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Carbohydrate Metabolism of *Paramecium aurelia*, Variety 4, Stock 47.8 (Sensitive)

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SUMMARY: Homogenates of *Paramecium aurelia*, var. 4, stock 47.8 (sensitive), grown in axenic culture contain enzymes of the glycolytic, the citric acid cycle, and the pentosephosphate pathway. The presence of the following enzymes has been demonstrated: hexokinase, phosphoglucomutase, phosphohexoisomerase, aldolase, triosephosphate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, pentosephosphate isomerase, citric acid oxidase, isocitric acid dehydrogenase, α -ketoglutaric acid dehydrogenase, succinic acid oxidase, malic acid dehydrogenase, glutamic acid dehydrogenase, fumarase, and acid phosphatase. The presence of phosphorylase, transaldolase and transketolase is indicated.

Studies on the metabolism of ciliates have been notably deficient in the investigation of the presence of the individual enzymes of the metabolic sequences. Most studies have been confined to adding compounds to media in which the protozoans were to be grown. Increased multiplication or an increased oxygen uptake measured in the conventional Warburg apparatus was taken to indicate that the compounds under investigation were metabolites. Humphrey & Humphrey (1948) found that succinic acid would cause a twofold increase in the respiration of *Paramecium caudatum*. In studies on the utilization of carbohydrates by the same organism Geddes & Humphrey (1951) established that glycogen, glucose, fructose, glucose-1-phosphate, glucose-6-phosphate and fructose-1:6-diphosphate were utilized. A few studies on the presence of enzymes of the electron transport system in *Paramecium*, notably cytochrome oxidase, have been made by Clark (1945) and Boell (1945), while Sato & Tamiya (1937) demonstrated the presence of cytochromes c, a and b in *Paramecium*. Simonsen & van Wagtenonk (1952) suggested that the respiration of killer stocks of *P. aurelia*, which, unlike the respiration of sensitive stocks, is not inhibited by KCN, may be almost entirely mediated by flavin systems. In a later study (Simonsen & van Wagtenonk, 1956), the same authors reported the presence in killer cells of an active succinoxidase system which is absent, or present only in small concentrations in the sensitives. The metabolism of *Tetrahymena pyriformis* has been extensively studied and the presence of many enzymes involved in the various phases of carbohydrate metabolism has been established. This work has been reviewed by Seaman (1955).

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While many enzymic studies on the metabolism of *Paramecium aurelia* can be carried out in bacterized media, it is imperative for certain experiments of long duration involving homogenates that these be free from contaminants. With the advent of an axenic medium for *P. aurelia* (van Wagtendonk, Conner, Miller & Rao, 1953; Miller & van Wagtendonk, 1956), an investigation of its carbohydrate metabolism became possible without the danger of interference by other organisms.

METHODS

Paramecium aurelia, var. 4, stock 47.8 (sensitive), was grown axenically in the medium given in Table 1 or in the medium described by Vloedman (1955). Large cultures were derived from cultures in test tubes by transferring to

Table 1. *Composition of axenic medium for Paramecium aurelia*

Component	Concn. ($\mu\text{g./ml.}$)	Component	Concn. ($\mu\text{g./ml.}$)
Vitamins		Amino acids	
Pantothenic acid	2.0	L-Arginine	200
Nicotinamide	2.0	L-Histidine	100
Pyridoxal	2.0	DL-Isoleucine	300
Biotin	0.03	L-Leucine	300
Riboflavin	2.0	L-Lysine	250
Folic acid	1.0	DL-Methionine	300
Thiamine	6.0	L-Phenylalanine	150
		DL-Serine	400
Salts		DL-Threonine	300
Sodium ethylenedinitrilotetraacetate	10.0	L-Tryptophan	100
MgSO ₄ .7H ₂ O	50.0	L-Tyrosine	75
Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	12.5	DL-Valine	150
MnCl ₂ .4H ₂ O	0.25	L-Alanine	50
ZnCl ₂	0.03	L-Aspartic acid	100
CaCl ₂ .2H ₂ O	25.0	Glycine	50
CuCl ₂ .2H ₂ O	2.5	L-Glutamic acid	150
FeCl ₃ .6H ₂ O	0.63	L-Proline	100
K ₂ HPO ₄	500.0	Yeast fractions (mg./ml.)	
KH ₂ PO ₄	500.0	Precipitate*	3.0
Sodium acetate	500.0		
Stigmasterol	0.2		

* The precipitate was obtained from a water extract of yeast (Conner & van Wagtendonk, 1953) by addition of an equal volume of acetone and centrifugation of the mixture (Conner & van Wagtendonk, unpublished).

increasingly larger volumes, up to 500 ml. in a 3 l. Erlenmeyer flask. At maximum population density (5000–10,000 organisms/ml.), the organisms were harvested by filtering the culture through a sterile Berkefeld filter. Further concentration was obtained by electromigration through a sterile salt solution according to the procedure described by van Wagtendonk, Simonsen & Zill (1952), followed by a final centrifugation at 850 g for 10 min. The resulting pellet was suspended in a suitable amount of buffer, homogenized with the apparatus described by Katzberg (1950), a hand homogenizer of the Potter-Elvehjem type, or by exposure to ultra-sound in a 10 kc. oscillator (water

cooled Raytheon), and made up to the desired volume with the same buffer. Cell-free extracts were prepared by centrifugation of the homogenates at 24,000 *g* in an angle centrifuge for 30 min. The protein content of the homogenates and the cell-free extracts was determined according to the method of Gornall, Bardawill & David (1949). In some instances the N content of the homogenates was determined with the micro-Kjeldahl method using the digestion mixture of Brüell, Holter, Linderstrøm-Lang & Rozits (1947).

Reduction of 2,3,5-triphenyltetrazolium chloride (TTC) was used for the determination of dehydrogenases. The determinations were carried out in conical graduated centrifuge tubes (15 ml.). The reaction mixture consisted of 0.5 ml. substrate at a suitable concentration; 0.1 ml. 0.001 % (w/v) diphosphopyridine nucleotide (DPN, 65 % pure, Sigma); 0.1 ml. 0.05 M-cysteine; 0.1 ml. 0.1 M-MgCl₂; 0.5 ml. 0.001 % (w/v) TTC; 0.5 ml. homogenate and 0.1 M-tris-(hydroxymethyl)-amino-methane (Tris) buffer to a final volume of 2.5 ml., depending upon the determination. The reaction mixture was incubated at 37° for various lengths of time and the reaction stopped by the addition of 1 ml. 10 % (w/v) trichloroacetic acid (TCA). The resulting precipitate, consisting of protein and reduced and unreduced TTC, was spun down at 850 *g* in a refrigerated centrifuge. The supernatant fluid was poured off, and the pellet extracted twice with 2 ml. acetone. One ml. 10 % TCA was added to the second acetone extract. (The extraction of the precipitate with chloroform-acetone as recommended by Green, Mii, Mahler & Bock (1954) was not applicable here, due to the large amounts of protein present in the precipitate.) Additional acetone was added to bring the final volume to 7 ml. The optical density was read on a Klett-Summerson colorimeter with filter no. 54 (540 mμ.). The readings were compared with those obtained from a standard curve, which was prepared by the reduction of TTC with sodium sulphide according to Black & Speer (1953). A few crystals of sodium sulphide were added to known amounts of TTC (10–100 μg.) dissolved in 1 ml. distilled water. The reaction was allowed to proceed for 1 hr., 1 ml. 10 % TCA solution was then added and the mixture brought to a final volume of 7 ml. with acetone. The optical densities were determined on the Klett-Summerson colorimeter using filter no. 54.

Determinations of other enzymic activities were made according to published procedures, and the compositions of the reaction mixtures are given in the table headings and the figure legends.

Demonstration of the presence of enzymic pathways

Phosphorylase. Homogenates of *Paramecium aurelia* stock 47.8 contain an active phosphorylase, the presence of which was demonstrated by the method described by Whelan (1955), except that glycogen was substituted for starch. The results are given in Table 2.

The liberation of orthophosphate in the absence of added glycogen is not due to the action of acid phosphatase, because in *Paramecium aurelia* this enzyme has a low activity at pH 6.0 (Fig. 9). Apparently enough stored

glycogen is present in *P. aurelia* to allow the reaction to proceed in the absence of added glycogen.

Hexokinase. This enzyme was determined by following the reduction of triphosphopyridine nucleotide (TPN) in a reaction mixture containing glucose, a cell-free extract of *Paramecium aurelia* and an added excess of glucose-6-phosphate dehydrogenase, according to the method of Wood & Schwerdt (1953). A cell-free extract was prepared by centrifugation of a homogenate at 24,000 *g* for 15 min. at 4° in an angle centrifuge. The results are given in Fig. 1.

Table 2. *Phosphorylase in homogenates of Paramecium aurelia, var. 4, stock 47.8*

The complete reaction mixture contained 0.2 ml. 4% (w/v) glycogen solution, 100 μ mole glucose-1-phosphate, 4.0 ml. homogenate containing 26.4 mg. protein and 0.1 M-citrate-NaOH buffer to make a total volume of 6 ml. pH 6.0, temp. 38°.

Reaction mixture	μ mole phosphorus liberated/mg. protein					
	Minutes					
	0	5	10	30	60	120
Complete	0	0.003	0.012	0.036	0.079	0.136
Minus glycogen	0.002	0.007	0.011	0.036	0.073	0.130
Minus glucose-1-phosphate	0	0	0	0	0	0
and glycogen						
Minus homogenate	0	0	0	0	0	0

Phosphoglucomutase. The presence of this enzyme in homogenates is indicated by the fact that glucose-1-phosphate could also act as substrate for the dehydrogenation by glucose-6-phosphate dehydrogenase (Fig. 1). The transformation of glucose-1-phosphate to glucose-6-phosphate was followed by the decrease in acid-labile phosphate (7 min. hydrolysis in 1.0 N-HCl at 100°) in a test system described by Najjar (1948) (Fig. 2).

Phosphohexoisomerase. The removal of fructose-6-phosphate from a reaction mixture and the formation of fructose reactive material from glucose-6-phosphate provided evidence for the presence of phosphohexoisomerase in homogenates (Sable & Calkins, 1953) (Fig. 3).

Aldolase. The presence of this enzyme was demonstrated by incubating a homogenate with the Mg-salt of fructose-1:6-diphosphate, using hydrazine as the trapping agent for the trioses formed (Sibley & Lehninger, 1949; Beck, 1955), and measuring the triosehydrazone by its conversion to the chromogen of the 2,4-dinitrophenylhydrazine derivative. The trapped triosehydrazone was also measured by determining the amount of alkali-labile phosphate formed (Meyerhof & Lohmann, 1934). The results are given in Table 3.

Triosephosphate dehydrogenase was determined in a reaction mixture containing Mg-fructose-1:6-diphosphate, homogenate and TTC. The reduction of TTC indicated the presence of a triosephosphate dehydrogenase (Table 4). The addition of hydrazine blocked the reduction of TTC, presumably by trapping the triose intermediates, and the addition of iodoacetic acid (IOA) prevented the same reaction presumably by inhibiting the triosephosphate dehydrogenase itself.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase react specifically with DPN in *Paramecium aurelia* and the change in absorption at 340 m μ . with time is a measure of the reaction. An active cell extract was prepared by twice-repeated freezing and thawing of a homogenate. The latter was prepared in a Potter-Elvehjem type homogenizer. The mixture was centrifuged for 30 min. at 850 g to prepare a cell-free extract. The reaction mixture of Wood & Schwerdt (1953) was used. The results are given in Fig. 4.

Fig. 1

Fig. 2

Fig. 1. Demonstration of the presence of hexokinase in cell-free extracts of *Paramecium aurelia*. The reaction mixture contained: 0.3 ml. cell-free extract (1.8 mg. protein), 5 μ mole $MgCl_2$, 50 μ g. TPN (Sigma, 80 % pure), 3 mg. glucose-6-phosphate dehydrogenase (Sigma, 0.15 Kornberg units/mg.), 3.3 μ mole substrate, 0.25M-glycylglycine buffer (pH 7.4) to a final volume of 3 ml. The change in optical density at 25° was determined with a Beckman DU spectrophotometer at 340 m μ . in cuvettes with 1 cm. light path. ○—○, glucose-1-phosphate; ●—●, glucose; ◐—◐, no substrate.

Fig. 2. Phosphoglucumutase activity of homogenates of *Paramecium aurelia*. The complete test systems consisted of: 3 ml. homogenate (19.2 mg. protein), 50 μ mole Tris buffer (pH 7.5), 10 μ mole $MgCl_2$, 10 μ mole glucose-1-phosphate (Schwarz Laboratories), 50 μ mole cysteine. Total volume 6 ml., temp. 32°. Reactions stopped at 10 min. intervals with 5 ml. of 6 % (w/v) perchloric acid and the reaction mixture hydrolysed for 7 min. in 1N-HCl in a boiling water bath. Liberated inorganic phosphate was determined according to Fiske & SubbaRow (1925). No easily hydrolysable phosphate was detectable in the absence of substrate.

Pentose metabolism. When ribose-5-phosphate was incubated in the presence of a homogenate of *Paramecium aurelia*, TTC and DPN, reduction of TTC was observed indicating the oxidation of ribose-5-phosphate. Addition of hydrazine prevented the reduction of TTC (Table 5). The course of this oxidation

could be further analysed with the same reaction mixture using the colour reactions described by Dische & Borenfreund (1951) and by Mejbaum (1939). An increase in absorption over the range from 450 to 700 m μ . of the chromogen formed in the Dische-Borenfreund reaction indicated the formation of keto-pentose, suggesting the presence of pentosephosphate isomerase (Fig. 5). This

Table 3. *The formation of chromogen by trapped triosehydrazone and alkali labile phosphate formed by the action of aldolase*

The reaction mixtures contained: 0.5 ml. homogenate (7.1 mg. protein), 12.5 μ mole Mg-fructose-1:6-diphosphate, 100 μ mole Tris buffer (pH 8.5), 140 μ mole hydrazine sulphate (pH 8.6), final volume 2.5 ml., temp. 37°. The reactions were stopped at various time intervals with 2 ml. 10 % (w/v) trichloroacetic acid and the chromogen developed in a 1 ml. sample. The optical density was measured in the Klett-Summerson colorimeter at 540 m μ . (filter no. 54) against the reaction mixture without substrate as a blank. Alkali labile phosphate was determined on a separate 1 ml. sample.

Time (min.)	μ mole alkali labile phosphate liberated/mg. protein		increase in optical density of chromogen at 540 m μ .	
	Experimental	Endogenous	Experimental	Endogenous
10	0.019	—	0.038	—
20	0.039	—	0.164	—
30	0.065	—	0.300	—
40	0.093	—	0.390	—
50	0.099	—	0.480	—
60	0.124	0.028	0.600	—

Table 4. *Triosephosphate dehydrogenase in homogenate of Paramecium aurelia*

The complete system contained: 0.5 ml. homogenate (10 mg. of protein), 25 μ mole Mg-fructose-1:6-diphosphate, 5 μ mole cysteine, 0.1 μ mole DPN, 20 μ mole K₂HPO₄, 1.5 μ mole TTC, 70 μ mole Tris buffer (pH 7.5), 2.5 μ mole MgCl₂. 50 μ mole hydrazine or 2 μ mole iodoacetic acid (IOA) were included where indicated—total volume 2.0 ml., temp. 38°.

	μ mole TTC reduced		
	30 min.	60 min.	90 min.
Complete system	0.037	0.054	0.088
Complete system plus IOA	—	0.010	0.007
Complete system plus hydrazine	—	0.019	0.008
Endogenous	—	0.017	0.024

Table 5. *Oxidation of ribose-5-phosphate by homogenate of Paramecium aurelia*

The reaction mixtures consisted of 14.7 μ mole ribose-5-phosphate, 1.5 μ mole TTC, 0.1 μ mole DPN, 10 μ mole MgCl₂, 20 μ mole K₂HPO₄, homogenate containing 15.5 mg. protein, 0.1 M-Tris buffer (pH 7.6) to 3.0 ml. The reactions were stopped at 15 min. intervals by adding 1 ml. 10 % (w/v) TCA.

	μ mole TTC reduced/mg. protein				
	Minutes				
	15	30	45	60	75
Complete	0.026	0.046	0.054	0.068	0.073
— Ribose-5-phosphate	0.020	—	—	—	0.023
+ Hydrazine	—	—	—	—	0.018

Fig. 3

Fig. 4

Fig. 3. Demonstration of the presence of phosphohexoisomerase in homogenates of *Paramecium aurelia*. The reaction mixture contained 3 ml. homogenate (18.2 mg. protein), 10 μ mole substrate (glucose-6-phosphate or fructose-6-phosphate), 50 μ mole Tris buffer (pH 7.5), 10 μ mole *p*-chloromercuribenzoate. Total volume 5 ml., temp. 32°. Volumes of 1 ml. were removed at intervals, and the reaction stopped by the addition of 5 ml. 6% (w/v) perchloric acid. Fructose reactive material in the supernatant after centrifugation was determined according to Roe (1934). In these analyses account was taken of the fact that fructose-6-phosphate gives considerably less colour than an equimolar amount of free fructose. The factor 0.75 was used to correct for this difference. ○—○, fructose-6-phosphate; ●—●, glucose-6-phosphate.

Fig. 4. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase in *Paramecium aurelia* extracts. The medium contained: 3.3 μ mole substrate, 25 μ g. DPN (Sigma, 65% pure), 5 μ mole $MgCl_2$ and 0.25 ml. *P. aurelia* extract (1 mg. protein). Tris buffer (0.1 M, pH 7.8) to 0.5 ml., temp. 27°. The optical density was measured in the Beckman DU spectrophotometer using cells with a light path of 1 cm. The readings were made against the above reaction mixture from which the substrate was omitted. There was no endogenous reduction of DPN. ○—○, glucose-6-phosphate; ●—●, 6-phosphogluconate.

is further corroborated by an analysis of the orcinol spectra of the reaction mixture at 0 hr. and at 2 hr. (Fig. 6). Concomitant with the decrease of the aldopentose peak at 670 $m\mu$, an increase in the ketopentose peak at 540 $m\mu$ is noted. At the same time aldopentose reactive material disappears from the reaction mixture (Table 6). According to Horecker and co-workers (Horecker, Smyrniotis & Seegmiller, 1951; Seegmiller & Horecker, 1952; Horecker, Smyrniotis & Klenow, 1953), the ratio D_{540}/D_{670} for aldopentose is 0.19 and for ribulose 0.90. Similarly, the ratio D_{670}/D_{580} for sedoheptulose is 0.29 and for pentose 3.78. The course of the change of these two ratios is given in Table 7, the results being consistent with the formation of ketopentose and, in addition,

Table 6. *Utilization of aldopentose reactive material by homogenates of Paramecium aurelia*

The reaction mixture was identical with that of Fig. 5.

	μ mole of aldopentose		
	0 min.	5 min.	120 min.
Experimental	18.43	17.21	11.83
Endogenous	2.63	2.98	4.09
Aldopentose remaining	15.80	14.23	7.74

Table 7. *Change in D_{540}/D_{670} and D_{670}/D_{580} ratios during incubation of ribose-5-phosphate with homogenate of Paramecium aurelia*

The reaction mixture was identical with that in Fig. 7.

Ratio	Minutes				
	0	10	30	60	120
D_{540}/D_{670}	0.27	0.32	0.36	0.61	0.63
D_{670}/D_{580}	4.51	4.17	3.61	2.11	1.92

Fig. 5

Fig. 6

Fig. 5. Formation of ketopentoses as indicated by the change in absorption spectra. The reaction mixture consisted of 15.80 μ mole ribose-5-phosphate, 375 μ mole Tris buffer (pH 7.0) and 27.4 mg. homogenate protein in a total volume of 4 ml., temp. 27°. 0.5 ml. samples were removed at various time intervals and added to 4.5 ml. 10% TCA (w/v). 1 ml. samples were used for the Dische-Borenfreund reaction and 0.2 ml. samples for the orcinol reaction. Absorption spectra were measured in the Beckman DU spectrophotometer in a cell with a 1 cm. light path. ●—●, experimental zero time; ○—○, experimental 120 min.; ●—●, endogenous zero time; ○—○, endogenous 120 min.

Fig. 6. Formation of ketopentoses by homogenate of *Paramecium aurelia*. The curves represent the change in absorption spectra over the range of 440–800 m μ . of the chromogens formed in the orcinol reaction. The reaction mixture was identical with that of Fig. 5. ○—○, zero time; ●—●, 120 min.

of heptose, as probable intermediates in the oxidation of pentosephosphate. This probability is strengthened by the observation that *Paramecium* homogenates form pentoses from sedoheptulose. A pentose peak appears in the orcinol spectrum of the reaction mixture (Fig. 7), while at the same time the concentration of sedoheptulose decreases (Fig. 8).

Fig. 7

Fig. 8

Fig. 7. Formation of pentoses from sedoheptulose by homogenate of *Paramecium aurelia*. The curves represent the absorption spectra of the chromogen formed in the orcinol reaction by the sedoheptulose and pentoses. The reaction mixture consisted of 9 μ mole sedoheptulose [kindly furnished by Dr B. L. Horecker as the Ba-salt (78 % pure)], 390 μ mole Tris buffer (pH 7.6), 0.1 mg. cocarboxylase complex (Difco), 2 ml. homogenate (41 mg. protein). Final volume 5 ml., temp. 37°. 1 ml. samples were removed at various times and added to 4 ml. 10 % (w/v) TCA. 0.5 ml. samples were used for the Dische-Borenfreund and orcinol reactions. ●—●, zero time; ◐—◐, 60 min.; ○—○, 120 min.

Fig. 8. Disappearance of sedoheptulose. The curves represent the absorption by the chromogen of sedoheptulose formed in the Dische-Borenfreund reaction at 0 and at 120 min. The composition of the reaction medium was identical with that of Fig. 7. ○—○, 0 min.; ●—●, 120 min.

Enzymes of the citric acid cycle. Of the tricarboxylic acid cycle acids, citric acid, isocitric acid, α -ketoglutaric acid, succinic acid, malic acid and glutamic acid can be oxidized by homogenates of *Paramecium aurelia* in the presence of DPN or TPN and TTC. The results are given in Table 8.

Since TTC is a sluggish electron acceptor, the oxidation of succinic acid and of α -ketoglutaric acid by homogenates was measured manometrically following the method of Eisenberg (1953). It can be seen from Table 9 that the oxidation of these two acids proceeds faster when other electron acceptors are used.

Fumarase. The presence of this enzyme was demonstrated by measuring the increase in optical density at 240 m μ . (Racker, 1950) due to the appearance of the double bond of fumaric acid in a reaction mixture containing malic acid and a homogenate. The fumarase activity was mostly found in the non-sedimentable fraction (at 10,000 g for 30 min.) of the homogenate (Table 10). This

Table 8. *Oxidation of citric acid cycle intermediates by Paramecium homogenates*

The reaction mixtures contained 10 μ mole substrate, 1 μ mole DPN or 0.3 μ mole TPN, 5 μ mole cysteine, 10 μ mole $MgCl_2$, 10 μ mole TTC, 150 μ mole Tris buffer (pH 7.4), 1 ml. *Paramecium* homogenate (9.7 mg. protein). Total volume 2 ml.; gas phase air; temp. 37°. Identical reaction mixtures were treated as described in the text at zero time, 30 and 60 min.

Substrate	μ mole TTC reduced	
	30 min.	60 min.
Citric acid	0.08	0.29
Isocitric acid	0.43	0.46
α -Ketoglutaric acid	0.004	0.008
Succinic acid	0.03	0.05
Malic acid	0.19	0.31
Glutamic acid	0.11	0.19
Endogenous	0.002	0.006

Table 9. *Oxidation of α -ketoglutarate and succinate by homogenate of Paramecium aurelia*

The reaction medium for the determination of the oxidation of α -ketoglutarate by homogenates contained 10 μ mole substrate, 50 μ mole Tris buffer (pH 7.3), 5 μ mole $MgCl_2$, 0.5 mg. DPN, 10 μ mole methylene blue, 3.5 Lipmann units of CoA and 0.5 ml. homogenate (4.6 mg. protein). The reaction medium for the determination of the oxidation of succinate contained 10 μ mole substrate, 50 μ mole phosphate buffer (pH 7.3), 0.6 μ mole cytochrome *c* (Sigma), and 0.5 ml. homogenate (4.6 mg. protein). For both determinations: final volume 1 ml.; gas phase air; temp. 32°; 0.2 ml. 10% KOH (w/v) in the centre well.

Substrate	μ mole O_2 /mg. protein	
	30 min.	60 min.
α -Ketoglutarate	3.7	6.2
Succinate	1.1	1.8
Endogenous	0.1	0.2

Table 10. *Distribution of fumarase activity in homogenates of Paramecium aurelia*

The reaction mixture consisted of 25 μ mole malate (pH 7.4), 125 μ mole Tris buffer (pH 7.5), homogenate preparation 0.1 to 0.5 ml. as required. Total volume 3 ml. The increase in optical density at 240 m μ . was measured with the Beckman DU spectrophotometer. The original homogenate was centrifuged in a Sorvall SS-1 angle centrifuge at 10,000 *g* for $\frac{1}{2}$ hr. at room temperature. A measured volume of the supernatant was used for the determination of the fumarase activity. The sediment was taken up in 10 ml. Tris buffer (0.1 M, pH 7.4) and the fumarase activity was determined on a measured volume.

Fraction	Total volume (ml.)	Protein/ml. (mg.)	Total protein (mg.)	Units*/ml.	Total units	Specific activity†
Original homogenate	6	10.81	64.86	133‡	798	12.3
Sediment	10	5.59	55.9	14	140	2.5
Supernatant	3.1	2.86	8.9	43	133.3	14.97

* One unit equals the amount of enzyme causing an increase in optical density of 0.001 per min.

† Specific activity equals number of units per mg. of protein.

‡ Activity determined on a 1:10 (v/v) dilution of the whole homogenate.

distribution is similar to that found in human liver (Shepherd, Li, Mason & Ziffren, 1955).

Phosphatase activity was measured at both acid and alkaline pH values using Na-phenolphthalein phosphate as substrate (Huggins & Talalay, 1945). Phosphatase activity could only be detected in the acid range, with an optimum activity at pH 4.5 (Fig. 9).

Fig. 9. Effect of pH value on activity of acid phosphatase in homogenate of *Paramecium aurelia*. The reaction mixtures consisted of: 5 μ mole Na-phenolphthalein phosphate, 0.3 mg. homogenate protein, 0.1M-Na acetate buffer to 5 ml. pH adjusted to desired value with 0.1N-HCl. Temp. 38°. The reactions were stopped with 1 ml. 1N-NaOH after 60 min., and the liberated phenolphthalein measured in the Klett-Summerson colorimeter using filter no. 54.

DISCUSSION

The quantitative aspects have not been stressed in this exploratory study. The picture that emerges from this investigation is that of a conventional metabolic pattern. *Paramecium aurelia*, var. 4, stock 47.8 (sensitive) can metabolize carbohydrates via the three main routes, the glycolytic, the citric acid cycle and the pentosephosphate pathways. Which of the two oxidative schemes, the citric acid cycle or the pentosephosphate pathway, is preferentially utilized has not been determined. An attractive postulate, which is at present under investigation, is that the three pathways are of equal importance for the organism, the glycolytic and citric acid pathways providing the required energy, and the pentosephosphate pathway serving mainly to supply the organism with pentoses. The pentose demand can conceivably be very high in view of the large amount of genetic material carried by *P. aurelia*, specifically in the macronucleus (Moses, 1950). The presence of pentosephosphate iso-

merase, transaldolase and transketolase has not been unequivocally proven, but the circumstantial evidence for the participation of these enzymes in the pentosephosphate pathway is strong. The reduction of TTC with ribose-5-phosphate as substrate and the formation of ketopentose reactive material from added ribose-5-phosphate are indicative of the presence of pentosephosphate isomerase. Similarly, the formation of pentoses from sedoheptulose-7-phosphate is evidence for the establishment of an equilibrium in which transketolase and transaldolase are participating enzymes.

Levine (1955) reported some enzymic studies carried out with *Paramecium aurelia*, var. 4, stock 51.8 (sensitive) and *P. aurelia*, var. 8, stock 135.15 (sensitive). His results are in several aspects at variance with those reported here. Levine was not able to demonstrate the first two steps in the pentosephosphate pathway, the oxidation of glucose-6-phosphate and the oxidation of 6-phosphogluconate. It was suggested that this apparent absence of the oxidative pathway for the utilization of glucose-6-phosphate was due to the availability of pentoses from the 'rather opulent diet the bacteria provide'. If this were the case, the presence of the pentosephosphate pathway in axenically cultured *P. aurelia* could be considered to be an adaptation to the environment. Another explanation, e.g. that there is a difference in enzymic make-up between different stocks, is plausible, although Levine was unable to find such differences between his two stocks.

Seaman (1955) states: 'Although purity of the culture and the availability of chemically defined media are prerequisites for growth and nutrition experiments, these considerations are not of prime importance in purely metabolic studies. If a protozoan can be cultured whatever the complexity of the medium—even with the presence of bacterial contaminants—it is eligible for investigation.' Our studies, however, again emphasize the importance of axenic cultures for biochemical and physiological studies of protozoa, although this emphasis might be tempered by the question: 'How many of the findings, biochemical, nutritional, or even genetic, can be ascribed to adaptation to environmental changes from a natural habitat, via dioxenic to axenic cultures?' Only further investigations might provide the answer.

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REFERENCES

- BECK, W. S. (1955). Determination of triosephosphates and proposed modifications in the aldolase method of Sibley and Lehninger. *J. biol. Chem.* **212**, 847.
BLACK, M. M. & SPEER, F. D. (1953). Relative dehydrogenase activity of kidney and liver slices from control and tumor mice. *Proc. Soc. exp. Biol., N.Y.* **82**, 289.

- BOELL, E. J. (1945). Respiratory enzymes in *Paramecium*: 1. Cytochrome oxidase. *Proc. nat. Acad. Sci., Wash.* **31**, 396.
- BRÜELL, D., HOLTER, H., LINDERSTRØM-LANG, D. & ROZITS, K. (1947). A micro-method for the determination of total nitrogen (accuracy 0.005 $\mu\text{g. N}$). *Biochim. biophys. Acta*, **1**, 101.
- CLARK, A. M. (1945). The effect of cyanide and carbon monoxide on the oxygen consumption of *Paramecium caudatum*. *Aust. J. exp. Biol. med. Sci.* **23**, 317.
- CONNER, R. L. & WAGTENDONK, W. J. VAN (1953). Steroid requirements of *Paramecium aurelia*. *J. gen. Microbiol.* **12**, 31.
- DISCHE, Z. & BORENFREUND, E. (1951). A new spectrophotometric method for the detection and determination of keto sugars and trioses. *J. biol. Chem.* **192**, 583.
- EISENBERG, M. A. (1953). The tricarboxylic acid cycle in *Rhodospirillum rubrum*. *J. biol. Chem.* **203**, 815.
- FISKE, A. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.
- GEDDES, M. & HUMPHREY, G. R. (1951). Glycolysis in *Paramecium caudatum*. *Aust. J. exp. med. Sci.* **29**, 187.
- GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.* **177**, 751.
- GREEN, D. E., MIL, S., MAHLER, H. R. & BOCK, R. M. (1954). Studies on the fatty acid oxidizing system of animal tissues. III. Butyryl coenzyme A dehydrogenase. *J. biol. Chem.* **206**, 1.
- HORECKER, B. L., SMYRNIOTIS, P. Z. & KLENOW, H. (1953). The formation of sedoheptulose phosphate from pentose phosphate. *J. biol. Chem.* **205**, 661.
- HORECKER, B. L., SMYRNIOTIS, P. Z. & SEEGMILLER, J. E. (1951). The enzymatic conversion of 6-phosphogluconate to ribulose-5-phosphate and ribose-5-phosphate. *J. biol. Chem.* **193**, 383.
- HUGGINS, C. & TALALAY, P. (1945). Sodium phenolphthalein phosphate as a substrate for phosphatase tests. *J. biol. Chem.* **159**, 399.
- HUMPHREY, B. A. & HUMPHREY, G. T. (1948). Studies in the respiration of *Paramecium caudatum*. *J. exp. Biol.* **25**, 123.
- KATZBERG, A. A. (1950). A micromacerator. *Science*, **112**, 339.
- LEVINE, M. (1955). An enzymatic comparison of two interbreeding but genetically isolated varieties of *Paramecium aurelia*. *J. cell. comp. Physiol.* **45**, 409.
- MEJBAUM, W. (1939). Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. *Z. physiol. Chem.* **258**, 117.
- MEYERHOF, O. & LOHMANN, K. (1934). Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. *Biochem. Z.* **271**, 89.
- MILLER, C. A. & WAGTENDONK, W. J. VAN (1956). The essential metabolites of a strain of *Paramecium aurelia* (stock 47.8) and a comparison of the growth rate of different strains of *Paramecium aurelia* in axenic culture. *J. gen. Microbiol.* **15**, 280.
- MOSES, M. J. (1950). Nucleic acids and proteins of the nuclei of *Paramecium*. *J. Morph.* **87**, 493.
- NAJJAR, V. A. (1948). The isolation and properties of phosphoglucomutase. *J. biol. Chem.* **175**, 281.
- RACKER, E. (1950). Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acid. *Biochim. biophys. Acta*, **4**, 211.
- ROE, J. H. (1934). A colorimetric method for the determination of fructose in blood and urine. *J. biol. Chem.* **107**, 15.
- SABLE, H. Z. & CALKINS, C. W. JUN. (1953). Phosphoglucomutase and phosphohexoseisomerase in Tunicates. *J. biol. Chem.* **204**, 695.
- SATO, T. & TAMIYA, H. (1937). Über die Atmungsfarbstoffe von *Paramecium*. *Cytologia, Tokyo* (Suppl. part 2), p. 1133.

- SEAMAN, G. R. (1955). Metabolism of free-living ciliates. In *Biochemistry and Physiology of Protozoa*, 2, 91. New York: Academic Press Inc.
- SEEGMILLER, J. E. & HORECKER, B. L. (1952). Metabolism of 6-phosphogluconic acid in liver and bone marrow. *J. biol. Chem.* **194**, 261.
- SHEPHERD, J. A., LI, Y. W., MASON, E. E. & ZIFFREN, S. E. (1955). The distribution of aconitase and fumarase in homogenates of human liver. *J. biol. Chem.* **213**, 405.
- SIBLEY, J. A. & LEHNINGER, A. L. (1949). Determination of aldolase in animal tissues. *J. biol. Chem.* **177**, 859.
- SIMONSEN, D. H. & WAGTENDONK, W. J. VAN (1952). Respiratory studies on *Paramecium aurelia*, variety 4, killers and sensitives. *Biochim. biophys. Acta*, **9**, 515.
- SIMONSEN, D. H. & WAGTENDONK, W. J. VAN (1956). The succinoxidase system of killer and sensitive stocks of *Paramecium aurelia*, variety 4. *J. gen. Microbiol.* **15**, 39.
- VLOEDMAN, D. A. (1955). Enzymological studies on *Paramecium aurelia*, variety 4, stock 47.8, sensitive. Ph.D. Thesis, Indiana University.
- WAGTENDONK, W. J. VAN, CONNER, R. L., MILLER, C. A. & RAO, M. R. R. (1953). Growth requirements of *Paramecium aurelia*, variety 4, stock 51.7, sensitives and killers in axenic medium. *Ann. N.Y. Acad. Sci.* **56**, 929.
- WAGTENDONK, W. J. VAN, SIMONSEN, D. H. & ZILL, L. P. (1952). The use of electromigration techniques in washing and concentrating cultures of *Paramecium aurelia*. *Physiol. Zool.* **25**, 312.
- WHELAN, W. J. (1955). *Methods in Enzymology*. Ed. S. P. Colowick & N. O. Kaplan, 1, 192. New York: Academic Press Inc.
- WOOD, W. A. & SCHWERDT, R. F. (1953). Carbohydrate oxidation by *Pseudomonas fluorescens*. I. The mechanism of glucose and gluconate oxidation. *J. biol. Chem.* **201**, 501.

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