DISCUSSION AND SUMMARY

Green seed peas were extracted with hot and cold saline buffer and with hot and cold water. The protein was removed from these extracts by trichloroacetic acid, ethanol, tungstic acid and colloidal iron; diffusates were also prepared by dialysis of the extracts. Cold water and saline extracted more nitrogen-containing compounds, but the best yields of non-protein nitrogen were obtained by hot-saline extraction followed by trichloroacetic acid precipitation. Ethanol and colloidal iron gave good recoveries after a similar extraction, but the amounts yielded by tungstic acid precipitation and by dialysis were less. This action of precipitants is similar to that observed by Steward & Street (1946) in their survey of the soluble-nitrogen fractions of the potato tuber.

The amount of free amino acid was high in the hot-saline extract after trichloroacetic acid precipitation but the highest values were obtained after tungstic acid precipitation.

Paper chromatography of the extracts showed the presence of numerous amino acids. Tungstic acid filtrates are unsuitable for chromatography.

It is suggested that, for a complete extraction of non-protein nitrogen from peas, hot saline-buffer extraction followed by trichloroacetic acid precipitation is the best procedure. Excess trichloroacetic acid may be easily removed by extraction with ether. The procedure of next choice would appear to be the use of ethanol.

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The Liberation of Aspartic Acid during the Acid Hydrolysis of Proteins

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Comparatively little is known about the relative rates of hydrolysis of the different peptide bonds in proteins, or about the effect on these rates of changes in hydrolytic conditions (cf. Acher, Jutisz & Fromageot, 1950; Desnuelle & Casal, 1948). Partridge & Davis (1950) found that when a number of proteins were hydrolysed with boiling weak acids, aspartic acid was liberated in the free state much sooner than other amino acids, and later Blackburn (1950*a*) showed that aspartic acid was also preferentially liberated during hydrolysis of proteins with dilute solutions of strong acids at the boil.

The present paper is concerned with the liberation of aspartic acid under various conditions of acid hydrolysis, and the extent to which this liberation occurs independently of the splitting of other linkages. A mechanism for the liberation is suggested.

MATERIALS AND METHODS

A '56s' quality virgin wool (Blackburn, Consden & Phillips, 1944) was used in the first experiments. As the supply of this wool became exhausted, a '64s A' quality Australian wool

was cleaned in a similar manner and used in the later experiments. Crystallized bovine plasma albumin was obtained from Armour Laboratories.

Partial hydrolysis and estimation of free amino acids. The wool or other protein was hydrolysed with HCl (100 ml./g.) under the conditions stated below, any undissolved wool filtered off and excess of acid removed by repeated distillation *in vacuo* below 40°, with successive additions of water. The hydrolysates were examined on phenol-NH₃, collidine, or two-dimensional collidine/phenol-NH₃ chromatograms, the concentration of the free amino acids being estimated by visual observation of the size and intensity of the amino acid spots on the chromatogram. The $\times$'s of Table 1 have the significance attached to them by Consden, Gordon, Martin & Synge (1947) and Consden, Gordon & Martin (1949), and give a semiquantitative estimate of the amounts of the amino acids. Proline was detected by the isatin reaction (Acher, Fromageot & Jutisz, 1950) and cystine by oxidation to cysteic acid with hydrogen peroxide (Dent, 1948). The concentration of free aspartic acid in some of the hydrolysates was measured by the method of Blackburn & Robson (1953). The peptides present in the partial hydrolysates had very small R_F values and were far removed from the aspartic acid spot, and other free amino acids were only present in traces. It was therefore possible to estimate the aspartic acid on one-dimensional phenol-NH₃ chromato-

grams of the hydrolysates. Determination of the aspartic acid by 'spot dilution' analysis (Polson, Mosley & Wyckoff, 1947) gave values of the same order of magnitude as those obtained by the radiochemical method. The figures in Table 2, however, are regarded as giving the order rather than an exact value for the free aspartic acid in solution.

Deamidation of wool. To remove the amide nitrogen, wool (1 g.) was either treated with nitrous acid in 2*N*-HCl for 24 hr. (Plimmer, 1925; Fraenkel-Conrat, Cooper & Olcott, 1945), or treated with 100 ml. of 0.05*M* cetylsulphonic acid (prepared according to McBain & Williams, 1933) at 65° for 24 hr. (Steinhardt & Fugitt, 1942).

Isolation of peptides and end-group estimations. Large polypeptides present in partial hydrolysates were isolated as follows (cf. Partridge & Davis, 1950). The solution was neutralized with Li₂CO₃ and 3 vol. of ethanol were added. After standing in the refrigerator for some time the precipitated peptides were removed by centrifuging. Amino-N (Van Slyke) determinations showed that these peptides had long chains. The dinitrophenylpeptides (DNP-peptides) were prepared as described previously (Blackburn, 1950*b*), insoluble peptides being treated with 1-fluoro-2:4-dinitrobenzene (FDNB) for 24 hr. and soluble peptides for 4 hr. To determine the *N*-terminal amino acids in partial hydrolysates, the hydrolysate was made alkaline with NaHCO₃, treated with FDNB, diluted and extracted with ether, made 5.7*N* in HCl and refluxed for 17 hr. after removing dissolved ether by warming. The DNP-amino acids liberated on acid hydrolysis were separated on buffered silica columns (Blackburn, 1949).

RESULTS

The liberation of free amino acids

Table 1 shows the relative amounts of free amino acids liberated during the hydrolysis of wool under various conditions. During 4 hr. hydrolysis with 0.1*N*-HCl at the boil, only aspartic acid is liberated in the free state. This preferential liberation of aspartic acid is not confined to HCl, for 0.1*N*-H₂SO₄ at the boil acts similarly. With increasing concentration of acid (HCl up to *N*), other amino acids are liberated, glycine being one of the first. Serine is liberated readily, valine, leucine and threonine slowly. The order is roughly that of increasing length of side chain of the amino acid; a similar order is observed when wool is hydrolysed with concentrated HCl at 37°. Cystine and proline are not liberated very readily. The cystine content of the undissolved residue obtained when wool is partially hydrolysed with dilute acid shows a marked increase above that of the original wool, e.g. after boiling with 0.5*N*-HCl for 8 hr. the residue amounted to 17 % of original weight of the wool and had increased in cystine content by 67 %. Similar increases have been observed when wool or other proteins are digested with enzymes (cf. Blackburn, 1950*b*). This slow liberation of cystine from the protein is probably due to the fact that four peptide linkages must be broken before cystine is set free, as compared with two for other amino acids.

Table 1. *The liberation of free amino acids during the hydrolysis of wool*

Conditions of hydrolysis		For significance of 'x' see Conden, Gordon, Martin & Syngé (1947).									
Reagent	Temp.	Time of hydrolysis (hr.)	Aspartic acid	Glutamic acid	Glycine	Alanine	Valine	Leucine	Serine	Threonine	Basic amino acids
0.1 <i>N</i> -HCl	Boiling	1	x								
0.1 <i>N</i> -HCl	Boiling	3	x								
0.1 <i>N</i> -HCl	Boiling	5	x								
0.25 <i>N</i> -HCl	Boiling	1	x								
0.25 <i>N</i> -HCl	Boiling	3	x								
0.5 <i>N</i> -HCl	Boiling	1	x								
0.5 <i>N</i> -HCl	37°	144									
0.5 <i>N</i> -HCl	50°	72									
<i>N</i> -HCl	Boiling	2	x	x	x	x	x	x	x	x	x
<i>N</i> -HCl	37°	72		x	x	x	x	x	x	x	x
<i>N</i> -HCl	37°	144	?	?	x	x	x	x	x	x	x
5 <i>N</i> -HCl	50°	72	x	x	x	x	x	x	x	x	x
5 <i>N</i> -HCl	37°	24	?	x	x	x	?	x	x	x	x
Conc. HCl	37°	24	x	x	x	x			x	x	x
Conc. HCl	37°	48	x	x	x	x			x	x	x
0.1 <i>N</i> -Ba(OH) ₂	Boiling	1	x	x	x	x			x	x	x
0.25 <i>N</i> -Ba(OH) ₂	Boiling	1	x	x	x	x			x	x	x

Table 2. *The free aspartic acid and N-terminal residues liberated during the hydrolysis of bovine plasma albumin with dilute acid at the boil*

		Time of hydrolysis (hr.)				
		2	4	6	8	24
Free aspartic acid (μ moles/g. protein) after hydrolysis with (a) 0.1 N-HCl and (b) 0.25 N-HCl	(a)	70	181	198	338	.
	(b)	163	257	400	666	.
<i>N</i> -terminal residues (μ moles/g. protein) after hydrolysis with 0.1 N-HCl						
Aspartic acid	.	.	53	.	132	270
Glutamic acid	.	.	26	.	37	82
Serine	.	.	9.5	.	16	53
Threonine	.	.	28	.	42	102
Glycine	.	.	Present	.	Present	Present
Alanine	.	.	Present	.	Present	Present
Valine	.	.	Present	.	Present	Present
Leucine + isoleucine	.	.	Present	.	Present	Present

Table 3. *The N-terminal residues of long-chain peptides from protein hydrolysates*

<i>N</i> -terminal residues (μ moles/g. DNP-peptide)	Wool	Bovine plasma albumin
Aspartic acid	3.5	3.9
Glutamic acid	6.4	13.6
Serine	19.9	5.9
Threonine	8.5	12.9
Glycine	Present	Present
Alanine	Present	Present
Valine	4.9	Present
Leucine + isoleucine	11.7	Present

As some of the aspartic acid residues in proteins are combined with ammonia as asparagine, it was of interest to determine the effect that removal of the amide groups would have on the liberation of aspartic acid. Wool was therefore deamidated with HNO_3 in HCl, or partially hydrolysed under conditions (cetylsulphonic acid at 65°; concentrated HCl at 37° for 1 or 2 days; or 0.1 N-HCl at the boil 24 hr.) where the amide-N is rapidly removed (cf. Steinhardt & Fugitt, 1942; Gordon, Martin & Synge, 1941). When these wools or partial hydrolysates were boiled with 0.1 N-HCl for 4 hr., chromatographic examination of the resulting hydrolysates showed that aspartic acid was still liberated at a rate exceeding that of other amino acids. Removal of the amide groups thus has no effect on the preferential liberation of aspartic acid.

Table 2 shows that, in addition to the liberation of aspartic acid, a large number of different *N*-terminal residues are formed when bovine plasma albumin is hydrolysed with 0.1 N-HCl for various times. No correction factor has been applied to these figures for the destruction of DNP-amino acids during hydrolysis, since its magnitude is rather uncertain and the relative rather than the absolute values are of importance to the present argument.

Two peptide linkages must be hydrolysed in order to set free an aspartic acid residue in a peptide chain; the first one to be hydrolysed may involve either the α -amino or α -carboxyl group of the residue and the partial hydrolysate should then contain relatively large amounts of either aspartyl or aspartic acid peptides. In an experiment to obtain evidence on this point the long-chain peptides from a partial hydrolysate of Australian '64sA' wool (7 g.) with 0.1 N-HCl for 8 hr. at the boil were precipitated as described above and converted into their DNP derivatives (0.141 g.). In a parallel experiment bovine plasma albumin (250 mg.) boiled for 4 hr. with 0.1 N-HCl gave 157 mg. of DNP-peptide. The free amino groups of both these peptide fractions were then determined (Table 3). The most noteworthy point is that the amount of *N*-terminal aspartic acid present is small compared with those of other *N*-terminal residues. The long-chain peptides represent an intermediate stage in the breakdown of protein to short peptides and at this stage, i.e. after 4 or 8 hr., aspartic acid is still being liberated preferentially compared with the other amino acids. These results may therefore indicate that the peptide linkage involving the α -carboxyl group of aspartic acid is the first to be broken. Desnuelle & Bonjour (1952) suggested that a partial hydrolysate of protein with oxalic acid might contain seryl and threonyl peptides, and that more aspartyl peptides than aspartic acid peptides should be present. In dilute HCl hydrolysates, seryl and threonyl peptides are certainly present but the amount of aspartyl peptides seems to be small. Table 2 and amino-N determinations on the hydrolysates showed that for every aspartic acid residue set free several other peptide linkages involving other amino acids are broken. The liberation of aspartic acid during dilute acid hydrolysis has thus only a limited specificity.

DISCUSSION

There is a marked difference in the rate at which free aspartic acid is liberated during hydrolysis with concentrated hydrochloric acid at 37° and dilute hydrochloric acid at the boil. Partridge & Davis (1950) considered that during the hydrolysis of proteins with weak acids such as oxalic acid, free carboxyl groups were released from asparagine and glutamine side chains at an early stage. These carboxyl groups then acted as proton donors to promote the hydrolysis of peptide linkages involving first the α -carboxyl groups and then the α -amino groups, liberating free aspartic or glutamic acid. The release of aspartic acid at an earlier stage than glutamic acid was attributed to the lower pK_1 and pK_2 values of aspartic acid as compared with glutamic acid. On this mechanism it would be expected that cysteic acid residues in a protein, which differ from aspartic acid residues in having a sulphonic group at the end of the chain instead of a carboxyl group, would be set free earlier than aspartic acid during hydrolysis with dilute acids. In fact, cysteic acid was liberated from wool oxidized with performic acid (Blackburn & Lowther, 1951) at a fairly early stage but at a slower rate than aspartic acid. Sanger & Tuppy (1951) similarly observed that large amounts of free aspartic acid, but only small amounts of free cysteic acid, were present in the digest when oxidized insulin was boiled with 0.1N hydrochloric acid.

Partridge & Davis further suggested that a similar type of argument should apply to the free carboxyl group at the end of a peptide chain. It is noteworthy, however, that when lysozyme, whose molecule contains a single amino acid chain with a C-terminal leucine residue (Thompson, 1952) was boiled with 0.1N hydrochloric acid, the short-chain amino acids, not leucine, were the first to be liberated after aspartic acid.

Since the liberation of free glutamic acid does not show anything like the variation with changes in hydrolytic conditions shown by aspartic acid, some

mechanism must operate which is applicable to aspartic acid and not to glutamic acid. Liberation of glutamic acid with subsequent ring closure to pyrrolidonecarboxylic acid does not seem to occur to a significant extent, since when a partial hydrolysate of wool with 0.1N hydrochloric acid for 4 hr. was further hydrolysed for 24 hr. with 5N hydrochloric acid after removal of insoluble protein material and examined chromatographically, glutamic acid was not present in amount noticeably greater than other amino acids. In addition, the preferential liberation of aspartic acid on boiling with dilute hydrochloric acid from wool already partially hydrolysed with concentrated hydrochloric acid at 37° means that the mechanism must apply to relatively short-chain peptides in addition to intact proteins.

One possible mechanism is that ring closure occurs between the β -carboxyl group of aspartic acid and the adjacent carboxyl group of the peptide linkage, fission of the peptide linkage then occurring (Fig. 1a). Barker (1953) observed that *N*-acetyl- or *N*-benzoyl-aspartic acid with acetic anhydride formed an internal anhydride rather than an oxazolone. Ring closure could possibly occur also in the other direction (Fig. 1b).

Baker & Ollis (1949) found that β -benzamidoisovaleric acid underwent ring closure on treatment with acetic anhydride to give 6-oxo-2-phenyl-4:4-dimethyl-5:6-dihydro-1:3-oxazine, and suggested that aspartic acid derivatives might react in a similar manner. The relative rates of the cyclization and hydrolysis reactions may be such that the ring compound never appears in solution (cf. Sanger, 1952). At 37°, acid of low concentration (0.1–0.5N hydrochloric acid) liberates glycine and serine preferentially, indicating that the principal factor in the formation of free aspartic acid at the boil with dilute acid is the higher temperature. This fact would suggest that a ring closure such as the above is favoured by a rise in temperature. Other instances where aspartic acid or asparagine residues in peptide linkage show exceptional lability (Waelsch, 1952; Weygand & Junk, 1951; Thompson, 1953)

Fig. 1. Suggested mechanisms for preferential release of aspartic acid from proteins.

are probably due to the presence of a reactive residue such as serine adjacent to the aspartic acid or asparagine residue (Thompson, 1953).

SUMMARY

Aspartic acid is preferentially liberated during hydrolysis of proteins with hot dilute acids but not with concentrated HCl at 37°. The specificity of the reaction and its mechanism are discussed.

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Effects of Nicotine on the Pyruvic Oxidase System in Brain

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The pharmacology of nicotine has been extensively studied, but comparatively little is known about its mode of action in the biochemical sense.

Nicotine, in common with other pyridine derivatives (Dickens, 1936*a*), inhibits the Pasteur effect and retards the enzymic degradation of cozymase (Baker, Fazekas & Himwich, 1938; McIlwain, 1950). It may interfere with coupled phosphorylation (Case & McIlwain, 1951) but does not affect the hexokinase reaction (Fahmy & Walsh, 1954). Nicotine is particularly toxic to nerve cells (Langley & Dickinson, 1889, 1890*a, b*); the toxicity towards living organisms parallels the degree of development of the nervous system (Greenwood, 1890), and the effects on nerve tissue are analogous to those resulting from oxygen deprivation (McIndoo, 1916; Wilson, 1928).

It is well known that nicotine inhibits cellular respiration and, since nerve is uniquely dependent

on carbohydrate (Dickens, 1936*b*) and is unable to metabolize nicotine (Werle & Müller, 1941; Miller & Larson, 1953), it seemed probable to us that the toxicity of nicotine towards nerve cells might be attributable to its ability to block essential steps in the oxidative metabolism of carbohydrate. In the present work we have made an analytical study of the effects of nicotine on the oxidation of pyruvate through the citric acid cycle.

MATERIALS AND METHODS

Substrates. Neutral solutions were prepared freshly as required from pure, crystalline materials. Sodium pyruvate was prepared as described by Price & Levintow (1952).

Homogenates. Glass homogenizers of Potter & Elvehjem's type were used. Tissues (rat-brain cerebral cortex, pigeon cerebral hemispheres or rat-kidney cortex) were homogenized in Krebs's phosphate-saline, with cooling, to give suspensions containing 100 mg. tissue/ml.