ethyl hydroperoxide to give 'green compounds' with two-banded absorption spectra. The exact positions of these bands depend on the pH and the amount of peroxide added. In the case of protopara-haematin additional transient absorption bands are observed under certain conditions.

7. The 'green compound' given by pyridine uropara-haematin and ethyl hydroperoxide reduces spontaneously and reversibly to a compound which differs from pyridine urohaemochromogen in that the positions of its absorption bands are nearer the red and in that it does not combine with carbon monoxide. The green compounds given by pyridine protopara-haematin with hydrogen peroxide (pH 6.5)

and ethyl hydroperoxide are probably of the same nature.

8. It is suggested that these 'green compounds' correspond to those given by pyridine protopara-haematin and hydrogen peroxide described by Haurowitz, but that they are oxidation products of the haematins and not compounds of the haematins with hydrogen peroxide.

9. Urohaematin-peroxide is so far the only example of a free haematin-peroxide compound where the porphyrin ring is unaltered.

I wish to thank Dr E. F. Hartree for a sample of ethyl hydroperoxide and Dr E. C. Slater for helpful discussions.

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A New Amino-acid Amide in the Groundnut Plant (*Arachis hypogaea*): Evidence of the Occurrence of γ -Methyleneglutamine and γ -Methyleneglutamic Acid

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A brief account of preliminary studies of a new metabolite detected in groundnut plants has already been given (Done & Fowden, 1951). The substance was isolated and found to be an unsaturated amino-acid amide. This earlier work is now described in more detail, together with subsequent investigations of the structure of the new compound.

The first material examined was the 'sap' produced when the turgid stems of young seedlings were incised between the cotyledons and the first leaf. About 20 μ l. of liquid exuded from the cut surfaces. It was found by paper chromatography that the main ninhydrin-reacting component of the exudate gave an orange-brown spot ($R_F = 0.66$ on

chromatograms run in phenol-ammonia). The colour of the ninhydrin spot varied somewhat on different occasions, but always it was clearly outside the range of colours obtained with known α -amino-acids.

A large number of exudates produced in the manner described have been examined, and the new substance (I) has invariably been the dominant ninhydrin-reacting component. When amounts of the order of 20 μ l. were chromatographed, much smaller spots due to asparagine, glutamine, aspartic acid, glutamic acid, alanine and other amino-acids sometimes appeared.

(I) has also been detected in homogenates of stems, leaves, hypocotyls and roots, but in these many other ninhydrin-reacting compounds were also present. The new compound could not be

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detected in seedlings less than 2 days after germination, but it occurred in all samples taken after the differentiation of the first leaf, e.g. in the exudate formed after the incision of the petiole of the fifteenth leaf of a main stem, and in the gynophores bearing immature fruits. The stem, root and hypocotyl of seedlings germinated and maintained in the dark for 12 days all contained (I). It was not detected in immature or ripe seeds, or in the hydrolysate of a commercial sample of arachin. Thus, the compound (I) appeared after germination of the seed and occurred in all the vegetative tissues examined.

A number of varieties of beans, peas and lupins, and the sprouts of potato tubers were examined, but no compound giving the type of ninhydrin reaction shown by (I) was observed on the chromatograms.

Preliminary examination of the chemical properties of (I)

The action of hydrochloric acid. Exudate was heated with 5N-hydrochloric acid at 105° for 16 hr. in a sealed tube; hydrochloric acid was removed by evaporation. (I) could not be detected on chromatograms, but an intense yellow-brown spot ($R_f = 0.30$ in phenol-ammonia) appeared. Here again, the colour of the spot was quite distinct from those obtained with known α -amino-acids. Similar results were obtained after treatments with 5N-hydrochloric acid for 3 days at 105° and with N-hydrochloric acid for 3 hr. at 100°. Spots due to asparagine and glutamine occurred on chromatograms of the exudate, but the intensity of each of these was less than one-tenth of the intensity of the main spot due to (I). These amides were hydrolysed to the corresponding acids by the hydrochloric acid treatments.

When a sample of exudate containing 6.74 $\mu\text{g. N}/\mu\text{l.}$ was treated with N-hydrochloric acid for 3 hr. at 100°, ammonia nitrogen equivalent to 2.7 $\mu\text{g.}/\mu\text{l.}$ was produced. The conditions of hydrolysis in this experiment were those used in the estimation of amide nitrogen (Borsook & Dubnoff, 1939), so the results could be explained by the hypothesis that amide I $\rightarrow$ acid II + NH_3 . This was supported by the chromatographic evidence. The ratio of the R_f of (I) to that of (II) in phenol-ammonia was 2.2. The corresponding ratios for asparagine and glutamine are 2.5 and 2.0 respectively (Dent, 1948).

(I) and (II) both combined with copper as shown by the copper acetylacetonate test (Wieland & Fischer, 1948); thus the presence of α -amino nitrogen was indicated.

Autolysis. When homogenized leaf tissue was allowed to autolyse for 3 hr. at 37° chromatograms showed that (I) had been converted to (II). Both compounds occurred in ethanolic extracts of fresh plant 'tops'.

Isolation of compound (I). (I) was retained when diluted exudates were run through a column of Zeo-Karb 215 (Partridge & Westall, 1949) and the compound was isolated after elution with ammonia solution. It was readily soluble in cold water and formed colourless plates on crystallization from aqueous ethanol. Elementary analysis gave an empirical formula $\text{C}_6\text{H}_{10}\text{O}_3\text{N}_2$. Half the nitrogen was released as ammonia by treatment with N-sodium hydroxide for 24 hr. at room temperature, and by treatment with N-hydrochloric acid for 3 hr. at 100°.

Isolation of compound (II). De-Acidite B (Partridge & Brimley, 1949) retained compound (II) when an autolysate of the plant 'tops' was run through a column of the material. The new acid was eluted by acetic acid followed by dilute hydrochloric acid, and it was isolated by recrystallization of the solid left after evaporation of fractions of the effluent from the column. The compound was only slightly soluble in cold water but much more soluble in hot water. It was therefore convenient to crystallize the compound from hot water. Elementary analysis gave an empirical formula $\text{C}_6\text{H}_7\text{O}_4\text{N}$. All the nitrogen was amino nitrogen as measured by the Van Slyke nitrous acid method. The equivalent weight found by titration with sodium hydroxide was 158 (formula weight 159). No optical activity was detected in an aqueous solution of the sodium salt.

The structure of the new compounds

The analyses of the new compounds confirmed the chromatographic evidence that (II) was an α -amino-acid derived from (I) by deamidation.

The presence of a double bond. Both compounds decolorized a solution of potassium permanganate at room temperature. Treatment with ozone gave a substance which could not be distinguished from aspartic acid by paper chromatography. The new amide therefore appears to resemble asparagine and glutamine in that the amide group is not attached to the α -carbon atom.

The product of the action of ozone on (II) was isolated and shown to be aspartic acid by exhaustive methylation to fumaric acid (Braunstein, Nemshinskaya & Vilenkina, 1947).

When (II) was hydrogenated in the presence of palladium-on-carbon catalyst, the amount of gas absorbed corresponded to that required for a reaction $\text{C}_6\text{H}_7\text{O}_4\text{N} \rightarrow \text{C}_6\text{H}_{11}\text{O}_4\text{N}$.

The reduced form of (II), compound (III). Elementary analysis of (III) gave the empirical formula $\text{C}_6\text{H}_{11}\text{O}_4\text{N}$. The compound formed a purple spot with ninhydrin at R_f 0.42 on chromatograms run in phenol-ammonia. The aqueous solution showed no optical rotation.

Since aspartic acid was produced by the action of ozone on (II), it seemed likely that the compound

contained $\text{COOH} \cdot \text{CH}(\text{NH}_2)\text{CH}_2\text{C}=\text{CH}_2$, and since a titratable carboxyl group has also been detected, possible formulae were

and

The corresponding saturated compounds are α -amino adipic and γ -methylglutamic acids respectively.

Substance (III) differed from DL- α -amino adipic acid in its solubility and melting point and in its position on paper chromatograms. α -Amino adipic acid is converted to ornithine by hydrazoic acid (Adamson, 1939). However, treatment of (III) with hydrazoic acid gave a substance which could be separated from ornithine on chromatograms run in aqueous *n*-butanol-acetic acid mixture (Partridge, 1948). It was therefore evident that (III) was not DL- α -amino adipic acid.

Fig. 1. X-ray powder photographs of (a) compound (III) and (b) synthetic γ -methylglutamic acid. Cu (Ni filtered) $\kappa\kappa$ radiation; specimen to film distance 5 cm.

On the other hand, it had been found that the melting point of (III) was low compared with those of most of the known α -amino-acids, and pyrrole derivatives had been detected in the vapour formed when (III) was fused with zinc dust. These facts suggested that the compound contained an α -amino and a γ -carboxyl group, and it was considered that (III) might be γ -methylglutamic acid.

Synthesis of γ -methylglutamic acid. Glutamic acid has been synthesized by Michael condensations: methyl acrylate and ethyl phthalimidomalonate were employed by Marvel & Stoddard (1938), and methyl acrylate and ethyl acetaminomalonate by Snyder, Shekelton & Lewis (1945).

Evidence of the synthesis of (III) by condensation of methyl methacrylate with ethyl phthalimidomalonate was obtained. However, better results

were obtained by the reaction between methyl methacrylate and ethyl acetaminomalonate:

Elementary analysis of the synthetic material gave the empirical formula $\text{C}_6\text{H}_{11}\text{O}_4\text{N}$; when fused with zinc dust it gave a positive pine-splinter test for pyrrole derivatives.

The position of the synthetic compound on paper chromatograms coincided with that of the natural acid (III). Both substances melted at $154\text{--}155^\circ$ and the melting point of a mixture was unchanged.

Prof. Kathleen Lonsdale, F.R.S., very kindly arranged for X-ray powder photographs of the natural and synthetic acids to be prepared (Fig. 1). She reported that the two samples gave identical results.

Oxidative degradation of (II). The synthesis of (III) had provided good evidence of the carbon skeleton of the new compounds. The possible position of the double bond had been indicated by the formation of aspartic acid by treatment of the unsaturated compounds with ozone. However, the yield of aspartic acid was low, and no non-nitrogenous fragment had been isolated. Accordingly, further experiments were made on the oxidative degradation of (II).

The compound reacted almost completely with an approximately equimolecular amount of potassium permanganate in ice-cold aqueous solution. Further oxidation with periodic acid gave, on different occasions, yields of formaldehyde (dime-done derivative) of 41, 27 and 25% (mol. basis). The main ninhydrin-reacting component resulting from permanganate-periodate oxidation could not be separated from aspartic acid on chromatograms run in phenol-ammonia, butanol-acetic acid

(Partridge, 1948) or collidine-lutidine solvents. Evidence that aspartic acid was formed in 32% yield was obtained by a quantitative chromatographic method (Fowden, 1951).

Exhaustive methylation (Braunstein *et al.* 1947) of the product of oxidative degradation after removal of formaldehyde gave fumaric acid, corresponding to a yield of 29% of aspartic acid.

Provisional structures of the new compounds are included in the following scheme, which also summarizes the main reactions described:

EXPERIMENTAL

All melting points are uncorrected.

Isolation of compound (I). Young plants (3000–4000) were grown in boxes containing either garden soil or gravel and nutrient salts. Exudates were obtained by cutting the main stems when four to eight leaves had developed. They were collected in capillary tubes, and stored in the refrigerator in 2–4 ml. batches. The precipitate which formed was removed by centrifugation. A total of approx. 40 ml. of material was obtained. Preliminary attempts to concentrate or crystallize (I) by extraction of dried exudate with ethanol and by other methods were not successful.

A column of Zeo-Karb 215 was prepared (Partridge & Westall, 1949), 15 cm. in length and 0.9 cm. in diameter. The exudate material was diluted with 250 ml. of water and passed through the column at the rate of 1 drop in approx. 4 sec. The resin darkened progressively from the top as the mixture passed through the column, until finally only a few mm. at the bottom remained unchanged. Distilled water (1 l.) was used to wash the column and then a solution of 0.15N- NH_3 was run through. Eighteen fractions of vol. 10–15 ml. were collected.

A light-coloured band moved down the column during elution. This was apparently due to (I), since when it 'broke through' fraction 9, 5 $\mu\text{l.}$ of the effluent gave a strong yellow to red colour when treated with ninhydrin on filter paper. Chromatograms of 50 $\mu\text{l.}$ amounts of the eluates were run in phenol- NH_3 . The results are given in Table 1.

Table 1. *Elution of compound (I) from Zeo-Karb 215 with dilute ammonia solution*

(For details, see text.)

Fractions (serial nos.)	Appearance of filter-paper chromatogram (Phenol- NH_3 -water)
1–8	Faint (I) spot
9	Fairly strong (I) spot
10–12	(I) spot about 10 times as strong as that in fraction 9. Faint pink spot at origin
13–17	(I) spot as in preceding fractions. Faint purple streaks before and after (I)
18	(I) spot about half as strong as that of fraction 9

Fractions 1–7 were discarded. Fractions 8–17 were evaporated to dryness over H_2SO_4 in a vacuum desiccator. 190 mg. of residue were obtained which yielded 120 mg. of material in flat colourless crystals after two crystallizations from aqueous ethanol. (Found: C, 46.0; H, 6.5; N (Dumas), 17.3. $\text{C}_4\text{H}_9\text{O}_2\text{N}_2$ requires C, 45.6; H, 6.3; N, 17.7%.) When heated in a sealed melting-point tube at the rate of about 3°/min., signs of decomposition appeared at 173° and increased until 182° had been reached. No melting or further change occurred up to 260°. The tube was cut open at both ends, and the vapour inside blown over the surface of a moist litmus paper which then gave a strong alkaline reaction.

(I) dissolved very readily in water, and the solution gave a slightly acidic reaction with bromothymol blue.

Estimation of amide nitrogen. The microdiffusion method of Dekker, Stone & Fruton (1949) was used, adapted to a small diffusion unit made of paraffin wax.

A solution (50 $\mu\text{l.}$) made by dissolving 2.223 mg. of (I) in 1 ml. of water was placed in one depression in the wax. 2N- NaOH (50 $\mu\text{l.}$) was placed in a second depression, and 3N- H_2SO_4 (100 $\mu\text{l.}$) in a third. The unit was covered by a glass plate and sealed by melting a little wax at the edges of the glass. The alkali was then shaken into the test solution, and the unit left at room temperature for 30 hr. The acid was removed to a test tube, and the alkaline residue digested with nitrogen-free H_2SO_4 and a selenium catalyst. Nitrogen in the mixtures and in a digest of the original solution was estimated colorimetrically after nesslerization. (Found: diffused N, 9.3; residual N, 9.1; total N, 18.2%.)

Following Borsook & Dubnoff (1939), who determined amide N by hydrolysis in N-acid, 1 ml. of solution as used in

the preceding experiment was heated for 3 hr. with 0.5 ml. 3N-H₂SO₄ at 100°. NH₃-N in the hydrolysate, as determined by nesslerization, was equivalent to 8.7% of (I).

Treatment with ozone. 5 mg. of (I) dissolved in water were treated with ozone. When the solution was concentrated in a vacuum desiccator, extensive decomposition occurred with the formation of carbonaceous matter. The main ninhydrin-reacting component gave a purple spot which coincided in position on paper chromatograms with that due to aspartic acid. It could not be separated from added aspartic acid on two-dimensional or long-developed one-dimensional chromatograms in aqueous phenol-NH₃ and *n*-butanol-acetic acid (Partridge, 1948).

The isolation of compound (II). The tops of the young plants from which the exudates had been obtained were broken up in water in a blender and then incubated overnight at 37° in the presence of a little thymol. The autolysates were filtered through muslin and stored in the refrigerator. The autolysates were pooled, charcoal was added and the mixture stirred for 30 min. at room temperature and filtered. The final volume was about 10 l.

The liquid was passed through a column of Zeo-Karb 215, which adsorbed all the ninhydrin-reacting substances. The column was washed with several litres of distilled water, and then a solution of 0.15N-NH₃ was run through. The effluent was evaporated under reduced pressure until the volume was 1.8 l., and then extracted several times with ether to remove fat. Dissolved ether was removed by aeration, and the liquid again treated with charcoal at room temperature and filtered.

A column of De-Acidite B was prepared (Partridge & Brimley, 1949) measuring 40 × 2.4 cm. The liquid was run through at the rate of 5–6 ml./min., and the effluent collected in fractions of approx. 250 ml.

Chromatograms showed that the column had removed the acidic components (aspartic and glutamic acids and compound II) from the first 1.1 l. of liquid. The later fractions (about 650 ml.) contained the acidic as well as other amino-acids.

The column was washed with 1.8 l. of 0.1N-acetic acid followed by 500 ml. of 0.25N-HCl, at the rate of about 100 ml./hr. Fractions were collected at 50–60 min. intervals.

Chromatograms of fractions 1–6 showed glutamic acid and only traces of (II), so they were discarded. Pooled fractions were evaporated by distillation at reduced pressure, and residual water was removed by drying over NaOH in a vacuum desiccator.

Fractional crystallization of the products from water yielded 1.063 g. of crystals which gave only spot (II) on chromatograms, and a number of samples of total weight 2.680 g. which were found to contain slight traces of glutamic acid. The former batch was used for analytical and other quantitative work. The other samples were used for qualitative examinations, and, after further crystallizations, for the preparation of derivatives.

Some properties of compound (II). When heated in a melting-point tube, decomposition commenced at 196°, after which the substance changed in colour through yellow to dark brown at 325°. With more rapid heating (i.e. greater than 2°/min.) the first change occurred at 204°, when the surface of the substance became 'moist'.

The acid had a low solubility in water. No optical activity was detected when 96.3 mg. were made neutral to phenolphthalein with NaOH in water (final vol. 1.82 ml.) and observed in a 1 dm. polarimeter tube. (Found: C, 44.1; H, 5.7; N, 8.6; amino N (Van Slyke nitrous acid method,

4 min. reaction), 8.4. C₆H₉O₄N requires C, 45.2; H, 5.7; N, 8.8%.) When 164 mg. of (II) was titrated with 0.735N-NaOH to a faint pink to phenolphthalein, the equiv. wt. was found to be 158 (formula wt. 159). CO₂ was evolved when (II) was dissolved in 10% (w/v) Na₂CO₃ solution.

Treatment with ozone. 100 mg. of (II) was suspended in 7 ml. of water and ozonized oxygen was passed through for 45 min. The product of the treatment was at first a clear solution, but extensive decomposition occurred when it was allowed to stand overnight. The main ninhydrin-reacting component found on chromatograms coincided in position with aspartic acid.

After removal of the charred material by filtration the solution was boiled with CuCO₃. The excess CuCO₃ was removed by filtration, and, on cooling, a copper compound of low solubility in water crystallized out. Copper was removed from this by H₂S, and 11.1 mg. of a white crystalline solid were obtained by crystallization from aqueous ethanol. This was shown to be aspartic acid by exhaustive methylation to fumaric acid (Braunstein *et al.* 1947). The product of exhaustive methylation, isolated from the reaction mixture with ether by means of a small continuous extraction apparatus, melted at 295° in a sealed tube. The melting point was unchanged when the product was mixed with an authentic sample of fumaric acid.

Oxidation with permanganate. KMnO₄ solution (1%, w/v) was added to about 1 ml. of a saturated solution of (II), until there was a slight permanent pink colour. This colour was discharged by adding 1 drop more of the solution of (II). The mixture was filtered and electrolytically desalted (Consden, Gordon & Martin, 1947).

Chromatograms run with aqueous *n*-butanol-acetic acid mixture showed a prominent spot which coincided in position with aspartic acid. Two other spots were visible: a well marked spot of lower *R_F*, and a faint spot of higher *R_F* than the aspartic acid.

In another experiment 96 mg. of (II) were dissolved in 4 ml. 10% (w/v) Na₂CO₃ solution. Finely powdered KMnO₄ was added until a slight excess could be detected. A few mg. more of (II) were then added. The mixture was warmed to about 60° for a few minutes, when coagulation of MnO₂ commenced. It was then filtered, and the precipitate washed. A portion of the filtrate was desalted and chromatograms showed a single spot coincident in position with aspartic acid. However, the latter could not be isolated as the Cu salt, presumably because of the high concentration of electrolytes in the mixture. Later, oxalic acid was isolated from residues from this experiment through the Ca salt (melting point of the di-*p*-toluidide 264°, unchanged when mixed with an authentic sample).

Oxidation with cold aqueous permanganate was studied in more detail later.

Reduction of compound (II) to compound (III) and investigation of the product. In one experiment, 28.9 ml. of H₂ were absorbed by 180 mg. of (II) suspended in 10 ml. water with 50 mg. palladium-on-carbon catalyst. (The theoretical amount for a reaction C₆H₉O₄N → C₆H₁₁O₄N under the same experimental conditions was 27.8 ml.)

After removal of the catalyst, the solution was evaporated in a vacuum desiccator. Solid separated when 1–2 ml. of solution remained. This was dissolved by addition of the minimum of water. Acetone was added and the mixture left overnight in the refrigerator. 154 mg. of product were obtained after filtration. (Found: C, 44.7; H, 6.8; N, 9.1. C₆H₁₁O₄N requires C, 44.7; H, 6.8; N, 8.7%.) Equiv. wt. by

titration with 0.103N-NaOH was 159 (equiv. wt. of monobasic $C_6H_{11}O_4N$, 161). Melting points varied from 154–165°, apparently depending on the rate of heating and the amount of material used.

2–3 mg. of (III) were shaken with conc. H_2SO_4 and excess of N_3H in $CHCl_3$ at 43–46° (Adamson, 1939). A sample of the reaction product was desalted and on paper chromatography the main ninhydrin-reacting component of the solution had a higher R_F value than ornithine in aqueous *n*-butanol-acetic acid mixture, being clearly separated from added ornithine.

Synthesis of γ -methylglutamic acid. Preliminary experiments were based on the glutamic acid synthesis of Marvel & Stoddard (1938), methyl methacrylate (containing quinol to inhibit polymerization) being used in place of the methyl acrylate. Methyl methacrylate and diethyl phthalimidomalonate were refluxed in absolute ethanol in which small amounts of sodium had been dissolved. Volatile components were removed after different periods of refluxing, and samples of the residues were hydrolysed overnight with 20% (w/v) HCl in sealed tubes at 105°. Chromatograms run in aqueous *n*-butanol-acetic acid showed the presence of glycine (presumably from free diethyl phthalimidomalonate) and another component coincident in position with compound (III) and presumably γ -methylglutamic acid. The glycine and phthalic acid also present could not be separated from this by crystallization from aqueous ethanol or aqueous acetone. A copper salt of low solubility in water was obtained from the hydrolysis product, which, after removal of the copper by H_2S , was found to yield only a trace of glycine on the chromatograms run in butanol-acetic acid solvent, together with an intense spot coincident in position with (III). 1.83 g. of the copper salt were obtained from 23.8 g. of diethyl phthalimidomalonate. However, preliminary experiments indicated that better results could be obtained by a different procedure.

Snyder *et al.* (1945) synthesized DL-glutamic acid from methyl acrylate and diethyl acetaminomalonate. In the experiment to be described, the conditions were similar to those in the above-mentioned synthesis except that sulphur was added to inhibit polymerization of methyl methacrylate and that an intermediate was isolated and recrystallized before hydrolysis.

Sodium (120 mg., 0.0052 g. atom) was dissolved in approx. 20 ml. ethanol in a 200 ml. flask. 100 mg. sulphur and 10.85 g. (0.05 mole) ethyl acetaminomalonate were added. An attachment bearing a mechanical stirrer, a reflux condenser, and a dropping funnel was fixed to the flask. The contents were refluxed and 10 g. (0.1 mole) of methyl methacrylate were added in drops during 4 hr. Refluxing was continued for a further hour.

The reaction mixture yielded 7.34 g. of colourless crystals after three crystallizations from ethanol. Melting point 109.5°, unchanged on recrystallization. (Found: N (Kjeldahl), 5.7, 5.8%). This substance was not, apparently, the primary condensation product of methyl methacrylate and ethyl acetaminomalonate, the N content of which would be 4.6%. A small sample was hydrolysed overnight with 20% (w/v) HCl in a sealed tube at 105°, and chromatograms run with *n*-butanol-acetic acid showed a spot coincident in position with (III), and no glycine. 2.78 g. of the intermediate was hydrolysed by refluxing in 20% (w/v) HCl, more HCl being added from time to time to replace the acid which had been used in the reaction.

The hydrolysate was evaporated almost to dryness at reduced pressure. The process was repeated three times after addition of 5 ml. amounts of water. The product was then dissolved in 50 ml. of water. Residual HCl was removed by two extractions of the solution with excess of a 5% (w/v) solution of trioctylamine in $CHCl_3$ (Lester Smith & Page, 1948). The aqueous layer was washed twice with $CHCl_3$ and evaporated in a vacuum desiccator until solid appeared. A further batch of the intermediate (3.62 g.) was hydrolysed and the acid-free product combined with that from the first batch. The crude product was dissolved in the minimum of water. Acetone was added, and the mixture cooled in crushed ice and left overnight in the refrigerator. The resulting solid was filtered off, dissolved in the minimum amount of water, and again treated with acetone, cooled and left overnight in the refrigerator. 2.82 g. crystalline solid were obtained (45% yield, based on the diethyl acetaminomalonate). (Found: C, 44.9; H, 6.8; N, 8.5. $C_6H_{11}O_4N$ requires C, 44.7; H, 6.8; N, 8.7%.)

Oxidative degradation of acid (II). 1.4 ml. of 0.735N-NaOH were added to 161 mg. of acid (II). The solution thus obtained gave a light-green colour with bromothymol blue. A solution of 158 mg. of $KMnO_4$ in 10 ml. water was added in drops over 45 min. The reaction was carried out in a cold room and the temperature of the mixture maintained at 5–6°. After the reaction, the mixture was allowed to stand for 30 min. at laboratory temperature. It was then warmed to 30–35° in a water bath for a few minutes to coagulate the MnO_2 . The precipitate was removed by centrifugation and washed with four lots of water (total vol. 10 ml.). A sample (0.5 ml.) was removed.

A faint turbidity developed when aqueous dimedone solution was added to part of the sample. The remainder of the sample was desalted. Chromatograms run with phenol- NH_3 and *n*-butanol-acetic acid solvents showed that the main ninhydrin-reacting component had a lower R_F than aspartic acid. Smaller amounts of aspartic acid, compound (II) and compound (III) were also present. (The compound (III) had probably been formed by reduction of compound (II) in the desalter.)

0.75 g. of $HIO_4 \cdot 2H_2O$ was added to the combined supernatants. A slight red precipitate formed. This was removed after 1 hr. by centrifugation and the mixture was allowed to stand for a further 30 min. CO_2 was slowly evolved during the periodate reaction. Periodate was precipitated as the Ba salt by addition of $Ba(OH)_2$ in slight excess. The precipitate was washed on the centrifuge, and the combined supernatants made slightly acid with H_2SO_4 and filtered.

The filtrate was distilled and the distillate collected in an aqueous solution containing approx. 260 mg. of dimedone. 71 mg. of a dimedone derivative were obtained. Its melting point was 189°, unchanged when mixed with an authentic sample of the formaldehyde derivative (yield corresponding to 25% of formaldehyde, mol. basis).

A sample of residue from the distillation was desalted. Chromatograms showed a heavy spot corresponding in its position with aspartic acid. The spot could not be separated from that due to added aspartic acid on further chromatograms run in phenol- NH_3 , *n*-butanol-acetic acid, or collidine-lutidine solvents. A desalted sample of the residue from the distillation was analysed by a quantitative chromatographic method (Fowden, 1951). The amount of amino N in the spot referred to above was found to correspond to a yield of 32% (mol. basis) from the amount in the original substance. A similar experiment gave 27% of formaldehyde.

377 mg. of (II) were oxidized in a third experiment. 370 mg. of KMnO_4 and 1.3 g. of $\text{HIO}_4 \cdot 2\text{H}_2\text{O}$ were used. After the treatment with $\text{Ba}(\text{OH})_2$, 330 mg. of dimedone dissolved in hot water were added. The mixture was left overnight at room temperature, and the resulting precipitate filtered off and washed with cold water. 301 mg. of formaldehyde derivative were obtained (mixed m.p. 189° , yield 41%). The filtrate was evaporated to small bulk, filtered, and treated with dimethyl sulphate and 33% (w/v) NaOH (Braunstein *et al.* 1947). 81 mg. of fumaric acid (29% yield) were obtained after continuous ether extraction (m.p. 292° in a sealed tube; 295° after crystallization from water, unchanged on admixture of an authentic sample).

DISCUSSION

The oxidative degradation of (II) and the synthesis of its reduced form have provided evidence on the basis of which the provisional structure

(γ -methyleneglutamic acid) is proposed. The identification of aspartic acid by paper chromatography after treatment of the amide with ozone indicates that the amide is

(γ -methyleneglutamine).

Steward & Thompson (1950) presented a synopsis of the results of paper-chromatographic examination of ethanol extracts of various plant materials. They suggested that amides may occur among a group of acid-labile substances detected. However, although asparagine was isolated by Vauquelin & Robiquet (1806) and glutamine by Schulze & Bossard (1883), no similar amino-acid amide has been isolated before the present investigation.

The detection and isolation of the new amino-acid amide (tentatively recognized as γ -methyleneglutamine) and the corresponding acid were made possible by the techniques of paper chromatography. Among other favourable circumstances were: the high concentration of the amide in the exudates, the distinctive ninhydrin reaction of the amide and acid, the low solubility of the acid in water, and the fact that autolysis caused only partial deamidation of asparagine and glutamine, but apparently almost complete deamidation of γ -methyleneglutamine.

The autolysate from which γ -methyleneglutamic acid was isolated contained a substance (IV) which gave a yellow-brown spot at R_f 0.85 in phenol-ammonia. Traces of the same substance were found in the end residues from the fractional crystallizations of the acid, along with a number of other amino-acids. (It is likely that non-acidic amino-acids were retained to some extent on the De-Acidite B and were eluted by the ammonia.) A small quantity of (IV) was isolated from chromatograms of these residues by elution of the appropriate parts of the paper. After treatment with ozone, the product contained a substance which could not be dis-

tinguished from β -alanine on chromatograms run in three different solvents. β -Alanine could be produced from α -methylene- γ -aminobutyric acid:

γ -Aminobutyric acid and β -alanine, the ω -amino-acids related to glutamic and aspartic acids respectively, are now known to be widely distributed among plants. The possible existence of an ω -amino-acid related to γ -methyleneglutamic acid is therefore of considerable interest.

Compounds (I), (II) and (IV) were converted, in the electrolytic cell of the desalter, to substances which gave purple ninhydrin spots. After such treatment, the substance formed from (II) could not be distinguished from compound (III) by paper-chromatographic methods. The R_f 's of the substances formed from (I) and (IV) differed only slightly from those of the original compounds. Apparently compounds (I) and (IV) are also reduced to substances which give a purple colour with ninhydrin. None of the reduced forms has been detected in ethanolic extracts of the plant material.

Application of modern methods to amino-acid analysis and experiments using isotopic tracers are providing increasing evidence that glutamine, asparagine, glutamic acid and aspartic acid are very active metabolites. Thus interesting results may be obtained from further work on the biochemistry of the new type of amide and the corresponding acid described in this paper.

SUMMARY

1. An unsaturated amino-acid amide and the corresponding dicarboxylic amino-acid have been isolated from vegetative material of groundnut plants (*Arachis hypogaea*). The acid showed no optical activity as its sodium salt in water.

2. The provisional structures

(γ -methyleneglutamine) and

(γ -methyleneglutamic acid) have been proposed.

3. The dicarboxylic amino-acid was reduced and the product agreed in properties with a synthetic preparation of γ -methylglutamic acid.

4. The new compounds appear to be normal metabolites of the plants, but the reduced forms were not detected in ethanolic extracts of fresh material.

5. Evidence was obtained that a third unsaturated amino compound was present in the autolysed material.

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The Carotenoids of the Berries of *Lonicera japonica*

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Although a great deal is known concerning the distribution and occurrence of carotenoids in fruit (see Karrer & Jucker, 1949; Goodwin, 1952*b*), there are still many gaps in our knowledge; these will have to be filled if a rationale of carotenoid distribution in fruit is eventually to be achieved. Furthermore, studies on the minor carotenoid components of fruit may well throw considerable light on the mode of biogenesis of carotenoids. Only one such investigation on tomatoes has been carried out (Porter & Lincoln, 1950); the results permitted the authors to suggest a working hypothesis for carotenogenesis in this fruit.

No precise information is available on the carotenoids of the berries of the family Caprifoliaceae, although early workers had indicated the presence of lipochromes (carotenoids) in *Lonicera tartarica* (Schimper, 1885), *L. xylosteum* (Schimper, 1885; Molisch, 1896; Kohl, 1902; Nowak & Zellner, 1921); *Sambucus nigra* (Nowak & Zellner, 1921), *Viburnum opulis* and *V. lantana* (Wisselingh, 1914; Nowak & Zellner, 1921; Kryz, 1919). When a small crop of ripe berries of a cultivated climbing honeysuckle (*Lonicera japonica*) became available an investigation was undertaken into the nature of the carotenoids present, with special emphasis on the minor components.

EXPERIMENTAL

Materials. The fully ripened berries were obtained from a garden in north-west Cheshire. Two portions of about 200 g. each were examined separately with identical results.

Extraction and separation of the carotenoids

The fresh berries were ground to a fine powder with Na_2SO_4 and the powder extracted with successive portions of diethyl ether (freshly distilled over reduced iron) until no further colour was extracted. The combined ether extracts were taken to dryness *in vacuo* at room temperature and the lipids saponified by adding to the residue 1 ml. of 60% (w/v) aqueous KOH and 5 ml. of ethanol, mixing and allowing to stand overnight at room temperature. The unsaponifiable matter was extracted into ether as described by Goodwin & Morton (1946) and the ether was removed *in vacuo*; the residue was dissolved in a small volume of light petroleum (b.p. 40–60°) and examined chromatographically. Petroleum, b.p. 40–60°, was used throughout this investigation.

Separation 1. A preliminary separation of the pigment mixture was first carried out on alumina (Spence, Grade 'O') deactivated with methanol (Goodwin, 1952*a*) using light petroleum containing 10% (v/v) of ether as developer. The resulting chromatogram is described in Table 1.

Separation 2. Fraction 1 (Table 1), which eventually percolates through the column, was collected in the filtrate, the mixed solvents removed *in vacuo* and the residue redissolved in light petroleum and chromatographed on activated alumina (Spence, Grade 'O'). Five fractions were obtained as recorded in Table 2.

Separation 3. Zone 6 (Table 1) was eluted with ethanol. The ethanol was removed *in vacuo* at 30° and the residue dissolved in a few drops of ether and made to approx. 10 ml. with light petroleum. This treatment is necessary because this fraction will not dissolve directly in light petroleum. Chromatography was carried out on a column of ZnCO_3 using benzene as developer. Four zones were obtained, two of which eventually moved down the column to be collected