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II. Isolation of a Dextrorotatory Urobilin from Human Fistula Bile.*

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The formation of urobilinogen or urobilin in infected bile has been noted repeatedly in the past.[1, 2] MacMunn[3] observed that the urobilin in the bile of humans, mice, cattle, hogs and sheep differed slightly in spectroscopic characteristics from urinary urobilin. There has been no subsequent investigation of this possible difference.

We have repeatedly observed that fistula bile becomes much lighter in color on standing at room temperature or in the incubator. Coincident with this lightening, the Ehrlich reaction for urobilinogen becomes much more intense, in some instances having been negative at the outset. These changes are associated with an obvious and marked increase in bacterial flora and with the appearance of a fecal odor. The urobilinogen which forms during this period is readily oxidized to a urobilin, which is strongly dextrorotatory, quite unlike stercobilin (laevorotatory), or urobilin IX,a (optically inactive). This dextrorotatory urobilin has been identified in 11 of 12 bile samples from 9 different patients. Since some of these samples exhibited a strong Ehrlich reaction as they left the fistula tube, it is likely that the formation of the dextrorotatory urobilinogen was proceeding in the infected biliary tract. The possibility exists, however, that the chromogen first excreted was mesobilirubinogen, since as will be seen in the next paper of this series, mesobilirubinogen is converted to d-urobilin in some samples of bile. The substance has been isolated in crystalline form from four of these samples. The method of isolation may be given briefly as follows: The bile is weakly acidified with 10% HCl, then extracted 5 times with $CHCl_3$. The latter is filtered through a $CHCl_3$ moistened paper and is then passed through a Tswett column of Al_2O_3† 8 x 2 cm. The urobilinogen and urobilin are adsorbed near the top of the column while

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1 Meyer-Betz, F., *Ergeb. d. inn. Med. u. Kinderheilk.*, 1913, **12**, 733.

2 McMaster, P. D., and Elman, R., *Ann. Int. Med.*, 1927, **1**, 88.

3 MacMunn, C. A., *Proc. Roy. Soc.*, 1881, **31**, 206.

† According to Brockmann; Merck & Company.

bilirubin wanders down slowly. The column is next washed with ethyl alcohol (30-50 cc) to remove $CHCl_3$. The urobilinogen and urobilin are then eluted completely with a 20% aqueous solution of acetic acid.‡ The acetic acid eluate is allowed to stand over night to permit oxidation of urobilinogen to urobilin. The further treatment is as follows: The eluate is washed repeatedly with ether, acidified further by adding a 1/5 volume of concentrated HCl, following which the urobilin hydrochloride is extracted with $CHCl_3$. The latter is filtered through $CHCl_3$ moistened paper and concentrated to dryness on the boiling water bath. Acetone is added to the hot residue with resultant immediate crystallization. The intense green fluorescence with alcoholic zinc acetate is the same as for stercobilin and urobilin IX,a. The absorption band in methyl alcohol containing a little HCl is identical with that of urobilin IX,a; *i. e.*, at a maximum of 4945 Å, while the stercobilin maximum is at 4920 (see Paper I). This undoubtedly explains the difference noted by MacMunn, since stercobilin predominates in feces and in many pathological urines. The specific rotatory activity is in the neighborhood of $[\alpha]_{20°}^{589\,m\mu} = +4000$. As yet the yields of this substance have not exceeded 1-2 mg, so that this value cannot be regarded as more than approximate. Stercobilin has been shown to be dextrorotatory in concentrated sulphuric acid and in formic acid.[4] We cannot relate these observations to the present d-urobilin, however, since in the case of sulphuric acid the laevorotatory activity is restored when the substance is taken into $CHCl_3$, and in the case of formic acid the dextrorotatory activity is undoubtedly due to salt formation,[4] while we have ascertained that the free d-urobilin of itself exhibits strong dextrorotation. The melting point of the hydrochloride is approximately that of stercobilin; *i. e.*, 142-144°C (from acetone), 128-131°C (from $CHCl_3$).§ The melting point of the free substance has not yet been ascertained.

A specific color reaction with dioxan-HCl has been noted and is receiving further study. When a solution of d-urobilin in dioxan + 1-2 drops of 10% HCl is heated in a boiling water bath for 2-5 minutes, the color changes from orange, to violet, to blue, to green. Stercobilin is unaffected by the same treatment; urobilin IX,a becomes violet after 5-10 minutes and soon becomes colorless. Bili-

‡ Bilirubin is not eluted by 20% acetic, but is removed subsequently by $CHCl_3$ containing 1% glacial acetic, from which mixture it crystallizes readily. This method of isolation of bilirubin from bile will be described in detail in a separate communication.

4 Fischer, H., and Libowitzky, H., *Z. physiol. Chem.*, 1939, **258**, 255.

§ These melting points were determined with a Fisher-Johns micro-apparatus.

rubin exhibits a transitory green color with this method.

It is believed best to designate this new substance tentatively as d-urobilin, at least until further evidence regarding its structure is obtained. This designation does not necessarily imply that d-urobilin is a stereo-isomer of stercobilin, although such a relationship is suggested by the identical melting points of the hydrochlorides of the two substances. Further studies including elementary analyses are in progress.

Summary and Conclusions. 1. A dextrorotatory urobilin has been identified in 11 of 12 infected fistula bile samples examined. 2. The initial urobilinogen content of infected bile samples is considerably increased and the bile becomes lighter in association with overgrowth of bacteria productive of a fecal odor. The crystalline urobilin isolated from four of such samples was dextro- rather than laevorotatory (as in the case of stercobilin isolated from the feces). 3. A specific color reaction and other characteristics of d-urobilin are described.

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III. Formation of D-Urobilin from Mesobilirubinogen in Human Bile.*

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The presence of a dextrorotatory urobilin in infected bile samples, as described in the preceding paper of this series, induced us to study the question of derivation of this substance. Since the simple reduction of bilirubin, *in vitro,* leads only to mesobilirubinogen, the first possibility requiring investigation was that d-urobilin was derived from mesobilirubinogen through some chemical activity peculiar to the bile. The following experiments were carried out with a view to answering this question.

1. *Addition of Mesobilirubinogen to Urobilin-free Bile.* Fifty mg of crystalline mesobilirubinogen were dissolved in 1 cc 95% ethyl alcohol and 5 cc of 0.025% NaOH. This mixture was added

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