Braithwaite, G. D. & Goodwin, T. W. (1957). *Biochem. J.* **66**, 31P
Chibnall, A. C. (1939). *Protein Metabolism in Plants*, p. 153. New Haven: Yale University Press.
Glover, J. (1956). In *Modern Methods of Plant Analysis*, vol. 1, p. 325. Ed. by Paech, K. & Tracey, M. V. Heidelberg: Julius Springer.
Goodwin, T. W. (1952). *The Comparative Biochemistry of the Carotenoids*, p. 292. London: Chapman and Hall.
Goodwin, T. W. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 272. Ed. by Paech, K. & Tracey, M. V. Heidelberg: Julius Springer.
Goodwin, T. W. (1958*a*). *Biochem. J.* **68**, 503.
Goodwin, T. W. (1958*b*). *Biochem. J.* **68**, 26P.
Kay, R. E. & Phinney, B. (1956). *Plant Physiol.* **31**, 226.
Loeffler, J. E. (1955). *Yearb. Carneg. Instn*, **54**, 159.
Menke, W. & Jacob, E. (1942). *Hoppe-Seyl. Z.* **272**, 227.
Smith, J. H. C. & Benitez, A. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 142. Ed. by Paech, K. & Tracey, M. V. Heidelberg: Julius Springer.
Strain, H. H. (1938). *Publ. Carneg. Instn*, no. 490.
Zalokar, M. (1955). *Arch. Biochem. Biophys.* **56**, 318.

Canavanine and Related Compounds in Leguminosae

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Canavanine (α-amino-δ-guanidinoxybutyric acid) occurs in the free state in the seeds of the jack bean, *Canavalia ensiformis*, and in the seeds of *C. lineata* and *C. obtusifolia* (Kitagawa & Tomiyama, 1929; Kitagawa, 1937; Damodaran & Narayanan, 1939). Fearon (1946) showed that compounds containing the guanidoxy grouping,

$$-O\cdot NH\cdot C(:NH)\cdot NH_2,$$

react with trisodium pentacyanoammonioferrate in aqueous solution at pH 7, to give colours which range from orange–red with *N*-methoxyguanidine to magenta with canavanine itself. With this reagent a representative selection of plants were tested for the presence of canavanine, and positive results were obtained with species of *Medicago*, *Ornithopus* and *Colutea*. Canavanine was isolated from *C. arborescens* (Fearon & Bell, 1955).

The inhibition of the pentacyanoammonioferrate colour reaction by such compounds as ascorbic acid and creatinine (Fearon & Bell, 1955) makes its direct application to biological tissues or extracts of limited value. This difficulty has been overcome by using paper chromatography and paper ionophoresis to separate pentacyanoammonioferrate-reacting compounds from others which inhibit or mask the colour reaction.

The occurrence of canavanine in concentrations of 3–5% of dry weight in the seeds of *Canavalia ensiformis* and *Colutea arborescens* suggests its function as a nitrogen-storage product and hence the existence of an enzyme system associated with its utilization. Damodaran & Narayanan (1940) reported the existence in the seeds of *Canavalia ensiformis* of an enzyme which brought about the hydrolysis of canavanine to canalin and urea. They also showed that this enzyme was identical with jack-bean arginase, though the optimum pH of hydrolysis was 9·4 for arginine and 7·5 for canavanine. It was suggested that canavanine might be the natural substrate of this arginase. In view of these facts, and of the occurrence of canavanine in plants other than *Canavalia*, it seemed likely that other known sources of arginase in the Leguminosae, such as vetch (*Vicia sativa*) and red clover (*Trifolium pratense*), might also prove to be sources of canavanine. This is now shown to be so. It is also shown that canavanine occurs in the seeds of many leguminous plants; quantitative determinations have been made on those seeds which give the strongest colour reaction with trisodium pentacyanoammonioferrate, and the amino acid has been isolated from two of them. The presence in leguminous seeds of compounds other than canavanine which also react with the pentacyanoammonioferrate at pH 7 has been demonstrated.

EXPERIMENTAL

Pentacyanoammonioferrate reagent (*PCAF*). A 1% (w/v) solution of PCAF (Fearon, 1946) in Cu-free distilled water was used.

Phosphate buffer (pH 7). This was prepared by mixing equal volumes of 0·066M-NaH_2PO_4 and 0·066M-Na_2HPO_4.

Determination of α-amino nitrogen. This was determined as α-amino acid carboxyl by the method of Van Slyke, MacFadyen & Hamilton (1941).

Specificity of the PCAF colour reaction for canavanine

Fearon (1946) found that, of the substituted guanidines which he tested, only the guanidoxy compounds reacted with PCAF to give orange and red colours in the range pH 5–7·5, and of these only canavanine gave a vivid magenta.

Reaction with deaminocanavanine. It has been found that deaminocanavanine (hexahydro-3-imino-1-oxa-2:4-diazepine-5-carboxylic acid), prepared by the method of Kitagawa & Tsukamoto (1937), also reacts with PCAF, at pH 7, to give a magenta colour. Canavanine and deaminocanavanine are readily separated chromatographically on Whatman no. 1 paper by the descending method at 18°; the R_F values in phenol–water (4:1, w/v) were 0·10 and 0·81 respectively, and in butanol–pyridine–acetic acid–water (4:1:1:2, by vol.), 0·10 and 0·33. The two compounds may be distinguished by the failure of deaminocanavanine to react with ninhydrin.

Qualitative analysis of leguminous seeds

Preparation of seed extracts. Finely ground seed (0·1–0·3 g.) was stirred with 2 ml. of 0·1 N-HCl at room temp. and kept for 1 hr. Drops of the supernatant liquid were transferred to paper for chromatography and ionophoresis.

Chromatography. Acid seed extract (0·2 ml.) was applied to Whatman no. 1 paper and chromatographed in phenol–water (4:1, w/v) and in the butanol–pyridine–acetic acid–water (4:1:1:2, by vol.) solvent used by Makisumi (1952) for the chromatography of guanidines. Each chromatogram was developed for 24 hr., then dried in air and sprayed successively with phosphate buffer (pH 7) and 1% PCAF solution. Pyridine was found to inhibit the development of PCAF colours, and on spraying with phosphate buffer was liberated from the papers which had been run in the solvent containing it. These chromatograms were therefore again dried in air after spraying with phosphate buffer before applying the PCAF reagent.

Ionophoresis. Single drops of acid seed extract were dried on Whatman no. 3 MM paper and ionophoresis was carried out in phosphate buffer (pH 7) at 5 v/cm. for 3 hr. Under these conditions canavanine migrated 7 mm. towards the cathode.

PCAF reaction negative. No compounds reacting to give colours with PCAF at pH 7 were detected in the extracts prepared from the seeds of *Acacia dealbata, A. farnesiana, Baptisia australis, Calpurnia aurea, Cassia corymbosa, C. didymobotrya, C. fistula, C. marylandica, Cytisus albus, Laburnum alpinum, L. anagyroides, Lathyrus latifolius, Lupinus arboreus, L. luteus romulus, Piptanthus laburnifolius, Poinciana regia* and *Thermopsis lanceolata.*

PCAF reaction positive for canavanine. A compound giving the magenta reaction of canavanine with PCAF at pH 7 and occupying the same position on the chromatograms and ionophoresis papers as authentic canavanine was detected in the extracts prepared from seeds of *Anthyllis vulneraria, Caragana arborescens, Coronilla emerus, C. valentia, Desmodium gyrans, Hedysarum coronarium, Indigofera gerardiana, Medicago echinus, M. lupulina, M. sativa, Ononis fructicosa, Trifolium dubium, T. hybridum, T. pratense, T. repens* and *Vicia sativa.*

PCAF reaction positive for compounds other than canavanine. Extracts prepared from seeds of the following plants contained one or more compounds which reacted with PCAF to give colours at pH 7. These compounds appeared as spots in positions distinct from canavanine on both chromatography and ionophoresis papers. *Anthyllis vulneraria, Amorpha fragans nana, A. fructicosa, Bauhinia acuminata, B. megalandra, B. purpurea, Caesalpinia pulcherrima, Coronilla emerus, C. valentia, Desmodium gyrans, Erythrina crista-galli, E. humeana, Medicago sativa, Swainsonia hybrida, Trifolium pratense* and *T. repens.*

All the reacting compounds gave the orange and red colours expected of the guanidoxy compounds except one common to the three species of *Bauhinia*, which gave a lemon-yellow reaction, another common to *Trifolium repens* and *T. pratense*, which gave a paler-yellow spot, and another from *Swainsonia hybrida*, which gave a blue reaction with PCAF.

Quantitative determination of canavanine

Seed was finely ground and dried at 110° for 20 hr. Portions of the dried seed (50–100 mg.) were stirred with 2 ml. of 0·1 N-HCl and kept for 24 hr. The mixture was neutralized with 2 ml. of 0·1 N-NaOH, stirred with 50 mg. of kieselguhr and filtered at the pump. To 1 ml. of the clear filtrate was added 0·5 ml. of 1% PCAF mixed with 0·5 ml. of phosphate buffer, and the whole was made up to 12 ml. with buffer. When the colour had fully developed (40 min.) the optical density was measured in an EEL photoelectric colorimeter and the concentration of canavanine read from a standard curve as described by Fearon & Bell (1955). The seed was extracted a second time and the determination repeated. As large a volume of the extract as possible was used for the second determination in order to obtain an appreciable reading on the colorimeter. The results are set out in Table 1.

Isolation and characterization of canavanine from Anthyllis vulneraria *and* Medicago sativa

Finely ground seed (100 g.) of *Anthyllis vulneraria* was extracted with light petroleum (b.p. 120°) in a Soxhlet apparatus for 5 hr. After being dried in a vacuum oven at 48° the defatted seed was stirred with 50% aqueous ethanol (400 ml.) and acidified to Congo red by the dropwise addition of 50% sulphuric acid. The acidified mixture was stirred vigorously for 0·5 hr. at room temp. and the extract was filtered at the pump. The residue was extracted with 200 ml. of 50% ethanol and filtered off (no further acid was added). To each filtrate was added 5 vol. of ethanol. The solutions were kept at 4° for 24 hr.; a heavy viscous precipitate separated from the first filtrate, and considerably less from the second. (Further extraction of the seed residue with 50% ethanol did not appreciably increase the yield.) The supernatant liquid was decanted from the two precipitates, which were combined, dissolved in the minimum volume of water and filtered. Flavianic acid (20 g. in 100 ml. of water) was added and the solution was

Table 1. *Concentration of canavanine in the seeds of Leguminosae*

	Canavanine (% of dry wt.)		
	First extract	Second extract	Total
Anthyllis vulneraria	2·1	0·12	2·22
Caragana arborescens	3·65	0·15	3·80
Coronilla emerus	2·36	0·16	2·52
Hedysarum coronarium	0·67	0·15	0·82
Indigofera gerardiana	2·16	0·17	2·33
Medicago sativa	1·46	—	1·46
Ononis fructicosa	0·74	—	0·74
Trifolium dubium	1·41	—	1·41

kept at 4° for 12 hr. The crystalline flavianate was filtered off, recrystallized five times from water and decomposed with $Ba(OH)_2$. The base was finally obtained in the form of its hydrated sulphate as previously described (Fearon & Bell, 1955). Yield 2·8 g., m.p. (after shrivelling at 162°) 170–171° (decomp.) not depressed when mixed with authentic canavanine sulphate (Found: C, 20·9; H, 5·5; N, 19·4; α-NH_2 N, 5·3. Calc. for $C_5H_{12}O_3N_4,H_2SO_4,H_2O$: C, 20·5; H, 5·5; N, 19·2; α-NH_2 N, 4·8%). $[\alpha]_D^{18}+18\pm2°$ in water (*c*, 10); canavanine sulphate monohydrate from *Colutea arborescens* (Fearon & Bell, 1955) had $[\alpha]_D^{18\cdot5}+18\cdot6$.

Finely ground seed (100 g.) of *Medicago sativa* was extracted as described for *Anthyllis vulneraria*. The two extracts were combined, concentrated to 200 ml. by distillation under reduced pressure and filtered before the addition of 5 vol. of ethanol. The amino acid was purified by recrystallizing its flavianate and finally isolated as the sulphate. Yield 1·5 g.; m.p. (after shrivelling at 162°) 172° (decomp.) not depressed when mixed with authentic canavanine sulphate (Found: C, 20·6; H, 5·4; N, 18·9; α-NH_2 N, 4·5%). $[\alpha]_D^{18}+18\pm2°$ in water (*c*, 2·2).

SUMMARY

1. Methods are described for the identification of canavanine in biological extracts containing compounds which mask or inhibit the guanidoxy colour reaction given by canavanine with trisodium pentacyanoammonioferrate.

2. It has been shown that canavanine occurs in the seeds of sixteen leguminous plants; quantitative values for eight are given. The acid has been isolated from two of them.

3. In addition to canavanine other compounds which, like the guanidoxines, react with trisodium pentacyanoammonioferrate at pH 7 have been detected in the seeds of leguminous plants.

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REFERENCES

Damodaran, M. & Narayanan, K. G. A. (1939). *Biochem. J.* **33**, 1740.
Damodaran, M. & Narayanan, K. G. A. (1940). *Biochem. J.* **34**, 1449.
Fearon, W. R. (1946). *Analyst*, **71**, 562.
Fearon, W. R. & Bell, E. A. (1955). *Biochem. J.* **59**, 221.
Kitagawa, M. (1937). *J. Biochem., Tokyo*, **25**, 23.
Kitagawa, M. & Tomiyama, T. (1929). *J. Biochem., Tokyo*, **11**, 265.
Kitagawa, M. & Tsukamoto, J. (1937). *J. Biochem., Tokyo*, **26**, 373.
Makisumi, S. (1952). *J. chem. Soc. Japan*, **73**, 737.
Van Slyke, D. D., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 671.

The Detection of Metabolic Products from Dimethylnitrosamine in Rats and Mice

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The toxic properties of dimethylnitrosamine have been described by Barnes & Magee (1954), Magee & Barnes (1956) and O'Leary, Wills, Harrison & Oikemus (1957). Doses of 20–40 mg./kg. produced acute liver necrosis and death in several species. Prolonged feeding of rats with lower doses led to malignant liver tumours.

Dimethylnitrosamine is metabolized rapidly *in vivo*. Magee (1956) showed that its half-life in rats was about 4 hr., and that its disappearance in rabbits followed an exponential law. Dutton & Heath (1956*b*) concluded that the compound was rapidly demethylated in rats and mice, since much of the ^{14}C administered in a labelled sample was expired as carbon dioxide. Since the metabolic products of demethylation are chemically much more reactive than the rather inert parent compound, it is possible that the toxic action may be exerted by metabolites. We have therefore made a search for metabolites in rats and, to a lesser extent, in mice, in the hope that this might throw some light on the primary biochemical lesion. The experiments were of a preliminary nature, aimed only at discovering systems likely to repay detailed study later, and consequently very few animals were used.

The problem has been approached in three ways. In the first, [^{14}C]dimethylnitrosamine was given, and the distribution of ^{14}C between the various tissues of rats and mice was found, to ascertain whether the ^{14}C not expired as carbon dioxide was particularly concentrated in any part of the body. In the other two approaches attention was concentrated on the liver, as this was the only organ damaged, and on the urine, which might contain a high proportion of metabolites, and in which, as it is less biochemically active, they were more likely to be preserved. Thus in the second approach we carried out distribution studies with dimethylnitros-