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SYNTHESIS OF α- AND β-*N*-OXALYL-L-α,β-DIAMINOPROPIONIC ACIDS AND THEIR ISOLATION FROM SEEDS OF *LATHYRUS SATIVUS*

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Abstract—The α- and β-*N*-oxalyl derivatives of L-α,β-diaminopropionic acid have been chemically synthesized and also isolated from seed extracts of *Lathyrus sativus*. Chemical and physical properties of the natural and synthetic isomers were in good agreement. The toxicity of the α-isomer to chicks was evaluated and compared with that of the β-isomer.

INTRODUCTION

The consumption of *Lathyrus sativus* seeds by human populations is correlated with the incidence of neurolathyrism, a chronic paralysing disease [1–3]. A number of substances acutely toxic to experimental animals have been isolated from *L. sativus* seeds, including β-*N*-oxalyl-L-α,β-diaminopropionic acid [4, 5]; *N*-β-D-glycopyranosyl-*N*-α-L-arabinosyl-α,β-diaminopropionitrile [6, 7] and phenolic material of unknown structure [8]. There is strong experimental evidence that β-*N*-oxalyldiaminopropionic acid is the principal causative agent in neurolathyrism [9, 10], but the mechanism of action of this compound is unknown and it is uncertain whether neurolathyrism is caused by the compound alone.

L. sativus seed also contains α-*N*-oxalyl-L-α,β-diaminopropionic acid [11–13], although the quantity in fresh seed extracts is small. However, the α- and β-*N*-oxalyl derivatives of α,β-diaminopropionic acid readily interconvert and the process is accelerated by heating [11]. Hence the α-isomer probably constitutes a significant proportion of the total quantity of *N*-oxalyldiaminopropionic acids in the seeds after cooking prior to human consumption. Similarly, isomerization could occur *in vivo* following ingestion of the β-isomer. It is not known whether the α-isomer is chronically toxic to animals, and such information is essential for a full understanding of the toxicity of *L. sativus* seed. A recent report [14] suggests that the α-isomer is not acutely toxic to 1-day-old chicks or to neonatal mice.

Progress in elucidating the mechanism of action of β-*N*-oxalyldiaminopropionic acid has been hampered by the unavailability of the compound in a pure form. Earlier synthesis gave very poor yields [4, 15], but a successful synthesis was reported by Rao [16]. A synthetic route to the α-isomer was recently described [14]. This paper reports alternative syntheses of the individual α- and β-*N*-oxalyl derivatives of L-α,β-diaminopropionic acid and a method by which the two isomers can be isolated from seed extracts of *L. sativus*.

RESULTS AND DISCUSSION

The methods described here provide relatively convenient procedures for obtaining simultaneously α- and β-*N*-oxalyl diaminopropionic acids either synthetically, or from natural sources. Both preparations rely upon a separation of a mixture of isomers by elution chromatography on a column of ion-exchange resin.

Preparative separation of α- and β-N-oxalyldiaminopropionic acids

It was established that pH 2.3 was optimal for operating the ion-exchange column. Greater pH values gave incomplete separation of the two substances, whereas lower pH values retarded the elution of the substances without increasing the resolution of the peaks. The retention volumes of the substances were dependent upon the volume of sample applied to the column. With a sample volume of 45 ml, containing a mixture of isomers derived from 1 g α,β-diaminopropionic acid, fractions 14–26 contained the β-isomer and fractions 30–45 the α-isomer. Larger sample volumes were necessary when the column was more heavily loaded with the isomers, because the substances had a relatively low solubility at pH 1.3, the pH at which the sample was applied to the column. Sample volumes up to 70 ml (representing about 1.5 g of mixed isomers) increased the retention volumes of the substances, but resolution was maintained. This quantity of amino acids is probably close to the maximum capacity of the column used. Larger capacity columns, up to 10 cm in diameter, have been used with success.

Chemical synthesis of α- and β-N-oxalyldiaminopropionic acids

That both isomers should be produced from the reaction of diethyl oxalate and α,β-diaminopropionic acid was anticipated from the data of Leclerc and Benoiton [17] who found that acylation of α,β-diaminopropionic

Table 1. Identity of natural and synthetic α- and β-*N*-oxalyl-L-α,β-diaminopropionic acids

	Natural α-isomer	Synthetic α-isomer	Natural β-isomer	Synthetic β-isomer
Elemental analysis*	C, 31.42; H, 5.27; N, 14.20%	C, 30.95; H, 5.04; N, 14.87%	C, 31.29; H, 5.03; N, 14.30%	C, 30.97; H, 5.48; N, 14.51%
Melting point	161–163° (dec.)	160–161° (dec.)	204–206° (dec.)	200–202° (dec.)
Specific rotation M HCl	−41.6°	−39.8°	−21.2°	−21.3°
5M HCl	−37.4°	−33.8°	−19.4°	−19.4°

*$C_5H_8N_2O_5.H_2O$ requires: C, 30.93; H, 5.19; N, 14.43%.

acid by variety of acylating agents was not selective at the α- or β- nitrogen atom. In preliminary experiments we found that with Cu^{2+} present at pH 5 [4], the extent of oxalylation by a number of reagents was very low and we were not able to reproduce the excellent level of oxalylation achieved by these workers. In the absence of Cu^{2+}, diethyl oxalate gave a quantitative conversion of α,β-diaminopropionic acid to a mixture of oxalyl derivatives. The extent of the reaction was markedly pH sensitive, being greater at pH 10 than at any higher or lower pH value. We have since found that isomerization of the *N*-oxalyl isomers is also maximal at pH 10, presumably due to the formation of an uncharged amino group about this pH value encouraging migration of the oxalyl group.

The yield of the β-isomer (*ca* 36%) was superior to that of Mehta [15], but inferior to that of Rao [16] who used a more direct method than that described here. A comparison of the yield of the α-isomer produced here (22%) with the method of Wu *et al.* [14] cannot be made directly as our intention was to synthesize both isomers simultaneously.

Isolation of α- and β-N-oxalyldiaminopropionic acids from seed extracts of L. sativus

The isolation of the β-isomer has been described on many occasions [4]. The mixture of α- and β-isomers which forms when ethanolic extracts of *L. sativus* seed are allowed to stand for some days [11] was the basis of the preparation described here. The ratio of β:α isomers recovered (5.7:1), would have favoured the α-isomer more had the substances been allowed to stand at pH 10, but this was not done for this preparation. The total recovery of *N*-oxalyldiaminopropionic acids, by this method, 5.69 g/kg seed, compares well with previous studies [4].

Analytical data

The results in Table 1 show good agreement between the chemically synthesized and naturally occurring isomers in their elemental analyses, melting points and specific rotations. The elemental analyses show that we have isolated the monohydrate of both isomers, whereas others have isolated the anhydrous β-isomer [16] and the hemihydrate of the α-isomer [14]. The difference in specific rotation of each isomer in M and 5M HCl confirms that the natural and synthetic isomers are of the L-configuration (Clough–Lutz–Jirgenson's rule [18]). The agreement in the specific rotation of each pair of isomers indicates that no racemization of the parent compound occurred during the chemical synthesis.

The TLC system which was developed is a useful additional analytical method for separating the α-isomer from the β-isomer, previously separable only by electrophoresis [11, 12]. The conditions used in the column chromatographic method have also been used successfully in analytical separations [P. B. Nunn, unpublished data].

Table 2. Chemical shift differences (Δδ) between (i) CH, (ii) CH_2 protons in free and acylated derivatives of various aminoacids in D_2O

$^-O_2C-CH-\overset{+}{N}H_3$		(i) $^-O_2C-CH-NH-CO-R$		Δδ ppm	$R'-CH_2-\overset{+}{N}H_3$		(i) $R'-CH_2-NH-CO-R$		Δδ ppm
example	δCH ppm	example	δCH ppm		example	δCH_2 ppm	example	δCH_2 ppm	
analine	3.80*	β-alanyl-alanine	4.17*	0.37	glycine	3.56†	aminoacylglycines	3.80‡ (±0.12)	0.24
	3.78†	aminoacyl-alanines	4.15‡ (±0.02)	0.37	lysine (ε—CH_2)	2.92†	Nε-acetyllysine	3.20*	0.28
leucine	3.70†	aminoacyl-leucines	4.25‡ (±0.02)	0.55					
	3.60†	aminoacyl-valines	4.05‡ (±0.03)	0.45					
α,β-diaminopropionic acid	4.02	α-*N*-oxalyl-L-α,β diaminopropionic acid	4.48	0.46	α,β-diaminopropionic acid	3.45	β-*N*-oxalyl-L-α,β diaminopropionic acid	3.83	0.38
β-*N*-oxalyl-L-α,β diaminopropionic acid	4.07				α-*N*-oxalyl-L-α,β diaminopropionic acid	3.40			

*Ref. [21]; †Ref. [19]; ‡Ref. [20]. Result cited incorporates values from 5 dipeptides.

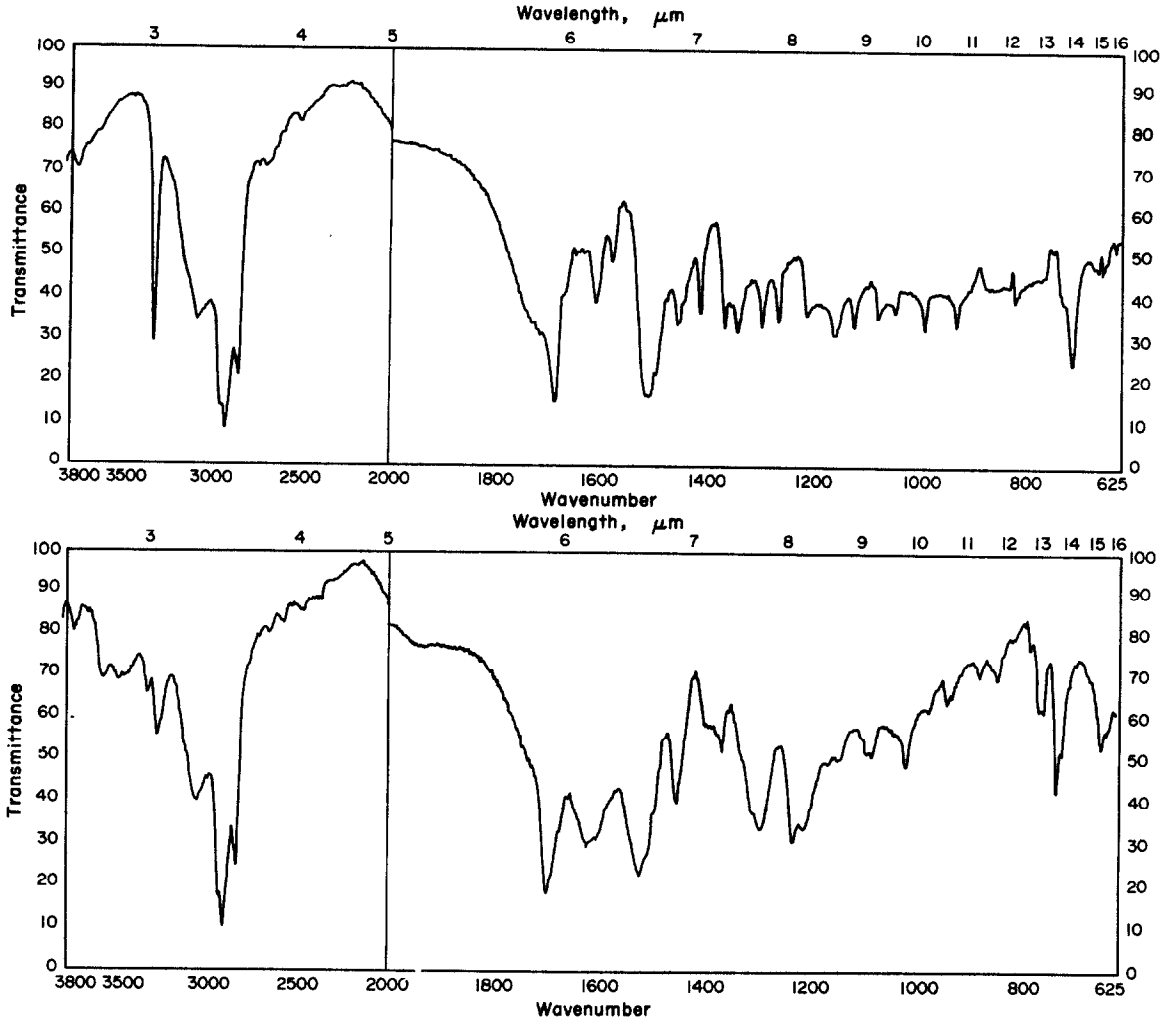

Fig. 1. Infra-red spectra of the α-, and β-*N*-oxalyl-L-α,β-diaminopropionic acids. Upper trace: β-isomer. Lower trace: α-isomer. Both spectra were obtained from preparations in Nujol.

The identity of each isomer, which was established by electrophoretic migration [11], was very clearly confirmed by PMR studies. PMR studies of amino acids and peptides have established [19, 20] that the α-CH resonance of the zwitterionic form of an amino acid is displaced downfield when the amino group is acylated. A similar downfield displacement is found with analogous comparisons of CH_2 protons. Table 2 compares examples of CH and CH_2 protons in α,β-diaminopropionic acid and its α- and β-*N*-oxalyl isomers. The strucutral assignments for the *N*-oxalyl isomers [11] accord perfectly with the empirical data in Table 2. The multiplet structure in all 3 spectra is that expected for an ABX spin system distorted towards an ABC system. The spectra have profiles almost identical with similar systems already recorded [21]: α,β-diaminopropionic acid with L-cystine; the α-oxalyl derivative with L-asparagine in $NaOD/D_2O$, and the β-oxalyl derivative with DL-serine in DCl/D_2O. The synthetic and natural isomers gave identical spectra.

IR spectra of the α- and β-*N*-oxalyl-L-α,β-diaminopropionic acids in Nujol preparations are shown in Fig. 1 and may be compared with that of the parent acid [4]. The fingerprint regions, 700–1400 cm^{-1} are the most diagnostic, being very different in all three compounds and therefore a reliable method of identification. The Nujol and KBr spectra of the α- and β- isomers were identical in this region. The 1500–1700 cm^{-1} and 2500–3500 cm^{-1} regions, though containing the strongest absorptions, are probably susceptible to variation with sample condition. Thus, whereas the spectra were very similar in both Nujol and KBr samples for the β-isomer, they differed somewhat for the α-isomer, especially in the region above 3000 cm^{-1}.

The spectrum for the β-isomer shows considerably better resolution than that previously published [4], and a particularly distinctive difference between the two spectra is that the β-isomer shows a maximum at 1420 cm^{-1} and a minimum at 1400 cm^{-1}, whereas the opposite is the case for the α-isomer.

Comparative toxicities of α- *and* β-N-*oxalyl-*L-α,β-*diaminopropionic acids*

Administration of the β-isomer to 22 chicks (12.5–205

nmol/bird) produced neurological symptoms consistent with previous results [4]. The α-isomer was not a similarly potent convulsant. Seven chicks which received 150–175 nmol of the substance showed no convulsant activity, but two of 5 birds which received 205 nmol of the α-isomer developed convulsions within 3 hr and subsequently recovered.

There is a number of possible interpretations of the results concerning the toxicity of the α-isomer. The substance might be a convulsant itself only when administered in very high doses. The maximum dose used here was *ca* twice that previously reported [14] to be non-convulsant in chicks. Several *N*-oxalyl amino acid derivatives cause neurological symptoms in chicks, an effect which is probably due to a common structural relationship to oxamic acid [22]. Possibly the high doses of the α-isomer were toxic for this reason. Alternatively, isomerization of high doses of the α-isomer to the β-isomer might occur *in vivo*, producing sufficient of the β-isomer to cause convulsions. (We have established that isomerization of the two pure compounds does occur at physiological pH and temperature *in vitro*). The purity of the α-isomer used in these experiments excludes the possibility that contamination by the β-isomer was responsible for the convulsions.

We observed in all 10 birds treated with 100–200 nmol of the α-isomer atypical behaviour; the adoption of a standing position while remaining immobile for periods of up to 30 min. These symptoms are of interest since they were also observed, before the onset of convulsions, in the 22 birds receiving doses of the β-isomer. No behavioural changes of this nature occurred in 10 birds injected with 0.24–0.66 M NaCl solutions.

These preliminary results show that the α- and β-*N*-oxalyl-L-α,β-diaminopropionic acids cause behavioural changes in chicks which may be distinct from the commonly observed convulsant activity of the β-isomer. Whether the non-convulsant effects of the two isomers are important factors in the toxicity of *L. sativus* seeds cannot be assessed at present.

EXPERIMENTAL

Qualitative and quantitative determination of amino acids. Amino acids were detected on PC and TLC using cadmium–ninhydrin [23]. Equal amounts of both isomers gave *ca* equal colour intensity when heated with the reagent at 100° for *ca* 3 min. For quantitative analyses in soln, the ninhydrin method of ref. [24] was used. Solns of the pure isomers (1 ml of 0.1 mM) were reacted with 1 ml of reagent. After colour development at 100° for 20 min, the colour yield compared with 0.1 mM leucine was 0.99 for the β-isomer and 0·61 for the α-isomer. The lower colour yield of the α-isomer is in agreement with results for other amino acids with free β-amino groups [24]. For quantitative determinations of column effluents, 50 μl of each fraction was diluted to 1 ml with eluting buffer and subsequently reacted with 1 ml of ninhydrin reagent.

High voltage electrophoresis. Performed on Whatman 3 MM paper essentially as described in ref. [25]. The plates of the instrument were maintained at 15° by a circulating refrigeration unit, while a potential gradient of 77 V cm^{-1} was applied for 20 min. Electrophoresis was performed in buffers of pH 1.9 (33.2 ml 98% HCO_2H, 147 ml 17 M HOAc diluted to 2 l. with H_2O) and at pH 3.0 (3.7 ml Py, 100 ml 17 M HOAc diluted to 2 l. with H_2O).

TLC. Performed on commercial cellulose ('Polygram CEL 300'). Chromatograms were developed for 2 hr in *n*-BuOH–Me_2CO–98% HCO_2H–H_2O (7:7:2:4), which clearly separated the two isomers. (R_f values: α-isomer, 0.31; β-isomer, 0.25).

Spectral analyses. PMR spectra were measured at 60 MHz in D_2O at ambient temp., using TSS as internal reference. IR spectra were determined in either Nujol or as KBr discs.

Optical rotation measurements. 10 cm cell length, Na D line at 25°; β-isomer c = *ca* 0.5, α-isomer c = *ca* 1.0. Both isomers were analysed in M and in 5 M HCl, immediately after dissolving the solid preparations, to minimize hydrolysis or isomerization of the compounds.

Synthesis of α- and β-N-oxalyldiaminopropionic acids. L-α,β-Diaminopropionic acid monohydrochloride (1 g) was dissolved in 18 ml H_2O and the soln adjusted to pH 10 using saturated LiOH. A mixture of 10 ml diEt oxalate in 10.8 ml EtOH was added dropwise with vigorous shaking to the soln of diaminopropionic acid maintained at 30°, during a period of about 2 hr. Satd LiOH soln was used to maintain the reaction mixture at pH 10 during this time. The time course of the reaction was followed using HVE at pH 3. When virtually all the diaminopropionic acid had been utilized (judged by colour intensity with ninhydrin) the reaction mixture was evaporated to dryness at 40° *in vacuo*. The dry material was suspended in 500 ml H_2O adjusted to pH 10 using LiOH soln and heated at 80° for 17 hr. This treatment served to hydrolyse esters of the oxalyl amino acids and *bis* compounds [26]. After cooling, the preparation was desalted by passing the soln onto a column (4 × 25 cm) of Zeo Karb 225 (8% crosslinked, H^+-form) and 10 ml fractions were collected. When the sample had been applied to the column, elution continued with 0.2 M HOAc. Fractions 54–200 inclusive, which contained ninhydrin positive material, were either freeze-dried or reduced to a min. vol. at 40° *in vacuo*. This material was free of Li^+ and oxalic acid, as determined by flame test and the $CaCl_2$/NaOAc test, respectively. HVE at pH 3 revealed that the only ninhydrin-positive substances present were α- and β-*N*-oxalyl diaminopropionic acids.

Extraction of α- and β-N-oxalyldiaminopropionic acids from seed of L. sativus. Seed (obtained from Bombay, India) (1 kg) was finely ground and extracted with 3 l. of 50% (v/v) EtOH for about 20 hr at 4°. The solid material was filtered off. The filtrate obtained was tested on filter paper for ninhydrin positive material, and the solid material was re-extracted as before. Seven such extractions removed the majority of the ninhydrin-positive material from the seed residue. The bulked extracts were freed from EtOH and concnd to a syrup by flash evaporation at 40°. The syrup was dissolved in H_2O to make 4 l., and passed onto a column (4 × 25 cm) of Zeo Karb 225 (8% crosslinked, H^+ form). Fractions (250 ml) were collected. When all the sample had entered the column, elution continued with 0.062 M HOAc. Fractions 16–44 inclusive contained ninhydrin positive material. These fractions were freeze-dried and electrophoresis at pH 3 showed that α- and β-*N*-oxalyl diaminopropionic acids were the only ninhydrin-positive substances present.

Separation of α- and β-N-oxalyldiaminopropionic acids. The two isomers were separated on a column (4 × 75 cm) of Zeo Karb 225 (8% crosslinked, pyridinium form). The resin was purified and fine particles removed [27, 28], after which the particle size in the H^+ form was 35–40 μm, judged microscopically. The pyridinium form of the resin was prepared from the H^+-form by washing with 2 l of M Py–HCO_2H, pH 4 (151.3 ml Py, 100 ml 98% HCO_2H, diluted to 2 l. with H_2O). Before use, the resin was washed twice in eluting buffer, 0.035 M Py–HCO_2H, pH 2.30 (28.2 ml Py, 250 ml 98% HCO_2H, diluted to 10 l. with H_2O and the final pH adjusted to within ∓0.01 if necessary). The column was packed in one step by suspending the resin in 2 l. of eluting buffer and allowing the resin to settle by gravity into the column from a large separatory funnel for 18 hr. The column was equilibrated for 30 min before use by pumping with eluting buffer at a flow rate of 25 ml/min using a positive displacement pump. The sample was suspended in about 25 ml H_2O and Py was added to dissolve. The vol.

was increased to either 45 ml (synthetic preparation) or 70 ml (seed extracts) and adjusted to pH 1.3 by adding 11 M HCl with vigorous stirring. (The oxalyl derivatives are not very soluble as the free acids and this procedure produced an almost saturated soln. To prevent crystallization of the substances on the column, it was necessary to equilibrate the sample at about 15° before proceeding further). The sample was allowed to enter the resin under gravity, after which elution was begun at a flow rate of 25 ml/min at 20–25°. Fractions (75 ml) were collected and immediately stored at 4° to prevent isomerization of the separated isomers. The substances were located either quantitatively by ninhydrin analysis or qualitatively on filter paper. The separated isomers were freeze-dried immediately after analysis and the identity of the eluted peaks was determined by electrophoresis at pH 3 [11]. Each isomer was suspended in H_2O, dissolved by adding Py to pH 4.5, treated with charcoal and rept. with Me_2CO after addition of 11 M HCl to pH 2. Following recovery, the solid material was dried over P_2O_5 *in vacuo* to remove residual Py. From 1 g α,β-diaminopropionic acid monohydrochloride (7.1 mmol), 0.54 g (2.77 mmol) of α-isomer the and 0.31 g (1.56 mmol) of the β-isomer were recovered. The yield of the isomers from the seed extract was: α-isomer 0.85 g/kg seed and β-isomer 4.84 g/kg seed. The purity of the solid substances was checked by electrophoresis at pH 1.9 and 3 and by TLC. By analysing known amounts of the purified isomers by electrophoresis at pH 3 for 35 min, the purity of each isomer with respect to the other isomer was established. 500 nmol of the α-isomer contained less than 2 nmol of the β-isomer (isomeric purity *ca* 99.6%). 200 nmol of the β-isomer contained less than 2 nmol of the α-isomer (isomeric purity *ca* 99%). No other ninhydrin-positive substances was detected during these experiments.

Toxicity experiments. Male 3-day-old chicks (Strain: Rhode Island × Sussex, obtained fron a commercial hatchery), were administered intraperitoneally solns of α- or β-N-oxalyl-L-α,β-diaminopropionic acids from the natural source, or saline solns. All solns were neutralized to pH 6–8 and sterilized by filtration before use. Birds were observed continuously when possible for *ca* 36 hr after injection.

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REFERENCES

1. Jimenez Diaz, C., De Landazur, E. O. and Roda, E. (1943) *Revista Clinica Espanola* **8**, 154.
2. Paissos, C. S. and Demopoulos, T. (1962) *Clin. Orth.* **23**, 236.
3. Barrow, M. W., Simpson, V. and Mitlar, C. F. (1974) *Quart. Rev. Biol.* **49**, 101.
4. Rao, S. L. N., Adiga, P. R. and Sarma, P. S. (1964) *Biochemistry* **3**, 432.
5. Murti, V. V. S., Seshadri, T. R. and Venkitasubramanian, T. A. (1964) *Phytochemistry* **3**, 73.
6. Rukmini, C. (1968) *Ind. J. Biochem.* **5**, 182.
7. Rukmini, C. (1969) *Ind. J. Chem.* **7**, 1062.
8. Nagarajan, V., Mohan, V. S. and Gopalan, C. (1965) *Ind. J. Med. Res.* **53**, 269.
9. Rao, S. L. N., Malathi, K. and Sarma, P. S. (1969) *World Rev. Nutr. Dietetics* **10**, 214.
10. Mani, K. S., Sriramachari, S., Rao, S. L. N. and Sarma, P. S. (1971) *Ind. J. Med. Res.* **59**, 880.
11. Bell, E. A. and O'Donovan, J. P. (1966) *Phytochemistry* **5**, 1211.
12. Roy, D. N. and Rao, B. S. N. (1968) *Curr. Sci. (India)* **37**, 395.
13. Nagarajan, V. (1969) *Ind. J. Med. Res.* **57**, 92.
14. Wu, G., Bowlus, S. B., Kim, K. S. and Haskell, B. E. (1976) *Phytochemistry* **15**, 1257.
15. Mehta, T., Hsu, A-F. and Haskell, B. E. (1972) *Biochemistry* **11**, 4053.
16. Rao, S. L. N. (1975) *Biochemistry* **14**, 5218.
17. Leclerc, J. and Benoiton, L. (1968) *Can. J. Chem.* **46**, 1047.
18. Greenstein, J. P. and Winitz, M. (1961) *Chemistry of the Amino Acids* Vol. 1, p. 85. Wiley, New York.
19. Nagai, M., Nishioka, A. and Yoshimura, J. (1970) *Bull. Chem. Soc. Japan* **43**, 1323.
20. Conti, F., Pietronero, C. and Viglmo, P. (1970) *Org. Mag. Res.* **2**, 131.
21. Pouchert, C. J. and Campbell, J. R. (1974) *The Aldridge Library of NMR Spectra* Volume III. Aldridge Chem. Co.
22. Rao, S. L. N., Sarma, P. S. (1966) *Ind. J. Biochem.* **3**, 5.
23. Heilmann, J., Barrollier, J. and Watzkie, E. (1957) *Z. Physiol. Chem.* **309**, 219.
24. Spackman, D. H., Stein, W. H. and Moore, S. (1958) *Anal. Chem.* **30**, 1190.
25. Gross, D. (1961) *J. Chromatog.* **5**, 194.
26. Greenstein, J. P. and Winitz, M. (1961) *Chemistry of the Amino Acids* Vol. 3, p. 2526. Wiley, New York.
27. Moore, S. and Stein, W. H. (1951) *J. Biol. Chem.* **192**, 663.
28. Moore, S. and Stein, W. H. (1954) *J. Biol. Chem.* **211**, 893.