STUDIES ON SANDAL SPIKE

Part VI. A Study of the Amino-Acid Make-up and Some Organic Acids of Sandal (*Santalum album* L.) in Health and Disease

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But for the report that the amino nitrogen is more in the spiked tissues than in the healthy[1] and the incidental analysis of the basic nitrogen into various fractions,[2] the amino-acid metabolism of sandal does not appear to have been studied in any detail. In continuation of the work in progress in this Laboratory on the physiology of sandal in health and disease, a study of the free amino-acids of the green foliage has been taken up; some of the important amino-acids and ketocarboxylic acids have been quantitatively estimated in order to elucidate the relationship between them.

Experimental

Extraction of amino-acids.—The sandal leaves used in these experiments were collected from two localities, namely, Forest Research Laboratory nursery (healthy area) and Kenchenahalli (spike-affected area). In both the cases representative samples were drawn from the leaves collected and the extraction for the amino-acids done separately according to the method of De Kock and Morrison.[3] The material (10 g.) was ground in a glass mortar and treated with 75% ethanol (90 ml.). After a thorough shaking, the mixture was kept overnight and filtered. The residue was then successfully extracted three times with 0·01 N hydrochloric acid, taking 50 ml. each time. This extract was added to the first after distilling off the alcohol. After neutralisation to pH 7, the combined extract was again extracted with ether and chloroform in order to remove chlorophyll and other pigments. The resulting brownish-yellow solution was concentrated to about 150 ml. in vacuum and passed through cation-exchange resin column (140 × 25 mm.) of Dowex 50W–X 8 (ionic form H^+, 20–25 mesh) at a rate of 8 to 10 drops per minute. The column was then washed twice with distilled water (50 ml.) and the eluant was used for the analysis of keto acids.

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The resin column which retained the amino-acids was then eluted gradiently with ammonia solution (0·5–2·0 N) till the eluant gave no more test for the amino-acids when treated with ninhydrin. The eluant containing the amino-acids was evaporated to dryness and taken in 10% isopropanol (10 ml.).

Chromatographic analysis of amino-acids.—The mixture of the acids was separated employing two-dimensional paper chromatography. The two solvent systems used were butanol-acetic acid-water (15: 3: 7 *v*/*v*) and phenol-ethanol-water (3: 1: 1 *v*/*v*) containing 0·05% of 8-hydroxyquinoline, in an atmosphere of ammonia. The first run was made with the first solvent using the descending technique of Consden *et al.*,[4] and it took about 16–18 hours at 25–27° C. to descend over 40 cm., while in the second run, which was made with the second solvent using ascending technique of Williams and Kirby,[5] the solvent front rose to 35 cm. during the same period. The developed chromatograms were air-dried thoroughly since the presence of even traces of phenol would interfere with the estimation of amino-acids. On spraying with 0·5% ninhydrin solution in 95% acetone and drying, the amino-acids developed the characteristic pink and bluish-pink spots. By comparison with a standard chromatogram the following amino-acids were detected in the extracts of both the healthy and the spiked leaves: alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, leucine, isoleucine, lysine, methionine, ornithine, phenylalanine, proline, hydroxy proline, serine, threonine and valine. In addition to the above normal constituents of proteins, α-aminobutyric acid was also found in both the healthy and diseased tissues. Sometime ago, while isolating hydroxy proline from sandal leaves, Radhakrishnan and Giri did not report some of the acids mentioned above.[6]

The quantitative estimation of alanine, glutamic acid and aspartic acid was made since these are the main amino-acids that establish the link with the Krebs cycle of respiration. Apart from these, α-aminobutyric acid, arginine and methionine were also estimated, since they appeared to be present in appreciable amounts. The estimations were done as detailed by Giri *et al.*[7] The ninhydrin-sprayed chromatograms were dried at 65° C. for 30 minutes. The corresponding colour bands were cut, extracted with 4 ml. of 75% ethanol containing 0·2 mg. of copper sulphate and the colour density measured in a Bausch and Lomb spectronic colorimeter at 540 mμ (millimicrons). The results are presented in Table I.

Extraction and estimation of α-ketocarboxylic acids.—The extraction of these acids from the tissues was carried out according to the procedure of Isherwood *et al.*[8]

TABLE I

Amino-acid	Healthy leaves mg./gm.	Spiked leaves mg./gm.
Alanine	0·150	0·075
Glutamic acid	0·406	0·219
Aspartic acid	0·125	0·156
Arginine	0·316	0·366
Methionine	0·025	0·500
α-Aminobutyric acid	0·125	0·025

A sample (10 g.) was frozen by keeping in deep freeze overnight and extracted with HPO_3 (0·6 M; 50 ml.). The mash was centrifuged and was extracted twice with cold HPO_3 (25 ml.) and the extracts combined. To this extract 20 ml. of 2:4-dinitro-phenylhydrazine dissolved in 5 N sulphuric acid was added and left aside for 45 minutes. The hydrazones were extracted with ether and from the ethereal solution they were re-extracted with 80 ml. of saturated sodium bicarbonate solution. The bicarbonate extract was then acidified with 3 N sulphuric acid to pH 2 and from this solution the hydrazones were extracted with chloroform containing 15% of ether. From this solution the hydrazones were recovered by evaporation. Taking the residue in ethanol (5 ml.) the hydrazones were separated by paper chromatography (ascending technique) using the solvent, *n*-butanol: ethanol: 0·5 N NH_4OH (7:1:2 *v*/*v*) as suggested by El Hawary and Thompson,[9] and estimations were carried out on the lines indicated by Seligson and Shapiro.[10] Each spot of the hydrazone on chromatogram after exposure to ultra-violet light was cut into different bits and placed in separate tubes. The hydrazone was eluted with N sodium hydroxide (5 ml.) by shaking for 10 minutes, filtered and estimated with the colorimeter at 455 mμ. If the hydrazone was present in very small quantities, which could not be detected under the U.V. light, its presance was indicated by spraying the chromatogram with 2% ethanolic potassium hydroxide, when it appeared as a red brown spot.[11] The results of the analysis are given in Table II.

TABLE II

Ketocarboxylic acid	Healthy leaves mg./gm.	Spiked leaves mg./gm.
Pyruvic acid	0·30	0·90
α-Ketoglutaric acid	0·60	0·31
Oxalacetic acid	0·20	traces

DISCUSSION

One route of amino-acid biosynthesis is by transamination of the corresponding α-ketocarboxylic acids. Thus the three key amino-acids, namely, alanine, aspartic acid and glutamic acid, are said to be produced from the corresponding α-ketocarboxylic acids, namely, pyruvic, oxalacetic and α-ketoglutaric acids which are the metabolic products of carbohydrates. Hence there should be a proportionality between the two groups of acids, if the assumed biosynthetic route is correct. The results now obtained show that spiked sandal tissues contain considerably smaller quantities of alanine and glutamic acid but an almost equal amount of aspartic acid as in the healthy tissues. On the other hand, the contents of the keto acids show a different picture. In the diseased sandal tissues as compared with the healthy ones, there is an abnormal accumulation of pyruvic acid, a lower amount of α-ketoglutaric acid and only traces of oxalacetic acid. The abnormal accumulation of pyruvic acid in the diseased tissues may be due to one or more of the following metabolic disturbances caused by the virus infection: (1) the Krebs cycle functioning in the diseased tissues may not be keeping pace with the glycolytic cycle (for utilization of pyruvic acid); (2) the alanine formed from pyruvic acid by transamination may be converting itself back, at least partly, to the keto acid by a process of deamination. Also the negligibly small amounts of oxalacetic acid noticed in the diseased tissues is suggestive of the poor incorporation of pyruvic acid in the Krebs cycle. It thus appears that in the spiked tissues of sandal the normal functioning of the Krebs cycle is affected with its consequential disturbance on the biosynthesis of the key amino-acids.

In this connection it may be worthwhile to note that the work of Longenlaker *et al.* shows that the activity of the transamination enzymes is enhanced

by metals like iron, copper, etc.,[12] while the investigations of DeKock indicate that the activity of aconitase (an enzyme involved in the Krebs cycle oxidations) is depressed when the supply of iron is low.[13] It may be noted that our earlier work has indicated a deficiency of iron in the diseased sandal tissues[14] and this may be a contributory cause for the disturbance in the Krebs cycle and transaminations.

However, it may be noted that, although oxalacetic acid is present only in traces, yet the corresponding amino-acid, namely, aspartic acid, is found in the diseased tissues in almost the same amount as in the healthy tissues. The reasons for this unexpected finding are still under investigation. It is possible that its origin may be from succinic acid through reductive amination.[15, 16]

Another noteworthy feature of the present findings is the depletion of α-aminobutyric acid, which is a non-protein amino-acid, in the diseased sandal tissues. It is known that succinic acid acts as a precursor for this amino-acid through reduction to succinic semi-aldehyde and subsequent transamination.[17] Apparently, the factors responsible for this conversion are adversely affected with the onset of the spike disease, and this may be a reason for the reported accumulation of succinic acid in the diseased sandal tissues.[18]

SUMMARY

The following amino-acids have been detected in the healthy and the diseased tissues of sandal (green foliage): alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine leucine, isoleucine, lysine, methionine, ornithine, phenylalanine, proline, hydroxy proline, serine, threonine, valine and α-aminobutyric acid.

Quantitative estimations have been made of alanine, aspartic acid and glutamic acid as also those of th (corresponding ketocarboxylic acids, namely, pyruvic, oxalacetic and α-ketoglutaric acids which are involved in the amino-acid and carbohydrate metabolisms, respectively. Distinct differences have been noticed in regard to the quantities of these acids present in the healthy and diseased tissues and their significance has been discussed.

REFERENCES

1. Narasimhamurthy, N. and Sreenivasaya, M. *J. Ind. Inst. Sci.*, 1929, **12 A**, 153.
2. Sreenivasa Rao, Y. V. .. *Ibid.*, 1933, **16 A**, 91.