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Received November 16, 1964. P.S.E.B.M., 1965, v118.

Dehydrogenases of Purine Metabolism in *Tetrahymena pyriformis*. (29985)

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Either guanine, guanosine or guanylic acid is required for the growth of *Tetrahymena pyriformis* due to its inability to synthetize the purine ring(1,2). Adenosine deaminase and purine nucleoside phosphorylase have been reported in *Tetrahymena*(3), but guanase and xanthine oxidase were considered to be absent from this ciliate by Kidder on the basis of nutritional studies(1). This was confirmed by Eichel with enzymatic experiments(3). Recently, however, Seaman showed that extracts from sonicated *Tetrahymena* contain adenase, xanthine oxidase, guanase, uricase and adenosine deaminase(4). We investigated the presence of guanase, xanthine oxidase and uricase as well as some effects of purines on the endogenous respiration of this ciliate in an effort to resolve these discrepancies.

In studying anaerobic reduction of triphenyltetrazolium chloride (TPTC) with some purines as substrates we found a marked activity with cell suspensions and homogenates of this organism. Incubation in air with these substrates showed that the activity producing uric acid was somewhat lower. These findings indicate that *Tetrahymena* is able to dehydrogenate actively

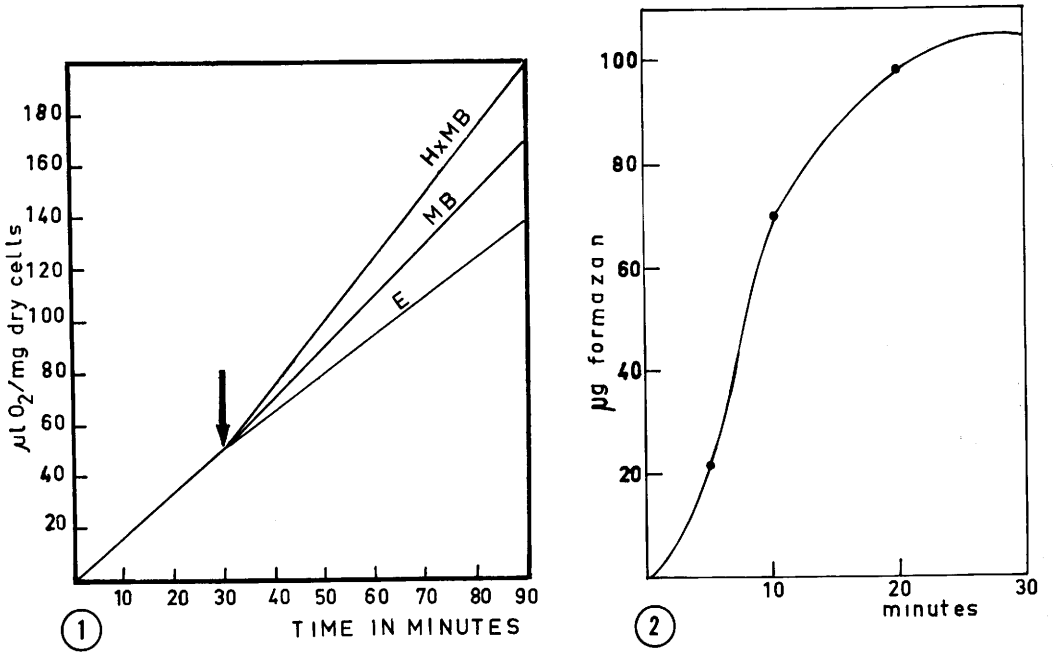

FIG. 1. Effect of methylene blue on oxygen uptake of washed cells. Each manometer contained 0.5 ml cell suspension (= 1 mg dry wt), 0.1 ml substrate, 0.05M and phosphate buffer, pH 7.2 to make 2 ml. Gas phase air; temperature 37°C, 0.15 ml methylene blue, 0.005M. Hypoxanthine was tipped after 20 min (arrow). E = endogenous respiration; MB = methylene blue; HX + MB = hypoxanthine plus methylene blue.

FIG. 2. Production of formazan in µg in relation to time of incubation in min. Substrate: Hypoxanthine 0.05M.

in anaerobiosis when an electron acceptor is present. The dehydrogenase was shown to be more active than the oxidase in the presence of methylene blue.

It is known that xanthine oxidase may have a different capacity to oxidize purine substrates in aerobiosis (xanthine oxidase) than in anaerobiosis (xanthine dehydrogenase)(5). Remy and coworkers showed that chicken liver has a low xanthine oxidase measured manometrically and a high dehydrogenase activity in the presence of methylene blue(6). Homogenates of *Tetrahymena* presented low O_2 uptake, measured in the Warburg respirometer with xanthine or hypoxanthine as substrates. However, with carriers such as methylene blue an increase in O_2 uptake was observed (Fig. 1). The predominant dehydrogenase of *Tetrahymena* in relation to its oxidase activity is possibly of the same type as that reported for the chicken liver preparation by Reichert *et al*(7).

Methods. Cell suspensions and homogenates were prepared with cells of *Tetrahymena pyriformis*, var. 1, mating type II, kindly supplied by Dr. S. H. Hutner, Haskins Laboratories, New York, to whom we are very thankful. The ciliate was grown in 1 liter Erlenmeyer flasks containing 100 ml of a medium composed of peptone (Difco), 2.0%; glucose, 0.5% and yeast extract (Difco) 0.1%. Each flask was inoculated with cells from cultures in the logarithmic phase of growth and incubated at 36°C for 48 or 72 hours. The ciliates were harvested by low-speed centrifugation, washed 3 times in distilled water, and resuspended in 0.02 M phosphate buffer, pH 7.0. Homogenates were prepared by shaking a thick suspension of cells vigorously for 2 minutes in a closed tube containing glass powder (prepared by grinding thin capillary glass tubes in a mortar until a fine powder was obtained) and centrifuging 5 minutes at 2000 × *g* at 4°C. This procedure was shown to be capable of yielding a complete breakage of cells when examined with a phase contrast microscope. Duplicate dry weights were taken at

the same time that samples were taken for the experiments. Protein nitrogen was determined by micro-Kjeldahl. Cell suspensions were adjusted by dilution to an optical density 1.2 (as measured colorimetrically with a 650 mμ red filter).

Dehydrogenases were assayed in evacuated Thunberg tubes (620 mm vacuum for 5 minutes) containing 0.5 ml washed cells or 0.2 ml of the cell-free homogenate and 1.0 ml of 0.01 M phosphate buffer, pH 7.0 The side arm had 0.3 ml of 0.1% TPTC and the substrate (0.05 M). The tubes were evacuated for 5 minutes, then tipped and immersed in a water bath at 37°C for 15 minutes. The red formazan produced was extracted with 5 ml petroleum ether after addition of 10 ml acetic acid. The colored petroleum ether layer was measured in a photocolorimeter or a spectrophotometer at 490 mμ; results were expressed as μg formazan as calculated from a standard curve based on pure formazan dissolved in petroleum ether(8). Blanks without purines were run for each set of experiments and showed a faint color or were colorless when measured with a colorimeter. Even when measured with a spectrophotometer (Zeiss PMQ II) the blank values were not significant (optical densities from 0.001 to 0.005 at 490 mμ). The products of guanase and xanthine oxidase action were assayed spectrophotometrically according to Kalckar (9). In these experiments the homogenates were incubated with the substrates for 25 minutes at 37°C, and 2 ml of the 0.01 M phosphate buffer pH 7.0. After this time 1.5 ml of 0.6 M perchloric acid was added and celite, the volume adjusted to 10 ml with water, filtered, and the filtrate assayed in a Zeiss PMQ II apparatus at 295 mμ. The results were expressed in μg uric acid formed.

To demonstrate the specificity of the dehydrogenase reaction measured with TPTC, the contents of the Thunberg tubes after the formazan was produced were treated with 1.5 ml 6.0 N $HClO_4$ and the volume adjusted to 10 ml with distilled water, filtered and an aliquot assayed spectrophotometrically at 295 mμ. The results are summarized in Table I. Oxygen consumption was measured in conventional Warburg respirometer (2 ml flasks) with air as gas phase. The homogenates, cell suspensions or extracts were added to the flasks in the volume of 0.5 ml in 0.02 M phosphate buffer, pH 7.0, 0.2 ml of 10% KOH in the central well and substrates, 0.05 M (0.1 ml) in the side arm. The total volume was adjusted to 2 ml with the buffer solution. Methylene blue, 0.005 M (0.15 ml) was tipped from the side arm. Results were expressed in O_2/mg dry cells (Fig. 1).

TABLE I. Uric Acid Produced Anaerobically in the Presence of TPTC (0.3 ml of a 0.1% solution).

Homogenates mg N	Substrate .05 *M* (.1 ml)	μg formazan produced	Uric acid produced (μg)
.9	Hypoxanthine	24	13
.9	Xanthine	29	16
.9	Blank	<1	—

Guanine, guanosine, were from Nutritional Biochemicals Corp.; hypoxanthine from California Corp. for Biochemical Research; xanthine from Eastman Kodak; Na guanylate from Schwartz Laboratories, Inc. and DPN from Sigma Chemical Co., St. Louis.

Results and discussion. Cell suspensions and homogenates of *Tetrahymena* can dehydrogenate anaerobically xanthine and hypoxanthine and convert guanine to xanthine which is measured as formazan produced (Table II). Dehydrogenase activity was related to the mass of cells and was manifest within a few minutes after addition of substrate. Fig. 2 shows formazan production upon incubation with a suspension equivalent to 25 mg dry cells; it is seen that almost 60% of the total reaction occurred after 10 minutes of incubation at 37°.

The dehydrogenase activity is unstable. Homogenates when stored in the refrigerator (4°C) showed a drop of 30 to 40% in activity, but are inactivated when frozen for

TABLE II. Purine Dehydrogenases of *Tetrahymena* Values Expressed in μg Formazan Produced Anaerobically.

Substrates (.05 *M*)	Microg formazan produced per mg ciliate	mg N
Guanine	20.0	4.0
Guanosine	26.1	4.3
Iso-guanine	16.3	3.2
Hypoxanthine	15.0	3.0
Xanthine	18.0	3.6
Blank without purine substrates	1.0	—

TABLE III. Uric Acid Produced in Aerobiosis. Homogenates incubated with purine substrates for 25 minutes at 37°C.

Homogenate mg dry cells	Substrate .05 *M*	μg substrate in reaction mixture	μg uric acid produced*	% substrate oxidized
15	Hypoxanthine	68	5.2	7.6
15	Xanthine	76	4.2	6.1

* Each value represents an average of 3 experiments.

TABLE IV. Reactivation of Xanthine Dehydrogenase by DPN.

Cell free homogenates mg protein	Substrate (.05 *M*)	DPN added (μg)	μg formazan produced in 15 min at 37°C	Conditions
8	Hypoxanthine	—	22.2	Immediately*
8	"	—	18.8	Ice box 1 hr†
8	"	—	0	Deep freezing 24 hr
8	"	—	1.2	" " 1 "
8	"	6.7	22.7	" " 24 "
9	Guanine	—	41.0	Immediately*
9	"	—	27.5	Ice box 1 hr
9	"	—	—	Deep freezing 24 hr
9	"	6.7	44.3	" " 24 "

* Cells disrupted in the cold (0°C) and used immediately after separation by centrifuging 2000 × *g* at 4°C for 5 min.

† The same stored in the ice box (4°C).

24 hours. It has been observed that addition of diphosphopyridine nucleotide (DPN, NAD) restored the activity completely. Homogenates containing 8-10 mg protein were incubated for 10 minutes with 45 μg DPN in a Thunberg tube; 0.3 ml TPTC and 0.1 ml of 0.05 M hypoxanthine or guanine in the side arm. The tubes were evacuated and tipped. DPN when added to homogenates without the purines does not reduce TPTC. Table IV summarizes some of these results. The decrease of activity is possibly due to DPNase present in *Tetrahymena*. This is now being investigated.

Seaman reported that his extracts were frozen and thawed 3 times to obtain a clear supernatant(4). This indicates that the oxidase activity was stable to such treatment. However, in our experiments the dehydrogenases were completely inactivated when frozen and thawed twice for one hour. It is possible that the oxidase is more resistant than the dehydrogenase indicating consequently a different behaviour in its activity. As was observed in the oxygen consumption experiments (Fig. 1), our findings showed that *Tetrahymena* dehydrogenates xanthine and hypoxanthine actively in anaerobiosis but exhibits a poor oxidase activity. Guanine is also promptly attacked and the xanthine formed oxidized to uric acid. Aged cultures of 92 hours have been shown to be poor in dehydrogenase activity; the more active homogenates were prepared with organism growing for 3 days.

For guanase assay, 1 ml of the homogenate (corresponding to 0.24 mg protein) was incubated with 50 μg of guanine (0.2 ml) and 2 ml of 0.02 M phosphate buffer pH 7.0 for 25 minutes at 37°C in a water bath. After this time 1.5 ml of 0.6 N perchloric acid and celite were added, the volume adjusted to 10 ml with water, and filtered. The filtrate was diluted 5 to 10 times and the optical density read at 250, 270 and 295 mμ in a Beckman DU spectrophotometer. Xanthine was assayed similarly using hypoxanthine instead of guanine. Blanks were run without incubation and prepared immediately after the reagents were mixed. An increase in optical density ($+ \Delta_{290}$) of 0.060 was observed with the homogenate incubated with 25 μg hypoxanthine and a $+ \Delta_{270}$ of 0.085 with 50 μg guanine. The optical density of the blanks was subtracted from the values obtained with the assay; the values represent, therefore, this difference.

Calculating the per cent conversion of

guanine to xanthine for 3 experiments by the formula of Rakovsky, Zimmerman & Beck(10):

$$\frac{\text{Ex(initial)} - \text{Ex(final)}}{0.9} \times 100 = \%\ \text{conversion}$$

$$\text{Ex} = \frac{\text{D(250 m}\mu\text{)}}{\text{D(270 m}\mu\text{)}}$$

a 22% conversion of guanine (10 μg) for 15 minutes of incubation was obtained. Table I shows results of some incubation experiments with hypoxanthine and xanthine as substrates; the uric acid produced was relatively low. Homogenates and cell suspensions when incubated for 30 minutes with uric acid showed no uricase activity. A blank not incubated was run simultaneously.

Some experiments in which guanine was added to ciliate suspensions and the μl O_2/mg measured in the Warburg respirometer showed that guanine in the concentration of 75 μM stimulates respiration slightly; the same was observed with iso-guanine, whereas higher doses (150 μM) are partially inhibitory. Azaguanine does not stimulate but inhibits strongly on the same molar basis.

Summary. Cell suspensions and homogenates of *Tetrahymena pyriformis* contain dehydrogenases which reduce triphenyltetrazolium chloride in anaerobiosis to formazan in the presence of some purine substrates (guanine, iso-guanine, hypoxanthine, xanthine). The dehydrogenase activity was also revealed in oxygen consumption experiments using methylene blue as hydrogen acceptor. Homogenates of *Tetrahymena* oxidized purine substrates in the air only weakly and were shown to be devoid of uricase activity. Guanine in lower concentration corresponding to the amount required for growth stimulated slightly the respiration of washed cells, but higher concentrations inhibited markedly the O_2 consumption. The instability of purine dehydrogenases and inequality between oxidase and dehydrogenase activities may account for some discrepancies in the literature.

We thank Dr. S. H. Hutner for suggesting this subject, Dr. Ottilia R. Affonso for the manometric experiments and Dr. Emilio Mitidieri for the illustrations.

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Received November 2, 1964. P.S.E.B.M., 1965, v118.

Absence of Cellular Localization of Antigen in Delayed Hypersensitivity. (29986)

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Although it is now well established that delayed hypersensitivity is mediated by cells and not by conventional circulating antibody (1), the relationship between the cells involved in the reaction and the eliciting antigen is not known. One suggestion has been that the antigen interacts directly with specific mononuclear cells by combining with a cell bound antibody or with substances near the cell surface(2). The existence of such a reaction remains speculative since it has not been demonstrated that the antigen is local-

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