A Study of the Reported Occurrence of Mesobilirubin in Human Bile.* (28161)

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Mesobilirubin, which is readily prepared by the catalytic hydrogenation of bilirubin(1), is presumed to be intermediary in the conversion of bilirubin to urobilinogen, *in vivo*. It has been demonstrated in small amount in the feces of patients receiving tetracyclins(2). Baumgärtel(3,4) and Allmendinger(5) reported its presence in gall bladder bile from pigs, dogs, and humans (post mortem). They based this statement on the results of $FeCl_3$ oxidation of chloroform extracts of bile. It was assumed that the blue color often noted as a result of this reaction was due to glaucobilin (mesobiliverdin) formed by dehydrogenation of mesobilirubin. Baumgärtel believed that this was effected in the gall bladder by the action of hepatic dehydrogenases. There are objections to the assumption of specificity of the $FeCl_3$ reaction, as discussed below.

The present study was undertaken to examine in more detail the question of the occurrence of mesobilirubin in human bile.

Material and method. Bile samples were obtained from t-tube drainage of 6 post-cholecystectomy patients, from one gall bladder at necropsy, from a patient with external biliary fistula, and from 2 gall bladders removed surgically. The ante mortem urine was studied in a fatal case of acute hepatic necrosis but at necropsy, bile could not be obtained as the patient had no gall bladder.

In preliminary studies, $FeCl_3$ oxidation as employed in this laboratory(6) was carried out on several chloroform extracts of bile which had been adjusted to pH 4.0-5.0 (acetic) prior to extraction. The resulting solutions were examined with the DK Beckman spectrophotometer. $FeCl_3$ oxidation and spectral analysis was also carried out on mixtures of crystalline bilirubin† and i-urobilin (H_{42}) and on bilirubin alone.

All of the bile samples were studied by a chromatographic method devised to permit separation of bilirubin and mesobilirubin. This depends on the greater solubility of mesobilirubin in petroleum ether.

Bile samples were divided into 2 equal portions. To one, crystalline mesobilirubin, prepared from bilirubin by catalytic hydrogenation(1,7), was added to provide a final concentration of 0.5 mg %. The two portions were then subjected to alkaline hydrolysis with 10 volumes of 2.5% NaOH for 30 minutes at room temperature. The pH was adjusted to 4-5 with glacial acetic acid. The solution was then filtered into a separatory funnel and extracted with 2 volumes of chloroform. The chloroform extract was washed twice with water, then concentrated in a Lyovac apparatus at room temperature to about 4 ml. This concentrate was spotted on Whatman #1 paper and ascending chromatography was carried out for 2 hours at room temperature, in a system of petroleum ether:chloroform, 90:10, in a vertical tank saturated in advance with the solvent system.

The paper was then dried and developed with saturated alcoholic zinc acetate followed by 0.1% iodine in 95% alcohol. The resulting oxidation of mesobilirubin gives rise to intense red fluorescence in ultraviolet light (8).

In this system mesobilirubin had an R_f of .6-.7 and bilirubin .2-.3. Control runs with pure compounds were carried out with each run of extracts of bile.

The remaining concentrate, amounting to about 3.5 ml, was mixed with 50 ml of petroleum ether which precipitated most of the bilirubin. The precipitate was collected on a filter paper and washed with petroleum ether to recover any precipitated mesobilirubin. This was combined with the filtrate and applied to a packed powdered (cane) sugar column first saturated with petroleum ether. The column was developed

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† Eastman Kodak Co.

with petroleum ether, then with a petroleum ether:chloroform mixture with increasing concentration of chloroform until the lower yellow band (mesobilirubin) was well down the column. The column was dried and the lower yellow band cut out, crumbled into a Buchner funnel, and eluted with chloroform until the eluate ran clear. The eluate was washed with water, then concentrated under reduced pressure to about 2 ml. Five-tenths of this concentrate was used for the diazo reaction(9). The remainder was combined with an equal volume of saturated alcoholic zinc acetate plus one drop of 10% alcoholic iodine. The oxidation products of mesobilirubin under these conditions (mainly glaucobilin = dehydromesobilirubin; smaller amounts of mesobilipurpurin) exhibit spectral absorption at 625-628 mμ(8). Measurement of absorption maxima was carried out with a Zeiss grating spectrometer. Under the same conditions, bilirubin yields an oxidation product (mainly biliverdin) whose zinc complex has an absorption band at 635-640 mμ (7).

Results. Most of the bile extracts gave blue colors upon $FeCl_3$ oxidation. These blue solutions had absorption maxima at 560 mμ and 660 mμ, corresponding respectively with mesobiliviolin-mesobilirhodin and biliverdin. Maximal absorption at 650 mμ characteristic of a preponderance of glaucobilin was not seen. Bilirubin alone, on $FeCl_3$ oxidation, gave a green solution with a single peak at 660 mμ. Bilirubin-i-urobilin mixtures, on $FeCl_3$ oxidation, exhibited variable color from blue-green to purple with increasing concentrations of i-urobilin. Spectral distribution curves of these solutions also showed maxima at 560 mμ and 660 mμ, while with the reaction products of mixtures of i-urobilin and mesobilirubin the maximum absorption in the red region was at 650 mμ.

By means of the chromatographic and spectroscopic methods described, mesobilirubin was easily separated from bilirubin, and was consistently detected in the samples to which it had been added in a concentration as low as 0.5 mg %.

Native mesobilirubin could not be detected in any of the bile samples. In the one urine sample studied (by the same method) mesobilirubin was definitely detected in the presence of a much higher concentration of bilirubin.

Discussion. Baumgärtel(3,4) believes that mesobilirubin is formed by the action of hepatic dehydrogenases, particularly during bile stasis in the gall bladder. This belief is central to his theory of the hepatogenous origin of i-urobilinogen (mesobilirubinogen)(4). As mentioned earlier, he bases the belief on the finding that $FeCl_3$ oxidation of chloroform extracts of bile often yields blue solutions, the blue color attributed to glaucobilin, which can "only be formed from the oxidation of mesobilirubin"(4).

Previous evidence as well as the present observations are not in accord with this belief. Bile is known to contain various mixtures of the urobilin group(6), in addition to bilirubin. d-Urobilin has been shown to yield predominantly glaucobilin with $FeCl_3$ oxidation, and i-urobilin has been shown to give variable mixtures of mesobiliviolin-mesobilirhodin and glaucobilin with $FeCl_3$ oxidation(10). Thus, it is evident that a blue color could be due to a mixture of pigments or, if it were due to glaucobilin alone, this glaucobilin could well be derived from d- or i-urobilin(ogen) rather than mesobilirubin.

In an earlier study reported from this laboratory(6), $FeCl_3$ oxidation of the urobilin group from urine and feces of 68 patients resulted in blue, aqua or blue purple colors in 25, under conditions which preclude the possibility that mesobilirubin was responsible for the color. In 18 of these cases, the color could be accounted for by a preponderance of d-urobilin; the others contained largely i-urobilin.

From these observations and the results of the present study, it is clear that a blue color after $FeCl_3$ oxidation is not specific for glaucobilin, and that the presence of glaucobilin after $FeCl_3$ oxidation is not specific for mesobilirubin. Identification of mesobilirubin in bile cannot depend upon the results of $FeCl_3$ oxidation; thus, the conclusion of Baumgärtel and Allmendinger is not confirmed.

By means of a chromatographic method permitting detection of mesobilirubin at the

0.5 mg % level, in bile, it has not been possible to demonstrate the presence of mesobilirubin in fistula, t-tube, or gall bladder bile from non-icteric humans. However, it was identified in the urine of a patient with severe hepatic failure and no gall bladder, thus indicating that a gall bladder is not essential to its production. In this situation, however, its occurrence is believed due to intestinal formation and enterohepatic circulation. Thus it is possible that mesobilirubin derived in this manner might on occasion be re-excreted in the bile, but none was observed in the present study.

Summary and conclusions. 1. Paper and column chromatographic methods of separating bilirubin and mesobilirubin are described. 2. Use of these methods permits detection of mesobilirubin at the 0.5 mg % level in the presence of much larger amounts of bilirubin. 3. The spectroscopic distinction of biliverdin and mesobiliverdin (glaucobilin), and of their respective zinc complexes, is discussed. 4. Glaucobilin is readily formed from d- or i-urobilin, in addition to mesobilirubin; glaucobilin formation on $FeCl_3$ oxidation is thus not specific for mesobilirubin as suggested by others. 5. Mesobilirubin has not been detected in human bile but was definitely identified in a urine sample containing larger amounts of bilirubin. Its occurrence in the urine is believed to relate to an enterohepatic circulation.

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Depression of Plasma Albumin by Steroid Therapy.* (28162)

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Albumin has been reported to bind estrogens avidly(1). This protein is responsible for 90 or more per cent of the orientation in human plasma of estradiol and estrone sulfate (2). It has been shown that Premarin (mixed, conjugated equine estrogens) increases survival of men with myocardial infarction(3). From these observations one might infer that a deficiency of certain estrogenic substances or of albumin exists in these patients. Since plasma albumin concentration is significantly depressed in the aging, arteriosclerotic subject(4), it was of interest to attempt to learn if the metabolism of the albumin molecule is altered by an increased plasma level of certain steroids.

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Methods and materials. Human albumin standard. A preparation of human albumin was donated by Cutter Laboratories, Berkeley, Calif. The preparation was globulin-free as determined by paper electrophoresis.

Albumin assay–electrophoretic. Separation of plasma albumin was made by the Durrum paper electrophoresis cell. The albumin band