Studies on the Dual Requirement for Phenylalanine and Tyrosine in Tissue Cultures

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Studies in several laboratories (1-4) have established that isolated tissues growing or surviving in vitro require both phenylalanine and tyrosine. This finding is in marked contrast to the well-known sparing effect of tyrosine on the phenylalanine requirement of the whole animal (5-7). In the latter case, this sparing effect has been attributed (8) to the action of "phenylalanine hydroxylase," an enzyme system consisting of two parts, Fraction I, found only in the liver, and Fraction II, found in most tissues studied (9). The dual requirement for phenylalanine and tyrosine by tissue cultures is in accord with the observations on phenylalanine hydroxylase distribution. It is also possible, however, that isolated tissue cells may resemble certain microorganisms (10) in which these two amino acids arise by separate metabolic pathways from a common precursor.

The survival of chick embryonic heart tissues cultivated in vitro in completely synthetic media has proved a sensitive tool for the study of amino acid interrelationships (11, 12). Accordingly, a study of the phenylalanine-tyrosine requirement in this system was undertaken to clarify the relationship between these two amino acids. The present communication also reports data on the stereospecificity of the amino acid requirement and the effect of two antagonists, β-2-thienylalanine and p-fluorophenylalanine. The results indicate that these two antagonists inhibit different reactions, and some of the characteristics which distinguish these reactions are discussed.

EXPERIMENTAL

Methods for preparation of chick heart cultures and depletion of their intracellular reserves have been described previously (3, 11, 13). The standard control medium was M 150 (14, 15) which contains 50.0 mg per liter of D,L-phenylalanine and 40.0 mg per liter of L-tyrosine. For individual experiments, various modifications of this medium were prepared as described in Table I. The amino acids used in these studies were checked for purity by paper chromatography. After the initial 3-day depletion period (11) in balanced salt solution, the synthetic media were added and were subsequently removed and replaced twice each week until death of the cultures. The experimental design and the method of evaluating the cultures have been described in detail in previous publications (3, 11, 13). The significance of differences in survival times was calculated by the alternate t test, where required.

RESULTS

Phenylalanine Requirement of Chick Embryonic Heart Fibroblasts—Two basic synthetic media were prepared, one containing no phenylalanine and the second containing neither phenylalanine nor tyrosine (M 739 and M 1029, Table I). To these media, graded concentrations of L- or D-phenylalanine were added and the effect on culture survival determined. The results of these experiments are summarized in Fig. 1. It is evident (Curve A) that a uniform response curve to L-phenylalanine is obtained when the culture medium contains tyrosine. When L-tyrosine is not present (Curve B), the response is less marked at lower phenylalanine levels but increases sharply at high concentrations. No response to D-phenylalanine was obtained either in the absence or presence of L-tyrosine (Curve C).

Tyrosine Requirement of Chick Embryonic Heart Fibroblasts—Two basic synthetic media were prepared, one without tyrosine and the second without tyrosine or phenylalanine (M 665 and M 1029, Table I). To these media, graded levels of L- or D-tyrosine were added and the effect on culture survival determined, as summarized in Fig. 2. It is apparent (Curve A) that, when the culture medium contains phenylalanine, graded levels of L-tyrosine cause a marked increase in culture survival. When phenylalanine is absent (Curve C), graded levels of L-tyrosine do not increase culture survival but cause a sharp toxicity. In the presence of phenylalanine (Curve D), the culture response to D-tyrosine is appreciably less than that effected by the L-isomer.

Inhibition by β-2-Thienylalanine and Its Reversal—The activity of β-2-thienylalanine as a phenylalanine analogue has been studied in the rat (16) and in bacteria (17) and it was considered of interest, therefore, to test its behavior in tissue cultures. Graded levels of this analogue were added to complete medium M 150 and to media deficient in either phenylalanine, tyrosine, or both (Table I). The effects on culture survival were then determined (Fig. 3). Low levels of thienylalanine, in the presence of both phenylalanine and tyrosine, caused a marked increase in culture survival (Curve A). This increase was followed by a progressive toxicity at higher analogue levels. In the absence of either tyrosine (Curve B) or phenylalanine (Curve C), the same pattern of stimulation at low levels and inhibition at high was found. In the absence of both phenylalanine and tyrosine (Curve D), no stimulation was observed, but toxicity was found at high concentrations of analogue.

To study the possible reversal of this toxicity, a synthetic medium was prepared (M 1502, Table I), containing neither phenylalanine nor tyrosine, but with the incorporation of 10.0 mg per liter of β-2-thienylalanine. To this toxic medium were added graded levels of L- or D-phenylalanine, L-tyrosine, or L-tyrosine in the presence of 0.1 mg per liter of phenylalanine. The effects of these media on culture survival are summarized in Fig. 4. It is evident that L-phenylalanine (Curve A) effects complete reversal of the toxicity, but that D-phenylalanine (Curve B) has no effect.

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is completely ineffective. L-Tyrosine (Curve B) shows only a moderate reversal activity at the highest level tested (100.0 mg per liter). When L-tyrosine is tested in the presence of 0.1 mg per liter of phenylalanine (Curve D) a slight reversal is observed at moderate tyrosine levels and no effect at higher concentrations.

Inhibition by p-Fluorophenylalanine and Its Reversal—This compound is known to be a competitive inhibitor of phenylalanine in Escherichia coli (18) and, accordingly, its behavior in the tissue culture system was investigated, with experimental media similar to those employed previously with β-2-thienylalanine (Table I). The effects on culture survival were then determined and are summarized in Fig. 5. In the presence of both phenylalanine and tyrosine (Curve A), low levels of fluorophenylalanine caused a moderate increase in tissue survival. This increase was followed by a marked toxicity at higher concentrations. In medium containing phenylalanine but no tyrosine (Curve B), the same pattern of stimulation at low levels and toxicity at high was found. In the presence of tyrosine and absence of phenylalanine (Curve C), no stimulation at low levels of fluorophenylalanine was found but, rather, a marked and progressive toxicity. In medium containing neither phenylalanine nor tyrosine (Curve D), the toxicity of fluorophenylalanine was not quite as marked and a slight stimulation was noted at the lowest level tested.

The possible reversal of fluorophenylalanine toxicity was studied by means of a series of media similar to those used previously with β-2-thienylalanine, except that the basic medium

### Table I

<table>
<thead>
<tr>
<th>Medium No.</th>
<th>Amino acid content</th>
</tr>
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<tbody>
<tr>
<td>M 150</td>
<td>Complete basal medium (contained 50.0 mg/l DL-phenylalanine and 40.0 mg/l L-tyrosine)</td>
</tr>
<tr>
<td>M 665</td>
<td>L-Tyrosine omitted</td>
</tr>
<tr>
<td>M 739</td>
<td>DL-Phenylalanine omitted</td>
</tr>
<tr>
<td>M 1029</td>
<td>Both DL-phenylalanine and L-tyrosine omitted</td>
</tr>
<tr>
<td>M 1502</td>
<td>M 1029 plus 10.0 mg/l β-2-thienylalanine</td>
</tr>
<tr>
<td>M 1718</td>
<td>M 1029 plus 1.0 mg/l p-fluorophenylalanine</td>
</tr>
</tbody>
</table>

* All other ingredients of these media were identical with the formula of M 150 (14, 15), which contained a complete supplement of essential and nonessential amino acids, vitamins, purines and pyrimidines, certain accessory growth factors, and inorganic ions. No serum or other uncharacterized substances were added to the synthetic medium at any stage in the experiments.

![Fig. 1](image1.png)  
**Fig. 1.** Response of chick embryonic heart cultures in deficient medium to graded levels of L-phenylalanine in presence of tyrosine (Curve A), L-phenylalanine in the absence of tyrosine (Curve B), and p-phenylalanine (Curve C).

![Fig. 2](image2.png)  
**Fig. 2.** Response of chick embryonic heart cultures in deficient medium to graded levels of L-tyrosine in the presence of phenylalanine (Curve A), DL-tyrosine in the presence of phenylalanine (Curve B), and L-tyrosine in the absence of phenylalanine (Curve C).

![Fig. 3](image3.png)  
**Fig. 3.** Effect of graded levels of β-2-thienylalanine on survival of chick heart tissues cultivated in complete synthetic medium (Curve A), in medium deficient in tyrosine (Curve B), in medium deficient in phenylalanine (Curve C), and in medium deficient in both phenylalanine and tyrosine (Curve D).

![Fig. 4](image4.png)  
**Fig. 4.** Effect of graded levels of L-phenylalanine (Curve A), L-tyrosine (Curve B), p-phenylalanine (Curve C), and L-tyrosine in the presence of suboptimal phenylalanine (Curve D) on survival of chick heart tissues cultivated in a medium containing a toxic level of β-2-thienylalanine.
These observations are in general accord with previous findings, employing completely synthetic media, that a tissue response to L-phenylalanine occurs, whether or not tyrosine is present, but that the magnitude of the response is greater in the presence of tyrosine. n-Rhenylalanine, or not n-tyrosine is present, but that the magnitude of the response is distinctly different. In the presence of phenylalanine, graded levels of L-tyrosine produce directly correlated survival time but, in the absence of phenylalanine, a progressive toxicity is exerted by increasing amounts of tyrosine. This toxicity does not appear to have been detected previously in other systems and serves to emphasize the value of cell cultures for uncovering new metabolic interrelationships. These data also support the concept that phenylalanine and tyrosine are not interconverted in isolated tissues, a concept in agreement with the observation by Eagle et al. (21) that C¹⁴-labeled phenylalanine and tyrosine are incorporated independently into the protein of cell cultures.

The observed toxicity of tyrosine in the absence of phenylalanine suggests a metabolic imbalance involving these amino acids comparable to that discussed by Cohen (22) in other systems.

The effect of the amino acid analogues, β-2-thienylalanine and p-fluorophenylalanine, was studied in the tissue culture system to determine whether their effects could be related to those reported previously with animals (16) and microorganisms (17). It was found that high concentrations of either β-2-thienylalanine or p-fluorophenylalanine markedly decreased tissue survival. On the other hand, both inhibitors caused a moderate increase in survival when incorporated in the medium at low concentrations. With β-2-thienylalanine, this stimulation was obtained when phenylalanine, tyrosine, or both were present in the medium but not when both were absent. With p-fluorophenylalanine, stimulation was found with all combinations of phenylalanine and tyrosine except when tyrosine was present and phenylalanine absent. To the best of the authors’ knowledge, the present observation appears to be the first report of stimulation by amino acid analogues in tissue culture, although stimulation by β-2-thienylalanine has been reported with E. coli (23).

Reversal studies have shown that the toxicity of β-2-thienylalanine can be reversed completely by L-phenylalanine, but that the d-isomer is completely ineffective. A moderate, but significant, degree of reversal was also effected by high concentrations of L-tyrosine. This observation is in contrast to the report by Ferger and du Vigneaud (16) that phenylalanine, but not tyrosine, reversed the inhibitory effect of this analogue in rats. Studies with p-fluorophenylalanine have shown that the toxicity of this compound can be reversed completely by L-phenylalanine and that L-tyrosine is completely inactive. It was also observed that a considerable degree of reversal was effected by d-phenylalanine. With E. coli (24), it has been found that p-fluorophenylalanine does not inhibit enzyme formation but is incorporated into the enzyme protein. In rat liver and kidney slices, this analogue has been shown to inhibit protein breakdown, as well as protein synthesis under some conditions (25). In most systems, therefore, p-fluorophenylalanine appears to function as a general inhibitor of amino acid utilization and the present findings are in agreement with this concept. The observation that tyrosine, by itself, is moderately effective in reversing the inhibition due to β-2-thienylalanine, but not that due to p-fluorophenylalanine, suggests that these antagonists may interfere with different systems.

A difficult factor to eliminate in the present experiments has been the possibility of cross-contamination in the samples of phenylalanine and tyrosine. The chromatographic methods in 

**Discussion**

The dual requirement of tissue cultures for phenylalanine and tyrosine affords a test system for studying the metabolic functions of these two amino acids under conditions free from the complex interconversions that occur in the intact animal. The present studies, employing completely synthetic media, have shown that a tissue response to L-phenylalanine occurs, whether or not L-tyrosine is present, but that the magnitude of the response is greater in the presence of tyrosine. D-Phenylalanine, on the other hand, is completely inert, whether or not tyrosine is present. These observations are in general accord with previous growth studies on phenylalanine in the rat (8), chick (19, 20), and human (5). With L-tyrosine, however, the pattern of tissue response is distinctly different. In the absence of phenylalanine, graded levels of L-tyrosine produce a directly correlated survival time, but, in the absence of phenylalanine, a progressive toxicity is exerted by increasing amounts of tyrosine. This toxicity does not appear to have been detected previously in other systems and serves to emphasize the value of cell cultures for uncovering new metabolic interrelationships. These data also support the concept that phenylalanine and tyrosine are not interconverted in isolated tissues, a concept in agreement with the observation by Eagle et al. (21) that C¹⁴-labeled phenylalanine and tyrosine are incorporated independently into the protein of cell cultures.

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A difficult factor to eliminate in the present experiments has been the possibility of cross-contamination in the samples of phenylalanine and tyrosine. The chromatographic methods in
use in this laboratory (26) employ solvent systems which provide a marked separation of phenylalanine and tyrosine. Application of a specific color reaction for phenylalanine (27, 28) and the use of the Pauly reaction for tyrosine, in conjunction with these solvent systems, failed to demonstrate cross-contamination of either phenylalanine or tyrosine. On the other hand, minor contamination of \( n \)-phenylalanine with \( L \)-phenylalanine could not be eliminated by the methods available. Such a minor contamination (5% or less) might have been responsible for the partial reversal of the toxicity of \( p \)-fluorophenylalanine by \( n \)-phenylalanine. This possibility is rendered somewhat unlikely, however, by the complete inability of high levels of \( n \)-phenylalanine to reverse the toxicity of \( \beta \)-2-thienylalanine or to support the survival of the tissue cultures in the absence of inhibitor.

The present experiments have shown that chick embryonic heart cultures possess an absolute requirement for \( L \)-phenylalanine but that the requirement for \( L \)-tyrosine can be demonstrated only in the presence of phenylalanine. This relationship is somewhat analogous to the previous demonstration (29) that chick heart cultures have an absolute requirement for \( L \)-cystine and only a supplementary requirement for \( L \)- or \( \beta \)-methionine. Although the interrelationships in these two groups of amino acids differ in some details, the findings serve to illustrate the importance of cell cultivation methods in evaluating supplementary nutritional factors.

**SUMMARY**

1. The dual requirement of tissue cultures for phenylalanine and tyrosine was studied in chick embryonic heart fibroblasts cultivated in vitro in completely synthetic media.

2. Average survival time was found to be an approximately direct function of \( L \)-phenylalanine concentration, whether or not \( L \)-tyrosine was present in the culture medium, but \( n \)-phenylalanine was completely inactive.

3. A response to \( L \)-tyrosine could be obtained only if phenylalanine was also present in the medium. In the absence of phenylalanine, increasing amounts of \( L \)-tyrosine caused a progressive toxicity.

4. Two analogues, \( \beta \)-2-thienylalanine and \( p \)-fluorophenylalanine, were both found to markedly inhibit tissue survival. Low concentrations of either of these antagonists caused moderate increase in culture survival.

5. The toxicity of \( \beta \)-2-thienylalanine was reversed completely by \( L \)-phenylalanine but not by the \( n \)-isomer; moderate reversal was effected by \( L \)-tyrosine. The toxicity of \( p \)-fluorophenylalanine was reversed completely by \( L \)-phenylalanine and partially by \( n \)-phenylalanine, while \( L \)-tyrosine was completely ineffective.

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**REFERENCES**

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