CONTRIBUTION TO THE CHEMISTRY OF LEUCAEMIC URINE

by

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MILLER and his associates[1] have isolated from human leucaemic urine substances which, when injected into guinea pigs, induced leucaemic infiltration into many organs. Their results have revived, after a long period of stagnation, the interest in the chemical investigation of leucaemic urine as a source of active compounds or of specific metabolites.

Researches in this direction have been reported by FRIEDMANN AND JACOBSON[2] who isolated, from leucaemic urine, three well characterised, apparently new, substances:

1. a crystalline purple pigment,
2. a yellow pigment and
3. a colourless crystalline substance, m.p. 176–177°.

In continuation of this work, we have been able to elucidate the structure of the purple pigment. This work was helped considerably by the results of RIMMINGTON, HOLIDAY AND JOPE[3] who isolated indirubin from pathological urine and who showed further, that this substance was responsible for the urorosein test[4].

The methods used by RIMMINGTON for the isolation of indirubin were, to some extent, similar to those used by us. Whilst RIMMINGTON isolated indirubin after hydrolysis with hydrochloric acid and extraction with chloroform, we allowed an acidified concentrate of leucaemic urine to stand and repeated the chloroform extraction at 5 day intervals. RIMMINGTON's final purification of chloroform extracts was effected by chro-

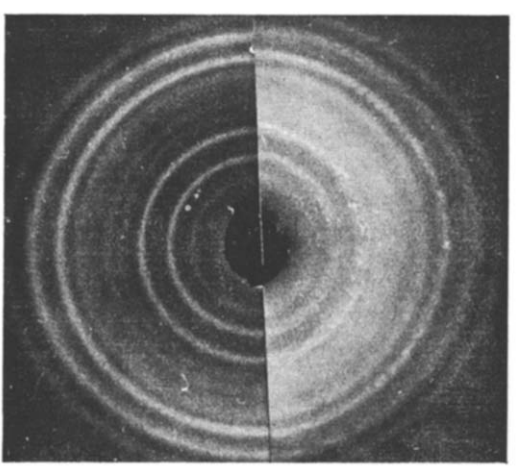
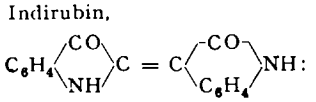


Fig. 1. Indirubin,



X-ray powder diagrams of the isolated purple pigment (left hand side) and authentic indirubin (right hand side). Camera 3 cm. radius (Cu $K\alpha$). The very small amount of crystalline material was suspended in methanol and filtered off on a plug of cotton wool in a Hysil capillary 0.02 mm wall thickness and 1 mm diameter. The powder diagram was then taken through the capillary.
It is regretted that by an error in the original communication[2] the two X-ray diagrams became interchanged.

References p. 52.

matography, whilst we used extraction with concentrated alkali, followed by dilution and ether extraction which allowed the pigment to be transferred back to the organic phase. By this procedure, material was obtained which, after recrystallization from methanol, was spectroscopically pure. The compound was identified as *indirubin* by comparison with an authentic sample by three methods: by X-ray diffraction, by spectroscopy, and by the development of a mixed chromatogram.

The results of X-ray and spectroscopic analysis of the purple pigment are shown in Figs. 1 and 2.

The colourless substance, m.p. 176–177° was next investigated. As many of the patients whose urine was collected received phenobarbitone as part of their treatment, and as the m.p. of phenobarbitone is reported as 174°, it was thought possible that the

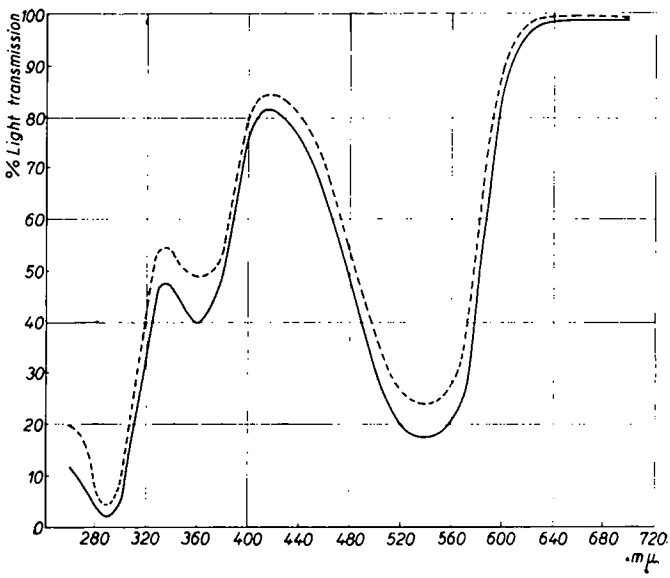


Fig. 2. Light transmission of isolated purple pigment and authentic indirubin. —·— Authentic indirubin; ---- Isolated purple pigment. Each specimen was dissolved in unknown concentration in chloroform and the percentage transmission read off against the wave length in a Beckmann Spectrophotometer. The difference in concentration accounts for the non-superimposition of the two curves.

isolated substance might be identical with phenobarbitone, but comparison of the X-ray powder diagrams of phenobarbitone and of the substance isolated from urine indicated that the two were different compounds. On one occasion, however, we were able to isolate phenobarbitone from leucaemic urine following the same isolation and identified it by mixed m.p. and by its X-ray powder pattern.

The inconclusive analytical figures so far obtained suggest that the colourless crystals may not represent a pure compound.

As the substance is not present in normal urine and there is, *at present*, no evidence that its occurrence in leucaemic urine is due to the application of some substance given as part of the treatment, the substance may be considered, at least for the time being, as an unknown constituent of leucaemic urine. The X-ray diagram given in Fig. 3 may assist in the future identification of this substance.

References p. 52.

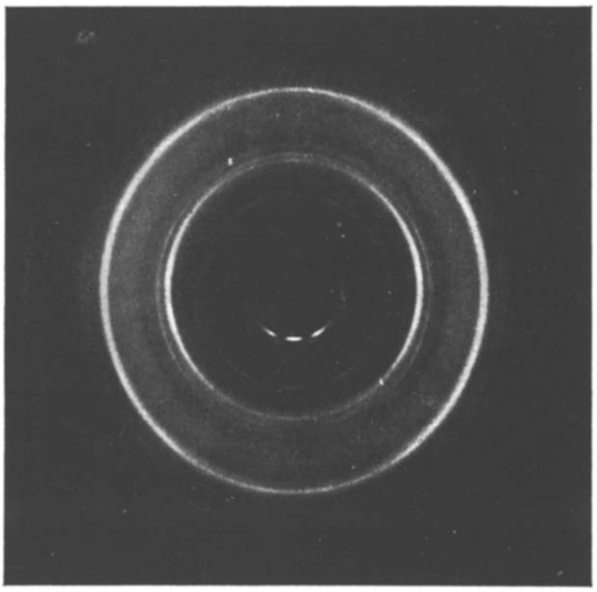


Fig. 3. *a*. X-ray diffraction picture of the substance m.p. 176 177°. Technical details as for Fig. 1

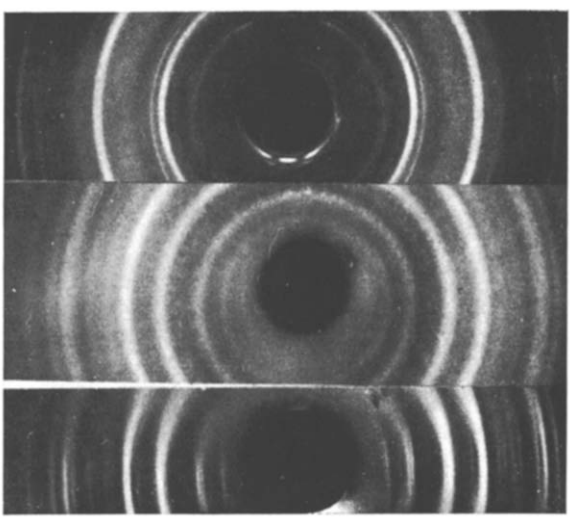


Fig. 3 *b*. X-ray diffraction pictures of the unknown substance (top) compared with authentic phenobarbitone (middle) and with phenobarbitone isolated from urine (bottom). The splitting of the lines in the bottom picture is due to the experimental conditions used and not a genuine feature of the diffraction pattern.

EXPERIMENTAL

Extraction of the leucaemic urine

1. *Extraction with 97% alcohol.* 20 litres of urine was brought nearly to dryness on the water bath, and the residue extracted on the water bath with alcohol, twice with 1 litre and twice with 500 ml. The alcohol must boil for some time before the extract is decanted. The residue was now solid and was treated with 300 ml and then 200 ml of cold alcohol and filtered by suction. The combined extracts were left overnight at room temperature when a residue settled out leaving a perfectly clear supernatant fluid which could easily be decanted. The extraction took about 1 hour.

2. *Alcoholic residue.* The alcohol was evaporated *in vacuo*. The residual syrup was heated on the water bath till all the alcohol was removed and, whilst hot, was dissolved in 100 ml water and acidified to Congo Red with syrupy H_3PO_4/H_2O (1 : 2 - - about 90 ml required).

3. *Chloroform extraction.* The solution was diluted to 800 ml and divided into 2 portions. Each portion was extracted 3 times with 100 ml chloroform and the extracts washed with water, filtered and evaporated. The residue was brown.

4. *Removal of porphyrins.* The chloroform residue was dissolved in 100 ml ether and the solution extracted 4 times with 10 ml 25% HCl. (A colloidal precipitate tends to emulsify at the interface). The HCl extract was extracted twice with 50 ml ether and the combined ethereal solutions were washed repeatedly with water. A considerable amount of white colloidal material was removed by filtration and the ether evaporated leaving a reddish residue.

5. *Isolation of the purple pigment.* The ethereal residue was dissolved in 2*N* NaOH solution (*e.g.*, for 0.8 g residue take 16 ml) giving a red alkaline turbid solution which was extracted 3 times with ether. The residual NaOH solution was treated under 6. below. The red ethereal extract was washed with water and extracted 3 times with 10 ml of Claisen KOH solution[5]. The violet extract was diluted with water, extracted twice with ether, the ether washed with water, dried over potassium carbonate and evaporated. Next day the residue was covered with a little ether and the ether quickly decanted. The *purple pigment* remained undissolved and was recrystallised from hot methanol.

6. *Treatment of the NaOH solution.* The solution was acidified with 10 ml 5*N*. H_2SO_4 giving a yellow oily precipitate. The whole was extracted 3 times with ether when a small amount of orange-red colloidal material separated at the interface. The ether extract was washed with water, dried over sodium sulphate and evaporated. The residue was a yellow varnish containing traces of benzoic acid.

7. *Bicarbonate insoluble fraction.* The residue was dissolved in 5 ml methanol and 50 ml 5% $KHCO_3$ solution added. The clear reddish solution was extracted twice with ether, the ether dried over sodium sulphate and evaporated. The residue was a yellow fat-like paste which was extracted with petrol ether. This dissolved only a part of it and caused the remainder to solidify. From the solidified mass, the *colourless crystalline substance* m.p. 176-177° was isolated (for details see later).

8. *Bicarbonate soluble acids.* The bicarbonate solution from 7 above was acidified with 5*N* H_2SO_4, extracted 3 times with ether, the ether washed, dried over sodium sulphate, evaporated and the residue investigated. In this fraction, the yellow pigment was found.

References p. 52.

The whole extraction was repeated from stage 3. (chloroform extraction) after 5, 10 and 20 days.

TABLE I

YIELDS AT DIFFERENT STAGES OF THE EXTRACTION OF 20 LITRES OF LEUCAEMIC URINE

Residue from Stage No.	0 days	+5 days	+5 days	+10 days
3. $CHCl_3$ extraction	2.5 g	1.0 g	0.8 g	0.9 g
4. after HCl extn.	0.8 g	0.2 g	0.2 g	0.3 g
6. total acids	0.6 g	0.2 g	0.2 g	0.15 g
7. Bicarb. insoluble fraction	66.8 mg	8.5 mg	19.3 mg	11.8 mg
Pet. ether soluble	41.2 mg		20.2 mg	
8. Bicarb. soluble acids	460 mg	105.5 mg	64.4 mg	70.2 mg
Pet. ether soluble	152.8 mg			
5. Neutral fraction	7.2 mg	2.5 mg	1.3 mg	Trace
Purple pigment	0.6 mg	1.2 mg	Trace	Trace

COMPARISON OF NORMAL URINE (20 LITRES) WITH THE FIGURES GIVEN FOR LEUCAEMIC URINE

Residue from Stage No.	0 days	10 days
3. $CHCl_3$ extraction	3.1 g	1.3 g
4. after HCl extraction	1.0 g	0.3 g
6. total acids	0.6 g	0.1 g
7. Bicarb. insol. fraction	85.5 mg	18.7 mg
8. Bicarb. soluble acids	393.6 mg	25.2 mg
5. Neutral fraction	90.6 mg	28.4 mg

The purple pigment

Preparation of an authentic sample of indirubin for comparison. An authentic sample was dissolved in warm benzene and put on a colum of activated alumina (Grade 0). Washing with benzene removed traces of a blue compound, while the indirubin was eluted by a mixture of benzene/chloroform (80/20). Evaporation of the solvents left indirubin as purple crystals. A mixed chromatogram of a sample of authentic indirubin and the isolated purple pigment could not be separated on such a column. The results of X-ray and spectrographic analysis have been given in Figs. 1 and 2.

Comparison of indirubin content of normal and leucaemic urines. 20 Litres of leucaemic urine (ML XI) and 20 litres of normal urine were extracted in the same way. The

References p. 52

dried ethereal fractions from stage 5 (taking the second extraction as standard) were made up to 50 ml with ether and viewed through a green filter (Chances o Gr. 1).

	%	Reading		
M.L. XI	100	20	20	20
	50	9.7	9.8	10.0
	25	5.0	4.9	5.0

	Reading			% of standard
Standard M.L. XI		20		100
Normal urine (2nd. extn)	6.5	6.6	6.6	33
M.L. XI (1st. extn.)	5.5	5.6	5.4	28
Normal urine (1st. extn)	1.8	1.8	2.0	9

Conclusions from the above measurements

1. The investigated leucaemic urine contained 3 times as much indirubin as normal urine.

2. Normal urine (2nd. extn.) : Normal urine (1st. extn.) = 3.7 : 1

3. Leucaemic urine (2nd. extn.) : Leucaemic urine (1st. extn.) = 3.6 : 1

The yellow pigment. The yellow pigment has been isolated from the benzoic acid fraction of leucaemic urine (Stage 8). Owing to lack of material, the investigation of this pigment has not advanced. We therefore refer to the description given by FRIEDMANN AND JACOBSON[2].

The colourless crystalline substance m.p. 176–177°. This substance was found in the bicarbonate insoluble fraction of leucaemic urine (Stage 7).

The slowly solidifying petrol ether extracted residue of this fraction was dissolved in 20 ml dry ether, and the slightly yellowish, clear solution was completely decolourized by boiling with charcoal. In order to remove traces of colloidal charcoal, the filtrate was treated with talc, filtered three times through the same small filter, and the filtrate concentrated to small bulk. Petrol ether was added till a slight cloudiness appeared. On standing, crystallization set in. When the solution had become clear, fresh petrol ether was added and this repeated until no further cloudiness is produced by the addition. After collecting the crystals, the mother liquors were brought to dryness, redissolved in a very little ether, and the petrol ether treatment repeated as described. The crystals so obtained were colourless, transparent needles and plates m.p. 176–177°.

Two samples from different preparations were analysed.

Sample 1. Found C, 60.4; H, 5.5; N, 11.1%. Mol. Wt. (Rast, camphor), 214.

Sample 2. Found C, 60.0; H, 5.8; N, 11.5%. Mol. Wt. 258.

$C_{13}H_{14}O_4N_2$ requires C, 59.5; H, 5.3; N, 10.7%. Mol. Wt. 262

Quantitative analysis ascertained the absence of sulphur, halogen and methoxyl groups.

The substance gives no reaction with ferric chloride or with sulphuric acid and formaldehyde; the urorosein reaction and the diazo reaction were also negative. Characteristic is the behaviour of the substance with MILLON's reagent. When boiled with this reagent it slowly develops an intense red colour which does not fade on standing for several weeks.

References p. 52.

Comparison of the isolated substance with phenobarbitone. Phenobarbitone, ($C_{12}H_{12}O_3N_2$, mol. wt. 232, m.p. 174°, C, 62.1; H, 5.2; N, 12.1%) was compared with the isolated substance, as some of the leucaemic patients received phenobarbitone gives the same colour reaction with MILLON's reagent as that described for the substance m.p. 176–177°. X-ray powder diagrams of the substance from leucaemic urine and of phenobarbitone were taken. Fig. 3 (b) shows elearly that the two powder diagrams are not identical.

Identity of the X-ray powder diagrams of two crystalline substances can be regarded as absolute proof of this chemical identity. On the other hand, if two substances give different powder diagrams, there is always the possibility that the chemical identity will be concealed by polymorphism of the crystals. Normally this can be tested by cross-seeding: if crystals grown under these conditions still give different powder diagrams the possibility of the chemical identity would be remote.

In the present case it was not possible to seed a solution of phenobarbitone with a crystal of the colourless substance obtained from leucaemic urine, as the substance had been used up for investigations of its chemical structure.

It was decided therefore, to take the next best course, and to crystallize phenobarbitone under the exact conditions used for the colourless compound. An X-ray powder diagram of this sample of phenobarbitone was different from that of the colourless substance, as is clearly shown in Fig. 3. However, in one case a substance was isolated from leucaemic urine, which had m.p. 174° undepressed on admixture with phenobarbitone. Furthermore, the X-ray powder diagram was identical with phenobarbitone (see Fig. 3 (b)).

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SUMMARY

1. A purple pigment previously isolated from leucaemic urine has been identified as indirubin.
2. A colourless crystalline substance of m.p. 176–177° previously isolated from leucaemic urine has been further investigated. X-ray powder diagrams indicated that the colourless crystalline substance m.p. 176–177°, found in the leucaemic urine, was not identical with phenobarbitone. It may be tentatively regarded as an unknown substance contained in leucaemic urine.

RÉSUMÉ

1. Le pigment pourpre, isolé des urines leucémiques a été identifié comme indirubine.
2. L'analyse à rayons X appliquée à la substance crystalline, non colorée, point de fusion 176–177°, trouvée dans l'urine leucémique, a montré que la substance n'est pas identique avec le phénobarbitone.

References p. 52.

ZUSAMMENFASSUNG

1. Das purpurfarbige Pigment, das aus leukämischem Harn isoliert worden ist, ist identisch mit Indirubin.

2. Die Röntgenanalyse zeigte, dass Pulverdiagramme der farblosen, kristallinischen Substanz, Schm.p. 176–177°, die in leukämischem Urin gefunden worden ist, mit Pulverdiagrammen von Luminal nicht identisch sind.

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