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Amino-acid Metabolism of Tissue Cells *in vitro*

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Ever since the negative results of the investigations by Burrows & Neymann (1917), Carrel & Ebeling (1924) and Baker & Carrel (1926, 1928) the amino-acids have been regarded as being able neither to prolong the life of cells nor to accelerate cell growth in tissue cultures. However, in these investigations normal culture media were used and such media already contain the necessary amino-acids. This point was realized by the present author, and a technique was devised (Fischer, 1941) whereby the compounds of the culture medium were dialyzed against a Ringer's solution containing glucose. It was found that the dialyzed culture medium was completely unable to maintain the life of the cells—they died and disintegrated within 24 hr. With this dialyzed medium as a basis it became possible to make a systematic analysis of the effect of substances of low molecular weight on the maintenance of cells grown *in vitro*.

A study was then made of the effect of an amino-acid mixture composed of nine amino-acids in the same relative proportions as found by Bergmann & Niemann (1936) in fibrin (Table 1). It was found that cystine plays a very important part. Without cystine, the remaining amino-acids are no longer able to save the cells from rapid disintegration within 24 hr. It was also observed that in the cultures of myoblasts and osteoblasts cystine cannot be replaced by methionine, though methionine is an essential acid for the organism as a whole whilst cystine is not. Moreover, both lysine and glutamic acid were found to be of great importance to the cells. Obviously, the amino-acids required by the whole organism are not the same as those necessary to a pure culture of tissue cells. For example, glutamic acid is essential to myoblasts, but not to the organism as a whole. Our technique of 7 years ago has since been improved, and the present paper presents some of the results obtained.

EXPERIMENTAL

The tissue cells in the present experiments belonged to a pure strain of myoblasts from a 9-day-old chicken embryo. The individual cultures were divided into two halves, one serving as control in the medium described below, the other as the experimental culture, in the same medium, but with the substance under investigation. At intervals during growth, drawings of the tissue cultures were made by means of an Edinger projector which magnified twenty times. The area of each drawing was measured by means of a planimeter and the results are given in Figs. 1–14, expressed as ratios according to the formula $(B-A)/A$, where A = initial area and B = growth area (Fischer, 1925).

The medium consisted of 0·5 ml. dialyzed chicken plasma, 1 ml. Tyrode's solution and 0·1 ml. of the mixture called the basic nutrient (Table 1). Coagulation was produced by adding 1 drop of dialyzed embryo juice. When coagulated, a liquid phase was introduced by adding 0·5 ml. dialyzed serum and 0·1 ml. of the basic nutrient. Carrel flasks type D-3 were used. The pH of the mixture was adjusted by introducing into the flask a gas composed of 8% CO_2, 12% N_2 and 80% O_2. The flasks were sealed with sterile rubber stoppers.

The basic nutrient mentioned (Table 1) was designed by G. Ehrensvärd, and is a mixture of substances known empirically to be important to the whole organism. It contains three main groups of substances; the first group consists of salts including salts of heavy metals and organic phosphates which can function as phosphorylating agents,

Table 1. *Basic nutrient of biologically active substances tested in tissue cultures*

(Mg. of substances contained in 1 l. solution.)

NaCl	7500
KCl	200
$CaCl_2$	200
$MgCl_2$	100
Na_2HPO_4	50
$NaHCO_3$	1000
$FeCl_2$	0·6
$CuCl_2$	0·2
$MnCl_2$	0·3
$ZnCl_2$	1·0
$CoCl_2$	0·01
Glucose	800
Mannose	100
Galactose	100
Inositol	20
Adenosine triphosphate	200
Fructosediphosphate	100
β-Glycerophosphate	100
Inosinic acid	30
Cozymase	5
Aneurin	3
Riboflavine	0·2
Pyridoxin	0·3
Lysine dihydrochloride	15·1
Arginine monohydrochloride	7·7
Tryptophan	5
Methionine	2·6
Histidine monohydrochloride	3·1
Glutamic acid	14·1
Aspartic acid	5·9
Proline	5·1
Cystine	1·5
DL-Methionine	6
Choline (as hydrochloride)	10
Creatine	10
Nicotinic acid	0·3
Glutathione	5
Pantothenic acid	0·07
Biotin	0·007
p-Aminobenzoic acid	1
Hypoxanthine	100
Sodium succinate	10
Sodium fumarate	10
Sodium malate	10
Sodium oxaloacetate	10
Ascorbic acid	2
Methylnaphthohydroquinone sulphate	0·005

another group comprises the amino-acids, substances acting as methyl donors or providing SH groups, and finally a third group containing the various vitamins, choline, creatine and the C_4 acids (functioning in the Krebs cycle). This basic nutrient is so designed that it is easy to eliminate any part of it and replace it by another.

RESULTS

Importance of amino-acids as a group

The first and fundamental experiment involved the question of the general importance of the amino-acids in connexion with the maintenance and growth of tissue cells. It was found, as one would expect, that if no amino-acids are present in the basic nutrient the cells die and disintegrate rapidly (Table 2). To be sure, there is a very slight growth on the first day, but that is due to residual amounts of amino-acids in the tissue itself at the time of transfer to the dialyzed, incomplete medium. The importance of the individual amino-acids could now, as mentioned above, be studied by omitting the amino-acid in question from the mixture.

Deficiencies of single amino-acids

Cystine. The absence of cystine and methionine in animal nutrition leads to death in a comparatively short time but, since the myoblasts are unable to utilize methionine in place of cystine, the withdrawal of this amino-acid from our medium has no effect on the growth of the cells. Cystine forms a structural constituent of the proteins of the cell. Fig. 1 and Table 3 show that the control culture in the medium containing the whole of the basic diet grows normally, whereas the experimental culture, with no cystine in the medium, does not grow at all. This confirms our earlier findings (Fischer, 1941) as to the basic significance of cystine. It was also found that methionine, in the absence of cystine, is insufficient (Fig. 1). When cystine was added to the deficient medium, the cells not only survived, but showed a small but definite increase in mass, and the cells had a normal appearance.

Cystine can, however, be replaced by glutathione (Fig. 2). It is believed that in addition to being of

Table 2. *Comparison of growth of myoblasts on the basic nutrient with and without amino-acids*

Exp. no.	Days	Superficial growth in medium with amino-acids (mm.²)	Superficial growth in medium without amino-acids (mm.²)	Ratio of area of growth with and without amino-acids
18213–14	7	855	86	9·9
18215–16	7	1267	154	8·2
18217–18	7	1076	95	11·3
18219–20	7	1096	191	5·7
18221–22	7	1116	98	11·3
18257–58	5	—	—	—
18259–60	5	—	—	—
17967–68	4	1051	190	5·5
17969–70	4	1005	212	4·7

importance as a building stone for the cytoplasm, cystine is also an activator for various proteolytic enzymes which enable the cells to split the plasma proteins in the surrounding medium. The break in the curve in Fig. 3 may be explained as being due to a liberation of split products from the plasma proteins of the medium, or possibly to the fact that glutamine, together with cystine and glycine, may enable the cells to produce other amino-acids for their maintenance.

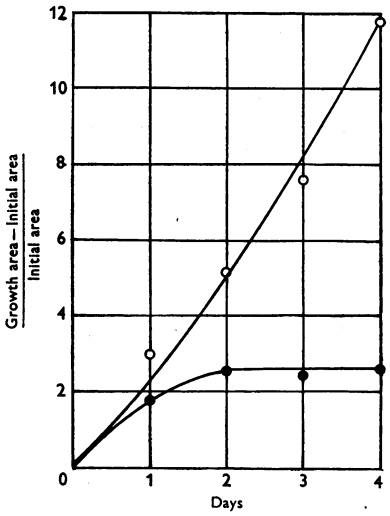

Fig. 1. Effect of cystine deficiency on growth of myoblasts. The circles indicate the growth in the control medium, the black dots the growth when cystine is omitted.

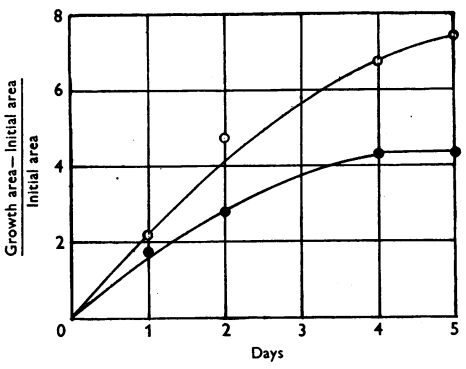

Fig. 2. The effect of the replacement of cystine by glutathione. The black dots indicate the growth in the control medium, the circles the growth when cystine is replaced by glutathione.

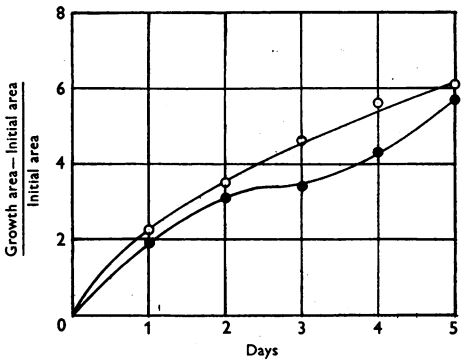

Fig. 3. Effect of a medium containing only cystine, glycine and glutamine. The circles indicate the growth in the control medium, the black dots the growth when the amino-acids comprise only cystine, glycine and glutamine.

Glutamic acid is known to be very important and to be associated with the transamination process. According to Rose (1937) this amino-acid has no effect on the growth rate of young rats. The growth of myoblasts on a nutrient without glutamic acid was slightly retarded in comparison with that shown with the basic diet (Figs. 4, 9). If glutamic acid was replaced by glutamine (0·5 mg./flask) the rate of growth increased enormously (Fig. 5). At the same time, the morphological appearance of the cells underwent a change and became perfectly normal, and gradually the fat vacuoles disappeared. Without glutamine the cells were extremely atrophic, having the appearance of small glass splinters. The process is reversible, i.e. cells which already are highly atrophic change into perfectly normal cells when glutamine is added to the medium.

Lysine. The absence of lysine from the amino-acid mixture had only a slight effect or none at all on the growth of myoblasts (Fig. 6); lysine thus resembles glutamic acid (Fig. 4). This may be connected with the circumstance that lysine cannot be regenerated by amination once it is deaminated (cf. Weissman & Schoenheimer, 1941). Osteoblasts seem to be more sensitive to lysine deficiency (Fig. 7).

Table 3. *Growth of fibroblasts on the basic nutrient with and without cystine*

Exp. no.	Days	Superficial growth with cystine (mm.²)	Superficial growth without cystine (mm.²)
18257–58	5	614	0
18259–60	5	805	0
18269–70	4	700	0
18271–72	4	571	0

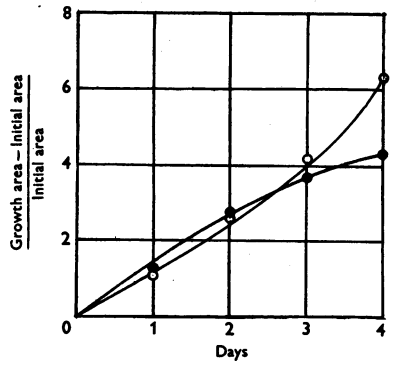

Fig. 4. Effect of a deficiency of glutamic acid on the growth of myoblasts (Exp. 18261–62). The circles indicate the growth in the control medium, the black dots the growth when glutamic acid is omitted.

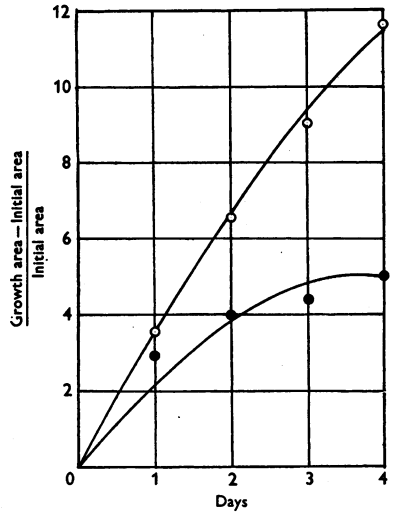

Fig. 5. Comparison of the effect of glutamine and glutamic acid (Exp. 18291–92). The black dots indicate the growth in the control medium, the circles the growth when glutamic acid is replaced by glutamine.

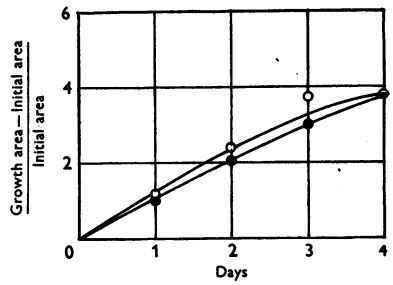

Fig. 6. Effect of lysine deficiency on the growth of myoblasts (Exp. 18375–76). The circles indicate the growth in the control medium, the black dots the growth when lysine is omitted. There is practically no difference.

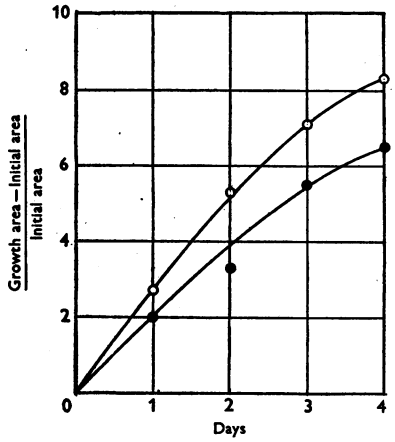

Fig. 7. Effect of lysine deficiency on the growth of osteoblasts (Exp. 18527–28). The circles indicate the growth in the medium containing all the amino-acids of the basic diet, the black dots the growth in the medium when lysine is omitted.

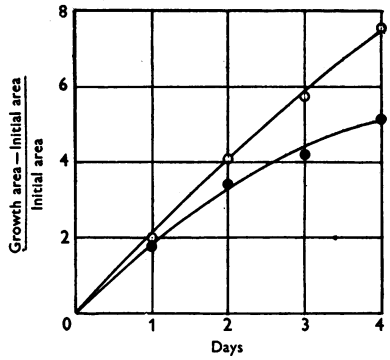

Fig. 8. Effect of the combined deficiency of lysine and glutamic acid on the growth of myoblasts (Exp. 18479–80). The circles indicate the growth in the control medium, the black dots the growth when both lysine and glutamic acid are omitted.

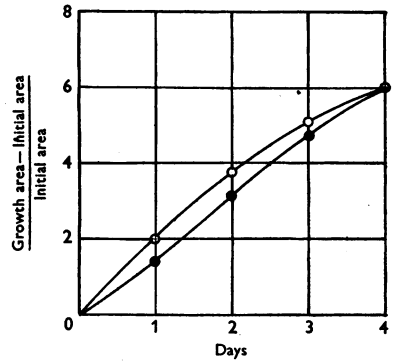

Fig. 9. Effect of glutamic acid deficiency on growth of myoblasts (Exp. 18263–64). The circles indicate the growth in the control medium, the black dots the growth when glutamic acid is omitted.

A medium lacking both lysine and glutamic acid has a growth-depressing effect (Fig. 8), which is more marked than if only one of these amino-acids is lacking (Figs. 4, 6 and 9). The morphological appearance of the cells was perfectly normal in the control, whereas in the lysine-deficient medium the cells were atrophic and of the above-mentioned glass splinter type.

Tryptophan. According to Osborne & Mendel (1914) tryptophan is an essential amino-acid; it may be deaminated *in vitro* under aerobic conditions by slices of kidney (Krebs, 1933). Fig. 10 shows the

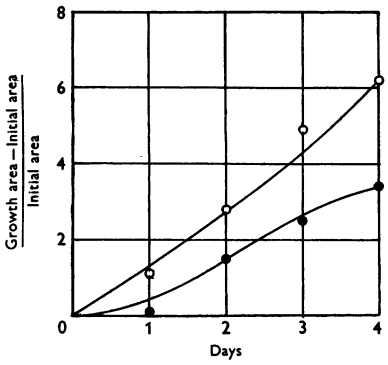

Fig. 10. Effect of deficiency of tryptophan on growth of myoblasts (Exp. 18379–80). The circles indicate the growth in the control medium, the black dots the growth when tryptophan is omitted.

effect of a nutrient deficient in tryptophan (Exp. 18379–80). The response of the cells was plain; in the control medium the cells were perfectly normal, while they showed atrophy in the deficient medium. Morphological differences between the cells on the two diets became manifest from the time the cells began to migrate out into the medium.

Arginine is claimed to be a dispensable amino-acid as far as the animal organism is concerned (Scull & Rose, 1930). According to Klose, Stokstad & Almquist (1938) the young chick seems to lack any ability to synthesize arginine. Since the absence of arginine causes an inhibition of the growth of myoblasts, this amino-acid must be indispensable to these particular cells which cannot, like the mammalian organism, synthesize arginine (Moss & Schoenheimer, 1940). Fig. 11 shows the pronounced effect of arginine deficiency. The cells in the arginine-free medium contained, curiously enough, less fat vacuoles than did the control cultures.

Histidine-proline. Rose & Cox (1924, 1926) have shown that histidine is indispensable to the growth of rats. Removal of proline from the diet is claimed to have no effect on the growth of young rats. Histidine-proline deficiency in the basic nutrient led to a marked depression of the growth of our experimental cultures (Fig. 12). The cells in these cultures were thin and atrophic, containing numerous fat vacuoles, whereas the cells in the control cultures, with all the amino-acids in the medium, were perfectly normal.

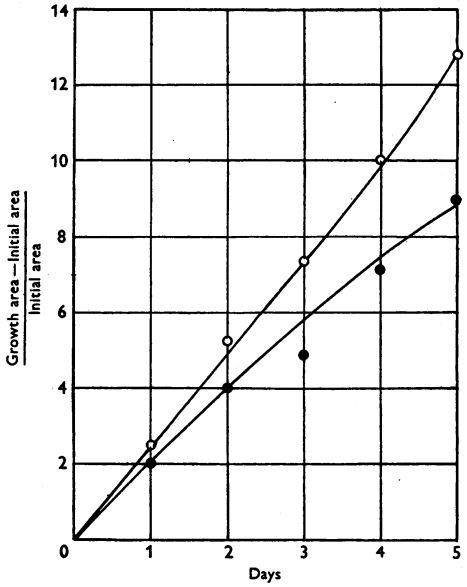

Fig. 11. Effect of arginine deficiency on growth of myoblasts (Exp. 18437–38). The circles indicate the growth in the control medium, the black dots the growth when arginine is omitted.

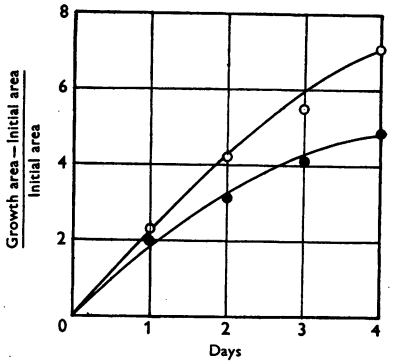

Fig. 12. Effect of combined deficiency of histidine and proline on growth of myoblasts (Exp. 18555–56). The circles indicate the growth in the control medium, the black dots the growth when histidine and proline are omitted.

The effect of media containing only few amino-acids

Medium containing only cystine, histidine, proline and aspartic acid. The growth of myoblasts in a medium, the amino-acids of which include only these amino-acids, was, as one would expect, very defective. There was practically no growth, the cells

were full of fat vacuoles, and disintegrated very rapidly.

Medium containing only cystine, glutamine and glycine. A medium containing only these amino-acids and all the other components of the basic diet showed a remarkable effect. The concentration of each of the components was the same as in the basic nutrient and 0·5 mg. glutamine was used in each flask. The cells looked perfectly normal, and no difference could be observed between the cells in this experimental medium and the cells in the control. Cystine alone was not capable of keeping the cells alive for any length of time (Exp. 18339–43). It is thought that the cells might be able to build other amino-acids by transamination of the three amino-acids mentioned.

Amino-acids and digests of proteins

Complete chemical analysis of several proteins has recently been undertaken by Brand (1946). These data make it possible to carry out a comparative investigation of the effect on tissue cells of, on the one hand, an amino-acid mixture approximating the composition of lactoglobulin and crystalline bovine serum albumin and, on the other, a peptic and tryptic digest of the same proteins. For the preparations of lactoglobulin and pure trypsin we are indebted to Prof. Linderstrøm-Lang. Armour's crystalline pepsin was used.

The crystalline enzymes were first dialyzed for 24 hr. The proteins to be digested were diluted with water 1 : 3 and toluene was added to prevent bacterial growth. The digestion was followed by heating for 5 min. on the water bath at 100°, cooling, filtration, evaporation *in vacuo* to remove the toluene. pH was adjusted by means of NaOH or HCl, depending on the enzymes used. The solutions were sterilized by filtration.

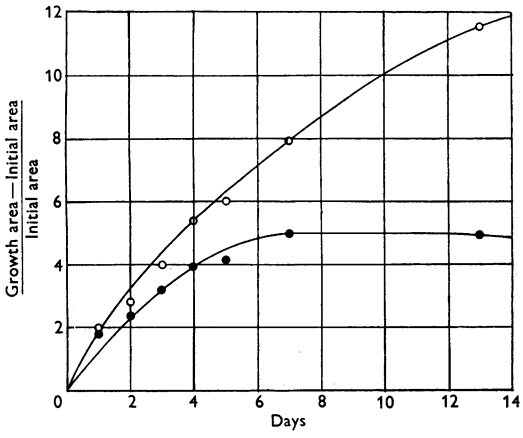

Fig. 13. Effect of a digest of lactoglobulin and an artificial amino-acid mixture resembling this protein (Exp. 18233–34). The black dots indicate the growth in the amino-acid medium, the circles the growth when the medium contains the enzymic digest of lactoglobulin.

Figs. 13 and 14 show the complementary effects on the deficient plasma medium of a mixture

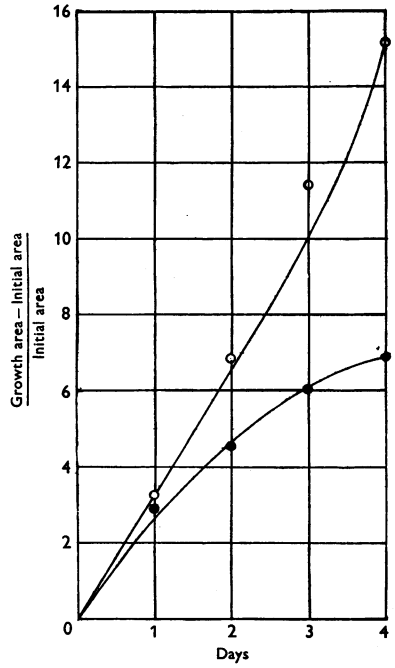

Fig. 14. Effect of a digest of bovine serum albumin and of an amino-acid mixture resembling this protein (Exp. 18455–56). The black dots indicate the growth in the amino-acid medium, the circles the growth when the medium contains the bovine serum albumin digest.

Table 4. *Amino-acid composition of lactoglobulin and bovine serum albumin*

Amino-acids	Lactoglobulin (mg. in 20 ml.)	Bovine serum albumin (mg. in 20 ml.)
Glycine	1·4	1·9
Alanine	6·2	—
Valine	5·83	6·5
Leucine	15·6	13·7
Isoleucine	8·4	2·9
Proline	4·1	5·7
Phenylalanine	3·54	6·2
Cysteine	1·11	1·11
Cystine	2·29	5·41
Methionine	3·22	0·81
Tryptophan	1·94	0·58
Arginine	2·88	6·2
Histidine	1·58	3·8
Lysine	11·4	12·4
Aspartic acid	11·4	10·6
Glutamic acid	19·5	16·9
Glutamine	1·5	1·05
Serine	5·0	4·5
Threonine	5·85	6·5
Tyrosine	3·78	5·49

of the amino-acids approximating in their relative proportions to the composition of the proteins, lactoglobulin and bovine serum albumin (Table 4).

The effects were pronounced. While the proteins in the form of amino-acids produce an excellent complementary effect on the deficient plasma medium, the digests cause besides an enormously increased rate of growth, a phenomenon known already from the work of Baker & Carrel (1928) when using proteoses from Witte's peptone.

For the same nitrogen content there is a remarkable difference in the effects of the amino-acids and the digests of the lactoglobulin. While the growth curve of tissue cells in a medium containing the amino-acids slowly approaches a maximum, the growth of the cells in the medium containing the digests proceeds almost logarithmically from the very beginning and reaches high values (Figs. 13, 14). In other words, the cells respond quite differently to amino-acids and to polypeptide-amino-acid mixtures.

DISCUSSION

Comparative experiments on the importance of amino-acids in the nutrition of pure strains of tissue cells *in vitro* demonstrate the significance of the individual amino-acids as far as growth and maintenance are concerned. Cystine occupies a key position and was found to be the only amino-acid, the absence of which leads to complete inhibition of growth even in the presence of all the other amino-acids, a fact which was already recognized by the author several years ago (1941) when he employed a nutrient composed of amino-acids only. Since then we have developed a complete basic nutrient containing all the substances necessary for satisfactory growth. Here again it has been confirmed that cystine cannot be replaced by methionine, as myoblasts are found to be unable to metabolize this amino-acid under the experimental conditions employed.

It is concluded that those amino-acids, which when withdrawn from the mixture cause a depression of cell growth, are normally metabolized by the cells in question and may therefore be regarded as indispensable to these types of cells.

The experiments also show that a nutritive evaluation of the individual amino-acids on the basis of their effect on strains of tissue cells is very rapid in comparison with other methods involving whole animals. For the investigation of the protein metabolism of cell types, the tissue-culture method is the only one that can be used. It is evident also that the amino-acid requirement of various types of tissue cells may not be the same, as is shown by a comparison of the response of myoblasts and osteoblasts to lysine deficiency (Figs. 6, 7). Perhaps we may soon be in a position to map the amino-acid diet necessary for the maintenance of other types of tissue cells.

An approximate rating of the amino-acids according to the degree to which they are essential to the cells of the cultures in the present experiments is as follows: cystine (most essential), arginine, tryptophan, glutamine and lysine.

SUMMARY

1. A basic nutrient, however elaborate, involving compounds found empirically to be of importance to the animal organism is inadequate for the maintenance and growth of tissue cells if it does not contain the necessary amino-acids.

2. A technique has been developed making it possible to evaluate accurately the importance of each of the amino-acids necessary to the life of the cells.

3. If the elimination of an amino-acid from the diet causes the inhibition of cell growth, in comparison with the growth of the control culture, this amino-acid must be, when present, metabolized by the cells and must be regarded as indispensable.

4. The degree to which an amino-acid is essential can be determined accurately by measuring the ratio between the growth of the controls in the medium containing all the amino-acids and the growth of the experimental cultures where the medium is deficient in this particular acid. The present experiments involved cystine, methionine, lysine, glutamic acid, aspartic acid, tryptophan, arginine, glutathione, histidine and proline.

5. The effect of the amino-acids constituting lactoglobulin and crystalline bovine serum albumin was compared with that of enzymic digests of these proteins. The differences are very marked and characteristic.

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