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Incorporation of L-2,5-Dihydrophenylalanine into Cell Proteins of *Escherichia coli* and Sarcoma 180

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L-2,5-Dihydrophenylalanine is extensively incorporated as a phenylalanine analogue into cell proteins. Phenylalanine-requiring *Escherichia coli* ATCC 9723f and sarcoma 180 grow at normal rates initially with the analogue and maximally replace 65 and 33%, respectively, of phenylalanine in the peptide residues of their cell protein without death. With the analogue alone growth of *E. coli* becomes non-steady-state and asymptotically inhibited. In mixtures of the analogue and phenylalanine, growth eventually becomes steady state or logarithmic. The logarithmic rate is inversely proportional to the extent of incorporation of this analogue or of *p*-fluorophenylalanine, and the projected maximal replacement is the same as that obtained asymptotically with the analogue alone. Thus, the toxicities in steady-state and non-steady-state growth are closely related. Moreover, it is proposed that single salient protein defects may determine the extent of growth rate reduction.

L-2,5-Dihydrophenylalanine ($pheH_2$) was reported first as a product of the Birch reduction of phenylalanine (phe) (10) and subsequently as an antibiotic synthesized by streptomyces (2, 8, 11). It is a growth antagonist of phe in microbes and the rat (3, 5, 10). In *Escherichia coli* it represses synthesis of the initial enzyme of aromatic biosynthesis, a function attributed to phe-transfer ribonucleic acid (3). The possibility that $pheH_2$ may ligate to transfer ribonucleic acid and thus incorporate into cell protein is now borne out in *E. coli* and sarcoma 180 (S-180). Further, steady-state and asymptotic growth responses to $pheH_2$ administration have been compared. Steady-state or logarithmic growth of phe-requiring *E. coli* could be maintained with balanced mixtures of phe and $pheH_2$, enriching the cell proteins randomly and equally with analogue. Administration of $pheH_2$ alone in contrast induces steadily decreasing or asymptotic growth, with separate populations of normal and $pheH_2$-substituted cell proteins. A simple relationship could be established between these two methods for the administration of $pheH_2$ and perhaps other phe analogues in *E. coli*. With analogues of additional amino acids and with other growth systems, however, the relationship may be more complex.

MATERIALS AND METHODS

A derivative of *E. coli* ATCC 9723f requiring phe, proline, methionine, and arginine was routinely cultivated in a minimal medium of salts, glucose, and required amino acids, as previously described (7). Tyrosine was also added at 10 μg/ml to eliminate the side effects of phe analogue on tyrosine metabolism (3, 4, 5). An inoculum in early growth (7) was washed on a cellulose membrane filter in phe-deficient medium, and 2×10^6 cells per ml was supplemented with phe and its analogues. Growth rates were estimated turbidimetrically (7) over a time period that would comprise 5 to 7.5 cell generations after inoculation. When analogue was substituted totally for phe, higher inocula were used and incubated overnight after growth had become stationary at mid-growth turbidities. For amino acid analyses the cells were boiled for 30 min in 0.1 N $HClO_4$, washed in 10% trichloroacetic acid, acetone, and then chloroform-methanol (2:1). Cell protein was hydrolyzed at 100 C overnight in vacuo, either in 6 N HCl for recovery of *o*- or *p*-fluorophenylalanine, or in 3 N KOH for recovery of $pheH_2$. Ratios of phe and its analogues in the hydrolysate and in various preparations were determined with a Beckman model 121 amino acid analyzer (1). For *o*-fluorophenylalanine analysis, the interfering peak of tyrosine was removed from the hydrolysate in 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0, for 1 h at room temperature with $C(NO_2)_4$ at 3% of the final volume, followed by removal of the reagent with ether and reacidification. Corrections were made for destruction of 14% of supplied $pheH_2$ and conversion of 2% to phe during alkaline hydrolysis, and for phe contamination of 1.1 μg/ml in 5% dialyzed horse serum and of 3 to 7% in different preparations of $pheH_2$.

To observe growth of S-180 cells, an inoculum of 5×10^4 cells was grown in stoppered test tubes (12 by 75 mm) for 4 days in two changes of 2 ml of minimal

Eagle medium with Hanks salts and 5% horse serum. Growth was determined by protein analysis (6) of the centrifuged washed cells. For incorporation of radiolabeled amino acid, the cells of confluent cultures were trypsinized, washed in the base of salts and glucose, and concentrated twofold in the Eagle medium without phe or valine and with 10% horse serum that had been dialyzed against saline for 3 h. One milliliter of the cell suspension was supplemented with uniformly labeled [^{3}H]phe at varying concentrations plus 0.4 mM uniformly labeled [^{14}C]valine, each at 0.32 mCi/mmol. After 2 h of incubation under air plus 5% CO_2 at 37 C in a Dubnoff shaker, the suspensions were diluted with excess medium, chilled, filtered on cellulose acetate membranes with saline followed by 10% trichloroacetic acid, dried at 60 C, combusted in a Packard model 306 Tri-Carb sample oxidizer, and assayed separately for ^{3}H and ^{14}C activities. L-pheH$_2$ was prepared from L-phe according to the procedure of Snow et al. (10). The other amino acid analogues were obtained from Nutritional Biochemicals Corp. Labeled amino acids were obtained from Schwarz Mann.

RESULTS

Growth of E. coli with phe analogues. When 0.1 mM pheH$_2$ replaced phe, the washed *E. coli* auxotroph grew normally for about 20 min, as judged from 5-min incorporations of [1-^{14}C]leucine supplied at 2 μg/ml. Thereafter, growth leveled off slowly and stopped after several hours, as judged either turbidimetrically or by colony counts on nutrient agar. Thus the viable cell number tripled and remained constant for 6 h. After 16 h, pheH$_2$ was found to replace 65% of the cell peptide phe. This represents the limit of incorporation at zero growth rate under conditions of non-steady-state growth. In terms of cell turbidity increase (Fig. 1), growth in pheH$_2$ is nonlogarithmic and becomes self-limiting. If the growth curve is plotted linearly, it still appears slightly convex rather than strictly linear and then presumable breaks and approaches an asymptotic limit. In contrast, at varying ratios of phe and pheH$_2$, a range of steady-state or logarithmic growth rates was obtained (Fig. 1). As many as four cell generations elapsed before growth attained these rates, judging from the time of appearance of minimal turbidity. An inverse linear correlation appears between the steady-state growth rates and the extent to which pheH$_2$ replaces peptide phe in cell protein (Fig. 2). Moreover, the line extrapolates to the non-steady-state limit of phe replacement at the ordinate. Thus the interpretation is made that toxicity during non-steady-state administration is basically no different from steady-state toxicity, and that

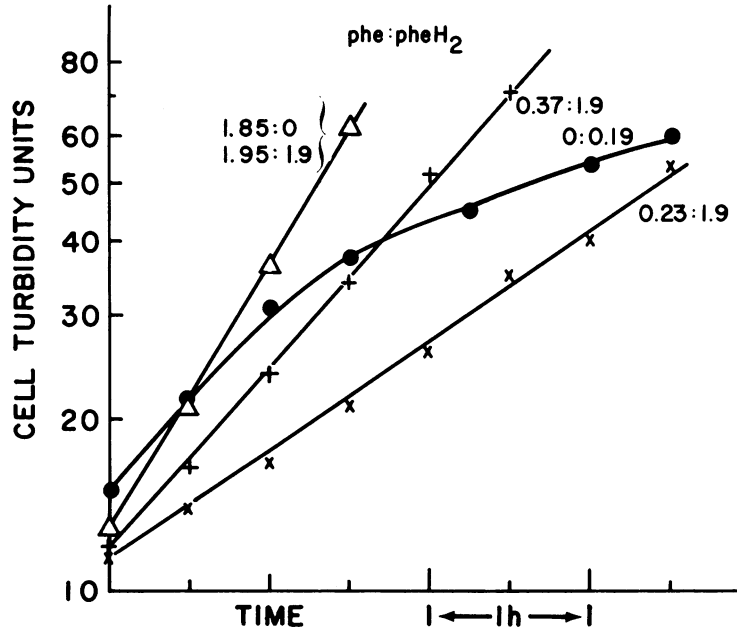

FIG. 1. *Growth of E. coli in mixtures of phe and pheH$_2$. Indicated on each curve is the concentration ratio mM phe:mM pheH$_2$ calculated at mid-growth. Minor corrections are made for phe contaminant in pheH$_2$ and for growth consumption of phe. In curve (●) contaminating phe was first removed by growing a small washed inoculum until growth stopped at 20 turbidity units. The medium was filtered and supplemented with fresh phe-deficient medium plus washed inoculum, and cell growth was followed immediately. The remaining curves were measured after cell growth had increased approximately 30-fold.*

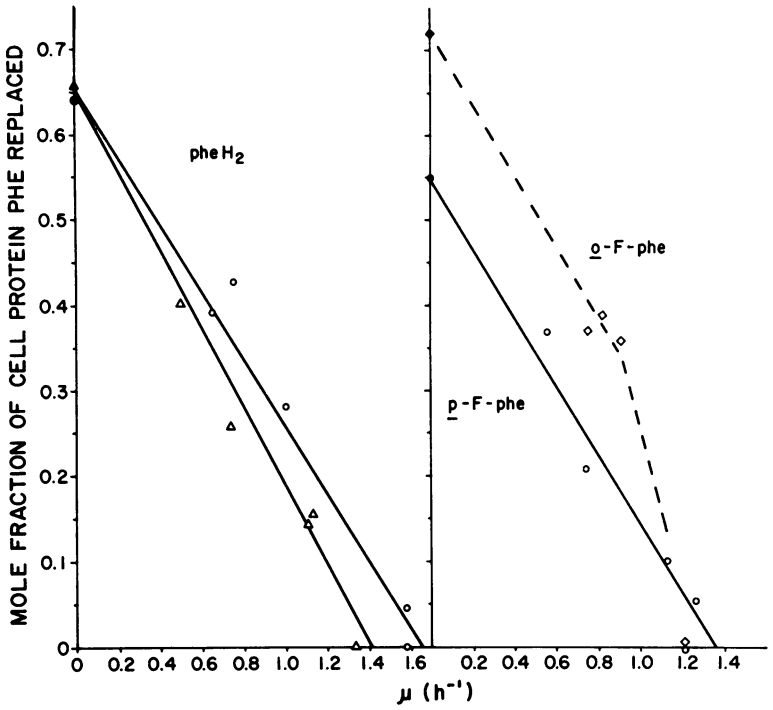

FIG. 2. *Steady-state growth rate of E. coli (μm = 1/doubling time) versus extent of analogue replacement of phenylalanine in cell protein: (A) (O and Δ) pheH₂ replacements in steady-state cultures shown in Fig. 1 and from an additional experiment; (B) (O) p-fluorophenylalanine and (◇) o-fluorophenylalanine replacement. Filled symbols on ordinates are maximal replacements after growth in pure analogue. Values on abscissas are growth rates in phe without analogue. All analogues were 2 mM (as L form) and phe varied from 0.05 to 2 mM.*

during non-steady-state toxicity growth slows down to an extent predictable from intermediate steady-state levels of incorporation until the growth eventually ceases.

$pheH_2$ was a more effective antagonist of phe than DL-*o*- or *p*-fluorophenylalanine. A threefold greater L-analogue-phe ratio of 30:1 was required for 50% growth inhibition when the latter analogues were supplied at 0.2 mM (as the L form). These and additional steady-state growth rates obtained by varying the phe level show varying linearity when plotted versus replacement of peptide phe, and the analogues resemble $pheH_2$ closely in their maximal or limiting replacement of peptide phe (Fig. 2). However, the relationship between partial peptide replacement by *o*-fluorophenylalanine and growth rate may more properly be described by a convex line (Fig. 2). Additional studies were made of L-norleucine as a substitute for methionine and of DL-3,4-dehydroproline and L-azetidine-2-carboxylate as substitutes for proline. The depressed growth rates in methionine antagonism appeared steady. In proline antagonism, however, growth tended either to recover to normal after a lag or to become increasingly depressed and to level off. Thus the antagonism between certain amino acids and their analogue may not lend itself to the present analytical approach.

Effects of $pheH_2$ incorporation on S-180 cells. During a comparatively short exposure of 2 h in $pheH_2$-phe mixtures, growth as judged from [^{14}C]valine incorporation remains at least at 75 to 80% of normal at the highest ratios, while incorporation of [^{3}H]phe is selectively depressed as much as 88% (Fig. 3). $pheH_2$ evidently can almost totally substitute for phe initially at nearly normal growth rates. Replacement of phe, selected at 45% of the maximum [^{3}H]phe incorporation, occurs at virtually the same $pheH_2$-phe ratio, 2.0:1, 2.2:1 and 2.3:1, at 0.03, 0.09 and 0.3 mM phe concentrations, respectively. $pheH_2$ is therefore competitive with phe over a 10-fold concentration range which is adequate for maximum growth.

Exposure to $pheH_2$-phe mixtures for 4 days gives complex growth kinetics. The reciprocals of growth rate versus phe concentration are plotted in Fig. 4. At comparatively low

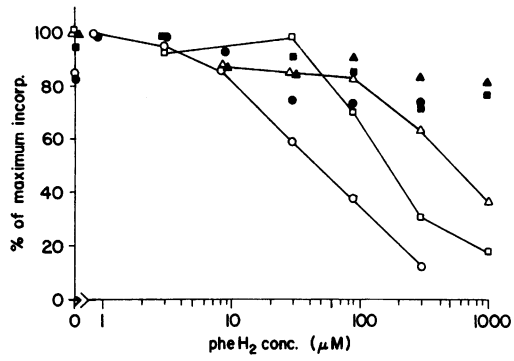

FIG. 3. *Short-term (2 h) interaction between pheH₂ and phe for amino acid incorporation by S-180 cell suspensions. Open symbols: [³H]phe incorporation. Filled symbols: [¹⁴C]valine incorporation. phe levels: (○, ●), 0.03 mM; (□ ■), 0.09 mM; (△, ▲) 0.3 mM.*

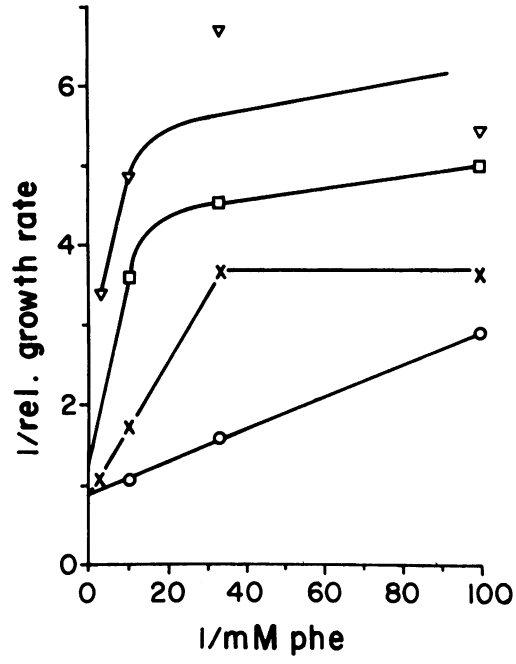

FIG. 4. *Competitive interaction between pheH₂ and phe for 4-day growth of S-180 cells. Ordinate scale: 1/log (final cell protein concentration/initial cell protein concentration). Cell protein ranges: 30.5 μg/ml (initial) to 331 μg/ml (final) without pheH₂. pheH₂ levels: (○), 0.01 mM; (×), 0.03 mM; (□), 0.1 mM; (▽), 0.3 mM.*

$pheH_2$-phe ratios the curves are linear and tend to converge at the ordinate, suggesting competitive interaction between $pheH_2$ and phe expressed by balanced changes in growth rate. Thus the $pheH_2$-phe ratios for 50% inhibition of growth are fairly constant at 0.23, 0.19, and 0.18 at 0.03, 0.1, and 0.3 mM phe, respectively. At higher $pheH_2$-phe ratios, growth is not proportionately suppressed. As corroborated by the leveling of the curves, cell protein still increases by a minimum of 50%, reflecting unbalanced incorporation of $pheH_2$ until zero growth rate is reached. When 0.3 mM $pheH_2$ was substituted completely for phe in S-180 cells at one-third confluence, they became rounded and ready to detach from the flask surface after 24 h. $pheH_2$ had replaced 33% of the peptide phe, in accordance with the 50% increase in protein just noted. The cells were still viable as judged by exclusion of trypan blue and by resumption of normal morphology and reinitiation of growth when phe was restored. In a mixture of 0.3 mM phe and 0.06 mM $pheH_2$, which would give a half-maximal growth rate, $pheH_2$ replaced 7% of peptide phe after a 10-fold cell increase and 5% after a repeated subculture. These peptide replacement levels are decidely less than half the maximum of 33% replacement obtained with incorporation of pure $pheH_2$. Thus, cell biosynthesis in S-180 is more susceptible to steady-state than to non-steady-state administration of $pheH_2$.

DISCUSSION

$pheH_2$ replaces phe virtually completely at normal or nearly normal growth rates in *E. coli phe* and S-180 cells. *E. coli* growth increased maximally threefold, as evidenced by a threefold increase in viable cell number and replacement of two-thirds of protein phe. Upon the latter criterion, $pheH_2$ resembles *o*- and *p*-fluorophenylalanine, and would thus appear exemplary of those amino acid analogues that approximate the molecular structure of the parent molecule sufficiently to duplicate it adequately at almost all peptide sites where it has a more than neutral role (4). In contrast, incorporation of a less accommodating amino acid analogue such as canavanine produces a comparatively abrupt cessation of protein biosynthesis and rapid lethality (9). The combined affinity of $pheH_2$ for cellular uptake and ligation to transfer ribonucleic acid is still high, at about half that of phe, in S-180. This is concluded from the 50% replacement of peptide phe after 2 h of incorporation at a 2:1 $pheH_2$-phe ratio, and 5% replacement after a 100-fold increase in cell number at a 0.2:1 $pheH_2$-phe ratio. In *E. coli* the corresponding $pheH_2$-phe ratio for 50% growth inhibition is 10:1. A low steady-state growth rate can still be maintained in *E. coli* when $pheH_2$ has replaced nearly half of the peptide phe of the cell. In this respect $pheH_2$ is not notably different from *o*- and *p*-fluorophenylalanine. Extensive analogue re-

placement of phe must give rise to a variety of biochemical defects. However, the high capacity for replacement at steady growth rates and the linear response of the growth rates to $pheH_2$ and *p*-fluorophenylalanine replacements suggest that growth is limited by one salient protein defect more than any other, possibly at a single critical peptide site. If multiple defects, either in several proteins or in several peptide sites in the same protein were needed to inhibit growth, they would be expected to interact cooperatively and produce a convex curve of growth response, perhaps as illustrated by *o*-fluorophenylalanine (Fig. 2). Even in this latter example cooperativity does not appear particularly striking. Moreover, the maximum peptide replacement by $pheH_2$ of *p*-fluorophenylalanine when supplied alone is the same as the replacement limit arrived at by extrapolating partial replacements in steady-state growth to the zero growth rate. Thus, growth is limited finally by the same net analogue replacement regardless whether the analogue is randomly distributed in the target site or restricted to some molecules of it. If multiple peptide defects were needed to affect the protein target, the growth inhibition should be more apparent in the latter, nonrandomized condition where cooperative intramolecular interactions would be more probable. None of these arguments can be more than suggestive, but they provide a predictive framework for examining additional physiological effects of these analogues. Thus, it might be anticipated that different congeners of an amino acid can have distinctive growth-limiting effects in spite of their potential for multiple nonspecific protein perturbation. *E. coli* would appear a particularly useful organism for study because of its high level of accommodation to $pheH_2$ substitution and the apparent simplicity of its growth kinetics. More complex kinetics are indicated in other systems such as *o*-fluorophenylalanine incorporation in *E. coli* where toxic interactions may be cooperative, and in $pheH_2$ incorporation in the S-180 cell, where cellular organization and structure may be too complex to allow steady-state analogue incorporation at levels that can be attained during acute administration. There are presently four known phe analogues: $pheH_2$, *o*- and *p*-fluorophenylalanine and β-thienylalanine which can substitute effectively for phe and allow significant cell growth. This would permit close and meaningful comparison of the effects of minor to moderate modifications of the phenyl moiety upon different enzyme and cell functions.

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