CCLIII. THE DIGESTION PRODUCTS FORMED BY THE ACTION OF PAPAIN ON EGG ALBUMIN.

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AN ultracentrifuge study of the action of papain on egg albumin was made in this laboratory [Svedberg & Eriksson, 1933; 1934], but with the centrifugal forces and methods of measurement then available it was not possible to separate effectively the digestion products obtained. It was therefore thought advisable to use the improved apparatus now in the laboratory to extend these observations. As the ultracentrifuge data still proved insufficient for definite conclusions measurements were also made by other physical methods.

Experimental procedure. The egg albumin was prepared by Sørensen's method [Sørensen, 1917; Sørensen & Høyrup, 1918]. It was crystallized three times, dissolved and dialysed against distilled water, and then electro-dialysed. The solution was evaporated *in vacuo* over sulphuric acid and over phosphorus pentoxide, and stored in the dry state in the dark.

The papain used was Merck's Papainotinum 1 : 350. Its activity was tested according to directions given by Willstätter & Grassmann [1924] and found to give results comparable with theirs.

The papain activation with HCN and the treatment of the digestion mixture of papain and egg albumin were carried out in the manner recommended by Willstätter & Grassmann [1924; 1926]. All digestion was done at 40° in acetate buffer of pH 5.0. The papain solution was in each case activated at 40° for 2 hours before it was added to the egg albumin. Two different sets of concentrations of digestion mixture and protein solution were used: (a) those used by Willstätter & Grassmann for papain activation tests (only slight digestion occurred with these proportions so they were not used in later work), and (b) those concentrations used by Willstätter & Grassmann in egg albumin digestion. These concentrations were as follows:

(a)	(b)
4.8 % egg albumin	4.0 % egg albumin
0.12 % papain	0.3 % papain
0.24 % hydrogen cyanide	0.1 % hydrogen cyanide
0.04 <i>N</i> acetate buffer	0.02 <i>N</i> acetate buffer

To prevent continued digestion solutions were kept at 4° after removal from the digestion oven. Further, the HCN was removed by reducing the air pressure above the solution; this was done over a water-bath to minimize solution evaporation. All dialysis took place at 4°.

I. *Ultracentrifuge measurements.* Descriptions of the theory and technique of ultracentrifuge experiments and details of the methods of calculating the results have already been given [Svedberg, 1933; McFarlane, 1935]. The absorption method of measurement was only used with undigested solutions and as a means of testing whether digestion had occurred. It requires far less time for the calculation of results than the refractive index method, but the latter is much more suitable for the separation of components of a mixture.

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The solutions used for centrifuge analysis contained 0.02 *N* acetate buffer pH 5.0, 0.2 *N* sodium chloride, and approximately 1 % protein. The sodium chloride was added to suppress the Donnan effect produced when charged particles move through a liquid under the action of a centrifugal field [Tiselius, 1932].

It was found that the papain caused the egg albumin to break up into two fractions. There was no observable change in the nature of these two fractions with length of time of digestion, only in their proportions. The concentration of the lighter fraction increased at the expense of the heavier one as digestion proceeded until the latter had completely disappeared.

The sedimentation diagrams of the heavier fraction showed no asymmetry indicative of heterogeneity. Its average sedimentation constant (S_{20})¹ was 3.30, but the deviations of individual values were a little larger than would have been expected from a consideration of the experimental errors involved. This suggests that the fraction was a mixture of materials with nearly equal sedimentation constants, the composition differing slightly under different conditions. The accepted value for the sedimentation constant of undigested egg albumin is 3.55. The results are shown in Table I.

Table I.

Method of measurement (absorption or ref. index)	Digestion time hours	$S_{20} \times 10^{13}$ sec.	Remarks
A	0	3.59	
RI	0	3.49	Average 3.54
		(Heavier component.)	Digestion proportions (a) (Only slight digestion occurred with these proportions)
RI	45	3.29)	Same solution
A	45	3.22)	
A	93	3.25	
RI	117	3.20)	Same solution
A	117	3.27)	
A	120	3.20	
			Digestion proportions (b)
RI	0.5	3.40	—
RI	5	3.36	—
RI	19.5	3.31)	Same solution
A	19.5	3.14)	
RI	24	3.14	—
RI	24	3.49? S_1	—
RI	24	3.58? S_2	—
RI	43	3.32 S_1	—
RI	43	3.41 S_2	$S_2 = S_1$ with the light component removed by dialysis
RI	45	3.33 S_1	—
RI	45	3.38 S_2	—
RI	48	3.15	—

Even centrifugal forces 410×10^3 times gravity were not sufficient to cause the peak of the sedimentation curve of the lighter fraction to break away from the meniscus, i.e. the material was so light that the sedimentation diagrams obtained were those of the early stages of sedimentation equilibrium rather than

¹ S_{20} —sedimentation velocity in pure water at 20° under unit force. It is always expressed in units of 10^{-13} seconds.

of sedimentation velocity experiments. It was therefore not possible to compute a sedimentation constant for this fraction, but calculation showed that it must be less than 0.2. The lighter fraction passed through a cellophane membrane so that it was possible to separate the two fractions of the digestion mixture by dialysis. This effect is illustrated in Figs. 1 and 2. Fig. 1 shows a sedimentation

Fig. 1.

Fig. 2.

diagram for a solution digested for 45 hours; Fig. 2 shows a corresponding sedimentation diagram for the same solution after the light component had been dialysed away through a cellophane membrane.

The light component gave no precipitate with salicylsulphonic acid, a slight precipitate with lead acetate, and a dense one with phosphotungstic acid.

Previous experiments had shown that keeping the egg albumin at 40° had no effect on its behaviour in the ultracentrifuge, nor had treating it with unactivated papain.

Except when the time of digestion was less than 5 hours, a reference scale made with a solution containing no papain gave better (i.e. more horizontal) base lines in the sedimentation diagrams than a reference scale made with a solution containing papain in the concentration present in the digestion mixture. This suggests the possibility that the material contained in the papain preparation was combined with the egg albumin molecules. No evidence could be found of heavy albumin-papain particles but their number would be so small and their weight such as to make separation from the principal component in the sedimentation diagrams very difficult. From reference scales made with and without papain it was possible to deduce that the sedimentation constant of the papain preparation was about 2.8.

II. *Refractive index measurements.* The purpose of these measurements was to study quantitatively the time variation of the relative amounts of the two fractions of digestion products. Seven samples of the same egg albumin-papain mixture (digestion proportions (b)) were placed in an oven at 40° and removed after various lengths of time of digestion. The two fractions in each sample were separated by dialysis through cellophane membranes. The refractive index

differences between each of these solutions and a standard buffer solution were measured with a Pulfrich refractometer ($\lambda = 546\text{ m}\mu$). Assuming these refractive index differences to be proportional to the concentrations the total amount of material in each solution was calculated. This assumption is not strictly justified [v. Hand, 1935]. The increase during digestion in the apparent total amount of material present is probably attributable to variations in refractive increment. The results have been plotted in Fig. 3.

Fig. 3.

As digestion proceeded the proportion of the lighter fraction increased at the expense of the heavier one. The process was a linear function of the time of digestion except for a short initial period, not greater than 5 hours, during which the action was somewhat more rapid.

III. *Diffusion measurements.* The diffusion apparatus was that developed by Tiselius & Gross [1934] and by Lamm & Polson [1936]. The method of measurement was of the refractive index type similar to that used to measure concentration gradients in the ultracentrifuge. Series of experiments were made using solutions digested for various lengths of time and carefully separated into two fractions by dialysis through cellophane membranes. All experiments were made at 20° in acetate buffer $0.02\text{ }N$ pH 5.0. Sodium chloride ($0.2\text{ }N$ in most cases) was used to depress the Donnan effect. The protein concentration was approximately 1%.

The homogeneity of the diffusing materials was tested by comparing concentration distribution curves with the corresponding ideal diffusion curves calculated by means of the formulae developed by Pearson [1894]. The diffusion constants (D') were calculated from the ideal curves using equation (1)

$$D' = \frac{\mu^2}{2t} \left(\frac{1-b}{1} \right)^2 \quad \text{.....(1).}$$

μ = half the distance between the two inflexion points. The position of the inflexion points was obtained by dividing the maximum height of the curve by $\sqrt{e}$.

t = the time since diffusion started.

l = the optical distance from the scale to the camera objective.

b = the optical distance from the scale to the centre of the diffusion tube.

These values were corrected for the viscosity of the salts present in the solution by means of equation (2)

$$D = D' \frac{n_s}{n_w} \quad \text{.....(2).}$$

n_s = the viscosity of the solvent at 20° .

n_w = the viscosity of distilled water at 20° .

For comparison with the values for the light component, the diffusion constants of two amino-acids and one tripeptide were measured. For these experiments no buffer solutions were used, but the materials were dissolved directly in freshly boiled distilled water in order to be as nearly as possible at their iso-electric points. As the magnitude of the charge on the particles was not known, a high sodium chloride concentration (0.5 *N*) was used to suppress Donnan effects.

Table II.

Material	Time of diffusion hours	D'	$D \times 10^7$ cm. ² /sec.
Light component:			
36 hours' digestion	1	51.3	
	4	50.4	52.1
45 hours' digestion	3	44.7	
	5	47.1	46.6
70.5 hours' digestion	2	57.2	
	3	55.3	56.5
70.5 hours' digestion	2	54.2	56.8
Heavy component:			
27 hours' digestion	13	7.52	
	21	7.71	7.80
36 hours' digestion	20	8.24	
	24.5	8.15	8.20
44.5 hours' digestion	9	8.38	
	19	8.42	8.40
Glycine (mol. wt. 75)	1.0	91.3	
	1.5	85.8	
	1.75	84.6	
	2.5	85.7	90.8
<i>l</i> -Tryptophan (mol. wt. 204)	1.5	53.8	
	2.0	55.7	
	2.5	55.8	57.3
<i>dl</i> -Leucyl-glycyl-glycine (mol. wt. 245)	1.5	43.9	
	2.5	43.6	46.1

The diffusion experiment results are given in Table II. Both light and heavy components were found to be heterogeneous, i.e. there was a marked difference between the experimental and the ideal diffusion curves. The heterogeneity was definitely larger than could be accounted for by incomplete separation of the two components in dialysis. The diffusion constants listed have been calculated from the ideal diffusion curves most nearly corresponding to the experimental data and must therefore be regarded as average values for the various substances present. There was some indication that in each case the average diffusion constant increased slightly with the time of digestion. The glycine, *l*-tryptophan, and *dl*-leucyl-glycyl-glycine gave ideal diffusion curves. From a consideration of these diffusion constants it is evident that the lighter fraction of the digestion mixture must have contained some substances having diffusion constants of the same order of magnitude as the amino-acids, and probably some of the order of magnitude of the lower polypeptides as well. The average diffusion constant of the heavier component (8.27 cm.²/sec.) was a little higher than that of undigested egg albumin (7.76 cm.²/sec.).

IV. *Cataphoresis measurements.* These measurements were made with the moving boundary apparatus developed by Tiselius [Tiselius, 1930; Pedersen, 1933]. Both absorption and refractive index methods of measuring the concentration gradients were available. The former method was used with the light component. Samples were tested after 27 hours' and after 45 hours' digestion

They were found to be so heterogeneous that calculations of mobility were not possible. At pH 5.0 the average mobilities were almost zero, but they contained components having anodic and some having cathodic motion.

The refractive index method was used to study the heavy fraction. This necessitated using 2% solutions. As mobilities of undigested egg albumin solutions of such high concentration had not been measured previously by this method, these were investigated first. Solutions were used which had been kept at 40° in buffer but without papain, to test whether the heat alone had any effect on the mobility of the egg albumin. The concentration distribution curves for this solution showed no trace of asymmetry. The mobility calculated from these curves was exactly that obtained for this pH by Tiselius [1930] using dilute solutions [v. however Smith, 1935; 1936]. At pH 5.01 the motion was anodic 4.5×10^{-5} cm./sec./volt/cm.

The heavy fraction gave concentration distribution curves which were very asymmetrical, showing marked heterogeneity. Consideration of these curves

Fig. 4.

Fig. 5.

shows that the heavy fraction was made up of several components. The principal component had an anodic mobility of 1.4×10^{-5} at pH 5.0 (digestion time 36 hours), and 1.9×10^{-5} at pH 5.4 (digestion time 24 hours). The other components all had mobilities less than these so that no material remained having a

mobility equal to that of the undigested egg albumin. A typical concentration distribution diagram of the undigested egg albumin is shown in Fig. 4, and a typical curve for the heterogeneous heavy fraction is shown in Fig. 5.

V. *Light absorption measurements.* The light absorption of the undigested egg albumin and of the light and heavy fractions of the digestion mixture were measured by means of a Judd-Lewis spectrophotometer. Tests were also made of the intensity of light λ 270 $m\mu$ (obtained by means of a mercury lamp and chlorine and bromine filters) transmitted by a quartz cell containing the liquids.

The results cannot be regarded as very definite as concentrations were measured by means of refractive index measurements, and no account was taken of possible variations in refractive increment. The light fraction of the digestion mixture appeared to have a distinctly higher and the heavy fraction a somewhat lower extinction coefficient than the undigested egg albumin. There was no change in the positions of the wave-lengths of maximum or minimum absorption. The absorption of light by egg albumin in the region 253–290 $m\mu$ is almost entirely due to the aromatic amino-acids phenylalanine, tyrosine, and tryptophan [v. Coulter *et al.* 1936]. Thus an increase in the extinction coefficient of the lighter component would mean that the relative concentration of one or all of these substances had increased.

DISCUSSION.

It seems probable that the digestion of egg albumin by papain takes place in at least two stages: (1) a change in all of the molecules, probably a loosening of bonds within the egg albumin molecule and (2) a gradual splitting off of small pieces from these modified molecules.

Evidence for the first stage is furnished by cataphoresis measurements: partially digested solutions contain no component having a mobility equal to that of undigested egg albumin. Further, the sedimentation constant of the material is decreased even before any appreciable amount of decomposition products has been formed. This is in agreement with the previous investigation of this problem [Svedberg & Eriksson, 1934] in which a fraction was isolated from the digestion mixture of unchanged molecular weight but having a decreased sedimentation constant.

All the experiments show that very small molecules are produced during the second stage of digestion. Ultracentrifuge and diffusion experiments show that these must be of the order of magnitude of amino-acids and lower polypeptides. If we neglect changes in partial specific volume—the changes may be quite large [v. Cohn *et al.* 1934]—we may get an approximate idea of the average molecular weights of the components formed during digestion using the relationship

$$\text{Mol. wt.} = \text{Mol. wt.} \times \frac{\text{Sed. const. (Fraction)}}{\text{Sed. const. (Egg albumin)}} \times \frac{\text{Diffn. const. (Egg albumin)}}{\text{Diffn. const. (Fraction)}}.$$

This gives a molecular weight of 38×10^3 for the heavy fraction and about 4×10^2 for the light fraction. The molecular weight of undigested egg albumin is 42,200. This suggests that certain definite parts are split off from the molecule rather than that some molecules are simply broken up into many small pieces. This is in agreement with the fact that the light component seemed to contain a higher percentage of the aromatic amino-acids than undigested solutions.

Refractive index measurements show that except for a short initial period the process of digestion is a linear function of the time (see Fig. 3). This corresponds

to a monomolecular reaction taking place always at saturation. The change of slope of the initial part of the curve points to the presence of some other factor during this part of the digestion process.

SUMMARY.

Under the action of papain the egg albumin molecule is split up into two groups of substances. Both fractions are heterogeneous. The light fraction consists of particles of the order of magnitude of the amino-acids and the lower polypeptides. The heavy fraction has an average sedimentation constant of 3.30 and an average diffusion constant of 8.27 cm.²/sec. The heavy fraction contains no unchanged egg albumin. It is suggested that the first stage in digestion consists in a loosening of bonds within the molecule and that subsequently small parts are split off from these modified molecules. Ultracentrifuge, refractive index, diffusion, cataphoresis and light absorption measurements have been used in arriving at these conclusions.

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