urobilinogen (Fig. 4) and both urine and faecal porphyrin excretions were high, on clinical improvement the fall in the excretion of urine and faecal porphyrin took place simultaneously. This sequence is similar to that reported before (Prunty, 1946) but is in sharp contrast to the findings of Gray, Rimington & Thomson (1948) in an unusual case of porphyria. In their case the faecal porphyrin excretion rose as the urine excretion fell, suggesting that the liver had temporarily been unable to excrete porphyrin via the biliary tract.

SUMMARY

1. The porphyrins of two cases of acute porphyria have been studied. Both uroporphyrin and coproporphyrin excretion in urine and faeces reached high values during an acute attack in the first of these cases. With clinical improvement there was a concurrent fall in these amounts and a decrease of porphobilinogen excretion in the urine.

2. Fractionation of the porphyrins present in the urine of the first case showed the presence of coproporphyrin I, a pentacarboxylic porphyrin and traces of porphyrins with six and seven carboxyl groups.

The uroporphyrin was similar to 'uroporphyrin III' of Waldenström, but proved to consist mainly of uroporphyrin I.

3. The porphyrins of the faeces in case 1, in addition to protoporphyrin IX and deuteroporphyrin IX, consisted of coproporphyrin I and a small amount of the type III isomer, a pentacarboxylic porphyrin similar to that in the urine, and traces of a porphyrin with 7 carboxyl groups. There was also isolated a uroporphyrin of similar properties to that in the urine.

4. The liver of the second case contained protoporphyrin IX, and fractions consisting of mixtures of coproporphyrin and a tricarboxylic porphyrin were obtained. A uroporphyrin with similar properties to that from the urine and faeces of the first case was isolated.

5. The importance of these findings in relation to porphyrin metabolism has been indicated.

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A Note on the Urinary Uroporphyrin in Acute Porphyria

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It is well known that the urine in acute porphyria contains a substance porphobilinogen which, on standing or heating with weak acid, is converted into a brown pigment, porphobilin. There is evidence (Waldenström & Vahlquist, 1939; Prunty, 1945) that porphobilinogen may be a precursor of porphyrin also. Accordingly, methods of pretreatment of the urine in this disease to bring about maximal formation of porphyrin have been recommended by various workers: e.g. Rimington (1943b) states that the same full porphyrin production may be obtained

by heating the weakly acidic urine on the water bath or by standing overnight with 1% (w/v) hydrochloric acid. It is, however, by no means clear to what extent such methods of pretreatment have been adopted by workers in this field or, in general, if the urine has been used fresh or after spontaneous darkening has been allowed to take place. If it could be shown that the porphyrin obtained after these treatments was identical with that present in the fresh urine, it would clearly be advantageous to apply such methods before beginning the isolation.

We are not aware, however, of any direct comparison of the porphyrins present in the fresh urine and those formed on heating.

During the course of a recent investigation of the urine from a case of acute porphyria (Gibson, Harrison & Montgomery, 1950), it was observed, as expected, that the yield of porphyrin was greatly increased by preliminary heating of the urine under weakly acidic conditions. A specimen of the uroporphyrin ester obtained after heat treatment, however, had a lower melting point than that isolated from the fresh urine, suggesting that the porphyrins might not be identical. We therefore decided to investigate further samples of urine from this patient, applying the two types of treatment mentioned above and comparing the products with the pigment preformed in the urine.

EXPERIMENTAL

Methods. Melting points were determined with a hot-stage polarizing microscope calibrated with reference substances. All melting points given in this paper are corrected. Absorption spectra were measured in a Hartridge reversion spectroscope (Beck) calibrated with a mercury arc. All solvents were dried and redistilled. Talc used for adsorption was 'acid-washed' (British Drug Houses Ltd.). Paper partition chromatography was carried out according to Nicholas & Rimington (1949), using 2:4- and 2:5-lutidine mixture and NH_3 .

RESULTS

Urine samples were collected daily and kept in the refrigerator. When 6 l. had accumulated, the samples were pooled, brought to pH 3.7 with glacial acetic acid and shaken with 120 g. talc (see Grinstein, Schwartz & Watson, 1945). The talc was filtered off and the filtrate treated with 60 g. talc. The total talc was washed with acetate buffer (pH 3.7) and the pigments eluted with $n\text{-NH}_3$ (150, 150, 30 ml.). The combined eluates were brought to pH 3.7 with conc. HCl. In order to show that all the preformed porphyrin had been removed from the urine, a further treatment with talc was carried out. The final yield of porphyrin ester from this control was only 2 $\mu\text{g./l.}$ urine.

The filtrate from the talc was divided into two parts, one half at pH 3.7 being heated to 100° and boiled for 45 min. Conc. HCl was added to the other half to give a final concentration of 1% (w/v) HCl; the mixture was allowed to stand overnight at room temperature, and then brought to pH 3.7 with NaOH. The talc treatment and elution was repeated on each half as for the fresh urine, using half quantities for each portion. The eluates from all three fractions were each extracted twice at pH 3.7 with 2 vol. 9:1 ether-light petroleum mixture (Rimington, 1943 a) to remove any coproporphyrin. Repeated extraction of the ether layers with 0.25% (w/v) HCl did not indicate the presence of any significant amount of coproporphyrin as judged by colour and fluorescence of the extracts in ultraviolet light.

The ether was allowed to evaporate from the aqueous phase of the urine filtrates, the porphyrin adsorbed from each fraction on successive small quantities of talc (total 2.5 g. talc/l. urine), and eluted with several small portions of

$n\text{-NH}_3$ (total vol. 10 ml./l. urine). The eluates were brought to pH 3.7 with conc. HCl and allowed to stand overnight. The precipitates were centrifuged off and the porphyrin in each esterified by standing overnight with 5 ml. methanol freshly saturated with HCl. The remaining insoluble residues were centrifuged off and retreated with fresh methanolic HCl. This treatment was repeated a third time. The combined acidic methanol fractions were mixed with an equal volume of CHCl_3 , shaken with an excess of ice water and centrifuged. The CHCl_3 was washed three times with water, once with dilute NH_3 and twice more with water. It was dried with anhydrous Na_2SO_4 , filtered and taken to dryness on the water bath. The residues were dissolved in a few drops of CHCl_3 , mixed with a few ml. benzene and chromatographed on chalk columns, development being carried out with CHCl_3 -benzene (1:20). The brown pigment forming the upper zone on each column was discarded and the porphyrins eluted with CHCl_3 . The latter was evaporated, the residues dissolved in benzene and rechromatographed on chalk.

The findings for the three fractions were as follows.

Fresh urine. On chalk a single porphyrin band was observed. It was eluted with chloroform and yielded 3.68 mg. ester from 6 l. urine, crystallizing from chloroform-methanol (1:3) as long matted hairs. After three further crystallizations these melted at $282\text{--}285^\circ$. In chloroform the absorption bands were centred at 625.5, 569.4 (max.), 535.1, and 501 $\text{m}\mu$. Paper chromatography of the free porphyrin made by saponification with hot 10% (w/v) sodium hydroxide gave a single spot corresponding in position with a uroporphyrin. Decarboxylation was carried out by heating to 185° in 1% (w/v) hydrochloric acid for 3 hr. The product was esterified with methanolic HCl, chromatographed from benzene-light petroleum (1:1) and developed with chloroform-benzene (1:20). The chromatogram showed one main band, which gave compact clumps of needles from methanol-chloroform (5:1), melting after two recrystallizations at $248\text{--}250^\circ$. In chloroform bands were present at 621.5, 596.2, 566.6 (max.), 534.1 and 498.5 $\text{m}\mu$. The free porphyrin gave one spot on paper chromatography, corresponding in position with a coproporphyrin. The material appears to be mainly coproporphyrin I, and this, coupled with the findings above, indicates that the main porphyrin originally present in the urine was uroporphyrin I. We did not find evidence for the presence of a heptacarboxylic porphyrin in the urine.

Boiled urine. On chromatography, three main bands were observed, one large red band and, further down the column, two smaller violet bands. The total weight of methyl ester from 3 l. urine was 12.46 mg., equivalent to seven times as much as from the fresh urine. (The increased yield on heating has been confirmed several times.) The main band was separated from the two smaller bands, and gave

short hair-like crystals melting after three recrystallizations from chloroform-methanol (1:3) at 264–265°. The ester in chloroform gave bands at 625·6, 565·6 (max.), 533·8, and 499 m μ . Paper chromatography of the free porphyrin gave two spots, one corresponding with uroporphyrin, and a weaker one with a slightly greater R_f value. After decarboxylation and esterification, three bands were obtained on a chalk chromatogram, one at the top of the column, then a narrow band, and finally a broad main band. The main band yielded needles melting after three crystallizations at 235–243°. The bands in chloroform were at 621·9, 595·9, 566·7 (max.), 533·2, and 499 m μ . The free porphyrin on paper gave two spots very close together and corresponding with coproporphyrins. Judging from the melting point the material contains much coproporphyrin I, but accompanied by some impurity. The results suggest that the porphyrin of this fraction contained uroporphyrin I mixed with some other porphyrin not separable on chalk. It appears to correspond closely with Waldenström Type B porphyrin (Watson, Schwartz & Hawkinson, 1945). The two minor violet bands from the uroporphyrin chromatogram, together containing less than 0·5 mg. ester, gave absorption bands in chloroform at 624·9, 569·8 (max.), 536·1, and 500 m μ . The material was freely soluble in hot methanol and deposited short needles on cooling. After four recrystallizations from methanol it melted at 233–235°, the final crystals being rosettes of small bent needles. Paper chromatography of the free porphyrin obtained by hydrolysis of the initial eluate of these minor bands with concentrated hydrochloric acid gave three rather indefinite spots, the main one of which appeared to correspond in position with a pentacarboxylic porphyrin and which may be similar to the porphyrin, m.p. 227–232°, described by McSwiney, Nicholas & Prunty (1949). The amount of material available was not sufficient for further characterization.

Hydrochloric acid-treated urine. This fraction gave a yield of only 75 μ g. porphyrin from 3 l. urine. It gave a typical uroporphyrin spectrum, but was not further identified.

DISCUSSION

Our results suggest that the porphyrin obtained after boiling is not a single substance, and that the main fraction is not the same as that obtained from the fresh urine. It seems possible that the changes taking place on allowing porphyria urine to stand and darken in colour might similarly be accompanied

by formation of porphyrin different from that present when it is fresh. The work confirms our previous finding that the main uroporphyrin in the urine of this patient is of type I and not of type III. The latter was generally considered to be characteristic of acute porphyria until the recent work of Watson *et al.* (1945). It may be noted that the main porphyrin ester from the boiled urine gave a melting point close to that usually ascribed to the Waldenström porphyrin ester. The result obtained on decarboxylation of this porphyrin did not, however, suggest that it contained any large proportion of uroporphyrin III, though the main product separated after decarboxylation was not pure coproporphyrin I. The melting point of the latter was lower than that obtained for the coproporphyrin derived from the fresh urine fraction, and it melted over a range rather than sharply.

It is perhaps of interest to record that we did not detect in this case the heptacarboxylic porphyrin (m.p. 208°) reported by Grinstein *et al.* (1945) in some samples of acute porphyria urine. It is possible that a trace of heptacarboxylic porphyrin was present in the boiled urine as shown by the weak spot given on paper chromatography with an R_f value slightly greater than that of uroporphyrin.

In general, our results point to the conclusion that while preliminary boiling of the weakly acid urine greatly increases the yield of the uroporphyrin fraction, this treatment has the drawback that it may give a false picture of the nature of the uroporphyrin originally present in the fresh urine. It is obviously impossible to draw any general conclusions from the study of a single patient but, in view of the rarity of porphyria, it seems desirable to record our observations in the hope that they may be supplemented by workers having access to a wider range of material.

SUMMARY

1. Uroporphyrin was isolated from the fresh urine of a case of acute porphyria and shown to be type I uroporphyrin. No other uroporphyrins were found.

2. Preliminary treatment of the urine by boiling in weakly acidic solution, until maximum development of colour, led to a seven-fold increase in the uroporphyrin fraction, but this newly formed fraction consisted of several different uroporphyrins and pure uroporphyrin I could not be isolated from it.

We wish to thank Dr D. A. D. Montgomery for obtaining the urine samples for us.

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The Site of Action of Penicillin

1. UPTAKE OF PENICILLIN ON BACTERIA

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Since the discovery of penicillin considerable data have been published about its mode of action. Among the findings which have a real bearing on the primary action of the drug on bacterial metabolism is the early observation that penicillin does not affect resting cells, but gradually inhibits the respiration of rapidly growing cells (Chain & Duthie, 1945). This would indicate that the killing effect is associated with the act of division, and for this reason studies with isolated enzyme systems are unlikely to progress far unless the enzymes studied are closely connected with cell division.

Gale & Rodwell (1949) have shown that by training a sensitive organism to dispense with certain amino-acids it becomes more resistant to penicillin without any contact with the drug. It appears that if an organism requires energy to transport preformed amino-acids from the external environment it will be sensitive to penicillin. In the case of one such amino-acid (glutamic acid, Gale & Taylor, 1947) it was found that penicillin prevented active transport across the cell wall. Active growth of the organism for several hours in the presence of penicillin is required for the prevention of amino-acid transport. This prevention may, therefore, be an end result of a series of reactions instituted by the penicillin, and is very probably still a secondary effect of the drug, although it may occur earlier than many other effects noticed. It remains to be seen whether this phenomenon is actually the cause of death of the organism.

The present studies aimed at examining the primary action of penicillin on the bacterial cell. The penicillin molecules which affect bacteria may

become attached to them. Hobby, Meyer & Chaffee (1942) were unable to show any definite removal of the drug by bacteria when using biological assay methods, and so a more sensitive method would be required to detect the minute amounts of penicillin which may be involved. The incorporation of a radioactive element in the antibiotic seemed a likely method of affording a high sensitivity, and had the added advantage that the fate of the penicillin could be followed after reaction with the organism. Radioactive ^{35}S was added to a synthetic penicillin medium, and the crude labelled penicillin extracted from the culture filtrate was utilized in our experiments. Initially some difficulties were encountered owing to the very small amount of penicillin removed by the bacteria, and to the very soft radiations of ^{35}S (Rowley, Miller, Rowlands & Lester Smith, 1948). However, by using penicillin of the highest specific activity and by directly measuring the radioactivity on thin layers of dried bacteria, consistent results have been obtained (Cooper & Rowley 1949).

EXPERIMENTAL

Preparation of radioactive penicillin

The chemically defined medium of Jarvis & Johnson (1947) for the growth of the mould has been successfully modified by decreasing the S content to about a quarter, thereby deliberately sacrificing the total yield of penicillin in favour of a higher yield calculated on the S. The radioactive S in most instances was supplied as Na_2S , which was converted to sulphate by boiling with ammonia and redistilled H_2O_2 . After inoculation with a loopful of vegetative mould suspension, the medium was shaken for about 4 days at 24° and