172. *Canavanine.*

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The amino-acids arginine and canavanine share the distinction of being the only substances known which yield urea directly in the mammalian body. Canavanine, isolated by Kitagawa and his collaborators (*J. Biochem., Japan*, 1929, **11**, 265; *Proc. Imp. Acad. Japan*, 1929, **5**, 380) from the jackbean (*Canavalia ensiformis*), has the molecular formula $C_5H_{12}O_3N_4$, and the problem of its constitution has chiefly been studied with the simpler amino-acid canaline, $C_4H_{10}O_3N_2$, which is formed together with urea by the fission of canavanine with the liver enzyme canavanase. The constitution (I) assigned to canaline by Kitagawa is largely based on its quantitative fission (Kitagawa and Monobe, *J. Biochem., Japan*, 1933, **18**, 333) by catalytic reduction to ammonia and a substance which was regarded as identical with synthetic α-amino-γ-hydroxybutyric acid (Fischer and Blumenthal, *Ber.*, 1907, **40**, 106; Sørensen and Andersen, *Z. physiol. Chem.*, 1908, **56**, 250). A comparison of the properties of this material and its derivatives with those of the synthetic compound does indeed suggest a close relationship, but does not for the following reasons give proof of the identity: certain minor discrepancies in properties may be detected, no direct comparison of the substances from natural and synthetic sources was made, and the probability that the α-amino-γ-hydroxybutyric acid obtained under such conditions from optically active canaline would also be active was apparently not considered by the Japanese authors.

$NH_2{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ (I.)

$NH_2{\cdot}C(:NH){\cdot}NH{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ (III.)

$NH_2{\cdot}CH_2{\cdot}CH(OH){\cdot}CH(NH_2){\cdot}CO_2H$ (II.)

$NH_2{\cdot}C(:NH){\cdot}NH{\cdot}CH_2{\cdot}CH(OH){\cdot}CH(NH_2){\cdot}CO_2H$ (IV.)

The structure (I)—a hydroxylamine derivative—is so unexpected in the group of amino-acids as to require confirmation, and our practical interest in the problem was also aroused by the possibility that canaline might be the isomeric αγ-diamino-β-hydroxybutyric acid (II), since we had recently confirmed (J., 1934, 1644) that such α-amino-β-hydroxy-acids—apart from serine—may occur in nature by preparing from casein β-hydroxyglutamic acid (Dakin, *Biochem. J.*, 1918, **12**, 290), the re-isolation of which had for long baffled other workers.

No complete structure for canavanine has yet been proposed, and Kitagawa and Yamada (*J. Biochem., Japan*, 1932, **16**, 339) have stated that "it cannot be considered likely that the guanidine group $NH_2{\cdot}C(:NH){\cdot}NH$ is present in canavanin, but it is possible to suggest that canavanin is more nearly related to guanidine derivative than to ureide derivative." Nevertheless the presence of a guanidine group would express most readily the properties of canavanine—its basic character, its fission into urea and an amino-compound, and the interaction of two only of its four nitrogen atoms with nitrous acid in Van Slyke determinations of amino-nitrogen (Kitagawa and Yamada, *loc. cit.*). The introduction of the guanidine group into (I) and (II) would lead, therefore, to (III) and (IV) as possible structures for canavanine. Our investigations have dealt solely with

canavanine, not with canaline, in view of the desirability of working with a material which has not been subjected to the action of the complex battery of enzymes of the liver, and a method is now described which is more practicable for the isolation of canavanine than those hitherto recorded.

Oxidation with chloramine-T is the most effective process for the detection of β-hydroxy-amino-acids, since it yields α-hydroxy-aldehydes. Oxidation of canavanine in this way, followed by precipitation of carbonyl compounds with *p*-nitrophenylhydrazine, yielded the *p*-nitrophenylosazones of glyoxal and tartronic semialdehyde (V). The glyoxal fragment was presumably formed as the result of decarboxylation, since its osazone alone was obtained when the oxidation and osazone formation were effected in acetic acid, whereas the osazone of (V) alone was isolated after the reactions had been carried out in dilute hydrochloric acid.

$CO_2H{\cdot}CH(OH){\cdot}CHO$ (V.) $CO_2H{\cdot}CH(OH){\cdot}CH(NH_2){\cdot}CO_2H$ (VI.) $CH_2Br{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ (VII.)

These facts appeared at first to strengthen the possibility that canavanine might be (IV), since Dakin (*Biochem. J.*, 1919, **13**, 419) obtained tartronic semialdehyde *p*-nitrophenylosazone by the same method from hydroxyaspartic acid (VI); in the present case oxidation of the guanidino-methylene group to carboxyl might occur. This possibility, however, was not supported by the observation that the solution obtained from the chloramine-T oxidation did not rotate polarised light, as would have been expected if it contained tartronic semialdehyde from (IV). This might have been due to racemisation or internal oxidation-reduction to the corresponding ketonic primary alcohol, though this seems improbable from the work of Berl and Fodor (*Z. ges. Schiess.-Sprengstoffw.*, 1910, **5**, 296). Alternatively, the osazones of glyoxal and (V) might have resulted from the oxidation products of aspartic acid, formed by oxidation of (III) at the $NH_2{\cdot}C(:NH){\cdot}NH{\cdot}O{\cdot}CH_2$ group; aspartic acid is known to yield glyoxal under these conditions (Dakin, *Biochem. J.*, 1917, **11**, 79).

The action of hot halide acids on canavanine seemed to offer a method of distinguishing between (III) and (IV), since *O*-ethers of hydroxylamine readily yield the alkyl halide and hydroxylamine, whereas arginine is remarkably stable to this treatment; (III) may be regarded as an *O*-ether of a *N*-substituted hydroxylamine; and (IV) is a hydroxylated, lower homologue of arginine. Hot concentrated hydrobromic acid converted canavanine into ammonia, guanidine, and a substance which must be regarded as α-amino-γ-butyrolactone hydrobromide in view of its properties. This substance could not be completely separated from admixed ammonium bromide and sodium bromide (from sodium carbonate used in the preparation), but when heated again with concentrated hydrobromic acid it yielded optically inactive *γ-bromo-α-aminobutyric acid hydrobromide* (as VII), identical with a specimen prepared by the same method from synthetic α-aminobutyrolactone (Fischer and Blumenthal, *Ber.*, 1907, **40**, 106). The racemisation at the α-carbon atom of canavanine which occurs during the treatment is paralleled by the racemisation of arginine by hot concentrated sulphuric acid (Kutscher, *Z. physiol. Chem.*, 1901, **32**, 478; Reisser, *ibid.*, 1906, **49**, 222).

These results prove canavanine to have the structure (III) and confirm the conclusions of Kitagawa and Monobe as to the structure of canaline.

Experimental.

Preparation of Canavanine.—Jackbean meal was freed from oil by extraction (Soxhlet) for 24 hours with acetone. The oil-free, air-dried meal (200 g.) was extracted twice with 50% (by weight) aqueous ethyl alcohol (1 l.) on a shaker for 1 hour. The combined extracts were concentrated under reduced pressure to a syrup, which was poured into vigorously stirred, absolute alcohol (15—20 vols.). Next day the alcohol was decanted from the sticky solid; this was dissolved in warm water (4 parts) and filtered slowly into mechanically stirred, cold absolute alcohol (15 vols.), and the mixture kept at 0° till next day. For the success of this precipitation it is essential to make the addition slowly and to use enough alcohol. The crude canavanine which had separated was collected, washed with absolute alcohol (when dried under

reduced pressure, the yield from 1·5 kg. was 180 g.), and dissolved in water (500 c.c.). The solution was acidified with dilute sulphuric acid and mixed with flavianic acid (250 g.) in water (400 c.c.). The mixture was kept at 0° for 12 hours, and the crystalline flavianate was collected, washed with dilute flavianic acid solution, and recrystallised twice from water. This removed a small amount of a sparingly soluble, deep red flavianate, which was discarded; the canavanine flavianate ultimately obtained formed yellow needles, m. p. 212° (Kitagawa and Yamada record that the flavianate becomes semi-fluid at 190—192° and melts with decomposition at 210—215°).

Kitagawa and his collaborators continued the isolation of canavanine by way of the picrate (m. p. 163—164°, Kitagawa and Yamada; m. p. 155—158°, Kitagawa and Tomita). We have prepared a picrate, m. p. 220°, from the neutralised hydrochloride of the pure amino-acid, but it is rather readily soluble in water, and we have been unable to follow the isolation used by Kitagawa owing to lack of experimental details of procedure.

Various methods of decomposing the flavianate—decomposition by concentrated hydrochloric acid, adsorption of the flavianic acid with wool in acid suspension (cf. Mueller, *Z. physiol. Chem.*, 1932, **209**, 207), decomposition by electrodialysis, precipitation of the flavianic acid as lead salt with lead oxide—proved unsuccessful or unsatisfactory, and the following procedure was finally adopted. A hot aqueous solution of the flavianate was mixed with a slight excess of warm baryta solution, the mixture cooled, and the barium flavianate collected and extracted twice with boiling water. The filtrate and washings were combined and freed from barium ions by titration with 0·1*N*-sulphuric acid, sodium rhodizonate being used as an external indicator (Giblin, *Analyst*, 1933, **58**, 752). The barium sulphate was removed and extracted with boiling water and the combined filtrate and washings were concentrated to small volume. The solution, still yellow, was mixed with excess of a concentrated aqueous solution of rufianic acid (Zimmermann, *Z. physiol. Chem.*, 1930, **188**, 180) and left at 0° for 24 hours. The crystalline rufianate, recrystallised from hot water, formed deep red, spherical aggregates (unmelted below 350°), which were dissolved in hot water and mixed with excess of warm baryta solution. The mixture was cooled and filtered from barium rufianate, and the almost colourless filtrate was freed from barium ions by titration with 0·1*N*-sulphuric acid and sodium rhodizonate. The barium sulphate, which carried down the last traces of colour, was collected and extracted with boiling water, and the colourless solution was concentrated to small volume under reduced pressure and poured into absolute alcohol (15 vols.). No immediate precipitation occurred, but after remaining over-night at 0°, a good yield of crystalline canavanine, m. p. 184°, had separated and a further amount was obtained by concentrating the mother-liquor. The canavanine formed colourless irregular crystals, m. p. 184°, $[\alpha]_D^{20°}$ + 7·9° in water (c = 3·2%) (Found: C, 34·2; H, 6·9; N, 30·7. Calc. for $C_5H_{12}O_3N_4$: C, 34·1; H, 6·8; N, 31·8%). Kitagawa and Yamada give m. p. 182—183° and $[\alpha]_D$ + 8·09°. It gave the characteristic colour reaction with irradiated sodium nitroprusside solution, a positive ninhydrin, and a negative Sakaguchi reaction. The failure to give the Sakaguchi reaction, characteristic of the guanidine group of arginine, is explicable on Poller's observation (*Ber.*, 1926, **59**, 1927) that, in the case of unsubstituted guanidine radicals, a positive test is obtained only when the radical is united directly to a carbon atom which is essentially part of an alkyl group.

Oxidations with Chloramine-T.—(i) *In dilute acetic acid.* A mixture of a saturated aqueous solution of chloramine-T (5·1 g.) and canavanine (0·92 g.) in water (10 c.c.), previously neutralised with acetic acid, was kept for ½ hour at room temperature, warmed at 60° for 1 hour, cooled, and filtered from *p*-toluenesulphonamide. The filtrate was heated on the water-bath for 1½ hours with *p*-nitrophenylhydrazine (3 g.) in alcoholic acetic acid and cooled. The precipitate was collected, extracted with hot nitrobenzene to remove a small amount of soluble material which was not an osazone (no blue colour with alkali), and recrystallised from pyridine. It formed scarlet needles, m. p. 309° alone or mixed with authentic glyoxal-*p*-nitrophenylosazone, and gave the blue coloration with sodium hydroxide characteristic of *p*-nitrophenylosazones.

(ii) *In dilute hydrochloric acid.* A mixture of chloramine-T (10 g.) suspended in water (25 c.c.) and canavanine (2 g.) dissolved in water (4 c.c.), previously neutralised with hydrochloric acid, was kept at room temperature for 1 hour, cooled, and filtered. The filtrate was optically inactive and remained so after being warmed at 60° for ½ hour. It was heated with *p*-nitrophenylhydrazine (6 g.) in dilute alcoholic hydrochloric acid for 1 hour, and the precipitate was collected, washed with water and alcohol, and extracted with boiling nitrobenzene to remove a small amount of soluble material which was not an osazone. The insoluble residue of tartronic aldehyde *p*-nitrophenylosazone crystallised from much nitrobenzene in red needles, m. p. 302° alone or mixed with an authentic specimen (m. p. 302°) (Dakin, *Biochem. J.*, 1919,

13, 398) (Found: N, 22·6. Calc. for $C_{15}H_{12}O_6N_6$: N, 22·6%). It gave a royal-blue colour when dissolved in acetone and treated with sodium hydroxide solution.

In connection with the constitution of canavanine, it is noteworthy that in both the experiments described above the solution became deep yellow during the oxidation with chloramine-T. Guanidine behaved similarly under the same conditions, whereas in experiments with methylguanidine and arginine, in which the guanidine group is directly united to carbon, the liquid remained colourless. Methylguanidine, moreover, yielded much methylamine, showing that the carbon–nitrogen linkage is not broken by the oxidation.

Action of Mineral Acids on Canavanine.—Preliminary experiments showed that canavanine is rapidly decomposed—no reaction with irradiated nitroprusside—by boiling 25% sulphuric acid, whereas it was recovered unchanged after being heated under reflux for 3 hours with 20% hydrochloric acid.

(i) *Dilute hydrobromic acid.* A solution of canavanine (0·2 g.) in hydrobromic acid (6 c.c., *d* 1·3) and water (3 c.c.) was heated for 3 hours in a sealed tube at 150°. The contents of the tube were evaporated to dryness under reduced pressure and dissolved in water. The solution, which gave a pronounced ninhydrin reaction but no coloration with irradiated nitroprusside, contained ammonia and urea, isolated as dixanthylurea, but gave no precipitate of the sparingly soluble guanidine picrate when neutralised and mixed with picric acid.

A parallel experiment under similar conditions with guanidine yielded ammonia and a small amount of urea, but only 12·5% of the guanidine was recovered unchanged as picrate.

(ii) *Concentrated hydrobromic acid.* A solution of canavanine (1·3 g.) in 60% hydrobromic acid (25 c.c., *d* 1·7) was heated in a sealed tube for 5 hours at 160°. The contents of the tube were evaporated to dryness under reduced pressure, and the residue was dissolved in water, neutralised to litmus with sodium carbonate, and mixed with an excess of a cold saturated aqueous solution of picric acid. The mixture was kept for 3 hours at 0°, and the guanidine picrate (18·8%), m. p. 315°, was collected, washed with water, and dried at 100°. After recrystallisation from water, it melted at 318° alone or mixed with an authentic specimen.

The filtrate from the picrate was acidified with hydrobromic acid and freed from picric acid by extraction with benzene. The aqueous layer was evaporated to dryness under reduced pressure; the product gave a pronounced ninhydrin reaction, but also contained ammonium and sodium bromides. It was repeatedly extracted with a cold mixture of equal parts of alcohol and chloroform, but scarcely any material dissolved. There can be little doubt that this insoluble product consisted essentially of α-aminobutyrolactone hydrobromide, since an authentic specimen gave a strongly positive ninhydrin reaction, was insoluble in a mixture of alcohol and chloroform, and yielded γ-bromo-α-aminobutyric acid hydrobromide when heated with hydrobromic acid. The crude lactone hydrobromide was heated with 60% hydrobromic acid (20 c.c.) in a sealed tube for 5 hours at 160°, and the contents of the tube were evaporated to dryness and extracted with cold ethyl alcohol–chloroform (1 : 3), which dissolved all the material giving the positive ninhydrin reaction, and left undissolved sodium bromide and most of the ammonium bromide. The filtered solution was freed from solvent and again extracted with alcohol–chloroform. The solution from this extract was evaporated, and the residue dissolved in dilute hydrobromic acid and concentrated under reduced pressure to a very small volume. The oily mass solidified to a crystalline mass, which was pressed on porous tile and crystallised twice from alcohol–chloroform. The resulting *γ-bromo-α-aminobutyric acid hydrobromide* formed fine colourless needles, m. p. 164° alone or mixed with a synthetic specimen (Found: C, 18·5; H, 3·6; Br, 60·9. $C_4H_8O_2NBr,HBr$ requires C, 18·3; H, 3·4; Br, 60·8%). An 8·2% aqueous solution showed no optical activity.

γ-Bromo-α-aminobutyric Acid Hydrobromide.—A solution of α-aminobutyrolactone hydrobromide (1 g.) (Fischer and Blumenthal, *loc. cit.*) in 60% hydrobromic acid (6 c.c.) was heated in a sealed tube at 160° for 5 hours. The contents of the tube were concentrated to small volume under reduced pressure and the crystalline mass was extracted with absolute alcohol, to remove traces of insoluble material. The residue obtained by evaporation of the alcohol was twice crystallised by precipitation from a very concentrated solution in hot alcohol with chloroform. γ-Bromo-α-aminobutyric acid hydrobromide formed fine square-ended needles, m. p. 164° (decomp.), which gave a strong ninhydrin reaction (Found: C, 18·4; H, 3·5; N, 4·9; Br, 60·3. Calc. for $C_4H_8O_2NBr,HBr$: C, 18·3; H, 3·4; N, 5·3; Br, 60·8%). It dissolved readily in water, ethyl and methyl alcohols, and glacial acetic acid, was less soluble in acetone, and insoluble in chloroform.

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