistent with evidence obtained from several other proteins (55). Immunological distances between ring-necked pheasant lysozyme and other bird lysozymes are consistent with the degree of sequence difference between ring-necked pheasant and other bird lysozymes and fit predictions based on the observed correlation between immunological cross-reactivity and sequence difference.

Table 1. Amino acid sequence differences and minimal mutation distances among animal lysozymes c (31, 37, 38, 43). The number of amino acid differences between any two lysozymes is given in the upper right-hand section of the matrix, while the minimal mutation distance appears in the lower left-hand section.

	Hen	California quail	Bobwhite quail	Japanese quail	Turkey	Ring-necked pheasant	Guinea fowl	Duck II	Duck III	Chachalaca	Baboon	Human	Cow	Rat
Hen		4	4	6	7	10	10	22	23	27	50	52	58	58
California quail	4		2	9	9	13	7	24	25	30	52	54	60	60
Bobwhite quail	4	2		10	11	14	8	23	24	31	51	53	59	59
Japanese quail	7	9	11		10	12	15	25	26	30	52	54	59	57
Turkey	7	9	11	10		10	16	22	23	28	53	54	59	60
Ring-necked pheasant	13	15	17	14	10		13	24	25	24	51	56	58	57
Guinea fowl	10	7	8	16	16	16		29	30	28	54	57	60	61
Duck II	27	29	28	29	27	28	33		6	30	50	53	58	56
Duck III	27	29	28	29	27	28	33	7		31	49	52	57	52
Chachalaca	30	33	34	32	32	29	32	36	36		50	55	56	57
Baboon	69	71	70	69	72	69	72	68	66	70		14	39	33
Human	70	72	71	72	72	74	75	69	67	75	14		41	37
Cow	74	76	74	72	77	75	77	74	71	71	46	47		55
Rat	82	84	83	79	84	80	84	77	71	75	45	51	69	

4.1.3. *The effect of a single amino acid substitution on the antigenic specificity: quail and duck lysozymes. The importance of the loop region*

Immunological studies were carried out with closely related lysozymes using anti-lysozyme and anti-loop antibodies. Of all the lysozymes of known amino acid sequence, the California quail and bobwhite quail lysozymes are the most similar (56). These two lysozymes differ from each other only at positions 68 (Arg/Lys) and 121 (His/Gln) and yet they are antigenically different (31). The implication is that one or both of these substitutions alters the antigenic structure of lysozyme. It was shown (57) that bobwhite quail lysozyme, although similar to chicken lysozyme in its overall reactivity with anti-lysozyme, is much less reactive than chicken lysozyme with antibodies specific exclusively towards the loop region of chicken lysozyme. The loop region, strictly speaking, consists of residues 64–80, with a disulfide bridge joining residues 64 and 80 (Fig. 1). Since the only difference between bobwhite quail and chicken lysozymes in the loop region is a substitution of lysine for arginine at position 68, it was inferred that this substitution, either directly or else indirectly due to a possible change in conformation, is the cause of the alteration in antigenic specificity.

A similar observation was made with the two Kaki-duck lysozymes II (34) and III (35) which were indistinguishable in the lysozyme-phage system (58): both are 380 times less efficient than hen lysozyme. Their behavior is however very different in the 'loop'-phage system, as only duck lysozyme II is capable of inhibition. This may be due to the known replacement of the glycine residue in position 71 of the duck lysozyme II sequence with an arginine residue in duck lysozyme III (Fig. 1). The introduction of a new basic amino acid might explain the immunological difference. Studies carried out (33) with three closely related lysozymes from Peking-duck egg-white allowed also to correlate small structural replacements with clear immunological differences (36).

4.1.4. *Mammalian lysozymes*

Only a small number of mammalian lysozymes have been completely sequenced, from human milk (39) and urine (40), from baboon (41) and rat (42): they all belong to the c type (Fig. 1). Bovine stomach lysozyme, another c type enzyme, will be discussed in a separate section (see Section 5).

4.1.5. *Reptilian lysozyme*

Jollès et al. (46) sequenced for the first time a reptilian (tortoise) egg lysozyme from *Trionyx gangeticus* and were able to demonstrate that the latter belongs to the c type. A phylogenetic tree was constructed and a closer relationship between tortoise and bird lysozymes c than between tortoise and mammalian lysozymes c was demonstrated.

4.1.6. *Insect lysozymes*

Recently Jollès et al. (47) demonstrated that the c type lysozyme occurs also among invertebrates, specifically among members of the insect order Lepidoptera, by sequencing the first 33 residues of the lytic enzyme from three different species. Thus the gene for lysozyme c was shown to be ancient in origin.

The three insect lysozymes clearly are homologous in their N-terminal halves to lysozymes of the chicken type. No homology could so far be detected with lysozymes of the goose type. Over the first 33 amino acids of their sequence the insect lysozymes share 40 ± 5% identical amino acid residues with chicken and human lysozymes. The *Galleria* enzyme is the lysozyme most similar in sequence to the mammalian lysozymes. Some of the noted substitutions have been identified for the first time in the lysozymes sequences. Two of them have already been encountered in α-lactalbumin, i.e. residues 2 (Gln of *Spodoptera* lysozyme) and 5 (Thr). Between the insect lysozymes and bovine α-lactalbumin, the percentage of identical amino acid residues is still around 25% in the N-terminal regions. The two half cystine residues encountered in the N-terminal moieties of the insect lysozymes are in phase with the half cystine residues of the other c type lysozymes, provided that a deletion of three amino acids be introduced in the sequences of the former.

The insect lysozyme c sequence information has enabled Wilson's group to carry out a phylogenetic analysis of invertebrate lysozymes c along with vertebrate lysozymes c and α-lactalbumins. They concluded that α-lactalbumin-like proteins could exist in non-mammals and even in non-vertebrates (59).

4.1.7. *Cystines and residues participating in catalysis*

c type lysozymes contain 129–130 amino acids: 40 positions have so far been found invariant among them.

The half-cystines of all the c type lysozymes are in

phase as the disulfide bonds play an important role in the formation and maintenance of their secondary structure (60). The refolding kinetics of unfolded hen lysozyme and the structural intermediates have been investigated in detail (61).

As a result of X-ray crystallographic studies, three of the ten carboxyl groups of hen lysozyme are found to be located in the active site cleft (5): two (Glu-35 and Asp-52) of these carboxyls participate in the well-known catalytic process (6) and occur in similar positions in all c type lysozymes. A third (Asp-101) is involved in a substrate binding interaction: it occurs in most but not all c type lysozymes (Table 1). In order to obtain more direct proof for the importance of these carboxyls, some chemical modifications of carboxyl groups have been developed. Derivatives singly modified at Asp-52 (62) or Glu-35 (63) have been prepared and were found to be enzymatically inactive. The mechanism for the selective modification of these carboxyls in lysozyme is related to the metal binding ability of lysozyme (64). These rather recent studies clearly indicate that Glu-35 and Asp-52 are essential for the lysozyme activity as postulated many years before (5).

4.2. *g type lysozymes*

Goose egg-white lysozyme has been discovered by Jollès (65) and Canfield (66). Prager et al. (52) detected immunologically this goose type (g type) in the egg-white of species representing nine different orders of birds: it has thus a much broader taxonomic distribution than has the c type lysozyme in bird egg-white. Only in the egg-white of the black swan (*Cygnus atratus*) two distinct lysozymes (a c type and a g type enzyme) have so far been detected together (67).

The g type lysozymes have a molecular weight of around 21 000 instead of 14 000 for the c type enzymes and do not cross-react immunologically with the latter (68). Only a limited number of structural results are available for g type lysozymes in comparison to the data established for c type enzymes (complete sequences of black swan (48), ostrich (49) and Emden goose (50) egg-white lysozymes) (Fig. 3).

The enzymes contain 185 amino acids: all the four half-cystine residues are situated in the N-terminal half of the chain and an unusually high occurrence of paired amino acids in the molecules is

```
                                                  10                                      20
Ostrich       Arg-Thr-Gly-Cys-Tyr-Gly-Asp-Val-Asn-Arg-Val-Asp-Thr-Thr-Gly-Ala-Ser-Cys-Lys-Ser-Ala-Lys-Pro-Glu-Lys-Leu-Asn-Tyr-
Black Swan            Asp             Asn             Ile                                 Thr                 Gly     Ser
Goose                 Asp             Asn             Ile                                 Thr                 Gly     Ser

                  30                                      40                                      50
Ostrich       Cys-Gly-Val-Ala-Ala-Ser-Arg-Lys-Ile-Ala-Glu-Arg-Asp-Leu-Gln-Ser-Met-Asp-Arg-Tyr-Lys-Ala-Leu-Ile-Lys-Lys-Val-Gly-
Black swan                Pro         Lys Thr                         Lys Ala                     Thr Ile
Goose                     Ser         Lys                                 Ala                     Thr Ile

                          60                                      70                                      80
Ostrich       Gln-Lys-Leu-Cys-Val-Asp-Pro-Ala-Val-Ile-Ala-Gly-Ile-Ile-Ser-Arg-Glu-Ser-His-Ala-Gly-Lys-Ala-Leu-Arg-Asn-Gly-Trp-
Black swan    Glu                 Glu                                                                 Val     Lys
Goose         Glu                 Glu                                                                 Val     Lys

                                  90                                      100                                     110
Ostrich       Gly-Asp-Asn-Gly-Asn-Gly-Phe-Gly-Leu-Met-Gln-Val-Asp-Arg-Arg-Ser-His-Lys-Pro-Val-Gly-Glu-Trp-Asn-Gly-Glu-Arg-His-
Black swan            Arg                                         Lys                     Gln     Thr                 Val
Goose                 Arg                                         Lys                     Gln     Thr                 Val

                                          120                                     130
Ostrich       Leu-Met-Gln-Gly-Thr-Glu-Ile-Leu-Ile-Ser-Met-Ile-Lys-Ala-Ile-Gln-Lys-Lys-Phe-Pro-Arg-Trp-Thr-Lys-Glu-Gln-Gln-
Black swan    Ile Thr             Thr         Thr Asp Phe         Arg                         Ser             Asp
Goose         Ile Thr             Thr             Asn Phe         Thr                         Ser             Asp

              140                                     150                                     160
Ostrich       Leu-Lys-Gly-Gly-Ile-Ser-Ala-Tyr-Asn-Ala-Gly-Pro-Gly-Asn-Val-Arg-Ser-Tyr-Glu-Arg-Met-Asp-Ile-Gly-Thr-Thr-His-Asp-
Black swan                                                Ala                         Ala
Goose                                                     Ala                         Ala

                      170                                     180                 185
Ostrich       Asp-Tyr-Ala-Asn-Asp-Val-Val-Ala-Arg-Ala-Gln-Tyr-Tyr-Lys-Gln-His-Gly-Tyr
Black swan
Goose
```

Fig. 3. Amino acid sequences of three g type lysozymes.

noted. Contrary to previously reported observations, a slight sequence homology between the g type and c type lysozymes could be suggested by Jollès et al. (49, 69) on the basis of sequence homology: Glu-73 and Asp-86 of the g type might correspond to Glu-35 and Asp-52 of the active center of the c type enzymes. This observation was corroborated by X-ray studies carried out by Matthews' group (70) (see Sections 8.1 and 10).

4.3. *Phage lysozymes: the most convincing example to date of the divergence of proteins with non-homologous sequences*

The bacteriophage lysozymes include 164 amino acids with a molecular weight of 18 700: their sequences are not homologous to the c type enzymes (71). However they have similar catalytic properties, although the cell walls of *E. coli* are digested much more rapidly be the phage enzymes than by hen lysozyme. The two enzyme types have been shown to be similar in their backbone conformations, in the location of bound substrates and in the specific details of substrate binding and catalysis: indeed the only amino acids which seem to have retained their identities are Glu-11 and Asp-20 of phage lysozymes corresponding to Glu-35 and Asp-52 of hen lysozyme which are involved in the enzymatic mechanism of action. These similarities strongly suggest that c type and phage lysozymes have diverged from a common precursor (70, 72) (see Section 10).

A computer search suggests that bacteriophage T4 lysozyme contains one region and that rabbit myosin light chains contain three regions similar, and supposedly homologous to the calcium binding region of carp muscle calcium binding parvalbumin (73).

4.4. *Plant lysozymes*

Lysozyme was detected in several plant tissues, particularly in some flowers, different roots and tubers. Crystalline lysozyme was prepared from papaya latex (74) and analyzed with some details (75). Jollès' group reported the purification of turnip lysozyme and discussed some of its biochemical properties (76): the enzyme seems to act as a chitinase rather than as a lysozyme.

4.5. *Fungus and bacterial lysozymes*

The fungus, *Chalaropsis* (Ch) species, produces an extracellular bacteriolytic enzyme: in addition to possessing β-1.4-N-acetylmuramidase activity as a typical lysozyme, it also possesses β-1,4-N,6-0-diacetylmuramidase activity. Thus lysozyme Ch is active against bacterial species that are insensitive to c type lysozymes. The sequence as well as size ($M_r = 22\,415$) of this enzyme differs markedly from other lysozymes whose sequences are known. The catalytic activity of lysozyme Ch is at least partially due to two acid amino acids, Asp-6 and Glu-33: the common feature between the c, g, phage and Ch lysozymes is that they all have acidic residues as part of their active site (77).

A species of lysozyme found in the culture broth of *Streptomyces erythraeus* (SE) appears also to differ from the c type, more particularly as, contrary to hen lysozyme, it hydrolyzes the cell walls of *Staphylococcus aureus* which contain N,6-0-diacetylmuramic acid (78). This observation explains that SE lysozyme presents some sequence homologies with lysozyme Ch. At 6 Å resolution, it was not possible to see any resemblance with c type or phage lysozymes (79).

4.6. *Invertebrate lysozymes*

As indicated above, Jollès et al. (47) characterized a c type lysozyme among members of the insect order Lepidoptera; others isolated from the insect *Ceratitis capitata* eggs a lysozyme with a molecular weight of about 23 000 whose N-terminal sequence showed a high homology with that of the phage lysozyme. Chitinase/muramidase specific activity ratio is around 350 times higher for the insect than for hen lysozyme (80). A lysozyme isolated from *Helix pomatia* possessed also a high molecular weight of about 24 000 (81).

Lysozyme-like enzymes occur in many marine invertebrates (82). Only a limited number of them has been completely purified and their enzymatic properties studied. The lysozyme from *Nephthys hombergii* (82, 83) can be classified in the category including the goose enzyme in regard to its very low action on N-acetylglucosamine polymers. However it resembles also the c type enzymes when several other criteria are considered, such as the variation of the initial velocity of lysis as a function of ionic

strength or the behaviour in the presence of urea (84).

A lysozyme isolated from *Asterias rubens* has been completely purified (85). It has a molecular weight of 15 500 ± 1 000 and its high sensitivity to ionic strength must particularly be noted. A long N-terminal sequence was reported and is not recognizably related to other known N-terminal lysozyme sequences: thus the lysozyme from *A. rubens* seems to be representative of a new type of lysozyme.

Lysozyme-like enzymes have been detected in many marine bivalves (86); the enzyme of the mussel *Mytilus edulis* (82) was purified: its molecular weight is about 18 000 and its pI of 9.2 is considerably lower than the isoelectric point of hen lysozyme (87).

5. Species adapt to new environments by regulatory changes affecting certain key proteins: bovine stomach lysozyme as an example

A quite interesting lysozyme is the bovine stomach lysozyme purified by Dobson et al. (88, 89). The enzyme was isolated from the true stomach mucosa of cattle and three distinct, closely related, non-allelic lysozymes c were found. The three enzymes account for 10% of the total protein extracted from mucosa at pH 6. Jollès' group (43) performed an extensive sequence study of the most abundant of these enzymes which had the following interesting properties: (a) increased resistance to pepsin digestion; (b) a more acidic pH profile; (c) low specific activity at neutral pH at physiological ionic strength.

Rumen bacteria are an important nutrient source of cattle but an important factor in their utilization is the ability of the animal to break open the bacteria. The latter are surrounded by peptidoglycans which contain glycosidic bonds which could be split by lysozyme. A logical place for a digestive lysozyme to be produced is the true stomach of ruminant-like mammals. The bacteria would be killed by the low pH in the true stomach and opened with the lysozyme secreted by the mucosal lining. By disrupting the peptidoglycan layer, lysozyme would make bacterial contents available for digestion by other enzymes in the stomach and intestines. This hypothesis predicts that only mammals with foregut fermentation should have high levels of stomach lysozyme (88, 89), and the evidence available to date indicates this to be the case.

The sequence of cow lysozyme 2 is indicated in Fig. 1: it differs from the sequences known for other lysozymes c at 39 to 60 positions at one of which there has been a deletion of one amino acid. Of the 40 positions previously found to be invariant among lysozymes c, only one has undergone substitution in the cow lineage. This modest number of changes at novel positions is consistent with the inference, based on tree analysis, that the tempo of evolutionary change in the cow lysozyme lineage has not been radically different from that in other lysozyme c lineages. When compared to other lysozymes c, replacements in cow lysozyme are much more frequent in the C-terminal than in the N-terminal part. However, the half-cystine residues producing the four disulfide bonds are at the same place and this might reflect, already at this stage, a more or less conserved three-dimensional structure: its detailed study will of course be of interest. Indeed it will thus be possible to test the hypothesis of Wilson et al. (90) that species adapt to new environments by regulatory changes affecting certain key proteins and to follow on a molecular level in the present case how an enzyme has undergone a major functional change as a result of becoming a digestive enzyme and to determine the amino acid substitutions that make the enzyme to function in the stomach environment, thus to increase its resistance to cleavage by pepsin. The latter is most likely the result of fewer pepsin-sensitive bonds and decreased electrostatic interactions with pepsin: when examining the distribution of charges on cow lysozyme (43), it is clear that all its faces bear more negative charges than the corresponding faces in hen and human lysozymes. The Glu replacement at position 101 and the deletion of Pro at position 102 eliminate the Asp-Pro bond that is present between these positions in all other mammalian lysozymes c tested. This bond appears to be the most acid-sensitive one in lysozyme at physiological temperature and its evolutionary loss may have contributed to the ability of cow enzyme to survive and function at low pH in the stomach fluid (43).

6. From the antigenic structure of lysozyme to that of proteins

Hen lysozyme has long served as a prototype protein for investigations concerning the specificity of immune recognition: they conducted to two opposing views of what constitutes a determinant on a protein antigen. How much of the hen lysozyme surface is antigenic?

6.1. *Determination of the antigenic structure by surface-simulation synthesis*

According to Atassi et al. (91) proteins contain a very limited number of sites which are intrinsically immunogenic irrespective of the host responding to the antigen. A large number of specific chemical derivatives of lysozyme have been prepared and characterized and their immunochemistry studied in detail (90). A useful approach in the delineation of protein antigenic structures depends on the isolation of a large variety of overlapping peptide fragments representing various parts of the protein molecule. Atassi et al. (91) proposed that only three antigenic sites exist in lysozyme and that they are discontinuous (I-residues 5, 7, 13, 14, 125; II-33, 34, 113, 114, 116; III-62, 87, 89, 93, 96, 97; see Fig. 1). The determination of the entire antigenic structure of native lysozyme was achieved by precise boundary, conformational and directional definition of its three antigenic sites by surface-simulation synthesis (91). Many general conclusions relating to antigenic structures of proteins were derived from previous studies carried out with sperm whale myoglobin. They were also applicable to lysozyme and include more particularly the small size of the antigenic sites and their sharp boundaries, their presence only in a limited number, their surface locations and their sensitivity to conformational changes.

An important difference was found to exist between the antigenic sites of myoglobins and those of lysozyme. The five antigenic sites of myoglobin are constituted of residues that are in direct peptide bond linkage whereas the three sites of lysozyme each constitutes spatially adjacent surface residues that are mostly distant in sequence, each describing a line which circumscribes part of the surface topography of the protein (91).

6.2. *A multideterminant-regulatory model*

A quite different model has been proposed by the group of Wilson et al. (31, 92). Recent studies which have re-examined the antigenic structure of hen lysozyme as defined by the total number of different specificities which can be detected in panels of monoclonal antibodies from several strains of inbred mice suggest that the Atassi structure is incomplete. Several lines of evidence of these studies support the conclusion that the capacity for antibody response to hen lysozyme is very broad, consisting of many different clonotypes which bind distinct, but often overlapping, determinants which in aggregate include most, if not all, of the hen lysozyme surface. Wilson et al. conclude that most of the accessible surface of any globular protein is potentially immunogenic, that one can define which sites are immunogenic only with respect to a particular responding individual and that the total antigenic structure of a protein is the sum of all sites recognized by a large variety of responding individuals and species. The multideterminant-regulatory model predicts that nearly all evolutionary substitutions would directly affect immunological cross-reactivity.

7. The lysozyme gene

7.1. *Hen lysozyme*

The gene for hen lysozyme has been isolated (93): the nucleotide sequence was determined for hen lysozyme mRNA and for the exons of the gene together with their flanking intron regions. Determination of the exact position of the exon/intron boundaries made it possible to correlate the structure of the gene to structure and function of the protein (94) (Fig. 4).

7.1.1. *Exons encode functional and structural units*

Protein chemists have divided hen lysozyme into four sequences: residues (a) 1–39; (b) 40–85; (c) 86–100; (d) 101–129 (Fig. 1). A deep crevice containing the active site divides the molecule into two halves: on one side is situated the β-structured (b) fragments whereas on the other side are the (a) (N-terminal) and (d) (C-terminal) helical fragments; fragment (c) with an α-helix joins the two halves.

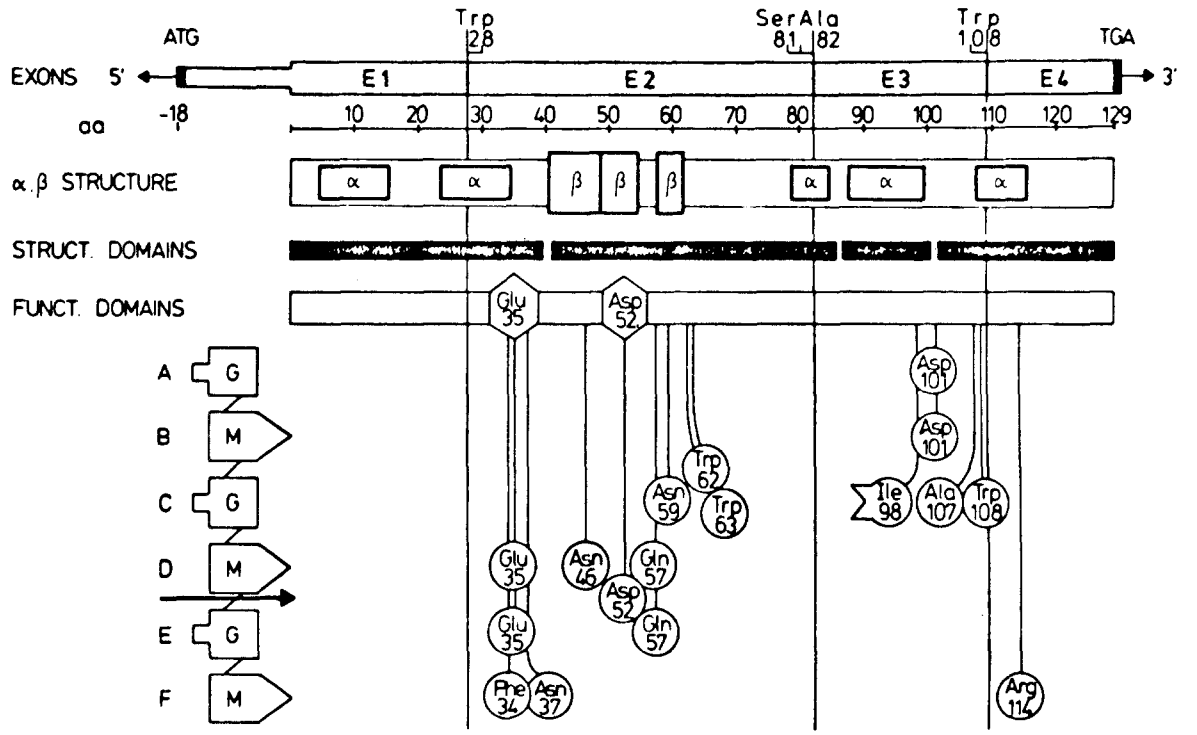

Fig. 4. Correlation between structural and functional parts of lysozyme and the exons of its gene. A linear diagram is shown of the structural gene parts encoded by exons, the α-helical and β-sheet structural elements (5) and the structural segments of the protein that have been described. Functionally important residues of the enzyme are delineated two-dimensionally in correlation with their contact points in ring positions A–F of the substrate, an alternating co-polymer of N-acetylglucosamine (G) and N-acetylmuramic acid (M) (reproduced from Jung et al. (94), with permission).

There is a reasonable correlation between the exon pattern and the structural units with, however, an exception of the first exon/intron boundary which occurs within fragment (a) at position 28 situated in an α-helix: no further interruption occurs at codon 40 (between fragments (a) and (b)). Each of the products of the four exons carries a main structural element which folds independently and does not penetrate very much into other exon units (95).

Exon 2 codes for a region that includes the catalytic center of the enzyme with Glu-35 and Asp-52, the surrounding functionally important elements from both sides of the crevice and part of the substrate binding site: exon 2 could possibly function as a primitive glycosidase.

Exon 3 codes for a fragment that, combined with the exon 2 region, completes the active site and could give additional substrate specificity and catalytic efficiency. Exons 1 and 4 code for translational signal sequences on the mRNA, for the signal peptide of prelysozyme and for the N- and C-terminal regions of the enzyme: they are not directly involved in the catalytic function but may have stabilizing or as yet unknown biological functions (95).

Lysozyme is structurally related to α-lactalbumin and it has been proposed that they have arisen from a common ancestral gene. The genes of the two proteins contain three introns at similar positions. The first three exons of the two genes have similar nucleotide sequences but the fourth exon of α-lactalbumin which partly codes for its C-terminal sequence, essential for its interaction with galactosyltransferase, is markedly different from the corresponding exon of the lysozyme gene (96).

7.1.2. *Exons, domains, modular structural units and function in hen lysozyme*

All the larger protein molecules seem to be composed of separate folded units called domains: fre-

quently it is possible to correlate a protein function with a structural domain of the protein. The view that proteins had evolved by the combination of domains carrying particular functions was in currency before the discovery of exons and introns (95). Gilbert (97) and Tonegawa (98) suggested that exons correspond to functional units of protein molecules and new functional proteins might evolve by selection of various combinations of the functional units that are produced by unequal crossing-over on introns. Blake (99) argued that if exons encode structural units as well as functional units the combinations of such exons would have the advantage of producing stable functional proteins.

Go (100) proposed a method to define compact structural units – also called modules – as least extended conformations in globular proteins. Five modules, M1–M5 (Fig. 5), have thus been identified in hen egg-white lysozyme (101): they consist of residues 1–30, 31–55, 56–84, 85–108 and 109–129, respectively (Fig. 1). Modules M1, M2 plus M3, M4 and M5 have been shown to correspond to exons 1, 2, 3 and 4 of the lysozyme gene, respectively. This finding led to the prediction that one more intron may have been present in an ancestral lysozyme at the position corresponding to the junction between modules M2 and M3. Localization of the catalytic sites Glu-35 and Asp-52 on the module M2 suggests that this module might have worked as a functional unit in a primitive lysozyme. The slight sequence homology observed between c and g type lysozymes (49, 69) concerns also module M2.

Quite recently Blake (102) pointed out that the Go analysis of the correlation of exons with protein structure does probably not have general applicability and that the catalytic function of enzymes such as lysozyme is encoded by a number of exons, not one.

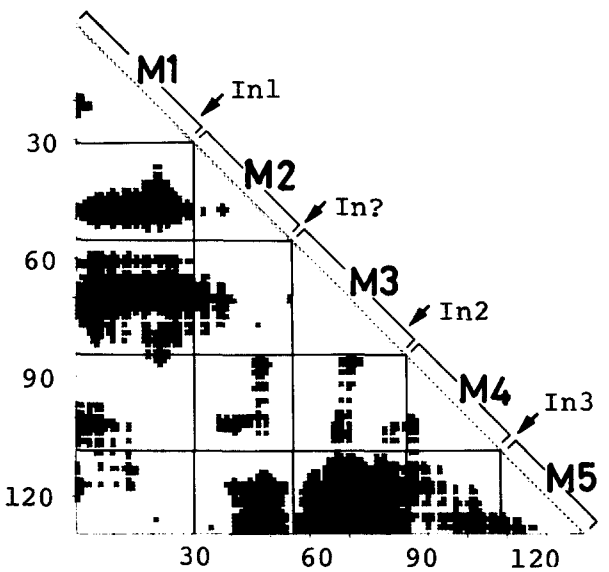

Fig. 5. Modules of chicken egg-white lysozyme and intron positions of its gene. The dark regions represent pairs of C^{α} atoms that are separated more than 23 Å. Both ordinate and abscissa are residue numbers. Five modules, M1–M5, are identified by using the least extended criterion – i.e., by drawing a pair of horizontal and vertical straight lines that meet on the diagonal in the map in such a way as to keep away from the dark regions. Intron positions (94) are marked by arrows, together with the predicted position of another intron at the junction between modules M2 and M3 (reproduced from Go (101), with permission).

7.2. *Phage lysozyme*

The nucleotide sequence of the lysozyme (e) gene of bacteriophage T4 and approximately 130 additional nucleotides on each side has been determined. Nucleotide sequence analysis of mutant e genes confirmed that three identified hot spots of frameshift mutations are runs of five A nucleotides in the wild-type gene. The endpoints of two deletions are direct repeats of eight base-pairs in the wild-type gene. Two frameshift mutations with high reversion frequencies are duplications of five or seven base-pairs (103).

8. Lysozymes' conformation, three-dimensional structure and mechanism of action

8.1. *Lysozymes' crystal structure at low temperature* (≤20 °C)

Hen egg-white lysozyme is known to crystallize in a great variety of forms depending upon the nature of the precipitating salt and the choice of pH. Of these, many have already been studied by X-ray techniques. The structure of the molecule in the tetragonal form has been solved to a resolution of 2 Å (6, 104, 105). The triclinic as well as the monoclinic and the low-temperature orthorhombic forms have also been the subject of extensive investigations (106–110). All these studies showed clearly that lysozyme has essentially the same conformation in these forms (temperature ≤20 °C).

More recent studies of the lysozymes c isolated from humans (111), turkey (112) and tortoise eggs (113) have shown that the conformation of the molecule is highly conserved in these different species and crystal structures. According to Artymiuk et al. (114) the patterns of atomic displacements in the crystals of hen and human lysozyme derived from independent crystallographic refinement are broadly similar. Analysis of the pattern indicates a close correlation with molecular structure, strongly suggestive of intramolecular motion. The active site of lysozyme is located in a region of high displacement. It is concluded that protein mobility may play a significant part in biological activity and that X-ray crystallography can contribute to its analysis.

Lysozymes from other classes have also been extensively studied: Matthews and Remington (115) reporten the three-dimensional structure of T4 phage lysozyme, Grütter et al. (70) the structure of goose lysozyme and Isaacs et al. (67) the structure of black swan lysozyme. Preliminary crystallographic data for lysozyme produced by *Streptomyces erythraeus* have also been established (116).

8.2. *The conformation of hen lysozyme in solution at low temperature*

The conformations of lysozyme in crystals and in aqueous solution have been discussed by Blake et al. (117). They showed that the basic conformation is similar in the two states and described by physical methods and chemical inspection the following new features: (i) the protein has a well-maintained fold in all conditions; (ii) there is mobility in various parts of the protein, particularly side-chain mobility, but also some general vibrations of the main chain; (iii) there is unconventional chemistry in the active site which is not just due to hydrophobicity but is peculiar to the juxtaposition of particular residues. This contrasts with the rather orthodox chemistry of the surface and the absence of chemistry of the interior; (iv) there are particular conformational changes associated with some chemical reactions which run some distance from the site of attack; (v) there is a general conformational change of the whole protein associated with changes of temperature and the binding of some reagents which cross-link the groove. The protein can act as a cooperative unit for energy in these reactions.

8.3. *Inhibitor and substrate binding: mechanism of action*

The complexes of the tetragonal form of hen lysozyme with saccharides provided a basis for approaching the nature of catalytic properties of the protein and enabled Blake et al. (105) to propose a mechanism of action. It accounts for the catalytic rate enhancement as resulting from distortion associated with binding of the saccharide ring that carries the glycosidic oxygen, participation of Glu-35 as a general acid catalyst and stabilization of the developing carbonium ion by Asp-52. The active site of lysozyme can accommodate six units of a polymeric substrate. The $\beta(1\rightarrow4)$-linked hexasaccharide of GlcNAc and the similar bacterial cell wall hexasaccharide (GlcNAc $\beta(1\rightarrow4)$ $MurNAc)_3$ are the best known substrates of lysozyme judged by the specificity of the enzymic hydrolysis (for the bond between the 4th and 5th units from the non-reducing end) and the maximum rate of reaction. Mono-, di- and trisaccharides, more particularly GlcNAc to $(GlcNAc)_3$ are known to be inhibitors of the enzymic reaction.

As defined by Blake et al. (105), lysozyme has six subsites for substrate binding: A, B, C, D, E and F, each corresponding to a saccharide unit and running from one end of the active site cleft to the other with catalytic groups Glu-35 and Asp-52 between sites D and E. Binding at the A, B and C sites have been directly observed by crystallographic methods involving binding of tri-GlcNAc in tetragonal lysozyme crystals (105) and D site binding has been observed for the lactone moiety of a lactone derivative of tetra-GlcNAc (118). Binding has not yet been directly observed in the E and F sites, although there is chemical evidence for F site binding as shown by a dye replaced by hexa-GlcNAc (119). In turkey lysozyme, however, binding of $(GlcNAc)_2$ or GlcNAc-MurNAc could be observed in subsite E and a new subsiste F′ different from F (112).

There are clear indications that the conformation of the enzyme changes in the crystals when inhibitors are bound. The changes extend through the molecule and are most evident in the movement of the loop between residues 70–75, in the clearer definition and reduced motion of Trp-62, and in the angular movement (7°) of the mean-square plane of Trp-63 relative to its position in native lysozyme (117). The changes seen in solution are similar. The

resonances of Trp-63 show that the region of protein around Trp-62 and Trp-63 now has a fixed conformation, i.e. there is no longer flapping of a tryptophan (117). The binding of inhibitors also alters the magnitude of the shifts on Trp-108 owing to the Glu-35. As Trp-108 senses strongly the ionization of both Asp-52 and Glu-35, the inhibitor connects the two sides of the groove in some cooperative way.

The activation energy of lysozyme for attacking its substrate is lowered by the use of the binding energy of the substrate in the distorsion of the sugar ring in the critical site D. The first change in emphasis lies in the observations on Glu-35. NMR data show clearly (117) that Glu-35 and Trp-108 shift relative to one another on ionization of Glu-35. Asp-52 has no strictly comparable behaviour but in the presence of substrate (inhibitor), ionization of Asp-52 causes a change in the interaction between Glu-35 and Trp-108. All the results indicate that the enzyme active site is in a specially energized condition. Thus lysozyme does not just distort the substrate on binding toward the transition state of its reaction. It provides a special acid, Glu-35, which is perhaps held close to its interaction with a special side-chain Trp-108. Blake's et al. (117) structural studies are fully consistent with the kinetic analysis of the lysozyme reaction by Banerjee et al. (120).

Some reports, however, do not entirely fit in with the above reported data. Kurachi et al. (121) were not able to bind $(GlcNAc)_3$ to lysozyme in the triclinic form. Furthermore a high number of enzymatic (122), chemical (123), theoretical (124–128) and physical (129) studies have been devoted to the importance of substrate distortion, but there is little direct crystallographic evidence on the conformation of a MurNAc residue bound at side D. Kelly et al. (130) presented the X-ray structure of the non-hydrolyzed trisaccharide MurNAc-GlcNAc-MurNAc bound in subsites B, C, D. Their interpretation of the 2.5 Å resolution difference map does not involve distortion of this residue in site D.

Pincus and Scheraga (131) predicted the three-dimensional structures of complexes of lysozyme with cell wall substrates. As with the homopolymer $(GlcNAc)_6$, the hexasaccharide $(GlcNAc\text{-}MurNAc)_2\text{-}(GlcNAc)_2$ binds preferentially on the 'left' side of the active site cleft, involving residues such as Arg-45, Asn-46 and Thr-47. The alternating co-polymer $(GlcNAc\text{-}MurNAc)_3$, however, binds with its F site residue preferentially on the 'right' side of the active site cleft, involving residues such as Phe-34 and Arg-114. The lactic acid side-chain prevents good binding to the F site on the left side. This result can explain the higher rate of catalysis for the cell wall substrate (the alternating copolymer). The relative affinities of the disaccharide GlcNAc-MurNAc for all sequential pairs of sites A-F (including E and F sites on both sides of the cleft) were determined. It was found that the highest affinity of this disaccharide is for sites C and D and 'right-side' sites E and F, in good agreement with experimental results of Sarma and Bott (112). The energy of the recently determined X-ray crystallographic structure of MurNAc-GlcNAc-MurNAc bound to the B, C and D sites of hen egg-white lysozyme has been minimized and found to lead to a conformation quite similar to one which was predicted previously for the trisaccharide $(GlcNAc)_3$. The D ring is undistorted and binds close to the surface of the active site cleft. The structure can be extended into sites E and F by addition of two GlcNAc residues, but only on the left side of the active site cleft. This indicates that polymers bound with their D site residues near the surface of the cleft must bind to sites E and F on the left side of the cleft, as also predicted previously.

Difference between various lysozymes in the mode of association with oligosaccharides has been observed. It was shown that while the α- and β-anomeric forms of GlcNAc compete for the same site of hen lysozyme, they have different affinities and bind in different orientations (132): on the contrary tortoise lysozyme does not distinguish between the two anomeric forms of GlcNAc (133).

The present trend in this kind of studies is to determine the crystal structure in the presence and absence of inhibitors at $T \leqslant 20\,^{\circ}C$ and very high resolution (<1.5 Å) in order to precise the atomic position of the residues, particularly those implicated in the mechanism of action and to study the water structure, ordered and disordered, around the molecule. The structure of the water in crystals of human and tortoise lysozymes which contain about 350 and 650 water molecules per protein molecule, respectively, has been described quite recently (134). Whatever the lysozyme (human, egg, tortoise), the crystal form studied and the water content of these crystals, it seems that the number of the ordered water molecules is identical, as if it

were a property of the lysozyme molecule and not of the crystal (134).

Finally it was tempting to slow down the reaction between lysozyme and its substrate(s) by working at sub-zero temperatures: such an experiment has been tried with human lysozyme and oligosaccharide substrates (135).

8.4. *Dynamics of enzymatic reactions*

A possible way to explore the dynamics of enzymatic reactions is to use computer simulation approaches. Warshel (136) had developed a method for simulating the molecular dynamics of a reacting enzyme–substrate complex: it allows one to explore what type of fluctuations are involved in enzymatic reactions and to evaluate entropic contributions to enzyme catalysis. In a preliminary study, this method was applied to lysozyme (136).

9. The high (physiological) temperature form of hen lysozyme

9.1. *A phase transition in a protein crystal*

In 1972, Jollès and Berthou (137) noticed that tetragonal crystals were unstable above 25 °C and especially at physiological temperatures (37–40 °C); under the influence of temperature alone they transformed into stable orthorhombic crystals. Later on it became clear (138, 139) that the other forms obtained at low temperature were able to transform into this same orthorhombic form, whatever the initial conditions of crystallization had been, the only difference being the transition point: this form seems thus to be the stable form at high, and more particularly physiological, temperature. The latter is stable over a broad range of temperatures and can be obtained until 55 °C. The possibility was considered that the enzyme molecule undergoes a conformational transition which could account for the existence of the two crystalline forms.

9.2. *Behaviour of the high temperature form in solution*

To this thermodependence of lysozyme crystallization there correspond particular properties in solution. Evidence for a specific temperature-dependent conformational transition between 20 and 30 °C was obtained by means of ^{13}C-nuclear magnetic resonance spectroscopy (140); a decrease in the affinity of lysozyme for N-acetylglucosamine was noted at neutral pH above the transition temperature (25 °C), when this sugar was known to be a strong inhibitor at 20 °C (141). Evidence in favour of the existence of a structural transition in lysozyme which does not involve denaturation, has also been provided by the finding of a sharp break in the Arrhenius plot at 25 °C; a pH dependence of this temperature-induced rearrangement was observed, which suggests a possible involvement of some carboxylic groups in the transition (142). As some importance has already been attributed to these carboxylic groups in the inhibitory action of N-acetylglucosamine at low temperature, Saint-Blancard et al. (143) decided to reinvestigate the action of this sugar at 40 °C (physiological temperature for birds) and at different values of pH; inhibitor-insensitive lysozyme forms were thus characterized. Finally it was recently determined (144) that, in the pH range 6.7–8.6 frequently used in experiments involving lysozyme, the pH optimum of lysis of *Micrococcus luteus* cells at low ionic strength (0.02–0.05) by the high temperature form was 1–2 units lower than that by the low temperature form. All these experiments suggested the existence of two different temperature-induced ranges in each of which lysozyme behaves differently, possibly because of different conformations. These were called the A and B forms (low and high temperature forms, respectively).

9.3. *Three-dimensional structure (high temperature form)*

Two independent 6 Å maps of the structure of the high temperature orthorhombic crystals were obtained by means of double isomorphous replacements (145) and molecular replacements (146). The initial model obtained ($R = 0.52$ at 6.0 Å) was refined first as a rigid body at 6.0 Å and then by restrained least-squares at 2.5 Å and later at 2.0 Å resolution (147). The final model ($R = 0.23$ at 2.0 Å) was compared with that of the tetragonal form: the structures are very similar with a root mean square difference in superimposed α-carbon coordinates of 0.46 Å. There are, however, differ-

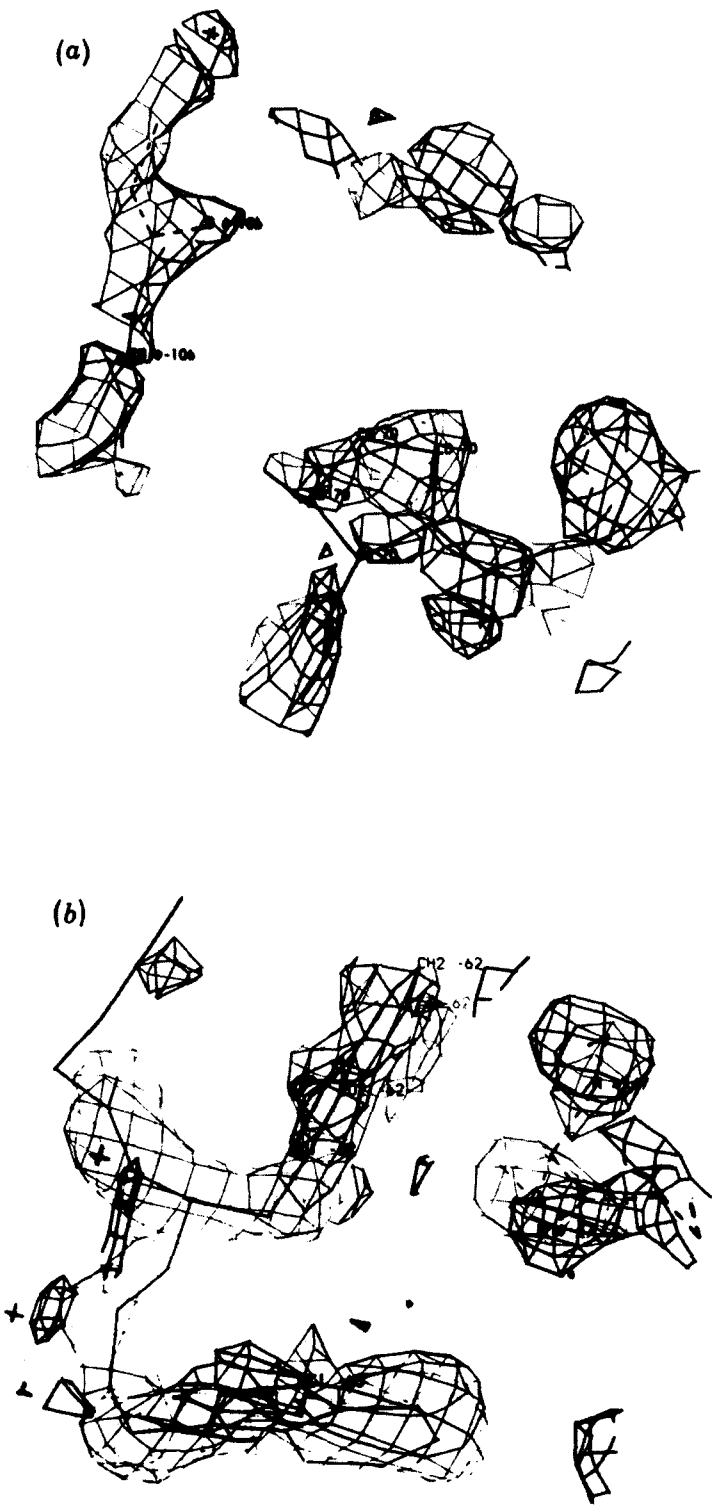

Fig. 6. Electron density around (a) Pro-70 and (b) Trp-62 (upper centre) and Trp-63 (bottom) as obtained at 2.0 Å resolution in the high temperature form crystals of hen lysozyme (reproduced from Berthou et al. (147), with permission).

ences which are caused by a crystal contact involving the upper part of this active site in the high temperature orthorhombic form. Because of this, residues Trp-62 and Pro-70 are much better ordered than in the tetragonal form where they are exposed to solvent (Fig. 6). These differences can partly explain the difficulty of inhibitor-binding in high temperature orthrohomic crystals, but do not seem to reflect the particular behaviour of lysozyme in solution at high temperature (147).

10. From crystallography to evolution

10.1. *An evolutionary link between lysozymes of different classes?*

The lysozymes of hen egg-white, goose egg-white and bacteriophage T4 have similar catalytic properties: two amino acids, Glu-35 and Asp-52, are involved in the active site of hen lysozyme (5, 118). Obviously Glu-73 of goose lysozyme and Glu-11 of phage lysozyme are counterparts to Glu-35 in hen lysozyme; similarly Asp-86 of goose and Asp-20 of phage lysozymes correspond to Asp-52 of hen lysozyme (49, 69, 70). The amino acid sequences of the three lysozymes are not homologous; nevertheless Jollès et al. (49, 69) noted that a sequence homology occurs between residues 35 → 54 of hen lysozyme (beginning of exon 2) and residues 73 → 88 of the ostrich (g type) enzyme. The beginning of exon 2 corresponds to Go's (102) module M2, a further indication that the latter might correspond to the 'primitive glycosidase'. The question therefore arises whether they are derived from a common ancestral protein or have arisen independently.

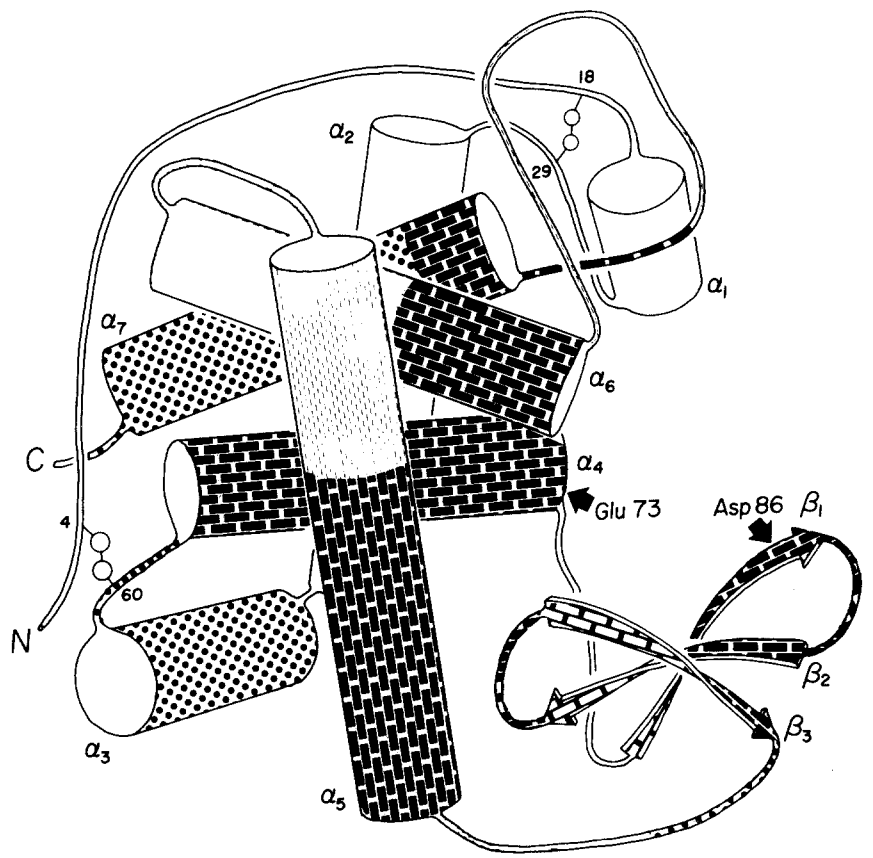

Fig. 7. Simplified drawing of goose egg-white lysozyme showing those parts of the structure that are common to hen and to phage lysozymes. Parts that are common to all three lysozymes are shown as bricks, parts common to goose lysozyme and hen lysozyme are shown dotted, parts common to goose lysozyme and T4 phage lysozyme are shown dashed, and parts that occur only in goose lysozyme are shown as open (reproduced from Grütter et al. (70), with permission).

It has been suggested that the amino acid sequence of a protein is much more variable and changes more rapidly than its tertiary structure and meaningful comparisons between distantly related proteins should therefore come from structural rather than from sequence homology studies. It has been shown that the three lysozymes are at least in part similar in the conformation of their backbones. The three-dimensional sequence of goose lysozyme is more similar to both hen and phage lysozymes than phage and hen lysozymes are to each other (70, 72). There are extended regions of goose lysozyme that are strikingly similar to hen but not to phage lysozyme. Also there are other regions of goose lysozyme that correspond closely to phage but not to hen lysozyme. This is not so for hen and phage lysozymes: there are no regions of extended similarity that are exclusive to these two enzymes (Fig. 7).

This pattern of structural similarities between the three lysozymes places restrictions on their probable linkage. One could imagine that a hen type or phage type lysozyme might evolve to (or from) a goose type lysozyme; direct evolution from a phage type to a hen type lysozyme seems however very unlikely. Several possibilities of divergence of the three lysozymes from a common precursor were proposed (70) (Fig. 8). The dissimilarity of the amino acid sequences of the three lysozymes allows to speculate on the evolutionary time since their divergence. According to Wilson et al. (91, 148), c type lysozymes have undergone a 1% change every 2.5 million years. In the absence of a detectable homology, it is assumed that the three sequences have changed relative to each other by at least 99%:

Fig. 8. Formalized representation of the relation between the structures of hen type, goose type and phage type lysozymes together with possible transitions that could have occurred between precursors of these lysozymes during the course of evolution. The symbols are not meant to represent distinct structural domains. Rather, the rectangles represent those structural elements that are common to all three lysozymes, the triangles represent features unique to goose lysozyme and hen lysozyme, the circles represent parts of the lysozyme structures that occur only in goose lysozyme and T4 phage lysozyme and the star represents the polypeptide segment that occurs only in goose lysozyme (see Fig. 7) (reproduced from Grütter et al. (70), with permission).

thus the estimated time since the divergence of the three lysozymes is at least 1.2×10^9 years, well before the separation of the mammals and reptiles (0.3×10^9 years) (70) and very likely before the evolution of the first eukaryotes ($1.3–1.9 \times 10^9$ years ago) (149).

10.2. *A lysozyme type mechanism in the β-1,4-glycoside hydrolases?*

The N-terminal sequence of an endo-β-1,4-glucanase from the cellulose complex of the white rot fungus *Schizophyllum commune* has quite recently been determined (150). It was homologous with the active site sequences of various c type lysozymes. The sequence homology offers evidence of a lysozyme type mechnism in enzymic hydrolysis of cellulose; it is also remarkable since it implies a closer ancestral link between the endoglucanase and c type lysozymes than between the latter and other types of lysozyme (goose ad T4 phage). In this respect the case of endoglucanase is analogous to that of α-lactalbumin.

10.3. *A substrate-induced evolution*

In the past, the search for an evolutionary connection between proteins or enzymes of various species and phyla has been attempted through the comparison of primary or three-dimensional structures or immunological cross-reactivity. In all of these comparisons it has been tacitly assumed that the substrate of the enzyme or ligand of the binding protein has not undergone any major evolution of its own. The variation in composition and structure of the bacterial cell wall from a highly complex multi-layered peptidoglycan as in Gram-positive bacteria to the mono- or bi-layered peptidoglycan of Gram-negative bacteria and further to the relatively simple external cell wall of fungi and insects containing chitin, may have had a direct bearing on the differences found in the specificity of lysozymes from different sources. These changes in cell wall structure could have provided the impetus for a type of '*substrate-induced evolution*' of lysozymes, perhaps by the independent evolution of these enzymes from non-lysozyme protein progenitors (151).

The various lysozymes can be classified according to their specificity requirements. c type lyso-

Table 2. Substrate specificity requirements of lysozymes (151). Digestibility of substrate: +++, well-digested; ++, fair; +, poor; –, not digested. Substrate types: I, peptide-substituted glycan; II, unsubstituted glycan; III, $(GlcNAc)_n$ = oligosaccharides (n = 3–6) isolated from chitin.

Lysozyme source	Substrate types			presumed main natural substrate (type)
	I $(GlcNAc\text{-}MurNAc)_n$ | peptide	II $(GlcNAc\text{-}MurNAc)_n$	III $(GlcNAc)_n$	
c type	++	+++	++	I, II, (III)
g type	+++	++	±	I, II, (III)
Papaya	±	±	++	III
T4 phage	+++	–	–	I

zymes appear to act equally well on peptide-substituted or unsubstituted peptidoglycan and also on chitin oligosaccharides, although slower than on the linear peptidoglycan. g type lysozymes have a similar specificity on the linear peptidoglycan as the c type enzymes, but does not act on chitin oligosaccharides nor is inhibited by the latter (152, 153). Furthermore, in contrast to the c type lysozymes which are capable of both hydrolysis and transglycosylation, g type enzymes act only as hydrolases. The specificity requirements of the phage lysozymes are much more stringent as they digest glycosidic linkages next to peptide-substituted N-acetylmuramic acid residues. Plant lysozymes have a quite distinct specificity and present a high activity on chitin oligosaccharides and cleave the peptide-substituted peptidoglycan very poorly (75, 76) (Table 2).

11. Biological role of lysozymes

Lysozymes are widespread enzymes occurring in many human tissues and secretions (Table 3) (154) and in those of various other vertebrates, invertebrates as well as bacteria, phages and plants. However despite extensive studies devoted to their structure, catalytic mechanism, relationship between structure and activity, phylogeny, immunology and genetics, their functions in higher vertebrates are still open to question, with the exception of their probable antibacterial role following Fleming's

Table 3. Lysozyme activity in various human body fluids (154).

Kind of fluid	Number	Lysozyme activity mg/l ($\bar{x} \pm \sigma$)
Serum of healthy adults	85	9.8 ± 2.90
Serum of healthy children	20	10.8 ± 2.31
Serum of healthy newborn	33	10.6 ± 1.70
Serum of umbilical blood	20	8.5 ± 2.53
Urine of adults and children	96	0.5 ± 0.45
Cerebrospinal fluid (without pathological signs)	25	0.5 ± 1.30
Gastric juice (fasting)	35	74.2 ± 31.40
Bile (fraction B)	3	0.4 ± 0.41
Saliva	35	8.8 ± 2.97
Mothers' milk	21	65.0 ± 10.20
Amniotic fluids	10	9.3 ± 3.02
Sperm	10	13.4 ± 5.38
Tears	5	1267 ± 58
Transudates	7	14.9 ± 26.2
Pus	3	150 ± 40.4

original hypothesis that lysozyme plays a part in the body's defence against infection. The possibility that this enzyme may have other important physiological functions and will find practical applications has been repeatedly suggested. Distinction must be made however between enzymatic and non-enzymatic activities of lysozymes. It should be pointed out that most of the biological studies have been performed so far with hen lysozyme. However one should not generalize the role(s) of lysozyme(s) from one animal to the next (human and hen lysozymes do not share all their properties) or from one microbial species to the next or from one environment to the next.

11.1. *Lysozyme's enzymatic activity: role in the body's defence mechanisms*

The literature abounds with evidence pertaining to the functional significance of lysozyme in various organisms as a defence mechanism against bacteria and chitin-covered pathogens. Lysozyme in invertebrates may perhaps serve as a rudimentary protective system since these organisms do not produce immunoglobulins. The specificity of the lysozymes in this case is against the bacterial cell wall. The papaya and fig proliferate in climates that are favourable to fungal growth. A large number of phytopathogenic fungi posses chitin in their cell walls. It seems reasonable that the papaya enzyme would act mainly as a chitinase and in conjunction with the proteolytic enzymes of these fruits offer protection from invasion. The phage lysozyme, which requires a peptide-substituted substrate, would also appear to be uniquely adapted to its host, *E. coli*, in which all the N-acetylmuramyl residues are substituted with peptides (151).

Lysozyme exists in high concentrations in the egg-white of many birds and two classes of the enzyme, c and g types, have been characterized (52). Although these vertebrates all contain immunological systems, the egg and developing embryo do not produce immunoglobulins until about 7 days before hatching (155). It is therefore possible that the egg has a high lysozyme content to maintain protective vigilance until the embryo has the capability to produce immunoglobulins.

Because it occurs so widely, lysozyme is considered to be a constituent of primitive unspecific defence mechanisms associated with the monocyte–macrophage system, phylogenetically older than the more specific lymphocyte–plasma cell–immunoglobulin system (156). However, evidence is accumulating for a close functional relationship between lysozyme and immunoglobulins, a relationship related mainly but not exclusively to bacteriolysis (157). Lysozyme and secretory immunoglobulins show a similar distribution in various body fluids. Both IgA and lysozyme are present in abundance in such external secretions as tears, saliva, tracheobronchial secretions and gastric juice and in only small amounts in such internal secretions as serum, pleural fluid and cerebrospinal fluid.

The role of lysozyme as an antibacterial agent appears to be mediated through its direct bacteriolytic action (158), as well as via stimulatory effects on macrophage phagocytic function (159). The significant role of lysozyme in mediating the microbial action of rat alveolar macrophages has been demonstrated (158): phagocytosis of intact bacteria did not occur, whereas lysozyme-degraded bacteria were rapidly phagocytized. It has also been demonstrated that lysozyme may directly enhance phagocytic activity of polymorphonuclear leukocytes (160) and macrophages (159). Studies with cultured monocytic cells showed that the addition of high concentrations of lysozyme to the media resulted in an enhanced phagocytosis of tubercle bacilli (159, 161).

From a study devoted to the action of lysozyme on oral microorganisms, several important observations concerning its antibacterial properties could be deduced (162). The studies were performed with oral microorganisms representing the seven serotypes of *Str. mutans, Veillonella alcalescens* and the virulent and avirulant strains of *Actinomyces viscosus* T14. The results indicated that different mechanisms may be responsible for the bacteriostatic, lytic and bactericidal properties of lysozyme and that the enzyme is a selective but effective antibacterial factor for oral microorganisms.

11.2. *Lysozyme in association with other biologically active proteins*

The mechanism of lysozyme's role in the defence from pathogens, summarized above, remains to be fully explained: most probably it is intricately in-

terrelated with other components for full effectiveness (163). Among them should be mentioned: complement, lactotransferrin which inhibits the growth of certain microorganisms by chelating iron, antibodies (see also section 11.1) such as milk sIgA which potentiates the antibacterial activity of lactotransferrin (164). Moreover lysozyme, lacto (or ovo) transferrin and immunoglobulins are found together in several natural secretions such as saliva, tears, certain milks (165), bronchial mucus or egg-white.

11.3. *Lysozyme and immunostimulation*

Jollès (166) suggested in 1976 that a possible physiological function of lysozyme might consist of an indirect effect arising in the course of its action on bacteria. Indeed lysozyme alone or associated with other enzymes finds itself *in vivo* in various tissues and compartments of the body (such as the respiratory tract and the digestive tract) or in inflammation sites in presence of various bacteria (Gram-positive or Gram-negative). Simple and small digestion or split products (from peptidoglycans) as well as similar compounds with a somewhat higher molecular weight can thus arise; they belong to a group of naturally widespread substances which are able to exert an adjuvant or immunostimulating activity: stimulation of the production of antibodies against a variety of antigens, induction of delayed hypersensitivity to the same or other antigens, enhancement of resistance against bacterial or viral infection, mitogenic activity, etc.

11.4. *Lysozyme as a marker in the course of some diseases*

Determination of serum and/or urinary lysozyme is useful in the diagnosis of several diseases or as a marker substance in their development.

Serum lysozyme levels have intensively been determined in the diagnosis of leukemias. The normal serum lysozyme concentration varies from 7 to 20 μg/ml. In acute lymphoblastic leukemia the serum lysozyme level was significantly decreased (4 μg/ml) whereas in chronic myelogenous leukemia (32 μg/ml) as well as in acute monoblastic (122 μg/ml) and myelomonocytic (33 μg/ml) leukemias the serum lysozyme concentration was greatly increased (167, 168). Evaluation of serum lysozyme in sarcoidosis was reported to be useful in determination of this disease activity (169).

More generally, increased serum lysozyme activity might be due (170) to (a) diminished enzyme degradation (impaired renal glomerular filtration) or to (b) increased enzyme production provoked by benign myeloid hyperproliferation (such as acute bacterial infection or leukemoid reaction), malignant myeloid hyperproliferation (acite or chronic granulocytic leukemia, myeloid metaplasia), benign monocyte hyperproliferation (sarcoid and tuberculosis, monocytic leukemoid reaction), malignant monocyte hyperproliferation (acute or chronic monocytic leukemia, myelomonocytic leukemia).

Reduced serum lysozyme activity might be due to (a) increased enzyme excretion (proximal tubular renal disease) or (b) decreased enzyme production provoked by benign myeloid hypoproliferation (aplastic anemia) or malignant myeloid hypoproliferation (leukemic reticuloendotheliosis, acute lymphoblastic and lymphosarcoma leukemias, most aleukemic leukemias).

Only small quantities of lysozyme are normally found in urine (0.10 mg/l) (171) because of a very efficient tubular transport mechanism. Urinary lysozyme measurement is therefore helpful in evaluating patients with renal disease, especially that associated with tubular dysfunction (172). Urinary excretion of the enzyme was also followed in the diagnosis of renal allograft rejection (173). Patients sustaining severe burns excrete greater amounts of lysozyme than patients with mild burns and normal controls: a correlation was found between the severity of the clinical situation and the increase of lysozyme concentration in urine (174).

Lysozyme determinations have also been performed in two further secretions. The degeneration of the tear gland in kerato conjunctivitis sicca results not only in a decrease in tear fluid production, but also in a decrease in tear lysozyme concentration (175). The presence of the enzyme in the cerebrospinal fluid has been claimed to be a sensitive index of inflammatory and neoplastic diseases of the central nervous system (176).

11.5. *Lysozyme, a basic protein*

Lysozymes are usually basic proteins that may interact with a series of acidic compounds. Interactions with acidic mucous glycoproteins has been

reported (177): they may influence gel structure and consequently the rheologic and transport properties of tracheobronchial mucus.

The action of rennin, a rather acidic enzyme, on κ-casein (milk clotting process) seems to proceed in a different manner when lysozyme is added to cow milk which normally is devoid of this enzyme.

Interaction of lysozyme with the quite acidic, sulfated, proteoglycans has been observed by the group of Kuettner (178, 179) in the course of their studies devoted to lysozyme in cartilage and calcifying tissues: epiphyseal cartilage is especially rich in lysozyme and it has been suggested that the enzyme might play a role in the calcification of this tissue where a correlation of proteoglycan biosynthesis and lysozyme activity has been observed (180). In the presence of these acidic substances, lysozyme might play the role of a local buffer. The formation of complexes between lysozyme and heparin has been reported and was used for the purification of the enzyme by affinity chromatography (181). Finally, lysozyme as a basic protein, is able to bind to anions of inorganic origin.

11.6. *Miscellaneous, sometimes unexpected properties of lysozyme*

Lysozyme tends to associate into dimers and higher polymers as a function of pH, concentration and temperature (182). Their biological properties and occurrence *in vivo* have so far not been studied in detail.

The binding of drugs to lysozyme has also been reported (183): it was anticipated that anionic drugs would easily interact; this was not observed. The weakest acid of three sulpha drugs, i.e. sulphathiozole, had the highest affinity constant whereas the strongest acid, sulphisoxazole, has a rather low affinity constant; chloramphenicol, neutral over the pH range investigated had a very high affinity for the enzyme.

When lysozyme is used in association with other antibiotics, it is important to avoid aminoglycosidic antibiotics, such as neomycin B, gentamicin C_{1a}, kanamycin A or dihydrostreptomycin, the structure of which is related to the saccharidic substrate of the enzyme: indeed its lytic activity was pH-dependent inhibited by these compounds (184).

The antibiotics thiostrepton and polymyxin B as well as cardiotoxin II of the Indian cobra exert a powerful, but incomplete and irreversible, inhibitory action on the lysis of *M. luteus* cells by the enzyme (185).

During a Symposium held in Milan in 1964 (186) several communications were devoted to the fact that lysozyme relieved rheumatic fever and pain and had a therapeutic effect on rheumatoid arthritis. Later, on several occasions, lysozyme was claimed to possess analgesic properties (187, 188, 189). Antinociceptive activity of the enzyme was recently determined against abdominal contractions provoked by irritants injected intraperitoneally into rats (189) and mice (190). Some of the mentioned properties might not be due to lysozyme as such but might be related to some peptides obtained after (enzymic) degradation of the molecule.

11.7. *Pharmaceutical applications*

Lysozyme, almost exclusively from hen egg-white, has been used either alone or in addition to a series of other components such as various antibiotics (tetracyclin, bacitracin), enzymes (α-amylase, papain), vitamins, etc. The presence of lysozyme was claimed (191) to be efficient in the treatment of various bacterial or viral (zona, herpes zoster) infections, colitis, various pains, allergies, inflammations as well as in pediatrics (maternization of cow milk by addition of lysozyme). However, it should be pointed out that in most cases molecular pharmacological studies have not yet been performed.

References

1. Fleming A: Proc Roy Soc Ser B93:306–317, 1922.
2. Jollès J, Jollès P: CR Acad Sci Paris 253:2773–2775, 1961.
3. Jollès J, Jauregui-Adell J, Bernier I, Jollès P: Biochim Biophys Acta 78:668–689, 1963.
4. Canfield RE: J Biol Chem 238:2698–2707, 1963.
5. Blake CCF, Koenig DF, Mair GA, North ACT, Phillips DC, Sarma VR: Nature 206:757–761, 1965.
6. Phillips DC: Sci Am 215:78–90, 1966.
7. Osserman EF, Canfield RE, Beychok S (eds), Lysozyme. Academic Press, New York and London, 1974.
8. Piszkiewicz D, Brince TC: Biochemistry 7:3037–3047, 1968.
9. Oliver CN, Stadtman ER: Proc Natl Acad Sci USA 80:2156–2160, 1983.
10. Jollès J, Jollès P: FEBS Lett 162:120–122, 1983.
11. Bienkowska K, Taylor A: Eur J Biochem 96:581–584, 1979.
12. Turner GA, Ghneim H, Freeman J: Ann Clin Biochem 16:51–53, 1979.

13. Yamada H, Imoto T: Carbohydr Res 92:160–162, 1981.
14. Yamasaki N, Tsujita T, Takakuwa M: Agr Biol Chem 37:1507–1508, 1973.
15. Jollès P, Charlemagne D, Petit JF, Maire AC, Jollès J: Bull Soc Chim Biol 47:2241–2259, 1965.
16. Stelzner A, Süss J: Z ges Hyg 28:681–686, 1982.
17. Yuzuriha T, Katayama K, Tsutsumi J: Chem Pharm Bull 26:908–914, 1978.
18. Thomas MJ, Russo A, Craswell P, Ward M, Steinhardt I: Clin Chem 27:1223–1226, 1981.
19. Maeda H: J Biochem 88:1185–1191, 1980.
20. Jollès P, Bonnafé M, Mouton A, Schwarzenberg L: Rev franç Et clin biol 12:996–998, 1967.
21. Barthelemy J-P, Bachard F, Fiet J, Brière J, Dreux C: Clin Chim Acta 50:257–264, 1974.
22. Plonet J, Madec Y: Clin Chim Acta 93:51–60, 1979.
23. Bailon P, Nishikawa AH: Prep Biochem 7:61–87, 1977.
24. MacKay BJ, Iacono VJ, Zuckerman JM, Osserman EF, Pollock JJ: Eur J Biochem 129:93–98, 1982.
25. Szewczyk B, Kur J, Taylor A: FEBS Lett 139:97–100, 1982.
26. Boesman-Finkelstein M, Finkelstein RA: FEBS Lett 144:1–5, 1982.
27. Fernandez-Sousa JM, Perez-Castells R, Rodriguez R: Biochim Biophys Acta 523:430–434, 1978.
28. Gachon A-M, Kpamegan G, Dastugue B: Clin Chim Acta 129:189–195, 1983.
29. Kaneda M, Kato I, Tominaga N, Titani J, Narita K: J Biochem 66:747–749, 1969.
30. Prager EM, Arnheim N, Mross GA, Wilson AC: J Biol Chem 247:2905–2916, 1972.
31. Ibrahimi IM, Prager EM, White TJ, Wilson AC: Biochemistry 18:2736–2744, 1979.
32. La Rue JN, Speck JC: J Biol Chem 245:1985–1991, 1970.
33. Jollès J, Leemputten E van, Mouton A, Jollès P: Biochim Biophys Acta 257:497–510, 1972.
34. Hermann J, Jollès J: Biochim Biophys Acta 200:178–179, 1970.
35. Hermann J, Jollès J, Jollès P: Eur J Biochem 24:12–17, 1971.
36. Kondo K, Fujio H, Amano T: J Biochem 91:571–587, 1982.
37. Jollès J, Ibrahimi IM, Prager EM, Schoentgen F, Jollès P, Wilson AC: Biochemistry 13:2744–2752, 1979.
38. Jollès J, Schoentgen F, Jolles P, Prager EM, Wilson AC: J Mol Evol 8:59–76, 1976.
39. Jollès J, Jollès P: Helv Chim Acta 54:2668–2675, 1971.
40. Canfield RE, Kammerman S, Sobel JH, Morgan FJ: Nature New Biol 232:16–17, 1971.
41. Hermann J, Jollès J, Buss DH, Jollès P: J Mol Biol 79:587–595, 1973.
42. White TJ, Mross GA, Osserman EF, Wilson AC: Biochemistry 16:1430–1436, 1977.
43. Jollès P, Schoentgen F, Jollès J, Dobson DE, Prager EM, Wilson AC: J Biol Chem 259: in press, 1984.
44. Gavilanes JG, Gonzales de Buitrago G, Martinez del Pozo A, Perez-Castills R, Rodriguez R: Int J Peptide Prot Res 20:238–245, 1982.
45. Riblet R: In: Osserman EF et al. (eds), Lysozyme. Academic Press, New York, 1974, pp 89–95.
46. Jollès J, Sen A, Prager EM, Jollès P: J Mol Evol 10:261–264, 1977.
47. Jollès J, Schoentgen F, Croizier G, Croizier L, Jollès P: J Mol Evol 14:267–271, 1979.
48. Simpson RJ, Begg GS, Dorow DS, Morgan FJ: Biochemistry 19:1814–1819, 1980.
49. Schoentgen F, Jollès J, Jollès P: Eur J Biochem 123: 489–497, 1982.
50. Simpson RJ, Morgan FJ: Biochim Biophys Acta 744: 349–351, 1983.
51. Jollès P: Angew Chem intern Ed 8: 227–239, 1969.
52. Prager EM, Wilson AC, Arnheim N: J Biol Chem 249: 7295–7297, 1974.
53. Palmiter RD, Gagnon J, Ericsson LH, Walsh KA: J Biol Chem 252:6386–6393, 1977.
54. Wetmore A: Smithson Misc Collect 139:1–37, 1960.
55. Nolan RA, Brush AH, Arnheim N, Wilson AC: Condor 77:154–159, 1975.
56. Ibrahimi IM, Eder J, Prager EM, Wilson AC, Arnon R: Mol Immunol 17:37–46, 1980.
57. Fainaru M, Wilson AC, Arnon R: J Mol Biol 84:635–640, 1974.
58. Maron E, Arnon R, Sela M, Périn J-P, Jollès P: Biochim Biophys Acta 214:222–224, 1970.
59. Prager EM, Wilson AC: Personal communication, 1981.
60. White FH: Biochemistry 21:967–977, 1982.
61. Kato S, Okamura M, Shimamoto N, Utiyama H: Biochemistry 20:1080–1085, 1981.
62. Eshdat Y, Dunn A, Sharon N: Proc Natl Acad Sci USA 71:1658–1662, 1974.
63. Yamada H, Imoto T, Noshita S: Biochemistry 21:2187–2192, 1982.
64. Perkins SJ, Johnson LN, Machin PA, Phillips DC: Biochem J 181:21–36, 1979.
65. Dianoux AC, Jollès P: Biochim Biophys Acta 133:472–479, 1967.
66. Canfield RE, McMurry S: Biochem Biophys Res Commun 26:38–42, 1967.
67. Isaacs NW, Machin KJ, Masakuni M: 8th Eur Crystall Meeting, Liège, Abstract, 1983, p 25.
68. Arnheim N, Hindenburg A, Begg GS, Morgan FJ: J Biol Chem 248:8036–8042, 1973.
69. Jollès J, Schoentgen F, Jollès P: CR Acad Sci Paris 292:891–892, 1981.
70. Grütter MG, Weaver LH, Matthews BW: Nature 303: 828–831, 1983.
71. Inouye M, Imada M, Tsugita A: J Biol Chem 245: 3479–3484, 1970.
72. Matthews BW, Grütter MG, Anderson WF, Remington SJ: Nature 290:334–335, 1981.
73. Tufty RM, Kretsinger RH: Science 187:167–169, 1975.
74. Smith EL, Kimmel JR, Brown DM, Thompson EOP: J Biol Chem 215:67–80, 1955.
75. Howard JB, Glazer AN: J Biol Chem 244:1399–1409, 1969.
76. Bernier I, Leemputten E van, Horisberger M, Bush DA, Jollès P: FEBS Lett 14:100–104, 1971.
77. Fouche PB, Hash JH: J Biol Chem 253:6787–6793, 1978.
78. Morita T, Hara S, Matsushima Y: J Biochem 83:893–903, 1978.
79. Sarma R, Harada S, Tanaka N, Kakudo M, Hara S, Ikenaka T: J Biochem 85:1765–1771, 1979.
80. Fernandez-Sousa JM, Gavilanes JG, Municio AM, Perez-

Aranda A, Rodriguez R: Eur J Biochem 72:25–33, 1977.
81. Takeda H, Strasdine GA, Whitaker DR, Roy C: Can J Biochem 44:509–518, 1966.
82. Jollès P, Jollès-Thaureaux J, Fromageot: CR Soc Biol 151:1368–1369, 1957.
83. Jollès P, Zuili S: Biochim Biophys Acta 39:212–217, 1960.
84. Périn J-P, Jollès P: Mol Cell Biochem 2:189–195, 1973.
85. Jollès J, Jollès P: Eur J Biochem 54:19–23, 1975.
86. McHenery JG, Birkbeck TH, Allen JA: Comp Biochem Physiol 63B:25–28, 1979.
87. McHenery JG, Birkbeck TH: Marine Biol Lett 1:111–119, 1979.
88. Dobson DE: Evolution of ruminant lysozymes. PhD Thesis, Univ California, Berkeley, 1981.
89. Dobson DE, Prager EM, Wilson AC: J Biol Chem 259: (in press), 1984.
90. Wilson AC, Carlson SS, White TJ: Ann Rev Biochem 46:573–639, 1977.
91. Atassi MZ, Habeeb AFSA: In: Atassi MZ (ed), Immunochemistry of Proteins, Vol 2. Plenum, New York, 1977, p 177.
92. Benjamin DC, Berzofsky JA, East IJ, Gurd FRN, Hannum C, Leach SJ, Margoliash E, Michael JG, Miller A, Prager EM, Richelin M, Sercarz EE, Smith-Gill SJ, Todd PE, Wilson AC: Ann Rev Immun 2 (in press), 1984.
93. Nguyen-Huu MC, Stratmann M, Groner B, Wurtz T, Land H, Giesecke K, Sippel AE, Schütz G: Proc Natl Acad Sci USA 76:76–80, 1979.
94. Jung A, Sippel AE, Grez M, Schütz G: Proc Natl Acad Sci USA 77:5759–5763, 1980.
95. Blake CCF: TIBS 8:11–13, 1983.
96. Quasba PK, Safaya SK: Nature 308:397–380, 1984.
97. Gilbert W: Nature 271:501, 1978.
98. Tonegawa S, Maxam AM, Tizard R, Bernard O, Gilbert W: Proc Natl Acad Sci USA 75:1485–1489, 1978.
99. Blake CCF: Nature 273:267, 1978.
100. Go M: Nature 291:90–92, 1981.
101. Go M: Proc Natl Acad Sci USA 80:1964–1968, 1981.
102. Blake CCF: Nature 306:535–537, 1983.
103. Owen JE, Schultz DW, Taylor A, Smith GR: J Mol Biol 165:229–248, 1983.
104. Blake CCF, Mair GA, North ACT, Phillips DC, Sarma VR: Proc Roy Soc London B 167:365–377, 1967.
105. Blake CCF, Johnson LN, Mair GA, North ACT, Phillips DC, Sarma VR: Proc Roy Soc London B 167:378–388, 1967.
106. Joynson MA, North ACT, Sarma V, Dickerson RE, Steinrauf LK: J Mol Biol 50:137–142, 1970.
107. Hodson JM, Siecker LC, Jensen LH: Ann Crystallogr Ass Spring Meeting, Abstract J8, 1974.
108. Moult J, Yonath A, Traub W, Smilansky A, Podjorny A, Rabinovich D, Saya A: J Mol Biol 100:179–195, 1976.
109. Hogle J, Rao ST, Mallikarjunan M, Beddell C, Mc Mullan RK, Sundaralingam M: Acta Crystallogr B 37:591–597, 1981.
110. Artymiuk PJ, Blake CCF, Rice DW, Wilson KS: Acta Crystallogr B 38:778–783, 1982.
111. Artymiuk PJ, Blake CCF: J Mol Biol 152:737–762, 1981.
112. Sarma R, Bott R: J Mol Biol 113:555–565, 1977.
113. Aschaffenburg R, Blake CCF, Dickie HM, Gayen SK, Keegan R, Sen A: Biochim Biophys Acta 625:64–71, 1980.
114. Artymiuk PJ, Blake CCF, Grace DEP, Oatley SJ, Phillips DC, Sternberg MJE: Nature 280:563–568, 1979.
115. Matthews BW, Remington SJ: Proc Natl Acad Sci USA 71:4178–4182, 1974.
116. Sarma R, Kakudo M, Hara S, Ikenaka T: J Mol Biol 131:409–410, 1979.
117. Blake CCF, Grace DEP, Johnson LN, Perkins SJ, Phillips DC, Cassels R, Dobson CM, Poulsen FM, Williams RJP: In: Molecular Interactions and Activity in Proteins, Ciba Foundation Symposium 60, 1978, pp 137–185.
118. Ford LO, Johnson LN, Machin PA, Phillips DC, Tjian R: J Mol Biol 88:349–371, 1974.
119. Rossi GL, Huller E, Kumar S, Rupley JA, Hess GP: Biochem Biophys Res Commun 37:757–766, 1969.
120. Banerjee SK, Holler E, Hess GP, Rupley JA: J Biol Chem 250:4355–4367, 1975.
121. Kurachi K, Sieker LC, Jensen LH: J Mol Biol 101:11–24, 1976.
122. Chipman DM, Grisaro V, Sharon N: J Biol Chem 242: 4388–4394, 1967.
123. Rand-Meir T, Dahlquist FW, Raferty MA: Biochemistry 8:4206–4212, 1969.
124. Levitt M: In: Blout ER, Bovey FA, Foodman M, Lotan N (eds), Proceedings of the Rehovot Symposium on Poly Amino Acids, Polypeptides and Proteins and their Biological Implications. Wiley, New York, 1974, pp 99–113.
125. Warshel A, Levitt M: J Mol Biol 103:227–249, 1976.
126. Vernon CA: Proc Roy Soc London B 167:389–401, 1967.
127. Doonan S, Vernon CA, Banks BBC: Progr Biophys Mol Biol 20:249, 1970.
128. Warshel A: Proc Natl Acad Sci USA 75:5250–5254, 1978.
129. Patt SL, Baldo JH, Boekelheide K, Weisz G, Sykes BD: Can J Biochem 56:624–629, 1978.
130. Kelly JA, Sielecki AR, Sykes BD, James MNG, Phillips DC: Nature 282:875–878, 1979.
131. Pincus MR, Scheraga HA: Biochemistry 20:3960–3965, 1981.
132. Dahlquist FW, Raftery MA: Biochemistry 7:3269–3276, 1968.
133. Chatterjee N, Banerjee KK, Sen A: FEBS Lett 159: 171–174, 1983.
134. Blake CCF, Pulford WCA, Artymiuk PJ: J Mol Biol 167:693–723, 1983.
135. Douzou P, Hui Bon Hoa G, Petsko GA: J Mol Biol 96:367–380, 1975.
136. Warshel A: Proc Natl Acad Sci USA 81:444–448, 1984.
137. Jollès P, Berthou J: FEBS Lett 23:21–23, 1972.
138. Berthou J, Jollès P: Biochim Biophys Acta 336:222–227, 1974.
139. Berthou J, Jollès P: Biochimie 60:209–210, 1978.
140. Cozzone P, Opella SJ, Jardetzky O, Berthou J, Jollès P: Proc Natl Acad Sci USA 72:2095–2098, 1975.
141. Jollès P, Saint-Blancard J, Allary M, Périn J-P, Cozzone P: FEBS Lett 55:165–167, 1975.
142. Saint-Blancard J, Clochard A, Cozzone P, Berthou J, Jollès P: Biochim Biophys Acta 491:354–356, 1977.
143. Saint-Blancard J, Mazurier J, Bournaud M, Maurel PP,

Berthou J, Jollès P: Mol Biol Rep 5:165–169, 1979.
144. Saint-Blancard J, Maurel JP, Constant JF, Berthou J, Jollès P: Biosci Rep 1:119–123, 1981.
145. Berthou J, Laurent A, Lifchitz A, Jollès P, Artymiuk P, Rice DW: 4th Europ Crystallogr Meeting, Oxford, 1977.
146. Harada Y, Lifchitz A, Berthou J, Jollès P: Acta Crystallogr A 37:398–406, 1981.
147. Berthou J, Lifchitz A, Artymiuk P, Jollès P: Proc Roy Soc London B 217:471–489, 1983.
148. White TJ, Mross GA, Osserman EF, Wilson AC: Biochemistry 16:1430–1436, 1977.
149. Dobzhansky T, Ayala FJ, Stebbins GL, Valentine JW: Evolution. WH Freeman and Co, San Francisco, 1977.
150. Yaguchi M, Roy C, Rollin CF, Paice MG, Jurasek L: Biochem Biophys Res Commun 116:408–411, 1983.
151. Schindler M, Mirelman D, Sharon N: Biochim Biophys Acta 482:386–392, 1977.
152. Allen AK, Neuberger A: Biochim Biophys Acta 235: 539–541, 1971.
153. Jollès P, Saint-Blancard J, Charlemagne D, Dianoux A-C, Jollès J, Le Baron TL: Biochim Biophys Acta 151:532–534, 1968.
154. Hankiewicz J, Swierczek E: Clin Chim Acta 57:205–209, 1974.
155. Moore MAS, Owen JJT: Dev Biol 14:40–51, 1966.
156. Osserman EF: Adv Pathobiol 4:98–102, 1976.
157. Reitamo S, Klockars M, Adinolfi M, Osserman EF: La Ricerca Clin Lab 8:211–231, 1978.
158. Biggar WD, Sturgess JM: Infect Immunol 16:974–982, 1977.
159. Thacore H, Willet HP: Am Rev Resp Dis 93:786–790, 1966.
160. Klockars MI, Roberts P: Acta Haematol 55:289–292, 1976.
161. Kokosis PL, Williams DL, Cook JA, Di Luzio NR: Science 199:1340–1342, 1978.
162. Iacono VJ, MacKay BJ, Di Rienzo S, Pollock JJ: Infect Immunol 29:623–632, 1980.
163. Adinolfi M: In: Lambert HP, Wood CBS (eds), Immunological Aspects of Infection in the Fetus and Newborn. Academic Press, London, 1981, pp 19–47.
164. Spik G, Montreuil J: Bull Eur Physiopath Resp 19: 123–130, 1983.
165. Jorieux S, Mazurier J, Montreuil J, Spik G: Protides of the biological Fluids, Proc. 31st Coll., 1984.
166. Jollès P: Biomedecine 25:275–276, 1976.
167. Jollès P, Sternberg M, Mathé G: Isr J Med Sci 1:445–447, 1965.
168. Osserman EF, Lawlor DP: J Exp Med 124:921–952, 1966.
169. Selroos O, Klockars M: Scand J Resp Dis 58:110–116, 1977.
170. Finch SC, Castro O, Lippman ME, Donadio JA, Perrilie PE: In: Osserman EF, Canfield RE, Beychok SA (eds), Lysozyme. Academic Press, New York, London, 1974, pp 335–345.
171. Houser MT: Clin Chem 29:1488–1493, 1983.
172. Zager RA: Invest Urol 18:197–202, 1980.
173. Ellis L, McSwiney RR, Tucker SM: Ann Clin Biochem 15:253–260, 1978.
174. Saint-Blancard J, Allary M, Drévillon L, Périn, J-P, Monteil R, Guilbaud J: Ann pharm franç 32:257–264, 1974.
175. Janssen PT, Bijsterveld OP van: Clin Chim Acta 121: 251–260, 1982.
176. Di Lorenzo N, Palma L, Ferrante L: Neurochirurgia 20:19–22, 1977.
177. Creeth JM, Bridge JL, Horton JR: Biochem J 181: 717–724, 1979.
178. Kuettner KE, Eisenstein R, Sorgente N: In: Osserman EF, Canfield RE, Beychok S (eds), Lysozyme. Academic Press, New York, London, 1974, pp 399.
179. Kuettner JE, Eisenstein R, Sorgente N: Clin Orthop 112:316–320, 1975.
180. Schmidt A, Rodegersdts U, Buddecke E: Calcif Tiss Res 26:163–172, 1980.
181. Boschetti E, Girot P, Secheresse JP, Saint-Blancard J: J Chromatogr 210:469–475, 1981.
182. Sophianopoulos AJ, Holde KE van: J Biol Chem 239: 2516–2524, 1964.
183. El-Nimr A, Hardee GE, Perrin JH: J Pharm Pharmacol 33:117–118, 1981.
184. Fernandez-Sousa JM, Gavilanes JG, Municio AM, Rodriguez R: Biochem Biophys Res Commun 75:895–900, 1977.
185. Shashidharan P, Ramachandran LK: Indian J Biochem Biophys 20:132–135, 1983.
186. Third Int Symp on Fleming's lysozyme. Museo della Scienza e della Tecnica, Milan, 1964.
187. Scolari EG: Médecine et Hygiène 22:983, 1964.
188. De Donder C, Morias J: Medikon 3:19–20, 1974.
189. Bianchi C: Eur J Pharmacol 71:211–221, 1981.
190. Bianchi C: Clin Exp Pharmacol Physiol 10:45–52, 1983.
191. Saubusse M: Critical Data Concerning the Therapeutic Use of Lysozyme. Med Dr Thesis (N° 42.55.76), Univ Paris V, 1976.

Received 11 April 1984.