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THE ISOLATION OF AMINO ACIDS FROM *PISUM SATIVUM*. IDENTIFICATION OF L(−)-HOMOSERINE AND L(+)-*O*-ACETYLHOMOSERINE AND CERTAIN EFFECTS OF ENVIRONMENT UPON THEIR FORMATION

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Abstract—L-aspartic acid, L-glutamic acid, γ-aminobutyric acid, L-alanine, L-valine and L-leucine were isolated from pea plants, crystallized and their identity established by comparison with authentic substances, and by analysis. Especial attention was also given to two others, L-homoserine and L-*O*-acetylhomoserine, which were also isolated in a pure state. The properties which confirm the occurrence of L(−)-homoserine and the evidence which critically establishes the identity of L(+)-*O*-acetylhomoserine are described. Whereas L-homoserine and L-*O*-acetylhomoserine exist as though they were in dynamic equilibrium in the pea plants, asparagine behaves differently. This is shown by the effects of growth (during 77 days) at different night temperatures, from 4 to 26°, under 16-hr nights on the amino acid composition of pea shoots. The lower the night temperature, the higher was the concentration of homoserine and *O*-acetylhomoserine, but the night temperature had the opposite effect on asparagine. The significance of these results is discussed.

INTRODUCTION

(a) *Homoserine*

HOMOSERINE was discovered in plants by Miettinen *et al.*[1] who isolated it from pea shoots in the form of its lactone hydrochloride without determining its optical configuration. As far as can be ascertained, this compound has not since been isolated from any other plant. Semi-quantitative analyses on the occurrence of homoserine in the pea at different stages of development, were made by Virtanen and Miettinen[2] and Virtanen *et al.*[3] These studies revealed that although homoserine is absent from dry pea seeds, it nevertheless is one of the most abundant of the free amino acids in the vegetative parts of the pea plant.

A quantitative paper chromatographic study by Grobbelaar[4] on the influence of environment on the free amino acid composition of four pea varieties, confirmed the occurrence of homoserine in pea plants. In general, it was found to be the most abundant of the free amino acids present, its concentration being of the same order as that of asparagine. Depending on

[1] J. K. MIETTINEN, S. KARI, T. MOISIO, M. ALFTHAN and A. I. VIRTANEN, *Suomen Kemistilehti* **B2**, 26 (1953).
[2] A. I. VIRTANEN and J. K. MIETTINEN, *Biochem. Biophys. Acta.* **12**, 181 (1953).
[3] A. I. VIRTANEN, A. BERG and S. KARI, *Acta Chem. Scand.* **7**, 1423 (1953).
[4] N. GROBBELAAR, Ph.D. Thesis, Cornell University (1955).

the environmental conditions in which the plants were grown, the concentration of homoserine in the shoots of the flowering pea plants varied between 1 and 12 mg/g of total dry matter. Thus, it represented from 3 to 36 per cent of the weight of the total free amino acid complement.

Rabson and Tolbert[5] reported that pea plants can convert aspartic acid to homoserine. Although this appears to be the extent of information on the metabolism of homoserine in higher plants, its role in the metabolism of micro-organisms[6,7] and of animals[8] has been studied more fully.

The present isolation of homoserine from pea shoots represents the first isolation of the amino acid in its free form permitting the optical configuration of the naturally occurring compound from the pea to be established as L(−).

(b) *O-Acetylhomoserine*

During a paper chromatographic study of the free amino acids of the shoots of pea plants,[4] an amino acid-like compound of unknown identity was detected in most extracts. This compound, referred to as unknown 200 by Grobbelaar,[4] was later isolated and conclusively identified as L(+)-*O*-acetylhomoserine. A preliminary report on this work has already been published.[9] *O*-Acetylhomoserine is absent from the dry seed and apparently also from the roots of mature plants. It is present in the stems, leaves and developing fruits but only attains the status of a prominent free amino acid in the young pods (i.e. pericarps of the fruit).[4]

In the shoots of four varieties of peas that were grown under various environmental conditions, *O*-acetylhomoserine was found in concentrations that varied from 0 to 7 mg per g of total dry weight of the shoots. Thus, it comprised from 0 to 16 per cent of the dry weight of the total free amino acid complement of the flowering pea shoots. As far as can be determined, *O*-acetylhomoserine has not yet been detected in any other plant species and no other work appears to have been done on its metabolism in plants. However, a similar compound (*O*-oxalylhomoserine) together with homoserine have been implicated in the metabolism of *Lathyrus* species by Przybylska and Pawelkiewicz.[10]

(c) *Other "Common" Amino Acids*

During the isolation of homoserine and *O*-acetylhomoserine, aspartic acid, glutamic acid, γ-aminobutryic acid, alanine, valine and leucine, were also obtained. In view of the relatively few instances in which these commonly detected amino acids have been actually isolated and critically identified from plants, they were obtained in the crystalline state and were fully characterized.

RESULTS

The eight afore-mentioned amino acids were isolated from extracts of pea shoots and pods, obtained in a crystalline state and fully characterised by standard procedures (see Experimental).

[5] R. RABSON and N. E. TOBERT, *Plant Physiol.* **32**, XXV (1957). Proceedings of the Plant Physiol. meetings held at Stanford University, California.
[6] H. J. TEAS, N. H. HOROWITZ and M. FLING, *J. Biol. Chem.* **172**, 651 (1948).
[7] H. J. TEAS, *J. Bacteriol.* **59**, 93 (1950).
[8] F. BINKLEY, *J. Biol. Chem.* **155**, 39 (1944).
[9] N. GROBBELAAR and F. C. STEWARD, *Nature* **182**, 1358 (1958).
[10] J. PRZYBYLSKA and J. PAWELKIEWICZ, *Bull. Acad. Polon. Sci.* Cl II, *Ser. Sci. Biol.* **13**, 327 (1965).

*The Influence of Night Temperature on the Concentration of Homoserine, O-acetyl Homoserine and Asparagine in Pea Shoots**

Four varieties of peas (Green Feast, Swartbekkie, Unica and Vinco) were grown for 77–78 days in a glass house in which the air temperature was maintained at 17° from 8 a.m. to 4 p.m. From 4 p.m. to 8 a.m., the plants were kept in darkrooms at air temperatures maintained at 4, 7, 10, 14, 17, 20, 23 and 26°, respectively, for the different treatments. The treatments commenced 7 days after the seeds were planted.

The shoots were macerated in about ten times their weight of 80% ethanol and after centrifugation the insoluble material was extracted twice with similar volumes of 80% ethanol at room temperature. The combined extract was concentrated near room temperature until free of ethanol and the residue was then extracted with chloroform until free of chlorophyll. The amino acid composition of the aqueous residue was determined by paper chromatography.[11] Apart from some unidentified compounds which were often detected as faint purple spots on the chromatograms, all extracts contained alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, glutamic acid, glycine, homoserine, leucine, lysine, proline, serine, threonine and valine. *O*-acetylhomoserine and pipecolic acid were found in nearly all extracts while phenylalanine and tyrosine were found less commonly, probably due to their rather weak colour production with ninhydrin.

The effect of the treatments on the absolute and relative concentration of asparagine, homoserine and *O*-acetylhomoserine in the shoots of the Unica plants are given in Figs. (1*a*) and (1*b*) respectively. The curves for the concentrations of the compounds in mg per unit dry weight of the shoots, are very similar to those which trace the changes in the relative concentrations of the compounds in the alcohol soluble (non-protein) nitrogen pool. Although only the data for the cultivar Unica are given, the corresponding curves for the Green Feast and Swartbekkie plants were generally very similar. The night temperature treatments had a clear influence on the absolute concentration and/or relative concentration of several other amino acids also, but these effects were generally smaller and were not consistent through all the varieties examined.

The contrasted effect of night temperature on the concentration of asparagine as compared to that of homoserine and *O*-acetylhomoserine, is compatible with the idea that these amino acids are competitively derived from a common precursor, such as aspartic acid. This view is supported because peas can convert aspartic acid to homserine.[5] In fact, Larson and Beevers[12] discuss the labelling of homoserine derived from aspartic acid. There should be little theoretical objection to assuming that *O*-acetylhomoserine is formed from homoserine in the pea since these two compounds are structurally so closely related. The fact that night temperature affects the concentration of homoserine and of *O*-acetylhomoserine in a similar manner suggests that these compounds occur *in vivo* in a dynamic state of metabolic equilibrium.

While the labile nature of *O*-acetylhomoserine might fit this compound to be a reactive acetyl carrier in metabolic reactions, it now seems to be implicated in other ways. Moore and Thompson[13] have described the enzymatic formation of methionine from *O*-acetylhomoserine and methyl mercaptan. Also *O*-acetylhomoserine and the appropriate sulphur

* The results given in this section are taken from the Ph.D thesis of one of us (N.G.); see Ref. 4.

[11] J. F. Thompson and F. C. Steward, *Plant Physiol.* **26**, 421 (1951).
[12] L. A. Larson and H. Beevers, *Plant Physiol.* **40**, 424 (1965).
[13] D. P. Moore and J. F. Thompson, *Biochem. Biophys. Res. Commun.* **28**, 474 (1967).

compounds may give rise, in spinach extracts, to homocysteine, methionine or ethionine,[14] and in *Neurospora* extracts to homocysteine and cystathionine.[15] Thus, current interest in *O*-acetylhomoserine and *O*-acetylserine, as well as their presumptive succinyl and oxalyl derivatives, appears to center around the role of acetyl CoA in the activation of homoserine and serine which could allow for their participation in reaction sequences which lead to methionine, *S*-methylcysteine, as well as to other important sulfur-containing compounds in plants.

The relationships between asparagine on the one hand and homoserine and *O*-acetylhomoserine on the other, which are so demonstrably a function of the environmental con-

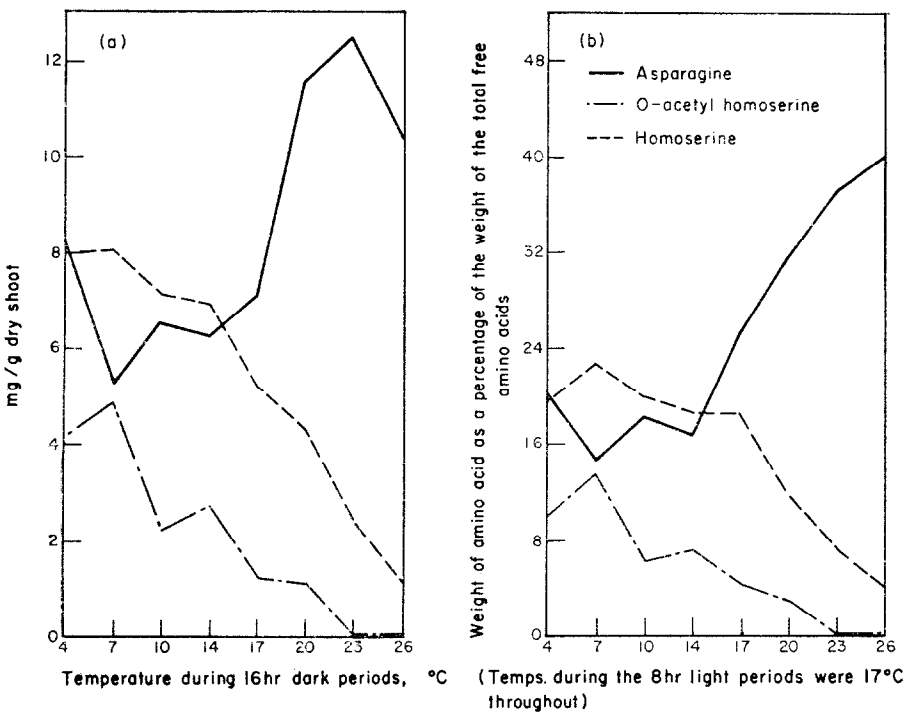

FIG. 1. THE INFLUENCE OF NIGHT TEMPERATURE APPLIED OVER A GROWING PERIOD OF 77 DAYS ON THE ABSOLUTE CONCENTRATION (a) AND RELATIVE CONCENTRATION (b) OF ASPARAGINE, HOMOSERINE AND *O*-ACETYLHOMOSERINE IN PEA SHOOTS

ditions that obtain during growth, provide an example of a widespread phenomenon. The biochemical outcome is not always, as indeed in this case, the consequence only of the genetically determined enzymic reactions which the plant can perform for there may also be overriding effects mediated by the interplay of environmental factors. This point was made earlier[16] in part upon the basis of the facts here reported. However, the means by which the environmental factors (such as night temperature) intervene to determine the metabolism of asparagine, homoserine and *O*-acetylhomoserine as alternative end products of metabolism

[14] J. GIOVANELLI and S. H. MUDD, *Biochem. Biophys. Res. Commun.* **31**, 275 (1968).
[15] D. S. KEER and M. FLAVIN, *Biochem. Biophys. Res. Commun.* **31**, 124 (1968).
[16] F. C. STEWARD, In *Environmental Control of Plant Growth*, (edited by L. T. EVANS), p. 206, Academic Press, New York (1963).

in peas must await further work. Since asparagine, on the one hand, and homoserine and its *O*-acetyl derivative on the other, seem to be alternative end products of metabolism in peas the further investigation of this system could help to elucidate the biosynthesis of asparagine, which is still not clear.

EXPERIMENTAL

Paper Chromatography

Whatman No. 1 filter paper, 18¼ in. × 22½ in., were used throughout except where otherwise mentioned. The full size of the papers was exploited to ensure the best possible resolution by using the descending technique and allowing the solvents to flow from the lower edge of the paper where necessary. Although various solvents were employed, phenol saturated with water and a 9:1:2·9 v/v mixture of *n*-butanol, acetic acid and water were routinely used. For two-dimensional paper chromatography, the phenol solvent was always used in the first, and longer, direction. For the qualitative detection of the amino acids on the chromatograms, the papers were irrigated with 0·1% ninhydrin in ethanol and heated for about 20 min at 60°. The method of Thompson and Steward[11] as standardized for later use in the laboratory of one of us (F.C.S.), was used for the quantitative determination of the amino acids.

Isolation Procedures

1·5 kg of freshly harvested shoots of flowering pea plants were macerated in 70% ethanol in a Waring blendor. The macerate was centrifuged, the extract concentrated at room temp. until free of ethanol and the aqueous residue was extracted with $CHCl_3$ until free of pigments. Alternatively, 4·5 kg of green peas in the pod, as marketed for human consumption, were shelled and only the pods (pericarps) were extracted by maceration in a Waring blendor with 70% ethanol yielding a filtrate which was concentrated. The aqueous residue was treated with an excess of a saturated solution of basic lead acetate. The resulting precipitate was removed by centrifugation and the supernatant treated with H_2S. The PbS that formed was removed by centrifugation and the supernatant was freed of excess H_2S by concentrating it at room temperature under reduced pressure.

In the preliminary work a small quantity of "Unknown 200" was isolated from the whole shoot extract by chromatographing a sample of the extract as a band in one direction on Whatman 3MM filter paper according to the method of Mueller.[17] The isolated band of substance 200 was eluted with water and the solution was concentrated. For the main isolation, the extracts were first fractionated on columns of ion exchange resins.[18] Although considerable fractionation was achieved by this procedure, it was necessary to chromatograph several of the fractions on cellulose powder columns using a 9:1:2·9 v/v mixture of *n*-butanol, acetic acid and water as the solvent, for complete separation of individual amino acids.

The isolated amino acids were in each case, dissolved in water, treated with charcoal at room temp. and the solutions were then filtered through celite. The filtrates were concentrated under reduced pressure until, by the gradual addition of one or more of the solvents methanol, ethanol, acetone and ether, crystallization commenced and was promoted by storage in the cold. The crystals were finally filtered and dried over $CaCl_2$.

Identifications and Criteria of Purity

The purity of all isolated samples was checked by paper chromatography in phenol-water, butanol-acetic acid-water, collidine-lutidine-water (1:3:2 v/v) and toluene-pyridine-water (2:15:7 v/v). In all cases the identity of the isolated compounds was established by co-chromatography with authentic synthetic materials. The m.p.s of all isolates which melted below 300° were compared with m.p.s of the corresponding authentic synthetic compounds determined on the same apparatus. I.r. spectra were obtained of all isolates and the corresponding synthetic L-isomeric forms of the compounds in question.

Analytical Data on Isolated Compounds

Homoserine. Yield=1·1 g, m.p. 218°. Found: C=40·09; H=7·50; N=11·86. Calc. for $C_4H_9O_3N$: C=40·33; H=7·62; N=11·76%. $[\alpha]_D^{20} = -8·5°$; conc. 214·9 mg/10 ml water.

Leucine. Yield=52 mg. Sublimes rapidly above 200°. Found: C=54·55; H=9·80; N=10·43. Calc. for $C_6H_{13}NO_2$: C=54·94; H=9·99; N=10·68%. $[\alpha]_D^{20} = +20°$; conc. 33 mg/10 ml HOAc.

Valine. Yield=50 mg. Does not melt below 300°. Found: C=50·89; H=9·02; N=11·50%. Calc. for $C_5H_{11}NO_2$: C=51·26; H=9·46; N=11·69%. $[\alpha]_D^{20} = +65°$; conc. 30 mg/10 ml HOAc.

Alanine. Yield=470 mg. Sublimes readily above 200°. Found: C=39·84; H=7·67; N=15·74. Calc. for $C_3H_7O_2N$: C=40·44; H=7·92; N=15·72%. $[\alpha]_D^{20} = +35°$; conc. 80 mg/10 ml HOAc.

[17] J. H. MUELLER, *Science* **112**, 405 (1950).
[18] C. W. HIRS, S. MOORE and W. H. STEIN, *J. Biol. Chem.* **195**, 669 (1952).

Aspartic acid. Yield=35 mg. Decomposes gradually without a clear m.p. Found: C=35·91; H=5·38; N=10·84. Calc. for $C_4H_7O_4N$: C=36·09; H=5·30; N=10·52%. $[\alpha]_D^{20}=+24·8°$; conc. 20 mg/10 ml 5N HCl.

Glutamic acid. Yield=105 mg, m.p. 213°. Found: C=40·52; H=6·14; N=9·47. Calc. for $C_5H_9O_4N$: C=40·82; H=6·17; N=9·52%. $[\alpha]_D^{20}=+33·9°$; conc. 58 mg/10 ml 5N HCl.

γ-Aminobutyric acid. Yield=229 mg, m.p. 216°. Found: C=45·96; H=8·58; N=13·46. Calc. for $C_4H_9O_2N$: C=46·59; H=8·80; N=13·58%. $[\alpha]_D^{20}=0°$; conc. 50 mg/10 ml H_2O.

Unknown 200 (*O-acetylhomoserine*). Yield=65 mg, m.p. 200°. Found: C=45·01; H=6·80; N=8·90; OAc=26·94. Calc. for $C_6H_{11}O_4N$: C=44·72; H=6·88; N=8·69; OAc=26·71%. $[\alpha]_D^{20}=+4·5°$; conc. 40 mg/10 ml H_2O.

The Properties of O-Acetylhomoserine Isolated from Peas

The colourless isolate gave a purple colour on paper chromatograms when treated with ninhydrin; it also gave a positive test for primary amino groups and a negative test for secondary amino groups.[19] An aqueous solution of the compound was neutral and its chromatographic behaviour on both paper and ion-exchange resin columns was consistent with its being a neutral amino acid. The compound was insoluble in camphor and consequently its mol. wt. could not be determined by the Rast method. The compound was unaffected in its paper chromatographic behaviour by H_2O_2 oxidation in the presence of ammonium molybdate.[20]

Samples of Unknown 200 were hydrolyzed with 6N HCl and a 5% solution of $Ba(OH)_2$ in sealed tubes for 16 hr at 100°. The excess acid was removed by concentration and the $Ba(OH)_2$ by precipitation as $BaSO_4$. Two-dimensional paper chromatograms of the hydrolysates were prepared both before and after the hydrolysates were oxidized with H_2O_2 in the presence of ammonium molybdate. Phenol and butanol-acetic acid were used as the solvents. The H_2O_2 oxidation did not have any effect on the hydrolysates. In the case of the alkaline hydrolysate, only one strong purple spot was found on the chromatograms in the position normally occupied by homoserine. In the case of the acid hydrolysate, a similar spot was found in the position normally occupied by homoserine, but there was also a long diffuse spot (designated 201) which extended from the location of homoserine almost to the phenol front.

Comparative chromatography of the hydrolysates and an authentic sample of synthetic homoserine, using the four standard solvents mentioned above, confirmed the identification of the one degradation product as homoserine. The other, diffuse spot (No. 201), obtained after acid hydrolysis, proved to be due to the lactone of homoserine. After heating a solution of homoserine in 6N HCl it gave a chromatogram that was similar to that obtained from the acid hydrolysate of Unknown 200, suggesting that spot 201 was derived from homoserine during the acid hydrolysis. Furthermore, comparative chromatography of the lactone of homoserine, which was furnished by Dr. J. K. Pollard, then working with one of us (F.C.S.), confirmed that spot 201 was in fact due to the lactone of homoserine.

When Unknown 200 was dissolved in 6N NH_4OH at room temperature and this solution was chromatographed on paper it was found that no ninhydrin-reactive spot could be detected on the chromatogram. Indeed, if an aqueous solution of substance 200 was spotted on filter paper and the spot was exposed to the vapours of conc. NH_4OH for a few seconds before chromatography, only a small fraction of the expected ninhydrin colour could be produced in the area normally occupied by 200 and no other ninhydrin reactive spots appeared on the chromatogram. This result is consistent with the observed decrease in the ninhydrin colour that is obtained when substance 200 is chromatographed on paper using alkaline solvents such as collidine-lutidine in contrast to such slightly acidic solvents as butanol-acetic acid.

All the evidence on the identity of Unknown 200 is consistent with its being an acetyl derivative of homoserine. Whereas the *N*-acetyl derivative should be acidic and react poorly, if at all, with ninhydrin on paper, the *O*-acetyl derivative should be neutral and should show a normal α-amino acid reactivity towards ninhydrin. Furthermore, the acetyl group of *O*-acetyl homoserine might migrate to the amino group at room temperature in alkaline solution (cf.[21]). Although all the evidence thus far was consistent with Unknown 200 being *O*-acetyl homoserine final identification, especially of the stereochemical configuration of the natural compound, required the synthesis of one of the optically active isomers and also a comparison with *N*-acetyl homoserine.

Synthetic O-*acetyl*-L-*Homoserine*

O-acetyl-L-homoserine was synthesized from 0·98 g of L-Homoserine (Calbiochem Corp.), by the general method of Sakami and Toennies[22] as adapted by Matsuo *et al.*[23] Crystallization of the *O*-acetyl-L-homoserine (900 mg) from the final reaction mixture was effected by the addition of acetone and ether. After several recrystallizations from water by the addition of acetone and ether, a final crop of colourless crystals with constant m.p. at 199° and the following properties was obtained: Yield: 400 mg. Found: C=44·63; H=7·47;

[19] F. FEIGL, *Spot tests Vol. II*, Elsevier, N.Y. (1954).
[20] C. E. DENT, *Biochem J.* **43**, 169 (1948).
[21] S. SAKAKIBARA, K. H. SHIN and G. P. HESS, *J. Am. Chem. Soc.* **84**, 4921 (1962).
[22] W. SAKAMI and G. TOENNIES, *J. Biol. Chem.* **144**, 203 (1942).
[23] Y. MATSUO, M. ROTHSTEIN and D. M. GREENBERG, *J. Biol. Chem.* **221**, 679 (1956).

N=8·76; OAc=26·53. Calc. for $C_6H_{11}O_4N$: C=44·72; H=6·88; N=8·69; OAc=26·71. $[\alpha]_D^{20} = +3·9°$; conc. 168 mg/10 ml H_2O.

The synthetic material was insoluble in camphor, gave a positive test for primary amino groups but a negative test for secondary amino groups. It was found to be neutral and to be chromatographically homogenous. The synthetic *O*-acetyl-L-homoserine was identical to Unknown 200 since (*a*) i.r. spectra of both compounds had the characteristic strong absorption peak for an ester linkage at 5·75 μ while the corresponding peak for a substituted amide at 6·00 μ was not present; (*b*) their paper chromatographic behaviour in four solvents was identical; (*c*) they were both unstable to acid and alkaline hydrolysis with the production of homoserine and were similarly unstable in alkaline solution at room temperature.

N-acetyl Derivatives of Homoserine, Glycine, Alanine, Methionine and Valine

The *N*-acetyl derivative of DL-homoserine (Calbiochem. Corp) was prepared by the method of du Vigneaud *et al.*[24] The *N*-acetyl derivatives of the other amino acids were purchased from Calbiochem Corp. These compounds all gave an acid reaction when dissolved in water, and although they could not be detected on chromatograms by the ninhydrin reaction, their location on chromatograms could readily be detected by spraying the chromatogram with pH indicators such as a 0·005% ethanolic solutions of neutral red or methyl red. Apart from their inactivity toward ninhydrin, all the *N*-acetyl amino acids could readily be separated from *O*-acetylhomoserine by paper chromatography. The identification of Unknown 200 from the shoots of the pea plant as L(+)-*O*-acetyl homoserine is, therefore, regarded as being complete.

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[24] V. DU VIGNEAUD and C. E. MEYER, *J. Biol. Chem.* **98**, 295 (1932).