A sensitive method for the colorimetric determination of urea

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SYNOPSIS A method is described for the direct colorimetric determination of urea in biological fluids. The method depends on the reaction (first described by Wheatley, 1948) between urea, diacetylmonoxime, and phenylanthranilic acid in the presence of controlled amounts of oxidant; chloride ions are included to sensitize the reaction; manganous ions stabilize the resultant colour; and phosphate enables reasonable reproducibility to be achieved.

The method is rapid and suitable for routine analytical purposes. Precision and accuracy are good; sensitivity is high for an activated acid reagent up to about one week old, and thereafter decreases.

A hypothetical mechanism for colour formation is presented.

Of the numerous methods published for the direct colorimetric determination of urea and its derivatives, the most investigated have been those based on the Fearon (1939) carbamido reaction between the urea compound and diacetyl monoxime (DAM), *iso*-nitrosopropiophenone, or diacetyl (Ormsby, 1942; Barker, 1944; Archibald, 1945; Wheatley, 1948; Natelson, Scott, and Beffa, 1951; Friedman, 1953; Koritz and Cohen, 1954; Rosenthal, 1955; Kawerau, (1946); Marsh, Fingerhut, and Kirsch, 1957; Le Mar and Bootzin, 1957; Sardou, 1958; Girard and Dreux, 1958; Crockaert and Schram, 1958; Holden, 1959).

The number of attempts which have been made to meet the difficulties encountered is indicative of the magnitude of the problem. The disadvantages which have to be overcome include the instability of the resultant colour to light and time; the differential effect of light on blanks and test solutions; low to moderate sensitivity; prolonged heating time for reaction; sensitivity of colour intensity to reaction temperature; the objectionable nature of the reagents, *e.g.*, concentrated hydrochloric acid, arsenic compounds; lack of proportionality between urea concentration and colour over a sufficiently wide range.

The present work is an attempt to develop reagents which minimize or obviate these various disadvantages. A discussion of the chemistry of the reactions involved is given.

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METHOD

REAGENTS The following are required:—

(1) *Zinc sulphate-sodium sulphate isotonic solution* Dissolve 2.6 g. zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) A.R. and 12 g. sodium sulphate anhydrous (or 27 g. decahydrate) in water and dilute to 1,000 ml.

(2) *Sodium hydroxide, 0.5 M.*

(3) *Sulphuric acid, 50% v/v (9 M) A.R.*

(4) *Manganous chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) A.R., 40% w/v.*

(5) *Sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) A.R.*

(6) *Sodium nitrate A.R. 0.05 M* Dissolve 0.4250 g. solid in water and dilute to 100 ml. and refrigerate.

(7) *Hydrochloric acid concentrated A.R. (10 M)*

(8) *Diacetylmonoxime (DAM) 1% in 0.02% v/v acetic acid* Refrigerate.

(9) *Phenylanthranilic acid (PAA) 0.1% in 20% v/v ethanol-1% sodium bicarbonate A.R.* Dissolve 100 mg. PAA in 20 ml. pure ethanol, add 1 g. sodium bicarbonate and about 60 ml. water. Dissolve and dilute to 100 ml. and refrigerate.

(10) *Acid phosphate stock reagent* To 312 g. sodium dihydrogen phosphate in a 3-litre flask add about 250 ml. water, followed by 1,230 ml. sulphuric acid 50% v/v. Add 100 ml. manganous chloride 40% and 40 ml. 10 M hydrochloric acid. Mix well and dilute to 2,000 ml. This reagent keeps indefinitely.

(11) *Activated acid phosphate reagent* Measure 4.0 ml. sodium nitrate 0.05 M into a 1,000 ml. flask; add rapidly 500 ml. acid phosphate stock reagent (10). Mix well and allow to stand at room temperature for 17 to 24 hours before setting up a calibration, although the reagent may be used immediately if standards and a blank are included with the test. This reagent keeps for about one week at

room temperature. Check regularly with the standards.

(12) *DAM—PAA reagent* Mix equal volumes of reagents (8) and (9). This reagent is best made in small quantities but keeps for several days in the refrigerator. The separate reagents appear to be stable for long periods, especially if refrigerated.

PROCEDURE FOR BLOOD SPECIMENS Measure 0.05 ml. blood, serum, or plasma into a tube containing 3.85 ml. reagent (1). Mix and add 0.1 ml. reagent (2). Mix and allow to stand for 2 min. Centrifuge or filter.

Measure 0.6 ml. protein-free solution into a Pyrex test-tube (6 in. $\times$ $\frac{3}{8}$ in.). Add 0.6 ml. reagent (12) and then run 5 ml. reagent (11) directly into the tube. The last one or two millilitres may be used to wash down the wall of the tube. Mix thoroughly. A purple colour appears if reagent (11) has matured for at least 15 hours.

Heat the tubes for 11 to 12 minutes in a bath made of metal and containing water boiling within the range 99 to 100.5°C. and equipped with an accurate thermometer. The purple colour disappears after about one minute's heating and a magenta colour slowly develops. After heating, cool immediately in running water to room temperature. Read in a spectrophotometer at 535 m μ or in a colorimeter using a yellow-green filter such as the Ilford filter No. 625.

PROCEDURE FOR URINE SPECIMENS The procedure is very simple. Dilute the urine 1 in 500 and 1 in 1,000. Take 0.6 ml. of each liquid and proceed as for blood analysis. At such high dilutions any interferences are usually of negligible proportions.

CALIBRATION Prepare a stock standard containing 62.5 mg. % urea A.R. Dilute 1, 2, and 3 ml. of this to 100 ml. These standards correspond to bloods containing 50, 100, and 150 mg. % urea. Take 0.6 ml. of each standard (containing 3.75, 7.5, and 11.25 μ g. urea) and proceed as for blood specimens. Carry out duplicate determinations. Measure against a water blank, although, if preferred, a reagent blank may be used. Thereafter check the reagent daily by including one standard in duplicate.

EXPERIMENTAL

PRELIMINARY VARIATIONS IN ACID REAGENT COMPOSITION Initially, many combinations of sulphuric, hydrochloric, phosphoric, perchloric, arsenic, and other acids were investigated in conjunction with a DAM—PAA reagent as first employed by Wheatley (1948). The reagents used fell into the following groups, namely, those which (a) produced colour which was proportional over too narrow a range of urea concentration; (b) lacked sensitivity; (c) lacked stability either of the reagent or of the colour produced; (d) gave erratic results; (e) caused precipitation.

Combinations of sulphuric, arsenic, and moderate amounts of hydrochloric acid were found to give the best results. Marsh *et al.* (1957) found that 15% sodium chloride in their sulphuric-arsenic acid reagent increased sensitivity but led to loss of proportionality between urea concentration and colour. Rosenthal, in an excellent paper, showed that high concentrations of hydrochloric

acid have a strongly inhibitory effect on colour formation from urea and DAM (PAA was not used by either of these workers.)

At a later stage of our work arsenic pentoxide was replaced by sodium arsenate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$) for greater ease in preparing the reagents. Two grades of arsenate were used, one of A.R. purity and the other of laboratory reagent grade. Surprisingly, the A.R. material gave a reagent of poor sensitivity while the other was satisfactory. Analysis of the two grades showed that the main difference lay in the nitrate content. The A.R. material contained <0.001% while the other had a content of $\sim 0.008\%$ NO_3^- . This implicated nitrate ion and not arsenate as the oxidant responsible for colour formation in our method. Omission of arsenate, but inclusion of small amounts of nitrate, gave good but erratic results, so it appeared that the arsenate had some stabilizing or 'buffering' effect. This suggested the use of other salts such as citrate, tartrate, and phosphate. Citrate and phosphate were both effective in producing intense and reproducible colours, but phosphate was finally selected since it is obtainable in a state of high purity. Phosphate-nitrate mixtures were found to give results as good as impure arsenate and were preferred since the amount of oxidant is controllable.

Other oxidants such as chlorate, perchlorate, bromate, iodate, and permanganate were investigated but proved unsatisfactory for various reasons.

Apart from PAA, other sensitizers were employed. These included other diphenylamine derivatives, quinones, and quinone imines. Of these only 4-aminodiphenylamine was found to be as effective as PAA, but since it is less stable, PAA was retained. Diphenylaminesulphonic acid (sodium salt) also produces good sensitivity but the concentration required is about four times that of PAA.

CERTAIN EFFECTS OF VARIATION IN THE REAGENTS FINALLY SELECTED When each constituent of the optimum reagents was varied in turn from the optimum used in the method, the following effects were produced:—

Acid phosphate reagent Reduction in molarity of the sulphuric acid below 5.5 M caused loss of sensitivity; above that level overoxidation of PAA, loss of hydrochloric acid, and marked turbidity were observed. Satisfactory results were obtained within the range 5 to 6 M.

The hydrochloric acid could be varied over the range 0.1 to 0.4 M without effect. Higher concentrations led to loss of colour and formation of a brown flocculent precipitate.

Increasing phosphate ion concentration up to 1M gave increasingly better reproducibility and colour stability but no change in sensitivity. At appreciably higher concentrations crystallization of salts is trouble-some.

Concentrations of manganese chloride below 0.1 to 0.2 M failed to produce satisfactory light- and time-stability. Higher concentrations yielded no further improvement.

The optimum range for nitrate ion concentration was found to be 0.32 to 0.48 mM. Higher concentrations produced higher blanks and eventually destruction of the colour formed. Sensitivity dropped rapidly below the 0.3 mM level.

Diacetylmonoxime The optimum range was found to be 0.8 to 1.2%. Below this range loss of sensitivity occurred and above it colour suppression was observed. Rosenthal (1955) found the same effects for his reagents. No special purification of the DAM was necessary.

Phenylanthranilic acid The optimum concentration range was 0.098 to 0.12%. Below this range there was loss of linearity at the higher urea levels and above it turbidities were produced. A slight opalescence is produced even within the optimum range but is not sufficiently serious to interfere with the colorimetric measurements.

Variation of relative volume of active acid reagent used in the test The ratio of the volume of active acid reagent to that of the remainder (1.2 ml.) could be varied over the range 6 : 1.2 to 3 : 1.2 without loss of sensitivity (after allowing for volume change). A ratio of 5 : 1.2 was chosen to give a convenient volume for colorimetric measurement.

TIME OF HEATING AT 100°C. Using the optimum reagents and the volumes described under Method, a series of urea standards was heated for periods ranging from 4 to 14 min. (Fig. 1).

The colour reaches a maximum in 12 min. and then begins to decrease, prolonged heating periods destroying the colour. A period of 11 to 12 min. is therefore suitable.

EFFECT OF REACTION TEMPERATURE ON COLOUR PRODUCTION Colour development was carried out over the range 90° to 105°C. in a bath controlled to $\pm 0.5^\circ\text{C}$. (Fig. 2). The optimum temperature range is 99° to 100.5°, so that a boiling water bath equipped with a calibrated

thermometer is ideal under the usual conditions of atmospheric pressure.

A bath constructed of metal is preferred since at 100°C. the coloured product is somewhat sensitive to light, values some 6% lower being observed when the reaction is carried out in daylight.

STABILIZATION OF COLOUR One of the main difficulties in the use of DAM methods (with or without the use of PAA) has been the sensitivity of the blanks and colours to light. This problem is more acute when sensitizers such as PAA are employed and this has probably been a significant reason for their avoidance.

In our search for suitable stabilizers a variety of metal ions was tried. Of these, only Sb^{3+} , Cu^{2+} , and Mn^{2+} were found to be effective. Manganese was selected because it contributes virtually nothing to the blank reading and is not toxic. With manganese chloride at the 0.2M level in the acid reagent it was found that both blanks and test colours could be irradiated at 20° to 25°C. in direct sunlight for 30 min. without significant change. In diffuse daylight or in darkness, at a level of 0.1M manganese chloride, the colour decreased by 5% per hour so that this concentration was considered adequate for normal working conditions.

SPECTRUM The absorption spectrum of the urea reaction product (curve 1, Fig. 3) has a broad band in the visible with λ_{max} at 535 m μ . The nature of the spectrum is such that accurate measurements may be made even with a colorimeter equipped with a moderately narrow band-pass filter.

FIG. 1. Effect of time of heating at 100°C. on colour production.

FIG. 2. Effect of reaction temperature on colour production.

FIG. 3. Curve 1: Absorption spectrum of reaction product from 0.25 μ mole urea.

Curve 2: Absorption spectrum of reaction product from 0.25 μ mole phenyl urea.

The curves were measured on the Beckman DK2 spectrophotometer using 1 cm. cells.

RESULTS

COMPARISON OF PRESENT METHOD WITH A UREASE METHOD Comparisons were made over a period of time under ordinary routine conditions between the present method and a method employing urease and Nessler's reagent (Connerty, Briggs, and Eaton, 1955). The results are presented in Table I and indicate a high specificity for urea in the present method, the average difference between the two being less than 3%. Single determinations only were made by each method for most of the blood specimens examined. In cases where duplicate or triplicate determinations were made by each method, closer

agreement between them was evident. No exhaustive investigation of possible interferences was therefore undertaken. This matter has been dealt with by previous workers (see, for example, Wheatley, 1948; Koritz and Cohen, 1954; Rosenthal, 1955). These authors have shown that for blood and urine the relative colour equivalents and concentrations of potential interferences are such that, in general, no significant contribution from them is likely to be encountered.

REPRODUCIBILITY Table II shows the variations encountered when repeated determinations were carried out on several standards, using two different batches of reagent and separate blanks. Under

TABLE II

REPRODUCIBILITY OF DETERMINATIONS

Reagent Batch No.	Urea (mg.%)	Colorimeter Readings	Mean Reading	Readings as % Mean
7(a)	40	0.25 0.28 0.28	0.270	92.6 103.7 103.7
5	40	0.28 0.29 0.28	0.283	98.9 102.5 98.9
7(a)	80	0.53 0.52 0.53 0.49 0.51	0.516	102.7 100.8 102.7 95.0 98.8
5	80	0.53 0.50 0.53	0.520	101.9 96.2 101.9
7(a)	100	0.61 0.62 0.58	0.603	101.1 102.8 96.2

$$\sigma = \pm 3.33\% \quad 3\sigma = \pm 10\%$$

TABLE I

COMPARISON OF THE PRESENT METHOD WITH ONE USING UREASE

Serum No.	Urea (mg.%)		% Difference Present-Urease	Serum No.	Urea (mg.%)		% Difference Present-Urease
	Present	Urease			Present	Urease	
1051	42	38	+10	1342	27	26	+4
1059	88	73	+20	1417	43	48	-10
1066	58	55	+6	1418	42	43	-3
1067	26	26	0	1425	30	26	+16
1090	28	26	+8	1492	32	34	-6
1310	25	28	-12	1493	63	63	0
1322	16	16	0	1601	56	56	0
1327	58	59	-2	1602	31	30	+3
1328	68	76	-12	1604	38	38	0
1329	34	33	+3	1678	123	125	-2
1331	40	34	+18	1608	69	63	+10
1332	33	32	+3	2237	424	381	+10

Average % difference + 2.7%

average conditions, and with urea levels as low as 40 mg. %, overall confidence limits of $\pm 10\%$ cover all results including variations in the blanks (Henry and Segalove, 1952).

RECOVERIES FROM SERUM Varying amounts of standard urea were added to blood sera and the tests carried out in the usual way. The results are shown in Table III. All determinations were carried out in duplicate and the mean values taken. Overall recoveries thus appear to be satisfactory. All measurements were made using a Beckman DK2 or a Unicam SP600 spectrophotometer.

TABLE III

RECOVERIES FROM BLOOD SERA

Serum Urea (mg. %)	Urea Added (mg. %)	Total Recovered (mg. %)	Added Urea Recovered (mg. %)	% Recovery
43	20	64	21	105
	30	72	29	97
32	53.7	89	57	106
	107.4	137	105	98
53	100	151	98	98
	300	366	313	104

Mean recovery $101 \pm 4\%$

CALIBRATIONS Three different instruments were used to measure a series of standards varying from 0 to 500 mg. % urea. The instruments were, in order of increasing order of optical resolution: (a) the Eel filter colorimeter, (b) the Unicam SP600 spectrophotometer, and (c) the Beckman DK2 ratio recording spectrophotometer.

Figure 4 shows typical calibration curves for these

FIG. 4. The relationship between the amount of urea and linear absorbance of the reaction product, when measured on three different instruments: 1 Eel colorimeter, 1.5 cm. diameter tubes; 2 Unicam SP600 spectrophotometer, 1 cm. cells; 3 Beckman DK2 ratio recorder, 1 cm. cells.

instruments. The results show clearly that there is a linear relationship between urea concentration and resultant colour up to a sample level of at least 20 $\mu\text{g.}$ urea. The range of observed linearity for any particular instrument is determined by the degree of resolution obtainable, being much lower for a filter instrument than for one possessing a monochromator. A filter instrument is satisfactory up to a level of about 150 mg. %; for higher sample values it is probably best with this instrument to repeat the test using a smaller aliquot of protein-free filtrate. The Beckman DK2 spectrophotometer gave linear results up to about 30 $\mu\text{g.}$ urea, corresponding to a blood urea level of at least 400 mg. %.

DISCUSSION

In investigations of the Fearon reaction as applied to urea, many workers have included, as did Fearon, an oxidant, the purpose of which usually has been stated to be to destroy the hydroxylamine produced by hydrolysis of the DAM. However, Archibald (1945), using *iso*-nitrosopropiophenone, and Holden (1959) both omit oxidant from their reagents and obtain reasonable sensitivity. We find that somewhat more of the yellow product of reaction between urea and DAM is formed with our unactivated acid reagent than with the activated one. Furthermore, the addition of moderate amounts of hydroxylamine has no effect on the sensitivity of the reaction either in the presence or absence of oxidant. It appears therefore that not only is oxidant unnecessary in the Fearon reaction for urea but also that it is somewhat deleterious.

It has been assumed also that diacetyl liberated by acid hydrolysis is the compound which reacts with the urea (Fearon called the reaction the 'carbamide-biacetyl reaction'). Indeed, under the conditions of our test, it may be shown spectroscopically that approximately 90% of the DAM is hydrolysed in ten minutes. However, if the DAM is replaced by diacetyl in varying concentrations, much less colour formation is evident; the addition of hydroxylamine actually enhances colour formation, but the combination is still much less effective than DAM. This compound, and not diacetyl, would appear therefore to be the one which reacts with the urea. This would also account for the fact that a relative excess of DAM is required for optimum colour formation.

Little appears to be known concerning the nature of the reaction between urea and DAM. The possibilities which suggest themselves are: (1) Straight chain condensation between one molecule of DAM and one molecule of urea; (2) the same, with a 2 : 1 ratio of DAM to urea; (3) a 1 : 1 ring condensation with formation of a substituted 1, 2, 4-triazine.

These reactions may be formulated thus:

(ii) as (i) followed by condensation with a further molecule of DAM to give

(iii)

3-hydroxy-5, 6, dimethyl-1, 2, 4-triazine

with possibly the addition of one or more protons to the triazine ring under the strongly acid conditions used.

Evidence for one or another of these possible mechanisms was sought by studying the reactions between mono- or disubstituted ureas and DAM (using colour formation as a criterion of reaction). Table IV shows the results which might be expected for the three types of reaction outlined above, according to the nature of the urea derivative used.

Monophenyl urea reacted to give approximately the same amount of colour as an equimolar amount of urea (Fig. 3, curve 2), the absorption band being shifted bathochromically by some 10 mμ. *sym*-Diphenyl urea failed to produce colour.

No account has been taken of possible steric or deactivating effects which could result upon substitution but as far as it goes the evidence indicates some kind of ring, rather than chain condensation. The formation of the triazine derivative is suggested as a tentative hypothesis on the basis of which no reaction would be expected to occur with *asym*. disubstituted ureas.

As mentioned earlier, numerous compounds were investigated as colour intensifiers and of these diphenylamine derivatives gave the most satisfactory results. This might indicate that the function of nitrate (acting either directly, or indirectly by oxidation of hydrochloric acid) in the acid medium is to oxidize the diphenylamines to diphenylbenzidines:

where R = 4-NH₂, 2-COOH or 4-SO₃H.

This is perhaps reasonable in that at higher nitrate and acid concentrations a 'diphenylamine blue' is formed almost immediately, but under the conditions used here, the oxidation apparently stops at the intermediate diphenylbenzidine stage. Confirmation of this suggestion was sought first by carrying out the reaction using N,N'-diphenylbenzidine

TABLE IV

POSSIBILITY OF REACTION BETWEEN SUBSTITUTED UREAS AND DAM

Urea Derivative	Nature of Reaction		
	One-step Chain	Two-step Chain	Ring Formation
Urea	+	+	+
Mono-substituted	+	+	+
<i>sym</i> . disubstituted	+	+	-
<i>asym</i> . disubstituted	+	-	-

and unactivated acid reagent; the formation of a magenta colour indicated that the reaction proceeded in the manner suggested. Secondly, PAA in 85% sulphuric acid was oxidized with sufficient nitrate to convert it to the intensely coloured diphenylamine blue derivative. The warm solution was then reduced to the dicarboxy derivative of N,N'-diphenylbenzidine by the dropwise addition of sodium dithionite until the colour was just discharged. Concomitantly any excess nitrate was reduced. The reaction with urea was carried out using this reduced solution and unactivated acid reagent. Again, a magenta coloured product resulted, more intense than that obtained with an equivalent amount of diphenylbenzidine. The comparative behaviour of these two benzidine derivatives as regards intensity of colour production thus parallels the behaviour of PAA and diphenylamine (or diphenylamine sulphonic acid) on oxidation and thus suggests strongly that it is the diphenylben-

zidine structure which interacts with the urea-DAM condensation product.

Since it is difficult to conceive of a ready mechanism whereby this product could react with diphenylbenzidines, it is suggested tentatively that the final step in colour production is the formation of a magenta-coloured molecular complex from the two components, stabilized by manganese ions.

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