Studies on the Metabolism of the Protozoa

3. METABOLISM OF THE CILIATE *TETRAHYMENA PYRIFORMIS* (*GLAUCOMA PYRIFORMIS*)

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During the past 20 years, numerous studies have been carried out on the nutritional requirements and metabolic activities of certain small, holotrichous ciliates, known by a variety of names, which can be grown in bacteria-free pure cultures. As a result of morphological studies carried out with twenty-nine different cultures of these ciliates, Corliss (1952) has concluded that they are nearly all different strains of one and the same organism, which should in his opinion be called *Tetrahymena pyriformis*. In the present paper, the '*Glaucoma piriformis*' of A. Lwoff (1932), M. Lwoff (1934) and Lawrie (1935) and the '*Tetrahymena geleii*' of Chaix, Chauvet & Fromageot (1947) will be referred to as *Tetrahymena pyriformis* (GL), the '*Colipidium campylum*' of Wilber & Seaman (1948) and Seaman (1949), and the '*Tetrahymena geleii*' of Seaman (1950, 1951) will be referred to as *Tetrahymena pyriformis* (S), the '*Colipidium campylum*' of Hall (1941) will be referred to as *Tetrahymena pyriformis* (H), the '*Tetrahymena geleii*' of Pace & Ireland (1945) and Pace & Lyman (1947) will be referred to as *Tetrahymena pyriformis* (W), while the '*Tetrahymena geleii*' of Baker & Baumberger (1941) and Niel, Thomas, Ruben & Kamen (1942) will be referred to as *Tetrahymena pyriformis* (T); Kidder & Dewey (1945) give the origins of some of these strains.

A striking chemical characteristic of *T. pyriformis* (GL) is the unusually high content of intracellular glycogen (Ryley, 1951; Manners & Ryley, 1952). In the present study, the anaerobic metabolism of *T. pyriformis* is shown to depend on a phosphorylative breakdown of this reserve of glycogen, with succinic, lactic and acetic acids as end products, while the aerobic metabolism is shown to depend on processes other than glycogen breakdown.

MATERIAL AND METHODS

Organism. The culture of *Tetrahymena pyriformis* (GL) was kindly supplied by Dr E. G. Pringsheim of the Botany School, Cambridge; it was the strain described by Lwoff

(1932) under the name *Glaucoma piriformis*. The organism was cultivated in a sterile medium (pH 7.3) containing 1% (w/v) 'Oxoid' brand bacteriological peptone and 0.4% (w/v) NaCl. Cultures were maintained in tubes at room temperature, sub-inoculations being made every 2 weeks. For experimental studies, cultures were grown at 30°, in flasks containing 2-4 l. medium, with an air space of 1 l. The organisms were harvested after 6 days' growth, using a small angle centrifuge; it was found necessary to use a centrifuge which came to rest quickly, and to remove the supernatant immediately by suction. With most centrifuges tried, although the ciliates were sedimented readily, by the time the centrifuge had stopped the organisms had swum back into the supernatant. The centrifuged cells were washed and suspended in either 0.45% (w/v) NaCl, a Ringer-phosphate solution containing 0.047 M-NaCl, 0.002 M-KCl, 0.001 M-MgSO₄ and 0.012 M-phosphate buffer (pH 7.3), or a Ringer-bicarbonate solution containing 0.05 M-NaCl, 0.003 M-KCl, 0.001 M-MgSO₄ and 0.016 M-NaHCO₃, which when used in conjunction with a gas mixture of 5% CO₂-95% N₂ (v/v), had a pH of 7.3. Homogenates were made by washing the organisms in water, and then shaking a thick aqueous suspension with glass beads in the Mickle disintegrator for 5 min. Cell-free preparations were obtained by centrifuging this homogenate for 15 min. at 11 000 rev./min. in the Servall angle centrifuge. The supernatant was a clear, light-brown fluid, free from microscopically visible particles.

Materials. Glucose oxidase and catalase were kindly supplied by Dr E. F. Hartree. The glycogen used was a purified specimen prepared from *Tetrahymena pyriformis* (GL), described by Manners & Ryley (1952). Adenosinetriphosphate (ATP) was prepared from the barium salt, kindly supplied by Dr E. C. Slater. Potassium glucose-1-phosphate and calcium fructose-1:6-diphosphate were gifts from Dr T. Mann.

Spectroscopic observations. These were made by means of a microscope fitted with a Zeiss microspectroscope ocular.

Purpurogallin number (P.Z.). This was determined by the method of Sumner & Gjessing (1943), slightly modified. A mixture of 2 ml. 5% (w/v) purpurogallin solution, 2 ml. 0.5 M-phosphate buffer (pH 6.0), 1 ml. 1% (v/v) H₂O₂ and 15 ml. water were warmed to 20° in a beaker. An aqueous suspension (1 ml.) of the ciliate, which had been twice frozen and thawed to disrupt the cells, was added, and the mixture incubated at 20° for 5 min.; the reaction was stopped by the addition of 1 ml. 2 N-H₂SO₄. As much protein was present, and interfered with the extraction of purpurogallin, 1 ml. 100% (w/v) trichloroacetic acid was added, and the mixture centrifuged. The supernatant was extracted twice with ether, and the extract diluted to 25 ml. with ether. Purpurogallin was estimated photoelectrically in the

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extract. The P.Z. is defined as mg. purpurogallin formed per mg. material under the standard conditions used.

Metabolic experiments. These were carried out in Warburg manometers, using cups (vol. 20–25 ml.) fitted with two side arms. Gas phases of air or 5% CO₂–95% N₂ (v/v) were used; the experiments were mostly carried out at 37°. The effect of CO on respiration was studied in Barcroft differential manometers at 19° and at 30°. Metabolic rates were based on the N content of the suspensions, being expressed as q_{O_2} and q_{CO_2} , i.e. as μ l. gas/mg. N/hr. It was found that 1 mg. ciliate N was equivalent to 8.9 mg. dry weight, or about 80 mg. wet weight of cells.

Estimation of metabolites. Glycogen was estimated by the method of Good, Kramer & Somogyi (1933). Reducing sugars was estimated iodometrically by the method of Somogyi (1945), in Ba(OH)₂–ZnSO₄ filtrates; glucose was determined from the loss in reducing value of such a filtrate after treatment with glucose oxidase and catalase (Mann, 1946), while fructose was estimated colorimetrically (Roe, 1934). Fructose estimations were also carried out in trichloroacetic acid filtrates to determine the total of free and phosphorylated sugar; values for phosphofructose were calculated from the difference in fructose content of these two filtrates. Phosphate fractions were estimated by the method of Fiske & Subbarow (1925); total phosphorus was estimated after digestion with 60% HClO₄ and a little HNO₃. Using trichloroacetic acid filtrates, pyruvic acid was estimated by the colorimetric method of Friedemann & Haugen (1943), and lactic acid by the method of Barker & Summerson (1941). Acetic acid was estimated by distillation of a tungstic acid filtrate (Buchanan, Sakami, Gurin & Wilson, 1947), while ethanol was determined by the bichromate method of Nicloux, le Breton & Doutecheff (1934). Succinic acid was estimated manometrically by means of a washed horse-heart preparation of succinic oxidase.

RESULTS

Aerobic metabolism

The free-living protozoan *T. pyriformis* (GL), although it occurs naturally at a much lower temperature, is able to maintain constant motility and metabolism at 37° for a period of several hours at least. It was thought preferable to work at this higher temperature, rather than 22° or similar temperatures utilized by previous workers, as this reduced the duration of the experiments. The organisms were cultivated at 30°, which was found to be the optimum temperature for growth; at 37°, although the ciliates flourished, growth was not quite as good as at 30°.

Respiratory activity. In a non-nutrient medium at 37°, the ciliate respired with a q_{O_2} of 480 (mean of seven values, ranging from 380 to 565). From Fig. 1 it can be seen that the respiration continued at an almost linear rate for 60 min.; other experiments have shown no marked decrease in respiration after 5 hr. in a similar system. Fig. 1 also shows that respiration proceeds at an almost linear, but reduced rate (q_{O_2} = 225) at 25°. Although the ciliate maintains a high endogenous respiration in the absence of extracellular nutrients, several added compounds were able to increase the O₂ uptake still further. Thus, as shown in Table 1, acetate, propionate, butyrate, and to a lesser extent pyruvate, increased the respiration, while glucose, ethanol, and a number of acids associated with the tricarboxylic acid

Fig. 1. Aerobic and anaerobic metabolism of *T. pyriformis* (GL). Each manometer cup contained 1.14 mg. ciliate N in a volume of 1.5 ml.; gas phase air or 5% CO₂–95% N₂; ●—●, O₂ uptake (μ l.) at 37°; ●—●—●, O₂ uptake (μ l.) at 25°; ○—○, anaerobic CO₂ production (μ l.) at 37° in bicarbonate buffer; ○—○—○, anaerobic CO₂ production (μ l.) at 25°.

Table 1. Effect of extracellular substrates on respiration

(Respiration of ciliates in Ringer-phosphate solution (pH 7.3) followed for 30 min.; 0.01 M substrate then added from side bulb, and respiration followed for a further 60 min. Table gives O₂ uptakes observed during this latter period. Total vol. 1.5 ml.; temp. 37°; gas phase air.)

Substrate added	O ₂ uptake (μ l./hr.)	Stimulation (%)
Exp. 1 (0.5 mg. ciliate N)		
None	206	0
Glucose	226	10
Ethanol	213	3
Sodium citrate	223	8
Sodium succinate	219	6
Sodium formate	218	6
Sodium acetate	385	87
Sodium propionate	400	94
Sodium butyrate	406	97
Exp. 2 (0.57 mg. ciliate N)		
None	265	0
Sodium succinate	300	13
Sodium malate	309	16
Sodium fumarate	275	4
Sodium malonate	276	4
Lithium lactate	308	16
Sodium pyruvate	368	39
Pyruvate + malonate	385	45
Pyruvate + malonate + fumarate	385	45
Sodium acetate	454	71

cycle had little effect. The dicarboxylic acids had even less effect on the respiration when tested at pH 6.0, or if added to organisms which had been starved prior to use by keeping in Ringer-phosphate solution for 20 hr. at room temperature. With one particular ciliate preparation, which during a period of 60 min. consumed 23 μ moles O_2 , it was found that 0.01 M-glucose increased the O_2 uptake by 1.96 μ moles or 8%, while 3.87 μ moles glucose had disappeared. The oxidation of 3.87 μ moles glucose would require a much greater quantity of O_2 than the extra uptake of 1.96 μ moles observed. This may indicate a sparing of the unknown substrate responsible for endogenous respiration, or may indicate an assimilation of the sugar.

18.5 μ moles glucose; had the oxidation been incomplete, the glycogen utilization would have been even greater.

Effect of inhibitors on respiration and motility. The sensitivity of endogenous respiration to a number of inhibitors was studied; the results are summarized in Table 2. The presence of cyanide (0.46×10^{-3} M), using the balanced KOH-KCN mixtures in the centre well as recommended by Robbie (1946), reduced respiration by only 18%; this inhibition was maintained over a period of 5 hr. On another occasion, in the presence of glucose, the same concentration of cyanide gave a 34% inhibition of O_2 uptake. CO ($CO/O_2=19$), had no inhibitory effect on respiration in the dark, at either 19 or 30°, but rather gave rise to a small

Table 2. *Effect of inhibitors on respiration*

(Each manometer cup contained about 0.6 mg. ciliate N in a total vol. of 1.5 ml.; gas phase air; temp. 37°. Inhibitor added from side bulb, and O_2 uptake over a period of 1 hr. compared with that of control.)

Inhibitor	Concn. (M)	Respiration (as % that of control)		
		pH 7.3	pH 6.0	pH 5.5
None	—	100	100	100
Potassium cyanide	0.00046	82	—	—
Carbon monoxide (30°)	(CO/ O_2 =19)	105	—	—
Sodium azide	0.01	—	17	40
Sodium azide	0.001	—	143	272
Sodium azide	0.0001	—	119	157
2:4-Dinitrophenol	0.001	—	28	—
2:4-Dinitrophenol	0.0002	—	65	—
2:4-Dinitrophenol	0.0001	—	144	—
2:4-Dinitrophenol	0.00002	—	143	—
Hydroxylamine	0.002	70	—	—
Sodium iodoacetate	0.0001	69	—	—
Sodium arsenite	0.01	38	—	—
Sodium fluoride	0.02	100	—	—
Phlorrhizin	0.01	88	—	—
2:2'-Dipyridyl	0.0003	96	—	—
4:4'-Dipyridyl	0.0003	95	—	—
8-Hydroxyquinoline	0.0003	83	—	—
Phenylthiourea	0.0003	100	—	—

Nature of endogenous respiration. A determination of the respiratory quotient was made, using the direct method of Warburg on organisms in a Ringer-phosphate medium; corrections were applied both for the initial and the final bound CO_2 in the medium. This gave an R.Q. value of 0.85, which suggests that endogenous respiration is probably not dependent on carbohydrate metabolism. A similar conclusion can be derived from experiments in which intracellular glycogen was estimated before and after a period of active respiration. Six manometers were set up, each containing 1 mg. cellular N in Ringer-phosphate solution. After manometric equilibration, 2 N- H_2SO_4 was tipped from the side bulb into three of the flasks, while the ciliates in the other three flasks were allowed to respire for a period of 150 min. Respiration was stopped by the addition of H_2SO_4 , and glycogen was estimated in the contents of each of the flasks. The initial glycogen present was equivalent to 45.2 μ moles glucose. During the incubation, a total of 111 μ moles O_2 was consumed by the ciliates in the three flasks, while the decrease in glycogen content amounted to only 1.79 μ moles glucose. Had the O_2 uptake observed been due to the complete oxidation of carbohydrate, there would have been a glycogen disappearance corresponding to

stimulation. Azide, another potent inhibitor of the cytochrome oxidase system, was used at pH 6.0 and 5.5, but acted as an inhibitor only at high concentrations. Indeed, at concentrations of 0.001 M and less, azide caused a pronounced stimulation of the O_2 uptake. Motility studies, however, showed that azide inhibited motility to a small extent at a concentration of 2×10^{-5} M, and that higher concentrations had a larger effect; the influence of azide on motility increased with the time of incubation. 2:4-Dinitrophenol had similar effects to azide on respiration; concentrations greater than 0.0002 M inhibited respiration at pH 6.0, while concentrations less than 0.0001 M stimulated respiration. In all cases, there was an adverse effect on motility; in the presence of 0.001 M-dinitrophenol, motility was abolished within 30 min. at room temperature, while in 0.0002 M-dinitrophenol, motility was gradually reduced, until the organisms were completely immotile after 230 min. The controls to these experiments showed that respiration at pH 6.0 was as good as that observed at pH 7.3, but that at pH 5.5, O_2 uptake was reduced by 30%.

Respiration and motility were insensitive to high concentrations of fluoride, while arsenite had little effect at a concentration less than 0.01 M; this concentration of

arsenite was sufficient to render the ciliates almost stationary after 100 min. at room temperature, while a concentration of 0.003 M had a smaller, but noticeable, effect after this time. Iodoacetate at a concentration of 3×10^{-5} M had no effect on respiration and motility, but 0.0001 M-iodoacetate reduced both considerably over a period of 60 min.; 0.0003 M-iodoacetate began to produce inhibitory effects after 15 min. incubation, and within 1 hr. the organisms were completely immotile. Phlorrhizin and the metal-binding substances tested were without noteworthy effect, save that 8-hydroxyquinoline caused a slight reduction in motility. In all cases, loss of motility was an irreversible process, being accompanied by damage to the cells.

Haem compounds

Cytochrome system. Direct spectroscopic examination of a suspension of *T. pyriformis* (GL) shows the presence of a number of haem pigments. Under conditions of good aeration, the most prominent feature of the spectrum is the α -band of oxyhaemoglobin (Keilin & Ryley, 1951). When reduced with a small amount of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), a cell suspension examined at room temperature showed: (a) A narrow, weak cytochrome *c* band at 550 m μ . (b) A strong cytochrome *e* band at 551–553 m μ . (c) A weak cytochrome *b* band at 563 m μ . (d) An exceedingly weak band at 605 m μ ., probably due to cytochrome *a*. (e) Double β -bands at 521 and 530 m μ . (f) A very weak and diffuse band at 580–590 m μ ., possibly due to cytochrome a_1 , or to incompletely reduced haemoglobin. This band moved to 568–578 m μ ., as a weak and very diffuse band, in the presence of CO; the other bands were not affected by CO. (g) A very faint band at 635 m μ ., probably due to methaemoglobin, derived by oxidation of some of the haemoglobin.

Manometric experiments showed that a homogenate of the ciliate was unable to catalyse the oxidation of *p*-phenylenediamine, either in the presence or in the absence of added cytochrome *c*. In connexion with this lack of cytochrome oxidase activity, the results of Table 2 concerning the effects of azide, cyanide and CO on respiration, should be noted.

Peroxidase. Lawrie (1935) reported that *T. pyriformis* (GL) contained a peroxidase. The peroxidase activity of the protozoan has been confirmed, and the purpurogallin number determined. Saturated ethanolic benzidine (1 ml.) was mixed with 1 ml. water, 6 drops glacial acetic acid, and 1 ml. of a suspension of the ciliate. A few drops of H_2O_2 were added; the mixture rapidly became blue in colour. Microscopic examination immediately after adding H_2O_2 showed that the cells became uniformly blue throughout, but that this colour soon diffused out of the cell. (Similar experiments with *Paramecium caudatum* showed a concentration of the blue colour in and around unidentified granules in the cell.) Boiled organisms gave only a slight production of colour, indicating that the benzidine reaction was mainly due to a thermolabile enzyme, rather than to the intracellular haematin as such. Similar colour-producing reactions were obtained with other phenolic compounds, such as pyrogallol, *p*-phenylenediamine, guaiacum or pyrocatechol, when tested with H_2O_2 in 0.1 M-phosphate buffer (pH 7.3). In each case, colour production was dependent on the addition to the system of H_2O_2 and cell suspension. During these experiments, a considerable production of gas was observed, due to decomposition of the peroxide by catalase. Using the modified method of Sumner & Gjessing (1943), it was found

that 14.4 mg. dry wt. of cells formed 0.475 mg. purpurogallin, giving a P.Z. of 0.033.

Cell homogenates were able to oxidize reduced cytochrome *c* peroxidatically. A small amount of dilute cytochrome *c* solution was reduced by adding a trace of *p*-phenylenediamine. Cell homogenate was then added, and the mixture examined spectroscopically. The intense band of cytochrome *c* at 550 m μ . did not fade in spite of aeration for at least 1 hr., indicating that no active cytochrome oxidase was present. When H_2O_2 was added to the system, the cytochrome *c* band quickly disappeared, reappearing when all the peroxide had been utilized; further addition of H_2O_2 caused the band to disappear once more.

Anaerobic metabolism

Under anaerobic conditions, *Tetrahymena pyriformis* (GL) is able to survive and maintain motility for prolonged periods of time. A suspension of the ciliate in Ringer-bicarbonate solution was placed in a Thunberg tube, which was evacuated and filled four times with an O_2 -free CO_2/N_2 mixture. Normal motility was maintained by a large proportion of the cells (examined microscopically with a $\frac{2}{3}$ in. objective while in the tube) for at least 72 hr., although a number of the organisms died during this time; a similar picture was obtained with a control suspension exposed to the air. Initially, all the cells stained a dark-brown colour with dilute iodine, but many of those which had been kept under anaerobic conditions for 72 hr. gave very little coloration with iodine; the cells kept for the same period of time under aerobic conditions still gave a good iodine stain. When suspended in a Ringer-bicarbonate medium under anaerobic conditions, the ciliate produced acid (Fig. 1). From six experiments carried out at 37°, a mean $q_{\text{CO}_2}^{\text{N}_2}$ of 180 was observed (range 123–238), while at 25°, a $q_{\text{CO}_2}^{\text{N}_2}$ value of 70 was obtained. This liberation of CO_2 was increased by 33% in the presence of 0.01 M-glucose, 35% of the added glucose being utilized.

Nature of anaerobic endogenous metabolism. Under anaerobic conditions the ciliate fermented its intracellular glycogen reserves and maintained a high degree of motility. Six manometers were set up, each with 1 mg. ciliate N in 1.5 ml. Ringer-bicarbonate solution, and a CO_2 - N_2 gas phase. After equilibration, acid was tipped into three of the flasks in order to estimate the initial bicarbonate and after 110 min. acid was tipped into the other flasks to estimate residual bicarbonate. Estimations showed that the glycogen reserves had been depleted by 32% during the fermentation, while 55.0 $\mu\text{equiv.}$ acid had been produced, and 7.05 μmoles CO_2 had been assimilated, i.e. for each glucose unit of glycogen metabolized, 2.75 equiv. of acid were formed, with the assimilation of 0.35 molecules of CO_2 .

A similar experiment was carried out on a somewhat larger scale, the flask contents being analysed for glycogen, succinic, lactic, pyruvic and acetic acids and ethanol. From Table 3 it can be seen that the glycogen was mainly converted to succinic acid, with smaller amounts of lactic and acetic acid. The metabolites estimated accounted for 91% of the carbon supplied, and for 97.5% of the CO_2 liberated by acid formation.

Effect of inhibitors on anaerobic metabolism. From Table 4 it can be seen that of the seven compounds tested as possible inhibitors of the fermentation, only iodoacetate had a marked effect. In the presence of 0.001 M-iodoacetate, CO_2 evolution was reduced by 75% over the period of 60 min.

studied; the metabolism stopped within 20 min. of adding the inhibitor. In contrast to the anaerobic metabolism of glucose by *Trypanosoma lewisi* (Ryley, 1951), which is highly sensitive to the metal-binding substances 2:2'-dipyridyl and 8-hydroxyquinoline, these same compounds were found to stimulate the rate of an aerobic acid production by *Tetrahymena pyriformis* (GL).

Table 3. *Anaerobic glycogen fermentation by Tetrahymena pyriformis (GL)*

(Ciliate N 8.8 mg. was incubated for 120 min. in a total vol. of 18 ml. Ringer-bicarbonate solution, using six manometers; gas phase 5% CO₂-95% N₂; temp. 37°. Reaction stopped, and residual bicarbonate estimated by adding 0.4 ml. 2N-H₂SO₄ to each flask. Contents of flasks neutralized, pooled, and deproteinized for estimation of metabolites. Yields expressed in terms of moles metabolite found per glucose unit of glycogen disappearing, from which values for carbon and acid recoveries have been calculated. During the fermentation, a total of 71.1 μ moles glucose (as glycogen) were used, and 28.9 μ moles CO₂ were fixed.)

Metabolite	Moles	Carbon (g. atoms)	Acid (g. equiv.)
Succinic acid	0.94	3.76	1.88
Lactic acid	0.47	1.41	0.47
Pyruvic acid	0.00	0.00	0.00
Acetic acid	0.32	0.64	0.32
Ethanol	0.01	0.02	0.00
Sum	—	5.83	2.67
C g. atoms supplied (as glycogen and CO ₂)	—	6.41	—
Acid yield (from bicarbonate destroyed)	—	—	2.74

Table 4. *Effect of inhibitors on anaerobic metabolism*

(Each manometer cup contained about 0.9 mg. ciliate N in a total vol. of 1.5 ml.; gas phase 5% CO₂-95% N₂; temp. 37°. Inhibitor added from side bulb, and CO₂ evolution over a period of 1 hr. compared with that in control.)

Inhibitor	Concn. (M)	CO ₂ evolution (as % that of control)
Sodium fluoride	0.02	86
Sodium arsenite	0.01	66
Sodium iodoacetate	0.001	25
2:2'-Dipyridyl	0.0003	127
4:4'-Dipyridyl	0.0003	100
8-Hydroxyquinoline	0.0003	121
Phenylthiourea	0.0003	100

Table 5. *Amylase and maltase activity in cell-free Tetrahymena pyriformis (GL) preparations*

(Each tube contained 3.9 mg. N in the form of cell-free preparation, 0.027 M-acetate-veronal buffer (pH 7.3), 0.017 M-NaCl, and starch, glycogen and maltose respectively, in a total volume of 9 ml. Tubes incubated at 37°, 2 ml. samples being removed at intervals. Reducing sugar expressed in terms of mg. hexose. All figures given in mg.)

Incubation time (min.)		Starch	Glycogen	Maltose
0	Substrate added	36	36	60.2
	Reducing sugar found	2.02	2.03	35.6
	Glucose found	1.74	1.86	4.4
30	Reducing sugar found	6.85	6.41	40.0
90	Reducing sugar found	15.47	16.75	48.1
	Glucose found	12.35	13.26	29.2

Intermediary carbohydrate metabolism

Amylase and maltase. Cell-free preparations of *T. pyriformis* (GL) were incubated with starch, glycogen or maltose at pH 7.3 in the presence of NaCl, and the reaction followed by estimating reducing sugar and glucose at suitable time intervals. From Table 5, it can be seen that starch and glycogen were hydrolysed at approximately the same rate to reducing sugar, 80% of which was found to be glucose. During the 90 min. incubation, 23.4 mg. maltose were broken down, and 24.8 mg. glucose were recovered. At the beginning of the experiment, the samples already contained a small amount of reducing sugar, due no doubt to hydrolysis of protozoal glycogen in the course of preparation of the cell-free extract.

Table 6. *Hexokinase activity of cell-free Tetrahymena pyriformis (GL) preparations*

(Each manometer cup contained 0.7 mg. N in the form of cell-free preparation, 0.026 M-NaHCO₃, 0.005 M-MgSO₄, 0.06 M-NaF, 0.05 M-KCl, 0.0045 M-glucose and 0.0031 M-ATP in a total volume of 2.5 ml. Mixture incubated 40 min. at 37° with gas phase of 5% CO₂-95% N₂.)

	Inorganic P [P _o] (μ moles)	Acid-labile P [P _i -P _o] (μ moles)	Reducing sugar (μ moles)
Initial	1.70	15.22	14.1
Final	11.81	4.01	14.5

P esterified = 1.10 μ moles. CO₂ liberated by acid production = 145 μ l. (6.48 μ moles).

Hexokinase. An attempt was made to demonstrate hexokinase activity in cell-free preparations. Warburg manometers were set up as indicated in Table 6, the reaction being followed by the liberation of CO₂ due to acid formation. During the experiment, 145 μ l. (6.48 μ moles) CO₂ were evolved; there was a large breakdown of ATP to inorganic phosphate, but about 10% was esterified. This was observed on two occasions. It was not possible to follow the hexokinase reaction by the disappearance of glucose since, during the experiment, there was a very slight increase in glucose, due no doubt to hydrolysis of polysaccharide present in the enzyme preparation. An identical system containing a similar homogenate of *Trypanosoma lewisi* showed marked hexokinase activity: 60% of the added glucose was utilized, and ATP was metabolized, equal amounts of inorganic and esterified P accumulating.

Phosphorylase. Although *Tetrahymena pyriformis* (GL) contains hydrolytic enzymes capable of degrading glycogen and starch, in a system containing protozoal glycogen and phosphate buffer cell-free preparations of the ciliate caused a breakdown of glycogen, accompanied by an uptake of inorganic phosphate. During the first 15 min. of incubation, over 50% of the inorganic phosphate disappearing was converted to an easily hydrolysable P compound (Fig. 2). On further incubation this fraction decreased, while the less easily hydrolysable phosphate fraction increased. Fructose estimations in trichloroacetic acid extracts and in $\text{Ba}(\text{OH})_2\text{-ZnSO}_4$ extracts showed that the process was accompanied by

Fig. 2. Phosphorylase activity of cell-free *T. pyriformis* (GL) preparation. Each tube contained 0.85 mg./ml. N in the form of cell-free preparation, 4 mg./ml. *Tetrahymena* glycogen and 0.025 M-phosphate buffer (pH 7.3) in a total volume of 6 ml.; temp. 37°; ●—●, inorganic phosphate; ○—○, [P₇-P₁] phosphate fraction; ▲—▲, [P₇-P₀] phosphate fraction.

the formation of fructose phosphate. After 120 min. incubation, there had been an esterification of 7.6 μg. atoms/ml. P, and an accumulation of 2.7 μmoles/ml. fructose phosphate. In a similar experiment (Table 7), it was found that cell-free preparations of the ciliate were able to phosphorylyse starch as well as glycogen.

Phosphoglucomutase and oxoisomerase. The ability of *T. pyriformis* (GL) to transform glucose-1-phosphate into a mixture of hexose-6-phosphates has been shown directly by

incubating Cori ester with a cell-free preparation of the ciliate. From Table 8 it can be seen that the easily hydrolysable phosphate fraction was reduced on incubation, 50% of the phosphate being recovered as inorganic phosphate, the other 50% being converted into less easily hydrolysable phosphate, part of it in combination with fructose, presumably as fructose-6-phosphate.

Table 7. *Phosphorylase activity of cell-free Tetrahymena pyriformis (GL) preparations*

(Each tube contained 0.5 mg./ml. N in the form of cell-free preparation, 4 mg./ml. starch or glycogen and 0.025 M-phosphate buffer (pH 7.3) in a total volume of 9 ml. Samples (2 ml.) deproteinized before and after 2 hr. incubation at 37°, with $\text{Ba}(\text{OH})_2\text{-ZnSO}_4$ or with 5% trichloroacetic acid (TCA). Reducing sugar, glucose and fructose estimated in Ba-Zn filtrates, and fructose, P₀ and P₇ in TCA filtrates. Results expressed as μmoles/ml. of enzyme system.)

	Glycogen	Starch
Inorganic P esterified	5.36	3.55
Glucose-1-phosphate formed	1.29	0.42
Glucose-6-phosphate formed	1.85	1.81
Fructose-6-phosphate formed	2.22	1.42
Reducing sugar liberated	8.10	8.24
Free fructose found	0.16	0.22

Table 8. *Phosphoglucomutase and oxoisomerase activity in cell-free Tetrahymena pyriformis (GL) preparations*

(Each tube contained 1 mg. N in the form of cell-free preparation, 0.002 M-MgCl₂, 0.023 M-acetate-veronal buffer (pH 7.3) and 3 mg. potassium glucose-1-phosphate in a total volume of 4.5 ml. Incubation carried out at 37°, and systems deproteinized with 1.5 ml. 10% (w/v) TCA.)

Time (min.)	[P ₀] (μg. atoms)	[P ₇ -P ₀] (μg. atoms)	[P ₁₈₀ -P ₇] (μg. atoms)	[P ₇ -P ₁₈₀] (μg. atoms)
0	1.45	8.64	0.10	0.29
10	4.91	3.26	0.77	1.55
60	5.39	0.74	1.87	2.48

Glucose-1-phosphate used = 7.90 μmoles. Inorganic phosphate liberated = 3.94 μmoles. Less easily hydrolysable phosphate ester formed = 3.96 μmoles. Fructose-6-phosphate found (as fructose) = 2.77 μmoles.

Enzymic degradation of fructose diphosphate. Table 9 shows that a cell-free enzyme preparation from *T. pyriformis* (GL), incubated anaerobically with potassium fructose-1:6-diphosphate (FDP), gave rise to triosephosphate (alkalilabile phosphate); the conversion was greater in the presence of cyanide, which acts as a keto fixative. Using a similar

Table 9. *Aldolase activity in cell-free Tetrahymena pyriformis (GL) preparations*

(Each manometer cup contained 0.4 mg. N in the form of cell-free preparation, 0.025 M-NaHCO₃, 0.06 M-NaF and 0.01 M-fructose-1:6-diphosphate (FDP) in a total volume of 2.5 ml.; gas phase 5% CO₂-95% N₂; temp. 37°. Mixtures incubated for 1 hr., and deproteinized with 1.5 ml. 10% TCA.)

System	Alkali-labile P formed (μmoles)	FDP used (μmoles)	Inorganic P liberated (μmoles)
Enzyme + FDP	8.95	6.35	1.3
Enzyme + FDP + 0.06 M-KCN	21.10	13.20	1.0

system, it was found that 0.001 M-2:2'-dipyridyl had no effect on aldolase activity at pH 7.3. However, at pH 6.0, aldolase activity was inhibited by 5% in the presence of 0.001 M-2:2'-dipyridyl, and by 34% in the presence of 0.005 M-dipyridyl.

metrically. Even in the absence of FDP, appreciable amounts of acid were formed, owing to the fermentation of some substrate present in the enzyme preparation itself; however, when FDP was added, CO₂ evolution was doubled.

Fig. 3. Fructose diphosphate metabolism by cell-free *T. pyriformis* (GL) preparation. Each manometer cup contained 0.73 mg. N in the form of cell-free preparation, 0.025 M-NaHCO₃, 0.008 M-fructose-1:6-diphosphate (FDP), 0.008 M-pyruvate, 0.0001 M-cozymase, 0.0018 M-phosphate and 0.06 M-NaF in a total vol. of 2.5 ml.; gas phase 5% CO₂-95% N₂; temp. 37°; ●—●, CO₂ evolution (μl.) in presence of FDP; ○—○, CO₂ evolution (μl.) in presence of FDP and 0.004 M-arsenate; ▲—▲, CO₂ evolution (μl.) in absence of FDP.

Fig. 4. Lactic oxidase activity of cell-free *T. pyriformis* (GL) preparation. Each manometer cup contained 1.0 mg. N in the form of cell-free preparation, and 0.04 M-phosphate buffer (pH 7.3), in a total volume of 2.5 ml.; temp. 37°; gas phase air; ●—●, O₂ uptake (μl.) in presence of 0.03 M-lactate; ○—○, O₂ uptake (μl.) in presence of 0.03 M-lactate and 0.00005 M-methylene blue; ○- - -○, O₂ uptake (μl.) in presence of 0.00005 M-methylene blue.

Speck & Evans (1945), using malaria parasite preparations, attempted to demonstrate the oxidation of triosephosphate, coupled with the simultaneous reduction of pyruvate, fluoride being added to the system in order to prevent the breakdown of phosphoglyceric acid. A similar system has been used in the present case of *T. pyriformis* (GL). Fig. 3 illustrates an experiment in which a cell-free preparation of the ciliate was allowed to act upon FDP under anaerobic conditions, acid production being followed mano-

In the presence of FDP, fermentation proceeded at the same rate in the presence as in the absence of added pyruvate or cozymase. Chemical analysis showed that at the end of this experiment about 40% of the added FDP had disappeared; triosephosphate formation accounted for 60% of the FDP disappearing, while the remainder was converted to lactic acid. The production of lactic acid was not influenced by the addition of extra pyruvate although, when present, pyruvate was utilized. Arsenate (0.004 M) doubled the rate of acid production in the presence of FDP, and there was a corresponding increase in FDP utilization. Similar experiments

Table 10. *Dehydrogenase activity in Tetrahymena pyriformis (GL)*

(Thunberg tubes contained homogenate or cell-free preparation and 0.04 M-phosphate buffer (pH 7.3), with 0.134 μmole methylene blue and substrate as indicated added from hollow stopper. Tubes evacuated and washed with pure N₂ four times; temp. 37°; total volume, 2.5 ml. Figures give time (min.) for 90% decolorization.)

Preparation	Additions				
	None	Lithium lactate (0.03 M)	Sodium succinate (0.03 M)	Glucose (0.03 M)	FDP (0.008 M)
Homogenate (2 mg. N)	45	33	11	37	—
Homogenate (3.9 mg. N)	33	27	8.5	23	—
Cell-free preparation (1.8 mg. N)	80	37	25	—	—
Cell-free preparation (1.1 mg. N)	150	65	37	—	61

showed that the endogenous fermentation of the preparation was insensitive to iodoacetate, but that acid formation in the presence of FDP, with or without arsenate, was reduced to the endogenous level by the addition of 0.001 M-iodoacetate.

As already stated, during glycogen fermentation by intact cells of *T. pyriformis* (GL), lactic and succinic acids accumulate. It seems probable that the production of these acids is a means of reoxidizing dihydrocozymase, which is formed from cozymase during the oxidation of phosphoglyceraldehyde. Table 10 gives the results obtained from a number of Thunberg tube experiments, using both whole and centrifuged homogenates of the ciliate. Succinate accelerated the reduction of methylene blue fourfold, while lactate, glucose and fructose diphosphate were less effective. The centrifuged preparation alone did not respire, but in the presence of 0.03 M-lactate, there was a vigorous O_2 uptake ($q_{O_2} = 125$; Fig. 4). On the other hand, succinate, which was shown in Thunberg tube experiments to accelerate methylene blue reduction, did not increase the O_2 uptake of homogenates when tested manometrically, either in the presence or in the absence of added cytochrome *c* or methylene blue.

DISCUSSION

The ciliate *T. pyriformis* (GL) is able to maintain active motility and respiration in the absence of extracellular substrates over a wide range of temperature, in contrast to the trypanosomes, flagellates which have become adapted to life in a specific and constant environment on which they rely for their source of energy. *T. pyriformis* (GL) respire with a q_{O_2} of 480 at 37°, and 225 at 25°. The nature of the intracellular substrates responsible for this endogenous respiration is an interesting matter for speculation. The present study indicates that respiration is relatively independent of the intracellular glycogen reserve. A respiratory quotient of 0.85 suggests that the ciliates may be oxidizing fat or protein, rather than carbohydrate. Pace & Ireland (1945) demonstrated the presence of fat droplets in the ciliate by staining techniques, while Wilber & Seaman (1948) showed that the organisms contain lipid, and a small amount of phospholipin. However, Pace & Lyman (1947) were unable by microscopical methods to observe any utilization of fat during the metabolism of *T. pyriformis* (W); it would be interesting to confirm this, carrying out chemical estimations of cell lipid before and after a period of active respiration.

Although *T. pyriformis* (GL) respire well in the absence of extracellular substrates, a number of compounds are able to increase the oxygen uptake. Chaix *et al.* (1947) found that the endogenous respiration of this strain was stimulated by short-chain fatty acids, but was little affected by citrate, succinate or malate, substances associated with the tricarboxylic acid cycle. The present study confirms these observations, except that the respiratory stimulations produced by fatty acids were larger;

this may well be due to the higher temperature used in the present work. In contrast to these findings, Seaman (1949) reported that the endogenous respiration of the S strain of *T. pyriformis* was doubled in the presence of 0.02 M-succinate, α -keto-glutarate, fumarate, malate or oxaloacetate, although citrate was without effect, and that the increase in respiration produced by pyruvate was reversed by malonate, this malonate inhibition being antagonized by fumarate. On the basis of these observations, Seaman (1949) suggested that respiration in the ciliate involved the tricarboxylic acid cycle. With the GL strain of *T. pyriformis* used in the present study it has not been possible to obtain such effects. Moreover, unlike Seaman (1950, 1951), we could also find no evidence for the oxidation of succinate by cell homogenates prepared from the ciliate. No oxidation of succinate was observed with cell homogenates or particle-free preparations, either in the presence or in the absence of added cytochrome *c*; such preparations, however, readily oxidized lactate.

According to Seaman (1950), *T. pyriformis* (S) also attacks added acetate, partly by oxidizing it, and partly by converting it to intracellular lipid and carbohydrate. However, since his carbohydrate analyses were carried out on filtrates prepared from cell suspensions deproteinized with barium hydroxide and zinc sulphate, it is difficult to assess the relation between these results and the total carbohydrate content of the cells. It would appear from Seaman's data, that the initial 'carbohydrate' content of the cells was of the order of 0.1%, a value which contrasts with a content of 20% glycogen demonstrated by us in the case of *T. pyriformis* (GL) (Ryley, 1951; Manners & Ryley, 1952).

In the present study, it was found that the respiration of *T. pyriformis* (GL) was not affected by the presence of carbon monoxide ($CO/O_2 = 19$) in the gas phase, and that cyanide at a concentration of 0.46×10^{-3} M inhibited endogenous respiration by only 18%. Lwoff (1934) found that respiration of the GL strain in a peptone solution was cyanide-sensitive, but that in Ringer-glucose solution, 0.0022 M-cyanide stimulated respiration by 36%. Using the H strain of *T. pyriformis*, Hall (1941) observed that respiration was inhibited 88% by 0.001 M-cyanide, or 35% by 0.0001 M-cyanide. Baker & Baumberger (1941), using the T strain of *T. pyriformis*, observed that respiration was blocked, and oxidation of the cytochrome bands prevented, in the presence of an unspecified concentration of cyanide. They also found that respiration was sensitive to carbon monoxide, but this effect was only observed at very high partial pressures of carbon monoxide ($CO/O_2 = 45$). It would seem that on the whole, while cyanide probably has some effect on the respiration of *T.*

pyriformis, the cyanide-sensitivity of the process is far below that of other cells with typical cytochrome oxidase activity. These facts, and the absence of any *p*-phenylenediamine oxidase activity in cell homogenates, are in agreement with the spectroscopic observations reported. We observed very little absorption at 605 m μ , the location of cytochrome *a*, usually involved in cyanide-sensitive respiration, but in contrast to this there was intense absorption in the green part of the spectrum. This was considered to be due to a strong cytochrome *e* band, with a weaker *b* band, and a weak *c* band. These results are in good agreement with the spectroscopic observations of Baker & Baumberger (1941) on the T strain, but they attributed their band at 552 m μ to cytochrome *c*, rather than to cytochrome *e*. They found absorption in the region 587–592 m μ , and in some cases at 616–617 m μ , but they were not able to observe the α -band of oxyhaemoglobin, which appears at about 582 m μ under conditions of good aeration.

It has been noted (Experimental section) that glucose stimulated respiration to a small extent, and that under aerobic conditions appreciable amounts of sugar were removed from the medium. Kidder & Dewey (1945) noted that most strains of *T. pyriformis* examined were able to ferment glucose, fructose, mannose, maltose, starch, dextrin and glycogen, but no other carbohydrates tested. Niel *et al.* (1942) examined the glucose metabolism of the T strain under anaerobic conditions, and showed qualitatively the production of succinic, lactic and acetic acids. They were able, by the use of radioactive carbon, to demonstrate the assimilation of carbon dioxide, and its incorporation in the carboxyl groups of succinic acid. The high intracellular carbohydrate content of *Tetrahymena* appears to have escaped the notice of all previous investigators, nor have they realized the significance of the intracellular carbohydrate reserve for the anaerobic metabolism of the ciliate. The present study shows that under anaerobic conditions, the ciliate *T. pyriformis* (GL) maintains a normal degree of motility at the expense of its vast glycogen store. At room temperature, under strictly anaerobic conditions, the ciliate maintained normal motility in the absence of any extracellular nutrient for at least 72 hr. Although it seems doubtful that the ciliate will reproduce under anaerobic conditions, yet, contrary to the findings of Pace & Ireland (1945) with the W strain, the organism will survive. It is interesting to compare these ciliates with the haemoflagellate *Trypanosoma lewisi* (Ryley, 1951); the latter organism is able to survive in the presence of extracellular substrate under anaerobic conditions, but motility is much less than that under aerobic conditions. With *Tetrahymena pyriformis* (GL), motility under anaerobic conditions is just as

vigorous as under aerobic conditions. There is a striking similarity between the anaerobic metabolic processes of the two organisms; in the trypanosome, extracellular glucose is fermented to a mixture of succinic, lactic and acetic acids, with the simultaneous assimilation of carbon dioxide; with the ciliate, intracellular glycogen is fermented with the formation of exactly the same products.

In cell-free preparations of the ciliate, the presence of phosphorylase, phosphoglucumutase and oxoisomerase has been demonstrated. The phosphorylase of the ciliate acts upon starch as well as glycogen. Hydrolytic enzymes capable of degrading these polysaccharides have also been demonstrated. Cell-free preparations of *T. pyriformis* (GL) readily convert fructose diphosphate to triosephosphate, by an enzyme system which does not seem to be sensitive towards 2:2'-dipyridyl. In this respect, the aldolase of the ciliate resembles the animal, rather than the yeast type of enzyme (Warburg & Christian, 1943). The occurrence of phosphorylative mechanisms in carbohydrate breakdown by *T. pyriformis* (GL) is also supported by the observations relating to the formation of lactic acid from fructose diphosphate by cell-free enzyme preparations, the stimulation of this process by arsenate, and its inhibition by iodoacetate.

SUMMARY

1. The free-living ciliate *Tetrahymena pyriformis* (*Glaucocoma pyriformis*) maintains metabolism and motility in the absence of extracellular nutrients for considerable periods of time at room temperature or at 37°.

2. Under aerobic conditions at 37°, the ciliate respire with a q_{O_2} of 480 and an R.Q. of 0.85. This endogenous respiration is practically independent of the intracellular glycogen, is only slightly stimulated by added glucose and a number of dicarboxylic acids, but is almost doubled by 0.01 M-acetate, propionate or butyrate.

3. High concentrations of azide or 2:4-dinitrophenol inhibit endogenous respiration, while low concentrations stimulate it; in all cases, motility in the presence of these substances is reduced.

4. The cytochrome system of the ciliate is composed of cytochromes *b*, *c* and *e*, but very little *a*; homogenates display no cytochrome oxidase activity towards *p*-phenylenediamine. Respiration of the ciliate is comparatively resistant to cyanide and carbon monoxide.

5. *T. pyriformis* contains a heat-labile peroxidase (purpurogallin number, 0.033); in the presence of hydrogen peroxide this peroxidase is able to oxidize a number of phenolic compounds, and reduced cytochrome *c*.

6. Anaerobic metabolism and motility depend on the fermentation of the intracellular glycogen; in

the absence of extraneous carbohydrate, the ciliate produces large amounts of succinic acid, with smaller quantities of lactic and acetic acid, and assimilates some carbon dioxide.

7. Cell-free, centrifuged homogenates contain enzymes capable of hydrolysing starch, glycogen and maltose to glucose; they are also able to phosphorylate glycogen and starch to form glucose-1-phosphate and contain phosphoglucomutase and oxoisomerase.

8. Cell-free preparations convert fructose-1:6-diphosphate to triosephosphate, which is fermented

to lactic acid. This process is stimulated by arsenate, and inhibited by iodoacetate.

9. Homogenates of the ciliate display both succinic and lactic dehydrogenase activity; in the presence of atmospheric oxygen, however, they oxidize lactic, but not succinic, acid.

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Glyoxalase: the Role of the Components

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Since the discovery by Lohmann (1932) that reduced glutathione (GSH) is the coenzyme of glyoxalase, several workers have reported that methylglyoxal and GSH form a compound and have suggested that this might be the true substrate for the enzyme or an intermediate in the reaction. Thus Jowett & Quastel (1932) observed that SH groups titratable by iodine decreased when methylglyoxal

was added to GSH solution and suggested a reaction sequence: