Changes in Oxo Acid Concentrations during the Growth of Groundnut Seedlings

By J. A. WEBB and L. FOWDEN
Department of Botany, University College, Gower Street, London, W.C. 1

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In recent years we have been investigating the occurrence, distribution and metabolism of unsaturated amino acids in groundnut plants (*Arachis hypogaea*). Three related amino acids have been identified, namely (i) γ-methyleneglutamic acid (γ-MGA), (ii) its amide, γ-methyleneglutamine (γ-MG), and (iii) its decarboxylation product, γ-amino-α-methylenebutyric acid (Done & Fowden, 1952; Fowden & Done, 1953). These substances are not ubiquitous in plant species, and have so far only been reported in tulips (Zacharius, Pollard & Steward, 1954), hops (Tatchell & Harris, 1953), and in a *Lilium* sp. (personal communication from Dr G. H. N. Towers, Montreal). A possible pathway for the biological synthesis of γ-MGA could involve its oxo acid analogue, γ-methylene-α-oxoglutaric acid (γ-MOG), as an intermediate stage (Fowden, 1954). Some support for this idea has resulted from the isolation of this oxo acid (as the 2:4-dinitrophenylhydrazine derivative) from tulips (Towers & Steward, 1954), and groundnuts (Fowden & Webb, 1955).

Investigations reporting the oxo acid concentration levels in higher plants have been few in number, and although Virtanen, Miettinen & Kunttu (1953), and Towers, Thompson & Steward (1954) have determined the concentrations of the main acids of some plant tissues, we believe that this is the first publication which relates the oxo acid levels in different plant organs to the stage of development of the plants. The quantitative distribution of γ-MOG and other oxo acids found in groundnut plants at different stages of their early seedling growth is reported. Since a similar study of the distribution of γ-MGA and γ-MG has already been made (Fowden, 1954), a comparison of the importance of the related types of γ-methylene compounds during seedling development can now be made.

EXPERIMENTAL

Material. The groundnut seeds were grown in moistened wood chippings in a greenhouse kept at about 30° during the latter part of summer. They were watered with Knoop's nutrient solution about every fourth day. Under these conditions growth was rapid and the plants were harvested for analysis at 1, 2, 4, 6, 10 and 17 days after planting. After 24 hr. growth the radicles were about 0·5 cm. in length and after 2 days had increased to 3–5 cm. At 4 days the cotyledons were above the growing medium and development was sufficient to divide the plants into three parts, namely immature leaves, cotyledons and roots. The first two leaves (for all practical purposes an opposite pair) were strongly developed at 6 days, and the third leaf was beginning to unfold but still had a completely undeveloped petiole. Further regular growth produced at 10 days 3 leaves and at 17 days 5 leaves which had attained maximum development, morphologically, whilst at each stage a further leaf was unfolding. The roots became increasingly branched and fibrous and the cotyledons gradually became smaller and were tending to become slightly withered in appearance at 17 days. All the plants were harvested for analysis in the late morning.

Determination of oxo acids. The oxo acids present in the plant materials were determined after their separation as 2:4-dinitrophenylhydrazine (DNPH) derivatives by paper chromatography. The procedure used for the conversion of the oxo acids into their DNPH derivatives was based on that published earlier (Fowden & Webb, 1955). The DNPH derivatives so formed were extracted into ethyl acetate by four successive extractions, using a ratio of 1 vol. ethyl acetate to 6 vol. of supernatant. This was followed by four successive extractions of the DNPH derivatives into 10% (w/v) Na_2CO_3 solution using a ratio of 1 vol. Na_2CO_3 to 10 vol. of the ethyl acetate extract. The combined Na_2CO_3 extracts were acidified at 0° by the addition of conc. HCl, and the DNPH derivatives once more extracted into ethyl acetate by four successive extractions using a ratio of 1 vol. ethyl acetate to 6 vol. of the acidified soln. The ethyl acetate extracts were combined and evaporated to dryness. The residues of DNPH derivatives so obtained, which were contaminated with darker impurities, were treated with small volumes of 4N-NH_3 soln., when the derivatives went into solution, leaving most of the impurities undissolved. After centrifuging, the supernatants were taken to dryness *in vacuo* and redissolved in 0·5N-NH_3 soln. Small measured volumes of these solutions were applied to the paper chromatograms of Whatman no. 3 filter paper.

The chromatograms were developed in *n*-butanol saturated with 6% (w/v) NH_3 soln. (Fincham, 1953) until adequate separation of the individual oxo acid derivatives was achieved. This period was usually about 24 hr. at laboratory temperature. Areas of paper containing individual oxo acid spots were cut out, and the derivatives eluted from the paper by soaking for 1 hr. in 4 ml. 10% (w/v) Na_2CO_3 soln. To 3 ml. of this Na_2CO_3 extract, 0·5 ml. of 3N-NaOH soln. was added, and the extinction coefficients of the final solutions were rapidly measured at $\lambda = 540$ mμ. using a Unicam SP. 500 spectrophotometer.

Weighed amounts of pure oxo acids were converted into DNPH derivatives and subjected in an identical manner to chromatographic analysis and colorimetric determination. In this way standard calibration lines were obtained for each oxo acid over the range 20–100 μg. of acid, from which the amounts of oxo acids in the plant materials could be interpolated. As pure γ-MOG is as yet available, so the determinations for this acid were based on the standard calibration line for α-oxoglutaric acid, assuming that an equal colour production results from equimolar amounts of the two acids.

When the above procedure is used for pyruvic and glyoxylic acids, each acid produces two DNPH derivative spots on the chromatogram. Glyoxylic acid gives spots at R_F values of 0·37 and 0·61, and pyruvic acid at R_F values of 0·49 and 0·70. In our experience, the spot of lower R_F for each acid was always the stronger of the two (compare Markees & Gey, 1953), and the ratio of the extinction coefficients measured for extracts of the two spots resulting from a single acid was virtually constant, irrespective of the total acid concentration, provided the procedure was standardized as above. Only the amounts of DNPH derivative present in the lower R_F spots for glyoxylic and pyruvic acids have then been measured in both the standard and experimental determinations.

To confirm the identity of the oxo acids detected in the plant tissues, the separated DNPH derivatives were converted into their amino acid analogues by catalytic hydrogenation by the procedure reported previously (Fowden & Webb, 1955), following the original method of Towers *et al.* (1954). The amino acids resulting were characterized by two-dimensional chromatography.

RESULTS

When ungerminated seeds were treated as described above DNPH spots corresponding to α-oxoglutaric (α-OG) and pyruvic acids were detected. A trace of the DNPH derivative of glyoxylic acid appeared to be also present. The identities of the three acids were confirmed by reduction of the derivatives to glutamic acid, alanine and glycine respectively. Quantitative assay indicated an α-OG concentration of 11 μg./g. fresh weight (f.w.), and a pyruvic acid level of 4 μg./g.f.w.

After 24 hr. growth, γ-MOG was also present in the seedlings. The DNPH derivative of this acid formed a spot moving just in advance of that of α-OG under the chromatographic conditions described. γ-Methylglutamic acid resulted after catalytic hydrogenation of the derivative. No attempt was made to dissect the seedlings at this stage, or after 2 days' growth, the internal oxo acid concentrations determined being recorded in Fig. 1*c*, where, for the convenience of graphical representation, the seedlings are considered to consist entirely of cotyledon material.

The oxo acid contents of the plants grown for 4 or more days are reported graphically in Fig. 1*a–c*, where separate values are recorded for leaves, roots and cotyledons respectively. The 17-day-old

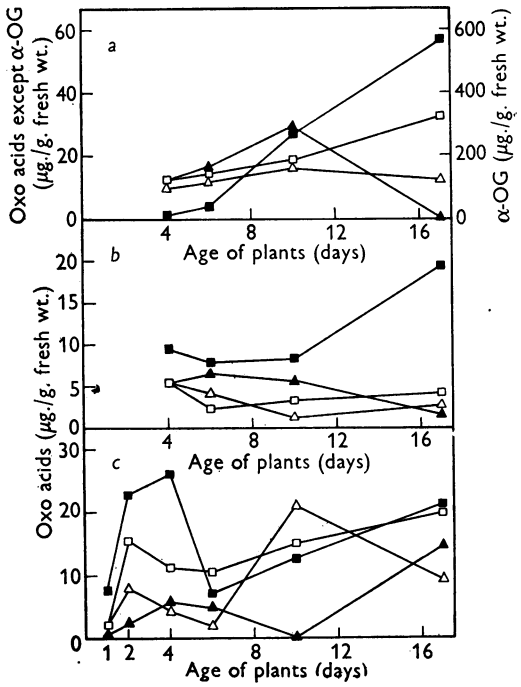

Fig. 1. Concentration of oxo acids expressed as μg. acid/g. fresh weight of the plant tissue at various stages of seedling growth; (*a*) oxo acid contents of oldest two leaves (serial nos. 1 and 2); (*b*) oxo acids of roots; (*c*) oxo acids of cotyledons; ■—■, α-OG; □—□, γ-MOG; △—△, pyruvic acid; ▲—▲, glyoxylic acid; note that in (*a*) the α-OG values shown are on a scale reduced ten times.

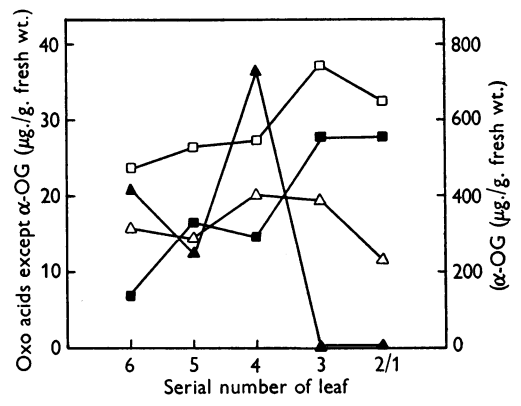

Fig. 2. Concentration of oxo acids expressed as μg. acid/g. fresh weight of leaf tissue for the leaves of different ages present on the 17-day-old plants; a higher leaf number represents a younger leaf; ■—■, α-OG; □—□, γ-MOG; △—△, pyruvic acid; ▲—▲, glyoxylic acid; note that the α-OG values are shown on a scale reduced twenty times.

plants, which had six leaves on the main stem, were further subdivided before analysis, the leaves of different ages being separately analysed. When the leaves on the main stem are serially numbered (nos. 1 to 6) from the base to stem apex, a lower leaf number represents an older leaf. The first two leaves (serial nos. 1 and 2), which developed together, were considered to be of the same age, and were analysed together. The results of leaf analyses are given in Fig. 2.

The most striking feature observed was the very marked rise in the concentrations of α-OG occurring in leaves as their age increased (see Figs. 1*a*, 2). Although α-OG tended to be present in slightly higher concentrations than the other oxo acids in all tissues examined, its increased concentrations (560 μg./g.f.w.) in older leaves, where it represented over 90 % of the total oxo acids present, was unparalleled by similar changes in other tissues, where α-OG concentration of about 20 μg./g.f.w. was maximal. In general, all the oxo acids were present in leaves in somewhat higher amounts than in the cotyledons, whilst roots contained even smaller amounts of the acids.

Extracts of cotyledons gave rise to additional DNPH derivatives. On chromatograms showing the highest degree of resolution, three spots were present flowing at R_F values lower than that of the α-oxoglutaric acid derivative ($R_F = 0{\cdot}10$). The natures of the oxo acids giving rise to the new spots are unknown, but although they appeared in the early germination stage, they could not be detected in the ungerminated seeds. One of the spots representing the unknown DNPH derivatives was usually more intense than the remaining two, and it was of similar intensity to those of the known oxo acids in the early stages of growth. After catalytic hydrogenation, this DNPH derivative yielded a substance that moved on paper chromatograms to a position between those occupied by aspartic and glutamic acids in water-saturated phenol ($R_F = 0{\cdot}23$), and which reacted with ninhydrin to give a brown spot. These additional DNPH derivatives were not detected in leaf or root extracts.

DISCUSSION

The fact that γ-MOG acid forms a measurable proportion of the total oxo acids present in seedlings grown for 1 day, whereas γ-MGA and γ-MG have not been identified amongst the amino acids present at this stage (unpublished results), may indicate that the formation of the amino compounds follows that of their oxo acid analogue; the oxo acid may result indirectly by respiratory breakdown of carbohydrate or fat, and may be converted into γ-MGA either by transamination or direct reductive amination. Since the three unidentified oxo acids were also produced during the first day's growth, it may be that one or more of these acids are themselves precursors of γ-MOG production. γ-MOG never accumulated in any tissue in amounts which were in excess of those of other oxo acids. This is in marked contrast to the distributions observed for the amino acid analogues, γ-MGA and γ-MG, each of which constituted a large proportion of the total free α-amino nitrogen of certain tissues.

Virtanen *et al.* (1953) have shown that the oxo acid content of pea plants was very dependent upon the degree of illumination of the plants just before harvesting and analysis. Although our plants were harvested at the same hour each day, and had therefore all been subjected to a similar period of illumination, the intensities of illumination received by sets of plants obviously varied with the brightness of the particular day of their harvest. Some of the smaller variations which we have observed in the contents of the particular oxo acids may be attributed to this cause. The reason for the much larger increase in the α-OG concentration during leaf ageing lies presumably elsewhere. One explanation depends upon the fact that as leaves develop, they pass from a state of rapid net protein synthesis during the period of their active growth, to one where the rate of protein synthesis is much reduced, and only counterbalances protein breakdown in the mature leaf. Since a major pathway for the entry of nitrogen into organic compounds is through α-OG, levels of this oxo acid would tend to remain low during active synthesis in growing leaves, and to increase when the need for α-oxoglutarate residues diminishes with the reduced rate of protein synthesis in mature leaves.

With the exception of the high values found for the α-OG content of older leaves, the levels of oxo acids found in groundnut plants (i.e. amounts up to 35 μg./g.f.w. of single oxo acids) are of the same order as those reported previously for other species. Virtanen *et al.* (1953) reported values of 40 μg. for α-oxoglutaric, 20 μg. for pyruvic, and 20 μg. for oxaloacetic acids per g.f.w. in pea plants, whilst Towers *et al.* (1954) also reported μg. amounts in a number of other species examined. The latter workers have noted that the α-OG concentration observed for mint leaves may, under certain conditions, be much increased, and approach 1 mg./g.f.w.

It is noteworthy that oxaloacetic acid has not been identified as a constituent of groundnut plants, either as its DNPH derivative or by reduction to give aspartic acid. Virtanen *et al.* (1953) have shown that decarboxylation of oxaloacetic acid occurred rapidly when extracts of pea plants were prepared using dilute sulphuric acid, which

completely converted oxaloacetic acid into pyruvic acid. In contrast, extraction of the plants with 2N-NaOH immediately inactivated the decarboxylase and stabilized the oxo acid levels, so that oxaloacetic acid could be reliably estimated. Although our plant extraction procedure involved slightly acid pH values this could not explain the apparent absence of oxaloacetic acid, since extractions using 2N-NaOH also failed to reveal the acid.

SUMMARY

1. The oxo acids present in groundnut plants include α-oxoglutaric, pyruvic, glyoxylic and γ-methylene-α-oxoglutaric acids, together with other unidentified oxo acids. All the acids were characterized chromatographically as their 2:4-dinitrophenylhydrazine derivatives, and by catalytic hydrogenation of the latter to the corresponding amino acids. Oxaloacetic acid has not been detected.

2. The amounts of these acids present in leaves, cotyledons and roots were determined at various stages of seedling growth. Concentrations of individual oxo acids were normally in the range of 0–40 μg./g. fresh weight of plant tissue, but α-oxoglutaric acid concentration showed a marked increase during the process of leaf ageing.

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Kinetic Studies of the Metabolism of Foreign Organic Compounds

7. TOLUIC ACIDS AND *N*-TOLYLUREAS*

BY J. J. BETTS, H. G. BRAY, W. V. THORPE AND K. WHITE
Physiology Department, Medical School, University of Birmingham

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Previous papers in this series (Bray, Humphris, Thorpe, White & Wood, 1952*b*, *c*, *d*; 1955; Bray, Thorpe & White, 1952*e*) have dealt mainly with the kinetics of reactions which *para*-substituted aromatic compounds undergo in the rabbit. It is known that in the dog and in the rabbit the conjugation reactions of *ortho*-, *meta*- and *para*-isomers are often qualitatively similar (e.g. see Quick, 1932*a*, *b*; Thorpe, 1950), although quantitatively there are gradations in the percentage of a dose conjugated corresponding to the position of the substituent. For example, the toluic acids are excreted by the rabbit mainly as conjugates of glucuronic acid and glycine; the amount of the former increases progressively from the *para*- to the *ortho*-isomer and that of the latter from *ortho* to *para* (Bray, Thorpe & Wood, 1949*b*; for the dog, see Quick, 1932*a*). With the tolylureas (Bray, Lake & Thorpe, 1949*a*) conjugation may occur through a carboxyl group formed by oxidation of the methyl group or through a new hydroxyl group introduced by nuclear hydroxylation; the extent to which oxidation of the methyl group occurs increases from the *ortho*- to the *para*-isomer, whereas the extent of hydroxylation does the reverse. It was, therefore, of interest to see whether these gradations were a reflexion of the magnitude of the velocity constants of the reactions involved.

Previous determinations of velocity constants have been made using compounds which are readily absorbed by the rabbit, so that after about 2 hr. after dosage the rate of absorption could be neglected. Several hours, however, are required for the absorption of the tolylureas, so that these compounds have provided an opportunity for studying the effect of slow absorption upon the kinetics of conjugation reactions.

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