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RECEIVED for review January 5, 1966. Accepted March 21, 1966.

Ultra Sensitive, Specific Method for Cyanide Using p-Nitrobenzaldehyde and o-Dinitrobenzene

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▶ p-Nitrobenzaldehyde reacts with cyanide specifically to give an active reductant, which is capable of effecting the reduction of various compounds to form highly colored products. The intermediate cyanohydrin reacts with o-dinitrobenzene to give a highly colored purple compound, the dianion of o-nitrophenyl hydroxylamine, or with triphenyl tetrazolium chloride to produce a red dye, triphenyl formazan. Because cyanide is regenerated, a catalytic reaction occurs, with corresponding increase in sensitivity. Adding isonitroso benzoyl acetone, as little as 1.3×10^{-9} gram of cyanide per ml. of total solution (3 nanograms total CN^-) may be detected. Only p-nitro- and p-cyanobenzaldehyde of over 30 aldehydes tested react at an appreciable rate to form the reductive cyanohydrin. Only cyanide of more than 35 anions tested is detectable by this procedure.

IN A PREVIOUS paper (*5*), we described a specific method for the detection and determination of cyanide using various quinone derivatives. Further experiments were conducted, in attempts both to improve the sensitivity of detection and to develop a water soluble system which might be applicable to enzymatic reactions.

Feigl (*2*) reported that a variety of reductants was capable of effecting the reduction of 1,2-dinitrobenzene(III) in the presence of alkali to *o*-nitrophenyl hydroxylamine. This reaction served as the basis for qualitative spot tests for such compounds as hydroquinone, ascorbic acid, benzoic acid hydrazide, and reducing sugars. See Reactions (1) and (2).

It was thought that a catalytic reaction involving aromatic aldehydes and cyanide would result in a benzoin condensation, whose products would reduce *o*-dinitrobenzene. The formation of the characteristic blue color of the *o*-nitrophenylhydroxylamine dianion (IV) could then be used to monitor the reaction. Aldehydes incapable of undergoing the benzoin condensation due to substitution by electron withdrawing groups (*p*-Br, *p*-NO_2), would not, therefore, be expected to participate in the reduction of *o*-dinitrobenzene (*3*, *4*). Nevertheless, it was found that *p*-nitrobenzaldehyde, in the presence of cyanide ion, rapidly effects this reduction.

p-Nitrobenzaldehyde readily forms a cyanohydrin (*1*) but does not undergo the benzoin condensation. Preliminary experiments indicated that this reaction can serve as the basis for a sensitive, specific method for the detection of cyanide. The cyanohydrin produced (II) reacts with *o*-dinitrobenzene(III) to give the highly colored blue compound(IV). Because cyanide is regenerated, a catalytic reaction occurs, with increased sensitivity.

Isonitrosobenzoyl acetone(IBA) serves as a further source for the production of the catalyst cyanide, permitting the determination of as little as 1.3 nanograms of cyanide. Only *p*-nitro and *p*-cyano benzaldehyde of over 30 aldehydes tested react at an appreciable rate to form the cyanohydrin, and only cyanide of over 35 anions tested is detectable by this procedure. No other ions interfere in concentrations up to 0.1*M*.

EXPERIMENTAL

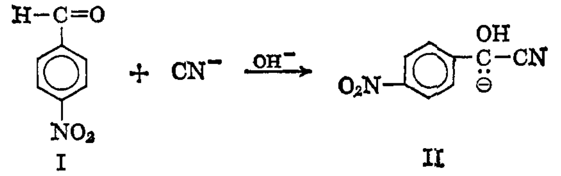
Reagents. All experiments were performed with reagent grade chemicals and pure solvents. All anions tested were added in the form of the C.P. sodium or potassium salt.

p-Nitrobenzaldehyde and *o*-dinitrobenzene solutions, 0.1*M*, were prepared by dissolving the C.P. compounds (Eastman Organics, Rochester, N. Y.) in methyl cellosolve.

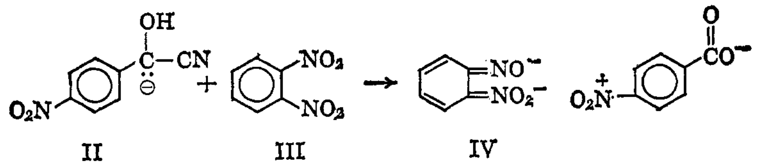
Triphenyl tetrazolium chloride, 0.1*M* solution in tris (hydroxymethyl)amino methane buffer, 0.1*M*, pH 8.5, was obtained from the Nutritional Biochemical Corp.

Isonitrosobenzoyl acetone solution, 0.02*M*, was prepared by dissolving the pure compound [prepared in these laboratories by the method of Wolf (*10*)] in pH 8.5 borax buffer, 0.1*M*.

Preparation of Pure *o*-Nitrophenyl Hydroxylamine Monoacetate. Two grams of *o*-dinitrobenzene (Aldrich Chemical Co.) (0.012*M*) was dissolved in 50 ml. of tetrahydrofuran with



(1)



(2)

slight heat and stirring. Zinc dust (2.0 grams) and ammonium chloride (3.0 grams) were then added and the stirring was continued for 3 hours at room temperature. The initial pale yellow solution turned deep brownish yellow. The suspension was filtered and 15 ml. of acetic anhydride–pyridine (3:1) was added. The solution now became yellow. After 0.5-hour acetylation time, 10 volumes of ice water were added and 1.8 grams (83% yield) of a yellow solid was obtained, which upon recrystallization from ether–petroleum ether (40–60° C. fraction), melted at 44–5° C. (uncorr.). The purity of the compound was checked by infrared (N—H, 3.01 microns, ester carbonyl 5.72 microns), and elemental analysis ($C_8H_8O_4N_2$, Calcd.: C, 48.9; H, 4.08. Found, C, 48.4; H, 3.9). Repeated attempts to obtain stable preparations of *o*-nitrophenylhydroxylamine by the method of Kuhn and Weigand (*7*) were unsuccessful.

The bisulfite addition product of *p*-nitrobenzaldehyde was prepared by conventional means.

Isolation of Product of Reaction of *o*-Dinitrobenzene, *p*-Nitrobenzaldehyde, and Cyanide. The products of reactions 1 and 2 have been shown (*6*) to be *p*-nitrobenzoic acid and *o*-nitrophenylhydroxyamine. Thus, it was reasoned that the reaction must proceed as per reactions 1 and 2.

Apparatus. A Beckman model DB spectrophotometer was used in all experiments. A constant temperature of 25° C. was maintained using a constant temperature room.

Procedure. Qualitative Detection of Cyanide. To a reagent solution, containing 1 ml. of $0.1M$ *o*-dinitrobenzene, 1 ml. of $0.1M$ *p*-nitrobenzaldehyde, 0.1 ml. of $0.02M$ IBA and 0.1 ml. of $0.50M$ sodium hydroxide, is added 0.1 ml. of the unknown ion (containing at least 3×10^{-9} gram of the anion to be tested). If a purple color develops, cyanide is present.

Determination of Cyanide. A mixture consisting of 1 ml. of $0.1M$ *p*-nitrobenzaldehyde, 1 ml. of $0.1M$ *o*-dinitrobenzene, and 0.1 ml. of $0.5M$ NaOH is placed in each of two cuvettes in a spectrophotometer, and the absorbance is adjusted to read zero. At zero time, 0.1 ml. of a solution of the cyanide to be determined (containing 0.1 to 1.0 μg. of cyanide) is added. The rate of change in the absorbance with time is then measured, and from calibration plots of $\Delta A/\Delta t$ *vs.* cyanide concentration (45-450 nanograms per ml. of solution used), the amount of cyanide present can be calculated.

DISCUSSION AND RESULTS

Detection of Cyanide Using Reduction of *o*-Dinitrobenzene. A number of substituted benzaldehydes were screened for their ability to effect the reduction of *o*-dinitrobenzene in the presence of cyanide to *o*-nitrophenyl hydroxylamine. The rates of reduction were followed spectrophotometrically by monitoring the change in the absorbance at 560 mμ, ascribed to the di-aci-form of *o*-nitrophenylhydroxylamine(II) [(*7*) (Table I)].

Table I. Relative Rates of Reduction of o-Dinitrobenzene by Substituted Benzaldehydes in Presence of Cyanide Ion

[*o*-Dinitrobenzene] = $0.1M$; [Aldehyde] = $0.1M$; $[CN^-] = 5 \times 10^{-6}M$

[Aldehyde]	Δ Absorbance/ Min.[a]
p-Nitro	0.20
o-Nitro	0.010
p-Cyano[b]	0.14
p-Me_2N; *p*-Et_2N; *m*-OH; 2,4,6-trihydroxy; 2-Cl, 4-Me_2N; *p*-isopropyl; *p*-H; *p*-methoxy; *p*-ethoxy; *p*-benzyloxy; 3,4 dimethoxy; 3,4,5 trimethoxy; *p*-acetimido; 2,4 dimethoxy; *p*-bromo; 2,4,6 trinitro[c]; 2,6 dichloro; *m*-methoxy; 2,4,6 trimethyl; 2-ethoxy 3-methoxy; *m*-fluoro; *p*-fluoro; 4-chloro 3-nitro[d]; *m*-nitro[d]; *o*-chloro	0

[a] A rate of zero indicated no reaction, regardless of the amount of cyanide added.

[b] Compound displays a green fluorescence.

[c] Turns red because of a pH effect.

[d] $2 \times 10^{-5}M$ cyanide needed to effect reaction.

p-Nitrobenzaldehyde reacted 20 times faster than *o*-nitrobenzaldehyde. The rate of reaction of the meta nitro derivative was immeasurably slow, but the rate is faster with higher concentrations of cyanide. The remaining aldehydes did not enter into the reaction, except for the *p*-cyano benzaldehyde, which had a rate approximately $^2/_3$ that of the *p*-nitro derivative. The 4-chloro-*m*-nitro derivative reacted at about the same rate as the *m*-nitro. The *p*-bromo and *p*-fluoro benzaldehydes did not react with cyanide at any appreciable rate to give a compound that effects the reduction of *o*-dinitrobenzene.

A kinetic study of the reaction (*6*) revealed a first order dependence on *p*-nitrobenzaldehyde and cyanide ion, and a zero order dependence on *o*-dinitrobenzene (Table II). In addition, the reaction is dependent on the amount of base present, being optimum at pH 9. The rate was measured by following the change with time of the absorbance at 560 mμ, which is the wavelength of maximum absorbance of the *o*-nitrophenylhydroxylamine anion.

Table II. Variation of Rate of Reaction Expressed as ΔA/min. on Concentration of o-Dinitrobenzene, p-Nitrobenzaldehyde, Cyanide, and pH (T = 23°C.).

o-Dinitrobenzene, *M*	*p*-Nitrobenzaldehyde, *M*	Cyanide, *M*	pH	Δ*A*/min.
5×10^{-2}	5×10^{-5}	5×10^{-5}	9	0.01
5×10^{-2}	1×10^{-4}	5×10^{-5}	9	0.02
5×10^{-2}	5×10^{-4}	5×10^{-5}	9	0.10
5×10^{-6}	5×10^{-2}	5×10^{-5}	9	0.60
2.5×10^{-5}	5×10^{-2}	5×10^{-5}	9	0.60
2.5×10^{-4}	5×10^{-2}	5×10^{-5}	9	0.60
5×10^{-2}	5×10^{-2}	5×10^{-7}	9	0.010
5×10^{-2}	5×10^{-2}	2.5×10^{-6}	9	0.040
5×10^{-2}	5×10^{-2}	5.0×10^{-6}	9	0.083
5×10^{-2}	5×10^{-2}	1.0×10^{-5}	9	0.17
5×10^{-2}	5×10^{-2}	2.0×10^{-5}	9	0.40
5×10^{-2}	5×10^{-2}	5×10^{-5}	8[a]	0.070
5×10^{-2}	5×10^{-2}	5×10^{-5}	7[a]	0.008
5×10^{-2}	5×10^{-2}	5×10^{-5}	9	0.70

[a] No correction was made for the effect of pH on molar absorptivity of the *o*-nitrophenylhydroxylamine. Also, at the lower pH values, the concentration of the cyanohydrin ion is lower.

Using the optimum concentrations of *p*-nitrobenzaldehyde and *o*-dinitrobenzene ($0.1M$ added) and sodium hydroxide (0.1 ml. of $0.5M$ = pH 9), the reaction was found to be approximately proportional to the cyanide concentration, allowing the determination of this anion with a deviation of about 8%. Under these conditions, a $1.7 \times 10^{-6}M$ (45 nanograms per ml. of solution) concentration of cyanide could be assayed.

Over 30 anions were tested for possible reaction with *p*-nitrobenzaldehyde to form a compound that might effect the reduction of *o*-dinitrobenzene. None of the ions tested had any effect on either the qualitative or the quantitative determination of cyanide: iodide, chloride, bromide, sulfite, carbonate, cyanate, bicarbonate, phosphite, tungstate, bisulfate, bismuthate, arsenite, arsenate, perborate, iodate, molybdate, ferricyanide, ferrocyanide, phthalate, thiosulfate, chlorite, chlorate, thiocyanate, fluoride, silicate, bromate, sulfate, perchlorate, citrate, tartrate, nitrate, hypochlorite, sulfide, tellurite, and various nitriles and isocyanates. Some of the compounds had slight solubilities under the reaction conditions described, giving a moderately high initial absorbance caused by cloudiness. In the presence of dichromate, the color of the cyanide detection reaction is purple-black, the color being slightly obscured by the yellow color of dichromate. All materials were tested in

Table III. Dependence of Rate of Reduction (ΔA/min.) of *o*-Dinitrobenzene by Cyanohydrin of *p*-Nitrobenzaldehyde Produced from the Spontaneous Decomposition of IBA

o-Dinitrobenzene = $5 \times 10^{-3}M$; *p*-nitrobenzaldehyde = $5 \times 10^{-2}M$ pH = 9.0, no cyanide added

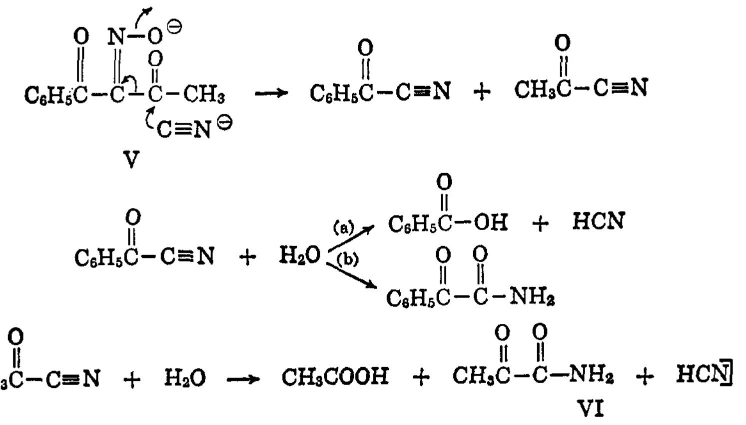
IBA, M	ΔA/min.	Time for cyanide production, min.[a]
8.6×10^{-5}	0.015	20
1.7×10^{-4}	0.030	14
3.4×10^{-4}	0.060	10
6.8×10^{-4}	0.120	9
8.7×10^{-4}	0.150	7

[a] Time required for first appearance of color.

concentrations up to $10^{-1}M$. Thus, the method is specific for cyanide.

Molar Absorptivity of Dye. In order to measure the molar absorptivity of the blue dye produced from the reduction of the *o*-dinitrobenzene by the cyanohydrin of *p*-nitrobenzaldehyde, a pure sample of *o*-nitrophenyl hydroxylamine monoacetate was prepared as described in the experimental section. Known concentrations of the purified monoacetate were then hydrolyzed with excess base to form the blue compound, and the absorbance was determined at different pH's at various time intervals until stable, maximum values were obtained. The absorbance increased with increasing pH, reaching a maximum at about pH 10, and remained constant over the range 10–14. A pK was calculated for the Dianion of 8.5, and at a pH of 9.0 (the pH at which all determinations were made), a molar absorptivity of 2.3×10^3 was obtained. Although the molar absorptivity at pH 9 is only about 70% of that at 10 (3.5×10^3), the instability of the blue color at pH's > 9 necessitated the use of a below maximum pH.

Addition of IBA. The sensitivity of the *o*-dinitrobenzene–*p*-nitrobenzaldehyde reaction for cyanide was found to be increased by the addition of the oxime isonitrosobenzoyl acetone (IBA). Adding 0.1 ml. of $0.02M$ IBA (overall $M = 8.7 \times 10^{-4}M$) to 1 ml. of $0.1M$ *p*-nitrobenzaldehyde and 1 ml. of $0.1M$ *o*-dinitrobenzene at a pH of 9, 1.30 nanograms of cyanide per ml. of total solution (3 nanograms total CN^- in the 2.3 ml. of solution used) can be detected. Catalysis by IBA(V) is believed due to the reaction of cyanide with IBA to form benzoyl and acetyl cyanide (reactions 3, 3a, and 3b). The hydrolysis of these two compounds then produces two molecules of cyanide for each original cyanide, or a further catalytic effect (*9*).



V (3)

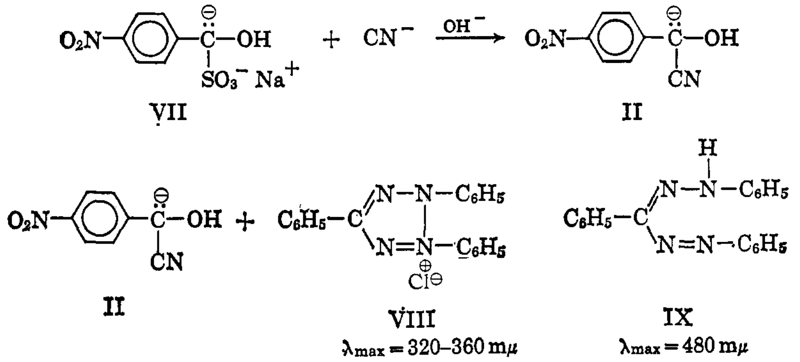
$$C_6H_5\overset{O}{\overset{\|}{C}}-C{\equiv}N + H_2O \xrightarrow{(a)} C_6H_5\overset{O}{\overset{\|}{C}}-OH + HCN \qquad \xrightarrow{(b)} C_6H_5\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-NH_2 \quad (3a)$$

$$CH_3\overset{O}{\overset{\|}{C}}-C{\equiv}N + H_2O \rightarrow CH_3COOH + CH_3\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-NH_2 + HCN \quad (3b)$$

VI

One disadvantage in the use of IBA as a sensitizer for the detection of cyanide, is that it is spontaneously decomposed at pH 9 to liberate cyanide after some time. The dependence of the rate of reduction of *o*-dinitrobenzene upon IBA, together with the time required for spontaneous production of cyanide, is indicated in Table III. Using the concentration of IBA recommended in the procedure for the most sensitive detection of cyanide, $8.7 \times 10^{-4}M$, 7 minutes is required before any significant purple color is observed to form in the absence of cyanide added. This is sufficient time to allow an analysis of cyanide. Also, the rate of reaction, ΔA/min., appears proportional to the concentration of IBA, thus providing a possible method for the assay of this compound (Table III).

Reduction of Triphenyl Tetrazolium Chloride. In attempting to apply the cyanohydrin reduction of *o*-dinitrobenzene procedure for detecting cyanide to the monitoring of enzymatic reactions that liberate cyanide, a completely water soluble system was sought. Compounds I and III are water insoluble, and could not be used. Hence a bisulfite addition product of I was prepared (VII), and the soluble dye triphenyl tetrazolium chloride(VIII) was used:



VII (4) II

II VIII $\lambda_{max} = 320–360$ mμ (5) IX $\lambda_{max} = 480$ mμ

Triphenyl tetrazolium chloride(VIII) is reduced by II to a red colored compound (IX), triphenyl formazan (*8*). Using the optimum conditions for analysis [1 ml. of $0.1M$(VIII) in tris buffer, $0.1M$, pH 8.5; 1 ml. of $0.01M$(VII) in water and 0.1 ml. of $0.50M$ NaOH, overall pH 8.50], as little as $5 \times 10^{-6}M$ cyanide can be detected. Because this procedure is not so sensitive as the reduction of *o*-dinitrobenzene method ($2 \times 10^{-7}M$ cyanide detectable), further work on it was not pursued.

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RECEIVED for review November 8, 1965. Accepted December 27, 1965.