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Metabolic Pathways in *Tetrahymena:* Distribution of Carbon Label by Reactions of the Tricarboxylic Acid and Glyoxalate Cycles in Normal and Desmethylimipramine-Treated Cells*

R. J. Connett and J. J. Blum

ABSTRACT: A metabolic scheme is presented showing the interaction of the tricarboxylic acid and glyoxalate cycles with gluconeogenesis, glycolysis, and lipogenesis in *Tetrahymena*. A steady-state kinetic analysis of the redistribution of label from acetate according to this scheme is developed. The following data were obtained from measurements under steady-state conditions with [1-^{14}C]acetate and [2-^{14}C]acetate as substrates: O_2 consumption; the incorporation of label into CO_2, lipid, and glycogen; cell glycogen content before and after incubation. The data were used in conjunction with the theoretical analysis to obtain estimates of the rates of component pathways within the metabolic scheme. For normal cells our results indicate that: total rate of acetyl-CoA production is approximately constant; the rate of the glyoxalate cycle varies from 20 to 75% of the rate of the tricarboxylic acid cycle in a manner dependent on culture age; the rate of lipogenesis is comparable to the rate of the tricarboxylic acid cycle except in stationary cultures, where the rate of lipogenesis falls. Data were also obtained for cells treated with desmethylimipramine. It was found that treatment with this drug depressed the rates of: O_2 consumption, the glyoxalate cycle, the tricarboxylic acid cycle, and glycogen synthesis. Desmethylimipramine generally increased the rate of oxidation of glyoxalate *via* glyoxalate oxidase. Possible modes of action of desmethylimipramine on *Tetrahymena* are discussed.

The pathways of intermediary metabolism in *Tetrahymena* resemble those in mammalian cells in many ways (Kidder, 1967; Hogg and Elliott, 1951; Ryley, 1952; Barber *et al.*, 1965), but one major difference lies in the ability of *Tetrahymena* to carry out the net synthesis of glycogen from 2-carbon precursors (Levy and Scherbaum, 1965). The ability to perform this net synthesis of glucose units from acetyl-CoA is due to the presence of the enzymes of the glyoxalate cycle in *Tetrahymena* (Hogg and Kornberg, 1963). The anaplerotic function of the glyoxalate cycle serves to replenish the 4-carbon compounds which are drained from the tricarboxylic acid cycle and thus permits gluconeogenesis to proceed without disruption of the energy-yielding steps of the tricarboxylic acid cycle (Kornberg, 1967).

The original work of Hogg and Kornberg (1963) demon-

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strated that isocitrate lyase and malate synthetase, the key enzymes of the glyoxalate cycle, were localized on particles under conditions where gluconeogenesis could occur. These particles, now known to be the peroxisomes (Müller *et al.*, 1968), also contain acetyl-CoA synthetase (Levy, 1970a,b) and thus clearly play a major role in gluconeogenesis in *Tetrahymena*. Therefore, in order to understand the factors which control carbohydrate metabolism and the rate of energy supply in *Tetrahymena*, one must deal with two organelles containing interconnected cyclic pathways and with reactions in the cytosol (*e.g.*, glycolysis, lipogenesis) leading into or out of these cycles.

Methods have been developed recently which permit computation of the rates of various pathways of intermediary metabolism from *in vivo* measurements on the distribution of isotope from labeled substrates (Landau *et al.*, 1964; Reich, 1968; Rognstad, 1969; Heath, 1968).

An example of this approach, which also serves as the basis of the method to be described below, is the work of Heath, who analyzed the distribution of carbon when precursors labeled in specific positions were supplied to liver cells. Heath derived equations predicting the distribution of carbon after each turn of the cycle. Iteration of these equations on a computer permitted the computation of the steady-state distribution, but explicit equations allowing computation of reaction rates from steady-state data were not derived. To extend this approach to a study of the metabolism in *Tetrahymena*, it was necessary to include a second cyclic pathway, the glyoxalate cycle. This makes the system considerably more complex than that dealt with by Heath and leads to involved equations for the distribution of carbon after a few turns of each cycle. For the present work, an approach is developed which permits one to write equations predicting the distribution of ^{14}C in metabolites of the system directly, without recourse to computer iteration procedures. It is assumed that the system (*i.e.*, the living cell) is supplied with a substrate and is in the steady state with respect to its metabolic processes when the same substrate, labeled in a specific position, is added. In a short time the system will also be in the isotopic steady state, *i.e.*, every carbon atom of every compound of the tricarboxylic acid cycle, the glyoxalate cycle, and their tributary pathways will have a specific activity determined by the speed of each pathway and by the stereochemistry of the chemical reactions involved. Equations are derived which define the specific activity of each carbon atom in such a system for substrates labeled in different positions. Such a model will obviously be applicable to many microorganisms and plant cells.

In this paper acetate labeled in each position is used to obtain data which, in conjunction with the kinetic analysis, yields estimates of the rates of the component pathways of carbohydrate metabolism in *Tetrahymena*.

In addition to the studies on normal cells, data have been obtained for cells treated with desmethylimipramine. This drug, used in the treatment of mental depression, is known to inhibit the uptake of catecholamines by nerve cells (Maxwell *et al.*, 1970) and was found to inhibit the growth of *Tetrahymena* (Blum, 1967). Since desmethylimipramine was also reported to deplete glycogen content and to decrease the isocitrate lyase activity of *Tetrahymena* (Blum, 1968), it was of interest to examine the effects of this drug on the rates of the pathways of carbohydrate metabolism.

Materials and Methods

Cells. Tetrahymena pyriformis, strain HSM, were grown axenically in erlenmeyer flasks with Morton closure tops at 25° with shaking. In all cases a 500-ml flask containing 100 ml of proteose peptone medium (1% proteose peptone and 0.05% liver extract in 0.02 M potassium phosphate at pH 6.5) was inoculated with 10–15 ml of a shaken culture. Cells were counted using a Coulter counter. Additions of drugs and substrates were made in water solutions in amounts ranging from 1 to 4 ml. Control cells received the same volume of water. Except for measurements of $[^{14}C]CO_2$ production, which are described below, all other incubations were made in 125-ml erlenmeyer flasks containing 18 ml of cells and 1.2 ml of substrate. The flasks were shaken in a water bath at 25°.

Protein was measured by the method of Lowry *et al.* (1951) using serum albumin as the standard.

Glycogen. Total glycogen was measured on an ethanol-washed pellet of cells. Glycogen was hydrolyzed to glucose as suggested by Bartley and Dean (1968). Glucoamylase (50 μg) (*Rhizopus niveus*, from Miles Laboratories) in 1 ml of 0.05 M acetate buffer (pH 4.5) was incubated with no more than 1 mg of glycogen for 90 min at 45°. Tris (2-ml of 0.5 M pH 7.0) was added and the solution spun to clarify. An aliquot was then taken for glucose assay by glucose oxidase.

The amylase step results in complete digestion of the glycogen to glucose, since identical results were obtained when the glycogen was hydrolyzed by treatment with 2 N H_2SO_4 at 100° for 1 hr.

Specific Activity of Glycogen. About 10^7 cells were washed twice with ice-cold 5% (w/v) trichloroacetic acid and then twice with 50% (v/v) ethanol. From 5 to 10% of the original glycogen in the pellet is lost by this procedure. The glycogen was solubilized by the glucoamylase procedure and an aliquot counted for radioactivity in [2,5-bis-2-(5-*tert*-butylbenzoxazolyl)thiophene–toluene]–Triton (20:13, v/v) (see below). Another aliquot was assayed for glucose by the glucose oxidase method. The specific activity of glycogen obtained by this method differed no more than 10% from that of glycogen which had been purified until free of all detectable protein.

CO_2 Production. For measurement of $[^{14}C]CO_2$ production, 3 ml of cells was placed into 50-ml erlenmeyer flasks equipped with removable center wells, and 0.2 ml of labeled acetate was added at zero time. The reaction was stopped by addition of 0.2 ml of 10 N H_2SO_4 through the sleeve-type rubber stopper. The vial in the center well then received 0.2 ml of Hyamine *via* a syringe, and the flasks were incubated 1 hr at 37° to allow trapping of the CO_2. The vial was then removed, and the contents were washed into a counting vial and counted in [2,5-bis-2-(5-*tert*-butylbenzoxazolyl)thiophene–toluene]–ethanol (13:10, v/v) scintillation solution.

Radioactive Counting. All counts were made in a Packard liquid scintillation counter using a scintillation fluid consisting of 16 g of 2,5-bis-2-(5-*tert*-butylbenzoxazolyl)thiophene/l. of toluene. Quenching was corrected for by the channels' ratio method.

Lipid Incorporation. Lipid was extracted by shaking approximately 2×10^6 cells with 15 ml of chloroform–methanol (2:1, v/v) solution, according to Folch *et al.* (1957). The lipid sample was then dissolved in 2,5-bis-2-(5-*tert*-butylbenzoxazolyl)-thiophene–toluene and counted.

Total Incorporation. An aliquot of approximately 2×10^6 cells was washed with 0.09 M NaCl, solubilized with 0.5 ml of Hyamine (1 M in methanol), and counted in [2,5-bis-2-(5-*tert*-butylbenzoxazolyl)thiophene–toluene]–ethanol (10:3, v/v).

Reagents. Acetate labeled with ^{14}C in the 1 or 2 position was purchased from New England Nuclear Corp. and adjusted to 0.05 M by addition of unlabeled acetate. The pH was adjusted

to 6.8 and the specific activity was approximately 20 mCi/mole.

Desmethylimipramine was a gift of the Geigy Pharmaceutical Co. All other chemicals were reagent grade.

Metabolic Scheme

T. pyriformis has the enzymes of the glyoxalate cycle (Hogg and Kornberg, 1963) and uses this pathway to achieve a net synthesis of glycogen from acetate and fatty acids (Levy and Scherbaum, 1965). These enzymes are localized in the peroxisomes (Müller *et al.*, 1968). Recently acetyl-CoA synthetase has also been shown to be present in the peroxisomes (Levy, 1970a). These particles, also called glyoxosomes, are bound by a single membrane which appears to be highly permeable to many substrates including isocitrate, malate, and acetyl-CoA (DeDuve, 1969). For the purposes of this work, it shall be assumed that there is complete equilibration between compounds in the peroxisome and in the cytosol. The metabolic scheme showing the interaction between the glyoxalate cycle, the tricarboxylic acid cycle, and the gluconeogenic pathway is seen in Figure 1.

Further information leading to this choice of the scheme is as follows. (1) Pyruvate carboxylase, which catalyses the formation of oxaloacetate from pyruvate, has not been detected in *T. pyriformis* (Schrago *et al.*, 1967; Van Niel *et al.*, 1942). Formation of phosphoenolpyruvate from pyruvate was also looked for and not detected (Munk and Rosenberg, 1969). It has been shown that the labeling pattern in glycogen obtained from *Tetrahymena* grown with several labeled substrates is inconsistent with a direct pathway for the incorporation of pyruvate into phosphoenolpyruvate (R. J. Connett and J. J. Blum, to be published). It thus appears that the only path from pyruvate to glucose is *via* pyruvate decarboxylase as shown in Figure 1. (2) The malic enzyme, which catalyzes the formation of phosphoenolpyruvate from malate, has not been found in *Tetrahymena* (Schrago *et al.*, 1967). (3) Although the enzymes unique to the glyoxalate cycle (isocitrate lyase and malate synthetase) are localized in the peroxisomes, the other enzymes of the glyoxalate cycle (citrate synthetase and aconitase) appear to be localized in the mitochondria (Müller *et al.*, 1968). This means that there is effectively a single pool of citrate and isocitrate. (4) By the same argument, since succinate oxidase is localized in the mitochondria, there is effectively one pool of succinate whether formed from isocitrate by the action of isocitrate lyase of the glyoxalate cycle or from α-ketoglutarate by the action of α-ketoglutarate dehydrogenase. (5) There is no phosphogluconate shunt in *T. pyriformis* (Conger and Eichel, 1966). This means that there is no CO_2 which comes asymmetrically from glucose (Landau *et al.*, 1964), and that the distribution of label in glucose is entirely and simply determined by the distribution of label in phosphoenolpyruvate. (6) *T. pyriformis* has been shown to lose lactate, alanine, glutamate, aspartate, and glycine to the medium during growth (Reynolds, 1970; Cann, 1968; Warnock and Van Eys, 1962), and the glutamate leak has been confirmed under our conditions (P. Porter, unpublished data). In order to allow for this, pathways to the medium have been designated for alanine and/or lactate from pyruvate and for glutamate from α-ketoglutarate. These appear to be the points of largest loss (Reynolds, 1970). (7) Glyoxalate oxidase is found in the peroxisomes (Müller *et al.*, 1968). This step is in principle an oxidative bypass of the tricarboxylic acid cycle. In terms of the labeling pattern, to be discussed later, the operation of this pathway results in labeled CO_2 production

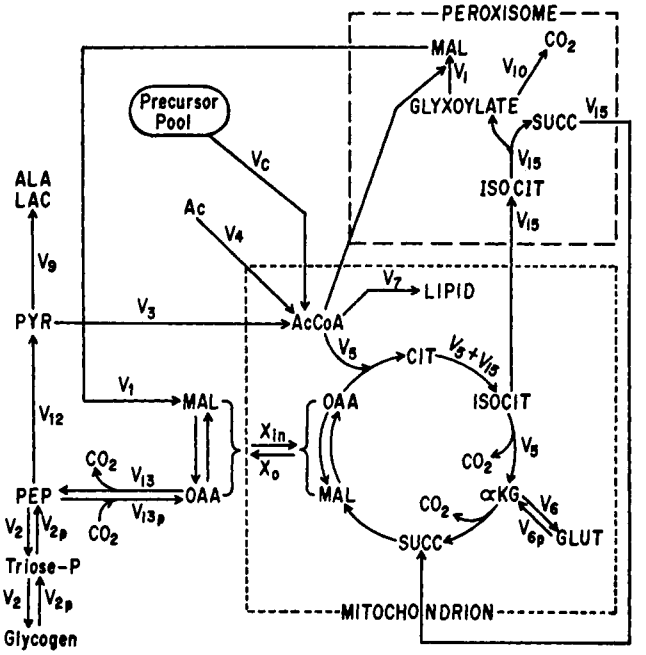

FIGURE 1: Pathways of carbohydrate metabolism in *Tetrahymena*. For convenience of illustration, lipogenesis and the glutamate to α-ketoglutarate steps are shown within the mitochondrial compartment.

from both carbons of acetate without an attendent shift of noncarboxyl label to a carboxyl position as occurs in the tricarboxylic acid cycle. This step was found to be necessary in obtaining solutions for some of our data. (8) One pool of acetyl-CoA is indicated in the scheme of Figure 1, although there is now some evidence to support the existence of two pools in *Tetrahymena*, as has been suggested for liver cells by DeLisle and Fritz (1967). Preliminary experiments indicate that although the ratio of incorporation of label into CO_2 to that into glycogen was the same for [1-^{14}C]acetate as for [2-^{14}C]pyruvate, the ratio of incorporation into lipid was different than that into CO_2 for these two substrates. The existence of two pools of acetyl-CoA would, however, be difficult to detect in experiments using acetate as the sole source of label, and for the present work it was unnecessary to introduce this complication. It should be emphasized, however, that there is no difficulty in extending the theoretical treatment developed in the Appendix to allow for the existence of two pools of acetyl-CoA. (9) The labeling patterns in oxaloacetate, malate, and fumarate are equivalent. Transfer of malate or oxaloacetate by way of aspartate between the intra- and extramitochondrial compartments thus serves to mix all three of these compounds. The parameter X in Figure 1 is a mixing parameter. A value of 0 indicates no mixing, while a value greater than unity indicates essentially complete mixing. Although mixing redistributes label between the compartments, it does not alter the position of the label within these compounds. In the mitochondria, however, as has been discussed in detail by Heath (1968), reversal of the succinic dehydrogenase step leads to randomization of the label between C-1 and C-4 and between C-2 and C-3 in fumarate, malate, and oxaloacetate. In an initial formulation of the kinetic treatment presented in the Appendix, equations were derived including X and a parameter α, equal to the fraction of mitochondrial malate randomized. Computer analysis of this formulation showed that for any glyoxalate cycle rates, V_1 =

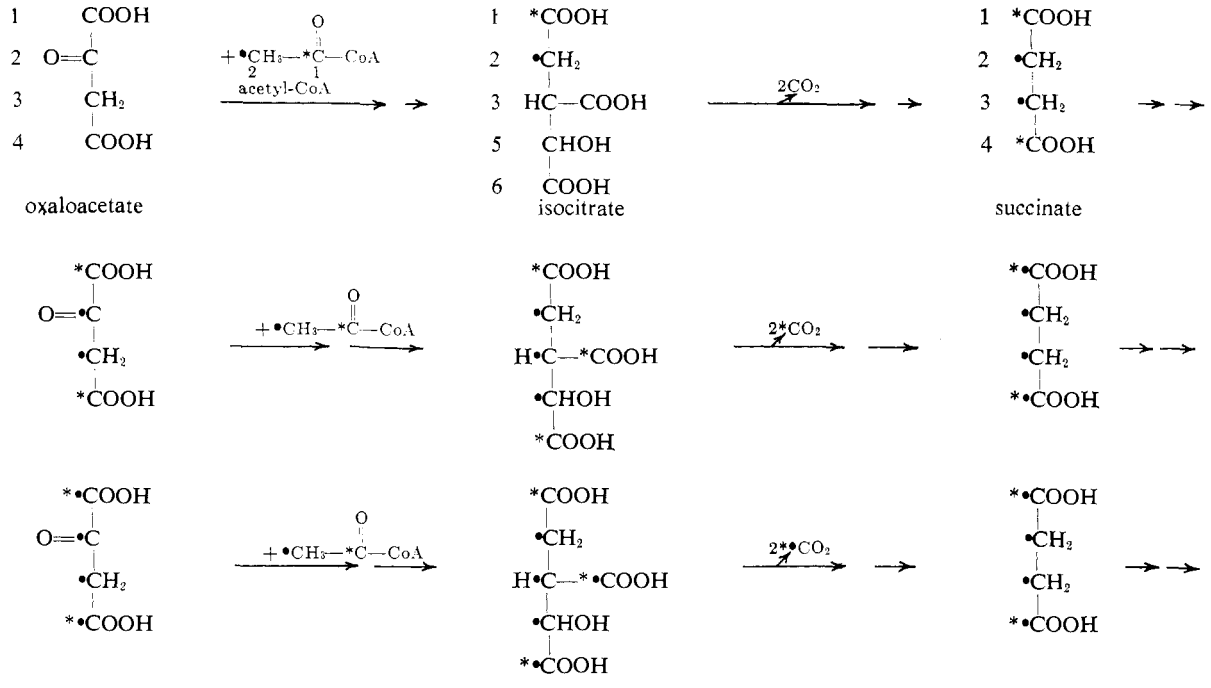

FIGURE 2: The distribution of labeled carbon atoms from acetyl-CoA during three turns of the tricarboxylic acid cycle.

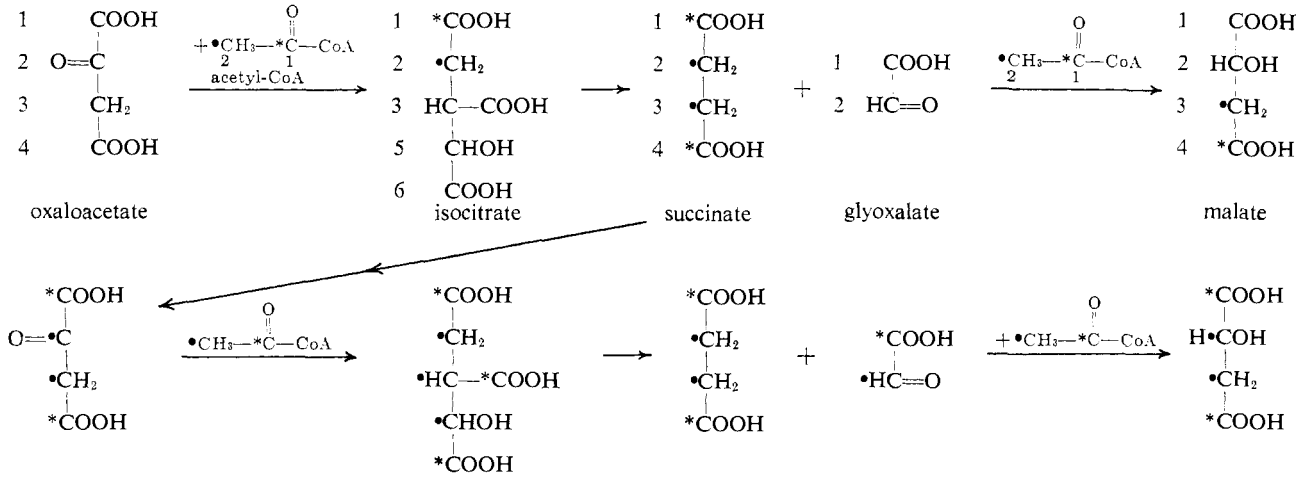

FIGURE 3: The distribution of carbon atoms from acetyl-CoA during two turns of the glyoxalate cycle.

>0.11, there was no difference between the solution obtained with $X = 0$ and $\alpha = 0$ and the solution with $X = \infty$ and $\alpha = 1$. Since all data (see below) required values of $V_1 = >0.16$, all further computations were made on the simplified model shown in the Appendix, in which complete mixing and complete randomization have been assumed.

Labeling Patterns. Figure 2 shows the labeling pattern to be expected from the operation of the tricarboxylic acid cycle when labeled acetyl-CoA is the only input. It can be seen that when [1-^{14}C]acetyl-CoA is the sole source of label only the carboxylate groups are labeled, whereas all carbons will be labeled (though to different extents) if [2-^{14}C]acetyl-CoA is the sole source of label.

Figure 3 shows the labeling pattern to be expected from the operation of the glyoxalate cycle. As with the tricarboxylic acid cycle, [1-^{14}C]acetyl-CoA gives rise to carboxylate labeling only. [2-^{14}C]Acetyl-CoA, however, gives internal (noncarboxyl) labeling only. The theory is predicated on this difference in the labeling pattern of the two cycles.

Figure 4 outlines the pathway for gluconeogenesis from oxaloacetate. All label in C-4 of oxaloacetate is lost as CO_2 in the first step.

Results

Establishment of Steady-State Conditions. Before data could be obtained in a routine manner, it was necessary to establish conditions under which the steady-state assumption which underlies the theoretical formulation holds. Under steady-state conditions, one should observe a linear rate of incorporation of label into any of the observed pools, *i.e.*, CO_2, glycogen, and lipid. From the labeling patterns seen in Figures 2 and 3, one would also expect [2-^{14}C]acetate to attain the isotopic steady state later than [1-^{14}C]acetate. This was observed.

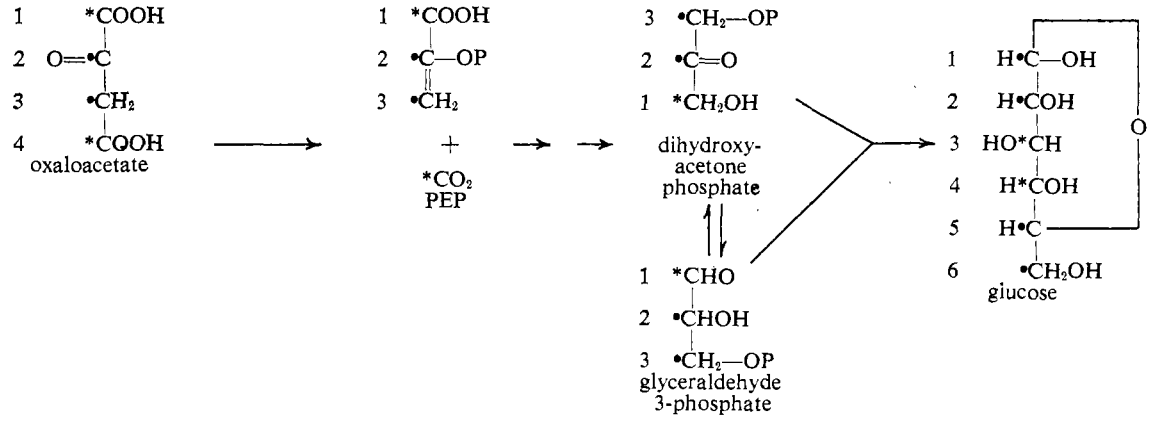

FIGURE 4: The distribution of carbon atoms arising from oxaloacetate (or malate) in glucose.

Figure 5 shows that even metabolism of [2-^{14}C]acetate is effectively in the steady state within 15 min after addition of the label and stays in the steady state for at least 75 min more. Similar results were obtained for cells treated with desmethylimipramine. It was also established that incorporation of acetate into lipid was linear with time for both normal and desmethylimipramine-treated cells. For all subsequent experiments, measurements were made 1 hr after addition of the labeled acetate to the cells.

Calculation of Parameter Values Using Data. In each experiment total incorporation of label from [1-^{14}C]acetate and [2-^{14}C]acetate into CO_2, glycogen, lipid, and whole cell were measured under steady-state conditions. All values were normalized by expressing the incorporation as a per cent of total utilization. A computer program was written to calculate GT, CT, and LT for varying values of the parameters from eq 32 to 34 of the Appendix. The input rate of acetate, V_4, was set at 1.0 so that the calculated values could be compared directly to the normalized data.

Three parameters were fixed for the final solutions: V_{13p}, V_{6p}, and V_{2p}. V_{13p} is the rate of CO_2 fixation *via* phosphoenolpyruvate carboxylase. Although extensive CO_2 fixation occurs in anaerobically grown cells (Van Niel *et al.*, 1942), there is no evidence to suggest appreciable CO_2 fixation in aerobically grown cells such as were used in the present experiments. Thus for calculation V_{13p} was set to 0. Figure 6 shows the effect of V_{2p}, the breakdown of glycogen, and V_{6p}, the input of cold glutamate, on the solution values for several parameters. The calculated incorporations at each point were within 2% of each other and differed from the experimental values by no more than 5%, which is well within the experimental variations of duplicate determinations. When $V_{2p} = 0.1$, all values appear to have stabilized except V_2. At this value of V_{2p}, V_5, $[O_2]_{min'}$, and V_2 also appear to be independent of V_{6p}. Thus for calculation, V_{2p} was set at 0.1. V_{6p} appears primarily to have a scaling effect on V_1 and V_7. Since *Tetrahymena* tend to produce glutamate rather than consume it, V_{6p} should be small. V_{6p} cannot be 0, however, since incubation with labeled glutamate leads to the production of labeled CO_2 (unpublished observations). Thus for the purposes of calculation, V_{6p} was set equal to 0.05. Although the data in Figure 6 refer specifically to expt C of Table IA, the same conclusions concerning V_{2p} and V_{6p} are obtained using any of the other experiments which were performed.

The comput am used was relatively simple in that no attempt wa to "solve" the equations. Instead, the computer was measured LT, and O_2 consumption, an estimate of V_3, and the values of V_{6p} and V_2 which have just been described. The computer was then given suitable ranges of V_c, V_6, V_{10}, and V_2 and asked to select a range of V_1 such that the calculated $[O_2]_{min}$ was compatible with the observed

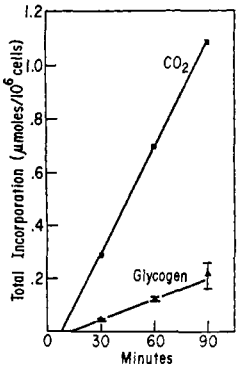

FIGURE 5: Incorporation of label from [2-^{14}C]acetate into CO_2 and glycogen in control cells. Cells were incubated as described in the Methods section, and samples taken at the indicated times.

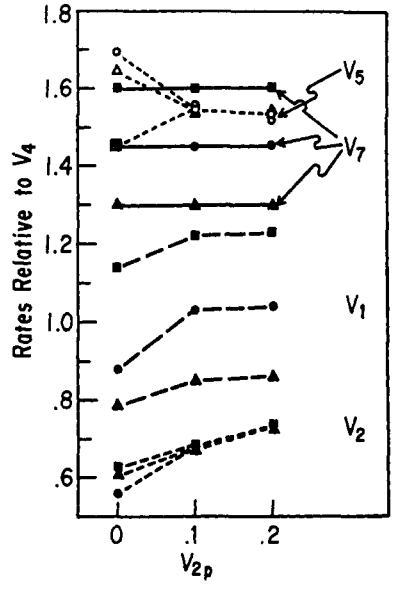

FIGURE 6: Effect of variation of V_{2p} (rate of glycolysis) on several rate parameters. For further details, see text.

TABLE I: Metabolic Distribution of Label from [1-^{14}C]Acetate and [2-^{14}C]Acetate in *Tetrahymena*.[a]

Expt	Cells/ml	N_f:N_i	CT_1	CT_2	GT_1	GT_2	LT_1	LT_2	Glycogen Content (μg/10^6 Cells) Initial	Glycogen Content (μg/10^6 Cells) Final	Protein (mg/10^6 Cells)	Acetate Utilization (μmoles/10^6 Cells)
						A. Control Cells						
A	446,500	23.0	0.666	0.377	0.024	0.117	0.263	0.310	53 ± 3	52 ± 4	1.34	1.46
			0.636	0.373	0.024	0.090	0.263	0.288				
B	502,360	11.7	0.667	0.367	0.033	0.121	0.293	0.291	68 ± 3	62 ± 1.6	1.38	1.47
			0.621	0.377	0.033	0.128	0.292	0.2				
C	635,700	4.9	0.608	0.226	0.080	0.266	0.287	0.31	219 ± 4	261 ± 9	1.25	1.73
			0.586	0.264	0.080	0.268	0.287	0.310				
D	676,700	0.93	0.758	0.529	0.029	0.128	0.143	0.156	421 ± 18	393 ± 28	1.42	1.24
			0.760	0.515	0.029	0.116	0.143	0.184				
	Mean O_2 consumption: 4.26 ± 0.43 μmoles/hr per 10^6 cells											
	Calculated O_2 consumption: $[O_2]_{max}$ = 4.94; $[O_2]_{min}$ = 4.58											
						B. 2.5 × 10^{-5} M Desmethylimipramine						
A	416,940	17.0	0.608	0.412	0.034	0.069	0.332	0.353	50 ± 4	47 ± 2	1.45	1.53
			0.588	0.420	0.034	0.075	0.332	0.356				
B	450,870	8.4	0.606	0.392	0.022	0.063	0.323	0.342	76 ± 0.3	67 ± 3.9	1.54	1.60
			0.586	0.411	0.022	0.066	0.323	0.338				
C	908,260	7.2	0.568	0.363	0.031	0.099	0.375	0.369	74 ± 2	68 ± 2	1.03	0.840
			0.544	0.363	0.031	0.097	0.372	0.372				
D	638,340	0.91	0.784	0.433	0.007	0.016	0.076	0.090	283 ± 28	313 ± 28	1.21	0.667
			0.799	0.436	0.007	0.022	0.076	0.080				
	Mean O_2 consumption: 3.16 ± 0.21 μmoles/hr per 10^6 cells											
	Calculated O_2 consumption: $[O_2]_{max}$ = 3.18; $[O_2]_{min}$ = 2.94											

[a] N_i and N_f are the cell densities at the beginning and the end of the approximately 17-hr growth period. $CT_{1,2}$, $GT_{1,2}$, and $LT_{1,2}$, represent the incorporation into CO_2, glycogen, and lipid, respectively, from 1- and 2-labeled acetate expressed as a fraction of total acetate utilization. Initial and final glycogen contents were measured at the beginning and end of the 1-hr incubation period. For each experiment the upper row of numbers are the measured data and the lower row are the values computed using the parameter values for that experiment shown in Table II.

O_2 consumption. The computer then calculated and printed the theoretical values of CT1, CT2, GT1, GT2, LT1, LT2, $[O_2]_{min}$, and $[O_2]_{max}$ for each combination of parameters. By direct inspection of the computer output or by interpolation when necessary one could then pick out a solution which matched the experimental data within satisfactory limits of error. When the process was complete, the parameters listed in Table II had been computed.

Distribution of Label from Acetate in Normal Cells. Table IA shows for four typical experiments the incorporation from [1-^{14}C]acetate and from [2-^{14}C]acetate into CO_2, glycogen, and lipid and the total acetate utilization, defined as the amount incorporated into the cell plus the amount appearing as [^{14}C]CO_2. Also shown are the protein content, the cell count at the end of the approximately 17-hr growth period, the ratio of final to initial cell count, and the glycogen content of the cell at the beginning and end of the 1-hr incubation period. When the cells enter the stationary phase of growth, as indicated by a small ratio, N_f:N_i, cell glycogen content increases markedly (Blum, 1967).

The amount of acetate utilized in the 1-hr incubation was fairly constant over the whole growth range. It can be seen that under these conditions the amount of label incorporated into glycogen ranges from 7 to 15% of the total incorporation from [2-^{14}C]acetate while approximately one-third of the label was incorporated into lipid. Using the parameter values listed in Table IIA, the values of GT1, GT2, CT1, CT2, LT1, LT2, and oxygen consumption shown in Table IA were computed. It is clear that the model can, within experimental error, quantitatively account for oxygen consumption and for the observed distribution of label into glycogen, CO_2, and lipid. It is also clear that the minimal estimate of oxygen consumption (see eq 35) is a closer approximation of the experimental data than is the maximal extimate given by eq 36.

The values of parameters listed in Table IIA represent the rates of the various pathways of carbohydrate metabolism shown in Figure 1. Some of these pathways appear to be relatively variable in the different experiments, but several trends can be discerned which probably reflect metabolic events. V_c, the rate of utilization of unlabeled acetyl-CoA percursors from the medium, is approximately constant and about twice the rate of utilization of acetate until the cells reach the stationary phase, when V_c falls. Cells in the stationary phase presumably stop growing because of lack of some crucial nutrient, and the result is a general lowering of metabolic rate which is reflected in a decreased utilization of substrates. V_9, the loss of label *via* alanine and/or lactate, remains approximately constant throughout the whole range of growth.

TABLE II: Computed Rates of Metabolic Pathways in *Tetrahymena*.[a]

Expt	$N_f:N_i$	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_9	V_{10}	V_{13}	V_c
					A. Control Cells							
A	23	0.74	0.35	0.23	1.46	1.80	0.07	1.17	0.32	0	0.74	2.77
B	11.7	0.53	0.35	0	1.47	1.86	0	1.20	0.40	0	0.60	2.65
C	4.9	1.05	0.75	0.21	1.73	1.49	0	1.45	0.36	0	1.14	3.11
D	0.93	0.45	0.26	0.06	1.24	1.93	0.06	0.47	0.24	0.75	0.45	1.99
					B. 2.5×10^{-5} M Desmethylimipramine							
A	17	0.40	0.26	0.14	1.52	0.52	0	1.11	0.23	0.92	0.47	1.68
B	8.4	0.41	0.20	0.09	1.57	0.54	0	1.09	0.35	0.94	0.49	1.72
C	7.2	0.34	0.21	0	0.84	0.81	0	1.19	0.26	0.50	0.38	2.35
D	0.91	0.57	0.06	0.08	0.67	1.07	0.02	0.20	0.52	0.20	0.57	1.87

[a] V refer to the pathways indicated in Figure 1 and are expressed as μmoles/hr per 10^6 cells. The letter for each experiment corresponds to that of Table I.

Effect of Desmethylimipramine on Utilization of Labeled Acetate. Measurements were made on cells treated overnight with desmethylimipramine. Most experiments were done with 2.5×10^{-5} M desmethylimipramine, but higher and lower concentrations were also used and successfully fit to the model.

One major effect of desmethylimipramine is a decrease in oxygen consumption. The decrease in oxygen consumption rate is linearly related to the dose of desmethylimipramine in the range studied (Figure 7).

Table IB shows some of the data obtained from cells treated with desmethylimipramine and the values computed from the model. The letters indicate the paired controls in Table IA. Total acetate utilization appears to be decreased, especially in older cultures. This does not appear to be due to leakage of intermediates during washing with NaCl, since there is a good fit of the theoretical values to the experimental data.

Table IIB lists the computed values of the parameters corresponding to the solution points of Table IB. A comparison of these values to the corresponding values for the control cultures (Table IIA) displays the effects of desmethylimipramine on the rates of the various metabolic pathways of Figure 1. In addition to the decrease in oxygen consumption already noted, it was found that most of the metabolic rates were lower in desmethylimipramine cells than in their controls, suggesting a general metabolic depression due to desmethylimipramine. An exception to this generalization is V_{10}, the rate of glyoxalate oxidation, which was usually close to zero in control cells and generally a real positive number in the desmethylimipramine-treated cells. This suggests that under these circumstances the peroxisomes may serve as an oxidative bypass for the tricarboxylic acid cycle, as postulated by DeDuve and Baudhuin (1966). V_9, the loss to alanine and/or acetate, appears to be unaffected by desmethylimipramine.

Culture Age. Although these experiments were not undertaken to analyze the effects of culture age on metabolic rates, it was possible to gain some information concerning this factor from data such as that shown in Table I. The variation of selected parameters with growth rate for cells treated with 2.5×10^{-5} M desmethylimipramine and for their paired controls is shown in Figure 8. The upper curve is the change in glycogen content during 1-hr incubation. The control experi-

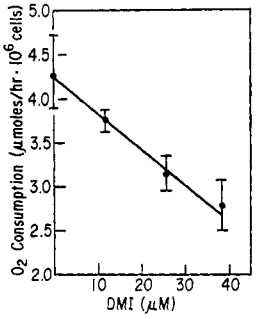

FIGURE 7: Effect of desmethylimipramine on O_2 consumption. Cultures were exposed for about 17 hr to desmethylimipramine at the concentrations shown on the abscissa. O_2 consumption was measured at 25° using a Yellow Springs Instrument Co. oxygen electrode. A minimum of three cultures at varying cell densities were used for each concentration. The bars indicate one standard error in each direction.

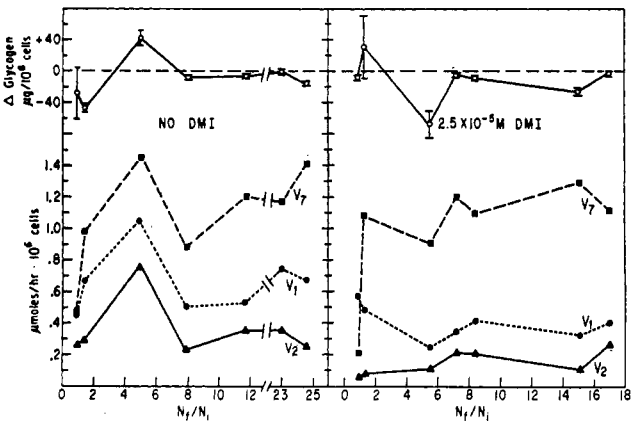

FIGURE 8: Variation of metabolism with culture age for cultures treated with desmethylimipramine and paired controls. The abscissa for each half of this graph is a measure of culture age. N_i and N_f are the initial and final cell densities, respectively, for the 17-hr growth period. The upper ordinate shows the change in glycogen content during the 60-min incubation with labeled acetate. The lower ordinate shows the computed rates of lipogenesis (V_7), the glyoxalate cycle (V_1), and gluconeogenesis (V_2), taken from Tables I and II and similar data.

ments show a peak of synthesis in cells going from log growth into the stationary phase. This is reflected in the calculated rates for the glyoxalate cycle, V_1, and in glycogen synthesis, V_2. The rate of lipid synthesis appears to follow a similar course with a sharp decline as cells go into the stationary phase. The desmethylimipramine-treated cells besides showing a general depression of metabolism do not have the synthetic peak during the transition although they do show the decline in lipid synthesis.

Discussion

Normal Metabolism. Several workers have developed kinetic models pertaining to restricted areas of carbohydrate metabolism and have defined the specific activities of intermediates that would be obtained from labeled substrates. Two approaches have been used in order to obtain and interpret data in terms of the theoretical model. One involves measuring the specific radioactivities or concentrations of selected intermediates and comparing such values to the predicted values (Threlfall and Heath, 1968, London, 1966). The other is to measure the radioactivity in various outputs from the system such as CO_2, lactate, glycerol, and fatty acids in adipose tissue (Rognstad, 1969). The second approach has been selected, *i.e.*, to measure the distribution of label from acetate into simple end products of metabolism: CO_2, glycogen, and lipid. With *Tetrahymena* measurements could be made under the steady-state conditions demanded by the theoretical analysis, and much new information has been obtained on the rates of metabolic pathways under normal conditions as well as in the presence of desmethylimipramine, a drug known to inhibit the growth and perturb the metabolism of *Tetrahymena*.

Justification for the metabolic scheme shown in Figure 1 has been given earlier in this paper. Nine primary pieces of data were obtained: O_2 consumption, CT1, CT2, GT1, GT2, LT1, LT2, total label incorporated, and the specific activity of glycogen.

As pointed out earlier, there are more than nine independent rates shown in Figure 1, and it was necessary to set three of these rates—V_{2p}, V_{6p}, and V_{13p}—in order to achieve parity between the number of parameters to be determined and the number of independent measurements. Fortunately, knowledge of the general features of the metabolism of *Tetrahymena* permits reasonable choices of these three parameters. CO_2 fixation under aerobic conditions, as mentioned earlier, is negligible. V_{6p}, input to the tricarboxylic acid cycle from glutamate, was set at 0.05 from considerations explained in conjunction with Figure 6. The computed values for V_6 (approximately 0.05) then accord well with the propensity of *Tetrahymena* to leak glutamate into the medium, as described. For convenience of computation, V_{2p} was chosen as 0.1× acetate utilization. In conjunction with the computed values for V_2, one would predict a slight net synthesis of glycogen during the 1-hr incubation. In fact, as shown in Figure 8, the cells usually showed no change in glycogen content or a net loss. Except for a scale factor, however, the trend of V_2 parallels the trend in Δ glycogen (Figure 8). In order to compute net loss from glycogen and account for the label incorporated into glycogen, one would have to use excessively high values of V_{2p}. It should be stressed, however, that the metabolic model used in this paper postulates a single triose phosphate pool. If, as suggested by Reich (1968) and Threlfall and Heath (1968), glycogenolysis and glyconeogenesis were compartmentalized, this problem would not arise. Since the change is computed as the difference between two large numbers, one need not insist upon a precise fit between the computed change in glycogen and the observed change.

When the metabolic rates computed for control cells are compared with the measured enzyme contents of *Tetrahymena*, one finds, with few exceptions, that the *in vivo* metabolic rates are much lower than the enzymatic capacity of the cell. This indicates that dynamic controls govern much of the enzyme activity. An apparent exception is the enzyme aconitase, for which a mean rate of 1.9 μmoles/hr per mg of protein is found. This *in vivo* value is just slightly less than the value of 2.4 μmoles/hr per mg of protein measured for aconitase in mitochondrial preparations (Müller *et al.* 1968). In liver, aconitase probably operates at equilibrium (Lowenstein, 1967), and primary control of the rate of the tricarboxylic acid cycle is thought to be at citrate synthetase. Although the citrate synthetase of *Tetrahymena* does not act at capacity and may therefore be rate controlling, it is also possible that aconitase serves as the rate-limiting step of the tricarboxylic acid cycle in this organism. The values computed for isocitrate lyase in the present study (0.5–1.2 μmoles/hr per mg of protein) are consistent with those measured by Levy and Wasmuth (1970) and Blum (1968) in homogenates. The agreement between the *in vivo* and the *in vitro* values for this enzyme suggests that isocitrate lyase operates near its maximal rate in *Tetrahymena* under aerobic conditions.

The possibility of defining two different levels of oxygen consumption arises from considerations of the compartmentalization of malate. A minimum value for O_2 consumption occurs when only the oxaloacetate used to form citrate is produced within the mitochondrion. The maximum value of O_2 consumption will occur when all malate (whether produced in the peroxisomes or from glutamate) is oxidized to oxaloacetate in the mitochondrial compartment. Comparison of the measured O_2 consumption to the values computed from the kinetic analysis shows that the minimal value is the one which best agrees with experiment. The kinetic solutions, however, were computed on the basis of a single pool of malate and oxaloacetate. Thus, although the labeling patterns may be insensitive to the number of pools, it appears that little if any of the malate formed in the peroxisomal compartment is oxidized in the mitochondrial compartment.

Perturbations of Metabolism by Desmethylimipramine. The effect of desmethylimipramine on *Tetrahymena* does not appear to be limited to a single locus. Perhaps most noticeable is the reduction in oxygen consumption. This reduction reflects a marked decrease in the tricarboxylic acid cycle which is accompanied by an increase in the direct oxidation of glyoxalate. The increased glyoxalate oxidation was observed in all but three of eleven experiments. In the three experiments in which no increase was observed, the control cells had significant glyoxalate oxidation (*e.g.*, expt D of Tables I and II). Thus in the presence of desmethylimipramine, glyoxalate oxidase serves as an oxidative bypass of the tricarboxylic acid cycle. The rate of loss of label to lactate and/or alanine (V_9) is not affected by desmethylimipramine. Pyruvate kinase activity (V_{12}) and pyruvate decarboxylase activity (V_3) are both decreased relative to control cells. It should be pointed out that V_3 is primarily dependent on the measured incorporation into lipids and is not appreciably dependent on the rates of other pathways in the metabolic scheme. It is therefore gratifying that V_3 derived from the present analysis agrees well with the values obtained by measurement of the rate of oxidation of pyruvate (R. J. Connett and J. J. Blum, to be published). Thus the reduction in V_3 observed in desmethyl-

imipramine-treated cells may reflect a loss of pyruvate decarboxylase activity, perhaps because of decreased enzyme content.

It was earlier reported (Blum, 1968) that exposure of *Tetrahymena* to desmethylimipramine resulted in a decrease in isocitrate lyase activity and in glycogen content, especially in the late logarithmic and early stationary phases of growth. The present data accord well with the earlier findings; at each stage of growth the values of V_1 (the rate of the glyoxalate cycle and, in the steady state, also the rate of isocitrate lyase) in the desmethylimipramine-treated cells are lower than those of control cells (Figure 8).

It should be pointed out that in control cells there appears to be an age-dependent peak in the activities of the glyoxalate cycle, of glycogen synthesis, and of lipid synthesis (Figure 8). These peaks of activity, occurring in the late log or early stationary phase cells, are reduced markedly by desmethylimipramine treatment. V_c, the rate of consumption of all unspecified sources of acetyl-CoA (*e.g.*, endogenous breakdown of lipids, input of certain amino acids), is also decreased in desmethylimipramine-treated cells.

Overnight exposure to desmethylimipramine interferes with the metabolism of acetyl-CoA—both production as reflected in V_3, V_4, and V_c, and utilization, as reflected in the fall of V_1, V_5, and V_7. In view of this widespread effect of desmethylimipramine on the rates of consumption and utilization of acetate, it seems unlikely that desmethylimipramine affects the metabolism of *Tetrahymena* by interference with a single biochemical reaction.

The most potent known effect of desmethylimipramine is on the uptake of catecholamines across nerve membranes (Hertting *et al.*, 1961). It seems unlikely that this is relevant to the effect of desmethylimipramine in *Tetrahymena*. Brizzi and Blum (1970) found that desmethylimipramine had no effect on serotonin content of *Tetrahymena* and noted (unpublished observations) that the content of catecholamines was variable but that desmethylimipramine still inhibited growth in cultures with negligible catecholamine content. Perhaps more relevant to an understanding of the effect of desmethylimipramine on *Tetrahymena* are the observations that imipramine inhibits the calcium-induced increase in ATPase activity and the rate of calcium uptake of fragments of sarcoplasmic reticulum isolated from rabbit skeletal muscle (Balzer *et al.*, 1968a) and binds to the lipids of these vesicular preparations (Balzer *et al.*, 1968b). Whatever the molecular mechanisms of action of desmethylimipramine on *Tetrahymena*, it is clear that this drug alters the rates of several metabolic pathways in at least two cell compartments.

Thus this type of steady-state analysis can demonstrate in living cells the effect of drug-induced changes in enzyme activity. In addition the analysis serves to indicate other metabolic control junctions for future study. The analysis can easily be generalized to include the effects of compartmentalization (*e.g.*, two pools of acetyl-CoA, separate gluconeogenic and glycolytic pathways, etc.) and to include the pentose cycle when present. Although in such more complex (and more realistic) models the algebra becomes cumbersome, it can nevertheless be performed without approximations. Use of other labeled substrates in addition to acetate increases the number of independent measurements and thus the number of rates which can be determined. Such experiments are in progress.

Appendix

In this appendix the steady-state analysis of labeled carbon distribution for the metabolic scheme shown in Figure 1 is derived in detail. As discussed in the paper, malate and oxaloacetate are treated as forming a single, completely randomized pool.

TABLE III: Definition of the Subscript i in $S_i(j)$.

Compound	Subscript
Acetate	a
Acetyl-CoA	ac
PEP[a]	pp
Pyruvate	p
Oxaloacetate	o
Malate	m
Succinate	s
Isocitrate	i
Glyoxalate	g
α-Ketoglutarate	k
Triose phosphate	tr

[a] PEP is the abbreviation for phosphoenolpyruvate.

Eighteen rates are defined in Figure 1. Not all of these are independent since in the steady state the flow into a pool must equal the flow out of it. Thus the rate of formation of pyruvate from phosphoenolpyruvate, V_{12}, must equal the rate of loss of pyruvate to acetyl-CoA, alanine, and lactate.

$$V_{12} = V_3 + V_9 \tag{1}$$

Similarly for glyoxalate, oxaloacetate, phosphoenolpyruvate, and acetyl-CoA, we write eq 2, 3, 4, and 5, respectively

$$V_{15} = V_1 + V_{10} \tag{2}$$

$$V_1 + V_{6p} + V_{13p} = V_{13} + V_6 \tag{3}$$

$$V_9 + V_3 + V_{13p} + V_2 = V_{13} + V_{2p} \tag{4}$$

$$V_c + V_3 + V_4 = V_5 + V_7 + V_{10} + 2V_1 \tag{5}$$

The specific activity in a compound is denoted by $S_i(j)$, where i is the letter symbol from Table III indicating the compound in question and j denotes the particular carbon atom of the compound which is of interest, *e.g.*, $S_{ac}(1)$ means the specific activity of acetyl-CoA in carbon 1.

In the metabolic and isotopic steady state

$$\text{specific activity} = \text{flow of label/flow of carbon} \tag{6}$$

Thus equations can be written defining the specific activity of any compound in terms of the specific activity of its percursors and the rates of all connecting steps. For isocitrate, which is formed only in the tricarboxylic acid cycle by condensation of acetyl-CoA with oxaloacetate

$$S_i(1) = S_{ac}(1) \tag{7}$$

$$S_i(2) = S_{ac}(2) \tag{8}$$

$$S_i(3) = S_o(2) = S_m(2) \tag{9}$$

$$S_i(4) = S_o(1) = S_m(1) \quad (10)$$

$$S_i(5) = S_o(3) = S_o(2) = S_m(2) \quad (11)$$

$$S_i(6) = S_o(4) = S_o(1) = S_m(1) \quad (12)$$

Glyoxalate is formed by the action of isocitrate lyase

$$S_g(1) = V_{15}S_i(6)/(V_1 + V_{10}) \quad (13)$$

$$S_g(2) = V_{15}S_i(5)/(V_1 + V_{10}) \quad (14)$$

From eq 2 it is evident that

$$S_g(1) = S_i(6) \text{ and } S_g(2) = S_i(5) \quad (15)$$

α-Ketoglutarate is formed from isocitrate and glutamate and is further metabolized to either succinate or glutamate. Thus

$$S_k(1) = R_5S_i(6) \quad (16)$$

$$S_k(2) = R_5S_i(5) \quad (17)$$

$$S_k(3) = R_5S_i(3) \quad (18)$$

$$S_k(4) = R_5S_i(2) \quad (19)$$

$$S_k(5) = R_5S_i(1) \quad (20)$$

where $R_5 = V_5/(V_5 + V_{6p})$.

Although succinate is formed from α-ketoglutarate in the tricarboxylic acid cycle and from isocitrate in the peroxisome, all further metabolism occurs in the mitochondria. Therefore one randomized pool is adequate for this compound

$$S_s(1) = S_s(4) = \{(V_5 + N_6)[S_k(2) + S_k(5)] + V_{15}[S_i(1) + S_i(4)]\}/[2(V_5 + V_{15} + N_6)] \quad (21)$$

$$S_s(2) = S_s(3) = \{(V_5 + N_6)[S_k(3) + S_k(4)] + V_{15}[S_i(2) + S_i(3)]\}/[2(V_5 + V_{15} + N_6)] \quad (22)$$

where $N_6 = V_{6p} - V_6$.

Malate is formed in the tricarboxylic acid cycle, the glyoxalate cycle, and by CO_2 fixation from phosphoenolpyruvate

$$S_m(1) = S_m(4) = \{2(V_5 + V_{15} + N_6)S_s(1) + V_1[S_g(1) + S_{ac}(1)] + V_{13p}[S_{co2} + S_{pp}(1)]\}/[2(V_{15} + V_5 + V_{13})] \quad (23)$$

$$S_m(2) = S_m(3) = \{2(V_5 + V_{15} + N_6)S_s(1) + V_1[S_g(2) + S_{ac}(2)] + V_{13p}[S_{pp}(2) + S_{pp}(3)]\}/[2(V_{15} + V_5 + V_{13})] \quad (24)$$

Similarly, one obtains the following equations for triose phosphates, phosphoenolpyruvate, pyruvate, acetyl-CoA, and CO_2

$$S_{tr}(n) = R_2S_{pp}(n) \quad (25)$$

where $R_2 = V_2/(V_2 + V_{2p})$.

$$S_{pep}(n) = [V_{13}S_m(n) + V_{2p}S_{tr}(n)]/(V_2 + V_{12} + V_{13p}) \quad (26)$$

$$S_p(n) = S_{pp}(n) \quad (27)$$

$$S_{ac}(n) = [V_4S_a(n) + V_3S_p(n + 1)]/(V_1 + V_7 + V_5 + V_{15}) \quad (28)$$

$$S_{co2} = \{V_{10}[S_s(1) + S_s(2)] + V_5S_i(4) + (V_5 + N_6)S_k(1) + V_4S_p(1) + V_{13}S_m(1) - V_{13p}S_{co2}\}/(2V_5 + N_6 + 2V_{10} + V_3 + V_{13} - V_{13p}) \quad (29)$$

Since the whole system is interconnected, the specific activity of any compound can be written in terms of the specific activity of a selected reference compound and the various rate constants. It was convenient to choose malate as the reference compound. Upon solving the above equations, one obtains the following expressions for the specific activity of each carbon of malate in terms of the input acetate.

$$S_m(2) = V_4 \cdot M2I \cdot S_a(2)/(V_1 + V_7 + V_5 + V_{15} - V_3R_{13}M2I) \quad (30)$$

where $M_2I = [V_1 + R_5(V_5 + N_6) + V_{15}]/[V_{15} + 2V_5 + 2V_{13} - V_1 - R_5(V_5 + N_6) - 2V_{13p}R_{13}]$, $N_6 = V_{6p} - V_6$, $R_5 = V_5/(V_5 + V_{6p})$, $R_{13} = V_{13}/(V_2 + V_{12} + V_{13p} - V_{2p}R_2)$, and $R_2 = V_2/(V_2 + V_{2p})$.

$$S_m(1) = \{[R_5(V_5 + N_6) + V_{10}R_{13p} + V_3R_{13}R_c]S_m(2) + [V_1 + R_5(V_5 + N_6) + V_{15}]V_4S_a(1)\}/\{V_5 + V_{10} + 2V_{13} - V_{13p}R_{13} - R_{13p}[V_{10} + V_5 + R_5(V_5 + N_6) + V_3R_{13} + V_{13}]\} \quad (31)$$

where $R_c = [V_1 + R_5(V_5 + N_6) + V_{15}]/(V_1 + V_7 + V_5 + V_{15})$ and $R_{13p} = V_{13p}/(2V_5 + V_{10} + N_6 + V_3 + V_{13})$.

From eq 30 and 31 and the definitions of all other compounds in terms of malate, equations can be written for the input into glycogen (GT), lipid (LT), and CO_2 (CT).

$$(GT) = V_2R_2R_{13}\sum_{i=1}^{3} S_m(i) \quad (32)$$

$$(LT) = V_7\{V_4[S_a(1) + S_a(2)] + 2V_3R_{13}S_m(2)\}/(2V_1 + V_5 + V_7 + V_{10}) \quad (33)$$

$$(CT) = \{[V_{10} + V_5 + R_5(V_5 + N_6) + V_3R_{13} + V_{13}]S_m(1) + V_{10}S_m(2)\}(1 - R_{13p}) \quad (34)$$

GT_1 and GT_2 will refer to the amount of label incorporated into glycogen from [1-^{14}C]acetate and [2-^{14}C]acetate, respectively, and similarly for LT_1, LT_2, and CT_1, CT_2.

Two levels of oxygen consumption can in theory be defined in terms of the scheme of Figure 1. If one assumes that only the oxaloacetate utilized to form citrate is oxidized in the mitochondria, a minimum estimate of oxygen consumption can be written

$$[O_2]_{min} = 2V_5 + 0.5V_{10} + V_{15} + N_6 + 0.5V_3 \quad (35)$$

If all the malate formed through the operation of the glyoxalate cycle plus that resulting from any input of glutamate is also oxidized in the mitochondria, a higher value results

$$[O_2]_{max} = 2N_5 + 0.5V_{10} + V_{15} + 1.5N_6 + 0.5V_1 + 0.5V_3 \quad (36)$$

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Hybridization of Membranes by Sonic Irradiation*

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ABSTRACT: Mixtures of membranes labeled with a density-labeled fatty acid and unlabeled membranes can be resolved by equilibrium centrifugation in sucrose density gradients. Subjecting the density-labeled and unlabeled membranes individually to sonic irradiation does not interfere with subsequent resolution by density gradient centrifugation. When a mixture of density-labeled and unlabeled membranes is treated by sonic irradiation, however, a new membrane species of intermediate density is formed. If a mixture of density-labeled and unlabeled membranes is sonicated in the presence of sucrose, only partial hybrid formation is observed, and the hybridized species is the lowest in particle weight of the membranes in the population. Controlled sonic treatment in the presence of sucrose allows a significant fragmentation of membranes with only limited hybrid formation.

Recently, Morrison and Morowitz (1970) have published radioautographic evidence which suggests that lipids are incorporated into the membrane of *Bacillus megaterium* KM at one or a few foci. Donachie and Begg (1970) have shown that *Escherichia coli* grow by an apparent extension from one pole of the organism and have extrapolated from their data to claim that the membrane of *E. coli* grows from some fixed focus with accompanying conservation of old and newly synthesized membrane during cellular growth and division.

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