Quantitative Analysis of the Change of Metabolite Fluxes along the Pentose Phosphate and Glycolytic Pathways in Tetrahymena in Response to Carbohydrates*

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A metabolic scheme of glycolysis and the pentose phosphate pathway has been constructed, assuming that the reactions occur in a single compartment. From this scheme, equations are written for a system in metabolic and isotopic steady state. These allow computation of the specific activity of every carbon atom of all the intermediates of the glycolytic and pentose phosphate pathways and consequently of the flux of carbon along each step of these pathways.

A sufficiently large number of well distributed measurements of incorporation of radioactive label from different positions of several substrates into intermediates or products must be made to determine all the fluxes. This is done by choosing a set of metabolic fluxes, calculating incorporation with the aid of a computer, and then manipulating the flux rates until the computed incorporations match the data. The model is used in this paper to analyze the metabolic flux rates until the computed incorporations match the predictions of the model, thereby independently corroborating the values obtained for the metabolic fluxes, and in particular the value of the futile cycle. There is an appreciable bidirectional flux through the nonoxidative portions of the pentose cycle, and, as expected from *in vitro* enzyme measurements, a very small flux through the oxidative portion.

A futile cycle between fructose-6-P and fructose 1,6-diphosphate amounting to ~70% of the forward carbon flux occurs throughout the incubation. The operation of this futile cycle is the only path for incorporation of label from [6-14C]glucose into carbon 1 of the glucose moiety of glycogen; measurement of the amount of label so incorporated matched the predictions of the model, thereby independently corroborating the values obtained for the metabolic fluxes, and in particular the value of the futile cycle. Late in the incubation, ATP expenditure in this futile cycle comprises up to 9% of the total ATP consumption of the cells. During the first 40 min of adaptation the cells utilize more ATP than is derived from catabolism of the carbohydrate mixture. Thereafter they are in positive net energy balance.

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even without utilization of other components of the medium.

Although a large number of well distributed measurements is required to determine all the in vivo fluxes, we have developed methods for estimating, from limited data, fluxes through hexose phosphate isomerase, triose phosphate isomerase, glyceraldehyde dehydrogenase, and through the exchange reactions of transaldolase and transketolase. Most previous models have required one or more assumptions about these reactions as well as the assumption that a futile cycle at phosphofructokinase and fructose-1,6-diphosphatase is absent. Since this model requires none of these assumptions, it can be used to test earlier models and to investigate the validity of their predictions when any of the assumptions are not justified. These considerations, including an analysis of extant data on rat mammary tissue, are presented in miniprint as Appendix I of this paper.

Much work has been done to characterize the in vivo behavior of many enzymes of intermediary metabolism with respect to substrates and modulators with the ultimate aim being to understand the in vivo operation and control of metabolism. Extrapolation from in vitro to in vivo behavior is hazardous, however, and studies of metabolism in living cells are of increasing importance. A method of quantitatively studying whole cell metabolism, developed in this laboratory, has previously been used to analyze acetyl-CoA metabolism in the ciliated protozoan, Tetrahymena pyriformis (see references in 1). Raugi et al. (1) constructed a model consistent with all known information on Tetrahymena's enzyme complement and compartmental structure, specifying in detail acetyl-CoA metabolism in the Krebs cycle, the glyoxylate bypass, β-oxidation, and related pathways. The model enabled computation of the specific activity of each carbon of any metabolic intermediate and hence computation of the mass fluxes along the pathways considered. In this earlier work, the segment of metabolism connecting the hexose and triose phosphates was greatly simplified to facilitate the analysis; there was assumed to be a direct exchange of label between phosphoenolpyruvate and the glycoool monomers of glycogen.

The present work is an expanded treatment of the in vivo carbohydrate metabolism of Tetrahymena, complementing the previous studies of Raugi et al. (2).

Although there have been numerous theoretical and experimental studies aimed at quantitating fluxes through glycolysis and the pentose phosphate pathway, the complexity of these pathways necessitated models based on simplifying assumptions which, while allowing reasonable estimates of flux through the oxidative steps of the pentose phosphate pathway, did not permit assessment of the fluxes through each reaction of both pathways. These methods were, in any case of limited applicability for studying the role of this pathway in the metabolism of Tetrahymena since the available evidence indicated that little if any glucose-6-P dehydrogenase or 6-phosphogluconate dehydrogenase was present in this organism, cated that little if any glucose-6-P dehydrogenase or 6-phosphogluconate dehydrogenase was present in this organism, and the glyceraldehyde dehydrogenase is absent. Since this model requires none of these assumptions, it can be used to test earlier models and to investigate the validity of their predictions when any of the assumptions are not justified. These considerations, including an analysis of extant data on rat mammary tissue, are presented in miniprint as Appendix I of this paper.

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Absence of the oxidative steps of the pentose-phosphate pathway in Tetrahymena raises many questions about the role of the nonoxidative reactions in this organism, making it of interest to assess the direction and magnitude of carbon flux in this pathway. Earlier work with Tetrahymena (5) had shown that the ratio of 14CO2 produced from 6-14Cglucose to that from [1-14C]glucose was between 1.2 and 1.5, which was interpreted as implying a role for transaldolase in glucose metabolism. In addition, the finding that reserpine reduced this ratio to nearly 1.0 suggested that studies of the pentose phosphate pathway in Tetrahymena might contribute to our understanding of the evolution of the regulation of carbohydrate metabolism in eukaryotes.

With these and other related questions in mind, a model of Tetrahymena metabolism was developed. Some general properties of this model and its application to other systems are described in detail in Appendix I, in miniprint format. The model, relatively free of simplifying assumptions, accounts for the metabolism of the substrates glucose, fructose, ribose, and glyceraldehyde, an expanded scheme of glycolysis, glycogen synthesis, the pentose phosphate pathway, and an abbreviated tricarboxylic acid cycle. The structure of the model is consistent with the known facts of Tetrahymena metabolism with the exception that the fate of acetyl-CoA is greatly simplified. The equations that have been derived permit computation of the incorporation of radioactivity from any carbon atom of the substrates into a variety of products. By making experimental measurements of the rates of 14C incorporation into products (CO2, lipid, glycogen, and RNA) and comparing these to the predictions of the model, the actual fluxes through the major metabolic pathways in the cell can be ascertained.

In contrast to the earlier work on acetyl-CoA metabolism in Tetrahymena, in the present study of carbohydrate metabolism, some of the observed rates of 14C incorporation into products from labeled substrates were time-dependent. This called into question the use of a model predicated on a metabolic steady state. Haut et al. (6) have used a kinetic model to analyze the role of the pentose cycle under non-steady state conditions in mammary tissue. To quantitate the flux in each step of the glycolytic and pentose phosphate pathways, however, would require an enormous number of assumptions or measurements or both. Therefore it was of great interest to develop a way of treating our time-dependent system within the framework of a steady state approach. The 1-h incubation during which the cells were studied was broken into three 20-min intervals. In each of these intervals, 29 incorporation measurements were made to determine the 18 independent rate parameters of the model. The resulting overdeterminacy of the system enhances the significance of the fact that the model could be successfully employed to fit the data and also

1 J. J. Blum, unpublished observation.
2 Two appendices are presented in a miniprint format immediately following this paper. Appendix I contains a description of the model and its application to situations where limited data are available. Appendix II presents information pertaining to the main body of this paper and deals with methods of collecting and organizing data, and with establishing the validity of the model in a non-steady state situation. Figs. 3 and 4 and Tables I and II of the main body of the paper will be found on p. 1695. For the convenience of those who prefer to obtain the supplementary material in the form of pages of full size photocopies they are available as JBC Document Numbers 76M-891 A and B. Orders should specify the title, authors and reference to this paper, the JBC Document Number, and the number of copies desired. Orders should be addressed to the Journal of Biological Chemistry, 8560 Rockville Pike, Bethesda, Md. 20014 and must be accompanied by remittance to the order of the Journal in the amount of $2.85 for Appendix I, and $2.10 for Appendix II, respectively.
3 It should be emphasized that since the model predicts the specific activity of each carbon atom of each metabolite via the glycolytic and pentose phosphate pathways, measurements may be chosen entirely for analytical convenience and may include 14C incorporation into particular atoms or whole molecules of any intermediates or products as well as or instead of those end products we chose.
makes it very likely that the picture of metabolism obtained is accurate.

In this paper, we describe the time course of flux changes through the reactions of glycogen synthesis, glycolysis, and the pentose phosphate pathway during the 1st h of adaptation to the presence of carbohydrate substrates. These changes are found to include an accelerating rate of hexose catabolism through glycolysis (most probably as a result of a steady rise in phosphofructokinase activity), alterations in bidirectional flux through the pentose phosphate pathway, and a rise in flux through the reaction catalyzed by fructose-1,6-diphosphatase commensurate with the increase in the phosphofructokinase reaction, resulting in a continuing "futile cycle" between fructose-6-P and fructose-1,6-diphosphate. A preliminary account of this work has been published (7).

**METABOLIC SCHEME**

*Tetrahymena* is known to possess the enzymes of glycogen synthesis (8, 9), of the glycolytic pathway (3, 10) and of the nonoxidative portion of the pentose phosphate pathway (4). These enzymes all appear to be completely cytosolic except for hexokinase (11), phosphofructokinase (12), 3-phosphoglyceraldehyde dehydrogenase (13), glyceraldehyde-P dehydrogenase (3), and lactate dehydrogenase (14, 15) which are in part bound to particles, presumably mitochondria. Although this raises the possibility that segments of the glycolytic pathway may be compartmented, as has been suggested for several other cells (16-19), we have chosen, in the absence of any evidence for multiple pools of glycolytic or pentose phosphate pathway intermediates in *Tetrahymena*, to represent the standard reactions of the glycolytic and pentose phosphate pathways as occurring in a single compartment, as shown in Fig. 1. The model allows for the oxidative steps of the pentose cycle, symbolized by Vγ, although it was expected that Vγ might be zero in view of the very low levels of glucose-6-P and 6-phosphoglucose dehydrogenases in this cell. The model allows for an input of P-enolpyruvate (Vε) from other pathways of intermediary metabolism, and for the formation of lactate (Vν), fatty acids ( Vσ), and lipid glycerol (Vπ) as outputs from the triose phosphates, and for the utilization of ribose-5-P for synthesis of glycogen (Vζ), nucleic acid (Vη), and lipids (Vθ).

**Fig. 1.** Metabolic scheme for glycolysis and the pentose phosphate pathway in *Tetrahymena* with *in vivo* fluxes during a 1-h incubation with glucose, fructose, ribose, and glycerol. Each V represents the reaction or sequence of reactions defined in Table IV. The smaller numbers represent the values for the fluxes, in nmol/10^6 cells/h, which give the best fit to the data under the experimental conditions described. For reactions with a constant flux throughout the hour of incubation, a single number is given. For those reactions which have changing rates, these are listed vertically: the top, middle, and bottom are those in the 0 to 20-min, 20 to 40-min, and 40 to 60-min intervals, respectively. For convenience of presentation some of the flux values have been rounded off; the exact values used in the computations of the model are listed in Table IV. Abbreviations: FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone-P; GAP, glyceraldehyde-3-P; PYR, pyruvate; PEP, phosphoenolpyruvate; Ru-5-P, ribulose-5-P; Xyl-5-P, xylose-5-P; Rib-5-P, ribose-5-P; Ery-4-P, erythrose-4-P; Sed-7-P, sedoheptulose-7-P; GOLP, Sn-glycerol-3-P; AcCoA, acetyl coenzyme A; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-6-P, fructose-6-phosphate.
nucleic acid synthesis ($V_{14}$). $V_{15}$, $V_{16}$, $V_{17}$, and the steps allowing for the utilization of pyruvate ($V_{16}$, $V_{17}$, $V_{18}$, and $V_{19}$) were considered as irreversible, and, although phosphorylase is present in *Tetrahymena* (9), it was assumed that no glycogen degradation occurred under conditions where glycogen deposition from glucose was occurring at a very high rate. Fructose was shown as entering metabolism only via hexokinase ($V_{1}$) since fructokinase was looked for but not found in this cell (11). All other steps of the glycolytic and pentose phosphate pathways were considered to be reversible. Of the 22 independent parameters in this scheme, 4 were set equal to zero ($V_{15}$, $V_{16}$, $V_{17}$, and $V_{18}$); justification for this is discussed in detail below.

**RESULTS**

**Choice of Substrates and Experimental Design**—Since a large number of measurements is needed in order to determine the 18 independent flux rates with a minimum of ambiguity, it was decided to use a total of 10 labeled substrates in the standard mixture of glucose (6 mM), fructose (6 mM), ribose (3 mM), and glycerol (3 mM). A series of initial experiments was performed on cells that were grown to transition phase and then briefly centrifuged and resuspended in an inorganic medium, but the data obtained from these experiments were too variable to allow determination of the flux values to the precision we desired. These experiments did, however, indicate that the utilization of glucose and ribose becomes independent of concentration in the range from 3 to 6 mM. Since glucose was utilized at a much faster rate than ribose, we chose 6 mM glucose and 3 mM ribose as the concentrations in our standard substrate mix. The concentration of fructose, which is consumed more slowly than glucose, was also chosen as 6 mM. The rate of glycerol utilization increased with concentration up to the highest concentration tested. It was convenient to use it at 3 mM. At these concentrations, less than 2% of the ribose, glycerol, and fructose, and less than 10% of the glucose was consumed during the course of a 1-h incubation, so that the concentration of each of the four substrates remained essentially constant during the course of these experiments. Since centrifuging the cells and suspending them in an inorganic medium caused an unacceptably large variance in the data, the experiments reported in this paper were performed by adding cells in their growth medium to the standard mixture of the four substrates, as described in detail under “Materials and Methods” (see Appendix II).

**Applicability of the Model under Non-Steady State Conditions**—In the derivation of the model (see Appendix I for details) the assumption is made that the system is in a metabolic steady state i.e. that net accumulation or destruction of any intermediate occurs. If the observed incorporations of label from substrates into products are linear with time, this constitutes presumptive evidence that the pool sizes of intermediates are not changing. Although incorporation of label into glycogen from all substrates was linear with time, we have been unable to detect any lactate production. From considerations presented in detail in Appendix II, it was concluded that an approximate steady state is achieved within the first few minutes after substrate addition and that the system remains in a quasi-steady state thereafter.

**Treatment of Data**—In previous experiments with substrates where label incorporation into product was linear with time, we established the linearity and the duration of the lag period, if any, with a few time course experiments (1). We then computed the average rate of label incorporation by making repeated measurements at a single time (usually 60 min). In an analogous manner, in the present investigation we used a few experiments to establish the “shape” of the curve of incorporation from each labeled substrate into each product. Details of this procedure are presented in Appendix II. The equations used to calculate the experimental data at 20, 40, and 60 min after the addition of the carbohydrate substrates are listed in Table II of Appendix II. The resulting data are listed with their estimated standard errors in Table III. A total of 29 measurements was made at each of the three time intervals. Since the data have been adjusted for the incorporation of label into fatty acids during the first 20 min and there was practically no label incorporated into fatty acids at later times, $V_{f}$, in Fig. 1 has been set to zero. $V_{17}$, the rate of lactate production, has also been set to zero since in previous experiments, we have been unable to detect any lactate production. $V_{i}$, which represents the input of P-enolpyruvate from the Krebs and glyoxylate cycles, was set to zero since the gluconeogenic flux in the presence of large amounts of glucose, fructose, glycerol, and ribose and the absence of added acetate or pyruvate is likely to be very small. It was found that when $V_{p} = 0$, the choice of $V_{15}$ was entirely arbitrary so that it, too, was set to zero. Thus the 29 incorporation measurements made at each time point provide considerable redundancy for determining the 18 independent parameters. These measurements, plus others to be described below, thus enable the model to be subjected to a stringent test.

* M. Eldan and J. J. Blum, unpublished observation.

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**Fig. 2.** Time course of incorporation of label into CO₂ and lipid from two substrates. Cells were grown and incubated in the standard mixture of substrates as described under "Materials and Methods." Panels A and B show the incorporation of label into CO₂ and total lipid, respectively, from substrates labeled as indicated. The points at 20 and 40 min show the data from individual experiments, while the points at 60 min show the mean ± S.E. for the number of experiments indicated in parentheses. The lines are drawn using the equations and coefficients shown in Table II. For further details, see text.
Choice of Solutions which Best Fit Data at Each Time—As discussed in detail earlier (1), a trial and error procedure was necessary to find an acceptable solution and then refine it to achieve the best fit. Because many of the flux values were clearly changing during the course of the incubation, an assumption of minimal complexity was made so that, where possible, the same rate was chosen for all three times. The numbers next to each arrow in Fig. 1 show the flux values, in nanomoles/h/10^10 cells, which gave the best fit to the data. The numbers are arranged vertically, the topmost of each set being the average flux during the 0 to 20-min interval, the next being that during the 20 to 40-min interval, and the bottom number that during the 40 to 60-min interval. The expected incorporation from each labeled substrate into each product at 20, 40, and 60 min, computed from these flux values (which are also listed in Table IV) is shown in Table III immediately below each measurement. With a few exceptions which are discussed below, the overall fit to the data is good, and the flux configurations shown in Fig. 1 therefore can be considered an accurate representation of the temporal pattern of metabolite flow in these pathways. It should be pointed out that the measurements of the incorporation of [1-^14C]fructose into lipids were not used in obtaining the fits. The closeness with which these values are predicted constitutes further evidence that the values in Fig. 1 reflect the in vivo carbon flows throughout the 1-h period.

Assessment of Flux Values and Probable Limits of Confidence—In spite of the large excess of measurements, not all of the 29 measurements are independent so that some flux values are more tightly determined than others. To establish the permissible range of variation for each parameter, the procedure employed was to vary a single parameter until the fit to the data worsened significantly and then attempt to re-establish a fit through variation of other parameters. No significant improvement on the best fits shown in Fig. 1 could be obtained in this way, in agreement with the definition of these values as the best fit values. Table IV presents the flux value for the best fit in each interval. (Some of these numbers were rounded off in Fig. 1 for convenience in presentation.) The numbers in parentheses show the acceptable range of variation, i.e., that within which a good fit was still attainable. Table IV shows the maximal in vitro rates reported in the literature for many of the reactions of Fig. 1. Clearly, most of the in vivo flux values computed here are consistent with the in vitro assays.

When the parameters directly associated with substrate utilization or product formation (V_0, V_1, V_2, V_3, V_4, V_5, and V_6) were varied the fit to the data failed rapidly and could not be restored by variation of any other parameter even with deviations of as little as 3 to 5% from the best fit values. Thus these values are fairly precisely determined. It is clear that the preferred substrate is glucose; it is used at about 5 times the rate of fructose and nearly 50 times that of ribose. That this glucose should be utilized more rapidly than fructose when the concentration of each is 6 mM is expected, since the hexokinase of Tetrahymena has the same V_max for these two hexoses but the K_m for glucose is about 4 x 10^{-3} M while that for fructose is about 7 x 10^{-3} M (11). The much lower rate of utilization of ribose accords well with the lower amount of ribokinase reported compared to hexokinase (Table IV). It can also be seen that while fructose and glyceroi are metabolized at essentially constant rates during the entire hour of incubation, the rates of ribose and, to a lesser extent, glucose utilization increased with time.

The bulk of the carbon utilized under these conditions winds up in glycogen, with V_12 accounting for about 85% of all products formed (given by V_12 + V_14 + V_16 + V_18). It should be noted that there was practically no change in the rate of glycogen synthesis throughout the 1-h incubation. The other

### Table III

Experimental and computed incorporation of radioactivity from ^14C-labeled substrates into measured products

From the coefficients in Table II (Appendix II), the best values for data values are shown as the upper number in each row, with an error proportional to the S.E. of the coefficient a in Table II. The lower part of each pair of numbers represents the value for incorporation in nanomoles/10^10 cells, computed from the best fit flux parameters presented in Table IV and Fig. 1. NM, not measured.

| SUBSTRATE | (U) | GLUCOSE | LIPID GLUCOSE | TOTAL GLUCOSE |
|-----------|-----|---------|--------------|--------------|
| [1-^14C]glucose | 6.749±0.36 | 23.81±3.3 | 49.5±2.5 | 53.5±17 | 67.0±24 | 30.7±1 | 0.567±0.007 | 1.250±0.02 | 2.40±0.03 | NM | NM | NM |
| [2-^14C]glucose | 5.58±0.49 | 25.3±2.3 | 62.4±5.6 | 28.3±7 | 58.5±14 | 48.1±1 | 0.466±0.049 | 1.43±0.10 | 2.62±0.20 | NM | NM | NM |
| [3-^14C]glucose | 4.67 | 19.8 | 41.9 | 6.4 | 62.3 | 62.3 | 0.417 | 1.28 | 2.55 | NM | NM | NM |
| [3-^14C]glycerol | 7.90±0.37 | 28.1±4.3 | 61.3±2.9 | 50.1±8 | 60.5±16 | 50.5±2 | 0.760±0.065 | 2.16±0.14 | 3.62±0.26 | NM | NM | NM |
| [6-^14C]glycerol | 4.88 | 36.9 | 64.2 | 59.9 | 64.1 | 64.1 | 0.722 | 1.90 | 3.45 | 0.90 | 2.65 | 4.89 |
| [6-^14C]fructose | 10.9±0.5 | 46.0±1.6 | 86.6±1.0 | 20.4±7 | 86.6±1.0 | 86.6±1.0 | 0.123±0.030 | 3.31±0.61 | 2.32±0.00 | 0.043±0.116 | 2.76±0.34 | 3.96±0.44 |
| [6-^14C]glucose | 6.93 | 26.1 | 55.0 | 56.2 | 66.6 | 66.6 | 0.560 | 1.59 | 3.00 | 0.905 | 2.95 | 5.53 |

* These values were measured after the best fit had been determined and hence may be regarded as predictions of the model. For further details, see text.
### Table IV

| Reaction | 0-20 min | 20-40 min | 40-60 min | V\text{max} | Ref. |
|----------|----------|-----------|-----------|-------------|------|
| *V_1* | G6PDH + 6PGDH | G6P → RU-5-P + CO\textsubscript{2} | 8 | 8 | 8 | 5-24 | [2, 3, 4] |
| *V_2* | Hexose-P-isomerase | G-6-P → F-6-P | 1000 (500-1200) | 2400 (1500-3200) | 3500 (2000-5000) | 12,000 | [10] |
| *V_3* | FPK | F-6-P → FDP | 63.33 | 208 | 450 | 360-31,000 | [3, 10, 12] |
| *V_4* | FPDase | FDP → DHAP + GAP | 48 (35-52) | 145 (100-190) | 350 (240-420) | 10,200 | [20] |
| *V_5* | Aldolase | FDP → DHAP + GAP | 500 | 500 | 500 | 1800-7200 | [3, 10, 21] |
| *V_6* | Triose-P isomerase | DHAP → GAP | 5000 (>1000) | 5000 (>1500) | 5000 (>2500) | 46,000; 360,000 | [3, 10] |
| *V_7* | Glycolysis | GAP → PEP | 80.13 | 171.5 | 243.5 | 3600; 6000 | [3, 10] |
| *V_8* | Ribulose-5-P epimerase | RU-5-P + XYL-5-P | 150 (>40) | 150 (>75) | 150 (>80) | 800 | [4] |
| *V_9* | Ribose-5-P isomerase | RU-5-P → RIB-5-P | 150 (>40) | 150 (>75) | 150 (>80) | 1400 | [4] |
| *V_10* | Transketolase | XYL-5-P + ERY-4-P | 25.67 | 25.5 | 25.5 | 900 | [4] |
| *V_11* | Transaldolase | F-6-P + ERY-4-P | 42 (55-60) | 85 (50-100) | 120 (70-170) | 330 | [4] |
| *V_12* | Transketolase | XYL-5-P → SED-7-P | 47.67 | 90.5 | 125.5 | |
| *V_13* | Glycerol-P-dehydrogenase | GOLP → DHAP | 135 (120-175) | 230 (200-300) | 350 (300-450) | 2300; 7200 | [3, 10] |
| *V_14* | Glycolynthesis | G-6-P → glycogen | 1112 | 1148 | 1148 | 400; 1500 | [8, 9] |
| *V_15* | Pyruvate kinase | PEP → PYR | 80.13 | 171.5 | 243.5 | 9000-11,000 | [3, 10, 22-24] |
| *V_16* | RNA synthesis | RIB-5-P → RNA | 8.5 | 16 | 22 | |
| *V_17* | Lipid glycerol synthesis | GOLP → LIPID | 11.2 | 12.0 | 14.9 | |
| *V_18* | Pyruvate dehydrogenase | PYR → AcCoA + CO\textsubscript{2} | 80.13 | 171.5 | 243.5 | |
| *V_19* | Krebs cycle | AcCoA → CO\textsubscript{2} | 80.13 | 171.5 | 243.5 | |
| *V_20* | Hexokinase | Glucose → G6P | 900 | 1008 | 1046 | 200-30,000 | [3, 8, 10, 11, 21] |
| *V_21* | Hexokinase | Fructose → F6P | 194 | 200 | 199 | |
| *V_22* | Glycerol kinase | Glycerol → GOLP | 56 | 52 | 52 | |
| *V_23* | Ribokinase | Ribose → RIB-5-P | 17.5 | 24.5 | 30.5 | 11 | [4] |
principle pathway of hexose utilization *i.e.* glycolysis with subsequent oxidation in the Krebs cycle, increases 3-fold, accounting for about 6% of substrate utilization at early times and increasing up to 17% by the end of the hour. The rates of production of lipid glycerol (V<sub>n</sub>) and RNA (V<sub>n</sub>) represent a much smaller fraction of the total. Lipid glycerol synthesis remains nearly constant, but the incorporation of ribose into RNA rises almost 3-fold during the hour. Since ribose utilization (V<sub>b</sub>) increases at about the same rate as V<sub>b</sub>, the amount of ribose entering the remainder of the metabolic pathway remains constant.

The flux through hexose phosphate isomerase (V<sub>2</sub> and V<sub>3</sub>) was fixed to within about 20% during the first 20-min interval and to within about 40% thereafter. The value of V<sub>2</sub> is not large, relative to V<sub>6</sub>, and the specific activities of glucose-6-P and fructose-6-P calculated by the model for the best fit values are not equal (data not shown), as expected since there is an upper limit to the acceptable values of V<sub>2</sub>. V<sub>2</sub> increased during the 1-h incubation, although the exact pattern of this increase is not clear because of the range of uncertainty in this parameter. In contrast to this, the flux through triose phosphate isomerase (V<sub>4</sub> and V<sub>5</sub>) could be made as large as one desired without disturbing the goodness of fit, and even at their lowest permissible levels was sufficiently large to ensure that dihydroxyacetone-P and glyceraldehyde-3-P were close to isotopic equilibrium throughout the incubation. Although the net flux through glycerol-3-P dehydrogenase (V<sub>n</sub> - V<sub>n</sub>) remains constant at about 40 nmol/10<sup>6</sup> cells/h in the direction of glycerol-P oxidation, both V<sub>n</sub> and V<sub>n</sub> increased significantly during the hour. In so far as we are aware this constitutes the first description of a seemingly pointless bidirectional flux of reduc-

Although there is at most a very small flux of metabolites through the oxidative steps of the pentose pathway, there is clearly an appreciable flux through all the nonoxidative steps. Despite the large number of measurements, the data are not sufficient to estimate all these fluxes with precision. In particular, varying any of the fluxes, V<sub>1</sub> (ribulose-P epimerase), V<sub>8</sub> (ribose-P isomerase), or V<sub>9</sub> and V<sub>11</sub> (transketolase), had qualitatively similar effects. Since the effects of raising any of these parameters could largely be compensated by lowering another, they were free to vary over a wide range. To determine the permissible ranges, V<sub>1</sub> and V<sub>8</sub> were varied as a pair, as were V<sub>9</sub> and V<sub>11</sub>. As shown in Table IV, V<sub>1</sub> and V<sub>8</sub> had no upper bound, but if they were taken to be large, V<sub>9</sub> and V<sub>11</sub> had to be lowered to their minimum value of about 10 nmol/10<sup>6</sup> cells/h. If V<sub>9</sub> and V<sub>11</sub> were kept at their best fit values (20 nmol/10<sup>6</sup> cells/h), then V<sub>1</sub> and V<sub>8</sub> had an upper limit of about 300 nmol/10<sup>6</sup> cells/h. Similarly, there was an obligatory association between the minimal acceptable values for V<sub>9</sub> and V<sub>11</sub> and the upper limits of V<sub>n</sub> and V<sub>n</sub> for the best fit, an intermediate value of 20 nmol/10<sup>6</sup> cells/h was chosen for V<sub>n</sub> and V<sub>n</sub> and this was found to be associated with values for V<sub>9</sub> and V<sub>11</sub> of 150. Although, as just discussed, this combination is not unique, several important conclusions may be drawn nevertheless. V<sub>2</sub> and V<sub>8</sub> must be at least 40 nmol/10<sup>6</sup> cells/h during the first part of the incubation and flux through these steps must be at least twice this at later times. Similarly, the fluxes V<sub>n</sub> and V<sub>n</sub>, which represent the "backward" reactions of the pentose cycle, must be at least as large as the net flux through these reactions at all times, no matter what values are chosen for V<sub>1</sub> and V<sub>8</sub>.

In contrast to the relative indeterminancy in the magnitude of flow through these reactions, the flux through the remaining nonoxidative reaction, catalyzed by transaldolase (V<sub>10</sub> and V<sub>10</sub>), could be determined fairly precisely. Flux through transaldolase was of the same magnitude as that through the other nonoxidative steps of the pentose phosphate pathway, but unlike the other reactions, the data required that flux through transaldolase increase about 3-fold during the hour. (From the range of acceptable values for V<sub>n</sub> it would seem that a smaller increase is possible than was chosen. This would, however, necessitate having certain calculated values for incorporation fall at one extreme of the data at one time point and at the opposite extreme at the next point. Thus although the increase in V<sub>n</sub> may not be quite as linear as depicted in Fig. 1, it is unlikely that the flux through transaldolase differs markedly from this.) It should also be noted that the bidirectional flux through transaldolase, like that through the other nonoxidative reactions, is large relative to the net flux.

Unlike methods based on the use of tritium-labeled substrates which are subject to error because of uncertainty in the extent of tritium exchange *in vivo* (25), the present method provides a relatively unambiguous estimate of the extent of futile cycling at the phosphofructokinase (V<sub>3</sub>/fructose-1,6-diphosphatase (V<sub>5</sub>) couple. Table IV shows that V<sub>3</sub> increases about 7-fold during the course of the 1-h incubation, and that this is accompanied by a comparable increase in V<sub>5</sub>. At each time interval, V<sub>3</sub> is at least two-thirds of V<sub>5</sub>, so that futile cycling represents an appreciable portion of the total flux through phosphofructokinase. It should also be stressed that there is a large increase in net flux in the direction of glycolysis through this pair of steps, going from ~15 nmol/10<sup>6</sup> cells/h at the beginning of the 1-h incubation to about 100 at the end. Precise determination of the limits of V<sub>n</sub> is complicated by the fact that manipulation of V<sub>1</sub> and V<sub>n</sub> has the same qualitative effect on the fit of the data as does manipulation of V<sub>n</sub>. For the best fit we assumed V<sub>1</sub> to be constant at 500 nmol/10<sup>6</sup> cells/h. For higher values of V<sub>1</sub>, the limits on V<sub>n</sub> drop somewhat, but even for a constant, very rapid exchange at aldolase, the minimal values for V<sub>n</sub> are 35, 90, and 150 nmol/10<sup>6</sup> cells/h at 0 to 20, 20 to 40, and 40 to 60 min, respectively. If V<sub>1</sub> is permitted to increase with time, then the increase in V<sub>n</sub> becomes less pronounced; but in order to have V<sub>n</sub> remain nearly constant, it is required that V<sub>n</sub> increase by 3 to 4 orders of magnitude during the hour, which is highly unlikely since aldolase is not generally considered to be regulated and since the activity of this enzyme as assayed *in vitro* is large (Table IV).

The conclusion that there is simultaneous operation of the
Analysis of Pentose Phosphate and Glycolytic Pathways

Incorporation of 6-14C-glucose into carbon 1 of glucose in glycogen

Cells were grown and incubated for times up to 1 h with the standard substrate mixture containing 6-14C-glucose. Glycogen was isolated and the glucose decarboxylated as described under "Materials and Methods." The percentage of radioactivity in glucose released by decarboxylation is given in the row labeled "Observed" for two experiments (labeled I and II); each value represents the mean of a triplicate determination. The reaction rates from Table IV were used with the model to compute the relative specific activity of the glucose-6-P pool, and the percentage of label calculated to be in Position 1 is given in the row marked "Calculated." The calculated amount of label appearing in Carbon 1 of glucose-6-P when 6-14C-glucose was the labeled substrate, because this is the only combination of reactions which will cause such a redistribution of carbon. Since glycogen is assumed to have glucose-6-P as its precursor, experiments were performed in which cells were incubated with 6-14C-glucose, and the glycogen was degraded to glucose and decarboxylated as described under "Materials and Methods" (Appendix II). The results (Table V) show that label does appear in Carbon 1 of glucose-6-P, thereby confirming the operation of a futile cycle at V2 and V3. That the measured incorporation of 6-14C-glucose into Carbon 1 of glucose-6-P is comparable to the amount predicted at each time from the flux values of Fig. 1 constitutes strong support for the values of V3 and V4, chosen in the best fit. It should also be recalled that the amount of label appearing in Carbon 1 of glucose-6-P also depends on other flux values. The agreement between predicted and measured values thus further supports the entire flux configuration of Fig. 1.

Although, on the whole, the model gives a close fit to the data, in some cases the predicted values for incorporation of label into product do not agree well with the observed values. For example, the model predicts that incorporation of 1-14C-glucose and 2-14C-glucose into both glycogen and lipid glycerol should be practically equal (see Table III) whereas about 20% more 1-14C-glucose than 2-14C-glucose was incorporated into glycogen and slightly less into lipid glycerol. No choice of fluxes was capable of producing this result. While it is possible that some additional pathway might be added which would account for this discrepancy, the magnitude of the difference is not, in our opinion, sufficient to warrant a conclusion that the metabolic scheme needs modification. Similarly, although the predicted values for incorporation of U-14C-fructose and 2-14C-fructose into both glycogen and lipid glycerol should be practically equal (see Table III) whereas about 20% more 1-14C-fructose than 2-14C-fructose was incorporated into glycogen and slightly less into lipid glycerol. No choice of fluxes was capable of producing this result. While it is possible that some additional pathway might be added which would account for this discrepancy, the magnitude of the difference is not, in our opinion, sufficient to warrant a conclusion that the metabolic scheme needs modification.

DISCUSSION

The present work quantitates the flux of carbon through the reactions of glycolysis and the pentose phosphate pathway in Tetrahymena, thus complementing earlier work analyzing the 2- and 3-carbon metabolism of this ciliate (1). In contrast to our earlier studies, however, the metabolism of Tetrahymena upon exposure to carbohydrates was time-dependent, i.e. the rates of incorporation of label from substrates into some of the products measured changed during the 1-h incubation. Although the model used to analyze our data was developed for steady state conditions, it was found to be applicable for several reasons. First, in each 20-min interval the incorporation data into all products from all labeled substrates was nearly linear, as expected for a system in the steady state. Second, measurements of the pool sizes of glucose-6-P and fructose-6-P (the two intermediates which might be expected to undergo the largest changes in amount) indicated that they change only slowly after the first 5 min of incubation. Therefore the
pools change size slowly relative to the flux through them, so that the conditions for isotopic and metabolic steady state are closely approximated. Third, that cycloheximide prevents the acceleration in rate of incorporation of label from glucose into CO₂ suggests that slow changes in enzyme levels are responsible for the observed nonlinearity. Therefore, at least after the first few minutes, the system can be treated as a quasi-steady state system in which changes in enzyme levels and pool sizes are so slow that the steady state derivation is applicable. Although we may expect that the flux values computed for the first 20-min interval to be less accurate than for the two subsequent intervals, the general closeness of the computed values to the data even in the first 20-min period supports the validity of the analysis. The patterns shown in Fig. 1 thus constitute a realistic picture of carbon flow along the glycolytic and pentose phosphate pathways in *Tetrahymena* during the 1st h of adaptation to addition of a mixture of glucose, fructose, glycero, and ribose to cells in the transition phase of growth.

**Glucose Utilization and Glycogenesis**—It is well known that *Tetrahymena* has a high capacity for glycogeneogenesis (21) and for glycogen synthesis from glucose (5, 8). Glucose uptake appears to be mediated by a phlorizin-sensitive, sodium-dependent, stereospecific carrier-mediated process (26). Glucose uptake was very high even during the first 20 min after addition of the substrate mixture and rose significantly with time (Fig. 1). Fructose, which is thought to be transported by the same carrier system (26), however, is used at a constant rate throughout the hour. The disparity between the constancy of fructose utilization and the small but important increase in glucose utilization is difficult to explain since it is thought that the two sugars are transported by the same carrier and phosphorylated by the same kinase. It is noteworthy that glycogen synthesis during the first 20-min interval proceeded at over 9% of the rate attained in the subsequent intervals. Thus, it appears that *Tetrahymena*, growing in the absence of carbohydrates, maintains a near maximal capacity for hexose transport, phosphorylation, and glycogen synthesis. If there are changes in the activities of the enzymes involved in glycogen synthesis from glucose-6-P in *Tetrahymena* they must occur very rapidly. It is highly unlikely, therefore, that the decrease in levels of adenyl cyclase and of cyclic AMP which occur in response to long term growth of *Tetrahymena* in proteose/peptone media supplemented with glucose (37), play any role in the regulation of glycogen synthesis from glucose in the present experiments.

**Glycolysis**—During the hour of incubation an ever increasing amount of carbon enters the glycolytic pathway. The observations that the appearance of label from glycero in CO₂ and of label from glucose and fructose in glycero are linear with time (Table II, Appendix II) localize the controlled step(s) between the hexose phosphates and the triose phosphates. Flux through phosphofructokinase, which is smallest of all the reactions of the upper portion of the glycolytic pathway (Fig. 1) increases 7-fold over the course of the hour. In view of the known regulatory properties of this enzyme (28), it seems likely that the increase in flux at this step is of primary importance in producing the acceleration of glycolysis. This cannot be the only control point, however, since the increase in flux through phosphofructokinase is accomplished without a decrease in the pool size of fructose-6-P (Table I, Appendix II). This indicates that an increase in input of this pool must occur. Part of this must result from the increase in glucose phosphorylation (Vc). Although this increase appears small (going from 930 to 1045 nmol/10⁶ cells/h), it is not accompanied by an increase in glycogen synthesis and thus must result in an increase in catabolism. It should be noted, however, that the net flux through hexose phosphate isomerase is in the direction of glycogen synthesis. Thus the increase in rate of glucose-6-P formation is accompanied by a decreasing utilization of fructose-6-P for glycogen synthesis and the increasing catabolism of this intermediate. It appears, therefore, that both hexokinase and phosphofructokinase mediate the augmentation of glycolysis, which is of interest since both enzymes have been reported to be rate-limiting in *Tetrahymena* (2, 10) and both enzymes are in large part localized on the mitochondria of this cell (11, 121). Several roles for this sort of partitioning are possible: (a) bound enzyme may be closer to the site of ATP production and hence more effective in phosphorylation; (b) bound enzyme may have different kinetics from the free enzyme; (c) adsorption to particles may make the enzyme inaccessible to the cytoplasmic pool of substrate, possibly creating separate pools of substrates. Experiments on cells grown to the logarithmic and stationary phases of growth with or without glucose supplementation failed to reveal any changes in intracellular distribution of phosphofructokinase (12) or hexokinase (11), but such experiments do not rule out the possibility of more rapid changes in localization or in kinetic properties of these enzymes. In view of these considerations and the finding that cycloheximide prevented the acceleration of glycolysis, further experiments on the intracellular distribution and properties of these enzymes are warranted.

Gumaa and McLean (29), in an extensive study of the pentose phosphate pathway in ascites tumor cells, found that prior to the addition of glucose, the ratio fructose-6-P/glucose-6-P was about 0.86. Within 10 min after glucose addition, a steady state was achieved in which this ratio dropped to 0.36, a value close to the apparent equilibrium constant (0.32 to 0.47; see Table 5 of Ref. 29) of hexose phosphate isomerase. In *Tetrahymena* the ratio in proteose/peptone was 0.14 (Table I, Appendix II). Within 5 min this ratio rose to 0.52 and by 30 min after the addition of the substrate mixture dropped slightly to 0.41. Thus the mass action ratio for hexose phosphate isomerase changes in the opposite direction to that observed in ascites cells (possibly because of the addition of fructose as well as glucose), but is never very far from the ratio expected if the reaction were close to equilibrium. The 3.5-fold increase in bidirectional flux through this step is presumably a consequence of the increase in pool sizes of both fructose-6-P and glucose-6-P. Initially, the flux in each direction is only about 5 times the net flux, but this increases to 16 times and 35 times the net flux in the 20 to 40- and 40 to 60-min intervals, respectively, so that one would anticipate that fructose 6-P and glucose-6-P would be at isotopic equilibrium. In fact, however, examination of the computed specific activities of each carbon of glucose-6-P and of fructose-6-P shows that even in the 40 to 60-min interval complete equilibration is not achieved. In the 40 to 60-min interval, with [1-¹⁴C]glucose as the labeled substrate, for example, the specific activities (relative to the 1-¹⁴C]glucose in the medium) in Carbon 1 of glucose-6-P and fructose-6-P are 0.73 and 0.71, respectively, and in Carbon 6, 0.062 and 0.067, respectively. Thus even a bidirectional flux 35 fold higher than the net flux does not yield isotopic equilibration when, as in the present case, there is a large influx of labeled substrate into one of the two pools under consideration. The effect of the large flux values on pool equilibration is not limited to the case when the label is on an immediate precursor of one of the pools. If the labeled substrate is [U-¹⁴C]ribose, for example, the relative specific activi-
ties in Carbon 1 of glucose-6-P and fructose-6-P are 0.0024 and 0.0031, respectively.

Futile Cycle at Phosphofructokinase Fructose-1,6-diphosphatase – In addition to the 7-fold increase in flux through phosphofructokinase there was also a 7-fold increase in flux through fructose-1,6-diphosphatase (Fig. 1). These increases were not only necessary to achieve a fit to the incorporation data of Table III but also were verified by the independent determination of the incorporation of label from [6-14C]glucose into the 1 position of the glucosyl moiety of glycogen (Table V). It must be emphasized that, contrary to the several uncertainties attending interpretation of tritium exchange measurements, as discussed in detail by Katz et al. (25), the present measurement of the magnitude of this apparently futile cycle depends only on the assumption that the reactions occur according to the scheme shown in Fig. 1 in a single compartment, an assumption which in any case is necessary for estimation of this futile cycle by tritium exchange methods. It can be seen from Fig. 1 that the amount of futile cycling between fructose-6-P and fructose 1,6-diphosphate (defined as 100· V' \text{,} V_0) is very large, ranging from 70 to 80% of the flux through the forward reaction. This is considerably larger than the values (ranging up to 40%) estimated for other cells (30, 31). The reason for such a large futile cycle is not known; it may be related to the very high glyconeogenic capacity of this cell. In cells grown identically but washed free of proteose/peptone and suspended in a mixture of acetate, pyruvate, glutamate, hexanoate, and bicarbonate, the glyconeogenic flux from P-enolpyruvate was 255 nmol/10^6 cells/h (1), which is considerably larger than the net glycolytic flux (V_0 - V') at the phosphofructokinase step measured here in the presence of glucose and fructose. It should be noted that Sato et al. (32) have provided evidence that label exchange via reversal of phosphofructokinase may occur in vivo. If such reversal occurs in vivo, the magnitude of the futile cycle would be correspondingly overestimated.

Glycerol Metabolism and Fatty Acid Synthesis – Whereas in many species carbohydrate is an excellent precursor of fatty acids we had earlier reported that practically no label from [1-14C]glucose appeared in the fatty acids of Tetrahymena (5). The present results extend this observation; even in the presence of glucose, fructose, ribose, and glycerol, fatty acid synthesis became undetectable after the first few minutes of incubation (Fig. 4). The reason for this cessation of fatty acid synthesis in the presence of carbohydrate is unknown, but it should be noted that triglyceride does not appear to function as a reserve fuel in Tetrahymena (33). Since earlier work has shown that Tetrahymena converts exogenous pyruvate to acetyl-CoA in a compartment not associated with lipogenesis (34), the present work demonstrates that the metabolism of pyruvate from 6- and 3-carbon precursors is similar to that of exogenous material. Although fatty acid synthesis stops soon after the substrate mixture is added, a small amount of lipid glycogen continues to be formed (Fig. 1). Most of the glycerol utilized, however, enters the glycolytic pathway at a rate (~40 nmol/10^6 cells/h) which does not change appreciably during the incubation. Although it is frequently assumed that the reaction catalyzed by glycerol-P dehydrogenase is near isotopic equilibrium, this is clearly not so during the first 20 min after addition of the substrate mixture, when the unidirectional fluxes (V_{0G}, V_{AG}) are less than 4 times the net flux (V_{0G} - V_{AG}). During the 40 to 60-min interval, equilibration is approached if the label is in glucose, fructose, or ribose. With [U-14C]ribose, for example, the relative specific activities of Carbon 3 of dihydroxyacetone-P and glycerol-P are 0.0063 and 0.0054, respectively, and with [1-14C]fructose as the labeled substrate, 0.079 and 0.088, respectively. If the labeled substrate is [2-14C]glycerol, however, the relative specific activity of Carbon 2 of dihydroxyacetone-P is 0.088 while that of glycerol-P is 0.22.

Pentose Phosphate Pathway – Previous work in our laboratory had indicated that the nonoxidative portions of the nutrient phosphate pathway played a role in the metabolism of carbohydrates (4, 5) even though the oxidative portion of this pathway appeared to be absent (2-4). In this work we have been able to quantitate the fluxes through the pentose phosphate pathway under a particular set of conditions. The results show that the in vivo flux through the oxidative portion (V_1) is very small, amounting to less than 1% of glucose utilization. During the first 20 min of incubation, however, it represents a significant fraction of the total glycolytic flux. The observed flux is very close to the barely measurable in vivo activity of glucose-6-P dehydrogenase. The function of this very small flux is unknown.

The role of the nonoxidative reactions of the pentose phosphate pathway in the synthesis of the pentose moieties of nucleic acid has been in dispute. The pattern of label incorporation from [2-14C]glucose into pentose led earlier workers to conclude that the nonoxidative steps were important (35, 36), but Katz and Rognstad (37) pointed out the necessity of distinguishing between incorporation of radioactivity via the exchange reactions of transketolase and transaldolase and net synthesis of pentose from hexose. Studies with organisms lacking the oxidative enzymes (38-41) show clearly, however, that under some conditions the nonoxidative reactions are capable of the net conversion of hexose to pentose. Our results with Tetrahymena demonstrate that although RNA is synthesized in significant quantities, and label from both ribose and glucose is incorporated into RNA, the net movement of carbon under these experimental conditions is from pentose to hexose.

Although the rate of nucleic acid synthesis is less than the uptake of ribose, a close link between the two processes is indicated by the temporal correlation between the increase in ribose uptake and the increase in RNA synthesis (Fig. 1). It would be interesting to see if the pentose moieties of RNA can be synthesized from glucose in the absence of added ribose.

It is generally recognized that the nonoxidative reactions of the pentose phosphate pathway can serve as a salvage pathway for ribose, and the ability of Tetrahymena to synthesize glycogen from added ribose (4, 42) clearly testifies to this role. In nature, where bacteria are thought to be the main food, this would permit utilization of the pentose moieties of the ingested nucleic acids. The nonoxidative portions of the pentose phosphate cycle may also play an important role during starvation, when large quantities of RNA are degraded (43, 44). Although much of the phosphate and bases were released into the medium, Leboy et al. (43) found that very little ribose was released unless iodoacetate was added. These results suggest that Tetrahymena can utilize the pentose moieties that are made available from RNA degradation during starvation and indicate another function for the nonoxidative portion of the pentose phosphate cycle.

The present work provides the first quantitative analysis of bidirectional flux through the nonoxidative reactions of the pentose phosphate cycle for any organism. Although measurements considerably in excess of the number of independent parameters were made and the measurements were fairly well distributed, we did not have sufficient data to determine the
ATP utilization that would have been realized if there had been no futile cycle (i.e. if the fructose-1,6-diphosphatase activity were zero but the same net flux had gone through the phosphofructokinase step). There would have been only a 1% reduction in ATP utilization in the first 20-min interval, but during the last 20-min interval a 9% reduction in ATP consumption would be realized if there were no futile cycling. Thus as the cell adapts to the catabolism of carbohydrates, a considerable portion of its consumption of ATP is apparently "wasted." Obviously, we are here neglecting the energy cost of nucleic acid synthesis, ion pumping, swimming, etc. If one could account for these modes of ATP consumption, then even during the 40 to 60-min interval, the fraction of total ATP consumption due to fructose-1,6-diphosphatase activity would be very small. In terms of the consumption of ATP within the framework of the pathways of intermediary metabolism encompassed in Fig. 1, however, the futile cycle does account for an appreciable amount of the ATP consumed in the latter part of the incubation.

Of considerable interest is the result that during the first 20 min after addition of the carbohydrate mixture, 583 nmol of ATP are used (per 10^8 cells) in excess of the amount produced. Since the content of ATP is about 17 nmol/10^8 cells (47), this deficit obviously could not be made up by depleting the cell ATP. Thus in the early stages of adaptation to the carbohydrate mixture the cell must use other fuels, probably amino acids from the medium, in order to maintain a positive energy balance.

The oxygen consumption results support this. The theoretical Q_0, due to oxidation of the added substrates can be computed from the fluxes of Fig. 1 since 0.5 mol of O_2 is required to reoxidize every mol of reduced pyridine nucleotide produced. The total production of NADH + NADPH (including NADH generated in the Krebs cycle) is given by the sum 2V_{12} + V_{24} + V_{ik} + V_{ok} - V_{ud} + 4V_{4}. The rate of O_2 consumption due to oxidation of sugars and glycerol is therefore 270 nmol/10^8 cells/h.

### Table VI

**Rates of formation and utilization of ATP during incubation of Tetrahymena with glucose, fructose, ribose, and glycerol**

Reactions (a) through (f) consume ATP at the rates shown for each interval during the 1-h incubation. The flux values are taken from Fig. 1. The sum of these values is shown as ATP consumption. Subtraction of V_3, corrects the net consumption of ATP to the value which would be computed if there were no futile cycling at the phosphofructokinase step. Reactions (h) through (i) produce ATP at the rates shown; subtraction of ATP consumption from ATP production then yields the net rate of production of ATP in each interval.

| Reaction | ATP Flux | Interval | nmoles ATP utilized/10^8 cells |
|----------|----------|----------|-----------------------------|
| (a) Hexokinase (glucose) | V_1 | 0-20 min | 930 |
| (b) Hexokinase (fructose) | V_r | 0-20 min | 194 |
| (c) Ribokinase | V_k | 0-20 min | 18 |
| (d) Glycerol kinase | V GL | 0-20 min | 55 |
| (e) Phosphofructokinase | V_4 | 0-20 min | 63 |
| (f) Glycogen synthesis | 2V_{12} | 0-20 min | 2220 |
| (g) Fructose-1,6-diphosphatase | V_3 | 0-20 min | 48 |

**ATP consumption if no futile cycle:**

| Reaction | ATP Flux | Interval | nmoles ATP formed/10^8 cells |
|----------|----------|----------|-----------------------------|
| (h) Triose phosphate → 3-phosphoglycerate | V_6 | 0-20 min | 80 |
| (i) NADH oxidation | 3V_6 | 0-20 min | 240 |
| (j) Pyruvate kinase | V_{24} | 0-20 min | 80 |
| (k) Aceto-CoA oxidation | 3V_4 | 0-20 min | 240 |
| (l) α-Glycero-phosphate dehydrogenase | 3(V_{ok} - V_{ud}) | 0-20 min | 132 |

**ATP production:**

**Net production of ATP:**

- 1748
- 234
- 916

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*V_{12} includes the conversion of G6P to G1P, the formation of UDPG, and the transfer of the glycosyl group to glycogen.

*It is assumed that the P:O ratio is 3 when NADH is oxidized in the mitochondria of Tetrahymena.*
h in the first 20-min interval and increases to 546 and 760 nmol/10^6 cells/h in the next two intervals. Since the measured Qo is about 5000 nmol/10^6 cells/h (see Appendix II), the oxidation of material present in the medium is clearly providing most of the ATP for the energy needs of the organism. The small difference between the Qo, in the presence and absence of added substrates is consistent with a small contribution of carbohydrate to the total oxygen consumption. It should be noted that at later times (see Table VI), the glycolytic flux has increased sufficiently so that carbohydrate oxidation alone would be sufficient to meet the energy needs of the cell.

Adequacy of Metabolic Scheme—Although the model employed gives a detailed description of *Tetrahymena* metabolism whose accuracy is demonstrated by the close match of most of the computed incorporations to the experimental data, a few of the measurements were not fit by the model. While some of these failures to achieve excellent fit may represent technical errors, it is also possible that some assumption is not satisfied. Aside from the assumption of the steady state, the validity of which has been discussed in detail, the only other assumptions relate directly to the structure of the metabolic system as depicted in Fig. 1. These fall into two classes—deliberate oversimplifications and possible errors.

Several oversimplifications are apparent in Fig. 1. Thus an input of P-enolpyruvate from reactions of the Krebs and glycolytic cycles and associated pathways has been neglected, and the fate of acetyl-CoA has been reduced to a single reaction. Possible consequences of these simplifications have been discussed where relevant under "Results" and will not be repeated here. The only way to ascertain whether any of the data that are not well fit could be more closely matched would be to set up a complete model in which no simplifications are made, i.e. one which combines the elements of Fig. 1 of this paper with those of Fig. 1 of Raugi et al. (1). Such work is currently in progress.

Of more fundamental concern is whether the scheme shown in Fig. 1 is structurally correct. As noted above, 5 of the glycolytic enzymes in *Tetrahymena* are known to be particulate as well as cytosolic (3, 11-15) and it is possible that compartmentation of intermediates, which has been shown to be important in the case of acetyl-CoA (34), plays an important role in the glycolytic pathway. The measurements which necessitate an increasing rate of hexose phosphate isomerase are those of the relative incorporation of fructose and glucose into glycogen. These data could be fit, at least qualitatively, by a model in which there were two pools of glucose-6-P, one fed by glucose and the other by fructose-6-P. If this were the case, the flux through hexose phosphate isomerase could be large and constant, and the observed data reproduced by a progressively greater mixing of the glucose-6-P pools. Although several authors have presented data indicating the presence of two pools of glucose-6-P (and, indeed, of several other glycolytic intermediates) in other tissues (16-19, 48) we believe that it would be premature to invoke the presence of multiple pools of any glycolytic intermediates in *Tetrahymena* until experiments using a full model with no simplifications have been performed and analyzed.

Kinetics of Adaptation to Presence of Carbohydrates—Since low concentrations of cycloheximide prevent the increase in rate of ^14CO₂ formation from labeled glucose whether the cycloheximide is added together with the substrate mixture or half way into the hour of incubation, it is reasonable to suppose that protein synthesis or protein modification is responsible in part for the adaptation process. One may therefore ask whether any simple model for the adaptation process would be consistent with the observed shapes of the curves of label incorporation into products with time (see Fig. 2).

Suppose that the activity of a rate-limiting enzyme is A₀ + A₁t, where A₀ is the activity at the start of the incubation and A₁ is a measure of the rate of increase of activity of this enzyme with time. The amount of product, P, formed by this enzyme would then be given by

\[ P = \int_{0}^{t} (A₀ + A₁t')dt' = A₀t + \frac{A₁t²}{2} \]  

If one assumes that the rate-limiting enzyme of glycolysis is phosphofructokinase, then one would expect a parabolic increase in the amount of label appearing in CO₂ after the addition of the carbohydrate mixture. We chose to fit the data to a power function of the form \( I = at^b \). It can be shown that a parabola of the form \( P = A₀t + A₁t²/2 \) when fit to a power function of the form chosen results in a value of \( b \) between 1 and 2, depending on the relative magnitudes of the linear and quadratic terms of \( P \). Since the average value for the exponent \( b \) in the equations for ^14CO₂ accumulation from [1-^14C]glucose and fructose is 1.86, it may be considered that the activity of phosphofructokinase increases linearly with time after addition of the carbohydrate mixture. This does not necessarily imply that the amount of this enzyme is increasing with time, since the rise in activity might be achieved by modification of the enzyme, synthesis of an activator, etc. Further experiments are obviously required to ascertain the factors controlling the increase in flux through phosphofructokinase.

The formalism of Equation 4 can also characterize the shapes of most of the incorporation of label versus time curves for the other products measured. Thus for the rate-limiting step in glycogen synthesis, the choice \( A₁ \sim 0 \), would predict a linear incorporation of label from all substrates into glycogen, as observed (Table II, Appendix II).

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Analysis of Pentose Phosphate and Glycolytic Pathways

Supplemental Material

Quantitative Analysis of the Pathways of Nucleotide and Nucleotide Metabolism in Human Fibroblasts

Michael J. Marullo, Robert B. Staal and Jacob J. van Heyningen

Abstract

The important role of nucleotides and the pentose-phosphate pathway in the synthesis of nucleotides has been well documented [1]. We have developed a method to determine the rates of nucleotide synthesis and the pentose-phosphate pathway in human fibroblasts. This method is based on the measurement of nucleotide synthesis rates and the determination of the rates of pentose-phosphate pathway.

For the 7th intermediate, in the metabolic analysis of the 7th intermediate, the data obtained from the 7th intermediate is analyzed to determine the relative rates of synthesis of the 7th intermediate. The results show that the relative rates of synthesis of the 7th intermediate are significantly different from those of the 6th intermediate. The results of the metabolite analysis are consistent with the observed differences in the relative rates of synthesis of the 7th intermediate.

Figure 1: Structure of pyrimidine and purine nucleotides. The relative rates of synthesis of the 7th intermediate are significantly different from those of the 6th intermediate. The results of the metabolite analysis are consistent with the observed differences in the relative rates of synthesis of the 7th intermediate.

Figure 2: Estimation of the relative rates of synthesis of the 7th intermediate. The results show that the relative rates of synthesis of the 7th intermediate are significantly different from those of the 6th intermediate. The results of the metabolite analysis are consistent with the observed differences in the relative rates of synthesis of the 7th intermediate.

Table 1: Effect of Perturbation of Selected Parameters of the Nucleotide and Nucleotide Pathways on the Rates of Nucleotide Synthesis

| Parameter | Effect of Perturbation | Rate of Nucleotide Synthesis |
|-----------|-----------------------|-----------------------------|
| GTP        | Increases by 50%       | +50%                        |
| ATP        | Increases by 25%       | +25%                        |
| ADP        | Increases by 75%       | +75%                        |
| AMP        | Increases by 100%      | +100%                       |
| Pyruvate   | Increases by 150%      | +150%                       |
| Glucose    | Increases by 200%      | +200%                       |
| Fructose   | Increases by 250%      | +250%                       |

These results show that the rate of nucleotide synthesis is significantly affected by the perturbation of selected parameters. The results are consistent with the observed differences in the relative rates of synthesis of the 7th intermediate.

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Analysis of Pentose Phosphate and Glycolytic Pathways

The table also depends on the rates of substrate utilization do not alone.

The effect of phosphoribosyl pyrophosphate (PRPP) on the pentose phosphate pathway was studied. PRPP, a key intermediate in the pathway, was found to inhibit the reaction at a site before glucose-6-phosphate dehydrogenase (G6PDH). This inhibition is thought to be a result of the nucleotide's ability to act as a competitive inhibitor for PRPP, leading to a decrease in the overall flux through the pathway.

The following equation is used to describe the inhibition of G6PDH by PRPP:

$$ V = V_{max} \frac{[S]}{K_s + [S]} $$

where $V$ is the steady-state velocity, $V_{max}$ is the maximum velocity, $[S]$ is the substrate concentration, and $K_s$ is the Michaelis constant. By measuring the inhibition constant ($K_i$), it is possible to determine the affinity of PRPP for G6PDH and thus understand the mechanism of inhibition.

To determine if $K_i$, Figures 4 and 5 show plots of the ratio of $V_{obs}$ to $V_{calc}$ at various substrate concentrations. The ratio is calculated as $V_{obs}/V_{calc}$, with $V_{obs}$ being the observed velocity in the presence of PRPP and $V_{calc}$ being the calculated velocity in the absence of PRPP. The ratio is plotted against the substrate concentration on a semi-log scale, and a straight line is fitted to the data points. The slope of the line is used to determine the inhibition constant, which is defined as:

$$ K_i = -\frac{dln(V_{obs}/V_{calc})}{dln[S]} $$

This method is used to determine the inhibition constant for PRPP on G6PDH, providing insights into the mechanism of inhibition and the role of PRPP in regulating the pentose phosphate pathway.

In summary, the data collected in this study demonstrate the role of PRPP in the pentose phosphate pathway and provide a quantitative measure of the inhibition constant. These findings contribute to our understanding of the metabolic pathways that regulate the production of pentoses and the utilization of carbohydrates in cells.
Analysis of Pentose Phosphate and Glycolytic Pathways

and of the fate of NADH and NADPH, the reactions of these pathways in cells are often described in terms of the redox potentials of the GSSG/GSH and NADH/NAD+ couples. However, the redox potentials of these couples at the cytoplasmic level are often close to their standard potentials, and the redox potentials of the intermediates of the pathways are often close to the redox potentials of the end products. Therefore, the main driving force for the reactions of these pathways is often the concentration gradients of the end products, rather than the redox potentials.

It is important to note that the reactions of the pentose phosphate pathway and the glycolytic pathway are often coupled, and the reactions of these pathways are often influenced by the concentrations of the end products of these pathways. For example, the reactions of the pentose phosphate pathway are often regulated by the concentration of NADPH, which is the end product of the pathway. Similarly, the reactions of the glycolytic pathway are often regulated by the concentration of ATP, which is the end product of the pathway.

It is also important to note that the reactions of the pentose phosphate pathway and the glycolytic pathway are often connected by the reactions of the tricarboxylic acid cycle, which is the main pathway for the oxidation of carbohydrates and fatty acids. Therefore, the reactions of these pathways are often connected by the reactions of the tricarboxylic acid cycle, and the reactions of these pathways are often influenced by the concentrations of the end products of the tricarboxylic acid cycle.

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Quantitative analysis of the change of metabolite fluxes along the pentose phosphate and glycolytic pathways in Tetrahymena in response to carbohydrates.

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