SUMMARY

The compartmentalization of acetyl-CoA metabolism in *Tetrahymena* has been studied by measuring the incorporation of label from [1-14C]acetate, [2-14C]pyruvate, and [1-14C]octanoate into CO2, lipids, glycogen, and glutamate in experiments in which these substrates were used singly, in pairs, and all together. The results of these studies require that there be at least three pools of acetyl-CoA in *Tetrahymena*, two in the mitochondria and one in the peroxisomes. A steady state model was developed which allows a quantitative analysis of the metabolic fate of acetyl-CoA derived from acetate, pyruvate, and octanoate. The model shows that pyruvate enters a different mitochondrial pool of acetyl-CoA than acetate or octanoate, which accounts for the relatively high proportion of [2-14C]pyruvate label incorporated into glycogen. About three-fourths of the acetate utilization occurs in the mitochondria, the remainder via the peroxisomal pool of acetyl-CoA for lipogenesis. Only part of the octanoate utilization is accounted for by β-oxidation in the peroxisomes; a pathway is also required allowing the incorporation of label from octanoate into lipids without prior oxidation to acetyl-CoA. Labeled acetyl-CoA used for glyconeogenesis comes from both the peroxisomal and the outer mitochondrial pools of acetyl-CoA, more of it coming from the former. The experiments were performed on well aerated cultures in both the logarithmic and transitional phases of growth. Differences in metabolic flux patterns between cells at these two stages of growth are small; lipogenesis was decreased and glutamate output and pyruvate utilization were increased in transition as compared to log cultures.

It has become apparent that compartmentalization of substrates and enzymes plays a major role in the regulation of metabolism in living cells. Connell et al. (1), on the basis of label incorporation studies, showed that there were at least two pools of acetyl-CoA in *Tetrahymena*, one in the peroxisomes and one in the mitochondria. This enabled Connell and Blum (2) to construct a model for carbon flow in *Tetrahymena* which appeared to conform to much of the known data on localization of enzymes in this cell. Although a good fit was obtained to most of their data, certain problem areas were apparent. Perhaps the most troublesome point was the utilization of acetyl-CoA for fatty acid synthesis. The model required that there be two pathways for lipid synthesis, one from the acetyl-CoA pool of the peroxisomes, and the other from the acetyl-CoA pool of the mitochondria, with the latter pool contributing an unexpectedly large fraction of the acetyl groups for lipogenesis. A second problem concerned the deduction that there were two pools of phosphoenolpyruvate but only the pool derived from the mitochondria was used for glyconeogenesis. Even with this assumption, it was not possible to obtain a good fit to the data on incorporation of label into glycogen in log phase cells.

Several authors have suggested that there are two pools of acetyl-CoA in the mitochondrion. Delisle and Fritz (3) and Fritz (4) suggested that one pool, derived primarily from pyruvate, was used for oxidation while the other, derived from fatty acids, was the primary source of acetocacetate. In mammalian mitochondria, where carnitine serves as an intermediate in the transport of long chain fatty acyl groups into the inner or matrix compartment, it appears almost self-evident that there should be two pools of acetyl-CoA, one in the intermembrane space and one in the matrix. Chase and Tubbs (5) from in vitro studies with inhibitors of fatty acid oxidation, recently concluded that there were two pools of CoA in rat liver mitochondria which serve as substrates for two pools of carnitine palmitoyltransferase. Although *Tetrahymena* contain neither carnitine nor carnitine palmitoyltransferase (6), it nevertheless seemed probable that the mitochondria of *Tetrahymena* might contain two pools of acetyl-CoA.

In this paper we have extended the approach first used by Connell et al. (1) to establish two pools of acetyl-CoA. Experiments were performed in which cells were exposed to octanoate, pyruvate, and acetate singly, in pairs, and all three substrates at once, with only one substrate at a time labeled with 14C in such a position that any acetyl-CoA formed would be labeled in the carboxyl carbon. The results of these experiments require that there be at least three pools of acetyl-CoA in *Tetrahymena* and lead to the construction of a model which quantitatively accounts for the flux of acetyl groups into the tricarboxylic acid cycle, the glyoxylate cycle, and lipogenesis.
All the experiments using three substrates were done as follows. Flasks containing 100 ml of medium at 26° for the experiments were phosphate buffer adjusted to pH 6.5 with NaOH. All stock cultures and 0.05% concentrated liver extract in 0.02M fluids routinely used were: Scintillation Solution A, BBOT-electrol (0.4% w/v, 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene in toluene and absolute ethanol (10:3 w/v)); Scintillation Solution B, BBOT-Triton (0.4% w/v BBOT in toluene and Triton X-100, 20:13, v/v); Scintillation Solution C, 4% w/v Cab-o-Sil thixotropic gel powder in Scintillation Solution A. Incorporation of label into CO₂ was measured as described previously (7), except that following injection of sulfuric acid into the flask to stop the reactions and hyamine to the center of the well to trap CO₂, flasks were kept at 0° without shaking for 1 hour to collect CO₂. This procedure was found to give much lower blanks than the 37° procedure but did not change the net number of counts collected. Incorporation of label into CO₂ was measured as described previously (7), except that following injection of sulfuric acid into the flask to stop the reactions and hyamine to the center of the well to trap CO₂, flasks were kept at 0° without shaking for 1 hour to collect CO₂. This procedure was found to give much lower blanks than the 37° procedure but did not change the net number of counts collected. Incorporation of label into CO₂ was measured as described previously (7), except that following injection of sulfuric acid into the flask to stop the reactions and hyamine to the center of the well to trap CO₂, flasks were kept at 0° without shaking for 1 hour to collect CO₂. This procedure was found to give much lower blanks than the 37° procedure but did not change the net number of counts collected. Incorporation of label into CO₂ was measured as described previously (7), except that following injection of sulfuric acid into the flask to stop the reactions and hyamine to the center of the well to trap CO₂, flasks were kept at 0° without shaking for 1 hour to collect CO₂. This procedure was found to give much lower blanks than the 37° procedure but did not change the net number of counts collected.

Incorporation into lipid, glycogen, and glutamate was measured following a 1-hour incubation of 9 ml of cells plus 0.6 ml of substrate at 26° with shaking in a 125-ml sealed Erlenmeyer flask. After the incubation 3 ml of the cell suspension from each flask were pipetted into 15 ml of ice-cold 2% (w/v) trichloroacetic acid for assay of incorporation into lipid, and into 8 ml of ice-cold 0.5% (v/v) ethanol for assay of incorporation into glycogen. The cells remaining in each set of triplicate flasks were chilled for about 15 min in ice and then centrifuged at 400 × g for 4 min at 0°. The supernatants were pooled and 8 ml were taken for assay of incorporation into glutamate, and, in representative experiments, into alanine and aspartate.

The cells in 2% trichloroacetic acid were centrifuged at 400 × g for 4 min at 0°. The pellet was washed twice with 0.5% (w/v) NaCl and resuspended in fresh sterile proteose peptone. Lipids were extracted from this mixture by the method of Folch (8). The chloroform-methanol mixture was evaporated under nitrogen and the lipids quantitatively transferred to counting vials in Scintillation Solution A.

The cells that had been pipetted into 95% ethanol were centrifuged and the pellet was washed twice with 8 ml of ice-cold 95% ethanol, and then stored at −15° until needed. After thawing the pellet was resuspended in 1 ml of 0.05 M sodium acetate buffer (pH 4.5) and glycogen was hydrolyzed by incubation with glucoamylase (Miles Laboratories) for 90 min at 45° as described by Connett and Blum (7). The samples were de-proteinized by the Somogyi method (9) and a 1-ml aliquot of supernatant was counted in 10 ml of Scintillation Solution B.

Incorporation of label into glutamate, aspartate, and alanine in the pooled supernatants was measured by column chromatography essentially as described by LaNoe et al. (10). One milliliter of 40% (w/v) trichloroacetic acid was added to the 8 ml of pooled supernatant and the mixture allowed to stand at 4° for about 2 hours. Following a brief centrifugation to remove the precipitate, the supernatant was extracted six times with equal volumes of diethyl ether and evaporated to dryness. It was ascertained that no alanine, aspartate, or glutamate was lost by this procedure. The residue was dissolved in 2 ml of water and applied to a Bio-Rad AG 1-X8 formate column (1 × 25 cm) and eluted with 40 ml of 0.01 M formic acid. The first 15 ml were discarded. Elution was continued with 60 ml of 0.3 M formic acid. The first 10 ml of this were combined with the last 25 ml of the 0.01 M formic acid, evaporated in a flash evaporator, and put on a thin layer plate as described below. The next 50 ml of 0.3 M formic acid eluate were collected and evaporated to dryness in a flash evaporator. The residue was dissolved in a small volume of water and applied to a Bio-Rad AG 1-X4 acetate column (1 × 25 cm) and eluted with 40 ml of 0.5 M acetic acid followed by 60 ml of 0.7 M acetic acid. Fractions of 2 ml were collected and aliquots from the regions corresponding to glutamate and aspartate were counted in Scintillation Solution B. The sum of all counts in each peak was taken as the amount of label incorporated into that amino acid.

The homogeneity of the peaks for glutamate and alanine was tested by thin layer chromatography. Fractions corresponding to glutamate, aspartate, or alanine were pooled and evaporated to dryness. The residue was redissolved in a small volume of water and spotted on a microcrystalline cellulose thin layer plate, 20 × 20 cm (Applied Science Laboratories) and subjected to two-dimensional development with Solvent 1 (diethyl ether-formic acid (90%)-water (7:2:1)) and Solvent 2 (phenol (90%)-water-formic acid (90%)) (83:17:1). For analysis of alanine a one-dimensional development using only Solvent 2 was necessary. A mixture of alanine, aspartate, and glutamate, each 10 mm, was run as a standard on a identical plate. The standard spots were visualized with ninhydrin spray. In corresponding regions on the plate containing the sample, 1-cm strips were taken, 1 ml of water was added to extract the amino acids and 10 ml of Scintillation Solution C were added to each.

Reagents—[1-¹⁴C]Acetate, [2-¹⁴C]Pyruvate, and [¹⁴C]Octanoate were obtained from Amersham-Searle. Octanoic acid was purchased from the Hormel Institute. Bovine serum albumin was purchased from Sigma. Sodium pyruvate was obtained from K & K. All other reagents were of high grade.

RESULTS

Table I shows the results of experiments using acetate, pyruvate, and octanoate singly and in pairs. The rates of oxidation for [1-¹⁴C]acetate, [2-¹⁴C]pyruvate, and [¹⁴C]octanoate were about 900, 100, and 50 nmoles per 10⁶ cells per hour, respectively. Acetyl-CoA derived from pyruvate and, especially, octanoate was preferentially used for lipogenesis as opposed to glycogenogenesis. Although 9 times more acetate was oxidized than pyruvate, less than 2 times as much acetate went to glycogen synthesis as pyruvate, and very little of the label derived from [¹⁴C]octanoate appeared in glycogen. It would appear from the single substrate data alone that more than two pools of acetyl-CoA might be present, but one could argue that the metabolic state in the presence of, say, acetate, was quite different than the state in...
Cells were grown overnight in a total volume of 54 ml in a 500-
m1 Erlenmeyer flask with shaking at 26°C. N; values for Experiments A and B were 139,000 and 192,000 cells per ml, respectively. After 17 hours aliquots were incubated for 1 hour in the presence of either 6.2 mm [1-14C]acetate (130 dpm per nmole) or 6.2 mm [2-14C]pyruvate (50 dpm per nmole) or 1.5 mm [1-14C]octanoate (500 dpm per nmole) (data shown under "Experiments using single substrates"). Additional aliquots were incubated similarly for 1 hour in the presence of 6.2 mm sodium acetate and 1.6 mm octanoate containing either [1-14C]acetate or [1-14C]octanoate at the same specific activities as above. Similarly, aliquots were incubated for 1 hour in the presence of 6.2 mm sodium pyruvate and 1.6 mm octanoate containing either [2-14C]pyruvate or [1-14C]octanoate, also at the specific activities above. Units are nanomoles of labeled substrate appearing per 10^6 cells per hour in the indicated products.

| Labeled substrate | Experiment | Amount of label per 10^6 cells per hr appearing in | nmoles |
|-------------------|------------|---------------------------------------------|--------|
|                   |            | CO2  | Lipid | Glycogen | Glutamate |
| Experiments using single substrates | | | | | |
| [1-14C]Acetate    | A          | 945  | 196   | 25.0     |           |
|                   | B          | 823  | 199   | 21.0     |           |
| [2-14C]Pyruvate   | A          | 114  | 44.3  | 14.0     |           |
|                   | B          | 93   | 40.3  | 14.5     |           |
| [1-14C]Octanoate  | A          | 55.4 | 46.5  | 1.4      |           |
|                   | B          | 52.9 | 37.3  | 1.5      |           |
| Experiments using pairs of substrates | | | | | |
| [1-14C]Acetate    | A          | 816  | 95.2  | 18.5     | 12.7     |
|                   | B          | 704  | 101   | 11.5     | 0.5      |
| [1-14C]Octanoate  | A          | 4.4  | 39.8  | 0.40     | <0.1     |
|                   | B          | 11.0 | 34.4  | 0.90     | <0.1     |
| Ratio of acetate to octanoate | A       | 174  | 2.7   | 46       | >120     |
|                   | B          | 64   | 2.9   | 38       | >60      |
| [2-14C]Pyruvate   | A          | 104  | 10.2  | 14.0     | <0.5     |
|                   | B          | 79.3 | 19.8  | 10.0     | <0.5     |
| [1-14C]Octanoate  | A          | 35.5 | 60.3  | 1.3      | 0.2      |
|                   | B          | 40.4 | 43.6  | 1.2      | <0.1     |
| Ratio of pyruvate to octanoate | A         | 2.9  | 0.27  | 10.7     |           |
|                   | B          | 2.0  | 0.45  | 8.3      |           |

the presence of say, octanoate, so that no deduction concerning pool number is possible. Experiments with pairs of substrates (with only one labeled) reduce the freedom of interpretation, since the cells were in the same metabolic state. It can be seen (Table I) that whereas acetate reduced the oxidation of [1-14C]-octanoate to 14CO2 by 80 to 90% (compared to the oxidation observed when [1-14C]octanoate was the only substrate), octanoate reduced the oxidation of [1-14C]acetate by only 10 to 15%. The incorporation of label into lipid was altered in an entirely different way; octanoate reduced incorporation of label from [1-14C]-acetate into lipid by about half, whereas acetate reduced incorporation of label from [1-14C]octanoate into lipid by only about 10%.

Similar experiments were done with octanoate and pyruvate as the paired substrates (Table I). Addition of octanoate caused a modest inhibition of pyruvate oxidation but a strong inhibition of incorporation of pyruvate into lipid. Addition of pyruvate decreased octanoate oxidation by 20 to 30% but slightly enhanced the incorporation of label from octanoate into lipid.

Although the amount of label appearing in glutamate was not measured in the experiments in which the substrates were used singly, it is clear that much more label is incorporated into glutamate from [1-14C]acetate than from either [2-14C]pyruvate or [1-14C]octanoate in the experiments with paired substrates. The ratios of acetate to octanoate oxidized to CO2 and of acetate to octanoate appearing in glutamate were about the same, and markedly different from the ratios of acetate to octanoate incorporated into either glycerogen or lipid. When pyruvate and octanoate were the paired substrates, the ratios of amounts incorporated into glycogen, lipid, and CO2 were also different from each other. The glutamate produced from either of these substrates was too small to permit an accurate measurement and the ratio for glutamate was not computed.

Since each substrate was labeled in each a way that only the carboxyl group of acetyl-CoA should be labeled, the data may be considered as measuring the distribution of acetyl-CoA derived from the various substrates. If the metabolism of two substrates contributes to the same homogeneous pool of acetyl-CoA, the specific activity of products synthesized from this pool should be proportional to the specific activity and contribution made by each substrate. Thus the observation that the ratios of CO2 and glutamate formed from acetate and octanoate are the same suggests that CO2 and glutamate are derived from the same pool of acetyl-CoA. The observation that all other ratios of products obtained from either acetate and octanoate or pyruvate and octanoate are different from the ratios of CO2 and glutamate and from each other strongly suggests that acetyl-CoA used for the ultimate synthesis of glycogen or lipid comes from different pools. Since it could be argued that cells in progress peptide supplemented with acetate plus octanoate are in a different metabolic state than cells supplemented with pyruvate plus octanoate, the paired substrate experiments cannot prove the point.

Table II shows the results of experiments in which cells in the log or transitional phase of growth were incubated for 1 hour in the presence of acetate, pyruvate, and octanoate, but with only one of these substrates labeled in each flask. Thus all measurements of label distribution were carried out on metabolically identical cells. Because the metabolic model to be developed below assumes that the cells were in a metabolic steady state, it was necessary to ascertain whether label output from each of the labeled substrates was linear with respect to time after addition of the three substrates. It has earlier been shown 1 that for any one substrate labeled in a mixture of acetate, glutamate, aspartate, and alanine, the rate of incorporation of label into CO2, glycerogen, or lipid was linear with time after about 10 min, indicating that a steady state was achieved about 10 min after addition of those substrates. Since neither pyruvate nor octanoate had been examined from this point of view, experiments were done on both log and transitional cells using acetate, pyruvate, and octanoate (with only one substrate labeled at a time). The rate of incorporation of label into CO2, lipid, and glycerogen was measured as a function of time up to 1.5 hours. For each substrate, incorporation of label into these end products of metabolism was linear after about a 5- to 15-min lag with one exception. The incorporation of label from octanoate into CO2 in log cells was not linear until after about a 30-min lag. This means that for log cells attainment of the steady state for octanoate.

1 R. J. Connett and J. J. Blum, unpublished data.
Cells were grown overnight in a total volume of 41 to 43 ml in a 500-ml Erlenmeyer flask with shaking at 26°. Cultures to be harvested in log phase were started by inoculating 2.5 ml of a late transition phase culture (Nf approximately 700,000 cells per ml) into 37.5 ml of proteose-peptone medium plus 1 to 3 ml of water. Cultures to be harvested in transition phase were started by inoculating 8.0 ml of a late transition phase culture into 32 ml of proteose-peptone medium plus 1 to 3 ml of water. After 17 hours aliquots were incubated for 1 hour in the presence of 6.2 mM sodium acetate, 6.2 mM sodium pyruvate, and 1.5 mM octanoate containing one of the radioactively labeled substrates. Final specific activities were 140, 50, and 500 dpm per nmole for [1-14C]acetate, [2-14C]pyruvate, and [1-13C]octanoate, respectively. Units are nanomoles per 10^6 cells per hour radioactivity appearing in product. (L), log phase cultures; (T), transition phase cultures. Values shown are means ± standard deviation.

**Table II**

| Labeled substrate | CO2 | Lipids | Glycogen | Glutamate |
|-------------------|-----|--------|----------|-----------|
| [1-14C]Acetate (L) | 481.2 ± 30.3 (9) | 160.1 ± 18.9 (9) | 14.7 ± 2.1 (9) | 4.2 ± 1.1 (9) |
| [1-14C]Acetate (T) | 496.0 ± 24.9 (8) | 142.2 ± 9.3 (8) | 16.1 ± 3.2 (8) | 7.6 ± 2.0 (8) |
| [2-14C]Pyruvate (L) | 62.4 ± 7.5 (9) | 2.3 ± 0.7 (9) | 1.8 ± 0.6 (9) | <0.5 (8) |
| [2-14C]Pyruvate (T) | 67.8 ± 4.0 (8) | 2.1 ± 0.5 (8) | 5.3 ± 1.5 (8) | <1.0 (7) |
| [1-14C]Octanoate (L) | 6.7 ± 2.1 (9) | 35.6 ± 3.1 (9) | 0.38 ± 0.06 (9) | <0.1 (8) |
| [1-14C]Octanoate (T) | 5.8 ± 1.8 (8) | 30.4 ± 3.0 (8) | 0.40 ± 0.13 (8) | <0.1 (7) |
| [1-13C]Pyruvate (L) | 77.1 ± 6.8 (5) | 83.2 ± 2.8 (2) |  |  |
| [1-13C]Pyruvate (T) |  |  |  |  |

a p < 0.05, b p < 0.01, c p < 0.02.

Additional text...
The model finally adopted and which, as will be shown below, accounts quantitatively for the data obtained for all 17 of the three substrate experiments, is presented in Fig. 1. Three pools of acetyl-CoA are shown, as required by the data presented in Tables I and II. A pool of CoA in the peroxisomes (Pool II) is required because of the presence of isocitrate lyase and malate synthase in the peroxisomes and the operation of the glyoxylate cycle in the peroxisomes. The model also shows β oxidation of octanoate occurring in the peroxisomal pool. This was necessitated in order to fit the data of Table II. Subsequently, it has been shown (11) that several of the enzymes of β oxidation and fatty acid activation are located in Tetrahymena in both the peroxisomes and the mitochondria, with the activities in the peroxisomes generally higher than the activities in the mitochondria. It is generally agreed (13-15) that the enzymes of the Krebs cycle are localized in the matrix of mitochondria. Since the data in Table I suggest that both glutamate and CO₂ come from the same pool, we have shown these two products as being derived from the inner mitochondrial pool of acetyl-CoA (Pool III), and pyruvate is shown as entering a second mitochondrial pool of acetyl-CoA, assumed to be localized between the inner and outer mitochondrial membranes. It is believed that both the outer mitochondrial membrane (13) and the peroxisomal membrane (16) are relatively permeable to small molecules. This cannot be true for both these membranes in Tetrahymena, since we require at least three pools of acetyl-CoA. It is possible, however, that either the peroxisomal membrane or the outer mitochondrial membrane are permeable to acetyl-CoA and thus are in equilibrium with any cytosolic acetyl-CoA. In the scheme shown in Fig. 1, glycogen is viewed as being derived from the peroxisomal pool as well as from the intermembrane mitochondrial pool, whereas lipid is derived by direct utilization of octanoate and from the peroxisomal pool of acetyl-CoA. Justification for these assumptions will be discussed below. Since only a negligible amount of label appeared in aspartate and alanine and since an excellent fit can be attained without assuming any uses for acetyl-CoA other than shown in the model, it was unnecessary to assume any other end product of quantitative importance.

**Fitting of Experimental Data to Model**

Table III presents sets of data obtained from two of the 17 experiments summarized in Table II and the parameters used to obtain the calculated values which are shown immediately below the observed values. The procedure used to obtain the calculated values was as follows. Equations were written (see "Appendix") for the steady state flow of acetyl-CoA according to the model shown in Fig. 1, and a computer program was written which would be suitable for trial and error fitting. After the data are entered, $V_i$, $V_3$, $V_5$, and $V_6$ are computed as the sum of the number of nanomoles of label from each substrate appearing in $CO_2$, glutamate, glycogen, and lipid, respectively, $V_5$, $V_6$, and $V_7$ are then computed, and the adjustable parameters $V_{10}$, $V_{14}$, $a$, $β$, $γ$, and $ε$ are set. From these $V_5$ and $V_{11}$ may be calculated from the conservation equations, and the specific activity of acetyl-CoA in each pool is then computed for each labeled substrate in turn. The calculated value of label appearing in $CO_2$ from $[1-^{14}C]$acetate, for example, is the product of $V_5$ and the specific activity of acetyl-CoA in Pool III when acetate is the labeled substrate. The parameter values are then adjusted until a satisfactory fit is obtained. In view of the virtually perfect fit obtainable, it was unnecessary to use any least square or other statistical techniques. It must be emphasized that the fits shown in Table III are typical fits and in no way unrepresentative of the fits obtained for the other 15 experi-

**Table III**

| Labeled substrate | Amount of label per 10⁶ cells per hr appearing in | nmoles |
|-------------------|---------------------------------------------|--------|
|                   | $CO_2$                                      | Lipids | Glycogen | Octanoate |
| **Experiment A**  |                                             |        |          |           |
| $[1-^{14}C]$Acetate | 473.3                                      | 161.4  | 16.7     | 3.0       |
| Observed          | 473.3                                      | 161.5  | 16.7     | 3.9       |
| Calculated        |                                             |        |          |           |
| $[2-^{14}C]$Pyruvate | 68.6                                       | 1.6    | 3.5      | <0.0      |
| Observed          | 68.6                                       | 1.6    | 3.5      | 0.6       |
| Calculated        |                                             |        |          |           |
| $[1-^{14}C]$Octanoate | 7.7                                        | 31.5   | 0.46     | <0.1      |
| Observed          | 7.8                                        | 31.5   | 0.46     | 0.06      |
| Calculated        |                                             |        |          |           |
| **Experiment B**  |                                             |        |          |           |
| $[1-^{14}C]$Acetate | 406.3                                      | 128.3  | 14.9     | 3.6       |
| Observed          | 406.2                                      | 128.3  | 14.9     | 3.6       |
| Calculated        |                                             |        |          |           |
| $[2-^{14}C]$Pyruvate | 56.9                                       | 1.2    | 2.9      | <0.0      |
| Observed          | 56.9                                       | 1.2    | 2.9      | 0.5       |
| Calculated        |                                             |        |          |           |
| $[1-^{14}C]$Octanoate | 10.0                                      | 29.0   | 0.46     | <0.1      |
| Observed          | 10.0                                       | 29.0   | 0.47     | 0.09      |
From 1-Octanoate. A change of 0.02 in the value of y deviation of the calculated fit for appearance of label in glycogen change of ~0.04 in the value of p (0.69) resulted in a 10 to 15% effort to find other solutions, none was found.

We shall consider Experiment B of Table III. A and of the fluxes of substrate and acetyl-CoA along the various pathways will be considered below.

There is at present no theoretical procedure known to determine whether the fit obtained with the particular set of parameters is unique. In each experiment 12 data points were obtained and 12 parameters used to fit these data points. Each set of 12 parameters can be considered as a point in phase space, and it is possible that another point in phase space might give almost as good a fit as the point selected. In the absence of data for each experiment. Instead Table II presents the average values and standard deviations for the parameters obtained from each experiment.

The physiological significance of these parameters and of the fluxes of substrate and acetyl-CoA along the various pathways will be considered below.

**Uniqueness of Fit and Limits of Reliability of Parameters**

There is at present no theoretical procedure known to determine whether the fit obtained with the particular set of parameters is unique. In each experiment 12 data points were obtained and 12 parameters used to fit these data points. Each set of 12 parameters can be considered as a point in phase space, and it is possible that another point in phase space might give almost as good a fit as the point selected. In the absence of theorems concerning the possibility of multiple minima in such problems, a trial and error search for other sets of parameters which would fit the data was conducted. Despite considerable effort to find other solutions, none was found.

The contribution of each adjustable parameter to the closeness of fit was estimated by varying each of them in turn. To be specific, we shall consider Experiment B of Table III. A change of ±0.04 in the value of β (0.69) resulted in a 10 to 15% deviation of the calculated fit for appearance of label in glycogen from [1-14C]octanoate. A change of ±0.02 in the value of γ (0.155) caused a 10 to 15% deviation of the calculated fit for appearance of label in glycogen from [2-14C]pyruvate but had little effect on the computed rate of appearance of label from [1-14C]-acetate into glycogen. Varying Vi0 from its value of 1.4 by ±0.6 caused about 50% variation in the computed value for [2-14C]pyruvate incorporation into lipid but caused no other changes except in V10. A change in V14 of ±5 had a significant effect only on V11. A change in α of ±0.04 caused a 20% change in the computed value for [2-14C]pyruvate incorporation into lipid and changed the computed values of Vi and V11 by approximately 20 nmoles per 10^6 per hour, but caused virtually no change in the computed values of [1-14C]acetate oxidation or incorporation into lipids. Changing ε by ±0.05 caused a 10% change in the computed values of [1-14C]octanoate label appearing in CO2 and lipid and a 20% change in [1-14C]octanoate label incorporation into glycogen. Thus most of the parameters are determined to within rather narrow limits. Although there are 12 independent measurements and 12 parameters to be determined, some of the parameters are slightly predetermined while others are slightly overdetermined. Vi and V10, for example, could be chosen larger than shown in Tables III and IV, and in this sense are not to be considered as necessarily reflecting the flux of acetyl-CoA between Compartments I and II in vivo. Rather, the values of Vi and V10 reflect the minimum rates of interchange of acetyl-CoA between these compartments that occur in these cells. Higher rates are possible, but, in any case, the approximate difference between Vi and V10 would have to be preserved. Removal of this small degree of uncertainty in the flux rates computed would require a larger number of independent measurements. For present purposes, however, this small degree of uncertainty in the parameters, which probably accounts for our
ability to fit the experimental data so closely, is of little consequence. Some uncertainty is also added because several of the glutamate measurements only place an upper limit on glutamate output and thus cannot be counted as a full measurement. This also contributes to our ability to obtain such close fits. Nevertheless, the main reason for attainment of close fit results from the properties of a three-pool system.

Other Models

In Fig. 1 we have presented a particular structural model for the required three pools of acetyl-CoA. The question naturally arises as to whether this configuration is unique or whether other configurations would also yield a quantitative fit to the data. The following models were considered.

Model A—Although the data require three pools of acetyl-CoA, we decided to see whether two pools could nevertheless be manipulated so that an acceptable fit would be obtained. Pool III was removed and \( V_{19} \) was set equal to zero. Acetate, octanoate, and pyruvate entered Pool I; acetate and octanoate also entered Pool II. Lipids and glutamate could be synthesized from both pools, but CO\(_2\) and glutamate only from Pool I. There was no direct incorporation of octanoate into lipid (i.e. \( \epsilon = 1 \) in Fig. 1). Steady state equations were written and a computer program prepared, and an attempt was made to fit the data from two experiments. Although it was possible to obtain close approximations to the CO\(_2\), lipid, and glutamate data, the maximum incorporation of label into glycerol from \([2-\text{H}]\)pyruvate was only 50\% of the observed value, from \([1-\text{H}]\)acetate was 20\% higher than observed, and from \([1-\text{H}]\)octanoate was 40 to 70\% too low.

Model B—Model B was also a two-pool model except that \( V_{19} \) was not set equal to zero and \( \epsilon \) was <1. This model also failed.

Model C—This model was structurally the same as the model used in Fig. 1, except that CO\(_2\) and glutamate came from Pool I instead of Pool III. This model represents the smallest change from a two-pool model and is really a two-pool model with a separate mitochondrial subcompartment for keeping acetyl groups from acetate and octanoate separate from acetyl groups derived from pyruvate. This model failed in a manner quantitatively similar to that of Models A and B.

Model D—Model D was identical with the model shown in Fig. 1 except that the mitochondrial contribution to glycerol was derived from Pool III instead of Pool I. This model failed, again primarily in the glycerol data, where the predicted values for incorporation from \([2-\text{H}]\)pyruvate were 50\% below the experimental values and those for label from \([1-\text{H}]\)acetate and \([1-\text{H}]\)octanoate were too high.

Model E—Model E differed from the model shown in Fig. 1 in that there was no direct incorporation of octanoate into lipid (i.e. \( \epsilon = 1 \)); lipid was derived both from the peroxisomal pool (Pool II) and the inner mitochondrial pool of acetyl-CoA (Pool III). The best over-all fit obtainable with this model gave a predicted value for \([1-\text{H}]\)octanoate incorporation into glycerol about 5 times higher than observed. Thus a direct incorporation of octanoate into lipid is required if one is to fit the experimental data. Since there are no other variations on the configuration shown in Fig. 1 which seem compatible with our knowledge of enzyme localizations in *Tetrahymena*, we conclude that the metabolic scheme adopted is unique in the sense that it is demonstrably superior to all other models we have been able to conceive.

**Flux Rates for Acetyl-CoA in Tetrahymena**

The average values of the parameters used to fit each of the log and transitional cultures are presented in Table IV, along with the average values for the flux of carbon along each pathway shown in Fig. 1. Values for two representative experiments have already been presented in Table III. The major difference between log and transitional cultures is the increase in \( V_3 \) (glutamate production) and a decrease in \( V_2 \) (lipogenesis from octanoate). Total pyruvate utilization (\( V_2 \)) increased slightly in the older cultures and this was associated with an increase in \( V_{19} \), the flow of acetyl-CoA from the outer mitochondrial pool to the inner mitochondrial pool.

**DISCUSSION**

This model for the intermediate metabolism of *Tetrahymena* differs from that of Connell and Blum (2), from which it is derived, in that it is a model of acetyl-CoA fluxes rather than carbon flow. This treatment therefore neglects the transfer of label between compartments via carriers such as malate, aspartate, or citrate, all which are taken collectively as \( V_2 \) and \( V_{19} \). Despite these limitations, or perhaps because of them, the present analysis allows a simple and almost exact description of the flow of labeled acetyl-CoA in this cell.

Acetate enters into both mitochondrial and peroxisomal pools of acetyl-CoA. Levy has shown (12) and we have confirmed (11) the presence of acetyl-CoA synthetase in the peroxisomes of *Tetrahymena*. It has also been shown that acetyl-CoA synthetase is present in the mitochondria of *Tetrahymena* (11), as required by the model. The localization of this enzyme in the inner compartment of the mitochondria is in accord with localization of medium and short chain fatty acid activating enzymes in the matrix of mitochondria from other species (17). Acetate is used for oxidation in Pool III at over 3 times the rate it enters into the peroxisomal pool and also at a much larger rate than the sum of inputs into Pool III from pyruvate and octanoate. Thus under the conditions of these experiments acetate is by far the largest labeled energy source for oxidative phosphorylation.

Octanoate is metabolized at a much lower rate than acetate, and enters about equally into the inner mitochondrial pool of acetyl-CoA and the peroxisomal pool. We have recently shown (11) that octanoyl-CoA synthase and several enzymes of the \( \beta \) oxidation pathway for fatty acids are localized in the peroxisomes as well as the mitochondria, as required by the model. The marked inhibition of octanoate oxidation by acetate (but not vice versa) would be expected since acetate enters Pool III at a much higher rate than octanoate and presumably reduces the amount of free CoA available to the octanoate. To our knowledge no previous studies of the metabolism of octanoate by *Tetrahymena* have been reported, although Dewey (18) observed that octanoate inhibits growth. We have confirmed this observation and also shown that the inhibition is completely reversible without any appreciable lag between dilution of the octanoate and resumption of growth. An unexpected feature of the present model is the rather large fraction of octanoate activity directly incorporated into lipid. It is not known whether this indicates direct incorporation of octanoate into neutral fat or phospholipid per se or whether the 8-carbon fragment undergoes a chain-lengthening process. In yeast it appears that medium chain length acyl-CoA moieties can exchange for those being synthesized on the fatty acid synthetase com-

---

2 J. J. Blum, unpublished data.
plex (19). If this is what is happening the source of acetyl-CoA
for chain elongation in *Tetrahymena* may be the peroxisomal
pool. Incorporation of long chain fatty acids such as palmitate
directly or after elongation into the lipids of *Tetrahymena* (20)
and rat brain myelin (21) has been described, and it may be that
this system is not limited to long chain fatty acids in *Tetra-
hymina*. It is not known whether there is any relationship be-
tween the direct incorporation of octanoate into lipid and the
ability of octanoate to inhibit growth, but further work on the
metabolism of octanoate by *Tetrahymena* is indicated and has
been initiated.

Pyruvate could, in principle, form phosphoenolpyruvate via
the action of pyruvate carboxylase or the malic enzyme, but
neither of these enzymes has been found in *Tetrahymena* (22,
23). Furthermore, the formation of phosphoenolpyruvate from
pyruvate has been looked for in *Tetrahymena* and not observed
(24). The labelling pattern observed when pyruvate was in-
corporated into glycogen was not consistent with any appreciable
direct incorporation of pyruvate into phosphoenolpyruvate (1).
The present experiments show that no significant amount of
pyruvate was converted into alanine by the alanine transaminase as a function of oxygen tension.

Pyruvate (26), as in birds, humans, guinea pigs, rabbits, cows, and cats (29), phosphoenolpyruvate carboxykinase is localized both in
the cytosol and the mitochondria. In guinea pigs, it has been
reported that this enzyme is in the matrix space of the mito-
chondria (29) ; in *Tetrahymena* it appears that it must be localized
so that it is either on the inner surface of the outer membrane,
the outer surface of the inner membrane, or in the intermem-
brane space. The utilization of acetyl groups from Pool I for
glyconeogenesis cannot occur as shown symbolically in the model.
Since, as discussed above, there is no pathway for the conversion
of pyruvate to phosphoenolpyruvate in *Tetrahymena*, there must
be a condensation pathway allowing the acetyl-CoA from Pool I
to generate oxalacetate, which, in turn, can be converted to
phosphoenolpyruvate by phosphoenolpyruvate carboxykinase.
The presence of part of the Krebs cycle enzymes in both the
matrix (where the complete cycle is involved in oxidative phos-
phorylation) and in (or facing into) the intermembrane space
would account for this pathway. The possibility that some of
the Krebs cycle enzymes are present in more than one location
in mitochondria is not excluded according to current compila-
tions of enzyme localization within mitochondria (13, 14).

Regardless of the mechanism by which acetyl-CoA from Pool I
gets converted into phosphoenolpyruvate, it is clear that about
one-third of the labeled glycogen synthesized under these growth
conditions must derive ultimately from acetyl groups originating
in Pool I even though most of the acetyl groups formed from
pyruvate do cross the inner membrane where they enter into
oxidative metabolism. It should be noted that *V*₁₁ was much
smaller than *V*₁₁. Thus once an acetyl group enters Pool III,
the probability that it will condense with oxalacetate and be
oxidized is very much larger than the probability that it will
return to the outer compartment.

In the present experiments there was remarkably little dif-
ference between log and transitional cells. This appears to
contradict the results obtained by Connett and Blum (2) among
others (23, 30) that glyconeogenesis via the glyoxylate bypass
is markedly increased in transition phase cells. It is well known,
however, that the controlling variable is not the phase of growth
per se but the oxygen tension in the culture, as recently studied
in detail by Malecki et al. (31). In the experiments of Connett
and Blum (2), 130 ml of culture were placed in a 500-ml capacity
Erlenmeyer flask with shaking. In the present experiments,
between 41 and 43 ml were present in the same capacity flask.
and the oxygen tension was therefore higher in the present growth
conditions than in those of Connett and Blum. Experiments
are being undertaken to ascertain the changes in the flux rates
as a function of oxygen tension.

The increase in glycogen content, which occurs even in well
aerated transition cultures, cannot be accounted for by the
present findings, which show no significant difference in *V*₁ as
a function of culture age. The magnitude of *V*₁ is about 20
mmoles of (labeled) acetyl-CoA entering glyconeogenesis per
hour per 10⁶ cells. If glyconeogenesis occurred at this rate
during the 17-hour growth period prior to the addition of ace-
tate, pyruvate, and octanoate, then the glycogen content
should have been about 20 μg per 10⁶ cells, which is of the order of 10% of
the amount present in log cells under the conditions of these
experiments. Thus unlabeled compounds from the medium
must contribute most of the carbon used for glyconeogenesis,
even in the presence of the added substrates. The present
model does not account for changes in the flow of unlabeled
acetyl-CoA or, especially, of other glyconeogenic precursors
which may form phosphoenolpyruvate directly. In fact, the
present model treats the system as if there were no other inputs,
although Connett and Blum (2) have shown that a large input of
unlabeled carbon into the Krebs cycle must occur in order to
account for the oxygen consumption of *Tetrahymena*. Thus
the computed flux rates refer to the flow of labeled acetyl-CoA
only; actual carbon flow along any pathway may be considerably
underestimated by this abbreviated model. Use of the struc-
tural information derived from this model in the more complete
model formulated by Connett and Blum (2) will presumably
permit computation of the intracellular flow of metabolites as
opposed to the flow of labeled acetyl-CoA alone.

It has been appreciated for some time that the peroxisomes of
*Tetrahymena* play an important role in glyconeogenesis (see
Ref. 2 and references therein). The present result show that
they account for a significant fraction of the β oxidation of added
octanoate and play a major role in lipogenesis as well. In this
sense the metabolism of *Tetrahymena* appears to resemble that
of castor bean seedlings (32, 33), which also have the capacity
to convert endogenous lipids into sugar moieties via a glyoxy-
late cycle and a β oxidation sequence localized in the glyoxy-
somes. One of the evolutionary advantages of this kind of compart-
mentalization may lie in the flexibility that arises, since relatively
large changes in the rates of oxidation, or biosynthesis of lipids,
or both, via the peroxisomal pool of acetyl-CoA can occur with
little effect on the rate of oxidative metabolism or of glyco-
genesis from non-lipid precursors via the mitochondrial gly-
coneogenic pathway.

Acknowledgment—We are grateful to Professor J. W. Moore
for generously allowing us virtually unlimited time on his PDP-
15 computer.

APPENDIX

Derivation of Steady State Equations for Model in Fig. 1

The conservation equations for this system are

\[ V_{11} = a V_6 + \phi V_4 + V_{14} - V_1 - V_7 \]  
\[ V_2 = \gamma V_3 + V_{10} + V_{14} - V_7 - V_{11} \]  

By definition

\[ s_I = \frac{V_{10}S_{III} + v_1S_p + v_2S_{II}}{V_9 + \gamma V_4 + V_{14}} \]  
\[ s_{II} = \frac{V_{10}S_I + (1 - \alpha)V_4S_p + \epsilon(1 - \beta)V_4S_3}{V_9 + (1 - \gamma)V_3 + V_8 - (1 - \epsilon)V_4} \]  
\[ s_{III} = \frac{V_{14}S_I + \alpha V_6S_p + \phi V_5S_5}{V_9 + \gamma V_4 + V_{14}} \]

where \( S_I, S_{II}, \) and \( S_{III} \) are the specific activities of \([1-^{14}C]\)acetyl-
CoA moieties in Compartments I, II, and III. \( S_6, S_p, \) and \( S_9 \)
represent specific activities of acetate, pyruvate, and octanoate,
respectively. Substituting Equations 4 and 5 into Equation
3 and rearranging, one obtains

\[ S_I = \frac{V_{10}S_{III} + v_1S_p + v_2S_{II}}{V_9 + \gamma V_4 + V_{14}} \]

where

\[ A_1 = V_{10} + \gamma V_4 + V_{14} \]
\[ A_2 = V_9 + (1 - \gamma)V_3 + V_8 - (1 - \epsilon)V_4 \]
\[ A_3 = V_1 + V_2 + V_{14} \]
\[ B_1 = 1 - \frac{V_4V_{14}}{A_1A_3} - \frac{V_5V_{14}}{A_1A_2} \]

Setting \( S_6 = 1 \) and \( S_p = S_9 = 0 \) allows computation of the
specific activity of acetyl-CoA in all three compartments when
acetate is the labeled substrate, for instance, and similarly for
the other two substrates. A copy of the computer program
used is available on request.

REFERENCES
1. Connett, R. J., Wittels, B., and Blum, J. J. (1972) J. Biol.
Chem. 247, 2037
2. Connett, R. J., and Blum, J. J. (1972) J. Biol. Chem. 247,
9199
3. Delisle, G., and Fritz, I. B. (1967) Proc. Nat. Acad. Sci.
U.S.A. 55, 790
4. Fritz, I. B. (1967) Perspect. Biol. Med. 10, 643
5. Chase, J. F. A., and Tubbs, P. K. (1972) Biochem. J. 129, 55
6. Wittels, B., and Blum, J. J. (1968) Biochim. Biophys. Acta
152, 229
7. Connett, R. J., and Blum, J. J. (1971) Biochemistry 10, 3290
8. Foch, J., Lees, M., and Sloan, Stanley, C. H. (1957) J.
Biol. Chem. 226, 497
9. Somogyi, M. (1945) J. Biol. Chem. 160, 69
10. LaRonde, K., Nicklas, W. J., and Williamson, J. R. (1970) J.
Biol. Chem. 245, 102
11. Blum, J. J. (1973) J. Protozool., in press
12. Levy, M. R. (1970) Biochem. Biophys. Res. Commun. 39, 251
13. Ernst, L., and Kuylenstierna, B. (1970) in Membranes of
Mitochondria and Chloroplasts (Racker, E., ed) p. 172, Van
Nostrand-Reinhold Books, New York
14. Ashwell, M., and Work, T. S. (1970) Ann. Rev. Biochem. 39,
457
15. Pette, D., Klingenberg, M., and Bucher, T. (1962) Bio-
chem. Biophys. Res. Commun. 7, 425
16. DeDuce, C. (1960) Proc. Roy. Soc. Biol. Chem. 179, 71
17. Haddock, B. A., Yates, D. W., and Garland, P. B. (1970)
Biochem. J. 119, 565
18. Dewey, G. C. (1967) in Chemical Zoology (Florkin, M., and
Scheer, B. T., eds) Vol. I, p. 161, Academic Press, New York
19. Schwabek, E., Lech, I., Knoepfl-Rueff, F., and Lynne,
F. (1970) Eur. J. Biochem. 15, 472
20. Thompson, G. A. (1967) Biochemistry 6, 2015
21. Dopheshwarkaar, G. A. (1973) Biochim. Biophys. Acta 296,
251
22. Warnock, L. G., and Van Eys, J. (1962) J. Cell. Comp. Phys-
iol. 60, 53
23. Shrago, E., Brech, W., and Templeton, K. (1967) J. Biol.
Chem. 242, 4060
24. Minke, N., and Rosenberg, H. (1969) Biochim. Biophys. Acta
177, 229
25. Porter, P., Blum, J. J., and Elrod, H. (1972) J. Protozool.
19, 375
26. Lowenstein, J. M. (1971) in Comprehensive Biochemistry
(Florkin, M., and Stotz, E. H., eds) Vol. 188, p. 1, Elsevier
Publishing Co., New York
27. Wexler, J. P. (1973) Ph.D. thesis, Duke University
28. Dieckhaupt, M. D., Hsieh, H.-C., Elson, C., Sallach, H.
J., and Shrago, E. (1972) J. Biol. Chem. 247, 2755
29. Garber, A. J., Ballard, F. J., and Hanson, R. W. (1972) in
Energy Metabolism and the Regulation of Metabolic Processes
in Mitochondria (Mehlman, M. A., and Hanson, R. W., eds)
p. 109, Academic Press, New York
30. Levy, M. R., and Scherbaum, O. H. (1965) Arch. Biochim.
Biophys. 109, 116
31. Malecki, M. T., Licko, V., and Eiler, J. J. (1971) Curr.
Mod. Biol. 3, 201
32. Cooper, T. G., and Beavers, H. (1969) J. Biol. Chem. 244,
3514
33. Hutton, D., and Stumpf, P. K. (1969) Plant Physiol. 44, 305
Structural Organization of Three Pools of Acetyl Coenzyme A in *Tetrahymena*
Gregory J. Raugi, Tony Liang and Jacob J. Blum

*J. Biol. Chem.* 1973, 248:8064-8072.

Access the most updated version of this article at [http://www.jbc.org/content/248/23/8064](http://www.jbc.org/content/248/23/8064)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/248/23/8064.full.html#ref-list-1](http://www.jbc.org/content/248/23/8064.full.html#ref-list-1)