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Studies upon the Photosensitisation of Animals in South Africa.

X. The Icterogenic Factor in Geel-dikkop. Isolation of Active Principles from *Lippia rehmanni* Pears.

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In the seventh contribution to this series of articles dealing with photosensitisation, the naturally occurring disease of sheep, "Tribulosis" or "Geel-dikkop" was more particularly considered and it was shown (Rimington and Quin 1934) that the condition of photosensitisation arises as a result of the presence of the chlorophyll derivative, phylloerythrin, in the blood of sheep, this pigment having entered the circulation with the bile. The plant *Tribulus* presumably contains some toxic substance causing a well-marked icterus, the liver becoming incapable of eliminating bile and it is this hepatic disturbance which must be looked upon as fundamental to the development of the symptoms characteristic of the disease.

Since the experimental production of geel-dikkop by the feeding of *Tribulus* is rarely satisfactory, we have had recourse to the plant *Lippia rehmanni* (*Verbenaceae*), found by Quin (1933) to cause, when administered to sheep, an icterus indistinguishable from that present in geel-dikkop.

In an article by Rimington and Quin (1935) an account was given of preliminary experiments directed to the isolation of the active principle from *Lippia rehmanni*. The icterogenic activity of the plant was found to reside in an ether and alcohol soluble but water insoluble oleo-resin, and by fractionation of this material it was demonstrated that activity was confined to the portion soluble in 50-75 per cent. alcohol. By working up comparatively large quantities of the resin, a series of crystalline materials was isolated

having acidic properties and certain of these were shown to be icterogenic when dosed to sheep. Finally, by an extension of the same technique of fractional crystallisation a material was isolated which was nitrogen free and to which the name " Icterogenin " was given. Two kinds of crystals were encountered both of which were active, the one consisting of prisms, M.P. 239°, the other irregular, fragile plates which melted at 155-160° but resolidified again to melt finally 230-6°. The preliminary change was associated with loss of weight and it was considered that alcohol of crystallisation was lost. Icterogenin when dosed in quantities of 1·5 gm. to a sheep *per os* caused distinct bilirubinaemia within 18 hours. Other physiological properties were also reported upon.

The method of isolation of Icterogenin left much to be desired. Fractional crystallisation could be expected, at best, to yield only a portion of the toxic principle actually present and the yields, moreover, were found to be variable. Since Icterogenin forms a sodium salt, attempts were made to better the procedure by shaking the crude ether extracts with aqueous sodium carbonate, but troublesome emulsions usually formed. On one occasion when salt had been added to break such an emulsion, the deposition was noticed in the liquid of a crystalline material which proved to be the sodium salt of Icterogenin and this observation provided a solution to the difficulties of isolation. Working over the sodium salt method we are now able to obtain quantitative isolation with perfect regularity and ease. A description of the technique is as follows.

Isolation of the active principle in the form of a sparingly soluble sodium salt.

The powdered plant material (500 gm.) is steeped in 96 per cent. alcohol (1,200 c.c.) overnight and after squeezing off in a press, the residue is subjected to a second similar extraction. The combined alcoholic extracts are shaken with sufficient decolourising charcoal (Kahlbaum, about 10 gm.) to remove all the chlorophyll, and the brown-yellow filtrate is fanned down to about $\frac{1}{2}$ or $\frac{1}{3}$ of its bulk at room temperature. An equal volume of ether is then added and much water, and the ether phase, which contains all the icterogenic material, is separated off in a large separatory funnel and washed with water. A small amount (15 to 20 c.c.) of 2·5 per cent. aqueous sodium carbonate solution is then added and, after shaking, the dark brown aqueous layer is removed. This first extract is worked up separately since, containing nearly all the dark pigments of the ether solution, the icterogenin obtained from it is not so pure as that from subsequent shakings.

When sufficient sodium carbonate solution has been used to remove all the Icterogenin from the ether phase, the combined alkaline liquids are again shaken once or twice with pure ether to free them from non-acidic substances. Finally, a layer of pure ether is left on the surface of the carbonate extract and then a few grams of sodium chloride stirred in. As the salt dissolves, the Icterogenin, if it be present in quantity, commences to separate in amorphous form at the interface. If the amount is small, crystalline sodium salt

forms slowly. In any case, the mixture is left in an ice-chest until next day and the precipitate then filtered off through an ordinary paper filter and washed by ether. Further crops can usually be obtained by increasing the concentration of sodium chloride present and setting the mixture once more in the ice-chest. The precipitates are spread upon unglazed porcelain to dry and form light, felted masses, almost white in colour and often consisting of long needles, M.P. 228°, very sparingly soluble in water but readily soluble in alcohol. The yield from 1 kilogram of powdered *Lippia* was approximately 2·5 grams.

To prepare the free acid, the sodium salt was dissolved in warm alcohol, an excess of hydrochloric acid added and the solution diluted to make an alcohol concentration of about 60 per cent. On cooling, a mass of beautiful, shining white crystals fills the vessel. A specimen of this acid, which was positively icterogenic in the sheep test, when suspended in water and stirred with the calculated quantity of decinormal sodium hydroxide necessary for exact neutralisation dissolved almost entirely but the solution was not very stable, the addition of sodium salts such as normal saline or even long standing causing the slow separation of the sparingly soluble sodium salt of the active principle.

Separation of Isomers present in the crude, free acid.

Fractional crystallisation of the crude acid material obtained from the sodium salt showed that it was not homogeneous. In addition to the two types of crystals described in the previous communication (Rimington and Quin 1935), there was also obtained an acid crystallising in regular, narrow plates and melting at 161° without resolidification. It was just as active icterogenically and in the other pharmacological tests as were the substances originally described.

A re-examination of material prepared by the older method of direct crystallisation revealed the fact that by this method also small quantities of the new constituent were obtained, usually in admixture with the irregular, plate-like crystals.

Owing to their closely similar properties, separation of these individuals was not easily accomplished. The substance crystallising in small, elongated prisms and melting without preliminary change at 239° dissolves to an inappreciable extent when stirred with dilute alkali whereas the plate-like material goes readily into solution. A separation from the mixture of crude crystalline acids could thus be effected fairly easily but the substance on recrystallisation can, according to conditions, separate in the form of prisms or as irregular plates melting at 158° then resolidifying to melt finally at about the same temperature as the prisms namely 239°. This is strictly in accordance with the previous experience recorded by Rimington and Quin (1935) and the preliminary melt is associated with loss of weight. The third material, long regular plates, melting at 161° without resolidification, or loss of weight, is mose effectively obtained by fractional crystallisation. It dissolves readily in dilute aqueous alkali.

Attempts to utilise differences in solubility in salt solutions of the sodium salts of these acids for purposes of separation lead to a separation but only after a good deal of labour and the final application of fractional crystallisation. Differing solubility in dilute alcohol has proved to be most helpful as indicated in the accompanying Tables I and II.

TABLE I.

Crude material separating from dilute alcohol. Boil up with 4 successive lots of 60 per cent. alcohol.

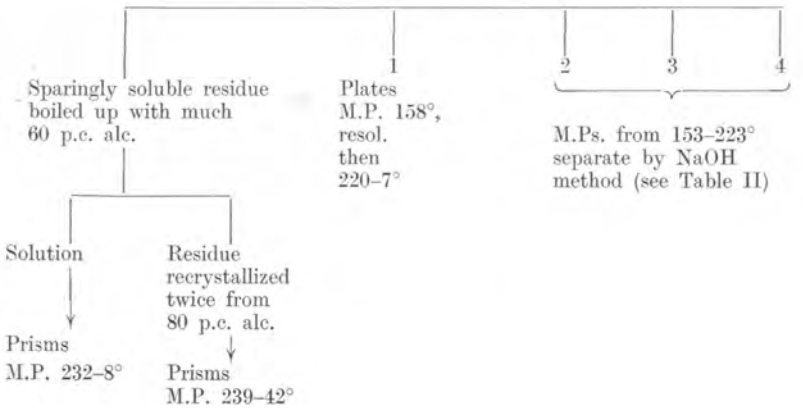
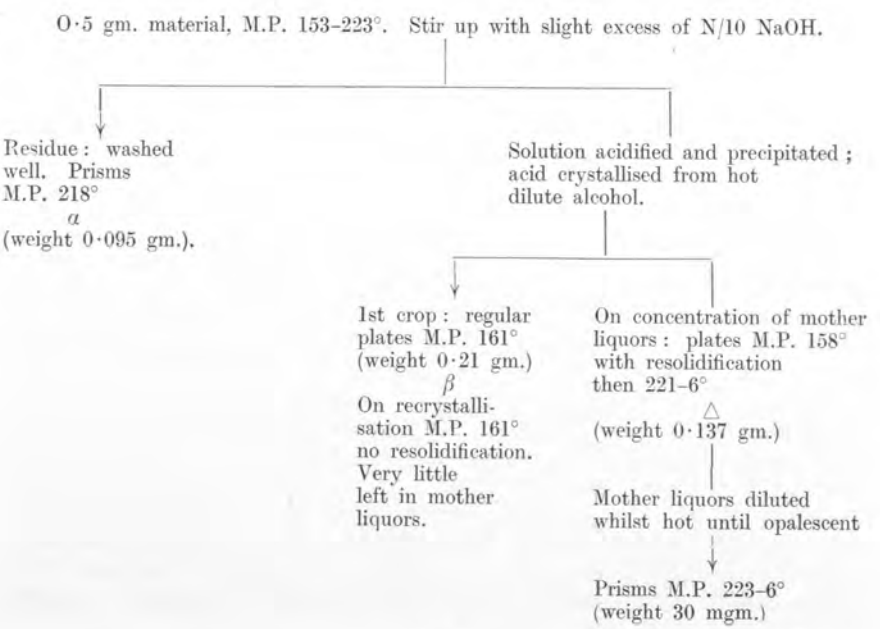


TABLE II.



Repeated recrystallisation finally afforded three distinct crystalline fractions with fairly constant melting points:—

1. Prisms M.P. 239° (see Fig. 1) " Icterogenin A ".
2. Regular elongated plates M.P. 161° without loss of weight; no resolidification (see Fig. 2) " Icterogenin B ".
3. Irregular plates M.P. 158°, resolidifies to melt at about 230° (see Fig. 3) " Icterogenin C ". Apparently one molecule of water, H_2O, is lost at the lower temperature.

These three materials, which are all icterogenically active, gave on analysis the following figures. Several preparations were analysed.

*Micro-analysis.**

	C.	H.	M. Wt. (Rast)
Prisms, M.P. 239°. Icterogenin A...	73·65	9·17	525, 535
	73·85	9·44	634, 630
	73·69	9·32	
	73·94	9·23	544, 515
$C_{34}H_{52}O_6$ requires.....	73·33	9·42	556·4
Plates, M.P. 161°, no resolidification, Icterogenin B.....	72·97	9·32	565, 530
	73·04	9·60	523, 506
	72·95	9·34	498, 494
	72·72	9·52	
	73·70	9·43	
Plates, M.P. 158° with resolidification. Icterogenin C.....	73·39	9·48	530, 525
	—	—	510, 500
	—	—	538, 570
After drying over P_2O_5 at 105° in vacuo.....	75·44	9·64	Loss of wt. 2·55 per cent.
$C_{34}O_{50}O_5$.....	75·78	9·36	

The difficulty of obtaining satisfactory analytical figures from substances of the nature of these resinic acids is well known. Isomers are extremely difficult to separate and we still feel that some caution must be exercised in drawing a conclusion as to the molecular formulae of the materials here described. It would appear from a consideration of the analyses of the acetyl derivatives and of the 2:4 dinitrophenylhydrazones described below, taken in conjunction with the figures here reported for the free acids, that the formula $C_{34}H_{52}O_6$ is that best meeting all requirements.

The molecular weight demanded by such a compound is 556·4, a figure not far removed from the determinations made by Rast's method upon our various preparations. The camphor method is not

* Micro-analysis by Dr. A. Schoeller, Berlin.

one, however, from which very accurate results can be expected when the molecular weight is so large as is the case here. A confirmation of the molecular size is afforded by titration experiments and more particularly by the percentage of nitrogen present in the 2:4 dinitrophenylhydrazones. In some instances, figures greater than 600 were obtained by the camphor method when applied to prism preparations. This circumstance prompts us to observe that we do not deny the possibility of further isomers or closely related resinic acids occuring in our crude sodium salt derived from *Lippia rehmanni*. Further work will be undertaken in an effort to clear up these discrepancies and to place beyond all doubt the molecular formula $C_{34}H_{52}O_6$ which we now put forward for all three materials isolated.

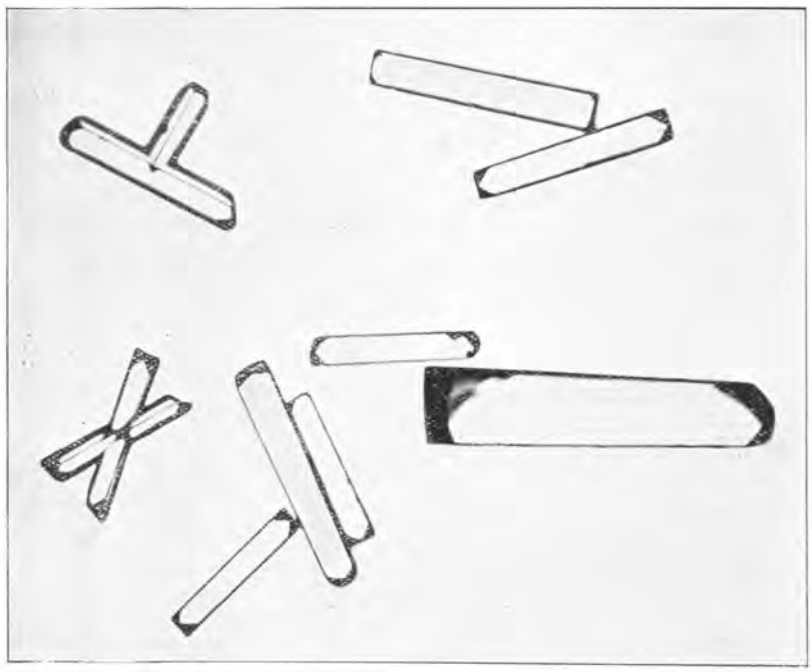


Fig. 1. Icterogenin A×300. Prisms M.P. 239°.

It would appear that the substances previously reported upon by Rimington and Quin (1935) were not quite pure and contained variable amounts of the third isomer which we now describe. For convenience and to avoid possible confusion at a later date, we propose to refer to the three active fractions we have now separated as Icterogenins A, B and C. All these materials are monobasic acids and possess, as will be shown later, one ketonic function. They give the Liebermann-Storch reaction with acetic anhydride and concentrated sulphuric acid, and a yellow colour with tetra-nitromethane, indicating the presence of ethylenic linkages. All are dextrorotatory to approximately equal extent thus precluding the use of rotation as an indication of purity.

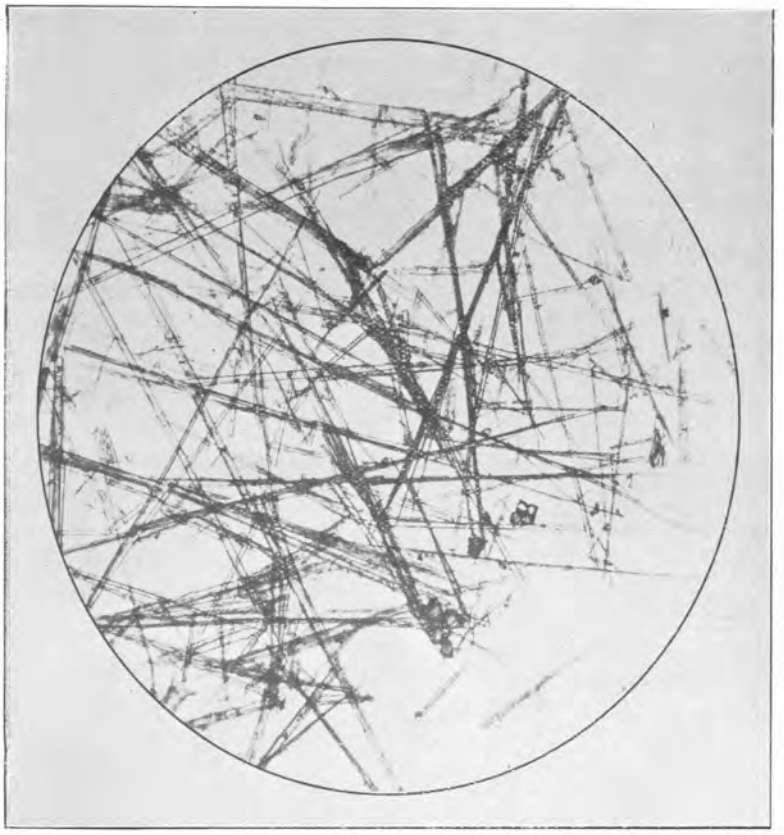


Fig. 2. Icterogenin B. M.P. 161°×125.

OPTICAL ROTATORY POWER.

Icterogenin A. 50 mgm. in 15 c.c. of absolute alcohol in a 2 dm. tube was found to have a rotation of +0·48°

$$\therefore \ [\alpha]_{D}^{25} = \frac{+ 0{\cdot}48 \times 100 \times 15}{2 \times 5}$$
$$= + 72{\cdot}0°.$$

Icterogenin B. 50 mgm. of the plates M.P. 161° dissolved in 15 c.c. of alcohol had a rotation of +0·46°

$$\therefore \ [\alpha]_{D}^{28} = \frac{+ 0{\cdot}46 \times 100 \times 15}{2 \times 5}$$
$$= + 69{\cdot}0°.$$

Ictogernin C. 50 mgm. of the plates M.P. 158° and 230°, under similar conditions, had a rotation of +0·47

$$\therefore \ [\alpha]_{D}^{28} = \frac{+ 0{\cdot}47 \times 100 \times 15}{2 \times 5}$$
$$= + 70{\cdot}5°.$$

Micro-titration.

Icterogenin A. 35·0 mgm. of prisms were dissolved in 5 c.c. of an alcoholic KOH solution (5 c.c.≡3·88 c.c. of 0·09709 N HCl), a drop of phenolphthalein added, and the mixture immediately back-titrated.

Acid back	3·23 c.c.
Neutralised	0·631 c.c. N/10.
Theory for 1 acid group in $C_{34}H_{52}O_6$	0·629 c.c. N/10.

A further 5 c.c. of alcoholic potash was added, the mixture refluxed for one hour and again back-titrated. It required 3·85 c.c. of acid indicating that no further acid groups develop under these conditions, i.e. the absence of lactone groupings. Similar results were obtained with all preparations.

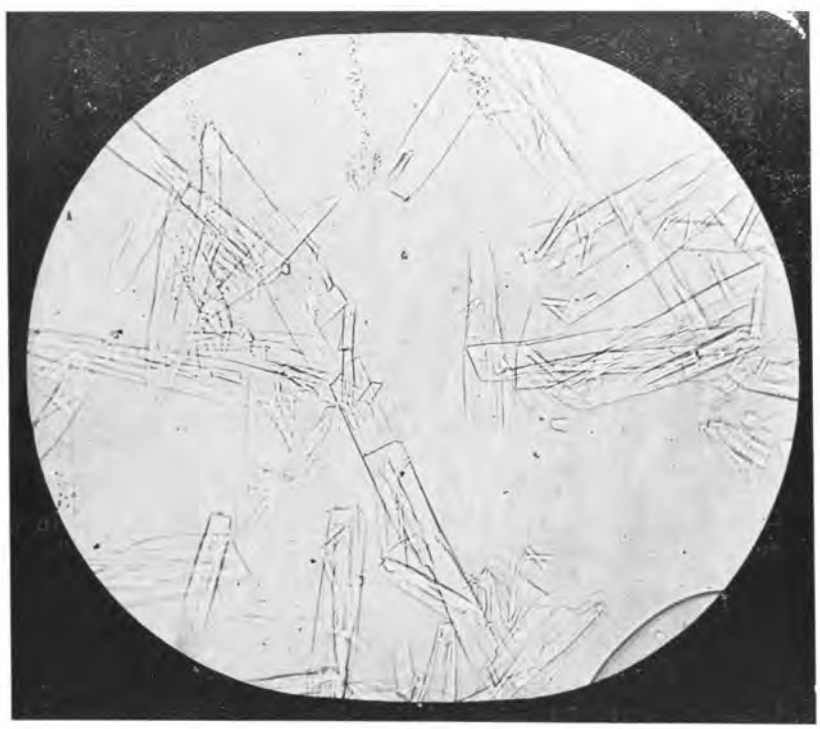


Fig. 3. Icterogenin C. M.P. 158° and 230°. ×55.

Preparation of Acetyl derivatives.

In order to test for the presence of hydroxyl groups in the Icterogenins, the following experiments were carried out.

0·95 gm. Icterogenin A (prisms) was refluxed for 2·5 hours with 2·5 c.c. of acetic anhydride and 0·25 gm. of sodium acetate. The pale yellow mixture was poured into a quantity of ice water when a solid material separated. This was well washed and then crystallised from hot 80 per cent. alcohol. It separated in fine needles M.P. 140-41° Yield 66·4 mgm. (see Fig. 4).

Micro-analysis.

	C.	H.	M. Wt. (Rast).
Found.....	72·74	9·08	593, 587
$C_{34}H_{51}O_6(CH_3CO)$ requires.....	72·19	9·09	598·4

0·1 gm. Icterogenin B, similarly treated yielded an acetyl derivative crystallising in small prismatic needles M.P. 142°.

Micro-analysis.

	C.	H.	M. Wt. (Rast).
Found.....	72·25	8·87	569, 550
$C_{34}H_{51}O_6(CH_3CO)$ requires.....	72·19	9·09	598·4

Each substance yields a monoacetyl derivative indicating the presence in their molecules of one hydroxyl group. The melting points were so similar that a mixed melt was carried out and it was found that there was no depression (M.P. of mixture 140-2°). They are therefore identical and it can be concluded that Icterogenins A and B are true isomers; whatever constitutional difference there is that distinguishes them, it is removed during the process of acetylation.

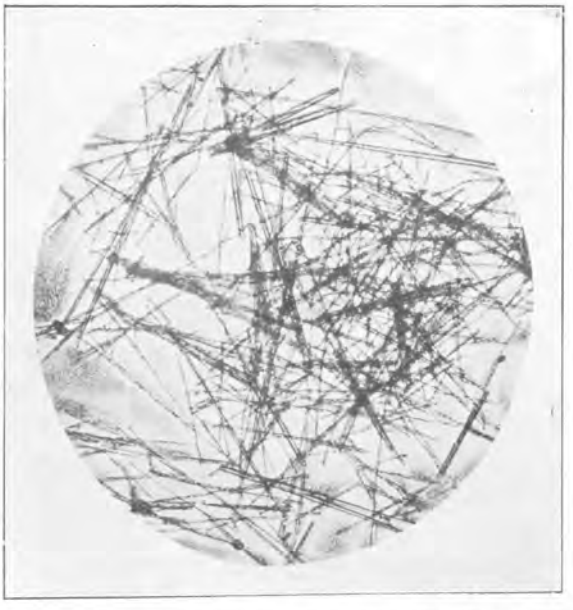


Fig. 4. Acetyl derivative of Icterogenin, M.P. 142°. ×135.

SAPONIFICATION OF THE ACETYL COMPOUNDS.

40 mgm. of acetyl-icterogenin A was dissolved in 5 c.c. of alcoholic KOH (5 c.c.≡3·88 c.c. of 0·09709 N HCl), a drop of phenolphthalein added and the mixture immediately back-titrated with the acid.

Acid back	3·12 c.c.
Neutralised	0·738 c.c. of N/10.
Theory for 1 free acid group	0·669 c.c. of N/10.

A further 5 c.c. of alkali was then added and, after refluxing for three hours, the mixture titrated as before.

Acid back 3·19 c.c.
Neutralised during saponification 0·670 c.c of N/10.

Theory for 1 acetyl group in $C_{36}H_{54}O_7$ 0·669 c.c. of N/10.

33·2 mgm. of acetyl-icterogenin B, similarly treated, afforded the following figures.

Titration before heating, neutralised 0·612 c.c. N/10.
Theory 0·555 c.c. N/10.
After 3½ hours saponification, neutralised 0·66 c.c. N/10.
Theory 0·555 c.c. N/10.

Preparation of the 2:4 dinitrophenylhydrazones.

20 mgm. of each material was dissolved in sufficient dilute alcohol and, after acidification with 0·5 c.c. of concentrated hydrochloric acid, 1·5 c.c. of hot Brady's reagent was added. The precipitates which formed were centrifuged off, washed well with 2 N hydrochloric acid and crystallised from hot, dilute alcohol. The 2:4 dinitrophenylhydrazones which separated were orange-yellow in colour and had the following properties.

Icterogenin A 2:4 dinitrophenylhydrazone. Aggregates of fine, needle-like prisms, M.P. 222-5°.

Derivative prepared from Icterogenin B. Aggregates of fine needle-like prisms, M.P. 226-9°.

Derivative prepared from Icterogenin C. Fine needle-like prisms, M.P. 224-8°.

Micro-analysis.

	C.	H.	N.
Derivative of Icterogenin A.....	63·74	7·50	7·44
,, ,, B.....	63·85	7·65	7·24
,, ,, C.....	63·65	7·55	7·09
$C_{34}H_{52}O_6 + C_6H_6N_4O_4$ requires.....	63·60	7·75	7·42

It will be noticed that allowance for elimination of H_2O is not made. We are at a loss at the present moment to explain why the derivatives should appear to possess H_2O more than is expected. Formulation of the parent substance with one more oxygen atom does not accord with analyses either of the free acids themselves or of their acetyl-derivatives.

Demonstration of the presence of an isopropyl side-chain.

0·4 gm. of the sodium salt of Icterogenin was placed with 2 c.c. of acetic acid in a Pregl micro-kjeldahl distillation flask and, whilst passing a brisk current of steam through the apparatus, an oxidation mixture of chromic acid in glacial acetic acid was run in drop by drop as required. The distillate, which was collected in an ice-cooled receiver, was neutralised and again distilled, the first 10 c.c. being

separately collected. To the distillate was added 2 c.c. of hot hydrochloric acid and 2 c.c. of hot Brady's reagent. The 2:4 dinitrophenylhydrazone which separated was well crystallised exhibiting the two types of crystals characteristic of the acetone derivative. It was centrifuged off, washed and recrystallised in the usual way and identified as acetone 2:4 dinitrophenylhydrazone.

The icterogenins therefore possess an iso-propyl side chain and the most probable general formula may consequently be written:

$$C_{29}H_{43}O_2 \begin{cases} -CO \\ -OH \\ -COOH \\ -CH < \begin{matrix} CH_3 \\ CH_3 \end{matrix} \end{cases}$$

The material remaining in the reaction flask after the oxidation was separated off, washed and recrystallised from hot dilute alcohol. It separated in clusters of white needles, M.P. 264-7°.

Physiological action of Icterogenin.

The most characteristic action of icterogenin is the production of an intense and somewhat persistant bilirubinaemia without, however, post mortem signs of severe or extensive liver damage such as one would expect to accompany so severe a jaundice. Animals dosed with large quantities of *Lippia rehmanni* or with crude extracts prepared from the dried and powdered plant material invariably suffer from a very severe chronic constipation in addition to exhibiting bilirubinaemia and, after some days, clinical jaundice. These symptoms resemble closely those seen in natural cases of geeldikkop. If for some time prior to the dosing of *Lippia* extracts, a plentiful supply of green feed is provided, typical photosensitisation supervenes but not photosensitisation follows when the basal ration consists of bleached, dry straw devoid of chlorophyll. There would thus seem to be a very close similarity in every respect between *Lippia* poisoning and Geeldikkop provoked by feeding on *Tribulus*.

In article No. 9 of this series (Quin 1936) the physiological action upon the living animal of crude *Lippia* extracts and *Lippia* dosing has been fully discussed.

Experiments performed with the purified principles upon animals and upon isolated tissues are now recorded below.

Since it was found that these isomers exerted identical effects, the preparations used in the majority of experiments were obtained by decomposing the crude sodium salt and crystallising the resultant acid mixture from alcohol. For the effect of individual isomers, see experiments upon isolated intestinal strips.

Dosing of Icterogenin *per os*.

In numerous instances, quantities of Icterogenin or Icterogenin sodium salt varying between 1 and 4 grams were given to normal sheep *per os*. On occasions, the dose was preceded by 3 c.c. of 10 per cent copper sulphate solution in order to close the oesophageal

groove and direct the material straight into the abomasum. In all cases, bilirubinaemia was noticeable within 24 hours and, with the higher doses, persisted for some days. A dose of 1 gm. is about the least quantity producing a distinctly positive bilirubinaemia in a 2 tooth sheep.

Where the jaundice was intense, it was usually noticed that on or about the third day after dosing there was present in the serum a small quantity of haemoglobin imparting to it an orange or brownish-yellow tint.

Following the oral administration of Icterogenin, a marked decrease in the number of ruminal movements per 5 minute interval was noticed and this effect persisted for some days (see below). In cases of animals with biliary fistulae, it was also observed that bile pigment rapidly disappeared and the bile eliminated had the characteristics of " white bile " as described in article No. 9 of this series.

Fig. 5. Sheep, 48 hours after dosing with 2 gm. of Icterogenin per os.

The following experiment summarised below, illustrates these observations in a typical manner.

Sheep 44892 (age 7 months) was observed for 6 days prior to dosing, the ruminal movements being counted thrice daily and the faeces collected and weighed. The dose given was 2 grams of Icterogenin suspended in 250 c.c. of water and administered by stomach tube. Twenty four hours later, photosensitisation was apparent and increased in intensity as shown in the photograph Fig. 5 taken 48 hours after dosing. Even after 13 days, the serum remained slightly yellow but an eventual recovery took place with sloughing of the necrosed skin from the face and ears.

TABLE III. *Effect of oral administration of 2 gm. of Icterogenin.*

Date.	Ruminal movements per 5 minutes. a.m. 9	p.m. 1	p.m. 4	Wt. faeces per 24 hours. gm.	Serum.	Observations.
23/8/35	7	12	12	321 Normal.....	Normal, water clear. Red ppt. 27 per cent.	Body wt. 42 lb.
24/8/35	7	10	—	360 Normal.....	Normal.	
26/8/35	—	11	11	505 Normal.....	Normal.	
27/8/35	5	12	11	462 sl. clumped..	Normal.	
28/8/35	7	12	10	312 Normal.....	Normal.	
	At 11 a.m. dosed 2 gm. Icterogenin by stomach tube.					
29/8/35	4	2	3	96 Soft.....	Yellow (++), clear. Red ppt. 26 per cent.	12 p.m. Restless, seeking shade, not feeding. 2 p.m. Definite swelling of face and ears.
30/8/35	4	0	2	230 badly formed	Darker yellow (+++), clear	9 a.m. Swellings of face and ears, feet sore, yellow serum oozing from skin of nose. Photographed 4 p.m. Very badly swollen.
31/8/35	3	3	0	100 badly formed	As above, urine deep yellow	Swellings sl. subsided, fluid gravitating to intermandibular space. Sl. clinical jaundice. Kept from sun.
1/9/35	—	—	—	221 badly formed	As above.....	Again badly swollen in sun.
2/9/35	2	7	8	61 badly formed, dry	As above.....	Depressed. Necrosis of skin commencing. Feeding slightly.
3/9/35	5	8	12	230 pellets.....	As above.....	Weight 35 lb.
4/9/35	6	9	10	232 pellets, dry..	As above.....	Improving.
5/9/35	10	5	10	332 pellets, dry..	sl. lighter.....	Improving.
6/9/35	5	9	8	313 pellets, dry..	sl. lighter.....	Improving.
7/9/35	—	—	—	—	Light yellow.....	Improving.
9/9/35	—			—	Faint yellow.....	Clinical jaundice has disappeared. Skin sloughing. Improved.
10/9/35	—			—	—	Wt. 37 lb. Improvement rapid.
19/9/35	—			—	—	Animal discharged.

In the following case (see Table IV), a biliary fistula was introduced prior to dosing. The animal was eliminating approximately 260 c.c. of clear, dark green bile daily when it was given 4 gm. of Icterogenin by stomach tube. The subsequent appearance of the bile and serum is shown in the accompanying plate Fig. 6.

TABLE IV.

Date.	Bile.	Serum.	Obersvations.
30/7/35	246 cc. Deep greenish black	Water clear.....	—
	10 a.m. dosed 4 g.m. Icterogenin by stomach tube.		
	4 p.m. No change in bile colour		
31/7/35	9 a.m. Very faintly greenish	Definitely yellow.	Faint clinical jaundice.
	4 p.m. Rather turbid. 404 cc. in all.		
1/8/35	380 cc. clear, very light yellowish	Deeper yellow....	Clinical jaundice. Photosensitive.
2/8/35	326 cc. pale, greenish yellow	Deep yellow.....	Not feeding.
3/8/35	256 cc. pale yellow.....	Deep yellow.....	Losing condition.
4/8/35	246 cc. pale yellow.....	Deep yellow.....	—
5/8/35	196 cc. greenish yellow....	Deep yellow.....	—
6/8/35	294 cc. pale yellowish.....	Deep Yellow.....	Poor condition.
7/8/35	Found dead.		

The post mortem revealed the lesions typical of *Lippia* poisoning, including complete stasis of the fore-stomachs and severe stasis and putrefaction in the large intestine.

ADMINISTRATION OF ICTEROGENIN INTRAVENOUSLY.

Some difficulty attended the intravenous injection of Icterogenin on account of the insolubility of the free acid in water and the comparatively low solubility of the sodium salt. It would appear, however, that Icterogenin given in this manner is exceedingly toxic, possibly on account of the fact that it possesses haemolytic properties (see below). A sheep which received 0·5 gm. dissolved in a little 70 per cent. alcohol showed no ill effects apart from a slightly hurried respiration. The serum, however, became slightly, but distinctly icteric within 48 hours of dosing.

Upon increasing the dose to 1 gm., very severe toxic effects became apparent. A sheep injected intravenously with such a dose at 10.30 a.m. was found dead at 1 p.m. At post mortem it showed pronounced hyperaemia of the spleen, hyperaemia and slight degenerative changes in the liver, with oedema of the gall bladder, and intense hyperaemia of the mucosa of the small intestine together with haemorrhages into the lumen.

HAEMOLYTIC ACTION OF ICTEROGENIN.

The icterus of geel-dikkop and *Lippia* poisoning is not an haemolytic icterus, nevertheless Icterogenin does exhibit a fair degree of haemolytic activity in vitro. Most probably, in the living animal, the liver eliminates the toxin from the portal circulation as fast as it is absorbed and destroys or excretes it via the bile. Should the assault upon the liver be sufficiently great, however, the cells of this organ become thrown out of action to such an extent that elimination of bile becomes impaired or impossible and a vicious circle may then be set up, i.e. an obstructive or regurgitative jaundice supervenes.

The in vitro haemolytic action of Icterogenin was demonstrated as follows. A suspension of washed sheep's cells in Ringer-Locke solution was made up by diluting 0·2 c.c. of cells to 100 c.c. 1 c.c. aliquots of this suspension were measured into test-tubes. The Icterogenin solution consisted of 10 mgm. of pure substance dissolved by the addition of the requisite amount of alkali (0·18 c.c of N/10 NaOH) in a total volume of 2 c.c. 1 c.c. of this solution was diluted to 25 c.c. to prepare the stock solution which thus contained 0·2 mgm. per c.c. The volumes added to the blood suspension varied from 0·15 to 0·4 c.c. and suitable controls showed that no haemolysis occurred within half an hour after the addition of the latter quantity of water alone.

The results obtained are presented below and in the curves Figs. 7 and 8. It will be seen that Fig. 8 is constructed by plotting log $1000/t$ against log $C \times 10^5$ where t is the time in seconds and C is the concentration of Icterogenin in grams per c.c. The points lie approximately upon the straight line $C^n t = \text{constant}$ until the last two points where a large increase in the quantity of Icterogenin added made little difference to the time taken for complete haemolysis. Clark (1933) points out that the relationship $C^n t = \text{constant}$ holds also for the action of saponins investigated by Ponder and Yeager (1931), ammonia, etc. upon blood cells and it appears to us that the deviation from this relationship at relatively very high concentrations can probably be explained by the intrusion of a fresh limiting factor. For example, the regular portion may represent the rate of combination of drug with cell, the latter portion, where a large excess of drug has been added, the time taken for the cells to swell up and burst or some such similar mechanism.

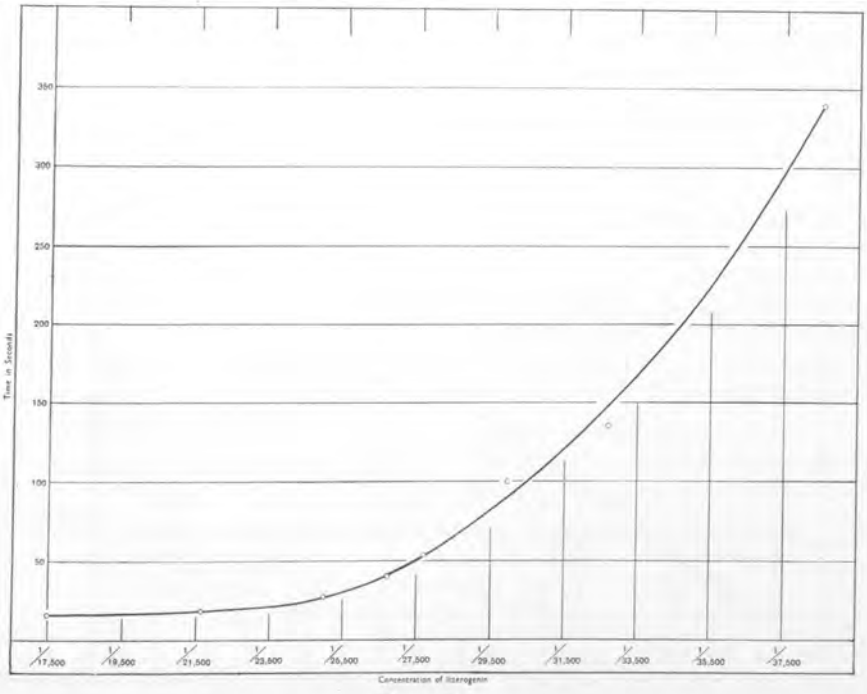


Fig. 7.

HAEMOLYTIC ACTIVITY OF ICTEROGENIN.

Vol. added.	*Quantity added.*	*Total volume.*	*Dilution.*	*Time for haemolysis.*		*Mean.*	Log 1000/t.	Log C x 10^5.
c.c.	*mgm.*	*c.c.*		*Secs.*		*Secs.*		
0·4	0·08	1·4	1/17,500	15	16	15·5	1·8097	0·7570
0·3	0·06	1·3	1/21,667	17	19	18	1·7447	0·6643
0·25	0·05	1·25	1/25,000	29	26	27·5	1·5607	0·6021
0·23	0·046	1·23	1/26,740	40	40	40	1·3979	0·5729
0·22	0·044	1·22	1/27,730	55	52	53·5	1·2716	0·5571
0·20	0·04	1·20	1/30,000	100	100	100	1·0000	0·5229
0·18	0·036	1·18	1/32,780	136	136	136	0·8665	0·4844
0·15	0·03	1·15	1/38,333	346	334	340	0·4685	·4164

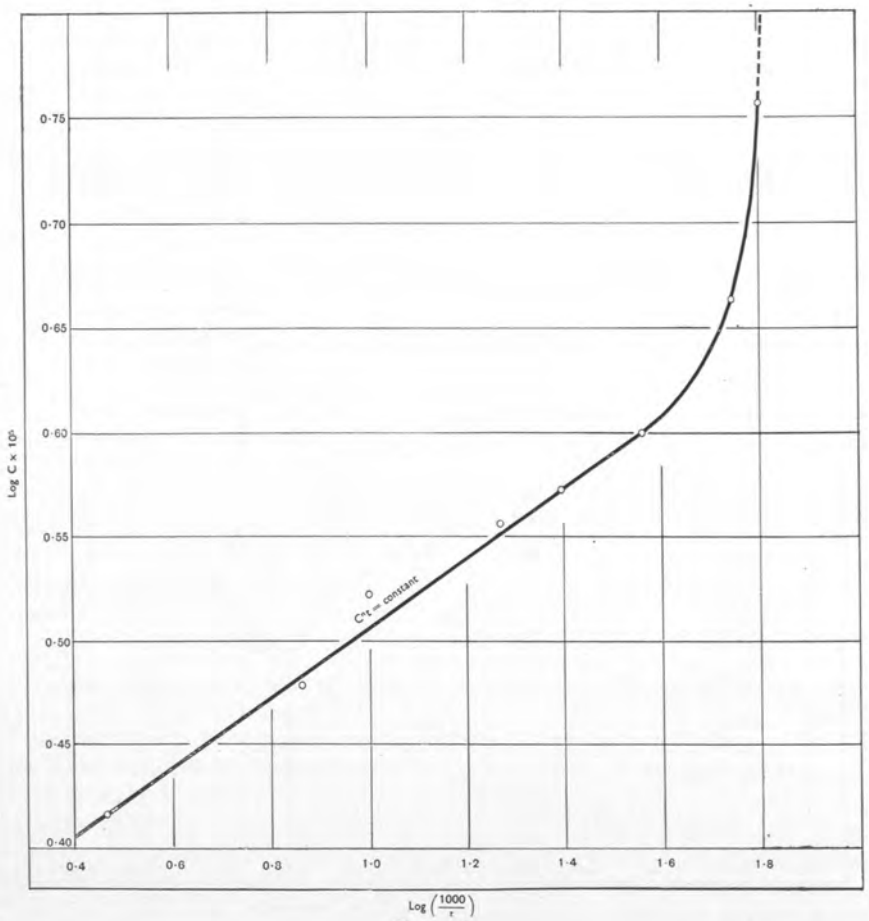


Fig. 8.

Action upon the isolated intestinal strip.

Strips taken from the small intestine of rabbits or sheep were suspended in Tyrode solution in the Dale bath and contractions kymographically recorded. The addition of Icterogenin A, B or C, as the neutral sodium salt dissolved in a small quantity of distilled water, was found to paralyse the tissue at concentrations as low as 1 in 200,000. The inhibition was permanent at higher concentrations but by removal of the bathing fluid and substitution of fresh Tyrode solution, intestinal strips poisoned by 1/200,000 icterogenin slowly recovered their normal rhythm. Repetition of the dose again caused inhibition. The muscle in every case remained fully *relaxed*. It is thus possible to visualise the manner in which the *Lippia* toxic principle brings about such intense constipation and ruminal inhibition in the living animal. See Figs. 9, 10, 11 and 12.

Blood pressure and pulse rate.

Injection of 0·1 gm. Icterogenin intravenously into the anaesthetised animal (dog) causes only a slight diminution in pulse rate accompanied by an increase in the pulse interval. There is no effect upon the general systolic pressure. See Fig. 13.

Perfusion of the isolated heart.

Rabbits' hearts were perfused with Ringer-Locke solution and neutral solutions of the sodium salt of Icterogenin added to the perfusion fluid. Quantities of 1 mgm. of Icterogenin caused a pronounced slowing of the heart beat and decrease in amplitude. The onset was gradual but the effect increased until the heart was arrested in systole. With larger quantities (1·5 mgm.) an almost immediate inhibition occurred, the muscle remaining fatally contracted in a systolic spasm —see Figs. 14 to 16. The exact concentration is difficult to give since the Icterogenin solution was added to the moving column of liquid.

Distribution of Icterogenin between leaves, stems and roots of *Lippia* plants throughout the year.

When examining a batch of *Lippia* plants growing in the poison garden at Onderstepoort, the observation was made that the roots of the plant possessed a thick, fleshy bark and by trial, crude Icterogenin was found to be present in this tissue in comparatively high concentration. Analysis of the different parts of the plant, using the sodium salt technique and working with care to ensure quantitative isolation, afforded the following figures in terms of sodium salt per 100 grams of air-dried material.

Onderstepoort poison garden *Lippia*, collected 6/4/36.

Leaves: 1·3 kilos yielded 1·251 gm. — 0·096 per cent.
Stems: 400 gms. yielded a trace — trace only.
Whole roots: 350 gms. yielded 2·5 gm. — 0·71 per cent.
Root wood: 120 gms. yielded a trace — trace only.
Root bark: 420 gms. yielded 8·556 gms. — 2·04 per cent.

It is clear from the above figures that the greatest concentration of this toxic material is to be found in the root bark, that of the leaves being in the particular sample investigated some 20 times

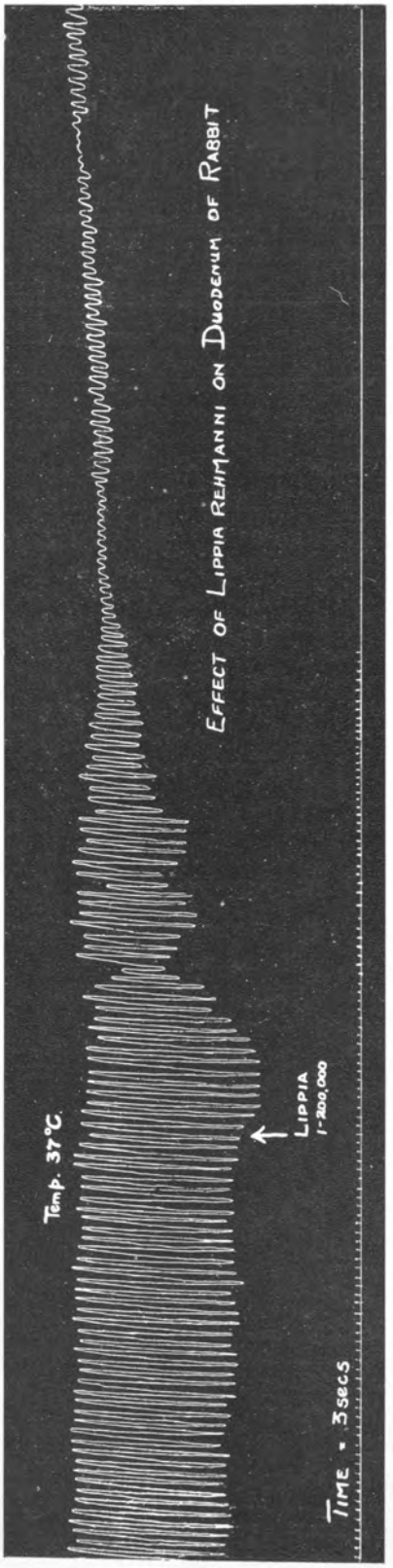
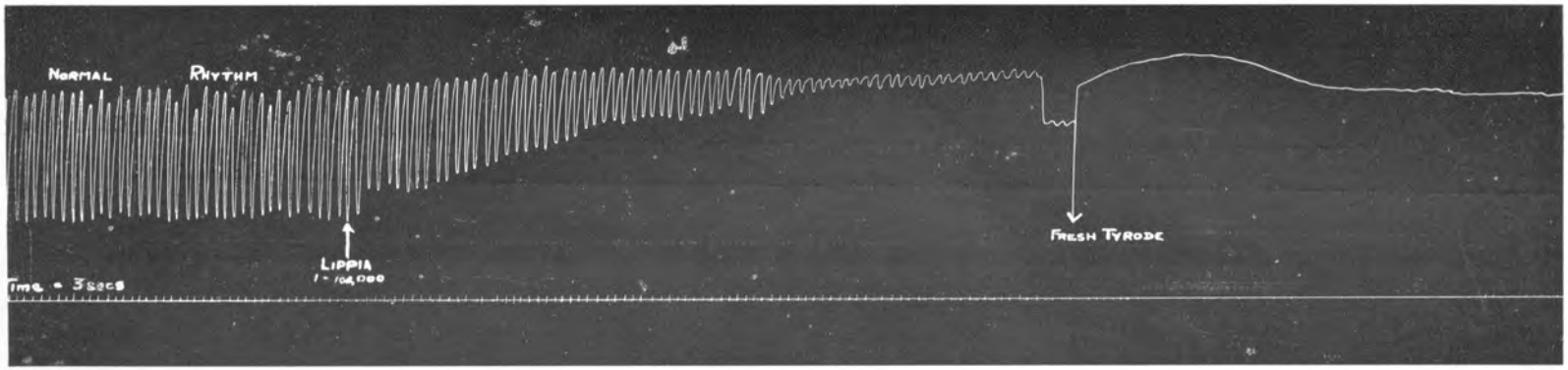


Fig. 9. Effect of Icterogenin 1/200,000 on rabbit duodenal strip.



Interval 146 seconds.

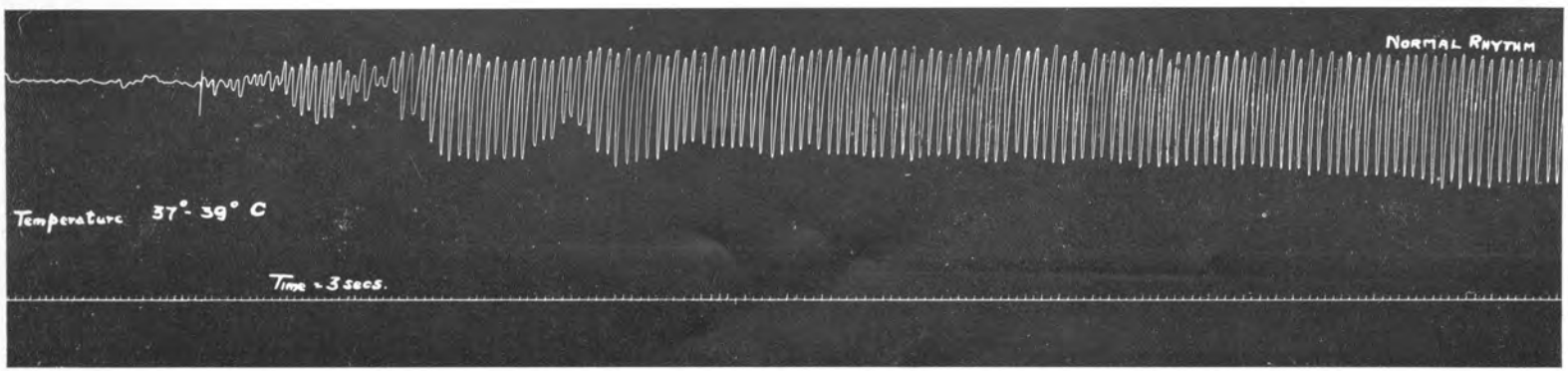


Fig. 10. Effect of Icterogenin 1/100,000 on rabbit duodenal strip showing inhibition and recovery.

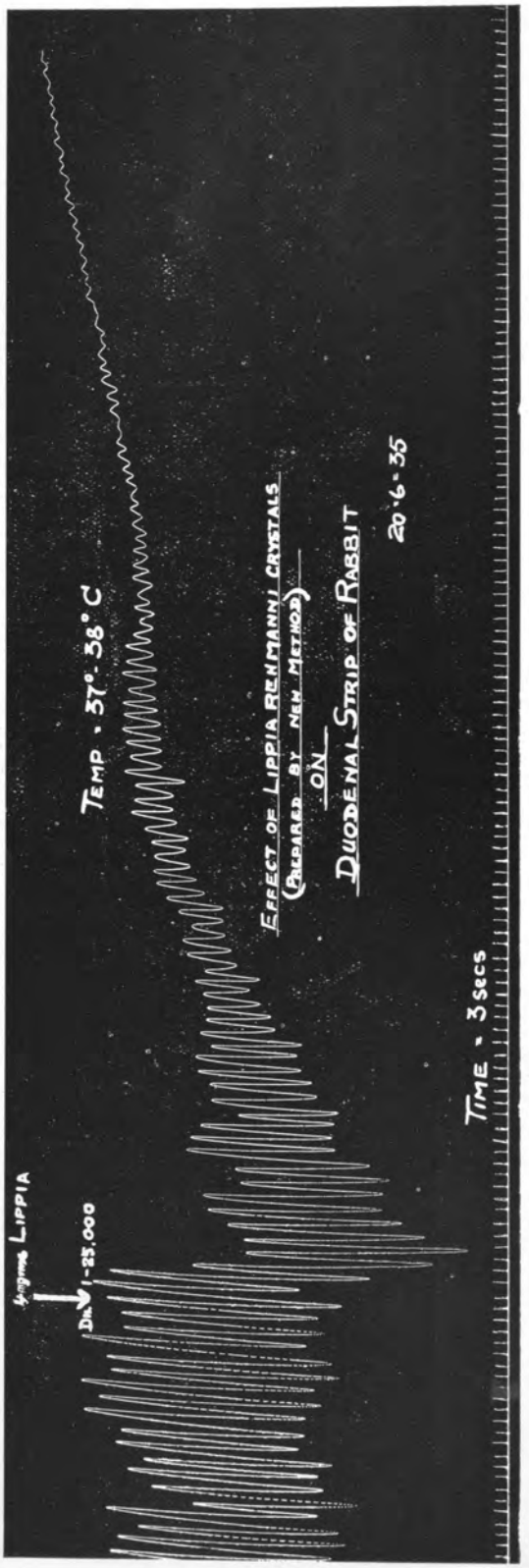


Fig. 11. Effect of Icterogenin B 1/25,000 (prepared by sodium salt method) on rabbit duodenal strip.

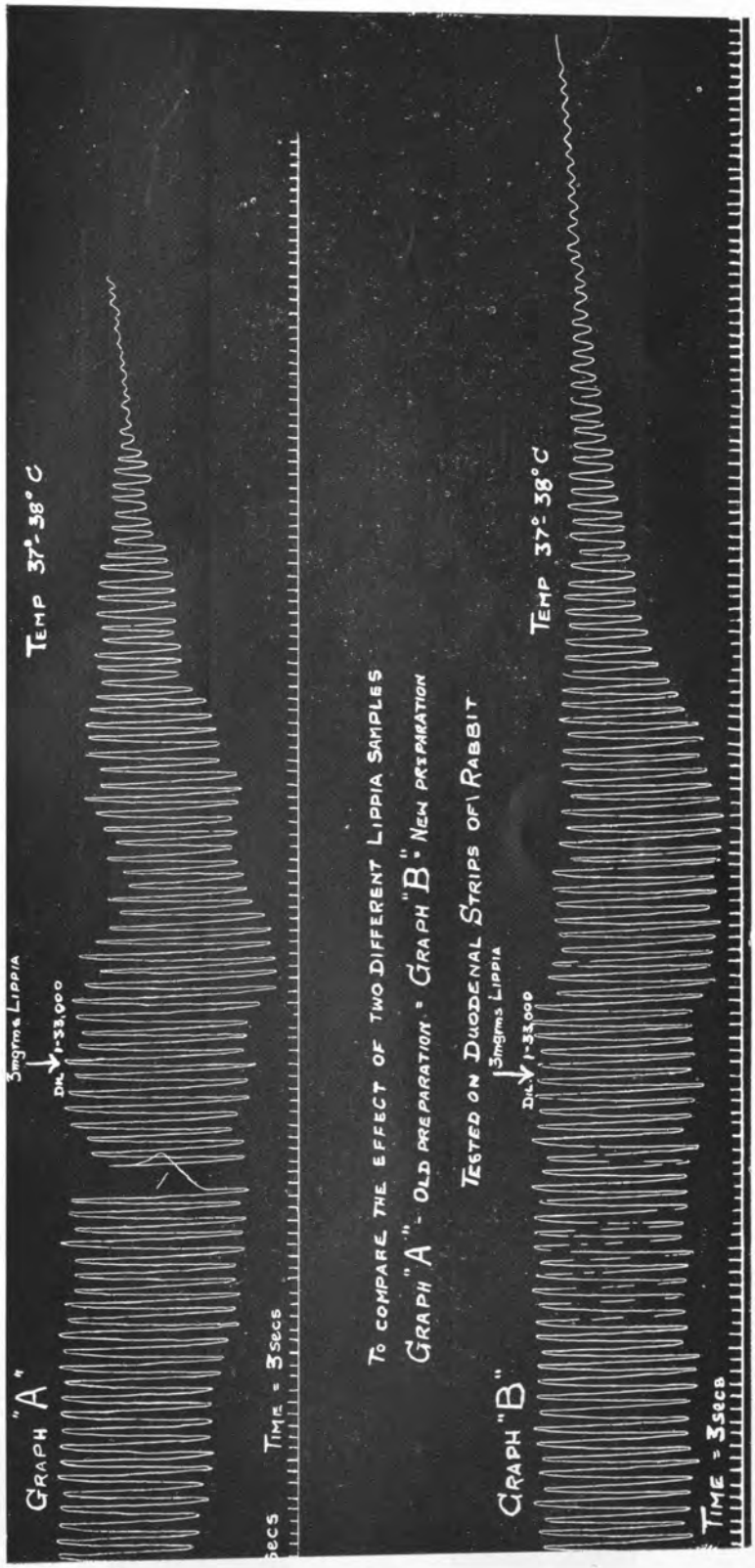
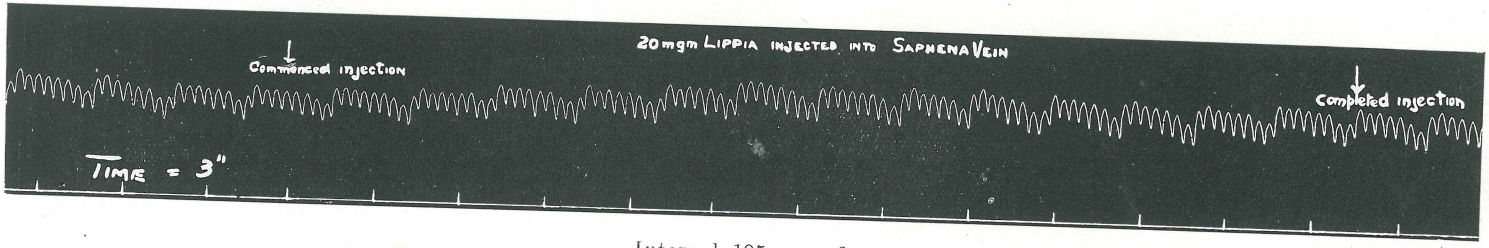


Fig. 12. Comparison of effects of Icterogenins A and B on rabbit duodenal strip.



Interval 195 seconds.

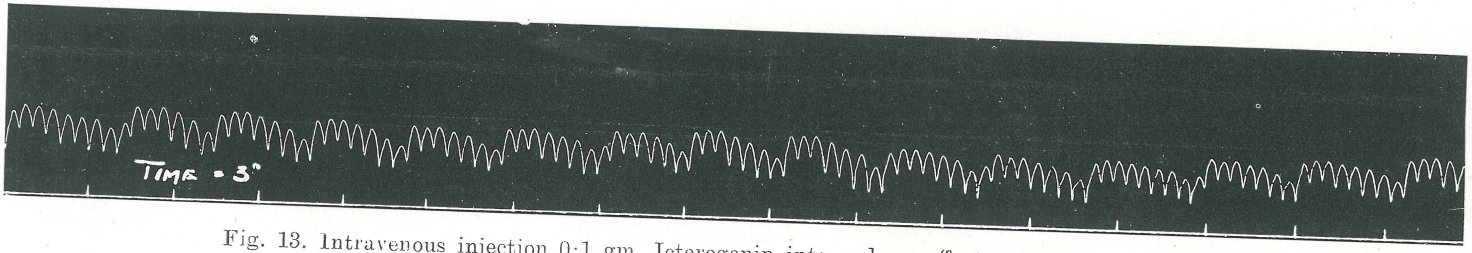


Fig. 13. Intravenous injection 0·1 gm. Icterogenin into a dog: effect on pulse rate and pressure.

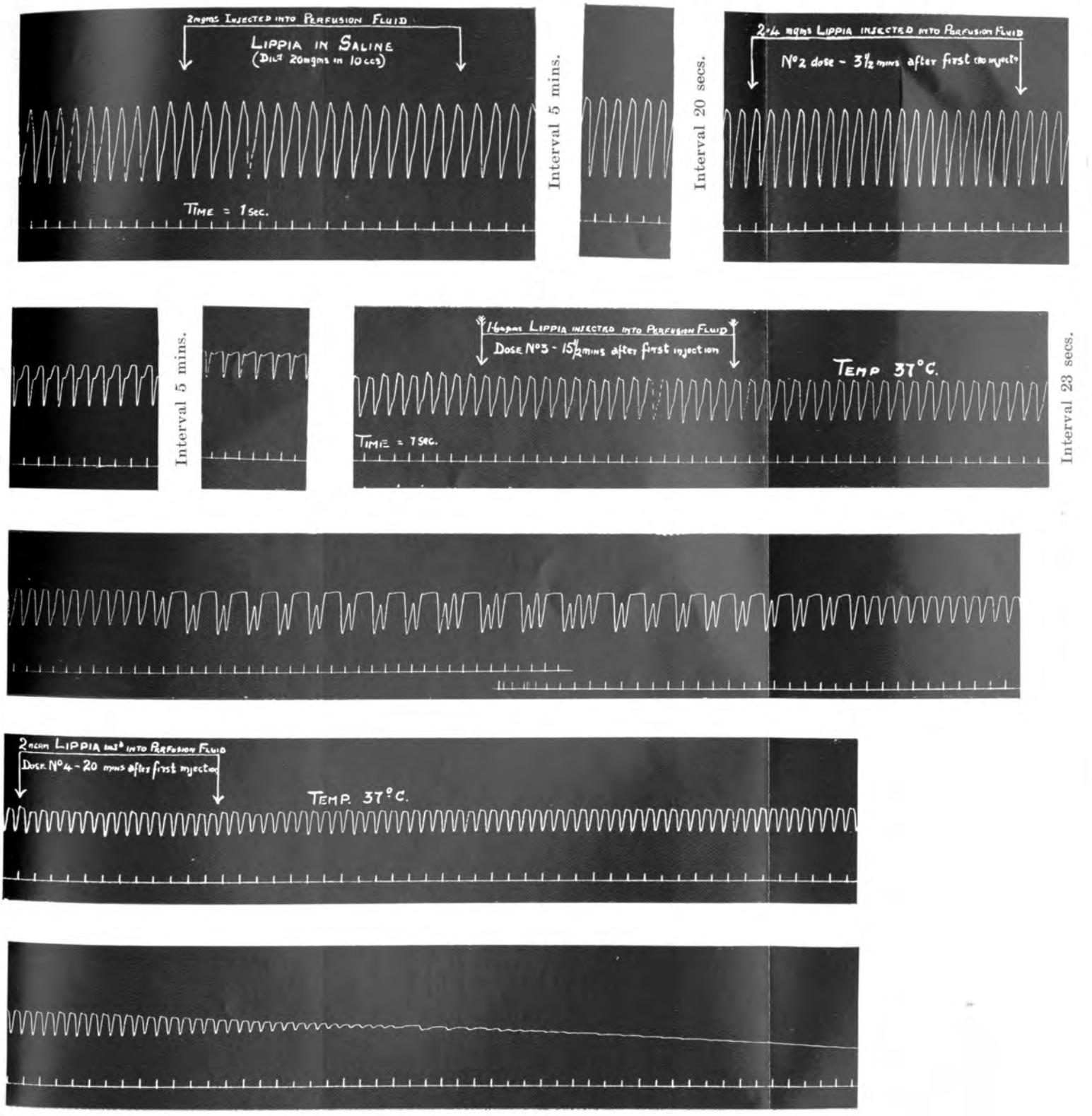


Fig. 14.—Effect of Icterogenin upon the isolated, perfused rabbit's heart.

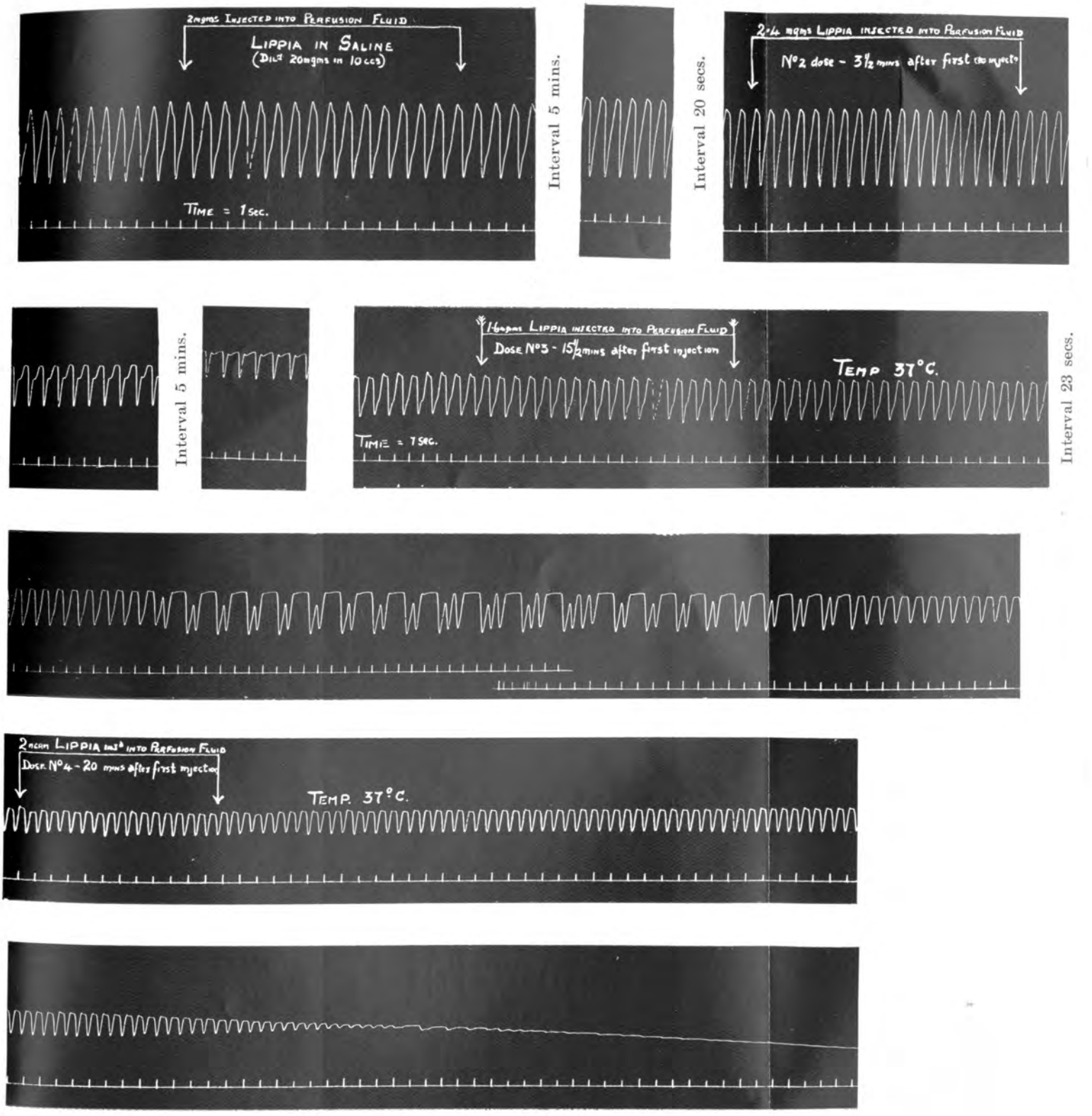


Fig. 14.—Effect of Icterogenin upon the isolated, perfused rabbit's heart.

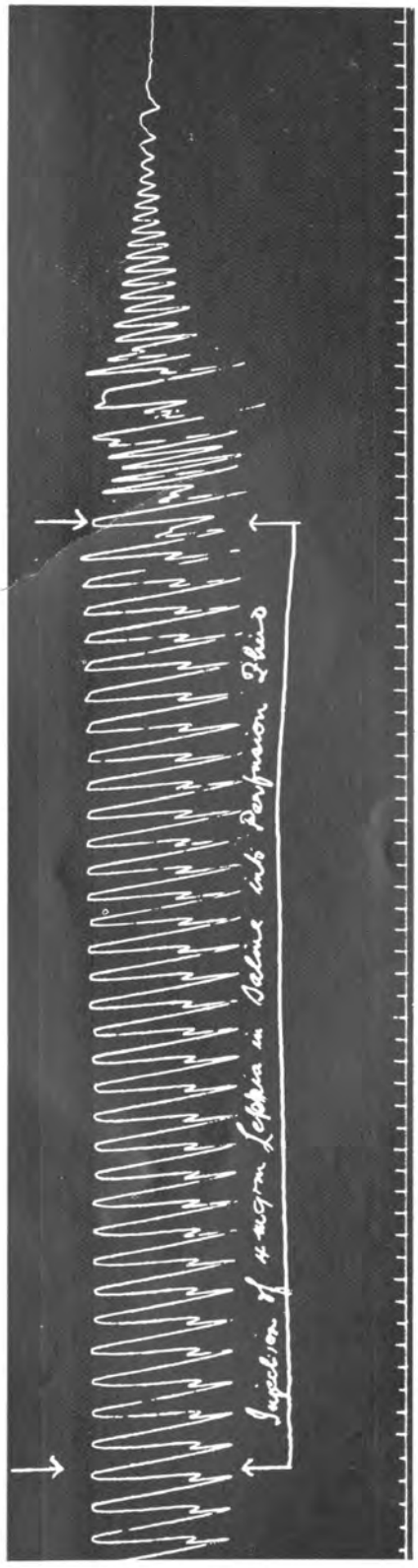


Fig. 15.—Effect of Iterogenin (4 mgm.) upon the isolated, perfused rabbit's heart. Time interval 1 sec.; temp. 35·7°.

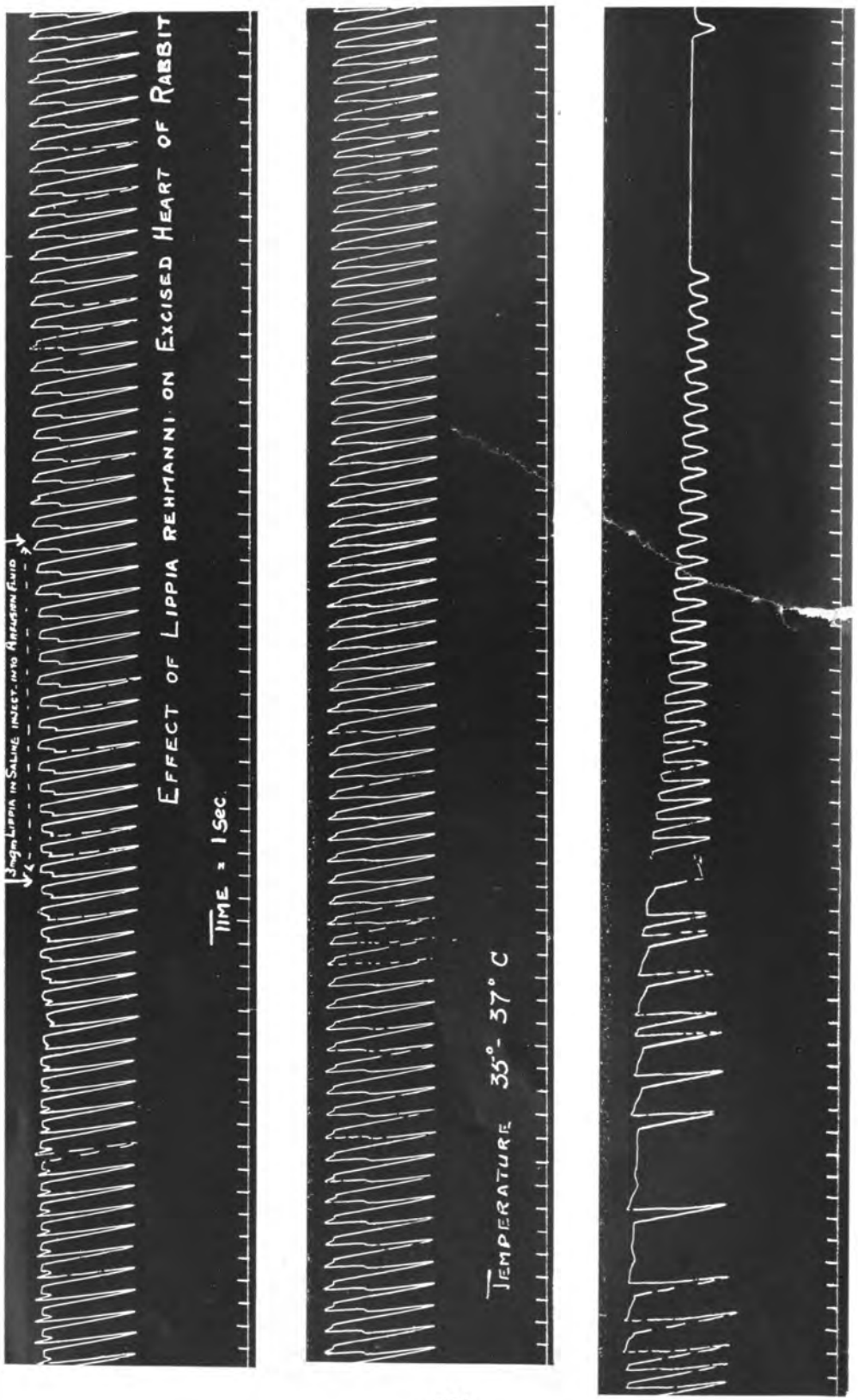


Fig. 16.—Effect of Ieterogenin (3 mgm.) upon the isolated, perfused rabbit's heart.

less, also that the woody portions both of stem and roots are virtually free from Icterogenin. It may be remarked that these plants were in the post-seeding stage when gathered and that heavy summer rains had fallen not long before.

Since this finding promised to throw considerable light on the fluctuations in toxicity throughout the year both of *Lippia* and possibly also of *Tribulus*, the cause of geel-dikkop, the following experiment was designed.

TABLE VI.

Distribution of Icterogenin in Lippia *plants during the year.*

Month.	Date of Collection.	Leaves gm/100 gm. air dry weight.	Root bark gm/100 gm. air dry weight.	State of growth.	Rainfall in inches.
1936.					
Mar....	—	—	—	—	8·50
April...	—	—	—	—	0·51
May....	6th	0·17	1·58	Very dry; seeds falling out	4·26
	duplicate	0·16	1·59		
June...	—	—	—	—	Nil.
July....	—	—	—	—	Nil.
Aug....	—	—	—	—	Nil.
Sept....	28th	0·28	1·01	Young, green shoots but ground very dry	0·46
Oct....	—	—	—	—	2·68
Nov....	5th	0·12	1·76	Plants fresh after good rains..	6·53
Dec....	10th	0·10	3·38	Period of drought prior to collection. Root bark shows spongy new growth	3·37
1937.					
Jan.....	—	—	—	—	4·51
Feb....	—	—	—	—	10·64
Mar....	11th	0·32	2·54	Bushes in good condition after heavy summer rains	1·38
April...	27th	0·26	1·92	(Unpruned bushes).....	2·20
	27th	0·96	4·40	(Leaves and root bark from bushes pruned March 11th, see text).	

These results are recorded graphically in Fig. 17.

A plot of natural, ungrazed veld, heavily overgrown with *Lippia rehmanni*, was kindly placed at our disposal by Mr. J. Wolmarans of the farm "Derdepoort," Silverton, Pretoria district. We wish to express our thanks to Mr. Wolmarans for his generous collaboration. At intervals during the year, expeditions were made to the farm and a sufficient quantity of *Lippia* plants uprooted to allow of chemical investigation. The roots were washed under a tap to remove adhering soil and the fleshy root bark then removed and spread out to dry: the leaves were easily plucked from the stems after sun-drying. Stems were not as a rule investigated after a preliminary experiment had shown that traces only of Icterogenin could

be isolated from them. Each material was finely ground and then extracted according to the technique described in this paper. It was found that the root bark could be directly extracted by boiling ether in a Soxhlet apparatus thereby shortening the procedure by one stage. The yields of sodium salt were recorded and it was found that duplicate analyses agreed well. In the above table (Table VI), the results are reproduced together with the monthly rain-fall figures measured at Onderstepoort.

An experiment was made on March 11th whereby it was hoped to ascertain if new growth was always to be associated with high Icterogenin content, and also whether or not Icterogenin was transferred from the leaves to the root bark. A number of bushes was stripped of leaves and pruned back very severely. At the date of the next collection, a month later, we were gratified to observe that this had had the desired effect of stimulating the plants to shoot. A mass of tender green leaves covered the old stems. These leaves were gathered, the plants then dug up and the bark from the roots also collected separately for comparison with the corresponding tissues of the untreated bushes. It was found that not only was the Icterogenin content of the leaves the highest which we have ever recorded (0·96 per cent.) but that the quantity in the root bark had also risen markedly. In the untreated plants, a fall was recorded in both leaves and bark.

We feel quite justified in drawing the conclusions therefore that (*a*) a transference of Icterogenin from leaves to root bark does normally occur and (*b*) that the growth of young leaves is associated with a big production of Icterogenin. It seems doubtful if any synthesis of icterogenin could take place in the root bark *in situ* so that the rise and fall occuring in this tissue during the course of the year must be occasioned by the transference of material to or from the leaves. The bark, of course, exhibits growth at certain seasons of the year, thus in December (mid-summer) the whole tissue was soft, fleshy and but poorly suberised, but since all quantities are calculated upon the dry weight basis, as a percentage, the resultant figure will embrace both of the two factors—the quantity of Icterogenin passed to the roots and the bulk of the bark itself.

Examination of the graph, Fig. 17, shows that the transference from leaves to root must be fairly rapid, in the early spring at least. About November, a fall in leaf Icterogenin is reflected by a rise in that of the root bark, as indicated by the crossing of the curves; later on (April) both values fall together. The precise influence of rainfall, apart from growth, if any such influence exists, will only become evident when we have a larger number of values extending over several seasons.

Whether or not a transference of Icterogenin in the reverse direction may take place, that is from root to the above ground portions of the plant, is a question we would like to leave open. We realise that hypothetical transference in this direction does not accord with generally accepted views, nevertheless, we do not wish to loose sight of the possibility.

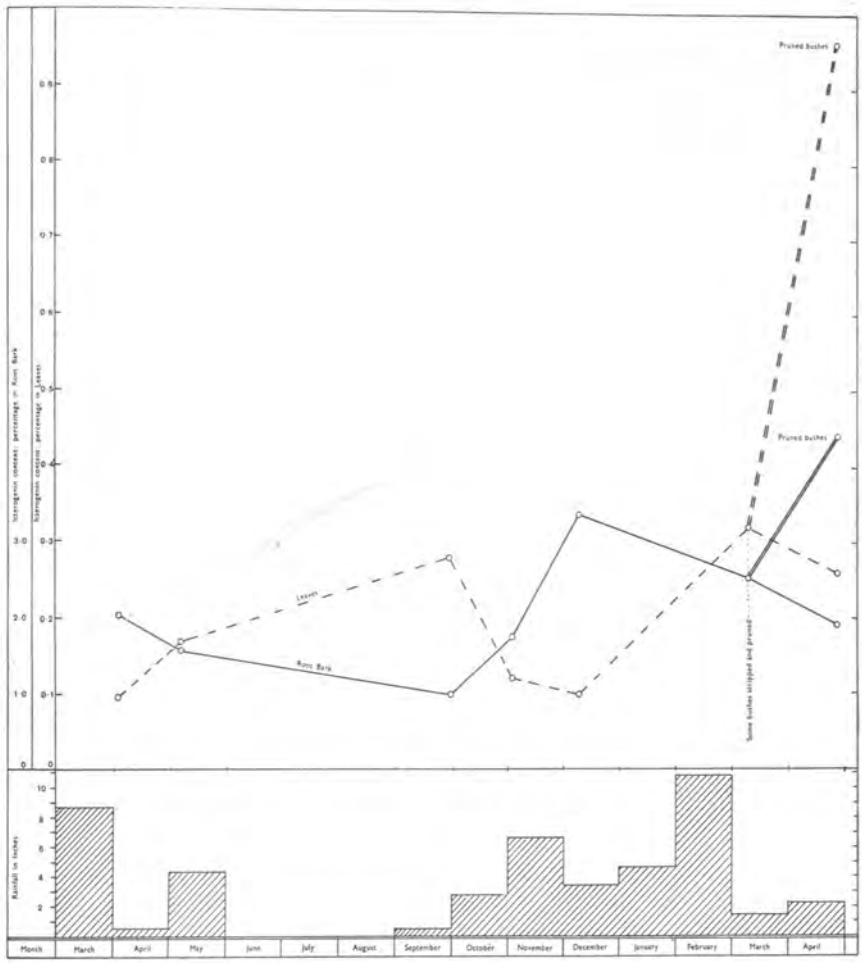


Fig. 17.

Finally, we would like to indicate briefly the bearing that these experiments may have upon the seasonal nature of geel-dikkop outbreaks and the well known tendency for this disease to disappear after good rains. Admittedly, many factors must contribute to the capricious nature of geel-dikkop, among which must be reckoned the scarcity or otherwise of other foodstuffs, the condition of the sheep, etc., but farming experience has shown that light rains leading to rapid growth of *Tribulus*, followed by a period of hot, dry weather, are indicative of danger from geel-dikkop. Such conditions, following the *Lippia* model, would be conducive to a rapid rise in the Icterogenin content of the leaves of this plant. December to March is also the period at which the danger from geel-dikkop is at its greatest.

Experiments are still in progress designed to reveal seasonal fluctuations in the toxicity of *Tribulus* and the root system of this plant is also now receiving due attention.

SUMMARY.

1. An improved method is described for the isolation of the icterogenic material from *Lippia rehmanni* Pears. (Verbenaceae). This takes advantage of the sparing solubility of the sodium salt of the active material in solutions containing sodium ions.

2. In addition to the two acids previously described, namely prisms M.P. 239° and irregular plates melting with loss of weight at 158°, resolidifying to melt ultimately at about 230°, a third active acid has been found to be present in the mixture of crude sodium salts. This material crystallises in elongated regular plates, M.P. 161° without resolidification or loss in weight. It is proposed to designate these materials Icterogenin A, C and B respectively.

3. Comparison of the analytical data afforded by the free acids and their acetyl derivatives with microtitration experiments and the nitrogen content of the 2:4 dinitrophenylhydrazones, leads to the formula $C_{34}H_{52}O_6$ as most probably representing the true composition of all three isomers. The possibility of further substances being present in the mixture is not excluded.

4. In alcoholic solution, the Icterogenins A, B and C have optical rotatory powers of +72·0°, +69·0° and +70·5° respectively.

5. Acetyl derivatives of Icterogenins A and B were prepared and found to be identical. The substance crystallised in needles M.P. 142°. Saponification showed that one hydroxyl group had been acetylated.

6. The presence of one ketonic group in the molecule was shown by the preparation of the 2:4 dinitrophenylhydrazones crystallising in orange-yellow needles with M.P.'s 222-5°, 226-9° and 224-8° respectively.

7. Chromic acid oxidation indicated the presence of an iso-propyl side chain. The formula of Icterogenin can thus be written

$$C_{29}H_{43}O_2\begin{cases} -\,CO \\ -\,OH \\ -\,COOH \\ -\,CH\begin{cases} CH_3 \\ CH_3 \end{cases} \end{cases}$$

8. Icterogenin, in a dose of 1·5 gm. or more *per os* to a sheep, causes bilirubinaemia within 24 hours, together with atony and stasis in the fore stomachs and large intestine.

9. By the intravenous route it was very toxic, quantities of 1 gm. causing death with shock-like symptoms. Lesser amounts cause bilirubinaemia and hurried respiration.

10. Upon the general systolic blood pressure, Icterogenin has no effect in doses of 0·1 gm. to a dog, but the pulse is slightly retarded and the pulse interval appreciably increased.

11. On the isolated heart, Icterogenin has a pronounced inhibitory effect in quantities of 1 mgm. added to the perfusion fluid. A systolic spasm gradually develops which, with larger doses, proves fatal.

12. The icterogenins inhibit the isolated intestinal strip in a concentration of 1 in 200,000, the muscle becoming relaxed.

13. Icterogen is haemolytic *in vitro* in a concentration of 1 in 35,000.

14. Examination of the root bark of *Lippia rehmanni* shows that the highest concentration of Icterogenin is present in this tissue.

15. A quantitative experiment extending over a year shows that a translocation of Icterogenin takes place from the leaves to the root bark in *Lippia* plants. The effect of growth, season, etc. upon this mechanism has been studied and the bearing of the results upon the incidence of geeldikkop is discussed.

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