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Chemical Investigations upon *Lotononis laxa* E. and Z.I. The Isolation of Pinitol, a Fatty Ester and Benzaldehyde.

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This plant was responsible for prussic-acid poisoning in stock on cultivated lands in the Lady Grey district, C.P., and was submitted to this laboratory for a chemical examination of the toxic principle. The dried and ground plant-material, after it had been for approximately six weeks at the laboratory still contained 259·2 mgm. HCN per 100 grams of dried plant (i.e. about 0·26 per cent. HCN), which obviously is a very high figure, making the plant extremely dangerous to stock.

Determination of Hydrocyanic-acid Content.

The hydrocyanic-acid determinations were carried out in the usual way with samples of 10 gms. of the dried and ground plant with the following results:—

Time of Maceration.	Medium.	Enzyme.	Temperature.	N c.c. — 50 $AgNO_3$.
4 hours	200 c.c. dist. H_2O.....	Plant only...	Room.....	12·1
6 hours	„ „	„	„	14·0
16½ hours	„ „	„	„	21·0
18½ hours	„ „	„	„	21·0
17 hours	200 c.c. pH6 (buffer solution)....	„	„	24·0*
19 hours	„ „ „	„	„	24·3*
24 hours	„ „ „	„	„	24·5*
25 hours	„ „ „	„	„	24·3*
20 hours	„ „ „	Plant enzyme and emulsin	„	23·5

* The maximum HCN content was obtained after about 18 hours maceration in a buffer solution of pH6 at room temperature (plant enzyme only).

Cyanhydrin-test.—10 gms. of the dried powdered plant was macerated with 200 c.c. of a buffer solution of pH = 6 for 20 hours (no emulsin). 50 c.c. Normal caustic soda was then added (distinctly

alkaline) and allowed to stand for 20 minutes (for hydrolysis of possible cyanhydrins). 20 gm. Tartaric acid was then added (distinctly acid) and the HCN distilled. On titration of the clear distillate 23·5 c.c. $\frac{N}{50}$ $AgNO_3$ was used.

Therefore neither the addition of emulsin nor the hydrolysis of possible cyanhydrins showed an increase in the total hydrocyanic acid content.

$\therefore$ The maximum HCN content $= 24 \cdot 0 \times 1 \cdot 08 \times 10$.
$= 259 \cdot 2$ mgm. HCN per 100 gms. dry plant.

In each case the distillate was collected in $\frac{N}{3}$ NaOH and had a strong aromatic odour, similar to that of bitter-almonds, and when the distillate was acidified to about 2 N-HCl and treated with a solution of Brady's reagent (2, 4-dinitrophenylhydrazone) in 2 N-HCl solution a strong precipitate was observed (see below for nature of precipitate). The aromatic substance had aldehydic properties.

Detection and Determination of Benzaldehyde.

When the dried and powdered plant was immediately steam distilled in either (1) a neutral solution, i.e. suspended in distilled water or (2) in an alkaline medium (i.e. about 20 gms. plant in 50 c.c. normal NaOH) distillates were obtained, which smelt strongly aromatic (bitter-almonds) and which gave strong orange-yellow precipitates with Brady's reagent.

The following experiment was conducted to determine the yield of the Brady's derivate:—

(1) 50 gm. of dried and ground plant-material (HCN content $= 0 \cdot 259$ per cent.) was suspended in 200 c.c. of a citrate buffer solution (pH $= 6$) and immediately steam distilled. The distillate was collected in a little ice-water. Hydrocyanic acid was freely liberated. The distillation was stopped after 50 minutes, which proved sufficient for the complete recovery of the aromatic substance.

The distillate (about 180 c.c.) was acidified with concentrated hydrochloric acid to about 2-normal and warmed. To the warm solution a slight excess of hot Brady's reagent (0·5 gm. in 15 c.c. 2 N-HCl) was added. An orange-yellow precipitate formed immediately which was centrifuged off after some time. The precipitate was thoroughly washed first with 2 N HCl and then with distilled water and finally dried to a constant weight at 110° C. Weight of precipitate $= 0 \cdot 2$ gm.

The crystalline precipitate was easily recrystallized from either acetone or alcohol and after two re-crystallizations from either solvent had a constant, clear melting-point of 235° C., formed fine

orange leaflets and was chemically pure. The crystals dissolved fairly easily in acetone and ethylacetate, dissolved with difficulty in hot absolute alcohol and were insoluble in water.

*Micro-analysis.**

5·191 mgm.....	10·400 mgm. CO_2; 1.720 mgm. H_2O.
3·373 mgm.....	0·562 c.c. N at 20·5° C. and 765 mm. Hg.
Found.....	C = 54·66%; H = 3·71%; N = 19·50%.
Calculated for $C_{13}H_{10}N_4O_4$.....	C = 54·56%; H = 3·52%; N = 19·57%.

i.e. Benzaldehyde—2, 4-dinitrophenylhydrazone or $C_6H_5\underset{H}{C}=N-\underset{H}{N}-C_6H_3(NO_2)_2$

* All micro-analyses by Dr. Ing. A. Schoeller, Berlin.

When this substance was mixed with an authentic specimen of benzaldehyde – 2, 4-dinitrophenylhydrazone prepared by the condensation of the components in HCl medium, no depression of the melting-point occurred. The authentic sample crystallized from acetone or alcohol and also had m.p. 235.

(11) The above steam disillation (1) was repeated and the distillate collected in ice-water. The distillate was then shaken with pure ether (Merck), the ethereal solution washed, dried over Na_2SO_4, filtered and allowed to evaporate at room temperature. A little absolute alcohol was then added to the residue, which smelt strongly of benzaldehdye, and the solution refluxed with 0·3 gm. semi-carbazide-hydrochloride for 30 minutes. The filtrate was then slightly evaporated and the micro-crystalline material re-crystallized from very dilute alcohol. Colourless slender needles separated which had a melting point of 214° C. This is also the m.p. of benzaldehyde-semi-carbazone.

An authentic specimen was therefore prepared from benzaldehyde (Merck) and semi-carbazide-hydrochloride. Crystallized from alcohol, the synthetic benzaldehyde-semi-carbazone (shining plates) had a m.p. of 214° C. The natural and synthetic specimens were mixed, and the m.p. of the mixture showed no depression.

The substance was therefore benzaldehyde-semi-carbazone.

As can be seen from the above the yield of benzaldehyde was very small. When the steam-distilled plant was therefore again macerated with emulsin in a buffer solution of pH = 6, the major portion of the hydrocyanic acid could be determined. It is thus clear that the major portion of the hydrocyanic-acid is present in the form of a substance capable of being hydrolised by an enzyme.

The Nature of the Cyanogenetic Glucoside.

With the small and inadequate quantity of plant material at our disposal the preliminary attempts to isolate the cyanogenetic glucoside failed. Only benzaldehyde, pinitol and a fatty ester could be isolated thus far and the isolation of the cyanogenetic constituent is reserved for a later date when more plant material will be available.

Preliminary results however would point to the possibility that the nature of the cyanogenetic glucoside may be that of a combination of benzaldehyde and hydrocyanic acid, e.g. with glucose or vicianose. Such examples are amygdalin, sambunigrin (see also Finnemore et al), vicianine (Bertrand), Prunasine (Fischer and Bergmann) and prulaurasine (Fischer and Bergmann). These glucosides are all highly toxic due to their ready hydrolysis to benzaldehyde, hydrocyanic acid and the sugar constituent.

Isolation of a Fatty Ester, Probably C_{42} H_{84} O_4.

When separate quantities (about 100 gm.) of the dried and ground plant were extracted in a Soxhlet apparatus with (*a*) ether, (*b*) acetone, (*c*) petroleum-ether and (*d*) ethyl-acetate, a fatty-like crystalline powder was obtained in each case. After re-crystallization from acetone the crystalline powder had a melting-point of 78° C. (clear).

The substance was insoluble in water, alkalis, mineral acids and dilute sodium carbonate solution. It was very soluble in chloroform, and soluble in absolute alcohol, ethyl-acetate and acetone. It was difficultly soluble in ether and petroleum-ether.

The substance gave no colouration with concentrated sulphuric-acid or with ferric-chloride solution. Phytosterol tests were negative. It is a neutral, non-acidic, non-phenolic, optically inactive substance and contained carbon, hydrogen and oxygen only.

Micro-analysis.

4·936 mgm.....	14·680 mgm. CO_2; 5·950 mgm. H_2O.
Found.....	C = 81·15%; H = 13·49%.
Calculated for $(C_{21}H_{42}O)_x$	C = 81·30%; H = 13·55%.

Molecular weight determinations in camphor (Rast) gave results for a molecular weight of 800 to 1200. Now $C_{42}H_{84}O_2$ (molecular weight = 620) is the cerylester of palmitic acid, the main constituent of opium wax; has a melting-point of 79° C. and is a crystalline powder (Beilstein, Heilbron).

It has the formula $CH_3.(CH_2)_{13}.CH_2.CO\text{-}O\text{-}CH_2.(CH_2)_{24}.CH_3$, which may be the same as the above substance isolated from *Lotononis laxa* E and Z.

Isolation of Pinitol.

The dried and powdered plant was extracted with acetone in a Soxhlet apparatus. When the neutral substance of m.p. 78° C. (see above) had separated out the acetone solution was filtered and decolourised with adsorbent charcoal. After filtration an aliquot was diluted with about an equal volume of benzene when crystals of m.p. 181-183° separated out. When the colourless acetone solution was allowed to stand in an ice-chest the same substance separated together with a syrupy liquid, which was positive for sugar and slightly positive for cyanogenetic glucoside.

However, very little cyanogenetic glucoside must have been extracted since the plant-residue still contained about 92 per cent. of the original hydrocyanic acid content. Neither did digestion with cold acetone, nor Soxhlet extraction with ether or petroleum-ether remove any of the cyanogenetic glucoside. Hot acetone and hot ethyl-acetate extracted some of the cyanogenetic glucoside, which can apparently be readily extracted with hot alcohol.

The sandy clusters of crystals which separated above had the appearance and properties of pinitol. When recrystallized the melting-point (189°) and the optical activity

$$\left[\alpha\right]_{D}^{22} = \frac{+0{\cdot}57 \times 100 \times 5}{0{\cdot}5 \times 8{\cdot}75} = +65{\cdot}1^{\circ}\ (H_2O)$$

were identical with that of pinitol, the mono-methyl ester of l-inositol.

Summary.

(1) Preliminary chemical investigations upon *Lotononis lava* E and Z, a dangerously toxic cyanogenetic plant to stock and occurring in the Lady Grey district, C.P., resulted in the isolation of a fatty ester, pinitol and benzaldehyde.

(2) The hydrocyanic-acid content of the dried and powdered plant was still very high, namely 0·26 per cent. and the view is expressed that the cyanogenetic glucoside may be constituted by the combination of a sugar, benzaldehyde and hydrocyanic-acid. Further results will follow when more of the fresh plant has been obtained.

Acknowledgement.

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