[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY, UNIVERSITY OF UPSALA]

Ultracentrifugal Study of the Action of Papain on Ovalbumin

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Measurements of the molecular weights of the native proteins by means of the ultracentrifugal technique worked out in this Laboratory[1] have brought to light certain regularities concerning the construction of the protein molecule.[2] It has been found that the molecular weight is in most cases a multiple (or submultiple) of the unit 34,500, which is the weight of the ovalbumin molecule. In view of this fact it is to the study of the simplest proteins such as ovalbumin we must direct our attention if we want to get an insight into the construction of this unit.

The ultracentrifugal investigation has shown that ovalbumin is homogeneous with regard to molecular weight and has a wide P_H stability range.[3] At the alkaline end of this region the molecular weight falls rapidly while at the acid border denaturation and aggregation to large particles takes place. A study of the alkaline disintegration might help to elucidate the build of the molecule but it is perhaps of still more interest to follow the changes brought about by an enzyme such as papain which has its optimum of action within the stability range near the isoelectric point of the protein.[4]

A number of determinations of the sedimentation constant and molecular weight have been carried out on products made by digesting samples of pure crystalline ovalbumin with unactivated and activated papain. By means of fractional dialysis and fractional precipitation with ammonium sulfate we have made an attempt to separate the different molecular species out of the mixture of disintegration products and study these fractions in the ultracentrifuge. Although the separation obtained so far is rather incomplete some definite information has been gained concerning the nature of these products.

Material and Experimental Procedure.—The ovalbumin used in these experiments was purified by means of crystallization in the usual way.[3] It showed a sedimentation constant of 3.4×10^{-13}. The papain preparation was the commercial product "Papaiotinum 1:350" from E. Merck. The activation was carried out by addition of hydrocyanic acid according to the directions given by Willstätter and Grassmann.[5] The enzyme action took place in acetate buffer at 40°.

A typical example may be mentioned. To 16 cc. of 0.6% potassium cyanide was added 14 cc. of 0.1 N hydrochloric acid (the accurate proportions determined by titration with methyl red as indicator). This solution was mixed with 18 cc. of 1.5% papain solution and 42 cc. of 0.2 N acetate buffer of P_H 5.0 and kept at 40° for two hours. To the activated enzyme solution was then added 90 cc. of a 4–5% ovalbumin solution and the mixture digested for twenty-four hours at 40°.

Sedimentation tests were carried out in the high speed ultracentrifuge on the untreated disintegration mixture after different times of reaction and on products obtained from this mixture by means of dialysis and fractional precipitation. Separation by dialysis was done in small collodion bags at +3° against an equal volume of buffer P_H 5.0 (0.02 N acetate + 0.1 N potassium chloride). The fractional precipitation was conducted on the following scheme. The disintegration mixture was saturated with ammonium sulfate and the precipitate centrifuged off, washed and dissolved in water. This solution was first half saturated with ammonium sulfate, the precipitate centrifuged off and the remaining solution saturated and the new precipitate removed. These two fractions were then each divided into two new fractions; the middle fractions were mixed and the three fractions thus obtained fractionated again, and so on.

Determinations of Sedimentation Constants and Molecular Weights.—In Table I the experiments with different times of reaction are given. The values have been corrected for the influence of viscosity and density of solvent. The percentage of non-centrifugible component has been computed from the light absorption and is therefore only a rough estimate.

(1) T. Svedberg, THIS JOURNAL (1926–1933); *Kolloid.-Z.*, **51**, 10 (1930).

(2) T. Svedberg, *Nature*, June 8, 1929.

(3) T. Svedberg and B. Sjögren, THIS JOURNAL, **52**, 5187 (1930).

(4) See Willstätter, Grassmann and Ambros, *Z. physiol. Chem.*, **151**, 307 (1926).

(5) Willstätter and Grassmann, *Z. physiol. Chem.*, **138**, 184 (1924).

TABLE I

ACTION OF PAPAIN ON OVALBUMIN

Protein concentration during reaction 2%; papain concentration 0.15%; ultracentrifugal runs on reaction mixtures ten times diluted with 0.2 normal acetate buffer of P_H 5.0; in last run diluted with 0.02 normal acetate buffer + 0.1 normal KCl; the controls had been kept for the same time at the same temperature without papain but with HCN.

No.	Enzyme, papain	Reaction at 40°, hours	Centrif. force, times gravity	$s_{20} \times 10^{13}$ for reaction mixture	Non-centr. compon., %	$s_{20} \times 10^{13}$ of control
1	Unact.	84	220,000	3.4	0	3.1
2	Activ.	12	220,000	2.9	14	3.4
3	Activ.	24	220,000	2.4	55	3.5
4	Activ.	46	220,000	2.5	59	3.3
5	Activ.	84	220,000	2.1	76	3.4
6	Activ.	24	400,000	0.8 and 3.1	32	...

Figures 1 and 2 give the sedimentation pictures of Expt. 3 and Fig. 3 the microphotometer curves of Expt. 2; Fig. 4 the microphotometer curves of

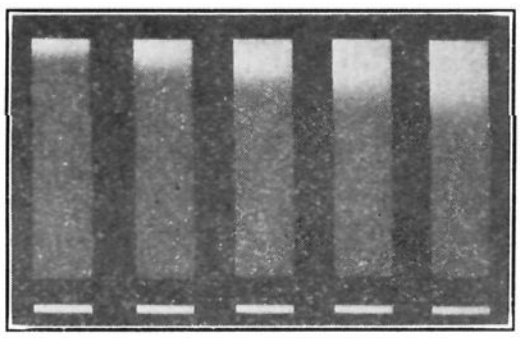


Fig. 1.—Sedimentation pictures of ovalbumin; centrifugal field 220,000 times gravity; time between exposures 15 min.

Expt. 6. Table I shows that the sedimentation of the reaction mixture is markedly lower than that of the original ovalbumin. The product is

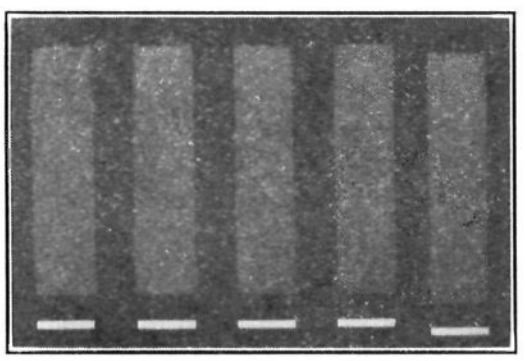


Fig. 2.—Sedimentation pictures of the product obtained by action of papain on ovalbumin; centrifugal field 220,000 times gravity; time between exposures 25 min.

polydisperse. This follows from an analysis of the photometer curves of Expts. 2–5 (Fig. 3) but is more clearly demonstrated by the shape of the curves of Expt. 6 (Fig. 4) in which case the centrifugal field was strong enough to cause distinct separation of the reaction mixture in two centrifugible components. The sedimentation

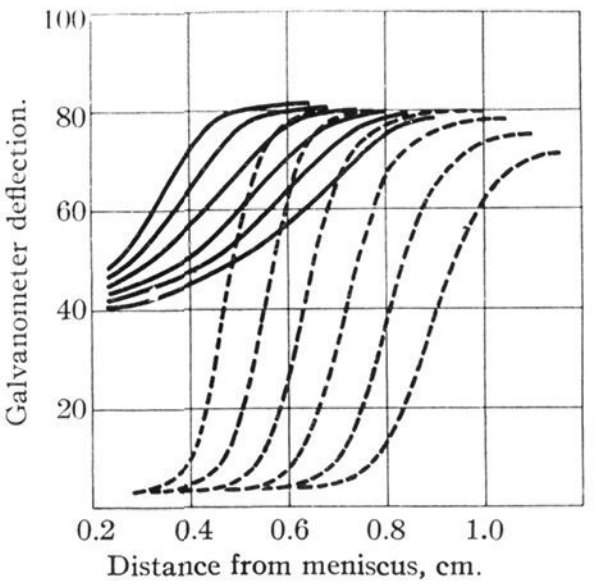


Fig. 3.—Photometer curves of the sedimentation of ovalbumin (lower curves) and of the product obtained by the action of papain on ovalbumin (upper curves); centrifugal field 220,000 times gravity; time between exposures 15 min.

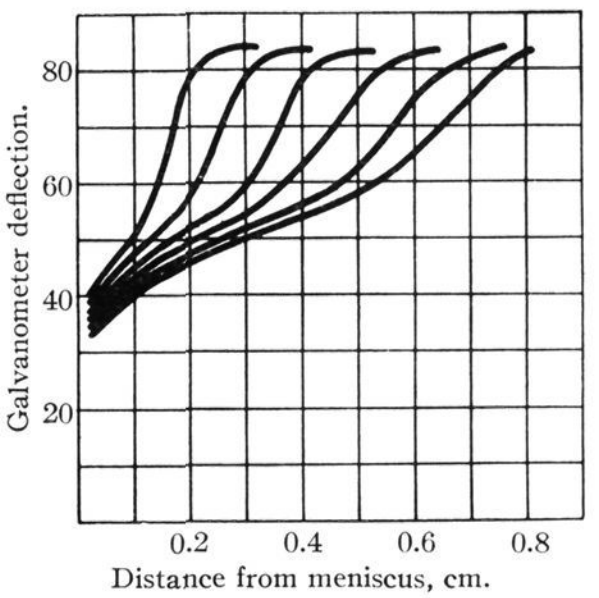


Fig. 4.—Photometer curves of the sedimentation of the product obtained by the action of papain on ovalbumin; centrifugal field 400,000 times gravity; time between exposures 10 min.

The diagram shows the presence of two molecular species of the sedimentation constant 0.8 and 3.1 $\times 10^{-13}$, respectively.

constant of the lower one is 0.8 $\times 10^{-13}$ and therefore of the same order as that of the protamines.[6] The higher component has a constant not far from that of the lowest proteins, *e. g.*, glia-

(6) Unpublished preliminary determinations by B. Sjögren and one of the present writers (I.-B. E.); see Waldschmidt-Leitz, *Z. physiol. Chem.*, **197**, 223 (1931).

din,[7] myoglobin[8] and certain erythrocruorins.[9] The non-centrifugible component may contain lower polypeptides and amino acids.

Unactivated papain has no influence on the sedimentation constant, nor is any non-centrifugible substance formed. Unactivated papain, therefore, does not attack the molecule of native ovalbumin.

The attempts to disentangle the mixture by dialysis and study the fractions in the ultracentrifuge are summarized in Table II.

TABLE II

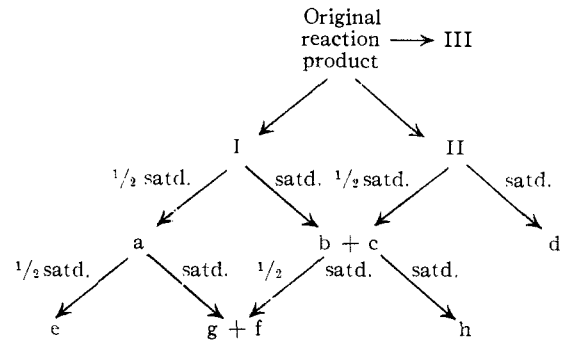
DIALYSIS EXPERIMENTS ON PAPAIN-OVALBUMIN REACTION MIXTURE

No.	Dialysis, hours	Centrif. force, times gravity	$s_{20} \times 10^{13}$ of inner soln.	$s_{20} \times 10^{13}$ of outer soln.
1	60	260,000	...	0.9 and 2.8
1	108	260,000	2.9	Outer liquid changed several times during last 48 hours
2	168	260,000	...	0.5
2	216	240,000	3.1	Outer liquid changed several times during last 48 hours
3	216	250,000	...	0.6
4	48	400,000	3.2	Uncentrif. comp. and lower centr. comp. present

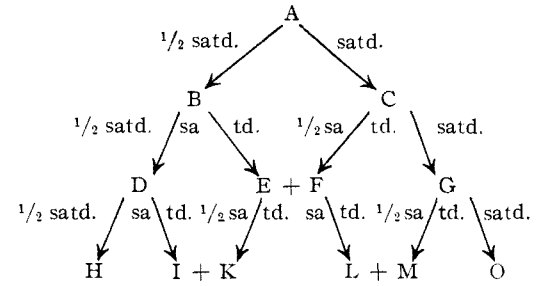
The collodion bags used were not uniform and it is therefore to be expected that the composition of the inner and outer liquid should vary somewhat from one experiment to another. The sedimentation data demonstrate the existence of two centrifugible disintegration products together with some unchanged ovalbumin. The direct ultracentrifugal analysis of Expt. 6 of Table I is thereby confirmed.

Two series of fractional precipitations with ammonium sulfate were carried out on reaction mixtures.

In the first series the original product which had been obtained by treating ovalbumin for thirty-six hours at 40° with activated papain was saturated with ammonium sulfate to 65% and centrifuged off (fraction I). The mother liquor was saturated with sulfate and the precipitate separated from the solution by centrifuging (fraction II). The new mother liquor (fraction III) was kept for ultracentrifugal examination. The fractionation was then continued according to the following scheme.



In the second series the time of reaction was twenty-four hours. The product was saturated with ammonium sulfate and the precipitate filtered off (fraction A). The further treatment was done in the following way.



In Table III the sedimentation data are collected.

TABLE III

FRACTIONAL PRECIPITATION EXPERIMENTS ON THE REACTION MIXTURE FROM THE ACTION OF PAPAIN ON OVALBUMIN

Substance	Sedimentation constant $\times 10^{13}$
First series, original ovalbumin	3.3–3.5
First series, fraction I	3.2
First series, fraction e	3.1
First series, fraction h	2.9
First series, fraction II	2.6
First series, fraction III	0.4
Second series, fraction O	2.8

The experiments summarized in Table III show that the systematic treatment of the reaction product with ammonium sulfate to half saturation leads to the isolation of a product rich in the original ovalbumin, while the fractionation of the mother liquors with sulfate to full saturation leads to a product of a constant between 2 and 3×10^{-13}. The mother liquor after the first full saturation contains the lower centrifugible substance of a sedimentation constant around 0.5×10^{-13}. Thus the three lines of attack, the direct sedimentation analysis, the dialysis and the fractional precipitation, have all of them led to the same result. The action of papain on native

(7) Unpublished determinations by Miss Laura Krejci.
(8) H. Theorell, *Biochem. Z.*, **252**, 1 (1932).
(9) T. Svedberg and Astrid Hedenius, *Nature*, **131**, 325 (1933).

ovalbumin gives rise to three kinds of disintegration products: a non-centrifugible substance which probably contains lower polypeptides and amino acids, a centrifugible substance of a sedimentation constant about 0.6×10^{-13}, which with regard to molecular weight is of the same order as the protamines, and finally a substance of a sedimentation constant about 2.7×10^{-13}. This latter product is of special interest because it probably represents the first step in the breaking up of the ovalbumin molecule. The sedimentation constant is almost identical with that of myoglobin.[8] Now myoglobin has the same molecular weight as ovalbumin but possesses a highly unsymmetrical molecule which finds its expression in the low sedimentation constant.[8] Indications of the existence of other protein molecules of a similar build have been found in the case of gliadin[10] and the red blood pigment of Myxine.[11] It would be of great interest to learn whether such is also the case with the disintegration product of ovalbumin of sedimentation constant 2.7×10^{-13}. In order to throw some light on this point two sedimentation equilibrium runs were carried out on fraction O of the second precipitation series. The values obtained for the molecular weight were 38,600 and 33,300, which figures are, within the limits of error, identical with the value 34,500 for normal native ovalbumin. In Table IV the complete data of one of these runs are given.

TABLE IV

REACTION PRODUCT: PAPAIN–OVALBUMIN OF SED. CONST. 2.75×10^{-13} SEDIMENTATION EQUILIBRIUM RUN

Solvent 0.02 normal acetate buffer of *P*H 5.0 + 0.9% NaCl; spec. vol. of solute 0.750; density of solvent 1.004; abs. temp. 289.6°; length of col. of soln., 0.55 cm.; thickness of col., 1.2 cm.; dist. of outer end of soln. from center of rot., 5.95 cm.; speed 12,210 r. p. m.; source of light, mercury lamp; light filter, chlorine and bromine; aperture of objective, F:33; Hauff x-ray plates; times of exposures 30, 40, 60 sec.; exposures made after 61, 68, 85 hours.

x_2	Distances, cm. x_1	Concn. ratio c_2/c_1	No. of expos.	Mol. wt.
5.85	5.80	1.176	9	33,100
5.80	5.75	1.205	12	38,500
5.75	5.70	1.194	12	36,900
5.70	5.65	1.153	12	29,900
5.65	5.60	1.155	12	30,500
5.60	5.55	1.165	12	32,600
5.55	5.50	1.162	12	32,300
5.50	5.45	1.162	12	32,800
			Mean	33,300

It is of interest to note that there is no drift in the values of the molecular weight with distance from the center of rotation, showing that the substance is practically homogeneous with regard to molecular weight. The first step in the disintegration of the ovalbumin molecule therefore probably consists in the loosening of some of the bonds within the molecule, thus causing it to assume a very unsymmetrical shape before breaking up into individual parts.

The expenses connected with this investigation have been defrayed by grants from The Rockefeller Foundation, the Nobel Foundation and The Foundation "Therese och Johan Anderssons Minne."

Summary

1. A number of determinations of the sedimentation constant and molecular weight have been carried out on products obtained by digesting samples of pure crystalline ovalbumin with activated and unactivated papain at 40°.

2. An attempt has been made to separate the different molecular species out of the mixture of disintegration products by means of fractional dialysis and fractional precipitation with ammonium sulfate. The fractions thus obtained have been studied in the ultracentrifuge.

3. The three lines of attack have all led to the result that the action of activated papain on native ovalbumin gives rise to three kinds of disintegration products: a non-centrifugible substance which probably contains lower polypeptides and amino acids, a centrifugible substance of a sedimentation constant about 0.6×10^{-13} which with regard to molecular weight is of the same order as the protamines and finally a substance of sedimentation constant about 2.7×10^{-13}. This latter product which has the same molecular weight as ovalbumin is probably formed by the loosening of some of the bonds within the ovalbumin molecule, thus causing it to assume a highly dissymmetrical shape. It may represent the first step toward the breaking up of the molecule into individual parts.

4. Unactivated papain has no influence on the sedimentation constant of ovalbumin, nor are any non-centrifugible products formed.

UPSALA, SWEDEN RECEIVED JULY 3, 1933

(10) Miss L. Krejci, unpublished determinations.

(11) Mrs. A. Hedenius and Miss I.-B. Eriksson, unpublished determinations.