Effect of Oxygen on the Regulation of Intermediate Metabolism in Tetrahymena*

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SUMMARY

Tetrahymena grown in poorly aerated cultures have a greater capacity to utilize oxygen than cells grown in relatively well aerated cultures. Paradoxically, the oxidation of [1-14C]-glucose was inhibited, while oxidation of [2-14C]pyruvate and [2-14C]glyoxylate was enhanced in cells grown under anaerobic conditions. Total glycogen content measured after 17 hours of growth was increased 30 to 60% in cells grown partially anaerobically. In 1-hour incubations at the end of this time, the capacity to incorporate label into glycogen from [1-14C]glucose was unchanged, but label incorporation from [2-14C]pyruvate and [2-14C]glyoxylate was increased several fold. The ratio of adenosine di- and triphosphates was nearly identical in the cells grown under different conditions of oxygenation, indicating that this ratio may not play a major role in regulating these changes. After 17 hours of growth in cultures of different depths, cells were also incubated with a mixture of acetate, pyruvate, and octanoate, with one substrate labeled at a time in such a way that [1-14C]-acetyl-CoA is generated at the initial step in the metabolism of each. These results were interpreted in terms of a previously developed three-compartment model of acetyl-CoA metabolism. Glyconeogenesis from peroxisomal and mitochondrial precursors was increased in cells grown in low oxygen tension, with the greater contribution coming from the peroxisomes. Oxidation of acetate and pyruvate was increased under these conditions, but appearance of [1-14C]-acetyl-CoA label in glutamate was decreased. Lipogenesis from labeled peroxisomal precursors was also increased in cells grown under relatively low oxygen tension.

After a shift down in O₂ tension there is a rapid rise in glyconeogenesis from the peroxisomes which levels off after about 4 hours, whereas the rate of oxidation in the Krebs cycle increases steadily for at least 8 hours following the transition to relatively anaerobic conditions. In response to a shift up in O₂ tension there is a decline in peroxisomal glyconeogenesis which continues for 8 hours, whereas the rate of oxidation in the Krebs cycle does not begin decreasing until about 4 hours after the increase in O₂ tension. Thus the flux of [1-14C]acetyl-CoA changes according to a different temporal pattern in mitochondria as compared to peroxisomes, and in each compartment the sequence of changes in response to a shift up in O₂ tension is not the mirror image of the sequence in response to a shift down.

The ciliate *Tetrahymena pyriformis* can synthesize over 20% of its dry weight as glycogen from noncarbohydrate precursors (1) and is thus an excellent model cell for study of the regulation of glyconeogenesis. During the transition from the exponential to the stationary phase of growth, the rate of glyconeogenesis from lipid precursors and glycogen content increase markedly (2). Levy and Scherbaum (3) found that the characteristic increase in glyconeogenesis of transition phase cultures could be induced merely by changing the conditions of growth from a shaken, well aerated state to a static, partially anaerobic state. The 8- to 13-fold increase in glyconeogenesis from trace levels of [2-14C]acetate was associated with a doubling in 3 hours of the specific activities of isocitrate lyase and malate synthetase which comprise the glyoxylate by-pass of the oxidative decarboxylation steps of the Krebs tricarboxylic acid cycle and thus permit the net synthesis of glycogen from acetate or other sources of acetyl-CoA in this cell. Levy (4) later found that although the increase in isocitrate lyase and malate synthetase activity could be prevented by inhibitors of RNA and protein synthesis, the increase in incorporation of label from [2-14C]acetate was not prevented, indicating that activation of the glyoxylate by-pass could be achieved in the absence of protein synthesis. Hogg (5) has similarly found that agitation of *Tetrahymena* at low partial pressure of oxygen leads to an increase in glyconeogenesis without a large increase in isocitrate lyase activity. In the absence of pyruvate carboxylase (6), oxaloacetate formed from the product of the malate synthetase step is converted into phosphoenolpyruvate by phosphoenolpyruvate carboxykinase, which is localized in both the cytosol and the mitochondria of *Tetrahymena* (7). The specific activity of the cytoplasmic enzyme was 20-fold higher in standing cultures than in shaken cultures, while the activity of the mitochondrial enzyme remained at a high level independent of the degree of aeration. In this respect *Tetrahymena* is analogous to guinea pig liver, in that both enzyme forms are present and it is the cytosol phosphoenolpyruvate carboxykinase which increases under all conditions in which there is increased glyconeogenesis.

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In early studies on the utilization of labeled substrates by cultures of various ages, Connett and Blum (2) found that in transition phase cultures there was a marked increase in glycolygen content and in glyconeogenesis. More recently, Raugi et al. (8) compared the intracellular fluxes of acetyl-CoA in logarithmically growing cultures with the flux rates in cultures entering the stationary phase of growth. They found few differences between the logarithmically growing and the stationary phase systems and suggested that the difference in oxygen tension between their conditions (shallow well-agitated cultures) and those of Connett and Blum (deep well-agitated cultures) accounted for the marked differences in metabolic flow patterns between the two sets of experiments. It therefore seemed likely that oxygen tension played a central role in the regulation of glyconeogenesis in Tetrahymena. We have recently established that there are three pools of acetyl-CoA in Tetrahymena and have developed a formalism which permits the quantitative estimation of acetyl-CoA flux rates along the major synthetic and oxidative pathways of intermediate metabolism in this cell (8).

In this paper, we report that the three-pool structure which was adequate to describe control cells and cells in which glyconeogenesis was inhibited by tolbutamide (9) or by AMP (10) is also adequate for the quantitative description of intermediate metabolism in Tetrahymena under conditions where glyconeogenesis is enhanced. We also describe the time course of adaptation to a step change in oxygen tension. We show that the temporal sequence of changes depends on the direction of change in oxygen tension and varies according to an individual pattern for each compartment.

**Materials and Methods**

**Cells—**Tetrahymena pyriformis, strain HSM, were grown axenically in a medium containing 1% proteose peptone (Difco) and 0.05% concentrated liver extract in 0.02 M potassium phosphate buffer adjusted to pH 6.5 with NaOH. All stock cultures were grown in a gyrotary water bath in 500-ml Erlenmeyer flasks containing 100 ml of medium at 26°C.

For all experiments, the initial cell density was about 140,000 cells per ml. Cultures were routinely grown in total volumes of about 40, 80, or 120 ml in 500-ml Erlenmeyer flasks at 26°C in a gyrotary water bath for 17 hours. For convenience, we will hereafter refer to cultures grown in total volumes of 40 ml, 80 ml, and 120 ml as "shallow," "medium," and "deep," respectively. Cultures were started by inoculating 8, 16, or 24 ml of a logarithmically growing culture into 500-ml Erlenmeyer flasks containing 100 ml of medium at 26°C.

**Measurement of Acetyl-CoA Flux Rates**—The fungus was harvested from cultures grown in total volumes of 40, 80, or 120 ml, and 0.5 ml of Tris-HCl, pH 7.5. After mixing thoroughly and standing on ice for about 10 min, samples were centrifuged at 400 X g for 4 min, 0.3 ml of the clear supernatant was diluted 8-fold in water, and 1 ml was taken for assay. Total adenosine diphosphate and tritophosphates were measured by converting the ADP into 4-fold diluted aliquot of the same supernatant to ATP as described before (14), except that the pyruvate kinase reaction was run for 10 min instead of 5. One-milliliter samples were assayed. Standards of ATP and ADP were prepared in fresh proteose-peptone medium and treated exactly as above. It was shown that a perchloric acid extract of a cell-free supernatant of a 17-hour-old shallow, medium, or deep culture did not affect the assay or the conversion of ADP to ATP.

An extract (Worthington) was stored at 4°C until used. One vial of extract was reconstituted in 500 ml of 0.04 M sodium phosphate buffer, pH 7.4, and 0.01 M MgSO4. Five milliliters of this solution, which was made fresh daily and kept cold and dark, was placed in a counting vial. The Packard scintillation spectrometer was set to the tritium channel, with the coincidence in the "off" mode and the external standard "out." After squirting in 1 ml of sample, total gross counts were recorded for five consecutive 6-s determinations, and the fifth count was used in all our computations. The results were shown to be linear over a wide range of ATP concentrations. The number of counts observed in 6 s at this time was stable for at least 1 or 2 min.

**Measurement of Total Glycogen**—The glucose oxidase method was used for determination of total glycogen in a twofold washed 95% ethanol pellet from 3 ml of cell suspension. Details of this procedure have been described elsewhere (8).

**Measurement of Enzyme Activities**—Glutamate dehydrogenase and aspartate transaminase activities were measured on crude sonicated preparations as described by Porter et al. (15).

**Incorporation of Labeled Substrates**—Samples were done in 9 ml of standard Tri-Carb scintillation spectrometer. Counting flasks routinely used are described elsewhere (8). Incorporation into lipid, glycogen, CO2, and glutamate was measured during a 1-hour incubation as described before (8). Cells grown overnight in various culture volumes were pipetted into 50-ml Erlenmeyer flasks equipped with removable center wells for determination of incorporation of radioactive label into CO2 or into 125-ml Erlenmeyer flasks for measurement of incorporation of label into lipids, glycogen, and selected amino acids. Measurements were done in triplicate for each experiment.

Separation of alanine from the crude protease peptone-liver extract cell-free supernatant was accomplished by adding 1 ml of 0.1 M sodium carbonate of supernatant; half was removed by a brief centrifugation, and the trichloroacetic acid was removed by extraction with diethyl ether. The residue obtained by flash evaporation was redissolved in about 2 ml of water and applied to a Bio-Rad AG 1-X8 formate column as described before (8). The first 15 ml of the 0.03 M formic acid eluate was discarded, and the next 25 ml plus the first 10 ml of 0.5 M formic acid were collected and redissolved in 0.5 ml of water. Fifty microliters of this solution were applied to a microcrystalline cellulose thin layer plate, 20 X 20 cm (Applied Science Laboratories), and developed with a diethyl ether-formic acid (90%)-water (7.2:1.1) solvent. A mixture of glutamate, aspartate, and alanine was separated in a parallel column. The standard spots were visualized with ninhydrin spray, and the spot corresponding to alanine was scraped off and counted as described before. The homogeneity of the alanine peak was tested by subjecting
a concentrated sample of the column eluate to two-dimensional chromatography on microcrystalline cellulose thin layer plates with the use of solvent 1 and solvent 2 as described by Kau et al. (8). The same sample was also subjected to two-dimensional thin layer chromatography on Silica Gel G plates with the use of phenol (90%)-water (85:17) followed by 1-butanol-acetic acid-water (4:1:1) as described by Brenner (10).

Measurement of Distribution of Pyruvate Label in Lipid—[2-14C]-Pyruvate (60 pmol per nmol) in final concentration of 3.1 mm with 3.1 mm acetate and 0.78 mm octanoate was incubated for 1 hour in a 125 ml flask at 26 °C with 0.0 ml of cells grown in a final volume of 126 ml under standard conditions. After the 1-hour incubation, the liquids were extracted by the Folch (17) procedure, evaporated to dryness under nitrogen, and redissolved in 1 ml of benzene. A small volume of HClO4 (10%, v/v, in methanol, Applied Science Laboratories) was added to the benzene-lipid solution in a sealed container and heated at 120 °C for 4 hours. After this period, the samples were evaporated to dryness and dissolved in 2 ml of water. The aqueous phase was extracted three times with equal volumes of diethyl ether. The ether phases were pooled, evaporated to dryness, and counted. After determining the volume of the aqueous phase, an aliquot of it was also counted.

Measurements of Time Course of Metabolic Adaptation to Step Change in Oxygen Tension.—For measurements of the time course of metabolic changes following a step down in oxygen tension (herein referred to as "adaptation"), cells grown for 17 hours in three shallow (40 ml) cultures were pooled to form a deep (120 ml) culture. For measurements of the time course of metabolic changes following a step up in oxygen tension (herein referred to as "de-adaptation"), cells grown for 17 hours in deep cultures were distributed among three flasks (40 ml in each flask, i.e. shallow culture conditions) and returned to the shaker bath. A separate deep culture was allowed to grow in the shaker bath and served as a control. For measurements of the time course of metabolic changes following a step up in oxygen tension (herein referred to as "de-adaptation"), cells grown for 17 hours in deep cultures were distributed among three flasks (40 ml in each flask, i.e. shallow culture conditions) and returned to the shaker bath. A separate deep culture was allowed to grow in the shaker bath and served as a control. At several times after the cells were transferred to the new culture conditions, measurements were made of the incorporation of label in a 1-hour incubation into CO2, glycogen, lipid, and glutamate from a mixture of [1-14C]acetate, [2-14C]pyruvate, and [1-14C]octanoate with only one substrate labeled at a time, as described above.

Reagents — Isotopically labeled compounds were purchased from Amersham-Searle. Octanoic acid was purchased from Hormel. 5’-AMP and bovine serum albumin were obtained from Sigma. All other reagents were of highest purity obtainable.

RESULTS

A variety of methods have been used to obtain partially anaerobic conditions of growth. Perhaps the most obvious method is to bubble gas of known composition through the culture at measured flow rates. Although this would appear to be the most reproducible method, this is probably not the case with *Tetrahymena*, since not only is it difficult to control the size of the bubbles (which in turn determines the rate of solubilization of oxygen into the medium), but also the intensity of bubbbling required to approach equilibration with the gas phase is exceedingly high, leading to frothing and cell damage. Malecki et al. (18) flowed gas mixtures of known composition over the surface of very shallow shaken cultures and obtained highly reproducible growth curves, but found that at low oxygen tensions growth was markedly inhibited by the process of shaking, thus obviating the use of this method for the present purposes.

Levy and Schroetermann (9), who have studied the rate of decline of oxygen tension during the growth of deep cultures, showed that flushing air over the surface of such cultures, even when they were in a shaker bath, did not increase the very low oxygen tension once the cell density was over 100,000 cells per ml. We have, therefore, chosen to use air as the gas phase and to vary only the depth of the liquid in the flasks (which were covered with Morton closures to ensure adequate entry of air into the flasks). Since the flasks were grown in the same shaker bath, the only difference between shallow, medium, and deep cultures was the surface to volume ratio and hence the average amounts of oxygen and of carbon dioxide dissolved per ml. We have found this method to give highly reproducible results, as will be seen below. In what follows, we shall assume that the metabolic changes which occur are due to changes in the average oxygen content of the cultures during the 17-hour incubation period, since this is certainly of greater influence than the changes in CO2 content. It must, however, be borne in mind that CO2 is fixed by *Tetrahymena*, especially under anaerobic conditions (19) so that the metabolic changes to be reported may result in part from changes in average CO2 content as well as in oxygen tension.

Effect of Culture Depth on Growth, Final Culture Glycogen Content and Oxygen Tension, and on Rate of Oxygen Consumption—Table I shows the results of measurements of cell growth, final oxygen tension, and the rate of oxygen consumption from cultures grown in total volumes of 40, 80, and 120 ml in 500-ml flasks, as described under "Materials and Methods." It can be seen that increasing culture depth from 40 to 80 ml had no effect on total cell growth, but a further increase to 120 ml resulted in a small but reproducible inhibition of growth. It was found that cells grown in deep cultures grew at the same rate as those in shallower cultures but reached stationary phase earlier.

| Culture volume | Nf (mg) | Pf (mg) | O2 consumption (μl/mg cell/hr) |
|----------------|--------|--------|------------------------------|
| 40             | 5.0 ± 0.6 (4) | 68 ± 12 (4) | 84 (2) 120 (2) |
| 80             | 5.3 ± 0.6 (4) | 73 ± 5 (5)  | 96 (1) 140 (2) |
| 120            | 4.4 ± 0.5 (4) | 11 ± 4 (4)  | 112 (2) 176 (2) |

Total glycogen content increased as expected with increasing depth, but was quite variable from experiment to experiment (data not shown), ranging from 160 to 280 μg/106 cells in 40-ml cultures. Values were 1.1 to 1.3 times higher in 80-ml cultures and 1.3 to 1.6 times higher in 120-ml cultures.

Finally, PO4 levels were measured in separate cultures with comparable Nf values to those on which the other measurements were made. It is noteworthy that even with only 40 ml in a shaken 500-ml flask the final PO4 is considerably less than that of a similar solution in equilibrium with room air. Increasing the culture volume to 80 ml decreased the final PO4 to about 12 mm Hg, which was not statistically different from the final PO4 of cultures of 120 ml. Since as will be shown below, there are marked differences in metabolism between medium and deep cultures, the time course of prior exposure to low oxygen tension must play a major role in determining the metabolic state of the culture at the end of the 17-hour incubation period.

Oxygen consumption experiments carried out in a Warburg
apparatus showed that cells grown in deep culture had a reproducibly greater rate of oxygen consumption than those grown in shallow cultures. Since the $Q_{O_2}$ measurements were made with a high rate of shaking and a total of 2 ml in 15-ml capacity Warburg flask, these measurements were made essentially under "shallow" conditions. Thus, the difference in $Q_{O_2}$ between cells from deep and shallow cultures presumably reflects an adaptation which lasts at least for 1 hour after transfer from deep to shallow conditions, although it is possible that the change in $Q_{O_2}$ also reflects a change in composition of the growth medium and the availability of oxidizable substrates during the 17-hour growth period. That the latter effect is not very important for the present experiments is suggested by the observation that although addition of a mixture of acetate, pyruvate, and octanoate to the Warburg flasks increased the $Q_{O_2}$ of all the cells, a clear difference remained in the oxidative capacity of cells grown under shallow, medium, and deep conditions. Thus, growth under conditions of decreasing oxygen tension causes an increase in oxidative capacity which lasts for at least 1 hour after transfer to conditions of high oxygen tension.

**Effect of Culture Depth on Oxidation and Incorporation into Glycogen of Label from [1-14C]Glucose, [2-14C]Pyruvate, and [2-14C]Glyoxylate**—Because of the apparent inverse relationship of oxygen tension to $Q_{O_2}$ and total glycogen content, it was of interest to determine whether these increases occurred at the level of acetyl-CoA fluxes or by the increased incorporation of glucosyl moieties into glycogen. According to the metabolic scheme shown in Fig. 1, [2-14C]pyruvate incorporation into glycogen should very nearly represent the contribution of the outer mitochondrial pool of acetyl-CoA to glyconeogenesis. Similarly, since glyoxylate is formed in the peroxisomes by the action of isocitrate lyase on isocitrate, incorporation of [2-14C]glyoxylate into glycogen should represent the peroxisomal contribution to glyconeogenesis.

From Table II, it can be seen that increasing culture depth (and therefore decreasing the time average oxygen tension of the culture during the 17-hour growth period) increased the rate at which [2-14C]pyruvate and [2-14C]glyoxylate were oxidized during the 1-hour assay, which was performed at high surface to volume ratios and with shaking. The rate of oxidation of [1-14C]glucose, however, was decreased, while there was little change in the incorporation of glucose into glycogen. The incorporation of [2-14C]pyruvate and [2-14C]glyoxylate into glycogen increased almost 2-fold and 4-fold, respectively, in deep as compared to shallow cultures. These results indicate that cultures grown under low surface to volume ratios develop a greater capacity to oxidize 2- and 3-carbon units and a reduced capacity to oxidize glucose when tested under shallow conditions. It is noteworthy that incorporation of label from glyoxylate into glycogen was increased more than that from pyruvate, indicating that the peroxisomal contribution to glyconeogenesis was more sensitive to changes in average oxygen tension than the mitochondrial contribution, a deduction which is sustained by the quantitative analysis presented below.

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Effect of culture depth on intracellular fluxes of acetyl-CoA. The three unbracketed numbers represent the average values obtained from experiments done on cells grown in shallow, medium, and deep cultures (top to bottom) and the value in parentheses represents the average values obtained from experiments done on cells grown in deep culture with 3.0 mM AMP.
Effect of culture depth on appearance of label in CO2 and glycogen from cells incubated with glucose, pyruvate, or glyoxylate

Cells were grown in 500-ml Erlenmeyer flasks in total volumes of 40, 80, and 120 ml, corresponding to shallow, medium, and deep cultures, respectively. At the end of the 17-hour growth period, aliquots were incubated for 1 hour with either [1-14C]glucose (6.2 nM, 6000 dpm per nml), [2-14C]pyruvate (6.2 mM, 200 dpm per nml), or [2-14C]glyoxylate (6.2 mM, 200 dpm per nml), and appearance of label in CO2 and glycogen was measured as described under "Materials and Methods." Each value represents the average and standard deviation from four experiments for glucose and glyoxylate and three experiments for pyruvate and is expressed as nanomoles of labeled substrate appearing as the indicated product per 10^6 cells per hour.

| Labeled substrate | Shallow | Medium | Deep | Shallow | Medium | Deep |
|-------------------|---------|--------|------|---------|--------|------|
| Glucose           | 93.8 ± 17.1 | 69.9 ± 15.9 | 37.2 ± 4.2 | 821 ± 32 | 752 ± 94 | 762 ± 52 |
| Pyruvate          | 018 ± 99 | 812 ± 135 | 880 ± 123 | 160 ± 8.0 | 67.0 ± 9.3 | 87.0 ± 12.7 |
| Glyoxylate        | 34.6 ± 3.4 | 66.8 ± 4.7 | 74.2 ± 11.0 | 29.6 ± 8.0 | 54.6 ± 10.9 | 112 ± 13 |

* p < 0.05 by Student's t test (paired data) between deep and medium.

b p < 0.02 by Student's t test (paired data) between deep and shallow.

p < 0.05 by Student's t test (paired data) between shallow and medium.

Effect of Culture Depth on Metabolism of [1-14C]Acetate, [2-14C]Pyruvate, and [1-14C]Oxaloacetate—To obtain quantitative measures of the changes in intracellular flux patterns of acetyl-CoA, experiments were carried out in which cells taken from cultures grown for 17 hours under shallow, medium, and deep conditions were exposed to a mixture of acetate, pyruvate, and oxaloacetate for 1 hour under shallow conditions, with only one substrate at a time labeled. It has been shown (8) that in *Tetrahymena* the labeled carbons from [1-14C]acetate, [2-14C]pyruvate, and [1-14C]oxaloacetate all become [1-14C]acetyl-CoA and that the further metabolism of labeled acetyl-CoA can be analyzed according to a structural model in which there are three pools of acetyl-CoA, as shown in Fig. 1. Table III shows the amount of label incorporated from each of the labeled substrates into CO2, glycogen, lipids, and glutamate for cultures grown under shallow, medium, and deep conditions. The values for cultures of 43-ml volume are taken from Raugi et al. (8). It can be seen that oxidation of [1-14C]acetate was increased almost 50% in deep compared to shallow cultures, and as in the experiments with single substrates (cf. Table II), oxidation of [2-14C]pyruvate was also increased. The oxidation of [1-14C]pyruvate increased with culture depth in a manner similar to that of [2-14C]pyruvate, thus demonstrating that conditions of low oxygen tension caused an increase in pyruvate dehydrogenase activity. The presence of acetate and oxaloacetate decreased the oxidation of pyruvate by 80 to 90%, an effect due primarily to the acetate (20). No change was observed in the oxidation of [1-14C]oxaloacetate as a function of culture depth. These results are consistent with the findings (cf. Table I) that oxygen consumption increased in cells grown under partial anaerobiosis and that oxidation of glyoxylate and pyruvate was increased under these conditions (cf. Table II).

There was a small but significant increase in incorporation of label from [1-14C]acetate into lipid but no significant changes occurred in the incorporation of label from pyruvate or oxaloacetate into lipid. In our original model (9), it was implicitly assumed that all the label derived from [2-14C]pyruvate that appears in lipid was in the fatty acid moieties of the lipids. Since more of the label from pyruvate appears in glycogen than in lipids, some of the label in the lipid could be in the glycerol moiety. It was therefore necessary to measure the fraction of label from [2-14C]pyruvate incorporated into the glycerol and fatty acid moieties of the lipids under the conditions of these experiments. It was found (Table IV) that 33 to 40% of the label was in the glycerol moiety and the remainder of the label was in the fatty acids. These findings are consistent with the observations of Connett et al. (21) that most of the label from labeled glucose appears in the glycerol moieties of the lipids. These results with [2-14C]pyruvate are also consistent with the three-compartment model (see Fig. 1) since one would expect [1-14C]acetyl-CoA derived from precursors such as pyruvate entering the outer mitochondrial pool to appear in the glycerol moiety of the lipids to a greater relative extent than acetate which is oxidized in the inner mitochondrial pool and mostly converted into fatty acids in the peroxisomal pool. Because the total amount of label incorporated into lipids from [2-14C]pyruvate in these three-substrate experiments is very small, we have not deemed it worthwhile to explicitly show in the model that about one-third of the label that enters into the lipids from [2-14C]pyruvate is in the glycerol moiety.

Incorporation of label from [1-14C]acetate and from [2-14C]pyruvate into glycogen was tripled and doubled, respectively, in deep as compared to shallow cultures, and there was a small but statistically insignificant increase in the incorporation of label from [1-14C]oxaloacetate as well.

As noted earlier (8), appearance of label into glutamate was measurable only when [1-14C]acetate was the labeled substrate. It was found that there was no change in label incorporation from acetate into glutamate in medium versus shallow cultures, but increasing culture depth from 86 to 129 ml halved the amount of label incorporated into glutamate. Incorporation of label into aspartate was not detected under any of these conditions. Table V shows that there was no significant change in the activity of aspartate transaminase or glutamate dehydrogenase in shallow versus deep cultures. Thus the halving of label incorporation from [1-14C]acetate into glutamate cannot be explained by changes of either of these activities as measured in vitro and changes in metabolic controls in the mitochondria must be postulated to account for these observations.

In shallow cultures, no label appeared in alanine from any of the labeled substrates. With increasing culture depth, label from [2-14C]pyruvate appeared in increasing amounts in alanine (see Fig. 1). Since alanine transaminase is localized exclusively...
transamination of pyruvate to alanine in the mitochondria. Since pyruvate enters primarily into the outer compartment of the mitochondria, it is probable that the alanine transaminase reaction occurs in this compartment, although it must be emphasized after 17 hours incorporation of label from [1-14C]acetate, strates were present in a given flask, only one substrate was measured only in representative experiments for each average & S.D. for four experiments at 86 ml, eight experiments that as oxygen tension decreases there is an increase in the concentrations and specific activities were as follows: [1-14C]acetate, 6.2 mM, 60 dpm per nmol. Although all three substrates were present in a given flask, only one substrate was labeled. Values for cultures grown in a total volume of 43 ml are from Raugi et al. (8) and represent the average ± S.D. for eight experiments. Values for cultures in larger volumes represent averages ± S.D. for four experiments at 80 ml, eight experiments at 129 ml total volume, and four experiments at 129 ml in the presence of AMP.

| Labeled substrate | Culture volume | Amount of label per 10^6 cells per hour appearing in |
|-------------------|----------------|--------------------------------------------------|
|                   |               | CO₂    | Lipids | Glycogen | Glutamate | Alanine |
| [1-14C]Acetate    | 43            | 496 ± 25a | 142 ± 9b | 16.1 ± 3.2a | 7.6 ± 2.0a | <1.0     |
|                   | 86            | 631 ± 64a | 183 ± 12b | 27.3 ± 6.4b | 7.2 ± 0.8b | <1.0     |
|                   | 129           | 723 ± 72  | 179 ± 19 | 47.1 ± 6.5c | 3.8 ± 1.4c | <1.0     |
|                   | 129'          | 624 ± 374* | 199 ± 184* | 24.1 ± 3.24* | 3.0 ± 0.64* | <1.0     |
| [2-14C]Pyruvate   | 43            | 678 ± 4.0a | 2.1 ± 0.39 | 5.3 ± 1.5a | <1.0      | <1.0a    |
|                   | 86            | 105 ± 9.0b | 1.7 ± 0.3b | 6.4 ± 1.5b | <1.0      | <1.0b    |
|                   | 129           | 133 ± 20  | 2.1 ± 0.3p | 9.7 ± 1.9c | <1.0      | 11.8c    |
|                   | 129d          | 127 ± 18d  | 1.6 ± 0.44 | 6.3 ± 0.74d | <1.0d     | <1.0d    |
| [1-14C]Octanoate  | 43            | 6.7 ± 2.1a | 30.4 ± 3.0a | 0.40 ± 0.13a | <0.1      | <0.2     |
|                   | 86            | 9.0 ± 2.0p | 30.8 ± 2.7p | 0.50 ± 0.11p | <0.2      | <0.2     |
|                   | 129           | 7.3 ± 1.5b | 34.5 ± 3.7 | 0.63 ± 0.20 | <0.2      | <0.2     |
|                   | 129d          | 3.9 ± 0.94* | 31.3 ± 1.94 | 0.97 ± 0.004* | <0.2d     | <0.2d    |
| [1-14C]Pyruvatef  | 40            | 94.8     | 135.5    | 5.3 ± 0.6d  | 3.0 ± 0.7d  | <1.0     |
|                   | 80            | 102.6    | 135.5    | 6.4 ± 1.5b  | 6.4 ± 1.5b | <1.0     |

Table III

Appearance of label in CO₂, lipid, glycogen, and glutamate from cells incubated with acetate, pyruvate, and octanoate

Cells were grown in 500-ml Erlenmeyer flasks in the volumes indicated. Initial cell densities (N₀) were about 140,000 cells per ml. After 17 hours incorporation of label from [1-14C]acetate, [2-14C]pyruvate, and [1-14C]octanoate in CO₂, lipids, glycogen, and glutamate, were measured in a 1-hour incubation. Appearance of label from [2-14C]pyruvate and [1-14C]octanoate in glutamate was measured only in representative experiments for each condition. AMP, when present, was 3.0 mM. Final substrate concentrations and specific activities were as follows: [1-14C]acetate, 0.2 mM, 150 dpm per nmol, [2-14C]pyruvate, 6.2 mM, 50 dpm per nmol; [1-14C]octanoate, 1.5 mM, 600 dpm per nmol; [1-14C]-pyruvate, 6.2 mM, 60 dpm per nmol. Although all three substrates were present in a given flask, only one substrate was labeled. Values for cultures grown in a total volume of 43 ml are from Raugi et al. (8) and represent the average ± S.D. for eight experiments. Values for cultures in larger volumes represent averages ± S.D. for four experiments at 80 ml, eight experiments at 129 ml total volume, and four experiments at 129 ml in the presence of AMP.

Table IV

Distribution of [2-14C]pyruvate label in water phase and fatty acid moieties of total extractable lipids

Following a 17-hour growth period under deep conditions, cells were incubated for 1 hour with a mixture of 3.0 mM acetate, 3.0 mM pyruvate, and 0.75 mM octanoate. Total lipids were extracted and subjected to the methanolysis procedure described under "Materials and Methods." Specific activity of [2-14C]pyruvate was 183 dpm per nmol in Experiment A and 62 dpm per nmol in Experiment B. The amounts of label recovered in the aqueous and other phases are designated "glycerol" and "fatty acids," respectively.

Table V

Effect of culture depth on glutamate dehydrogenase and aspartate transaminase activity

Cultures were grown under standard conditions containing a total volume of 40 or 120 ml. At the end of the 17-hour growth period, cells were harvested, sonicated, and assayed for these enzyme activities according to Porter et al. (15). Values represent means ± S.D. for four separate experiments.

| Enzyme              | Activity |
|---------------------|----------|
|                     | Shallow  | Deep    |
| Glutamate dehydrogenase | 12.6 ± 1.3 | 13.4 ± 1.8 |
| Aspartate transaminase      | 4.67 ± 0.44 | 4.27 ± 0.52 |

in the mitochondria in Tetrahymena (15), this finding suggests that as oxygen tension decreases there is an increase in the transamination of pyruvate to alanine in the mitochondria. Since pyruvate enters primarily into the outer compartment of the mitochondria, it is probable that the alanine transaminase reaction occurs in this compartment, although it must be emphasized that the site of the transamination cannot directly be deduced from the formalism of the three-pool model.

Effect of Culture Depth on Intracellular Distribution of Acetyl CoA—For each experiment shown in Table III, computer fits to the data were obtained as described in detail by Raugi et al. (8). In each case, the fits obtained were essentially as good as those described earlier, and it is therefore unnecessary to present examples of these fits. Fig. 1 presents the average of the individual computer fits obtained for the experiments summarized.
in Table III. Table VI shows the average values for the distribution parameters in Fig. 1.

It can be seen that the inputs of acetate and pyruvate (V4 and V5, respectively) increased with each increment of culture depth, but total octanoate input (V6) was relatively independent of culture depth. Total 14CO2 production (V3) increased 56%, but [14C]glutamate output (V2) decreased 36%, indicating that control mechanisms other than simple pool dilution must be operative in the inner mitochondrial compartment. Lipogenesis (V8) increased slightly with culture depth, the bulk of the increase being derived from peroxisomal acetyl-CoA (V1 - (1 - e)V6) rather than from the direct pathway of incorporation of octanoate into lipids.

Total glyconeogenesis (V4) increased 56% and 179% in medium and deep cultures, respectively. As the average oxygen tension decreased, glyconeogenesis increased from both the peroxisomal and the outer mitochondrial compartments, with the contribution from the peroxisomal compartment becoming more dominant in deep than in shallow cultures. This shift is expressed in the parameter γ, which changed from 0.25 to 0.188 to 0.165 with increasing culture depth.

In contrast to the marked effects on glyconeogenesis, there was no consistent pattern for the effect of reduced oxygen tension on the β oxidation of octanoate. Although the distribution of acetate between the mitochondrial and peroxisomal compartments (governed by α) did not change with culture depth, the amount of acetate entering each compartment increased with decreasing oxygen tension via the increase in V6. Almost all of the increase in lipogenesis and glyconeogenesis from the peroxisomes is accounted for by the increase in input of acetate to the peroxisomes. About 23% of the increase in 14CO2 production from the inner mitochondrial compartment (V3), however, was due to the increased flux of labeled acetyl-CoA from the outer mitochondrial compartment (V4). Virtually all of the increase in V4 is attributable to an increase in V4, reflecting an increase in the activity of pyruvate dehydrogenase. No significant changes in any of the other interpool fluxes (V8, V10, V11) were observed, but, as discussed earlier (8), these flux rates are very poorly determined with the data obtained.

**Effect of Culture Depth on ATP and ADP Levels**—The results so far presented show that the pattern of distribution of acetyl-CoA changes in each of the three acetyl-CoA containing compartments identified in *Tetrahymena* in response to decreasing oxygen tension, and that in addition there was an unexpected decrease in the rate of oxidation of glucose. Since the effects of changing the ratio of ATP to ADP on glycolysis and on the tricarboxylic acid cycle are well known in other systems (22), it was of interest to measure the levels of ATP and ADP as a function of culture depth, especially since Rooney and Eiler (13) reported that exposure of *Tetrahymena* to seven hypoxic shocks caused an increase in respiration rate (as observed here in response to continuous growth at low oxygen tension) and a 50% reduction in ATP content. Although Rooney and Eiler used a very sensitive procedure which did not require centrifugation of the cells, their values for ATP (about 2 to 4 mmol/106 cells) were considerably below the values we have earlier reported (9, 10) with a fluorimetric assay in which the cells had to be centrifuged prior to assay in order to obtain enough ATP. Because of the ATP:ADP ratios obtained, it was possible that appreciable conversion of ATP to ADP had occurred during the centrifugation step, and it was decided to reinvestigate the question of ATP:ADP levels with the use of the sensitive procedure essentially as described by Rooney and Eiler (13), except that the measurements were made immediately after the perchloric acid was neutralized, since we found that about half of the ATP in standards was lost if the neutralized perchloric acid extract was allowed to stand in ice for 4 hours. With this method, the values we obtain for the ATP + ADP content of *Tetrahymena* are comparable to the values reported earlier by us (9, 10) but the ATP:ADP ratios are higher than previously reported and comparable to those usually obtained in mammalian tissues. There was no difference in the ATP:ADP ratio between cultures grown overnight under shallow, medium, or deep conditions (Table VII). We have repeated the measurement of ATP and ADP levels on cells grown under shallow conditions with either tolbutamide or AMP and find (Table VIII) that although the ATP:ADP ratios are higher than previously reported neither tolbutamide nor AMP caused any change in the ATP:ADP ratio, as stated earlier (9, 10).

**Effect of β-AMPA on Metabolism of [1-14C]Acetate, [2-14C]Pyruvate, and [1-14C]Octanoate on Cells Grown in Deep Culture**—We have reported that the addition of AMP to the culture medium of cells grown without shaking results in an inhibition of growth and an increase in glycogen content (23), whereas when shallow cultures were grown with shaking and then assayed with radioactive substrates to deduce the intracellular flux patterns, it was found that AMP caused an inhibition of glyconeogenesis (10). It was therefore of interest to perform a set of experiments on deep cultures in which AMP was present during the 17-hour growth period (3.0 ml) and during the 1-hour assay. The experimental data are shown in Table III and the intracellular

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### Table VI

**Effect of culture depth on distribution parameters of metabolites**

The metabolic significance of these distribution parameters is evident from Fig. 1.

| Distribution parameter | Culture volume |
|------------------------|---------------|
|                        | 43 ml | 85 ml | 120 ml | 120 ml* |
| α                      | 0.758 | 0.750 | 0.761  | 0.735*  |
| β                      | 0.58  | 0.605 | 0.71   | 0.61*   |
| γ                      | 0.26  | 0.188 | 0.166  | 0.206*  |
| e                      | 0.27  | 0.32  | 0.24   | 0.18*   |

* Measurements made on cells grown 17 hours in 3.0 mM AMP.

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### Table VII

**Effect of culture depth on ATP and ADP levels in *Tetrahymena***

Cells were grown in the volumes indicated in 500-ml Erlenmeyer flasks. After 17 hours, ATP and total adenosine di- and triphosphates were measured as described under "Materials and Methods." Values for ADP levels were obtained by difference. Each value represents the average ± S.D. Numbers in parentheses indicate the number of experiments performed.

| Culture volume | ATP | ADP | ATP:ADP | ATP + ADP |
|----------------|-----|-----|---------|-----------|
|                | nmol/106 cells | nmol/106 cells |
| 40 ml          | 12.2 ± 1.8 (4) | 1.8 ± 0.8 (4) | 6.9 | 14.0 |
| 80 ml          | 14.6 ± 2.1 (5) | 1.5 ± 0.6 (5) | 9.5 | 10.1 |
| 120 ml         | 17.1 ± 3.0 (4) | 1.8 ± 1.4 (4) | 9.5 | 18.9 |

1 Table IV, page 8076 of Ref. 9 and Table V, page 8084 of Reference 10 should, therefore, be replaced by Table VIII of the present paper. These changes in ATP:ADP ratio do not alter the conclusions reached in either of the earlier papers.
The major effect of AMP, as was the case in shallow cultures (10), was an inhibition of glyconeogenesis; incorporation of label from $[^{1-14}C]$acetate and $[^{1-14}C]$octanoate was decreased by about 50% and from $[^{2-14}C]$pyruvate by about 35%. The increase in $\gamma$ shows that the contribution of the peroxisomal acetyl-CoA pool to glyconeogenesis was more strongly inhibited by AMP than was the contribution of the outer mitochondrial pool. Thus, the peroxisomal contribution to glyconeogenesis is more sensitive to inhibition by AMP and to activation by low oxygen tension than is the mitochondrial contribution. AMP had little if any effect on the interpool fluxes, on alanine production from pyruvate, on label incorporation into glutamate, or on total lipid synthesis.

**Time Course of Adaptation to Step Change in Oxygen Tension**—Although it is relatively well established that oxygen tension plays a major role in regulating the metabolism of *Tetrahymena* (6), relatively little work has been done on the time course of the metabolic changes that occur in response to a change in the level of aeration. Furthermore we have shown that for at least 1 hour after a sudden change in oxygen tension the cells are metabolically quite stable when tested under highly aerobic conditions, irrespective of their prior growth history. It was therefore of interest to determine the temporal sequence of adaptation to a step change in oxygen tension and in particular what quantitative contribution to the net effect was made by each metabolic compartment. It should be noted that in the course of these experiments, the steady state assumption is sometimes violated. However, because the time required for complete adaptation to occur is considerably longer than the period during which the measurements are made (4 to 8 hours versus 1 hour), it seemed that this type of analysis might nevertheless be suitable. That this assumption is warranted is partially borne out by the extremely close fit to the observed data obtained by our computer model. Nonetheless, where we have represented a particular metabolic rate as a point in time, we mean the average metabolic flux determined in a 1-hour period beginning at that point.

In what follows formal statistical evaluation of the results will not be presented because of the limited number of measurements made at some of the time points. If more experiments had been done, it might have been possible to draw more conclusions about the minor changes in certain fluxes but little would be added to the over-all picture which emerges. We have therefore confined our statements to those measurements in which appreciable changes were apparent between control and experimental cells. In the legends to Figs. 2 and 4, we have presented information relevant to the reproducibility of those measurements.

**Adaptation to Hypoxia**—The values obtained for the incorporation of label into CO$_2$, glycolate, glutamate, and lipid in a 1-hour incubation and for control cells maintained under shallow conditions are shown in Fig. 2. Incorporation of label from $[^{1-14}C]$acetate into CO$_2$ increased slightly within 2 hours after transfer to hypoxic conditions and increased steadily thereafter (Fig. 2C). The increase in acetate conversion to glycolate, however, was already noticeable within 1 hour after transfer to hypoxic conditions and the fully adapted level was achieved within 4 hours (Fig. 2D). Incorporation of label from $[^{2-14}C]$pyruvate into both glycolate (Fig. 2E) and CO$_2$ (Fig. 2H) increased with a similar time course, rapidly at first and then steadily. The significance of the changes during the first few hours are uncertain, however, because of increases in the control cultures which apparently occur at this stage of growth.

There was no significant change in the ratio of incorporation of label from acetate into lipids until 4 hours after transfer to hypoxic conditions when an increase in lipogenesis relative to the shallow (aerobic) controls became apparent (Fig. 2A). The amount of label incorporated into lipid from pyruvate is too small for reliable deductions to be made concerning any changes with time, and this information is not presented in Fig. 2, although it was used to obtain the computer fits which serve as the basis for the metabolic parameters presented in Fig. 3. The rate of label incorporation from octanoate into lipid increased in the control and possibly in the adapting cultures up to about 4 hours, but was the same in both the well aerated and hypoxic cultures at the end of the 8-hour period (Fig. 2C). The rate of appearance of label from acetate into glutamate increased markedly as a function of culture age, a trend which was either stopped or even slightly reversed during the first 4 hours after transfer to hypoxic conditions.

The points shown in Fig. 2 are the average values obtained from two to three experiments except that only one experiment was done for 1-hour adaptation. By means of these average values, single computer fits were obtained to the metabolic model described earlier (see Fig. 1). Fig. 3 summarizes the information so obtained and presents a quantitative picture of the changing metabolic flux pattern as a function of 8 hours of

**Table VIII**

*Effect of tolbutamide and δ'AMP on ATP and ADP levels in *Tetrahymena*

|                | ATP    | ADP    | ATP:ADP | ATP + ADP |
|----------------|--------|--------|---------|-----------|
|                | mmol/10^9 cells | mmol/10^9 cells |
| $N_1-N_2$     |         |         |         |           |
| Control        | 12.5 ± 1.1 | 3.3 ± 1.5 | 3.8 | 15.8 |
| Tolbutamide-treated | 13.7 ± 0.9 | 3.0 ± 2.0 | 4.6 | 16.7 |
| AMP-treated    | 14.7 ± 0.5 | 3.6 ± 0.6 | 4.1 | 18.3 |
| Transition phase cultures |
| Control        | 9.8 ± 1.2 | 2.4 ± 0.7 | 4.1 | 12.2 |
| Tolbutamide-treated | 11.8 ± 1.0 | 2.7 ± 0.4 | 4.3 | 14.5 |
| AMP-treated    | 13.7 ± 1.3 | 3.0 ± 1.3 | 3.5 | 17.6 |

* One standard deviation.

Each measurement is the average value of five experiments. The ATP:ADP ratios in tolbutamide-treated or in AMP-treated cells are not significantly different than the control ratios. The concentrations of tolbutamide and AMP during the 17-hour growth period were 0.24 mM and 3.0 mM, respectively.

**Log phase cultures**

|                | ATP    | ADP    | ATP:ADP | ATP + ADP |
|----------------|--------|--------|---------|-----------|
| Control        | 9.4    | 3.3    | 3.8      | 15.8      |
| Tolbutamide-treated | 5.9    | 3.0    | 4.6      | 16.7      |
| AMP-treated    | 7.7    | 3.6    | 4.1      | 18.3      |

**Transition phase cultures**

|                | ATP    | ADP    | ATP:ADP | ATP + ADP |
|----------------|--------|--------|---------|-----------|
| Control        | 4.7    | 2.4    | 4.1      | 12.2      |
| Tolbutamide-treated | 3.3    | 2.7    | 4.3      | 14.5      |
| AMP-treated    | 4.8    | 3.0    | 3.5      | 17.6      |
FIG. 2. Incorporation of label from [1-14C]acetate, [2-14C]pyruvate, and [1-14C]octanoate into CO2, glycogen, lipid, and glutamate in cells adapting to hypoxic growth conditions.

Cultures were grown at a total volume of 40 ml in 500-ml Erlenmeyer flasks with shaking for 17 hours. The initial and final cell densities, $N_i$ and $N_f$, were about 140,000 and 600,000 cells per ml, respectively. At zero time, three cultures were pooled into a single 500-ml flask and replaced in the shaker bath. A control flask containing 40 ml of cells was also left in the shaker bath. At the times indicated on the abscissa, cells from the experimental and control flasks were assayed for their capacity to incorporate label from [1-14C]acetate (130 dpm per nmol), [2-14C]pyruvate (50 dpm per nmol), and [1-14C]octanoate (500 dpm per nmol) into CO2, lipid, glycogen, and glutamate in a 1-hour incubation by the methods described in the text. All units shown on this graph are in nanomoles of label incorporated per 10^6 cells per hour. ○ control culture maintained under shallow conditions; ● culture adapting to the transfer to deep conditions.

The points shown are those from a single experiment at 1 hour, and the mean values of three experiments at 2 hours, two experiments at 4 hours, and three experiments at 8 hours. Incorporation of label into glutamate was not, however, measured in every experiment. The mean values shown were used to generate the computer fits presented in Fig. 3. The difference between the pairs or triplets of experimental values and the mean was less than ±15% except for the following points: C, 4-hour experimental, ±17%; D, 2-hour control, ±25%; E, 2-hour control, ±28%; G, 8-hour experimental, ±22%; H, 8-hour control, ±24%; I, 4-hour experimental, ±19%, 8-hour control, ±22%, 8-hour experimental, ±29%.
aging under shallow conditions or of 8 hours of aging plus adaptation to the transfer from shallow to deep culture conditions.

CO₂ production from the Krebs cycle in the inner mitochondrial compartment ($V_1$) did not change in control cells, but increased steadily in the cells adapting to hypoxia, going from a value of about 600 nmol/10⁶ cells per hour at the start of the experiment to about 900 after 8 hours of hypoxia (Fig. 3A). Glutamate formation ($V_2$), which also occurs from the inner mitochondrial compartment, was reduced almost immediately after transfer to hypoxic conditions, remained constant for about 4 hours, and then increased at the same rate as it increased in control cultures (Fig. 3B).

The glyconeogenic flux of labeled acetyl-CoA from the outer mitochondrial ($\gamma V_2$) and peroxisomal ($\alpha = (1 - \gamma) V_2$) pools was unchanged in control cells (Fig. 3C). The mitochondrial contribution to glyconeogenesis increased from about 3 nmol/10⁶ cells per hour to about 8 nmol/10⁶ cells per hour between the second and eighth hours after transfer from shallow to deep conditions. The peroxisomal contribution, however, increased from about 14 nmol/10⁶ cells per hour to about 28 nmol/10⁶ cells per hour.

Fig. 3. Changes in metabolic parameters during adaptation to hypoxic growth conditions. The distribution parameters $\alpha$, $\beta$, $\gamma$, and $\epsilon$ are unitless. All other values are in nanomoles per 10⁶ cells per hour. For further details, see legend to Fig. 2.
cells per hour within the first 4 hours of adaptation to hypoxia and remained at this level for the next 4 hours. The increase in total rate of glyconeogenesis \( V_\beta \) was thus largely achieved within the first 4 hours after transfer to deep conditions. Because the rates of glyconeogenesis from both pools increased, there was relatively little change in \( \gamma \), the fraction of the glyconeogenic flux derived from the mitochondria (Fig. 31). The rate of \( \beta \) oxidation of octanoate (Fig. 3D) in the inner mitochondrial compartment \( (\alpha V_\lambda) \) declined from about 10 nmol/10^6 cells per hour to about 4 nmol/10^6 cells per hour in both the control cultures and in those adapting to the transfer to low oxygen tension, but there was only a slight decline in the rate of \( \beta \) oxidation in the peroxisomal compartment \( (\epsilon(1-\beta)V_\epsilon) \). Thus \( \epsilon \), the fraction of octanoate \( \beta \)-oxidized, declines more or less steadily through the adaptation period (Fig. 31).

The total rate of acetate utilization by the peroxisomes \( ((1-\alpha) V_\lambda) \) was almost constant with time in both the control and adapting cultures, but there was a steady rise in the rate of acetate utilization by the mitochondria \( (\alpha V_\lambda) \) by the adapting cells (Fig. 3E). As can be seen by comparing Fig. 3, E and H, most of the rise in mitochondrial acetate utilization is due to an increase in \( V_\lambda \) rather than a change in the distribution of acetate \( (\alpha) \) between the mitochondrial and peroxisomal compartments.

Pyruvate utilization \( (V_\gamma) \) and the flux of acetyl-CoA from the outer to inner mitochondrial compartment \( (V_\delta) \) increased during the first 4 hours in both the control cultures and those adapting to low \( O_2 \) but then declined in the controls and continued to increase in the cells adapting to low \( O_2 \) (Fig. 3G).

**Change in Metabolic Flux Pattern during Deadaptation from Hypoxia**—The experiments so far described characterize the temporal sequence of changes in the flux rates of labeled acetyl-CoA in response to a sudden transition from relatively high oxygen tension to partial anaerobiosis. It was of interest also to examine the temporal sequence of metabolic changes in response to an abrupt increase in oxygen tension, a process we shall refer to for brevity as deadaptation. The measured values for incorporation of label into \( CO_2 \), glycogen, lipids, and glutamate during a 1-hour incubation with labeled precursors at various times during the deadaptation process are presented, along with the values obtained for control cultures maintained under deep conditions in Fig. 4. The values shown are the average values from two to three experiments except that only one experiment was done at zero hours.

There was little change in the oxidation of \([1-^{14}C]\)acetate during the first 4 hours of deadaptation. After 4 hours, there was a marked reduction in oxidative capacity (Fig. 4G). The rate of oxidation of \([2-^{14}C]\)pyruvate during the first 4 hours of deadaptation was the same as the control, but it is clear (Fig. 1H) that there was an increase in the capacity of cells maintained under hypoxic conditions to oxidize pyruvate whereas the capacity of the deadaptating cells decreased considerably by the end of the 8-hour period.

There was no change during 8 hours of aging in the rate of glyconeogenesis from acetate in control cells (Fig. 4D), but the rate of glyconeogenesis began declining rapidly immediately upon transfer of the cells grown in deep cultures to shallow conditions. Glyconeogenesis from \([2-^{14}C]\)pyruvate increased from about 9 to 13 nmol/10^6 cells per hour in control cultures aging under hypoxic conditions for 8 hours; deadaptating cells paralleled the controls during the first 4 hours after transfer to well aerated conditions, then declined markedly in the subsequent 4 hours.

The incorporation of label from acetate into glutamate increased markedly after 2 hours of deadaptation (Fig. 4B), while there was no significant change in label incorporation into glutamate in the control cultures.

The experimental data shown in Fig. 4 were analyzed according to the three-pool model of metabolism as described above and the parameters obtained are displayed in Fig. 5. Only the salient features of these graphs will be described.

There was little change in \( V_\lambda \), the rate of \( CO_2 \) production, from the Krebs cycle for 4 hours in the control and deadaptating cells, but a fall in oxidative capacity was seen between 4 and 8 hours in the latter (Fig. 5A). Glutamate production, \( V_x \), stayed relatively constant in control cells but increased steadily after 2 hours under high oxygen tension (Fig. 5B). There was little change in glycogen synthesis from the outer mitochondrial pool \( (\gamma V_\delta) \) in either control or deadaptating cells, or in the peroxisomal contribution \(((1-\gamma)V_\epsilon) \) to glyconeogenesis in the control cells aging under hypoxic conditions, but a steep steady decline in \(((1-\gamma)V_\epsilon) \) from the beginning of the deadaptation process (Fig. 5C) was observed. Total acetate utilization \( (V_\lambda) \) and acetate uptake into the inner mitochondrial compartment \( (\alpha V_\lambda) \) followed the same temporal pattern as did \( V_\lambda \) (cf. Fig. 5E with Fig. 5A), since \( \alpha \), the parameter governing distribution of acetate between mitochondria and peroxisomes, did not change (Fig. 5H). The fraction of octanoate \( \beta \)-oxidized in the mitochondria, \( \beta \), increased during aging of the control cultures but decreased during the first 4 hours of deadaptation and then stayed constant (Fig. 5H). The fraction of total glyconeogenic flux contributed by the mitochondria, \( \gamma \), increased steadily with time of deadaptation but did not change with time of aging in the control cells (Fig. 5I).

**DISCUSSION**

It was suggested by Raugi et al. (8) that the quantitative discrepancies between their results and those of Connett and Blum (2) were due to differences in growth conditions, and, specifically, in the difference in oxygen tension between the deep cultures used by Connett and Blum and the shallow ones used by Raugi et al. In the present experiments, cultures were grown under identical conditions of shaking, temperature, and initial cell density but with varying surface to volume ratios. The flux parameters measured in this study reflect the capacity of the cells to perform some metabolic activity and, as discussed earlier, are not necessarily indicative of the flux of unlabeled metabolites derived from the proteose-peptone medium. If growth for 17 hours at various culture depths left no persistent changes in the cells, then, since all the assays with the radioactive substrates were performed under highly aerobic conditions, one should have detected only minimal changes between the cells grown in deep versus shallow cultures. Such changes could result from alterations in the composition of the culture medium caused by different metabolic patterns during growth under conditions of varying oxygen tension. That this is probably not a major factor is indicated by Levy's finding (24) that the changed enzyme content which occurs during growth under static conditions reverts to that typical of cells grown under shaken conditions in the absence of cell division, and by our finding that the metabolic flux rates revert to those of cells grown in shallow cultures within a few hours.

The 30% increase in the capacity of *Tetrahymena* to consume oxygen after growth under deep conditions parallels the increase in appearance of label from \([2-^{14}C]\)pyruvate or \([2-^{14}C]\)glyoxylate in \( CO_2 \) as well as the increase in \( ^14CO_2 \) production from labeled acetate and pyruvate in the presence of these two substrates.
Fig. 4. Incorporation of label from [1-\textsuperscript{14}C]acetate, [2-\textsuperscript{14}C]pyruvate, and [1-\textsuperscript{14}C]octanoate into CO\textsubscript{2}, glycogen, lipid, and glutamate in cells grown under hypoxic conditions and transferred to aerobic conditions.

 Cultures were grown at a total volume of 120 ml in 500-ml Erlenmeyer flasks with shaking for 17 hours. At zero time, 2 aliquots of 40 ml each were transferred to empty sterile 500-ml flasks, and the three shallow cultures were then kept in the shaker bath for up to 8 hours. A control deep culture was also kept in the shaker bath. At the times indicated on the abscissa, cells from the experimental and control flasks were assayed exactly as described in the legend to Fig. 2. All units are nanomoles of label incorporated per 10\textsuperscript{6} cells per hour. •—•, control culture, maintained under deep conditions; ○—○, experimental culture, transferred from deep to shallow conditions at \( t = 0 \).

 The points shown are those from a single experiment at zero time, and the mean values of two experiments at 2 hours, three experiments at 4 hours, and two experiments at 8 hours. The difference between the pairs or triplets of experimental values and the means was less than \( \pm 15\% \) except for the following points: D, 4-hour experimental, \( \pm 17\% \); E, 4-hour control, \( \pm 28\% \); F, 8-hour control, \( \pm 37\% \); G, 8-hour experimental, \( \pm 32\% \); H, 2-hour experimental, \( \pm 27\% \); I, 4-hour control, \( \pm 28\% \); J, 4-hour experimental, \( \pm 34\% \); K, 8-hour experimental, \( \pm 23\% \); L, 4-hour control, \( \pm 34\% \); M, 4-hour experimental, \( \pm 18\% \), 8-hour control, \( \pm 70\% \).
Fig. 5. Changes in metabolic parameters during deadaptation from hypoxic growth conditions. The distribution parameters $\alpha$, $\beta$, $\gamma$, and $\varepsilon$ are unitless. All other values are in nanomoles per $10^6$ cells per hour. For further details, see legend to Fig. 4.

plus octanoate. Levy and Wasmuth (25) found that the specific activities of several enzymes, including isocitrate lyase and succinic dehydrogenase, increased in Tetrahymena grown under static versus shaken conditions which accords well with our findings of increased oxidative capacity in cells grown under relatively anaerobic conditions. An adaptive increase in oxidative capacity in response to hypoxia is not limited to Tetrahymena. In rats, for example, hypoxic acclimatization caused increases in succinic dehydrogenase activity in mitochondria from heart and kidney of about 41% and from liver of about 135%, on a per mg of mitochondrial protein basis (26). Recent evidence (27) confirms the increase in succinic dehydrogenase activity and suggests that it may reflect activation by an effector rather than increased enzyme content.

In contrast to the increase in capacity to oxidize glyoxylate, acetate, and pyruvate, the rate of oxidation of [1-14C]glucose to 14CO2 was decreased in cells grown in deep cultures. The finding by Levy and Wasmuth (25) that pyruvate kinase increased in static cultures renders it unlikely that glucose oxidation was limited by this reaction, and the reduction in glucose oxidation
capacity caused by growth under partially anaerobic conditions is probably due to a reduction in the enzyme activity between the hexokinase and pyruvate kinase steps. Since phosphofructokinase, localized largely on the mitochondria of *Tetrahymena* (28), appears to be the rate-limiting enzyme of the glycolytic sequence (22), it is likely that partial anaerobiosis reduces the activity of this enzyme *in situ* despite the fact that no change was observable when this enzyme was assayed *in vitro* from cells grown in deep or shallow cultures.

The effect of low oxygen tension during growth in causing a decrease in the capacity to oxidize glucose is contrary to the well known Pasteur effect described in *Tetrahymena* (29). Under conditions of complete anaerobiosis, *Tetrahymena* rapidly deplete their glycogen reserves (29), indicating a marked acceleration of the glycolytic pathway, whereas under the present conditions of partial anaerobiosis there was an increase in total glycolytic content as compared to well aerated cultures. This suggests that at lower oxygen tensions than were studied here there is a switch from net glyconeogenesis to net glycolysis. In all systems so far studied the Pasteur effect seems to be mediated via the regulatory effects of adenosine nucleotides on phosphofructokinase (22). The failure of the ATP:ADP ratio to change is thus not inconsistent with the lack of a Pasteur effect under these conditions of growth. It should be noted, however, that the measurements of adenosine di- and triphosphate were done on the original culture in contrast to measurements of label incorporation which were done after transfer to a highly aerobic environment.

The increased capacity to oxidize 2- and 3-carbon atom moieties after growth in deep cultures was reflected in an increase in $V_a$, the total acetate flux measured after the addition of the three substrates. Since $V_a$ was increased in each compartment, it would appear that either both the peroxisomal and the mitochondrial acetyl-CoA synthetases (30) increased or there was a greater availability of CoA in both the inner mitochondrial and peroxisomal compartments. Levy and Scherbaum (31), however, showed that there was no difference in total acetyl-CoA synthetase in cells exposed to static conditions for 3 hours, so the former explanation is unlikely. Total lipogenesis, $V_s$, also increased slightly in deep as compared to shallow cultures. There was no significant change in the pathway for direct incorporation of octanoate into lipids.

A key effect of growth under reduced oxygen tension was the large increase in flux of acetyl-CoA directed to glyconeogenesis, both from the outer mitochondrial compartment and from the peroxisomal compartment. Although both isocitrate lyase and malate synthetase increase upon transfer of cells from shaken to static conditions (31), the increase in glyconeogenesis can occur even when the increase in activity of these two enzymes is prevented (4, 32). The increase in the cytosol form of phosphoenolpyruvate carboxykinase (6, 33) observed in static versus shaken cultures of *Tetrahymena* may not be essential either since the lowest measured activity of this enzyme under either condition is much larger than either the rate of glyconeogenesis (see Fig. 1) or the activity of either of the glyoxylate cycle enzymes. Voichick *et al.* (34) recently found that the total 3':5'-monophosphate (cyclic AMP) content of a culture of *Tetrahymena* decreases during the induction of the stationary phase in standing cultures. It seems highly probable, then, that cyclic AMP levels are lower in deep as compared to shallow cultures, and this may play a major role in controlling the flow of metabolites along the glyconeogenic pathway. It is unlikely, however, that cyclic AMP is involved in all the changes reported here, and further experimentation on the role of cyclic AMP in the regulation of *Tetrahymena* metabolism is obviously necessary.

Following a report (23) that 5'-AMP increases the glycogen content of cultures of *Tetrahymena* grown without shaking, we investigated the effect of 5'-AMP on several aspects of metabolism (10). Because the experiments with the labeled substrates were done on shallow cultures, and the assays also performed under relatively aerobic conditions where glyconeogenesis is small, it was of interest to determine the effect of AMP on cells grown under hypoxic conditions. The finding that the effect of AMP was essentially the same in deep as in shallow cultures indicates that the inhibition of glyconeogenesis by AMP does not depend on oxygen tension. The observation that treatment with AMP reduces $V_s$ and $V_q$ while increasing $V_t$ is important in view of the fact that decreasing oxygen tension increased all three. This means that the mechanism of action of 5'-AMP is different than that for oxygen.

These experiments show that reductions in the average oxygen tension during growth cause changes in the pattern of flow of acetyl-CoA along the pathways of intermediate metabolism which last for at least 1 hour after the cells are transferred to aerobic conditions. In particular, peroxisomal function in *Tetrahymena* appears to be very sensitive to alterations in oxygen tension, and in the range of oxygen tension studied here the changes in glyconeogenesis and in glycolysis observed appear to be unrelated to the Pasteur effect which, presumably, comes into play under even more anaerobic conditions.

**Changes of Metabolism with Culture Age**—Cells grown for 17 hours with shaking attain cell densities in excess of 500,000 cells per ml whether grown under "shallow" or "deep" conditions. There is, of course, nothing special about 17 hours, a number chosen entirely for our convenience. It is to be expected, therefore, that even in cells maintained for 8 additional hours with no change in culture depth, metabolic changes will occur as the composition of the medium changes and as the cells make the transition from the logarithmic phase of growth to the stationary phase. The changes that occur with age in deep cultures when exposed to a mixture of labeled acetate, glutamate, aspartate, and alanine have been described earlier (2). During the 8-hour interval studied here, which corresponds to the time between the transitional cells and early stationary cells studied by Connett and Blum (2), there was a decline in glycogen, and probably in the oxidation of the octanoate as well. The oxidation of pyruvate and glyconeogenesis from pyruvate, however, increased with age. Thus, in cells maintained under low oxygen tension, octanoate utilization in the peroxisomes decreased while pyruvate utilization in the outer mitochondrial compartment increased during the interval from 17 to 25 hours after the start of growth.

During this same interval (i.e., from 17 to 25 hours after the start of growth) cultures maintained under shallow conditions exhibited little change in pyruvate oxidation, in pyruvate conversion to glycogen, or in glyconeogenesis from octanoate. Oxidation of octanoate, however, declined. Thus, in contrast to cells maintained under deep conditions, where octanoate utilization apparently declined in the peroxisomes, this decline occurred in the mitochondrial compartment in cells maintained at relatively high oxygen tension.

In shallow cultures, incorporation of label from acetate into glutamate increased with time during the 8-hour interval under
discussion. In deep cultures, however, there was little change in the incorporation of label from acetate into glutamate during this interval. The output of glutamate from rat heart mitochondria depends on the balance between citrate synthetase, aspartate transaminase, and α-ketoglutarate dehydrogenase, and is controlled in part by the ATP:ADP ratio and by the NADH:NAD + ratio (35). In Tetrahymena, α-ketoglutarate dehydrogenase and glutamic dehydrogenase are also involved in determining the outflow of glutamate, but, except for the observation that the ATP:ADP ratio does not change as a function of degree of aeration, nothing is known concerning the regulation of glutamate output.

Metabolic Responses to Changes in Oxygen Tension—In cells adapting to a sudden decrease in oxygen tension, the rate of oxidation in the Krebs cycle, V, increases steadily during the next 8 hours (Fig. 3A); a transition from low to high oxygen tension, however, does not cause a decrease in V, until about 4 hours after the deadaptation process begins (Fig. 5A). Similarly, acetate utilization in the inner mitochondrial compartment (vV) increases fairly steadily during adaptation to low oxygen (Fig. 3E), whereas αV does not begin decreasing until 4 hours after a change from low to high oxygen tension (Fig. 5E). Thus, in the mitochondria at least, the temporal changes during adaptation are not the mirror images of those seen during deadaptation. Indeed, because of the differences (described above) which occur during the interval from 17 to 25 hours after inoculation even in cultures maintained at constant depths, one would not expect the temporal pattern of metabolic changes during adaptation to low oxygen to be the same as that during deadaptation from low to high oxygen tension. A similar observation had been made for lactic dehydrogenase, localized in the mitochondria of Tetrahymena (15), and for pyruvate kinase, localized in the cytosol (33). These two enzymes increase at the same rate following a reduction in oxygen tension but did not decrease for at least 8 hours after shaking of the static cultures was resumed (24). Although it is known that succinic dehydrogenase increases in static cultures (25), the time course of this change was studied only during intervals of days. Acetyl-CoA synthetase does not appear to change in response to a decrease in oxygenation (31). Thus the mechanism for the observed changes in flow of labeled acetyl-CoA in the mitochondria are at present obscure, but it is clear that in this compartment the response to a step change in oxygen tension happens at different times for different processes and, furthermore, the rate of response is different for a step increase than for a step decrease in oxygen tension.

In response to an abrupt decrease in oxygen tension, there is a rapid rise in glyconeogenesis from the peroxisomes (11 - γ)ψl which levels off about 4 hours after the drop in oxygen level (Fig. 3C); in response to the reverse transition the decline in peroxisomal glyconeogenesis also begins soon after the change in oxygen tension, but continues declining throughout the 8-hour period (Fig. 5C). Thus in the peroxisomes there is also not a mirror image relation between the time course of change in flow of labeled acetyl-CoA into glyconeogenesis in cells adapting to a decrease in oxygen tension as compared to cells responding to an increase in oxygen tension. A doubling in the activities of isocitrate lyase and malate synthetase is achieved within 3 hours of a reduction in oxygen tension (4, 31), and a halving of the isocitrate lyase activity was observed about 3 hours after an abrupt increase in oxygenation (24). These changes are within the right time scale to account for the rates of change of peroxisomal glyconeogenesis reported here. It must be empha-

ized, however, the full increase in glyconeogenesis occurred even when the increase in enzyme content was prevented by inhibitors of protein and RNA synthesis (4).

The experimental results presented here are the first studies which permit one to describe the sequence of changes in the flux of labeled acetyl-CoA from each of the three pools known in this cell during adaptation to changes in oxygen tension. To within the error of the measurements, it can be seen that changes occur in each compartment according to an individual temporal pattern, which in itself depends upon the direction of the shift in oxygen tension. That these changes occur within a range of oxygen tension which alters the rates of glyconeogenesis but does not activate the Pasteur effect suggests that the cytosol, mitochondria, and peroxisomes of this cell are each sensitive to different processes and, furthermore, the rate of response is different for a step increase than for a step decrease in oxygen levels. Much further work will be necessary to unravel the complexities of this system.

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