References

Bate-Smith, E. C., and Westall, R. G. (1950), *Biochim. Biophys. Acta 4*, 427.
Birkofer, L., Kaiser, C., Nouvert, W., and Thomas, U. (1961), *Z. Naturforsch. 16b*, 249.
Corse, J. (1953), *Nature 172*, 771.
Corse, J., Lundin, R. E., and Waiss, A. C., Jr. (1965), *Phytochemistry 4*, 527.
Hanson, K. R. (1963), *Chem. Ind. (London)*, 1691.
Hanson, K. R. (1965), *Biochemistry 4*, 2719 (this issue; previous paper).
Hanson, K. R., and Zucker, M. (1963), *J. Biol. Chem. 238*, 1105.
Harborne, J. B., and Corner, J. J. (1961), *Biochem. J. 81*, 242.
Haslam, E., Haworth, R. D., and Makinson, G. K. (1961), *J. Chem. Soc.*, 5153.
Herrmann, K. (1956), *Pharmazie 11*, 433.
Maier, V. P., Metzler, D. M., and Huber, A. F. (1964), *Biochem. Biophys. Res. Commun. 14*, 124.
Marcinkiewicz, S., Green, J., and McHale, D. (1963), *J. Chromatog. 10*, 42.
Martin, A. J. P. (1950), *Biochem. Soc. Symp. 3*, 4.
Marvel, C. S., and Rands, R. D., Jr. (1950), *J. Am. Chem. Soc. 72*, 2642.
Meister, A. (1952), *J. Biol. Chem. 197*, 309.
Rúveda, E. A., Deulofeu, V., and Galmarini, O. L. (1964a), *Chem. Ind. (London)*, 239.
Rúveda, E. A., Deulofeu, V., and Galmarini, O. L. (1964b), *Anales Asoc. Quim. Arg. 52*, 237.
Scarpati, M. L., and Esposito, P. (1963), *Tetrahedron Letters 18*, 1147.
Scarpati, M. L., and Guiso, M. (1964), *Tetrahedron Letters 39*, 2851.
Sondheimer, E. (1958), *Arch. Biochem. Biophys. 74*, 131.
Sondheimer, E. (1964), *Botan. Rev. 30*, 667.
Waiss, A. C., Jr., Lundin, R. E., and Corse, J. (1964), *Chem. Ind. (London)*, 1984.
Williams, A. H. (1958), *Chem. Ind. (London)*, 1200.

The Molecular Biology of *Euglena gracilis*. III. General Carbon Metabolism*

E. S. Kempner and J. H. Miller

ABSTRACT: Glutamic acid, supplied as a sole carbon source, enters the metabolic pathways of *Euglena gracilis* principally *via* transamination by glutamic–oxalacetic transaminase.

Synthesis of purines and pyrimidines and the interconversions of amino acids suggest that general carbon metabolism is similar to that of most microorganisms.

The development of a simple growth medium and standard culture conditions for *Euglena gracilis* have been reported (Kempner and Miller, 1965a). The gross chemical composition of the cells and the kinetics of carbon assimilation among several cellular fractions were also determined (Kempner and Miller, 1965a,b). The continuing studies of the molecular biology of *Euglena gracilis* have now been extended to a very general study of carbon metabolism.

The biochemical pathways in *Euglena* have not been fully elucidated, although several reports have appeared in recent years concerning selected aspects of carbon metabolism. Two reports (Danforth, 1953; Hurlbert and Rittenberg, 1962) have been particularly significant with respect to intermediary metabolism. These studies have shown the existence of the Krebs cycle and Embden–Meyerhof and hexose monophosphate pathways. Under our growth conditions, glutamic acid is supplied as a sole carbon source; the question of which routes of entry into the various major cell pathways were utilized by the glutamic acid and some information about the synthesis of nucleic acids and amino acids have been considered. Most of the experimental techniques were those developed in the studies of the bacterium *E. coli* at the Carnegie Institution of Washington. On the basis of the published data and the results given here, a general picture of the carbon flow in *Euglena gracilis* can be drawn.

Materials and Methods

Composition of the growth medium and details of culture conditions for *Euglena gracilis* strain z have been reported previously (Kempner and Miller, 1965a). Radioactive compounds were obtained from the Nuclear

* From the Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received August 10, 1965.*

Chicago Corp., New England Nuclear Corp., and Calbiochem. All compounds were checked for radiochemical purity by two-dimensional paper chromatography. GOT,[1] GPT, and DPN-dependent GDH enzyme determinations were made using kits supplied by Biochemica Boehringer, Mannheim, Germany. Techniques of cellular extraction, paper chromatography, and radioautography are those described by Roberts *et al.* (1955).

Results

Gross Utilization of Glutamic Acid Carbon. Sealed cultures of *Euglena gracilis* containing uniformly labeled L-glutamic acid-^{14}C were grown to a 60-fold increase in cellular mass. Evolved CO_2 was trapped in NaOH solution, precipitated as the barium salt, and detected by radioactivity. Table I shows the results of a typical experiment. All of the radioactive carbon originally supplied is accounted for. Of the carbon utilized by the cells, about 55% is given off as CO_2, while 45% remains as cellular carbon.

TABLE I: Utilization of L-Glutamic Acid-^{14}C by *Euglena gracilis*.[a]

Carbon supplied as L-glutamic acid[b]		178.8
Carbon left in medium at end of growth[b]	132.3	
Carbon in cells[b]	27.1	
Carbon evolved as CO_2[b]	33.9	
Total carbon recovered[b]	193.3	

[a] Yield 65.15 mg dry cells. [b] In micrograms.

Entry of Glutamic Acid into Cellular Metabolism. The pathways by which glutamic acid carbon enters the metabolic pathways of *Euglena gracilis* were examined by two different techniques. The presence of three enzymes was studied directly, while three other pathways were followed by growth competition methods.

ENZYME DETERMINATIONS. Glutamic–oxalacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT), and glutamic dehydrogenase (GDH) were tested in cell extracts. Cell rupture was accomplished by homogenization with a Tenbroeck tissue grinder, grinding with alumina, or passage through a French pressure cell at 2000 psi. All extractions were performed with Tris–magnesium succinate buffer at 0°. The same enzymes were also examined by Drobnica and Ebringer (1963) using a freeze–thaw method of cell rupture. The results of these studies are summarized in Table II.

TABLE II: Enzyme Determinations in *Euglena gracilis*.

Cell Breakage Method	GOT	GPT	GDH (TPN)	GDH (DPN)
Micromoles of Substrate per Min per mg of N				
Freeze–thaw[a]	425	0	—	0
Micromoles of Substrate per Min per mg of protein				
Tissue grinding	1213	69	6	0
Alumina grinding	1414	112	19	0
Pressure cell	874	44	5	0

[a] Drobnica and Ebringer (1963).

COMPETITION EXPERIMENTS. Cultures of *Euglena gracilis* were grown in duplicate in normal growth medium containing glutamic acid and equimolar concentrations of L-proline, L-ornithine, or γ-aminobutyric acid. In each experiment, one culture was supplied with radioactive glutamic acid and in the duplicate the competitor was made radioactive. After 5-days' growth, the cultures were harvested and the radioactivity was measured. The results are given in Table III. None of the radioactive competitors appreciably altered the growth rate or the utilization of glutamic acid by *Euglena*. The major pathway of glutamic acid entry in cellular metabolism appears to be *via* transaminases, with GOT about 15-fold more active than GPT.

TABLE III: Competition with Glutamic Acid in *Euglena gracilis*.

Labeled Compd	Nonradioactive Competitor	^{14}C Compd Supplied (mg)	^{14}C Compd in Cells (mg)
L-Glutamic acid	None	438	40
L-Glutamic acid	γ-Aminobutyric acid	444	46
L-Glutamic acid	L-Ornithine	444	45
L-Glutamic acid	L-Proline	435	45
γ-Aminobutyric acid	L-Glutamic acid	322	0.2
L-Ornithine	L-Glutamic acid	496	10.8
L-Proline	L-Glutamic acid	338	1.3

Carbon Sources for Nucleic Acid Purines and Pyrimidines. The synthesis of the organic skeleton of purines and pyrimidines was compared with the pathways which had been found in *E. coli* (Roberts *et al.*, 1955).

[1] Abbreviations used in this work: GOT, glutamic–oxalacetic transaminase; GPT, glutamic–pyruvic transaminase; GDH, glutamic dehydrogenase; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; and UDP, uridine diphosphate.

TABLE IV: Incorporation of ^{14}C-Amino Acids[a] by *Euglena gracilis*.

	Ala	Asp	Gly	His	Lys	Phe	Pro	Ser	Thre	Tyr	Val
Total cpm supplied	4.493	8.950	58.261	5.324	2.096	5.400	4.637	1.196	2.495	2.933	1.236 $\times 10^6$
Total cpm taken up by *Euglena*	1.409	3.094	34.392	0.154	0.004	3.584	0.238	0.582	0.069	0.019	0.174 $\times 10^6$
Per cent ^{14}C distribution:											
Water extract	0.3	0.6	0.6	0.3	3.1	0.1	0.2	0.9	1.0	1.9	0.3
Cold trichloroacetic acid soluble	2.5	7.8	9.8	7.9	8.0	0.3	7.1	10.1	8.6	5.9	2.4
Ethanol–ether soluble	30.1	20.9	10.9	14.0	12.1	10.7	10.2	15.3	7.2	7.0	10.4
Hot trichloroacetic acid soluble	4.4	11.1	21.7	20.8	10.3	3.0	12.1	14.9	7.3	7.9	3.8
Residue	62.8	61.2	56.9	57.0	66.5	85.8	70.2	58.8	75.9	77.4	83.1

[a] Tracer compounds supplied carrier free.

In the bacterium glycine, aspartic acid, formate, and CO_2 were reported to be the major precursors.

Cultures of *Euglena* were grown in the presence of carrier-free ^{14}C-glycine or ^{14}C-aspartic acid (Table IV). The nucleic acids were extracted in hot trichloroacetic acid, hydrolyzed, and placed on paper for two-dimensional chromatography. The spots were identified by ultraviolet absorption and radioactivity was detected by autoradiography. As in *E. coli*, glycine was found to supply carbon to adenine and guanine, but not to the pyrimidines. Aspartic acid is utilized by *E. coli* for the pyrimidines and to a lesser extent for the purines; in *Euglena*, all four bases are labeled about equally with carbon from aspartic acid.

Carbon Flow in Amino Acid Synthesis. Cultures of *Euglena* were grown in media containing tracer quantities of uniformly labeled amino acids. After a 50-fold cellular mass increase, the protozoa were harvested and the radioactivity in the cell fractions (Kempner and Miller, 1965b) was determined. Table IV summarizes the distribution of ^{14}C from exogenous amino acids among these fractions. Only very small quantities of lysine and tyrosine were utilized by the cells; histidine, proline, threonine, and valine also were only sparingly incorporated. Excellent incorporation was obtained with aspartic acid, glycine, and phenylalanine.

The lipids and other materials extracted in hot ethanol–ether were heavily labeled with carbon from alanine and aspartic acid. The nucleic acids obtained radioactive carbon especially from glycine, serine, and, surprisingly, also histidine. This latter finding has not been examined further. The bulk of the radioactivity from all eleven amino acids is found in the proteins of the residue fraction.

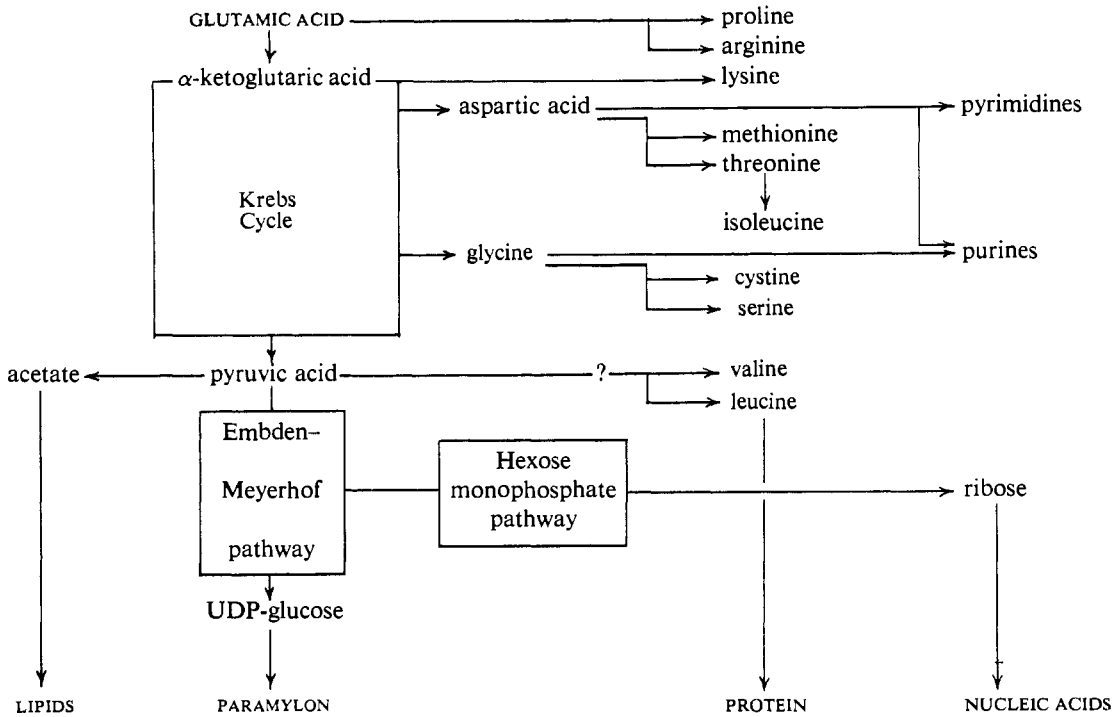
The biochemical relationship among several amino acids was examined in these samples. The residue fractions were hydrolyzed and the amino acids separated by two-dimensional chromatography. The chromatograms were placed against film for 1–3 weeks and the radioactive spots were identified by comparison with pure amino acids. Table V indicates the labeled amino acid initially supplied and the protein amino acids which were found to be radioactive for both *E. coli* (Roberts *et al.*, 1955) and *Euglena gracilis*.

Discussion

Rapid exponential growth of pure *Euglena* cultures can be obtained on a completely synthetic, defined medium utilizing glutamic acid as a sole source of carbon. Approximately as much carbon is utilized for cellular end products as is given off as carbon dioxide.

The major routes of glutamic acid metabolism involve transaminases, dehydrogenases, and decarboxylases. GOT and GPT have been detected by enzymatic means. Using three different methods of cell disruption, GOT appears to be 15 times more active than GPT. Small quantities of TPN-dependent GDH are present, but no evidence for the DPN-dependent enzyme was found. After cell disruption by freeze–thaw methods, Drobnica and Ebringer (1963) found GOT, but neither GPT nor GDH (DPN) could be detected in green *Euglena gracilis* cells. These authors felt that GDH was probably present but that their assay conditions were not suitable, while the presence of GPT was uncertain. It is possible that the freeze–thaw techniques inactivated the GPT in their samples, but it seems unlikely that GDH (DPN) is present in *Euglena*.

Three other pathways of glutamic acid utilization were examined by competition experiments. Equimolar concentrations of γ-aminobutyric acid, L-ornithine, or L-proline were added to the glutamic acid (2×10^{-2} M)



SCHEME 1: Carbon Flow in *Euglena gracilis*.

medium. There was no observable effect on the growth rate or on the quantity of glutamic acid utilized. In reciprocal labeling experiments, it is shown that the competitors were taken up by the cells, but only in small quantities.

These data indicate that the pathway of glutamic acid utilization in *Euglena gracilis* is principally by transamination *via* GOT and to a lesser extent by GPT. In both reactions, as well as dehydrogenation by GDH (TPN), the carbon skeleton of glutamic acid is used *in toto* to form α-ketoglutaric acid. Exogenously derived carbon is then transmitted through the Krebs cycle compounds (Danforth, 1953), the Embden–Meyerhof, and the hexose monophosphate pathways (Hurlbert and Rittenberg, 1962).

The synthesis of nucleic acid purines and pyrimidines was briefly examined. As in *E. coli*, glycine donates carbon to the purines but not to the pyrimidines. Aspartic acid is a carbon precursor for all four bases in *Euglena*. Formate and CO_2 were also found to supply carbon to the bacterial nucleic acid; in *Euglena*, nucleic acid was labeled when the cells were exposed to ^{14}C-formate (Britten, 1963). From these results, it is concluded that purine and pyrimidine synthesis in *Euglena gracilis* follows pathways similar to those well known in *E. coli*.

The utilization of exogenous amino acids by *Euglena* cultures has been examined. Lysine, tyrosine, and threonine were not readily incorporated by the cells, perhaps due to a permeability barrier. Alanine, aspartic acid, and serine supplied radioactive carbon to the lipid substances extracted in ethanol–ether solution. Nucleic acids were extracted in hot trichloroacetic acid and were labeled when the cells were grown in the presence of ^{14}C-glycine, serine, and also histidine. In all cases studied, the exogenous amino acids were principally utilized for protein synthesis. Phenylalanine in particular appears to be useful as a tracer for protein synthesis since it is massively incorporated and over 85% of the label is ultimately found in the proteins.

In these experiments, tracer quantities of a uniformly labeled ^{14}C-amino acid was added to a culture of *Euglena gracilis* growing in minimal medium containing 3 mg of glutamic acid/ml. The proteins were extracted in the residue and were hydrolyzed in HCl. Chromatography then allowed the identification of radioactive amino acids derived from the original ^{14}C-amino acid. Experiments with *E. coli* have been reported (Roberts *et al.*, 1955) which were similar, except that the bacteria were growing in a medium containing glucose rather than glutamic acid. From the results of the bacterial studies, amino acids were grouped into biochemically related "families." Comparison of the results of the protozoan and bacterial experiments (Table V) indicate many similarities. The major difference appears to be in the aspartic acid family, but in part this reflects the variation in culture media. In the *E. coli* studies, the ^{14}C-aspartic acid "spilled over" into the glutamic acid family (glutamic, proline, arginine); in the present *Euglena* studies this family was not labeled, probably due to the presence of large quantities of nonradioactive glutamic acid available. In addition, lysine, which is in the aspartic acid family in bacteria, is synthesized from α-ketoglutaric acid in *Euglena* (Vogel, 1959) and consequently was not labeled with ^{14}C from aspartic acid by the protozoan.

The glutamic acid family, the aspartic family (aspartic, threonine, isoleucine, methionine), the glycine

TABLE V: Fate of Exogenously Supplied Amino Acids.[a]

^{14}C-Amino Acid Added to Medium	Major ^{14}C-Amino Acids Found in Protein Hydrolysates	
	Euglena gracilis	*E. coli*[b]
Alanine	Alanine	Alanine
Aspartic acid	Aspartic Glycine Serine Alanine Isoleucine Threonine	Aspartic Glutamic Lysine Arginine Proline Threonine Isoleucine
Glycine	Glycine Serine Cysteine Lysine	Glycine
Histidine	Histidine	
Proline	Proline	Proline
Phenylalanine	Phenylalanine	Phenylalanine
Serine	Serine Glycine Cysteine	Serine Glycine
Threonine	Threonine Isoleucine Leucine Glycine	Threonine Isoleucine Leucine Glycine
Tyrosine	Tyrosine	Tyrosine
Valine	Valine	Valine Leucine

[a] Uniformly Labeled ^{14}C-amino acids added in tracer amounts. [b] Roberts *et al.* (1955).

family (glycine, serine, cysteine), and the valine family (valine, leucine) are probably the same in both species. In addition, tyrosine, phenylalanine, and histidine seem to be synthesized independently of the other amino acids in these organisms.

The major components of dried *Euglena* cells have been shown (Kempner and Miller, 1965a) to be proteins and free amino acids (48% of dry weight), nucleic acids (4%), polysaccharides and free sugars (23%), and lipid substances (19%). Some information concerning the last two classes of compounds in *Euglena* has been reported in the literature. The bulk of the *Euglena* polysaccharide is found in granules of paramylon, whose structure (Clarke and Stone, 1960) and synthesis (Goldemberg and Marechal, 1963; Marechal and Goldemberg, 1964) have been described. Glucose 1-phosphate is converted to UDP-glucose which is utilized directly for paramylon synthesis (Marechal and Goldemberg, 1964). Lipid synthesis has been followed using ^{14}C-acetate (Hulanicka *et al.*, 1964), and the relationships among the polyunsaturated fatty acids have been studied (Hulanicka *et al.*, 1964; Korn, 1964).

The general carbon flow from glutamic acid in *Euglena gracilis* can therefore be drawn in rough outline. The major biochemical relationships are indicated in the figure. Although details of this plan may be unique to *Euglena* and closely related species, the over-all description is quite similar to many microorganisms. From a study of the phosphorylated compounds in *Euglena gracilis*, Albaum *et al.* (1950) concluded that the phosphorus metabolism of this protozoan presented nothing unusual; in general, this appears to be true for carbon also.

Acknowledgment

Assays of TPN-dependent GDH were kindly performed by Dr. Gordon Tomkins.

References

Albaum, H. G., Schatz, A., Hutner, S. H., and Hirshfeld, A. (1950), *Arch. Biochem. 29*, 210.

Britten, R. J. (1963), *Carnegie Inst. Wash. Yearbook 62.*

Clarke, A. E., and Stone, B. A. (1960), *Biochim. Biophys. Acta 44*, 163.

Danforth, W. (1953), *Arch. Biochem. Biophys. 46*, 164.

Drobnica, L., and Ebringer, L. (1963), *Folia Microbiol. (Prague) 8*, 56.

Goldemberg, S. H., and Marechal, L. R. (1963), *Biochim. Biophys. Acta 71*, 743.

Hulanicka, D., Erwin, J., and Bloch, K. (1964), *J. Biol. Chem. 239*, 2778.

Hurlbert, R. E., and Rittenberg, S. C. (1962), *J. Protozool. 9*, 170.

Kempner, E. S., and Miller, J. H. (1965a), *Biochim. Biophys. Acta 104*, 11.

Kempner, E. S., and Miller, J. H. (1965b), *Biochim. Biophys. Acta 104*, 18.

Korn, E. D. (1964), *J. Lipid Res. 5*, 352.

Marechal, L. R., and Goldemberg, S. H. (1964), *J. Biol. Chem. 239*, 3163.

Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., and Britten, R. J. (1955), *Carnegie Inst. Wash. Publ. 607.*

Vogel, H. J. (1959), *Biochim. Biophys. Acta 34*, 282.