attacked at comparable speeds by the reduced bacterial flora remaining after washing out the gut. It also appears unlikely that the amino-acids are oxidized in the gut, since the intestinal mucosa has not been reported to be a rich source of amino-acid oxidases. It should be stressed, however, that the experiments described here do not establish that the amino-acids are in fact transferred to the blood stream, and it is for this reason that the term amino-acid disappearance has been used in preference to absorption.

It is uncertain how the rate of amino-acid disappearance is influenced by concentration. If the simplest assumption is made, namely that the rate of disappearance is proportional to the concentration, it seems quite unjustifiable to draw quantitative conclusions from the relative rates of disappearance of the different amino-acids, because the percentage of each which disappeared in the experimental period was not the same in every case. The figures for the L/D ratio (Table 3) are open to a similar criticism. They have, however, the merit of being minimum figures. Using, for example, the assumption just mentioned, it is easy to calculate that the L/D ratio for alanine would be 3.2 instead of 2.2 if it were possible to make an instantaneous measurement of the relative rates for the two isomers. On the other

hand, to obtain the greatest absolute difference in the amount of the two isomers disappearing, a long experimental period is desirable. If k_1 and k_2 were the velocity constants for the disappearance rates of the two isomers, the optimum length of experiment would be given by

$$t = -\frac{1}{k_1 - k_2} \log k_2/k_1.$$

Thus for DL-histidine the optimum length of experiment would appear to be about 1.25 hr., but the L/D ratio would be only 3.8 as compared with the observed value, for an experimental period of 0.5 hr. of 6.0.

SUMMARY

1. The rate of disappearance of the D- and L-isomers of thirteen amino-acids has been studied in an isolated loop of rat small intestine.
2. In each case the L-isomer disappeared faster than the D-isomer.
3. This is regarded as evidence for an active process in the absorption of amino-acids.

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REFERENCES

- Bender, A. E. & Krebs, H. A. (1950). *Biochem. J.* **46**, 210.
 Burton, K. (1950). (Unpublished observations.)
 Chase, B. W. & Lewis, H. B. (1934). *J. biol. Chem.* **106**, 315.
 Cori, C. F. (1925). *J. biol. Chem.* **66**, 691.
 Gale, E. F. (1941). *Biochem. J.* **35**, 66.
 Gale, E. F. & Epps, H. M. R. (1944). *Biochem. J.* **38**, 232.
 Höber, R. & Höber, J. (1937). *J. cell. comp. Physiol.* **10**, 401.
 Kratzer, F. H. (1944). *J. biol. Chem.* **153**, 237.
 Krebs, H. A. (1951). *Biochem. J.* **47**, 605.
 Macpherson, H. T. (1946). *Biochem. J.* **40**, 470.
 Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.

Methods for Isolating ω -Amino-acids: γ -Aminobutyric Acid from Rye Grass

By R. L. M. SYNGE

The Rowett Research Institute, Bucksburn, Aberdeenshire

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Work is in progress here on the chemical reactions which are undergone by dietary constituents, especially those from green leaves, in the course of digestion in the ruminant. The occurrence of ω -amino-acids in plant juices and as products of microbial degradation of common amino-acids in the rumen necessitates systematic procedures for the isolation of these compounds.

Three methods are now described which in suitable combinations permit systematic isolation of the ω -

amino-acids. These procedures can be used on the micro scale for metabolic studies with isotopic tracers. They are additional to the ion-exchange methods of Westall (1950) and Lederer & Pavlov (see Lederer, 1949). The present methods have so far been used for isolating γ -aminobutyric acid from among the dialysable constituents of rye grass (*Lolium perenne* L.), as described below, and in studying the production of δ -aminovaleric acid in the rumen of the sheep (cf. Shazly & Synge, 1950).

The γ -aminobutyric acid isolated was fully characterized and identified, in agreement with the studies of Reed (1950) and of Westall (1950), who independently isolated and identified the same substance from yeast extract and red beetroots respectively. Other data on the natural occurrence of γ -aminobutyric acid are discussed at the end of this paper.

EXPERIMENTAL

Materials

The following synthetic ω -amino-acids were used for testing the isolation procedures and for comparison with the naturally occurring materials: β -alanine (Light and Co., Wraysbury); γ -aminobutyric acid (DeWitt, 1937); δ -aminovaleric acid (according to Wallach (1900), from cyclopentanone oxime (cf. Fox, Dunn & Stoddard, 1941)); ϵ -aminocaproic acid (Eck, 1937).

Isolation procedures for ω -amino-acids

(a) *Use of diaphragm cell.* A three-compartment cell of the type described by Theorell & Åkeson (1942) fitted with two cellophan diaphragms and with platinum electrodes was used. Each compartment contained about 50 ml. of liquid. After adjusting the pH of the plant juice or other solution to be analysed to 6.5–7.0 (bromothymol blue) with NH_3 or acetic acid, it was placed in the middle compartment. The anode compartment was filled with 0.1N- H_2SO_4 and the cathode compartment with 0.2% (w/v) aqueous NH_3 . 200 V. was applied to the electrodes, and the pH of the middle compartment was maintained at 6.5–7.0 by addition of drops of 3% (w/v) aqueous NH_3 as required. After 2–3 hr. the catholyte was removed and replaced with fresh 0.2% (w/v) NH_3 . This first catholyte contained most of the alkali metals (as hydroxides) and organic bases, etc. from the solution analysed, but only relatively small quantities of neutral amino-acids. It was discarded. Addition of NH_3 to the middle compartment was now discontinued and the pH fell because the plant acids, dicarboxylic amino-acids and possibly other acidic substances did not enter the anode compartment. The catholyte was removed and replaced with fresh NH_3 solution at half-hourly intervals. The successive catholytes were examined by filter-paper chromatography (see below). With plant juices the middle-compartment solution quite quickly reached a low pH, and the rate at which ω -amino-acids entered the cathode compartment then only moderately exceeded that for the neutral α -amino-acids. All the ω -amino-acids present were usually collected in the first three or four changes of catholyte. They could be very greatly concentrated relative to the α -amino-acids that had accompanied them by evaporating the combined ω -amino-acid-containing catholytes to suitable volume *in vacuo* and analysing the resulting solution a second time in the three-compartment cell. After completing the pH-controlled run to remove traces of non-volatile basic substances, acetic acid was added to the middle compartment to a concentration of about 0.1% (w/v). Under these circumstances the pH of the middle compartment did not fall much below 4 and ω -amino-acids left it much faster than did the neutral α -amino-acids.

With some grass juices it was found that the cathode membrane became impregnated with brown material and

impermeable to amino-acids. This was presumably caused by protein or other positively charged material altering the surface potential of the pores of the membrane. The trouble could be avoided by using only that fraction of the juice that dialysed freely through cellophan, or by subjecting the juice to preliminary hydrolysis with acid. The resultant humin collected on the anode membrane.

(b) *Use of jelly ionophoresis.* The procedure of Consden, Gordon & Martin (1946; method C) was used. Silica jelly strengthened with paper powder was prepared as described by these authors and, after setting, was washed overnight with a solution of acetic acid (0.1M) and ammonium acetate (0.005M). Fractions enriched in ω -amino-acids from the diaphragm cell were evaporated to small volume *in vacuo* and packed with paper powder into a gutter cut close to the anode end of the jelly. On applying 4 V./cm. for 19 hr. (washing the electrodes with the same acetic acid-ammonium acetate buffer) it was found that the α -amino-acids had migrated 11 cm. towards the cathode as a compact, symmetrical band, while the ω -amino-acids formed a broad, faster-moving band with a diffuse front and sharp rear edge. The form and position of the bands was revealed on a print coloured with ninhydrin as described by Consden *et al.* (1946). The jelly containing the ω -amino-acids was removed from the trough, dried in a vacuum desiccator over H_2SO_4 and soda lime at room temperature (finally with high vacuum, to complete removal of ammonium acetate), powdered, packed into a chromatogram tube and extracted by percolation with water. A trough 4 cm. broad and 0.5 cm. deep would conveniently separate 5–10 mg. of ω -amino-acids from an equal weight of neutral α -amino-acids, sugars, etc. Under favourable conditions the product was free from α -amino-acids, but contamination often resulted from electroendosmotic leaking of solution along cracks between the jelly and the lid or walls of the trough.

(c) *Use of partition chromatography with filter paper and with cellulose powder.* The homologous ω -amino-acids from β -alanine to ϵ -aminocaproic acid were studied on filter-paper chromatograms individually and in mixtures. With *n*-butanol-water and with commercial collidine (Yorkshire Tar Distillers Ltd., Cleckheaton)-water the compounds were scarcely resolved. With phenol-aqueous NH_3 , R_F values were too great for good resolutions; with *n*-propanol-water (2:1, v/v) and with *m*-cresol-water (with or without added NH_3) there was some resolution, but much 'tailing'. However, the system *n*-butanol-water-acetic acid (Partridge, 1948) gave sharply defined spots and good resolutions. The R_F values found with various phase pairs of this system are shown in Table 1; values found for alanine, valine and leucine are included for reference. Reed (1950), using *n*-butanol-water-acetic acid (4:1:1, by vol.), found that γ -aminobutyric acid moved faster than alanine. Chromatography on filter paper has proved useful throughout this work for identification of ω -amino-acids and for controlling purifications. Colouring was effected by spraying with ninhydrin solution in the usual way.

For preparative purposes columns were made with a highly purified Whatman cellulose powder (kindly provided by H. Reeve Angel and Co., 9 Bridewell Place, London, E.C. 4). In a typical experiment 90 g. of air-dry powder was made to a slurry with the top phase obtained immediately after mixing and equilibrating 8 vol. *n*-butanol, 10 vol. water and 1 vol. acetic acid. The slurry was poured into a chromatogram tube (5.4 cm. diameter) and when the column had settled

Table 1. R_f values of ω -amino-acids on filter paper with *n*-butanol-acetic acid-water mixtures

(The chromatograms were run downwards on Munktell OB filter paper (Grycksbo Pappersbruk AB, Grycksbo, Sweden) using as solvent the upper phase of a freshly prepared mixture made with the volumes of solvents indicated. The chromatograms were run in an atmosphere saturated by the presence of both phases of the solvent mixture.)

<i>n</i> -Butanol : water : acetic acid (by volume)	R_f			<i>n</i> -Butanol-water
	(8 : 10 : 1)	(4 : 5 : 1)	(2.67 : 3.33 : 1)	
β -Alanine	0.17	0.32	0.65	0.05
γ -Aminobutyric acid	0.24	0.39	0.70	0.05
δ -Aminovaleric acid	0.32	0.50	0.75	0.05
ϵ -Aminocaproic acid	0.38	0.61	—	—
Alanine	0.19	0.33	0.65	—
Valine	0.41	0.53	0.77	0.24
Leucine	0.58	0.68	0.82	0.42

it was washed with 300 ml. of the same mixture. The mixture studied resulted from applying procedure (a) to a hydrolysed grass juice. It contained about 25 mg. of γ -aminobutyric acid contaminated with β -alanine and neutral α -amino-acids, among which alanine, valine, leucine and proline predominated. This was dissolved in 5 ml. of the solvent mixture, washed in with small portions and developed with the same solvent. Successive 25 ml. portions of effluent were collected. Samples from each fraction (numbered serially) were examined by paper chromatography. γ -Aminobutyric acid uncontaminated with other amino-acids was found in fractions 15–23 inclusive, which were pooled and evaporated to dryness *in vacuo*. The syrupy residue was dissolved in water. This led to the separation of some gummy, water-insoluble material, presumably extracted from the cellulose as it was found in effluent from blank columns run with solvent only. The mixture was treated with a little charcoal and filtered. The filtrate was evaporated *in vacuo* and the residue brought to crystallization in aqueous ethanol.

The cellulose powder from the column was washed repeatedly on a Büchner funnel with dilute aqueous HCl and then with distilled water. It was dried at room temperature and could be used repeatedly.

Isolation of γ -aminobutyric acid from rye grass

Two-dimensional filter-paper chromatograms made in this laboratory from plant extracts (grasses, clovers, sugar beet) have in every case shown a strong spot colouring with ninhydrin and corresponding in position and colour with γ -aminobutyric acid (Dent, 1948).

For the present work, juice derived from rye grass by the other procedure (Chibnall, 1939) was used, since other non-protein nitrogenous constituents of this juice are being studied here. By the kindness of Mr R. Bain of the North of Scotland College of Agriculture, rye grass (strain S23) was obtained from the grass plots of the College at Craibstone on various dates between May and November 1949. There were no obvious variations in the amounts of γ -aminobutyric acid present at different stages of the season, as far as could be judged from the paper chromatograms. Although preparations of γ -aminobutyric acid were obtained without dialysis or acid hydrolysis, these treatments aided purification by eliminating material that interfered with operations in the diaphragm cell (see (a) above) as well as peptide material and glutamine, which interfered somewhat with purification procedures at various stages.

Freshly picked grass was placed in ether for 20 min. and the resulting flaccid mass was squeezed in a hand press. The

resulting juice was filtered through paper pulp. The clear brown filtrate usually contained about 0.8 mg. N/ml. (Kjeldahl). Only about 12% of this N remained inside a cellophan sac after exhaustive dialysis against CHCl_3 water at 0°. Part of the fraction that had passed through cellophan (containing 150 mg. N) was evaporated *in vacuo* to small volume and refluxed 24 hr. in 6*N*-HCl. Excess HCl was evaporated *in vacuo*. The residue was dissolved in water and treated (in four portions) according to procedure (a) above (first stage only). The combined product was subjected to procedure (b) using a trough 15 cm. broad, and the resulting product was concentrated to dryness *in vacuo* and chromatographed according to procedure (c). The final product, after crystallization from ethanol-water, weighed 30 mg.

Characterization of γ -aminobutyric acid isolated from rye grass

The isolated product was homogeneous on paper chromatography with phenol-1% (w/v) aqueous NH_3 , collidine-water and *n*-butanol-acetic acid-water systems (cf. Table 1), colouring the chromatograms in each case with ninhydrin. Mixed chromatograms with authentic γ -aminobutyric acid showed in every case a single spot. M.p. (uncorr.) was 191°, which was not depressed on admixture of synthetic material (m.p. 198°): $[\alpha]_D^{19}$, $0^\circ \pm 1^\circ$ in water (c, 2.1). The material was analysed after drying to constant weight *in vacuo* at 100° over P_2O_5 . (Found: C, 46.3; H, 8.4; N (Kjeldahl), 13.3. Calc. for $\text{C}_4\text{H}_9\text{O}_2\text{N}$: C, 46.6; H, 8.7; N, 13.6%). The product gave completely negative results in the amino-acid determination with copper phosphate according to Pope & Stevens (1939) and in the ninhydrin- CO_2 determination according to Van Slyke, Dillon, MacFadyen & Hamilton (1941) using citrate buffer at pH 2.5. The same results were found with the synthetic material. In the Van Slyke amino N determination (30 min. reaction time at 19°) 90% of the N reacted. With the synthetic material the amount of amino N found was 87, 93 and 94% of total N after 4, 10 and 30 min. reaction, respectively, at 19°. Dunn & Schmidt (1922) found that γ -aminovaleric and δ -aminovaleric acids reacted completely in 10–15 min. at 23°. Aberdhalen & Kautzsch (1912) found 95% of the N of γ -aminobutyric acid available in the Van Slyke reaction (see also Gale, 1940). Since it was thought that the present low values might be due to cyclization by lactam formation occurring progressively on keeping in solution, determinations (30 min. reaction time) were done using aqueous solutions that had been kept at room temperature for periods up to 5 days, and material that had been kept

dissolved in acetic acid-*n*-butanol (1 : 10, v/v) overnight and then evaporated to dryness *in vacuo* at 60°. These treatments did not diminish the amino N, and the same was true for δ -aminovaleic acid, which yielded 92–96% of its N before or after such treatment.

Specimens of the natural and synthetic γ -aminobutyric acid were compared crystallographically by Dr D. Crowfoot Hodgkin and Miss P. Cowan who write: 'We had difficulty in identifying γ -aminobutyric acid by crystallographic methods owing to the crystallization of the material in two different modifications, *A* and *B*, which have the following characteristics:

' *γ -Aminobutyric acid A* crystallizes in tetragonal needles elongated along *c* and forms characteristic feathery growths when rapidly crystallized on slides. The lattice constants are: $a = 11.95$ Å, $c = 15.25$ Å. The space group is $I4cd$ with $n = 16$, in agreement with the observed density of approximately 1.3.

' *γ -Aminobutyric acid B* crystallizes in small monoclinic tables or plates on {021}, usually fairly equally developed. The lattice constants are approximately: $a = 7.1$ Å, $b = 10.1$ Å, $c = 8.2$ Å, $\beta = 68^\circ 30'$. The space group is $P2_1/c$ and $n = 4$, corresponding again with the observed density of 1.25–1.26.

'When rapidly crystallized on slides γ -aminobutyric acid *B* forms platy crystals less feathery than those of *A*, from which they can be distinguished by the fact that the direction of maximum growth tends to be parallel to the greatest refractive index in the plane of the plate in the case of *B* and to the least in the case of *A*. Experiments on recrystallization of one form on microscope slides in the presence of the other, produced no apparent interconversion of the two varieties. The synthetic sample, as supplied by Dr R. L. M. Synge, had crystallized in modification *A* and gave this modification again on recrystallization, both by slow evaporation from ethanol-water and on precipitation by ethanol from aqueous solution as described by Reed (1950). In two cases, however, crystals of form *A* which had been left exposed to the air were observed to deliquesce and then to crystallize after some days or weeks in form *B*.

'All the early samples of γ -aminobutyric acid from natural sources crystallized at first in form *B* and consequently gave X-ray photographs not identical with those given by the original synthetic sample. Only the final specimen sent to us had crystallized spontaneously in form *A* and this again, after standing for some weeks, partly recrystallized in form *B*.

'There are certain similarities between the two crystal structures which are probably best illustrated by the powder patterns in Pl. 3, but easy passage of one into the other does not appear possible. In particular, the arrangement of molecules in structure *A* is of a highly symmetrical and complex variety. Again, neither of the powder patterns of *A* or *B* is exactly the same as that produced by Reed, to which, however, both bear a family resemblance. It seems quite likely therefore, that γ -aminobutyric acid like glycylglycine (Hughes & Moore, 1949), can crystallize under apparently similar, if not identical, conditions in three different crystalline modifications. The appearance of one modification rather than another is probably affected by traces of impurities, but the stability relations of the three certainly need further investigation.'

Amount of γ -aminobutyric acid: occurrence of other ω -amino-acids in rye grass

A rough quantitative determination of γ -aminobutyric acid was made using juice expressed from grass picked

30 November 1949, which contained 0.98 mg. diffusible N/ml. The diffused material (21 mg. N) was hydrolysed and subjected to analysis by procedures (a) (single treatment) and (c) in succession. The chromatographic fractions found to contain γ -aminobutyric acid were pooled, and N was determined on a portion. After allowing for a trace of N shown in a blank experiment to be extracted by the solvent from the cellulose, it was calculated that the fraction contained 1.3 mg. N, i.e. γ -aminobutyric acid N was 6.2% of the dialysable (non-protein) nitrogen; the freshly expressed juice thus contained 0.45 mg. γ -aminobutyric acid/ml. Westall (1950) isolated 0.16 mg./g. from fresh beetroot, and Steward, Thompson & Dent (1949) estimated 0.54 mg./g. to be present in fresh potato. Small amounts of what appeared to be β -alanine (judged by chromatographic rates and the clear blue colour with ninhydrin after paper chromatography with collidine; Dent, 1948) were always found in the grass juice and were concentrated with the γ -aminobutyric acid in procedures (a) and (b) and eliminated during procedure (c) (cf. Steward *et al.* 1949; Westall, 1950). The amount of β -alanine increased after acid hydrolysis; more detailed study (to be published later) showed that at least some of this newly formed material came from an acidic fraction, in which such compounds as pantothenic acid would occur.

δ -Aminovaleic acid and other ω -amino-acids would have been concentrated with the γ -aminobutyric acid and detected chromatographically. No evidence for their occurrence was obtained.

DISCUSSION

Methods for isolating ω -amino-acids

Methods (a) and (b) depend for their usefulness on the ionization of the carboxyl groups of the α -mono-amino-monocarboxylic acids being stronger (pK 1.8–2.8) than that for the corresponding ω -compounds whose pK values range from 3.6 for β -alanine to 4.4 for ϵ -aminocaproic acid (Cohn & Edsall, 1943, pp. 84, 99). The ionization behaviour of γ -aminobutyric and δ -aminovaleic acids was studied in detail by Neuberger (1937) and used by Gale (1940) for distinguishing between α - and γ -aminobutyric acids in solution. Consden *et al.* (1946) have discussed in detail how to select the pH at which electrical migration methods will give the best results in separating compounds whose ionization properties differ. Under the conditions used here, the carboxyl groups of the ω -amino-acids are less ionized than those of the α -amino-acids; there is, therefore, a greater net positive charge on the ω -amino-acid molecules, with the result that they migrate more rapidly towards the cathode. The asymmetrical character of the ω -amino-acid bands in the jelly ionophoresis is due to the low buffering capacity of the mixture of acetic acid and ammonium acetate, chosen for the sake of its volatility. Thus at higher concentrations of ω -amino-acid the pH rises and migration is slowed, giving a broad band with a diffuse front and a sharp rear edge.

It is presumably likewise the ionization behaviour of their carboxyl groups that causes β -alanine and

X-ray powder photographs of γ -aminobutyric acid specimens (Cu radiation; camera diameter 5.73 cm.). (a) Synthetic preparation in form A; (b) natural preparation in form A; (c) natural preparation as first crystallized in form B. ($\times 1$).

γ -aminobutyric acid to occupy positions between the neutral and the basic amino-acids on the cation-exchange displacement columns used by Westall (1950). It is not clear whether the same property determines the separations of Lederer & Pavlov on acid-washed alumina at pH 4.0 in the presence of formaldehyde or whether, as Lederer (1949) suggests, the effect is determined by more directly steric factors. Failure of ω -amino-acids to form complexes with cupric ions (Schimkoff, 1906; Tschugaeff, 1907; Fischer & Zemlén, 1909; Ley, 1909; Karrer & Widmer, 1926) seems to be determined by purely steric factors and has been used analytically for detecting ω -amino-acids by Crumpler & Dent (1949).

Regarding the behaviour of the ω -amino-acids in partition chromatography, it is noteworthy that in a number of solvent systems R_f values do not increase in the regular manner that would be expected from simple theory (cf. Martin, 1949; Bate-Smith & Westall, 1950) in passing up the homologous series from β -alanine to ϵ -aminocaproic acid. This is well illustrated for the solvent system n -butanol-water by the data in Table 1. A likely explanation is that the increasing molecular dipole moments of these homologues (see Cohn & Edsall, 1943, pp. 140–154) decrease their solubilities in less polar solvents relative to their solubilities in water. The data tabulated by Cohn & Edsall (1943, pp. 201, 203) for the solubilities of α - and β -alanines and α - and ϵ -aminocaproic acids in ethanol and water show the effect well. The acetic acid added to the system n -butanol-water presumably increases the polarity of the non-aqueous phase and may also form mixed chelate double molecules with the carboxyl groups of the ω -amino-acids; as seen in Table 1, the R_f values of the ω -amino-acids increase so as to approach quite closely those of the α -isomers. Since Partridge (1948) first used n -butanol-acetic acid-water systems for chromatography of sugars many workers have found them useful in chromatography of amino-acids, but this special usefulness in the ω -amino-acid series does not seem to have been remarked before.

The Whatman cellulose powder used in the present work for preparative purposes gave similar fractionations to those described by Peterson & Reineke (1949) in the analysis of amino-acids from circulin. These workers used a fine grade of the commercial cellulose 'Solka Floc' (Brown Company, Berlin, New Hampshire). The resolutions achieved were in neither case comparable with those obtained on starch chromatograms (cf. Stein & Moore, 1950), which were used by Reed (1950) for his isolation of γ -aminobutyric acid. In fact resolutions on columns of this type are not as good as those on filter-paper chromatograms. This may be due to the relatively high rate of percolation of solvent caused by the loose packing

of the powder when the columns are poured wet. Results with cellulose-powder columns packed dry may well be better. However, columns of the type used here are convenient to prepare and use, do not exhibit channelling or unevenness of flow, and may be recommended for the less exacting sorts of preparative work especially if it is desired to save time.

γ -Aminobutyric acid in nature

Work in recent years in a number of laboratories has now demonstrated that γ -aminobutyric acid is a normal major component of the 'non-protein nitrogen' fraction of the tissues of higher plants, and therefore an important constituent of the diet of many animals (Dent, Stepka & Steward, 1947; Allsopp, 1948; Steward *et al.* 1949; Joslyn & Stepka, 1949; Jones, 1949; Hulme & Arthington, 1950; Westall, 1950). It was probably the great solubility of the substance, the effects of quite small amounts of impurities in delaying and modifying its crystallization (see note by Dr Hodgkin and Miss Cowan above) and the high solubilities of all the derivatives and salts employed for isolation that prevented its recognition by the pioneer students of plant juices. The application of chromatographic methods has now overcome these difficulties. However, too few of those working with these techniques have appreciated the continuing need for preparing and fully characterizing the novel substances which they encounter.

γ -Aminobutyric acid was isolated by Ackermann (1910) after incubating a mixture of glutamic acid, salts, glucose and peptone with a little putrefying pancreas. The new 'aporrhagma' (a term coined by Ackermann & Kutscher (1910) to describe breakdown products of amino-acids which resulted from metabolic processes in animals and plants) was further characterized by Engeland & Kutscher (1910), who subjected it to exhaustive methylation and showed that the resulting betaine (sometimes known as actinine) was identical with the 'ptomaine' isolated by Brieger (1886) from putrefying horse flesh and by Takeda (1910) from the urine of a dog poisoned with phosphorus, and since found to be widely distributed in nature. These observations led Ackermann & Kutscher (1910) to regard γ -aminobutyric acid as an intermediate of the normal catabolism of glutamic acid in higher as well as in lower organisms. First attempts by Abderhalden & Kautzsch (1912) to repeat Ackermann's bacterial degradation of glutamic acid were unsuccessful, but later trials (Abderhalden, Fromme & Hirsch, 1913) led to the successful isolation of γ -aminobutyric acid by ester distillation from a putrefaction mixture in which staphylococci and yeasts were prominent. Subsequent work (reviewed by Gale, 1946) has shown that many, but by no means all, micro-organisms are capable of decarboxylating glutamic

acid; Gale (1940) showed γ -aminobutyric acid to be the main reaction product. More recently the production of γ -aminobutyric acid by micro-organisms has been studied directly, and has led to similar conclusions (Proom & Woiwod, 1949; Woiwod & Proom, 1950; King & Fletcher, 1950).

As concerns the occurrence of γ -aminobutyric acid in micro-organisms, Work (1949*a, b*) noted weak spots corresponding with it on filter-paper chromatograms made with extracts from *Corynebacterium diphtheriae*. The same has been found in this laboratory with a number of micro-organisms. Bremner (1950) found that acid hydrolysis was necessary to liberate the corresponding substance from soils. This seems to be the only evidence so far for the natural occurrence of chemically bound γ -aminobutyric acid. Reed (1950) noted that the amount of γ -aminobutyric acid in yeast increased greatly during storage in the cold and on autolysis.

In animals, chromatographic studies have revealed a wide distribution of γ -aminobutyric acid in the body fluids, though the concentrations found have been low (e.g. Dent, 1948, 1949; Dent & Schilling, 1949; Gordon, 1949; Udenfriend, 1950). Subbarow & Hitchings (1940) reported its isolation from liver, but gave no experimental details.

At present, facts are lacking about the metabolic origin and fate of γ -aminobutyric acid. It seems firmly established that in micro-organisms glutamic acid may serve as a precursor. Hasse & Schumacher (1950) cite evidence for the decarboxylation of glutamic acid in some higher plants, and isolated γ -aminobutyric acid after allowing a preparation from radish roots to act on L-glutamic acid. Steward & Thompson (1950) found little glutamic acid decarboxylase activity in potato tubers which nevertheless contained and actively metabolized γ -aminobutyric acid. Roberts & Frankel (1950) observed the formation of γ -aminobutyric acid from glutamic acid by brain. Schotten (1883), who first prepared γ -aminobutyric acid, found that it was non-toxic on administering 0.3 g. subcutaneously to a rabbit. The observations of a number of other authors suggest

that it is fairly quickly metabolized in the animal body (Thomas, 1913; Thomas, Milhorat & Techner, 1932, 1933; Corley, 1926; Keil, 1932). Emmerling (1902) showed it to be an excellent source of carbon and nitrogen for a number of fungi. Further work by modern methods is needed for tracing the metabolic pathways on which γ -aminobutyric acid lies.

SUMMARY

1. ω -Amino-acids may be separated from α -amino-acids by electrical-migration methods depending on differences in the net molecular charge at weakly acid pH. Procedures using respectively a diaphragm cell and ionophoresis in silica jelly are described. These procedures are suitable for use with fluids of biological origin for systematic isolation of ω -amino-acids on the micro scale.

2. The separation of β -alanine, γ -aminobutyric, δ -aminovaleric and ϵ -aminocaproic acids on filter-paper chromatograms is described, and some anomalies in the chromatographic behaviour of these substances are discussed. For larger scale separations cellulose-powder chromatograms were used.

3. γ -Aminobutyric acid has been isolated from rye grass and identified with authentic material prepared synthetically. Dr D. C. Hodgkin and Miss P. Cowan report crystallographic data on the crystal modifications encountered. A rough quantitative determination showed γ -aminobutyric acid to account for 6% of the 'non-protein nitrogen' of the grass. Small amounts of β -alanine but no other ω -amino-acids were found.

4. A brief survey is made of what is known about the distribution, origin and fate of γ -aminobutyric acid in nature.

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REFERENCES

- Abderhalden, E., Fromme, G. & Hirsch, P. (1913). *Hoppe-Seyl. Z.* **85**, 131.
 Abderhalden, E. & Kautzsch, K. (1912). *Hoppe-Seyl. Z.* **81**, 294.
 Ackermann, D. (1910). *Hoppe-Seyl. Z.* **69**, 273.
 Ackermann, D. & Kutscher, F. (1910). *Hoppe-Seyl. Z.* **69**, 265.
 Allsopp, A. (1948). *Nature, Lond.*, **161**, 833.
 Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. Biophys. Acta*, **4**, 427.
 Bremner, J. M. (1950). *Biochem. J.* **47**, 538.
 Brieger, L. (1886). *Untersuchungen über 3 Theil Ptomaine*, part 3, p. 27. Berlin: Hirschwald.
 Chibnall, A. C. (1939). *Protein Metabolism in the Plant*. New Haven: Yale University Press.
 Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*. New York: Reinhold.
 Consden, R., Gordon, A. H. & Martin, A. J. P. (1946). *Biochem. J.* **40**, 33.
 Corley, R. C. (1926). *J. biol. Chem.* **70**, 99.
 Crumpler, H. R. & Dent, C. E. (1949). *Nature, Lond.*, **164**, 441.
 Dent, C. E. (1948). *Biochem. J.* **43**, 169.
 Dent, C. E. (1949). *Biochem. Soc. Symp.* **3**, 34.
 Dent, C. E. & Schilling, J. A. (1949). *Biochem. J.* **44**, 318.

- Dent, C. E., Stepka, W. & Steward, F. C. (1947). *Nature, Lond.*, **160**, 682.
- DeWitt, C. C. (1937). *Organic Synth.* **17**, 4.
- Dunn, M. S. & Schmidt, C. L. A. (1922). *J. biol. Chem.* **53**, 401.
- Eck, J. C. (1937). *Organic Synth.* **17**, 7.
- Emmerling, O. (1902). *Ber. dtsh. chem. Ges.* **35**, 2289.
- Engeland, R. & Kutscher, F. (1910). *Hoppe-Seyl. Z.* **69**, 282.
- Fischer, E. & Zemplén, G. (1909). *Ber. dtsh. chem. Ges.* **42**, 4878.
- Fox, S. W., Dunn, M. S. & Stoddard, M. P. (1941). *J. org. Chem.* **6**, 410.
- Gale, E. F. (1940). *Biochem. J.* **34**, 392.
- Gale, E. F. (1946). *Advanc. Enzymol.* **6**, 1.
- Gordon, A. H. (1949). *Biochem. J.* **45**, 99.
- Hasse, K. & Schumacher, H. W. (1950). *Chem. Ber.* **83**, 68.
- Hughes, E. W. & Moore, W. J. (1949). *J. Amer. chem. Soc.* **71**, 2618.
- Hulme, A. C. & Arthington, W. (1950). *Nature, Lond.*, **165**, 716.
- Jones, T. S. G. (1949). *Discuss. Faraday Soc.* **7**, 295.
- Joslyn, M. A. & Stepka, W. (1949). *Food Res.* **14**, 459.
- Karrer, P. & Widmer, A. (1926). *Helv. chim. Acta*, **9**, 886.
- Keil, W. (1932). *Hoppe-Seyl. Z.* **207**, 248.
- King, H. K. & Fletcher, L. I. (1950). *J. gen. Microbiol.* **4**, 238.
- Lederer, E. (1949). *Discuss. Faraday Soc.* **7**, 329.
- Ley, H. (1909). *Ber. dtsh. chem. Ges.* **42**, 354.
- Martin, A. J. P. (1949). *Biochem. Soc. Symp.* **3**, 4.
- Neuberger, A. (1937). *Proc. roy. Soc. A*, **158**, 68.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Peterson, D. H. & Reineke, L. M. (1949). *J. biol. Chem.* **181**, 95.
- Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.
- Proom, H. & Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 319.
- Reed, L. J. (1950). *J. biol. Chem.* **183**, 451.
- Roberts, E. & Frankel, S. (1950). *Fed. Proc.* **9**, 219.
- Schimkoff, J. P. (1906). Dissertation, Moscow. (Quoted by Tschugaëff, L., 1907.)
- Schotten, C. (1883). *Ber. dtsh. chem. Ges.* **16**, 643.
- Shazly, K. & Synge, R. L. M. (1950). 18th Int. physiol. Congr., Copenhagen, Abstr., p. 445.
- Stein, W. H. & Moore, S. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 179.
- Steward, F. C. & Thompson, J. F. (1950). *Ann. Rev. Plant Physiol.* **1**, 233.
- Steward, F. C., Thompson, J. F. & Dent, C. E. (1949). *Science*, **110**, 439.
- Subbarow, Y. & Hitchings, G. H. (1940). *J. biol. Chem.* **133**, c.
- Takeda, K. (1910). *Pflüg. Arch. ges. Physiol.* **133**, 365.
- Theorell, H. & Åkeson, Å. (1942). *Ark. Kemi Min. Geol.* **16 A**, no. 8.
- Thomas, K. (1913). *Hoppe-Seyl. Z.* **88**, 465.
- Thomas, K., Milhorat, A. T. & Techner, F. (1932). *Hoppe-Seyl. Z.* **205**, 93.
- Thomas, K., Milhorat, A. T. & Techner, F. (1933). *Hoppe-Seyl. Z.* **214**, 121.
- Tschugaëff, L. (1907). *J. prakt. Chem.* **183**, 153.
- Udenfriend, S. (1950). *Fed. Proc.* **9**, 240.
- Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.
- Wallach, O. (1900). *Liebigs Ann.* **312**, 171.
- Westall, R. G. (1950). *Nature, Lond.*, **165**, 717.
- Woiwod, A. J. & Proom, H. (1950). *J. gen. Microbiol.* **4**, v.
- Work, E. (1949a). *Bull. Soc. Chim. biol., Paris*, **31**, 138.
- Work, E. (1949b). *Biochem. Soc. Symp.* **3**, 50.

Formation by Formaldehyde of a Cross-link between Lysine and Tyrosine Residues in Wool

By P. ALEXANDER*, D. CARTER AND K. G. JOHNSON

Research Department, Wolsey Limited, Leicester

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The reaction of formaldehyde with proteins has been extensively investigated (French & Edsall, 1945), although the general picture is not yet clear since the nature of the reaction is dependent on the conditions employed. The reaction with wool keratin has been studied by a number of workers. Stoves (1943) used a 2% (w/v) solution of formaldehyde at 100° and, from measurements on the mechanical properties of treated fibres, deduced that the disulphide bond had been attacked with formation of $-\text{S}-\text{CH}_2-\text{NH}-$ and $-\text{S}-\text{CH}_2-\text{S}-$ cross-links. At high pH values, hydrolysis of the disulphide bond occurs, and Ratner & Clarke (1937) showed that formaldehyde then reacts with the cysteine residue produced to form a residue of thiazolidine-4-carboxylic acid. This reaction, however, appears to be reversible, since

Middlebrook & Phillips (1942) found that the formaldehyde bound by the cystine residues at high temperatures was removed by dilute phosphoric acid at 100°. Middlebrook (1949) showed that at pH 1 and 100° part of the formaldehyde which combines is not removed by boiling acid, and he suggested that the irreversibly combined formaldehyde has reacted with the asparagine amide groups to form 6-hydroxytetrahydropyrimidine-4-carboxylic acid, which is stable to boiling acid.

It is well known that formaldehyde reacts with amino groups to form a methylol derivative with a different pK value from that of the original base. Such compounds are, however, very unstable, and after removal of the formaldehyde they immediately dissociate. In the case of wool, all the ϵ -amino

* Present address: Department of Inorganic and Physical Chemistry, Imperial College of Science, London, S.W. 7.