THE PURIFICATION AND PROPERTIES OF LYSOZYME

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Fleming (1) in 1922 reported the occurrence of a bacteriolytic principle, lysozyme, in egg white, tears, and other animal fluids. Since then much work has been devoted to its bacteriological behavior, but the only work of importance on its purification and chemical nature was that of Wolff (2). Wolff precipitated diluted egg white with colloidal iron, evaporated the filtrate to a small volume, precipitated the lysozyme with acetone, and dialyzed the aqueous solution of the precipitate. The last two steps were repeated several times and finally a white powder was obtained, soluble in water, but insoluble in the common organic solvents. It was reported to give no Molisch reaction and to be free of sulfur, phosphorus, and nitrogen, although the biuret reaction was weakly positive.

In the present work an attempt was made to obtain the lysozyme from egg white in a purified form and to study its nature.

Preparation—The starting material for purification was a dry powder obtained by precipitation of native egg white with 9 volumes of ice-cold acetone. After the mixture had stood cold overnight, the precipitate was filtered off, washed well with acetone and ether, and dried in a heated vacuum desiccator over P₂O₅. Usually 14 to 15 per cent of the total weight of the egg white was thus obtained as a dry white powder with no loss of potency. Acetone precipitation at the original alkaline pH of egg white renders the greater part of the proteins insoluble, while the lysozyme retains its solubility in aqueous media.

In a procedure similar to that of Wolff, 40 gm. lots of the egg white powder were extracted with three 500 cc. portions of 0.9
per cent NaCl. To the combined extracts 120 cc. of commercial colloidal iron were added. If any colloidal iron remained in the solution after centrifuging, it was brought down with solid K$_2$HPO$_4$. The lysozyme in the supernatant liquid was then precipitated almost quantitatively by flavanic acid. However, the losses by adsorption on the iron precipitate were great, and the procedure was abandoned.

In the method finally adopted, 10 gm. lots of the egg white powder were extracted with 200 cc. of 50 per cent alcohol containing 10 per cent acetic acid at 60-70° for 20 to 30 minutes. These conditions were not injurious to the enzyme. The cooled mixture was filtered, and the filtrate evaporated under reduced pressure to a small volume. This was taken up in water and precipitated with 5 volumes of alcohol. The mixture stood cold overnight, and the precipitate was removed, washed with alcohol, taken up in 100 cc. of slightly alkaline water, and acidified with H$_2$SO$_4$ to maximal precipitation. The lysozyme was then precipitated from the supernatant solution by flavanic acid. The mixture stood cold for 24 to 48 hours, and the yellow precipitate was centrifuged off and washed abundantly with alcohol until the washings were colorless. The flavianate was in some instances dissolved in water made just pink to phenolphthalein with NaOH, then reprecipitated with dilute H$_2$SO$_4$ in the presence of a trace of flavanic acid. From the alcohol-wet precipitate the dye was removed by repeated extractions with 200 cc. portions of ice-cold 90 per cent alcohol containing 0.5 per cent NH$_3$. The residue was washed free of ammonia with alcohol and ether and dried. The yield was 100 to 150 mg. from 10 gm. of egg white powder; the activity was 2000 to 6000 units per mg.

1 Flavanic acid was previously used successfully in the purification of hypophyseal hormones. Its removal by alcoholic ammonia was worked out at that time (3).

2 The following procedure was employed in determining activity. Progressive dilutions (0.5 cc.) of the preparations in 0.9 per cent NaCl were mixed with 0.5 cc. of a saline suspension of the test organisms and incubated at 37°. Readings were usually made after 1 hour and 18 to 20 hours, the latter reading being regarded as more significant. The highest dilution where complete lysis occurred was taken as the end-point, although partial lysis was usually evident at much higher dilutions. The unit of activity was defined as the smallest amount of lysozyme causing complete
A further concentration was obtained by extraction with small amounts of water acidulated with acetic acid, followed either by reprecipitation with flavianic or picrolonic acid or by evaporation in a high vacuum over \( P_2O_5 \) while frozen. The flavianate or picrolonate could be further purified by recrystallization from 50 per cent aqueous pyridine. In this way, preparations of crystalline appearance have been obtained with an activity of 32,000 units per mg. Efforts to devise a standard procedure for crystallization met with failure.

The principal contaminant of the different lysozyme preparations was an egg mucoid; the mucoid, however, was not precipitated by flavianic acid, and could be easily obtained from the flavianic acid supernatant solution by alcohol precipitation. Such a precipitate contained about 11 per cent nitrogen and 25 per cent reducing sugar after hydrolysis, and was almost devoid of any lysozyme activity.

Properties—The purest lysozyme preparations were basic in nature, being soluble only in acidified aqueous media and insoluble in pure organic solvents. They contained about 15 per cent nitrogen, a small amount of sulfur present as sulfhydryl, and a small amount of phosphorus. A highly purified preparation which appeared crystalline under the polarizing microscope had the following composition (in per cent): C 48.65, H 6.44, N 15.33, ash 3.31, P 0.25, S 0.64. With phosphorus and sulfur as a basis, the minimum molecular weight is about 25,000.

The biuret, glyoxylic acid, Greenberg phenol (5), and nitroprusside reactions were positive, and the Molisch negative. Bromine in glacial acetic acid was readily decolorized. Lysozyme solutions were not precipitated by trichloroacetic or sulfosalicylic acids and only incompletely by tungstic acid. Perchloric acid
Lysozyme

precipitated the enzyme. Some salts of heavy metals (as gold and silver) precipitated lysozyme with simultaneous inactivation. Lysozyme is very stable toward heat and acid (6). Solutions in 2 per cent acetic acid were kept at 100° for 45 minutes with no loss of lytic activity. Neutral preparations treated in this way lost all activity. At pH 9, heating for 5 minutes at 100° destroyed most of the activity. At room temperature, treatment with 0.01 N NaOH for 10 minutes lowered the activity from 3000 to 80 units per mg.

**Table I**

Reversible Inactivation of Lysozyme by Iodine and Cuprous Oxide; Reactivation by Hydrogen Sulphide

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Final dilution of mixtures, 1 to</th>
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<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>A</td>
<td>4</td>
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<tr>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
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<td>D</td>
<td>4</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
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The destructive action of peroxides was observed during work with an ordinary vacuum-distilled sample of dioxane as a precipitant; the activity was completely destroyed. After the removal of peroxides (7), the dioxane had no deleterious effect.

The sensitivity of lysozyme to alkali and peroxide suggested the necessity of an intact sulfhydryl group in the molecule. Furthermore, iodoacetic acid inactivated the enzyme. Soziodolic acid, used by Ackermann (8) for the crystallization of amines, gave in acid solution a complete precipitation of the ferment; a solution of the precipitate was inactive. Precipitation and inactivation
were also obtained when lysozyme solutions were iodized with iodine in potassium iodide. Cuprous oxide, which reacts with sulfhydryl groups to form mercaptides (9), decreased the activity of lysozyme. The inactivation by iodine or cuprous oxide was at least partially reversible, as Tables I and II indicate.

In Table I hydrogen sulfide was the reactivating agent; in Table II, sulfite. The latter experiment further differed in that the only variant throughout the tubes of a given series was the lysozyme concentration. In the second experiment, after incubation 1 drop of 40 per cent NaOH was added to each tube to clear up the turbidity imparted to the lysozyme solution by the more concentrated iodine; the alkali had no effect on the appearance of the organisms alone. Hydrogen cyanide was also found partially to reactivate iodine-inactivated preparations.5

## Table II

### Reversible Inactivation of Lysozyme by Iodine; Reactivation by Sulfite

Each series contained 0.25 cc. of progressive dilutions of lysozyme with the following additions.

<table>
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<tr>
<th>Addition</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>0.25 cc. 2.5% KI + 0.25 cc. saline + 0.75 cc. test organisms</td>
</tr>
<tr>
<td>B</td>
<td>0.25 cc. 0.1 N I₂ in 2.5% KI + 0.25 cc. saline + 0.75 cc. test organisms</td>
</tr>
<tr>
<td>C</td>
<td>0.25 cc. 0.1 N I₂ in 2.5% KI + 0.25 cc. 9% Na₂SO₃ (neutralized with HCl) + 0.75 cc. test organisms</td>
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In Mixtures B and C, the I₂ solution was allowed to act for 10 minutes before further additions were made. Reading made after 1 hour at 37°. 4 indicates complete clearing; 0 indicates no lysis. A control of 0.50 cc. of saline + 0.25 cc. of sulfite + 0.75 cc. of test organisms showed no lysis.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Final dilution of lysozyme solution, 1 to</th>
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<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>A</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
</tr>
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DISCUSSION

With the process of purification described a substance was obtained with the properties of a basic polypeptide having 15.3 per

5 Attempts to attack otherwise non-susceptible bacteria with lysozyme activated with HCN have been unsuccessful.
cent nitrogen. Wolff (2) claimed that lysozyme was nitrogen-free, but gave a weak biuret reaction. His purification method consisted in the removal of impurities with colloidal iron and precipitation by acetone. In our experience the use of colloidal iron caused no change in the nitrogen content of the product. Wolff's preparations, like ours, gave no Molisch reaction and no reducing sugar after hydrolysis. In his first paper (2) he stated that lysozyme was destroyed by tryptic digestion, but subsequently he reported that a 3 hour incubation with trypsin did not destroy it. It is difficult to conceive a nitrogen-free, non-sugar neutral substance of high molecular weight, soluble in aqueous solutions only and insoluble in all organic solvents. Wolff stated, however, that lysozyme was extracted from aqueous solutions by vegetable oils and that it was very difficult to remove the lysozyme from the oil. On repeating this, we found lysozyme in the interphase, but it could easily be reextracted with a concentrated NaCl solution after dilution with petroleum ether.

It may be of interest to compare the potency of our preparations with those of Wolff. The latter used a different organism (Micrococcus lysodeikticus) and a different incubation temperature (56°) which he found to be the optimum. Native egg white had in Wolff's test a potency about 40 times greater than in our test. Allowing for this factor and calculating in our terms, his best preparations had an activity of 20,000, 6,400, and 11,300 units per mg.; that is, about the same as our purest, 16,000 to 32,000.

According to Shwachman, Hellerman, and Cohen (10), pneumococcus hemolysin can be reversibly oxidized and reduced, the oxidized form being inactive. We found the same to be true for lysozyme. This suggests the possible rôle of peroxide in many bacteria as a defense against lytic agents. It may also throw light on the decreased resistance to infection in avitaminosis A in which it seems to be difficult for the body to maintain substances in the reduced form.6

SUMMARY

The preparation of lysozyme from acetone-dried egg white was studied. Lysozyme can best be freed from the tenaciously ad-

6 It has been shown that the lysozyme content is greatly diminished in the tears of experimental animals (11) and human patients (12) deficient in vitamin A; the keratomalacia disappears following local treatment with lysozyme.
hering mucoid in the extracts by precipitation with flavianic acid. Lysozyme is apparently a basic polypeptide, having a nitrogen content of 15.3 per cent and giving a number of protein reactions. From the presence of sulfhydryl, its inactivation by alkali, peroxide, iodine, and cuprous oxide, and its reactivation by hydrogen sulfide, sulfite, and hydrogen cyanide, it is concluded that lysozyme acts only in the reduced state.

BIBLIOGRAPHY

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