THE NUTRITION OF ANIMAL TISSUES CULTIVATED IN VITRO

IV. Amino Acid Requirements of Chick Embryonic Heart Fibroblasts

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Plates 26 and 27

(Received for publication, November 5, 1956)

The chemically defined media devised to support the survival of chick embryonic heart tissues cultivated *in vitro* (1–3) contain a complete supplement of twenty-one amino acids. In the initial experiments leading to the formulation of these media (1), the amino acids were incorporated as a group, at the observed optimal total level of 110 mg. per cent. The proportions were based on analyses of tissue proteins (4), but no studies were conducted to determine which of the individual amino acids were actually essential. The recent development of a nutritional depletion technique (5) that intensifies the tissue culture response to essential growth factors made it possible to determine the specific effect exerted by each individual amino acid in the complex synthetic media. The results reported in the present communication list the essential and non-essential amino acids for the survival of chick embryonic heart cultures and relate these findings to the nutritional patterns reported for other types of tissue cultures and for whole animals (6–11).

Materials and Methods

All cultures were prepared from the heart muscle of 11-day old chick embryos. The tissue was chopped to a fine pulp, and portions of 1 to 2 mg. wet weight (12) were transferred to standard pyrex test tubes and cultivated directly on the glass surface without the use of plasma clots. Depletion of intracellular nutrients was accomplished by cultivating the tissues in Hanks's balanced salt solution (13) for an initial 3 day period previously shown to be effective for this purpose (5). Following this depletion period, the extent of proliferation and health of individual cells were determined by microscopic examination, the salt solution was removed, and the experimental synthetic media were added. The cultures were maintained in the experimental media, with twice-weekly replacement of fluids and frequent microscopic examination, until living cells could no longer be demonstrated. The cultures were kept stationary during the initial 3 day depletion period and thereafter rotated by the conventional roller tube method (14). Cumulative average survival times were calculated for the large groups of cultures in each test medium. The significance of differences in survival times was calculated by the alternate t test. Full details of these culture procedures have been reported previously (2, 3, 5).

The basic synthetic medium used was M 150 (1, 2), the amino acid composition of which is shown in Table I. Deficient media were prepared by omitting individual amino acids from

this formula. In certain experiments, special media were prepared in which the total amino acid concentration of M 150 was altered or L-isomers only were used. All media and solutions were prepared from reagent grade chemicals and sterilized by passage through UF fritted glass filters.

RESULTS

Effect of Omission of Individual Amino Acids on Culture Morphology and Survival

In these studies, each experiment used approximately eighty uniform cultures, prepared from the same tissue pool and divided among five test media plus a control (complete M 150). Each test group contained from ten to thirteen cultures, which were maintained in the experimental media, with twice-weekly

TABLE I

*Amino Acid Composition of Synthetic Medium M 150**

Amino acid	Mg. per liter	Amino acid	Mg. per liter
L-Arginine	70.0	DL-Leucine	120.0
L-Histidine	20.0	DL-Isoleucine	40.0
L-Lysine	70.0	DL-Valine	50.0
L-Tyrosine	40.0	DL-Glutamic acid	150.0
DL-Tryptophan	20.0	DL-Aspartic acid	60.0
DL-Phenylalanine	50.0	DL-Alpha-alanine	50.0
L-Cystine	20.0	L-Proline	40.0
DL-Methionine	30.0	L-Hydroxyproline	10.0
DL-Serine	50.0	L-Glutamine	100.0
DL-Threonine	60.0	Glycine	50.0
L-Cysteine	0.1		

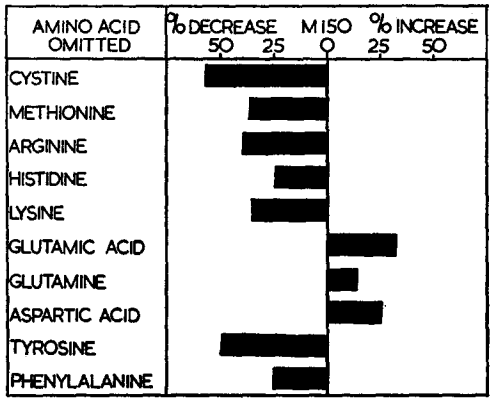
* Total amino acid content, 1100 mg. per liter, made up of 9 L-forms and 11 DL-forms.

fluid replacements, until death. All experiments were repeated four times. Accordingly, each line of Text-figs. 1 and 2 represents an average value based on the survival time of at least forty cultures. These values are expressed as the percentage difference from the control value which itself is based on more than two hundred cultures. Survival values after typical essential, non-essential, and inhibitory amino acids were omitted are listed in Table II.

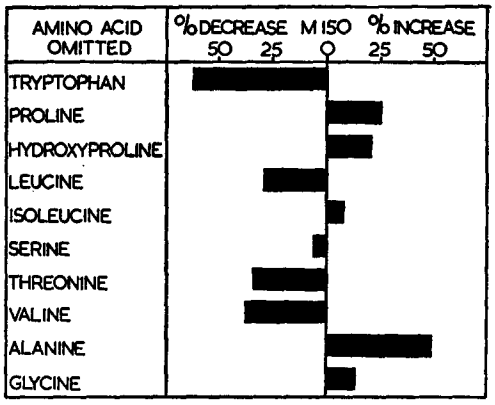
Each of the twenty-one amino acids was omitted in turn from the formula of M 150 and the effect on survival of nutritionally depleted chick embryonic heart cultures determined. Results of these experiments are summarized in Text-figs. 1 and 2. It is apparent that omission of cystine, methionine, arginine, histidine, lysine, tyrosine, phenylalanine, tryptophan, leucine, threonine, or valine has caused a marked reduction in culture survival. Omission of glutamine, serine, isoleucine, or glycine has had no significant effect but the omission

of glutamic acid, aspartic acid, proline, hydroxyproline, or α-alanine has increased culture survival.

The effect of individual amino acid omissions on the morphology of representative chick embryonic heart cultures is illustrated in Figs. 1 to 12. Each of



TEXT-FIG. 1. Effect of omission of individual amino acids from medium M 150 on survival of nutritionally depleted chick embryonic heart cultures. All values calculated as percentage difference from the survival time of control cultures in complete M 150.



TEXT-FIG. 2. Effect of omission of individual amino acids from medium M 150 on survival of nutritionally depleted chick embryonic heart cultures. All values calculated as percentage difference from the survival time of control cultures in complete M 150.

these cultures has been depleted by cultivation in Hanks's solution for 3 days, followed by cultivation for 7 days in M 150 deficient in one amino acid. The control culture in complete M 150 (Fig. 1) shows a population of large, healthy appearing cells with their margins nearly confluent, giving the whole a sheet-like appearance. The outgrowth of cells is from the entire edge of the original

fragment. In contrast, sister cultures in media deficient in tyrosine, arginine, cystine, or valine (Figs. 2, 3, 5, 6) show only a scattered outgrowth of small, thin, irregular and very granular cells. Sheet growth is completely absent in these cultures and the edge of the original fragments appears retracted and possibly dead. The culture in M 150 with glutamic acid omitted (Fig. 4) shows extensive sheet formation, with very large and healthy appearing cells. Omission of methionine, leucine, tryptophan, or lysine (Figs. 8, 9, 11, 12) results in the outgrowth of only small, scattered, and granular cells, with no sheet formation and a dark granularity in the original fragments. Omission of α-alanine or

TABLE II

Effect of Omission from Medium M 150 of Representative Essential, Non-Essential, and Inhibitory Amino Acids

Medium No.	Amino acid omitted	Average survival*
		days
M 150	Complete medium M 150 control	32.2 ± 10.9
M 665	L-Tyrosine	15.6 ± 8.1
M 666	DL-Tryptophan	12.6 ± 6.2
M 763	L-Cystine	12.8 ± 3.8
M 741	DL-Serine	31.5 ± 11.2‡
M 744	DL-Isoleucine	34.5 ± 12.6‡
M 746	DL-Glutamic acid	42.1 ± 14.0
M 759	DL-Alanine	48.3 ± 10.9

* All experimental values (average survivals and standard deviations) calculated from approximately forty cultures. Control value calculated from approximately two hundred cultures.

‡ Differences from control value not significant statistically.

isoleucine (Figs. 7 and 10), still permits excellent sheet formation with large healthy cells. These two cultures compare favorably with the control culture in complete M 150 (Fig. 1) at 7 days.

Effect of Total Amino Acid Level and Optical Configuration on Survival of Chick Embryonic Heart Cultures

Six variations of medium M 150 were prepared: one contained the usual proportion of 9 L- and 11 DL-amino acids, but the concentration of each was 100 mg. per liter; a second variation contained a similar mixture but at an individual concentration of 50 mg. per liter; a third medium was composed of entirely L forms, each at 100 mg. per liter; in the fourth variation, entirely L isomers were used at 50 mg. per liter; a fifth medium contained only L-amino acids, but in the concentrations found in M 150; the sixth medium was M 150 from which glutamic acid, alanine, and proline had been omitted. The effect of

these variations in amino acid concentration and optical configuration on the survival of chick embryonic heart cultures is summarized in Table III.

The only marked alterations in survival time are shown with M 494 and M 894. The presence of high levels of the L- and DL-isomers, as used in M 150 (M 494), has considerably reduced survival time. This decrease was found to be significant by the alternate *t* test. On the other hand, omission of glutamic acid, proline, and α-alanine (M 894) has only slightly increased survival time (see effect of individual omissions, Text-figs. 1 and 2), but the increase is not statistically significant. Since M 496, which contains only L-amino acids, at the same concentrations as the mixed isomers in M 494, supports a normal survival

TABLE III

Effect of Total Amino Acid Concentration and Optical Configuration on Survival of Chick Embryonic Heart Cultures

Medium No.	Amino acid composition	Average survival
		days
M 150	Control medium	35.6 ± 11.7
M 494	M 150 mixture of L- and DL-, 100 mg./1.	25.5 ± 6.4
M 495	M 150 mixture of L- and DL-, 50 mg./1.	37.8 ± 13.4
M 496	Entirely L-forms, each at 100 mg./1.	38.9 ± 15.1
M 497	Entirely L-forms, each at 50 mg./1.	39.5 ± 15.4
M 498	M 150 levels, but entirely L-forms	37.4 ± 14.5
M 894	M 150, no glutamic acid, proline, alanine	41.4 ± 19.7

* All values (average survivals and standard deviations) calculated from large groups of cultures.

time, it would appear that the inhibitory effect of M 494 must be attributed to a toxicity from one or more of the eleven D isomers in the medium. Further experiments are in progress to identify this toxic isomer.

Comparative Amino Acid Requirements of Various Types of Tissue Cultures, of the Chick, and of Man

Determination of the specific amino acid requirements of chick embryonic heart fibroblasts made it of interest to compare these findings with the requirements previously reported by other workers for various types of tissue cultures (6–8, 15, 16), for the growing chick (9, 10), and for man (11). This comparison is presented in Table IV. It is apparent from these data that certain similar patterns of amino acid requirements exist among the various types of tissue cultures but that certain points of difference occur that may be related to the specific tissues under cultivation. In general, the amino acid requirements of cultures of single tissues appear to be more exacting than those of the whole animal, as might be expected.

TABLE IV

*Comparative Amino Acid Requirements of Various Types of Tissue Cultures, of the Chick, and of Man**

Amino acid	Chick (10)	Chick heart fibroblasts	L strain‡ (6, 15)	HeLa‡ (7, 15)	Walker‡ 256 (16)	Rabbit fibroblasts‡ (8)	Man (11)
Arginine	+	+	+	+	+	+	−
Histidine	+	+	+	+	+	+	−
Lysine	+	+	+	+	+	+	+
Tryptophan	+	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+
Tyrosine	−	+	+	+	+	+	−
Cyst(e)ine	−	+	+	+	+	+	−
Methionine	+	+	+	+	+	+	+
Serine	−	−	−	−	−	+	−
Threonine	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+
Isoleucine	+	−	+	+	+	+	+
Valine	+	+	+	+	+	+	+
Glutamic acid	−	x	−	−	−	−	−
Aspartic acid	−	x	−	−	−	−	−
α-Alanine	−	x	−	−	−	−	−
Proline	−	x	−	−	−	−	−
Hydroxyproline	−	x	−	−	−	−	−
Glycine	+	−	−	−	−	−	−
Glutamine		−	+	+	+	+	
Asparagine					+		

* + indicates essential, − indicates non-essential, x indicates inhibitory.

‡ The media used to determine these requirements contained various proportions of whole or dialyzed serum.

DISCUSSION

The results of the present experiments, which are based on more than 1600 cultures over a 12 month period, have shown that arginine, histidine, lysine, tyrosine, tryptophan, phenylalanine, cystine, methionine, threonine, leucine, and valine are essential to support the survival of chick embryonic heart cultures in completely synthetic media. Omission of any one of these eleven amino acids causes characteristic degenerative changes leading to rapid death of the cultures. The omission of serine, isoleucine, glycine, or glutamine was without effect on culture survival, while the omission of glutamic acid, aspartic acid, α-alanine, proline, or hydroxyproline was beneficial. It is of interest that the amino acids found to be essential in this study have also been shown to disappear from the medium during the cultivation period (17). Conversely, glutamic acid, aspartic acid, and α-alanine, whose omission from the medium is

beneficial, have been found to accumulate in the used culture fluids (17). These observations suggest that the chick embryonic heart cultures possess an active transamination system which may cause the accumulation of excessive and possibly inhibitory amounts of certain amino acids. Some support for this suggestion in indicated by the work of Westfall, Peppers, and Earle (18), showing that α-keto acids increase in the used culture medium.

Studies on the effect of amino acid concentration have shown that the total level in the medium is not a critical factor in maintaining the survival of chick embryonic heart cultures. The optimal level appears to be in the neighborhood of 100 mg. per cent, as determined previously (1), but considerable variation in this concentration may be made without altering appreciably the survival time of the cultures. Studies on the optical configuration of the amino acid mixtures suggest that DL forms may be employed without harmful effects, unless excessive quantities are used.

An unexpected result of these experiments was the failure of M 894, which contains no glutamic acid, proline, or α-alanine, to increase culture survival significantly beyond that supported by M 150, even though omission of these three amino acids individually was beneficial. These results suggest that complex interrelationships exist in the amino acid requirements of tissue cultures and that many enzyme systems must be functioning even in a tissue such as heart muscle that has not been regarded as a particularly rich source for enzyme or coenzyme isolations. The fact that repeated washing of the tissues with Hanks's salt solution did not alter the survival of cultures on their subsequent return to the complete synthetic medium (5) would indicate that these enzymes are firmly bound in the cell.

When the requirements of chick embryonic heart fibroblasts are compared with those established for the L strain (6, 15), HeLa cell (7, 15), rabbit fibroblast (8), and Walker 256 carcinosarcoma (16), a remarkable similarity in the pattern of essential and non-essential amino acids is seen. In general, arginine, histidine, lysine, tryptophan, phenylalanine, tyrosine, cystine, methionine, threonine, leucine, isoleucine, and valine are essential for all cultures. Exceptions to this common pattern are to be noted in the lack of requirement for isoleucine with chick fibroblasts, the necessity for serine with rabbit fibroblasts (8), the requirement for glutamine with the L strain, HeLa cell, and Walker 256 carcinosarcoma (15, 16), and the necessity for asparagine with the Walker 256 cell (16). These specific patterns suggest that comparative biochemistry may ultimately prove of value in characterizing different cells in tissue culture.

Two major differences are apparent between the requirements of tissue cultures and those of the adult human (11). All cultures so far tested require both phenylalanine and tyrosine and also both methionine and cystine. The mechanism of these dual requirements is under study in this Laboratory (19,

20) and may, it is hoped, add to our knowledge of alternate or abnormal metabolic pathways.

SUMMARY

1. The amino acid requirements of freshly explanted chick embryonic heart tissues cultivated in completely synthetic media have been determined, employing a nutritional depletion technique. Arginine, histidine, lysine, tyrosine, tryptophan, phenylalanine, cystine, methionine, threonine, leucine, and valine were found to be essential. Serine, isoleucine, glycine, and glutamine were found to be non-essential. Glutamic acid, aspartic acid, α-alanine, proline, and hydroxyproline were found to be inhibitory in this test system.

2. A total amino acid level of approximately 100 mg. per cent was found to be optimal and DL-amino acids were found to be non-toxic, unless used in high concentrations.

3. A comparison has been made of the amino acid requirements of various types of tissue cultures, of the chick, and of man and certain differences in these requirements have been discussed.

The technical assistance of Miss J. Gagnon, Mrs. D. Gordon, Miss P. Maheu, Miss S. Rinfret, and Miss P. Robert is gratefully acknowledged. Statistical analyses were performed through the courtesy of Mr. E. J. Hamilton, of this Laboratory.

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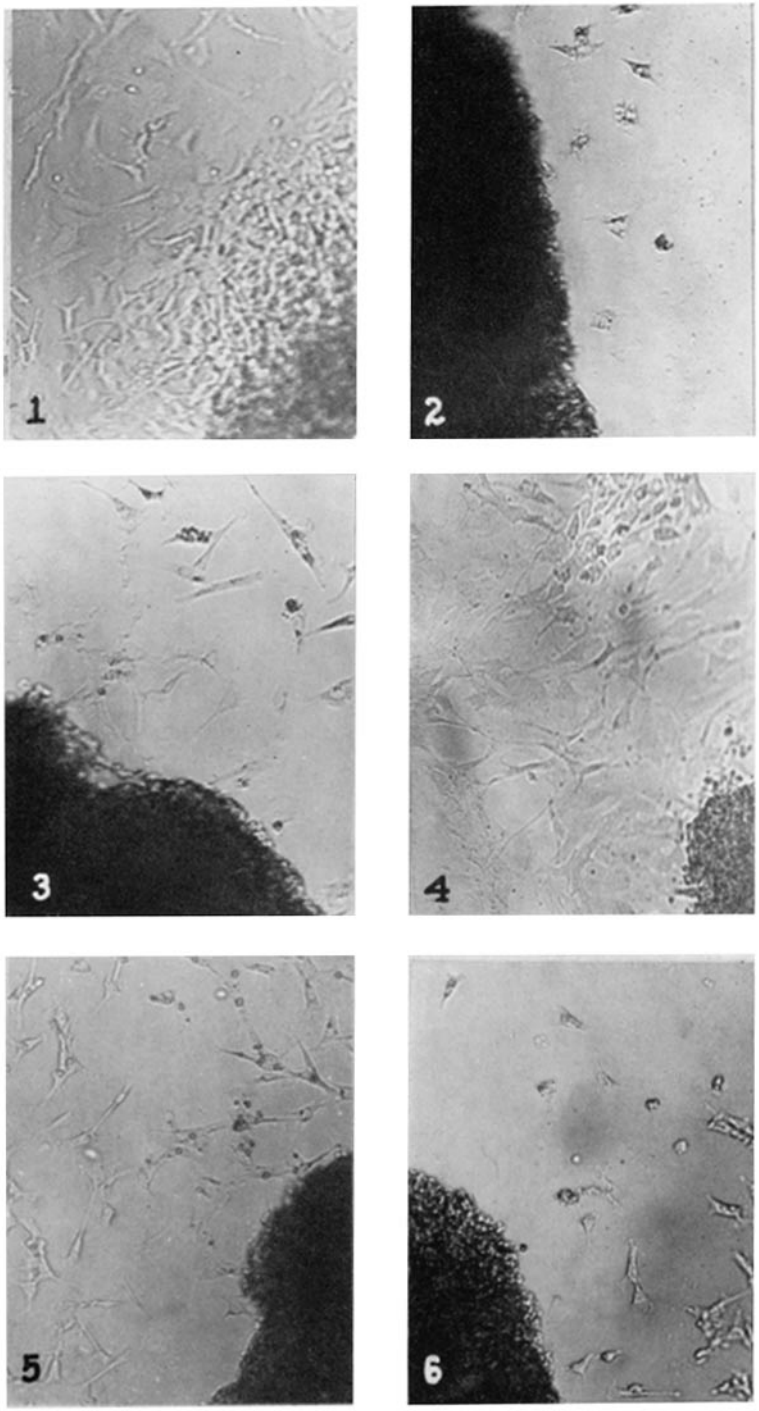
EXPLANATION OF PLATES

PLATE 26

Effect of omission of individual amino acids from medium M 150 on morphology of nutritionally depleted chick embryonic heart cultures. All cultures photographed at 7 days. × 105.

FIG. 1. Complete M 150 control.
FIG. 2. No tyrosine.
FIG. 3. No arginine.
FIG. 4. No glutamic acid.
FIG. 5. No cystine.
FIG. 6. No valine.



(Morgan and Morton: Nutrition of animal tissues *in vitro*)

PLATE 27

Effect of omission of individual amino acids from medium M 150 on morphology of nutritionally depleted chick embryonic heart cultures. All cultures photographed at 7 days. × 105.

FIG. 7. No alpha-alanine.

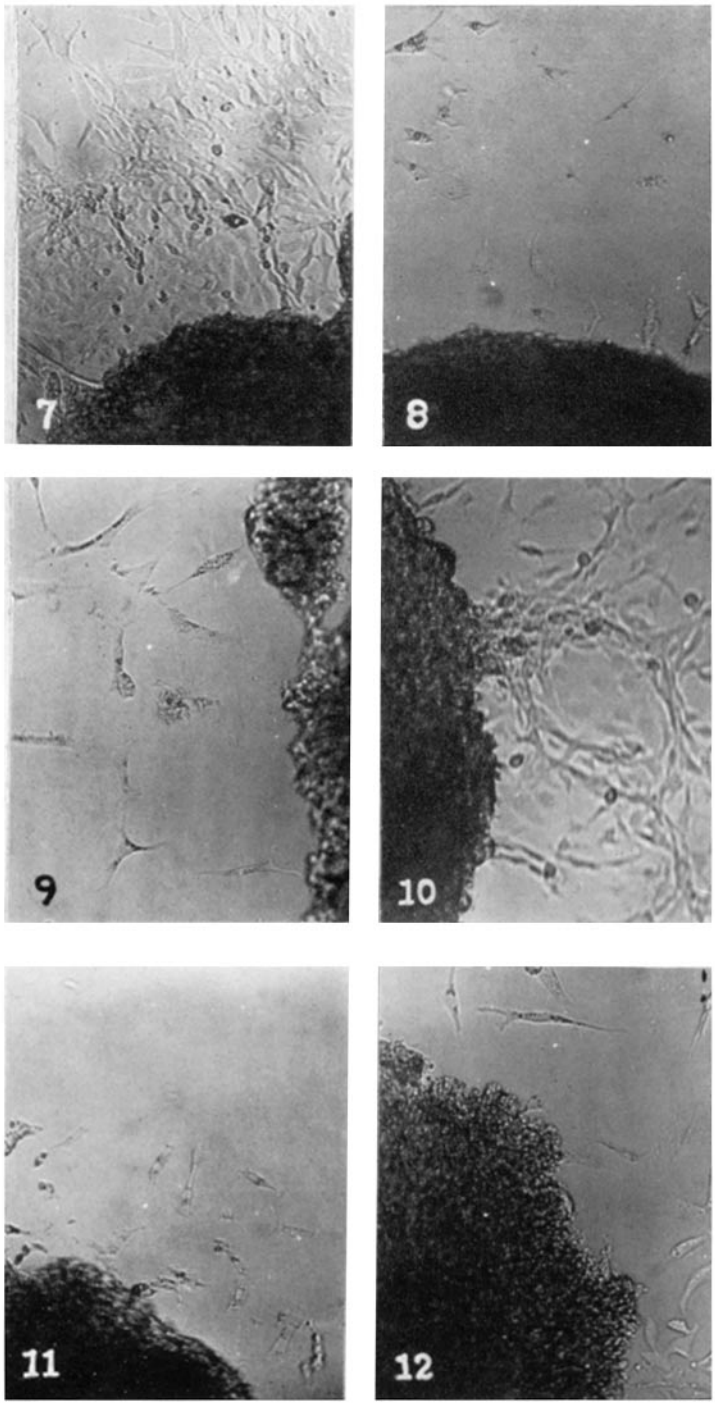
FIG. 8. No methionine.

FIG. 9. No leucine.

FIG. 10. No isoleucine.

FIG. 11. No tryptophan.

FIG. 12. No lysine.



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