ENZYME SYSTEMS IN TETRAHYMENA GELEII S*

I. Anaerobic Dehydrogenases Concerned with Carbohydrate Oxidation

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In view of the present increased interest in the metabolism of the ciliate, *Tetrahymena geleii*, an investigation of the relative activity of the anaerobic dehydrogenases which have a direct role in carbohydrate oxidation was undertaken as the first phase of a general survey of enzymatic activity in this organism.

The presence in the ciliate of the dehydrogenases active in the tricarboxylic acid cycle was inferred from the results obtained in previous investigations. Chaix, Chauvet, and Fromageot (2) obtained slight increases in oxygen uptake if *Tetrahymena* was incubated with succinate and with citrate. A more conclusive demonstration of succinate oxidation by the organism was obtained by Seaman (3) and by Seaman and Houlihan (4); added citrate, however, was without effect. In these previous investigations no attempts were made to assay for maximal activity of the systems.

Methods

Pure cultures of *Tetrahymena geleii* S were maintained in 2 liter Erlenmeyer flasks containing 1 liter of 1.5 per cent Difco proteose-peptone solution. Inoculations (25 ml.) were made with the aid of sterile pipettes. After 48 hours of growth at 25°C., the organisms were concentrated by gentle centrifugation (750 R.P.M., R = 16.2 cm.), and were washed three times with distilled water. Cells were allowed to stand in each wash solution for 10 minutes. Finally the cells were concentrated to a volume of about 3 ml. The cell concentrate was homogenized at 0°C. for 90 seconds according to the method of Potter (5) and an aliquot of the homogenate was added to the Warburg vessels. Another aliquot was placed in a tared tinfoil pan and was dried to constant weight at 80°C.

Microscopical examination of the cells obtained after the final centrifugation indicated that the method of washing resulted in very little, if any, cell damage

* The history of this strain is discussed in a previous publication (1).

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(less than 5 per cent of the cells were either broken or non-motile). The contention of Chaix, Chauvet, and Fromageot (2) that washing of *Tetrahymena* in distilled water results in cell lysis was thus not confirmed. In fact, organisms of the strain used in the present investigation retain motility in distilled water, without cell breakage, for periods of at least 72 hours.

When dried at 80°C. the weight of *Tetrahymena* was found to be 14.3 mg./10^6 cells. Ormsbee (6) obtained a value of 8.3 mg./10^6 cells when *Tetrahymena* was dried at 100°C. Table I shows that when the organisms used in the present investigation were dried to constant weight at this higher temperature, the

TABLE I

Determination of Dry Weight of Tetrahymena at 100°C. and at 80°C.

Cell populations were estimated by diluting a sample of the cell concentrate with distilled water and counting, with the aid of a microscope, the number of organisms in a small aliquot of the diluted suspension. All cell counts are the average obtained from at least 6 dilutions of each suspension.

Cells dried at 100°C.			Cells dried at 80°C.		
No. cells	Weight of cells	Weight of 10^6 cells	No. cells	Weight of cells	Weight of 10^6 cells
× 10^6	*mg.*	*mg.*	× 10^6	*mg.*	*mg.*
4.9	32.7	6.7	2.4	33.4	13.9
4.5	29.8	6.7	1.7	24.8	14.6
6.9	48.6	7.0	3.0	44.1	14.7
3.9	38.4	9.8	2.6	35.4	13.6
4.8	39.2	8.2	1.9	26.4	14.4
5.2	38.5	7.4	2.4	33.4	13.9
3.7	34.5	8.9	3.2	47.4	14.8
4.2	39.5	9.4	2.9	42.3	14.6
Average....		9.1	Average....		14.3

mean value obtained is in close agreement with that reported by Ormsbee (6). The table also shows that when the higher temperature is used, the value obtained for the weight of 10^6 cells varies from 6.5 mg. to 9.8 mg. When cells are dried at 80°C. the range is from 13.6 mg. to 14.8 mg. Therefore, by using the lower temperatures more consistent values are obtained.

Dehydrogenase activity was ascertained by measuring the oxygen uptake of the homogenate in mixtures containing all the necessary components of the specific dehydrogenase systems. Endogenous values were determined simultaneously, in each case, in mixtures containing all the components of the system under study, except the specific substrate, and these values were subtracted from those obtained with the complete system. In all cases, two con-

centrations of the homogenate were used in the assay of each system. All assays were carried out in quadruplicate. The average of the best 10 minute values obtained from each assay was used in calculating dehydrogenase activity as μl. O_2 consumed/mg. dry weight of homogenate/hr.

The Warburg vessels were immersed in an ice bath while adding the contents of the reaction mixtures. All experiments were carried out in a gas phase of air at a temperature of 25.6°C., with a rate of shaking of 120 oscillations/min. The equilibration period was 10 minutes in all cases. The two levels of homogenate always used were 0.5 and 1.0 ml.; 1.0 ml. contained 20 to 30 mg. dry weight of cells. All concentrations are indicated as final concentration. The concentrations of DPN, TPN, and cytochrome *c* are expressed on the basis of 100 per cent purity. The buffers and the specific substrates were always adjusted to pH 6.8 before use.

Conditions for the optimal activity of each system in *Tetrahymena* were ascertained by observing the rate of oxygen uptake in the presence of various concentrations of each component of the respective dehydrogenase systems studied. The reaction mixtures found to be optimal for the assay of the cytochrome-linked dehydrogenases are as follows (in addition to the homogenate and 0.2 M Na_2HPO_4):—

Succinic Dehydrogenase (7)[1].—Na succinate (3.7×10^{-2} M); cytochrome *c* (1×10^{-5} M); $CaCl_2$ (3×10^{-4} M); $AlCl_3$ (4.2×10^{-5} M).

α-Glycerophosphate Dehydrogenase (8).—Na α-glycerophosphate (3.7×10^{-2} M); cytochrome *c* (1×10^{-5} M).

The quantities of the components of the two cytochrome linked systems are well in excess; the rate of oxygen uptake is proportional to the amount of enzyme present (Fig. 1).

The activity of the DPN dependent dehydrogenases was measured by linking the cytochrome system with the coenzyme. The optimal reaction mixtures for the assay of these dehydrogenases are as follows (in addition to the homogenate and 0.2 M Na_2HPO_4):—

Malic Dehydrogenase (9).—Na malate (8.2×10^{-2} M); DPN (1.4×10^{-3} M); cytochrome *c* (1×10^{-5} M); nicotinamide (2.5×10^{-2} M); Na glutamate (2.5×10^{-2} M).

Lactic Dehydrogenase (10).—Na lactate (3.9×10^{-2} M); DPN (7×10^{-4} M); cytochrome *c* (4×10^{-5} M); nicotinamide (2.5×10^{-2} M); Na glutamate (2.5×10^{-2} M).

Glucose Dehydrogenase (11).—Glucose (1.3×10^{-2} M); DPN (4×10^{-4} M); cytochrome *c* (1×10^{-5} M).

[1] References 7 to 16 contain reports of concentrations of the necessary components of the specific dehydrogenase systems which have been found to be optimal for the assay of these systems in mammalian tissue.

Glutamic Dehydrogenase[2] (12).—Na glutamate (5×10^{-2} M); DPN (4×10^{-4} M); cytochrome *c* (1×10^{-5} M); semicarbazide hydrochloride (5×10^{-2} M). All attempts to demonstrate the presence of a DPN linked α-glycerophosphate dehydrogenase in *Tetrahymena geleii* S were unsuccessful. No additional oxygen uptake was obtained when flavoprotein, methylene blue, or cytochrome *c* were added to flasks containing K or Na α-glycerophosphate, DPN, and homogenate.

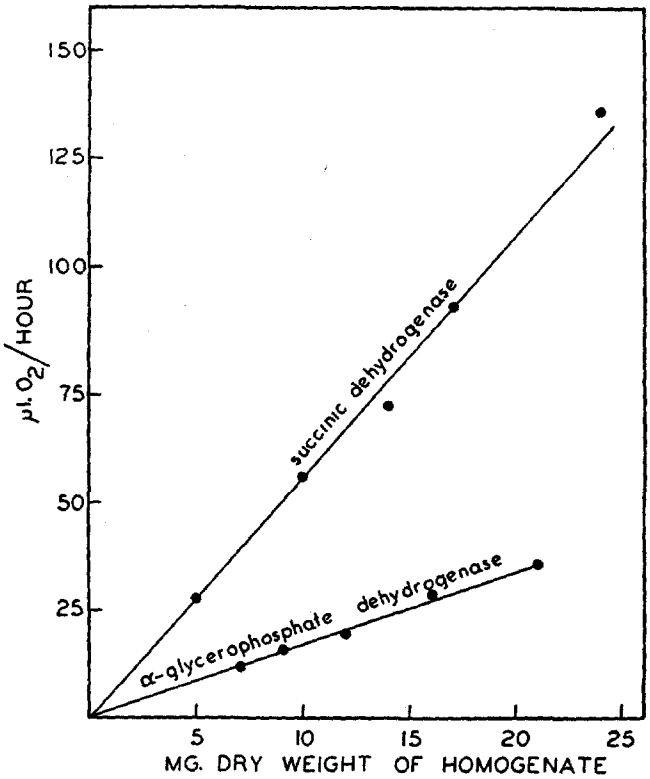

FIG. 1. Relation of oxygen uptake to homogenate concentration in the assay of cytochrome linked dehydrogenases. Gas phase, air temperature, 25.6°C. Flask contents as described in text.

Triphosphopyridine nucleotide dependent dehydrogenases were assayed by linking the TPN with phenazine methosulfate (13). The reaction mixtures found to be optimal for the assay of these systems are (in addition to homogenate and 0.17 M veronal):—

[2] Although this enzyme cannot be properly classified as concerned primarily with carbohydrate metabolism, the role of glutamate in transamination reactions make it of interest to include this system in the study of dehydrogenases participating directly in the oxidation of carbohydrates.

Isocitric Dehydrogenase (14).—Na isocitrate (1×10^{-2} M); TPN (5×10^{-5} M); phenazine methosulfate (5×10^{-3} M); $MgCl_2$ (6×10^{-2} M).

Glucose-6-Phosphate Dehydrogenase (13, 15).—Na glucose-6-phosphate (1.2×10^{-3} M); TPN (1×10^{-5}M); phenazine methosulfate (2.5×10^{-3} M); $Ca(CN)_2$ (1.9×10^{-3} M).

6-Phosphogluconic Dehydrogenase (16).—Ca 6-phosphogluconate (1.1×10^{-2} M); TPN (5×10^{-5} M); phenazine methosulfate (2.5×10^{-3} M); $Ca(CN)_2$ (1.9×10^{-3} M).

In vessels containing cyanide, the center well contained $Ca(CN)_2$–$Ca(OH)_2$ mixtures as recommended by Robbie (17).

In the coenzyme I and II linked systems, the components of each assay mixture were in sufficient excess and, as was the case with the cytochrome

TABLE II

Activity of Dehydrogenases in Tetrahymena

Gas phase, air: temperature, 25.6°C. Flask contents as described in text.

Dehydrogenase	Enzyme activity
	µl. O_2/mg. dry wt./hr.
Lactic	11.2 ± 0.6
Isocitric	9.1 ± 0.8
Succinic	7.0 ± 0.8
Glucose	6.2 ± 0.5
Glucose-6-phosphate	4.5 ± 0.2
6-Phosphogluconic	4.1 ± 0.3
Malic	3.7 ± 0.1
Glutamic	3.3 ± 0.2
α-Glycerophosphate (cytochrome linked)	2.8 ± 0.3

dependent dehydrogenases, the rate of oxygen utilization was proportional to the amount of enzyme (homogenate) present.

All substrates, except isocitrate, were obtained commercially. The dimethyl ester of isocitric lactone was a generous gift from Dr. H. B. Vickery. This lactone was hydrolyzed with dilute NaOH for use. Cytochrome *c* and DPN were obtained commercially and the latter was found to be 62 per cent pure when assayed by the method of Jandorf, Klemperer, and Hastings (18). Cytochrome *c* was standardized spectrophotometrically (5). The TPN was a gift from Dr. G. A. LePage and was 91.5 per cent pure. Phenazine methosulfate was prepared according to the method of Hillemann (19). Flavoprotein was prepared from beef heart according to the method of Straub (20).

RESULTS

Table II shows that the rates of activity of the assayed dehydrogenase systems in *Tetrahymena*, in order of activity are: lactic > isocitric > succinic =

glucose > glucose-6-phosphate = 6-phosphogluconic = malic > glutamic = α-glycerophosphate (cytochrome linked) dehydrogenase.

While the rate of oxygen uptake of all the systems studied was constant for periods of at least 15 minutes, the rate of falling off of activity of each system varied greatly. This is illustrated in Fig. 2. The succinic, lactic, α-glycer-

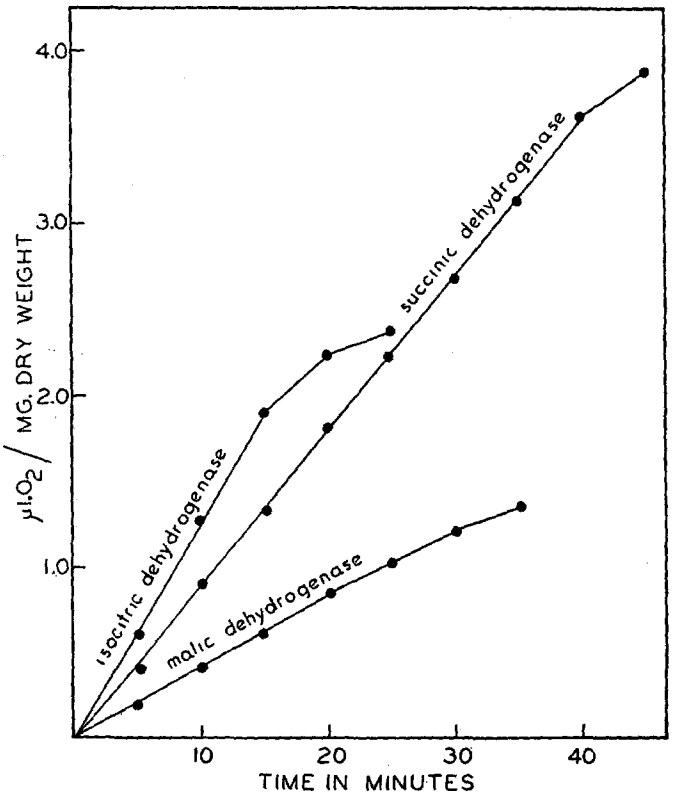

FIG. 2. Rate of falling off of activity of dehydrogenase systems. Gas phase, air; temperature, 25.6°C. Conditions for each assay as described in text.

ophosphate, glucose-6-phosphate, glucose, malic, and 6-phosphogluconic systems show rates of constant activity for at least 30 minutes. At the other extreme, the activity of the isocitric and glutamic dehydrogenases falls off sharply at the end of 15 minutes.

It was thought that perhaps the rapid decrease in activity of the glutamic system was due to either too low a concentration of semicarbazide, and thus incomplete removal of α-ketoglutarate, or to a rapid breakdown of DPN in the reaction mixture. However, increasing the amount of semicarbazide to

fivefold was without effect. The addition of nicotinamide (2.5×10^{-3} M to 1.2×10^{-2} M) failed to stabilize the dehydrogenase activity. Attempts to maintain the rate of activity of the isocitrate system were likewise without effect. Neither increasing the concentration of $MnCl_2$, nor the addition of methylene blue (12) or nicotinamide had any effect on the initial rate of oxygen uptake or on the rate of falling off of the activity.[3]

DISCUSSION

Potter (5) has pointed out that the concentrations of components of assay mixtures which have been found to be optimal in mammalian tissue should not be assumed to be optimal for other types of tissue. This point is illustrated by the present investigation. For example, the optimal concentration of DPN for the mammalian malic dehydrogenase is 6×10^{-4} M (9) whereas the dehydrogenase in *Tetrahymena* requires a final DPN concentration of 1.4×10^{-3} M (values calculated on basis of 100 per cent purity). The glutamic system of mammalian tissue has an optimal DPN requirement of 2.1×10^{-3} M (12). In *Tetrahymena*, however, the optimal concentration is somewhat lower: 4×10^{-4} M. In like manner, the optimal cytochrome *c* concentration for the mammalian α-glycerophosphate dehydrogenase is 3×10^{-5} M; the optimum in the *Tetrahymena* system is 1×10^{-5} M. On the other hand, the DPN and cytochrome *c* requirements of the lactic dehydrogenase are the same in mammalian tissue (10) and in *Tetrahymena*.

It is of interest to compare the activity of dehydrogenases in *Tetrahymena* with values reported for other protozoa. Since reports of metabolic studies made with other protozoa have been calculated on a population rather than on a dry weight basis, the values obtained with *Tetrahymena* must be appropriately converted. Since the dry weight of 10^6 cells of *Tetrahymena* is 14.3 mg. (Table I) it can be readily calculated that the activity of the succinic dehydrogenase in this organism is 1.0 μl. O_2/hr./10^4 cells. This may be compared with the value of 2.9 reported for *Paramecium* (21). The temperature at which these experiments with *Paramecium* were carried out was not stated by the authors. However, it may be assumed to have been close to 25°C., the usual temperature for investigations on protozoa.

The relatively high rate of activity of lactic dehydrogenase in *Tetrahymena* was not unanticipated in view of the observation that the cytoplasm of the organism contains rather large amounts of lactate (22), and that lactate is one of the main products of glucose fermentation by the organism (23).

[3] Since this paper was submitted for publication, it has been found that ATP (2×10^{-3}M) stabilizes the isocitric dehydrogenase activity of *Tetrahymena* for periods of approximately 30 minutes; it does not alter the initial rate of activity. The addition of ATP was based on the recent experiments of Hogeboom and Schneider (25). ATP is without effect on the glutamic dehydrogenase of *T. geleii* S.

In the literature there are apparently conflicting data concerning the oxidation of succinate and of citrate by *Tetrahymena*. Chaix, Chauvet, and Fromageot (2) found that added succinate results in an increase of only 8 per cent in the oxygen consumption of living cells, and they conclude that the organism thus utilizes succinate. On the other hand, Seaman (3) reported that the addition of succinate in living *Tetrahymena* causes an increased oxygen uptake of 105 per cent. It was later reported (4) that while succinate is readily oxidized by cell homogenates, the living cell is actually impermeable to this compound. In the earlier investigation (3), since cells were centrifuged at a speed of 2,200 R.P.M. (R = 16.2 cm.), it appears that in reality, the suspension used was a "mild" homogenate. Thus, in all subsequent investigations, centrifugal speeds of 750 R.P.M. (R = 16.2 cm.) were always used (1, 4). This slower rate of centrifugation does not cause apparent cell damage.

TABLE III

Effect of $MgCl_2$ Concentration on Activity of Isocitric Dehydrogenase in Tetrahymena

Gas phase, air: temperature, 25.6°C. Flask contents as described in text with exception that the concentration of $MgCl_2$ used was varied as noted.

$MgCl_2$ concentration	Enzyme activity
$\times 10^{-2}$ M	$\mu l.\ O_2/mg.\ dry\ wt./hr.$
1.0	0.9 ± 0.3
2.0	1.2 ± 0.4
4.0	8.4 ± 0.7
5.0	9.0 ± 1.3
8.0	9.3 ± 1.2

Chaix, Chauvet, and Fromageot (2) also found that added citrate results in an increased oxygen uptake of 11 per cent. Seaman (3) found no significant change in oxygen utilization when *Tetrahymena* was incubated with citrate in concentrations ranging from 2×10^{-4} M to 1×10^{-2} M. In addition he found that citrate was unable to release malonate inhibition. The apparent discrepancy between the inactivity of the citrate system in *Tetrahymena* as reported in the previous investigations and the rather high activity of the system as reported in the present study may be explained by the phenomenon of ion-binding (24). In the present work the concentration of Mg^{++} in the reaction mixture is three times as great as was used in the previous investigations. Table III shows that in the presence of 2×10^{-2} M $MgCl_2$, the concentration used in the previous investigation, the isocitric system shows very slight activity. However, in the presence of concentrations of $5\text{-}8 \times 10^{-2}$ $MgCl_2$ the activity of the system reaches the value reported above.

The absence of a DPN linked α-glycerophosphate dehydrogenase in *Tetrahymena* is of interest. It is claimed (8) that this enzyme rather than the cyto-

chrome linked dehydrogenase is the one which is active in the glycolytic process. It thus appears that in this organism the cytochrome linked dehydrogenase takes over the function which is performed by the DPN linked system in mammalian tissue and in yeast.

SUMMARY

The rates of activity of the dehydrogenase systems in *Tetrahymena*, which are concerned with carbohydrate oxidation, in descending order of activity are: lactic > isocitric > succinic = glucose > glucose-6-phosphate = 6-phosphogluconic = malic > glutamic = cytochrome linked α-glycerophosphate dehydrogenase.

No evidence was obtained to indicate the presence of DPN linked α-glycerophosphate dehydrogenase.

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