THE RELATIONS EXISTING BETWEEN BILIRUBINEMIA, UROBILINURIA AND UROBILINEMIA.*

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I. THE QUANTITATIVE DETERMINATION OF BILIRUBIN IN BLOOD SERUM.

When one considers the frequency of jaundice as a clinical manifestation and its importance as a diagnostic sign in various diseases, it is somewhat surprising that its detection and measurement should so long have escaped the refinements which come from the application of accurate laboratory methods.

It is the every-day experience that both of the usual means of recognizing the presence of bile pigment in the blood—the visible pigmentation of the skin and mucous membranes and the reaction for bilirubin in the urine—are crude and often unsatisfactory. In the lesser grades of cholemia the urine does not react for bile pigment, and the appearance of the skin and membranes is often such as to leave one wholly in doubt as to whether or not a jaundice really exists.

The examination of the blood itself—the method that would seem to be the most direct and reliable—has failed thus far to come into anything like general use.

A number of tests for bilirubin in blood serum have, however, been devised and used. The simpler of these, such as the methods of Hamel¹ and Posselt,² depend on a comparison of the color of the serum to be tested with that of normal serum, and are crude and not entirely trustworthy. Of the several qualitative chemical tests the modification by Syllaba of Gerhardt's procedure is perhaps the best.³ Until recently no satisfactory quantitative test for bilirubin in the blood was available. In 1903, however, Gilbert and his pupils developed a method of measuring the bilirubin content of blood serum which is simple enough for use in the clinical laboratory and seemed to them to give reasonably accu-

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1. Hamel: *Deutsch. med. Wehnschr.*, 1902, xxviii, 1702.

2. Posselt: *Zentralbl. f. inn. med.*, 1907, xxviii, 489.

3. The test recently recommended by Biffi (*Folia hæmatologica*, 1906, iii, 189) we have not used.

rate results. The method consists in an adaptation of the familiar Gmelin test to fluids rich in albumin, and a standardization of it so that it can be used as a quantitative test.

In such albuminous media the complete Gmelin reaction, that is, the characteristic play of colors, occurs only when bilirubin is present in relatively strong concentrations, for example, between 1 to 3,000 and 1 to 5,000. In strengths of from 1 to 7,000 to 1 to 11,000 the reaction appears as a distinct blue-green ring, but without the color play ("Hayem's reaction"). In weaker solutions of bilirubin the blue ring at the point of contact becomes finer, and has a violet rather than a green reflection, but remains distinct up to a dilution of about 1 to 40,000, beyond which point it ceases to disappear.

Working with well-known strengths of pure bilirubin, Gilbert, Herscher and Posternak⁴ have found the limit of this reaction—the point beyond which no distinct blue ring is to be seen—to represent constantly a dilution of approximately 1 to 40,000. Furthermore, they believe that they have proved that this blue ring is characteristic of bilirubin and is not produced by any other substances in the blood, such as albumin, hemoglobin, indican or lutein. It is on this assumption that the limit of the Gmelin reaction in the blood represents a definite strength of bilirubin, that they have based their method of measuring the bilirubin content of blood serum.

DETAILS OF THE METHOD OF "CHOLÉMIMÉTRIE."

The plan consists in making a series of known dilutions of the blood serum to be tested, in tubes of standard size, underlying the fluid in each tube with fuming nitric acid and noting the tube in which only a faint blue ring is visible at the end of a certain period. The bilirubin concentration in this tube is then assumed to be 1 to 40,000, and, the degree of dilution being known, it is easy to calculate the bilirubin concentration in the undiluted blood serum.

APPARATUS REQUIRED.

1. Eight or ten flat-bottomed cylindrical glass tubes 4 or 5 cm. long, with an inside diameter of 1 cm. (It is convenient to have a block of wood or a frame into which these tubes can be set in a row.)
2. Three pipettes: (a) One holding 1.5 c.c. and graduated accurately to 1/20 c.c., for measuring the blood serum; (b), one holding 2 c.c. and graduated to 1/4 c.c., for measuring the diluting fluid ("artificial serum"); (c), one with a tapering point, to measure approximately 1/4 c.c. of the nitric acid reagent.

REAGENTS REQUIRED.

1. The artificial serum: The whites of several eggs are added to an equal volume of 0.7 per cent. sodium chlorid solution; thoroughly mixed, and allowed to stand on ice for twenty-four hours. The liquid is then decanted and to it is

4. Gilbert, Herscher and Posternak: Sur la reaction de Gmelin dans les milieux albumineux, etc., *Compt. rend. Soc. d. biol.*, 1903, *lv*, 530, 574, 1587.

added caustic soda, in the proportion of 0.5 gm. to 100 c.c. It is then allowed to stand three or four days before using, during which time a precipitate forms which includes the coloring matters of the egg-white. When used the liquid should be perfectly colorless in the thickness of 1 cm. This artificial serum in its fluidity, albumin content and alkalinity approximates blood serum. It should be kept cool, and should be freshly made at least every month.

2. The nitric acid reagent.

Pure HNO_3	200 c.c.
Distilled water.....	100 c.c.
Sodium nitrite.....	0.06 gm.

The bottle must be kept carefully stoppered.

TECHNIC.

Distribute in each of, say, 6 glass tubes with the pipette B exactly 0.5 c.c. of artificial serum; then add with pipette A increasing amounts of blood serum (e. g., 1/20 c.c. in the first tube, 2/20 in the second tube, 3/20 in the third, 5/20 in the fourth, 8/20 in the fifth and 12/20 in the sixth tube) and mix by shaking. Underlay the fluid in each tube with 1/4 c.c. of the acid reagent with the pipette C. After standing for thirty minutes the tubes are examined. For this good daylight (not direct sunlight) is needed. The examiner's back should be to the light, the eyes should be somewhat above or below the tubes, rather than on a level with them, and the tubes should be held against a white background. If the blood serum contain sufficient bilirubin, in some of the tubes at the line of contact between the acid and the serum will be seen a clear blue ring, the ring being more pronounced in the tubes containing the larger amounts of blood serum. The tube in which the first faint but distinct blue ring is seen, therefore, is assumed to contain bilirubin in the dilution of 1 to 40,000. (See plate.) The strength of bilirubin in the initial blood serum can then be readily obtained by the following formula.

$$\frac{x = 10 + a}{a} \chi$$

χ represents the fraction 1/40000; a = the number of twentieths of cubic centimeters added.

Example:—If the ring is first seen in the tube to which 5/20 c.c. of blood serum has been added, then:

$$a = 5 \text{ and } x = \frac{10 + 5}{5} \chi = 3/40000 = 1/13333.$$

The blood serum, therefore, contains bilirubin in the ratio of about 1 to 13,300.

If 30/20 c.c. of blood serum are needed to show the ring, then $a = 30$ and the bilirubin content will be 1 to 30,000. If 60/20 c.c. are required the bilirubin content will be 1 to 34,300.

In this case it will be found convenient to use only 30/20 c.c. of the blood serum and add it to 1/4 (instead of 1/2) c.c. of the artificial serum. The resulting mixture will be the same and a will still equal 60. On the other hand, if bile pigment is present in large amount and the tube containing 1/20 c.c. of blood serum gives too strong a reaction, it will be necessary to mix 1/20 c.c. of blood serum with 1 or even 2 c.c. (instead of 1/2) of artificial serum. Then $a = 1/2$ or $1/4$ and the bilirubin content will be, respectively, 1 to 1900 or 1 to 975.

If undiluted blood serum is needed to give the faint blue ring, the bilirubin content will of course represent a dilution of 1 to 40,000. If bile pigment is present in smaller amounts than this, no blue line can be detected.

In case the blood serum to be tested is of pale color, or is believed to contain only a small amount of bilirubin, instead of beginning the series with 1/20 c.c. of the serum, it is well to begin with a considerably larger quantity and to increase the amount rapidly; for example, in the first tube 16/20 c.c., in the

second 18/20, in the third 20/20, in the fourth 25/20, in the fifth 30/20, in the sixth 40/20. A little experience will enable one to select the dilutions so that the end-reaction shall usually fall somewhere within the series. If it does not, other tubes must be prepared, for it is necessary to have at least one tube in which no blue line can be seen. It is important that the examination be made just half an hour after the tubes have been prepared, as the reaction tends to become stronger on standing and tubes which fail to give a reaction within the specified time may do so after an hour or two.

Using this method, Gilbert and his pupils have established the average bilirubin content of the blood in a large series of normal individuals and in a number of conditions associated with more or less distinct jaundice, such as simply family cholemia, cholelithiasis, pneumonia, chronic interstitial nephritis, advanced heart disease, alcoholic and biliary cirrhosis, icterus gravis, etc.⁵ Since the blue ring is found to occur regularly in the undiluted serum of normal persons, Gilbert and Herscher⁶ are led to the belief that normal human blood serum contains a minute quantity of bilirubin, such as is known to be present normally in the blood of certain of the lower animals. They found this physiologic cholemia to correspond to a bilirubin strength which varies between 1 to 28,000 and 1 to 40,000, with an average of 1 to 36,500.

Our own experience with this method of measuring the bilirubin content of blood serum covers a period of fifteen months and includes upward of 160 examinations made in about 120 cases. The list of subjects examined includes normal individuals, many patients with pneumonia, nephritis, typhoid fever, heart affections, cancer, tuberculosis, appendicitis, rheumatism and a great variety of affections of the liver and bile passages. In general, our own results, which were constantly controlled by other, qualitative tests, correspond closely with those of Gilbert and his followers.

We have found it advisable, however, to make two slight modifications of the method as above described: first, in making the artificial serum, when sodium hydroxid is added in the strength prescribed (0.5 per cent.) it is frequently impossible to prevent the egg albumin from forming a jelly-like mass. This we have been able to avoid by reducing the strength of the sodium hydroxid to 0.3 per cent. Second, we have found that a much sharper and clearer line of contact between the acid and the serum can be obtained by first putting the acid reagent in the tube and then overlaying this with the mixed serum. This procedure requires the mixing of the artificial and the blood serum before they are

5. See numerous publications in *Compt. rend. Soc. d. biol., Paris*, during the years 1903, 1904 and 1905.

6. Gilbert and Herscher: *Compt. rend. Soc. de biol.*, 1905, lviii, 899.

placed in the tubes and, therefore, takes a little more time, but it increases materially the distinctness of the reaction.

We have found that one quickly acquires facility in recognizing the end-reaction and have been surprised to see how closely the independent readings of the same tests by different persons agree. The test is open to the objections inherent in all quantitative tests which depend on fine distinctions of color, and the figures can not, of course, be looked on as other than approximate. In control tests, with solutions of pure bilirubin, we have found, however, that the limit of the reaction corresponds, quite uniformly, with a dilution of about 1 to 40,000 when read at the end of half an hour, and that a distinction between such dilutions as, say, 1 to 32,000 and 1 to 35,000, can be made without difficulty. In the application of the test one possible source of error has been encountered. In the very few cases in which we could demonstrate a considerable amount of urobilin in the blood (but no bilirubin) this test gave a peculiar smoky purplish color at the point of contact which by careful inspection, however, was readily distinguishable from the blue ring of bilirubin.

We have found, with Gilbert and Herscher, that a faint blue ring is present, usually, in undiluted normal blood serum, and that it is indistinguishable from that obtained in control tests with very weak bilirubin solutions. We are, therefore, inclined to agree with these writers, that the blood normally contains a minute quantity of bilirubin, although we have not yet obtained positive proof of this fact.

The test, we believe, is of distinct clinical value. Slight grades of jaundice and fluctuations in jaundice, which are quite imperceptible to the eye, are readily detected and measured. We have several times seen it of service as an aid in distinguishing between appendicitis and cholecystitis. In the differentiation of gallstone attacks from gastric or renal affections it seems likely to be of help, although we have as yet had no opportunity to try it.

The time required for making the test is not great and the chief obstacle to its general use is the difficulty of obtaining a sufficient quantity of blood. This difficulty, however, we have found to be much less than might be supposed, and in hospital practice at least the blood can be obtained with very little trouble. The method used by us is as follows:

After the upper arm has been bound so as to obstruct the venous return and the skin about the front of the elbow has been made aseptic, a sterile aspirating needle of medium size is thrust through the skin over the median cephalic or basilic vein, and then, with a second slight thrust, is made to penetrate the vein. From 15 to 30 c.c. of blood are al-

lowed to flow directly into a clean, large test-tube, and then the arm ligature is removed before the needle is withdrawn. If the needle is bright and smooth and the procedure is done briskly, the blood will usually flow freely, and there will be no clotting in the needle. The tube of blood is allowed to clot in the open air and is then placed on ice to permit the serum to separate. This separation may be hastened somewhat by placing the tube for a short time in a thermostat. The yield of clear serum is usually 40 to 50 per cent. of the volume of whole blood.

II. THE RELATION OF UROBILINURIA TO BILIRUBINEMIA.

In connection with their studies of the bilirubin content of the blood serum in various diseases by the method just described, Gilbert and Herscher⁷ investigated the condition of the urine with regard to urobilin. They found that in all cases in which the urine contained pathologic amounts of urobilin, or its precursor, urobilinogen, the blood regularly showed a pathologic amount of bilirubin. They were not able, however, to demonstrate urobilin in the blood, even when it was present in the urine in very large quantity. The constant relation between bilirubinemia and urobilinuria, the absence of urobilin from the blood, and the results of certain experimental work led them to believe that the urobilin which appeared in the urine was not simply the result of the excretion by the kidneys of urobilin carried to them by the blood, but was actually produced in the kidneys themselves, by a process of reduction from the bilirubin of the blood, and that urobilinuria was, therefore, only the index of a cholemia.

This theory of the renal origin of the urinary urobilin had been suggested by von Leube⁸ many years before, as a result of his findings in a case of jaundice in which the urine showed only urobilin, whereas the sweat, obtained by pilocarpin injections, contained only bilirubin. Von Jaksch⁹ also was inclined to accept this view of the origin of urobilinuria, as were certain Italian investigators (Patella and Acorimboni,¹⁰ Mya¹¹).

Ever since the discovery of urobilin by Jaffé¹² (1868) much uncertainty and much difference of opinion have existed concerning both its origin and its clinical significance. It is known to occur in the urine

7. Gilbert and Herscher: *L'urobilinurie*, Presse méd., 1902, x, 843. Also Herscher: *Origine rénale de l'urobiline*. Thesis, Paris, 1902.

8. von Leube: *Beitr. zur Lehre von Urobilinikterus*, Sitzungsber. d. phys. med. Gesellsch. z. Würzburg, 1888, p. 120.

9. Von Jaksch: *Ztschr. f. Heilk.*, 1895, xvi, 48.

10. Patella and Acorimboni: *Rev. clin.*, 1891, 465.

11. Mya: *Arch. ital. di clin. med.*, 1891.

12. Jaffé: *Zentralbl. f. d. med., Wissensch.*, 1868, vi, 241.

in a great variety of pathologic conditions¹³ and to be present often in small amount in normal urine which has stood for some hours in the light. In freshly passed normal urine, however, urobilin itself is not found (Saillet¹⁴). There has been much discussion, also, over the relationship between the urobilin of the urine, the stercobilin of the feces and a body, having the same characteristics, which Maly obtained by the reduction of bilirubin and named hydrobilirubin. Although complete agreement is still lacking, the weight of evidence is strongly in favor of the identity of these three substances. The attempts also of MacMunn,¹⁵ Jolles¹⁶ and others to distinguish between "physiologic" and "pathologic" urobilin, chiefly on the ground of slight spectroscopic differences, seems to be without justification (Garrod and Hopkins,¹⁷ Hildebrandt,¹⁸ Huber¹⁸).

It was early noticed that many urines which were light colored when passed and gave no reaction for urobilin, after standing a few hours in the light, would grow darker in color and present the characteristic reactions of urobilin. The conclusion was natural that such urines must contain some colorless chromogen which, by the action of light, was gradually converted into urobilin. Such a colorless body (urobilinogen), which, when acted on by sunlight, was convertible into urobilin, was produced by Hoppe-Seyler¹⁹ by the strong reduction of hemoglobin, and by Disqué²⁰ by a similar reduction of bilirubin. The change of urobilinogen into urobilin was shown by Disqué to be a process of oxidation. Further valuable information concerning the chemical nature of urobilinogen was furnished by Nencki and Zaleski,²¹ who were able to produce it from hematin. They found that it had the formula $C_8H_{13}N$ and gave the pyrrol reaction, and they named it, therefore, hemopyrrol.

Further evidence of the pyrrol nature of urobilinogen has recently been supplied by Neubauer.²² Moreover, it has been shown by various

13. For details of the clinical aspects of urobilinuria see article of Hildebrandt: Studien über Urobilinurie und Ikterus, Ztsch. f. klin. Med., 1906, lix, 351.

14. Saillet: Rev. de méd., 1897, xvii, 109.

15. Mac Munn: Jour. of Physiol., 1890, x, 71.

16. Jolles: Zentralbl. f. inn. med., 1895, xvi, 1161.

17. Garrod and Hopkins: Jour. of Physiol., 1896, xx, 112.

18. Huber: Ueber die Ursache und der Bedeutung der Urobilinurie, Charite-Ann., 1906, 49.

19. Hoppe-Seyler: Ber. d. deutsch. chem. Gesellsch., 1874, vii, 1065.

20. Disqué: Zeitschr. f. physiol. chem., 1878, ii, 259.

21. Nencki and Zaleski: Ber. d. deutsch. chem. Gesellsch., 1901, xxxiv, 997.

22. Neubauer: Sitzungsh. d. Gesellsch. f. Morphol. u. Physiol. in München, 1903, ii, 32.

investigators that urobilinogen occurs in the urine, feces and bile under the same conditions as, and usually in association with, urobilin.

Since there is now general agreement that urobilinogen is simply a precursor of urobilin and has the same source and the same significance, we shall, for the sake of convenience, in what follows, use the term urobilin to include both urobilin proper and urobilinogen, unless it is made evident from the context that the term is used in its more restricted sense.

The renal theory of the origin of urobilinuria, so vigorously advocated by Gilbert and Herscher, while attractive and plausible, is yet so contrary to the generally accepted views that it has seemed to us desirable to verify, if possible, the assertions of these investigators that urobilinuria occurs only when an abnormal quantity of bilirubin is present in the blood, and that, even when urobilin is abundant in the urine, it can not be found in the circulation. Moreover, it was desirable to know if anything like a quantitative relationship could be established between the urobilin in the urine and the bilirubin in the blood, as one might reasonably expect if the former were merely the result of the latter.

With these objects in view we have, in most of the cases in which the bilirubin content of the blood was determined, examined the urine simultaneously for urobilin. In many of the cases, also, urobilin has been sought for in the blood.

Before giving the results of these investigations it is necessary to describe in some detail the methods employed, since the conflicting results of different writers, especially those relating to the demonstration of urobilin in the blood, seem to be due, in large part, to differences in method and technique.

METHODS EMPLOYED.

For the demonstration of bilirubin and urobilin in blood serum we have relied chiefly on Hedenius' and Syllaba's methods, the latter slightly modified.

Hedenius' method:²³ This consists in the precipitation of from one to three c.c. of serum with two volumes of 95 per cent. alcohol, acidification with 5 drops of 10 per cent. hydrochloric acid and filtering, after having heated twice to boiling. Filtrate and sometimes precipitate, are green in the presence of bilirubin.

Syllaba's modification of D. Gerhardt's method:²⁴ "About 5 c.c. of serum is doubly diluted with water and coagulated with the addition of sodium sulphate and acidification with acetic acid. The bilirubin will be completely carried down by the coagulated albumin, while the urobilin will remain in solution. Filter. In order to make sure that coagulation is complete the filtrate is boiled. If there is no urobilin present, the filtrate is clear, colorless, and spectroscopically free from the absorption band. If urobilin is present the filtrate is reddish colored and shows spectroscopically a fine stripe between C and F. The precipitate col-

23. Hedenius: *Malby's Jahresberichte*, 1894-5, xxiv, 385.

24. Syllaba: *Folia hæmatologica*, 1904, 1, 636.

lected on the filter-paper is white in the absence of bilirubin but is of a pale yellow color if it is present. The precipitate is then washed with hot water, boiled with alcohol acidified with sulphuric acid and filtered. In the presence of bilirubin the alcohol is colored a bright green and the precipitate on the filter paper is also green. In the absence of bilirubin the alcohol is colorless and the precipitate is white."

In working with Syllaba's method we found that frequently some of the urobilin was carried down with the precipitated albumin and appeared only in the filtrate obtained after the precipitate had been boiled with acid alcohol. On this account we slightly modified Syllaba's procedure as follows: 5 c.c. clear serum, diluted with 10 c.c. distilled water, were coagulated by short heating in water bath after the addition of about 0.5 gm. powdered sodium sulphate and 1 c.c. of 5 per cent. acetic acid. The filtrate from this was either practically colorless or faintly yellow or faintly pink. The color of filtrate, we found, could not always be taken as an index of the presence or absence of urobilin and all filtrates must be carefully examined spectroscopically after the addition of two or three drops of Lugol's solution. We employed a large spectroscope and absorption cells from 1 to 4 cm. in depth. (A small pocket spectroscope gives fairly satisfactory results if filtrates are clear.) After neutralization, the filtrate was tested with Schlesinger's zinc acetate solution for confirmatory green fluorescence. This was allowed to stand twenty-four hours before being pronounced negative. The precipitate, usually very faintly yellow or white, was boiled in water bath a few minutes with 20 to 30 c.c. of 5 per cent. acid alcohol (hydrochloric acid 5; 95 per cent.; alcohol, 95). The filtrate from this was sometimes colorless, sometimes green, sometimes yellowish pink. The colorless filtrates contained neither urobilin nor bilirubin. The green filtrates contained bilirubin, and on spectroscopic examination occasionally showed a band of urobilin. The pink filtrates contained only urobilin. Syllaba does not mention the possibility of obtaining a pink acid alcohol filtrate, so it is probable that he had no opportunity to work with sera containing as large quantities of urobilin as those we encountered.

The confirmatory green fluorescence may readily be obtained, after neutralization, in the pink filtrates, but this reaction is not so satisfactory in the green filtrates, as biliverdin will give a confusing fluorescence. This fluorescence of biliverdin with zinc acetate solution in our experiments, seemed to bear a direct relation to the amount of biliverdin present. Occasionally the precipitate from 5 per cent. acid alcohol is green, at other times pearly white or pearl blue. A further boiling of this precipitate with 20 per cent. acid alcohol will yield a filtrate similar to that obtained with 5 per cent. acid alcohol if the amount of pigment was originally large. The sera in which by this method we were able to demonstrate urobilin only, and no bilirubin, gave after coagulation an almost colorless, or faintly yellow or pink filtrate as described above, showing characteristic absorption band and giving fluorescence with zinc acetate. The precipitates from these sera were bluish-white and on boiling with 5 per cent. acid alcohol they gave a pink filtrate showing an absorption band, and fluorescence with zinc acetate. In the specimens showing large amounts, reboiling with 20 per cent. acid alcohol gave a similar but much fainter reaction. No green filtrate could be obtained.

Ehrlich's para-dimethyl-amido-benzaldehyd reagent (2 per cent. solution in 5 per cent. hydrochloric acid) gave a positive reaction in three of our cases. In two the characteristic red color developed immediately and in the other after fifteen minutes. As shown by Neubauer,²² this substance reacts to all bodies containing the pyrrol ring. That in these cases the body responsible for the reaction was urobilinogen was readily shown by the demonstration of the absorp-

tion band of urobilin, as well as that of the benzaldehyd, on the completion of the reaction.

Attempts to separate the urobilin directly from the serum by means of amyl alcohol were unsuccessful because of the tendency to the formation of an emulsion, from which the alcohol separated very slowly and incompletely.

Braunstein's²⁵ test was found inapplicable, failing in several sera known to contain urobilin. It gave a positive pink chloroform extract in one serum in which all other tests for urobilin were negative.

Schlesinger's direct application of his zinc acetate test without previous coagulation gave a positive fluorescence and band in one case in which we had previously demonstrated urobilin. Blood is received directly into a 0.5 per cent. solution of calcium oxalate in 200 c.c. 0.7 per cent. sodium chlorid solution and corpuscles separated by centrifuge. The zinc acetate solution is added to the resulting fluid. The possibility of bilirubin giving rise to a deceptive fluorescence should be borne in mind and results confirmed by spectroscope.

Urobilin and bilirubin in urine: The characteristics relied on for the demonstration of urobilin in the urine were its absorption spectrum, a single band between C and F near F and the green fluorescence developing in the presence of certain zinc salts. The chromogen urobilinogen has no characteristic spectrum but it is readily, and apparently quantitatively, converted into urobilin by the addition of a few drops of Lugol's solution. The fluorescence may be readily developed by the addition of an equal volume of a 10 per cent. solution of zinc acetate in absolute alcohol as suggested by Schlesinger. This is a simpler and more sensitive method than the older one in which zinc chlorid and ammonia were used. Ehrlich's benzaldehyd reagent was not used for demonstration of urobilin because the many substances containing the pyrrol ring which may occur in the urine necessitate the spectroscopic confirmation of each positive reaction.

For the quantitative estimation the simple method was used of diluting the specimen to the point of disappearance of the absorption band when observed in a layer 1 cm. deep. The complete daily volume of urine was not taken into account in these estimations. (The highest value we encountered in this series required a dilution of 100 times before the disappearance of the band.)

For the demonstration of bilirubin Huppert's test and its various modifications were sometimes used, as was also Hedenius' test, but we relied chiefly on Rosenbach's modification of Gmelin's test. For this several sheets of soft Swedish filter-paper were used and a large volume of urine slowly filtered through them. On the pigment thus collected a drop of yellow nitric acid gave the most satisfactory play of colors.

Simultaneous quantitative examinations of the blood serum for bilirubin and the urine for urobilin were made in 121 cases. The results of the examinations of the urine have been grouped into five classes, according as urobilinuria was absent, slight, moderate, marked or extreme. Similarly the results of the examinations of the blood for bilirubin have been divided, arbitrarily, into five groups, representing different degrees of bilirubinemia.

The mutual relations between the two series of groups can best be appreciated by their graphic representation, as in the accompanying chart.

25. Braunstein: *Ztschr. f. Krebsforsch.*, 1903, i, 15.

CHART SHOWING RESULTS OF EXAMINATION OF BLOOD SERUM FOR BILIRUBIN AND URINE FOR UROBILIN.

	From	To
Group 1. —————	Bilirubin concentration in blood serum.....1 to 35,000	1 to 40,000 or negative.
Group 2. - - - - -	Bilirubin concentration in blood serum.....1 to 30,000	1 to 35,000
Group 3.	Bilirubin concentration in blood serum.....1 to 20,000	1 to 30,000
Group 4. -	Bilirubin concentration in blood serum.....1 to 10,000	1 to 20,000
Group 5. —	Bilirubin concentration in blood serum.....1 to 1,000	1 to 10,000

Considering, first, the group of cases (50 in all) in which the bilirubin content of the blood may be regarded as normal (1 to 35,000 or less), it will be seen that the urine was entirely free from urobilin in two-thirds of the cases and that in all the remaining ones urobilin was present in only slight amount. A study of Groups 2, 3 and 4 will show that, as the bilirubin content of the blood increases, there is also a steady rise in the average of urobilin in the urine, although the individual cases of each group show well-marked variations in the degree of urobilinuria. Group 5, however, which contains the highest grades of bilirubinemia and represents the cases of marked jaundice, shows a striking departure from this general rule. Of the 19 cases included in it, 10 showed no urobilin in the urine and 3 others only a small quantity, but in all of these 13 cases the urine gave a strong reaction for bilirubin (indicated by the letter B in the chart). In the remaining 6 cases of this group the urine contained very large quantities of urobilin, but no bilirubin. (It should be added that in no other cases of the entire series was bile pigment found in the urine.)

It has long been known that in many of the severest cases of jaundice the urine is entirely free from urobilin. The usual explanation for this peculiarity is that in such cases there is complete occlusion of the common or hepatic duct and, because no bile can enter the intestines, no urobilin (stercobilin) is formed. Indeed, it is just this class of cases that has furnished one of the strongest arguments of the supporters of the theory of the enterogenous origin of urobilinuria. Gilbert and Herscher attempt to harmonize these facts with their renal theory by supposing that, when the concentration of bilirubin in the blood reaches a certain point the kidneys are, as it were, overwhelmed by it and are no longer able to convert it into urobilin and that, therefore, bilirubin passes unchanged into the urine.

If we except the cases just referred to, it is evident from the chart that a very close relationship between bilirubinemia and urobilinuria does exist. When the bilirubin content of the blood is normal, urobilin is either absent altogether, or is present in the urine only in very small quantity. With the increase of the bilirubin concentration there is a well-marked tendency for the urobilinuria to become more and more pronounced. But this quantitative relationship is by no means apparent in every case. In each of the Groups 2 and 3 there are two or three cases in which the urine contained no urobilin at all. On the other hand, the last case in Group 2 showed a degree of urobilinuria greatly in excess of that of the other cases of that group. It is evident, therefore, that, while it may be stated as a general rule that bilirubinemia and

urobilinuria go hand in hand and usually bear a rough quantitative relation to each other, the exceptions to this rule are so frequent and important that it can hardly be said to furnish much support to the renal theory of the origin of urobilinuria.

III. UROBILIN IN THE BLOOD.

Very conflicting opinions have existed concerning the presence of urobilin in the blood. Herscher,⁷ in 55 examinations in cases of marked urobilinuria, was never able to demonstrate it; and these negative results were used to support the renal theory of urobilinuria. Schlesinger,²⁶ using better methods, failed, in 15 cases of marked urobilinuria, to detect urobilin in the blood, and endorses the views of other writers (Gerhardt, F. Müller), that, in fresh blood, urobilin in any considerable quantity does not occur. On the other hand, Huber¹⁸ states that in the 40 cases examined he was always able to find urobilin in the blood when it was present abundantly in the urine. He ascribes Herscher's negative results, and his own earlier ones, to the use of unsatisfactory methods. He insists that with Schlesinger's method its demonstration in the blood is easy. It is necessary to convert the urobilinogen into urobilin by adding one or two drops of Lugol's solution, and this, he thinks, is probably the reason why Schlesinger himself failed to find it. Biffi,²⁷ in a very recent article, states that urobilin (or its chromogen) can be demonstrated not infrequently in the blood and that in certain diseases, e. g., croupous pneumonia, it is to be found "almost always" in considerable amounts.

Our own experience does not tally exactly with that of any of the writers cited. In spite of repeated and careful efforts, in patients with marked urobilinuria, our results were uniformly negative until we chanced to examine blood serum from a patient with a very severe case of lobar pneumonia with beginning pulmonary edema, on whom venesection had been done. The results in this case were in striking contrast to those of our other cases. Urobilin could be demonstrated readily by every method and could be easily measured by the spectroscope. In 9 other cases of the same sort—patients with very severe pneumonia who soon afterward died—exactly the same results were obtained. (In several of these cases urobilin was present in such quantity that the untreated blood serum showed a pronounced green fluorescence.) In these

26. Schlesinger: Zum klinische Nachweis des Urobilin. Deutsch. med. Wehnschr., 1903, xxix, 561.

27. Biffi: Ueber das Vorkommen einer bedeutenden Menge von Urobilin im Blute menschlichen Leichen, *Folia hæmatologica*, 1907, iv, 533.

cases there was nothing in the urobilin content of the urine to distinguish them from many other pneumonias. While the urobilin was abundant in the urine (the dilution values varied between 6 and 40), it was less in amount than in several cases of pneumonia which showed no urobilin in the blood, and was much less than in certain cases of cirrhosis in which, likewise, the blood failed to show urobilin.

Another surprising fact in these 10 fatal cases of pneumonia was that, while urobilin was present in large amount, no bilirubin could be found in the blood. In 3 severe cases of pneumonia in which the patients eventually recovered, the blood contained a small quantity of urobilin and also some bilirubin. Still another severe case showed only bilirubin on the first examination. On the next day the blood contained both bilirubin and urobilin. A day or two later the patient died, without another blood examination having been made. In contrast to the blood findings in these severe cases of pneumonia, there were 15 cases with recovery in which no urobilin could be demonstrated in the blood. In all but one of these the blood contained an increased quantity of bilirubin.

The constant appearance of urobilin in the blood in pneumonia patients shortly (from one to three days) before death seemed to present such a notable exception to the general rule (in our examinations) that no urobilin can be found in the blood, even when it is present abundantly in the urine, that efforts were made to find some explanation for this peculiarity. The apparent disappearance of bilirubin from the blood suggested the possibility that the growth and multiplication of pneumococci in the blood might bring about the transformation of the circulating bilirubin into urobilin. Another possibility was that, by the action of the bacteria, urobilin might be formed directly from the hemoglobin of the blood. Added plausibility was lent to this idea by the well-known fact that the growth of pneumococci in blood-containing media is often associated with the development of a greenish color, as, for example, in the green-tinted vegetations in acute pneumococcic endocarditis. In order to determine these points, agar plates, to which had been added varying quantities of blood, were inoculated with pneumococci obtained from some of the fatal cases; a second series of plates was inoculated with *Streptococcus mucosus capsulatus* obtained from the patient in a fatal case of septicemia with pneumonia and urobilinemia. In addition, cultures of these organisms were made in bouillon and glucose bouillon to which had been added varying amounts of hemoglobin and bilirubin. In none of these cultures, however, could urobilin be demonstrated. A partial reduction of the hemoglobin was obtained, but no urobilin.

Rabbits were also inoculated—two with the pneumococcus and one with the *Streptococcus mucosus*—but their blood, although swarming with the bacteria, failed to show urobilin. Portions of consolidated lung from patients showing urobilinemia, when washed free from blood and ground in a mortar, likewise failed to show the presence of urobilin. We were, therefore, forced to the conclusion that the marked urobilinemia observed in the very severe forms of pneumonia is not to be explained on the ground either of bacterial transformation of hemoglobin or bilirubin into urobilin in the blood, or the formation of urobilin in the hepatized lung itself.

A recent statement by Biffi,²⁷ which has come to our notice only since the completion of this work, has a direct bearing on this subject. He found that postmortem blood almost invariably contained urobilin in considerable quantity. In 96 such examinations he could demonstrate urobilin in the heart blood in all but two cases, and these two were cases of sudden death in persons up to that time in good health. That the urobilinemia was due neither to a postmortem diffusion of the intestinal stercobilin nor to bacterial reduction of the plasmochrome (bilirubin?) into urobilinogen he thinks probable from the fact that it was found in cases examined a very short time after death. On the other hand, in several of his cases the blood had been examined for urobilin only a few days before death, with negative results. The condition was observed after death from all manner of diseases and at all seasons of the year. Biffi suggests, therefore, that the development of the urobilinemia occurs during the last few hours of life as an antemortem change, irrespective of the nature of the fatal disease. We have as yet had no opportunity to verify this view. In our cases of pneumonia, while there was regularly the appearance of a urobilinemia in the fatal cases during the last day or two of life, urobilin also appeared, in small amount, in three severe cases which ended in recovery. It can not, therefore, be purely an antemortem change. This subject will be referred to again later.

In the light of our own experience it is difficult to understand the statement of Huber, that he was always able to demonstrate urobilin in the blood in cases of marked urobilinuria. In over 60 examinations, most of them in cases with a high grade of urobilinuria, we were able to demonstrate urobilin in the blood serum only in the cases of severe pneumonia mentioned above. In these cases, however, the reactions for urobilin were so distinct and so easily obtained that it seems very unlikely that our failures in other instances were due to faulty technic.

We have found that the zinc acetate test (Schlesinger's), in the presence of bile pigment, is unreliable, as a distinct green fluorescence may

be produced by biliverdin. In blood serum containing bilirubin, therefore, reliance must be placed on the spectroscope and Syllaba's test. In view of the fact that Huber's results were obtained with Schlesinger's test, and the further fact that most cases of marked urobilinuria are associated with bilirubinemia, it seems probable that some of his positive reactions may have been caused by biliverdin rather than by urobilin.

IV. THE SOURCE OF URINARY UROBILIN.

A number of theories have been advanced to explain the origin of the urobilin of the urine, and much difference of opinion and uncertainty still exist concerning it. The subject is complicated by the fact that, as has been said, there is still some conflict of opinion concerning the identity of the urinary urobilin with the stercobilin of the feces, although the weight of opinion strongly supports the view that they are identical.

Briefly stated, these theories are as follows:²⁸

The Hepatogenous Theory.—This has gained widespread acceptance in France, chiefly through the advocacy of Hayem²⁹ and his school, and is well defined in the dictum of Hayem: "L'urobiline est le pigment du foie malade." When the functional activity of the liver is disturbed the coloring matter of the blood is manufactured, not into bilirubin as usual, but into urobilin. Urobilinuria is, therefore, an indication of hepatic insufficiency. This insufficiency may be absolute, when the liver itself is at fault, or relative, when, owing to the excessive amount of blood pigment carried to the liver, this organ can no longer convert it all into bilirubin.

The Hematogenous Theory.—In this the blood itself, rather than the liver, is looked on as the direct source of the urobilin. Urobilinuria is known to occur with all sorts of blood extravasations and in various conditions in which there is extensive destruction of red cells, for example, in paroxysmal hemoglobinuria, scurvy, blackwater fever, pernicious anemia, sulphonal poisoning, etc. Moreover, urobilin has been found locally in blood extravasations, it (urobilinogen) has been produced by a process of reduction from hematin, and is said to have formed in a solution of hemoglobin which had been for a long time protected from bactericidal action (Winter).

The Histogenous Theory.—This has as its basis the fact that in many forms of jaundice, especially in conditions of fading jaundice, the urine

28. The whole question is discussed fully by Weintraub in von Noorden's *Metabolism and Practical Medicine*, Am. ed., 1907, ii, 241.

29. Hayem: *Sur la valeur diagnostique et pronostique de l'urobiline*, *Gaz. d. hôp.*, 1889, lxii, 1314.

contains very large quantities of urobilin and no bilirubin. It was, therefore, assumed that the bilirubin deposited in the various tissues might, by the action of those tissues, be converted gradually into urobilin which, because of its great diffusibility, would be taken up promptly by the blood and excreted in the urine.

The Renal Theory.—This is really only the application of the histogenous theory to a particular type of tissue, and has already been referred to.

The Enterogenous Theory.—This assumes that the only source of urobilin in the body is the intestines, where it is formed in large quantity, and that urobilin appears in the urine when, for any reason, it is absorbed from the intestines in larger amount than usual.

Many facts, both clinical and experimental, support the view that a close relationship exists between the urobilin of the intestine and that of the urine. Urobilinogen and urobilin (stercobilin) are formed in large amount in the intestine by the reducing action of the putrefactive bacteria on the pigment of the bile. Urobilinogen is found chiefly in the small intestine, urobilin chiefly in the colon. Normally these substances are to some extent absorbed from the intestine and carried to the liver, where they are, in part at least, excreted in the bile. When no bile enters the duodenum, as in occlusion of the common duct, urobilin disappears from the intestine, bile and urine. In a case of this sort Friedrich Müller³⁰ found that by administering bile by mouth urobilin could be made to appear in the urine and that it disappeared soon after the feeding of bile ceased. Newborn infants during the first few days of life, that is, before putrefactive bacteria have invaded the intestine, show urobilin neither in the stools, bile nor urine. In many disturbances of the liver associated with marked urobilinuria, an unusually large quantity of bile pigment is furnished to the intestine (pleiochromia) and the conditions are favorable there for the production of an increased quantity of urobilin. These facts, on the one hand, and, on the other, the absence of convincing evidence that urobilin is produced elsewhere in the body, have led to the wide acceptance, especially in Germany, of the idea that the urobilin of the urine is produced in the intestine and is simply the expression of its increased absorption from the intestine. Increased absorption does not necessarily imply an increased production in the intestine. Ladage³¹ has shown that when urobilin is introduced into the upper part of the small intestine most of it is absorbed and ap-

30. Müller (F.): Ueber Ikterus, Verhandl. d. schles. Gesellsch. f. vaterl. Kultur, 1892.

31. Ladage: Bijdrage tot de kennis der Urobilinurie, Thesis, Leyden, 1899.

pears in the urine. Conditions which favor the rapid conversion of bilirubin into urobilin in the small intestine might, therefore, be assumed to favor the absorption of the latter. Efforts to demonstrate a parallelism between urobilinuria and increased intestinal putrefaction, however, have thus far not been successful (Fischler³²).

But the intestinal theory of the origin of urobilinuria fails satisfactorily to explain many of the clinical facts. There is abundant evidence, for example, that a marked urobilinuria may exist when it is certain that much less than the normal quantity of bile is entering the intestine. For these and other reasons which can not here be discussed, it seems very unlikely that conditions in the intestines constitute the sole, or even the chief, factor in the production of urobilinemia and urobilinuria.

The fact that normally the urobilin which is absorbed from the intestine is almost completely abstracted from the portal blood by the liver and returned to the intestine has led to the view that the failure of the liver properly to remove the urobilin brought to it by the portal vein may explain the appearance of urobilin in the general blood stream and in the urine. Such a failure might result from some disturbance of the liver itself, when only the usual quantity of urobilin was brought to it, or from the fact that the portal blood contained an amount of urobilin so great that even the normal liver was unable to abstract it all.

This combined intestinal and hepatic theory of the origin of urobilinuria seems at present to be the one which best explains most cases of urobilinuria. That it will, however, account for all cases of urobilinemia and urobilinuria is by no means certain. Of the several other possible sources it may be said first, that there is no proof whatever that urobilin is ever formed in the body tissues from the bile pigment deposited there. The renal theory, also, attractive and plausible as it seems, has little evidence to support it and fails to explain many of the clinical facts. We have shown that the relation between bilirubinemia and urobilinuria is not sufficiently constant to accord with such a view. It has been shown also that urobilin does occur in the blood. The theory, moreover, fails to explain satisfactorily the important class of cases of common duct occlusion in which, although bilirubin is present in the blood in large amount, no urobilin appears in the urine. Finally, the theory is contradicted by the experiment of F. Müller and by the later ones of Fischler,³² in which perfusion of the living kidney with solutions of bilirubin failed to show any transformation of bile pigment into urobilin.

32. Fischler: *Das Urobilin und seine klinische Bedeutung*, Thesis, Heidelberg, 1906.

The view that urobilin may sometimes be formed directly from the hemoglobin of the blood can not be dismissed so readily. It is known that urobilin can be produced *in vitro* directly from hematin. There is at present, however, no proof that such a transformation occurs in the circulating blood. Our efforts to demonstrate such a source for the urobilinemia of certain cases of pneumonia were altogether unsuccessful. The possibility, however, of the hematogenous origin of urobilin must still be acknowledged.

Can the liver itself produce urobilin? And is there any basis for Hayem's dictum, that urobilin is the pigment of the diseased liver? A categorical answer to these questions can not be given at the present time. There are a multitude of clinical facts which point to the liver as the dominant factor in the production of urobilinuria. In no other class of disorders is urobilinuria so frequent and so excessive as in those associated with disturbances of the liver functions. In almost every variety of acute and chronic disease of the liver, urobilinuria is a common and conspicuous symptom. It seems probable that, in most such cases, this urobilinuria is caused by the failure of the disordered liver properly to arrest the urobilin carried to it from the intestine, and until within the past year or two no convincing evidence had been adduced to show that the liver, healthy or diseased, has the power of actually producing urobilin. Recently, however, Fischler,³² in a series of interesting and carefully performed experiments, has obtained data which seem to prove that, under certain circumstances, the liver can, in fact, manufacture this substance. He found that in dogs, in which he had severed the common bile duct and had made a complete bile fistula and in which, therefore, no bilirubin could enter the intestine, the bile, which normally contains a considerable amount of urobilin, promptly became completely free from it and, at the same time, the stools became colorless and lost all but a small fraction of their urobilin. The urine also remained free from urobilin. He then produced changes in the liver by the administration of well-known liver poisons (amyl alcohol, phosphorus, etc.) and found that the bile immediately showed enormous quantities of urobilin and that urobilin appeared in the urine. With the subsidence of the poisonous effects, the bile and urine again became free from urobilin. In the face of these experiments it is difficult to escape the conviction that the liver can, under certain circumstances, produce urobilin.

How important a rôle this purely hepatogenous urobilin may play in the production of the clinical forms of urobilinuria and urobilinemia must be left for future study to determine. Fischler himself is inclined to believe that such an hepatogenous form of urobilinuria is exceptional.

It is possible, however, that these new facts may throw some light on the antemortem form of urobilinemia to which Biffi has called attention and which seems to correspond in most respects with the marked urobilinemia found during life in our cases of fatal pneumonia. When one considers that even in the extreme grades of urobilinuria it is usually impossible to demonstrate urobilin in the blood, it seems probable that the concentration of urobilin in the blood in these cases of pneumonia must have been exceptionally great—so great, indeed, that with the failing circulation of approaching death it is not easy to imagine the absorption from the intestine of a sufficient quantity of urobilin to explain it. In this connection reference should again be made to the striking fact that in these 10 cases of pneumonia with marked urobilinemia no bilirubin could be found in the blood, although it was almost constantly present in the other cases of pneumonia.

The constant association of these two exceptional conditions can hardly be a mere coincidence, and it suggests that both the marked urobilinemia and the absence of a bilirubinemia may have as a common cause the disturbed metabolism of a failing liver.

SUMMARY.

By a method devised and used extensively by Gilbert and his pupils we have made some 160 determinations of the bilirubin content of the blood serum in a variety of diseases, and have become convinced that this method of *cholémimétrie* is sufficiently simple and reliable for clinical use, and that by it one may readily recognize and measure slight grades of icterus which fail to disclose themselves either by discoloration of the skin or by the presence of bile pigment in the urine. The method has, we believe, a distinct field of usefulness as an aid in the differential diagnosis between affections of the bile passages and those of other organs, such as the stomach, appendix and kidney.

Simultaneously with the examination of the blood serum for bile pigment the urine was tested quantitatively for urobilin. The mutual relations between bilirubinemia and urobilinuria are shown in the foregoing chart. It will be seen that when the blood serum was normal as regards bilirubin, the urine either was free from urobilin or contained only minimal quantities. With the increase in the concentration of bile pigment in the blood there was, in general, an increase in the urinary urobilin. But this rule was by no means invariable. A number of important exceptions occurred, notably among the cases of marked jaundice (Group 5), in many of which no urobilin could be demonstrated in the urine; on the other hand, in some cases the grade of urobilinuria was extreme. Our results, in the light of these exceptions, seem, therefore,

to offer additional evidence against the view that the urinary urobilin is formed in the kidneys from the bilirubin carried to them by the blood and is, therefore, only an index of the degree of cholemia.

In sixty cases of urobilinuria, some of them of extreme grade, the blood serum was examined for urobilin, with uniformly negative results except in the case of a group of ten fatal cases of pneumonia in which, during the last two or three days of life, urobilin was present in the blood in considerable amount, although its quantity in the urine was by no means unusually large. In fifteen pneumonia patients who recovered no urobilin could be found in the blood.

Our efforts to show that the marked urobilinemia in the fatal cases of pneumonia was the result of transformation of the hemoglobin or bilirubin of the blood into urobilin by the action of the circulating bacteria were entirely unsuccessful, as was also the attempt to demonstrate urobilin in the hepatized lung tissue. These negative results, in connection with certain other facts and with some recent experiments of Fischler, suggest the possibility that in these cases the urobilin of the blood may have a direct hepatic origin.

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