amounts of Sb(III). Fifty milliliters of the reagent mixture were added to 100 ml. volumetric flasks which contained 0, 2.0, 4.0, and 8.0 ml. of 2000 p.p.m. methanolic SbCl₃ and were diluted to the mark. Spectra are shown in Figure 6. The bathochromic shift can be attributed to an antimony interaction with the molybdate aggregate.

A yellow compound can be prepared by mixing SbCl₃ in HCl with aqueous sodium molvbdate. Spectrographic analysis indicates that this material contains only antimony and molybdenum in major amounts. Efforts are being made to characterize this substance more completely.

Apparently the role of ascorbic acid is not unique, because enhancement can be obtained with other reducing agents. but the mechanism of oxidation of ascorbic acid may lead to products which do have unique properties.

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Specific Detection and Determination of Cyanide Using Various Quinone Derivatives

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▶ p-Benzoquinone, N-chloro-p-benzoquinoneimine, and O-(p-nitrobenzene sulfonyl) quinone monoxime are suitable reagents for a simple, direct method for the specific detection of small quantities of cyanide. Addition of as little as 0.2 μ g. of cyanide per ml. of total solution to these reagents yields a highly fluorescent product. The fluorescence produced in the reaction of p-benzoquinone with cyanide is proportional to the cyanide concentration over the range 0.2 to 50 μ g. No other ions interfered in concentrations up to 0.1M. The order of the rate of reaction and sensitivity increased in the general order O-(pnitrobenzene sulfonyl) quinone monoxime < N-chloro-p-benzoquinoneimine < p-benzoquinone. The order of fluorescence intensity increased in the order of solvents used: dioxane < ethyl formate < 2-methoxy ethanol < methanol < acetonitrile < dimethylformamide < dimethylsulfoxide. A series of derivatives of the quinone monoxime, O= —N—- $O - SO_2 - \phi - R'$ was prepared,

where $R' = CH_3$, H, NO₂, Br, and OCH₃. A Hammett plot of log k vs. σ was constructed, and a value of +0.785was obtained for ρ . In general, substitution on the quinone ring decreased the fluorescence of the product, as well as the rate of reaction.

W E DESCRIBED, in a previous com-munication (4), a specific fluorometric method for the detection of cyanide. In that procedure, quinone monoxime benzene sulfonate ester (VI) reacted with cyanide specifically to give a green fluorescent compound. In attempting to improve the sensitivity of detection, a number of guinones and quninone derivatives have been investigated-namely, p-benzoquinone, N - chloro - p - benzoquinoneimine, and substituted quinone monoxime benzene sulfonate esters and ethers.

The present study provides data on the reaction of these compounds and their derivatives with cyanide to give fluorescent products. The effect of substituents, solvent, pH, and interferences in the reaction as a means of

detecting cyanide is discussed. Evidence is advanced in elucidating the formula of the fluorescent products formed. All compounds react specifically with cyanide. No other ions interfere in concentrations up to 0.1M.

EXPERIMENTAL

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

p - Benzoquinone and N - chloro-p - benzoquinoneimine were obtained from Eastman Organics, Rochester, N. Y. The *p*-benzoquinone was purified by sublimation before use.

The sulfinimine derivatives were prepared by the method of Killmer and Gamson (7).

The various substituted benzene sulfonate esters of quinone monoxime were prepared by reacting equimolar quan-tities (0.01M) of the appropriate nitrosophenol(sodium salt) and the substituted benzene sulfonyl chloride in tetrahydrofuran and pyridine with stirring for 1 hour. The product was then isolated by pouring the reaction mixture into ice water. The precipi-

	Table I.	Analytical Data of	Various C	ompounds	Prepared	as Substra	ates for C	.N−		
		M.P.	Carbon		Hydrogen		Nitrogen		Other	
Compou	nd	°C.	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found
0=N-0502-		130-131	54.7	54.0	3.42	3.5				
	Br VIII	151-152	42.1	42.4	2.34	2.3			Bron 23.4	mine: 23.6
	O-NO ₂ IX	164-165	46.7	46.4	2.6	2.5	9.09	9.0		
0=NOSO2 <	⊙-осн₃ х	170–171								
	⊙≻—сн₃ хі	132	56.3	56.1	4.0	3.9	5.05	5.03		
	⊃>−сн₃ хи	156-157	57.8	57.4	4.5	4.6			Sul 11.0	lfur: 10.9
	⊃}−och, xi	E 160–161	54.7	54.5	4.2	4.1				
0 = NOCH2 - (⊙}—a xiv	101-102	63.0	62.9	4.1	4.3				
0= NOCH2	Q _{NO2} xv	113–114	60.4	60.3	3.9	4.2				
0= NOCH2-	XVI 🚫	97–98	63.0	63.2	4.1	4.2				
0=	⊃>-no₂ xvi	I 157–158	60.4	60.4	3.9	4.0				

Table II. Reaction of Various Compounds with Cyanide Ion Substrate = $3.4 \times 10^{-4}M$ in DMSO; $CN^- = 3 \times 10^{-5}M$

		Properties of	of Produ	ct	Lowest detectable limit
Compound	λ _{ex} , mμ	$\lambda_{em}, m\mu$	F_{\max} , units	$\Delta F/\min.,$ F.U./min.	of CN-, µg./ml.
0=<	400-420	480-490	90	88	0.2
о	410–430	470	40	8.0	0.2
o=√N−ci iv	440	500	75	60	1.0
$0 \rightarrow P \rightarrow $	420	500	10	0.3	2.5
¢	410-420	480	10	14	1.0
VI VIII IX XX XI XII XIII XIV XV XVI XVI	$\begin{array}{c} 440\\ 430-440\\ 450\\ 440\\ 430-440\\ 420\\ 410-420\\ 440\\ 440\\ 440\\ 440\\ 440\\ 440\\ \end{array}$	$500 \\ 500 \\ 500 \\ 500 \\ 490 \\ 490 \\ 500 $	60 50 30 60 23 60 60 60 60 60	$\begin{array}{c} 33.6\\ 33.5\\ 37.8\\ 20\\ 22.2\\ 3.0\\ 10\\ 0.45\\ 0.80\\ 0.51\\ 0.90\\ \end{array}$	$1.0 \\ 1.0 \\ 1.0 \\ 1.0 \\ 1.0 \\ 2.0 $

tate was filtered, dried, and recrystallized from carbon tetrachloride. The analytical data on the compounds prepared are given in Table I.

The quinoneimine ethers were prepared by reacting equimolar concentrations of the sodium salt of p-nitrosophenol (0.1M) with either $p-\alpha$ -dichlorotoluene, α -chloro-m-nitrotoluene, $o-\alpha$ -dichlorotoluene, or p-nitrobenzyl chloride, respectively (Table I), with shaking overnight. The solvent employed was acetone. The product was isolated by pouring into ice water, and the precipitate was filtered, dried, and recrystallized from ethanol.

Procedure: To 3 ml. of $3.4 \times 10^{-4}M$ solution of *p*-benzoquinone in dimethyl sulfoxide is added 0.1 ml. of the solution to be analyzed, containing 0.6 to 150 µg. of cyanide. If a green fluorescence develops, cyanide is present. This green fluorescence is read in an Aminco-Bowman spectrophotofluorometer at excitation and emission wavelengths of 400 and 480 mµ, respectively. From calibration plots of fluorescence readings *vs.* cyanide concentration, the amount of cyanide present in the unknown may be calculated.

DISCUSSION AND RESULTS

Substrates for the Detection of Cyanide. In a previous communication (4), we described a specific, direct method for cyanide based on the reaction with quinone monoxime

benzene sulfonate ester (VI) to give a green fluorescent product. A number of related sulfonate esters and ethers were prepared in an attempt to improve the sensitivity of detection, and the results obtained are indicated in Table II. In this table, some of the properties of the final green fluorescent product(wavelength of excitation and emission and fluorescence maximum), as well as the rate of reaction as indicated by the rate of production of fluorescence with time, $\Delta F / \Delta t$, and the lowest detectable limit of cyanide (in micrograms per milliliter of total solution used) are given.

A series of derivatives of the quinone monoxime benzene sulfonate esters was prepared (Tables I and II, compounds VI and VIII to XIII), in which various substituents were placed on the quinone and aryl sulfonate rings. A Hammett plot of log k vs. σ was constructed (Figure 1), and a value of ± 0.785 was obtained for ρ . This is the value to be expected for a substitution reaction (5) (Equation 2). In general, all the products had approximately the same excitation and emission wavelengths, 440 and 500 m μ , respectively. One might expect also, the



Figure 1. Hammett plot of log k vs. σ for reaction of various substituted tosylates with cyanide

of the inhibition of the 1,4-addition of the second cyanide to compound VIa in forming compound VIb, due to the presence of the R group.

A series of quinoneimine ethers were prepared (Tables I and II, compounds XIV to XVII) as possible substrates



same total fluorescence, because the same product VII is believed to be produced. Evidently, when an electron withdrawing group is present on the tosylate, a side reaction occurs namely, the alkaline hydrolysis of the tosylate to the quinone monoxime. This compound has been shown experimentally not to react with cyanide to yield the fluorescent product VII. Hence, the total fluorescence observed is less when $\mathbf{R}' = \mathbf{NO}_2$ or Br (Table II).

Substitution on the quinone ring decreased the fluorescence of the product, as well as the rate of reaction. Also, the excitation and emission wavelengths were shifted to shorter wavelengths when R groups were placed on the ring. This is presumably because for cyanide. These compounds all react slowly with cyanide to give a product which has the same λ_{ex} , λ_{em} and fluorescence (probably VII). A value indicating a substitution reaction, +0.785, was obtained for ρ from a Hammett plot similar to Figure 1. None of these compounds is suitable for the detection of cyanide.

In addition, a series of sulfinimines (XVIII), in which R = Br, Cl, or H, and R' = R'' = H, COOH, or COCH₃, was screened for possible reaction with cyanide. All of these compounds exhibited brilliant color changes from red to blue to green, but no fluorescence was observed. These reactions were not investigated further because of the



greater sensitivity of the fluorescence measurements. However, these observations may prove useful to laboratories not equipped for fluorescence.

In 1900 Thiele and Meisenheimer (10) described the formation of 2,3dicyanohydroquinone (II) by reaction of HCN with quinone. Preliminary experiments indicated that this is a rapid reaction, and one which can serve as the basis for the most sensitive test for cyanide (Table II). The excitation and emission wavelengths are hypsochromically shifted, and the product is more fluorescent (Table II) than that obtained from the quinone monoxime Substitution of sulfonate esters. chlorine on the quinone ring (III) decreases the rate of reaction, and hypsochromically shifts the emission wavelength and bathochromically shifts the excitation wavelength of the final fluorescent product (probably 2,3-dicyano-6-chloro hydroquinone). This decreased rate is presumedly caused by a displacement of one Cl by a cyanide, because Reynolds and Van Allan (9) have shown that the product of the reaction of 2,3-dichloro-1,4-naphthoquinone with NaCN is 2,3-dicyano-1,4naphthalenediol.

A similar compound, 1,4-naphthoquinone reacts much more slowly with cyanide to give a product that has similar excitation and emission wavelengths, but a lower fluorescence. The product is presumably the same as that isolated by Reynolds and Van Allan (9).

Finally, N-chloro-p-quinoneimine (IV) and its bromine derivative (V) were tested as substrates. The parent compound reacts quickly with cyanide to give a compound that has the same characteristics as VII. The Br derivative displays a marked decrease in reactivity, and gives a compound which has a weaker fluorescence and is excited at a lower wavelength.

The fluorescence produced in the reaction of *p*-benzoquinone with cyanide, as well as the initial rate, $\Delta F/\Delta t$, is proportional to the cyanide concentration over the range of concentrations 0.2 to 50 μ g. per ml. of solution. From calibration plots of $\Delta F/\Delta t$ or fluorescence (maximum produced in 3 minutes) vs. cyanide concentration, the amount of cyanide present may be determined with a deviation of about 5%. The reagent *p*-benzoquinone appears to be the most satisfactory substrate for the detection and determination of cyanide, based on

sensitivity (Table II), reproducibility, and accessibility.

Effect of Solvent. A number of different solvents were tested for use in this reaction. The order of fluorescence intensity and rate of reaction increased in the order dioxane < chloro-form < ethyl formate < methyl cellosolve < ethanol < methanol < aceto-nitrile < dimethylformamide < dimethylsulfoxide. This parallels the order of the dielectric constants of the solvents used (Figure 2). With the equation reported by Frost and Pearson (3, 6).

$$\ln k = \ln k_0 - [(D-1)/(2D+2)] [(N/RT)] \times \left[\frac{\mu_A^2}{r_A^3} + \frac{\mu_B^2}{r_B^3} - \frac{\mu_{\pm}^2}{r_{\pm}^3}\right]$$

where k = the rate constant for an ionization reaction in a solvent of dielectric constant D; k_0 is the rate constant in a solvent of unit dielectric constant, r and μ are the radius and dipole moment of species A and B, and \hat{N}, R , and T have standard meanings, a plot of $\ln k vs. (D-1)/(2D+2)$ should give a straight line, if the activated complex is more polar than the reactants (as would be true if the products are ions). Laidler and Eyring (8) pointed out that this equation should hold true only if mixtures of two solvents are used, in which the dielectric constant can be varied. This latter condition was observed in these studies because water (3%) was held constant, and the dielectric constant was varied by using different second solvents (97%). A plot of $\ln k vs.$ (D-1)/(2D+2) gave a straight line, indicating an ionic mechanism is involved.

A similar effect of solvent on the fluorescence was observed, the total fluorescence increasing with increasing dielectric constant. This effect should be observed, because the fluorescent species is an ionic molecule and solvents favoring the ionic form should be best.

Table III. Effect of pH and Water on Reaction of p-Benzoquinone with Cyanide					
$[p-\text{Benzoquinone}] = 3.4 \times 10^{-4}M;$ $[\text{CN}^{-}] = 3 \times 10^{-5}M$					
pH, buffer	$\mathbf{F}.\mathbf{U}./\min.$				
6.5, Phosphate 7.0, Phosphate 7.0, H ₂ O 7.5, Phosphate ^a 7.5, Tris 8.0, Tris 10.0, Tris 7.5, Phosphate ^b 7.5, Phosphate ^c 7.5, Phosphate ^d	$23 \\ 25 \\ 4.8 \\ 26 \\ 0 \\ 0 \\ 8 \\ 26 \\ 25 \\ 6.6$				
^a Total water = 0.1 = 0.2 ml. ^c Total ^d Total water = 0.5 m	ml. ^b Total water water = 0.3 ml. hl.				



Figure 2. Variation of rate of reaction of p-benzoquinone with cyanide as function of dielectric constant of solvent used

Because the rate and maximum fluorescence are obtained in dimethylsulfoxide, this solvent was used in all determinations with *p*-benzoquinone.

Effect of Interferences and pH. Over 30 anions were tested for possible reaction with *p*-benzoquinone to give a fluorescent product. In addition to the anions reported in the previous communication (4), tungstate, arsenate, and molybdate and various nitriles and isocyanates were tested for reaction. Only cyanide reacted with *p*-benzoquinone to give a green fluorescence. Hence, the method is highly specific for cyanide.

The effect of these various anions on the quantitative determination of cyanide was then tested. The cyanide used was at a concentration of 3 \times $10^{-5}M$; the p-benzoquinone, 3.4 \times $10^{-4}M$ in dimethyl sulfoxide. All anions were added at a concentration of 0.1M, their overall concentration being $3 \times$ $10^{-3}M$, in aqueous solution. The following ions had no effect: chlorate, perchlorate, nitrate, iodide, iodate, bromide, bismuthate, sulfate, tartrate, fluoride, chloride, phosphite, citrate, phthalate, and thiocyanate. The ions phosphate, bicarbonate, molybdate, cvanate, carbonate, borate, perborate, thiosulfate, bismuthate, tungstate, arsenate, sulfite, silicate, bromate, ferricyanide, ferrocyanide, dichromate, and sulfide interfere in the determination because of a pH effect. When these compounds were added in pH 7.5 phosphate buffer, no interference was observed in the determination.

The effect of pH on the solution added is given in Table III. If the pH of the added solution (0.1 ml.) is held at 6.5 to 7.5, the reaction will proceed smoothly. More acidic or basic conditions will tend to slow the reaction. At pH's of 8 to 10, the solution will turn yellow because of the occurrence of side reactions, and no fluorescence is observed. This observation is similar to that of Reynolds and Van Allan (9), who identified the yellow solution resulting from treating 2,3-dicyano-1,4naphthalenediol with base as 2-cyano-3-hydroxy-1,4-napthoquinone.

The cyanide may be added in a water solution (Table III), provided basic interferences are known to be absent (silicate, phosphate, etc). Best results are obtained at pH 7.5 using phosphate buffer, and it is recommended that the sample be prepared in this medium if possible. Water has a deleterious effect on the rate of reaction at high amounts (>0.3 ml.); the rate decreases with increasing quantities of this solvent (Table III). For this reason, the sample is added in 0.1 ml. of solution.

Besides serving as a specific spot test for cyanide, this reaction can be used to detect specifically quinones and quinoneimine compounds.

Identification of Products. PROD-UCT OF REACTION OF *p*-BENZOQUINONE WITH CYANIDE. The structure of the green fluorescent compound derived from I is believed to be II. Compound II was prepared by treating *p*-benzoquinone in dimethylsulfoxide with KCN. The material was isolated by extraction with ether, and was recrystallized from ether and petroleum ether. The m.p. was 161-3°, and the elemental analysis agrees well with the structure proposed (C calcd, 60.0; found, 59.9; H calcd, 2.5; found, 2.4; N caled, 17.5; found, 17.5). The infrared spectra showed a strong OH band at 3280 and a moderate CN band at 2440 cm.⁻¹ The ultraviolet spectrum indicated a λ_{max} at 245 m μ . This closely resembles the spectrum of hydroquinone. A 3.4 \times $10^{-5}M$ solution of II in dimethylsulfoxide and base exhibited a fluorescence identical to the compounds produced by direct reaction of cyanide with *p*-benzoquinone, $\lambda_{ex} = 410 \text{ m}\mu$, $\lambda_{em} = 470-480 \text{ m}\mu$, and a fluorescence coefficient of 4.4×10^6 (fluorescence in units per concentration in M). The pK's of the compound (II) were determined by titration to be 0.50 and 5.9 corresponding to the reactions



This proposed formula (II) is identical to that previously reported by Thiele and others (2, 10).

PRODUCT OF REACTION OF QUINONE MONOXIME TOLUENE SULFONATE ESTER WITH CYANIDE. Compound VII was prepared by reacting quinone monoxime toluene sulfonate ester (VI where $\mathbf{R'}$ = CH₃) with KCN in dimethylsulfoxide. The product was isolated by extraction with ether and was recrystallized from ether and pet ether. The ultraviolet spectrum indicated a compound similar in structure to phenol, with a λ_{max} at 290 and 240 and a λ_{\min} at 260 m μ . The infrared showed a strong OH band at 3200-3375 m μ and a strong cyanide band at 2220 cm⁻¹. A solution of VII in dimethylsulfoxide and base exhibited a fluorescent compound similar to that produced by direct reaction of cyanide with VI, λ_{ex} = 440 mµ, λ_{em} = 500 m μ , and a fluorescence coefficient of 3.2×10^6 . The structure was postulated because of the similarity of observed properties to compound II. The pK's of the compound(VII) were likewise similar to II, being 0.70 and 5.95.

Further evidence for structure VII was obtained from the elemental analysis (C caled, 66.7; found, 66.5;



H caled, 2.78; found, 2.7; N caled, 19.4; found, 19.2) and the isolation and identification of the other product of VI with KCN as toluene sulfonic acid.

The structure of VII is also postulated on the basis that the maximum fluorescence is obtained at pH > 5.9, corresponding to an ionizable phenolic hydroxyl group. Also, Adams and Moje (1) found that 1,4-naphthoquinone dibenzene sulfonimide reacted with hydrogen cyanide to yield 3,4-dicyano-1-naphthalene benzenesulfonamide. Analogously, one is lead to postulate the sequence of reactions indicated in Equation 6 following the scheme of Adams and Moje.

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 $\lambda_{em} = 500 \text{ m}\mu$

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Chemical Microscopy of Urinary Calculi

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A combination of crystal morphology and simple optics is used to identify components of urinary calculi. Mechanically separated fragments are subclassified by observation with a dispersion staining objective. This provides presumptive evidence for the presence of cystine, calcium oxalate, calcium hydrogen phosphate, and uric acid. Chemical confirmation is achieved by formation of characteristic crystals or simple chemical reaction products. Cystine is confirmed by formation of cystine hydrochloride. Uric acid and urates are confirmed by formation of characteristic uric acid crystals on acidification of a basic solution of uric acid. Calcium is confirmed by formation of calcium sulfate; phosphate is converted to ammonium phosphomolybdate, oxalate is confirmed by formation of oxalic acid dihydrate crystals. Magnesium is converted to a colored lake and ammonia is converted to ammonium chloride. These techniques are rapid and applicable to any sample size to be expected in the clinical laboratory.

UALITATIVE ANALYSIS of urinary calculi is accomplished by chemical (4), optical crystallographic (9-12), x-ray diffraction (5, 12), and infrared spectroscopic methods (1, 6, 13, Although the instrumental 17). methods identify specific compounds and are applicable to the whole range of sample sizes encountered, their use in the clinical laboratory is limited because of other factors. Among these are high instrument cost, long time per analysis, and the necessity for a degree of technical training not normally present in the clinical laboratory. Consequently, chemical methods are usually employed despite their lack of specificity for individual compounds and their frequent failure to detect trace substances such as the material comprising the nucleus of the stone. This work was undertaken to develop an analytical procedure for urinary calculi that possesses the advantages of instrumental methods, yet retains the simplicity and economics of chemical methods. Chemical microscopy employing known reactions when possible was selected to accomplish this objective.