the sample are made in the same way as the diffraction measurements, without moving the sample, which is an advantage over the method proposed by Leroux, Lennox, and Kay (*5*). Furthermore, in all respects the method gives the results predicted by theory, without the need for determining an empirical correction factor as was found necessary by Leroux *et al.*

When using copper radiation, the method is restricted to a certain extent to the analysis of materials of fairly low absorption, and the lower limit of concentration that can be estimated is rather high. However, the use of molybdenum radiation should eliminate both these defects somewhat.

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Determination of Blood Urea with p-Dimethylaminobenzaldehyde

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▶ **A simple, rapid, accurate procedure is described for the routine determination of urea in protein-free filtrates of blood plasma or serum with an acidified solution of p-dimethylaminobenzaldehyde in ethyl alcohol. More than 1000 plasmas were analyzed; only sulfa drugs interfered. A simple screening technique to detect contaminated filtrates is described and a procedure to circumvent the contamination is given. The effects of such variables as concentrations of reagents, temperature, and time are discussed. The standard curve is linear to 100 mg. of urea nitrogen per 100 ml. Higher values may be determined by dilution with the reagent.**

THE colored solution formed by urea and a reagent consisting of *p*-dimethylaminobenzaldehyde (PDAB) in ethyl alcohol and hydrochloric acid has been introduced as a colorimetric procedure for the determination of urea in samples containing urea, hydrazine, semicarbazide, and ammonium ion (*5*). A similar reagent was used to determine urea in an enzymatic, urea-synthesizing system (*4*). This report describes a procedure for the routine determination of urea in protein-free filtrates of blood serum or plasma with a *p*-dimethylaminobenzaldehyde reagent.

REAGENTS

Reagents are analytical grade unless otherwise indicated.

Zinc Sulfate, 10%. Dissolve 100 grams of zinc sulfate heptahydrate in distilled water and dilute to 1 liter.

Sodium Hydroxide, approximately 0.5*N*. Dilute a saturated solution of carbonate-free sodium hydroxide to provide 1 liter of approximately 0.5*N* sodium hydroxide. The zinc sulfate and sodium hydroxide solutions should be balanced as follows: Dilute 10 ml. of the zinc sulfate solution to about 50 ml. with water and titrate slowly with the sodium hydroxide to the phenolphthalein end point. Ten ± 0.05 ml. should be required. If necessary, dilute the stronger solution with water and titrate again.

p-Dimethylaminobenzaldehyde – Sulfuric Acid Solution. Dissolve 5 grams of *p*-dimethylaminobenzaldehyde (Eastman No. 95) in 95% ethyl alcohol (U.S.P. or reagent grade) and dilute to 100 ml. with 95% ethyl alcohol. Most lots of PDAB were used as received; occasionally however, a lot would produce such a high blank that it had to be recrystallized (*1*). In every instance the alcoholic solution had to be filtered to make it clear.

Slowly add 5 ml. of concentrated sulfuric acid to about 50 ml. of the PDAB solution in a 100-ml. volumetric flask, mix, and allow to cool to room temperature. Dilute to the mark with more of the solution. This reagent has a fairly intense yellow color. It is stable for many weeks at room temperature.

Alcoholic Buffer Solution. Dissolve 10 grams of sodium acetate trihydrate and 1 gram of (ethylenedinitrilo)tetraacetic acid, disodium salt, dihydrate (disodium EDTA) in about 50 ml. of water in a 100-ml. volumetric flask. Add 30 ml. of 95% ethyl alcohol and dilute to the mark with water. The pH should be approximately 6.8.

Urease Solution. Add 20 ml. of the alcoholic buffer solution to 0.5 gram of defatted jack bean meal (Sigma Chemical Co.) in an Erlenmeyer flask. Shake for 5 minutes and filter. A sediment forms on standing, but the supernatant fluid may be used. This urease retains its activity for several days, if stored in a refrigerator.

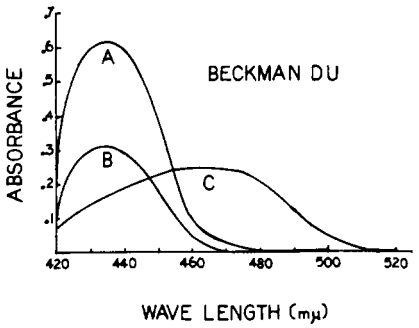

Figure 1. Absorption spectra of p-dimethylaminobenzaldehyde reagent

A. Urea equivalent to 50 mg. of urea N/100 ml.
B. Urea equivalent to 25 mg. of urea N/100 ml.
C. Sulfathiazole equivalent to 2 mg./100 ml. Blank solution was equal volumes of water and color reagent

Urea-Free Plasma. Pooled plasma (or serum) free of urea is required for a blank of the unknowns. The following preparation has been found convenient: Pool several plasmas that have normal urea concentrations and do not contain sulfa drugs to obtain about 5 ml. Add 3 or 4 drops of the urease preparation, cover, and allow to stand overnight at room temperature. (The presence of sulfa drugs can be detected as described in the procedure.)

Standard Urea Solutions (50 mg. of urea nitrogen per 100 ml.). Dissolve 107 mg. of urea in water in a 100-ml. volumetric flask and dilute to the mark. Prepare other standards as required by diluting aliquots of the standard with water.

APPARATUS

A Beckman DU spectrophotometer with 1-cm. Corex cells and a Bausch & Lomb Spectronic 20 spectrophotometer with 0.5-inch round cuvettes were used.

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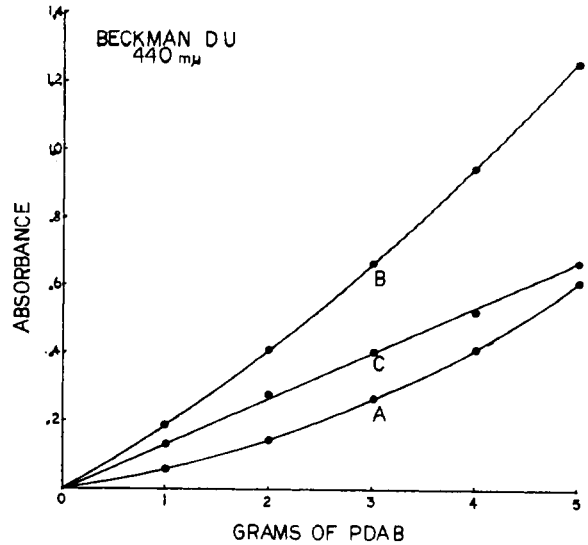

Figure 2. Effect of increasing concentration of PDAB in color reagent

A. Equal volumes of color reagent and water *vs.* water blank
B. Equal volumes of color reagent and 50 mg. urea N standard *vs.* water blank
C. The corresponding solutions from *A* used as blanks to determine absorbances of solutions from *B*

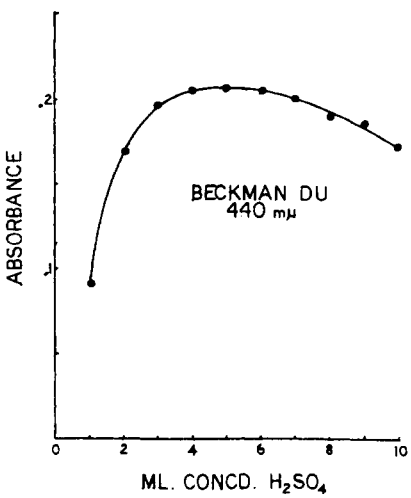

Figure 3. Effect of increasing concentration of sulfuric acid in the PDAB reagent

Equal volumes of color reagent with increasing sulfuric acid content and 20 mg. of urea N standard measured against color reagent–water blanks with corresponding amounts of acid

Because of the intense color of the PDAB reagent, the Coleman Junior spectrophotometer could not be used.

PROCEDURE

Pipet 1 ml. of water (blank for standards), 1 ml. of urea-free plasma (blank for unknowns), 1-ml. aliquots of the standards, and 1-ml. aliquots of the unknown plasmas or serums into appropriately labeled test tubes. Add 7 ml. of water to each and mix. Put 1 ml. of the zinc sulfate solution in each and mix thoroughly. Place 1 ml. of the sodium hydroxide solution in each test tube and mix thoroughly. Filter into appropriately labeled beakers. Transfer 2-ml. aliquots of each filtrate to test tubes or cuvettes that are labeled to correspond to the beakers. Add 2 ml. of the *p*-dimethylaminobenzaldehyde–sulfuric acid color reagent to each and mix thoroughly. Allow to stand for 10 minutes. Measure the absorbance in a spectrophotometer at 440 mμ, setting the instrument at zero absorbance with the water blank for the standards, and with the urea-free plasma blank for the unknowns. Also measure the absorbance of the unknowns at 480 mμ *vs.* the urea-free plasma blank.

Any of the unknowns that has an absorbance at 480 mμ greater than about 10% of that at 440 mμ is contaminated by one of the sulfa drugs and must be treated as follows: Pipet 2-ml. aliquots of the blank filtrates, the 50-mg. urea nitrogen standard, and the suspected serum or plasma filtrate into test tubes. Add 2 drops of the urease solution to each tube, mix and let stand for 30 minutes at room temperature. Add 2 ml. of the color reagent to each, mix and let stand for 10 minutes. Determine the absorbance of each in the spectrophotometer at 440 mμ, using the appropriate blanks. The 50-mg. standard should have the same absorbance as its blank (thus assuring the activity of the urease preparation). Subtract the absorbances of the contaminated specimens from the absorbances of the respective unknowns to obtain the absorbance due to urea.

Plot the absorbances against the concentrations of the standards. Determine the concentrations of the unknowns from the standard curve.

An unknown with urea nitrogen concentration considerably higher than 50 mg. per 100 ml. may be diluted with an equal volume of the urea-free plasma blank. The absorbance is measured, the amount of urea nitrogen is determined, and it is multiplied by 2. Greater dilutions may be made if required. If a specimen of high urea concentration is also contaminated with a sulfa drug, one must be certain that the urease preparation is potent enough to hydrolyze all the urea.

DISCUSSION

Absorption Spectra. The absorption spectra of the urea standards with the *p*-dimethylaminobenzaldehyde–sulfuric acid reagent are shown in Figure 1, curves *A* and *B*. The maximum absorption is at about 435 mμ. It drops off sharply and has only slightly more absorbance than the blank at 480 mμ.

The absorption spectrum of a sulfathiazole solution equivalent to 2 mg. per 100 ml. mixed with the color reagent is also plotted in Figure 1, *C*. There is a much broader absorption maximum and nearly the same absorbance at 440 as at 480 mμ.

Those specimens that are contaminated by sulfa drugs are thus easily detected by the simple screening procedure of reading all unknowns at 480 mμ. If the unknown reads considerably higher than the blank at 480 mμ, the absorbance cannot be due to urea and a urease-treated aliquot of the specimen must be used as a blank to correct for the contamination.

The PDAB reagent blank itself has such an intense absorbance below 420 mμ that the spectrophotometer cannot be set at zero absorbance. With increasing wave length, the blank absorbance decreases sharply, but at 440 mμ it still has an absorbance that is equivalent to approximately 45 mg. of urea nitrogen per 100 ml. of plasma (Figure 2, *A*). At higher wave lengths the absorbance of the reagent blank decreases, but the absorbance of the urea-PDAB reagent also decreases.

The choice of 440 mμ as the operating wave length is a compromise to obtain a reasonably low blank, sufficient sensitivity to permit the determination of a convenient range of values for clinical purposes, and adequate sensitivity for the detection of sulfa drug contamination.

Standard Curve. The calibration curve is linear up to 100 mg. of urea nitrogen per 100 ml. with the Beckman DU at 440 mμ. Linearity with the Bausch & Lomb Spectronic 20 extends only to about 70 mg.

Effect of *p*-Dimethylaminobenzaldehyde Concentration. A series of color reagent solutions with increasing amounts of PDAB was prepared. A volume of color reagent was mixed with a volume of distilled water and the absorbance was measured, using the solution without PDAB as a blank (Figure 2, *A*). The result of substituting the urea standard, equivalent to 50 mg. of urea nitrogen per 100

ml., for the water is shown in Figure 2, *B*. The absorbance of the urea-PDAB complex alone is shown in curve *C*. The absorbance of the color reagent is subtracted by using the water–color reagent solutions with appropriate concentration of PDAB as blanks. The absorbance of the complex increases linearly with increasing concentrations of PDAB. By using high concentrations of PDAB the sensitivity with urea is increased, but the absorbance of the blank increases at an even greater rate. A study of the mechanism involved in the reaction between acidified *p*-dimethylaminobenzaldehyde and urea has indicated that it is an easily reversible, equilibrium reaction involving the combination of equimolar amounts of reactants (*2*).

Because the color reagent itself has such a marked absorbance particular care must be used in pipetting it. Otherwise small differences in the quantity of color reagent will lead to significant errors.

Effect of Acid Concentration. Hydrochloric acid was first used in the color reagent, but if the test tubes containing it and urea were left uncovered, a ring soon appeared near the open end. It was probably ethyl alcohol condensing, but it developed a yellow color that became more intense with time. Substituting sulfuric acid for the hydrochloric acid prevented the volatilization of the yellow complex.

A solution containing 6.25 grams of *p*-dimethylaminobenzaldehyde diluted to 100 ml. with 95% ethyl alcohol was prepared. Twenty-milliliter aliquots were added to a series of 25-ml. volumetric flasks. Increasing quantities of concentrated sulfuric acid were added, the solutions were mixed and allowed to cool to room temperature, and 95% ethyl alcohol was added to the marks on the flasks. Calculated on the basis of a 100-ml. volume, each flask contained 5 grams of PDAB and from 1 to 10 ml. of concentrated sulfuric acid. One volume of each color reagent was mixed with one volume of a urea standard equivalent to 20 mg. of urea nitrogen per 100 ml. and read against a blank made for each acid concentration by substituting water for the standard.

The results are plotted in Figure 3, and although there is a rather broad optimum concentration for acid, the 5 ml. per 100 ml. was chosen because it is about the maximum.

Effect of Temperature. The effect of temperature was not investigated in this study. However, Watt and Chrisp (*5*) reported a temperature coefficient of 0.6% absolute transmittancy per 1° C. over the range of 20° to 40° C. with urea solutions of 80 to 160 p.p.m. and with their less concentrated color reagent. They found the effect to be completely reversible. If standards are used with each series of unknowns, and if all the samples in a series are allowed to come to room temperature before reading, the temperature will not affect the results, providing that no large fluctuations occur in room temperature while reading.

Table I. Results with Two Methods for Urea

Sample No.	Mg. Urea Nitrogen/100 Ml. Plasma	
	Diacetyl	PDAB
1	35	32
2	8	8
3	22	24
4	19	18
5	13	12
6	27	24
7	16	18
8	10	9
9	27	26
10	27	26
11	21	19
12	19	20
13	23	23
14	82	84
15	40	38
16	41	42

Effect of Time. The color formed by the PDAB reagent and the urea solutions develops immediately and increases slowly for a few minutes. After about 10 minutes there is very little additional increase in absorbance. The blank made by adding equal volumes of color reagent and water also shows a slow increase in absorbance, but it increases negligibly after 10 minutes, provided the cuvette is stoppered and the temperature is constant.

Protein Precipitants. The zinc sulfate–sodium hydroxide system was chosen to precipitate proteins, because it yields a neutral filtrate that may also be used for enzymatic glucose determinations. Tungstic acid filtrates developed turbidities when the PDAB color reagent was added and were, therefore unsuitable. Trichloroacetic acid filtrates of mouse liver homogenates were used by Nadai (*4*), but such filtrates cannot be used for glucose determinations.

Comparison with the Diacetyl Procedure. Necessity for Urea-Free Plasma Blank. The described procedure was used to determine the concentrations of urea nitrogen in samples from the routine clinical laboratory that had been analyzed by a diacetyl method (*3*), modified for use with automatic pipets. Results with the PDAB procedure ran lower by 1 to 2 mg. of urea nitrogen per 100 ml.

Filtrates of the water blank, standards, and unknowns without sulfa drugs were then treated with the urease solution as described in the procedure, and the color reagent was added. The unknowns had absorbances that averaged about 0.012 (range about 0.005 to 0.02) unit lower than the water blank and standards. The reason for the decrease in the color of the plasma filtrates was not determined. Such an effect was also noted for trichloroacetic acid filtrates (*4*). When a urea-free plasma blank was used for the unknowns, the results agreed more closely with the diacetyl method. A typical day's results are given in Table I.

More than 1000 routine determinations were made by both the diacetyl and PDAB procedures, and only the sulfa drugs were found to interfere. As described in the procedure, this contamination is easily detected by the simple technique of reading at two wave lengths.

In addition about 300 specimens were checked for contamination with other substances by treating the filtrates with urease, adding the color reagent, and reading against a urea-free plasma blank. Again, the only interfering substances found were the sulfa drugs.

Advantages. The method is rapid if no sulfa drug contamination is present. Sulfa drug contamination is easily detected, and the additional time required to circumvent contamination is no greater than that required by the classical methods employing urease and direct nesslerization. Ammonia does not interfere.

The standard curve is linear over a much wider range of values than that from the diacetyl procedure, and higher values may be determined by simple dilution of the colored solution. One need not start again with dilutions of the original filtrates.

No heating or cooling is required. The reagents are stable. The sensitivity is adequate for the clinical ranges of urea concentrations. The same filtrate may also be used for glucose determinations.

Disadvantages. Not all colorimeters are suited for the method described. Small variations in addition of the color reagent can cause significant errors. Sulfa drugs interfere. A special blank is required for the plasma filtrates, which are needed in large amounts.

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