An Improved Diacetyl Reaction for the Estimation of Urea in Blood

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The diacetyl or carbamido reaction of Fearon (1939) has been utilized by Ormsby (1942) and by Barker (1944) for the estimation of urea in biological fluids. Archibald (1945) has used the homologue of diacetyl, α -isonitrosopropiophenone (the monoxime of benzoyl-acetyl) for a similar method which has the advantage that the colour obtained is red instead of yellow. By analogy with the Barritt (1936) reaction, in which α -naphthol is used to enhance the colour obtained from creatine and diacetyl in alkaline solution, it was considered possible similarly to enhance the diacetyl-urea reaction. The observation of Barker (1944) that thymol interfered with this reaction lent support to the hypothesis. Phenols were first tried but produced precipitates. With aromatic amines the colour produced was orange or red and not yellow, whilst diphenylamine and its derivatives produced an intense magenta colour. The reaction with *N*-phenylanthranilic acid (diphenylamine-2-carboxylic acid) has been studied in detail and adapted for the estimation of urea in blood.

EXPERIMENTAL

Effect of added substances on the carbamido reaction

The effect of the addition of various substances to the carbamido reaction was tested in the following manner. To 2 ml. of a solution of urea (0.1 mg.) 0.25 ml. of 3% diacetyl monoxime was added, followed by a few milligrams of the substance under test and finally by 4 ml. of 18*N*-H₂SO₄. The solution was placed in a boiling water bath for 10 min., and immediately after removal from the bath 0.25 ml. of 1% potassium persulphate was added. The nature of the colour was then observed. With α -naphthol and thymol a precipitate was produced which dissolved on the addition of ethanol to give a red coloration. Aniline, α -naphthylamine and α -naphthylidimethylamine gave a red or orange-red colour and clear solutions. With amidopyrin, an immediate violet colour was obtained which faded rapidly and was slowly succeeded by red. Diphenylamine and phenylanthranilic acid gave intense magenta colours.

The nature of this effect was further investigated by examination of the absorption spectra. Those obtained with α -naphthylidimethylamine, diphenylamine, and phenylanthranilic acid are given in Fig. 1. That produced by added α -naphthylidimethylamine is characteristic of the simple aromatic amines; the spectrum shows two maxima, one in the blue and one in the green, and the intensity of absorption is much less than that for the yellow colour obtained

in the absence of added amine. With the diphenylamine derivatives there is only one absorption band in the green, and the intensity is much greater than that of the original yellow colour; in the case of phenylanthranilic acid more colour was obtained with 0.02 mg. of urea than with 0.1 mg. using the diacetyl reaction.

Fig. 1. Absorption spectra of solutions obtained in the diacetyl test for urea: (1) with no added amine, 0.1 mg. of urea; (2) with added α -naphthylidimethylamine, 0.1 mg. of urea; (3) with added diphenylamine, 0.1 mg. of urea; (4) with added *N*-phenylanthranilic acid, 0.02 mg. of urea.

Investigation of reaction conditions with phenylanthranilic acid

Amount of reagents. Two molecules of phenylanthranilic acid per molecule of urea were required for maximum colour production, and in order to keep the blank as low as possible minimal amounts were used. The diacetyl monoxime and potassium persulphate could be varied within wide limits without alteration of the colour produced; the amounts eventually chosen were those used by Barker (1944). The concentration of H₂SO₄ for the condensation could be varied between 9 and 14*N* without alteration of the intensity of the colour, although the weaker acid solutions

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were more turbid and the colour faded more rapidly. Here again the conditions of Barker were retained by using 4 ml. of 18N-H₂SO₄.

Time of heating. The reaction appears to reach completion after 10 min. heating in the water bath; no further increase occurs even if the heating is continued up to 30 min. (Fig. 2).

Fig. 2. Effect of varying times of heating in water bath on the intensity of colour produced in the improved diacetyl test for urea using *N*-phenylanthranilic acid.

Development of colour. After the addition of persulphate the colour develops rapidly and reaches a fairly steady value in 10–15 min. (Fig. 3); the colour then remains stable for at least 2 hr. The persulphate must be added as quickly as possible after the tubes are removed from the water bath, otherwise colour production is poor.

Fig. 3. Rate of development of colour after the addition of persulphate, in the reaction between urea and diacetyl in the presence of *N*-phenylanthranilic acid.

Calibration curve. Calibration curves have been prepared both with the Leifo photometer and the Spekker absorptiometer (Fig. 4). The colour obeyed Beer's law up to a urea concentration of 20 µg./ml.; beyond this the readings were erratic possibly due to difficulties in measurement of the high extinctions obtained.

Specificity. In specificity this reaction is similar to the carbamido test but with certain important differences;

ammonia, glycine, histidine, tyrosine, cystine, caffeine, uric acid, barbiturates, acetamide, asparagine, creatine, sulphonamides and thiouracil all give negative reactions. Proteins and monosubstituted ureas, e.g. phenylurea, give red colorations. Semicarbazide gives a magenta colour

Fig. 4. Calibration curve for the improved diacetyl test with known amounts of urea. Dots are values obtained with Spekker photoelectric absorptiometer (spectrum green filter together with calorex heat filter); crosses are results with Leifo visual photometer (filter 530).

similar to that obtained with urea, different from the bright red obtained in the carbamido reaction and of lower intensity. Biuret gives a brown colour. Creatinine gives a positive reaction, and this is an important difference between the two tests; but fortunately the sensitivity in this case is only one hundredth of that with urea, so that creatinine will not interfere with the estimation of urea in biological fluids.

BLOOD UREA METHODS

Reagents

Zinc sulphate solution, 2% (w/v).

Sodium hydroxide, 0.1N.

Isotonic sodium sulphate. 30 g./l. of anhydrous salt.

Diacetyl monoxime, 3% (w/v) in water; this solution is stable indefinitely if kept in the refrigerator and for a considerable time at room temperature.

N-Phenylanthranilic acid, 0.005M. Phenylanthranilic acid (0.106 g.) dissolved in water containing Na₂CO₃ (0.05 g.); when dissolution is complete the solution is diluted to 100 ml.

Sulphuric acid: 18N, made by mixing equal vols. of concentrated acid and water.

Potassium persulphate, 1% (w/v). This is stable for 2 months in the refrigerator.

Urea standards. Urea of B.P.C. purity, m.p. 130–132°, is adequate for the preparation of standards. The stock standard, 0.1% (w/v), will keep for a month or more in the refrigerator. From this the working standards are prepared at frequent intervals by suitable dilution. For visual

colorimetry three standards should be used containing respectively 3, 6 and 12 $\mu\text{g. urea/ml.}$; with the procedure for 0.1 ml. samples these are equivalent to blood ureas of 30, 60 and 120 mg./100 ml. If a photoelectric colorimeter is used a single standard containing 5 $\mu\text{g. urea/ml.}$ is most suitable; this gives an extinction of c. 0.40 with the Ilford spectrum green filter.

Procedure for 0.1 ml. samples

Into a centrifuge tube containing 7.9 ml. of isotonic Na_2SO_4 pipette 0.1 ml. of blood. Add 1 ml. of 2% ZnSO_4 followed by 1 ml. of 0.1N-NaOH, mix and filter. Sufficient filtrate is obtained to enable the determination to be performed in triplicate if desired. Pipette 2 ml. of the filtrate into a test tube. Into similar tubes pipette 2 ml. of the standards, and, if a photoelectric colorimeter is used, prepare a blank using 2 ml. of water. To each tube add 0.25 ml. of 3% diacetyl monoxime and 0.25 ml. of 0.005M-phenylanthranilic acid followed by 4 ml. of 18N- H_2SO_4 . Mix thoroughly and place in a boiling water bath for 10 min. Remove from the bath and without cooling add at once 0.25 ml. of 1% potassium persulphate. Mix thoroughly and allow the tubes to stand for 15 min. for the colour to develop; when a visual colorimeter is used 10 min. suffices. Compare using the Ilford spectrum green filter. For a blood urea over 150 mg./100 ml. it is best to repeat the determination using 1 ml. of the filtrate, although a fairly reliable result can be obtained by diluting with water before comparing.

Procedure for 0.02 ml. samples

Into a centrifuge tube containing 2.5 ml. of isotonic Na_2SO_4 pipette 0.02 ml. of blood, add 0.2 ml. of 2% ZnSO_4 followed by 0.2 ml. of 0.1N-NaOH. Mix and centrifuge. Pipette a 2 ml. sample (equivalent to 0.0137 ml. of blood) into a clean tube, taking precautions to avoid contamination by any of the precipitate, and treat exactly as described for 0.1 ml. samples. The same standards can be used, and the result is multiplied by 1.46 to allow for the extra dilution.

RESULTS

The method has been compared with the manometric hypobromite method of Van Slyke & Kugel (1933), and fairly good agreement has been obtained (Table 1). In 80% of cases the difference between the two methods was less than 3 mg./100 ml. urea,

Table 1. *Comparison of the improved diacetyl reaction for urea with the Van Slyke manometric hypobromite method*

Range of differences (mg./100 ml.)	Percentage of cases
- 10 to - 6	8.6
- 6 to - 4	2.9
- 4 to - 2	17.1
- 2 to + 2	54.4
+ 2 to + 4	5.7
+ 4 to + 6	8.6
+ 6 to + 10	2.9

but in 10% of cases errors of 8–10 mg./100 ml. were obtained. In spite of this, these results compare favourably with other colorimetric urea methods in current use, and the method can be recommended for routine analysis where only small quantities of blood are available. It is not sufficiently accurate for urea clearance tests. It has the disadvantage that the final solution is strongly acid. Attempts to overcome this were unsuccessful since dilution with water causes precipitation of the colour complex, whilst dilution with ethanol causes the colour to fade. If acetone is used as diluent the colour develops in the absence of persulphate, but unfortunately only reaches its maximum value after 3 hr. standing.

SUMMARY

1. When the condensation of urea with diacetyl is carried out in the presence of an aromatic amine a red colour is produced, instead of the usual yellow. With diphenylamine derivatives an intense magenta colour is produced.

2. The reaction with *N*-phenylanthranilic acid has been studied in detail and a method for the determination of urea in blood has been derived.

3. The estimation can be performed with as little as 0.02 ml. of blood, and results sufficiently accurate for most clinical purposes are obtained.

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