CXXVIII. THE RATE OF LIBERATION OF ARGININE IN TRYPTIC DIGESTION[1].

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(*Received July 1st, 1930.*)

When a protein is subjected to the hydrolysing action of trypsin[2], its constituent amino-acids are liberated at different speeds and to different extents. Such differences of behaviour are the consequence, one may presume, of differences in the mode of combination, or in the order and arrangement, of the individual amino-acids within the protein molecule. Their study, therefore, could it be prosecuted in detail and with quantitative exactness, might be expected to throw some light upon the complex problem of protein constitution.

Unfortunately, in the medley of substances presented by a tryptic digest few amino-acids can be estimated with anything like precision. There are, indeed, but three—tyrosine, tryptophan and glutamic acid—of which it has been possible hitherto to follow the appearance by quantitative methods. Brown and Millar [1906] and, later, Plimmer and Eaves [1913], found that tyrosine was completely liberated in from 1 to 6 hours of tryptic digestion. In the experiments of Abderhalden and Reinbold [1905] and Abderhalden and Voegtlin [1907], its appearance was less rapid, but within two to three days equally complete. The behaviour of tryptophan would seem to be similar; for Ragins [1928] employing a colorimetric method of estimation, found that of the total amount potentially present from one-half to three-fourths was set free within 1 hour and the remainder within 1 to 5 days. On the other hand Fürth and Lieben [1920], who used a different colorimetric method, came to

[1] The experimental data of this paper are taken from a thesis presented by James A. Dauphinee in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Toronto.

[2] Throughout this paper we use the term "trypsin" in its original sense as a collective designation for the protein- and peptide-splitting enzymes of the activated pancreatic secretion. These, it has within recent years been shown [Waldschmidt-Leitz and Harteneck, 1925, 1, 2; Waldschmidt-Leitz, Schäffner and Grassmann, 1926; Waldschmidt-Leitz, Balls and Waldschmidt-Graser, 1929; Waldschmidt-Leitz and Purr, 1929], include no less than four distinct and highly specific hydrolysing agents—a proteinase, two polypeptidases and a dipeptidase. The crude preparations which we (and others before us) have employed may have contained any or all of these enzymes. In the effects which we are about to describe the proteinase probably played the predominant part; for it alone is capable of acting upon native proteins. How far its action may have been modified or supplemented by accompanying peptidases must be left for the present uncertain.

the conclusion that tryptophan is liberated very slowly and incompletely. Glutamic acid has been shown to behave quite differently [Abderhalden and Reinbold, 1905; Abderhalden and Voegtlin, 1907]; its liberation is a very gradual process, and the maximum yield, which may not be attained for 2 to 3 weeks, falls decidedly short of complete hydrolysis.

With respect to the remaining amino-acids little more is known than that some (like proline and phenylalanine) are not released at all by trypsin acting alone [Fischer and Abderhalden, 1903; Abderhalden and Reinbold, 1905], and that the behaviour of others (including alanine, valine, leucine, aspartic acid and the hexone bases) resembles more or less closely that of glutamic acid [Abderhalden, 1922, p. 464].

The work now to be reported was planned in the hope of adding arginine to the small list of amino-acids, concerning the liberation of which by trypsin we have precise quantitative information. That free arginine does actually appear in tryptic digests was shown by Kutscher [1898, 1899] and by Kossel and Mathews [1898]. Its presence has been noted also among the products arising from the proteolytic action of certain enzymes of the spleen [Leathes, 1902; Cathcart, 1905]. On the other hand there are on record a number of observations (to be cited later) which suggest that part of the arginine of proteins resists indefinitely the action of trypsin. Quantitative data with respect to either aspect of the case are entirely lacking; and, although it has been generally thought that arginine is liberated rather slowly, there is no experimental basis for this belief. Our own experiments indicate that as a matter of fact the liberation of arginine, though never complete, may sometimes be just as rapid as that of tryptophan or tyrosine.

Methods.

(*a*) *General.*

The general plan of our experiments was to bring an approximately 10 % solution of protein to a p_H of 8·2 (or thereabout) and a temperature of 37°, and to treat it with enough of a commercial trypsin preparation to give a concentration of 0·4 %. The mixture (protected by toluene against bacterial contamination) was allowed to digest at 37°, while at stated intervals samples were removed and boiled. On each sample we then determined total nitrogen (by Kjeldahl), free amino-nitrogen (by the micro-method of Van Slyke), ammonia (by treatment with potassium carbonate and aeration) and arginine (by the method discussed below). The total arginine content of each protein studied was determined on a separate sample hydrolysed by boiling for 4–24 hours with 20 % hydrochloric acid [Hunter and Dauphinee, 1930]. In some cases we determined also the free amino-nitrogen of both the original and the completely hydrolysed protein; in others (as will be indicated in the tables) we accepted for these the values given in the literature.

Certain variations of detail in treatment and in sampling, necessitated by

the different behaviour of the individual proteins, will be mentioned later at appropriate points.

(b) Determination of arginine.

In determining the free arginine of each sample of digest we employed the method of Jansen [1917, 1, 2], in which the arginine is hydrolysed by the enzyme arginase and the resulting urea is determined by the use of urease. Elsewhere [Hunter and Dauphinee, 1930], we have demonstrated the accuracy of this procedure and have described in detail its application to the determination of arginine in completely hydrolysed proteins. In that application it was found convenient to use, as the source of arginase, a crude glycerol extract of liver clarified by a brief period of heating at 60°. As such an extract is itself capable of liberating arginine from partly digested proteins, it was deemed necessary in the present investigation so to treat it that, retaining as much as possible of its power to decompose arginine, it should lose all or most of its proteolytic activity. A sufficiently satisfactory method of reaching this result was found in the use of aluminium hydroxide.

The aluminium hydroxide was prepared according to the directions of Willstätter and Kraut [1923], and was used only after it had been converted by long standing into the highly active C_γ modification [Willstätter, Kraut and Erbacher, 1925]. Preliminary experiments showed that this adsorbent, applied at p_H 5·0, would remove from our liver extracts practically all the protease, but comparatively little of the arginase. The latter, however, is subject at the p_H specified to a rather rapid auto-destruction. The following, therefore, was the method finally adopted to obtain active arginase preparations suitable for our purpose[1]. 30 cc. of an ice-cold crude extract of liver, prepared in the manner described by Hunter and Dauphinee [1930, pp. 632–3], are treated with enough *N* HCl (usually about 0·9 cc.) to bring the p_H to approximately 5·0. 15 cc. of an 11·5 % suspension of alumina C_γ are added, and the mixture is quickly centrifuged. The supernatant liquid is treated with another 15 cc. of the alumina suspension, and centrifuged again. The second supernatant liquid is then adjusted to neutrality by addition of the required amount of *N* NaOH. To minimise the destruction of arginase all these operations are carried out as rapidly, and as nearly at the freezing point, as possible. Much time may be saved by determining beforehand the exact quantities of *N* HCl and *N* NaOH that will produce the p_H changes desired. The procedure described should yield about 42 cc. of a product containing from 30 to 40 units[2] of arginase per cc.

In applying the purified arginase to the determination of arginase in tryptic digests we followed a procedure the same in essence as that described

[1] That aluminium hydroxide may be applied to the preparation of protease-free arginase has been shown already by Edlbacher and Simons [1927]; but, although their results are not inconsistent with ours, their method of utilising them was quite different.

[2] The unit here meant is that defined and used by Hunter and Dauphinee [1930, p. 630].

by Hunter and Dauphinee [1930, pp. 649–653] as suitable for the direct determination of the substance in total hydrolysates without previous removal of either humin or ammonia. The samples to be analysed, measuring either 5 or 10 cc., were treated with a drop of phenolphthalein indicator and enough *N* NaOH to give a permanent light pink colour. To each were then added at least 50 units of arginase (usually 2 cc. of alumina-treated extract). The further addition of a buffer was not found necessary. The enzyme was allowed to act for 10 to 12 hours at 37°[1], and the urea formed was then determined by the urease method of Van Slyke and Cullen. Each determination was performed in duplicate, and was accompanied by the appropriate blank controls. For details concerning these reference may be made to the earlier paper just cited.

(*c*) *Trypsin controls.*

The "trypsin" added to each solution of protein undergoes itself a fairly extensive digestion, with liberation of arginine, so that control experiments are always necessary. In these controls 2 g. of each trypsin preparation used, together with enough Na_2CO_3 to give p_H 8·2, were dissolved in 500 cc. of water at 37°. The total nitrogen of the solution was determined on a 15 cc. sample. Samples of 50 cc. were withdrawn at convenient intervals, heated to boiling, and cooled. Arginine (and ammonia) were then determined on 15 cc. portions, amino-nitrogen on 2 cc. portions, of each. The results were calculated to 100 cc. of the original solution.

Materials.

The proteins or protein derivatives included in the investigation were gelatin, caseinogen, edestin, egg-albumin, fibrin and Witte-peptone. For the first of these we used the Bacto-gelatin of the Digestive Ferments Company. The egg-albumin and fibrin bore the label of Merck. The caseinogen was prepared from a commercial product (already fairly pure) by dissolving in dilute NaOH, precipitating with acetic acid, repeating the process, and drying the final product with alcohol and ether. The edestin was extracted from defatted hempseed with warm 5 % sodium chloride, and precipitated by pouring the extract into 10 volumes of water; the product was redissolved and reprecipitated thrice, washed free from salt, and dried with alcohol and ether.

For the digestion of these proteins we used two different highly active brands of commercial "trypsin." In the description of the experiments these will be distinguished as trypsin-D and trypsin-P.

[1] The conditions adopted would have ensured the quantitative decomposition of several times as much arginine as any of our samples could have contained.

Experiments and results.

(a) *Experiments with caseinogen.*

Exp. 1. Ten successive 5 g. portions of caseinogen were ground in a mortar, each with 50 cc. of 0·06*N* NaOH. The solutions were combined, and the mixture was treated with so much 5*N* NaOH that it gave a just perceptible pink colour with phenolphthalein (p_H 8·2). 500 cc. of the clear solution of caseinogen thus obtained were placed in a flask, brought in a water thermostat to a temperature of 37°, and at a given instant treated with 2 g. of trypsin-P. The trypsin was quickly dissolved by shaking, 10 cc. of toluene were added, and the flask was returned to the thermostat. At intervals of 1, 3, 9 and 24 hours, and of 3, 7 and 14 days, a 50 cc. portion of the well shaken digest was pipetted into a 100 cc. volumetric flask containing 8 drops of 20 % HCl, 30 cc. of water were added, and the mixture was heated until the separated tyrosine had dissolved and the enzyme had been destroyed. After cooling to room temperature the solution was neutralised and made up to 100 cc.

For analysis 3 cc. were taken for the determination of total nitrogen, 10 cc. for ammonia and 10 cc. for arginine. For the free amino-nitrogen 4 cc. of the first three samples, or 2 cc. of the later ones, were further diluted to 10 cc., and the determination was carried out on duplicate or triplicate portions of 1 cc.

The results are shown in Tables I and II. The figures in the last 2 columns of Table II—recorded as "percentage hydrolysis of peptide" or "arginide links"[1]—are calculated according to the formula $\frac{100\,(A-A_0)}{A_1-A_0}$, in which A signifies the observed value at any given time, A_0 the value for the intact protein before hydrolysis, and A_1 the value after complete hydrolysis[2].

Table I. *Tryptic digestion of caseinogen. Exp. I.*

(Original data for 10 cc. portions of the diluted samples, each portion containing 68·03 mg. N, and corresponding to 5 cc. of the original digest.)

		Mg. ammonia-N				
Time of digestion	Mg. free amino-N	(*a*) in untreated sample (amide blank)	(*b*) in sample treated with arginase and urease	(*c*) yielded by blank controls*	(*d*) derived from liberated arginine (*b*)–(*c*)	Mg. arginine-N liberated†
1 hr.	13·37	0·28	1·93	0·86	1·07	2·17
3 hrs.	17·67	0·35	2·44	1·04	1·40	2·84
9 „	22·67	0·59	3·07	1·31	1·76	3·57
24 „	25·67	0·73	3·36	1·50	1·86	3·78
3 days	28·70	1·27	4·28	2·26	2·02	4·10
7 „	31·20	1·85	4·76	2·68	2·08	4·22
14 „	32·00	2·52	5·24	3·06	2·18	4·43

* This includes the "amide blank" of column (*a*). See Hunter and Dauphinee [1930].

† Calculated by doubling the quantity in column (*d*) and adding the empirical correction of 1·5 % [Hunter and Dauphinee, 1930].

[1] Here, and elsewhere throughout this paper, these expressions are used solely as a descriptive convenience, and without prejudice to the question whether other than peptide links play any part in the structure of the protein molecule.

[2] In the case of arginine A_0 of course is always zero.

Table II. *Tryptic digestion of caseinogen.*

Exp. 1. Trypsin-P 0·4 %. Total N in 100 cc. digest = 1·361 g.; of which substrate-N = 1·314 g.
Exp. 2. Trypsin-D 0·4 %. Total N in 100 cc. digest = 1·576 g.; of which substrate-N = 1·529 g.
Exp. 3. Trypsin-D 0·8 %. Total N in 100 cc. digest = 1·296 g.; of which substrate-N = 1·202 g.

(All results are calculated to 100 cc. digest.)

Time of digestion	Mg. free amino-N in whole digest	Mg. free amino-N in trypsin control	Mg. free amino-N derived from substrate	Mg. arginine-N liberated in whole digest	Mg. arginine-N liberated in trypsin control	Mg. arginine-N liberated derived from substrate	Percentage of total substrate-N as free amino-N	Percentage of total substrate-N as arginine-N	Percentage hydrolysis of peptide links	Percentage hydrolysis of arginide links
					Exp. 1.					
0 hrs.	—	—	—	—	—	—	5·73*	0·00	0·0	0·0
1 ,,	267·4	18·0	249·4	43·4	1·1	42·3	19·0	3·22	19·9	40·4
3 ,,	353·4	19·5	333·9	56·8	3·5	53·3	25·5	4·06	29·6	50·9
9 ,,	453·4	22·0	431·4	71·4	4·5	66·9	32·8	5·09	40·6	63·8
24 ,,	513·4	24·5	488·9	75·6	4·7	70·9	37·2	5·40	47·2	67·7
3 days	574·0	25·6	548·4	82·0	5·0	77·0	41·7	5·86	54·0	73·4
7 ,,	624·0	28·1	595·9	84·4	5·0	79·4	45·4	6·04	59·4	75·7
14 ,,	640·0	28·2	611·8	88·5	5·7	82·8	46·6	6·30	61·3	79·0
Complete hydrolysis	—	—	—	—	—	—	72·4*	7·98	100·0	100·0
					Exp. 2.					
14 days	760·7	31·3	729·4	105·6	6·9	98·7	47·7	6·45	63·0	80·9
21 ,,	—	—	—	109·6	6·9	102·7	—	6·72	—	84·2
35 ,,	—	—	—	110·9	6·8	104·1	—	6·81	—	85·3
					Exp. 3.					
3 hrs.	425·0	43·6	381·4	71·4	11·6	59·8	31·7	4·97	39·0	62·3
24 ,,	560·0	50·0	510·0	86·0	13·2	72·8	42·4	6·06	54·9	76·0
7 days	645·0	58·6	586·4	91·0	13·8	77·2	48·8	6·42	64·6	80·4
14 ,,	694·0	62·6	631·4	93·0	13·8	79·2	52·5	6·59	70·2	82·6

* Hunter and Smith [1925].

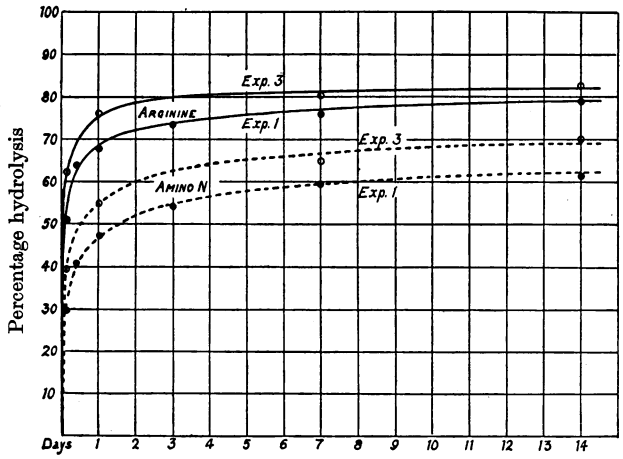

Fig. 1. Tryptic digestion of caseinogen. Liberation of arginine and amino-nitrogen under different concentrations of enzyme.

A graphical representation of the results of Exp. 1 will be found in Fig. 1, in which percentage hydrolysis is plotted against time. From this curve, as from the data of the tables, it appears that the initial velocity of arginine liberation in the case of caseinogen is very great. In a relative sense it surpasses even the general rate of polypeptide cleavage. Thus in the first hour,

during which only 20 % of the total peptide linkages were split, as much as 40 % of the total available arginine was set free. In 3 hours the proportion freed had risen to a half, in 24 hours to two-thirds. From this point the rate of liberation rapidly fell off, until during the second week hydrolysis seemed to have all but come to a standstill. On the 14th day, when the experiment was terminated, the proportion of originally bound arginine which had been freed was almost 80 %. The free amino-nitrogen in the meantime had risen to only 61 % of the possible maximum.

The question at once presented itself, whether with the liberation of just four-fifths of the total arginine the reaction had reached a definite and reproducible end-point. In order to test this Exps. 2 and 3 were carried out.

Exp. 2. This was a repetition of Exp. 1 with the following differences. (1) A new preparation of caseinogen was employed. (2) Trypsin-D was substituted for trypsin-P. (3) Arginine was determined only at the end of 2, 3 and 5 weeks of digestion. (4) Amino-nitrogen was determined only at the end of the 2nd week.

The results are shown beside those of Exp. 1 in Table II. It will be noted that at the 14th day both amino-N and arginine had reached practically the same values as in Exp. 1, so that the two trypsins used were of almost identical potency. In the 3 weeks following the free arginine did definitely increase, but the rate of increase, especially in the last 14 days, was exceedingly slow. The proportion of arginine ultimately liberated was 85 %.

Exp. 3. In this experiment we followed again the same general procedure as before, but used enough trypsin-D to give a concentration of 0·8 instead of 0·4 %. Samples were removed at 3 and 24 hours, and at 7 and 14 days. The results are again incorporated in Table II, and in Fig. 1 are compared graphically with those of Exp. 1.

As was to be expected, the higher concentration of enzyme led to a more rapid proteolysis. More than 60 % of the arginine was free at the end of 3 hours, and as much as 75 % at the end of 24. Later the arginine curves of the two experiments converge, and at the end of 2 weeks the higher enzyme concentration has raised the total amount of arginine liberated at the most by some 4 %.

Exps. 2 and 3 do not provide an altogether unambiguous answer to the question which they were designed to settle. In each the percentage of arginine liberated did reach an appreciably higher level than in Exp. 1. But in each case the effect was small, and its significance is doubtful. The enzyme preparations used contained almost certainly (see p. 1128, footnote), along with their trypsin, a certain proportion of ereptic enzymes. For the main effect, culminating apparently in the second week of digestion, the former, as the predominant factor, may be presumed to be responsible; the succeeding much more gradual increase of free arginine reveals perhaps the influence, at what was doubtless a much lower concentration, of the latter. Possibly, also, the slight alkalinity of the digests was in itself sufficient to bring about, at 37°,

a slow continuous hydrolysis. Such considerations, hypothetical as they are, lead us to question the importance of fractional increases taking place only upon prolonged incubation. On the whole, therefore, we are inclined to interpret our results as indicating that the activated protease of the pancreas sets free just 80 % of the total arginine of caseinogen[1]. We admit the somewhat arbitrary nature of this conclusion, and the desirability of testing it by further experiments with a purified enzyme.

(*b*) *Experiments with gelatin.*

Exp. 4. 50 g. of gelatin were dissolved by warming in 500 cc. of water, and the reaction of the solution was adjusted with NaOH to p_H 8·2. Of this solution 500 cc. were brought to 37°, and treated with 2·0 g. of trypsin-D. From this point the procedure followed was the same as in Exp. 1 with caseinogen. The only difference of detail was that in the present case there took place, of course, no separation of tyrosine.

Exp. 5. This was a repetition of Exp. 4 with the difference that only arginine determinations were made, and these only after 2, 3 and 5 weeks of digestion. The object was simply to check the result of the first experiment with respect to the maximum amount of arginine liberated.

In reporting the results of these, as of all subsequent experiments, we omit, for the sake of brevity, the original analytical data (such as were shown for caseinogen in Table I), as well as the corrections (exemplified in Table II)

Table III. *Tryptic digestion of gelatin.*

(Corrected values for 100 cc. of digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
		Exp. 4. Total substrate-N = 1·363 g.				
0 hrs.	43·1	—	3·16	0·00	00·0	00·0
1 „	170·8	97·6	12·53	7·16	12·8	45·2
3 „	222·9	113·6	16·35	8·34	19·5	52·6
9 „	262·4	129·4	19·25	9·49	23·8	59·9
24 „	336·5	142·8	24·69	10·48	31·8	66·1
3 days	391·4	153·3	28·71	11·25	37·7	71·0
7 „	416·9	165·7	30·58	12·16	40·5	76·7
15 „	451·8	170·9	33·15	12·54	44·3	79·1
21 „	466·8	175·8	34·25	12·90	46·0	81·4
63 „	—	180·5	—	13·24	—	83·5
Complete hydrolysis	—	—	70·8	15·85	100·0	100·0
		Exp. 5. Total substrate-N = 1·491 g.				
14 days	—	189·3	—	12·70	—	80·1
21 „	—	196·4	—	13·17	—	83·1
35 „	—	202·6	—	13·59	—	85·7

[1] Some years ago we reported briefly preliminary experiments not only with caseinogen but also with gelatin [Hunter and Dauphinee, 1925; Hunter, 1925]. In both cases we obtained a maximum arginine yield of only two-thirds instead of, as now, four-fifths. The records of these early experiments were unfortunately lost in a laboratory fire, and we are unable at present to offer an explanation of the discrepancy.

which have to be made for the self-digestion of the trypsin. Table III contains therefore the results of Exps. 4 and 5 as calculated for 100 cc. of digest and already corrected by the corresponding trypsin controls.

A comparison of Tables II and III shows that, if the increase of free amino-nitrogen be taken as the measure of proteolysis, gelatin will appear to be digested more slowly and less completely than caseinogen. With respect, on the other hand, to the rate and the extent of arginine liberation the behaviours of these two proteins are practically identical, so that the curve of arginide hydrolysis given for caseinogen in Fig. 1 would serve almost equally well for gelatin. The very slightly different curve actually obtained for the latter appears with others in Fig. 2. Its initial part is produced again, on a different time scale and for a special purpose, in Fig. 3. We have thought it unnecessary to complicate Fig. 2 by introducing into it curves of peptide hydrolysis; but for the case of gelatin such a curve accompanies the curtailed arginine curve of Fig. 3.

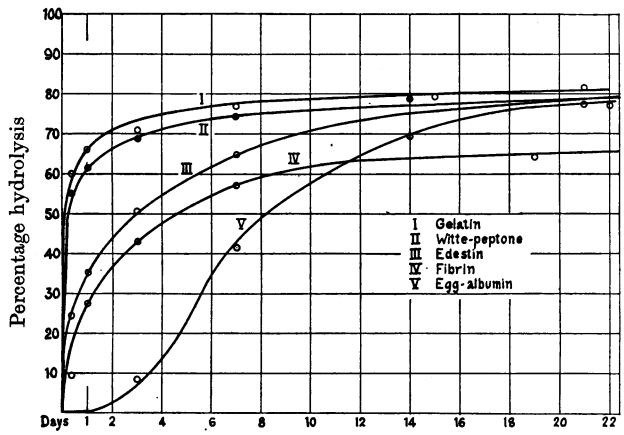

Fig. 2. Liberation of arginine in tryptic digestion of various proteins.

Exps. 4 and 5 are in accord in suggesting that the end-result of the action of trypsin on gelatin is the liberation of four-fifths of its total content of arginine.

(*c*) *Experiments with edestin.*

Exp. 6. 25 g. of edestin were dissolved by grinding in a mortar with 250 cc. of 0·25 % sodium carbonate, and by the cautious addition of HCl the reaction of the solution was so adjusted that it gave a just perceptible pink colour with phenolphthalein. The solution having then been brought to 37° was at a given instant treated with 1 g. of trypsin-P and 5 cc. of toluene. Since the earlier samples of this digest coagulated upon heating, the procedure of sampling and analysis hitherto employed was modified as follows. At selected intervals portions of 25 (or for those taken at the 29th and 35th days, 10) cc. were removed, and treated, for the immediate inhibition of tryptic action, with 4 (or 2) drops of 20 % HCl. The samples taken at the 1st and 3rd hours

were left undiluted, the others were made up with water to a volume of 50 cc. Aliquots of 5 cc. each were then measured into urea tubes, heated in boiling water for the final destruction of the enzyme, and used, without separation of any resulting coagulum, for the determination of ammonia and of arginine. Free amino- and total nitrogen were determined on suitable unheated aliquots.

The results were calculated to 100 cc. of digest, and in that form, after suitable correction for the added trypsin, are exhibited in Table IV. The rate of liberation of arginine is shown graphically in Fig. 2.

Table IV. *Tryptic digestion of edestin.*

(Corrected values for 100 cc. digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
			Exp. 6. Total substrate-N = 1·626 g.			
0 hrs.	—	—	1·8*	0·00	0·0	00·0
1 „	111·2	58·2	6·84	3·58	8·3	13·4
3 „	175·5	80·1	10·8	4·93	14·8	18·5
8 „	239·2	105·9	14·7	6·51	21·1	24·4
24 „	283·5	153·6	23·6	9·41	35·7	35·3
3 days	584·4	219·1	—	13·47	—	50·5
7 „	656·9	281·6	40·4	17·32	63·3	64·9
22 „	801·8	334·5	49·3	20·57	77·9	77·0
29 „	—	357·7	—	22·00	—	82·4
35 „	863·5	360·5	53·1	22·17	84·1	83·0
Complete hydrolysis	—	—	62·8†	26·7	100·0	100·0

* Van Slyke and Birchard [1914]. † Van Slyke [1912].

If the data of Table IV be compared with those of Tables II and III it will be evident that the behaviour of edestin under the action of trypsin differs considerably from that of caseinogen or gelatin. From both of the latter the amino-groups in general and the arginine in particular are liberated with a speed which may almost be described as explosive. Under identical conditions the hydrolysis of edestin is in each respect much more gradual. The contrast between edestin and gelatin is made very evident in the curves of Fig. 2. It was equally conspicuous in certain earlier experiments, of shorter duration than the present, which have been briefly reported elsewhere [Hunter, 1925; Hunter and Dauphinee, 1925]. The difference is therefore not accidental, but truly characteristic.

It was 3 or 4 weeks before the liberation of arginine in the present experiment came to an apparent standstill. The proportion then free was approximately 80 %. In this respect there would appear to be little, if any, difference between edestin and the two proteins previously discussed[1].

[1] It ought, however, to be mentioned that in another experiment, conducted upon a rather small scale and with a specimen of unknown origin, edestin appeared to yield a distinctly higher proportion of free arginine.

(*d*) *Experiments with egg-albumin.*

Exp. 7. 500 cc. of an approximately 10 % filtered solution of egg-albumin were digested at 37° and p_H 8·0 with 2 g. of trypsin-P. The sampling and the analyses were carried out in the same way as with edestin, except that none of the samples was diluted (Table V and Fig. 2).

Table V. *Tryptic digestion of egg-albumin.*

(Corrected values for 100 cc. digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
Exp. 7. Total substrate-N = 1·113 g.						
0 hrs.	54·8	—	4·92	0·00	0·00	0·00
3 „	56·1	1·76	5·04	0·16	0·16	1·54
9 „	66·0	—	5·93	—	1·35	—
24 „	89·9	—	8·08	—	4·24	—
3 days	168·6	9·61	15·2	0·86	13·8	8·32
7 „	432·0	47·8	38·8	4·29	45·5	41·4
14 „	641·8	79·6	57·7	7·15	70·8	69·2
21 „	685·8	89·3	61·6	8·02	76·0	77·4
37 „	701·8	97·0	63·1	8·72	78·0	84·1
Complete hydrolysis	—	—	79·5*	10·36	100·0	100·0

* Van Slyke [1912].

The peculiar course of the digestion in this experiment exemplifies the well-known resistance of uncoagulated egg-albumin to the initial attack of trypsin [Vernon, 1904; Bayliss, 1908, p. 67][1]. This resistance has usually been ascribed to the influence of an antitrypsin, and it has been supposed that only as the antitrypsin disappears does the trypsin itself gradually become effective. The curve of proteolysis is in this way delayed and distorted. Under the existing conditions the rate of liberation of arginine, from the 4th day onward, is represented by a curve which shows a general resemblance to that obtained with edestin. The final result is the liberation, once again, of approximately four-fifths of the total arginine. To this figure it would be unwise in the present instance to attach too much significance; for the albumin used was probably far from pure.

(*e*) *Experiments with fibrin.*

Exp. 8. 50 g. of dried fibrin were reduced to powder, suspended in 500 cc. of water, brought to a temperature of 37°, and treated with 1 g. of sodium carbonate, 2 g. of trypsin-P and 10 cc. of toluene. At the end of the 1st hour of digestion much of the fibrin was still in suspension. The first sample analysed was taken at the 3rd hour, when practically all of the fibrin had gone into solution. The early samples coagulated on heating, and were treated therefore in the same way as those of edestin and egg-albumin.

[1] For further references to the literature of this phenomenon see Bateman [1916].

Exp. 9. This was a repetition of Exp. 8 with these differences—a new lot of fibrin was taken, trypsin-D replaced trypsin-P, and samples were taken only after 2, 3 and 5 weeks (Table VI and Fig. 2).

Table VI. *Tryptic digestion of fibrin.*

(Corrected values for 100 cc. digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
		Exp. 8. Total substrate-N = 1·215 g.				
0 hrs.	—	—	1·6*	0·00	0·0	0·00
3 „	142·9	11·9	11·8	0·98	13·0	6·85
9 „	240·4	16·2	19·8	1·33	23·2	9·29
24 „	349·5	47·7	28·8	3·93	34·7	27·5
3 days	484·4	74·6	39·9	6·14	48·8	42·9
7 „	631·9	99·3	51·9	8·17	64·1	57·1
19 „	691·8	111·2	56·9	9·15	70·5	63·9
29 „	—	114·5	—	9·42	—	65·8
Complete hydrolysis	—	—	80·0*	14·31	—	—
		Exp. 9. Total substrate-N = 1·231 g.				
14 days	—	114·5	—	9·30	—	65·0
21 „	—	111·9	—	9·09	—	63·5
35 „	—	109·3	—	8·88	—	62·1

* Levene and Bass [1929].

Whether one judges the rate of proteolysis by the increase of free amino-nitrogen or by the liberation of arginine, it will appear from Exp. 8 that fibrin is digested much less rapidly than gelatin or caseinogen. In the gradual progress of the reaction it rather resembles edestin. Nevertheless the proportion of peptide groups ultimately hydrolysed is very high. On this criterion fibrin would in fact seem to be quite as susceptible to the hydrolytic action of trypsin as any of the proteins already examined. It is otherwise with respect to the liberation of arginine; with fibrin the fraction liberated is decidedly smaller. Both experiments are in agreement in setting it at about two-thirds. (The apparent diminution of free arginine after the second week in Exp. 9 is probably accidental.)

(*f*) *Experiments with Witte-peptone.*

The experiments with fibrin having shown that a large fraction of its arginine resisted liberation by trypsin, it seemed of interest to ascertain whether the proteoses and peptones derivable from it would exhibit in this respect any greater susceptibility. As a preliminary test of this possibility we carried out, therefore, the following experiments with Witte-peptone (Table VII and Fig. 2).

Exp. 10. An approximately 10 % filtered solution of Witte-peptone was digested at 37° and p_H 8·2, with 0·4 % of trypsin-P. In sampling this digest and in analysing the samples we followed exactly the same procedure as in Exp. 1 with caseinogen.

Exp. 11. A different lot of Witte-peptone was digested under the same conditions with trypsin-D; but the samples were taken only at the end of 2, 3 and 5 weeks, and were analysed for arginine alone.

Table VII. *Tryptic digestion of Witte-peptone.*

(Corrected values for 100 cc. digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
		Exp. 10. Total substrate-N = 1·325 g.				
0 hrs.	184·0	0·0	13·9	0·00	0·0	0·0
1 „	338·0	51·2	25·6	3·86	18·9	27·9
3 „	430·5	79·7	32·6	6·01	30·0	43·4
9 „	468·0	101·1	35·5	7·62	34·7	55·0
24 „	555·5	111·0	42·0	8·52	45·3	61·5
3 days	604·4	126·5	45·8	9·53	51·3	68·8
7 „	641·9	136·7	48·6	10·30	55·8	74·4
14 „	671·7	144·9	50·8	10·92	59·3	78·8
28 „	671·7	146·6	50·8	11·05	59·3	79·8
Complete hydrolysis	—	—	76·1	13·85	100·0	100·0
		Exp. 11. Total substrate-N = 1·465 g.				
14 days	—	161·2	—	11·01	—	79·5
21 „	—	167·7	—	11·45	—	82·7
35 „	—	170·5	—	11·64	—	84·1

(*g*) *The liberation of arginine in acid hydrolysis.*

The hydrolysis of proteins by acids is known to follow in several respects a different course from that of tryptic digestion. It seemed therefore worth while to compare, in at least one instance, the curves of arginine liberation in these two processes. With this object we carried out the following experiment, in which gelatin was subjected to the action of N HCl at 100°. The conditions were so chosen that the proportion of arginine liberated within 24 hours would not be greatly different from that liberated within the same period by 0·4 % of trypsin at 37°.

Exp. 12. A flask containing 300 cc. of $2N$ HCl was immersed in a boiling water-bath, and allowed to come to temperature equilibrium. There were then added to it 300 cc. of a 10 % solution of gelatin already raised to the boiling point. The flask was fitted with a reflux condenser, and hydrolysis was allowed to proceed, at a temperature of approximately 100°, for a period of 80 hours. At given intervals samples were removed from the flask, added to a quantity of $5N$ NaOH calculated to neutralise the acid, cooled, and made up to a convenient volume. Suitable aliquots were then used for the determination of total nitrogen, free amino-nitrogen, and arginine[1]. The results as calculated to 100 cc. of hydrolysate are shown in Table VIII. In Fig. 3 they are

[1] The determination of arginine involved of course a determination of ammonia. But as the ammonia results merely confirmed those of Pittom [1914] and Vickery [1922] in showing that the amide-N of proteins is completely liberated at a very early stage of acid hydrolysis, we have not recorded these separately.

exhibited graphically side by side with the corresponding parts of the curves for the tryptic digestion of gelatin (Exp. 4).

Table VIII. *Acid hydrolysis of gelatin. Exp. 12.*

(The data are for 100 cc. hydrolysate, containing 693·8 mg. total nitrogen.)

Time of hydrolysis	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total N		Percentage hydrolysis	
			as free amino-N	as arginine-N	of peptide links	of arginide links
0 hrs.	—	—	3·16	0·00	0·0	0·0
1 „	154·9	21·1	22·3	3·04	28·3	19·2
2 „	217·2	25·0	31·3	3·60	41·6	22·7
6 „	304·3	38·6	43·8	5·56	60·1	35·1
18 „	373·3	65·8	53·8	9·48	74·9	59·8
32 „	400·3	75·1	57·7	10·82	80·6	68·3
80 „	440·8	92·1	63·5	13·27	89·2	83·7
Total hydrolysis	—	—	70·8	15·85	100·0	100·0

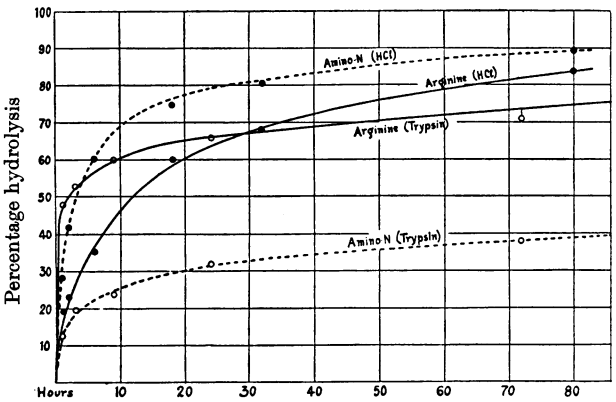

Fig. 3. Comparison of tryptic digestion with acid hydrolysis of gelatin.

The curves reveal at once decided differences between the two types of hydrolysis. As has been noted already, the effect of trypsin upon gelatin is to liberate arginine at a speed greatly in excess of the general rate of hydrolysis as measured by the increase of free amino-groups. Under the action of hydrochloric acid this relation is reversed. Another difference is that the initial rate of arginine liberation is much lower with the acid than with the enzyme. On the other hand the action of the enzyme slackens almost as suddenly as it starts, and is limited (as has been shown) to a partial hydrolysis; whereas that of the acid falls off much less abruptly, and continues until the liberation of arginine and other amino-acids is complete. The consequence is that after a time (in the present experiment about 30 hours) the two arginine curves cross one another, and the acid hydrolysate comes to contain the greater proportion of free arginine.

A full interpretation of these differences is for the present impossible; but their existence emphasises the selective character of enzymic, as contrasted with acid, hydrolysis.

Discussion.

The experiments which have been described demonstrate the liberation during tryptic digestion of a substance (or substances) susceptible to the action of arginase.

Up to this point it has been tacitly assumed that this substance is simple arginine. The correctness of this assumption, it must now be stated, is by no means certain. Although arginase is without action upon α-methylarginine, δ-methylarginine, dibenzoylarginine or arginine methyl ester, it liberates urea from α-monobenzoylarginine. Felix, Müller and Dirr [1928] conclude accordingly that any change in the guanidine or the carboxyl group of arginine, or the introduction into its amino-group of an alkyl radical, protects it from the action of arginase; but that the enzyme can still attack those derivatives in which the only substitution is that of one hydrogen of the amino-group by an acyl radical. If this generalisation is correct, arginase might be expected to act upon certain peptides of arginine—upon all those, namely, in which the arginine is bound only through its amino-group. Peptides of this type might conceivably enough be liberated during the course of tryptic digestion. It is therefore not impossible that the "arginine" determined in our experiments was still, in whole or in part, in a state of combination.

This argument rests of course upon a hypothesis, and there exists at present no direct evidence in support of the proposition to which it leads. The idea that peptide-bound arginine may in certain cases be susceptible to the action of arginase seemed indeed to be confirmed by the observation of Edlbacher and Bonem [1925] that Fischer's "arginylarginine" yields to the enzyme, as the theory would predict, one half of its total guanidine-nitrogen as urea. Unfortunately, as Zervas and Bergmann [1928] have shown, the supposed dipeptide of arginine is really an anhydride, and its apparent susceptibility to arginase was due, in all probability, to an admixture of free arginine. We possess as yet, therefore, no actual proof that any arginine peptide whatsoever is attacked by arginase.

Still less have we any proof that susceptible arginine peptides do actually appear under the action of trypsin. We have ourselves been unable, in spite of many attempts, to separate from tryptic digests any product, other than arginine itself, which would yield urea when treated with arginase. The only evidence which we have secured for the presence of such products is contained in the observation that flavianic acid, which forms a nearly insoluble compound with arginine, does not precipitate from a tryptic digest all of the material which is capable of reacting with arginase. Thus, in a particular 2-hour digest of gelatin the arginine determined by arginase amounted to 9·05 % of the total nitrogen, while the nitrogen precipitated by flavianic acid was only 7·24 %. In another digest, 18 days old, arginase indicated 12·25 % of arginine-nitrogen, flavianic acid only 11·32 %. In the first case therefore 19·8 %, in the second 7·6 %, of the arginine determined by arginase failed

to be precipitated by flavianic acid. This reagent was found to give in each case complete recovery of added arginine. The results might therefore be interpreted as indicating that the material susceptible to arginase, especially perhaps that of the earlier stages of digestion, included something other than free arginine. Unfortunately we cannot at present exclude the possibility that they depend simply upon an enhancement of the solubility of arginine flavianate by the more complex products of digestion.

A complete answer to the question at issue must be left to future work. In the meantime we must be content to recognise that what we have determined in our experiments may be either simple arginine, or some particular type of arginine-complex, or a mixture of both. In any case it is arginine which has been liberated from those connections which, in the intact protein, protect it from the action of arginase.

The rapidity with which this liberation takes place varies, as our experiments have shown, with the nature of the substrate. It is greatest in the cases of caseinogen and the two protein derivatives, gelatin and Witte-peptone. In all of these a few hours of digestion suffices to detach half at least of the total arginine from the parent molecule. The arginine of edestin, fibrin and egg-albumin is set free much more slowly, the liberation of 50 % requiring 3, 4½ and 8 days respectively. These differences are characteristic, but there is nothing about them that is specific for arginine. Very similar differences were found by Ragins [1928] in the rate of liberation of tryptophan from different proteins. Moreover, with each of the proteins studied in the present paper the curve of arginide hydrolysis did but parallel that of general peptide hydrolysis. For the cases of caseinogen and gelatin this is made evident in Figs. 1 and 3 respectively; and if the data for free amino-N had been plotted in Fig. 2, it would have been seen to be equally true for the others. The hydrolysis of the arginide links proceeds, therefore, simply *pari passu* with that of all the other peptide bonds actually susceptible, in any given instance, to the action of trypsin. This would indicate that differences in the rate of liberation of arginine are determined by differences (perhaps physical rather than chemical) in the general character of the molecule—not, as might otherwise have been supposed, by specific differences in the mode of union of arginine itself.

But, although the arginine curve and that for free amino-nitrogen run always parallel, it is only in the cases of edestin and egg-albumin that they show an approach to exact coincidence. With caseinogen (Fig. 1), with Witte-peptone and, still more conspicuously, with gelatin (Fig. 3) the arginine curve lies higher than the other. With fibrin, as the data of Table VI make sufficiently evident, it is distinctly lower. These differences, it is to be supposed, depend upon characteristic differences of structure; but until we know the precise nature of the grouping determined as "arginine," it would be idle to speculate more closely upon their significance.

None of the differences observable in the behaviour of different proteins

affects the main conclusion to be derived from our experiments. This is that in the tryptic digestion of proteins the rate of liberation of arginine (or of some complex containing it) is not exceeded by that of any other single amino-acid concerning which we possess exact information. This would suggest that much of the arginine (or of the particular complex in which it is bound) is situated, as tyrosine and tryptophan have been supposed to be, in a particularly exposed position within the molecule, or that it is held by links of a particularly susceptible configuration. It might, for instance, be imagined to lie at the ends of polypeptide chains. Such an idea receives a certain amount of support from the observations of Skraup and his co-workers, who showed that with certain proteins (though not with all) deamination [Skraup, 1908] or methylation [Skraup and Krause, 1909; Skraup and Böttcher, 1910] decreased by about 90 % the yield of arginine obtainable after hydrolysis. Since both treatments affected (or were assumed to affect) only amino-groups, Skraup concluded that in such cases the amino-group of arginine is for the most part free, and that the arginine occupies an external position in the molecule. Unfortunately for this argument the results of Skraup with deaminised caseinogen find but a partial confirmation in those of Steudel and Schumann [1929] and none at all in those of Dunn and Lewis [1921] and Wiley and Lewis [1930]. It has, moreover, been shown [Kossel and Weiss, 1909, 1, 2; 1910; Dakin, 1912; Dakin and Dudley, 1913] that racemised proteins yield upon total hydrolysis only inactive arginine; and, since "it appears likely that the conditions for racemisation of an amino-acid group require the attachment of other groups to both amino- and carboxyl radicals" [Dakin, 1912], this observation makes the hypothesis of a terminal position for arginine rather uncertain. On neither side is the evidence conclusive, and the precise reason for the ready liberation of arginine by trypsin remains therefore a matter of speculation.

While the liberation of arginine by trypsin is just as rapid as that of tyrosine or tryptophan it is apparently not so complete. In all of our experiments a certain definite proportion—with fibrin one-third, with the other proteins studied one-fifth—resisted indefinitely the hydrolytic action of the enzyme. In this respect our experiments are in accord with certain earlier observations. Siegfried and Lindner [1910], for instance, after submitting caseinogen to the action of trypsin for 20 days, isolated from the digest a "peptone" which upon total hydrolysis still yielded arginine; and similar trypsin-resistant and arginine-containing peptones have been prepared from gelatin and fibrin [Siegfried, 1902, 1, 2; 1903; Müller, 1903; Krüger, 1903].

The fact that only part of the arginine of proteins is rendered by trypsin accessible to the action of arginase, suggests that arginine exists within the protein molecule in at least two different modes of combination. Both Felix [1922] and Edlbacher [1924] have already, on quite other grounds, been led to consider this possible. Simms [1928, 1, 2] has suggested that much of the arginine obtained by acid hydrolysis does not exist in the parent molecule as

such, but as a "pre-arginine" group of unknown (possibly cyclic) nature. There is, however, no quantitative correspondence between our results and the proportions of arginine and "pre-arginine" reported by Simms. In edestin, for instance, he finds evidence for the presence of "pre-arginine" only. It would seem therefore that tryptic as well as acid hydrolysis results in the transformation of "pre-arginine" into arginine. The nature of the trypsin-resistant union remains quite unknown. One might speculate on the possibility that it involves the guanidine group in arginine, the occasional participation of which in the linking of amino-acids has been thought probable by Felix [1922, 1925; Felix and Harteneck, 1927]; but the views of Felix, which are based chiefly upon a study of the peptic digestion of histone, are not supported by the work of Waldschmidt-Leitz and Künstner [1927], and the available evidence is mostly in favour of the idea that all of the guanidine groups within the protein molecule are uncombined [Kossel and Kennaway, 1911; Kossel and Cameron, 1912; Sakaguchi, 1925].

If it is true that exactly four-fifths and no more of the arginine of gelatin can be liberated by trypsin, it follows that the number of arginine groups in the molecule of gelatin is five or a multiple thereof. The same conclusion may be drawn with respect to caseinogen, edestin and egg-albumin. On the other hand, in fibrin, which appears to yield only two-thirds of its arginine to trypsin, the number of arginine groups indicated is a multiple of three. These deductions are not affected by the question whether the arginine we determined was or was not entirely free, but they do of course depend upon the correctness of our estimates of the maximum amount that trypsin can liberate. As intimated in the experimental part of the paper all of these estimates are subject to a possible revision; the calculations based upon them possess therefore for the present a merely provisional value. It may however be pointed out that conclusions consistent with our own have been reached, in an entirely different way, by Cohn, Hendry and Prentiss [1925]. Considering the composition of proteins in relation to the problem of their molecular weight, these authors are led to infer that the minimum number of arginine groups in the molecule must be for gelatin five, for egg-albumin ten, and for fibrin six. For caseinogen they give a minimum of three, corresponding to a molecular weight of 12,800; but if, as they believe, the molecular weight of caseinogen is really 192,000, the actual number of its arginine groups must be 45, a multiple not only of 3 but of 5. As for edestin, its probable molecular weight—29,000 [Cohn *et al.*, 1925]—and arginine content—26·7 % of the total nitrogen [Hunter and Dauphinee, 1930]—are consistent with the assumption that it contains 25 (5×5) arginine groups. We would not be understood as laying undue stress upon these correspondences; for it is obvious that by assuming a molecular weight sufficiently large it would be possible to harmonise almost any pair of estimates of the kind under consideration.

One point more deserves perhaps some comment. In fibrin only two-thirds of the arginine appeared to be susceptible to the action of trypsin, and even

that was liberated rather slowly. From Witte-peptone, a product derived from fibrin by peptic digestion, the arginine is liberated not only much more rapidly, but also to a greater extent. That the fraction set free should be almost exactly four-fifths may be more or less fortuitous, for Witte-peptone is of course not a single substance. The point of interest is that arginine linkages, which in the original protein are inaccessible to trypsin, are rendered accessible by a previous treatment with pepsin. One of us is now engaged in a further study of this interesting phenomenon.

SUMMARY.

1. A study has been made of the rate at which arginine (or an arginine complex susceptible to the action of arginase) is liberated during the tryptic digestion of caseinogen, gelatin, edestin, fibrin, egg-albumin and Witte-peptone.

2. In the case of the first two the initial rate of liberation is so great that half of their total potential arginine may easily be set free within 3 hours. The speed with which Witte-peptone yields its arginine is but slightly inferior. With the other proteins the appearance of free arginine is a much more gradual process. Egg-albumin exhibits a quite peculiar behaviour, in that the liberation of arginine (like that of free amino-groups in general) commences only after a latent period of one or two days.

3. In no case did trypsin liberate from a protein the total amount of arginine obtainable on complete hydrolysis. The yield with fibrin was only two-thirds of the possible maximum, with each of the others four-fifths. These fractions appear to be characteristic.

4. In the acid hydrolysis of gelatin arginine is liberated less explosively and at a more regular rate than in its tryptic digestion.

5. The possible bearing of these findings upon certain problems of protein structure is discussed.

The expenses of this investigation were met in part by a grant, hereby gratefully acknowledged, from the Banting Research Foundation.

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