[CONTRIBUTION FROM CHEMISTRY DEPARTMENT, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

The Acid Hydrolysis of Egg Albumin. II. Molecular Weight Distribution of Peptides

BY HENRY B. BULL AND J. WILFRID HAHN

This paper describes a new method for the estimation of the molecular weight distribution of the products resulting from the partial acid hydrolysis of proteins. The hydrolysate is spread on the surface of an ammonium sulfate solution and compressed. The peptides are forced into the substrate solution. The film is re-expanded, and the average molecular weight of the peptides remaining in the film determined by the application of the gas laws in two dimensions. By successive compressions to higher and higher pressures, it is possible to estimate the molecular weight distribution of the peptides in the hydrolysate. The hydrolysates have been described,[1] and were obtained by the action of 7.95 N hydrochloric acid on egg albumin at 30, at 45 and at 60°.

The Method

The general film technique has already been described.[2] The Wilhelmy slides consisted of two thin microscope cover glasses suspended from an arm of a Chain-o-Matic analytical balance and were located at one end of a well paraffined cast aluminum tray which was 65 cm. by 14 cm. One milligram of weight was equivalent to 0.0409 dyne per centimeter film pressure.

The substrate solution was a 5% ammonium sulfate solution which had been treated with activated charcoal and filtered. Glycerol which had been exhaustively extracted with petroleum ether was added to the ammonium sulfate to the extent of 2% by volume. It is not easy to obtain solution surfaces sufficiently free of surface active impurities, and care has to be exercised. Spreading was done with a Blodgett pipet whose delivery volume was 0.0850 cc. From 20 to 30 micrograms is a convenient weight of film for the size of tray used.

One minute after spreading, pressure readings were made as a function of film area up to a pressure of about one dyne per centimeter. The film was then compressed to 5 dynes per centimeter and held at this pressure for five minutes by moving the barrier in as part of the film passed into solution. The film was then re-expanded and a series of pressure readings below one dyne per centimeter was made as a function of the film area. The film was then compressed to 10 dynes per centimeter and held at this pressure for five minutes. The film was re-expanded and the low pressure measurements made. This process was repeated at 15, at 20 and at 25 dynes per centimeter pressure. It was possible to determine the complete molecular weight distribution of an hydrolysate in about an hour and a half.

When the film pressure is multiplied by the film area and this product plotted against the film pressure a straight line results, the equation of which is

$$FA_1 = \alpha_1 F + \beta_1 \qquad (1)$$

At zero film pressure, FA_1 is equal to β_1 and when A_1 is expressed in square meters per milligram, β_1 is equal to NRT which at 25° is equal to $24.6 \times 10^2/M$. The slope is equal to α_1 which is the area occupied by the gaseous film molecules.

Before we can calculate the molecular weight we must know the weight of the film. To find this weight we proceeded in the following manner.

A known weight of intact egg albumin was spread on the surface and compressed to 5 dynes per centimeter, and it was found that 1.11 milligram occupies one square meter at this pressure. An hydrolysate film was compressed to 5 dynes for five minutes. The film was then re-expanded and low pressure readings made. The area of the gaseous film was then determined with the use of equation 1. The relation between the gaseous area and the area at 5 dynes per centimeter for many different films is shown in Fig. 1. Evidently, there is a close relation between these two areas. The average ratio between the gaseous area and the 5 dyne per centimeter area is 1.065. If we assume that the area per milligram of the hydrolysate film at 5 dynes per centimeter is the same as that of the film of intact egg albumin, we can calculate the weight of the hydrolysate film by multiplying the area of the gaseous film by 1.035.

Shown in Fig. 2 is a typical plot of FA_1 against F, and demonstrates the reversibility of the pressure–area relation.

The weight of the Wilhelmy slides tends to increase with time. This increase amounted in some cases to as much as 5 mg. in the course of one and one-half hours and tends to give a nonlinear relation when FA_1 is plotted against F, the apparent pressure being too small. It can be determined what weight must be added to the apparent pressures to yield a straight line when FA_1 is plotted against F, and such values have been accepted as valid provided the weight which had to be added was what was to be expected from the apparent increase of the surface tension of the clean surface during the same interval of time.

The return of some of the displaced peptides to the surface after the expansion of the film to low pressures would not invalidate this method, but would render the fractionation of the hydrolysate

(1) Bull and Hahn, THIS JOURNAL, **70**, 2128 (1948).

(2) Bull, *ibid.*, **67**, 4, 8 (1945); **68**, 745 (1946); *Adv. Prot. Chem.*, **3**, 95 (1947). See also Guastalla, *Cahiers phys.*, 2nd ser., **10**, 30 (1942).

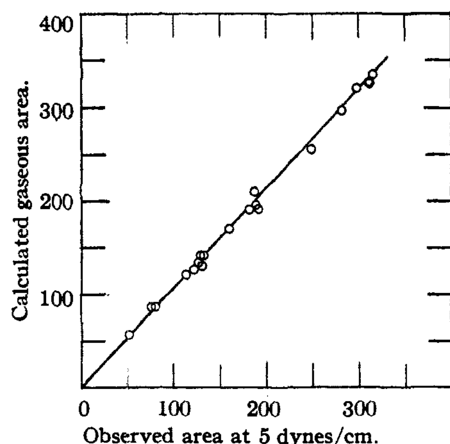

Fig. 1.—Calculated gaseous areas (α_1) in square meters plotted against the observed film area at 5 dynes per centimeter pressure.

inefficient to the extent to which such return occurs. To obtain a measure of this return to the surface the following experiment was done.

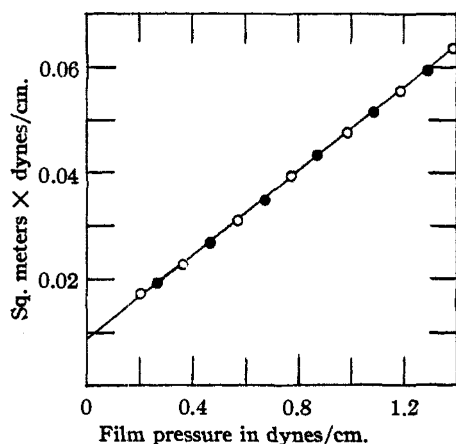

Fig. 2.—FA_1 in dynes per centimeter–square meter plotted against F in dynes per centimeter: open circles compression; filled circles, expansion.

An hydrolysate film was compressed to 25 dynes per centimeter and held at this pressure for five minutes. It was then re-expanded and after a few minutes compressed to 5 dynes per centimeter. This expansion and compression to 5 dynes per centimeter was repeated several times and the results plotted in Fig. 3.

The return of peptides to the film is probably small during a determination of the molecular weight distribution. Judging from the results shown in Fig. 3, roughly 2 to 3% of the displaced peptides would be expected to return to the film during an actual measurement of the molecular weight.

No answer can be given as to the accuracy of the present method because no other method exists for the resolution of peptides on the basis of their molecular weights, and it is not practical to synthesize a series of long chain peptides to calibrate the method. As shown previously,[2] results on the determination of the molecular weight of intact proteins were satisfactory and in accord with the results from accepted methods. As shown in Fig. 4, results from duplicate analyses of the molecular weight distribution of peptides are in reasonable agreement.

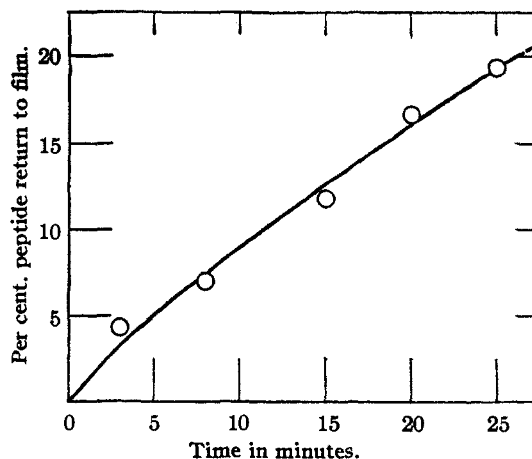

Fig. 3.—Per cent. of return of peptides to the surface as a function of time: 60° hydrolysate with 1.41 millimoles of peptide bond per gram of protein hydrolyzed.

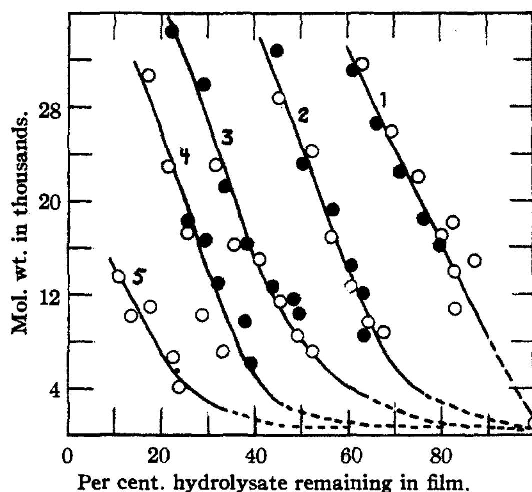

Fig. 4.—Number average molecular weights of peptides remaining in the film plotted against the per cent. of the total hydrolysate remaining in the film. The per cent. of the total peptide bonds hydrolyzed were as follows: Curve 1, 9.9; curve 2, 11.14; curve 3, 15.85; curve 4, 21.45; curve 5, 24.75. Open circles represent one series of measurements, while the filled circles are duplicates; 60° hydrolysates.

Results and Discussion

The partial hydrolysates used have been described[1] and were prepared for spreading by transferring 5 cc. of the hydrolysate resulting from the sodium bicarbonate neutralization to a 250 cc.

volumetric flask. A few drops of concentrated hydrochloric acid were added and after water was added nearly to volume, the flask was warmed to bring all of the hydrolysate into a clear solution. After cooling and making to volume the solutions were ready for spreading.

The primary data resulting from this investigation are very extensive, and it is not practical to report them in detail. It was found that the molecular weight distribution is, within limits of experimental error, the same for 30°, for 45° and for 60° hydrolysates for a given extent of hydrolysis and we are confining our report mainly to results for the 60° hydrolysates.

Shown in Fig. 4 are the number average molecular weights in thousands of the material remaining in the film after being subjected to compression, plotted as a function of the per cent. of the total amount of material in the hydrolysates. The individual curves are identified as representing material from different extents of hydrolysis at 60°. The open circles correspond to one series of measurements and the filled circles to results obtained several weeks later during a second series of measurements. While there is not perfect agreement between the two series, the agreement is reasonably satisfactory.

In order to express the results in Fig. 4 in a more understandable form, we have proceeded in the following manner:

The total number of moles of peptides remaining on the surface at any given compression have been calculated and these values plotted against the weight of the peptides in the film. The slopes of the smooth curves drawn through the points were measured with a Bausch and Lomb Tangent Meter. The reciprocal of these slopes is evidently equal to the molecular weight of the peptides being displaced from the film. These molecular weights were then plotted against the weight percentage of the peptides to give integral weight distributions which are shown in Fig. 5.

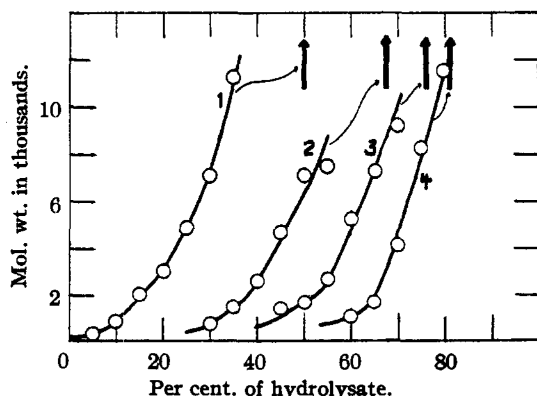

Fig. 5.—Integral molecular weight distribution curves for peptides resulting from the acid hydrolysis at 60°. The number of the curves corresponds to those shown in Fig. 4. Vertical lines at top of figure are the percentages of heat coagulable material at the isoelectric point.

It will be noted from Fig. 5 that the amount of isoelectric heat coagulable material is consistent with the molecular weight distribution of peptides. All curves in Fig. 5 are smooth, and there is no evidence for any appreciable accumulation of a peptide of a particular molecular weight.

A 30°, two hour hydrolysate was subjected to isoelectric heat coagulation. The percentage of peptide bonds hydrolyzed in the soluble peptides was 12.7. The integral distribution curve for these peptides is shown in Fig. 6.

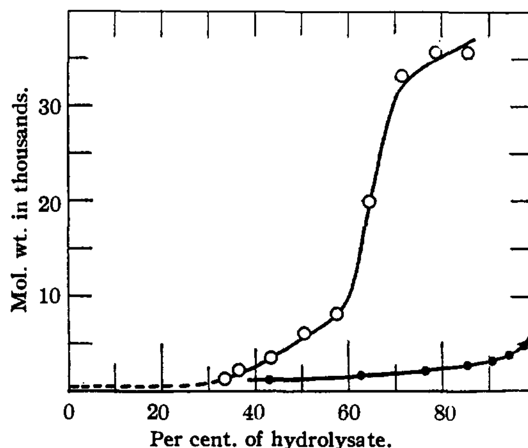

Fig. 6.—Integral molecular weight distribution curve of peptides after removal of isoelectric heat coagulable material: two hour hydrolysate at 30°. Also shown is the distribution of peptides on the basis of random hydrolysis for the same extent of hydrolysis (filled circles).

The molecular weight distribution of the pepides has been calculated for random hydrolysis with the theory of Montroll and Simha.[3] In these calculations the molecular weight of the monomer was assumed to be 111.3[4] and the number of residues to be 400. The fraction hydrolysis was 0.127. Figure 6 shows the results of these calculations, and it is clear that the hydrolysis of peptide bonds in egg albumin is far from being a random process.

Comparing the results shown in Fig. 6 with some of the conclusions drawn in the first paper of this series,[1] we can say the following: The 56 fast hydrolyzing bonds must be rather evenly distributed throughout at least part of the protein molecule, otherwise there would not be such a large fraction of low molecular weight peptides. On the other hand, there is a fairly large amount of peptides with molecular weights between 1,000 and 10,000. There is no way of knowing whether this heavier material is simply an intermediate in the fast reaction or is an end-product of this reaction. It is perhaps significant that there is little material whose molecular weight is between 10,000 and 30,000. This seems to indicate that the initial attack occurs on the ends of the peptide chains rather than in the middle.

(3) Montroll and Simha, *J. Chem. Phys.*, **8**, 721 (1940).

(4) Chibnall, *Proc. Roy. Soc.* (*London*), **B131**, 152 (1942).

In order to obtain the relation between the molecular weight of the peptides forced into the substrate solution and the pressure exerted on the film, the percentages of the hydrolyzed material remaining in the film were plotted against the pressures exerted. The corresponding molecular weights were then interpolated from Figs. 5 and 6. The logarithms of the molecular weight have been plotted against the pressures exerted and are shown in Fig. 7.

The points in Fig. 7 extrapolate at zero pressure to log molecular weight of about 3. This means that peptides whose molecular weight is about 1,000 or less pass spontaneously into the substrate solution and will not form a film.

The techniques described in this paper are being applied to the study of the molecular weight distribution of peptides resulting from the action of enzymes on proteins. These results will be reported in due time.

It is a pleasure to acknowledge the generous assistance granted this research by Corn Products Refining Company.

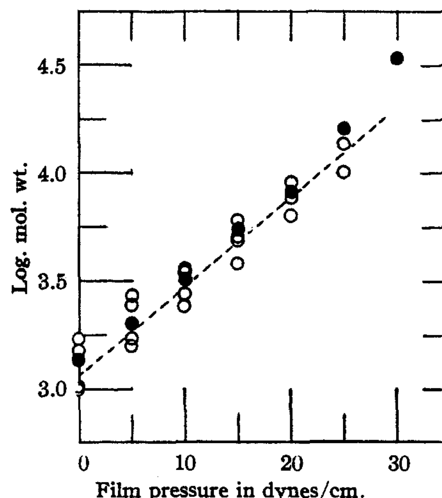

Fig. 7.—Logarithm of molecular weights of displaced peptides plotted against pressure exerted on the film. Open circles are 60° hydrolysates and filled circles two hour hydrolysate at 30° (protein free).

Summary

1. A new method has been described for the determination of the molecular weight distribution of peptides in a partial acid hydrolysate of a protein.

2. An hydrolysate is spread as a monomolecular layer on a 5% ammonium sulfate solution and is compressed to progressively increasing pressures. The logarithm of the molecular weight of the displaced peptides is proportional to the pressure exerted on the film. The molecular weight of the peptides remaining in the film after exposure to a given pressure is determined by the application of the gas laws in two dimensions.

3. The molecular weight distribution of egg albumin hydrolyzed by hydrochloric acid at several degrees of hydrolysis has been reported.

4. It is found that while the molecular weight distribution of peptides departs greatly from that expected for a random hydrolysis, there is no evidence for the accumulation of any considerable amounts of a peptide of a given molecular weight.

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[CONTRIBUTION FROM THE UNITED SHOE MACHINERY CORPORATION RESEARCH DIVISION]

Partition Chromatography of Amino Acids with Applied Voltage

BY GOTFRED HAUGAARD[1] AND THOMAS D. KRONER

In the one dimensional partition chromatography developed by Consden, Gordon and Martin[2] it is often very difficult to detect the bands of amino acids whose R_F values lie close together. To overcome this difficulty, two dimensional chromatography employing two solvents was developed by these workers.

In our work, we encountered overlapping of R_F values between the basic, acidic and certain neutral amino acids. We have effected a two dimensional chromatography by the passage of current through paper treated with phosphate buffer at *p*H 6.2.[3] The negatively charged acids—aspartic and glutamic—move toward the anode; the basic acids—lysine and arginine—migrate toward the cathode and the neutral amino acids are unaffected by the voltage gradient at the *p*H close to their isoelectric point.

(1) Present address: National Dairy Corporation, Oakdale, Long Island, New York.

(2) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).

(3) R. R. Goodall and A. A. Levi, *Nature*, **158**, 675 (1946).

Experimental

The papers used in the chromatograms were prepared as follows. Whatman no. 1 paper was dipped in *M*/15 phosphate buffer at *p*H 6.2 and the excess fluid was removed by pressing with a photographic roller over a glass plate. The paper strips (570 × 120 mm.) were air dried before use. We have employed aluminum, nickel and platinum as electrodes and have found little difference between them. The nickel ribbon (6.35 × 0.025 mm.) is woven into slits cut into the edges of the paper and the electrodes extend not more than one half the length of the paper. The electrodes may also be attached to the paper by stapling. The mixture of amino acids consisted of two dicarboxylic acids—aspartic and glutamic; two basic acids—lysine and arginine; and six neutral amino acids—serine, glycine, alanine, valine, leucine and proline. The concentration of the individual amino acids in the