THE ACTION OF EGG WHITE LYSOZYME ON OVOMUCOID AND OVOMUCIN

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Three fractions may readily be observed in the white of a hen's egg, (a) an outer layer of thin or liquid white, (b) a middle sac of thick white which encloses (c) an inner portion of thin white surrounding the yolk. According to SHARP[1] there is also a very thin layer of gelatinous white surrounding the yolk and continuous with the chalazae.

Thick egg white has a much higher mucin content than the thin white fractions (MCNALLY[2]) and the gel nature ot the thick white is due to the highly swollen condition of the mucin fibres. During storage of eggs in shell this gel structure breaks down, the thick white decreases both in amount and firmness and there is a simultaneous increase in the amount of thin white. The extent of this change is governed mainly by the temperature and time ot storage but is influenced to some extent by other factors, for example, pH. BALLS AND HOOVER[3] have shown that after thinning of the thick white has occurred the total mucin can be recovered from the thin white.

Egg white has the property of causing lysis of certain microorganisms (LASCHTSCHENKO[4]); FLEMING[5] established the enzymatic nature of the lytic agents in egg white and other biological fluids and applied to them the term "lysozyme". Egg white lysozyme has been shown[6] to be identical with the globulin designated G_1 by LONGSWORTH, CANNAN AND MACINNES[7].

Lysis of susceptible organisms has been shown by MEYER, PALMER, THOMPSON AND KHORAZO[8] and by EPSTEIN AND CHAIN[9] to be accompanied by an increase in reducing substances in the solution and the work of these authors and of MEYER AND HAHNEL[10] confirmed the suggestion of HALLAUER[11] that the action of the lysozyme is on the mucoid constituents of the organisms. MEYER and his co-workers also claimed to have demonstrated an increase in reducing substances when lysozyme from *Sarcinae* acted on ovomucoid fractions, and indicated that the primary change produced by the lysozyme probably consists in a depolymerisation of the substrate.

These observations suggested the possibility of egg white lysozyme being involved in the breakdown of the thick white gel referred to above. The experiments reported in this paper have failed to show any action of egg white lysozyme on ovomucoid but have demonstrated a rapid destruction of the gel structure of mucin by strong lysozyme solutions.

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MATERIAL AND METHODS

Lysozyme

Lysozyme was prepared from the white of fresh eggs by the method of ALDERTON AND FEVOLD[12], and recrystallised from dilute acetic acid as described by these authors. Before use the solutions were dialysed against running distilled water for 48–72 hours until free from reducing substances. The slight precipitate which separated during dialysis was discarded.

Mucoid

Samples of ovomucoid were prepared (a) as described by MEYER, THOMPSON, PALMER AND KHORAZO[13], and (b) by the method of MEYER, PALMER, THOMPSON AND KHORAZO[8] and (c) by the method usually employed in this laboratory which is a slight modification of that described by YOUNG[14]. These samples gave homogeneous electrophoretic patterns and contained 13.4–13.6% nitrogen.

Mucin

The majority of the experiments were carried out with mucin prepared as follows: white from fresh eggs, strained through muslin to remove chalazae, particles of shell etc. was finely minced to break up the thick white and poured into three times its volume of water. The precipitate was collected and repeatedly washed with 2% potassium chloride solution until the recovered potassium chloride solution gave no opalescence with trichloracetic acid. The mucin, now in a swollen state, was then washed several times with water to remove the bulk of the salts.

Some experiments were carried out with samples further washed with water containing a few drops of hydrochloric acid, dilute sodium bicarbonate solution, water, and finally dried with alcohol and ether. The dried material had a nitrogen content of 12.2–12.3% and readily swelled in water to a translucent mass.

Micrococcus lysodeikticus

The organism was grown on nutrient agar (Lemco) in Roux bottles. When required the cells were washed off the agar with distilled water, the suspension filtered through glass wool and dialysed against running distilled water till free from dialysable reducing substances.

Reducing substances

These were estimated by the HAGEDORN AND JENSEN ferricyanide method and are expressed in terms of equivalent amount of glucose. Ovomucoid was incompletely precipitated by the zinc sulphate-sodium hydroxide procedure and inconsistent results were obtained. In experiments with ovomucoid, therefore, zinc hydroxide precipitation was omitted (cf. MEYER *et al.*[8]). The ovomucoid solutions even after prolonged dialysis had considerable reducing power, presumably due to reducing groups in the protein.

EXPERIMENTAL RESULTS

In all experiments the activity of the lysozyme solutions used was estimated by the increase in reducing substances following lysis of *M. lysodeikticus*. The figures obtained in a typical experiment are shown in Table I.

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TABLE I

INCREASE IN REDUCING SUBSTANCES FOLLOWING LYSIS OF *M. lysodeikticus*

Time at 37° C (hours)	mg reducing substances (as glucose) per ml			
	Lysozyme	*Micrococcus*	Lysozyme + *Micrococcus**	
			Not lysed**	Lysed
0	0.01	0.12	0.13	0.14
5	0.02	0.14	0.14	0.38
24	0.01	0.12	0.12	0.39

* 9 ml *Micrococcus* suspension (45 mg/ml dry weight) plus 2 ml lysozyme solution (2.5 mg/ml dry weight) p_H 6.0.
** Calculated the from values determined or lysozyme and *micrococcus* solutions separately.

Action of lysozyme on ovomucoid

The effect of lysozyme on numerous ovomucoid preparations was tested under a variety of conditions and particularly those described by MEYER *et al.* The results of three experiments are shown in Table II and it is clear that no increase in reducing substances resulted from the action of the lysozyme.

TABLE II

mg REDUCING SUBSTANCES PER ml OF MIXED MUCOID + LYSOZYME*

Method of preparation of mucoid	Time (hours) at 37° C		
	0	5	24
MEYER *et al.*[8]	0.41	0.41	0.42
MEYER *et al.*[13]	0.61	0.60	0.61
L.T.R.S.	0.63	0.62	0.62

* Equal volumes of 1% mucoid and 1% lysozyme solution p_H 6.0.

Further, ovomucoid solutions after incubation with lysozyme for 24 hours at 37° C gave negative tests for amino-sugars[15] and there was no change in the viscosity.

Action of lysozyme on ovomucin

The swollen mucin "gel" prepared as described above could be suspended in *M*/20 phosphate buffer, p_H 7.0, without visible change; on gentle centrifuging the mucin separated as a clearly defined jelly-like layer. Addition of active lysozyme to the mucin in phosphate buffer led to a rapid and marked change. The translucent mucin layer first became opaque, then changed into a stringy compact mass, which usually floated to the surface of the liquid. On centrifuging, this material sedimented as an opaque precipitate of smaller volume than the original mucin, centrifuged under identical conditions. Lysozyme inactivated by heat had no action on the mucin. The appearance of the solution in a typical experiment is shown in Fig. 1, the volumes of the precipitates in another experiment are recorded in Table III.

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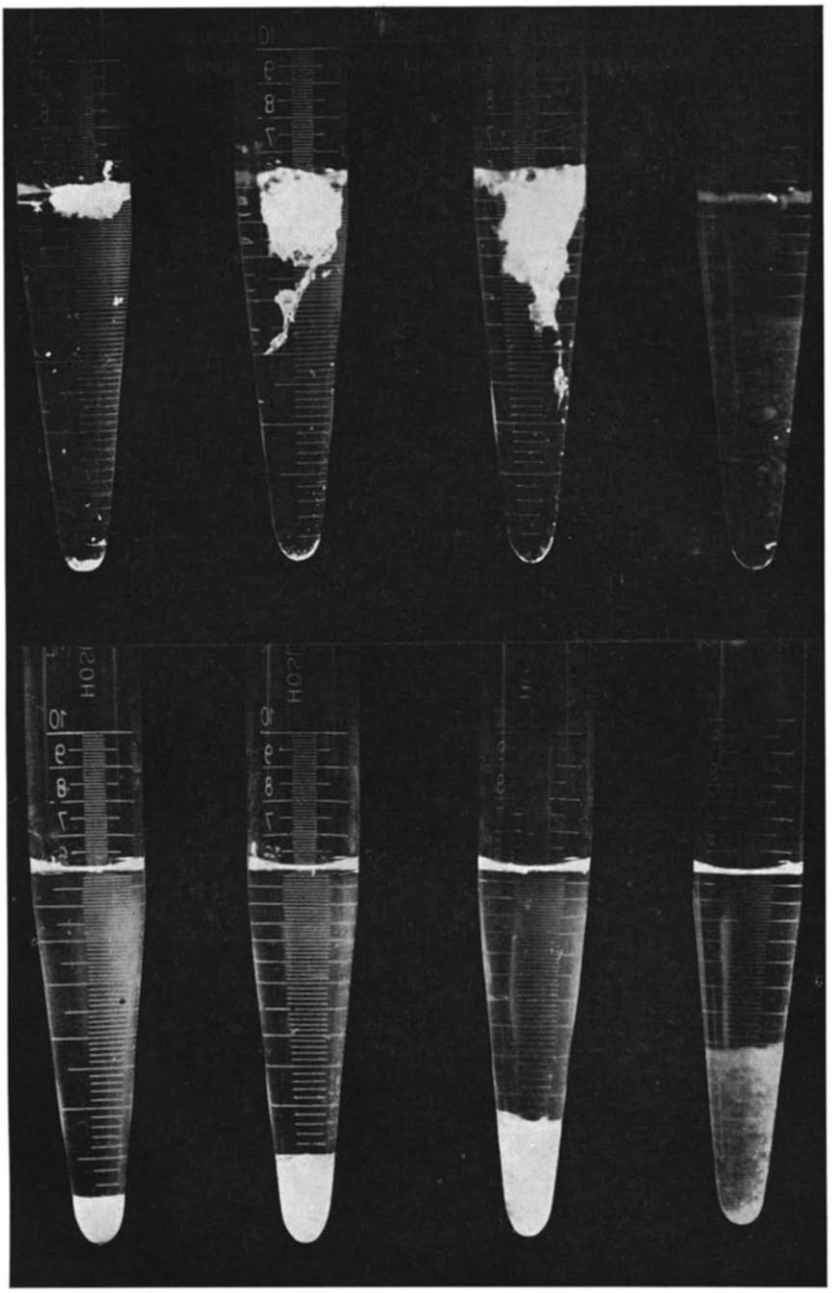


a b c d

Fig. 1. Effect of lysozyme on ovomucin. The tubes contained 2 ml swollen mucin, 2 ml $M/20$ phosphate buffer, p_H 7, and (a) 2 ml (b) 1 ml (c) 0.5 ml of 1% solution of active lysozyme (d) 2 ml of heat inactivated lysozyme solution. Top: not centrifuged. Bottom: centrifuged.

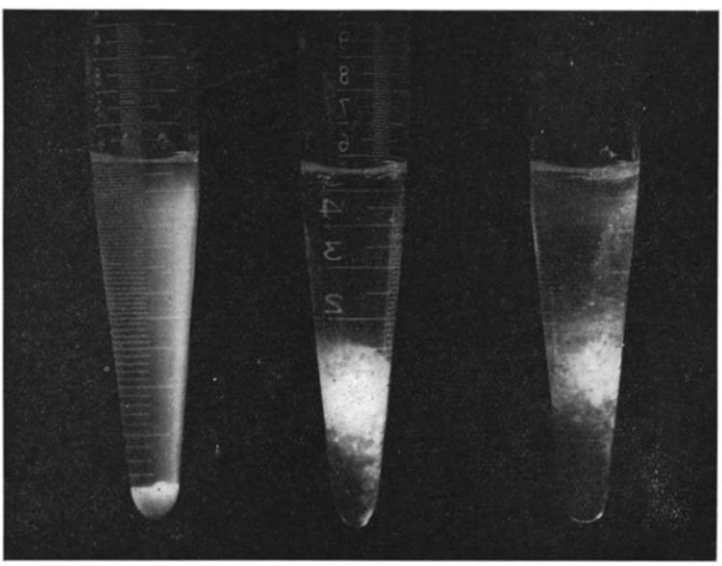
TABLE III

Lysozyme added (ml)	Volume of precipitate after centrifuging (ml)
1.5 active	Floated on surface
1.0 ,,	0.25
0.75 ,,	0.35
0.5 ,,	0.60
0.25 ,,	0.90
2.0 inactive	1.69

2 ml mucin gel (21 mg/ml dry weight)
2 ml phosphate buffer pH 7, 1% lysozyme solution
Total volume 6 ml. Incubated 1 hour at 25° C.

The contraction in volume of the mucin "gel" is proportional to the quantity of active lysozyme added, as is shown in Table III. This was also demonstrated in the following experiment in which the lysozyme was partially inactivated (or precipitated) by heating, the remaining activity being measured by following the lysis of *M. lysodeikticus*:

Replicate tubes containing 1% lysozyme solution in M/20 phosphate buffer, pH 7, were placed at 3 minute intervals in a boiling water bath. After a maximum of thirty minutes heating the tubes were removed, cooled and centrifuged. Aliquots of 0.25 ml of the supernatants were added to 2 ml of *M. lysodeikticus* suspension (11 mg/ml dry weight) and a second aliquot of 2 ml added to 2 ml mucin (20 mg/ml dry weight). After 1 hour's incubation at 37° C (1) lysis of the micrococcus was determined by measuring the transmission of light through the suspension (Spekker photoelectric absorptiometer,



a b c

Fig. 2. Effect of lysozyme on thick egg white. The tubes contained 2 ml thick white, 2 ml *M*/20 phosphate buffer, pH 7, and (a) 2 ml of 1% active lysozyme solution (b) 2 ml of heat inactivated lysozyme solution (c) no lysozyme. Centrifuged.

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2 mm cell, H508 filters) and by estimating the total reducing substances formed and (2) the mucin was centrifuged and the volumes of the lower layers recorded.

The results are shown in Table IV and it is clear that decrease in lytic action on *M. lysodeikticus* is parallel to the decrease in the action on mucin.

TABLE IV

EFFECT OF HEATED LYSOZYME ON *M. lysodeikticus* AND ON MUCIN

Time of heating lysozyme solution (mins)	Volume of centrifuged mucin (ml)	*Micrococcus lysodeikticus*	
		Log. I_0/I	Reducing substances (mg/ml)
0	0.3	0.36	0.25
3	0.9	0.54	0.17
6	1.3	0.83	0.14
9	1.5	1.10	0.13
12	1.7	1.35	0.11
15	1.8	1.44	0.10
18	1.7	1.45	—
21	—	1.56	0.10
24	—	1.60	—
30	—	1.58	0.10

Chemical Changes

After incubation of mucin with lysozyme as described in the above experiments no increase in free reducing sugars (HAGEDORN AND JENSEN method) nor in the total carbohydrate (orcin method of HEWITT[16]) in the supernatant solution could be detected. Tests for amino-sugars (ELSON AND MORGAN[15]) were negative and after addition of 3 volumes of alcohol to precipitate soluble proteins no ninhydrin reaction was observed.

Action of Lysozyme on Thick Egg White

The mucin preparations used in this work were free from lysozyme and remained apparently unchanged on incubation at 37° C for several days. Thick white, in contrast, has a marked lytic action on *M. lysodeikticus* and the separated thick white thins rapidly; the rate of thinning is increased by any manipulation. Attempts to demonstrate an increase in the rate of thinning in presence of added lysozyme by measuring the "viscosity" of a fine dispersion of thick white in buffer gave inconsistent results. With strong solutions of lysozyme an effect on thick white gel similar to that with isolated mucin can be obtained; this is shown in Fig. 2.

Recovery of Lysozyme from the Mucin Precipitate

FLEMING[5] showed that on addition of lysozyme to a suspension of a susceptible organism the lysozyme is rapidly fixed on to the cells; BOASSON[17] further demonstrated that during lysis there is no inactivation of the lysozyme but that it may be recovered *in toto* from the solution of lysed organism. KLOTZ AND WALKER[18] have demonstrated that lysozyme can form complexes with other materials. That lysozyme can be recovered from the compact precipitate which separates when lysozyme is added to swollen mucin was shown in the following manner:

Swollen mucin prepared as already described was washed with 2% potassium chloride solution. Neither the mucin nor the recovered potassium chloride solution had

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any lytic action on *M. lysodeikticus*. 4 ml of the washed mucin was then added to 5 ml of 0.1% solution of lysozyme and after 1 hour's incubation at 37° C the precipitate was separated by centrifuging and the supernatant (S_1) put aside. The precipitate was washed twice with 5 ml of water and the supernatants (S_2, S_3) preserved. The washed precipitate was then stirred with 5 ml of 2% potassium chloride solution; the opaque material became translucent and swelled somewhat though not to the original volume of mucin added. The potassium chloride solution (S_4) was again separated from the precipitate. S_1, S_2 and S_3 were then made 2% with respect to potassium chloride and to 1 ml of each of the four solutions S_1–S_4 was added 1 ml of a suspension of *M. lysodeikticus* and the mixtures incubated at 37° C. After 15 minutes lysis was determined by measuring the turbidity of the solutions with a Spekker absorptiometer (2 mm cell, H508 filters). The results are given in Table V and it is clear that lysozyme was extracted from the precipitate by washing with 2% potassium chloride solution.

TABLE V

Solution added to *M. lysodeikticus* suspension	Log I_0/I
Nil	0.65
S_1	0.125
S_2	0.50
S_3	0.55
S_4	0.148

DISCUSSION

The spontaneous breakdown of the mucin gel in egg white has engaged the attention of several workers but the cause of the change is still unknown. Evidence suggesting that the responsible agent is a proteolytic enzyme[19] has been shown to be invalid (BALLS AND HOOVER[3], VAN MANEN AND RIMINGTON[20]) and it seemed that the change might be another example of the colloidal syneresis which occurs in such relatively simple gels as those of dibenzoyl cystine or dilute silica. The results of the present work suggest a different explanation although no direct proof has been obtained that lysozyme is wholly or partly responsible for the shrinkage of thick white in the intact egg.

The chemical changes which accompanied the lysis of *M. lysodeikticus* were not apparent during the destruction of a mucin gel *in vitro* and there is therefore no direct evidence at present that lysozyme acts as an enzyme in the latter case; if it does it would seem that a different substrate and mode of action are involved in the two cases. It is possible however that the structural change is caused by a protein-protein interaction of somewhat similar type to the fibrinogen-fibrin or myosin-actin interactions and that further search might reveal the participation of specific chemical groups.

There is some slight evidence that the change is not caused by the weakening of the attractive forces (usually believed to be of the coulombic or Van der Waals type) at the points of contact in the gel structure by the strongly basic lysozyme. With a non-specific electrostatic interaction of this type it would be expected after removal of the major part of the lysozyme from the coagulum by washing with 2% potassium chloride solution that the mucin would swell to its original volume. The fact that only a partial swelling occurs may indicate that the structural elements have been permanently changed.

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SUMMARY

1. No increase in reducing substances, decrease in viscosity or production of amino-sugars was observed when ovomucoid preparations were incubated with egg white lysozyme.

2. Addition of lysozyme to swollen ovomucin resulted in the separation of a compact precipitate of mucin plus lysozyme.

3. The data suggest that the thinning of the thick white gel which occurs during storage of eggs may, in part, be due to the action of lysozyme on mucin.

RÉSUMÉ

1. Quand des préparations d'ovomucoid sont incubées avec du lysozyme de blanc d'œuf, on n'observe aucune augmentation de substances réductrices, aucune diminution de la viscosité et aucune production de sucres aminés.

2. L'addition de lysozyme à l'ovomucine gonflée conduit à la séparation d'un précipité compact de mucine et de lysozyme.

3. Ces résultats semblent montrer que la liquéfaction du gel de blanc d'œuf, observée pendant l'entreposage des œufs, est due, partiellement au moins, à l'action du lysozyme sur la mucine.

ZUSAMMENFASSUNG

1. Bei der Bebrütung von Ovomucoid-präparaten mit Lysozym aus Eiklar wurde keine Zunahme der reduzierenden Substanzen, keine Abnahme der Viskosität und keine Entstehung von Aminozuckern beobachtet.

2. Ein dichter Niederschlag von Mucin plus Lysozym entstand nach Zufügen von Lysozym zu gequollenem Ovomucin.

3. Diese Beobachtungen lassen vermuten, dass die Verdünnung des dicken Eiklars, die während der Lagerung von Eiern stattfindet, teilweise durch die Einwirkung von Lysozym auf Mucin erfolgt.

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