The Arginine Requirement of Tissue Cultures

I. INTERRELATIONSHIPS BETWEEN ARGININE AND RELATED COMPOUNDS

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(Received for publication, May 8, 1958)

The recent development of synthetic (1, 2) and semisynthetic (3) media that support the survival and propagation of mammalian tissue cultures has made it possible to determine the specific amino acid requirements of many cell lines. A remarkably similar pattern of essential and nonessential amino acids has been found for chick embryonic heart fibroblasts (4), Strain L cells (3), HeLa cells (5), Walker carcinosarcoma No. 256 (6), rabbit fibroblasts (7), and human and malignant cells (8). With nearly all cell types, arginine, histidine, cystine or cysteine, methionine, leucine, isoleucine, lysine, phenylalanine, tyrosine, threonine, tryptophan, and valine were required. A consistent finding in all these investigations has been that arginine is required for the survival and propagation of all tissue cultures so far tested.

Recent studies on the metabolism of amino acids and related compounds in rats (9-11) have emphasized the key role of arginine in preventing the toxicity resulting either from excessive quantities of essential amino acids or from ammonia. This importance of arginine under conditions in vivo prompted a detailed study of its metabolic function in tissue cultures. The results reported in the present communication confirm in a quantitative manner the previous observation that arginine is essential for the survival of chick embryonic heart fibroblasts (4) and show that arginine can be replaced by high levels of citrulline but not by ornithine. Inhibition by canavanine has been demonstrated and shown to be reversed specifically by arginine, and the relationship of urea and ureidosuccinic acid to the arginine requirement investigated.

MATERIALS AND METHODS

Tissue cultures were prepared from the heart muscle of 11-day-old chick embryos by the method described previously (4, 12). The tissues were cultivated in standard Pyrex test tubes in completely synthetic media (1, 2) without the addition of plasma, serum, or other uncharacterized substances. Each culture contained approximately 1 mg. of tissue, wet weight, as determined by total protein measurement (13). The standard control medium was M150 (2), a modification of Morgan's 199 (1), which contains 70 mg. per liter of L-arginine hydrochloride in addition to a complete supplement of essential and nonessential amino acids. Various other synthetic media were prepared by the omission of certain amino acids from the basic formula of M150 (1, 2). The composition of these media is described in Table I. All media were freshly prepared at frequent intervals and sterilized by passage through ultrafine filtered glass filters. The arginine, citrulline, and ornithine used in these studies were tested on paper chromatograms and shown to be chromato graphically pure.

Throughout these experiments, the nutritional depletion technique (14) was used, since this procedure has been shown to intensify the tissue culture response to essential growth factors. Following the initial 3 day period 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 (4, 12, 14). The significance of differences in survival times was calculated by the alternate \( t \) test, where required.

Arginine was determined by the Sakaguchi reaction, as modified by Weber (15). Citrulline and urea were measured by the method of Kawerau (16). Ornithine was determined by the method of Chirnaw (17). In addition to these determinations, paper chromatographic studies on the used culture media were made (18, 19).

RESULTS

Arginine Requirement of Chick Embryonic Heart Fibroblasts—A basic synthetic medium was prepared, containing no arginine (M629, Table I), and to this medium were added graded levels of arginine, citrulline, or ornithine. The results of these experiments are summarized in Fig. 1. It is evident (Curve A) that a uniform response curve is obtained with increasing concentrations of arginine. Graded levels of citrulline (Curve B) show no effect on culture survival until a concentration of 10 mg. per liter is reached. Above this figure, a marked increase is shown and at 1000 mg. per liter the culture survival is equal to that effected by an equivalent amount of arginine. With ornithine (Curve C), no effect on culture survival was found at any concentration.

Metabolism of Arginine, Citrulline, and Ornithine by Chick Heart Fibroblasts—The used media were aspirated from the cultures during the twice-weekly fluid renewals and arginine, citrulline, and ornithine determined by colorimetric chemical methods (15-17). A rapid decrease in the arginine content of the medium was found. At the period of greatest culture activity (18), this loss totaled 85 to 90 per cent of the initial concentration. When citrulline, rather than arginine, was present in the medium, it also showed a marked decrease, totaling 80 per cent at the maximum period. Under comparable conditions, little, if any, decrease in the ornithine content of the medium could be detected. Parallel chromatographic studies on these media showed that decrease of arginine was not accom-
panied by formation of citrulline or ornithine. These results suggest that disappearance of arginine from the culture medium represents an actual uptake by the cells and cannot be attributed to degradation resulting from arginase activity.

Inhibition by Canavanine and Its Reversal—Since canavanine has been shown to be a competitive antagonist for arginine in other systems (20), its behavior in tissue cultures was investigated. Graded levels of this analogue were added to synthetic medium M150, containing arginine, and to M629, containing no arginine, and the effect on culture survival determined (Fig. 2). When the culture medium contains arginine (Curve A), a progressive inhibition of culture survival is obtained, even at low concentrations of canavanine. This inhibition becomes very sharp at canavanine levels greater than 10 mg. per liter. When arginine is not present in the culture medium (Curve B), no inhibition is detected until the canavanine concentration exceeds 1 mg. per liter. Higher concentrations produce a progressively greater inhibition.

A synthetic medium was prepared (M1383, Table I) containing a toxic concentration of canavanine (10 mg. per liter). To this medium were added graded levels of arginine, citrulline, or ornithine and the effect on culture survival was determined (Fig. 3). It is evident that the toxicity of canavanine is completely reversed by arginine (Curve A), but that neither citrulline nor ornithine showed any reversal activity (Curves B and C).

Effect of Urea on Survival of Chick Heart Cultures—In view of the demonstration that urea may have a positive role in arginine synthesis under suboptimal conditions (21), its effect on the survival of chick embryonic tissue was investigated. Graded levels of urea were added to the complete medium M150, to a medium deficient in arginine, and to a medium containing a toxic level of canavanine (Table I). The effect of these various media on culture survival is summarized in Fig. 4.

The addition of urea to the complete medium (Curve A) causes a moderate increase in culture survival, with optimal activity exerted at a concentration of 100 mg. per liter. When arginine is omitted from the medium (Curve B), urea again causes a moderate increase in culture survival. In this case, however, the optimal activity is exerted at a much lower concentration (0.01 mg. per liter). In the presence of a toxic level of canavanine (Curve C), urea, at any concentration, is completely unable to effect reversal of the toxicity.

Effect of Ureidosuccinic Acid (Carbamyl Aspartate) on Survival of Chick Heart Cultures—The possible relationship between the cell requirement for arginine and pyrimidine biosynthesis was investigated through studies on the effect of ureidosuccinic acid. The results of these experiments are presented in Fig. 5. The incorporation of graded amounts of this compound in a synthetic medium deficient in arginine (Curve A) caused a moderate increase in tissue culture survival. The most effective level was found to be 0.01 mg. per liter. When the synthetic medium contained a low level of arginine (Curve B), a moderate response to ureidosuccinic acid was again obtained, with maximum effect at the same concentration. In the presence of citrulline (Curve C), the response to ureidosuccinic acid is very slight and requires a higher concentration (0.1 mg. per liter). When the synthetic medium contains ornithine (Curve D), ureidosuccinic acid does not prolong culture survival. Ureidosuccinic acid also proved completely unable to reverse the toxicity of canavanine (Curve E).

Since the synthesis of arginine from guanidoacetic acid has been shown to occur in rats (22), the behavior of this compound was investigated in the present test system. No replacement of arginine could be demonstrated at any concentration studied.

### Table I

**Amino acid composition of basic synthetic media used**

<table>
<thead>
<tr>
<th>Medium No.</th>
<th>Amino acid content*</th>
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<tbody>
<tr>
<td>M150</td>
<td>Complete basal medium. Contains 70.0 mg. per liter L-arginine HCl</td>
</tr>
<tr>
<td>M629</td>
<td>M150 with arginine omitted</td>
</tr>
<tr>
<td>M1558</td>
<td>M629 plus 0.1 mg. per liter L-arginine</td>
</tr>
<tr>
<td>M1551</td>
<td>M629 plus 1 mg. per liter L-citrulline</td>
</tr>
<tr>
<td>M1557</td>
<td>M629 plus 100 mg. per liter L-ornithine</td>
</tr>
<tr>
<td>M1583</td>
<td>M150 with arginine omitted and 10 mg. per liter canavanine added</td>
</tr>
</tbody>
</table>

* All other ingredients of these media were identical with the formula of M150 (1, 2), which contains a complete supplement of essential and nonessential amino acids, vitamins, purines and pyrimidines, certain accessory growth factors, and inorganic ions.
Arginine Requirement of Tissue Cultures. I

DISCUSSION

Arginine has been established as an essential amino acid for the survival and propagation of many mammalian tissues cultivated in vitro (3-8) and has recently been shown (23) to maintain active rates of cellular proliferation without renewal of medium. Despite these observations, the exact metabolic function of arginine in such systems has not been elucidated. The present studies have shown that chick embryonic heart cultures require arginine for maximal survival in a suboptimal synthetic medium and that the arginine requirement can be replaced by high levels of citrulline but not by ornithine. It should be noted that these observations were made in a synthetic medium which contains a complete supplement of both essential and nonessential amino acids, including aspartic acid, glutamic acid, and glutamine, as well as adenosine triphosphate, magnesium ions, and ammonium ions. Additional experiments in this laboratory have indicated that the arginine response curve is markedly affected by the concentrations of glutamic acid and/or glutamine in the culture medium. This finding is in agreement with recent studies on amino acid metabolism in rats (9-11) and suggests that further investigation on the metabolism of arginine and related compounds in tissue culture is warranted.

In microbiological systems (20, 24, 25), the toxicity of canavanine has been found to be completely reversible by arginine and, to some extent, by citrulline and ornithine. In the present system, the toxicity of canavanine can be reversed completely by arginine, but citrulline and ornithine are entirely ineffective. These results suggest that canavanine inhibits the conversion of citrulline to arginine by the tissue cultures. This suggestion is in agreement with the results obtained with a kidney transamidinase preparation (26), in which it was demonstrated that canavanine interferes with arginine synthesis from citrulline by competitively inhibiting the synthesis of argininosuccinic acid. In this step of metabolism, therefore, chick embryo heart cultures appear to exhibit the same pattern of activity as mammalian kidney tissue.

Urea has been shown to be utilized as a nitrogen source by rats on limited amino acid diets (21). In the present studies, urea was found to have a beneficial effect in the presence of arginine, to be effective at lower concentrations in the absence of arginine, and to have no effect on the toxicity of canavanine. It appears probable, therefore, that in this test system urea is functioning as a general nitrogen source rather than as a member of the arginine cycle.

Ureidosuccinic acid, which is thought to be an intermediate in the biosynthesis of pyrimidines from arginine (27), was found to enhance culture survival whether or not the medium contained arginine or citrulline, but no effect was observed when the medium contained only ornithine. No effect on the toxicity of canavanine was observed at any concentration. It should be noted that the stimulation by ureidosuccinic acid occurred in a nutrient medium that contained thymine and uracil but not orotic acid or uridylic acid. The relationship of this finding to the comparative value of preformed and biosynthesized pyrimidines in cell nutrition remains to be determined.

The results reported here emphasize the importance of arginine in cell nutrition studies and indicate that only part of the and the compound proved completely unable to reverse the toxicity of canavanine.
Krebs-Henseleit cycle is functioning in chick embryo heart muscle. Since the criterion employed in these experiments has been survival rather than rapid proliferation, the conditions may be considered as somewhat analogous to nitrogen balance studies in man (28). Thus, it would appear that tissue cultures provide a sensitive test system for determining the metabolic interrelationships of specific amino acids.

SUMMARY

1. Arginine has been shown to be essential for the survival of chick embryonic heart fibroblasts cultivated in vitro in completely synthetic media containing a complete supplement of both essential and nonessential amino acids.

2. The arginine requirement of the tissue cultures could be replaced by high concentrations of citrulline but not by ornithine. Urea and ureidosuccinic acid were slightly stimulatory but could not replace the arginine requirement.

3. Canavanine was found to be strongly inhibitory to tissue culture survival. The toxicity of this compound could be completely reversed by arginine, but not by citrulline, ornithine, urea, or ureidosuccinic acid.

4. The results are interpreted as indicating that the arginine cycle is functioning only in part in chick embryonic heart tissue.

Acknowledgment—We wish to acknowledge our indebtedness to Miss D. McKay, Mrs. S. Papineau, Mrs. J. Schryer, and Mrs. M. Thomas, whose technical assistance made the completion of this work possible.

REFERENCES
