display, very broad peaks with long retention times are compressed in width, and the distance between late peaks is decreased markedly. Any number of decades may be used on the abscissa and the retention time per decade may be calibrated in any units of time so that very long or very short chromatograms all have the same standard format of display.

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Cyanolysis and Spectrophotometric Estimation of Trithionate in Mixture with Thiosulfate and Tetrathionate

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A sensitive method for estimating trithionate is described, which gives a quantitative assay of trithionate in the presence of thiosulfate and tetrathionate. The conditions affecting cyanolysis of the three sulfur compounds were examined, including a brief examination of the catalysis of thiosulfate cyanolysis by cupric ions. The method could be extended to estimate trithionate in mixture with higher polythionates.

IN RECENT YEARS there has been a growing interest in the chemistry and biology of thiosulfate and the polythionic acids (*1–3*), and sensitive methods for the estimation of these compounds in mixtures have become increasingly desirable. Methods have been described for the titrimetric analysis of mixtures of thiosulfate, trithionate, and higher polythionates (*4–6*). We have found these methods to be relatively insensitive for the analysis of the small amounts of these materials frequently encountered in experiments on bacterial sulfur compound oxidation (*7–9*), and that values obtained for trithionate in mixtures were not always reliable. Sensitive colorimetric methods for estimating thiosulfate and tetra-, penta- and hexathionates have been described (*10–16*), but no similar consideration appears to have been given to the colorimetric determination of trithionate. We have devised a sensitive method for estimating trithionate in mixtures, based on the procedures of Koh *et al.* (*10–13*) and Sörbo (*16*). The method depends on the alkaline cyanolysis of thionates to yield thiocyanate which can be estimated colorimetrically. Tetrathionate, thiosulfate, and trithionate react with cyanide according to Equations 1, 2, and 3.

$$S_4O_6^{2-} + 3CN^- + H_2O = S_2O_3^{2-} + SO_4^{2-} + 2HCN + SCN^- \quad (1)$$

$$S_2O_3^{2-} + CN^- \xrightarrow[\text{catalyst}]{Cu^{2+}} SO_3^{2-} + SCN^- \quad (2)$$

$$S_3O_6^{2-} + 3CN^- + H_2O = SO_3^{2-} + SO_4^{2-} + 2HCN + SCN^- \quad (3)$$

The present method depends on the fact that Reaction 1 occurs spontaneously at low temperatures, Reaction 2 occurs at low temperature in the presence of cupric ions, while Reaction 3 takes place only at high temperatures. The factors affecting the reaction of thiosulfate, trithionate, and tetrathionate under our assay conditions are described.

EXPERIMENTAL

Assay procedure for a mixture of thiosulfate, trithionate, and tetrathionate. Three replicate reaction mixtures were prepared in 25-ml volumetric flasks. The sample to be analyzed (containing up to 8 μmoles total thionate) was added to 4 ml of NaH_2PO_4–NaOH buffer (*10*), pH 7.4, and water added to give a total volume of 10 ml. The replicates were separately treated as follows.

I. The mixture was cooled to 5 °C; 5 ml of 0.1*M* KCN was added and mixed rapidly, thereby giving 0.033*M* cyanide and raising the pH to pH 9.65. The mixture was maintained at 5 °C for 20 min.

II. A replicate mixture was cooled to 5 °C and 5 ml of 0.1*M* KCN added. After maintaining at 5 °C for 10 min 1.5 ml of 0.1*M* $CuSO_4$ was added with rapid mixing, thereby lowering the pH to pH 7.35 and giving a concentration of 0.0091*M* Cu^{2+}. The mixture was maintained at 5 °C for 10–15 min.

III. A third replicate was mixed with 5 ml of 0.1*M* KCN and heated in a boiling water bath for 45 min, then cooled to 5 °C and 1.5 ml of 0.1*M* $CuSO_4$ rapidly mixed. The mixture was maintained at 5 °C for 10–15 min.

Finally, 3 ml of 1.5*M* ferric nitrate in 4*N* $HClO_4$ was added to each replicate with continuous agitation; the flask contents were warmed to room temperature with constant shaking to redissolve any precipitate, then made up to 25 ml with distilled water. The ferric thiocyanate color which developed was read at once at 460 mμ in 10-mm round cuvettes in a Coleman Junior spectrophotometer. Samples and thiocyanate standards were read against a sulfur-free reagent blank prepared as in treatment I. Thiocyanate standards were prepared in the same mixture and gave optical density readings deviating only

(1) M. Goehring, *Fortschr. Chem. Forsch.*, **Bd2,** 444 (1952).
(2) N. Kharasch, Ed., "Organic Sulphur Compounds," Vol. 1, Pergamon Press, 1961, Chapters 2 and 9.
(3) P. A. Trudinger, *Rev. Pure Appl. Chem.*, **17,** 1 (1967).
(4) R. R. Jay, ANAL. CHEM., **25,** 288 (1953).
(5) A. Kurtenacker, in "Handbuch der Anorganischen Chemie." R. Abegg, F. Auerbach, and I. Koppel, Eds., Vol. 4, part 1, 1st half, S. Hirzel, Leipzig, 1927.
(6) R. L. Starkey, *J. Gen. Physiol.*, **18,** 325 (1935).
(7) D. P. Kelly and P. J. Syrett, *Biochem. J.*, **98,** 537 (1966).
(8) D. P. Kelly, *Aust. J. Sci.*, **31,** 165 (1968).
(9) P. A. Trudinger, *Aust. J. Biol. Sci.*, **17,** 459, 738 (1964).
(10) T. Koh, *Bull. Chem. Soc. Japan*, **38,** 1510 (1965).
(11) T. Koh and I. Iwasaki, *ibid.*, **38,** 2135 (1965).
(12) T. Koh and I. Iwasaki, *ibid.*, **39,** 352 (1966).
(13) T. Koh and I. Iwasaki, *ibid.*, p 703.
(14) O. A. Nietzel and M. A. De Sesa, ANAL. CHEM., **27,** 1839 (1955).
(15) N. H. Schöön, *Acta. Chem. Scand.*, **13,** 525 (1959).
(16) B. Sörbo, *Biochim. Biophys. Acta*, **23,** 412 (1957).

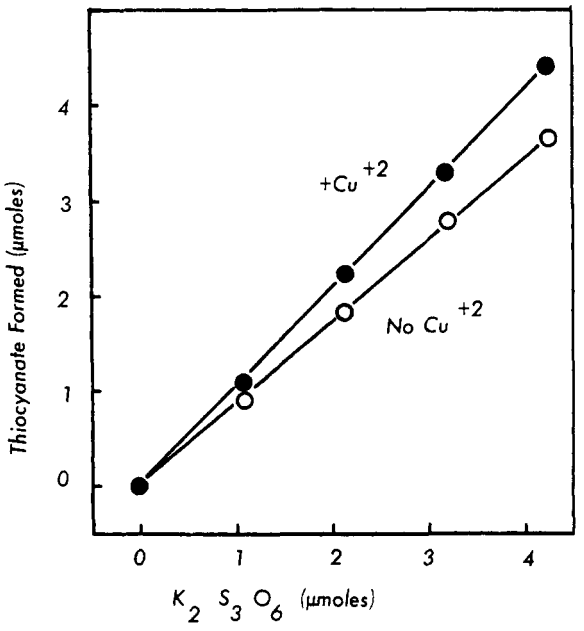

Figure 1. Effect of adding Cu^{2+} to trithionate mixtures after treatment III

○ — ○, no Cu^{2+} added; ● — ● Cu^{2+} added as in treatment III

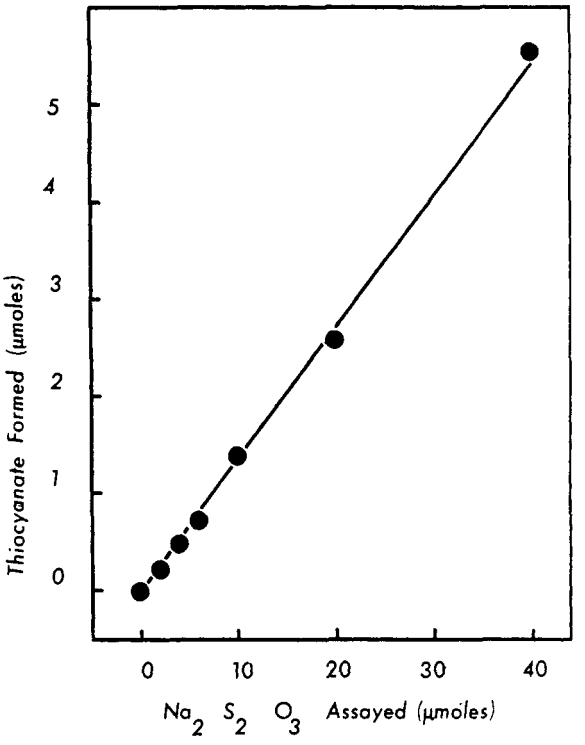

Figure 2. Hot cyanolysis of thiosulfate

The indicated amounts of thiosulfate were heated for 45 min at 100 °C as in treatment III, but thiocyanate formation *without* Cu^{2+} was estimated

slightly from Beer's law; 3.2 and 6.8 μmoles of thiocyanate gave absorbance values of 0.5 and 1.0, respectively. The same optical density readings were obtained if blanks or standards were treated as in treatment II or III. The color developed with the ferric nitrate–$HClO_4$ reagent was stable for at least 45 min, so no stabilizing agents were required. The intensity of the color developed was unaffected by the phosphate or by the cupric ions. Temperatures between 2°–10 °C were suitable for treatments I and II.

The three sulfur compounds thus gave the following equivalents of thiocyanate in the three procedures (see Equations 1–3):

I $S_4O_6^{2-} \equiv 1\ SCN^-$

II $S_4O_6^{2-} \equiv 2\ SCN^-$; $S_2O_3^{2-} \equiv 1\ SCN^-$

III $S_4O_6^{2-} \equiv 2\ SCN^-$; $S_2O_3^{2-} \equiv 1\ SCN^-$; $S_3O_6^{2-} \equiv 1\ SCN^-$

Two equivalents of thiocyanate were formed from tetrathionate in treatments II and III by copper-catalyzed cyanolysis of thiosulfate formed by its initial cyanolysis.

The trithionate content (μmoles) of a mixture was given directly as the difference in thiocyanate (μmoles) formed by procedures III and II. Tetrathionate was given directly by procedure I, and thiosulfate by subtracting twice the value for I from the value for II.

Titrimetric analysis of thionates. Classical procedures were used to estimate the purity of standard sulfur compounds. (i) Thiosulfate was estimated by titration in 5% acetic acid with standard iodine and by iodometric titration with standard iodate; (ii) thiosulfate and polythionates were estimated separately and in mixture by titration in 6*N* HCl with iodate (*17*); (iii) tetrathionate was determined by cyanide and sulfite degradation as described by Foss (*18*).

In addition to method (ii), the following methods were also used to estimate the purity of standard trithionate: (iv) acid production after treatment with mercuric chloride (*6*) was determined by titrating to a standard pH 5.0 with 0.1*N* KOH; (v) approximately 0.01*M* $K_2S_3O_6$ was heated with 0.2*M* $CuSO_4$ at 70 °C for 15 hr and the CuS formed was weighed as described by Riesenfeld, Josephy, and Grünthal (*5*); and (vi) approximately 0.005*M* trithionate was hydrolyzed with 1.25*N* KOH in a boiling water bath for 30 min before cooling and titrating thiosulfate with standard iodine after adding formaldehyde and excess acetic acid (*5*, *6*). Using these methods, the composition of mixtures could be determined, for example, as described by Kurtenacker (*5*) and Starkey (*6*).

Reagents. AnalaR $Na_2S_2O_3 \cdot 5H_2O$ was used without further purification; $K_2S_4O_6$ was prepared as described by Trudinger (*19*); and $K_2S_3O_6$ by the method of Stamm and Goehring (*20*). NaH_2PO_4–NaOH buffer, pH 7.4, was a mixture of 0.2*N* NaOH and 0.2*M* NaH_2PO_4 (*10*).

RESULTS AND DISCUSSION

Standard assay procedure. Table I shows the results of analyzing known mixtures, and demonstrates good recoveries of small amounts of added trithionate.

The method was extremely reproducible. Variation among replicates was generally insignificant and the deviation around the mean recovery for the three compounds separately or in mixtures never exceeded ±0.3%.

Reactivity of trithionate with cyanide. Under the standard assay conditions, trithionate produced no detectable thiocyanate at 5 °C, either in the presence or absence of cupric ions. Its presence does not, therefore, interfere with the estimation of thiosulfate or tetrathionate. At 40 °C, however, about 23% of added trithionate was converted to thiocyanate in 30 min. Using the conditions employed by Koh and Iwasaki (*13*), some 5.1% of the added trithionate underwent cyanolysis

(17) A. I. Vogel, "Quantitative Inorganic Analysis," 2nd ed., Longmans, London, 1951, p 358.
(18) O. Foss, *Det Kgl. Norske Vidensk. Selskabs Skr.*, **1945**, Nr. 2, Bruns, Trondheim, 1947.
(19) P. A. Trudinger, *Biochem. J.*, **78**, 680 (1961).
(20) H. Stamm and M. Goehring, *Z. Anorg. Allg. Chem.*, **250**, 226 (1942).

Table I. Colorimetric Analysis of Mixtures of Tetrathionate, Thiosulfate, and Trithionate

The mixtures used contained the following amounts of tetrathionate, thiosulfate, and trithionate (μmoles), respectively: *Expt. 1 and 4*, 1, 1, and 1; *Expt. 2*, 0.4, 1.6, and 0.4; *Expt. 3*, 0.8, 3.2, and 0.8.

		Thiocyanate formed (μmoles)			Recoveries (μmoles)		
		Treatment I	Treatment II	Treatment III	I $S_4O_6^{-2}$	II $S_2O_3^{-2}$	III $S_3O_6^{-2}$
Expt. 1	Found	1.08	2.96	4.00	1.08	0.80	1.04
	Expected	1.00	3.00	4.00	1.00	1.00	1.00
Expt. 2	Found	0.48	2.43	2.82	0.48	1.47	0.39
	Expected	0.40	2.40	2.80	0.40	1.60	0.40
Expt. 3	Found	0.98	4.80	5.66	0.98	2.84	0.86
	Expected	0.80	4.80	5.60	0.80	3.20	0.80
Expt. 4	Found	1.14	3.10	4.09	1.14	0.82	0.99
	Expected	1.00	3.00	4.00	1.00	1.00	1.00

(Table II). Trithionate would thus cause positive interference in the assay of thiosulfate or higher polythionates by Koh's procedure or in assays employing elevated temperatures.

Trithionate underwent cyanolysis at a decreasing rate on heating at 100 °C as in treatment III (Table III) and less than 100% of the expected thiocyanate was obtained in 45 min. The low recovery was attributed to possible hydrolysis of some of the trithionate to thiosulfate. This possibility was tested by heating trithionite with cyanide, cooling, and assaying thiocyanate with and without the prior addition of cupric ions (Table IV). Cu^{2+} catalyzed the cyanolysis of any thiosulfate formed from the trithionate and the results show that thiocyanate formation was complete provided that copper ions were added. In the *absence* of cyanide, trithionate heated at 100 °C for 45 min in KOH at the assay pH 9.6 was hydrolyzed by about 55% to thiosulfate (Table IV).

Heating at 100 °C at pH 9.6 with cyanide thus produced both thiocyanate and thiosulfate from trithionate, but the ratio of products was independent of the trithionate concentration (Figure 1) and some 87% of the trithionate was converted to thiocyanate.

Cyanolysis of tetrathionate and thiosulfate in the standard assay. Tetrathionate reacted completely and essentially instantaneously at 5 °C to give two or one moles of thiocyanate per mole of tetrathionate in the presence and absence of cupric ions, respectively. Thiocyanate recovery was unaffected by heating at 100 °C for 45 min.

Thiosulfate (1–6 μmoles) gave no detectable thiocyanate with cyanide at 5 °C unless cupric ions were added [Sörbo (*16*)]. On heating at 100 °C, thiosulfate was slowly cyanolyzed (Table V). The proportion of thiosulfate converted to thiocyanate in 45 min was only slightly increased by increasing the thiosulfate concentration, and about 12–13% of the added thiosulfate was cyanolyzed (Figure 2).

Effect of cupric ions on the cyanolysis of thiosulfate. As previously shown (*13*, *16*), complete cyanolysis of thiosulfate depended on the ratio of cyanide to cupric ions used. This relationship for our assay conditions is shown in Figure 3. As the cyanolysis of trithionate required a relatively high cyanide concentration, routinely KCN and $CuSO_4$ to give final concentrations (in 15 ml and 16.5 ml, respectively) of 0.033*M* and 0.0091*M*, respectively, were used. It is noteworthy that the proportion of thiosulfate cyanolyzed by a limiting amount of copper was independent of the amount of thiosulfate used when the cyanide concentration was constant (Table VIa). The reaction with cupric ions to produce thiocyanate was virtually instantaneous and no progressive thiocyanate formation occurred in the presence of a limiting amount of copper (Table VIb). Presumably the cyanolytic process is complex and depends on reaction of the copper with a preformed thiosulfate–cyanide complex. The recovery of thiocyanate was unaffected by heating thiosulfate with cyanide at 100 °C for 45 min prior to cooling to 5 °C and adding cupric ions as in treatment III.

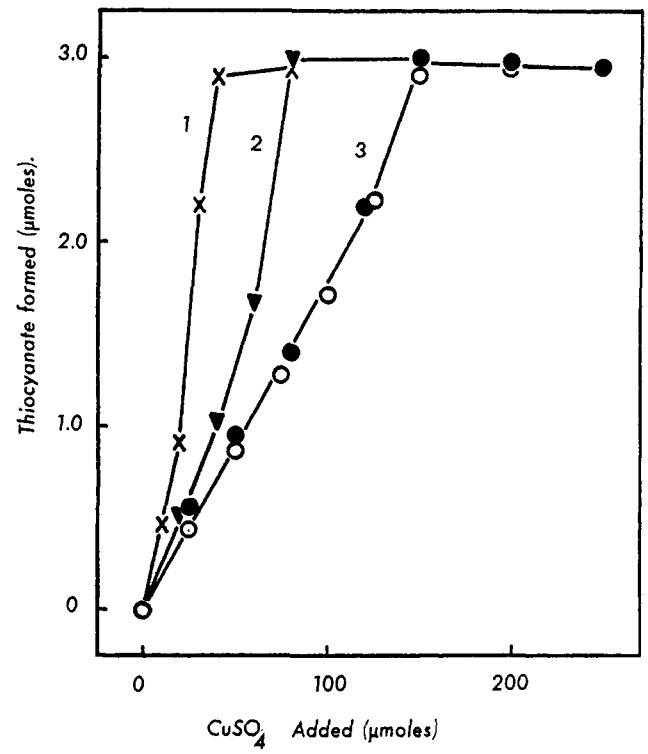

Figure 3. Effect of cupric ions on the cyanolysis of thiosulfate (3 μmoles) at three concentrations of cyanide, basically by treatment II

Curve 1. 0.00667*M* KCN before Cu^{2+} added
Curve 2. 0.01667*M* KCN before Cu^{2+} added
Curve 3. 0.033*M* KCN (standard assay concentration) before Cu^{2+}, ● and ○ denote results of two experiments

Application of the method to the analysis of complex mixtures. Clearly this procedure gives accurate estimates of trithionate in mixtures also containing thiosulfate and tetrathionate. It has also been routinely used to estimate trithionate in bacterial culture media during the growth of *Thiobacillus*. The method was unaffected by any compounds present in the small quantities of media (0.1–0.4 ml) used for the analysis. It should be possible to use this procedure to estimate trithionate in mixture with polythionates higher than tetra-

Table II. Partial Cyanolysis of Trithionate at 40 °C

Procedure A of Koh and Iwasaki (*13*) was used, using 2.5 ml of 0.1*M* KCN instead of the 5 ml used in our procedure

$K_2S_3O_6$ added (μmoles)	KSCN formed (μmoles)	Amount of $K_2S_3O_6$ converted (%)
2	0.10	5.0
3	0.14	4.7
4	0.20	5.0
5	0.26	5.2
6	0.34	5.7
	Average	5.1

Table III. Time Course of Trithionate Cyanolysis at 100 °C

$K_2S_3O_6$ (3.1 μmoles) was heated as in Treatment III for the indicated time before estimating thiocyanate without adding copper ions

Period of heating (min)	KSCN formed (μmoles)	% of theoretical 3.1 μmoles KSCN
0	0.05	1.6
5	1.28	41.3
15	2.32	74.8
30	2.63	84.8
45	2.75	88.7

Table IV. Thiosulfate Formation during Trithionate Cyanolysis[a]

Treatment	KSCN formed (μmoles)	% of initial $K_2S_3O_6$ recovered as KSCN
KCN only	2.77	86.6
KCN, then Cu^{2+}	3.30	103.1
KOH, then KCN and Cu^{2+}	1.75	54.7

[a] Replicate samples of $K_2S_3O_6$ (3.2 μmoles) were heated with KCN as for treatment III and parallel triplicate samples assayed for thiocyanate with and without prior treatment with Cu^{2+} at 5 °C. Further samples were heated at 100 °C for 45 min with 0.0064*N* KOH, pH 9.6, without cyanide, cooled to 5 °, then KCN and $CuSO_4$ added as in treatment II. Mean values for triplicates agreeing within ± 0.015 μmoles KSCN are given.

thionate as the higher polythionates would also be completely cyanolyzed by treatments I and II in the Experimental Section. The method could find application in the analysis of polythionate-containing mixtures, such as biological fluids in medicine; in the evaluation of inorganic sulfur compounds in soils; and possibly in the analysis of sewage and gas works effluent liquors. It has the advantages over existing methods of great simplicity, a much greater sensitivity than volumetric methods, and an unequivocal discrimination between trithionate and other polythionates or thiosulfate. Our procedure can also be used to estimate the amounts of thiosulfate and tetrathionate in unknown mixtures (Table I), but it was noted that recoveries of tetrathionate in known mixtures tended to be high and recoveries of thiosulfate to be correspondingly low. This discrepancy was found only when mixtures were analyzed, the recoveries using individual compounds were always close to the theoretical values. An erroneous high recovery for tetrathionate could occur if thiosulfate and tetrathionate reacted to yield trithionate and significant amounts of pentathionate (*5*) as shown in Equation 4:—

$$2\,S_2O_3^{2-} + S_4O_6^{2-} \rightleftharpoons S_5O_6^{2-} + S_3O_6^{2-} \qquad (4)$$

Table V. Time Course of Hot Cyanolysis of Thiosulfate

$Na_2S_2O_3$ (20 μmoles) was heated as in treatment III for the indicated time, cooled, and thiocyanate determined, without adding copper ions

Heating period (min)	NaSCN formed (μmoles)	% of initial $Na_2S_2O_3$ transformed
0	0.39	1.95
15	0.87	4.35
30	1.93	9.65
45	2.60	13.00

Table VI. Partial Cyanolysis of Thiosulfate in the Presence of Limiting Amounts of Cu^{2+} Ions

(a) $Na_2S_2O_3$ was treated by procedure II, except that only 0.8 ml of 0.1*M* $CuSO_4$ was added, to give a concentration of 0.00506*M* in 16.5 ml

$Na_2S_2O_3$ added (μmoles)	NaSCN formed (μmoles)	% of theoretical maximum NaSCN
1	0.42	42.0
2	0.87	43.5
3	1.34	44.7
4	1.82	45.5

(b) $Na_2S_2O_3$ (3μmoles) was treated as in (a), but the ferric nitrate–$HClO_4$ reagent was added at the indicated times after the addition of the $CuSO_4$

Length of incubation with Cu^{2+} at 5 °C (min)	Thiocyanate formed
1	1.44
5	1.32
10	1.38
20	1.37

Pentathionate would react spontaneously with cyanide to yield two thiocyanate ions, thus raising the apparent tetrathionate content of the sample. However, the recovery of thiosulfate plus tetrathionate (treatment II) would be lowered, and the recovery of trithionate raised above the expected values. As this was not the case, pentathionate formation by the reaction shown in Equation 4 was presumably negligible, and unlikely to be the cause of the discrepancy. The discrepancy persisted when lower concentrations of cyanide and copper were used and may presumably be due to some interaction of cyanide with a thiosulfate–tetrathionate association complex of the kind possibly involved in isotope exchange reactions between thiosulfate and tetrathionate. If this is the case, other cyanolytic methods for determining thiosulfate in mixture with polythionates would be subject to similar errors —*e.g.* Ref. *16*. It should be noted that these discrepancies are no greater than those reported by Koh and Iwasaki (*13*) for the recovery of higher polythionates in mixtures with each other, using copper ion catalyzed cyanolysis.

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