SUMMARY

Human embryonic diploid lung fibroblasts were capable of growing in a medium consisting of Eagle's MEM vitamins plus 0.1 µg/ml biotin, Earle's salt solution, 4 mM glutamine, 0.4 mM cystine, 0.5 mM tyrosine, 10% undialysed calf serum and 1 mM pyruvate. The growth rate and percent cell attachment in this medium was slightly less than in Eagle's MEM but the longevity was about the same. Addition of 0.5-1.0 mM histidine stimulated growth but addition of 0.2 mM phenylalanine, methionine or valine decreased longevity markedly.

Maintaining a constant pH of about 7.5-7.6 with 20 mM of the organic buffers HEPES, TES or TRICINE yielded a growth rate equal to or better than that of cells growing in unbuffered medium. Some buffers gave good growth initially but eventually the longevity decreased (20 mM BIS-TRIS, 20 mM BICINE, 10 mM TRIS). In almost all cases the initial exposure to the buffers yielded the best growth results.

INTRODUCTION

The amino acid requirements of almost all mammalian cells, diploid or heteroploid, grown in vitro have been shown to be served by 13 amino acids (Eagle, 1955a-c, 1959; Eagle, Oyama, Levy, Horton & Fleischman, 1956; Eagle, Oyama & Levy, 1957), although the relative utilization of each amino acid may be different with each cell strain (McCarty, 1962). These amino acids are: glutamine, arginine, histidine, lysine, tryptophan, valine, leucine, isoleucine, methionine, threonine, phenylalanine, tyrosine and cystine. Attempts to eliminate any single amino acid from these 13 results in a rapid cessation of growth (Eagle, 1955,a,b; Eagle et al. 1956, 1957; Freed & Schatz, 1969; Litwin, 1972). However, Jacobs (1966) described a medium containing only glutamine and cystine as amino acid source, which could support the growth of human embryonic diploid lung fibroblasts. This report seemed incongruous with what had been published earlier on the nutritional requirements of human cells (Eagle et al. 1957) and stimulated the present survey on the effect individual and combinations of amino acids have on growth and longevity of these cells in media deficient in essential amino acids.
MATERIAL AND METHODS

Cells

Human embryonic diploid lung fibroblasts (HEDLF) cells were grown from the trypsinized lung tissue of human embryos aborted in the third month of gestation. Two independent strains were used with similar results. Most experiments were started with cells that were in their 7th passage, approximately 20 to 25 cell divisions old. The growth patterns of these cells are similar to those described by Hayflick & Moorhead (1961) and Hayflick (1965).

Medium

The growth of HEDLF cells in Eagle's minimum essential medium (MEM) (Eagle, 1959) was used as comparison for the test media. The components common to all the test media were Earle's salt solution and the vitamin mixture used in MEM but containing 0.1 mg/l. biotin (Eagle, 1955c). These 2 components will be referred to as VS (vitamin, salts) in the text. Earle's salts were modified by using 0.9 % NaCl instead of 0.68 % and twice the glucose concentration recommended. These changes were found to yield slightly better growth of HEDLF cells in MEM. Glutamine was used at a concentration of 4 mM in all cases and only the L-isomers of amino acids were used (Sigma Chemical Co., St Louis, Mo., U.S.A.). The media were supplemented with 10 % calf serum, 100 I.U./ml penicillin and 100 µg/ml streptomycin. Sodium bicarbonate was used to adjust the pH. The serum was neither dialysed nor inactivated by heating. The initial pH of the media in most experiments varied between 7.0 and 7.2. The day following the initiation of the cultures the pH was adjusted to about 7.5 with NaHCO₃.

Culture conditions

The cells were grown in Jena glass culture bottles with a growth surface of 46 cm². Each bottle was seeded with 2 x 10⁵ cells in a volume of 20 ml medium. The following day the number of cells which attached to the glass surface was estimated by counting the attached cells in randomly selected fields of view with an inverted microscope (Litwin, 1971). A more accurate estimate of the number of cell divisions occurring with each passage could be obtained by using the number of attached cells as the starting cell density instead of the number of cells added to the culture vessel. The average attachment value given in the text was calculated from the percent attachment from all passages over the entire in vitro cultivation of each culture. When the cells grew to a confluent monolayer they were trypsinized off the glass in 0.25 % trypsin at room temperature, centrifuged, resuspended in 5 ml of fresh medium of the type used for their growth and counted with a haemocytometer. In more recent experiments an electric cell counter was used (Celloscope, Sweden). When most cultures reached senescence they were incubated for about a month with weekly changes of medium. If the cell layer did not become confluent during this time the cells were counted and discarded. To eliminate variations in growth due to inconsistent dishwashing the same bottle was used for all passages without intermittent washings in all experiments except those mentioned in the text. Prior experience has shown that this procedure gives as good growth as with cultures grown in clean bottles with each passage, and has no influence on the longevity of diploid fibroblasts.

RESULTS

Although HEDLF cells stopped growing within 2 to 4 cell divisions when any one of the amino acids in Eagle's MEM was omitted (Litwin, 1972), when all of the amino acids, with the exception of glutamine, were withdrawn some cells could be observed in mitosis after as long as 1–2 weeks of incubation. A survey was made of which amino acid or combination of amino acids would stimulate growth when added to a basal medium containing Eagle's vitamins and Earle's salts (VS) plus 4 mM glutamine, 1 mM sodium pyruvate and 10 % calf serum. The following combinations of amino acids...
Amino acids required for diploid fibroblast growth

acids at a concentration of 0.2 mM, except cystine at 0.1 mM, permitted some cell growth: cystine, tyrosine + cystine, phenylalanine + cystine, phenylalanine + cystine + lysine, phenylalanine + cystine + threonine, phenylalanine + cystine + valine, tyrosine + cystine + histidine, tyrosine + cystine + threonine. In these experiments clean bottles were used for each passage and growth was relatively poor. When these combinations were investigated in greater detail, we began to re-use the same bottle with each passage and the growth results improved considerably.

Using the base medium VS + 1 mM pyruvate very little growth occurred with only

Fig. 1. The effect of varying some of the conditions in the medium VS + 1 mM sodium pyruvate on the growth of HEDLF cells. x • • • • x, 4 mM glutamine added (20%); O ——— O, 0.2 mM tyrosine + 0.1 mM cystine, no glutamine (18%); x ——— x, 4 mM glutamine + 0.2 mM tyrosine + 0.1 mM cystine (38%); △ ——— △, 4 mM glutamine + 0.5 mM tyrosine + 0.1 mM cystine (62%); • ——— •, 4 mM glutamine + 1.5 mM tyrosine + 0.1 mM cystine (62%). The values in parentheses represent the average percentage cell attachment to the glass surface.

Fig. 2. The effect of varying some of the conditions in the medium VS + 4 mM glutamine, 0.1 mM cystine, 1 mM sodium pyruvate on the growth of HEDLF cells. x ——— x, Eagle's MEM control (69%); △ ——— △, medium given above (45%); • ——— •, 0.2 mM tyrosine (42%); O ——— O, 0.2 mM phenylalanine (50%); □ ——— □, 0.5 mM phenylalanine (57%); + ——— +, 1.5 mM phenylalanine (45%). The values in parentheses represent the average percentage cell attachment to the glass surface.
glutamine or cystine + tyrosine added (Fig. 1). However, significant growth was seen when all 3 were present. The optimal concentration of tyrosine appeared to lie between 0.5 and 1.5 mM. With 2 mM tyrosine the cells degenerated after 6 or 7 cell divisions.

The base medium of VS + 4 mM glutamine + 0.1 mM cystine + 1 mM pyruvate produced good growth initially but the cells stopped growing after 34 cell divisions (Fig. 2). This base medium plus 0.2 mM tyrosine gave as good a longevity (58 cell divisions) as cells grown in Eagle's MEM (57 cell divisions), although the growth rate of the former slowed inexplicably between 18 to 30 cell divisions. When 0.2 mM phenylalanine was used instead of tyrosine the growth became slower after 25 cell divisions and eventually stopped after 44 cell divisions. Higher concentrations of

Fig. 3. The effect on the growth of HEDLF cells of 0.2 mM of various essential amino acids added to medium containing VS + 4 mM glutamine, 0.4 mM cystine, 0.5 mM tyrosine, 1 mM sodium pyruvate. × × ×, Eagle's MEM control (69%); ○ — ○, histidine (44%); Δ — Δ, arginine (37%); □ — □, methionine (58%); + — +, phenylalanine (27%). The values in parentheses represent the average percentage cell attachment to the glass surface.

Fig. 4. The effect on the growth of HEDLF cells of 0.2 mM of various essential amino acids added to medium containing VS + 4 mM glutamine, 0.4 mM cystine, 0.5 mM tyrosine, 1 mM sodium pyruvate. × × ×, histidine (44%); ○ — ○, threonine (44%); Δ — Δ, tryptophan (34%); □ — □, isoleucine (37%); • • •, leucine (34%); + — +, valine (36%). The values in parentheses represent the average percentage cell attachment to the glass surface.
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phenylalanine reduced growth rate and longevity; with 2 mM the cells degenerated after 9 cell divisions.

A study on the influence of various concentrations of cystine in VS containing 4 mM glutamine, 0.5 mM tyrosine and 1 mM pyruvate showed that the optimum concentration was 0.4 mM. Cystine was toxic at 0.8 mM (data not shown). Therefore, the following medium is defined: VS + 4 mM glutamine + 0.4 mM cystine + 0.5 mM tyrosine (VSGCT medium) containing 1 mM pyruvate.

Table 1. The effect of various organic buffers and pH on the growth and longevity of HEDLF cells

VSGCTH medium was adjusted to pH 7.2 initially. After overnight incubation the medium was adjusted to pH 7.6. One culture with 20 mM TRICINE was adjusted initially to pH 7.9 and kept at this pH.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Total no. of cell divisions</th>
<th>Cell divisions at senescence</th>
<th>Day senescence occurred</th>
<th>Cell division time, days*</th>
<th>Total days</th>
<th>Average % cells attached</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate alone</td>
<td>30</td>
<td>30</td>
<td>140†</td>
<td>0.21</td>
<td>140</td>
<td>43</td>
</tr>
<tr>
<td>10 mM HEPES + 10 mM TES</td>
<td>33</td>
<td>31</td>
<td>85</td>
<td>0.37</td>
<td>147</td>
<td>38</td>
</tr>
<tr>
<td>20 mM TRICINE</td>
<td>29</td>
<td>29</td>
<td>113†</td>
<td>0.25</td>
<td>113</td>
<td>56</td>
</tr>
<tr>
<td>20 mM TRICINE, pH 7.9</td>
<td>24</td>
<td>24</td>
<td>141</td>
<td>0.17†</td>
<td>172</td>
<td>41</td>
</tr>
<tr>
<td>10 mM TRIS</td>
<td>18</td>
<td>17</td>
<td>50</td>
<td>0.34</td>
<td>100</td>
<td>38</td>
</tr>
<tr>
<td>20 mM BIS-TRIS</td>
<td>20</td>
<td>18</td>
<td>49</td>
<td>0.37</td>
<td>100</td>
<td>46</td>
</tr>
<tr>
<td>20 mM BICINE</td>
<td>19</td>
<td>18</td>
<td>63</td>
<td>0.29</td>
<td>90</td>
<td>48</td>
</tr>
<tr>
<td>20 mM HEPES</td>
<td>18§</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>70</td>
<td>41</td>
</tr>
<tr>
<td>10 mM HEPES</td>
<td>24</td>
<td>24</td>
<td>119</td>
<td>0.2</td>
<td>135</td>
<td>56</td>
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<tr>
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<td>28</td>
<td>106†</td>
<td>0.26</td>
<td>106</td>
<td>53</td>
</tr>
<tr>
<td>10 mM MES</td>
<td>24</td>
<td>24</td>
<td>126†</td>
<td>0.19</td>
<td>126</td>
<td>40</td>
</tr>
<tr>
<td>20 mM MES</td>
<td>16</td>
<td>16</td>
<td>84</td>
<td>0.19</td>
<td>119</td>
<td>50</td>
</tr>
</tbody>
</table>

* Calculated from the ratio of the number of cell divisions at senescence to the time senescence occurred.
† Culture contaminated at senescence.
§ Cell div./day = 0.23 if one does not include the long lag period of 63 days.
§ Culture discontinued accidentally.

Pyruvate appeared to have a stimulatory influence on growth of HEDLF cells in the various deficient media studied (data not shown), although it did not influence cell growth in Eagle's MEM. One and 2 mM pyruvate gave similar results but 10 mM was inhibitory.

The influence of other amino acids added to VSGCT medium is seen in Figs. 3 and 4. In this experiment 0.2 mM histidine yielded better longevity and 0.2 mM threonine gave as good results as Eagle’s MEM. Isoleucine reduced longevity without a decrease in growth rate, but arginine and leucine reduced the growth rate as well as longevity. A greater reduction in longevity was found when 0.2 mM methionine or valine was added to the medium even though the growth rate for the first 3 to 4 passages was good. However, phenylalanine was the most inhibitory to both growth and longevity.
When histidine was re-examined, growth at concentrations from 0.2 to 2.0 mM was similar and 0.5 mM was selected for use in future experiments (VSGCTH medium).

However, cell growth in these media varied considerably in different experiments. This variation also would occur, but to a lesser extent, with cultures growing in Eagle's MEM. One possible explanation of this variation may be that cells growing in deficient medium are very sensitive to slightly inhibitory substances which may be present in varying concentrations in different batches of serum (Litwin, 1970). Another factor which can affect growth is the rapid changes in pH which occur while the cells are actively growing, requiring the frequent addition of bicarbonate. The buffering capacity of the media studied was much less than MEM because they contained so few amino acids. Therefore, some of the various buffers recommended by Williamson & Cox (1968) and demonstrated by Ceccarini & Eagle (1971) and Eagle (1971) to increase the maximum cell density of human diploid cell growth at pH 7.6 were added to the medium to help stabilize the pH. The buffers were adjusted initially to pH 7.2 regardless of the pKₐ because it was found that better cell attachment was obtained at this pH than occurred at higher pH levels. After overnight incubation the pH was adjusted to 7.5-7.6 with bicarbonate and kept close to this level by daily inspection and adjustment when necessary. The results in Table 1 show that the addition of some buffers indeed increased the growth rate of HEDLF cells in VSGCTH medium (10 mM HEPES + 10 mM TES, 20 mM BIS-TRIS, 10 mM TRIS). Some buffers gave good initial growth but the longevity was shortened (20 mM BIS-TRIS, 10 mM TRIS, 20 mM BICINE). TRIS at 20 mM was inhibitory to growth after only 10 cell divisions (50 days incubation) but during the first 2 passages produced good growth. Most buffers yielded the same growth rate as unbuffered VSGCTH medium, but in no case was the longevity of the cells increased. The cells required about 60 days to adapt to 20 mM TRICINE adjusted to pH 7.9 at the start of the cultures, but they grew without any lag at pH 7.6. Once the cells were adapted to or selected by the higher pH, they grew at a rate comparable to unbuffered VSGCTH but stopped at an earlier age. It appeared that only TRICINE, HEPES and TES could be used over extended periods of time without causing a decrease in longevity.

The average attachment of cells on the glass surface after overnight incubation is given in the various figure legends and Table 1. The range in most of these experiments varied between 10 and 100%. In most cases, the cell attachment in the various deficient media was less than in Eagle's MEM (69%). The presence of glutamine and cystine in the media appeared to be necessary for optimal attachment. No other amino acid and none of the buffers produced a significant increase in cell attachment.

**DISCUSSION**

The amino acid requirement of cells growing in vitro have long been divided into 2 groups: essential and non-essential. An amino acid is considered essential if cells are unable to grow in its absence. Eagle (1963) and Eagle & Piez (1962) have shown that cells are capable of synthesizing most of these essential amino acids but at concentrations too low to compete with dilution when the ratio of medium to cell volume is large. The requirement for many of these amino acids, therefore, becomes population dependent,
but in most cases the cell population would have to be of the order of 2-4 \times 10^4 \text{ cells/cm}^2 (0.5-1 \times 10^5 \text{ cells/ml}) or higher depending on the experimental conditions. However, the range of cell attachment in our experiments varied between 4 \times 10^2 \text{ and } 4 \times 10^3 \text{ cells/cm}^2 \text{ or } 10^4 \text{ and } 10^5 \text{ cells/ml}. Thus, the cell density on the glass surface at the beginning of each passage was low enough so that the amino acid synthesis in the cells probably could not compete against the dilution factor of the medium. Furthermore, the media used in our experiments were deficient in more amino acids than media used in the experiments reported by Eagle & Piez (1962).

It is most probable that the amino acids supplied by the serum played a significant role in the ability of HEDLF cells to grow in the various deficient media studied. The serum was not dialysed in our experiments because dialysis also removes small-molecular substances, other than amino acids, which may stimulate cell growth (Eagle, 1960; Griffiths, 1972; Ham, 1963; Metzgar & Moskowitz, 1960; Sato, Fisher & Puck, 1957). In addition one of the purposes of this study was to develop a simplified medium which would be of practical use in laboratories which would not routinely use dialysed serum for large-scale cell cultivation. It was found earlier (Litwin, 1972) that upon removal of any one of the amino acids in MEM containing undialysed calf serum, all cell growth ceased within 2-4 cell divisions, in agreement with the results of Eagle (1955a, b, d) and Freed & Schatz (1969) using dialysed serum. Thus the amino acids in the serum were not at high enough concentration to rescue the cells from inhibition of growth under these conditions. Jacobs (1966) who reported good growth of human diploid fibroblasts in a medium containing only glutamine and cystine used undialysed serum, also. However, we did not obtain as good growth with these 2 amino acids as was claimed by Jacobs (1966). The free amino acids available in serum, the continual release of amino acids and peptides into the medium by serum proteolytic enzymes (Piez, Oyama, Levintow & Eagle, 1960), the ability of cells to concentrate many of these amino acids (Eagle, 1959; Eagle, Piez & Levy, 1961; Piez & Eagle, 1958) and the synthesis of amino acids by these cells may explain how the diploid fibroblasts were capable of growing in a medium containing only 3 of the 13 essential amino acids. Thus, it was apparent that HEDLF cell growth similar to that in Eagle’s MEM was obtained in the presence of 4 mM glutamine, 0.4 mM cystine and 0.5 mM tyrosine plus the amino acids supplied by 10% calf serum at the following concentrations estimated from the serum amino acid values given by Piez et al. (1960) and Jacobs (1966): 0.016 mM threonine, 0.025 mM valine, 0.002 mM methionine, 0.007 mM isoleucine, 0.013 mM leucine, 0.006 mM phenylalanine, 0.017 mM lysine, 0.01 mM histidine, 0.008 mM arginine, and 0.0003 mM tryptophan. The cells must be able to concentrate these amino acids to a level which will support protein synthesis, 0.01-0.1 mM (Eagle et al. 1961). The function of glutamine, cystine and tyrosine under the conditions stated above is not clear, but they may have a sparing effect on the cell requirement for the other amino acids. The question that remains to be answered is: under these conditions how low can the concentration of the other amino acids be made before they become growth limiting? However, some of these results suggest that the balance of amino acids in the medium is a more important factor than their concentration.

The addition of other amino acids to the VSGCT medium gave highly variable
results. Histidine and to a lesser extent threonine was stimulatory for growth and longevity but methionine, valine and isoleucine reduced longevity without affecting the growth rate. Arginine and leucine reduced both growth rate and longevity, but phenylalanine was the most inhibitory of all the amino acids tried. The effects of these amino acids emphasize the value of following the growth response of HEDLF cells over long periods of time. Most of the effects did not become apparent before 30–60 days of cultivation (at least 3–4 passages).

The decreased buffering capacity of the deficient media permitted rapid changes in pH during the growth period, which probably affected cell growth adversely. Since Eagle (1971) showed that the optimum pH for human diploid fibroblast growth was 7.6, we adjusted all buffers tried to this range after allowing the cells to attach to the glass at pH 7.2. In agreement with Eagle (1971), TRICINE, TES and HEPES gave the best growth results over long periods of cultivation. The maintenance of a stable and optimum pH had no influence on cell longevity.

It is known that withdrawing any of the 13 amino acids from Eagle’s MEM results in rapid cessation of growth (Eagle, 1955a–c; Eagle et al. 1957; Freed & Schatz, 1969; Litwin, 1972) and that good growth occurs in medium containing only VS + glutamine, cystine and tyrosine. It can be predicted, therefore, that as one continues to add amino acids to these 3 there should arise mixtures which will be growth inhibitory. A partial growth inhibition was already observed with phenylalanine, methionine, valine and to a lesser extent leucine, isoleucine and arginine. The fact that some amino acids such as histidine and threonine improved growth and longevity suggests that we may be able to find eventually the optimum amino acid composition for HEDLF cells.

The influence of amino acid balance on cell longevity may be of some importance in view of the recent findings and theories on the relationship between cell ageing and mistakes in protein synthesis (Lewis & Tarrant, 1972; Orgel, 1973). Holliday & Tarrant (1972) had shown that certain enzymes in aging human fibroblasts were more thermostable than the same enzyme in younger cells. Furthermore, Gershon & Gershon (1970, 1973) had shown that significant amounts of inactive enzymes are produced in ageing organisms. One possible way in which these mistakes could occur is that the wrong amino acids are selected for certain proteins. Lewis & Holliday (1970) reported that the amino acid analogue ethionine increased the rate of ageing in a mutant of Neurospora. Since many amino acids found in cells are structurally similar, differing only by a methyl or hydroxyl group, it is possible that as cells age their mechanism for selecting amino acids becomes less sensitive and cannot differentiate between very similar molecules. Thus, increasing the concentration of some amino acids may accentuate the mistakes made by ageing cells and reduce longevity, as we have seen occurs with phenylalanine, methionine, valine, leucine, isoleucine and arginine. On the other hand, if an amino acid is particularly important for the cell then an excess may reduce the incidence of mistakes and increase longevity, as occurred with excess tyrosine and histidine in Eagle’s MEM (Litwin, 1972).

Further information on the effect of amino acid balance on the growth and longevity of human diploid fibroblasts may be of value in studying the factors responsible for cell ageing.
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