Effects of Low Concentration of Cyanide on Growth and Respiration in *Pelomyxa carolinensis*-Wilson. (18424)

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The inhibitory effect of cyanide on respiration in animal cells in general is now an accepted fact. Chiefly because of technical difficulties, the rather irregular results obtained in early investigations with protozoan cells gave the impression that ciliates were, perhaps, an exception in that they did not appear to be cyanide sensitive. One of the earliest reports on the effects of cyanide on protozoan species was that of Lund(1). He maintained that KCN did not affect respiration in *Paramecium caudatum*. These results appeared to be confirmed by those of Shoup and Boykin(2). Peters(3) did not find inhibition of respiration in *Colpidium colpoda* in solutions containing KCN, while Pitts(4) actually observed a 25% increase in oxygen uptake in *Colpidium campylum*. Lwoff(5) reported increases of as much as 30 percent above normal oxygen consumption by *Glaucoma piriformis* in M/1000 KCN in Ringer's solution. However, more recently, Boell working with *Paramecium calkinsi*(6), Pace with *P. caudatum* and *P. aurelia*(7), and Clark with *P. caudatum*(8) found that, when proper precautions are taken, cyanide inhibits respiration in these forms. In the latter investigations, balanced KOH-KCN absorption mixtures [Krebs(9)] were used in the flasks of the Warburg respirometer. Under these conditions, it was found that solutions of 10^{-5}M KCN concentration, or greater, inhibited respiration. The results indicate that much of the work reported previously was at fault in that these precautions were not taken and that cyanide was actually lost from the test solution by vaporization and absorption in the alkali. Pace and Belda(10) and Pace and Kimura(11) have shown that *Pelomyxa carolininsis* is also sensitive to cyanide. The sensitivity appears to be of the same order as that for the ciliate species. In solutions containing 10^{-5}M KCN or more, there is approximately a 65% inhibition in respiration.

The increase in respiration as found by Pitts and Lwoff(4) for certain protozoan species has not, as yet, been explained. Since they did not use KOH-KCN balanced absorption solutions it is evident that there must have been a continuous loss of cyanide from the solution containing the organisms. The final concentration of cyanide in solution may have been comparatively very low. The following investigation was carried out to ascertain the effects that low concentrations of cyanide might have on growth and respiration.

Materials and methods. The large multinuclear amoeba, *Pelomyxa carolinensis* Wilson, was used in this study. It was cultured in the special solution given in Table I.

Stock cultures of the organism were made up by adding approximately 150 ± 50 pelomyxae to 400 ml of the culture solution in stacking dishes of 20 cm diameter. Excellent growth occurred in 14 to 20 days if the

TABLE I.
Culture Solution for the Growth and Maintenance of *Pelomyxa carolinensis* Wilson.

K_2HPO_4	67 mg
NaH_2PO_4	39 "
$CaCl_2$	100 "
$MgCl_2$	22 "
Distilled water to	1000 ml

1. Lund, E. J., *Am. J. Physiol.*, 1918, v45, 365.
2. Shoup, C. S., and Boykin, J. T., *J. Gen. Physiol.*, 1931, v15, 107.
3. Peters, R. A., *J. Physiol.*, 1929, v68, 2.
4. Pitts, R. F., PROC. SOC. EXP. BIOL. AND MED., 1932, v29, 542.
5. Lwoff, M., *Compt. Rend. Soc. Biol. Paris*, 1934, v115, 237.
6. Boell, E. J., *Proc. Nat. Acad. Sci.*, 1945, v31, 396.
7. Pace, D. M., *Biol. Bull.*, 1945, v89, 76.
8. Clark, A. M., *Austral. J. Exp. Biol.*, 1945, v23, 317.
9. Krebs, H. A., *Biochem. J.*, 1935, v29, 1620.
10. Pace, D. M., and Belda, W. H., *Biol. Bull.*, 1944, v87, 138.
11. Pace, D. M., and Kimura, T. E., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 223.

TABLE II. Effect of Low Concentrations of KCN on Growth of *Pelomyxa carolinensis* Wilson.

Molar conc. KCN	No. of cultures	Age of cells	Avg No. cells/culture after 7 days	% inhibition	% increase
0	3	Not recorded	166*		
10^{-5}	3	" "	90*	54	
0	23	Young	187		
10^{-7}	23	"	181	3.7	
0	20	Old	156		
10^{-7}	20	"	145	8.4	
0	18	Young	96		
10^{-9}	20	"	131		49.3
0	20	Old	125		
10^{-9}	20	"	142		17.0
0	19	Young	205		
10^{-11}	20	"	218		6.3
0	20	Old	129		
10^{-11}	20	"	128		0.9

* Cultures counted at the end of 6 days.

organisms in the cultures were fed every 4 days. The food organism, *Paramecium caudatum,* was grown in glass jars containing 500 ml of culture solution to which 0.2 g of proteose peptone (Difco) and 0.1 g of glucose had been added. After four to seven days, when the cultures were flourishing, 10 ml of the liquid containing the organisms were withdrawn from the edges at the top of the solution, filtered through loose cotton and by means of a hand centrifuge washed by centrifugation at the rate of 8 revolutions per second for 30 seconds. Paramecia contained in the equivalent of 10 ml were added to a pelomyxa stock culture at each feeding. These paramecia cultures could be used for feeding purposes for 3 to 5 weeks.

Results. Effect of low concentrations of cyanide on growth in pelomyxa. Growth tests were conducted as follows: 25 pelomyxae were added to 100 ml of the culture solution containing the equivalent of 10 ml of paramecium culture. For all tests, adequate portions of a concentration of washed paramecia which had been placed in a smaller amount of solution were added so that the number of paramecia was constant for all cultures. In those tests in which the highest concentrations of cyanide (10^{-5}M) were used no effort was made to prevent loss of cyanide by diffusion. However, for all other concentrations culture dishes were sealed to prevent excessive loss of cyanide as HCN. Results in lower concentrations of KCN showed that no appreciable difference in growth resulted when culture dishes were sealed with vaseline or placed in sealed desiccators above a cyanide solution 10 to 100 times the concentration of that used in the test solutions. Therefore, the results of all tests were tabulated together.

The cultures were placed in a dark chamber in which the temperature was maintained at 26 ± 2°C and did not change more than 1°C over a 24 hour period. The effect upon "young cells" was ascertained by using pelomyxae from 2- to 4-day stock cultures and for "old cells" stock cultures 10 to 12 days old were used. The number of pelomyxae in test cultures at the end of the growth period was ascertained by counting with a low power magnifying lens. The results are presented in Table II.

There is a definite inhibition of growth in *Pelomyxa* in solutions containing 10^{-5}M KCN as compared to the controls. In solutions containing 10^{-9}M KCN, there is a significant increase in growth when both "young" and "old" pelomyxae were used, although in 10^{-11}M KCN, there is no significant difference.

Aside from cyanide itself, potassium was the only other variable in the above tests. Because of the possibility that this might have had an effect on growth the following tests were carried out. In these tests potassium chloride was added to the solution in place of potassium cyanide and in the same concentrations. The results are given in Table III.

TABLE III. Effect of Low Concentrations of KCl on Growth of *Pelomyxa carolinensis* Wilson.

Molar conc. KCL	No. of cultures	Age of cells	Avg No. cells/culture after 7 days	% inhibition	% increase
0	8	Young	122		
10^{-7}	4	"	124		2.1
0	4	Old	85		
10^{-7}	5	"	84	1.7	
0	8	Young	122		
10^{-9}	4	"	125		3.1
0	4	Old	85		
10^{-9}	4	"	89		6.7
0	5	Young	204		
10^{-11}	5	"	202	0.9	
0	4	Old	85		
10^{-11}	5	"	85		

The results are remarkably similar in the controls to those of the test cultures. The slight differences that do exist are not significant. It is evident that very low concentration of potassium has very little, if any, effect on growth in pelomyxa.

Effect of low concentrations of cyanide on respiration in pelomyxa. Pace and Belda(10) found that potassium cyanide inhibits respiration in *Pelomyxa;* the lowest concentration tested by them was 10^{-5}M. Because of the results obtained on growth with much lower concentration of cyanide as reported above, it seemed expedient that observations be made upon the effect of these low concentrations on respiration. The Barcroft-Warburg micro-respirometer was used for this study. Pelomyxae were counted and washed in fresh solution containing the desired concentration of KCN (Table I) the day before each test. They remained in this solution overnight. At the time of the test, the organisms were washed again and placed in the Warburg flask in 5.0 ml of the solution. Into the side-arm of each flask 0.3 ml of M HCl was introduced. The center well contained 0.2 ml of the proper KOH-KCN mixture.* Observations were made over a period of from 4 to 6 hours for each test. The results of these experiments are presented in Table IV.

Respiration is inhibited by about 50% in 10^{-7}M KCN and by 15% in 10^{-9}M. A definite increase in respiration is shown by *Pelomyxa* in the lowest concentration (10^{-11}M KCN) used in these studies. The increase in rate of respiration seems to be greater for the "old" pelomyxae than for the "young" which is just the reverse of that obtained in the growth studies. The significance of this difference is as yet not known.

Discussion. Pace and Belda(10) and Pace and Kimura(11) used cyanide in concentrations that inhibited respiration in *Pelomyxa,* and from their results, it appears that up to 70% of its oxygen consumption is dependent upon a cytochrome-cytochrome oxidase system. Results presented in this paper indicate that growth processes are also inhibited in 10^{-5}M KCN. This might be anticipated since general metabolic activity including growth would be affected by loss of the CCO system.

The increased respiration in low cyanide concentration, (10^{-11}M KCN), offers some evidence to indicate that increased respiration in ciliates as observed by Pitts(4) and Lwoff (5) might possibly have been due to the fact that proper precautions were not taken to prevent excessive cyanide loss. Thus, as more cyanide was lost from their test solutions, the concentration approached the level which caused accelerated respiration, or at least did not inhibit it. That this effect is due to cyanide, and not potassium ion concentration, is demonstrated by substituting KCl for KCN.

*The KCN and KOH concentrations of the absorption solution varied with the KCN concentration of the experimental culture fluid as follows:

Molar conc. of KCN in culture sol.	Absorption solution in inset
10^{-7}	10 ml M KOH; .05 ml M KCN
10^{-9}	10 ml M KOH; .01 ml M KCN
10^{-10} or 10^{-11}	10 ml M KOH; .01 ml M/10 KCN

TABLE IV. Effect of Low Concentration of KCN on Respiration in *Pelomyxa carolinensis* Wilson.

Molar conc. KCN	No. of tests	Duration of tests	Avg O_2 consumption, in mm^3/million/hr	% inhibition	% acceleration
0	3	4 min.	12060		
10^{-7}	3		5930	50.9	
0	13	4 hr	7430		
10^{-9}	11		6290	15.3	
0	3	6 hr	4030		
10^{-10}	3		4290		6.5
0*	9	4 hr	13670		
10^{-11}*	7		18140		32.7
0†	10	4 hr	9450		
10^{-11}†	12		14115		49.3

* Young cells, 2-3 days.
† Old cells, 7 days.

There is a direct relationship between respiratory processes (furnishing energy) and processes by which new protoplasm is synthesized. One would therefore expect that inhibitions or accelerations of growth or respiratory processes would occur at similar concentrations of cyanide. The fact that this did not hold true in these investigations may be due to some loss of cyanide from the solution in the dishes used for studying growth. Therefore, the concentration of cyanide put into the solution that supported maximum growth (10^{-9}M KCN) is probably not the concentration that exists after the dishes have been sealed for a few hours. It possibly approaches the concentration that was found optimum for oxygen consumption (10^{-11}M).

Further investigation is necessary to attempt to ascertain the reason for acceleration of growth and respiration in low concentrations of cyanide, although several hypotheses may be stated from the data obtained. For example, there is some evidence that cyanide is detoxified and at the same time has a reducing action on glutathione [Voegtlin, Johnson, and Dyer(12)]. This reduction may enhance the action of glutathione as an agent associated with the respiratory process. Such an action would not be evident in higher concentrations of cyanide because the increased action of glutathione would be nullified by the great decrease in the action of the CCO mechanism. The presence of glutathione in *Pelomyxa* was suggested by the work of Pace and Belda(10) and also by positive nitro-prusside tests (unpublished results). It has been suggested that about 30% of the respiration in *Pelomyxa* is associated with glutathione.

It is also possible that there may be regulatory mechanisms within cells which tend to retard action in other systems and which are even more sensitive to cyanide than the CCO system. These mechanisms would be inactivated first and if the concentration of cyanide is low enough there would be no action on cytochrome oxidase, resulting in an actual increase in metabolism. It is hoped that our knowledge on the possible mechanisms involved will be enlightened by future work.

Summary. 1. Concentrations of KCN from 10^{-7}M and higher cause inhibition of growth in *Pelomyxa carolinensis*. 2. In solutions containing 10^{-9}M KCN there is on the average approximately a 50% increase in growth in "young" *Pelomyxa* and a 17% increase in "old" *Pelomyxa*. 3. These effects are not caused by increase or decrease in potassium concentration. Potassium, alone, has very little effect, if any, in very low concentration. 4. Concentrations of KCN from 10^{-9}M and higher cause inhibition of respiration in *Pelomyxa*. 5. In solutions containing 10^{-11}M KCN, there is an approximate 32 and 50% increase in rate of respiration in "young" and "old" pelomyxae respectively, as compared to controls without cyanide.

12. Voegtlin, C., Johnson, J. M., and Dyer, H. A., *J. Pharmacol. and Exp. Therap.*, 1926, v27, 467.

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