Urea

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Urea, the diamide of carbonic acid, is the most important degradation product of protein metabolism in humans, other mammals and certain other animal species. Urea, which is formed in the liver, composes the major fraction of the organic substances present in urine.

Urea can be identified and determined by many methods. The qualitative identification according to [1] or [2] is unspecific. Of the quantitative methods, the determination with urease or with xanthydrol is the most successful. The latter determination can be performed gravimetrically[3], by *Kjeldahl* nitrogen estimation[4], colorimetrically[5], oxidimetrically[6,7] or by nephelometry[8].

The reaction with urease *) produces carbon dioxide and ammonia (equation 1). Both reaction products can serve to determine the urea content of the sample. The CO_2 can be determined gasometrically[16–20], the NH_3 can be determined directly[21–28], after distillation into a receiver[22–50], or by

*) refer, for example, to[9–15]. The older work is mainly quoted according to *H. Stetter*: Enzymatische Analyse. Verlag Chemie, Weinheim/Bergstr. 1951.

1) *H. K. Barrenscheen* and *D. Weltmann*, Biochem. Z. *131*, 591 [1922].
2) *W. R. Fearon*, Biochem. J. *33*, 902 [1939].
3) *P. C. Wenger*, *C. Limeran* and *A. Maulbetsch*, Mikrochemie *8*, 132 [1933/34].
4) *J. F. Barrett* and *E. B. Jones*, Biochem. J *26*, 1246 [1932].
5) *M. H. Lee* and *E. M. Widdowson*, Biochem. J. *31*, 2035 [1937].
6) *L. Cuny* and *J. Robert*, Bull. Soc. Chim. biol. *12*, 171 [1930]; *13*, 1167 [1931].
7) *F. W. Allen* and *J. M. Luck*, J. biol. Chemistry *82*, 693 [1929].
8) *M. Viré*, Bull. Soc. Chim. biol. *22*, 185 [1940].
9) *O. Folin* and *A. Svedberg*, J. biol. Chemistry *88*, 77 [1930].
10) *W. Ohlsson*, Acta physiol. scand. *1*, 278 [1940].
11) *H. W. Norbert*, J. Lab. clin. Med. *26*, 405 [1940].
12) *E. J. Conway*, Biochem. J. *27*, 419, 430 [1933].
13) *E. J. Conway* and *E. O'Malley*, Biochem. J. *36*, 655 [1942].
14) *C. J. Gentzkow*, J. biol. Chemistry *143*, 531 [1942].
15) *H. Pichler*, Wien. Z. inn. Med. *29*, 562 [1948].
16) *S. Patros*, Biochem. Z. *103*, 292 [1920].
17) *Z. Aszodi*, Biochem. Z. *128*, 391 [1922]; *134*, 546 [1923].
18) *A. Mishin*, J. Lab. clin. Med. *8*, 50 [1922].
19) *D. D. van Slyke*, Proc. Soc. exp. Biol. Med. *22*, 486 [1925].
20) *D. D. van Slyke*, J. biol. Chemistry *73*, 695 [1927].
21) *A. Hahn* and *J. Saphra*, Dtsch. med. Wschr. *40*, 430 [1914].
22) *A. Hahn*, Dtsch. med. Wschr. *41*, 134 [1915].
23) *E. L. Kennaway*, Brit. J. exp. Pathol. *1*, 135 [1920].
24) *Y. Morimoto*, J. Biochem. *1*, 69 [1922].
25) *G. Revoltella*, Biochem. Z. *134*, 347 [1923]; *144*, 229 [1924].
26) *F. W. Fox*, Biochem. J. *22*, 545 [1928].
27) *S. W. Cole*, Biochem. J. *25*, 1653 [1931].
28) *L. D. Scott*, Brit. J. exp. Pathol. *21*, 93 [1940].
29) *O. Folin*, Hoppe-Seylers Z. physiol. Chem. *37*, 161 [1903].
30) *E. K. Marshall* jr., J. biol. Chemistry *15*, 487 [1913].
31) *D. D. van Slyke*, *G. Zacharias*, and *G. E. Kullen*, Dtsch. med. Wschr. *40*, 1219 [1914].
32) *D. D. van Slyke* and *G. E. Kullen*, J. biol. Chemistry *19*, 211 [1914]; *24*, 117 [1916].
33) *H. S. White* and *J. G. Williams*, Pharmaceutic. J. (*4*) *42*, 323 [1916].
34) *C. H. Fiske*, J. biol. Chemistry *23*, 455 [1915].
35) *R. Bahlmann*, Nederl. Tijdschr. Geneesk. *64*, I, 473 [1920].
36) *K. L. Gad-Andersen*, J. biol. Chemistry *39*, 267 [1919]; *51*, 373 [1922].
37) *G. M. Wishart*, Biochem. J. *17*, 403 [1923].
38) *G. Revoltella*, Biochem. Z. *144*, 229 [1924].
39) *F. C. Koch*, J. Lab. clin. Med. *11*, 774 [1926].
40) *W. Laubender*, Biochem. Z. *186*, 162 [1927].
41) *F. Rappaport* and *A. Glaser*, Klin. Wschr. *11*, 814 [1932].
42) *F. Rappaport*, Mikrochemie *14* (*N. F. 8*), 49 [1933].

titration after diffusion[12,13,51-57]. The ammonia can also be determined colorimetrically with *Nessler*'s reagent after separation by distillation[58-62] or by ion exchange adsorption[63,64].

The simplest method is the direct colorimetric determination of NH_3 in the hydrolysis mixture with *Nessler*'s reagent after deproteinization[9,10,50,66-71]. This method is just as accurate[65] as those described above if the necessary blanks are carried out.

Principle

Urease catalyses the reaction:

(1) $$H_2N-CO-NH_2 + H_2O \longrightarrow CO_2 + 2\,NH_3$$

The reaction proceeds virtually completely from left to right. The amount of ammonia formed is the equivalent to the urea present. The ammonia is determined colorimetrically with *Nessler*'s reagent at 436 mμ or a similar wavelength. As the *Nessler* reaction is very sensitive and the colour depends on several factors, the measured value is related to a standard.

Reagents*)

1. Ammonium sulphate, A. R.
2. Glycerol, A. R.
3. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
4. *Nessler*'s reagent, $K_2[HgI_4]$
   preparation, see for example[72]; obtainable ready-made (*e.g.* Merck, Darmstadt, Germany).
5. Urease
   dry powder, commercial preparation, see p. 1000.

*) Complete reagent kits are available commercially, see p. 1036.

43) *J. Y. Yee* and *R. O. E. Davis*, Ind. Engng. Chem., analyt. Edit. *7*, 259 [1935].
44) *P. B. Rehberg*, Biochem. J. *19*, 278 [1925].
45) *J. Patterson*, Biochem. J. *19*, 601 [1925].
46) *W. W. Kay* and *H. L. Sheehan*, Biochem. J. *28*, 1784 [1934].
47) *A. E. Sobd*, *H. Yusha* and *J. Cohen*, J. biol. Chemistry *118*, 443 [1937].
48) *G. L. Peskett*, Brit. J. exp. Pathol. *15*, 306 [1934].
49) *L. C. Murphy* and *R. C. Jenkins*, J. Lab. clin. Med. *13*, 1049 [1928].
50) *B. Reichert* and *H. Schwebs*, Pharmazie *2*, 348 [1947].
51) *B. v. Horváth* and *H. Kadletz*, Dtsch. med. Wschr. *42*, 414 [1916].
52) *G. E. Gibbs* and *P. L. Kirk*, Mikrochemie *16*, (*N. F. 10*), 25 [1934].
53) *J. Abelin*, Biochem. Z. *297*, 203 [1938].
54) *E. Kaweran*, Sci. Proc. Roy. Dublin Soc. *22*, 405 [1941].
55) *F. Rappaport* and *M. Gutmann*, Klin. Wschr. *14*, 1325 [1935].
56) *G. Nørby*, Acta med. scand. Suppl. *78*, 794; cf. Chem. Zbl. *1928*, I, 2599.
57) *V. E. Kinsey* and *P. Robison*, J. biol. Chemistry *162*, 325 [1946].
58) *O. Folin* and *H. Wu*, J. biol. Chemistry *38*, 81 [1919].
59) *E. W. Hindmarsch* and *H. Priestley*, Biochem. J. *18*, 252 [1924].
60) *A. Kliesiecke*, C. R. Séances Soc. Biol. Filiales Associées *95*, 899 [1926].
61) *A. Kliesiecke*, Biochem. Z. *176*, 490 [1926].
62) *O. Folin* and *A. Svedberg*, J. biol. Chemistry *88*, 77 [1930].
63) *O. Folin* and *R. D. Bell*, J. biol. Chemistry *29*, 329 [1917].
64) *J. Ellinghaus*, Hoppe-Seylers Z. physiol. Chem. *150*, 211 [1925].
65) *H. Südhof*, *F. Tischendorf* and *F. Scheler*, unpublished.
66) *O. Folin* and *W. Denis*, J. biol. Chemistry *26*, 497, 501, 505 [1916].
67) *A. Grigaut* and *F. Guérin*, C. R. Soc. Biol. Filiales Associées *82*, 25 [1919].
68) *O. Folin* and *G. E. Youngburg*, J. biol. Chemistry *38*, 111 [1919].
69) *J. B. Sumner* and A. *Bodansky*, J. biol. Chemistry *38*, 57 [1919].
70) *C. E. May* and *H. P. Ross*, J. Amer. chem. Soc. *43*, 2574 [1920].
71) *A. M. Roman*, J. Urolog. *4*, 531 [1921].
72) *P. B. Hawk*, *B. L. Oser* and *W. H. Summerson:* Practical Physiological Chemistry. The Blakiston Company, Philadelphia and Toronto 1947, p. 1229.

Purity of the enzyme preparation

The urease must not contain arginase; it should be free from ammonium ions. The specific activity should be at least 3 units*)/mg.

Preparation of Solutions

I. Urease (50 mg. protein/ml.):
Suspend 250 mg. urease in 5 ml. 50% (v/v) glycerol-doubly distilled water[74].

II. Ammonium sulphate standard solution (1.22×10^{-3} M):
Dissolve 161 mg. ammonium sulphate in doubly distilled water and make up to 1000 ml.

III. Perchloric acid (*ca.* 4% w/v):
Dilute 3.3 ml. 70% $HClO_4$ (sp. gr. 1.67) to 100 ml. with doubly distilled water.

IV. *Nessler*'s reagent:
With the commercially available products, just before use mix equal parts of solution A (HgI_2-KI) and solution B (NaOH). *Nessler*'s reagent which has aged often gives rise to turbidity and therefore to errors in the measurements.

Stability of the solutions

Store the urease solution and the ammonium sulphate standard solution, stoppered, at 0–4°C. Prepare the urease solution freshly each month**). *Nessler*'s reagent does not keep for more than 1 hour.

Procedure

Experimental material

Blood, plasma or serum are the main experimental material of the clinical laboratory. In serum, urea makes up 50% of the non-protein nitrogen. The daily excretion of urea in the urine is 20–35 g.

Urine: Use a 24 hour specimen of urine. This may contain considerable amounts of ammonia which will result in the blank of the sample being too high. Remove the ammonia by adsorption on a cation exchange resin: add 1 spatula tip of Permutit[79,+] to 10 ml. of urine, shake and decant from the resin. Dilute the urine 1:100 with distilled water before the determination.

Plasma or *serum:* Obtain from whole blood (for plasma add oxalate or citrate) which is as fresh as possible; do not use blood containing fluoride, because fluoride inhibits urease[80,81]. If necessary, the fluoride inhibition can be overcome by addition of magnesium, or better

*) Measured according to[73]; a unit is the amount of enzyme which converts 1 μmole of substrate in 1 min.

**) Aqueous solutions of urease (*e.g.* according to[58]) are unstable, therefore *Delaby*[75] has recommended the use of urease papers (preparation, see[19,50,76–78]).

+) Ammonia free, according to *Folin*, *e.g.* from Schuchardt, Munich, Germany.

73) *J. B. Sumner* and *V. A. Graham*, Proc. Soc. exp. Biol. Med. *22*, 504 [1925].
74) *J. Koch*, Lab. Clin. Med. *11*, 776 [1926].
75) *R. Delaby*, Bull. Sci. Pharmacol. *27*, 372 [1920].
76) *P. B. Hawk*, *B. L. Oser* and *W. H. Summerson:* Practical Physiological Chemistry. The Blakiston Company, Philadelphia and Toronto 1947, p. 499.
77) *J. W. Cook*, J. Assoc. off. agric. Chemists *31*, 797 [1948].
78) *J. W. Cook*, J. Assoc. off. agric. Chemists *35*, 544 [1952].
79) *G. E. Youngburg*, J. biol. Chemistry *45*, 391 [1921].
80) *R. A. Kilduffe* and *E. G. Springer*, Arch. Pathology Lab. Medicine *4*, 396 [1927].
81) *A. E. Osterberg* and *E. v. Schmidt*, J. biol. Chemistry *76*, 749 [1928].

still by caffeine-magnesium salicylate[82]. Plasma or serum have the same urea content as whole blood. If whole blood is being examined, haemolyse with 7 volumes of doubly distilled water. After the enzymatic reaction deproteinize with perchloric acid and neutralize the supernatant with KOH. Allow to stand for 10 min. in an ice bath, filter off the $KClO_4$ and use 1 ml. of filtrate + 4 ml. water (instead of 0.2 ml. deproteinized sample and 4.8 ml. water) for the colorimetric measurements. Allow for the volume changes in the calculations.

At an early date urea was also determined in other experimental material with urease (*e.g.* muscle[83], foodstuffs[84,85], fertilizers[86–88] and drinking water[89]).

Homogenize *tissue*. In order to eliminate the formation of urea by the arginase of the tissue the samples must be deproteinized before the determination: for example, homogenize liver with eight volumes of 6% (w/v) perchloric acid solution, centrifuge and add 1.75 ml. 1 M K_3PO_4 solution to 4 ml. of the supernatant. After allowing to stand for 10 min. in an ice bath filter off the $KClO_4$ and use 1 ml. of the filtrate for the estimation.

Extract *foodstuffs* according to their composition, with water, methanol, ethanol or even, for example, butanol[90], or use directly for the determination as suspension in water or buffer (pH *ca.* 7) (*e.g.* milk, soft cheese[91]).

Enzymatic reaction

For each sample prepare a "sample blank" to obtain the initial ammonia content and for each series prepare an ammonium sulphate standard and a "reagent blank".
Pipette into 10 ml. centrifuge tubes:

Sample	*Sample blank*
1.00 ml. sample	1.00 ml. sample
0.10 ml. urease (solution I)	—

Mix by inversion and allow to stand for 15 min. at room temperature. With a small glass rod mix in

3.00 ml. perchloric acid (solution III)	3.00 ml. perchloric acid (solution III)
	0.10 ml. urease solution (I),

centrifuge for 5—10 min. at *ca.* 3000 g. Pour the supernatant into a dry test tube. Take 0.2 ml. of this solution for the colorimetric determination of ammonia.

Colorimetric measurements

Wavelength: 436 mμ (400 to 450 mμ); light path: 1 cm.; final volume: 5.1 ml.; room temperature.

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82) *Ch. F. M. Rox*, Brit. J. exp. Pathol. *14*, 339 [1933].
83) *J. B. Sumner*, J. biol. Chemistry *27*, 95 [1916].
84) *M. Schmoll* and *F. D. Schmalz*, Cereal Chem. *23*, 600 [1946].
85) *A. E. Perkins*, Ind. Engng. Chem. *10*, 69 [1938].
86) *E. J. Fox* and *W. J. Geldard*, Ind. Engng. Chem. *15*, 743 [1923].
87) *M. P. Wowk*, *E. S. Kogan* and *A. I. Achromeiko*, Betriebs. Lab. (russ. Sawodskaja Laboratoija) *1933*, No. 10, p. 8.
88) *J. Y. Yee* and *R. O. E. Danis*, J. Assoc. off. agric. Chemists *20*, 104 [1937].
89) *C. Engelhard*, Z. ges. Brauereiwesen *54*, 160 [1931].
90) *H. D. Kay*, Biochem. J. *17*, 275 [1923].
91) *J. Schormüller* and *H. Tänzler*, Z. Lebensmittel-Unters. u. -Forsch. *109*, 234 [1959].

Pipette into test tubes:

	Sample and Sample blank	*Standard*	*Reagent blank*
doubly distilled water	4.8 ml.	4.8 ml.	5.0 ml.
deproteinized supernatant	0.2 ml.	—	—
$(NH_4)_2SO_4$ standard solution (II)	—	0.2 ml.	—
Nessler's reagent (solution IV)	0.1 ml.	0.1 ml.	0.1 ml.

Mix thoroughly by inversion, pour part of the solutions into cuvettes and measure within 5 min.:

Standard against the reagent blank ($E_{Std.}$), sample against the sample blank ($E_{Sam.}$).

With $E_{Sam.}$ values above 0.650 dilute the sample 1:10 with doubly distilled water and repeat the measurements.

Calculations

The optical density of the sample ($E_{Sam.}$) is related to the optical density of the standard ($E_{Std.}$). This contains 0.448 μmoles ammonia, which corresponds to 0.244 μmoles urea. Since 0.2 ml. of the 4.1 ml. of the enzymatic reaction mixture are taken for the colorimetric measurements ($^1/_{20.5}$), the standard corresponds to $0.244 \times 20.5 = 5$ μmoles urea per ml. of the sample.

Therefore

$$\frac{E_{Sam.}}{E_{Std.}} \times 5 = \mu\text{moles urea/ml. sample}$$

This value multiplied by 60 gives the μg. urea/ml. sample and multiplied by 6 gives the urea content of the sample in mg. %.

With samples which have been diluted (*e.g.* urine) it is necessary to multiply by the dilution factor.

Example

Determination of urea in serum. 1 ml. of serum was analysed and the following optical densities were measured: $E_{Std.} = 0.200$: $E_{Sam.} = 0.230$

$$\frac{E_{Sam.}}{E_{Std.}} \times 5 = \frac{0.230}{0.200} \times 5 = 5.75\ \mu\text{moles urea/ml. serum}$$

or

$$5.75 \times 6 = 34.5\ \text{mg.\% urea in the serum.}$$

Normal Values

Serum: 13.8—39.8 mg.%, average 26.8 mg.%[92,93].
Urine: 20—35 g./24 hour urine[92].

Specificity

Urease is completely specific for urea[94,95].

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92) *A. C. Kibrick* and *S. Skupp*, Proc. Soc. exp. Biol. Med. *73*, 432 [1950].
93) *R. H. Owings, E. E. Mandel* and *F. Jones*, Proc. Soc. exp. Biol. Med. *78*, 363 [1951].
94) *J. B. Sumner:* The Enzymes, 1st. ed., Academic Press, New York 1951, Vol. I, p. 886.
95) *J. E. Varner* in *P. D. Boyer, H. Lardy* and *K. Myrbäck:* The Enzymes, 2nd ed., Academic Press, New York 1960, Vol. IV, p. 251.

Sources of Error

If the urease preparation has lost part or all of its activity, the urea will not be completely hydrolysed. *Nessler*'s reagent which has aged causes turbidity of the coloured solutions and therefore results in inaccurate and unreproducible values. *Gentzkow*[14] has recommended a stabilized *Nessler*'s reagent, which must be used within 15 min., but which gives colours which are stable for at least an hour.

In experimental material which is not absolutely fresh ammonia may be formed, resulting in too high a sample blank and therefore to a reduction in the accuracy of the measurements. If the ammonia comes from the urea of the sample, the results will be too low.

According to[66] creatinine contained in urine interferes with the colorimetric reaction. This interference does not occur if the measurements are made against a sample blank.