human blood cells belonging to group O were added. The tubes were kept again for 15 minutes at room temperature and centrifuged.

Table III shows that the agglutination of human group O cells by anti-O beef sera is inhibited by the gastric juice and saliva of patient Durk., proving the presence of O substance in both. Patient Dun. apparently did not secrete the O substance in the gastric juice, since the agglutination of O cells is inhibited only slightly by the gastric juice of this patient. The examination of his saliva, also, showed a complete absence of O specific substance, indicating that this patient belongs to the group of non-secretors.

Conclusion. The gastric juice of patients suffering from pernicious anemia contained blood group specific substances at least in the same large amounts as normal gastric juice in spite of the fact that there was no free hydrochloric acid or pepsin present in the specimens. Eleven of the 12 patients were proved to belong to the so-called secretor group, while one was a non-secretor.

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I. Nature of Urobilin Obtained after Amalgam Reduction of Human Fistula Bile.*

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The reduction of crystalline bilirubin either by amalgam or catalytic methods is known to yield the well-defined chromogen, mesobilirubinogen.[1-4] The *in vitro* oxidation of mesobilirubinogen leads to urobilin IX,a,† not to stercobilin.[5, 6, 7] The latter substance as

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1 Fischer, H., *Z. physiol. Chem.*, 1911, **73**, 204.

2 Fischer, H., and Meyer-Betz, F., *Z. physiol. Chem.*, 1911, **75**, 232.

3 Fischer, H., and Hess, R., *Z. physiol. Chem.*, 1931, **194**, 193.

4 Fischer, H., and Adler, E., *Z. physiol. Chem.*, 1931, **200**, 209.

† This substance was first described under the name of K-urobilin.[6] The designation IX,a simply indicates a configuration corresponding with mesoporphyrin IX, the porphyrin ring being opened at the a-methene bridge.

5 Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1508.

6 Watson, C. J., *Z. physiol. Chem.*, 1935, **233**, 39.

7 Siedel, W., and Meier, E., *Z. physiol. Chem.*, 1936, **242**, 101.

isolated in crystalline form from human feces and "urobilin"-containing urines, is optically active, strongly laevorotatory, while urobilin IX,a is optically inactive.[8, 9] The reduction of stercobilin yields stercobilinogen and not mesobilirubinogen.[5, 6, 9, 10]

In considering the *in vivo* transition of bilirubin to stercobilin, the question arose as to whether 2 bilirubins might be present in the bile, one the mother substance of mesobilirubinogen and urobilin IX,a, the other of stercobilinogen and stercobilin. Fischer and Libowitzky[11] recently isolated bilirubin from human bile. Reduction of this bilirubin led only to mesobilirubinogen. This finding, however, did not exclude the possibility that the bilirubin in its natural state in the bile was in some way modified so that if reduction were carried out on the bile directly, stercobilinogen might be formed.

In the present investigation, 7 samples of human fistula bile were used. Samples 1-4 did not contain native urobilin or urobilinogen, and were therefore subjected at once to amalgam reduction. This consisted in shaking the diluted bile with an excess (50-200 g) of 3.5-4% sodium amalgam, until nearly colorless. The Ehrlich reaction had now become strong. The samples were acidified weakly with glacial acetic acid and were then extracted repeatedly with petroleum ether. Samples 5-7 contained native urobilinogen and were therefore acidified and extracted with petroleum ether prior to the amalgam reduction. The latter was then carried out on the aqueous fraction and after acidification the newly formed urobilinogen was extracted with petroleum ether. The term urobilinogen is used here to indicate the sum of any mesobilirubinogen, and stercobilinogen, or other Ehrlich reacting chromogen that might have been formed. The petroleum ether extracts before amalgam reduction, and containing the native urobilinogen are designated in the following as 5-7 a, while those of the bile after reduction are 5-7 b.

The petroleum ether extracts in each instance were further treated as follows: Filtered through petroleum ether-treated paper—allowed to stand in the light for several days until the Ehrlich reaction was weak or negative—precipitated urobilin collected and redissolved in a small amount of $CHCl_3$—urobilin reprecipitated by pouring into 6 volumes of petroleum ether—dry precipitate dissolved in 1% Na_2CO_3—washed with ether, then acidified with acetic acid—washed with ether, then made 7% HCl by addition of sufficient

8 Fischer, H., Halbach, H., and Stern, A., *Ann. d. Chem.*, 1935, **519**, 254.

9 Fischer, H., and Halbach, H., *Z. physiol. Chem.*, 1936, **238**, 59.

10 Watson, C. J., *Z. physiol. Chem.*, 1932, **208**, 101.

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

25% HCl—urobilin hydrochloride removed by repeated extraction with small amounts of $CHCl_3$—dried and concentrated to a small volume *in vacuo*. When crystallization could not be achieved directly by cooling and scratching it was brought about by concentrating further to dryness and washing the residue with cold acetone.[7] This permitted separation of dark impurities from the light orange urobilin which was then easy to crystallize from hot acetone or from $CHCl_3$-acetone. Crystals were obtained from samples 1, 2, 5b, 6b, and 7b. The additional step of washing the initial $CHCl_3$ residue with cold acetone would probably have permitted crystallization from samples 3 and 4 as well, but at the time these were studied, the value of the procedure was not recognized. In each instance the crystals were of the boat-shaped type characterizing urobilin IX,a from acetone.[7] The melting points‡ were likewise indicative of this substance rather than of stercobilin. This may be seen in Table I.

Optical activity was determined for each sample, either for the crystals dissolved in $CHCl_3$ or for the final concentrated $CHCl_3$ solution from which crystallization had not occurred. For the sake of control it was repeatedly determined that there was measurable laevo-rotation in less concentrated solutions of crystalline stercobilin (isolated from the feces). Our measurements for stercobilin agree with those of Fischer and coworkers,[8, 9] $[\alpha]_{589}^{20} = -3300°$ ($CHCl_3$). Relative concentration was determined by means of absorption intensity as measured with the Evelyn photo-electric colorimeter, filter 500. Determinations of optical activity were made with a Schmidt-Haensch polarimeter, employing a sodium lab-arc as light source. In each instance the urobilin obtained after the amalgam reduction of the bile, was optically inactive (samples Nos. 1, 2, 3, 4, 5b, 6b, and 7b). Strong dextrorotatory activity was en-

TABLE I.
Melting Points of Urobilin IX,a, Stercobilin, and Crystals Isolated from Present Samples.

			Present samples			
	Urobilin IX,a	Stercobilin	1	2	6b	7b
Hydrochloride (from $CHCl_3$)	145-150°C	125-128°C		145-152°C		
Hydrochloride (from acetone)	165-170°C	146-149°C			167-170°C	165-168°C
Free substance (from acetone)	176°C (7)	236°C (9)	140-150°C (one crystallization only)		174-176°C (reeryst.)	

‡ With exception of sample No. 1, in which an ordinary macro-method was used, the melting points were determined with a Fisher-Johns micro-apparatus. The melting point of the crystals from sample 5b was not determined.

TABLE II.

Stercobilin	Urobilin IX,a	Crystals, 6b
4922 Å	4952 Å	4948 Å
4921	4951	4952
4925	4950	4948
4922	4947	4952
Avg 4923	Avg 4950	Avg 4950

Solvent: 5 cc methyl alcohol + 2 drops 10% HCl.

countered in the native urobilins present in the final $CHCl_3$ solutions from samples 5a and 7a. The presence of a dextrorotatory urobilin in infected fistula bile will be described in a separate communication.

The spectroscopic studies confirmed Lemberg's observation[12] that a slight difference exists in the position of the visible absorption band of stercobilin and urobilin IX,a. Table II contains a series of values for crystalline stercobilin (from feces), crystalline urobilin IX,a (from mesobilirubinogen), and the crystals obtained from sample 6b.

A difference of the same magnitude, *i. e.*, 25 Å, was observed both by measurement and superimposition of absorption spectra with the solutions of the crystals obtained from samples 1, 2, 5b, and 7b.

Discussion. The present results reveal clearly that simple reduction of the bilirubin present in human bile does not lead to the formation of stercobilinogen and hence to stercobilin. The lability of the urobilin which is obtained, together with its manner of crystallization, melting point, absorption spectrum, and total lack of optical activity are regarded as proof of identity with urobilin IX,a. Thus there is no reason to assume the presence of a second or modified bilirubin in the bile. This would have been a prominent possibility if stercobilin had appeared following these *in vitro* reductions. The present results also constitute further evidence that in the process of formation of bile pigment the protoporphyrin ring in the hemoglobin molecule is opened only at the a-methene bridge. Without this evidence the occurrence of differing tetrapyrrylmethenes, either bilirubins or bilirubinoid in character, could not be excluded, since it was conceivable, *a priori,* that the porphyrin ring might open at one of the other methene bridges. The present results, in agreement with those of Fischer and Libowitzky,[11] indicate that this is very unlikely.

Since there can be no doubt that stercobilin is a bilirubin derivative,[9] it becomes evident that it must owe its formation to a further

[12] Lemberg, R., Lockwood, W. H., and Wyndham, R. A., *Austral. J. Exp. Biol. and Med. Sc.*, 1938, **16**, 169.

chemical activity peculiar to the lumen of the intestinal tract. Thus we cannot agree with the suggestion of Lemberg and coworkers[12] that the term stercobilin be discarded, since in view of the present results it appears to be most descriptive insofar as stercoraceous mode and site of origin are concerned. Further studies of the transition of bilirubin to stercobilin will be described in separate communications of this series.

Much confusion has arisen with respect to the terminology of the natural bilirubin derivatives. In earlier papers it was stated[5, 6, 13, 14] that stercobilin was identical with natural urobilin as isolated from the urine. This was misinterpreted[7] to mean that stercobilin was identical with urobilin (IX,a) as derived from mesobilirubinogen, although the distinction of these substances had been demonstrated repeatedly.[5, 6, 15] The point of confusion, of course, was the term urobilin. We believe that confusion will be avoided in the future if the term urobilin IX,a is used to designate the optically inactive urobilin formed by simple dehydrogenation of mesobilirubinogen. The term stercobilin should apply only to the laevorotatory type of urobilin as originally isolated from feces.[10, 16]

Summary and Conclusions. The amalgam reduction of the native bilirubin in 7 samples of human fistula bile led only to mesobilirubinogen which in turn yielded urobilin IX,a, on suitable oxidation. No evidence of stercobilin formation was obtained. The formation of stercobilin is thus shown to depend upon a further chemical activity which is peculiar to the intestinal tract.

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13 Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1210.

14 Watson, C. J., *Z. physiol. Chem.*, 1933, **221**, 145.

15 Watson, C. J., *J. Biol. Chem.*, 1936, **114**, 47.

16 Watson, C. J., *Z. physiol. Chem.*, 1932, **204**, 57.