LXXVII. SOME PROPERTIES OF EGG-WHITE LYSOZYME

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AN enzyme capable of lysing certain bacteria, and particularly *M. lysodeikticus*, was discovered in nasal mucus by Fleming [1922] and called by him *lysozyme*. This, or a similar substance, is present in a number of animal tissues and fluids, the richest source being hen's egg-white.

An attempt to isolate lysozyme from egg-white was made by Wolff [1927] by removing inert protein with colloidal iron and precipitating the active material with acetone; the preparation obtained in this manner was said to contain no nitrogen or sulphur. Meyer *et al.* [1936] used an entirely different procedure. Acetone-dried egg white was extracted with 50 % alcohol containing 10 % acetic acid and the lysozyme precipitated from the concentrated solution with alcohol. It was purified by precipitation as the flavianate and decomposition of the latter with cold alcoholic ammonia. These investigators recognized the protein nature of the substance and considered that its lytic action involved the hydrolysis of a sugar linkage of certain mucoids present in the bacteria. The method of isolation was further improved by Roberts [1937]. Owing to the difficulty of decomposing the flavianate this part of the preparation was replaced by a fractional precipitation with acetone from a solution of the right acidity. The lysozyme was concentrated largely in the fourth and final fraction, which was said to behave in all respects like a homogeneous product, and had an activity, as determined by the method of Goldsworthy & Florey [1930], of about 2000 units per mg. This preparation was moderately soluble in water, showed most of the characteristic protein reactions, and its isoelectric point was thought to be near pH 7.0. Its dialysability through cellophane suggested that it was a protein of relatively low molecular weight.

The lysozyme used in the work described here was obtained as a white powder, dried with acetone and ether, by the method of Roberts. It had an activity of about 2000 units per mg. and contained 16.4 % N and 3.2 % S, calculated on a moisture- and ash-free basis. Ultra-centrifugal measurements by Mr J. St L. Philpot, using the absorption method, indicated that the material was homogeneous and was one of the group of proteins whose molecular weights are close to 18,000. Crystalline lysozyme was obtained from this material by Abraham & Robinson [1937] and appeared to have about the same activity as the amorphous substance, but unfortunately it has so far proved difficult to obtain crystals in sufficient quantity for chemical investigation.

In the meantime the properties of the amorphous substance, which was believed to possess a considerable degree of homogeneity, were further investigated. After an analysis had been made of certain of its constituent amino-acids it was decided to carry out an electrometric titration. Preliminary experiments in this connexion showed that the material was not completely homogeneous but that a certain fraction of it had a lower activity and much lower solubility than the rest.

COLORIMETRIC ESTIMATION OF CERTAIN AMINO-ACIDS

Experimental

1.26 g. of the protein enzyme (prepared by the method of Roberts) were hydrolysed by boiling with 100 ml. of 20 % HCl for 6 hr. and most of the acid removed by evaporating the solution several times to dryness. After removing the humin by the method of Keilin & Hartree [1937] the concentrated solution of amino-acids was diluted to 100 ml. and divided into two parts (solutions A and B).

Tyrosine was estimated in solution A by the method of Folin & Ciocalteu [1927] and cystine by the method of Folin & Marenzi [1929].

Solution B was evaporated *in vacuo* almost to dryness and the residue dissolved in 50 ml. of a solution containing 10 ml. of 33 % HCl and heated on the water bath. 50 ml. of hot 15 % phosphotungstic acid solution were then added and the mixture digested on the water bath for half an hour. After standing for 2 days the precipitate of diamino-acids was filtered and washed with 100 ml. of a 7.5 % phosphotungstic acid solution containing 10 ml. of 33 % hydrochloric acid. The precipitate and filter paper were then transferred to a beaker, dissolved in just sufficient 3 % NaOH, and the solution filtered into a volumetric flask and diluted to 500 ml. (solution C).

Arginine was estimated in solution C by the method of Jean [1934] and histidine by the method of Jorpes [1932]. The values obtained were corrected for the solubilities of the phosphotungstates, using the figures given by Van Slyke [1911].

The total N in solution C was determined by the micro-Kjeldahl method and the lysine present estimated by subtraction from the total N of the arginine-, histidine- and cystine-N. To obtain a value for the latter a cystine determination was made on solution C.

Results

The values obtained in this manner were: arginine 11.6 %, cystine 7.0 %, lysine 5.8 %, tyrosine 4.4 %, histidine 2.6 %.

The value for cystine may be high as the starting material gave a weak positive test for sulphydryl groups [Mirsky & Anson, 1936].

Calculated for a molecular weight of 18,000 the number of basic groups per molecule, due to arginine, lysine and histidine, is about 22.

Isolation of arginine

1.2 g. of protein were hydrolysed with HCl and the diamino-acids separated by precipitation as their phosphotungstates in the usual manner. 150 ml. of 1 % HCl were added to the precipitate and the phosphotungstic acid extracted with 1 : 1 ether-amyl alcohol. The aqueous solution was evaporated to dryness, the residue dissolved in 15 ml. of water, heated to 90°, and 15 ml. of a solution containing 1 g. of flavianic acid added. After standing for 24 hr. in the ice chest the precipitate was centrifuged and crystallized from 110 ml. of boiling water containing a trace of flavianic acid (220 mg.). It was recrystallized from 60 ml. of hot water and dried at 100° over P₂O₅. (Found: N, 17.47 %. (C₁₀H₆N₂SO₈) (C₆H₁₄O₂N₄) requires N 17.2 %.)

Van Slyke estimation of amino-N

The estimation of free amino groups in proteins by the method of Van Slyke is complicated by the slow reaction of nitrous acid with other groups (e.g. the guanidyl group of arginine). For this reason the most satisfactory procedure

consists in plotting the N_2 evolved against the time of reaction and extrapolating the final portion of the curve, due to side reactions, to zero time [Kekwick & Cannan, 1936; Rutherford *et al.* 1937]. The curve shown (Fig. 1) was obtained with a micro-volumetric apparatus. If the molecular weight of the material is

Fig. 1.

taken to be 18,000 it indicates the presence of nearly nine amino groups per molecule, and, on the assumption that only one of these is an α -amino group, of eight residues of lysine. The value of 5.8%, which was previously obtained for the lysine content of the substance, corresponds to seven residues per molecule.

SOLUBILITY OF THE MATERIAL; ITS SEPARATION INTO TWO ACTIVE FRACTIONS

It was stated by Roberts [1937] that lysozyme was moderately soluble in water but more soluble in dilute acetic acid, being partially precipitated from the latter solution on neutralization. A closer examination of the solubility showed that this was due to the presence of a small amount of an insoluble component. The material dissolved to the extent of about 90% in ten times its weight of water, but the residue was sparingly soluble, and after washing well with water dissolved, according to a micro-Kjeldahl estimation, only to the extent of 0.024% at room temperature. Its solubility was relatively small over a wide range of pH —4.5–10.5—but it was soluble, except for a negligible residue, in 5% NaCl solution. For this reason concentrated solutions of the original substance, which had been cleared by centrifuging, became slightly opalescent on dilution. The opalescence disappeared on the addition of a drop of NaCl solution.

A further small amount of protein separated from the soluble fraction, which comprised the main bulk of the material, on dialysing its solution, in fish swim bladders, against distilled water, at 0–5°, for 3 days. After centrifuging, the activity of the protein in the resulting clear solution was not distinguishable, by the present method of estimation, from that of the original material. It was reported to be homogeneous in the ultracentrifuge and to have a sedimentation

constant of 1.8×10^{-13} . The protein could not be obtained from the dialysed solution by precipitation with acetone, or evaporation to dryness, without some denaturation. It was completely precipitated by ammonium sulphate between 45 and 66% saturation. Fig. 2 shows the amount of protein in solution plotted

Fig. 2.

against the logarithm of the ammonium sulphate concentration ($\log S$), at pH 4.6. Estimations were carried out by adding an equal volume of 5% trichloroacetic acid to the solutions, heating to 70° for 5 min., washing the precipitate at the centrifuge until free of ammonia, and determining its total N by the micro-Kjeldahl method.

The activity of the insoluble fraction appeared to be about one-third of that of the original substance. Ultracentrifugal measurements showed that it was less homogeneous and contained material of molecular weight lower than 18,000. Its sedimentation constant was given as 1.63×10^{-13} . This, together with the fact that it gave a positive nitroprusside test for sulphhydryl groups and that its nitrogen and sulphur contents (N 16.1, S 3.1%) were very similar to those of the original material, suggested that it might consist of soluble, active, lysozyme adsorbed on "denatured" lysozyme formed during the preparation. No support, however, was obtained for this view from preliminary immunological experiments which were kindly carried out by Dr B. G. Macgraith in the School of Pathology. An antiserum was obtained from rabbits injected with a solution of lysozyme from which the insoluble fraction had been removed by dialysis and centrifuging. This showed a definite precipitin reaction with the antigen solution at a dilution of the latter of 10^4 , but gave no reaction with the insoluble fraction at a dilution of 10^3 . If the latter contained an amount of adsorbed soluble protein corresponding to its activity a positive reaction would have been expected under these conditions. This preliminary result thus indicates that the lytic activity is connected with two different proteins.

ELECTROMETRIC TITRATION

In order to obtain more information about the amphoteric nature of the preparation and the relation of the insoluble fraction to the rest of the material electrometric titrations were carried out.

Experimental

The apparatus consisted of a hydrogen electrode in a rocking cell, and was very similar to that used by Harington & Neuberger [1936] for the titration of insulin. It was standardized against 0.1 *N* HCl, the *pH* of this solution being taken as 1.096 [Scatchard, 1925].

Solutions of the soluble protein were prepared for titration in the following way: 600 mg. of lysozyme, prepared by the method of Roberts, were dissolved in 65 ml. of water, the insoluble fraction removed by centrifuging and the clear solution dialysed in a fish swim bladder against distilled water, at 0–5°, for 72 hr. The small amount of protein which separated during the dialysis was centrifuged off, and 12 ml. of the resulting solution, containing 70–80 mg. of protein, were used for each titration. A certain amount of the material (*c.* 15 %) appeared to be lost during dialysis. The protein concentrations of these solutions were determined by evaporating 5 ml. portions to dryness, drying at 110°, and weighing.

The insoluble fraction was washed well with water, redried with acetone and ether, and about 75 mg. portions, in 12 ml. of water, used for each titration.

Results

For several reasons, but especially because of the difficulty of making adequate blank corrections, accurate titrations are usually confined to a *pH* range of approximately 2.5–11.5.

In the case of egg albumin [Kekwick & Cannan, 1936] and insulin [Harington & Neuberger, 1936] about 80 % of the total acid binding took place at *pH* 2.5, but an end-point was obtained in the first case by extending the range to below *pH* 2.0 and in the second case by titrating in 80 % alcohol.

In the alkaline range the situation is less satisfactory. The guanidyl group of arginine cannot be completely included in the range to which accurate titration is limited, but, nevertheless, in the titration of insulin Harington & Neuberger found that, probably because no activity correction could be made, the base binding at *pH* 11.5 appeared unreasonably high in comparison with the analytical data.

In the present case the blank corrections were calculated on the assumption that the concentration of hydrogen ion was equal to its activity. Under the conditions of measurement a small amount of denaturation occurred and it was not possible to keep the solutions quite clear. This was probably a surface phenomenon: Roberts [1937] remarked on the ease of surface denaturation of lysozyme. Nevertheless, it appeared unlikely that any measurable change in the acid- and base-binding capacities of the material occurred, as the titrations in both the acid and alkaline ranges were reversible. After titration, especially in the alkaline range, the material appeared to be slightly less active than it was originally, but owing to the inaccuracy of the method of estimation it was only possible to say that no great loss in activity had occurred. Lowering the temperature of titration from 25 to 20° had very little effect on the denaturation.

Fig. 3 shows titration curves of dialysed solutions of the soluble protein at 25 and 20°, and an acid titration curve in 70 % alcohol at 25°. The figures for one titration are given in Table I. The acid- and base-binding per molecule have been calculated for a molecular weight of 18,000.

Fig. 3.

The pH of the dialysed solution should be close to the isoelectric point of the protein. This lay in a region where there was little buffering, and was 7.85 at 25° and 7.98 at 20°.

As would be expected the change in temperature made practically no difference to the results in the acid range. There was no indication of a maximum acid-binding at pH 2.5 in water, and at this point the curve shows the binding of about 19 groups per molecule of protein. It would be expected from the analytical data that there would be relatively little buffering in the range in which histidine is titrated, and in fact about 4 groups were titrated between the isoelectric point and pH 5.5.

Titration in 70 % alcohol was only possible in the acid range at pH values lower than 4.5, owing to the limited solubility of the protein in this solvent. There is difficulty concerning the definition of acidity in aqueous alcoholic solutions, and the term pH is used, in this case, in the sense indicated by Neuberger [1934]. The curve obtained lay about one pH unit above that given

Table I. *Titration of dialysed lysozyme solution*

A. With acid. 80 mg. protein (moisture and ash free); 12 ml. water. Temp. 25°.

ml. 0.1 <i>N</i> HCl	pH	ml. 0.1 <i>N</i> HCl corrected	Equiv. 10 ⁵ H ⁺ bound per g. protein	Equiv. H ⁺ bound per g. mol. protein
—	7.850	—	—	—
0.05	6.765	0.050	6.2	1.1
0.10	6.195	0.100	12.5	2.2
0.20	5.327	0.199	24.9	4.5
0.30	4.718	0.298	37.2	6.7
0.50	3.821	0.481	60.1	10.8
0.70	3.211	0.622	77.7	14.0
0.90	2.848	0.717	89.6	16.1
1.10	2.636	0.797	99.6	17.9
1.30	2.484	0.864	108.0	19.4
1.40	2.416	0.886	110.8	19.9

Reversed titration

ml. 0.118 <i>N</i> NaOH	pH	ml. 0.1 <i>N</i> HCl corrected	Equiv. 10 ⁵ H ⁺ bound per g. protein	Equiv. H ⁺ bound per g. mol. protein
0.20	2.585	0.810	101.3	18.2
0.40	2.822	0.720	90.0	16.2
0.60	3.237	0.611	76.4	13.7
0.80	4.024	0.443	55.4	10.0
1.00	5.208	0.219	27.4	4.9
1.10	6.190	0.102	12.7	2.3

B. With alkali. 80 mg. protein; 12 ml. water. Temp. 25°.

ml. 0.118 <i>N</i> NaOH	pH	ml. 0.1 <i>N</i> NaOH corrected	Equiv. 10 ⁵ OH ⁻ bound per g. protein	Equiv. OH ⁻ bound per g. mol. protein
0.05	8.712	0.058	7.2	1.3
0.10	9.304	0.115	14.4	2.6
0.20	9.981	0.224	28.0	5.0
0.30	10.404	0.321	40.1	7.2
0.50	10.976	0.466	58.2	10.5
0.70	11.285	0.570	71.2	12.8
0.90	11.486	0.648	81.0	14.6
1.00	11.538	0.710	88.7	16.0
1.10	11.617	0.730	91.3	16.4

Reversed titration

ml. 0.1 <i>N</i> HCl	pH	ml. 0.1 <i>N</i> NaOH corrected	Equiv. 10 ⁵ OH ⁻ bound per g. protein	Equiv. OH ⁻ bound per g. mol. protein
0.20	11.486	0.672	84.0	15.1
0.40	11.318	0.604	75.5	13.6
0.60	11.072	0.526	65.7	11.8
0.80	10.675	0.429	53.6	9.6
1.00	10.032	0.280	35.0	6.3

in aqueous solution, and at pH 3.0, where the acid-binding under these conditions would be expected to be nearly complete, the latter corresponded to 22–23 groups.

In the alkaline range no indication of maximum base-binding was obtained at pH 11.5. At this point the curve at 25° corresponded to binding by about 15 groups and that at 20° by about 11 groups per molecule of protein. This difference may be assigned to the temperature coefficients of the dissociation constants of the phenolic and guanidyl groups of tyrosine and arginine respectively.

The titration of the insoluble fraction from the original lysozyme was only possible below pH 4.5 and above pH 11. At pH 4.5 the acid-binding differed very little from that of the soluble protein, but with increasing acidity it showed greater acid-binding, the value at pH 2.5 being 24 groups per 18,000 g. At pH 11.5 the base-binding corresponded to about 21 groups. Thus at these pH values it appeared to bind about five more equivalents of acid and base than the soluble protein.

DISCUSSION

The colorimetric estimations showed that lysozyme prepared by the method of Roberts contained a considerable quantity of arginine and lysine and an unusually large proportion of cystine. In spite of its apparent homogeneity in the ultracentrifuge, however, the lysozyme prepared by this method is not a single protein: a certain fraction of it has a much lower solubility, a lower activity and a somewhat larger acid- and base-binding capacity, than the rest. No material is present of molecular weight higher than 18,000, but while the main, soluble, portion is homogeneous as regards molecular size, some inhomogeneity is revealed in the insoluble fraction due to particles of lower molecular weight. The latter are not present in sufficient quantity in the original material to be detected in the ultracentrifuge.

These facts would be understandable if the active proteins in both fractions were very similar in molecular size and amphoteric properties, but the insoluble fraction also contained products of partial degradation. Immunological experiments by Roberts *et al.* [1938] indicated that egg, cat and human lysozymes were not identical; but the presence of two non-identical active proteins in material obtained from one source, which is suggested by the preliminary experiments carried out in this case, is more surprising. Further experiments are required in this connexion.

SUMMARY

1. Lysozyme prepared by the method of Roberts appeared to be homogeneous in the ultracentrifuge and to have a molecular weight of about 18,000. A colorimetric estimation of certain amino-acids and a Van Slyke estimation of amino-nitrogen were carried out, and arginine was isolated in the form of its flavianate.

2. The solubility of the material subsequently showed that it was not completely homogeneous but that a fraction could be separated from it of lower activity and much lower solubility than the rest.

3. Electrometric titration of the soluble lysozyme showed that this was a basic protein with an acid-binding capacity not far from 23 groups per molecule. At pH 11.5 the base-binding in water, at 25° , corresponded to about 15 groups per molecular. The acid- and base-binding capacities of the insoluble fraction were somewhat larger.

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