FRONTAL ANALYSIS STUDIES ON THE ADSORPTION OF CRYSTALLINE EGG ALBUMIN AND ITS CLEAVAGE PRODUCTS

by

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The hydrolysis of proteins has been intensively studied for a long time, and a great number of different methods have been used in the investigation of the mechanism of the breakdown and the products formed. Ultracentrifugation, electrophoresis, titration, and precipitation methods are among those most frequently used. It is often only possible to observe either the increase of the breakdown products or the decrease of the unchanged protein. Chromatography and adsorption analysis seem to be advantageous in certain cases, as they are suited for the study of both products with low and high molecular weight. In this publication adsorption analysis and paper chromatography have been employed for studying the hydrolysis of crystalline egg albumin, first with pepsin and then with strong hydrochloric acid.

The adsorption analysis developed by TISELIUS and co-workers has been described in detail by TISELIUS[1, 2] and by CLAESSON[3] and only a few words will be mentioned here, mostly concerning the three different ways in which an analysis can be carried out. In all cases the principle of the liquid chromatogram is used, in which the concentration of the solution just leaving the adsorbent is determined continuously by measuring the refractive index either with a microinterferometer or with a self-recording apparatus built for that purpose.

FRONTAL ANALYSIS

The solution to be analysed is forced through the filter with adsorbent. The concentration or the refractive index of the solution leaving the filter is plotted against the corresponding volume that has passed the filter. The resulting curves are step-like, each step indicating a new component (Fig. 1). The volume that has passed the filter before a certain step appears is called the retention volume for the component causing that step. Only the first, least adsorbed component is obtained in pure form, the subsequent steps consist of mixtures. If there is no adsorption displacement of the components, the concentrations of the steps immediately give the composition of the original solution. For closely related compounds displacement is the rule; even in this case, however, the composition may often be calculated by using formulas, to be discussed later. Irreversible adsorption does not cause any appreciable errors in frontal analysis.

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ELUTION ANALYSIS

A small amount of the mixture to be analysed is adsorbed in the top of the filter with the adsorbent and then eluted by a suitable solvent. The curves obtained when plotting refractive index versus volume show peaks, each one indicating one pure component, the area of the peak being proportional to the amount of substance. On account of tailing of the peaks and irreversible adsorption this method often gives unsatisfactory results.

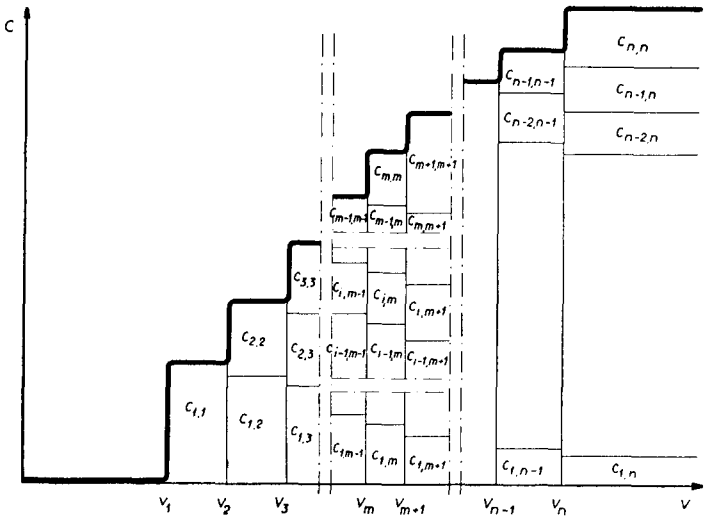

Fig. 1. Schematic frontal analysis diagram

DISPLACEMENT DEVELOPMENT

As in elution analysis a small amount of the mixture is first adsorbed in the top of the filter, then a solution of a solute that is more strongly adsorbed than any of the components in the mixture is forced through the filter. The components are now displaced by this solute and also displace each other. The curves from these experiments are step-like, each step indicating only one component and the last step the developer only. For a given concentration of the developer, the step for a component always has the same constant height and its length is proportional to the amount present. Both the qualitative and quantitative composition are thus immediately obtained from the diagram. The only drawback of this very elegant method is that irreversible adsorption may cause losses of material. Naturally it is confined to mixtures of substances that displace each other.

TISELIUS[2, 4, 5] has found that amino acids and peptides show large mutual differences in adsorption when using activated carbon as adsorbent. Both frontal analysis, elution, and displacement development have been used. The two latter methods present difficulties in many cases on account of the strong irreversible adsorption, and frontal analysis does not immediately give the correct concentrations of the components due to displacement*.

* In this connection it may be mentioned that the present author obtained improved results when eluting a mixture of a weakly adsorbed amino acid and a strongly adsorbed one by using the

As it was anticipated that a mixture of peptides from the partially broken down egg albumin would show stronger adsorption than the amino acids and also stronger irreversible adsorption, the present author decided to use only frontal analysis in the investigation, if possible. As only a small percentage of the total material is adsorbed on the carbon, neither the irreversible adsorption nor a possible oxidation of the substance will cause serious errors. A correction must, however, be made for the displacement effect. CLAESSON[3,6] when investigating mixtures of fatty acids where conditions are somewhat similar, worked out certain formulas for the determination of the composition from the frontal analysis diagram.

If the adsorption isotherms for the components may be written

$$a_i = f_i(c_1, c_2, \ldots, c_n) = k_i \cdot c_i \cdot r(c_1, c_2, \ldots, c_n) \tag{1}$$

where c_i is the concentration, a_i the amount adsorbed, and k_i the adsorption coefficient for the i : th component, the function r arbitrary, but supposed to approach the limit 1 when the concentrations tend to zero, then the following recursion formula for the concentration of the i : th component in the (m + 1) : th step is valid (see Fig. 1).

$$c_{i,\,m+1} = c_{i,\,m} \frac{1 - \dfrac{k_i}{k_m} \cdot \dfrac{v_m}{v_{m+1}}}{1 - \dfrac{k_i}{k_{m+1}}} \tag{2}$$

where v_m is the retention volume for the component giving rise to step m.

From this it is obvious that if the *ratios* of the adsorption coefficients are known the composition of the mixture may be calculated. If the qualitative composition is known the case is easy as the ratios of the k-values may be derived either from the isotherms of the pure components or from experiments with made up mixtures of known composition.

Even for a mixture where it is only known that the components are members of the same group of compounds, it is often possible to obtain a fairly good value of the ratio in the following way.

It has been shown[3] that the k-value of the component causing the i : th step always is given by the expression

$$k_i = \frac{v_i}{r(c_{1,i}, c_{2,i}, \ldots, c_{i,i})} \tag{3}$$

which however is of little practical value as it stands. By using a suitable approximation for the function r(c), e.g. $r(c) = \dfrac{1}{1 + l_i c_i}$ (LANGMUIR isotherm) we get

$$k_i = v_i \left(1 + \sum_{j=1}^{i} l_j c_{j,i}\right) \tag{4}$$

For closely related substances the l-values often do not differ very much from each

following procedure. The least adsorbed component was first eluted, then the filter (constructed specially for this purpose) was turned upside down to enable the second component to be eluted in the opposite direction. In this way the strongly adsorbed substance only passes part of the filter and the losses become lower.

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other, making it possible to use an average value known beforehand. The k-values can then be calculated immediately by the simple formula

$$k_i = v_i \left(1 + l \sum_{j=1}^{i} c_{j,i}\right) \tag{5}$$

as $\sum_{j=1}^{i} c_{j,i}$ is the total concentration of the i : th step.

In order to minimize the influence of the error in l on the k-value, the product $l \sum c_{ji}$ must be small as compared to 1 or at most of the same order of magnitude. This means that the l-value can not be too large and that low concentrations are desirable.

The k-values derived in this way may naturally be somewhat erroneous, however the error is mostly very much reduced in their ratio, which is the only value necessary for the calculation.

At the time of this investigation no higher peptides or mixtures of these resembling a mixture resulting from a pepsin digestion of egg albumin were available to the present author. The only possibility was to use amino acids and a few lower peptides as comparison material, assuming that the adsorption conditions would be rather similar. Some orientation experiments were carried out with these substances in order to determine

1. if the LANGMUIR isotherm could be used as an approximation, and in that case the k- and l-values, resulting therefrom;

2. the displacement effect at different concentrations and the k-values derived from mixtures;

3. if the composition could be calculated by using equation (2).

The LANGMUIR isotherm may be written

$$\frac{1}{c} = k \cdot \frac{1}{a} - l \tag{6}$$

and accordingly by plotting 1/c versus 1/a, a straight line is obtained from which k and l are immediately found. By performing frontal analyses with the pure substances the amount adsorbed, a, at the concentration, c, is obtained from the equation

$$a = v \cdot c \tag{7}$$

Fig. 2 shows the values of 1/a from frontal analyses plotted versus 1/c for some amino acids and one peptide (glycyl-tyrosine), the latter with very strong adsorption. The points fall close to straight lines indicating that the LANGMUIR isotherm can be used. In Table I the displacement effect in binary mixtures of amino acids is obtained by comparing $c_{1,1}$ with the original true concentration for the first, least adsorbed component; the ratios of the k-values are also given. For concentrations below 0.2–0.25% the displacement becomes less marked. Table II gives k- and l-values derived both from the isotherms and from the experiments with mixtures. The k-values vary from 1 to 200 but the l-values only from 6 to 9. In Table III are also given k-values for some substances when using an average l-value of 7.5.

From the frontal analysis diagrams of made up mixtures of three different amino acids the composition was first calculated using the correct ratio of the adsorption coefficients. The total concentration of the steps was both determined from the nitrogen

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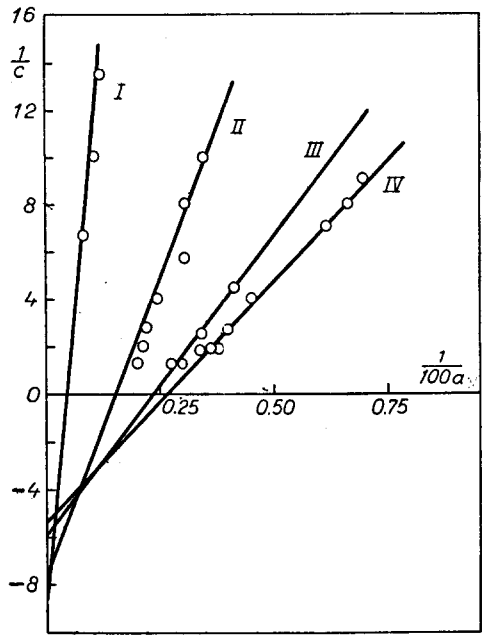

Fig. 2a, I, II, III, IV are the LANGMUIR isotherms for glycyl-tyrosine, methionine, isoleucine and leucine; c is expressed in g/100 ml and a in g/g adsorbent

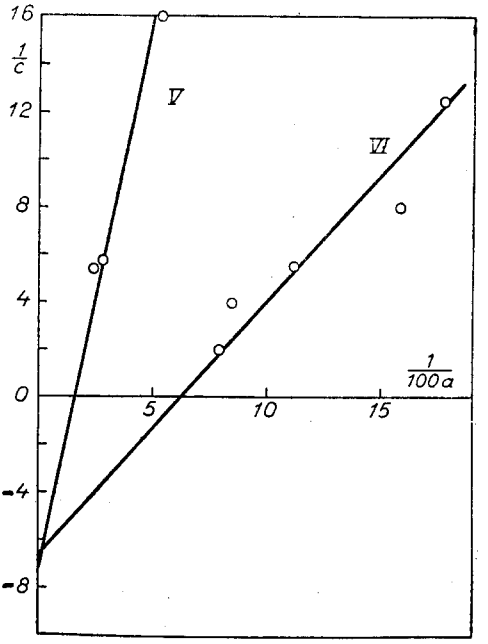

Fig. 2b, V and VI are the LANGMUIR isotherms for proline and alanine; c is expressed in g/100 ml and a in g/g adsorbent

TABLE I

RESULTS OF FRONTAL ANALYSES ON BINARY MIXTURES OF AMINO ACIDS. ADSORBENT: CARBO ACTIV

Mixture	Original conc. (g/100 ml)	Spec. retent. vol. v (ml/g ads.)	$c_{I,I}$ (g/100 ml)	k_1/k_2 calc. from equ. (2)	k_1/k_2 from isotherm	k_1/k_2 mean value
Leucine Methionine	0.100 0.100	11.8 23.6	0.125	0.375		
Leucine Methionine	0.123 0.124	11.4 19.9	0.179	0.379		
Leucine Methionine	0.152 0.151	9.1 16.0	0.215	0.389	0.400	0.391
Leucine Methionine	0.252 0.250	6.7 11.8	0.367	0.371		
Leucine Methionine	0.376 0.377	5.0 8.4	0.558	0.400		
Leucine Isoleucine	0.100 0.080	11.4 13.2	0.138	0.810	0.800	0.805
Leucine Tyrosine	0.100 0.140	12.2 56.1	0.110	0.140		
Proline Leucine	0.169 0.155	2.3 6.8	0.186	0.262	0.250	0.256

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TABLE II

Substance	k	l
Alanine	1	6.6
Proline	5	7.4
Leucine	20	5.4
Isoleucine	25	5.9
Methionine	50	7.5
Tyrosine	140	7.1
Glycyltyrosine	200	8.8

TABLE III

Substance	k for l = 7.5
Aspartic acid	5
Arginine	82
Glycylglycine	4
Leucylglycylglycine	79

content and from the height of the steps, that is the refractive index. The last procedure is possible owing to the fact that the refractive index increments for most amino acids apparently do not differ very much from each other. This is seen from Fig. 3 where the refractive index increments for a large number of amino acids have been plotted versus the concentration, giving a calibration curve. The values for egg albumin and some peptides are also included, showing that the same curve may be used for these substances. From Table IV it is seen that the composition calculated in both cases is in good agreement with the correct one. Some slight losses of material amounting to a few percent usually occur, the reason for which may be oxidation, although treatment of the carbon with HCN has not eliminated the effect.

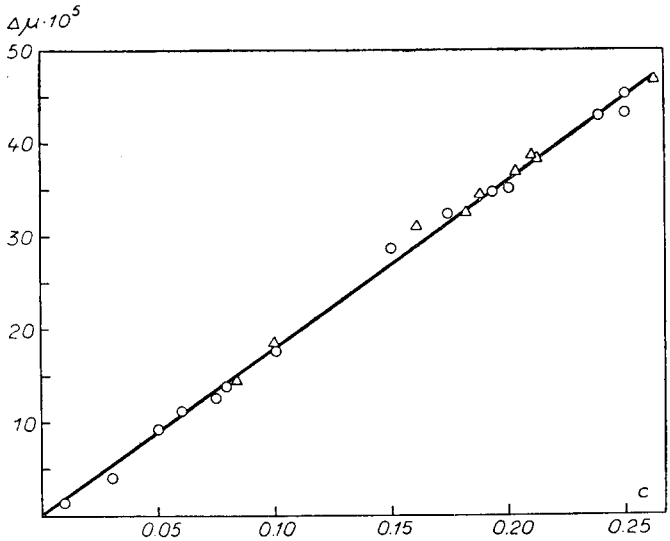

Fig. 3. The refractive index increment $\triangle\mu$ plotted against the concentration c in g/100 ml for amino acids and a few lower peptides (o) and for crystalline egg albumin ($\triangle$)

The composition was then also determined using the k-values calculated from equation (5) with l = 7.5. The concentrations of the steps were the ones derived from the refractive index. It is seen that the ratio of the adsorption coefficients at low concentrations is good enough to give a fairly accurate value of the composition even if the k-values themselves differ appreciably from the correct values. All the data are given in Table IV.

Before starting the investigation of the digestion products the adsorption of the

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pure crystalline egg albumin had to be studied. By means of frontal analysis it was found that the adsorption on carbon was very low (comparable with that of alanine). Furthermore the protein did not seem to be entirely homogeneous, one first large step was followed by two or three very small steps. Very similar frontal analysis diagrams were found with three different preparations*, which all appeared to be homogenous in the ultracentrifuge and showed the usual pattern with two peaks in the electrophoresis diagram. No fractionation of the egg albumin before digestion was attempted, a thing which however would be of interest to try in the future, especially as large filters for preparative work have come into use during the last year**[7].

The fact that egg albumin with high molecular weight is adsorbed less on carbon than most amino acids and peptides with low molecular weight is not altogether surprising, as it has previously been found that high polymers with sufficiently high molecular weight show decreasing adsorption with increasing molecular weight[8].

As it was now evident that any unchanged egg albumin in the digested mixture would interfere in the frontal analysis experiments, it was necessary to remove it in some way. TISELIUS[9] had found that crystalline egg albumin exhibits strong adsorption on a slightly acid aluminum hydroxide suspension (commercial name Mataki gel) amino acids and peptides on the other hand are hardly absorbed at all (at a concentration of 0.2% the retention volume for aspartic acid is 0.5 ml, for arginine 0.4 ml, and for leucine 0.3 ml on a 250 π mm^3 filter). This adsorbent was now tried in the hope that the breakdown products would show low adsorption and could therefore be easily separated from the unchanged protein. This indeed proved to be the case. Fig. 4 shows frontal analysis diagrams for different times of hydrolysis. Only two steps appear, the first evidently consisting of a mixture with very low adsorption, the second due to the egg albumin present which in itself appears homogenous on this adsorbent. Unfortunately pepsin shows low adsorption and will therefore be present in the first step. However, as the adsorption is somewhat higher than that of the breakdown products, it is possible to get rid of most of the pepsin by using large filters and only collecting part of the first fraction. By repeating this procedure several times, enough solution is obtained for the frontal analysis experiments on carbon.

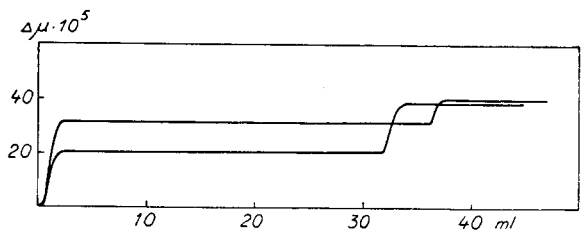

Fig. 4. Frontal analyses of egg albumin hydrolysed 17 hours (lower curve) and 71 hours (upper curve) Filter 250 π mm^3 filled with Mataki gel

The experiments in which two different crystalline egg albumins were used, A, five years old, and B, freshly prepared, are now carried out as follows. The egg albumin, conc. 0.4–0.5%, is digested with pepsin (PARKE, DAVIS 1 : 10000, the ratio of pepsin to egg albumin = 0.05) at 38° C in 0.01-n HCl during different lengths of time. The p_H changed somewhat during the hydrolysis but did not exceed 2.4 even after 240 hours. Buffer and strong HCl solutions were avoided as it was desirable to keep the salt content

* One of these preparations was made by *fil. lic.* CLAES WEIBULL, and I want to thank him for letting me use it.

** One preliminary electrophoresis experiment with the first fraction showed only one peak However, as the concentration was low no definite conclusions can be drawn as yet.

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TABLE

RESULTS OF FRONTAL ANALYSES ON TERNARY

Mixture	Original conc. (g/100 ml)	Spec. retent. vol. (ml/g ads.)	Ratio of k-values $\frac{k_1}{k_2}$, $\frac{k_1}{k_3}$, $\frac{k_2}{k_3}$	Step no.	Conc. from nitrogen values of comp. 1	2	3
Alanine	0.082	0.7	0.050	1	0.083		
Leucine	0.083	10.1	0.020	2	0.081	0.102	
Methionine . .	0.082	20.8	0.391	3	0.081	0.086	0.075
Alanine	0.175	0.5	0.050	1	0.177		
Leucine	0.164	8.0	0.020	2	0.175	0.229	
Methionine . .	0.165	14.5	0.391	3	0.174	0.169	0.153
Alanine	0.250	0.5	0.050	1	0.259		
Leucine	0.249	6.6	0.020	2	0.252	0.345	
Methionine . .	0.251	11.9	0.391	3	0.250	0.251	0.238
Proline	0.065	3.0	0.256	1	0.065		
Leucine	0.084	10.6	0.100	2	0.063	0.105	
Methionine . .	0.088	20.2	0.391	3	0.061	0.082	0.088
Proline	0.166	2.1	0.256	1	0.175		
Leucine	0.165	8.1	0.100	2	0.174	0.226	
Methionine . .	0.169	15.2	0.391	3	0.167	0.173	0.155

as low as possible in all the subsequent adsorption experiments. The enzyme action is stopped by adding an equal volume of 0.01-n NaOH, at the same time diluting the solution to a concentration of 0.2–0.25%. A frontal analysis on Mataki gel is then performed with the solution (see Fig. 4), giving the two mentioned steps. On account of the extremely low adsorption of the first step (around 0.3 ml for 250 π mm^3 filter) the adsorption displacement should be negligible and the egg albumin in the second step should be adsorbed as if it were alone in the solution.

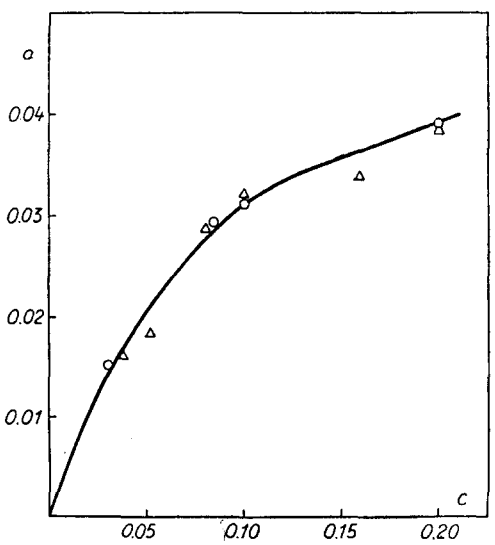

Fig. 5. Adsorption isotherm of crystalline egg albumin (o) and of the second component (△) obtained from frontal analysis of a hydrolysate on Mataki gel. c is given in g/100 ml and a in g

For comparison some frontal analyses were then carried out on 250 π mm^3 filter with pure egg albumin at different concentrations and the amount adsorbed calculated; see Table V. The adsorption isotherm is drawn in Fig. 5, the scattering of the points being due to the variation of the weight of adsorbent as Mataki gel is packed wet and not weighed each time. In Table VI the amounts adsorbed of the second component from the digestion experiments are given and in Fig. 5 they are plotted versus the concentration, which has been determined from the refractive index, using the curve in Fig. 3. It is seen

IV

MIXTURES OF AMINO ACIDS. ADSORBENT: CARBO ACTIV

Conc. from refractive index of comp.			k-values for $l = 7.5$	Ratio of k-values for $l = 7.5$ $\frac{k_1}{k_2}$, $\frac{k_1}{k_3}$, $\frac{k_2}{k_3}$	Conc. from refractive index using $l = 7.5$ and corresponding calc. k-values of comp.		
1	2	3			1	2	3
0.081			1.1	0.048	0.081		
0.079	0.100		23.6	0.019	0.079	0.100	
0.079	0.084	0.082	59.0	0.400	0.079	0.086	0.080
0.178			1.2	0.038	0.179		
0.176	0.222		31.9	0.017	0.176	0.222	
0.174	0.185	0.142	68.9	0.463	0.174	0.185	0.142
0.260			1.5	0.041	0.260		
0.253	0.336		35.8	0.019	0.250	0.336	
0.251	0.244	0.249	78.4	0.456	0.249	0.275	0.220
0.066			4.5	0.192	0.066		
0.064	0.099		23.5	0.082	0.059	0.104	
0.062	0.077	0.091	55.0	0.472	0.058	0.086	0.086
0.175			4.9	0.161	0.175		
0.174	0.208		31.3	0.070	0.155	0.227	
0.167	0.159	0.163	71.0	0.440	0.152	0.189	0.148

TABLE V

FRONTAL ANALYSES OF CRYSTALLINE EGG ALBUMIN DISSOLVED IN 0.005-n NaCl

FILTER: 250 π mm^3 ADSORBENT: MATAKI GEL

Conc. (g/100 ml)	Retent. vol. (ml)	Amount adsorbed a (g)
0.030	50.1	0.0150
0.084	35.0	0.0294
0.100	31.2	0.0312
0.200	19.6	0.0392

that the points fall on or close to the isotherm, giving evidence that the second step is caused by egg albumin.

The ratios of amino nitrogen to total nitrogen were determined for the first step, the second step, and the original solution. The values for the last two were the same within the experimental error, and so only the values for the peptide mixture (first step) and the original solution are given in Table VII. Correction for pepsin was always applied. From the data it is obvious that the breakdown process is the same both for egg albumin A and B, though in case of the freshly prepared B the partially broken down products seem to desintegrate more rapidly at the very earliest stage of the hydrolysis. The maximum value of approx. 0.25 attained for the ratio of amino nitrogen to total nitrogen is in close agreement with that one obtained under similar conditions by CALVERY[10], and CALVERY, BLOCK AND SCHOCK[11].

The split percentage, x, that is the percentage of the original egg albumin that is broken down at a certain time (100% when no egg albumin is detectable any

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TABLE VI

FRONTAL ANALYSES OF SOLUTIONS RESULTING FROM HYDROLYSIS OF CRYSTALLINE EGG ALBUMIN (0.4 g/100 ml 0.01–n HCl) WITH PEPSIN, THEN DILUTED WITH AN EQUAL VOLUME OF 0.01–n NaOH MEDIUM: 0.005–n NaCl. FILTER: 250 π mm^3. ADSORBENT: MATAKI GEL

Time of hydrolysis (hours)	Conc. of second component (g/100 ml)	Retent. vol. of second component (ml)	Amount adsorbed a (g)
0.0	0.200	19.3	0.0386
0.5	0.158	21.5	0.0340
17	0.100	32.1	0.0321
22	0.080	36.0	0.0288
71	0.051	36.2	0.0185
142	0.037	43.8	0.0162

TABLE VII

RESULTS OF EXPERIMENTS WITH EGG ALBUMIN (0.4 g/100 ml) HYDROLYSED WITH PEPSIN (0.010 g/100 ml) IN 0.01–n HCl AT 38° C

Substance	Time of hydrolysis in hours	Ratio of amino-N to total-N in		Split percentage calculated from			
		the hydrolysed solution	the solution of cleavage products (peptide fract.)	1. formula (8)	2. amount of unchanged protein	3. the total-N in the peptide fraction and in the hydrolysed solution	Mean value
Egg albumin A	0.5	0.060	0.146	26.2	21.0	23.3	24
	17	0.098	0.160	52.3	50.0	51.0	51
	22	0.124	0.175	63.0	60.0	60.9	61
	71	0.149	0.188	75.5	74.5	77.8	76
	120	0.169	0.201	80.7	80.0	81.6	81
	142	0.183	0.211	84.5	81.5	82.6	83
	257	0.201	0.222	88.9	88.1	89.2	89
	456	0.229	0.242	94.0	94.3	95.3	94
	816	0.262	0.258	101.6	99.8	97.6	100
Egg albumin B	3.5	0.081	0.207	26.7	25.3	24.7	26
	13	0.124	0.204	52.6	52.5	53.3	53
	44	0.136	0.209	58.0	59.4	57.1	58
	188	0.180	0.225	76.4	73.6	74.0	75
	552	0.245	0.250	97.7	99.9	96.6	98

more and the ratio of amino nitrogen to total nitrogen is constant, appr. 0.25), is calculated from the following formula which is easily derived

$$\frac{x \cdot a}{100} + \frac{(100 - x) \cdot d}{100} = b \qquad (8)$$

a, b and d are the ratios of the amino nitrogen to the total nitrogen in the first step, in the hydrolysed solution (the same as in the second step) and in the original egg albumin respectively. No knowledge of adsorption displacement is necessary for these calculations. The values are given in Table VII.

Assuming that there is only negligible displacement the split percentage is directly obtained, as both the concentration at the start and the concentration of the still unchanged egg albumin are known, the latter from the second step in the frontal

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analysis diagram. To use the ratio of the heights or concentrations of the first and second step is not correct, as the concentration of the cleavage products has increased slightly by uptake of water. The values are given in Table VII. The agreement with those previously calculated is quite good and favours the assumption that there is no displacement.

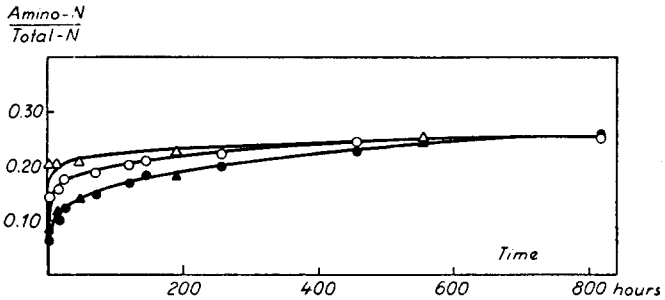

Fig. 6. Amino-N to total-N plotted against time of hydrolysis
● = unfractioned hydrolysate of egg albumin A
○ = peptide fraction from hydrolysate of egg albumin A
▲ = unfractioned hydrolysate of egg albumin B
△ = peptide fraction from hydrolysate of egg albumin B

Finally the split percentage has been calculated as the ratio between the total nitrogen content per ml of the split products in the first step and of the hydrolysed solution (Table VII) and Fig. 7.

It is seen that egg albumin A is hydrolysed more rapidly than B, where on the other hand as mentioned above the cleavage products have a higher ratio of amino nitrogen to total nitrogen at corresponding times.

The ratio of amino nitrogen to total nitrogen in the peptide mixture increases during the digestion from approx. 0.10 to 0.25 (see Fig. 6). This means that peptides with 10 or more amino acid residues are formed in the earlier stages of hydrolysis and then gradually broken down until finally the mixture mainly consists of tripeptides.

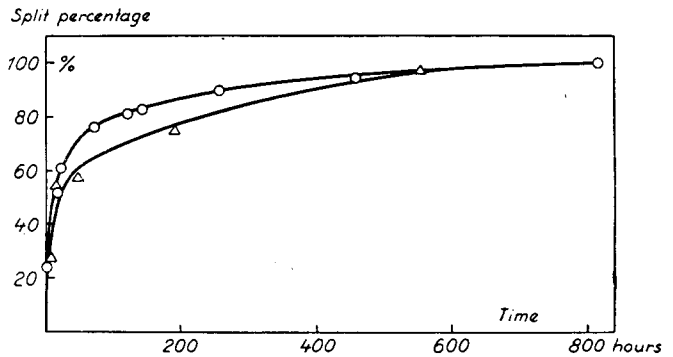

Fig. 7. The split percentage plotted against time of hydrolysis
○ = egg albumin A
△ = egg albumin B

In these last calculations the ratio of amino nitrogen to total nitrogen of the completely hydrolysed egg albumin is taken as 0.75 in accordance with CALVERY[10] and a correction for the amount of amino nitrogen present in the intact protein is naturally also made.

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The mixture of cleavage products resulting from repeated frontal analysis on Mataki gel, thus freed from all of the egg albumin and all or practically all of the pepsin, was finally investigated using carbon as adsorbent. Fig. 8 shows a typical diagram. Mostly 3 or 4 steps were observed except for short digestion times when they were fewer.

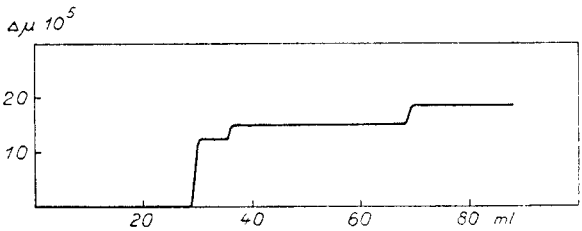

Fig. 8. Frontal analysis of the peptide fraction resulting from 17 hours hydrolysis of egg albumin. Filter: 175 π mm^3 containing 0.27 g Carbo Activ

From the retention volumes of the components it was directly seen that the strength of adsorption was generally comparable with that of tyrosine; only in a few cases stronger adsorption than that of glycyl-tyrosine was observed. This fact seemed to justify the use of the same average l-value 7.5, as in the case of the experiments described above with amino acids, when calculating the adsorption coefficients by means of equ. (5). As has been pointed out before, even if the error in the l-value is rather large it will not cause too great an error in the k-values and the ratio of the k-values will be still less affected. The composition of the mixtures is calculated using equ. (2). All concentrations of the steps are derived from the refractive index (Fig. 3). The ratio of amino nitrogen to total nitrogen was also determined for each step, and knowing the concentrations of the components in the steps it has been possible to calculate at least approximately the ratio of amino nitrogen to total nitrogen for each component. In these calculations the amount of total nitrogen has been assumed to be constant for all fractions (the variation is only slight). In Table VIII all data mentioned are given.

The ratio of amino nitrogen to total nitrogen ranges from approx. 0.35 to 0.10, higher values corresponding to lower adsorption. The ratios are plotted versus the k-values in Fig. 9. In Fig. 10 the percentage of the original egg albumin that has been hydrolysed is plotted against the corresponding k-values. In the earlier stages of the digestion only strongly adsorbed components with low ratio of amino nitrogen to total nitrogen are formed, probably with 10 or more amino acid residues, for longer digestion times the ratio is higher, at last even dipeptides seem to be present and the amount of strongly adsorbed components becomes too small to be observed in the diagrams. That they would be non-existant is not probable as some must always be formed until all protein is broken down, but they will be present in decreasing amounts for decreasing egg albumin concentrations. The same kind of products seem to result from the

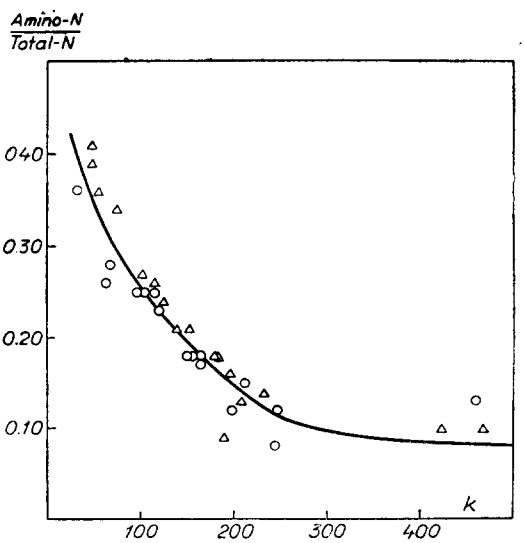

Fig. 9. Amino-N to total-N plotted against the adsorption coefficient k
○ = egg albumin A △ = egg albumin B

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digestion of both egg albumin A and B though the rate of formation is somewhat different.

In some experiments the original digestion mixture was directly subjected to frontal analysis on carbon. In these cases the egg albumin appeared long before any of the cleavage products. The unchanged character of the protein obtained in this manner followed from the fact that the total nitrogen and the amino nitrogen contents were the same as for the original crystalline product.

It is rather surprising that so few components show up in the frontal analysis diagrams; evidently each step must be a mixture of a large number of different cleavage products with very similar adsorption on carbon. In order to obtain further information on this question it was decided to try paper chromatography.

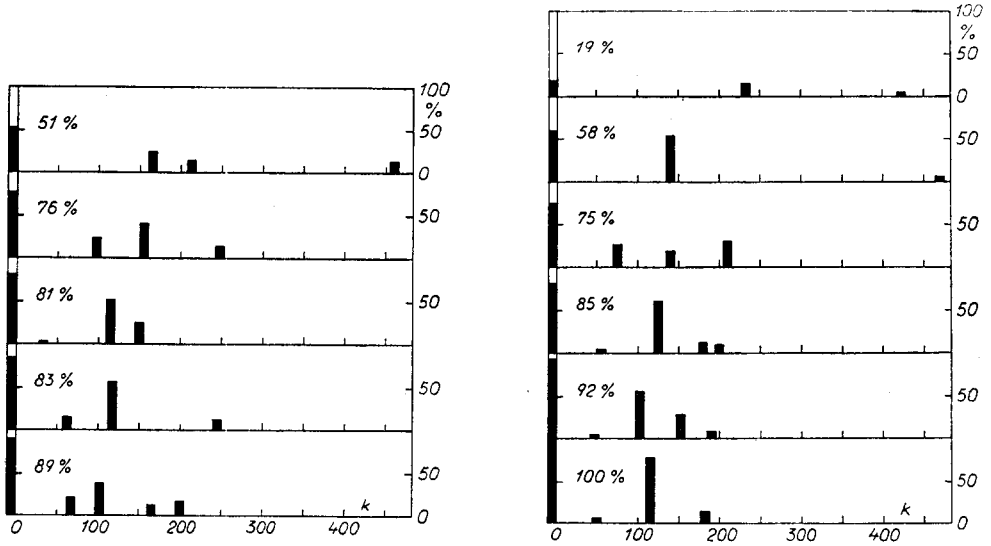

Fig. 10a and b. Per cent of original egg albumin transformed into cleavage products plotted against corresponding k values at different split percentages, the latter represented by the column to the very left on the figures. a) for egg albumin A (the left diagram) and b) for egg albumin B (the right diagram)

Paper chromatography as worked out by CONSDEN, GORDON AND MARTIN has been described in detail by these authors[12]. The methods is naturally most suited for investigating mixtures of amino acids and lower peptides for which the characteristic positions on the filter paper are already known beforehand. For mixtures of peptides resulting from a protein hydrolysis it is difficult to determine the qualitative composition because of lack of material for comparison, and the only information derived in those cases will be the number of different components as indicated by the spots.

The experimental arrangements used by the present author were similar to those described in the above-mentioned paper[12]. In the two-dimensional procedure the two systems water-phenol and water-collidine were mostly used and the filter papers were the Swedish Munktell OA and OB.

The fractions from the above-mentioned frontal analyses on carbon showed both distinct spots of normal size and larger spots that had remained unresolved on account of their very slow movement. The first fraction usually showed two or three distinct spots apart from the already mentioned large one, the next fraction these same spots but also a few additional ones and so on. The spots only showed up after heating even

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TABLE

RESULTS OF FRONTAL ANALYSES OF PARTIALLY

ADSORBENT: CARBO ACTIVE

Substance	Split-percentage	Fraction no.	Conc. of fraction g/100 ml	Spec. retent. vol. v ml/g ads.	k calc.	Ratio of amino-N to total-N
Egg albumin A . .		1	0.070	109	165	0.181
	51	2	0.084	131	213	0.170
		3	0.104	255	459	0.160
		1	0.082	60	97	0.249
	76	2	0.137	77	156	0.202
		3	0.153	155	247	0.188
		1	0.014	29	32	0.363
	81	2	0.145	55	115	0.206
		3	0.168	66	150	0.201
		1	0.061	43	63	0.256
	83	2	0.147	57	119	0.234
		3	0.164	110	246	0.211
		1	0.090	40	67	0.277
		2	0.144	49	103	0.258
	89	3	0.164	74	165	0.240
		4	0.182	84	199	0.222
Egg albumin B . .		1	0.041	178	233	0.139
	19	2	0.048	311	423	0.131
		1	0.110	76	139	0.212
	58	2	0.118	248	468	0.209
		1	0.074	48	74	0.343
	75	2	0.110	76	139	0.281
		3	0.149	98	208	0.225
		1	0.028	47	56	0.358
		2	0.164	52	126	0.248
	85	3	0.176	78	181	0.237
		4	0.183	83	198	0.231
		1	0.035	37	47	0.411
		2	0.186	43	103	0.275
	92	3	0.218	58	152	0.256
		4	0.227	70	189	0.243
		1	0.053	35	49	0.386
	100	2	0.231	43	116	0.264
		3	0.248	64	182	0.253

when using as large amounts of material as 1–2 mg, which is rather surprising as di- and tripeptides such as leucylglycine and leucyl-glycyl-glycine are detected as easily as the amino acids. Free amino acids seem to be present in very small amounts if at all; one might have expected tyrosine and cystine to show up. From these experiments only the confirmation of the assumption that the fractions consisted of several components was

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VIII

HYDROLYSED EGG ALBUMIN (THE PEPTIDE FRACTION)

MEDIUM: 0.005–n NaCl

Calc. conc. (g/100 ml) of the fractions of component 1	2	3	4	Component no.	Conc. of component g/100 ml	Components in % of total peptide fraction	Percent of original egg albumin hydrolysed into comp.	Ratio of amino-N to total-N calc.
0.070				1	0.051	48	24	0.18
0.053	0.031			2	0.028	27	14	0.15
0.051	0.028	0.026		3	0.026	25	13	0.13
0.082				1	0.046	30	23	0.25
0.048	0.089			2	0.080	52	40	0.18
0.046	0.080	0.027		3	0.027	18	14	0.12
0.014				1	0.009	5	4	0.36
0.009	0.136			2	0.107	64	52	0.25
0.009	0.107	0.052		3	0.052	31	25	0.18
0.061				1	0.030	18	15	0.26
0.031	0.116			2	0.110	67	56	0.23
0.030	0.110	0.024		3	0.024	15	12	0.08
0.090				1	0.046	25	22	0.28
0.049	0.095			2	0.079	43	38	0.25
0.047	0.084	0.033		3	0.024	13	12	0.17
0.046	0.079	0.024	0.033	4	0.033	18	16	0.12
0.041				1	0.039	81	15	0.14
0.039	0.009			2	0.009	19	4	0.10
0.110				1	0.108	92	53	0.21
0.108	0.010			2	0.010	8	5	0.10
0.074				1	0.054	36	27	0.34
0.059	0.051			2	0.035	24	18	0.21
0.054	0.035	0.060		3	0.060	40	30	0.13
0.028				1	0.009	5	4	0.36
0.009	0.155			2	0.130	71	60	0.24
0.009	0.136	0.031		3	0.023	13	11	0.18
0.009	0.130	0.023	0.021	4	0.021	11	9	0.16
0.035				1	0.008	4	4	0.41
0.008	0.178			2	0.137	60	55	0.27
0.008	0.141	0.069		3	0.062	27	25	0.21
0.008	0.137	0.062	0.020	4	0.020	9	8	0.09
0.053				1	0.016	6	6	0.39
0.016	0.215			2	0.197	79	79	0.26
0.016	0.197	0.035		3	0.035	14	14	0.18

obtained, even the exact number of spots was difficult to determine. The only way left was then to hydrolyse the fractions completely down to amino acids. This was done with concentrated hydrochloric acid during 24 hours at 100° C. The fractions now gave the typical amino acid spots, usually with 0.2 mg of material put on at the start. Only fractions obtained from egg albumin B were investigated. In Table IX the results of these experi-

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TABLE IX

RESULTS OF INVESTIGATIONS WITH PAPER CHROMATOGRAPHY OF THE COMPLETELY HYDROLYSED EGG ALBUMIN (+ MARKS PRESENCE IN MOST CASES)

Composition of egg albumin according to CHIBNALL[13]		Amino acids present in fractions with ratio amino-N to total-N			
aminoacids	N as % protein-N	> 0.30	0.25–0.30	0.20–0.15	0.15–0.10
Glutamic acid	9.7	+	+	+	+
Aspartic acid	5.4	+	+	+	+
Glycine	3.8	+	+	+	+
Alanine	6.7	+	+	+	+
Valine	3.5	+	+	+	+
Leucine, Isoleucine	10.8		+	+	+
Proline	2.5			+	+
Tyrosine	2.0				+
Tryptophan	1.2				
Phenylalanine	4.0			+	+
Methionine	3.1			+	+
Cystine	1.2			+	+
Serine	6.9		+	+	+
Threonine	3.0			+	+
Arginine	11.7			+	+
Histidine	4.1			+	+
Lysine	7.4			+	+

ments are given. For the fractions with lower adsorption and higher ratio of amino nitrogen to total nitrogen the most abundant amino acids seem to be those which themselves show rather low adsorption. The lower peptides then seem to be built up mainly of glutamic- and aspartic acid, glycine, valine, and leucine, the other amino acids forming part of the structure of higher peptides.

DISCUSSION

The breakdown process of a protein by a proteolytic enzyme may proceed mainly along two different lines. Either all the molecules are attacked at the same time and then gradually broken down, leaving no intact protein molecules as soon as the hydrolysis has started, or only part of the molecules are attacked at first and broken down relatively quickly, leaving intact molecules until the very end of the hydrolysis. The first type of reaction has been observed for instance by ANNETTS[14], the second by, for instance, TISELIUS AND ERIKSSON-QUENSEL[15], PETERMANN[16], HAUGAARD AND ROBERTS[17] and WINNICK[18]. In several of the last-mentioned cases it has also been reported that the cleavage products formed are the same during the whole hydrolysis.

The results from the investigation described in this paper indicate that the hydrolysis of crystalline egg albumin with pepsin is a reaction of the second type mentioned above. Intact protein has been observed all the time until the ratio of amino nitrogen to total nitrogen became constant. However, the composition of the peptide mixture was not the same all the time, in the earlier stages of hydrolysis peptides with 10 or more amino acid residues are most frequent, while later penta- and tetra-peptides seemed to be prevalent until finally at constant ratio of amino nitrogen to total nitrogen the main part consists of tripeptides (see Fig. 10 and 9). This is somewhat in contrast

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with the findings of TISELIUS AND ERIKSSON-QUENSEL[15]; however, it may partly be due to the fact that different kinds of pepsin were used. The lower peptides probably consist of glutamic and aspartic acid, valine, glycine, alanine and leucine. A more detailed study both of the higher and lower peptides will probably be carried out in the future.

EXPERIMENTAL

The two different egg albumins used were: Egg albumin A, prepared 5 years earlier, containing 15.5 % nitrogen (Kjeldahl) and having a ratio of amino-nitrogen to total nitrogen of 0.035.

Egg albumin B, freshly prepared according to LA ROSA[19], containing 15.1 % nitrogen (Kjeldahl) and with a ratio of amino nitrogen to total nitrogen of 0.031.

Adsorbents used. Slightly acid aluminium-hydroxide suspension, Mataki gel of A. B. MATAKI, Malmö, Sweden. (To 100 ml of the suspension was added 10 g of filter cel).

Carbo Activ of Merck Darmstadt, Germany, treated in the way described by CLAESSON[3].

Experiment 1. 0.400 g of egg albumin A dissolved in 100 ml 0.01 n HCl containing 0.020 g pepsin (PARKE, DAVIS 1 : 10000) was digested at 38° for 17 hours. The reaction was stopped by adding 100 ml 0.01 n NaOH. The solution was subjected to frontal analysis on Mataki gel using a 250 π mm^3 filter. The filter is previously washed through with 0.005 n NaCl. The diagram showed 2 steps the retention volume for the first step was only 0.3 ml, for the second 32.1 ml. The total refractive index increment was $38.5 \cdot 10^{-5}$, of which approximately 1 was due to pepsin and 18.3 due to the second component. According to the calibration curve the latter corresponded to a concentration of 0.100 g/100 ml and the amount adsorbed was 0.0321 g, assuming no displacement. The split percentage is obviously $100\left(1 - \frac{0.1}{0.2}\right) = 50\,\%$, 0.2 g/100 ml being the concentration of the egg albumin if no hydrolysis has taken place. The ratio between the amino nitrogen in the first step, in the second step and in the original hydrolysed solution was 0.159, 0.102 and 0.100, the last two agreeing within the limits of error. From these the split percentage was calculated with formula (8) and the value 52.3 % was obtained. The total nitrogen content per ml in the first step was 0.158 mg, in the hydrolysed solution 0.310 mg and their ratio 0.510, that means the split percentage is 51.0 %. In all these calculations a correction has been made for total nitrogen and amino nitrogen due to the pepsin (a 0.010 g/100 ml pepsin solution contained 0.014 mg Kjeldahl nitrogen and about 0.0003 mg amino nitrogen). From frontal analysis with a solution of 0.2 g/100 ml egg albumin and 0.010 g/100 ml pepsin in 0.005-n NaCl where no enzyme reaction had taken place, it had been found that the pepsin had a retention volume of 30–35 ml on a 1250 π mm^3 filter. On account of this several frontal analyses were performed with the original hydrolysed solution on 1250 π mm^3 filter with Mataki gel, taking care to collect only the first 30–35 ml each time. The fractions, somewhat diluted by this procedure, were put together and with this solution a frontal analysis on a 175 π mm^3 filter containing a weighed amount of Carbo Activ was performed, the filter having previously been washed with 0.005–n NaCl. Three steps appeared with total refractive index increments of 12.5, 15.0 and $18.7 \cdot 10^{-5}$, corresponding to concentrations of 0.070, 0.084 and 0.104 g/100 ml. The specific retention volume (ml/g adsorbent) were 108.5, 130.8 and 255 ml/g absorbent. The ratios of amino nitrogen to total nitrogen in the fractions were 0.181, 0.170 and 0.160 respectively For further calculated data see Table VIII. When performing a frontal analysis on 175 π mm^3 with Carbo Activ directly with the original hydrolysed solution the egg albumin already appeared at 3.0 ml, giving a ratio of amino nitrogen to total nitrogen of 0.034.

EXPERIMENTS WITH PAPER CHROMATOGRAPHY

The chamber used in the experiments had the top and two largest walls made of glass. The tray into which the filter papers were dipping was placed in a holder which could be easily brought in and out of the chamber even when the papers were put in position, which was especially convenient when the papers had to be sprayed with phenol before starting the experiment. Both trays of glass and aluminium were used though in case of phenol solution the glass trays were preferred. Several filter papers, both Swedish and German, were tried and several proved to be very good. The Swedish Munktell OB, previously used by EDMAN[20] and filtering about twice as rapidly as the English Whatman No. 1[12], and Munktell OA, a very fine but a little slower paper than OB and giving nice compact spots with ninhydrin, were mostly used in the experiments. Collidine-water usually seems to be a better system than amylalcohol-pyridine-water[20] and was used in the two-dimensional runs together with phenol-water as second system. The experiments with phenol-water were often carried out in an atmosphere containing ammonia and carbon monoxide. In a few one-dimensional experiments butanol-water was used.

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Experiment. Egg albumin B was hydrolysed during 408 hours, 92 % was split, and the experiment carried out as described above. The data may be seen in Table VIII. Then fractions of each step from the frontal analysis on Carbo Activ were investigated with paper chromatography. When only 0.2 mg in total was brought on the paper no spots could be seen even after heating. Increasing the amount of material to 1–2 mg in total, pink spots were seen after heating. It was possible to distinguish three spots in the first fraction; in the later fractions the number of spots increased with 2 to 3 for each fraction. However, some of them were close together and had not moved on the paper. By hydrolysing the separate fractions with strong HCl for 24 hours at 100° these were broken down to amino acids. Fraction 1, ratio of amino nitrogen to total nitrogen = 0.41, showed glutamic acid, aspartic acid, glycine, valine, alanine and traces of possibly leucine and cystine. Fraction 2 showed all the ones mentioned for fraction 1 and also serine and proline. Fraction 3 also showed threonine and the group phenylalanine, methionine, arginine and traces of lysine and histidine (the basic amino acids showed up rather poorly). In fraction 4 at last some tyrosine also seemed to be present.

The author wishes to express sincere thanks to Professor ARNE TISELIUS for suggesting this investigation and for all his help and advice.

The author also wants to thank Professor THE SVEDBERG for the privilege of working at his Institute and for his interest in this work.

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SUMMARY

1. The possibility of using frontal analysis on activated carbon in order to determine the composition of amino acid mixtures has been investigated. It has been found that the LANGMUIR isotherm can be used and that the adsorption displacement can be compensated for by using certain formulas.

Binary and ternary mixtures of amino acids have been studied and their composition has been determined with fair accuracy. The conclusions drawn from these experiments served as a guide for the subsequent investigation of unknown peptide mixtures obtained from the hydrolysis of egg albumin.

2. Crystalline egg albumin and the products obtained from partial hydrolysis of crystalline egg albumin with pepsin have been investigated by frontal analysis on aluminium hydroxide (Mataki gel) and on carbon. The cleavage products are obtained separately by using the first mentioned adsorbent, and the unchanged protein by using the second. The ratio of amino nitrogen to total nitrogen of the different fractions and the split percentage have been calculated for different digestion times. Concerning the breakdown process of the egg albumin the following was concluded. Only part of the protein molecules are attacked at first and intact protein is observed until the very end of the reaction. The cleavage products at the beginning consist of deca or higher peptides but at the end tripeptides are the average constituents.

3. The fraction containing the cleavage products only (peptide fraction)was investigated by frontal analysis on carbon and the composition determined in the same way as for the amino acid mixtures mentioned above. The number of fractions observed was less than five. The component with the lowest adsorption had the highest ratio of amino nitrogen to total nitrogen. In the earliest stages only fairly large products seemed to be formed, while in the final mixture even some dipeptides were indicated.

4. The peptide fractions were investigated with paper chromatography, showing more fractions than could be detected on carbon. No amino acids seemed to be present. After complete hydrolysis with strong hydrochloric acid, the fractions were again investigated on paper. The amino acids in the fraction showing the lowest adsorption on carbon were mostly amino acids that themselves show low adsorption on carbon. The lower peptides are accordingly mainly made up of glutamic and aspartic acid, glycine, valine, alanine, and leucine.

RÉSUMÉ

1. On a examiné la possibilité d'employer l'analyse frontale par chromatographie sur charbon activé pour déterminer la composition de mélanges d'amino-acides. On a trouvé qu'on peut utiliser l'isotherme de LANGMUIR et que le déplacement de l'adsorption peut être corrigé, grâce à certaines formules.

Les mélanges binaires et ternaires d'amino-acides ont éte étudiés et leur composition a été déterminée avec une bonne précision. Les conclusions tirées de ces expériences servent ensuite de guide dans les recherches sur les mélanges inconnus de peptides provenant de l'hydrolyse de l'albumine de l'œuf.

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2. L'albumine de l'œuf cristallisée et ses produits d'hydrolyse partielle par la pepsine ont été étudiés par l'analyse frontale sur l'hydroxide d'aluminium (gel MATAKI) et sur le carbone. On obtient les produits de coupure séparement en employant le premier adsorbant et les protéines non touchées en employant le second. On a determiné le rapport azote aminé — azote total pour les différentes fractions et le rendement en produits de coupure pour différents temps de digestion.

On a tiré les conclusions suivantes quant au processus de coupure de l'albumine de l'œuf. Une partie seulement des molécules protéiques est attaquée au début et la protéine intacte est présente jusqu'à la fin de la réaction. Les produits de coupure sont au début constitués en moyenne de décapeptides et de peptides plus élevés mais, à la fin, surtout de tripeptides.

3. La fraction contenant seulement des produits de coupure (fraction peptidique) a été examinée avec l'analyse frontale sur charbon et la composition déterminée de la même façon que pour les mélanges d'amino-acides déjà mentionnés. Le nombre de fractions trouvées est plus petit que cinq. Le composant d'adsorption la plus faible a le rapport azote aminé — azote total le plus élevé. Dans le stade initial il semble qu'il ne se forme que des produits dont le poids moléculaire est encore essez élevé, tandis que dans le mélange final on peut trouver jusqu'à des dipeptides.

4. Les fractions peptidiques soumises à la chromatographie sur papier ont montré plus de fractions qu'on en avait trouvées par le charbon. Il ne semble pas y avoir d'amino acides. Les fractions complètement hydrolysées par de l'acide chlorhydrique fort ont été de nouveau chromatographiées sur papier. Les amino-acides dans les fractions montrant la plus petite adsorption sur charbon sont surtout les amino acides qui eux-mêmes montrent une faible adsorption sur charbon. Par suite les peptides les plus bas sont surtout constitués d'acides glutamique et aspartique, de glycine, de valine, d'alanine et de leucine.

ZUSAMMENFASSUNG

1. Die Möglichkeit, Frontanalyse an aktiver Kohle zur Bestimmung der Zusammensetzung von Aminosäuregemischen zu benutzen, wurde untersucht. Hierbei wurde festgestellt, dass die LANGMUIR-Isotherme benutzt werden kann, und dass die Adsorptionsverdrängung durch Verwendung bestimmter Formeln ausgeglichen werden kann.

Binäre und ternäre Aminosäurengemische wurden untersucht und ihre Zusammensetzung mit ziemlicher Genauigkeit bestimmt. Die Schlussfolgerungen aus diesen Experimenten dienten danach als Leitfaden für die Untersuchung unbekannter Peptidgemische, die bei der Hydrolyse von Eialbumin erhalten wurden.

2. Kristallisiertes Eialbumin und die bei partieller Hydrolyse kristallisierten Eialbumins mit Pepsin erhaltenen Produkte wurden mit Hilfe der Frontanalyse an Aluminiumhydroxyd (MATAKI-Gel) und Kohle untersucht. Die Spaltprodukte werden durch Verwendung des ersten Absorbens getrennt erhalten, das unveränderte Eiweiss unter Benutzung des zweiten Adsorbens. Das Verhältnis Aminostickstoff-Gesamtstickstoff der verschiedenen Fraktionen und der Spaltprozentsatz wurden für verschiedene Verdauungszeiten berechnet. Bezüglich des Abbauprozesses des Eialbumins wurden die folgenden Schlüsse gezogen. Nur ein Teil der Eiweissmoleküle wird erst angegriffen, und intaktes Eiweiss ist bis zum Ende der Reaktion noch zu beobachten. Die Spaltprodukte bestehen am Anfang aus Deka- oder höheren Peptiden, während am Ende Tripeptide die Durchschnittsbestandteile sind.

3. Die Fraktion, die nur die Spaltprodukte enthielt (Peptidfraktion) wurde durch Frontanalyse an Kohle untersucht und ihre Zusammensetzung in gleicher Weise wie bei den obenerwähnten Aminosäurengemischen bestimmt. Die Zahl der wahrgenommenen Fraktionen betrug weniger als fünf. Der Bestandteil mit der geringsten Adsorption hatte das höchste Verhältnis Aminostickstoff/Gesamtstickstoff. In den ersten Stadien scheinen nur ziemlich grosse Produkte gebildet zu werden, während im Endgemisch sogar einige Dipeptide nachgewiesen wurden.

4. Die Peptidfraktionen wurden durch Papierchromatografie untersucht, wobei mehr Fraktionen als mit Kohle entdeckt werden konnten. Keine Aminosäuren schienen vorhanden zu sein. Nach vollständiger Hydrolyse mit starker Salzsäure wurden die Fraktionen wiederum mit Hilfe von Papier untersucht. Die Aminosäuren in der Fraktion mit der geringsten Adsorption an Kohle waren meistens Aminosäuren, die selbst geringe Adsorption an Kohle zeigen. Die niedrigen Peptide bestehen also, wie damit übereinstimmt, hauptsächlich aus Glutamin- und Asparaginsäure, Glykokoll, Valin, Alanin und Leucin.

REFERENCES

[1] A. TISELIUS, *Arkiv Kemi, Mineral. Geol.*, 14 B, (1940) No. 22.
[2] A. TISELIUS, *Advances in Protein Chemistry*, Vol. III, (1947) 67–93.
[3] S. CLAESSON, *Arkiv Kemi, Mineral. Geol.*, 23 A (1946) No. 1.
[4] A. TISELIUS, *Ibid.*, 15 B (1941) No. 6.

[5] A. TISELIUS, *The Svedberg* 1884–1944 (1944) p. 370–378.
[6] S. CLAESSON, *Arkiv Kemi, Mineral. Geol.*, 24 A (1946) No. 7.
[7] S. CLAESSON, *Ibid.*, 24 A (1947) No. 16.
[8] I. CLAESSON AND S. CLAESSON, *Ibid.*, 19 A (1944) No. 5.
[9] A. TISELIUS, Unpublished data, privately communicated.
[10] H. O. CALVERY, *J. Biol. Chem.*, 102 (1933) 83.
[11] H. O. CALVERY, W. D. BLOCK, AND E. D. SCHOCK, *J. Biol. Chem.*, 113 (1936) 21.
[12] R. CONSDEN, A. H. GORDON, AND A. J. P. MARTIN *Biochem. J.*, 38 (1944) 224.
[13] A. C. CHIBNALL, *J.I.S.L.T.C.*, 30 (1946) 1.
[14] M. ANNETTS, *Biochem. J.*, 30 (1936) 1807.
[15] A. TISELIUS AND I.-B. ERIKSSON-QUENSEL, *Biochem. J.*, 33 (1939) 1752.
[16] M. PETERMANN, *J. Biol. Chem.*, 144 (1942) 607.
[17] G. HAUGAARD AND R. M. ROBERTS, *J. Am. Chem. Soc.*, 64 (1942) 2664.
[18] T. WINNICK, *J. Biol. Chem.*, 152 (1944) 465.
[19] LA ROSA, *Chem. Anal.*, 16 (1927) 3.
[20] P. EDMAN, *Arkiv Kemi, Mineral Geol.*, 22 A (1945) No. 3.

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