THE INSENSITIVITY OF PARAMECIUM TO CYANIDE AND EFFECTS OF IRON ON RESPIRATION

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Some recent work, notably that of Dixon and Elliott,[1] has indicated that not all cells and tissues are completely cyanide-sensitive in the sense that they reduce their rate of respiration in the presence of cyanides. Allen[2] has pointed out that not all the respiratory exchange in *Planaria* may be inhibited by cyanides. This has indicated that there may be other oxidation systems in the cells besides those affected by cyanides. Lund[3] found some years ago that KCN had little or no effect on oxygen consumption by *Paramecium*. We proposed to reinvestigate this question and to determine if iron is present in the protoplasm of *Paramecium*, acting as the respiration catalyst according to the theory of Warburg, and to note if addition of iron would cause an increase in the rate of respiration in the *Paramecium*.

All of the present experiments have involved the measurement of oxygen consumption in the Thunberg-Winterstein microrespirometer. Using the type of respiratory vessel illustrated in Fig. 1, it was a simple matter to remove or add organisms in various culture media and solutions. In all experiments a preliminary determination was made of the rate of oxygen consumption before cyanide was added to the sample of *Paramecium*. The comparative rates of oxygen consumption indicated the intensity of total oxidation within the cells under experimental conditions.

To test the apparatus and the methods employed, two experimental runs were made on the same sample of *Paramecium* suspended in tap water. It was found that they consumed the same amount of oxygen over the same period of time. When 1 cc. of a 2 cc. sample of organisms was removed, and replaced with 1 cc. of distilled water without organisms, the respiration rate of the sample was re-

[1] Dixon, M., and Elliott, K. A. C., *Biochem. J.*, 1929, **23**, 812–830.

[2] Allen, G. D., *Am. J. Physiol.*, 1919, **48**, 93.

[3] Lund, E. J., *Am. J. Physiol.*, 1918, **45**, 365.

duced 50 *per cent*, approximately one-half of the total number of cells being removed by this method. When a sample of *Paramecium* was removed after a normal run, washed by centrifuging in distilled water, and replaced in the respirometer, no change in the respiratory rate was observed. It was perfectly safe to remove experimental samples from the apparatus, wash them in a solution, and replace in the apparatus, without significant loss of cells. The actual experiments carried through in this way were divided into two groups: (1) those in which the organisms were not suspended in distilled water nor tap water, but were in culture media with a normal food supply, and (2) those in which the cells were washed in distilled water to remove food supply and allow the organisms to come to a "basal" state for experimental purposes.

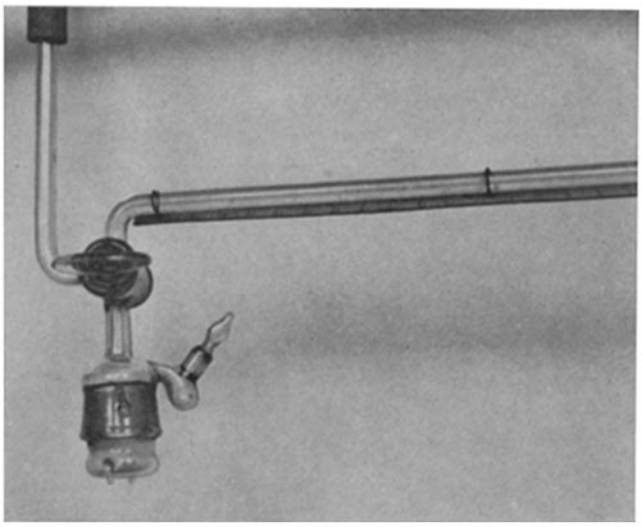

FIG. 1. Type of respiration chamber used in the microrespirometer

In the first group of experiments determinations of the oxygen consumption of samples of *Paramecium* in culture media were made, then the cells were centrifuged out of the media, suspended in KCN solutions of different strengths, and experimental determinations made of the effects of the KCN on the oxygen consumption. In all such cases there were reductions in the rate of respiration from 10 per cent to 50 per cent, when the medium was replaced by cyanide concentrations of M/200, M/300, M/500, M/1000, M/2000, M/4000, M/5000, or M/10,000.

It is interesting to note in this connection that Lund[3] points out that the rate of oxidation in the cell decreases simultaneously with the disappearance of available food. When the *Paramecium* in the above experiments were placed in the solutions of pure cyanide they were deprived of their food supply. It was possible

that this might be the real cause of the pronounced decrease in the respiration rate, rather than the actual effect of the KCN. The reduction in the oxygen consumption was indicated not to be produced by the action of KCN by the following experiments:

The respiration rate of samples of *Paramecium* in culture media was taken and then the same samples were washed with distilled water, suspended in the same volume of distilled water as the culture media they were previously in, and respiratory determinations again made on the organisms freed of their natural food. In such cases there was an average reduction of respiration of near 44 per cent in the absence of nutrient media. On further washing and replacement of the distilled water with an equal volume of M/4000 KCN, no further decrease in the respiratory rate was observed, but actually an increase of 6 per cent over the run in distilled water where food was absent. All of the cells remain in a normal condition and retain their activity with such treatment. It can be seen that the presence or absence of a nutrient medium had the marked effect on oxygen consumption, whereas the KCN had little or no effect.

All of the later and significant experiments were performed on *Paramecium* in which the normal preliminary determination of oxygen consumption was made in the absence of culture media, with washed *Paramecium* suspended in distilled water or in phosphate buffer solutions, followed by experimental runs on the identical sample of organisms in the KCN solutions of the concentrations used. The total results from this group of experiments showed a slight increase in the amount of oxygen consumed in the presence of buffered or unbuffered KCN solutions, rather than the marked decrease in oxygen consumption expected on the basis of experiments with other organisms and tissues.

Paramecium allowed to stand in M/1000 KCN solutions for 4 hours before being tested for respiratory rate, still showed no decrease in their rate of oxygen consumption as measured in the microrespirometer. In all the experiments performed, no decrease in the respiration rate was observed in any concentration of KCN from M/200 to M/10,000. In fact most experiments indicated a slight increase in the amount of oxygen consumed in the presence of the cyanide.

The average pH of the culture media in which the *Paramecium* were grown was found to be 7.3. The pH of a M/5000 KCN solution is about 8.8, and of a M/1000 KCN solution 9.7, an increase in alkalinity that is quite marked in unbuffered solutions.[4] The question arose

[4] *Cf.* Bodine, J. H., *J. Gen. Physiol.*, 1924, **7**, 19–23.

regarding the effect of pH on the oxygen consumption of the *Paramecium*. Buffer mixtures of KH_2PO_4 and Na_2HPO_4 adjusted to a pH of 7.3 in M/100 concentrations held added KCN of high concentration in a constant pH range, varying only 0.1–0.2 pH in electrometric measurement. In all cases when these buffer mixtures were substituted for distilled water or tap water as the suspension medium, no effect was observed on the addition of KCN of various concentrations in any way differing from that when the protozoa were sus-

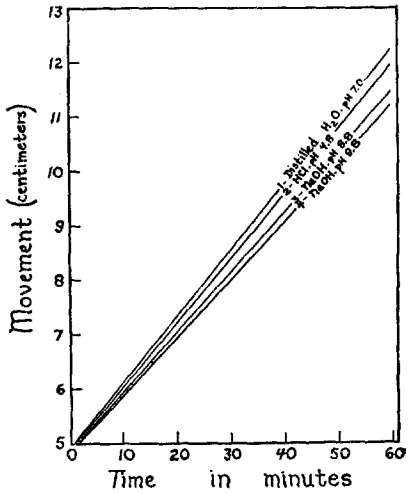

FIG. 2. Effect of HCl and NaOH on the respiration rates of a sample of *Paramecium*.

pended in an unbuffered non-nutrient medium. These experiments agree with those of Lund,[5] who added hydrochloric acid to his cyanide solutions.

The alkalinity of a pure KCN solution does not seem to affect *Paramecium*. Experiments performed with these organisms in the presence of NaOH at pH values from 8.0 to 9.8 caused an average 11 per cent decrease in the rate of oxygen consumption under that observed in distilled water. These were the pH values of some unbuffered KCN solutions used. This seems to add strength to the assumption that a change to an alkaline condition comparable to that

[5] Lund, E. J., *Am. J. Physiol.*, 1921, **57**, 336.

found in pure cyanide solutions does not inhibit or affect to a marked degree the action of the cyanide. The NaOH solutions really cause a slight decrease in the oxygen consumption while pure cyanide caused a slight increase in oxidation rate at the same pH values. We are of

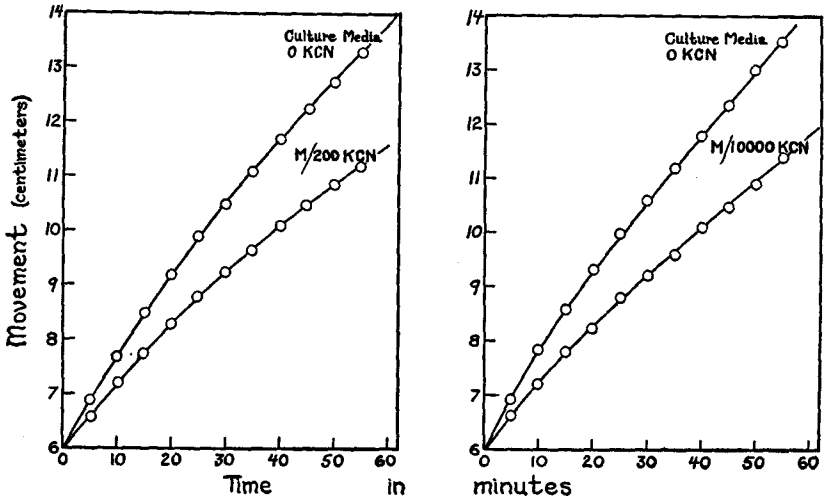

FIG. 3. Slight effect of different concentrations of KCN on oxygen consumption (explanation in text).

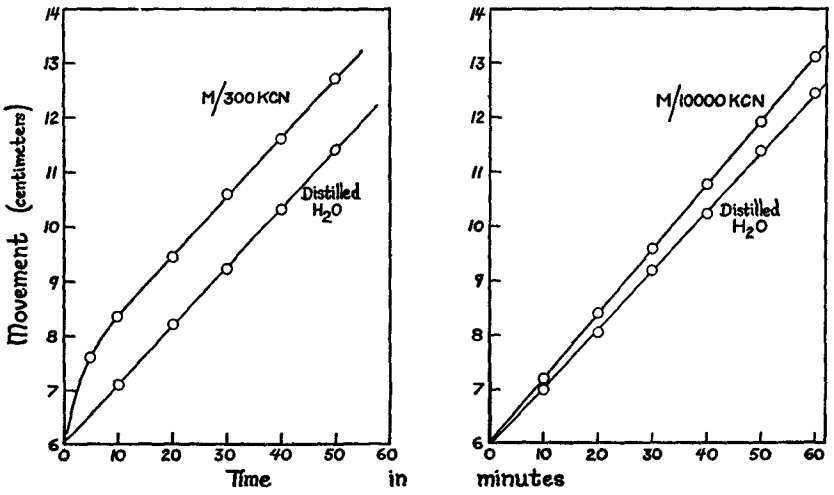

FIG. 4. Lack of inhibition of respiration by potassium cyanide (explanation in text).

the opinion that it is unnecessary to strictly control the hydrogen ion concentration of the media surrounding the *Paramecium* in order to obtain an indication of the insensitivity of this protozoan to cyanide. In Fig. 2 is shown the result of a series of experiments on the effects of hydrochloric acid and sodium hydroxide on the oxygen consumption of a sample of *Paramecium*. The region of change of the rate of oxygen consumption with change in pH is very narrow indeed. We have found that acid solutions are much more injurious to *Paramecium* than alkaline substances, a condition not encountered when using KCN.

Fig. 3 shows the results of two interesting experiments on two samples of *Paramecium* with nearly the same number of organisms in each sample. The preliminary determination of oxygen consumption in each case was made with the cells in culture media. This was removed and replaced with KCN solutions of M/200 concentration in one experiment and M/10,000 concentration in the second. Here it is interesting to note that even in widely differing concentrations of cyanide, the percentage reduction of oxygen consumption remains nearly identical, *i.e.*, 26 per cent reduction in the one case and 29 per cent reduction in the other.

It can be shown, as mentioned above, that if the natural medium is first removed from the organisms, and they are suspended in a buffered medium or in distilled water, there will be no resulting reduction in respiration on the addition of KCN whatever, but instead, a slightly increasing rate of respiration over a short period. The whole difference in the rates of oxygen consumption in the two experiments is apparently due to the amount of available food material or to the effect of a changed immersion medium, and in no way connected with inhibition by cyanide. An example of such a condition is indicated in Fig. 4. In each case the normal rate of respiratory activity is approximately the same for the two samples of organisms suspended in distilled water. On the addition of KCN in the indicated concentrations (M/300 and M/10,000) there was no decrease in the oxygen consumption. One notices instead a temporary increase in the oxygen consumption on the addition of M/300 KCN. We are inclined to disregard the first portion of this curve, on the assumption that equilibrium had not been established in the apparatus, and to regard the straight por-

tion of the curve as significant. It will be noted that no decrease in respiratory activity is indicated during nearly an hour of exposure of the cells to the high concentration of KCN. Similarly, on replacing the distilled water with M/10,000 KCN, no decrease in oxygen consumption was indicated, but instead a slight increase comparable to the effects of low concentrations of pure non-toxic salts.

In Fig. 5, the relative effects of the presence or absence of a nutrient medium may be compared with the effect on total oxygen consumption of M/4000 KCN when used as the immersion medium. It

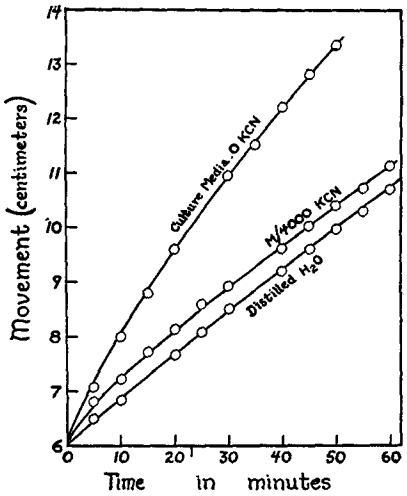

FIG. 5. Comparative effects of nutrient and non-nutrient media on the oxygen consumption of a sample of *Paramecium*, together with absence of inhibition of respiration by KCN (explanation in text).

will be seen that removal from a nutrient medium to a non-nutrient one produces a decrease in the rate of oxygen consumption in these protozoa, and addition of M/4000 KCN instead of the non-nutrient medium has no inhibitory effect.

We have found that sometimes, in a completely unbuffered medium, cyanide of the concentration of M/200 will injure and rupture the protozoan cell, and always that great injury results if concentrations of M/100 and higher are used. This rupture with death of the cells results in a decrease in the oxygen consumption of the sample of organisms, but it is reasonable to expect that if the cyanide is acting

in any way as a true respiratory poison, it would inhibit the respiration of the cells without their rupture, and at concentrations showing no inhibitory effect whatever in these experiments. Gerard and Hyman[6] have recently found *Paramecium* relatively insensitive to NaCN in high concentrations, to much the same extent as we find for KCN.

We have found it practically impossible to demonstrate the actual penetration of KCN into the cells of *Paramecium* when in low concentrations of cyanide. There is absolutely no change in the microscopic appearance of the cells, but only an increased activity and an occasional increase in vacuole formation. It is an interesting fact that although KCN in concentrations greater than M/200 causes swelling of the cells and seems to enter them, there is no reduction in the respiratory activity of the organisms until the cells are actually ruptured and destroyed, whereupon respiration completely ceases. Lund (1921) has pointed out that cytolized and disintegrated *Paramecium* always cease to show respiratory activity perfectly independently of the presence or absence of cyanide. Further work is now under way to determine the extent and speed of the penetration of KCN into the *Paramecium* cell.

Effects of Iron Compounds

The experimental indications have been set forth in this paper that *Paramecia* are stable with respect to their oxygen consumption in the presence of KCN. Some evidence exists that cells sensitive to cyanide (*Paramecium* seem to be insensitive) show an increase in their oxygen consumption in the presence of iron, particularly iron in the form of more soluble iron salts which may be capable of augmenting the respiration-catalyst in the cell, if iron may be considered as such. We have conducted a series of experiments to determine the effects of various concentrations of ferric oxide, ferrous chloride, ferric sulfate, ferrous phosphate, ferrous nitrate, and ferrous ammonium sulfate, on the rate of oxygen consumption in *Paramecium*. These substances were prepared in solutions or suspensions in distilled water. Careful check was made of the pH values of the solutions added to the organisms in the microrespirometer, replacing the distilled water or

[6] Gerard, R. W., Private communication, 1931.

tap water suspension medium in which preliminary determinations of oxygen consumption were made.

Table I summarizes and presents the data from these experiments on iron compounds. Ferric oxide, being insoluble in water, was taken into the gullet of every *Paramecium*, and became concentrated in vacuoles, sometimes accumulating to so great an extent as to alter the movement of the cell in swimming about. In high concentrations (M/1000) the cells were killed and ruptured, with consequent loss in respiratory rate. In lower concentrations (M/5000) of ferric oxide, a reduction of respiratory rate could be obtained from 10 to 30 per cent. On being returned to distilled water, the organisms recovered their normal rate of oxygen consumption. Several interesting experiments in this connection involved replacement of the M/5000 ferric oxide with M/1000 KCN, whereupon the protozoa returned to their normal respiratory rate, perfectly independent of the presence of the KCN, as though the medium contained no foreign substance whatever. There was no tendency for the ferric oxide taken in by the cell to counteract the lack of effect of cyanide, and both of these substances act in the same manner as when used separately. It is the opinion of some workers that iron and cyanide act as antagonists to each other but no indication of this effect is apparent in these experiments.

It is an interesting fact that M/5000 ferric oxide produces the same reduction in the rate of respiration whether in a buffered or an unbuffered solution, so long as approximately the same number of cells are present.

Ferrous chloride ($FeCl_2$) is extremely toxic for *Paramecium*. The organisms live for only a few seconds in an M/1000 solution. M/10,000 solutions of ferrous chloride produce marked reductions in the rate of respiration with no apparent effect on the microscopic appearance of the cells. Ferrous chloride solutions are quite acid in reaction (M/5000 $FeCl_2$ = pH 4.7), but experiments were conducted with *Paramecium* in distilled water and then in HCl of pH 4.8 without change in oxygen consumption from the normal rate in the solution near neutrality. If however, solutions of ferrous chloride are neutralized with phosphate buffer, they have less effect in lowering the oxygen consumption of *Paramecium*. In a similar manner, on addition of NaOH to solutions of ferrous chloride, neutral solutions could be produced

which did not markedly alter oxygen consumption, although large quantities of ferrous chloride were present. In these cases heavy precipitates were produced, probably insoluble ferric hydroxide that

TABLE I

Substance	Solubility	Concentration	pH	Change in O_2-cons.	Effects on cells	Condition of cells
				per cent		
Ferric oxide Fe_2O_3	Insol. in H_2O	M/ 1,000	5.9	−62	Sl. toxic	Swollen or killed
		M/ 5,000	6.3	−32	No effect	Normal
		M/10,000	6.5	−30	No effect	Normal
Ferrous chloride $FeCl_2$	160.1[10°] in H_2O	M/ 1,000	4.3	—	Toxic	All killed
		M/ 5,000	4.7	−60	Toxic	Swollen and ruptured
		M/10,000	4.9	−43	No effect	Normal
Ferric sulfate $Fe_2(SO)_4)_2$	Very sol. in cold H_2O	M/ 1,000	2.4	−100	Toxic	Killed instantly
		M/ 5,000	2.8	−100	Toxic	Killed
		M/10,000	3.2	−100	Toxic	Killed in 2 min.
Ferrous phosphate $Fe_3(PO_4)_2$	Insol.	M/ 5,000	5.9	−43	No effect	Normal
Ferrous nitrate $Fe(NO_3)_2$	200.0° in cold H_2O	M/ 5,000	3.4	−75	Toxic	Many dead. No cells ruptured
Ferrous ammonium sulfate $FeSO_4(NH_4)_2SO_4$	18.0° in cold H_2O	M/ 1,000	4.6	—	Toxic	All killed
		M/ 5,000	5.0	None	No effect	Normal
		M/10,000	5.6	None	No effect	Normal
		M/20,000	5.8	None	No effect	Normal

could not be taken into the cells and produce reduction in respiratory activity.

Ferric sulfate was found to be extremely toxic for *Paramecium*, killing the organisms within a few minutes in concentrations as low as M/10,000. This salt is very soluble and produces an acid solution. *Paramecium* exposed to the action of very dilute ferric sulfate, move

about rapidly until death. When their activity ceases they show no external change of form, but have a larger distribution of opaque particles within the cell.

Ferrous phosphate was found similar to ferric oxide and ferrous chloride in its reduction of respiration in *Paramecium*. The cells became inactive in the presence of this salt with no change in microscopic appearance.

Ferrous nitrate is quite toxic for *Paramecium*. M/5000 concentration causes marked reduction in respiratory activity (75 per cent reduction) with death of the cells within 1 hour.

Paramecium live for about 30 minutes in M/1000 ferrous ammonium sulfate and become enlarged and ruptured. In M/5000 solution of this complex salt, a 1 per cent increase in oxygen consumption over the normal in distilled water may be obtained. M/10,000 and M/20,000 ferrous ammonium sulfate produce no significant change in oxygen consumption, the whole action of this substance being scarcely perceptible. These results agree with those of Rosenthal and Voegtlin[7] with respect to this particular salt on the respiration of rat tissue. In no instance did they obtain an increase in oxygen consumption by addition of iron compounds to the tissue. For the *Paramecium* cell simple iron salts appear to be toxic in proportion to their solubility and in about the same manner as other heavy metal salts on most cells.

Following the method for the detection of minute amounts of iron in biological material given by Elvehjem and Hart,[8] we have attempted to obtain an indication of iron in the protoplasm of *Paramecium*.[9] Comparison of the color reaction in the *Paramecium*-ash solution with that in a standard control solution containing 0.1 mg. of iron, showed the faintest tint in contrast with the deep red of the standard control.

[7] Rosenthal, S. M., and Voegtlin, C., *Pub. Health Rep.*, 1931, **46**, 521.

[8] Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1926, **67**, 43.

[9] The method involved the evaporation of a mass of *Paramecium* (collected from their culture media by centrifuging) of 0.5 gm. to dryness, ashing, the taking up of the ash in dilute HCl, filtering, and treating the filtrate with 40 per cent NaOH, boiling for 1 hour. When hydrolysis was complete, the solution was made acid with HCl and diluted to a volume of 50 cc. to form an unknown solution for testing. 10 cc. of the unknown solution and 10 cc. of a standard iron solution containing 0.1 mg. of Fe, were tested for their color reaction with 5 cc. of a standard solution of 20 per cent potassium sulfocyanate.

When the procedure was repeated using a mass of *Paramecium* carefully washed many times in distilled water, no color reaction whatever could be evoked with this delicate treatment. We are inclined to believe that the only iron present in the organisms came from the culture medium and was eliminated from the mass of cells by repeated washing. Any remaining iron was possibly bound in the vacuoles and in the gullet of the cell in insignificant quantity. It is possible that a cyanide-sensitive iron-containing catalyst for respiration is not present in *Paramecium*, and any oxidation catalyst present represents some other type of system, the nature of which remains to be determined. Iron contained in these cells seems not combined in the form of a respiration catalyst.

SUMMARY

1. The effects of KCN and iron salts on oxygen consumption has been studied in the cell of *Paramecium caudatum* by manometric methods.

2. KCN solutions of strengths from M/200 to M/10,000 have been shown to produce no decrease in oxygen consumption, but have in most cases produced a very slight increase in the respiration rate.

3. The pH values were found to have little or no effect on these results.

4. Iron salts produce either no effect or a great diminution of oxygen consumption, in no case causing stimulation of rates of respiration.

5. Iron salts in neutral solutions do not penetrate the *Paramecium* cell nor do they cause so marked an effect as in an acid state.

6. The iron-content of *Paramecium* was found to be extremely small and not demonstrable by delicate tests. It is believed that iron is not combined in the cell in the form of a respiration-catalyst sensitive to cyanide.