A DIRECT COLORIMETRIC METHOD FOR THE DETERMINATION OF UREA IN BLOOD AND URINE

By ANDREW A. ORMSBY

(From the Department of Biochemistry, School of Medicine, University of Texas, Galveston)

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At the present time urea is usually determined by one of three methods; *viz.*, the aeration and titration procedure of Van Slyke and Cullen (1), the gasometric technique of Van Slyke (2), or one of the many modifications of the nesslerization procedure. The Van Slyke and Cullen procedure is accurate but requires 3 ml. of whole blood and is time-consuming; the gasometric method is also accurate but requires special equipment which is often not available in clinical laboratories. The chief fault of the nesslerization procedures is their inaccuracy, due primarily to the readiness with which the final solution becomes turbid and to a difference in quality of the color produced in the standard and sample. Gentzkow (3) claims to have overcome these errors in a recent modification of the direct nesslerization technique. All of these methods, with the exception of the gasometric procedure, also suffer from the disadvantage that ammonia is an interfering substance and must be determined separately.

The method to be described has the following advantages: (1) it is a colorimetric reaction which is applied directly to urine or to blood filtrate, no aeration or distillation being necessary; (2) ammonia does not interfere with the reaction; (3) a degree of accuracy is possible which surpasses that of most nesslerization procedures and is at least equal to that of the Van Slyke techniques; (4) the method is believed to be the simplest and most rapid yet described.

The method is based on a reaction first described by Fearon (4). When urea is heated with biacetyl monoxime in acid solution, a yellow color develops, deepening on subsequent oxidation with potassium persulfate. Many substituted ureas give a red color, but only urea yields a yellow pigment. Fearon used the reaction for the approximate determination of citrulline in casein, Gornall and Hunter (5) later modifying the procedure for the accurate determination of citrulline in tissues. Abelin (6) applied the reaction to the determination of urea in serum, but, since the reaction was carried out in neutral solution and no oxidizing agent was added, only a very rough approximation to the amount of urea present was given.

Fearon (4) has studied the specificity of the reaction. Urea was the

only substance giving a yellow to orange color but the following compounds gave rise to a red color, similar to that obtained with citrulline: methylurea, butylurea, phenylurea, β-naphthylurea, dimethylurea, allantoin, semicarbazide, citrulline, and all higher proteins examined. The test was found by Fearon to be negative with ammonium salts, hydrazine, carbamate, cyanate, acetamide, diphenylurea, guanidine, methylguanidine, creatinine, creatine, glycocyamine, uroxamic acid, uric acid, indole, and all amino acids examined (glycine, glycine ester, sarcosine, alanine, cystine, tyrosine, tryptophane, arginine, histidine, lysine, proline, hydroxyproline, asparagine, aspartic acid). Fearon (4) concluded that "the test is positive with compounds containing the system $R_1 \cdot NH \cdot CO \cdot NH \cdot R_2$, where R_1 is either hydrogen or a simple aliphatic radicle, and R_2 is not an acyl radicle." Gornall and Hunter (5) added some α-carbamido derivatives to the list of substances giving a positive test. Allantoin has been further tested in our own laboratory and was found to give only a very feeble positive reaction. Although human urine was used throughout in the experiments to be described, the feeble color given by allantoin in animal urines would introduce no appreciable error. The small amount of color introduced by allantoin would be further minimized by the use of the proper filter in the colorimeter.

EXPERIMENTAL

The method given below was found to be that which would combine an optimum stability with an optimum intensity of color. The conditions necessary for maximum color development from urea are very similar to, although not identical with, those which are used in the determination of citrulline (5).

Method

Reagents—

1. Concentrated HCl.

2. Biacetyl monoxime; a 3 per cent aqueous solution. This solution appears to last indefinitely when kept in the refrigerator.

3. Potassium persulfate; a 1 per cent aqueous solution. The solution is somewhat unstable, and should be made up weekly and kept in the refrigerator. Actually it is good for a somewhat longer period, but solutions 4 weeks old were found to give definitely lower results than did those which were freshly prepared.

4. Standard urea solution. A solution was prepared such that 1 ml. contained 0.1 mg. of urea. A little chloroform was added as a preservative.

Procedure—The volume of sample used should contain preferably from 0.1 to 0.2 mg. of urea (extreme limits, 0.05 to 0.3 mg.) and must not exceed 3 ml. For most samples of human urine 0.01 ml. (1 ml. of a 1:100

dilution), and for blood 3 ml. of the usual Folin-Wu filtrate were sufficient. Place appropriate volumes of sample in test-tubes, 1 ml. and 2 ml. of urea standard in similar tubes, and 3 ml. of distilled water in another tube to serve as a blank. The volume in each tube is then made up to 3 ml. with distilled water.

Add to each tube 5 ml. (accurately measured) of concentrated HCl. Follow this with 0.5 ml. of 3 per cent biacetyl monoxime.

Mix the contents by rotation and place the tubes in a vigorously boiling water bath for *exactly* 10 minutes. During the heating it is necessary to prevent evaporation. This may be accomplished by covering the tubes with hollow glass bulbs, small funnels, or some similar device. In this laboratory ordinary glass marbles were used successfully.

Remove the tubes simultaneously and cool for 2 minutes in running water.

Add slowly 0.25 ml. of 1 per cent potassium persulfate, so that a separate layer is formed. After this reagent has been added to all of the tubes, stopper, and mix simultaneously by inverting a few times.

The intensity of color is then read at intervals in a Klett photoelectric colorimeter, with a No. 42 filter. The time required for development of the maximum color depends on the concentration of urea. If the sample contains 0.1 mg. of urea, the maximum develops in about 5 minutes after mixing with the persulfate; 0.2 mg. requires 10 to 15 minutes, and 0.3 mg. from 25 to 30 minutes. Since the color fades at a very slow rate, it is sufficient, for most purposes, to take readings at, say, 5, 15, and 25 minutes. With a little experience one can easily judge the approximate time at which the maximum will occur by noting the color of the tubes when removed from the water bath.

The results may be calculated in the usual way by reference to the reading of the standard. Since, however, the color-concentration curve is not a straight line throughout the whole range, some error is introduced by this method of calculation if the standard and unknown readings are too far apart. Better results are obtained if the values are taken from a calibration curve (Fig. 1). Although the weekly changes in persulfate solution have no effect on the curve, it was found that a new curve had to be constructed for each solution of biacetyl monoxime. Fortunately the latter reagent is stable and can be prepared in large amounts, so that one curve will last for a long period.

The method as outlined was designed for use with the Klett photoelectric colorimeter, but results sufficiently accurate for clinical use can be obtained with the ordinary visual colorimeters.

Comparison with the Van Slyke-Cullen Method. Urine Analyses—Table I shows the results on a number of urines analyzed by both methods. The values are in good agreement, especially when results by the biacetyl

monoxime method are obtained from the calibration curve. In subsequent tables all values given were obtained by reference to the curve.

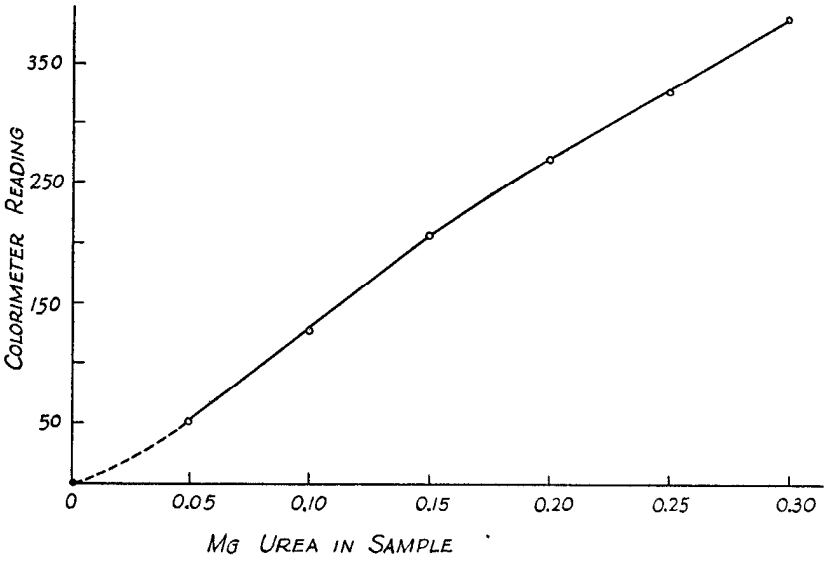

FIG. 1. Calibration curve showing the relation between the amount of urea in the sample and readings on the Klett photoelectric colorimeter, with No. 42 filter.

TABLE I

Comparison of Biacetyl Monoxime Method with Van Slyke-Cullen Procedure for Determination of Urea in Urine

All values are expressed as gm. of urea per 100 ml. of urine.

Specimen No.	Van Slyke-Cullen method	Biacetyl monoxime method	
		Calculated from standard	From curve
1	2.50	2.51	2.54
2	2.16	2.12	2.13
3	2.26	2.21	2.26
4	2.82	2.90	2.82
5	2.51	2.65	2.58
6	2.21	2.26	2.18

Blood Analyses—The values found by the two methods did not agree as closely for blood as had been the case with urine (Table II). It was felt, however, that, at least in our hands, the more accurate results were those obtained with the biacetyl monoxime method. In the first place, checks on duplicate samples showed much better agreement by the present

method than by the Van Slyke-Cullen procedure. Secondly, excellent recoveries of added urea were obtained by the biacetyl monoxime method and, finally, the potential errors involved in the application of the Van Slyke-Cullen technique to blood analysis are certainly greater than when that method is used for urine, even though Van Slyke (2) was able to obtain the same results from whole blood and from the Folin-Wu filtrate, using the aeration and titration method in the one case and the gasometric technique in the other. Cells of whole blood have been shown to contain arginase (7, 8). With sufficient time, there will be some urea formation from the action of this arginase on the arginine of the blood. During the incubation of whole blood with urease one must, therefore, strike a happy medium between too short an incubation, giving low results because of incomplete conversion of urea to ammonia and CO_2, and too

TABLE II

Comparison of Biacetyl Monoxime Method with Van Slyke-Cullen Procedure for Determination of Urea in Blood

All values are expressed as mg. of urea per 100 ml. of whole blood.

Specimen No.	Van Slyke-Cullen method	Biacetyl monoxime method
1	35.2	34.2
2	29.5	28.0
3	29.8	30.0
4	34.7	36.5
5	34.1	34.2
6	21.6	21.2
7	40.1	40.4

long an incubation, giving high results because of the formation of urea from arginine.

Therefore, whereas at least 15 minutes (we have used 30) are advised for the incubation of urease with urine, only 5 minutes are recommended for blood. In a study of the effect of blood cells on the determination of urea by the Van Slyke-Cullen method, Behre (8) concluded that the true urea content of blood can be obtained only by the analysis of blood filtrates. It must be admitted that the color obtained with blood filtrates treated with biacetyl monoxime is slightly different, qualitatively, than that obtained with the standard urea solution, owing to the presence of small amounts of citrulline or other material giving the carbamido reaction. The colors given by urea and by carbamido compounds are quite different. Fig. 2 shows the per cent transmission of light between 400 and 650 mμ, as determined with the Coleman spectrophotometer, for the colors produced by urea and by citrulline. The No. 42 filter, supplied with the Klett

photoelectric colorimeter, allows passage of light between 400 and 465 mμ. It is in this range that the two absorption curves diverge most. The use of this filter, then, eliminates a considerable part of the small error introduced by the foreign color. If this color were the source of the lack of agreement between the two methods, the results by the present method should be consistently higher than those obtained by the Van Slyke-Cullen procedure, and this was not the case.

Some preliminary work with the Coleman spectrophotometer indicated that this instrument offers definite advantages when the present method

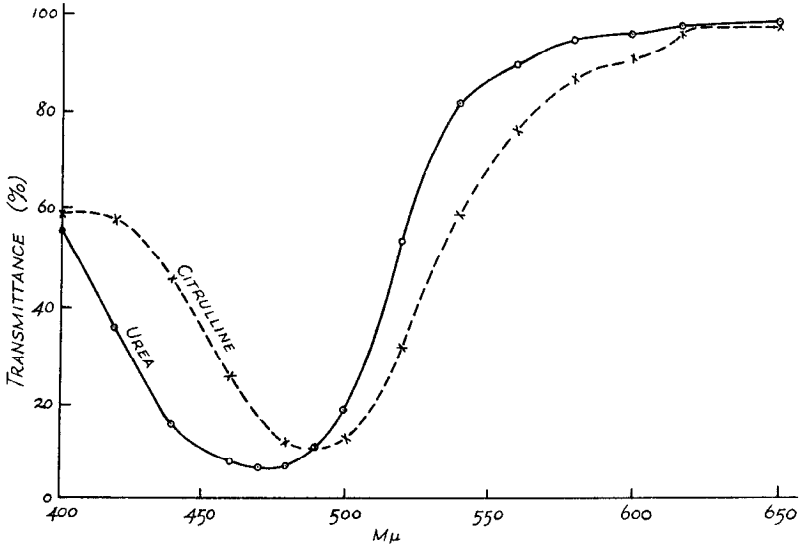

FIG. 2. Curves showing the per cent transmission of light between 400 and 650 mμ by colored solutions obtained by treating urea and citrulline with biacetyl monoxime in acid solution, followed by persulfate.

is applied to blood analysis. The method becomes more sensitive; so that low blood ureas, which are near the lower limit for the method as outlined above, give satisfactory readings, and the foreign color found in blood filtrates is perhaps more adequately eliminated by the use of a more nearly monochromatic light source.

Recovery of Added Urea—Recovery of urea added to urine is shown in Table III. In the last three specimens the urine was treated with urease to remove urea already present, the urease then being removed by acidifying, boiling, and filtering the urine. It was found, however, both by the present method and by the Van Slyke-Cullen procedure that the resulting urine still contained 28 mg. of urea per 100 ml.

Recovery of urea added to blood is shown in Table IV. The original urea content of the blood is much lower than in the samples of Table II. The blood specimens of Table II were mixed samples, some being pathological, from the clinical chemistry laboratory, whereas those of Table IV were from normal individuals.

TABLE III

Recovery of Urea Added to Urine

All results are expressed as mg. of urea per 100 ml. of urine.

Specimen No.	Urea originally present	Urea added	Urea found	Per cent of theoretical
1	1710	211	1940	101.6
1	1710	306	2065	102.4
2	1360	518	1864	99.2
3	2258	289	2516	98.8
4	1750	521	2214	97.5
5	28*	1626	1656	100.1
5	28*	1832	1884	101.3
5	28*	2027	2088	101.6

* Urea largely removed by treatment with urease.

TABLE IV

Recovery of Urea Added to Blood

All results are expressed as mg. of urea per 100 ml. of whole blood.

Specimen No.	Urea originally present	Urea added	Urea found	Per cent of theoretical
1	20.5	7.5	28.1	100.4
2	20.3	15.0	36.1	102.3
2	20.3	25.0	45.2	99.8
3	27.8	15.0	42.6	99.5
4	26.8	20.0	47.8	102.2
5	20.8	20.0	40.6	99.5

Time of Maximum Color Development—The rate of development of color with various concentrations of urea is indicated in Fig. 3. The time required to reach the maximum color is seen to increase with the concentration of urea. If it is necessary to obtain the greatest possible accuracy, successive readings must be taken frequently enough so that the true maximum will be observed. Since, however, the rate of fading is slow, it is possible to obtain fairly accurate results, suitable for most purposes, by taking readings at 5 and 10 minutes when the concentration appears low, and at 20 and 30 minutes for samples with a higher concentration of urea.

Effect of Time of Heating—The amount of color developed is dependent on the length of time in which the tubes are in the boiling water bath. The intensity of color increases rapidly up to 9 minutes boiling, and then increases more slowly. 10 minutes were selected as a reasonable length of time which would give nearly the maximum amount of color obtainable. In order to get reproducible results it is necessary to adhere strictly to a given time of heating. In addition to this it is also advisable to reproduce as closely as possible the other conditions of heating. To this end we have used test-tubes of a uniform diameter and have avoided superheating by employing a wire rack which supports the tubes vertically, about 2 inches

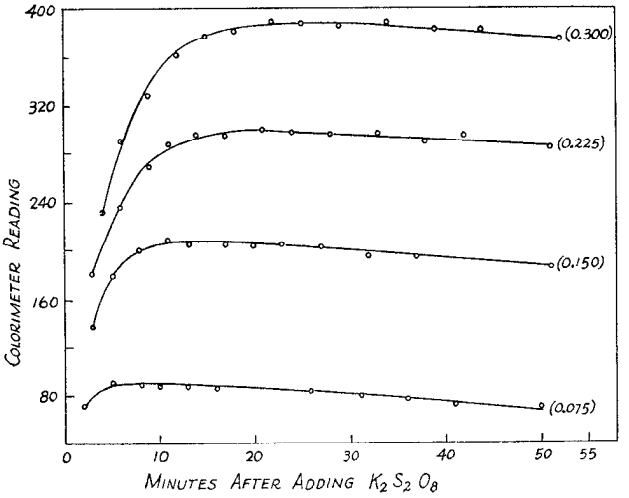

FIG. 3. Curves showing the relationship between colorimeter readings and time elapsed after addition of persulfate for various concentrations of urea. The figures in pare ntheses indicate the number of mg. of urea in the sample.

from the bottom of a large water bath. The water in the bath must, of course, be boiling vigorously throughout the entire heating period. Lower temperatures (37°, 45°, 60°) were found to give such a slow development of color as to be entirely impracticable.

Amount and Kind of Oxidizing Agent—Oxidation is necessary in order to get the maximum intensity of color, but an excess of oxidizing agent will cause a rapid destruction of the pigment. Fearon found that, whereas most of the commoner oxidizing agents would develop the color, potassium persulfate was less destructive to the formed pigment. Different amounts of persulfate were tried and it was determined that the optimum effect, maximum color with slowest fading, was obtained by using 0.25 ml. of a 1 per cent solution.

Effect of Light—During the early part of this work a set of tubes was inadvertently placed, after addition of persulfate, in the direct sunlight, and was found to fade very rapidly. Further experiments disclosed that only direct sunlight had this effect. Curves, showing development of color against time, constructed for samples carried through the procedure on a bright day in the ordinary light of the laboratory were identical with curves for other samples carried through the procedure in darkness, the latter being brought into the light only for the addition of reagents. When the samples are placed in the sunlight *after* the addition of persulfate, the only effect noted is a rapid fading. If, however, two similar sets of tubes are carried through the procedure, one set being placed in a boiling water bath in direct sunlight, while the other is heated in the ordinary light of the laboratory, it is readily seen that the effect of sunlight at this stage is to *increase* the color, the deep yellow color produced being apparently the same as that formed on the addition of persulfate in the regular procedure. This color, however, fades very rapidly and it was not possible to determine the maximum intensity by colorimetric readings. It appears, then, as if direct sunlight has, at least qualitatively, the same effect as does the addition of an excess of potassium persulfate.

Other Factors—The amount of biacetyl monoxime solution to be used was determined experimentally and 0.5 ml. of a 3 per cent solution was found to give the best results. Most samples of biacetyl monoxime are not pure white but are slightly tinged with yellow. The amount of yellow color may increase in stored bottles. Apparently this has no effect on the determinations, the foreign color being taken care of by the blank. It was found, however, that the calibration curve changed materially when a new batch of biacetyl monoxime solution was prepared. However, as mentioned previously, the reagent is stable and can be prepared in rather large amounts, thus obviating the necessity of constructing new calibration curves at frequent intervals.

Fearon (4) states that "H_2SO_4, H_3PO_4 or trichloroacetic acid may be used as condensing agents instead of HCl, but appear to offer no advantages." This was with reference to the color produced with citrulline. We have found that the only acid which could be substituted for HCl in the determination of urea was sulfuric acid, and that even in this case the intensity of color was distinctly less than when HCl was used. The quantities of sample and acid employed in the procedure given above were designed as being particularly suited for the Klett photoelectric colorimeter.

SUMMARY

A direct colorimetric procedure for the determination of urea in blood filtrates and in urine has been presented.

The procedure is based on the reaction between urea and biacetyl monoxime in acid solution to give a yellow color, deepening to a yellow-orange on subsequent oxidation with potassium persulfate.

The method is believed to possess the following advantages: (1) it is rapidly and simply carried out, requiring no special apparatus or particular degree of skill; (2) the determination can be carried out with 3 ml. of blood filtrate; (3) ammonia offers no interference; and (4) the degree of accuracy compares well with that of the best previously published procedures.

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