Application of the Phenol–Hypochlorite Reaction to Measurement of Ammonia Concentrations in Kjeldahl Digests of Serum and Various Tissues

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Conditions have been defined that enable the use of nitroprusside as a catalyst for the phenol–hypochlorite reaction when the latter is applied to measurement of nitrogen in Kjeldahl digests containing mercury. This is accomplished by lowering the concentration of mercury to a range where it remains effective in accelerating the destruction of organic matter during digestion but no longer reacts with nitroprusside. In addition, the use of minimal concentrations of nitroprusside diminishes further the possibility of interference by mercury and has the added advantage of decreasing the pH dependence of indophenol blue formation. The sensitivity of the reaction makes possible measurement of nitrogen in very small samples with relatively short digestion times.

Additional Keyphrases *Berthelot reaction* • *colorimetry* • *Hg catalyst*

Application of the phenol–hypochlorite reaction to analysis of total nitrogen in Kjeldahl digests has been handicapped by interference caused by metal catalysts, although these have been shown to be the most effective accelerators of destruction of organic matter. We have found that the concentration of mercury, the preferred catalyst, can be adjusted so as to avoid interference with the color reaction without diminishing its activity as Kjeldahl catalyst. A procedure is described that is particularly useful for measurement of nitrogen in serum and other tissues, in which the quantities of phenol, nitroprusside, and alkaline hypochlorite required for optimal color production have been adapted to the presence of mercury.

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Preliminary Experiments

Nitroprusside concentration. We found that nitroprusside concentrations of 250 mg/liter, generally used by others to catalyze the phenol-hypochlorite reaction, caused an increased blank without improving the yield of color. The adoption of 100 mg/liter of nitroprusside in place of the 250 mg concentration lowered the blank without decreasing net absorbance. There was also a diminished sensitivity to variations in pH in the range of pH 10.5 to 11.5, where the optimal yields of indophenol blue were obtained (Figure 1).

Effect of metal ions and SeO_2. Copper sulfate and selenium dioxide inhibited the phenol–hypochlorite reaction. So also did mercury in the concentrations ordinarily used for digestion by the Kjeldahl method. However, we found that concentrations of mercury could be lowered sufficiently to avoid interference with the phenol–hypochlorite reaction while maintaining its effectiveness as a catalyst of oxidation (Table 1). In these experiments, the phenol–nitroprusside

Table 1. Effect of Mercury Concentration on Color Produced by the Phenol–Hypochlorite Reaction

Mercury, μg/ml	0	0.54[a]	1.30	2.61	3.99	5.23	6.53	13.07	65.4
Absorbance, %[b]	100	100	100	88	79	76	64	62	57

[a] The concentration used in the procedure.
[b] Percent of absorbance yielded by 2 μg of N.

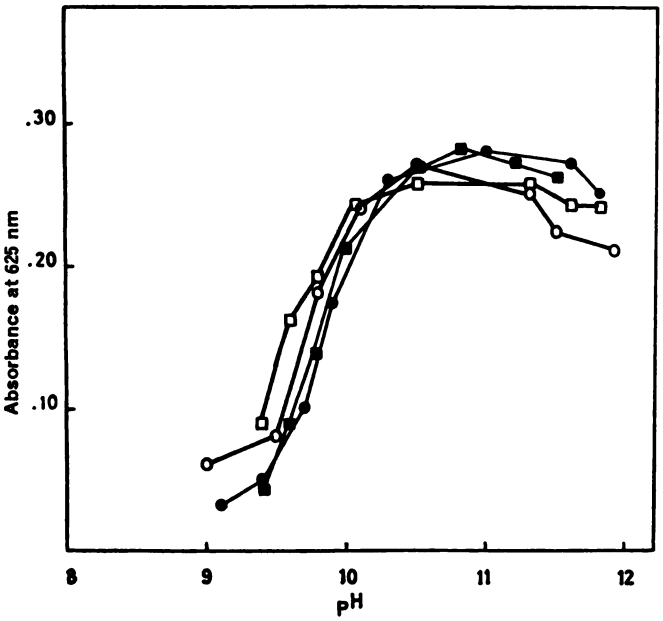

Fig. 1. Effect of pH on yield of indophenol blue from 2 μg of ammonium-N treated with phenol-hypochlorite reagent

Color was developed as described in the text in solutions adjusted to the pH values shown, by using phenol-nitroprusside reagent containing 100 or 250 mg of nitroprusside per liter in the presence or absence of a 0.20M borate buffer (76.3 g of $Na_2B_4O_0 \cdot 12H_2O$ dissolved in 0.2M NaOH and adjusted to pH 10.7 ± 0.1). 1.0 ml of the buffer was added to the reaction system after the phenol-nitroprusside reagent. Note the broadening of the absorbance maximum and the insignificant effect of the addition of buffer when the lower concentration of nitroprusside is used. ●: 100 mg of nitroprusside per liter. ■: the same in presence of buffer. O: 250 mg of nitroprusside per liter. □: the same in the presence of buffer

reaction was carried out in tubes containing 2 μg of N in the presence of the concentrations of mercury listed. Notice that the concentration of mercury used is well below that needed to depress color yield.

It is also evident from Table 2 that there is no gain in effectiveness of mercury catalysis, as measured by yield of nitrogen for a given time of digestion over a 5-fold range of mercury concentrations, when bovine serum albumin is used as a test substance. After 15- to 20-min digestions, 95% of the nitrogen was recovered, and 98% and 99% after 25 and 30 min. Moreover, the yield at 15 to 20 min was sufficiently consistent to permit its use in conjunction with a small correction factor to estimate total nitrogen. In these experiments, compressed propane served as fuel for the digestions. Microburners suitable for fuel of 1800–2200 Btu content were used.

The proposed method gives results in close agreement with those of a titrimetric Kjeldahl procedure (*1*) in which duplicate samples of 100 μl were digested 20 min (Table 3).

Total N of tissues. 0.1-milliliter samples of homogenates of tissues, homogenized in 10 volumes of water, were digested for 15 to 60 min. Digestion for 15 min converted 97% of the nitrogen of kidney and intestinal mucosa homogenates to ammonia. Liver homogenate required 20 min to reach the same stage. Whole intestine required a longer time.

NPN of serum. Nitrogen was totally converted to ammonium in 10 min in the presence of digestion mixture[2]; 15 min was necessary when it was omitted. The final volume of the digest is made to 10 ml instead of the 50 ml used with other materials.

Procedure for Total Nitrogen

Reagents

Phenol–nitroprusside reagent. Ten milligrams of sodium nitroprusside (nitroferricyanide; disodium pentacyanonitrosyloferrate), dissolved in 100 ml of phenol solution (50 g/liter).

Alkaline–hypochlorite reagent. Twenty-five milliliters of 2.5N sodium hydroxide solution and 2 ml of sodium hypochlorite solution (Clorox Co., Oakland, Calif.), 52.5 g/liter, were diluted to 100 ml with water.

Standard ammonium sulfate solution. Ammonium sulfate, 0.472 g, was diluted to 1000 ml with water; 1.0 ml contains 0.1 mg of N. A working standard (2 μg of N per ml) is prepared by diluting 1.0 ml to 50 ml.

Digestion mixture. This consists of 10N H_2SO_4 solution containing 0.5 g of $HgCl_2$ and 200 g of Na_2SO_4 per liter.

Ammonia-free water. Ammonia is removed from water used for reagents and in the procedure

[2] One milliliter of a filtrate of serum prepared by adding 0.2 ml of serum to 1.6 ml of N/12 H_2SO_4 and 0.2 ml of sodium tungstate solution (100 g/liter).

Table 2. Time of Digestion: Relationship to Mercury Concentration

Total Hg^{+2}, μg	Hg^{+2}, μg/ml	Digestion time, min					
		5	10	15	20	25	30
		mg N/g albumin					
170	0.26	136	142	142	145	150	148
340	0.52	130	140	139	145	147	151
425	0.65	133	140	150	147	145	149
510	0.77	134	140	142	146	146	151
680	1.03	139	143	143	142	147	147
850	1.30	123	144	150	143	152	160
	Mean	133	142	144.3	144.7	148	151

0.600 mg of bovine serum albumin dried in calcium chloride to constant weight, containing 15.2% N, was digested in the presence of the quantities of mercury shown for increasing periods of time. Time is measured from the start of boiling.

by means of a cation-exchange resin (Amberlite IR 120, H^+ form; Rohm and Haas, Philadelphia, Pa.). A mixed-bed resin may be used.

Technique

Twenty-five microliters of serum is added to 2.5 ml of sodium chloride solution in a test tube (13 × 100 mm). One milliliter of this dilution is pipetted into a digestion tube (25 × 200 mm, Corning Glass Works) having a heavy wall and graduated at 50 ml. One milliliter of digestion mixture is added together with two glass beads. The tube is heated in a perpendicular position on a digestion rack, until water has been removed and fumes of SO_3 fill the tube. It is loosely covered with a glass stopper and the digestion is continued for 25 min after the contents have begun to boil. The tube is allowed to cool, but while it is still warm to the touch, and before Na_2SO_4 crystallizes, about 10 ml of ammonia-free water is added. Four milliliters of 2.5N NaOH solution is added to neutralize the digestion mixture. The final volume is made to 50 ml with water.

One milliliter of the diluted digest is pipetted, after thorough mixing, into a test tube (16 × 150 mm), and to this 1 ml of phenol–nitroprusside reagent is added. One milliliter of sodium hypochlorite reagent is mixed gently with the contents of the tube by tapping. A standard is prepared by measuring 1.0 ml of the ammonium sulfate solution (2 μg of N per/ml) into a similar tube. A blank substitutes 1 ml of water for the sample. One milliliter of phenol–nitroprusside reagent and, after mixing, 1 ml of sodium hypochlorite reagent are added to both. Unknowns, standard, and blank are incubated at 37°C for 20 min; 10 ml of water is added to each tube, and the contents are mixed.

Absorbances are measured at 625 nm.

Calculation. Total N, g/100 ml = $A_u/A_s \times 2 \times 50 \times 2.5/0.025 \times 100/1000 \times 1/1000 = 1 A_u/A_s$, where A_u is the absorbance of the unknown and A_s that of the standard.

Conformity to Beer's Law occurs over a wide range of concentrations. Ammonia, pyridine, or other volatile reactants must be excluded from the room in which analyses are made.

Table 3. Results of Phenol–Nitroprusside (Berthelot) and Titrimetric Kjeldahl Methods for Total N Compared in 24 Samples of Human Serum

	Berthelot	Titrimetric
	N, g/100 ml	
Mean	1.108	1.113
SD	0.112	0.103
SE	0.0229	0.0211

Discussion

The sample sizes prescribed might be further decreased because of the remarkable sensitivity of the Berthelot reaction. However, we have not found the use of very small samples to be advantageous, partly because of the absence of compelling need in most circumstances, but also because of the greater care and effort demanded by precise measurement of such samples. The increasing importance of interference by ambient ammonia in very small samples is yet another consideration.

Shortened digestion time resulting from use of relatively small samples is a distinct advantage. Thus, the digestion time may be decreased from the 60 min or longer specified for serum in conventional micro-Kjeldahl analysis to 25 min or less, provided one can accept the slight underestimate of N that results. The latter is sufficiently consistent to permit the experimental results obtained by 25-min digestion to be increased by 2.5%, to give a true value.

However, Mann (*2*) observed that mercury interferes with the catalysis of the phenol–hypochlorite reaction by nitroprusside and we

found this to be true also of copper and selenium. Among the expedients proposed for enabling mercury to be used, addition of powdered zinc has been suggested (*2*, *3*). However, this increases the technical manipulation required and introduces new hazards of contamination. Bohley (*4*) substituted acetone for nitroprusside to make possible the use of mercury. However, acetone is a less effective catalyst of indophenol blue formation than is nitroprusside (*4*), so that this also is an unsatisfactory remedy.

The indophenol blue produced is an indicator with an apparent pK near pH 9.5 to 10.0. It is essential that sufficient alkali be added to the digest to neutralize the sulfuric acid of the digestion mixture, and to provide enough surplus NaOH to bring the pH to approximately 11. However, there is also some decrease in absorbance if the pH exceeds 11.5. At one stage of our study we found it helpful to introduce a buffer to stabilize the pH and to decrease the likelihood of variability of absorbance owing to this cause. However, decreasing the concentration of nitroprusside catalyst to 100 mg per liter instead of 250 mg decreased the sensitivity to pH changes and made it unnecessary to include the buffer. Horn and Squire (*6*) found maximal absorbance between pH 10.6 and 11.2. Their experiments also were carried out with lower concentrations of nitroprusside than those used by most workers.

The conditions of color development yield a color that remains stable for days. Nakagawa (*7*) proposed the use of EDTA to prevent inhibition of color development by mercuric ions. We have shown that additions are not needed if mercury concentrations are properly adjusted.

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