METABOLIC POOLS AND THE UTILIZATION OF AMINO ACID ANALOGS FOR PROTEIN SYNTHESIS

ELLIS S. KEMPNER AND DEAN B. COWIE

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. (U.S.A.) and

Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D.C. (U.S.A.)

(Received January 11th, 1960)

SUMMARY

Studies of the kinetics of analog and amino acid incorporation into metabolic pools and proteins of yeast and *E. coli* have shown that at least two processes are involved in the selection of the natural amino acid in preference to the analog. These selection steps occur in the formation of the metabolic pools, the first occurring in the concentrating system of the cell, which accumulates exogenous amino acids or analogs into the cell at levels exceeding external concentrations. In the yeast, the final distinction between analog and amino acid occurs at the time of entry into a second metabolic pool (internal pool). In this pool conversion of amino acids to the required end products occurs. Once incorporated into this pool no further selection occurs; the ratio of analog to amino acid in this pool and in protein is the same.

INTRODUCTION

The demonstration that amino acid analogs could be incorporated into bacterial proteins[1–9] raised many questions concerning (a) the nature of the protein synthesized and (b) the actual mechanism of analog incorporation. It has been shown that the substitution of the analogs norleucine or selenomethionine for methionine in the proteins of *E. coli* did not result in the synthesis of radically different molecular species, but that the macromolecules formed had physicochemical properties similar to the proteins normally synthesized[1,10]. Furthermore, each methionine site in the proteins seemed to have the same susceptibility for analog substitution, that is, norleucine[10] (and selenomethionine[1]) replaced methionine *in the same proportion* in all of the proteins examined. Recently it has been shown that the substitution of ethionine for methionine in a single protein (α-amylase) was the same for each individual methionine site[11].

As has been pointed out by YOSHIDA AND YAMASAKI[11], no evidence has been presented for the mechanism of analog incorporation into protein. It is evident that the bacterial cell possesses certain mechanisms for selecting the natural amino acid and rejecting, at least partially, the amino acid analog. With norleucine a large environmental pressure was required in order to effect a relatively small change in protein composition; a ratio in the medium of norleucine to methionine of 100 resulted

in only a 40 % replacement of methionine by the analog[10]. Studies of the kinetics of analog incorporation into the metabolic pools and proteins of yeast and *E. coli* were made to determine where these selections among amino acids and their analogs occurred.

PROCEDURES

Wild type *E. coli* ML 30 and the yeast, *Candida utilis* were used in these experiments. Both types of cells were cultured in vigorously aerated C medium* with maltose as the carbon source for *E. coli* and fructose employed with *Candida utilis*.

DL-[3-^{14}C]-*p*-fluorophenylalanine was obtained from the Volk Radiochemical Company, Chicago, Illinois (79.2 mg/mC). Uniformly-labeled L-[^{14}C]phenylalanine was obtained from Nuclear-Chicago Corporation, Chicago, Illinois (16 mg/mC). These materials were always added directly to exponentially-growing cultures of cells with appropriate carrier compound to bring the concentration to the desired levels.

Culture samples of *E. coli* were withdrawn at various times and the cells removed by centrifugation. The cell pellet was washed by resuspending in 40 ml C medium and centrifuged. The resulting pellet was resuspended in 5 ml of 5 % cold trichloroacetic acid (TCA) and an appropriate aliquot measured to determine the total radioactivity taken up by the cells. The remaining suspension was centrifuged and the radioactivity of the TCA-soluble fraction (containing the metabolic pool) and of the TCA-insoluble fraction (containing the proteins) were measured to determine the distribution of radioactivity between pool and protein.

The extracting process used for *Candida utilis* was the same as that given above, except where it was desired to separate the two metabolic pools (see below) found in the yeast[15]. After washing in C medium the concentrating pool was extracted by resuspending the pellet of cells in 20 ml cold distilled water for 20 min.

An appropriate aliquot of this suspension was measured for the total radioactivity taken up by the cells. The remaining suspension was centrifuged to separate the water soluble fraction containing the expandable pool material. The pellet was treated with 5 % TCA and the distribution of tracer between the internal pool and protein was determined from measurement of radioactivity in the TCA soluble and insoluble fractions respectively.

RESULTS

E. coli

Kinetics of amino acid analog incorporation: The kinetics of incorporation and utilization of amino acid analogs are in many respects similar to those observed with the natural amino acids[12–16]. For example, at low external concentrations the analogs can be accumulated in the metabolic pool of the cell up to levels exceeding their external concentrations. This accumulation is rapid and precedes the appearance of the analog in the protein. Fig. 1 shows the time course of incorporation of tracer quantities of DL-[3-^{14}C]-*p*-fluorophenylalanine (*p*FPhe) into the pool and protein**.

* C Medium: 2 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 3 g NaCl, 0.01 g Mg as $MgCl_2$, 0.026 g S as Na_2SO_4, 100 ml 10 % maltose or fructose, and 900 ml distilled H_2O.

** *p*-fluorophenylalanine has been shown by MUNIER AND COHEN[9] to replace only phenylalanine in the proteins of *E. coli*.

The limited exogenous supply of the analog is soon exhausted and incorporation into protein rapidly depletes the analog in the pool.

At higher external concentrations of analog a proportionally larger quantity of accumulated material is observed in the cells. Fig. 2 (upper curve) shows the quantity of analog contained in the cold TCA-soluble fraction as a function of external concen-

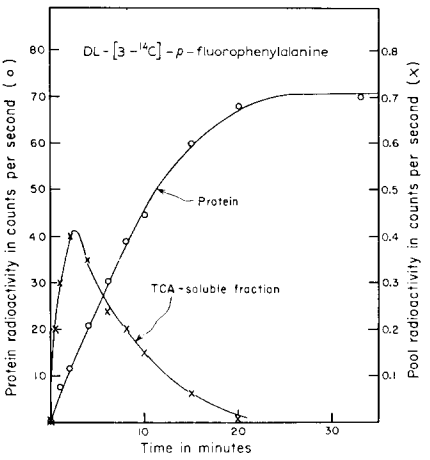

Fig. 1. Kinetics of uptake of tracer quantities of DL-[3-^{14}C] *p*-fluorophenylalanine (0.002 mg/ml medium) into pool and protein of *E. coli*.

tration. Two processes appear to be involved in the uptake of *p*FPhe. One, a process concentrating analog in the cell in excess of the external concentration, appears to saturate at low concentrations. At exogenous levels exceeding 0.03 mg *p*FPhe/ml medium a second process of incorporation is observed. The quantity of material taken into the pool through this second process is directly proportional to the external concentration (dashed curve, Fig. 2). Pool accumulation to levels exceeding the external concentrations does not occur through this process. Incorporation of the analog into protein, however, as shown in Table I, depends directly upon the total quantity of pool material available, regardless of the mechanisms of uptake.

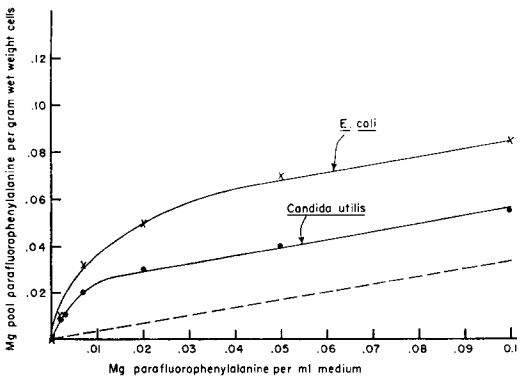

Fig. 2. Maximum level of *p*-fluorophenylalanine pool observed in *E. coli* and *C. utilis* as a function of external concentration of analog. Dashed curve shows component of accumulation which is directly proportional to exogenous concentration.

TABLE I

DISTRIBUTION OF PARAFLUOROPHENYLALANINE IN *Escherichia coli*

Medium mg pFPhe/ml (at t = o)	*Pool* mg pFPhe/g wet weight cells*	*Protein** mg pFPhe/Δ g wet weight cells*	*Protein/pool****
0.002	0.008	0.2	25
0.007	0.032	0.8	25
0.02	0.050	1.2	24
0.05	0.068	1.5	22
0.10	0.085	2.2	28

* Maximum level observed.

** Value calculated from steady state rate of incorporation of *p*-fluorophenylalanine per unit quantity of newly formed cells.

*** Δ gram wet weight cells = mass of cells grown after the addition of the tracer material.

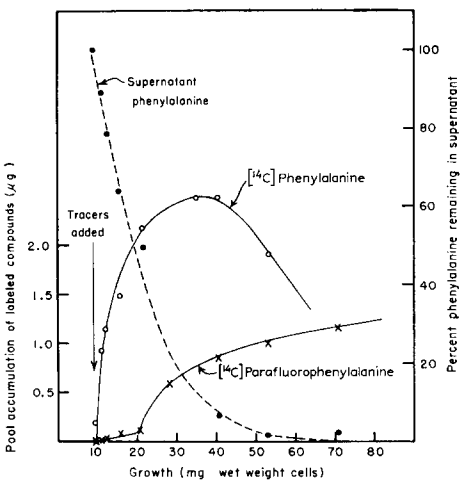

Fig. 3. ○, accumulation of L-phenylalanine; ×, DL-*p*-fluorophenylalanine in the pool of *E. coli*. An exponentially growing culture of cells was divided. Culture 1 (○) was supplied with L-[^{14}C]-phenylalanine and an equimolar quantity of DL-[^{12}C] *p*-fluorophenylalanine. Culture 2 (×) was treated identically, but with reciprocal labeling. Initial exogenous concentration of each compound was 0.0067 mg/ml medium. ●, concentration of phenylalanine remaining in medium. Growth of the two cultures was identical.

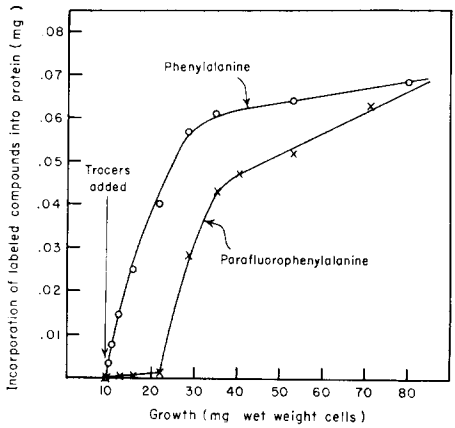

Fig. 4. ○, incorporation of L-phenylalanine; ×, incorporation of DL-*p*-fluorophenylalanine into proteins of *E. coli*. Conditions as described in Fig. 3.

Competitive utilization of amino acids and amino acid analogs: Kinetics of analog incorporation were significantly influenced by the presence of natural amino acids in the medium. Figs. 3 and 4 show the kinetics of pool accumulation and protein incorporation of phenylalanine and *p*FPhe. Initially very little of the analog was accumulated in the metabolic pool or incorporated into protein. On the other hand, phenylalanine was immediately concentrated by the cell and utilized for protein synthesis. When phenylalanine accumulation no longer continued at the maximal rate, due to depletion in the supply, accumulation of the analog into the pool occurred. Subsequently, the analog was incorporated into protein.

The analog, however, was not completely excluded from the cell, even during these early stages of phenylalanine incorporation. Fig. 5 shows the relative rates of

protein incorporation of DL-[^{14}C]-*p*-fluorophenylalanine in the absence or presence of equimolar quantities of L-phenylalanine. The rate of protein incorporation of *p*FPhe was reduced by a factor of 100 when phenylalanine was present.

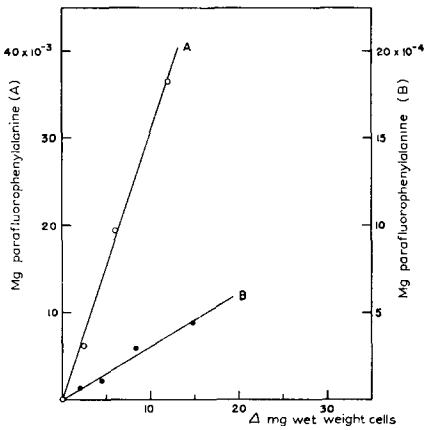

Fig. 5. Protein incorporation by *E. coli* of *p*-fluorophenylalanine. A, supplied in the absence of an equimolar quantity of phenylalanine; B, in the presence of an equimolar quantity of phenylalanine.

Candida utilis

Kinetics of amino acid analog incorporation: Similar experiments have been done with the yeast, *C. utilis*. In this organism *two* functionally distinct metabolic pools of amino acids have been found[15]. The first accumulates the amino acids within the cell to levels exceeding their external concentration. This pool has been called the expandable (or concentrating) pool[15] and has many of the characteristics of the amino acid-concentrating system observed in *E. coli*. The size of this pool varies with external concentration and the pool is only evident when amino acids (or analogs) are present in the medium. Material in this pool is sensitive to osmotic shock and readily exchanges with external amino acids.

A second pool, called the internal (or conversion) pool[15] is always found in exponentially-growing cells. The amino acids contained in this pool are on the main line of synthetic events. It is here that the conversion of one amino acid to others occurs, furnishing the appropriate molecules for protein incorporation. Once in this pool, these amino acids do not exchange with exogenous or accumulated amino acids, nor are they sensitive to osmotic shock. In the absence of exogenous amino acids (or analogs), this pool is formed solely from the carbon source (sugar). During exponential growth the size of this pool remains fixed and contains 13 % of the total cellular carbon. The presence of exogenous or accumulated amino acids in the cell does not alter the pool size.

In addition to these functional differences, the two pools can be extracted separately. The expandable pool alone is extractable with cold water; both pools are extractable with cold trichloroacetic acid.

Despite the existence of such amino acid pools, very little *p*FPhe can be *concentrated* by the yeast cells. Fig. 2 shows the total quantity of this analog contained in both pools (TCA-soluble fraction) as a function of exogenous analog concentration. These data are similar to those obtained with *E. coli* (Fig. 2), except that in the

yeast cell saturation of the concentrating system occurs at a lower external level. Very little *p*FPhe is found in the metabolic pools in excess of the external concentration. In the yeast, as in *E. coli*, protein incorporation of the analog was found to be directly dependent upon pool concentration. As a consequence of this reduced capacity for accumulation, analog replacement in the yeast cell requires a higher external concentration than in *E. coli* for the same degree of protein substitution.

Competitive utilization of amino acids and amino acid analogs: In the yeast our principal interest has centered on the internal pool of amino acids, because of its necessary and essential role in the synthesis of protein. Kinetic interrelationships among the internal pool, the expandable pool and the proteins are difficult to measure, however, at low external concentrations of *p*FPhe because of the possibility of saturation of the concentrating system for the analog.

At high concentrations of DL-*p*FPhe and L-phenylalanine (10^{-3} *M* and 10^{-4} *M* respectively) added simultaneously to a growing culture of *Candida utilis*, both compounds are taken up by the cells in easily measurable quantities. Two identical flasks with reciprocal labeling ([^{12}C]Phe + [^{14}C]*p*FPhe in one and [^{14}C]Phe + [^{12}C]*p*FPhe in the other) were inoculated with cells from an exponentially-growing culture of *C. utilis*. The distribution of the analog and phenylalanine was obtained by following kinetics of incorporation of the two labeled compounds in the expandable pool, the internal pool, and the protein. The results of a typical experiment are shown in Table II.

TABLE II

DISTRIBUTION OF PHENYLALANINE AND *p*-FLUOROPHENYLALANINE IN *Candida utilis*

External ratio of DL-*p*-fluorophenylalanine to L-phenylalanine was 10:1.

	Expandable pool (mμmoles/g wet weight cells)	*Internal pool (mμmoles/g wet weight cells)*	*Protein* (mμmoles/A g wet weight cells)*
*p*FPhe	23.9	14.0	7.25
Phenylalanine	4.58	6.1	3.05
Ratio *p*FPhe/Phe	5.2	2.30	2.38

* *A* gram wet weight cells = mass of cells grown after the addition of the tracer materials.

The yeast cells, given an external molar ratio of analog to amino acid of 10/1, contained these materials in the expandable pool at a ratio of 5.2/1. The ratio of the analog to amino acid in the internal pool was found to be 2.3/1 *and was identical to the ratio obtained in the proteins.* It appears that no further selection occurs by the processes through which the materials of the internal pool are made into protein.

DISCUSSION

The results obtained from the studies of the kinetics of utilization of *p*FPhe indicate that in yeast at least two processes exist selecting the natural amino acid in preference to the analog for protein synthesis. It is possible to correlate these results with schemes[14–16] describing the flow of endogenously synthesized amino acid carbon, and to show the alterations in the carbon flow produced by exogenous amino acid. Fig. 6 summarizes some of the essential details.

In the absence of exogenous amino acids (or analogs), all of the amino acid requirements of the cell are derived from the sugar carbon and CO_2. "Families"[17] of amino acids are formed from "parent" members: the parent is synthesized in the amino acid production system (Fig. 6) with the conversion of the parent to related family members occurring in the internal pool[14,15].

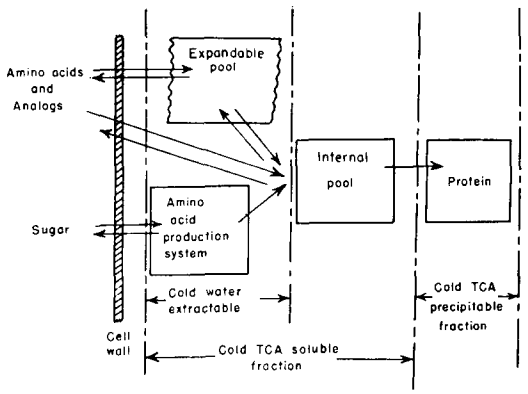

Fig. 6. Carbon flow in *C. utilis*.

The addition of amino acids or analogs to the medium results in their rapid cellular accumulation by a concentrating system (expandable pool) to levels exceeding (a) the external concentration, and (b) the intracellular level usually maintained through endogenous amino acid production from sugar. The accumulated amino acids mix with those amino acids endogenously formed, and from this mixture are withdrawn the molecules required for the internal pool and protein synthesis.

One of the first steps in the selection between an exogenous amino acid and an analog occurs in the concentrating system: With *E. coli*, accumulation of exogenous *p*FPhe is markedly reduced by the addition of equimolar quantities of phenylalanine (Fig. 3). It is evident that phenylalanine has a greater affinity for the concentrating system than *p*FPhe has. With *C. utilis*, this preference for phenylalanine is also observed (expandable pool, Table II), although not to the same extent as measured with *E. coli*.

Another process appears to be involved in the uptake of *p*FPhe, by-passing the concentrating system as shown in Fig. 6. Analog accumulation in the cell in excess of the external concentration does not occur beyond certain levels of exogenous *p*FPhe. However, with increasing external concentrations, larger and larger quantities of *p*FPhe are found in the cold TCA soluble fraction of the cell. After saturation of the concentrating system, the *p*FPhe taken up per unit volume of cells is proportional to, but smaller than, the external level (Fig. 2). Evidence supporting this interpretation is found in the data of HALVORSON AND COHEN[16]. These authors suggested that in *Saccharomyces cerevisiae* exogenous amino acids could be used for protein synthesis without equilibrating with the expandable pool.

Thus materials available for utilization in the internal pool are dependent upon a number of environmental and cellular factors. These are: (a) The ability to concentrate exogenous materials. This process depends upon the kind of exogenous supplements available, the relative specific affinity of each substrate for the concentrating mechanism, and the degree to which each substance can be accumulated (pool satu-

ration). (b) An entry process where cellular uptake is directly proportional to the external concentration. (c) Endogenous amino acid production.

Incorporation into the internal pool of materials made available from the above processes, provides a second opportunity for the selection between an amino acid and its analog for protein synthesis. Further competition among these materials occurs at the time, or prior to the time, of incorporation into the internal pool. Once an amino acid, or an acceptable analog, has been incorporated into this pool no further dilution or exchange with other cellular or extracellular amino acid carbon occurs[14,15].

The yeast cells (Table II), given an external ratio of analog to amino acid of 10/1, contained these materials in the expandable pool at a ratio of 5.2/1. The ratio of the analog to amino acid in the internal pool was found to be 2.3/1, and was identical to the ratio obtained in the proteins. *The final selection between natural amino acid and analog for protein incorporation thus must occur at the time of formation of the internal pool.*

Environmental conditions may markedly affect the degree to which analog substitution occurs, but the amounts of analog in the internal pool appear closely related to the final protein composition. One wonders whether the amino acids and analogs contained in the internal pool have not already been selected by the protein-forming templates, but have yet to be linked together in polypeptide strands.

ACKNOWLEDGEMENT

This work was done while E. S. KEMPNER was a guest at the Department of Terrestrial Magnetism, Carnegie Institution of Washington.

REFERENCES

1 A. B. PARDEE, V. G. SHORE AND L. S. PRESTIDGE, *Biochim. Biophys. Acta*, 21 (1956) 406.
2 R. MUNIER AND G. N. COHEN, *Biochim. Biophys. Acta*, 21 (1956) 592.
3 G. N. COHEN AND D. B. COWIE, *Comptes rend.*, 244 (1957) 680.
4 D. B. COWIE AND G. N. COHEN, *Biochim. Biophys. Acta*, 26 (1957) 252.
5 G. BRAVERMAN AND M. YCAS, *Arch. Biochim. Biophys.*, 68 (1957) 112.
6 A. B. PARDEE AND L. S. PRESTIDGE, *Biochim. Biophys. Acta*, 27 (1958) 330.
7 R. S. BAKER, J. E. JOHNSON AND S. W. FOX, *Biochim. Biophys. Acta*, 28 (1958) 318.
8 G. N. COHEN AND R. MUNIER, *Biochim. Biophys. Acta*, 31 (1959) 447.
9 R. MUNIER AND G. N. COHEN, *Biochim. Biophys. Acta*, 31 (1959) 378.
10 D. B. COWIE, G. N. COHEN, E. T. BOLTON AND H. DE ROBICHON-SZULMAJSTER, *Biochim. Biophys. Acta*, 34 (1959) 39.
11 A. YOSHIDA AND M. YAMASAKI, *Biochim. Biophys. Acta*, 34 (1959) 159.
12 R. J. BRITTEN, R. B. ROBERTS AND E. F. FRENCH, *Proc. Natl. Acad. Sci., U.S.*, 41 (1955) 863.
13 *Annual Report, Dept. Terrestrial Magnetism, Carnegie Inst. Wash.*, Washington, D.C., 1957.
14 D. B. COWIE AND B. WALTON, *Biochim. Biophys. Acta*, 21 (1956) 211.
15 D. B. COWIE AND F. T. MCCLURE, *Biochim. Biophys. Acta*, 31 (1959) 236.
16 H. O. HALVORSON AND G. N. COHEN, *Ann. inst. Pasteur*, 95 (1958) 73.
17 R. B. ROBERTS, P. ABELSON, D. B. COWIE, E. T. BOLTON AND R. J. BRITTEN, *Studies of Biosynthesis in* E. coli, Carnegie Inst. Wash. Publ. No. 607, Washington, D.C., 1955.