A SIMPLE METHOD OF ISOLATION OF CRYSTALLINE STERCOBILIN OR UROBILIN FROM FECES*

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The methods described two decades ago (1–3) for the isolation of crystalline stercobilin or urobilin from feces or urine are relatively slow and cumbersome. It is often desirable to obtain a few mg of crystalline material quickly, either to permit determination of the preponderant type (whether stercobilin, urobilin, or *d*-urobilin) or calibration of a quantitative method. More recently, the studies of Shemin and associates (4–8), based on the essential nature of glycine and acetate in the biosynthesis of the heme pigments, have made it highly desirable to have at hand a simple method permitting rapid and repeated isolation of stercobilin from feces in order that serial observations of N^{15} content may be made, following administration of N^{15}-glycine. Of special interest in this connection is the question of exact significance of the early appearance of N^{15} in the stercobilin molecule, a finding indicating that a certain fraction, varying under normal and pathological conditions, has a derivation other than the hemoglobin of red blood cells which have survived a normal life span (6, 7). Since determinations of the atom per cent excess of N^{15} in the mass spectrometer require but 10 mg. of pure substance, the method to be described in the following is particularly adapted to studies of this type. This method depends in considerable part on the dehydrogenation of stercobilinogen or urobilinogen with iodine, in petroleum ether solutions, as described in the preceding paper (9). It may be noted that Lichtenstein and Terwen, in 1925 (10), used petroleum ether to extract a urobilinogen from an aqueous filtrate of a feces-ferrous hydroxide mixture. The petroleum ether was then allowed to stand in the light, with resultant formation and precipitation of an amorphous urobilin. The latter was not crystallized, but was evidently relatively pure. It probably consisted in the main of the levorotatory stercobilin. We have, in fact, been able to isolate crystalline sterco-

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bilin from material prepared in this way, but the yield is relatively poor and the method very slow.

Method

The initial treatment of the feces is entirely similar to that used in the quantitative determination of urobilinogen (11), except that larger amounts are employed. 50 to 100 gm. of feces are thoroughly ground in a mortar with small amounts of distilled water. The mixture is further diluted with water to a volume of 200 to 400 cc., in proportion to the original amount of feces. All of this is poured into a 1 liter Erlenmeyer flask. From 100 to 200 cc. of freshly prepared 20 per cent ferrous sulfate ($FeSO_4 \cdot 8H_2O$) solution are then added and well mixed. The amount of ferrous sulfate solution used is in the proportion of 50 cc. for each 25 gm. of feces. The same amount of 10 per cent NaOH is then added slowly with constant swirling, the resultant ferrous hydroxide becoming entirely homogenized with the fecal emulsion. The flask is corked and set aside for an hour or longer. The reduction of any native urobilin to urobilinogen (using these terms in a group sense) may be regarded as complete if the filtrate is light in color and exhibits little or no urobilin type of absorption (maximum at 508 mμ) as viewed in a test-tube with a hand spectroscope. As in the ordinary quantitative procedure (11), reduction is usually complete or nearly so at the end of 1 hour, but in some instances, especially when there are high concentrations, longer periods are required and at times a complete reduction is not achieved. In so far as the present isolation is concerned this is of small consequence. The Ehrlich aldehyde intensity of the filtrate is then determined, the value being expressed in Ehrlich units[1] per 100 cc. (12). The yield of crystalline urobilin which may be anticipated amounts to about 10 to 18 per cent of the Ehrlich units in the filtrate. Depending on the approximate amount desired, a volume of filtrate is chosen and placed in a separatory funnel of about 10 times this volume. 4 volumes of petroleum ether (b.p. 30 to 60°) are added and the filtrate is weakly acidified by addition of buffered glacial acetic acid (4 parts of glacial acetic to 1 part of saturated aqueous sodium acetate solution). The two phases are then shaken together briskly and allowed to separate. The aqueous phase is separated and extracted twice more with 1 volume of petroleum ether. The combined petroleum ether is filtered and a quantitative Ehrlich determination is carried out on a 1 cc. portion, this being simply diluted with additional petroleum ether and treated exactly as the petroleum ether stage in the regular quantitative method (11). The entire petroleum ether solution in a large separatory funnel is now subjected to the iodine dehydrogenation-water extraction described in the preceding paper (9). The same ratio of iodine to urobilinogen, *i.e.*, 0.45:1.0 is used.

[1] 1 Ehrlich unit = the color equivalent of 1 mg. of urobilinogen aldehyde per 100 cc.

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The aqueous solution of urobilin is washed twice with ethyl ether to remove lipide impurities, which otherwise may contaminate the crystalline material to be obtained. This, furthermore, has the advantage of removing mesobiliviolin and mesobilirhodin. The further method of handling the urobilin solutions and of crystallization is the same as for the optically inactive urobilin. In the case of *d*-urobilin, however, the solubility in $CHCl_3$ is relatively small, and the material often begins to crystallize from a large volume of $CHCl_3$ while still hot. If the presence of *d*-urobilin is suspected because the individual providing the feces has received aureomycin or terramycin in recent months, it is wise to note whether crystallization from the $CHCl_3$ occurs before wide concentration. If not, it may be concentrated to a small volume and mixed with hot acetone, as described in the accompanying paper (9). Stercobilin and urobilin both crystallize much more satisfactorily from acetone than does *d*-urobilin. It has recently been found that a highly satisfactory and rapid method of recrystallization is from methyl alcohol-ethyl acetate. This is equally useful for urobilin and *d*-urobilin. The material is simply dissolved in a few cc. of methyl alcohol. This is concentrated further in a boiling water bath and a small amount of hot ethyl acetate is added. After further concentration under reduced pressure, the solution is allowed to cool and crystallization commences. The stercobilin hydrochloride crystals thus obtained are in the form of rectangular leaves or plates; m.p. 157–160°. The free substance prepared in the usual way (1) and after repeated recrystallization from acetone melted at 234–236°. Optical activity of the hydrochloride[2] in $CHCl_3$, $[\alpha]_D^{20} = -4000°$. The absorption maximum in dioxane or methyl alcohol (containing a little HCl) is at 4925 ± 2 A. The physical characteristics of the *d*-urobilin obtained by this method will be described in detail in a separate communication. Those of the optically inactive urobilin have already been referred to in the accompanying paper.

For larger amounts of feces ranging from 100 to 1000 gm., the following preliminary treatment has the advantage of keeping the volumes of fluid relatively small. The feces are ground in a mortar with 95 per cent alcohol. After standing a short time, this is decanted and filtered either on a sintered glass funnel or, preferably, through a small amount of infusorial earth[3] on a Büchner funnel with suction. Repeated extraction is carried out with additional amounts of alcohol, until the Ehrlich reaction is weak or negative. The combined alcoholic filtrate is run through a column of Al_2O_3,[4] the urobilin and urobilinogen largely remaining on the column and carotenoids and lipides passing through. The column is then eluted with

[2] Recrystallized repeatedly from acetone and dried to constant weight over P_2O_5.

[3] Hyflo, Johns-Manville.

[4] Baker and Adamson. (Merck's for chromatographic analysis is too retentive for this purpose.)

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distilled water, which is collected in an arbitrary series of fractions, each of which is tested with Ehrlich's reagent. Those fractions exhibiting significant reactions are pooled and filtered into a large separatory funnel. From this point on, the further treatment is exactly as described above for the aqueous filtrate obtained by the FeOH method. The water is weakly acidified with buffered glacial acetic acid and extracted with petroleum ether. The chromogen is dehydrogenated by iodine, and the resulting urobilin or stercobilin is then obtained in crystalline form.

The alcohol-Al_2O_3-water method as just described has been used to good advantage in a series of isolations of N^{15}-*d*-urobilin from a patient with hemolytic jaundice given terramycin and N^{15}-glycine. The results of this study will be described in detail elsewhere. The yields of crystalline *d*-urobilin which were obtained have ranged from 8.5 to 64 per cent (average 38.1) of the initial concentration of urobilinogen in Ehrlich units, both in this and other cases in which it was present. The wide range of yields is probably due at least in part to variable proportions of *d*-urobilin at different periods. As already noted, this substance is less soluble and crystallizes more completely from $CHCl_3$. It is also true, however, that some of the variation in yield is related simply to the thoroughness of the initial alcohol extraction. In general, the larger the amount of urobilinogen in the feces, the lower the percentage yield of crystalline material.

It is quite likely that in many instances, at least, the first crop of crystalline material obtained by either of the above methods consists of a mixture of the preponderant pigment of the sample at hand, with a smaller amount of one or two of the others. Thus, on occasion, we have noted significant changes in optical activity on recrystallization, indicating that a minor fraction of the first crop was either optically inactive or of opposite type. We have not encountered any instance thus far in which there was not sufficient preponderance to permit easy separation.

The method has also been applied very satisfactorily to urine. The chromogen is extracted by petroleum ether at pH 4.0, after which the preponderant urobilin or stercobilin, when excreted in adequate amount, is obtained by the same means as for feces.

SUMMARY

1. A simple method of isolation of stercobilin or urobilin from feces is described. This is especially advantageous for serial studies relating to N^{15} content after administration of N^{15} glycine.

2. The method embodies the principle of dehydrogenation of urobilinogen with iodine in petroleum ether solution, followed by extraction of the resulting stercobilin or urobilin with water, crystallization from chloroform-acetone, or, in the case of *d*-urobilin, from chloroform and recrystallization of either from methyl alcohol-ethyl acetate.

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3. Alternative preliminary treatments leading to the petroleum ether stage are described, one being identical with the method of quantitative determination of stercobilinogen or urobilinogen, the other depending on alcohol extraction, adsorption on alumina, and elution with water. The former permits simultaneous quantitative determination and is best suited for smaller amounts of feces, up to 100 gm. The latter can be employed with smaller or larger amounts.

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