Phytochemistry, 1975, Vol. 14, pp. 1591–1596. Pergamon Press. Printed in England.

HYDROGENATION AS AN APPROACH TO STUDY OF REACTIONS OF OXIDIZING POLYPHENOLS WITH PLANT PROTEINS

ROGER DAVIES, WILLIAM M. LAIRD and RICHARD L. M. SYNGE
Agricultural Research Council's Food Research Institute, Colney Lane, Norwich NR4 7UA, England

(*Received* 14 *December* 1974)

Key Word Index—Hydrogenation; lysine; polyphenols; potato; proteins; quinonoids; rhodium.

Abstract—Coupling of oxidation products of *o*-diphenols with $-NH_2$ groups of plant proteins can damage nutritional availability of lysine residues. Relevant model coupling products (before or after reductive acetylation or permethylation) are unstable to acid hydrolysis. Hydrogenation over Rh/Al_2O_3, at room temperature and atmospheric pressure, gave cyclohexane derivatives stable to hydrolysis and retaining, with only partial hydrogenolysis, all groups originally attached to the aromatic nucleus. Plant bulk proteins were hydrogenated with substantial conversion of their aromatic amino acids; their S-containing amino acids were desulphurized. The technique is therefore promising for study of the fate of lysine residues in "enzymically browned" proteins.*

INTRODUCTION

o-Diphenols are ubiquitous in plant materials and readily undergo enzymic or spontaneous oxidation to *o*-semiquinone radicals or to *o*-quinones. These products, besides themselves undergoing polymerization accompanied by further oxidation, can couple covalently with various functional groups found in proteins [1,2]; these include amine, thiol, thioether [3,4] and indole groups, perhaps also imidazole groups and peptide bonds [5]. Such reactions thus particularly involve the nutritionally important amino acid residues lysine, cyst(e)ine, methionine and tryptophan. Oxidation by semiquinones or quinones of thiol, disulphide, thioether and indole groups may also be envisaged; one possible product of such reactions, the cysteinesulphinic acid residue, would be expected to couple with quinones to give a sulphone, and coupling reactions by residues of methionine sulphoxides [6–8] or of "dehydromethionine" [9–12] are conceivable.

Despite these known and conceivable chemical reactions, this type of damage to proteins has been completely ignored in textbooks of nutrition. Direct evidence for it has been produced by Horigome and colleagues from experiments with casein [5,13 *cf.* 14]. Indirect evidence has been obtained in this laboratory with leaf-protein concentrates [15]. One of us has recently reviewed other relevant literature [16].

RESULTS AND DISCUSSION

Because content and availability of lysine are critical in the protein-rich supplements to basal cereal diets used for pig and poultry feeding, we decided to concentrate our studies on reactions of primary aliphatic amines. Coupling products of *o*-benzoquinone with simple alkylamines have been studied by one of us [17]. Cranwell and Haworth [18 *cf.* 19] have studied adducts of quinones with amino acid and peptide esters, and have pointed out that the bond between the cyclic nucleus and the imino group is a vinylogous

---

* In a Supplementary Publication, W.M.L. describes N^{δ},N^{1}-diacetyl-DL-5-(δ-cyclohexylaminobutyl)hydantoin, DL-N^{ϵ}-phenyllysine and its hydantoin (Annex 1); MS results are reported by R. Self and J. Eagles (Annex 2) and ion-exchange chromatography by A. M. C. Davies (Annex 3). SUP XXXXX (18 pp) deposited at the National Lending Library, Boston Spa, Yorkshire LS23 7BQ, England, from whom copies can be obtained.

amide bond and, as such, liable to hydrolysis. Under the conditions used for hydrolysis of proteins, Cranwell and Haworth got 20–86% recovery of the thus-linked amino acids, along with some ammonia etc. They got better recovery of an amino acid residue second in the peptide chain (*cf.* Ref. 20). We hoped that, by conversion of the Fischer–Schrader adduct **1** to **2** (no longer a vinylogous amide) by reductive acetylation [18,21], we could then hydrolyse and obtain the *bis*-imino acid **3**. In a sealed evacuated tube, the acid hydrolysate of **2** remained colourless, but darkened rapidly on opening; amino acid analysis revealed much glycine and NH_3, but no novel amino acid. Permethylation [22] of **1** or **2** did not, in our hands, lead to any well-defined product. Brieskorn and Mosandl [23] tried to stabilize the glycine adduct of 4-methyl-1,2-benzoquinone by reduction with borohydride followed by methylation. However, the N–C bond of the glycine moiety split, apparently by Strecker degradation, and formaldehyde and 4,5-dimethoxy-2-methylaniline resulted.

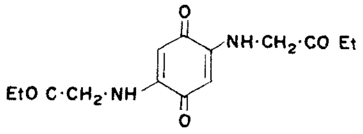
In view of this lability of the linkage of amine moieties with aromatic nuclei, and in the hope of preventing hydrolytic cleavage and oxidative attack at this point, we considered converting the aromatic nuclei to cyclohexane rings by catalytic hydrogenation. This would have the disadvantage of introducing unpredictable diastereoisomerism. However, we intend to rely chiefly on electrophoresis, LLC or GLC, followed by MS, as analytical techniques, and none of these is very sensitive to diastereoisomerism. A more serious expected trouble would be hydrogenolysis of amino and hydroxyl groups from the cyclohexane ring, with consequent loss of information about the original aromatic structure. We could also envisage that cyclohexane rings heavily substituted with amino and hydroxyl groups would be unstable under hydrolytic conditions, but in this matter we are encouraged by the great acid stability of such residues in a number of aminocyclitol antibiotics [24,25].

Reviews on catalytic hydrogenation [26,27] suggest that rhodium and ruthenium catalysts convert aromatic structures with minimal hydrogenolysis. We tried Rh–Al_2O_3 in 70% (v/v) aq. acetic acid with various model compounds, and achieved encouraging results using H_2 at room temperature and atmospheric pressure.

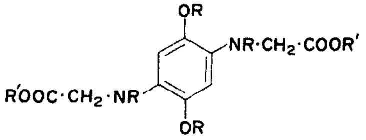
The *acetylated hydantoin of* DL-N$^{\varepsilon}$-*phenyllysine* (**4**) hydrogenated slowly to the corresponding *N*-cyclohexyl compound, which was identified with synthetic material prepared by a different route.

The *Fischer–Schrader adduct* (**1**) slowly took up more than the theoretical 4 mol prop. H_2 to give **5**, accompanied by some glycine ester etc.; **5** was acetylated to **6** for MS study. The free imino acid **7**, obtained from **5** by acid or cold-alkaline hydrolysis, was partially characterized. By contrast, *reductively-acetylated* (**1**) (**2**) was completely inert, giving no H_2 uptake, nor could any **6** be detected by MS of the reaction product, whose MS did not differ from that of **2** [18]. Perhaps substitution of the ring with two pairs of bulky *O*-substituents prevented its approach to the catalyst surface. However, this did not prevent reaction of *OO'*-diacetylcaffeic acid nor of **9** (see below).

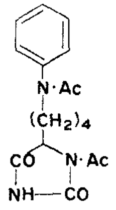
Caffeic acid hydrogenated quickly to give 3-(3',4'-dihydroxycyclohexyl)propionic acid, which



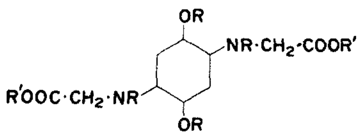
(**1**)



(**2**) R = Ac; R' = Et
(**3**) R = R' = H



(**4**)



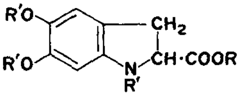
(**5**) R = H; R' = Et
(**6**) R = Ac; R' = Et
(**7**) R = R' = H

was characterized by MS before and after *O*-acetylation. No hydrogenolysis of the phenolic groups had occurred. By contrast, *OO′-diacetylcaffeic acid* took up more than theoretical H_2; MS of the reaction product suggested the additional presence of 3-cyclohexylpropionic acid, indicating partial hydrogenolysis of the acetoxy groups.

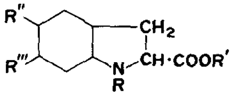
Acetylated DL-*cyclodopa methyl ester* (**8**) hydrogenated quickly and showed partial hydrogenolysis of the –*N*– and –*O*– groupings attached to the C_6 ring. MS showed no unconverted starting material and gave evidence for the occurrence, in addition to the expected **9**, of **10**–**16**. Ion-exchange chromatography of an acid hydrolysate of the reaction mixture gave a complicated series of ninhydrin-brown and ninhydrin-purple zones corresponding respectively (and in likely positions) to diastereoisomers of the imino and amino acids that would be produced by removal of all acetyl and methyl groups from **9** to **16**.

We tentatively conclude that acetyl groups close to the aromatic nucleus slow down reaction and promote hydrogenolysis. They should therefore be avoided unless absolutely necessary for stabilization of the group under investigation. Perhaps **17** hydrochloride [28,29] would have shown less hydrogenolysis than did **8**. In no case was any substituent group *completely* lost by hydrogenolysis.

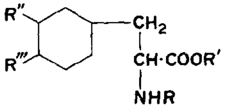
Model studies on the aromatic amino acids of proteins showed that: 3-*phenylalanine* hydrogenated smoothly to 3-cyclohexylalanine (**18**); *tyrosine* (which was hydrogenated as its N-*acetyl derivative* for reason of solubility) smoothly to diastereoisomers of 3-(4′-hydroxycyclohexyl)-alanine (**19**) with partial hydrogenolysis to **18** (*cf.* Refs. 30, 31, 44); *tryptophan* sluggishly to diastereoisomers of octahydrotryptophan, with perhaps partial lactamization to **20**.



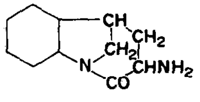
(**8**) R = Me; R′ = Ac
(**17**) R = R′ = H



(**9**) R = Ac; R′ = Me; R″ = R‴ = AcO
(**10**) R = Ac; R′ = Me; R″ = H ;= R‴ = AcO
(**11**) R = Ac; R′ = Me; R″ = AcO; R‴ = H
(**12**) R = Ac; R′ = Me; R″ = R‴ = H



(**13**) R = Ac; R′ = Me; R″ = R‴ = AcO
(**14**) R = Ac; R′ = Me; R″ = H; R‴ = AcO
(**15**) R = Ac; R′ = Me; R″ = AcO; R‴ = H
(**16**) R = Ac; R′ = Me; R″ = R‴ = H
(**18**) R = R′ = R″ = R‴ = H
(**19**) R = R′ = R″ = H; R‴ = HO



(**20**)

In attempting to apply this treatment to proteins, we took account especially of the work of Keil and colleagues [31–3]. They had to desulphurize with Raney nickel before they could use PtO_2 for hydrogenating the aromatic amino acid residues. Our impression has been that our Rh catalyst was initially poisoned by the S compounds of protein, while desulphurizing them; cyst(e)ine was replaced by alanine and methionine by 2-aminobutyric acid. Repeated additions of small lots of catalyst resulted in 30–70% conversions of the aromatic amino acid residues. The desulphurization makes this approach *prima facie* unsuitable for studying quinone reaction products of cyst(e)ine or methionine.

Laird *et al.* [34] have developed a convenient procedure for isolating bulk-protein fractions from plant material, which gives the protein in solution in phenol–acetic acid–H_2O. We have found that phenol can conveniently be removed by dialysis of such solutions in cellophan against 70% (v/v) aqueous acetic acid. The protein remains well in solution and, after hydrogenation, not too much of it is lost on the catalyst. Hydrogenation of bulk protein of potato tuber is described in the Experimental; the above-mentioned changes of amino acid composition were observed. With protein isolated from plant materials in which "enzymic browning" had occurred, further novel components have been noted after hydrogenation and hydrolysis. It is hoped to describe these in future publications.

Such a technique may also prove useful for

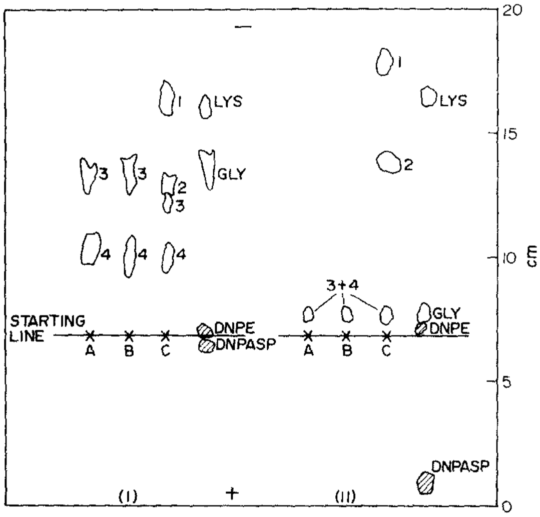


Fig. 1. High-voltage filter-paper electrophoresis of hydrogenated **1** after various treatments. Each spot was derived from 7·5 μg **1**. The hydrogenation mixture was: A, refluxed 18 hr in 6 N HCl, with subsequent evaporation to dryness; B, saponified in 0·06 N $Ba(OH)_2$ at room temp. overnight with subsequent addition of H_2SO_4 equiv. to the $Ba(OH)_2$ before evaporation to dryness; C, not further treated. Electrophoresis [43] was for 15 min at 5 kV in: (I) aq. soln of pyridine (2·5% v/v) and HOAc (0·3% v/v) (pH *ca* 6); (II) aq. HOAc (8·7% v/v) contg. 89% (w/v) formic acid (2·5% v/v) (pH *ca* 1·8). A mixed soln of *N*-2,4-dinitrophenylaspartic acid (DNPASP), *N*-2,4-dinitrophenylethanolamine (DNPE), glycine and DL-lysine dihydrochloride was used as marker. Yellow visible spots are hatched. Other spots appeared after spraying with ninhydrin soln, allowing time for colour development at room temp., and heating next day 5 min at 110°. 1 (brown) are attributed to glycine ethyl ester; 2 (pale pink, only visible after heating) to **5**; 3 to glycine; 4 (pale pink, only visible after heating) to **7**.

recognizing residues of cinnamic acid congeners which are so linked in plant materials as to be unstable to ordinary hydrolytic procedures [45].

EXPERIMENTAL

Materials. **1** and **2** were gifts from Prof. R. D. Haworth. The N^{ϵ}-substituted lysine derivatives were synthesized by W.M.L. (Annex 1). *OO'*-Diacetylcaffeic acid [35] was a gift from Mr. E. I. Mbadiwe. **8** was a gift from Prof. H. Wyler. *N*-Acetyl-L-tyrosine was prepared from L-tyrosine [36]. The hydrogenation catalyst was "rhodium 5% on alumina; catalyst grade" (Koch–Light). Other chemicals were obtained commercially.

Methods: Hydrogenation. A ground-glass-jointed Erlenmayer flask (5 ml, equipped with polypropylene-sheathed magnetic stirrer) was connected, through a vaseline-greased cone mounted on narrow-bore glass tubing, to the side tube of the reaction chamber of the Van Slyke–Neill [37] manometric apparatus (minimal length of rubber pressure tubing at joint). The specimen for hydrogenation [in 1 ml 70% (v/v) aq. HOAc] and a charge of catalyst (45 mg) were placed in the flask, which was then connected to the apparatus and evacuated by 5 strokes of Hg through the reaction chamber. In the course of evacuation, gas vol. in side arm and flask was determined. H_2 (*ca.* 30 ml) was then admitted from a Hempel pipette over dil. aq. $CuSO_4$, and initial gas vol. ascertained manometrically (Hg at 50 ml mark). H_2 pressure was returned to atmospheric and stirring commenced, from which moment reaction time was reckoned. Stirring was stopped at intervals for reading uptake (Hg at 50 ml mark). Vols. were all reduced to N.T.P. Loss of H_2 by diffusion through the rubber joint was 0·2 ml/day and one charge of catalyst could show immediate sorption of up to 0·9 ml H_2 in absence of any hydrogenation. Unless otherwise stated, H_2 uptakes have been corrected for these losses. At the end of reaction, the mixture was rinsed, using portions of 70% HOAc amounting to 9 ml, into a stoppered polypropylene centrifuge tube, the catalyst was centrifuged off, and portions of the supernatant taken for further study. *Acetylation* was done on reaction products etc., evaporated to dryness, as for ascorbalamic acid [38]. *Evaporations* were conducted *in vacuo* below 40° (except HCl hydrolysates). N was determined by a micro-Kjeldahl procedure.

Details of hydrogenations: The hydantoin of DL-N^{ϵ}-*phenyllysine* (76·3 mg) was acetylated; **4** did not crystallize, so the crude product was taken for hydrogenation. After 5 days, 2·7 mol prop. H_2 had been taken up. MS of the reaction product showed no starting material; the spectrum closely agreed with that of diacetyl-5-(δ-cyclohexylaminobutyl)hydantoin but showed a few lines >M. There was no sign of hydrogenolysis to diacetyl-lysine hydantoin (Annex 2). An impurity was precipitated from the reaction product in ethanol by ether. The filtrate gave crystals from EtOH–H_2O (31 mg, mp 127–9° uncorr), which did not on admixture depress the mp of the directly prepared cyclohexyl compound (Annex 1). Hydrolysis (96 hr in 6 M HCl at 110°) [39] of the reaction product from a less complete hydrogenation gave, on ion-exchange chromatography, zones corresponding both with N^{ϵ}-phenyllysine and N^{ϵ}-cyclohexyllysine (Annex 3).

The Fischer–Schrader adduct (**1**) (14·9 mg) was not readily soluble in the hydrogenation medium, but after 10 min all red colour and crystals had disappeared. After 2 days, 4·6 mol prop. H_2 had been taken up. On acetylation, MS was obtained consistent with **6** not obviously contaminated with hydrogenolysis products; however, immediately after insertion the spectrum of the more volatile *N*-acetylglycine ethyl ester [40] was observed (Annex 2). On high-voltage filter-paper electrophoresis, the hydrogenation mixture and its hydrolysis products (cold alkali and hot acid) gave the results shown in Fig. 1. By contrast, **2** (12·2 mg) showed no H_2 uptake; the reaction product after 4 days gave MS indistinguishable from that of the starting material [18].

Caffeic acid (20·0 mg) took up 4·0 mol prop. H_2 at a steady rate over 2·5 hr, after which there was no further uptake. A sample of evaporated reaction product was extracted from 0·1 M HCl into ether and subjected to MS. A further portion of this ether extract was acetylated previous to MS. *OO'-Diacetylcaffeic acid* (29·7 mg) took up 5·4 mol prop. H_2 over 3·2 hr, after which there was no further uptake. A sample of the reaction product was subjected to MS. There was no MS evidence for unchanged starting material in either hydrogenation product (Annex 2).

*Triacetyl-*DL*-cyclodopa methyl ester* (**8**) (9·8 mg) took up 4·1 mol prop. H_2 over 2·5 hr, with no further uptake. A portion of the reaction product was taken for MS (Annex 2). A further portion was hydrolysed, after evaporation to dryness, in a sealed evacuated tube in 6 M HCl for 24 hr at 110°. The pale-pink hydrolysate was evaporated and subjected to ion-exchange chromatography (Annex 3).

DL-3-*Phenylalanine* (46·7 mg) took up 2·7 mol prop. H_2 at a steady rate. After leaving overnight there was no further uptake. A portion of evaporated reaction product was converted to the methyl ester [41], then acetylated and the product subjected to MS (Annex 2). A further portion of evaporated reaction product was subjected to ion-exchange chromatography (Annex 3).

N-*Acetyl*-L-*tyrosine* (40·4 mg) took up 2·95 mol prop. H_2 at a steady rate over 2 hr., after which there was no further uptake. A portion of evaporated reaction product was derivatized and subjected to MS as for the product from phenylalanine above (Annex 2). A further portion was hydrolysed in 6 M HCl 24 hr at 110°. The hydrolysate was evaporated and subjected to ion-exchange chromatography (Annex 3).

DL-*Tryptophan* (40·7 mg) took up 3·71 mol prop. H_2 over 3 days, after which there was no further uptake. A portion of evaporated reaction product was derivatized and subjected to MS as for the product from phenylalanine above (Annex 2). A further portion was subjected to ion-exchange chromatography (Annex 3).

Bulk protein of potato tuber. This fraction (CV *Orion*, 1973 harvest) (9·0% of tuber DM; 43·3% of tuber N; N, 9·18% of fraction DM) in PhOH–HOAc–H_2O [34, 42] was dialysed in cellophan tube against successive replacements of 70% (v/v) aq. HOAc A.R. until phenol was absent from the diffusate. The bag contents (DM, 48% of DM taken for dialysis; N, 14·9% of fraction DM) were slightly opalescent. An amount of soln containing 20·5 mg DM was evaporated to small vol. and made to *ca* 1 ml 70% (v/v) aq. HOAc by appropriate additions of HOAc and H_2O. The opalescence cleared somewhat. On hydrogenation, 0·54 ml H_2 (uncorr) was taken up in the first 30 min, after which there was no further uptake for 24 hr. On adding a fresh charge of catalyst, 1·6 ml H_2 (uncorr) was taken up over 5 days. (In subsequent work with plant proteins, charges of catalyst were added daily, with at first increasing H_2 uptakes, until uptake substantially diminished; hydrogenation of phenylalanine and tyrosine residues could exceed 70% under these conditions.) A portion of the reaction supernatant was hydrolysed, after addition of 1·5 vols. 10 M HCl, in a sealed evacuated tube for 24 hr at 110°. The hydrolysate was subjected to amino acid analysis (Annex 3).

Acknowledgements—We thank Messrs. J. Eagles and R. Self for MS (Annex 2); Mr. A. M. C. Davies for ion-exchange chromatography (Annex 3); Dr. A. J. Bailey, Prof. R. D. Haworth, Dr. S. M. Partridge and Dr. W. S. Pierpoint for valued discussions; Prof. R. D. Haworth, Mr. E. I. Mbadiwe, Prof. W. T. J. Morgan and Prof. H. Wyler for gifts of specimens; Miss Mary M. Gilmour and Mr. P. Winsworth for skilful assistance.

Supplementary Publication Scheme—Information which supplements this article has been deposited with the National Lending Library, Boston Spa, Yorkshire LS23 7BQ, England. This supplementary information is available as microfiche or as enlargements from the Library's photocopying services.

REFERENCES

1. Pierpoint, W. S. *Rep. Rothamsted Exp. Stn. for* 1970, Part 2, 199.
2. Horspool, W. M. (1969) *Quart. Rev. Chem. Soc.* **23**, 204.
3. Vithayathil, P. J. and Murthy, G. S. (1972) *Nature New Biology* **236**, 101.
4. Bosshard, H. (1972) *Helv. Chim. Acta* **55**, 32.
5. Horigome, T. (1973) *Eiyo To Shokuryo* **26**, 257.
6. Kuhn, R. and Trischmann, H. (1958) *Ann. Chem.* **611**, 117.
7. Smith, S. G. and Winstein, S. (1958) *Tetrahedron* **3**, 317.
8. Kamiyama, K., Minato, H. and Kobayashi, M. (1973) *Bull. Chem. Soc. Japan* **46**, 3895.
9. Lavine, T. F. (1945) *Federation Proc.* **4**, 96.
10. Lavine, T. F. (1949) U.S. Pat. 2, 465, 461.
11. Gensch, K.-H. and Higuchi, T. (1967) *J. Pharm. Sci.* **56**, 177.
12. Möhrle, H. (1967) *Dtsch. Apotheker-Ztg* **107**, 781.
13. Horigome, T. and Kandatsu, M. (1968) *Agr. Biol. Chem. Tokyo)* **32**, 1093.
14. Wehr, H. M. (1973) *Dissertation Abstr. B.* **33**, 5666.
15. Allison, R. M., Laird, W. M. and Synge, R. L. M. (1973) *Brit. J. Nutr.* **29**, 51.
16. Synge, R. L. M. (1975) *Naturw. Rundschau*; *Qualitas Plant. Mater. Vegetabiles* in press.
17. Davies, R., unpublished work.
18. Cranwell, P. A. and Haworth, R. D. (1971) *Tetrahedron* **27**, 1831.
19. Morgan, W. T. J. *J. Chem. Soc.* **1926**, 79.
20. Perry, D. R. and Adams, W. A. (1971) *Biochem. J.* **125**, 29P.
21. Brockmann, H. and Budde, G. (1953) *Chem. Ber.* **86**, 432.
22. Eagles, J., Laird, W. M., Self, R. and Synge, R. L. M. (1974) *Biomed. Mass Spectrom.* **1**, 43.
23. Brieskorn, C. H. and Mosandl, A. (1973) *Arch. Pharm.* **306**, 164.
24. Dutcher, J. D. (1963) *Advan. Carbohydrate Chem.* **18**, 259.
25. Kojima, M., Inouye, S. and Niida, T. (1973) *J. Antibiotics (Tokyo)* **26**, 246.
26. Augustine, R. L. (1965) *Catalytic Hydrogenation*, Edward Arnold, London.
27. Rylander, P. N. (1967) *Catalytic Hydrogenation over Platinum Metals*, Academic Press, New York.
28. Wyler, H. and Chiovini, J. (1968) *Helv. Chim. Acta* **51**, 1476.
29. Kaiser, A., Koch, W., Scheer, M. and Wölcke, U. (1973) *Swiss Pat.* 542, 202.
30. Billman, J. H. and Buehler, J. A. (1953) *Proc. Indiana Acad. Sci.* **63**, 120.
31. Keil, B., Zikán, J., Rexová, L. and Šorm, F. (1962) *Collection Czech. Chem. Commun.* **27**, 1678.
32. Keil, B. and Šorm, F. (1962) *Collection Czech. Chem. Commun.* **27**, 1673.
33. Keil, B., Morávek, J., Dlouhá, V. and Filip, J. (1962) *Collection Czech. Chem. Commun.* **27**, 1687.
34. Laird, W. M., Mbadiwe, E. I. and Synge, R. L. M., unpublished work.
35. Tiemann, F. and Nagai, N. (1878) *Ber. Deut. Chem. Ges.* **11**, 646.
36. Vigneaud, V. du and Mayer, C. E. (1932) *J. Biol. Chem.* **98**, 295.
37. Peters, J. P. and Van Slyke, D. D. (1932) *Quantitative Clinical Chemistry*, Vol. 2, p. 267, Bailière, Tindall and Cox, London.
38. Couchman, R., Eagles, J., Hegarty, M. P., Laird, W. M., Self, R. and Synge, R. L. M. (1973) *Phytochemistry*, **12**, 707.
39. Stark, G. R. and Smyth, D. G. (1963) *J. Biol. Chem.* **238**, 214.
40. Andersson, C.-O., Ryhage, R. and Stenhagen, E. (1962) *Arkiv. Kemi*, **19**, 417.
41. Senn, M., Venkataraghavan, R. and McLafferty, F. W. (1966) *J. Am. Chem. Soc.* **88**, 5593.

42. Davies, A. M. C. and Laird, W. M., unpublished work.
43. Bailey, J. L. (1962) *Techniques in Protein Chemistry*, p. 40, Elsevier, Amsterdam.
44. Phillips, M. B. and Anker, H. S. (1957) *J. Biol. Chem.* **227**, 465.
45. Van Sumere, C. F., Albrecht, J., Dedonder, A., de Pooter, H. and Pé, I. (1975) *Chemistry and Biochemistry of Plant Proteins* (Harborne, J. B. and Van Sumere, C. F., eds.), p. 211, Academic Press, London.