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FATE OF CYANIDE AND RELATED COMPOUNDS IN AEROBIC MICROBIAL SYSTEMS—I.

CHEMICAL REACTION WITH SUBSTRATE AND PHYSICAL REMOVAL

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Abstract—The chemical reaction of cyanide with substrate was investigated in sealed glass ampoules using glucose as substrate and inorganic buffers. The reaction was found to be pseudo-first order and pH dependent, with an optimum pH near 11.0. The cyanide–glucose reaction products were found to be biodegradable by both acclimated and unacclimated heterogeneous cultures in shake flask and BOD bottle systems.

Adsorption onto microbial solids was investigated using sealed, stirred glass reactors containing bacteria and potassium cyanide in water buffered at pH 7.0 with inorganic buffers. Very little adsorption occurred on a starved non-flocculating pure culture of *Bacillus megaterium*, although up to 15% adsorption occurred in systems containing a stirred flocculent heterogeneous culture.

Stripping was investigated from a starved heterogeneous culture in an aerated microfermenter at neutral pH. Hydrogen cyanide and carbon dioxide in the off-gas were trapped in sodium hydroxide solution, separated and analyzed. Stripping removed up to 80% of original cyanide, and tests using $K^{14}CN$ revealed that a small amount of cyanide had been metabolized.

INTRODUCTION

Although cyanide itself can hydrolyze or polymerize in aqueous solution, it has been shown that for cyanide concentrations below 650 mg/l in the case of polymerization and temperatures below 100°C in the case of hydrolysis, these reactions are relatively insignificant (Sanchez *et al.*, 1967). Consequently, at the low temperatures and cyanide concentrations found in most wastewaters, removal of cyanide by hydrolysis or polymerization can be considered unlikely. In the presence of sugars, however, a third mechanism for removal, by chemical reaction, becomes possible. Cyanide can react with the open chain forms of aldoses to form cyanohydrins which then hydrolyze to the corresponding aldonic acids. This reaction has been used extensively as the Kiliani synthesis (1886) for lengthening the aldose carbon chain. The intermediary cyanohydrins usually are difficult to isolate since they are rapidly hydrolyzed *in situ* (Pigman, 1973). Shen and Nordquist (1974) hypothesized that polymerization of the cyanohydrins can also occur. Although the reaction between cyanide and glucose has been known for many years to proceed most rapidly in basic solution (Militzer, 1949), no systematic data on the pH dependence appears to have been reported, and this study was undertaken in part to provide this information. The major reactions of cyanide in aqueous solution with and without glucose are summarized in Fig. 1. The reaction products were also investigated for biodegradability by acclimated and unacclimated mixed bacterial cultures of the type found in domestic waste waters.

Adsorption has been proposed as a means of removing cyanide from waste streams entering sewage treatment plants (Zintgraff, 1969). A patent has been issued describing cyanide removal by treatment with

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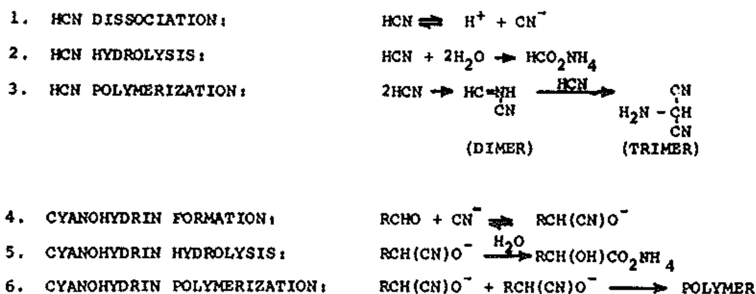


Fig. 1. Major reactions with cyanide in aqueous solution with and without glucose present. Cyanohydrins are shown in deprotonated form.

anaerobic digester solids followed by aerobic degradation of the wastes (Howe, 1903). The anaerobic sludges are reported to adsorb cyanide, rendering the feed non-toxic to the subsequent aerobic process. Since the reduced toxicity may occur due to mechanisms other than adsorption, experiments were designed to test the adsorption hypothesis.

Very little vapor–liquid equilibrium data exists for aqueous solutions containing low concentrations of cyanide. Since cyanide stripping has been reported to be significant in aerated biological treatment of cyanide-containing wastes (Ludzack, 1960), this removal mechanism was also investigated to determine its relative importance.

EXPERIMENTAL

Analytical techniques

Cyanide was estimated by the modified Liebig titration method after adjusting samples to pH 12 (APHA 1973). No detectable cyanide decrease was noted within a 30-min period after addition of 1000 mg/l glucose to a sample containing 10 mg/l cyanide at this pH, indicating that there was no glucose interference in the analysis. Since the cyanide concentration decreased eventually through reaction with the glucose, however, all samples were titrated within 30 min of the base addition.

Glucose was estimated by the Glucostat test (Worthington Biochemical Corporation, 1972), an enzymatic determination specific for D(+) β-glucose. Cyanide was found to interfere with the test, and where necessary was stripped from the sample to be analysed (typically 20 ml) by adjusting to pH 7 and sparging with dry filtered air at a flow rate of 200 cc/min for 3 h. After making up to the original volume with distilled water, the glucose content could then be analysed without interference.

Kinetic studies

Preliminary experiments involving reactions between glucose and cyanide at pH 10.0 in open containers indicated that pH decreased over a 24–48 h period due to carbon dioxide absorption from the atmosphere. To prevent this and cyanide loss due to volatilization at low pH subsequent reactions were carried out in 20 ml sealed glass ampoules.

A typical reaction solution was prepared by dissolving 0.5 g dextrose in 300 ml of the appropriate 0.1 M phosphate or borate buffer solution (Weast, 1968), adding 2 ml of freshly prepared 2.5 mg/ml of CN^- KCN solution, and making up to 500 ml with further buffer. The resulting glucose concentration of 1000 mg/l, compared to 10 mg/l for the cyanide, amounted to a 14-fold excess on a molar basis, and was therefore assumed to be effectively constant throughout the reaction. The contents of the make-up flask were poured into a beaker and drawn immediately into a syringe.

A series of the glass ampoules were then filled with the reaction solution from the syringe, sealed with a minimum vapor space, and placed in a 30° incubator. Periodically an ampoule was removed from the incubator, one end clipped off and inserted below the surface of 4 ml in NaOH in a small beaker, and the other end clipped to allow rapid drainage into the alkali. This procedure was found to minimize stripping loss at low pH. Cyanide was determined by titration as described above, and the volume of the total titrated sample measured. After correcting for volumes of alkali, indicator and titrant, the sample volume and hence, the sample cyanide concentration, could be calculated.

Since one proposed mechanism for bacterial metabolism of cyanide (Castric, 1969) involved an initial reaction between cyanide and serine, this reaction was briefly investigated to see if the reaction could occur extracellularly and without enzymatic catalysis. The experiment was conducted exactly as those involving glucose, with the reactants being studied in sealed glass ampoules. Initial concentration of L-serine (Cal-Biochem) was 1000 mg/l. The reaction was studied only at pH 7.0. There was no evidence of reaction after 22 days at 30°C.

Biochemical metabolism of glucose/cyanide reaction products

Three sets of growth experiments were performed with the aldonic acid products of the glucose–cyanide reaction. To synthesize these reaction products in high concentration, glucose and excess cyanide were reacted for 10 days at pH 11.0, and the cyanide was then stripped from the solution at pH 7. Subsequent analyses for glucose and cyanide were both negative. The solution was characterized by measuring its soluble organic carbon (SOC) content.

In the first experiment three shake flasks were prepared containing equal carbon amounts of reaction products, glucose, and reaction products plus glucose, respectively. Each flask also contained inorganic nutrients, and was inoculated with unacclimated seed consisting of settled primary effluent, filtered through Whatman No. 2 filter paper to remove protozoa. The flasks were stoppered with cotton gauze and shaken at 200 rev/min and 30°C in a New Brunswick Psycrotherm incubator. Growth was monitored indirectly by absorbance at 660 μm.

A second set of shake flask experiments was run in a manner identical to the first except that the flasks were inoculated with 1 ml of acclimated seed obtained from the first set of experiments. Twenty-four-hours following termination of the first experiment, all shake flask contents were combined, mixed, providing the inoculum for the second set of experiments. Growth was monitored in this set of experiments by following absorbance at 610 μm.

A third set of experiments monitored oxygen uptake to follow cell growth indirectly. Manometric BOD bottles (Hach Chemical Company) were filled with 70 ml of reaction products containing 18.1 mg SOC, 5 ml of acclimated seed from the second set of shake flask experiments, and 82 ml of dilution water containing inorganic nutrients and saturated with oxygen. The same ratio of nutrients was used as in the other experiments. Additional BOD bottles containing the same quantities of carbon (as glucose), nutrients, and seed were used as controls. Two BOD bottles were filled with 5 ml of seed and 152 ml of dilution water containing nutrients to provide seed blanks.

Adsorption experiments

In the first experiment, a pure culture of a nonflocculating bacterium, *Bacillus megaterium*, ATCC Strain 2300, was used since this organism is cyanide-resistant and exists as single rods in cyanide-containing media. The organism was cultured in a 1% tryptic soy broth solution at 30°C and harvested after 24 h of incubation by centrifugation at 15,000 × g at 15°C for 10 min, then washed four times with physiological saline. Concentrated cells were then drawn into a 100 ml glass syringe and injected into a 225 ml stirred glass reactor containing a known quantity of cyanide in pH 7.0 phosphate buffer. The reactor was stoppered with a serological stopper through which the solids injection was made. A second syringe needle was used as a vent during solids injection and when samples were removed for cyanide analysis. Solids were added such that the reactor was initially completely filled with liquid. Twelve milliliter samples were periodically removed from the reactor using a 15 ml glass syringe, and after centrifugation in a filled, sealed test tube at 10,000 × g for 5 min at 15°C, 10 ml samples of the supernatant were removed

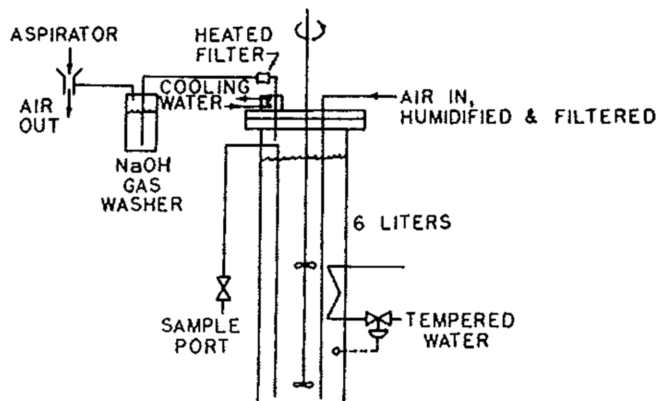


Fig. 2. Microfermenter used in cyanide stripping and metabolism experiments.

and analyzed for cyanide by the modified Liebig titration method.

The second experiment was identical to the first except that heterogeneous flocculent bacteria, acclimated to cyanide, were utilized. In both experiments, 200 ml of cyanide solution was prepared in pH 7.0 phosphate buffer by adding 2 ml of 2.55 mg/l (as CN^-) KCN to 198 ml of buffer in a 200 ml volumetric flask. After mixing, the solution was added to a 255 ml glass reactor (including magnetic stirring bar and stopper), and three 10 ml samples were removed for analysis by the modified Liebig titration method.

Stripping experiments

A New Brunswick Scientific Company Model MMF-07 Microferm Fermenter was used for stripping experiments so that temperature, air flow rate, and mixing could be carefully controlled. Humidified filtered air was used for stripping, and off-gas cyanide and carbon dioxide were captured in two 250 ml Fisher–Milligan gas traps connected in series, each filled with 200 ml of 0.1 N NaOH. A heterogeneous flocculent culture was grown on a 0.5% tryptic soy broth–1% glucose solution plus inorganic nutrients (C/N ratio of 4/1 and C/P ratio of 50/1). The cells were harvested after 24 h, centrifuged at 15,000 × g at 15°C for 10 min, washed four times with physiological saline solution, and then resuspended in deionized water buffered at pH 7.0 using inorganic phosphate buffer. Stripping was investigated at several different solids concentrations so that any effects due to solids would be ascertainable. All tests were conducted at 30°C and at 700 rev/min. Air flow was 2 cc/min at standard temperature and pressure. The reactor system is shown in Fig. 2.

After adding buffered culture solution to the reactor, standardized KCN solution was added, and initial samples for cyanide were taken (after one minute of mixing). Air flow was then started and at predetermined intervals, a sampling valve on a siphon was opened. The off-gas tubing was momentarily pinched shut, approximately 10 ml of sample were wasted into a graduated cylinder (in order to clear the sample line of previous sample) and approximately 20 ml of sample were withdrawn into a 50 ml centrifuge tube containing 4.0 ml of 0.1 N NaOH. The sampling valve was then closed to prevent siphoning back into the reactor, and the off-gas tubing was released. The entire sampling procedure required approximately 20 sec. The wasted amount was recorded and the sample portion was centrifuged at 15,000 × g for 10 min at 15°C. Twenty milliliters of supernatant were then titrated for cyanide using the modified Liebig titration method. The remaining sample volume (cells) was then measured and the total sample volume recorded as additional wastage. Tests were run to determine the amount of cyanide lost during centrifugation by measuring cyanide before and after centrifugation from the same test tube. The amount lost was undetectable.

In one of the stripping experiments, $H^{14}CN$ solution was added in order to determine if the starved cells were utilizing cyanide as a carbon source with consequent production of $^{14}CO_2$. The method for separating $H^{14}CN$ and $^{14}CO_2$ was patterned after that of Brysk (1969).

RESULTS AND DISCUSSION

Chemical reaction

The reaction of cyanide with glucose appears first order in cyanide with a pH optimum near 11.0 (Fig. 3). Most of the reaction solutions sealed in the ampoules were colorless for the duration of the experiments, but those at pH 12.0 turned increasingly straw yellow after 2–3 days. An additional test at pH 13.0 was attempted, but the straw yellow color developed after only 9.5 h and became progressively darker. The rate of cyanide removal, shown in Table 1, was slower than expected at this pH.

Enolization of glucose takes place only under strongly alkaline conditions (Pigman, 1972). The

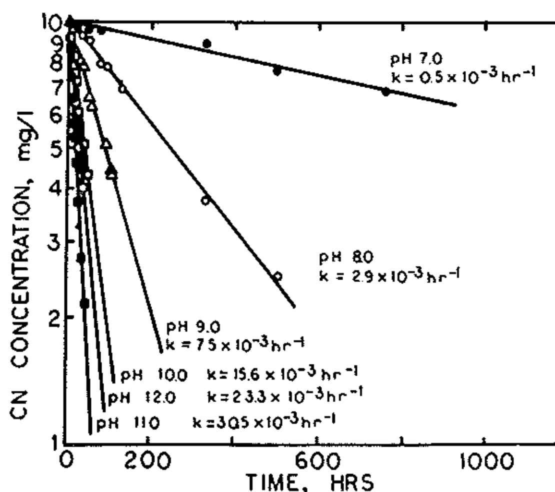


Fig. 3. Cyanide–glucose reaction in sealed ampoules at 30°C.

Table 1. Cyanide glucose reaction in sealed ampoules at 30°C, pH 13.0

Time, h	CN^- conc (mg/l)
0	11.6
2.7	10.7
9.5	10.3
19.2	10.0
27.2	10.0
50.1	9.5

negatively charged intermediate species formed during this base catalyzed reaction would not be amenable to nucleophilic attack by CN^-. This may explain the decrease in rate of cyanide removal, and the apparent occurrence of cyanide polymerization reactions rather than reaction of cyanide with glucose, at pH above 11.

Biodegradation studies

Results of the first set of experiments indicate that the glucose–cyanide reaction products are biodegradable (Fig. 4). The presence of two plateaux for the glucose–reaction product mixture suggests sequential metabolism. As expected, glucose and the mixture were metabolized most readily, followed by the reaction product alone. The results of the second set of experiments (Fig. 5) indicate that organisms can easily acclimate to the reaction products and that the reaction products are non-toxic to acclimated organisms. The fact that the shake cultures metabolizing the substrate mixture demonstrated a slightly shorter lag than the other cultures could be due to selection for dual substrate organisms or could merely be statistical variance since only two shake cultures of each substrate were investigated. The difference in plateau could be due to lower net synthesis from metabolism of the reaction products, or due to lesser absorbance by organisms growing on the reaction products. The difference in lag times between the first two experiments is not particularly significant since inocula used for the second experiment probably contained more bacteria. It is significant that the lag time for all three systems were grouped more closely in the second experiment, offering further evidence of acclimation. Results of the oxygen uptake experiments (Fig. 6) further indicate that acclimated organisms readily degrade the reaction products. The following equation for balanced growth in a BOD bottle at plateau (Busch, 1971) was used to predict oxygen uptake due to conversion of soluble substrate:

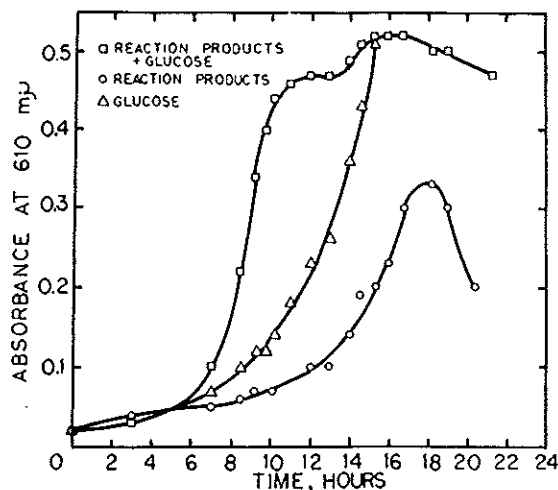
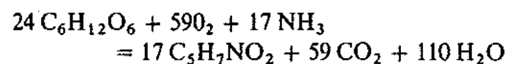


Fig. 5. Biodegradation of cyanide–glucose reaction products by acclimated shake-flask cultures.



$$24\,C_6H_{12}O_6 + 59O_2 + 17\,NH_3 = 17\,C_5H_7NO_2 + 59\,CO_2 + 110\,H_2O$$

Based on this stoichiometry, the glucose plateau BOD was expected to be 129 mg/l. A plateau BOD of 114 mg/l was obtained, indicating close agreement. Assuming the reaction products are aldonic acids, these would be expected to exert a lower BOD due to a higher percentage of molecular oxygen than glucose for an equal carbon amount.

Adsorption

Results of the two experiments are given in Tables 2 and 3. No significant cyanide reduction occurred

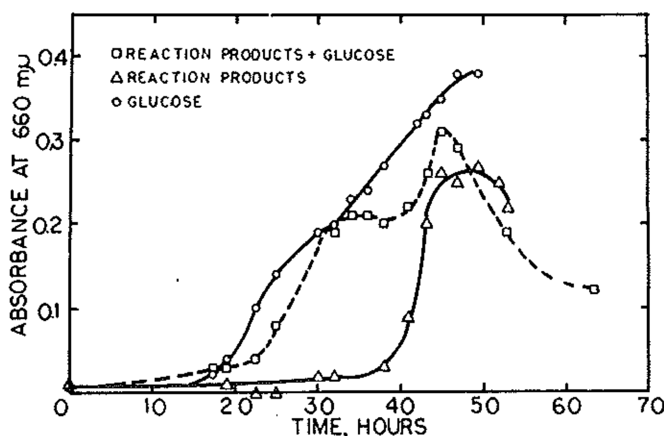


Fig. 4. Biodegradation of cyanide–glucose reaction products by shake-flask cultures of unacclimated sewage organisms.

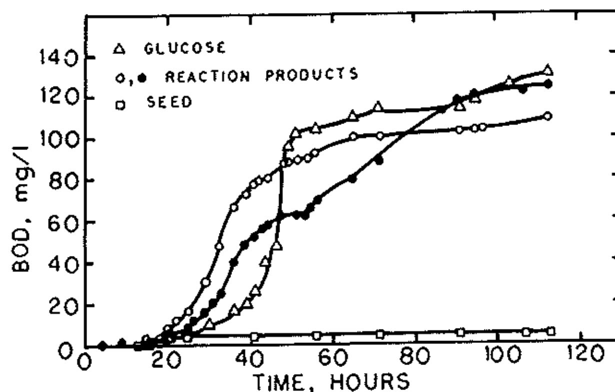


Fig. 6. BOD exerted by acclimated sewage organisms metabolizing glucose and cyanide–glucose reaction products.

Table 2. Adsorption of cyanide by a nonflocculating culture of *Bacillus megaterium*

Contact time (min)	CN^- (mg/l) Replicate 1	Replicate 2
3	19.8	20.6
20	20.6	20.8
40	20.0	20.6
60	20.0	

Initial conditions: CN^- conc, 20.0 mg/l. Dry biological solids 6000 mg/l.

Table 3. Adsorption of cyanide by a flocculant culture heterogeneous bacteria

Contact time (min)	CN^-, (mg/l)
1	16.0
13	15.2
25	15.3
38	14.7
48	14.5
60	14.1

Initial conditions: CN conc 16.0 mg/l. Dry solids 7260 mg/l.

with nonfloculating cells, but up to 12% removal occurred in a one hour contact period with flocculating cells. These results suggest that surface characteristics of the cells determine the degree of cyanide adsorption. Since a monolayer of cyanide ions could adsorb onto the cells without appreciably decreasing the observed cyanide concentration, these experiments do not rule out adsorption but rather suggest that it plays a rather insignificant role in overall cyanide removal observed in biological treatment plants. A patent has been issued (Howe, 1963) describing cyanide removal by treatment with anaerobic digester solids followed by aerobic bio-oxidation of the wastes. The anaerobic fermentation sludges are reported to adsorb cyanide, rendering it non-toxic to the subsequent aerobic process. Results of these experiments suggest that the observed reduced toxicity is probably due to other phenomena such as chemical reaction or stripping during aerobic treatment.

Stripping

Results of the experiments are given in Figs. 7–9. As seen in Fig. 7, virtually 100% cyanide recovery was obtained with no biological solids present. However, Fig. 8 indicates over 16% of the cyanide was unaccounted for at the termination of the experiment when large amounts of biological solids were present. Stripping rates at various solids concentrations were very similar except for an initial decrease when solids were present (Fig. 9). This decrease (approximately

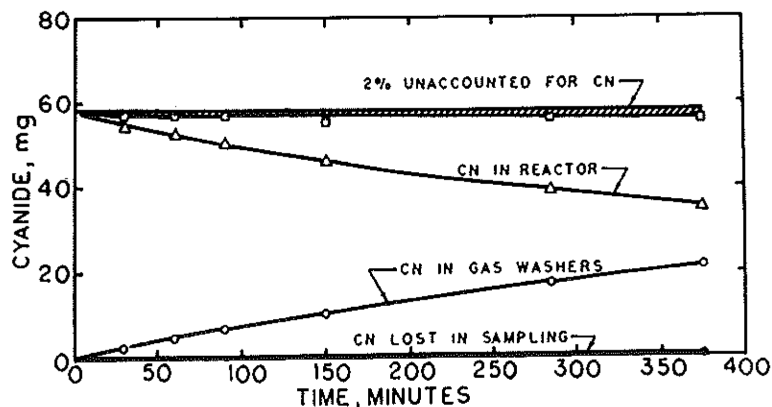


Fig. 7. Stripping of cyanide with no solids present.

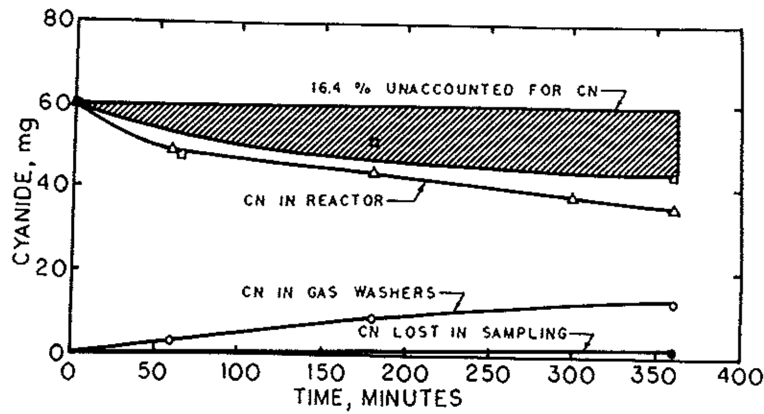


Fig. 8. Stripping of cyanide with 3130 mg/l solids present.

15%) could be attributed to physical adsorption, metabolism, or even cyanide reaction with biological polymers. Due to the rapid cyanide decrease, physical adsorption seems the more plausible explanation, especially in light of the earlier adsorption experiment using heterogeneous cultures. As seen in Fig. 4, within the accuracy of the cyanide test used, stripping is first-order with respect to cyanide. Results of the test using $K^{14}CN$ and 3130 mg/l biological solids are given in Figs. 8 and 10. Significant ^{14}C was found in the washed cells at the termination of the stripping experiment. This, in conjunction with the $^{14}CO_2$ produced (see Fig. 10), suggests that ^{14}CN was being metabolized as a substrate to a small degree. This accounts for a portion of the cyanide decrease that would otherwise be attributed to physical adsorption.

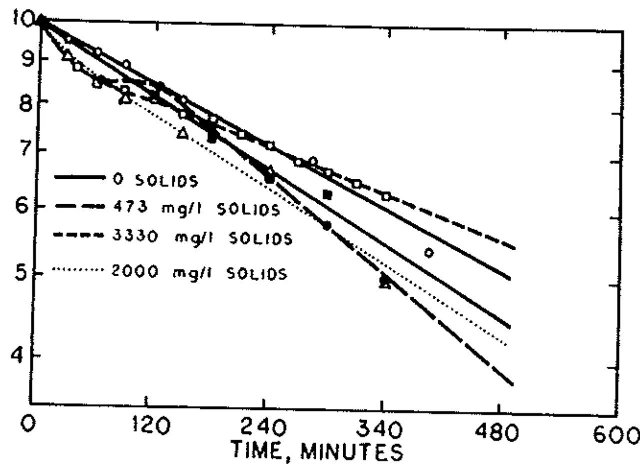


Fig. 9. Cyanide stripping at neutral pH with biological solids present.

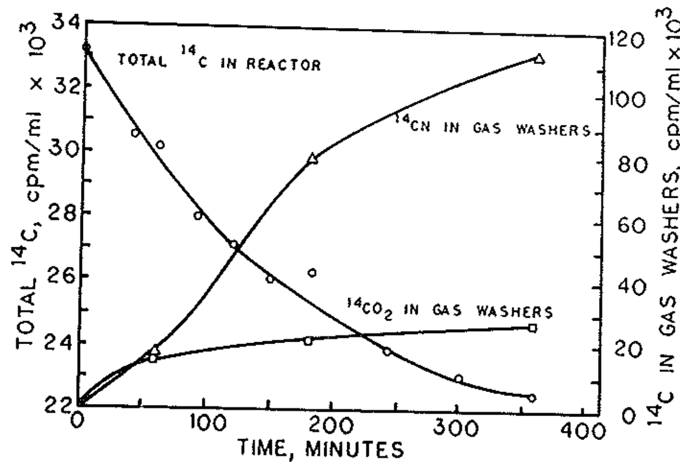


Fig. 10. $H^{14}CN$ stripping with 3130 mg/l biological solids present.

CONCLUSIONS

Significant reactions between cyanide and aldoses such as glucose can occur above pH 8, indicating that alkaline storage of cyanide samples containing aldoses can result in errors in analysis of cyanide. The reaction with glucose is pseudo-first order, with an optimum pH of 11.0. The reaction products are biodegradable, offering a possible method for detoxifying cyanide-containing wastes, particularly when cheap sources of aldose carbohydrates are available such as wastes from textile mills, canneries, breweries, beet sugar plants, cereal grain processing plants and pulp and paper mills.

In an aerated biological system in which cyanide removal is occurring, cyanide stripping is an important removal mechanism. Adsorption on biological floc is of lesser importance, and appears to be influenced by the extracellular composition of bacterial cells.

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