and mixed cultures of rat caecum contents and on the acid production by *Lactobacillus arabinosus* has been studied.

2. Growth of *Bact. coli* or of mixed cultures was not markedly affected by any one of the methyltryptophans in the concentrations used; that of *Lb. arabinosus* was considerably inhibited by the 7-methyl compound.

3. Nicotinamide synthesis from ornithine or ammonium lactate by *Bact. coli* was completely inhibited by 2 mM and correspondingly less by lower concentrations of 2-, 4-, 5- and 7-methyltryptophans, but not by abrin. Nicotinamide synthesis by mixed bacteria from the rat caecum contents was either completely or partly inhibited by the former at 2 mM and not affected by the latter compound. These findings suggest that tryptophan is involved in the bacterial biosynthesis of nicotinamide.

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The Linkage of Glutamic Acid in Protein Molecules

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The experiments presented in this paper were carried out to answer the question as to whether the γ-carboxyl groups of glutamic acid are involved in the formation of bonds in protein molecules. The possible existence of such bonds in proteins is suggested by the fact that γ-linked glutamic acid residues have been found in glutathione, in the capsular substance of *Bacillus anthracis* (Bovarnick, 1942; Hanby & Rydon, 1946) and in folic acid (Boothe, Mowat, Hutchings, Angier, Waller, Stokstad, Semb, Gazzola & Subbarow, 1948). Different modes of combination of the γ-carboxyl groups of glutamyl residues in proteins can be considered: (1) formation of a peptide bond with the terminal amino group of a peptide chain, (2) ester linkages with hydroxyl groups of hydroxy amino-acids and (3) thio-ester linkages with cysteine molecules of a peptide side chain (Chibnall, 1942). Since the γ-substituted glutamyl residues could give rise to branching of the main peptide chain, the problem of γ-substitution is of great importance.

In order to test proteins for the presence of γ-substituted glutamyl residues we subjected them to the following series of procedures: (*a*) partial digestion with trypsin, (*b*) oxidation, (*c*) extraction of the acidified solution with ether (ether extract 1), (*d*) total hydrolysis, (*e*) extraction of the acid hydrolysate with ether (ether extract 2) and (*f*) determination of succinic acid in the last ether extract. Succinic acid found in ether extract 2 was considered as originating from the γ-glutamyl residues. The above mentioned method is based on the fact that the γ-peptide bond of glutathione is more resistant to trypsin than are the normal α-peptide bonds (Grassmann, Dyckerhoff & Eibeler, 1930; Kendall, Mason & McKenzie, 1930). It was expected, therefore, that at least a part of the α- and γ-substituted glutamyl residues (formula I) would be transformed into γ-glutamyl peptides (formula II) by the action of trypsin.

(I) $R^1.CO.CH(NHR^2).CH_2.CH_2.CO.R^3$
Trypsin ↓
(II) $HOOC.CH(NH_2).CH_2.CH_2.CO.R^3$
NaOBr ↓
(III) $HOOC.CH_2.CH_2.CO.R^3$
HCl ↓
(IV) $HOOC.CH_2.CH_2.COOH$

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The reactions (*b*)–(*e*), by which the γ-glutamyl peptide (II) is oxidized to the succinyl peptide (III) and hydrolyzed to succinic acid (IV), have been used by Quastel, Stewart & Tunnicliffe (1923) and by Kendall *et al.* (1930) in their work on glutathione. Their methods had to be modified because of the small amounts of succinic acid to be expected.

EXPERIMENTAL

Partial hydrolysis with trypsin. Casein (Merck) and edestin (Schuchardt) were commercial preparations. Ovalbumin was prepared according to Kekwick & Cannan (1936), serum albumin from ox serum by fractional precipitation with $(NH_4)_2SO_4$, and haemoglobin from horse blood by the ethanol method (Hoppe-Seyler, 1878). Since native ovalbumin and serum albumin are scarcely attacked by trypsin, these proteins were denatured by keeping their aqueous solutions in a boiling water bath for 30 min. We are grateful to Dr H. N. Rydon (Birkbeck College, University of London) for a sample of the capsular substance of *B. anthracis*. Each of the proteins (2 g.), dissolved or suspended in water, was mixed with 0·2 g. of trypsin (Merck) and 1 ml. of toluene. The pH was adjusted to 8·5 by the addition of N-NaOH and the volume brought to 100 ml. by adding water. The mixtures were kept at 38° for 24 hr., those containing the albumins for 48 hr. The pH was kept near 8·5 by adding small amounts of N-NaOH from time to time. The rate of hydrolysis was determined in control tests run under the same conditions with 200 mg. of protein and 20 mg. of trypsin. At 0, 24 and 48 hr., 2 ml. of neutralized 30% (w/v) formaldehyde and 0·2 ml. of a 1% solution of phenolphthalein were added and the amount of hydrolysis determined by titration with 0·1 N-NaOH. The volumes of NaOH recorded in Table 1 include those added to the protein samples to maintain the pH at 8·5.

Table 1. *Action of trypsin on proteins*

(Protein (200 mg.); trypsin (20 mg.); extent of hydrolysis determined by formol titration using 0·1 N-NaOH. Figures in brackets show increases in NaOH titres over those at zero time.)

Protein	0·1 N-NaOH used for neutralization		
	0 hr. (ml.)	24 hr. (ml.)	48 hr. (ml.)
Casein	2·8	8·5 (5·7)	10·8 (8·0)
Edestin	0·64	6·5 (5·9)	7·6 (7·0)
Ovalbumin	0·85	4·9 (4·0)	7·2 (6·3)
Serum albumin	2·2	6·5 (4·3)	8·0 (5·8)

The amount of the capsular substance of *B. anthracis* was too small to permit formol titration. All of this substance (50 mg.) was treated with 5 mg. of trypsin in 5 ml. of water for 24 hr.

Oxidation. The tryptic hydrolysate from 2 g. of protein was mixed at 0° with 240 ml. of a cold 1% (v/v) solution of Br_2 in 0·5 N-NaOH. After 5 min. the mixture was acidified with glacial acetic acid, and 20 g. of KI were immediately added. The volume of the solution was reduced to 40–50 ml. by evaporation *in vacuo* and HCl was added until congo red paper gave a blue colour. The solution was extracted continuously with ether for approximately 100 hr. (ether extract 1). It was then mixed with 2 vol. of concentrated HCl and refluxed for 4 hr. The hydrolysate was evaporated to a small volume, diluted with water and extracted with ether for 15 hr. (ether extract 2).

Determination of succinic acid. The ether extracts were evaporated, dissolved in a small volume of water, filtered and evaporated to dryness. To the residues, dissolved in 1 ml. of water, an excess of finely powdered $Ba(OH)_2.8H_2O$ and 9 ml. of ethanol were added. The insoluble Ba succinate was centrifuged, dissolved in dilute HCl, evaporated to dryness and extracted repeatedly with 3–5 ml. of ether. The ether extracts were evaporated, dried, weighed and analyzed for their succinic acid content according to Krebs (1937), washed pigeon-breast muscle being used as source of enzyme. The results of the analyses are shown in Table 3.

Control experiments. In Exps. 1–7 and 10 (Table 4) no trypsin was used. The substrates were oxidized directly by NaOBr. Casein (Exp. 1) was dissolved in 100 ml. of water by adding the required amount of N-NaOH. Casein hydrolysate (Exp. 2) was obtained by boiling 0·5 g. of casein with 20 ml. of 20% (w/v) HCl for 4 hr., concentrating and neutralizing the aqueous solution. In Exps. 8 and 9 the substrates were treated with one-tenth of their weight of trypsin and kept at 38° for 24 hr. The amino-acid mixture (Table 4, Exp. 10) contained 100 mg. of each of the following: glycine, alanine, serine, cystine, valine, leucine, isoleucine, aspartic acid, phenylalanine, tyrosine, arginine and histidine, together with 50 mg. of lysine, 50 mg. of tryptophan and 100 mg. of glucose. In all these experiments (see Table 4) the oxidation, extraction and hydrolysis with HCl were carried out as described in the preceding sections. In another control experiment glutamic acid (100 mg.) was boiled with 20 ml. of 23% (w/v) HCl containing 1 g. of KI and approximately 0·1 g. of I_2. After 4 hr. the solution was extracted continuously with ether and the extract examined in the usual manner. No succinic acid was found.

Determination of glutamine in the tryptic hydrolysate. Each of the hydrolysates (0·5 ml. equivalent to 10 mg. of protein) was brought to pH 3·5 by the addition of 0·1 N-HCl and kept

Table 2. *Determination of glutamine and of pyrrolidone carboxylic acid in the tryptic hydrolysate*

Protein	Consumption of 0·04 N-HCl		Found in 2 g. of protein		
	Before heating (ml.)	After heating (ml.)	Pyrrolidone carboxylic acid (mg.)	Glutamine (mg.)	Total glutamic acid (mg.)
Casein	0·025	0·060	26	41	440*
Edestin	0·020	0·062	21	49	410*
Ovalbumin	0·106	0·190	109	99	320*
Serum albumin	0·011	0·035	11	28	340†
Haemoglobin	0·010	0·032	10	26	170‡

* Chibnall (1946). † Shemin (1945). ‡ Foster (1945).

in a boiling water bath for 2·5 hr. The amount of NH_3 present before and after boiling was determined according to Conway (1933), 0·04N-HCl being used for the titration of the NH_3 absorbed by the boric acid (Table 2). Asparagine is not split noticeably under these conditions (Chibnall & Westall, 1932).

RESULTS

Table 1 shows that 35–40 % of the peptide bonds of the proteins examined were split by the action of trypsin under the conditions of our experiments. Since γ-glutamyl bonds are slowly hydrolyzed by water at 38°, the duration of the digestion was reduced by employing relatively large amounts of trypsin. Table 4 (Exp. 9) shows that a considerable part of the glutathione resisted hydrolysis under the same conditions, but that another part was split as shown by the yield of succinic acid in ether extract 1.

The oxidizing agents used by Quastel *et al.* (1923) and by Kendall *et al.* (1930) were hydrogen peroxide, hypobromite or chloramine T. When the methods of these authors were applied to proteins, large amounts of succinic acid were found in the ether extract 2 (Haurowitz & Vardar, 1944). Later investigations showed that the destruction of hydrogen peroxide and of hypobromite is not complete in these methods, so that a further oxidation occurs during the hydrolysis with hydrochloric acid (Schwerin, Kara & Tanasoglu, unpublished results). Much lower results were obtained with chloramine T, which can easily be removed from the reaction mixture; but since the action of chloramine T requires temperatures of 40°, and since γ-glutamyl bonds are unstable at these temperatures in acid solutions, we preferred to use hypobromite at 0°. Goldschmidt & Strauss (1929) removed the excess of hypobromite by adding the calculated amount of hydrogen peroxide. It is hardly possible, however, to avoid a small excess of either peroxide or hypobromite. We therefore destroyed the peroxide by an excess of potassium iodide added in slightly acid solution. The bulk of the iodine formed was removed by the first ether extraction, so that it could not act as an oxidant during the subsequent acid hydrolysis.

Table 3 shows that 2 g. of protein on oxidation furnished 8–29 mg. of succinic acid before hydrolysis with hydrochloric acid (ether extract 1), and only 0·8–1·7 mg. of succinic acid after hydrolysis with acid (ether extract 2). The succinic acid found in ether extract 1 originates from the oxidation of glutamic acid and glutamine, both present in the tryptic hydrolysates of proteins. Only the succinic acid in ether extract 2 can arise from γ-glutamyl peptides of the formula II.

In view of the small quantities of succinic acid found in the ether extracts 2, it was very important to remove all of the succinic acid which had been

Table 3. *Determination of succinic acid in partially digested and oxidized proteins before and after hydrolysis with hydrochloric acid*

		Ether extract 1 (before HCl hydrolysis)			Ether extract 2 (after HCl hydrolysis)		
			Succinic acid in			Succinic acid in	
Protein	Wt. (g.)	Wt. (mg.)	2 g. of protein (mg.)	10^5 g. of protein (mol.)	Wt. (mg.)	2 g. of protein (mg.)	10^5 g. of protein (mol.)
Casein	2·0	88	29	12	15	1·5	0·64
Edestin	2·0	121	24	10	18	1·1	0·47
Ovalbumin	2·0	92	14	6	30	0·8	0·34
Serum albumin	2·0	138	23	10	12	1·1	0·47
Haemoglobin	2·0	120	8	3	39	1·7	1·4
Capsular substance of *B. anthracis*	0·05	3	0·3	5	1·6	0·48	8·1

Table 4. *Determination of succinic acid in control experiments*

			Hypobromite solution added	Ether extract 1		Ether extract 2	
Exp. no.	Substrate	Wt. (g.)	(ml.)	Total wt. (mg.)	Succinic acid (mg.)	Total wt. (mg.)	Succinic acid (mg.)
1	Casein	5·0	200	5	0·4	6	0·6
2	Casein hydrolysate	0·5	190	85	37	0	0
3	Glutamic acid	0·09	24	63	45	0	0
4	Proline	0·05	5	3	1·0	0·4	0
5	Hydroxyproline	0·1	10	0·4	0	0	0
6	Methionine	0·1	27	—	0·12	0	0
7	Pyrrolidone carboxylic acid	0·2	20	9·7	4·6	0·6	0·07
8	Glutamine	0·1	26	28	18	0·97	0·01
9	Glutathione	0·128	30	9·4	4·7	11·2	8·0
10	Amino-acid mixture	1·4	140	35·7	2·0	—	—

formed before hydrolysis. For this reason the extraction with ether was continued for several days, until the extracts were free even of traces of succinic acid. Under these conditions no succinic acid passed into ether extract 2 after the oxidation and hydrolysis of glutamic acid, proline, hydroxyproline, methionine and of the amino-acid mixture examined (Table 4, Exps. 2–6). On the other hand, glutathione gave rise to large amounts of succinic acid in extract 2..

Glutamine and pyrrolidonecarboxylic acid gave traces of succinic acid in ether extract 2 (Table 4, Exps. 7 and 8). It was necessary, therefore, to determine both these substances in the tryptic hydrolysates. Since pyrrolidonecarboxylic acid is formed from glutamine, its amount is approximately equivalent to the quantity of ammonia found before heating at pH 3·5, while the amount of glutamine is equivalent to the ammonia formed during the heating. According to Table 2 the amount of pyrrolidonecarboxylic acid varies from 10 to 109 mg., that of glutamine from 26 to 99 mg./2 g. of protein digested. The amounts of succinic acid to be expected from these quantities of glutamine and of pyrrolidonecarboxylic acid, according to Table 4, are so small that they would not noticeably affect the values of succinic acid passing into the ether extract 2 (Table 3).

DISCUSSION

The amount of succinic acid found after hydrolysis of the oxidized peptides is very small (Table 3). If molecular weights of 100,000, 309,000, 44,000, 68,000 and 68,000 are assumed for casein, edestin, ovalbumin, serum albumin and haemoglobin respectively, the number of γ-glutamyl bonds per protein molecule would be 0·64, 1·45, 0·15, 0·32 and 0·95. These figures, however, have to be considered as minimum values. The actual number of γ-glutamyl bonds is certainly higher for the following two reasons: (*a*) a considerable part of the γ-glutamyl bonds is split during the treatment with trypsin at 37°, as shown by the experiment on glutathione (Table 4); (*b*) less than one molecule of succinic acid is formed, when glutamic acid or glutathione are oxidized (Table 4, Exps. 3 and 9). We are thus unable to calculate the real number of γ-glutamyl bonds per protein molecule, and we have to content ourselves with the result that such bonds are present and that their number in the proteins examined is very low. The assumptions of Chibnall (1942) and of Haurowitz & Vardar (1944), that some of the γ-carboxyl groups of glutamic acid are linked to other groups in protein molecules, are supported by these results. Much larger quantities of succinic acid were found in the ether extracts 2 of the capsular substance of *B. anthracis*. According to Hanby & Rydon (1946) this substance has a molecular weight of 50,000 or more, and is built up exclusively of chains of 50–100 α-glutamyl residues interlinked by γ-glutamyl bonds. Our results agree with this view. The problem of chain branching is not solved by our results for the γ-linked glutamyl residues can be bound according to the formula $R.NH.CH(COOH).CH_2.CH_2.CO.R$, without any ramification of the peptide chains.

SUMMARY

1. Casein, edestin, ovalbumin, serum albumin, haemoglobin and the capsular substance of *Bacillus anthracis* were partially digested with trypsin, and the resulting peptide mixture was tested for the presence of γ-glutamyl peptides by oxidizing it with sodium hypobromite. Succinic acid was removed by ether extraction and the succinyl peptides, insoluble in ether, were hydrolyzed by hydrochloric acid.

2. The amounts of succinic acid obtained by this procedure indicate the presence of a small number of γ-glutamyl bonds in the proteins examined, and of a much higher number of such bonds in the capsular substance of *B. anthracis*.

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