

THE CARDIAC GLYCOSIDE FROM *URGINEA RUBELLA* BAKER.
PART II.—HYDROLYSIS OF RUBELLIN AND PRO-RUBELLIDIN.

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In the previous communication on rubellin (Louw, 1949), it was reported that hydrochloric acid hydrolysis of the glycoside yielded the crystalline product pro-rubellidin, which was considered to be the probable pro-genin of rubellin, together with a sugar which could not be identified.

This paper deals with further hydrolysis experiments conducted with rubellin and pro-rubellin and investigations on the sugar moiety of the glycoside.

Hydrolysis of rubellin.

Hydrochloric acid hydrolysis of rubellin under various conditions yielded pro-rubellidin as before, and no crystalline osazone could be obtained from the completely hydrolysed sugar solutions. On addition of phenylhydrazine to the cold sugar solution, an orange precipitate formed immediately which became oily on boiling the mixture. The immediate precipitation of the orange product suggested the formation of an insoluble hydrazone.

The sugar solutions were strongly reducing towards Fehling's solution, did not exhibit any fermentation with baker's yeast, gave positive tests for hexoses (skatol and indole reactions) and negative reactions for pentoses (negative tests for furfural and methyl-furfural in the hydrochloric acid distillate of the sugar solution).

The sugar solutions which oxidised alkaline iodine solutions (aldose) failed to form a crystalline osazone which fact suggested the presence of a desoxy-sugar, but both rubellin and the sugar solutions gave negative Keller-Kiliani tests for desoxy-sugars.

After neutralisation of the hydrochloric acid hydrolysis mixture of rubellin with dilute sodium hydroxide and separation of pro-rubellidin and sodium chloride, a product was obtained as hygroscopic needles from absolute alcohol on complete evaporation of the solvent in a vacuum exsiccator. This product which was thought to be a sugar of rubellin was eventually found to be a sodium derivative.

Sugar Acid.

Hydrolysis of rubellin with 0.5 per cent sulphuric acid and neutralisation of the reaction mixture with barium carbonate yielded the same hydrolysis product as before, viz. pro-rubellidin. The aqueous mother liquor from which the pro-rubellidin was separated quantitatively, reacted strongly positive for reducing

sugars. It was evaporated to a thick consistency and then treated with absolute alcohol when the entire contents solidified. The product was thoroughly digested with alcohol, leaving a light-yellow product which on ignition left a residue of barium and gave a white precipitate of barium sulphate when a solution of this product was treated with dilute sulphuric acid.

The barium compound was purified by repeated precipitation from aqueous solution with alcohol. In this way a white non-crystalline barium compound was obtained.

The barium compound reduced Fehling's solution, was negative for acetate and had a reducing action on alkaline iodine solutions.

Dried over P_2O_5 *in vacuo* at 100° C., the barium compound had a barium content of 38.90, 39.20 per cent.

From these results it was evident that a most unusual sugar component is present in rubellin. The possibility of acetate was eliminated. A barium content of 26.25 per cent is required for the barium salt of a hexuronic acid, while the barium salts of dicarboxylic acids, $C_6H_8O_8Ba$, have a barium content of 39.77 per cent.

The hydrolysis of 10 gm. rubellin yielded 7.35 gm. pro-rubellidin and 4.1 gm. of the barium derivative, against the calculated values of 7.79 and 4.36 gm. for pro-rubellidin ($C_{30}H_{38}O_{11}$) and $C_6H_8O_8Ba$ respectively.

As rubellin does not contain free carboxyl groups, the dicarboxylic acid, obtained upon hydrolysis, was therefore most probably present in rubellin as a dilactone.

The barium compound did not crystallise and could be purified by precipitation with alcohol only. The free acid was prepared by careful precipitation of the barium with the calculated amount of dilute sulphuric acid. The acid was obtained as a syrup which failed to crystallise. Neither could it be converted to a crystalline lactone. The quinine, cinchonine and brucine salts of the acid were then prepared but these failed to crystallise.

Hydrolysis of pro-rubellidin.

Pro-rubellidin which still contained carbohydrate and was therefore considered to be the pro-genin of rubellin, was subjected to acid hydrolysis under various conditions. In all the hydrolysis experiments with pro-rubellidin, the aqueous extracts were strongly reducing towards Fehling's solution, gave positive tests for carbohydrates but did not yield crystalline osazones.

Apart from the isolation of unchanged pro-rubellidin no crystalline product could be separated from the hydrolysis mixtures, yellow amorphous products with low carbon contents usually being obtained.

Hydrolysis of pro-rubellidin by the method of Mannich and Siewert (1942), yielded a crystalline compound, the analysis of which pointed to decomposition rather than hydrolysis of pro-rubellidin. Other hydrolysis experiments conducted with pro-rubellidin yielded only yellow amorphous products which also had low carbon contents.

In every case there was a marked change in the colour of the hydrolysis mixture from colourless to yellow, orange or sometimes red, which was suggestive of the decomposition of pro-rubellidin.

Paper Chromatography of sugar Hydrolysates.

The aqueous sugar filtrates after hydrolysis of rubellin and pro-rubellidin were chromatographed on paper by the method of Partridge (1948), using phenol as solvent.

The sugar solution obtained on hydrolysis of rubellin with 0.5 per cent H_2SO_4 followed by neutralisation with barium carbonate and removal of pro-rubellidin, gave only one distinct component with Rf value of 0.14-0.16, along with two faint components with Rf values 0.58 and 0.86.

The component with low motility was found to be the barium salt of the probable dicarboxylic sugar acid, the Rf value of the pure barium compound being 0.14-0.16.

Chromatography of the sugar solution obtained by hydrolysis of pure pro-rubellidin with sulphuric acid, gave only one component with Rf value 0.28.

After separation of the barium compound from the rubellin hydrolysate, the solution was concentrated to small bulk and on chromatography one main sugar component with Rf value 0.27-0.29 was observed. A faint component with Rf value 0.14 was due to some of the barium compound which was not completely removed. Again the two indistinct components with Rf values 0.58 and 0.86 were obtained.

The hydrolysates of pro-rubellidin always had one component with Rf value 0.28 only, which accounted for the second component which appeared in the hydrolysate of rubellin after removal of the barium compound and subsequent concentration of the residual solution.

Partridge (1948) found that the carboxylic acid group gives rise to a low Rf value as indicated by the slow movement of the uronic acids. He found the Rf values for d-galacturonic-, d-glucuronic- and l-ascorbic acids to be 0.13, 0.12 and 0.16 respectively using phenol as solvent.

EXPERIMENTAL.

1. *Hydrolysis of Rubellin with Hydrochloric Acid.*

2.0 gm. rubellin were hydrolysed in 50 per cent methanol solution with hydrochloric acid as described before. The hydrolysis product which crystallised from the aqueous solution was re-crystallised from ethyl acetate when it was obtained as small transparent plates with m.p. 267-8° C. (decomp.).

Pro-rubellidin gave a positive carbohydrate test with the Molisch sugar test. With the Liebermann test it gave a reddish colour which changed to purple, blue, green and finally brown.

Analysis.

Found C=62.73, 62.55 per cent.

H=6.64, 6.69 per cent.

Calculated for $C_{30}H_{38}O_{11}$.

C=62.70, H=6.66 per cent.

The aqueous hydrolysate after quantitative removal of pro-rubellidin was neutralised with 0.1N NaOH and the mixture evaporated to dryness leaving a brown residue which had a sweet smell suggestive of fructose.

* Micro-analysis by Drs. G. Weiler and F. B. Strauss, Oxford, England.

All the products for analysis were dried over P_2O_5 at 100° C. and 3 mm. Hg.

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To 100 mg. of the sugar residue dissolved in 5 ml. water, 1 ml. glacial acetic acid was added. On addition of 0.5 ml. phenylhydrazine an immediate orange precipitate formed which became oily when the mixture was heated in a waterbath to promote osazone formation. No crystalline product separated from the mixture. The oily product was removed, washed and treated with solvents but failed to crystallise. A chloroform solution of the product was chromatographed on aluminium oxide without success of obtaining crystalline products.

Titration of the sugar solution with iodine.

The sugar hydrolysate of 2 gm. rubellin was made up to 50 ml. and 2 ml. of this solution were treated with iodine in alkaline solution by the method of Auerbach-Bodländer (1923). After acidification, the smell of iodoform was clearly perceptible.

ml. 0.1N iodine consumed by 2 ml. aliquot = 2.10, 2.00.

∴ mg. aldose (as $C_6H_{12}O_6$) present = 18.91, 18.01.

Control of 16 mg. glucose used 1.75 ml. 0.1N iodine = 15.75 mg. glucose.

∴ Amount of aldose ($C_6H_{12}O_6$) hydrolysed from 2 gm. rubellin = 473.5, 450 mg.

Theoretical amount for one mol. glucose in 2 gm. rubellin = 489 mg.

Sodium salt of sugar acid.

The residual aqueous hydrolysate of rubellin after quantitative separation of pro-rubellidin by fractional crystallisation and extraction with ethyl acetate, was dried in a vacuum exsiccator and extracted with absolute alcohol which left the sodium chloride undissolved. The alcoholic solution was then evaporated to dryness in vacuum when hygroscopic needles were obtained. The crystalline product was washed with ether and acetone when it was obtained as a white hygroscopic powder.

This product was at first considered to be a sugar that was hydrolysed off, but although it had reducing properties it failed to form a crystalline osazone. Later it was found to be a sodium derivative.

A further amount of this sodium compound was prepared by decomposition of the barium salt obtained by hydrolysis of rubellin with sulphuric acid and neutralisation with barium carbonate. It was obtained as a light-yellow granular product which dissolved easily in ethyl alcohol, methyl alcohol, and acetone.

Analysis.

Na = 19.74, 19.29 per cent.

The disodium salt of a dicarboxylic sugar acid, $C_6H_8O_8Na_2$ requires:
Na = 18.10 per cent.

Hydrolysis of rubellin with sulphuric acid.

10.0 Grams rubellin were powdered and suspended in 1200 ml 0.5 per cent sulphuric acid and the mixture heated for one hour in a waterbath at 90° C. with continual stirring. The rubellin dissolved and the solution turned yellow.

The mixture was cooled and carefully neutralised with a suspension of barium carbonate. The excess carbonate and precipitated barium sulphate were centrifuged off leaving a yellow supernatant which was evaporated by fanning. On concentration a colourless product crystallised and was separated. The mother liquor was further concentrated until the crystallisation of the colourless compound was complete. In this manner 7.35 gm. of the crystalline product were obtained.

On re-crystallisation from ethyl acetate the product was obtained in small transparent plates which melted at 267–8° C. (decomp.) and when mixed with pro-rubellidin, no depression of melting point was found.

Yield of pro-rubellidin from 10 g. rubellin = 7.35 gm.

Theoretical yield of pro-rubellidin ($C_{30}H_{38}O_{11}$) from 10 gm. rubellin ($C_{36}H_{48}O_{16}$) = 7.79g m.

Barium salt.

The aqueous concentrate from which pro-rubellidin had been removed as quantitatively as possible by careful fractional crystallisation, had a brown colour and a sweet sugary smell suggestive of fructose. The concentrate was evaporated to small bulk in a vacuum exsiccator and then treated with absolute alcohol when the entire contents became solid. It was thoroughly digested with hot absolute alcohol yielding a yellowish product (4.1 gm.) The alcoholic extract contained 0.25 gm. solids on evaporation.

The yellow hydrolysis product which had a strong reducing action on Fehling's solution and gave a positive reaction for carbohydrate with the Molisch test, gave a residue of barium on ignition.

The barium product was purified by repeated precipitation from concentrated aqueous solution by the addition of alcohol, yielding a white non-crystalline product.

Analysis.

Found: Ba = 38.90, 39.20 per cent.

The barium salt of a dicarboxylic sugar acid, $C_6 H_8 O_8 Ba$, requires Ba = 39.77 per cent.

Absence of acetate in the barium compound.

21.0 Mg. purified barium salt were dissolved in one ml. water and treated with 0.1 N H_2SO_4 —a little under the theoretical amount required to decompose the barium compound. The mixture was then transferred into a Pregl micro-acetyl apparatus and distilled for acetic acid.

ml. 0.01 N NaOH used by distillate = 0.18,

Calculated for 21 mg. barium acetate: 16.45 ml. 0.01 N NaOH.

2. Acetylation of rubellin and pro-rubellidin.

(a) *Absence of acetyl groups in rubellin.*

224.3, 240.5 Mg. rubellin were dissolved in 3 ml. methanol and 3 ml. 30 per cent methanolic potash added and the mixture refluxed for one hour. On cooling 20 ml. of 10 per cent phosphoric acid were added. The mixture was

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distilled to small bulk and the distillate collected. Another 20 ml. water were added and the mixture again distilled down to small volume. The distillates were then titrated with 0.1 N NaOH.

ml. 0.1 N NaOH used = 0.28, 0.25.

Calculated for one acetyl group in rubellin, ml. 0.1 N NaOH required:
3.047, 3.268.

(b) *Hexa-acetyl rubellin.*

500 Mg. rubellin were dissolved in 10 ml. pyridine and 7.0 ml. acetic anhydride added. The mixture was left at room temperature for 24 hours when it was poured into excess ice-water, left in a refrigerator for one hour and the white precipitate then filtered off, washed and dried. Re-crystallised from methanol, the acetyl-derivative was obtained in small prisms which melted at 270.2° C. (decomp.) $[\alpha]_D^{25} = +37.19^\circ$ (CHCl₃)

Analysis.

Found: % C = 56.78, % H = 6.78, % CH₃CO = 27.39.
56.54, 6.56, 26.70.
57.15, 6.60, 25.30.

Calculated for hexa-acetyl rubellin, C₄₈ H₆₀ O₂₂ H₂O: (1 lactone group + 6 acetyl groups): % C = 57.25, % H = 6.20, % CH₃CO = 25.64.

Titration of acetyl derivative.

(i) 61.2, 64.3 Mg. acetyl rubellin (vac. dried over P₂O₅) were dissolved in 20 ml. methanol and 10 ml. 0.1 N NaOH added, stoppered and left in the dark for 48 hours, when the excess alkali was back-titrated.

ml. 0.1 N NaOH used = 3.98, 4.14.

(ii) 59.4 Mg. acetyl rubellin (vac. dried over P₂O₅) dissolved in 20 ml. methanol were refluxed for 90 minutes on a waterbath with the exclusion of carbon dioxide. The solution which became yellowish-brown turned yellow on cooling. The excess alkali was then back-titrated.

ml. 0.1 N NaOH used = 3.45.

Found: ml. 0.1 N NaOH used—

(i) 3.98, 4.14;

(ii) 3.45.

Calculated for hexa-acetyl rubellin—

(i) 4.33, 4.55;

(ii) 4.20.

(c) *Hexa-acetyl pro-rubellidin.*

The acetyl derivative of pro-rubellidin was prepared similarly to that of rubellin. Re-crystallised from methanol it was obtained as blunt opaque prisms which melted at 275-280° C. (decomp.) $[\alpha]_D^{24} = -66.39^\circ$ (CHCl₃)

Analysis.

Found: % C = 60.84, % H = 6.49, % CH₃CO = 31.07.

61.05,	6.32,	30.50.
60.56,	6.03,	33.3.
60.59,	6.21,	33.1.

Calculated for hexa-acetyl pro-rubellidin, C₄₂ H₅₀ O₁₇ (1 lactone group + 6 acetyl groups): % C = 60.99, % H = 6.09, % CH₃CO = 31.24.

Titration of acetyl pro-rubellidin.

69.8, 73.6 Mg. acetyl pro-rubellidin (vac. dried over P₂O₅) were dissolved in 20 ml. methanol, 10 ml. 0.1 N NaOH added, stoppered and left in the dark for 48 hours when the excess alkali was back-titrated.

ml. 0.1 N NaOH used = 5.61, 5.92.

Calculated for hexa-acetyl pro-rubellidin: 5.91, 6.23.

3. Paper chromatography of the sugar hydrolysates of rubellin and pro-rubellidin.

The hydrolysates of rubellin and pro-rubellidin, after separation of the water-insoluble products were chromatographed on paper by the method of Partridge, (1948).

Whatman No. 100 filter paper strips were used and chromatography by capillary ascent using phenol-water as solvent was employed. Silver nitrate-ammonia solution was used to locate the reducing components.

The results are summarised in Table I.

TABLE I.

Chromatography of Sugar hydrolysates of rubellin and pro-rubellidin.

Hydrolysate.	Results.
1. Rubellin hydrolysed with 0.5% sulphuric acid, neutralised with BaCO ₃ .	(a) Main component with Rf value 0.14-0.16. (b) Indistinct components with Rf values 0.58 and 0.86.
2. Hydrolysate of (1) after removal of the barium sugar compound and concentration to small bulk.	(a) Weak component with Rf value 0.14. (b) Main component with Rf value 0.27-0.29. (c) Indistinct components with Rf values 0.58 and 0.86.
3. Purified barium compound.....	Only one component with Rf value 0.14-0.16.
4. Hydrolysis of pro-rubellidin with— (a) 1% sulphuric acid..... (b) hydrochloric acid in 50% alcohol.....	Only one component with Rf value 0.28. Only one component with Rf value 0.28.

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SUMMARY.

1. The isolation of an unusual sugar component, viz. a sugar acid derivative, obtained by the hydrolysis of rubellin, is reported.

2. Hydrolysis of pro-rubellidin under various conditions gave products which pointed to the decomposition of pro-rubellidin.

3. Paper chromatography revealed two distinct sugar constituents with low Rf values, in the hydrolysate of rubellin. One constituent which had Rf value 0.14-0.16 was the barium salt of the sugar acid obtained by hydrolysis of rubellin, and the other with Rf value 0.28, was obtained on hydrolysis of pro-rubellidin.

4. The acetylation products of rubellin and pro-rubellidin are described.

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