The Influence of Hydrogen Ion Concentration and 2:4-Dinitrophenol on Orthophosphate Accumulation in *Tetrahymena pyriformis*

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SUMMARY

The relationship of the pH value of the suspension medium to inorganic phosphate accumulation by *Tetrahymena pyriformis*, strain W, in the presence and absence of 2:4-dinitrophenol was studied. Investigations were conducted to ascertain whether variations of the conditions of culture influenced phosphate accumulation. The pH value of the suspension medium had an influence on the amount of radio-phosphate accumulation. The pH optimum, under the conditions used, was 6.5. The pH value of the culture medium, in the range tested, had little or no influence on the pH optimum observed in the suspensions, although differences in total phosphate accumulation were noted. Approximately 70 % of the orthophosphate accumulated by the organisms was in the form of inorganic phosphate, which leads to the postulate of an active transport mechanism for phosphate accumulation in *Tetrahymena*. 2:4-Dinitrophenol was most effective in lowering radiophosphate accumulation in an acidic medium, and became less effective with increasing alkalinity. The results can be explained on the basis of the penetration of only the undissociated molecule into the organisms. It is suggested that 2:4-dinitrophenol interferes with the transport mechanism involved in phosphate entry.

INTRODUCTION

The growth of *Tetrahymena pyriformis* has been reported to be attenuated in the presence of 2:4-dinitrophenol (DNP). This growth inhibition is annulled by the addition of stigmasterol or related sterols to the medium (Conner, 1957). Numerous investigations concerning the effects of DNP on a variety of biological systems indicate that metabolic activities which require a constant supply of energy are adversely affected. DNP retards or attenuates active transport of cations in yeast (Conway, 1955), protein synthesis in normal and malignant rat livers (Frantz, Zamecnik, Reese & Stephenson, 1948), cleavage in *Arbacia* eggs (Clowes & Krahl, 1936), photosynthesis and photo-reduction in *Scenedesmus* (Gaffron, 1942), and sodium ion extrusion from the squid axon (Caldwell, 1957). The widespread effects of DNP on cellular activities can be explained in terms of an adverse effect on the

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enzymic reactions which lead to the production of energy-rich phosphate groupings such as adenosine triphosphate (ATP; Loomis & Lipmann, 1948). The formation of such groupings involves the esterification of orthophosphate (Hunter, 1951), and if such reactions were blocked, it might be expected that less orthophosphate would tend to enter the cell, either because of a lowered orthophosphate demand or a lowered availability of energy necessary for accumulation. In some cases it has been shown that the extent to which DNP influences metabolic processes is related to the pH value of the solution used for suspending the biological material (Caldwell, 1957; Simon, 1958; Hamburger & Zeuthen, 1957). This indicates the necessity of controlling the pH value when evaluating the effect of a given concentration of DNP on a biological system. The extent to which DNP affects phosphate metabolism may depend on the intracellular concentration of the substituted phenol, which in turn might depend on the pH value of the suspension medium. Also, the influence of the pH value on the amount of phosphate accumulated is an important consideration.

This paper deals with the influence of the pH value of the medium on DNP inhibition of growth of *Tetrahymena*, on orthophosphate accumulation in the presence and in the absence of DNP, and with the relationship of orthophosphate accumulation to the pH of the medium in which the organisms were cultured. The distribution of the accumulated phosphate between organic and inorganic forms was also determined.

METHODS

The organisms, *Tetrahymena pyriformis*, strain W, were cultured under axenic conditions in a medium composed of (% w/v): 2, proteose peptone (Difco); 0.1, yeast extract (Difco); 1, glucose; 0.2, sodium acetate; 0.15 tris buffer (2-amino-2-hydroxymethyl propane-1:3 diol methane; Sigma 121). In all cases, glucose was autoclaved separately and then added to the medium. For the various experiments the media were adjusted at the time of inoculation to pH values of 5.5, 6.5, 7.0 and 7.5 as determined by the Beckman pH meter. The organisms were incubated for 72 hr. in the dark at $25^{\circ} \pm 2^{\circ}$, harvested by centrifugation (200 g), washed three times with distilled water, and suspended in water to give an optical density reading of 0.5 on a Lumetron Colorimeter (Model 401A) equipped with a 650 m μ filter. The packed organisms of 1 ml. of this suspension had a volume of 0.015 ml.

The phosphate accumulation experiments were carried out in flasks containing the following: 0.012 M-tris buffer, 0.01 M-sodium acetate, 1.5×10^{-8} M-radiophosphate (0.008 μ c./ml. final concentration), 1×10^{-4} M-DNP when appropriate, and 1 ml. of *Tetrahymena* suspension. The final volume in all flasks was 10 ml. In the experiments involving fractionation of the phosphates, twice the number of organisms was added and a final volume of 20 ml. used.

The organisms were allowed to stand for 1 hr. in the presence of tris buffer; then sodium acetate and DNP were added to the appropriate flasks. Fifteen minutes later all flasks were charged with radiophosphate. After incubation for 2 hr. with radiophosphate the organisms were removed by filtration or centrifugation.

In the experiments involving the fractionation of the accumulated phosphate into acid-insoluble, acid-soluble organic and inorganic portions the following procedure was used. After the 2 hr. incubation period with the radiophosphate, 5 ml. of

suspension were filtered to remove the organisms. A 10 ml. portion of the suspension was added to an equal volume of 10% (w/v) trichloroacetic acid (TCA), the mixture allowed to stand for 1 hr., followed by centrifugation. To a 10 ml. portion of the TCA supernatant fluid the following reagents were added in the order given: 1.0 ml. KH_2PO_4 (0.2%, w/v); 2.0 ml. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (15%, w/v); 1.5 ml. ammonia (sp.gr. 0.880). The contents were mixed thoroughly, covered, and allowed to stand overnight. The following morning the precipitate of magnesium ammonium phosphate was sedimented by centrifugation, washed with dilute ammonia (sp.gr. 0.880, diluted 1/10) and resedimented. The supernatant fluid was discarded, the tube drained by inversion and the precipitate dissolved in 1 ml. concentrated HCl, and the volume adjusted to 5 ml. with water. The temperature of all solutions was maintained at 5° during the fractionation procedure.

The radioactivity of three 1 ml. samples was determined by using a thin window Geiger-Muller tube and scaler. Constant geometry was maintained and all counts were corrected for background. The solutions on which counts were taken to assess radioactivity are designated as follows: measure of initial radioactivity in the suspension medium, T_0 ; suspension medium after removal of organisms, T_2 ; TCA supernatant fluid, TCA; inorganic phosphate, P_i . The P_i counts were taken at 103% to compensate for the decay which occurred over the period of about 18 hr.

Direct counts were not made of the TCA-soluble organic or the TCA-insoluble fractions because quantitative recovery could not be achieved. To determine the values of these fractions and to correct for dilution the following calculations were made: total radiophosphate removed from the suspension medium (accumulated phosphate), $T_0 - T_2$; TCA insoluble, $T_0 - 2\text{TCA}$; TCA soluble organic, $2(\text{TCA} - P_i)$; inorganic phosphate, $2P_i - T_2$. Variance was computed for each set of original counts, corrected for background:

$$\frac{\Sigma(X - \bar{X})^2}{N - 1},$$

where X is count value, $\bar{X}$ is mean value, N is number of samples counted.

Standard deviation of the calculated count values was determined as:

$$\sqrt{\frac{s_x^2 + s_y^2}{N - 1}},$$

where s_x^2 is variance of the 1st term, s_y^2 is variance of the 2nd term. (When x or y was increased by a factor of two to correct for dilution, s^2 was doubled for determination of standard deviation.)

Percentage deviation was found by taking the extreme values as given by standard deviations:

$$\frac{X + s_x}{Y - s_y} - \frac{X}{Y},$$

where X is the calculated count value for a given fraction, Y is either T_2 counts or accumulated phosphate in the fractionation experiments.

RESULTS

The influence of 2:4-dinitrophenol and pH value of the culture medium on growth

The results of the growth experiment (see Fig. 1) indicate that the rate of multiplication of the organisms decreased with increasing DNP concentration. At a

given concentration of DNP, the rate of growth decreased with increasing acidity. In the absence of DNP, no pH effect was seen in the final concentrations of organisms, confirming the findings of Kidder (1941).

Tetrahymena pyriformis used for the phosphate accumulation studies was grown in proteose-peptone medium adjusted to the desired pH value. The pH values of the media after growth for 72 hr. are given in Table 1. In all cases the pH value of the media moved towards neutrality. Orthophosphate accumulation was similar in organisms which had been grown in media initially at pH 5.5 and 6.5, but was somewhat lower in the case of organisms grown in media initially at pH 7.5. In fifteen experiments there was no exception to the pattern of radiophosphate accumulation, although differences in the absolute amounts of orthophosphate accumulated were noted from experiment to experiment. In general, a shift in the pH value of the suspension medium towards neutrality took place during the experimental period. However, in spite of this tendency to approach neutrality, the initial pH value of the suspension medium had an influence on the degree of orthophosphate accumulation. A pH optimum of 6.5 was found regardless of the conditions of culture used.

Fig. 1. Effect of 2:4-dinitrophenol (DNP) in media at different pH values on the growth of *Tetrahymena*. (x) 5×10^{-5} M, (O) 1×10^{-4} M, (Δ) 2×10^{-4} M-DNP. The media and conditions were as reported previously (Conner, 1959).

Orthophosphate accumulation by *Tetrahymena pyriformis* was inhibited by DNP. The maximal inhibition for a given concentration of DNP occurred when the suspension medium was acidic (see Fig. 3). In the presence of DNP, the pH optimum at 6.5 was abolished.

Since the pH value of the suspension medium had an effect on phosphate accumulation in absence of DNP, the data require clarification. When the amount of radio-

Table 1. pH Value of culture media before and after growth of *Tetrahymena*

The protozoans were maintained at $25^{\circ} \pm 2$ for a period of 72 hr.

Initial pH of culture media	pH after growth for 72 hr.
5.5	6.3-6.4
6.5	6.7-6.8
7.5	7.2-7.3

phosphate accumulated at each pH value in the presence of DNP is compared with a control at that pH value, i.e. % DNP accumulation/% control accumulation, a better description of the influence of the pH of the suspension medium with regard to DNP inhibition is obtained (see Table 2). The distribution of accumulated phosphate (taken as 100%) was TCA-insoluble $19.8\% \pm 3.8$, organic TCA-soluble $11.3\% \pm 3.7$, inorganic phosphate $68.9\% \pm 3.5$. In these experiments the organisms were grown at pH 6.5 and the pH value of the suspension medium was 7.0. The phosphate removed from the suspension medium by the *Tetrahymena* was $75\% \pm 5$ (seven experiments). In a few experiments the organisms were incubated in a suspension medium at pH of 6.5 or 7.5. There was no significant variation in the distribution of phosphate under these conditions.

Fig. 2

Fig. 3

Fig. 2. Influence of pH value of the suspension medium on radiophosphate accumulation by *Tetrahymena* grown in proteose peptone media of pH 5.5 (x), 6.5 (Δ) and 7.5 (O). Each point represents the mean % accumulation for five experiments.

Fig. 3. The influence of the pH value of the suspension medium on radiophosphate accumulation by *Tetrahymena* in the presence (x) or absence (O) of 1×10^{-4} M-DNP. The organisms were grown in the proteose peptone medium (pH 6.5).

Table 2. Data from representative experiments on accumulation of radiophosphate by *Tetrahymena* in the presence and absence of 10^{-4} M DNP under various conditions of pH of culture medium and suspension medium

Organisms grown in medium at pH 5.5 (see Table 1)					
Initial pH value of suspension medium (T_0)	6.0	6.5	7.0	7.5	7.8
Terminal pH value of control (T_3)	6.4	6.75	6.95	7.4	7.7
Terminal pH value in presence of DNP	6.55	6.8	6.95	7.4	7.7
% Accumulation control (no DNP)*	47.4	70.8	61.6	84.8	80.6
% Accumulation in presence of DNP*	1.4	11.2	35.0	39.1	27.9
% DNP/% Control	3	16	57	112	91
Organisms grown in medium at pH 6.5					
Initial pH value of suspension medium (T_0)	6.0	6.5	7.0	7.5	7.8
Terminal pH value of control (T_3)	6.6	6.8	6.9	7.3	7.6
Terminal pH value in presence of DNP	6.4	6.7	6.9	7.2	7.5
% Accumulation control (no DNP)*	74.7	85.7	78.7	56.7	41.6
% Accumulation in presence of DNP*	6.0	15.4	25.9	35.3	38.6
% DNP/% Control	8	18	33	62	93
Organisms grown in medium at pH 7.5					
Initial pH value of suspension medium (T_0)	6.0	6.5	7.0	7.5	7.8
Terminal pH value of control (T_3)	6.4	6.6	7.0	7.5	7.6
Terminal pH value in presence of DNP	5.7	6.75	7.0	7.35	7.65
% Accumulation control (no DNP)*	83.2	60.0	35.9	29.3	21.7
% Accumulation in presence of DNP*	0.0	11.8	23.8	20.4	18.4
% DNP/% Control	0	19	66	70	85

* In all cases the standard deviations were less than 2%.

DISCUSSION

The data presented indicate that the accumulation of orthophosphate varied with the pH value of the suspension medium, with a maximum at pH 6.5. Van den Honert (1933) found that phosphate accumulation in sugar cane varied with the pH value of the medium in a manner suggesting that the rate of accumulation was dependent on the concentration of the monobasic phosphate ion. He offered an explanation based on the assumption that the dibasic ions could not be absorbed. In support of such a hypothesis, van den Honert found that accumulation at high phosphate concentrations was independent of changes in the acidity of the medium. The data concerning phosphate accumulation by *Tetrahymena pyriformis* cannot be explained solely on the basis of the ionization state of the phosphate ion. Such an explanation would not account for the sharp maximum in accumulation at pH 6.5. Even though monobasic phosphate ions are in greater concentration at pH 6.0, accumulation is lower. The pH optimum could be explained if the mechanism concerned with orthophosphate entry involves specific sites in the cellular membrane which are sensitive to hydrogen ions. Thus, it is possible that two pH-dependent factors might be involved, the state of the phosphate ion and that of a carrier, the combined effect of these producing an optimum at pH 6.5. Goodman & Rothstein (1957) studied the effect of extracellular pH value on radiophosphate accumulation in yeast and reported that accumulation was maximal when the pH value of the suspension medium was 6.5.

The amount of radiophosphate accumulated by *Tetrahymena* cannot be accounted for on the basis of free diffusion or catalysed permeability. The total volume of organisms used in these experiments was about 0.080 ml., while the volume of the suspension medium was 20 ml. At the end of the 2 hr. incubation period about 30 % of the radiophosphate remained in the medium; thus phosphate had been concentrated by the organisms by a factor greater than 900. It was shown by Slater (1957) and confirmed in this laboratory (Conner, 1958) that no detectable efflux of radiophosphate occurred from *Tetrahymena* under the conditions used. This removal of radioactivity from the medium probably represents true accumulation and not phosphate ion exchange. This is an accumulation against a concentration gradient and can be explained on the basis of an active transport mechanism.

In *Tetrahymena* the most pronounced influence of a given concentration of DNP in inhibiting growth or in lowering radiophosphate accumulation occurred in the acid range. Since DNP is a weak acid and is less dissociated in an acidic medium, it seems possible to correlate the degree to which DNP lowers phosphate accumulation with the concentration of free acid. The experiments of DeDeken (1955) and Cross, Taggart, Covo & Green (1949) indicate that the ability of substituted phenols to influence biological processes is a function of the phenolic configuration, the mobility of the phenolic proton, and an inverse function of pK.

Hamburger & Zeuthen (1957) reported DNP to have a greater influence on respiration of *Tetrahymena pyriformis* in an acidic medium and suggested that the free acid is the physiologically active form. It seems, however, that an equally plausible explanation might be to assume that undissociated DNP has the greater ability to penetrate. If only the free acid were active or if both forms penetrated the cellular membrane to an equal extent, it would be necessary to postulate that intracellular pH varies with the pH value of the suspension medium in order to explain the observed results on respiration, growth and phosphate accumulation. Caldwell (1957) found that the extent to which DNP affected metabolic activity in the giant axons of *Loligo forbesi* depended on the concentration of the substituted phenol in the axoplasm, which in turn was dependent on the pH value of the solution bathing the axon. A similar situation might exist with *Tetrahymena*.

The pH optimum for phosphate accumulation was not seen when DNP was added. Thus in the presence of DNP some other factor becomes rate limiting. A lowered utilization or an intracellular increase of orthophosphate could account for the lowered phosphate accumulation and for the disappearance of the optimum in accumulation at pH 6.5. Such an explanation would be compatible with the current concept of the action of DNP as an uncoupling agent of oxidative phosphorylation and as a stimulant of ATPase activity (Lardy & Wellman, 1953). However, there are other reasonable explanations that could account for the results obtained. It is possible that DNP interferes with an active transport mechanism involved in phosphate entry: directly by combining with a carrier or indirectly by decreasing the supply of energy required for active transport (Conway, 1955). Work is in progress to test the validity of the above hypotheses.

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