BLOOD UROBILIN.

THE UROBILIN CONTENT OF NORMAL HUMAN BLOOD.
DESCRIPTION OF A METHOD.

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Normal urobilin content of human blood has not been described. Present knowledge of the normal metabolism of urobilin deals indirectly with that phase which concerns the blood. The physiology of urobilin in animals also deals indirectly with the subject of blood urobilin. Elman and McMaster (1), in their experimental studies in urobilin physiology, had no method for blood urobilin.

The exact nature of urobilin is, perhaps, not known; but with the available methods for bile, feces, and urine, a large amount of fact about urobilin has accumulated and there is no serious discord in interpretation. Practically all urobilin studies have been done with one or the other of two methods; namely, the Schlesinger fluorescence method and the Ehrlich aldehyde test. The Ehrlich test is generally found to be not very sensitive, incapable of detecting urobilin in the blood of normal man or animal, and, furthermore, subject to the criticism that it reacts to tryptophane (Marechal (2)). The Schlesinger test is known to be very sensitive (von Fisher (3), 0.0048 mg. per 100 cc.) and highly specific.

Garrod and Hopkins (4), Biffi (5), Meyer-Betz (6), Obermayer (7), Grigaut (8), von Fisher (3), and Palmer (9) have worked with urobilin and accepted this test as specific, but no one has uniformly found the test to be positive in human blood, although urobilin is always present in urine.

A few methods for blood urobilin based on the Ehrlich test or Schlesinger test have been reported, the authors finding urobilin in abnormal conditions such as pneumonia, nephritis, or cirrhosis of the liver, where, for various reasons, a high concentration of urobilin was present. Brule and Weiss-
man (10), Grigaut (8), Weltmann and Löwenstein (11), Eppinger and Ranzi (12), Hildebrandt (13), Rodillon (14), Winternitz (15), Blankenhorn (16), and Royer (17) comprise the entire literature on the subject. Schlesinger's test has been greatly improved by the work of Marcussen and Hansen (18), who were particularly concerned with the nature of its fluorescence and described the optimum conditions for fluorescence with urobilin and zinc salts. The work of Elman and McMaster (1), who made the Schlesinger test quantitative by developing a standard, together with that of Marcussen and Hansen, has made possible the work reported here.

The method we describe is the application of the fluorescence test to blood serum, modified to make it more sensitive by refinements in two main principles; namely, to provide absolute clear supernatant solutions in which to develop fluorescence, and to examine these solutions in a dark room with an intense beam of light. By these refinements in technique it has been possible to detect fluorescence in practically every normal human blood. This fluorescence has been measured quantitatively by using the Elman and McMaster (1) standard. The details of the technique are as follows: Blood is obtained by venepuncture and allowed to clot for at least 12 hours. Oxalate and fluoride plasma have been used, but clearing seems more difficult than when serum is used. The clot is freed from the walls of the tube and centrifuged to give a clear serum without hemolysis. 2 or 3 cc. of clear serum are accurately pipetted off and placed in a special tube on about 0.5 gm. of finely powdered C.P. zinc acetate (the zinc is an excess and need not be measured carefully). This is shaken and given time to mix so that acid coagulation of the serum results. In a minute or two, 3 volumes of absolute alcohol are added and the specimen well mixed. The tube is then corked and placed in the ice box for 24 hours. After 24 hours the specimen is centrifuged, if clear, and the supernatant liquid is poured off into a similar tube and the urobilin estimated by the method described below. Certain precautions are observed to guarantee perfectly clear supernatant liquids; viz., uniform mixing of specimen so that a good flocculent coagulation is formed; specimens are not centrifuged until a good coagulation with clumping or flocculation has resulted. It has been found best to pour off the supernatant liquid carefully without carrying over any of the sediment, since it is not necessary to pour off all of the supernatant liquid. Absolute alcohol must be used because all other varieties of alcohol,
regardless of percentage or purity, contain a blue fluorescent substance which interferes with readings and is got rid of with great difficulty. It is necessary to keep the specimens in the ice box and to centrifuge immediately on removing from the ice box, so that specimens can be read at a slightly higher temperature than the temperature at which they were cleared. Specimens must be corked to prevent evaporation. Great care must be taken in the preparation of all glassware in order to prevent any turbidity of the supernatant liquid. It is frequently unnecessary to pour it off, and specimens can sometimes be read in the original tube. This is desirable because pouring into a new tube frequently causes turbidity for some strange reason—possibly minute particles of dust, perhaps slight changes in pH due to the action of glass. In general, the less specimens are handled the more likely they are to remain clear. Filtering of specimens is never employed because of the turbidity that arises from various causes; i.e., dust on the filter paper and lint from even the hardest of papers. Furthermore, repeated filtering has been found entirely to remove the fluorescent substance, possibly by adsorption to the paper.

If turbidity persists regardless of all precautions, specimens can occasionally be cleared by adding as much protein as is contained in a platinum loop of the serum originally employed. This minute addition introduces a negligible error and may turn the trick.

Thick-walled, culture tubes, \( \frac{4}{2} \times 4 \) inches, are carefully cleaned in acid cleaner and many rinsings of distilled water, dried in a drying oven, and then carefully protected from dust while in storage. These tubes are used for no other purpose and subsequent cleaning is done with Dutch Cleanser and water. Tubes are “banded” about the top so as to cover the entire lip with black paint. For this we use Service Seal black paint (sold by John A. Steen, Varnish Company, Chicago). All tubes must be carefully matched as to size, thickness of wall, and color of glass.

Comparison with the standard is made under carefully controlled circumstances, aimed to detect the most minute traces of fluorescence. The same conditions which detect minute traces of fluorescence also detect minute turbidity, or particulate matter of any origin which by causing a Tyndall phenomenon obscures a reading.
Fig. 1. A, side view of the entire apparatus; viz., lamp, housing, condenser, focusing tube, and comparing chamber. B, focusing tube and comparing chamber enlarged. C and D, details of comparing chamber, showing arrangement permitting the operator to examine a pair of tubes in a single beam of light. E and F, details of focusing diaphragm to be used in adjusting the single beam of light prior to making a comparison. This diaphragm and the comparing chamber are interchangeable on the end of the focusing tube.
An arc lamp, model D, haloptican (made by Bausch and Lomb Optical Company, Rochester, New York) is used as source of the light—this being a carbon arc in a light, tight housing and with condensing lens to bring the light to a sharp focus. At this focus, in a box of special design, two tubes can be examined by being rapidly alternately placed in the path of the beam of light as illustrated (Fig. 1). Upon working in a very dark room with this intense light, minute traces of fluorescence can be detected and estimated quantitatively by direct comparison with the standard.

The standard used is a dilute aqueous solution of neutral acriflavine (Boots Pure Drug Company) as described by Elman and McMaster (1). A parent solution is made of 0.1 gm. in 100 cc. of double-distilled water, this making a dilution of 1:1000. The parent solution keeps for 6 months if stored in an ice box. From this parent solution a stock solution is made once a week by adding 1 cc. to 99 cc. of double-distilled water. This makes a dilution of 1:100,000. The standard actually employed for the reading is made from the stock solution each day and should be used at once, because a marked loss of fluorescence can be noted in several hours at room temperature. The standard as used is a series of ten tubes, the strongest of which is a solution of 1 part in 10,000,000, and the weakest a solution of 1 part in 200,000,000. The intermediate tubes in the series are respectively, 30,000,000, 50,000,000, 70,000,000, 90,000,000, 110,000,000, 130,000,000, 150,000,000, and 170,000,000. This series was found by many trials to be as long and as regular as could be used; that is to say, that any further dilutions of the dye could no longer be seen at all, and any greater concentration, stronger than 1:10,000,000, could not be recognized as being more green. Furthermore, when a greater number of tubes was used in the series (that is more steps) there was confusion because differences of color were too slight to be recognized as steps. As a check on the standard, each series was proved by carrying out the dilution to the disappearing point and each series arranged in proper order without any reference to the labels—that is to say, the standard was considered correct when any further diluting beyond 1:200,000,000 dispelled all green color, and when there was enough color difference in each tube in the series so that each could be arranged...
Blood Urobilin

in proper order as to intensity of green color. In working with this standard, as well as the unknowns to be measured, it must be borne in mind that colors seen as a result of fluorescence do not behave as do colors of ordinary colored solutions. We are relying on the work of Elman and McMaster (1) to establish the correctness of the method as well as the value of the standard. It must be noted here that since publishing their method, these authors have corrected their publication by changing the figure 0.009, on page 511, to read 0.0105.

Fig. 2 represents among other things the strength of the standard in dilutions of acriflavine. This chart is arranged for direct determination of urobilin values; that is, all figures on the curve representing urobilin values have been multiplied by 4 on account of the usual dilution in preparation of the specimen. It
can be noted that in the greater dilutions, that is in all but the three strongest solutions, the strength of color is virtually a straight line function of the concentration of the dye. Nearly all specimens were diluted so as to be read on this part of the curve. In actual operation the method must necessarily be modified in order to bring the unknowns within the scope of the standard, because the standard cannot be made stronger. In order to accomplish these dilutions and not produce cloudiness in the specimens, the dilutions must be made before the entire precipitation of protein is accomplished. This somewhat difficult task must be anticipated and determined by the appearance of the specimen after the first precipitation. Inspection of the specimen is then made and the intensity of fluorescence estimated. If fluorescence is very intense further dilution must be made in order to bring the final product within the range of the standard. It has not been feasible to add alcohol to specimens after the final clearing, because it is impossible to add alcohol without either producing turbidity or else destroying the fluorescence by too great dilution of the zinc factor. With care and experience every specimen can be brought within the range of the standard and read. All normal blood specimens fall well within the lower dilutions.

All specimens were read after 24 hours standing in the ice box to precipitate before clearing in the centrifuge. The first readings were recorded and the specimens put back into the ice box unaltered, to allow further development of fluorescence, and to be read again the 2nd day and again on a 3rd day. The highest reading was taken as final. It has been found usually on the 3rd day.

Fig. 2 is a summary of 128 determinations of urobilin on human blood presumably normal. Of the 128 specimens but two were negative. In twelve instances no test could be made; this on account of inability to clear specimens. With greater experience, it is probable that no specimen will be found negative, and with good technique there should be very few with which no test can be made. The average of our series gives 0.28 mg. per 100 cc. This average may be slightly greater than is justifiable, due to including a few abnormal specimens. Blood for these tests was taken from unselected patients in a general medical clinic, where Wassermann tests were made. Blood was taken from no indi-
individual with any definite abnormality except occasionally a positive Wassermann, and such hypothetical disorder as may result from the administration of salvarsan.

Numerous pathological specimens were measured which go as high as 33 mg. per 100 cc.—the highest values being in patients with nephritis, malaria, pneumonia, and tuberculosis with fever; and one patient with complete obstruction of the gall duct and with complete intestinal acholia was found to be clearly negative. This is in harmony with current ideas of urobilin metabolism.

Since this series was described, the technique has been simplified by use of a smaller and simpler series for the standard as follows:

**Parent Solution.**—1 gm. of acriflavine in 1000 cc. of distilled water.

**Stock Solution.**—1 cc. of parent solution in 99 cc. of distilled water. To be made up weekly.

**Standard.**—Make a 1:100 dilution of the stock solution, which is Tube 1 in the series, being a 1:10,000,000 solution of the dye. The remaining five tubes of the series are made from Tube 1 by use of a graduated 1 cc. pipette for the solution and a graduated 10 cc. pipette for the water, worked into 10 cc. tubes, and with dilutions as follows: 1:3, 1:5, 1:7, 1:10, and 1:20. The final series of six tubes must contain about 3 cc. so that all solutions and unknowns are of equal depth when readings are made. From our computation, Elman and McMaster values being used and allowance made for the 1:4 dilution of the unknown, each tube of the standard has the following value in mg. of urobilin per 100 cc. of blood.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Value (mg. per 100 cc.)</th>
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**BIBLIOGRAPHY.**

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