COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY

VOLUME XIV

Amino Acids and Proteins

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FOREWORD

When *Amino Acids and Proteins* was selected as the topic of this year's symposium, it was realized that there would be some duplication with the sixth symposium of our series, held in 1938, which dealt with *Protein Chemistry*. During the intervening decade, however, such rapid progress had been made in research with proteins that it seemed desirable to renew the discussion of this subject.

The program for this symposium was organized with the help of A. Mirsky, J. S. Fruton, and D. Shemin. The editorial work was done by Katherine Brehme Warren.

Meetings were held from June 8 to June 16, 1949. The registered attendance was 186. Expenses of the symposium, particularly those connected with foreign guests, were covered by a grant received from the Carnegie Corporation of New York.

M. DEMEREC

LIST OF PREVIOUS VOLUMES

Volume I (1933) Surface Phenomena, 239 pp.
Volume II (1934) Aspects of Growth, 284 pp.
Volume III (1935) Photochemical Reactions, 359 pp.
Volume IV (1936) Excitation Phenomena, 376 pp.
Volume V (1937) Internal Secretions, 433 pp.
Volume VI (1938) Protein Chemistry, 395 pp.
Volume VII (1939) Biological Oxidations, 463 pp.
Volume VIII (1940) Permeability and the Nature of Cell Membranes, 285 pp.
Volume IX (1941) Genes and Chromosomes: Structure and Organization, 315 pp.
Volume X (1942) The Relation of Hormones to Development, 167 pp.
Volume XI (1946) Heredity and Variation in Microorganisms, 314 pp.
Volume XII (1947) Nucleic Acids and Nucleoproteins, 279 pp.
Volume XIII (1948) Biological Applications of Tracer Elements, 222 pp.

CONTENTS

HYDROLYSIS OF PROTEINS

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The hydrolysis of proteins has been of concern to chemists for a great many years. In spite of the large amount of work expended, important features of this reaction are still obscure; it is inherently a most complex sequence of reactions with which one has to deal.

So far, we in our laboratory have studied the kinetics of hydrolysis of egg albumin by hydrochloric acid and the molecular weight distribution of the peptides resulting therefrom, using a new spread film technique which we have been able to develop. We have also investigated to a limited extent the partial hydrolysis of egg albumin by barium hydroxide. We have been able to arrive at an approximate idea of the intrinsic relative rates of release of amino acids during the early stages of hydrolysis. For this purpose we have used filter paper chromatography. We have studied the kinetics of the hydrolysis of egg albumin by pepsin in some detail, and we are also prepared to make a preliminary report on the molecular weight distribution of the peptides resulting from the peptic hydrolysis of egg albumin.

The Peptide Bond

It is useful to review briefly some of the properties of the peptide-bond. This bond as it exists in peptides has an abnormally short length for a carbon-nitrogen bond. Corey (1948) assigns it a length of 1.33 A while the normal distance is about 1.47 A. This indicates that the peptide bond has considerable double bond character and is stabilized by resonance.

Massive groups attached to the α-carbons must impose considerable steric hindrance to the approach of a water molecule preparatory to hydrolysis of the peptide chain, and there are, no doubt, only certain configurations of the peptide chain which will permit such an approach.

From the dielectric constant studies of Marcy and Wyman (1941) and of Conner, Clarke and Smythe (1942), it can be concluded that while peptides show a marked degree of rigidity due to the high energy barriers for rotation about the various bonds in a peptide, a large measure of randomness in peptide configuration results.

Huffman (1942) has calculated the free energy change involved in the hydrolysis of some dipeptides and finds the energy change to be from about −3,000 to −4,000 calories per mole at 25°. This means, of course, that the equilibrium point is far in the direction of hydrolysis.

Haugaard and Roberts (1942) estimate that about 2,000 calories of heat are evolved per peptide bond when the peptide bonds in β-lactoglobulin are hydrolyzed by the action of pepsin.

It is to be expected that the two structural factors which would have the most important influence on the rate of hydrolysis of peptides would be 1) the nature of the amino acid residues adjacent to the peptide bond and 2) the length of the peptide chain.

Levene and co-workers (1932) investigated the rate of hydrolysis of a series of dipeptides by 0.5N NaOH at 25° and found the rate to be very much dependent on the size of the amino acid residues; the larger the residue, the slower the rate of hydrolysis. The size of the residue on the carbonyl side of the peptide bond was most important. This fact probably indicates that the water molecule attaches itself to the carbonyl group. More recently Synge (1945) has studied the rate of hydrolysis of a similar series of dipeptides in the presence of acid. His results parallel those of Levene closely. The influence of the size of the residue on the rate of hydrolysis was, however, less pronounced for acid than for alkali; alkali is apparently a more discriminating agent.

Kuhn, Molster and Freudenberg (1932) and later Freudenberg, Piazole and Knoevenagel (1938) studied the rate of hydrolysis of a series of glycine peptides of increasing length in the presence of one normal sodium hydroxide at 20°. They found that the rate of hydrolysis increases with the length of the peptide. A study of their data shows, however, that while the rate per peptide bond is substantially constant through the tetrapeptide, beyond this length the rate per bond progressively decreases.

Hydrolysis of Proteins by Acids and by Bases

In the hydrolysis of a protein either by acids or by bases, there is undoubtedly a large measure of randomness. It can be shown from statistical theory (Montroll and Simha, 1940) that under such circumstances there will be an accumulation of short chain peptides and the greater the degree of hydrolysis, the shorter will be the most favored length for the peptides. After hydrolysis of 10 to 15 percent of the peptide bonds, the peptides in greatest concentration will be di- and tripeptides.

It is true, however, that neither acids nor bases lead to complete randomness of hydrolysis. For example, the approximate intrinsic relative rates of release of various amino acids from egg albumin by barium hydroxide and by sulfuric acid during the early part of the hydrolysis reaction have been determined. These determinations were made by filter paper chromatography technique (Bull, Hahn and

Baptist, 1949). The results of these studies are shown in Table 1.

A relative intrinsic rate of unity means that the amino acid is liberated at a rate proportional to its concentration in the protein while a rate greater than unity means that it is preferentially liberated;

TABLE 1. INTRINSIC RELATIVE RATES OF RELEASE OF AMINO ACIDS BY 5.0N H_2SO_4 AND BY 3.7N $Ba[OH]_2$ FROM EGG ALBUMIN AT 60°

Amino Acid	Intrinsic relative rate of release	
	H_2SO_4	$Ba[OH]_2$
Aspartic	1.56	.57
Ornithine	—	.26
Glutamic, lysine, serine (average)	.94	1.29
Threonine, arginine (average)	.51	4.07
Alanine, tyrosine (average)	1.36	.67
Valine, leucines (average)	.79	.55

the slower rate of release of valine and the leucines is notable. Conspicuous also is the differential effect of acid and of alkali.

A comparison of the relative rate of release of total free amino acids by alkali (1.45N NaOH at 66°, Warner, 1942) with the rate with acid (7.95N HCL at 60°, Bull and Hahn, 1948) for egg albumin reveals that the ratio of free amino acids released to total peptide bonds hydrolyzed is, during the first part of the reaction, about 3.5 times greater for alkali than for acid.

Over a substantial part of the hydrolysis reaction of egg albumin by 7.95N HCL there appears to be a nearly constant ratio between the number of peptide bonds hydrolyzed and the amount of peptide which cannot be heat coagulated at the isoelectric point of egg albumin. This ratio corresponds to about 55 peptide bonds per mole of egg albumin. This constant ratio means that the peptide bonds in protein hydrolyze at a much faster rate than do the peptide bonds in peptides produced from the protein hydrolysis. It may well be that the more coherent structure of the protein molecule is more conducive to the activation of peptide bonds. The hydrolysis of a peptide bond is an exothermic process, and it is possible that the heat evolved tends to activate neighboring peptide bonds in the protein molecule.

While, as noted above, upon acid hydrolysis of egg albumin the molecule splits on an average into 55 fragments, these fragments are not of equal size. Figure 1 shows the molecular weight distribution of the peptides resulting from the hydrolysis of egg albumin by 7.95N HCL at 30° after the isoelectric heat-coagulable has been removed (Bull and Hahn, 1948). This distribution was determined by the spread film technique.

As can be seen from Figure 1, there is no evidence for the accumulation in the hydrolysate of any considerable quantity of peptide of a given molecular weight; there is a spread of molecular weights. About 40 percent of the hydrolysate has a molecular weight below 1,000, and about 20 percent is between 1,000 and 10,000. There is little material whose molecular weight is between 10,000 and 30,000. A most curious feature is the relatively large amount

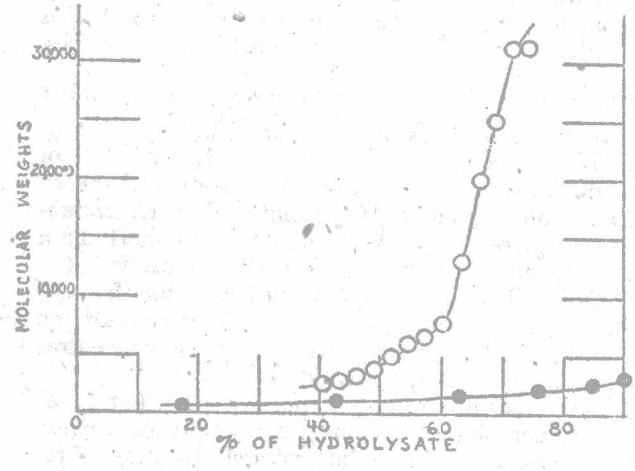

FIG. 1. Molecular weight distribution of peptides after removal of isoelectric heat coagulable material; two hour hydrolysate, 12.7 percent peptide bonds hydrolyzed at 30°. Also shown is the distribution of peptides on the basis of random hydrolysis for the same extent of hydrolysis (filled circles).

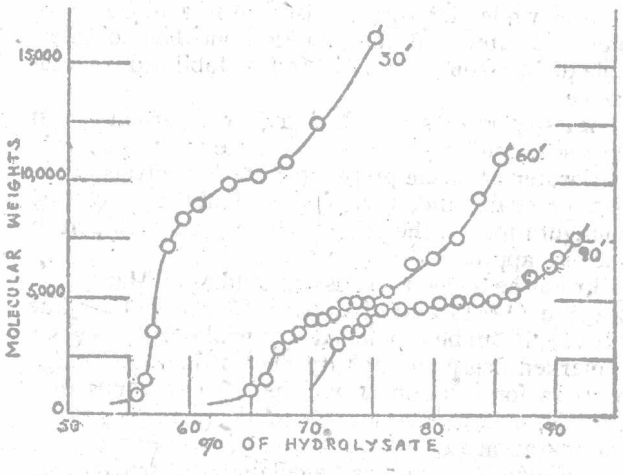

FIG. 2. Molecular weight distribution of peptides resulting from the action of 3.7N $Ba[OH]_2$ on egg albumin at 60° after removal of isoelectric heat-coagulable material for indicated times of hydrolysis.

of peptide of quite high molecular weight. The hydrolysis of egg albumin by acid departs very greatly from a random process; the greatest depar-

ture is by way of this high molecular weight peptide fraction.

Figure 2 shows the molecular weight distribution of peptides resulting from the action of 3.7N $Ba[OH]_2$ at 60° on egg albumin.

It appears from Figure 2 that there is an accumulation of peptides in the alkaline hydrolysate whose molecular weight is about 5,000. The very high molecular weight peptides are missing from the alkaline hydrolysate.

Peptic Hydrolysis

We have concerned ourselves with the action of pepsin on egg albumin, and have studied certain kinetic aspects of this problem along with determinations of the molecular weight distribution of peptides resulting (Bull and Currie, 1949).

The extent of the action of pepsin on egg albumin was estimated from the amount of egg albumin which cannot be heat-coagulated at its isoelectric point. The maximum velocities (V) are expressed in moles of egg albumin solubilized per second per mole of pepsin. The molecular weight of egg albumin was taken as 45,000 and that of pepsin as 35,000. These maximum velocities were estimated by the appropriate Michaelis-Menten plots. The variation of the maximum velocities with the pH of the reaction mixture are shown in Figure 3.

According to the theory of absolute reaction rates, the rate of decomposition of the activated complex should be

$$\gamma C^* \frac{kT}{h}$$

Where γ is the transmission coefficient whose value approaches unity, C^* is the concentration of the activated complex, k is Boltzmann's constant, h is Plank's constant and T is the absolute temperature. The important question arises as to whether or not the Michaelis-Menten complex is identical with the activated complex. If this is true, then V should equal $\gamma kT/K_m h$ where K_m is the Michaelis-Menten dissociation constant. Setting γ equal to unity and substituting the value of K_m (3.4×10^{-4}) at pH 2.0 and at 30° C. and the values for the constants, we calculate that V should be 1.9×10^{17} moles per second per mole of pepsin. The experimental value for V is 0.077 moles per second per mole. Evidently, the Michaelis-Menten complex is not the activated complex; its concentration is very much greater than that of the activated complex. It is, therefore, necessary that the Michaelis-Menten complex pass over into an activated complex before the egg albumin can hydrolyze into peptides.

The Michaelis-Menten treatment also neglects the influence of the hydrogen ions. Since the reaction rate is so dependent on the concentration of hydrogen ions, its seems reasonable to assume that they enter directly into the catalytic reaction.

There are, no doubt, a variety of ways to formulate the above considerations; three simple possibilities are:

$$1)\ H^+ + E \rightleftarrows H^+E;\quad H^+E + S \rightleftarrows H^+ES \rightleftarrows [H^+ES]^* \rightarrow E + P$$
$$2)\ H^+ + S \rightleftarrows H^+S;\quad H^+S + E \rightleftarrows H^+ES \rightleftarrows [H^+ES]^* \rightarrow E + P$$
$$3)\ E + S \underset{K_1}{\rightleftarrows} ES;\quad ES + H^+ \underset{K_2}{\rightleftarrows} H^+ES \underset{K_3}{\rightleftarrows} [H^+ES]^* \rightarrow E + P$$

In the above E represents the enzyme, S the egg albumin, H^+ the hydrogen ions, P is the peptide and $[H^+ES]^*$ is the activated complex.

A consideration of the first and second formulation reveals that the maximum velocity of the reaction should be independent of the hydrogen ion concentration; this is contrary to experience so that both of these formulations must be rejected. The third formulation will be considered in detail.

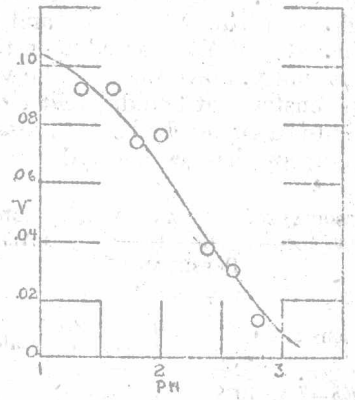

Fig. 3. Plot of experimental points for the maximum velocity of digestion of egg albumin against pH for 30°. The solid line has been calculated from Equation 5.

Now if we assume that the slowest reaction is the formation of the activated complex such that all steps leading up to this reaction are in substantial equilibrium, we can formulate the kinetics of the peptic hydrolysis quite simply. The velocity of the decomposition of the activated complex is

$$v = \gamma[H^+ES]^* \frac{kT}{h} \qquad (1)$$

Substituting the value of the equilibrium constants for the various steps in Equation 1, we have

$$v = \frac{\gamma \times H^+ \times E \times S}{K_1 K_2 K_3} \qquad (2)$$

We know that

$$E = E_0 - ES - H^+ES - [H^+ES]^* \qquad (3)$$

where E_0 is the total amount of enzyme added to the hydrolysate. Substituting Equation 3 in Equation 2 and rearranging, we find

$$1/v = \frac{K_1K_2K_3h}{\gamma kTSE_0} + \frac{K_2K_3h}{\gamma kTHE_0} + \frac{K_3h}{\gamma kTE_0} + \frac{h}{kTE_0} \qquad (4)$$

Evidently, when E_0 is unity and $1/v$ is plotted against $1/S$, $K_1K_2K_3h/kT$ is equal to the slope of the line and is equal to K_m/V. The intercept on the Y-axis is

$$1/v = \frac{K_2K_3h}{\gamma kTH^+} + \frac{K_3h}{\gamma kT} + \frac{h}{\gamma kT} \qquad (5)$$

When $1/V$ is plotted against $1/H^+$, the slope of the line is $K_2K_3h/\gamma kT$ and the intercept is $K_3h/\gamma kT + h/\gamma kT$. It is thus possible to evaluate all the dissociation constants of the third formulation, and these are given in Table 2 for 30° and in Table 3 for 45°. The value of K_3 is based upon the assumption that γ is unity. Since we have the value of the dissociation constants at two different temperatures, it is possible to calculate the heats of dissociation as well as the entropy changes involved.

TABLE 2. DISSOCIATION CONSTANTS AND ENERGIES FOR STEPS INVOLVED IN THE DIGESTION OF EGG ALBUMIN BY PEPSIN AT 30° C

Constant	ΔF 30 Calories	ΔH Calories	ΔS E.U.
$K_1 = E \times S/ES = 7.4 \times 10^{-4}$	4,400	− 1,500	−19.2
$K_2 = H^+ \times ES/H^+ES = 7.2 \times 10^{-6}$	3,000	11,100	26.6
$K_3 = H^+ES/[H^+ES]^* = 5.7 \times 10^{13}$	−19,200	−31,400	−40.5

TABLE 3. DISSOCIATION CONSTANTS FOR STEPS INVOLVED IN THE DIGESTION OF EGG ALBUMIN BY PEPSIN AT 45°

$K_1 = E \times S/ES = 6.6 \times 10^{-4}$
$K_2 = H^+ \times ES/H^+ES = 1.7 \times 10^{-2}$
$K_3 = [H^+ES]/[H^+ES]^* = 4.9 \times 10^{12}$

As can be seen from Table 2, the heat of dissociation of the proton from the enzyme-substrate complex is about 11,000 calories and its pKa is about 2.14. The dissociation constant is about what is to be expected from the ionization of a carboxyl group, but the heat change involved is much too large for a single carboxyl group. It can be seen that the plot of the maximum velocity against pH is essentially a titration curve of the complex. It is possible that a number of carboxyl groups are involved and there may be multiple binding of protons in the formation of the activated complex; if there is considerable overlapping of the binding constants without electrostatic interference, the dissociation of the protons could still be approximately treated by the mass law on the assumption that only one proton was bound.

Figure 3 shows a comparison of the experimental values for the maximum velocity plotted against the pH of the hydrolysate. Also shown are the values calculated according to Equation 5 (solid line). The agreement is satisfactory.

The large heat needed for the creation of the activated complex is very nearly equal to the heat of activation for the denaturation of egg albumin which, at 30° C and in the acid region, is about 35,000 calories (Cubin, 1929). The creation of the activated complex quite possibly involves changes in the entire egg albumin molecule and probably is not confined to a single peptide bond.

As we have formulated the hydrolysis of egg albumin by pepsin, the collision rate between the pepsin and the egg albumin becomes secondary. It appears, however, that the Smoluchowski theory (1916) of the precipitation of colloidal solutions provides a more realistic approach to the calculation of the collision rate than does the gas collision formula.

According to Smoluchowski, the number of unit particles in a colloid at any time, t, is given by

$$n = \frac{n_0}{1 + 8\pi r D n_0 t} \qquad (6)$$

where r is the radius of the unit particle, D is the diffusion constant, n_0 is the number of unit particles at the beginning of the reaction. Evidently, the rate of disappearance of unit particles is twice as great as the collision rate and is at zero time equal to $-8\pi r D n_0^2$. Then the collision rate is $4\pi r D n_0^2$. Calculations show that the rate of collision of pepsin molecules with egg albumin molecules under the conditions which we have worked is about 1/200th of the rate as given by the gas collision theory.

Comparison of the number of peptide bonds hydrolyzed as determined by the Van Slyke amino nitrogen with the amount of protein which cannot

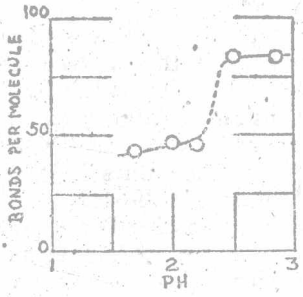

FIG. 4. Peptide bonds hydrolyzed per mole of egg albumin attacked by pepsin at 30° as a function of pH.

be heat-coagulated at the isoelectric point gives the average number of peptide bonds hydrolyzed per molecule of egg albumin attacked. We have determined this ratio as a function of pH, and these results are shown in Figure 4.

Including and above pH 2.5, the number of peptide bonds hydrolyzed per mole of egg albumin is about 84 while below pH 2.2 the ratio is about 44 bonds per egg albumin molecule. So far as can be judged, these ratios are independent of the reaction time; measurements were started as soon after the beginning of the reaction as feasible.

Shown in Figure 5 are the molecular weight distributions of the peptides resulting at pH 1.7 and pH 2.7.

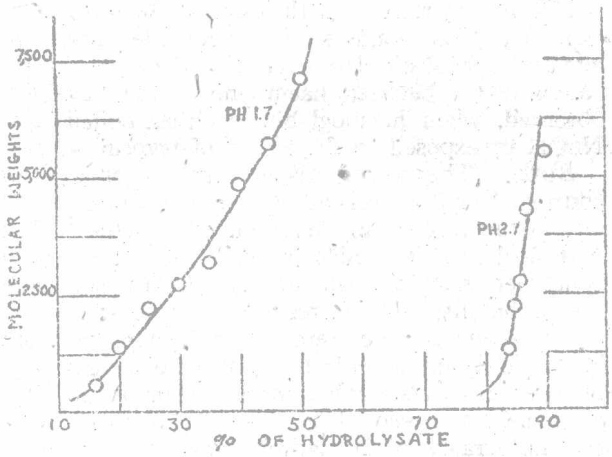

FIG. 5. Molecular weight distribution of peptides resulting from the action of pepsin on egg albumin at 30° after removal of isoelectric heat-coagulable material. Curve 1, pH 1.7. Curve 2, pH 2.7.

The reason for the lower ratio of bonds hydrolyzed per egg albumin molecules attacked, in the lower pH range, no doubt resides in the fact that there is a large quantity of high molecular weight material in the peptic hydrolysate at the lower pH values.

At pH values in excess of 2.5, the molar ratio between the bonds hydrolyzed and the molecules attacked is, as we have seen, about 84. This means that on the average, the egg albumin molecule is split into tetra- and pentapeptides, and since there are few or no free amino acids released and practically all the peptides have a molecular weight below 1,000, the tetra- and pentapeptides must be in abundance. It must also be noted that this extensive bond breakage per molecule occurs during the beginning of the hydrolysis reaction where there can hardly be any question of peptide rearrangements and of co-substrate formation. Quite evidently, the Bergmann (1942) specificity requirements for pepsin are, as far as the hydrolysis of egg albumin is concerned, meaningless. Pepsin simply hydrolyzes every fourth or fifth bond in the egg albumin molecule.

Our research on the hydrolysis of proteins has been supported by Corn Products Refining Company, Swift and Company, The Abbott Laboratories, and the Division of Grants of the U. S. Public Health Service.

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DISCUSSION

ANSON: There has been much evidence, both from the studies of acid hydrolysis by Bull and of enzymatic hydrolysis by Tiselius and others, that

the first pieces of proteins produced by hydrolysis are rapidly hydrolyzed further. In the usual course of hydrolysis one thus obtains a mixture of intact protein and small pieces. I have, however, obtained some suggestive, but quite inconclusive evidence that, despite the usual explosive character of hydrolysis, it is possible to isolate big pieces of proteins from the first products of enzymatic digestion.

If a protein is digested by a proteinase to the extent of only a few percent and the undigested protein is precipitated by 0.2N trichloracetic acid, half or more of the digestion products not precipitated by 0.2N trichloracetic acid can be precipitated by 1N trichloracetic acid. As digestion proceeds further, the fraction of the products of digestion precipitated by 1N but not by 0.2N trichloracetic acid becomes less and less (Anson, 1940, J. gen. Physiol. *23*:695; see page 699). It remains to be found out by direct experiment whether or not the fraction of slightly digested protein precipitated by 1N trichloracetic acid does in fact contain the much desired big pieces of proteins. This could be done by measurements of end groups and by the usual molecular weight measurements.

BULL: In the case of acid hydrolysis as well as of peptic hydrolysis a fair fraction of the hydrolysate has, during the early stages of hydrolysis, a molecular weight in excess of 30,000 as revealed by film balance studies. This high molecular weight material cannot, however, be heat coagulated. This is no doubt the material which Dr. Anson has encountered in his work. The nature of this large peptide fragment is obscure. Alkalin hydrolysis does not give rise to this high molecular weight peptide.

BUTLER: Is Dr. Bull sure that all the heat precipitated material is undigested protein? Peptic digests of proteins easily deposit, at some pH's, insoluble materials (plasteins). Does anything analogous occur under his conditions?

BULL: The question of whether or not some of the peptide is brought down during the heat coagulation of the intact egg albumin is dealt with in my reply to the discussion of Dr. Neurath, where it is shown by recovery experiments that such precipitation, under the conditions of our experiments, is small.

If the filtrate resulting from the heat coagulation of a peptic hydrolysate be concentrated to about a third of its volume in a sausage casing by evaporation at room temperature, a slight precipitate results.

BUTLER: With regard to the specificity of the action of the enzyme, we have found with chymotrypsin that there is an initial rapid specific action followed by a slow, less specific proteolysis. Has Dr. Bull found any indication that the bonds broken in the initial stages may be different from those broken toward the end of the reaction?

BULL: Such information as we have does not indicate that pepsin behaves like chymotrypsin in regard to bond specificity. The ratio of bonds hydrolyzed to egg albumin attacked is, as nearly as we can judge, constant over a broad range of the reaction extending to a point as early in the reaction as it is practical to make measurements.

HAUROWITZ: Could the larger split products of peptic hydrolysis not arise by disaggregation of ovalbumin? Are they not sub-units of a larger polymeric molecule?

BULL: Egg albumin, unlike some proteins, does not easily dissociate into sub-units. I am inclined to believe that the fragments which are produced by hydrolysis arise principally if not exclusively from the hydrolysis of peptide bonds.

HAUROWITZ: Can secondary closure of peptide bonds not interfere with the results? Such a formation of peptide bonds seems inprobable in non-enzymatic hydrolysis. However, the formation of covalent bonds between haem and globin has been observed, when haemoglobin is either boiled with NaOH or exposed to the action of trypsin.

BULL: The resynthesis of peptide bonds is, of course, always a possibility and nothing which we have done throws any light on this problem. The hydrolysis studies which we have made have been concerned with the early stages of hydrolysis where the probability of such resynthesis is least.

HAUROWITZ: Are there any indications of a core in the ovalbumin molecule? If there were a difference between superficial and interior parts of the molecule, they should be revealed by the paper chromatography of the hydrolysates.

BULL: We have no evidence for the existence of a core in the egg albumin molecule.

NEURATH: I am under the impression that Dr. Bull's most interesting results are predicated on the accuracy of the method which he has used in differentiating between unhydrolyzed protein and split products. The method of isoelectric heat coagulation may not be entirely valid in this respect not only because of the coprecipitation of split products with the heat coagulum, as already mentioned in this discussion, but also because it is known from Kleczkowski's work that the heat coagulation of a protein may be inhibited by other proteins which do not coagulate themselves under the same conditions. Thus, protein fragments of different isoelectric points, formed during the initial phase of hydrolysis, may conceivably mask the heat coagulation of unchanged egg albumin.

BULL: We hydrolyzed egg albumin by pepsin and prepared solutions of peptide free from egg albumin, the protein being removed by isoelectric heat coagulation and filtration. We then added this peptide to egg albumin solutions without the addition of pepsin. Aliquots of these solutions were removed and heat-coagulated at the isoelectric point of egg albumin in the usual manner. These solutions were

made up to volume and filtered. Total nitrogens were run on aliquots of the filtrates and the amount of peptide in the filtrates calculated. The recoveries of the peptides are shown in the table below:

GRAMS OF PEPTIDE RECOVERED FROM EGG ALBUMIN SOLUTIONS AFTER ISOELECTRIC HEAT COAGULATION

Grams protein per 100 cc.	Grams peptide added per 100 cc.	Grams peptide recovered per 100 cc.
6.10	0.159	0.159
1.89	0.049	0.046
1.02	0.026	0.025
0.62	0.016	0.017
4.46	1.62	1.63
3.96	1.62	1.64
2.63	1.62	1.67
0.98	1.62	1.63

Evidently, the method of isoelectric heat coagulation is capable of yielding good recoveries of added peptide. It is, accordingly, probable that no large error is involved in following the course of the digestion of egg albumin by pepsin by means of this method.

NEURATH: I should like to ask Dr. Bull also whether the hydrolysis of amide bonds in the protein occurs and if so, whether he has applied the proper corrections in his calculations.

BULL: We have been unable to detect any production of ammonia during the hydrolysis of egg albumin by pepsin. In the case of the acid hydrolysis of egg albumin, the ammonia has been determined and corrections applied.

NEURATH: Since I shall not be in attendance of the Symposium when other papers on proteolytic enzymes will be presented, I should like to offer now a few comments on the significance of the calculations based on the determination of the temperature dependence of hydrolysis rates. Such measurements have been carried out in our laboratory in great detail, primarily of the chymotryptic hydrolysis of specific peptide and ester substrates but also of the hydrolysis of specific peptides and esters by carboxypeptidase and trypsin. These have been calculated for the rate-determining step of the activation of the Michaelis-Menten complex, and corrected to a standard state defined by the moles of substrate activated per mole of enzyme-substrate complex, per second, at the pH optimum of each enzyme at 25°. The calculated heats of activation are uniformly lower than those given here by Dr. Bull, *i.e.*, ranging from about 10 to 16 kcals. per mole, as compared to Bull's value of 31 kcals. The changes in entropy of activation are likewise lower than those reported here for the peptic hydrolysis of egg albumin, *i.e.*, about −10 E.U., as compared to Dr. Bull's value of −40 E.U. Our values are comparable to those previously reported by Dr. Butler.

I can conceive of two explanations for the considerably higher values presented by Dr. Bull: First, Bull's values are only slightly higher than those found in the literature for the acid- or base catalyzed hydrolysis of specific peptides and thus may be indicative of the acid hydrolysis of egg albumin, though I am aware that the standard states for these catalytic reactions and for enzymatic catalysis need not necessarily be comparable. Nevertheless, one should recognize the fact that the measurements of the peptic hydrolysis of egg albumin were performed in solutions of high acidity and that the enzymatic contribution to the energetic constants can only be evaluated by comparison with analogous measurements in the absence of the enzyme.

The other possible explanation is that the energetic constants given by Dr. Bull refer not to protein hydrolysis but to the peptic denaturation of egg albumin. This suggestion is being offered because of the resemblance of the present energetic constants to those found for protein denaturation in general and also because of the widely-held notion that the first step in the enzymatic hydrolysis of proteins is denaturation. Since the measured rates are those of the slowest step, all we would have to assume is that in the present case, and perhaps in every such case, the rate of denaturation is slower than the rate of proteolysis. Whichever explanation is more nearly correct, I believe that calculations of energetic changes accompanying the enzymatic hydrolysis of peptide or ester substrates, which will be published in full elsewhere, represent more nearly the true state of affairs.

BULL: The value of −31 kcals per mole for the heat and −40 E. U. per mole apply to the reverse reaction; to compare these values with those obtained by the usual formulation, the signs should be reversed. As stated, these large values probably indicate that the entire egg albumin molecule is activated before it can undergo hydrolysis. Undoubtedly, this activation can be regarded as a form of denaturation, although how profitable such a point of view may turn out to be is questionable.

Dr. Neurath's experiments on the influence of temperature on the rate of hydrolysis of specific peptides by chymotrypsin were conducted at the pH optimum of the enzyme at 25° C. The heat of ionization of the enzyme-substrate complex has thus, by necessity, been neglected. This neglect has introduced an element of ambiguity into his calculations of the energies of activation. It is, therefore, difficult to compare our values in which the heat of ionization has been taken into account with those of Neurath and of Butler.

Under the conditions which we have worked, the amount of hydrolysis of egg albumin in the absence of pepsin (acid hydrolysis) is so small that it is undetectable.

NEURATH: Finally, I should like to ask Dr. Bull whether he would care to comment on the effect of enzyme concentration on hydrolysis rates, with particular consideration of the competitive influence of reaction products on the hydrolysis of remaining protein substrate.

BULL: We have made no detailed investigation of the influence of varying enzyme concentration on the rate of digestion. It has been found that the addition of peptide obtained from a peptic hydrolysate to a fresh enzyme-substrate system greatly inhibited the digestion of egg albumin by pepsin.

THE FRACTIONATION OF PROTEINS BY ELECTROPHORESIS-CONVECTION

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A method of fractionation of proteins in solution utilizing a combination of electrophoretic and convective transport of the components was first suggested by Kirkwood in 1941 and investigated experimentally by Nielsen and Kirkwood (1946) several years later. More recently an electrophoresis-convection apparatus of improved design has been described and successfully used to fractionate ovalbumin, horse diphtheria antitoxin pseudoglobulin, the bovine serum proteins and bovine γ-globulin (Cann, Kirkwood, Brown and Plescia, 1949; Cann, Brown and Kirkwood, 1949).

The method is based upon the same principles as those of the Clusius column, except that horizontal electrophoretic transport instead of transport by thermal diffusion is superimposed on convective transport in a vertical convection channel. The fractionation scheme may be briefly described as follows. Two reservoirs connected by a vertical channel, of width sufficiently small to ensure laminar flow, contain a solution of the protein to be fractionated. Upon application of a horizontal electric field, differential transport of the mobile components across the channel takes place, producing a horizontal density gradient depending upon the composition gradients. Under the action of gravity, the density gradient induces convective circulation in the channel with a velocity distribution qualitatively similar to that of the Clusius column. The result of the superposition of the horizontal electrophoretic transport and vertical convective transport is movement of the mobile components from the top reservoir to the bottom reservoir at rates depending on their mobilities, with a relative enrichment of the top reservoir with respect to the slow components and of the bottom reservoir with respect to the fast components. In order to avoid contamination of the solution by electrolysis products, the walls of the convection channel are constructed of semi-permeable membranes, separated from the electrodes by buffer solution. The electric field across the channel is maintained by the electric current carried by the ions of the buffer electrolyte, to which the membranes are permeable. The exterior buffer solution is replenished by a circulation system at rate sufficient to prevent electrolysis products from reaching the membranes.

The purpose of this paper is to point out the essential features of electrophoresis-convection and to illustrate the utility of the method in protein fractionation.

THE ELECTROPHORESIS-CONVECTION APPARATUS

The electrophoresis-convection apparatus may be considered as being composed of five principal parts: (1) the fractionation cell consisting of a cell block, two face plates, and semi-permeable membranes; (2) the box housing the fractionation cell and electrodes; (3) the electrode assemblies; (4) the buffer circulating system; and (5) the power pack. A photograph of an unassembled fractionation unit is presented in Figure 1. On assembly the cell consists essentially of a narrow vertical channel connecting upper and lower reservoirs. That portion of the channel effective in fractionation is formed by the rectangular space between two sheets of semi-permeable membrane. The cell, containing a buffered solution of the protein to be fractionated, is immersed in buffer solution to within about an inch above the bottom of the upper reservoir. The electrode assemblies are placed in the box on opposite sides of the cell, and an electric field applied across the channel of the cell. During operation electrolysis occurs which tends to change the pH of the buffer solution. To counteract this, buffer is circulated vertically around the cell. The temperature of the system is regulated in a constant temperature cold room operating at 4° C.

The material used in the construction of the fractionation cell and its housing must be electrically non-conducting, exhibit no swelling when in contact with aqueous solutions, possess dimensional stability, and be readily machined with high precision. Lucite appeared to be the material most suitable for this purpose and was chosen as the construction material.

The cell block consists of an upper and lower reservoir between which is a vertical rectangular slot. The reservoirs are connected to the central slot by means of narrow vertical channels passing through the body of the block. A recess is milled around the edge of the rectangular slot on both faces of the cell block. The cell block is supplied with two small legs. The reservoirs are supplied with valves for sampling; and the top reservoir is open to the atmosphere. The capacities of the upper and lower reservoirs of the apparatus used in these studies are 100 and 50 ml., respectively. The top and bottom reservoir capacities of the apparatus pictured in Figure 1 are 15 and 10 ml., respectively.

The face plates are frames which fit into the recesses around the periphery of the central slot of the cell block. Around the inner edge of the frame is a

shoulder constructed to fit into the central slot. The face plates clamp the sheets of semi-permeable membrane into place against the bottom of the recess. Since the membranes form the face-walls of the effective portion of the channel, the height of the shoulders control the channel wall separation. In the apparatus described here the wall separation is 0.037". The face plates are bolted to the cell block with machine screws. If metal screws are used it is necessary to insulate electrically the heads and tips of the screws. The use of plastic screws would, of course, eliminate the necessity for electrical insulation. Sheets of semi-permeable membrane are made from cellulose sausage casing.

The inside dimensions of the box which houses the fractionation cell and electrode assemblies are such that a snug fit is obtained between the sides of the box and the edges of the cell block. This minimizes loss of electric field by leakage around the cell. The box is supplied with buffer inlet and outlet tubes.

Each electrode assembly consists of 2 mil platinum foil mounted in a lucite frame. The dimensions of the platinum foil correspond to those of the inner periphery of the face plates. The dimensions of the frames are such that the strips of platinum and the effective channel of the cell are aligned when the cell and electrode assemblies are housed in the box. Since platinum occludes large quantities of electrolytic hydrogen it is advisable to alternate the polarity of the electrodes from run to run.

Circulation of buffer around the cell is by gravity flow. The buffer flows from an aspirator bottle into the box housing the cell and electrodes. After circulating vertically around the cell the buffer is discharged into a flask. A centrifugal pump periodically pumps the circulated buffer back into the aspirator bottle. It is desirable to operate under conditions of field strength and current density which do not lead to electrolytic decomposition of the buffer anions. Under these conditions the original pH of the buffer solution is restored on mixing, and the buffer may be recycled in the circulating system without replenishment.

Operation of the Electrophoresis-Convection Apparatus

Depending upon the electrophoretic properties of the particular protein system under investigation, fractionation can be accomplished by three modes of operation. In the first method the operating pH is such that all the components of the heterogeneous protein are either on the alkaline or acid side of their isoelectric points. Under these conditions the components are differentially transported out of the upper and into the lower reservoir, the fractionation depending upon the difference in mobilities of the constituent proteins. The greatest separation of components is obtained by so choosing the operating time that half of the major component is transported out of the upper reservoir. This method is suitable for the separation of components differing but slightly in their isoelectric points.

The separation of a protein mixture possessing discrete mobility and isoelectric point spectra, *e.g.*, serum, into its constituent proteins is accomplished by the isoelectric procedure. In this method of operation one of the constituents of the heterogeneous protein is immobilized by operating at its isoelectric point. The mobile components are transported out of the upper and into the lower reservoir, leaving the immobilized component in the upper reservoir. Complete exhaust of the mobile components from the top reservoir, although closely approach under ideal conditions is sometimes inhibited by various disturbing factors. The most important disturbing factors appear to be osmotic transport of solvent from the exterior buffer solution into the cell and the establishment of a stationary state before exhaust when the mobilities of some of the components are of opposite sign at the operating pH. Optimum operating conditions must therefore be determined by pilot fractionations. When applicable this procedure is far more efficient than the first method of operation.

The successive separation of the components of a protein mixture can be accomplished by the isoelectric procedure as follows. The component with the most alkaline or acid isoelectric point is first separated from the other in several successive stages by operating at its isoelectric point. The composite of the top cuts of these stages is further processed to purify the desired component. The bottom cut of the last of these stages is a concentrate of the mobile components. The process is repeated until the mixture has been resolved.

In the case of a protein which migrates as a single boundary in an electric field but possesses a specified mobility distribution as revealed by reversible electrophoretic boundary spreading, *e.g.* γ-globulin, fractionation is accomplished by means of a modified isoelectric procedure. In this procedure the fractionation is carried out at a pH displaced by an arbitrary amount from the mean isoelectric point of the heterogeneous protein. Transport in the apparatus leads to a redistribution of the protein ions such that the fractions withdrawn from the top and bottom reservoir possess mobility distributions differing from that of the original protein. Fractions possessing different mean mobilities and isoelectric points are obtained by proper choice of the operating pH's. Transport proceeds to a stationary state in which the top fraction is isoelectric at the operating pH.

The application of these methods of fractionation to particular heterogeneous proteins will be discussed below.

Fractionation of Ovalbumin

Within the pH range of 5 to 10, crystalline ovalbumin is resolved electrophoretically into two components A_1 and A_2, A_2 being the slower moving

component (Longsworth, Cannan and MacInnes, 1940). The relative concentration of A_2 in freshly prepared material is reported to vary from 15 to 25 percent. A third component, A_3, has also been found to be present in small amounts (MacPherson, Moore and Longsworth, 1944; Alberty, Anderson, and Williams, 1948). A_3 is less mobile than A_1 and A_2 at pH 6.8. At its average isoelectric point pH 4.58 and ionic strength 0.1 (Longsworth, 1941), ovalbumin is not resolved electrophoretically into its components (Alberty, Anderson and Williams, 1948).

Since the components of ovalbumin possess approximately the same isoelectric point and since the differences in the mobilities of the components are relatively small, the partial fractionation of this material affords a good example of the application of the first method of operation described above to a naturally occurring protein. Although component A_1 converts irreversibly into A_2, the rate of conversion is slow enough to be negligible in the present investigation (MacPherson, Moore and Longsworth, 1944).

Fractionation experiments were carried out in phosphate buffer, pH 6.8 and ionic strength 0.05. At this pH and ionic strength the mobility of component A_1 is -7.08×10^{-5} cm^{2}sec^{-1}volt^{-1}, A_2, -5.93×10^{-5}, and A_3, -5.2×10^{-5}. In these experiments the three components were differentially transported out of the top and into the bottom reservoir at rates depending on their mobilities, with a relative enrichment of the top reservoir with respect to components A_2 and A_3 and of the bottom reservoir with respect to component A_1. The results of a series of single stage fractionations are presented in Table 1. The tabulated relative concentrations of A_2 were obtained by extrapolation of the apparent electrophoretic distributions to zero protein concentration at a constant ionic strength of 0.1 and pH 6.8. The contribution of component A_3 to the electrophoretic pattern is included with that of component A_2. The efficiency of separation is expressed in terms of the top separation factor defined by the relation

$$f_T = \frac{x_t}{x_i^0} \cdot \frac{1 - x_i^0}{1 - x_t}$$
$$x_i = \frac{C_i}{\Sigma_j C_j} \qquad (1)$$

In this case x^0_i and x_t are the initial and final ratios of component A_2 concentration to total protein concentration in the upper reservoir, respectively.

The field strength employed in these experiments was varied from 0.4 to 3.1 volts/cm. The duration of the runs were such as to transport about half of component A_1 out of the top reservoir. It will be noted that under the condition of half transport the top separation factor is independent of the field strength. It was also found that at constant field strength and time, the fraction of protein transported out of the top reservoir and the separation factor are independent of the initial protein concentration within the range 1 to 3 percent. These observations and the magnitude of the top separation factor are in agreement with the predictions of a mathematical theory of transport in the electrophoresis-convection channel.

TABLE 1. FRACTIONATION OF OVALBUMIN BY ELECTROPHORESIS-CONVECTION AT pH 6.8 AND IONIC STRENGTH 0.05

Run	E_0[a] volt/cm.	t[b] hr.	Total protein concentration % by wt.			Percent component A_2			
			Initial	Top	Bottom	Initial	Top	Bottom	f_T
1	0.4	2	1.2	0.8	1.6	30	34	28	1.20
2	1.2	1	1.1	0.4	1.4	30	35	29	1.26
3	1.2	1	1.6	0.9	2.1	30	34	26	1.20
4	1.2	1	3.3	1.6	3.8	32	38	31	1.30
5	1.6	$\frac{3}{4}$	1.3	0.7	1.7	33	37	29	1.19
6	3.1	$\frac{1}{2}$	1.0	0.5	1.4	30	34	27	1.20

[a] Nominal field strength.
[b] Duration of run.

The average separation factor of 1.23 obtained in this series of runs indicated the possibility of accomplishing considerable enrichment of the slow moving components by successive batch fractionations, the top cut of each stage of fractionation serving as the starting material for the succeeding stage. Thus, starting with a solution of ovalbumin containing 20 percent A_2, after ten stages of fractionation the relative concentration of A_2 in the final top cut would be 67 percent. Such a three-stage batch fractionation was carried out at a field strength of 1.2 volts/cm and an operating time of 1 hr. The initial total protein concentration was 4.8 percent, and the initial relative concentration of A_2 was 22 percent. The solution withdrawn from the top reservoir after the third stage of fractionation analyzed 0.9 percent total protein and 31 percent A_2. This represents an over-all top separation factor of 1.59.

These experiments demonstrate that the separation of a mixture of proteins possessing nearly the