Effects of Inhibitors of RNA and Protein Synthesis on Activation of Glyconeogenesis in *Tetrahymena* [1]

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ABSTRACT   The effects of actinomycin D, puromycin, and p-fluorophenylalanine on the activation of glyconeogenesis in *Tetrahymena* were studied.

The extent of activation of glyconeogenesis in cultures containing inhibitor was as great as or greater than in the controls, as was the uptake of tracer levels of acetate into glycogen. These increases occurred despite a partial or complete inhibition of synthesis of isocitrate lyase, a glyconeogenic enzyme in *Tetrahymena*. Washed cells from these cultures could convert tracer or substrate levels of acetate to glycogen at enhanced rates.   When glyconeogenesis was activated in starved cells in the presence of inhibitor, there was a negligible increase in the amount of isocitrate lyase, but a significant increase in the rate of glyconeogenesis.   The data indicate that glyconeogenesis in *Tetrahymena* can be activated in the absence of enzyme synthesis.

In the ciliate, *Tetrahymena pyriformis*, the glyoxylate bypass of the tricarboxylic acid cycle is responsible for the conversion of lipids to glycogen. Cells capable of converting acetate to glycogen were found to have elevated levels of malate synthase and isocitrate lyase (Hogg and Kornberg, '63), and the location of C^{14} in glucose moieties of glycogen was consistent with metabolism of lipids *via* this pathway (Hogg, '59). In addition, activation of glyconeogenesis leads to an increase in the relative amount of acetate entering cellular metabolic pathways at the level of malate, as compared with that entering at the level of citrate (Levy and Scherbaum, '65b).

Glyconeogenesis in *Tetrahymena* is readily stimulated by a simple change in growth conditions. When well-shaken, peptone-grown cultures of this organism are transferred to static conditions, increases in the rate of glyconeogenesis and in the ability to convert acetate to glycogen (Levy and Scherbaum, '65a), and in the specific activities of isocitrate lyase (5-10-fold in the E strain, 2-fold in the GL strain) and malate synthase occur within three hours (Levy, Scherbaum and Hogg, '64; Levy and Scherbaum, '65b). In the present paper, the effects of an amino acid analogue and of inhibitors of RNA and protein synthesis on the activation of glyconeogenesis are tested. Results suggest that activation of this process is not primarily dependent upon synthesis of isocitrate lyase.

MATERIALS AND METHODS

Cultural procedures

Cultures of *Tetrahymena pyriformis* were grown at 28° in 2% (w/v) proteose peptone (Difco) with 0.1% (w/v) K_2HPO_4 and 0.1% (w/v) liver fraction. The E strain was used in all experiments except that shown in table 1, in which the GL strain was used. Cultures were grown in 2 l penicillin flasks, having a surface area of 225 cm^2. To insure good aeration, flasks contained only 250 ml of medium, and were shaken at 70-80 excursions/min on a New Brunswick gyratory shaker. Procedures for harvesting and washing of cells have been described (Levy and Scherbaum, '65a). For experiments on washed cells, organisms were suspended in an inorganic, phosphate-buffered medium (Ryley, '52).

Activation of glyconeogenesis

Activation of glyconeogenesis was accomplished by transferring well-shaken log phase cultures (or washed cell suspensions from such cultures) to smaller vessels, which were then maintained without shaking for 3-4 hours. Surface to volume ratios were chosen so as to limit respiration to about 5% of capacity (Levy and Scherbaum, '65a). This treatment also leads to

the onset of the stationary growth phase. Before and after transfer, the rate of glyconeogenesis was determined by shaking cells in air. In the case of cultures, duplicate 20-25 ml portions were shaken in 250 ml Erlenmeyer flasks for 30 or 60 minutes. Reactions were terminated by plunging cells into an equal volume of cold 95% ethanol, and organisms were collected by gentle centrifugation. Glyconeogenesis in washed cells was assayed by shaking duplicate 2.5 ml portions in 50 ml flasks, with or without 2.5 mg sodium acetate. Approximately 0.05 μc of sodium-2-^{14}C acetate (sp. act. 20.5 mc/mmole) were included in each flask. Incubations in this case were for two hours, and reactions were terminated by plunging cells into 60% KOH. Results are expressed in μmoles glucose formed/hr/mg protein. Under these conditions, when tracer amounts of acetate are added, the level of C^{14} in glycogen reaches its peak within one hour and then remains constant, at least for another hour, indicating that there is little or no turnover of glycogen (Levy and Scherbaum, '65a). Since this value represents a final level, it is given as the percentage of added isotope that is recovered in glycogen. When substrate levels of acetate are present, C^{14} incorporation continues linearly during the two hour assay period. In this case, the value is expressed as counts incorporated into glycogen/mg protein of cell suspension, as well as the percentage of the total isotope recovered. The latter values have been normalized to the cell suspension having the highest protein concentration for that particular experiment.

Analytical procedures

Methods for the isolation and estimation of glycogen and for the counting of radioactivity have been described (Levy and Scherbaum, '65a). Protein was determined by the method of Lowry et al. '51, on material that was insoluble after digestion for 15 minutes at 90° in 3% perchloric acid. Isocitrate lyase was assayed at three enzyme levels on total cell homogenates. Details have been given previously (Levy and Scherbaum, '65b).

Source of chemicals

Puromycin was purchased from the Nutritional Biochemical Company. Actinomycin D was a gift from Merck, Sharpe, and Dohme Laboratories. Sodium 2-^{14}C acetate was obtained from the Volk Radiochemical Company. Other chemicals were obtained from the California Corporation for Biochemical Research or from the Sigma Chemical Company.

RESULTS

In initial experiments, cultures of T. pyriformis, strain GL, were shaken for very brief periods with tracer levels of acetate after 0 and three hours under static conditions. Isocitrate lyase activity and C^{14} uptake into glycogen were determined (table 1). Static controls incorporated about 15 times as much tracer into glycogen as did the 0 hour controls. This increase has previously been shown to result from an increase in the relative amount of acetate metabolized via the glyoxylate bypass (Levy and Scherbaum, '65b) and to be accompanied by an increased ability to con-

TABLE 1

Effects of inhibitors on isocitrate lyase induction and incorporation of
C^{14} acetate into glycogen

Addition	Radioactivity		Isocitrate lyase	
	Zero hour	Three hour static	Zero hour	Three hour static
	counts/minute in glycogen/ 10^6 cells		μmoles glyoxylate formed/ hour/mg protein	
None	664	9,870	1.1	1.8
Actinomycin D, 10 μg/ml	—	9,260	—	0.9
D,L-p-fluorophenylalanine, 2.5 mM	—	16,000	—	1.0
Puromycin, 40 μg/ml	—	27,200	—	1.0

Glyconeogenesis was activated by transferring 100 ml portions of a well-shaken, exponentially growing culture into 500 ml Erlenmeyer flasks, and maintaining these for three hours without shaking. Inhibitors were added as indicated. At zero and three hours, 25 ml portions of culture were shaken for 15 minutes with 1.25 μc of sodium acetate-2-^{14}C (sp. act. 20.5 mc/mmole).

vert substrate levels of acetate to glycogen (Levy and Scherbaum, '65a). The presence of Act D,[3] PM, or pFPA during the activation period did not prevent this increase from taking place. In fact, the rate of acetate incorporation in the treated cells was usually even greater than in the activated controls. Other experiments have shown that incorporation of acetate into glycogen in the 0 hour samples is not affected by the inhibitors. In each case, the inhibitor prevented the increase in the specific activity of isocitrate lyase.

In the next set of experiments, rates of net glyconeogenesis in peptone were meas-

ured by shaking cultures for a 30 or 60 minute period after 0 and 3 hours under static conditions (table 2). The E strain of *T. pyriformis* was used in this and all subsequent experiments. Transfer of cultures of this strain from shaken to static conditions leads to a much greater increase of isocitrate lyase. In the three experiments shown, the rates of glycogen synthesis in the 0 hour (log phase) samples were 0.03 μmoles glucose formed/hr/mg protein or less, while rates in the static controls ranged from 0.13 to 0.23. In each case

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[3] Abbreviations: Actinomycin D, Act D; Puromycin, PM; D,L-p-fluorophenylalanine, pFPA.

TABLE 2

Effects of inhibitors on isocitrate lyase induction and activation of glyconeogenesis

Exper.	Inhibitor	Net glyconeogenesis			Isocitrate lyase		
		Zero hour control	Three hour static	Three hour static + inhibitor	Zero hour control	Three hour static	Three hour static + inhibitor
		μmoles glucose formed/hr/mg protein			*μmoles glyoxylate formed/hr/mg protein*		
1	Actinomycin D, 9 μg/ml	0.03	0.18	0.21	0.8	4.4	1.6
2	D,L-p-fluorophenyl-alanine, 2.5 mM	−0.07	0.23	0.31	—	—	—
3	Puromycin, 48 μg/ml	0.03	0.13	0.16	0.8	3.9	1.5

Glyconeogenesis was activated as described in the legend for table 1. Net glyconeogenesis was tested by shaking duplicate 20 ml portions of culture for one hour (experiments 1 and 3) or for 30 minutes (experiment 2).

TABLE 3

Effects of puromycin on activation of glyconeogenesis

	Zero hour	Three hour static	Three hour static + puromycin
	Net glyconeogenesis		
	μmoles glucose formed/hr/mg protein		
Cultures	0.03	0.13	0.16
Washed cells			
Endogenous	0.11	0.17	0.16
+ 1 mg/ml sodium acetate	0.10	0.20	0.18
	Incorporation of acetate-2-C[14] into glycogen in washed cells		
Tracer acetate only (% incorporation)	24	60	51
+ 1 mg/ml sodium acetate			
(% incorporation)	9	36	31
(counts/minute/mg protein)	674	2690	2330
	Isocitrate lyase		
	μmoles glyoxylate formed/hr/mg protein		
	0.8	3.9	1.5

Glyconeogenesis was activated as described for the previous experiments. Glyconeogenesis in cultures was tested by shaking duplicate 20 ml portions for one hour. Glyconeogenesis in washed cells was tested as described in the *Methods* section, after cells had been harvested, washed twice, and resuspended in inorganic medium.

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however, static samples treated with inhibitor formed glycogen at an even greater rate than the activated controls, although the increase in the specific activity of isocitrate lyase was largely suppressed.

The last of these experiments was carried out in greater detail (table 3). Before and after the activation period, portions of culture were harvested, washed, and suspended in inorganic medium. Glyconeogenesis was tested by shaking these cells for two hours with tracer or substrate levels of acetate. The rate of synthesis in the presence of acetate in the static controls was twice that of the 0-hour cells, and the rate in the puromycin-treated cells had also nearly doubled. In this particular experiment high endogenous rates of glycogen synthesis were observed. However, in the static samples, acetate was the major precursor when present at substrate levels as indicated by the high percentage of isotope recovered in glycogen. When only tracer levels of acetate were added, over 50% was incorporated into glycogen, both in static controls and in static cells treated with PM. Such high levels are consistent with

metabolism of acetate *via* the glyoxylate bypass. Thus, PM largely prevented the increase in isocitrate lyase, but not the increased ability to convert acetate to glycogen. Results similar to these have been obtained with cultures treated with Act D and pFPA.

In experiments such as the one just discussed, substantial increases in the specific activity of isocitrate lyase took place, even in the presence of inhibitors. In addition, the endogenous rates of glycogen synthesis were often nearly as great as those obtained in the presence of acetate. The use of starved cells served to eliminate these problems. There was virtually no synthesis of isocitrate lyase when inhibitor was present and the endogenous rates of glyconeogenesis were lowered so that a substantial stimulation was achieved when cells were incubated with substrate levels of acetate. Such a stimulation tends to justify the use of C^{14} incorporation as an index of net glyconeogenesis from acetate.

In the experiment shown in table 4, cells from a log phase culture were harvested, washed, and shaken in non-nutrient medium

TABLE 4
Effects of D,L-p-fluorophenylalanine on activation of glyconeogenesis in starved cells

	"Zero" hour	"Three" hour		"Three" hour	
		Shaken	Shaken + pFPA	Static	Static + pFPA
		Net glyconeogenesis			
		μmoles glucose formed/hr/mg protein			
Endogenous	0.017	< 0	< 0	0.13	0.064
+ 1 mg/ml sodium acetate	0.075	0.03	0.003	0.27	0.15
		Incorporation of acetate-2-C14 into Glycogen			
Tracer acetate only (% incorporation)	4.9	1.5	1.1	26	24
+ 1 mg/ml sodium acetate (% incorporation)	4.7	4.4	0.3	22	11
(Counts/min/ mg protein)	1160	1100	70	5510	2650
		Isocitrate lyase			
Specific activity (μmoles glyoxylate/hr/mg protein)	1.1	1.0	0.6	2.5	1.4
Units/ml cell suspension Initial		0.46	0.54	0.50	0.48
Final		0.36	0.27	1.0	0.53

Washed cells from growing cultures were suspended in inorganic medium and preincubated by shaking for three hours. Cells were then collected and resuspended in inorganic medium. One aliquot was tested immediately for glyconeogenesis in the presence of substrate or tracer levels of acetate, as described for table 3. The other portions were each diluted to 120 ml and were either shaken or maintained under static conditions for three hours, in the presence or absence of 0.25 mM D,L-p-fluorophenylalanine. In the shaken samples, 30 ml of cell suspension were transferred to each of four 500 ml flasks. For the static samples, 120 ml were placed in a single 500 ml flask. The initial protein concentration was about 0.45 mg/ml. After three hours, samples were collected and rates of glyconeogenesis were tested.

for three hours prior to treatment. After this preincubation cells were either shaken for an additional three hours or maintained under static conditions for three hours in the presence or absence of pFPA (table 4). Cells were then washed and tested for glyconeogenesis in the usual manner, by shaking for two hours in the presence of tracer or substrate levels of acetate. Cells *shaken* either with or without inhibitor showed a decreased rate of glyconeogenesis as compared with the "0" hour control. The rate of net synthesis in the presence of acetate decreased from a value of 0.075 in the "0" hour sample to 0.03 in the shaken controls, and to 0.003 in cells shaken with inhibitor. Cells from these suspensions had little ability to incorporate substrate or tracer levels of acetate into glycogen. In the shaken cells, both the specific activity and the amount of isocitrate lyase decreased. In contrast, *statically* maintained cells showed a greatly increased capacity for glycogen synthesis when compared with the "0" hour sample and especially when compared with the cells that were shaken for three hours. The rate of synthesis in the presence of acetate was 0.27 μmoles glucose/hr/mg protein in the static controls and 0.15 μmoles glucose/hr/mg protein in cells treated with inhibitor. The incorporation of tracer and substrate levels of acetate into glycogen also increased greatly. It can be seen that the static controls were able to synthesize a substantial amount of isocitrate lyase, even after several hours in non-nutrient medium, and despite a net decrease in total cell protein. The presence of pFPA greatly limited the increase in both the specific activity and the net amount of isocitrate lyase. Thus, the increase in glyconeogenic capacity resulted mainly from an activation of the glyoxylate bypass. The inhibitor itself did not lead to an increased rate of glyconeogenesis, since cells that had been shaken for three hours with pFPA had a negligible rate.

It would appear that the greater rate of glyconeogenesis in the static controls may be the result of synthesis of isocitrate lyase and, possibly, other glyconeogenic enzymes. Thus, in both the uninhibited and the inhibited samples, glyconeogenesis was activated, but the increase in glyconeo-

genic enzymes in the uninhibited cells permitted this process to proceed at a greater rate. The fact that in the pFPA-treated cells the final level in glycogen of C^{14} from *tracer* levels of acetate was about the same as in the static controls further shows that the shift in metabolism took place.

DISCUSSION

These results suggest that the amount of enzyme of the glyoxylate bypass is not the primary factor controlling glyconeogenesis in *Tetrahymena*. Thus, addition of Act D, PM, or pFPA to cultures or to washed cell suspensions at the beginning of the period of restricted aeration greatly limited the increase of isocitrate lyase. However, a considerable increase in the capacity for glyconeogenesis could still take place. In fact, when rates of glycogen synthesis were measured in cultures, cells treated with inhibitor usually exhibited an even greater increase than did the activated controls.

When freshly washed cells from activated cultures were shaken with acetate a considerable proportion of this substrate (over 50%) was converted to glycogen, as shown in table 3. The presence of PM, at levels which prevented most of the increase in isocitrate lyase activity only slightly inhibited this conversion. In experiments not shown here, high levels of radioactivity from 2-^{14}C acetate were found in glycogen after cells had been activated in the presence of Act D of pFPA.

More recent experiments also suggest that glyconeogenesis in *Tetrahymena* can be stimulated by activation mechanisms alone. Thus, incubation of cells under atmospheres of 0.5 – 2% oxygen leads to an activation of glyconeogenesis, but little increase in the specific activity of isocitrate lyase or malate synthase is usually observed (unpublished data).

Although glyconeogenesis can be activated without enzyme synthesis, the data of table 4 show that a greater rate may sometimes be obtained if enzyme synthesis does occur. The same experiment tends to rule out the possibility that increases in enzymes other than those of the glyoxylate bypass govern the activation of glyconeogenesis in *Tetrahymena*. It has been pointed out (Krebs and Kornberg, '57) that for a net reversal of glycolysis to occur, the

phosphofructokinase and pyruvate kinase steps must be circumvented. An induction of fructose-1,6-diphosphatase, which by-passes the first of these steps occurs when various microorganisms are grown on non-carbohydrate-containing medium (Gotto and Pogell, '62; Cancedo, Salas, Giner and Sols, '65). It also increases in mammalian tissue under the influence of glucocorti-coids (Weber, Singhal, Stamm, Fisher and Mentendiek, '64). Similarly phosphoenol-pyruvate carboxykinase has been shown to increase in mammalian livers when ani-mals are in a gluconeogenic state (Shrago, Lardy, Nordlie and Foster, '63). Although variations in the specific activities of these enzymes have not been tested in Tetrahy-mena,[4] it is unlikely that substantial in-creases could have occurred under the experimental conditions of table 4, where glyconeogenesis was activated in starved cells that were treated with pFPA. In fur-ther support of this view is the finding that activation of glyconeogenesis in this organ-ism involves a change in the initial reac-tions of acetate, relatively more of this compound appearing initially in malate as compared with citrate. In mammalian sys-tems, gluconeogenesis can also be activ-ated without increase in the amount of enzyme. Thus, hydrocortisone could stim-ulate gluconeogenesis when phosphopyru-vate carboxykinase increase was prevented by Act D (Ray, Foster and Lardy, '64). Similarly, this inhibitor did not abolish a triamcinolone-induced gluconeogenesis, although enzyme levels were not measured (Haynes, '65).

It has previously been suggested (Hogg and Kornberg, '63) that glyconeogenesis in Tetrahymena is controlled by changes in the intracellular distribution of isocitrate lyase and malate synthase. However, little difference was found in the relative distri-bution of these two enzymes when glyco-neogenesis was stimulated by the methods described here (unpublished data).

The role of the glyoxylate bypass in glyc-ogen storage, as well as its possible role in circumvention of the tricarboxylic acid cycle under conditions of oxygen limitation have been discussed in terms of the adap-tive value to the cell (Hogg and Kornberg, '63); Levy and Scherbaum, '65a and b; Hogg, '65). The experiments presented

here further indicate the powerful capacity of Tetrahymena to activate this pathway.

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