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The Occurrence of Cyanogenetic Glucosides in South African Species of *Acacia*.

II. Determination of the Chemical Constitution of Acacipetalin. Its Isolation from *Acacia stolonifera*, Burch.

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STEYN AND RIMINGTON (1935), in the first paper of this series, reported the occurrence in several South African species of *Acacia* of significant quantities of cyanogenetic substances. Among the species investigated, *Acacia lasiopetala* Oliv., and *Acacia stolonifera*, Burch. were found to yield the largest quantities of prussic acid.

The glucoside was isolated from the former species and shown to correspond with none of the known cyanogenetic glucosides. It was proposed to name it *Acacipetalin*.

In the present communication are recorded improvements in the method of preparation and also the isolation of Acacipetalin from *Acacia stolonifera* (see Fig. 1).

The constitution of Acacipetalin has been elucidated. It is the glucose ether of dimethylketenecyanhydrin.

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix} > C = C < \begin{matrix} CN \\ O\text{–}C_6H_{11}O_5 \end{matrix}$$

The only reference to the presence of cyanogenetic glucosides in species of *Acacia*, other than those studied by Steyn and the present writer, is contained in the work of Finnemore and Gledhill (1928) and Finnemore and Cox (1930). The latter isolated the glucoside sambunigrin, benzaldehyde-cyanhydrin glucose ether, from the Australian species *Acacia glaucescens* and *Acacia cheelii*. As pointed out by Steyn and Rimington, the South African species of *Acacia* belong, botanically, to a different group in the genus than do the Australian species, and it is therefore of very great interest to find that the glucoside contained in *Acacia lasiopetala* and *Acacia stolonifera* (the only two species so far studied) is in no way related to sambunigrin.

Accompanying acacipetalin, the substance *Pinit*, inositol monomethyl ether, was found in *Acacia lasiopetala*.

Fig. I.—*Acacia Stolonifera, Burch.* ½ natural size.

Improvements in the Method of Isolation of Acacipetalin.

It is presumably the presence of Pinit in the *Acacia* species studied which renders the isolation of the cyanogenetic glucoside so difficult. Pinit, or inositol monomethyl ether, has solubilities and physical properties very closely resembling those of Acacipetalin. Thus, both are very soluble in water, sparingly soluble in hot ethyl acetate and insoluble in ether, chloroform or benzene. Whilst the glucoside is sparingly soluble in hot absolute alcohol, however, pinit is practically insoluble in this solvent and it was by means of this difference that their separation was originally achieved before the identity of the non-glucosidal substance was known.

Since pinit can be precipitated by basic lead acetate and ammonia, it was hoped that a more rational separation could be accomplished by the use of these reagents. The surmise was

justified although some glucoside was lost in the lead precipitate. Thus, a residual syrup, from *Acacia stolonifera*, giving strongly positive glucosidal reactions, but from which nothing could be induced to crystallise, was dissolved in water and 1 gm. of cadmium nitrate added, followed by 5 c.c. of saturated basic lead acetate solution and sufficient ammonia to cause complete precipitation. The precipitate was removed and excess of lead precipitated from the filtrate by hydrogen sulphide. The filtered liquid was neutralised, a little solid calcium carbonate added and evaporated to dryness upon the water bath. The residue was exhausted with hot ethyl acetate from which a quantity of glucoside separated in crystalline form as the solution cooled.

Another modification tried out was the process described by Hérissey (1932) as a general one for the extraction of glucosides, successful in many cases where ordinary methods failed.

An extract of *Acacia lasiopetala* was made with boiling 96 per cent. alcohol, the alcohol removed in the presence of a little calcium carbonate by vacuum distillation and the residue taken up in water (volume equal to the weight of plant taken) and filtered. By trial upon an aliquot, diluted, the quantity was determined of basic lead acetate solution necessary for complete precipitation. This amount was then added to the main bulk plus about 10 per cent. in excess. Anhydrous sodium sulphate was then stirred in, using 1 gm. to every 0·75 c.c. of basic lead acetate solution (or 1 gm. to each 1 c.c. of original extract) followed by calcium carbonate in the proportion 0·25 to 0·5 gm. for each 1 gm. of sodium sulphate. The mixture was well stirred at intervals and when sufficiently solid was spread out on a large tray to dry. Dehydration was completed in the vacuum desiccator. The powdered mass was then introduced into a Soxhlet thimble and continuously extracted with boiling ethyl acetate. From the extract, some glucoside crystallised on cooling and a further quantity was obtained by evaporating to dryness, redissolving the deeply coloured residue in water, decolorising by charcoal, evaporating and again extracting the dry residue with successive portions of boiling ethyl acetate. Hérissey's method gave a yield slightly better than that previously obtained.

Possibly the best procedure was a simple modification of the method outlined (Steyn and Rimington, 1935), but even so, the yield fell far short of that theoretically obtainable.

The lead precipitation was carried out in the presence of cadmium nitrate and ammonia and the clarified extract treated as previously described. Having obtained the ether-alcohol filtrate and evaporated off the solvents, a little water was added to the residue followed by much decolorising charcoal and a little calcium carbonate. The mass was then dried as thoroughly as possible, powdered, introduced into a Soxhlet thimble and extracted by hot ethyl acetate. A perfectly colourless ethyl-acetate solution was thus obtained, from which crystals separated on concentrating and cooling. They were recrystallised from absolute alcohol-ether.

Pinit the impurity accompanying the glucoside in the final ethyl-acetate solutions was separated by means of its insolubility in boiling absolute alcohol. It crystallised in wedge-shaped prisms and had M.P. 184°. Micro-analysis* afforded the following figures:

	C	H	N
Found.....	43·41	7·06	Nil
$C_7H_{14}O_6$ requires.....	43·30	7·22	—

The optical rotatory power was determined in a 2 dm. tube using an aqueous solution:

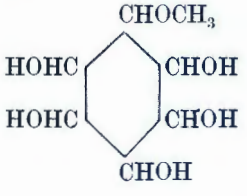
Wt. of substance..... 0·0978 gm.
Volume of solution..... 15 c.c.
Rotation observed..... + 0·78°

$$\therefore \left[\alpha\right]_D^{22} = + \frac{0{\cdot}78 \times 100 \times 15}{2 \times 9{\cdot}78}$$

$$= + 59{\cdot}8°$$

The constants for pinit are M.P. 186° and $\left[\alpha\right]_D^{25} = + 65{\cdot}3$. The isolated material was found to reduce ammoniacal silver nitrate on heating. Pinit occurs in the following plants: *Pinus lambertiana* Dougl.; *Abies pectinata*, D.C. *Cassia angustifolia* Vahl., *Landolphia madagascariensis*, Schum., *Sequoja sempervirens* Endl.

Its constitution is:—



ISOLATION OF ACACIPETALIN FROM *Acacia stolonifera*, BURCH.

Qualitative tests performed by Steyn and Rimington (1935) showed that both the fresh leaves and immature pods of *Acacia stolonifera* contained appreciable quantities of cyanogenetic substances.

A larger batch of material was gathered in the same locality (Wonderboompoort, near Pretoria), on 20th April, 1934, when the tree was in the seeding stage. The leaves, when dried and ground, yielded 62 mgm HCN per 100 gms. 1 kg. of the powdered material was exhausted with hot 96 per cent. alcohol in the presence of a considerable quantity of calcium carbonate. The extract was concentrated under reduced pressure and then by the fan to dryness. The residue was taken up in water, filtered, basic lead acetate added and then 20 gm. of cadmium nitrate, dissolved in water, and sufficient ammonia to produce complete precipitation. The liquid was

* Micro-analyses by Dr. Backeberg, of the University of the Witwatersrand, to whom I wish to express my thanks.

filtered, neutralised by acetic acid and excess of lead removed by hydrogen sulphide. After removal of the lead sulphide and excess of gas, the clear solution was concentrated in presence of calcium

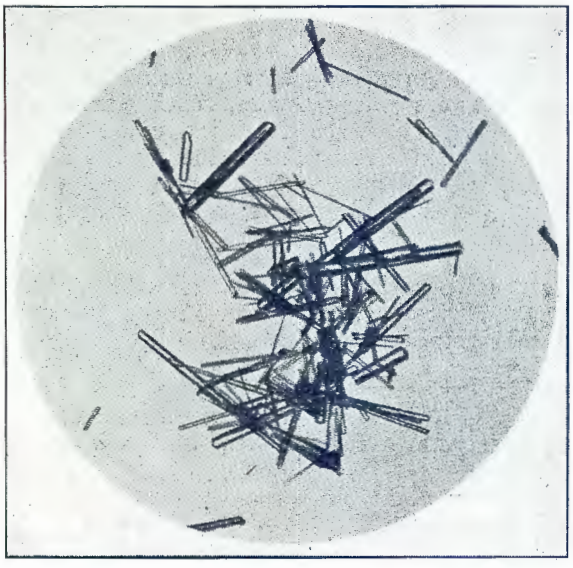


Fig. II (*a*).—*Acacipetalin* crystallised from abs. alcohol-ether × 65.

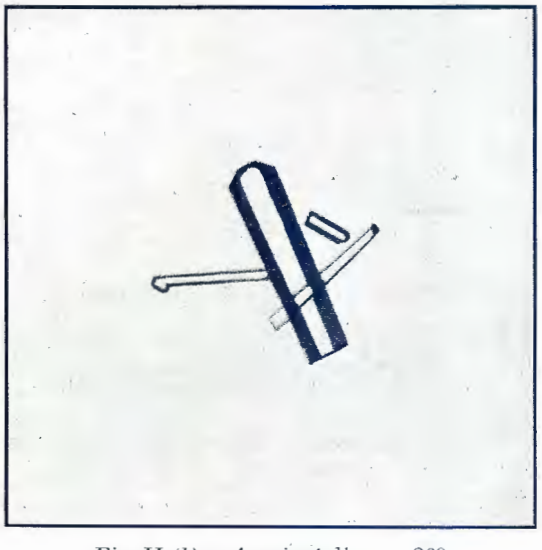


Fig II (*b*).—*Acacipetalin* × 260.

carbonate, first in vacuo and then by the fan, until it formed a thin syrup. A considerable quantity of decolorising charcoal and calcium carbonate were now stirred in until the mass was no longer sticky.

It was spread out in thin layers and dried first in the air and then in vacuo over sulphuric acid. It was finally powdered, introduced into a soxhlet extraction thimble and continuously extracted by boiling ethyl acetate for two days. As a precautionery measure some calcium carbonate was placed in the bottom of each extraction flask.

The pale, straw-coloured extracts were combined and concentrated. On cooling a large crop of crystalline glucoside separated and further crystals were obtained after concentration of the mother liquors. The material was washed with dry ice-cold ethyl acetate and dried in vacuo. Final recrystallisation by the absolute alcohol-ether method yielded an analytically pure substance with M.P. 176-7°. The yield was 1·12 gm. or about 20 per cent. of that theoretically possible.

From a further large batch of young leaves gathered from the same tree on 31st November, 1934 (i.e. in the spring), a yield of 10·69 gm. of Acacipetalin was obtained.

The glucoside crystallised in colourless six-sided prisms (see Figs II) had M.P. 176-7° and possessed a bitter taste. It resembled in all respects Acacipetalin from *Acacia lasiopetala.*

Micro-analysis:—

	C	H	N
Found.....	51·17	6·72	5·35
$C_{11}H_{17}O_6N$ requires.....	50·78	6·59	5·43

The optical rotatory power, determined in a 2 dm. tube, agreed with the value (-35·96) previously found for Acacipetalin from *Acacia lasiopetala* (see Steyn and Rimington).

C = 0·25 gm. in 15 c.c. water.
$\alpha = -1{\cdot}22°$

$$\therefore \left[\alpha\right]_D^{26} = \frac{-1{\cdot}22 \times 100 \times 15}{2 \times 25}$$

$$= -36{\cdot}60°$$

Constitution of Acacipetalin.

Acacipetalin possesses a somewhat remarkable constitution. On account of the unstable nature of the aglucone, it does not yield, on hydrolysis, an aldehyde or ketone together with hydrogen cyanide as do most cyanogenetic glucosides. Alkaline, followed by acid hydrolysis was found ultimately to provide the key to the solution of its structure. The various steps by which the final conclusion was arrived at are detailed below.

1. *Empirical formula.*

Micro-analysis of different preparations afforded figures agreeing most closely with $C_{11}H_{17}O_6N$. This was also shown to be the molecular formula from molecular weight determinations (see below).

Such a formula would demand that the aglucone formed when one molecule of glucose and one molecule of hydrogen cyanide are removed should have the composition C_4H_6O. This represents an unsaturated compound, there being some five or six isomeric possibilities. Had the original formula for the glucoside contained two more hydrogen atoms, making $C_{11}H_{19}O_6N$, a saturated aglucone C_4H_8O would be indicated by suitable calculations. Only three possibilities would then have to be considered, normal and iso-butyl aldehydes and methylethylketone.

Degradation experiments proved conclusively that neither of these three substances was formed so that the formula $C_{11}H_{17}O_6N$, can be accepted as correct. The analytical data agree more closely with this than with $C_{11}H_{19}O_6N$, thus:—

	C	H	N	
$C_{11}H_{19}O_6N$ requires.	50·39	7·31	5·38	
$C_{11}H_{17}O_6N$,,	50·78	6·59	5·43	
Found...	50·94	6·68	5·45	(*A. lasiopetala*)
	51·17	6·72	5·35	(*A. stolonifera*).

Methoxyl groups, CH_3O, were tested for but found to be absent.

2. *Molecular Weight.*

This was determined in water by the cryoscopic method and found to support the simple formula $C_{11}H_{17}O_6N$

Weight of glucoside.....	0·250 gm.
Weight of solvent.....	14·9 gm.
Observed depression.....	0·120; 0·125

$\therefore$ M. Wt. = 258·6; 248·3

$C_{11}H_{17}O_6N$ requires 259

3. *Identification of Glucose and Hydrogen Cyanide: enzyme hydrolysis.*

0·1460 gm. of Acacipetalin was dissolved in 15 c.c. of water and about 1 mgm. of an active emulsin preparation added. The tube was stoppered, including a piece of sodium picrate paper, and left overnight in an incubator at 37°.

The picrate paper was rapidly turned reddish-brown as hydrolysis proceeded. Hydrogen cyanide was also detected by the prussian blue test.

The hydrolysis mixture was filtered into a 2 dm. tube and its optical rotatory power measured. It was found to be +0·71° and from this the quantity of sugar present was calculated on the assumption that it was entirely glucose.

$$\frac{0{\cdot}71 \times 15}{52{\cdot}5 \times 2} = 0{\cdot}1014 \text{ gm. glucose present.}$$

Theory requires 0·1007 gm.

The osazone was prepared in the usual way and isolated as sheaves of yellow needles; wt. 18 mgm.

It was recrystallised from dilute alcohol-pyridine and obtained in stellate clusters of needles, M.P. 204·5-205°.

Mixed with authentic glucosazone of M.P. 204°, the melting point was undepressed 204-5°.

Micro-analysis:—

	N
Found.....	15·39
$C_{18}H_{22}O_4N_4$ requires.....	15·64

It is thus clear that Acacipetalin contains one molecule of glucose and is a β-glucoside since it is readily hydrolysed by the enzyme emulsin.

4. *Detection of acetone among the products of enzymic hydrolysis.*

On the expectation that Acacipetalin would yield a simple aldehyde or ketone on hydrolysis, derived from the break-up of a cyanhydrin, attempts were made to isolate this material as the 2:4 dinitrophenylhydrazone.

A solution of 60 mgm. of glucoside in 10 c.c. of water was hydrolysed by emulsin and the mixture distilled from a small distilling flask into an ice-cooled receiver containing a little water. Sufficient hydrochloric acid was added to the distillate to bring to 2N concentration and then 5 c.c. of hot Brady's solution (0·5 gm. 2:4 dinitrophenylhydrazine dissolved in 30 c.c. of 2N hydrochloric acid).

A small quantity of precipitate formed. This was centrifuged down, washed well with 2N acid and then with water and finally recrystallised from hot 60 per cent. alcohol. The material separated in the form of long orange-coloured needles together with plate-like crystals of a lighter colour. This appearance is characteristic of the 2:4 dinitrophenylhydrazone of acetone. The yield was 10 mgm.

The material had M.P. 124°.

Mixed with authentic acetone 2:4 dinitrophenylhyrazone of M.P. 124° it had M.P. 123-4°.

Micro-analysis:—

	C	H	N
Found.....	45·23	4·30	22·87
$C_9H_{10}O_4N_4$ requires....	45·37	4·20	23·53

The substance present was therefore acetone.

Since this result was somewhat surprising, its accuracy was checked by running a control experiment in which emulsin and water were incubated. On distillation no trace of any substance reacting with Brady's reagent could be detected. The acetone had not originated therefore from the enzyme.

A second experiment was performed in which the distillate from a hydrolysis mixture was treated with p-nitrophenylhydrazine. A small quantity of a micro-crystalline p-nitrophenylhydrazone was obtained which when recrystallised from dilute alcohol separated in the form of yellow needles with M.P. 139-144°. Authentic acetone p-nitrophenylhydrazone prepared for comparison had M.P. 144-6° and the mixture melted without depression at 139-145°.

The β-glucoside of acetonecyanhydrin is the well known substance linamarin $C_{10}H_{17}O_6N$, whose properties are quite different from those of Acacipetalin. The latter, moreover, contains one more carbon atom in its molecule. The production of acetone on enzymic hydrolysis was at this stage difficult to interpret, but it was noted that the yield was very small and that other substances of an acidic nature were produced. It was considered possible that the cyanide group might be attached to the glucose molecule in the form of glucose-cyanhydrin (as in the naturally occurring cyanogenetic glucoside Lotusin), although the ready hydrolysis of Acacipetalin by emulsin rendered this hypothesis somewhat improbable. The aglucone would have to be attached to the glucose in some other way than through the aldehyde group and no compound of such configuration is known to be hydrolysed by emulsin.

Nevertheless, alkaline hydrolysis of Acacipetalin was carried out and heptogluconic acid sought for. No trace of this substance could be isolated.

A further possibility entertained was that the acetone isolated after distillation of the enzymic hydrolysate was being formed from some or other heat-labile precursor. Accordingly an experiment was carried out in which emulsin was added to a solution of glucoside and after the hydrolysis at 37° the solution was filtered and hydrochloric acid and Brady's reagent added without any previous distillation. The 2:4 dinitrophenylhydrazone which separated was small in amount and somewhat difficult to crystallise but was finally identified with certainty as acetone. It crystallised from dilute alcohol in the characteristic manner and had M.P. 122·5°. When mixed with authentic material of M.P. 124°, it had M.P. 119-124°.

5. *Tetra-acetylacacipetalin.*

The acetyl derivative of Acacipetalin was next prepared and this shown to have the normal composition, thus demonstrating that Acacipetalin contains only the four free hydroxyl groups of the glucose residue.

0·18 gm. of glucoside was dissolved in a mixture of 0·5 c.c. of acetic anhydride and 1·5 c.c. of pyridine. After five days at room temperature, the mixture was poured into ice water. The precipitated crystalline acetyl compound was washed well with ice water and recrystallised, first from absolute alcohol and then from dilute alcohol. The yield was 0·207 gm. or approximately 70 per cent.

Micro-analysis:—

	C	H	N
Found.....	54·06	6·24	3·69
The tetra-acetyl derivative $C_{11}H_{13}(CH_3CO)_4O_6N$ requires...	53·37	5·90	3·28

Tetra-acetylacacipetalin crystallises in long flattened prisms of M.P. 104° (see Fig. III). It is easily soluble in absolute alcohol and in ethylacetate, insoluble in petroleum ether and in water.

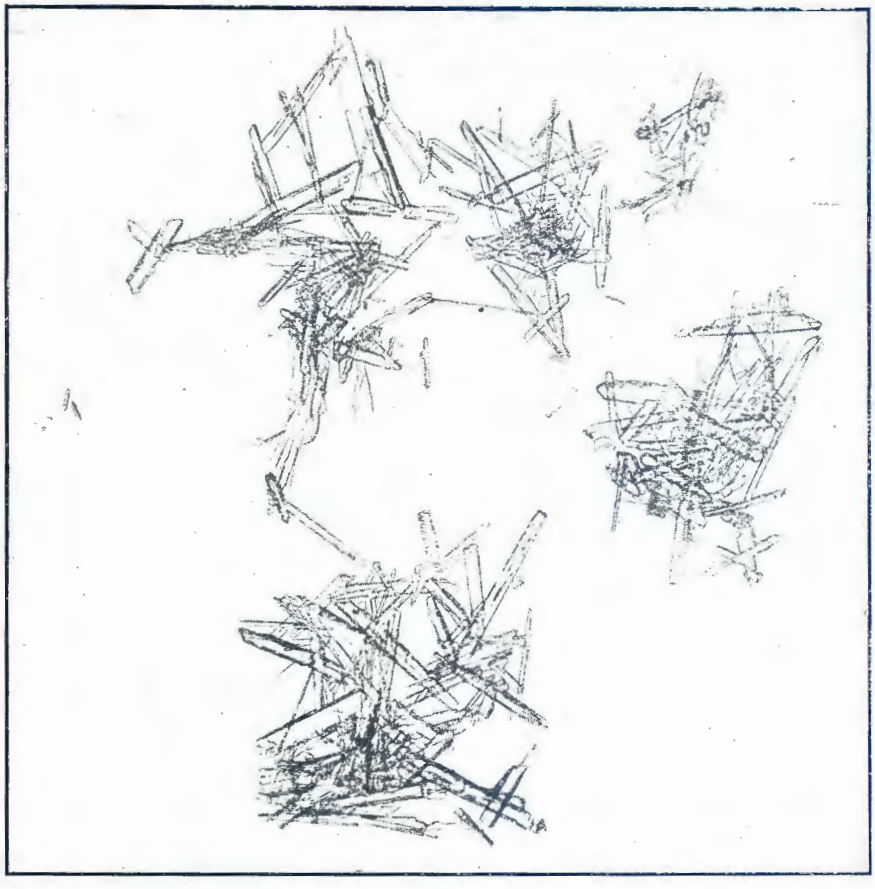


Fig III.—*Tetra-Acetylacacipetalin* × 200.

Emulsin added to a suspension of the substance in water produced no change in 24 hours at 37°, but dilute sulphuric acid at water bath temperature slowly liberated hydrogen cyanide.

The optical rotatory power was determined in absolute alcholic solution.

Weight of tetra-acetylacacipetalin.....	0·0972 gm.
Volume of alcohol.....	15 c.c.
Rotation observed.....	−0·21°

$$\therefore \left[\alpha\right]_{D}^{26} = \frac{-0{\cdot}21 \times 15 \times 100}{2 \times 9{\cdot}72}$$

$$= -16{\cdot}20°$$

6. *Alkaline followed by acid hydrolysis—Isolation of isobutyrylformic acid.*

Preliminary experiments having shown that the products of acid hydrolysis of the glucoside were unusual, no aldehyde or ketone being formed, attention was directed towards the action of alkalis. The glucosidic linkage being fairly stable towards boiling baryta, it is possible to hydrolyse the -CN group to carboxyl -COOH in fairly good yield without removing the glucose residue. Acid hydrolysis then gives an aglucone possessing acidic properties which may be used for its isolation and identification. Since hydrogen cyanide as a decomposition product of acacipetalin had only been determined qualitatively and by its physiological action, the opportunity was taken during the alkaline hydrolysis of isolating and identifying the ammonia formed (as the chloroplatinate) thereby confirming the presence of the -CN grouping.

1·0 gm. acacipetalin was refluxed for nine hours with 10 c.c. of saturated baryta in a flask connected by a ground glass joint to an upright condenser carrying at its upper end a trap bulb containing 2 c.c. of normal sulphuric acid. At the conclusion of the hydrolysis, the cooling water was run out of the condenser and all ammonia driven up into the acid trap. This was emptied into a beaker, a little hydrochloric acid added and then an excess of platinic chloride solution. The separation of ammoniumchloroplatinate commenced immediately and was completed in the ice chest. The bipyramidal crystals were collected on the centrifuge, washed with a little ice-water, dried and analysed with the following result, which confirmed their identity as the ammonium salt.

Analysis.	*Pt residue.*
Found.....	43·75
$(NH_4)_2PtCl_6$ requires.....	43·95

The baryta hydrolysate gave no precipitate, under the proper conditions, with Brady's reagent. It was acidified, while still slightly warm, with sulphuric acid, added to produce a final concentration of approximately 2 per cent., the barium sulphate centrifuged off, washed with 2 per cent. acid and the washings and supernatant returned to the flask. The contents was boiled under reflux condenser for two hours. The acid liquid was found to give a crystalline precipitate with Brady's reagent, but this was soluble in sodium carbonate solution.

Before working up the acid liquid, the quantity of glucose present was determined polarimetrically.

Volume of solution..... 55 c.c.
Rotation in a 2 dm. tube..... $+1{\cdot}02°$

$$\therefore \text{ Quantity of glucose} = \frac{1{\cdot}02 \times 55}{2 \times 52{\cdot}5}$$

$= 0{\cdot}5343$ gm.

This represents a yield of 77 per cent. since 1 gm. of glucoside would yield 0·6950 gm. glucose, assuming that no hydrolysis and destruction had taken place during the boiling with baryta.

The osazone was prepared from an aliquot and found to have M.P. 204°. Mixed with glucosazone (204°) M.P. 204°.

In order to examine the acid hydrolysate for neutral aldehydic or ketonic compounds, it was made slightly alkaline with baryta, filtered and distilled. The distillate was divided into two portions, the one treated with Brady's reagent and the other tested with sodium nitroprusside and ammonia and the iodoform reaction also applied. In neither case was a positive result forthcoming: there was no precipitate on adding Brady's reagent. Acetone was therefore absent from the distillate.

The main solution was now rendered acid with sulphuric acid, barium sulphate removed and the clear liquid extracted repeatedly with ether. The ether layer was washed once with water and then shaken with a little sodium carbonate solution, which immediately removed the material giving the dinitrophenylhydrazone precipitate.

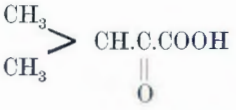
The sodium carbonate extract was aerated to remove ether, hydrochloric acid added to a final concentration of 2N and then a slight excess of hot Brady's reagent. The crystalline precipitate which formed was centrifuged down, washed with 2N acid and then with water and finally recrystallised from hot 60 per cent. alcohol. It was obtained in the form of orange-yellow, square-ended rectangular prisms. They had M.P. 188-190° unchanged by recrystallisation. The material was soluble in sodium carbonate solution, from which it could be reprecipitated in the crystalline condition (M.P. 188-191) by excess of hydrochloric acid, this behaviour strongly suggesting that the material was the 2:4 dinitrophenylhydrazone of an aldehydic or ketonic acid. The supposition was confirmed by analysis, which showed it to be the derivative of a substance having the formula $C_5H_8O_3$.

Micro-analysis:—

	C	H	N
Found.....	45·19	4·40	18·40
$C_{11}H_{12}O_6N_4$ requires....	44·60	4·05	18·92

Beilstein (4th Ed.) lists nine isomeric oxo-acids having the formula $C_5H_8O_3$ but of these, considering the isolation of acetone from the products of enzymic hydrolysis of the glucoside, as

previously recorded, that which appeared most probable was isobutyrylformic acid (α-oxo- β methylpropan- α-carboxylic acid; dimethylpyruvic acid) possessing the following structure:—



Accordingly, this acid was synthesised, following Franke and Kohn (1899), from isobutylideneacetone and also by the more acceptable method via isobutyrylcyanide (Tschelinzeff, 1929; Craig 1934) and its 2:4 dinitrophenylhydrazone prepared. This compound crystallised in long, slightly orange-yellow rectangular prisms, which, after repeated recrystallisation from dilute alcohol, had M.P. 190°. Mixed with the material from the glucoside (M.P. 188-190°) the mixture melted without depression at 189-190°, thereby proving the identity of the two materials and establishing the degradation product of acacipetalin as isobutyrylformic acid.

Synthesis of Isobutylideneacetone and of Isobutyrylformic acid.

Some details of the method employed may be reproduced here since Franke and Kohn's paper contains only a very meagre description of the technique they adopted.

57 c.c. of isobutyraldehyde and 46 c.c. of acetone were shaken in a separatory funnel with 100 c.c. of a 10 per cent. aqueous solution of sodium hydroxide. The mixture became hot and developed a pale yellow colour. After standing for several days with occasional shaking, the lower layer was removed and discarded, the oil being washed twice with distilled water, after which it was fractionated and the liquid distilling below 155° discarded. The main fraction passed over at 155-158° (uncor.), after which the temperature rose rapidly. The isobutylideneacetone so obtained was purified by redistillation. It was colourless but gradually acquired a pale yellow colour on standing, probably owing to polymerisation. The 2:4 dinitrophenylhydrazone was prepared and found to crystallise in long, orange-red prismatic needles of M.P. 163-5°.

Microanalysis.	N
Found.....	18·60
$C_{13}H_{16}O_4N_4$ requires.....	19·17

For the preparation of the ketonic acid, 10 gm. of isobutylideneacetone was mixed with about 100 c.c. of water and a cold 1 per cent. solution of potassium permanganate (700 c.c.) slowly run it to the ice-cooled and mechanically stirred mixture. This is considerably less permanganate than was used by Franke and Kohn, but when following their method, a considerable excess remained unreduced and great difficulty was experienced in isolating any isobutyrylformic acid at all. After filtering, traces of unchanged starting materials

which still remained were removed by extraction with ether. The mixture was then rendered strongly acid with sulphuric acid and again extracted by ether. The ethereal solution on evaporation left the ketonic acid as an oil, which was purified by mixing with water, shaking with a little charcoal and filtering. The aqueous solution was used for the preparation of the 2:4 dinitrophenylhydrazone, which had to be recrystallised repeatedly before a satisfactory melting point was obtained. Synthesis via butyrylcyanide yielded pure material at the first crystallisation, M.P. 189-191°.

Micro-analysis.	N
Found.....	18·82
$C_{11}H_{12}O_6N_4$ requires.....	18·92

7. *Hydrolysis by acid.*

The products of acid hydrolysis of the glucoside were next examined. As previously stated it had been found in preliminary experiments that no more than a possible trace of aldehyde or ketone was produced.

0·1 gm. Acacipetalin was refluxed with 10 c.c. of 1 per cent. sulphuric acid for 1 hour. Hydrogen cyanide was evolved. The cooled liquid was then made slightly alkaline with sodium hydroxide and distilled. To the distillate were added hydrochloric acid to 2N and 4 c.c. of hot Brady's reagent. A very slight turbidity formed, which settled into a flocculent precipitate after some hours, and this was centrifuged off and washed. When recrystallised it had M.P. 158-162°, but the quantity was so small that further identification was impossible. Formaldehyde 2:4 dinitrophenylhydrazone melts at 161°, and it is possible that this may have been the material isolated.

The residue in the distilling flask was diluted, acidified and again distilled. The distillate gave no trace of turbidity with Brady's reagent, but it was noticed that it reacted acid to litmus.

The contents of the distilling flask was made up to a solution of 15 c.c. and its rotation determined in a 2 dm. tube.

Rotation observed..... $+ 0{\cdot}48°$

$$\therefore \text{ Quantity of sugar as glucose} = \frac{0{\cdot}48 \times 15}{52{\cdot}5 \times 2} = 0{\cdot}0686 \text{ gm.}$$

Theoretical yield from 0·1 gm. glucoside.. = 0·0695 gm.

The osazone prepared in the usual way had M.P. 201-5°. Mixed with glucosazone, M.P. 203-5°. In a second experiment, 1 gm. of glucoside was hydrolysed by refluxing for two hours with 25 c.c. of 0·5N sulphuric acid. When cold, the mixture was extracted repeatedly with ether. The ethereal solution on evaporation left a

liquid, acid residue with a butyric-like smell. On titration it neutralised 8·4 c.c. of N/10 sodium hydroxide, and the sodium salt obtained by evaporation to dryness weighed 90·8 mgm.

∴ Equivalent weight by calculation.....	86·0
$C_4H_8O_2$ requires.....	88

The sodium salt crystallised in flat irregular plates and was slightly hygroscopic. For further identification the p-toluide was prepared, a control being simultaneously worked up, starting from 80 mgm. of sodium isobutyrate.

The sodium salt was transferred into a pyrex test tube (6″ × 0·5″) hanging by the lip from a small sheet of asbestos, 0·1 c.c. of concentrated hydrochloric acid and 0·2 gm. of p-toluidine were added and the tube heated by a micro-burner at such a rate that the vapours of the toluidine condensed in a ring about half way up the tube. Heating was maintained for one hour. The contents were then extracted by hot absolute alcohol and poured into 10 c.c. of boiling water. The liquid was boiled down rapidly to a volume of about 2 c.c. when a pinch of decolorising charcoal was added and the mixture filtered. The filtrate was concentrated to dryness, extracted by boiling benzene, the pale yellow benzene solution evaporated to dryness and the partly crystalline residue boiled with 2 c.c. of water in successive 1 c.c. portions, the clear colourless aqueous solution being filtered from insoluble tarry impurities and allowed to concentrate slowly in a shallow basin placed in an unexhausted calcium chloride desiccator. A small crop of large lustrous plates separated. These were dried on a porous tile and found to be similar in appearance to those from the control sodium isobutyrate experiment.

The identity of the two p-toluides was proved by melting point determination, thus—

p-toluide of acid from Acacipetalin M.P. 102°

control isobutyr-p-toluide M.P. 102-102·5°

Mixed M.P. 102°.

The products of the acid hydrolysis of Acacipetalin are thus, hydrogen cyanide, glucose and isobutyric acid, with possibly a trace of formaldehyde.

The Constitutional Formula of Acacipetalin.

In arriving at a decision concerning the constitution of Acacipetalin the following facts had to be considered:—

(1) One molecule of glucose was identified quantitatively and qualitatively after enzymic, acid, or alkaline, followed by acid hydrolysis.

(2) No heptogluconic acid could be found as a decomposition product.

(3) Acacipetalin yields a tetra-acetyl derivative.

(4) Emulsin readily hydrolyses the glucoside with liberation of HCN.

It is clear therefore that Acacipetalin must be a β-glucoside in which the nitrile group resides outside of the glucose residue in the aglucone.

(5) The aglucone from its derived empirical formula must be unsaturated in character.

(6) It is probably unstable, breaking up under acid hydrolysis conditions to yield isobutyric acid.

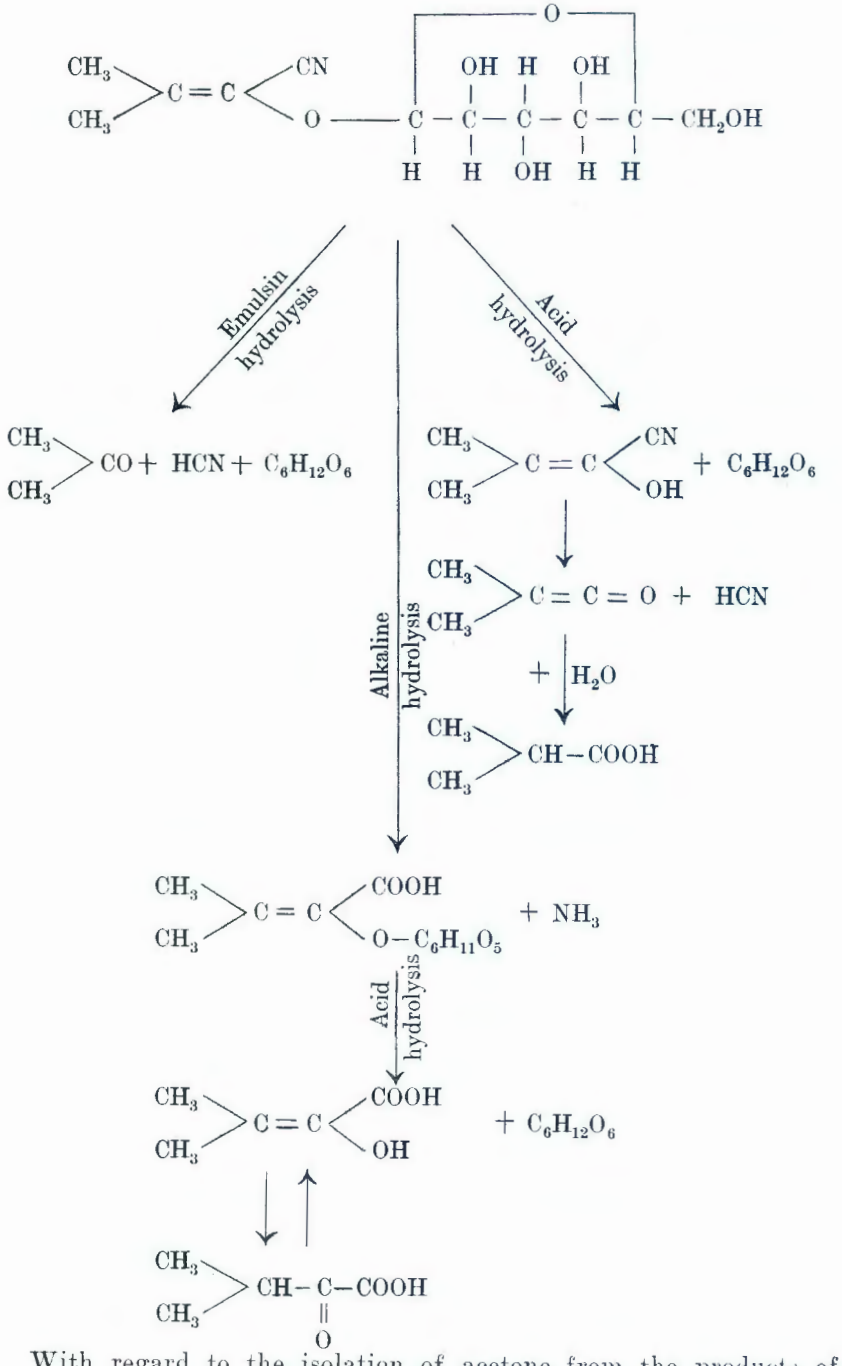
(7) Alkaline hydrolysis converts the -CN group into -COOH and NH_3. When the glucose residue is subsequently removed an α-ketonic acid is formed.

Usually such procedure leads to the production from cyanogenetic glucosides of α-hydroxyacids but owing to the keto-enol tautomerism, the ketonic acid may also be regarded as an α-β unsaturated- α -hydroxy acid.

(8) Acid hydrolysis would thus lead to a ketene cyanhydrin which would be expected to break up into hydrogen cyanide and the corresponding ketene. Dimethyl ketene is unstable and in contact with water yields isobutyric acid (Beilstein, 4th Ed., Vol. I, Syst. No. 90) which latter was identified after acid hydrolysis of the glucoside.

(9) The production of acetone in small yield (together with volatile acidic substances) among the products of enzymic hydrolysis is also susceptible of explanation (see below).

It is considered that all reactions of the glucoside can be adequately explained on the basis of the following structure which is therefore proposed as representing the constitution of Acacipetalin.



With regard to the isolation of acetone from the products of enzymic but not of acid hydrolysis, some discussion is warranted since these results are at first sight somewhat surprising.

It must be emphasised that the conditions are very different in the two cases. In the former, dimethyl-ketene appears in an acid medium at the boiling temperature, whilst in the latter it is formed only slowly *in statu nascendi* in a neutral medium at 37°.

The production of acetone from the ketene might occur as the result of an oxidation at the ethylenic linkage. A crude enzyme preparation such as emulsin is likely to contain oxido-reductive ferments in addition to those concerned in the liberation of glucose and production of free HCN from the cyanhydrin. What the action of these might be it is impossible to foretell.

There is evidence from a purely chemical standpoint, however, that ketenes on decomposition yield appreciable amounts of ketones. Thus Hurd and Dull (1932) discussing the preparation of ketenes by the pyrolysis of acylphthalimides state that a certain proportion of acids or their corresponding anhydrides and also ketones were formed in appreciable quantity, notwithstanding the fact that the starting materials had been dried with the utmost care. Thus, for example, among the products of the pyrolysis of propionylphthalimide were found propionic anhydride, diethyl ketone and carbon dioxide. Ketenes being very reactive and unstable materials may, at the moment of their formation, undergo changes in various ways among which must be numbered the production of ketonic bodies.

Attempts to hydrogenate the double bond in Acacipetalin were unsatisfactory. Several trials were made using a colloidal palladium catalyst and hydrogen as in the Paal process. A fairly slow but steady uptake of hydrogen was observed in each case, but the reaction was not quantitative, and it was soon evident that changes of a deep-seated nature were taking place. In the reaction mixture were detected small quantities of glucose and amines but no hydrogen cyanide.

It is clear that hydrogenation in the presence of palladium brings about a fairly extensive destruction of the glucoside; the reactions were much too complicated to be used as evidence of structure (compare Skita, 1909, 1915; Skita and Meyer, 1912; Paal and Gerum, 1909, etc.).

Biological Relationships.

Finnemore and Cox (1930) isolated from the Australian *Acacia* species, *A. glaucescens* and *A. cheeli*, the cyanogenetic glucoside sambunigrin. Sambunigrin is a benzaldehydecyanhydrin glucose ether and bears no relationship to Acacipetalin. The South African species of Aacacia belong to a different group in the genus, bearing true leaves, whilst those investigated by the Australian workers are phyllodineous. This circumstance probably accounts for the very different chemical findings.

Acacipetalin differs from typical cyanogenetic glucosides only in possessing an unsaturated aglucone which leads to a variety of secondary decomposition products when the ketene is set free by hydrolysis.

Unsaturated aglycones are met with in the glucosides, Gluconapin from *Brassica rapus* and many other mustard oil glucosides. Isobutyl mustard oil occurs in *Cochlearia officinalis*.

Helicin, the glucoside of salicylic aldehyde,

$$C_6H_{11}O_5.O.C_6H_4.CHO$$

has been combined with acetone (Tiemann and Rees, 1885) to form the unsaturated synthetic glucoside

$$C_6H_{11}O_5.O.C_6H_4.CH.=CH.CO.CH_3$$

Coniferin, the glucoside of coniferyl alcohol and occurring in conifers, also contains an ethylenic linkage

$$C_6H_{11}O_5.O.C_6H_3(OMe).CH=CH.CH_2OH.$$

Syringin is closely similar. Such examples might be multiplied, but sufficient has been said to indicate that unsaturated aglycones are not uncommon although previously not encountered in naturally-occurring cyanogenetic glucosides.

With regard to the possible mode of formation of Acacipetalin in the plant, a tentative suggestion might be made, with all reserve, that acetone and formaldehyde or formaldehydecyanhydrin present themselves as not unlikely starting points.

Such a condensation as that pictured below is not without its counterpart in the chemistry of living materials and is not objectionable from the purely chemical standpoint.

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!> CO + H_2C \!\!< \begin{matrix} CN \\ OH \end{matrix} + C_6H_{12}O_6$$

$$\downarrow$$

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!> C=C \!\!< \begin{matrix} CN \\ O—C_6H_{11}O_5 \end{matrix}$$

Summary.

1. The cyanogenetic glucoside Acacipetalin has been isolated by an improved method from *Acacia stolonifera* Burch. The constants were found to be M.P. 176-7°; $\left[\alpha\right]_D^{26} = -36{\cdot}60°$.

2. Tetra-acetylacacipetalin has been prepared. It has M.P. 104° and $\left[\alpha\right]_D^{26} = -16{\cdot}20$.

3. Pinit, inositol monomethyl ether, has been identified as a constituent of *Acacia stolonifera*.

4. The constitution of Acacipetalin has been elucidated. It is the glucose ether of dimethylketenecyanhydrin.

5. The facts upon which this conclusion is based are recorded and include the identification of the following breakdown products of the glucoside. On enzymic hydrolysis, hydrogen cyanide, glucose, acetone and acidic substances. On acid hydrolysis, hydrogen cyanide, glucose and isobutyric acid. After alkaline followed by acid hydrolysis, ammonia, glucose and isobutyrylformic acid, isolated as the 2:4 dinitrophenylhydrazone. This latter substance was prepared synthetically and found to have M.P. 190°.

The 2:4 dinitrophenylhydrazone of isobutylideneacetone crystallises in orange-red prisms and melts at 163-5°.

6. Catalytic hydrogenation using colloidal palladium as catalyst leads to deep-seated changes in the glucoside. Among the reaction products were detected acetone, glucose and amino substances.

7. A comparison with other glucosides containing unsaturated aglycones is made and a suggestion put forward as to the possible mode of origin of Acacipetalin in the plant.

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REFERENCES.

CRAIG, L. C. (1934). Isobutyrylformic acid phenylhydrazone. *J. Amer. Chem. Soc.*, Vol. 56, pp. 2008-9.

FINNEMORE, H., AND COX, C. B. (1930). Cyanogenetic glucosides in Australian plants. *Jl. Proc. Roy. Soc. N.S. Wales*, Vol. 62, p. 369. (Sydney University Reprints, Series III, Vol. 1, 1927, pp. 369-378.)

FINNEMORE, H., AND GLEDHILL, W. C. (1928). *Austr. Jnl. Pharm.*, 1928, N.S. 9, p. 174.

FRANKE AND KOHN (1899). *Monatsch*, Vol. 20, pp. 878 and 884.

HÉRISSEY, H. (1932). Sur une technique permettent l'extraction facile de certains hétérosides. *Jn. de Pharm. et de chim.* (8), Vol. 16, pp. 513-516.

HURD, C. D., AND DULL, M. F. (1932). Ketenes from acylphthalimides and other substituted amides. *J. Amer. Chem. Soc.*, Vol. 54, pp. 2432-2440.

PAAL AND GERUM (1909). *Ber.*, Vol. 42, p. 1553.

SKITA (1909). *Ber.*, Vol. 42, p. 1627.

SKITA (1915). *Ber.*, Vol. 48, p. 1494.

SKITA AND MEYER, H. (1912). *Ber.*, Vol. 45, p. 3593.

STEYN, D. G., AND RIMINGTON, C. (1935). The occurrence of cyanogenetic glucosides in South African species of *Acacia*, I. *Onderstepoort Jnl. Vet. Sc. and An. Ind.*, Vol. 4, No. 1, p 51.

TIEMANN AND REES (1885). *Ber.*, Vol. 18, p. 1955.

TSCHELINZEFF (1929). *Ber.*, Vol. 62, p. 2210.