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

* Aided by a grant from the John and Mary R. Markle Foundation, New York City.

to 10 cc of urobilin and urobilinogen-free fistula bile, which did not have a fecal odor. After thorough mixing, the bile and mesobilirubinogen in a small glass container were placed in the upper portion of an ordinary vacuum jar. The glass container rested on a wire gauze above the lower compartment which was partially filled with water. As a control, 10 cc of the same bile without added mesobilirubinogen but with 1 cc of 95% alcohol and 5 cc of 0.025% NaOH were similarly placed in the jar. The jar was then evacuated to a point where the bile commenced to rise, after which it was placed in an incubator at 37° C. Five days later the samples were removed and subjected to the following treatment: 100 cc of 7% HCl were added to each bile sample after which it was allowed to stand over night at room temperature. The Ehrlich reaction, which had been intense, was now weak. The diluted HCl-bile was now filtered and extracted repeatedly with $CHCl_3$. The latter was filtered through $CHCl_3$-treated paper to remove moisture after which it was concentrated to dryness on the boiling water bath. Acetone was at once added and crystallization then occurred in each instance. In the first, to which mesobilirubinogen had been added, the crystals were those of a urobilin together with a small amount of crystalline bilirubin. A solution of these crystals in 60 cc of $CHCl_3$ exhibited +0.13° (corr.†) optical activity with sodium light. The limit of error with the Schmidt-Haensch polarimeter used was ± 0.015 . The solution exhibited strong urobilin absorption and green fluorescence with alcoholic zinc acetate. The crystals obtained from the control, on the contrary, appeared to be solely bilirubin, exhibiting neither optical activity, urobilin absorption, nor green fluorescence with zinc.

2. *Addition of Mesobilirubinogen to Urobilin Containing Bile.* In a similar experiment 58 mg of mesobilirubinogen were added to 10 cc of infected bile with fecal odor, containing 5.5 mg of native urobilinogen. This was placed in a partly evacuated jar in the incubator, as described above. 150 cc of the same bile were placed in the refrigerator, to be used as a control. The latter was studied further after 3 days and the 10 cc incubated sample after 12 days. Both were subjected to the chromatographic method described in Paper II. The urobilin crystals obtained from each sample were dissolved in 15 cc of $CHCl_3$. The solution from the control (150 cc) sample exhibited +0.30° rotation, while that from the sample (10 cc) to which mesobilirubinogen had been added had +0.21°

† The actual readings with this apparatus are 3 times greater than the corrected values for degrees of optical activity.

rotation. If no mesobilirubinogen had been added, the expected rotation on the basis of the control would have been only +0.02°. The objection might be raised that the tenfold increase which was observed was due to conversion of bilirubin to d-urobilin. It is believed, however, that the amount of bilirubin in the 10 cc sample was much too small to be of significance. To account for the difference the sample would have had to contain 55 mg of bilirubin, and all of this would have had to be reduced, which was obviously not the case since crystalline bilirubin was obtained following chromatographic analysis.

3. *The Effect of Acholic Feces on Urobilin-free Bile and on Mesobilirubinogen.* (a) Control sample. A 10 g sample of feces containing 1.4 mg urobilinogen by quantitative method.[1] While not absolutely acholic, this represents a high degree of exclusion of bile from the intestinal tract. This was incubated in an evacuated jar for 6 days, as described above. Ground and extracted with 7% HCl. Filtered. Filtrate extracted with $CHCl_3$. Filtered through $CHCl_3$-moistened paper and concentrated to dryness. Acetone added. Amount too small to crystallize. Optically inactive.

(b) 10 g of same feces as in (a) + 46 mg crystalline mesobilirubinogen. Thoroughly mixed. Incubated and further treated as in (a). Crystalline urobilin IX,a isolated. Optically inactive.

(c) 10 g of same feces as in (a) + 50 mg mesobilirubinogen + 5 cc urobilin-free bile. Mixed, incubated, and further treated as in (a). Crystalline d-urobilin obtained. Solution of crystals in 15 cc $CHCl_3$ exhibited (+) 0.07° rotation (corr.).

(d) 20 cc urobilin-free bile + 1 g of the same feces as in (a). Mixed, incubated, and further treated as in (a). Crystalline d-urobilin obtained. Crystals in 20 cc $CHCl_3$ exhibited optical activity of (+) 0.13° (corr.).

4. *The Effect of Bile on Stercobilin.* The question arose as to whether stercobilin or stercobilinogen was converted in the bile to d-urobilin. This has not yet been answered with certainty, but the following observations indicate that it is not.

(a) Crystalline stercobilin was isolated‡ on one occasion from a sample of bile from a patient with incomplete bile fistula (T-tube without suction). The method used was essentially the same as the chromatographic procedure described in Paper II of this series. The observed laevorotation of the crystals in $CHCl_3$ was (−)0.10° (corr.).

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[1] Watson, C. J., *Am. J. Clin. Path.*, 1936, **6**, 458.

‡ We are indebted to Dr. Edmund Flink for this isolation.

(b) 50 mg of stercobilin hydrochloride were injected intravenously[2] in a patient having a complete (suction) external biliary fistula.[3] The bile obtained just before the injection gave negative Ehrlich and Schlesinger tests for urobilinogen and urobilin respectively. The sample collected during the first hour after injection was strongly positive for both substances. During the ensuing 11 hours the reactions became gradually weaker and were negative thereafter. By employing the chromatographic procedure described in the preceding paper, it was possible to obtain crystalline stercobilin from the combined bile samples for the 12-hour period after the injection. In $CHCl_3$ an optical activity of $(-)0.045^\circ$ was observed.

Discussion. The present results indicate that mesobilirubinogen is converted to d-urobilin in bile whether the latter is initially urobilin-free or urobilin-containing. In the preceding paper it was noted that the formation of urobilinogen in bile standing at room temperature or in the incubator is associated with the growth of bacteria productive of a fecal odor. This type of activity is evidently necessary for the reduction of bilirubin to a urobilinogen (either mesobilirubinogen or d-urobilinogen). It is just as evident, however, that if mesobilirubinogen is added to bile it may be converted to d-urobilin without this type of bacterial activity. Studies are now in progress to determine, if possible, the relation of bacteria to this problem, and also to the problem of stercobilin formation from mesobilirubinogen, as discussed in Paper IV of this group.

It is important to note that in the present study and in additional experiments that will be described subsequently, acholic feces alone did not convert mesobilirubinogen either to stercobilin or d-urobilin. Acholic feces + bile in 2 instances brought about conversion of mesobilirubinogen to d-urobilin. This was significant inasmuch as the control sample on incubation failed to develop urobilinogen or urobilin. As will be noted in Paper IV, the conversion is not uniformly in this direction and it appears that the direction with any given mixture of feces and bile depends upon a combination of factors as yet unknown. It will be shown, however, that with normal feces, or in the normal intestinal tract, mesobilirubinogen is converted to stercobilin rather than d-urobilin.

Summary and Conclusions. 1. Mesobilirubinogen added to both urobilin-free and urobilin-containing bile was converted at least in part to d-urobilin. No evidence of stercobilin formation was ob-

[2] Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 377.

[3] Layne, J. A., and Bergh, G. S., *Surgery*, 1941, **10**, 563.

tained. 2. Mesobilirubinogen added to bile and acholic feces was converted at least in part to d-urobilin. In one instance the addition of acholic feces to urobilin-free bile was instrumental in the development of d-urobilin.

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IV. Formation of (Laevorotatory) Stercobilin from Mesobilirubinogen in Human Feces.*

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In Paper I of the present series it was shown that stercobilin could not be obtained by the amalgam reduction of native bilirubin in various bile samples. This led only to mesobilirubinogen and, on oxidation, urobilin IX,a. Nor was stercobilin obtained by incubating mesobilirubinogen with acholic feces, or with bile, as described in Paper III. In these experiments, the hitherto undescribed d-urobilin was formed.

In the present study the following experiments were carried out in order to determine whether mesobilirubinogen is converted to stercobilinogen in the intestinal tract, or when incubated *in vitro* with normal feces.

1. 210 mg of crystalline mesobilirubinogen were dissolved in 50 cc of dilute Na_2CO_3 (0.2%) to which was added 0.33 g bile salts (Bilien, Abbott). The solution was diluted further to 100 cc with physiological saline. This was administered over a period of one-half hour through a Miller-Abbott tube, into the duodenum of a patient having a T-tube in the common bile duct, and with a continuous suction fistula.[1] The urobilinogen excretion in the feces was determined for two-day periods before and after the administration of mesobilirubinogen as shown in Table I. The feces for the various periods indicated by the brackets on the left in Table I were subjected to the chromatographic isolation method as described in Paper II of this series, with the one exception that the primary extraction and adsorption was carried out with 95% ethyl alcohol rather than with $CHCl_3$. The relative advantages of various isola-

* Aided by a grant from Mr. A. C. Randall, Minneapolis.

1 Layne, J. A., and Bergh, G. S., *Surgery*, 1941, **10**, 563.