NUTRITIONAL REQUIREMENTS FOR THE PRODUCTION OF POLIOVIRUS TYPE II, COXSACKIE B3, AND VACCINIA VIRUSES BY CONTINUOUS ANIMAL CELL CULTURES[1]

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Study of the nutritional requirements for the support of virus production by various animal cells has been greatly facilitated by the development of continuous animal cell cultures. Darnell and Eagle (1958) have shown that HeLa cell cultures are capable of supporting maximal production of poliovirus type I in a balanced salt + glucose + glutamine medium. Mouse fibroblast cell cultures latently infected with psittacosis virus were shown by Bader and Morgan (1958) (Morgan and Bader, 1957) to require the addition of glucose, water soluble vitamins, and nine amino acids for active infection to occur. A Hanks' balanced salts + cysteine medium was shown by Rappaport (1956) to support poliovirus production in primary monkey kidney cell cultures. Dubes (1956) has also shown cystine to be a requirement in the production of poliovirus by monkey kidney cells in culture. Chang (1959) has shown that Coxsackie B1, vaccinia, and to some extent, poliovirus production are inhibited by a bicarbonate depletion in certain cell cultures.

This report concerns the importance of L-cystine in the production of Coxsackie B3 virus, vaccinia virus, and to a lesser extent, poliovirus type II in four continuous cell cultures.

MATERIALS AND METHODS

Cell cultures. The four continuous cell cultures employed in this study were rabbit kidney (Ludwig, 1959, *unpublished data*), chick embryo vascular endothelium (Ludwig, 1959, *unpublished data*), monkey heart, and HeLa. The rabbit kidney and chick embryo vascular endothelium cell cultures were developed in this laboratory. The monkey heart and HeLa cell cultures were obtained from the Virus Research Laboratory, University of Pittsburgh.

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Viruses. Coxsackie B3, vaccinia, and poliovirus type II were employed in this study. Twelve different virus pools were prepared by inoculating bottle cultures of each of the four cell lines with each of the three viruses. The inoculated cultures were incubated at 37 C with 10 ml of medium 199 per bottle as the supporting medium.

Growth medium. The medium employed for supporting active growth of the various cell cultures was of the following composition: enzymatic lactalbumin (10 per cent) hydrolyzate (Nutritional Biochemical), 5 ml; bovine serum, 10 ml; Hanks' balanced salt solution, 85 ml; penicillin G, 10,000 units; dihydrostreptomycin, 10,000 μg; polymyxin B, 5,000 units; mycostatin, 5,000 units.

Maintenance medium. Medium 199 (Morgan, Morton, and Parker, 1950) diluted to one part in three with Hanks' balanced salt solution was used routinely as a cell culture maintenance medium, as the supporting medium for all virus titrations, and for various test purposes. All test media composed of the various components of medium 199 were prepared in such a manner that the concentrations of the components were equivalent to those found in a one part in three dilution of complete medium 199. The antibiotics employed and their concentrations in these media are the same as those employed in the growth medium.

Virus production. All of the experiments in this study which tested the ability of various media to support virus production in the different cell lines were carried out in tube cultures containing approximately 7×10^4 cells per tube. Groups of five tubes were routinely used for each test system. The tube cultures were thoroughly washed and starved in glucose-free balanced salt solution 4 to 6 hr prior to use. A virus inoculum of 100 $TCID_{50}$ prepared in Hanks' balanced salt solution was employed in all such experiments. Virus production in these systems was determined

by harvesting the supernatant fluids from the inoculated cultures and subsequently titrating these harvests to determine the virus yield. The 50 per cent end points of these titrations were calculated by the method of Reed and Muench (1938). In some experiments the supernatant fluids were harvested and replaced with fresh test media at various intervals over an 18 to 96 hr period. In other experiments a single harvest was made for each test system. The final harvests in all cases were made after the culture tubes were frozen and thawed.

RESULTS

Effect of various components of medium 199 upon virus production in various cell-virus systems. Medium 199 had been shown in preliminary experiments to be capable of supporting virus production in the 12 cell-virus systems. Thus the first series of experiments was designed to test various components of medium 199 for their ability to support virus production in these systems. The four media employed in these experiments were as follows: balanced salt solution + glucose + L-glutamine; balanced salt solution + the 20 amino acids of medium 199; balanced salt solution + glucose + L-glutamine + the 20 amino acids of medium 199; and medium 199.

Cell cultures were overlaid with each of the above media and inoculated with 100 $TCID_{50}$ of the appropriate virus. In the case of Coxsackie B3 and poliovirus type II production, the supporting medium of each group of test cultures was harvested at 18, 24, 42, and 66 hr. The media of the cell cultures supporting vaccinia virus production were harvested at 18, 42, 72, and 96 hr. The resultant titers of these various harvests are shown in figures 1 through 12. All uninoculated control cells overlaid with the four test media were negative for virus.

It is evident from figures 1 through 12 that the combination of the amino acid group with glucose and glutamine will support virus production in all twelve cell-virus systems to a level comparable to that of medium 199.

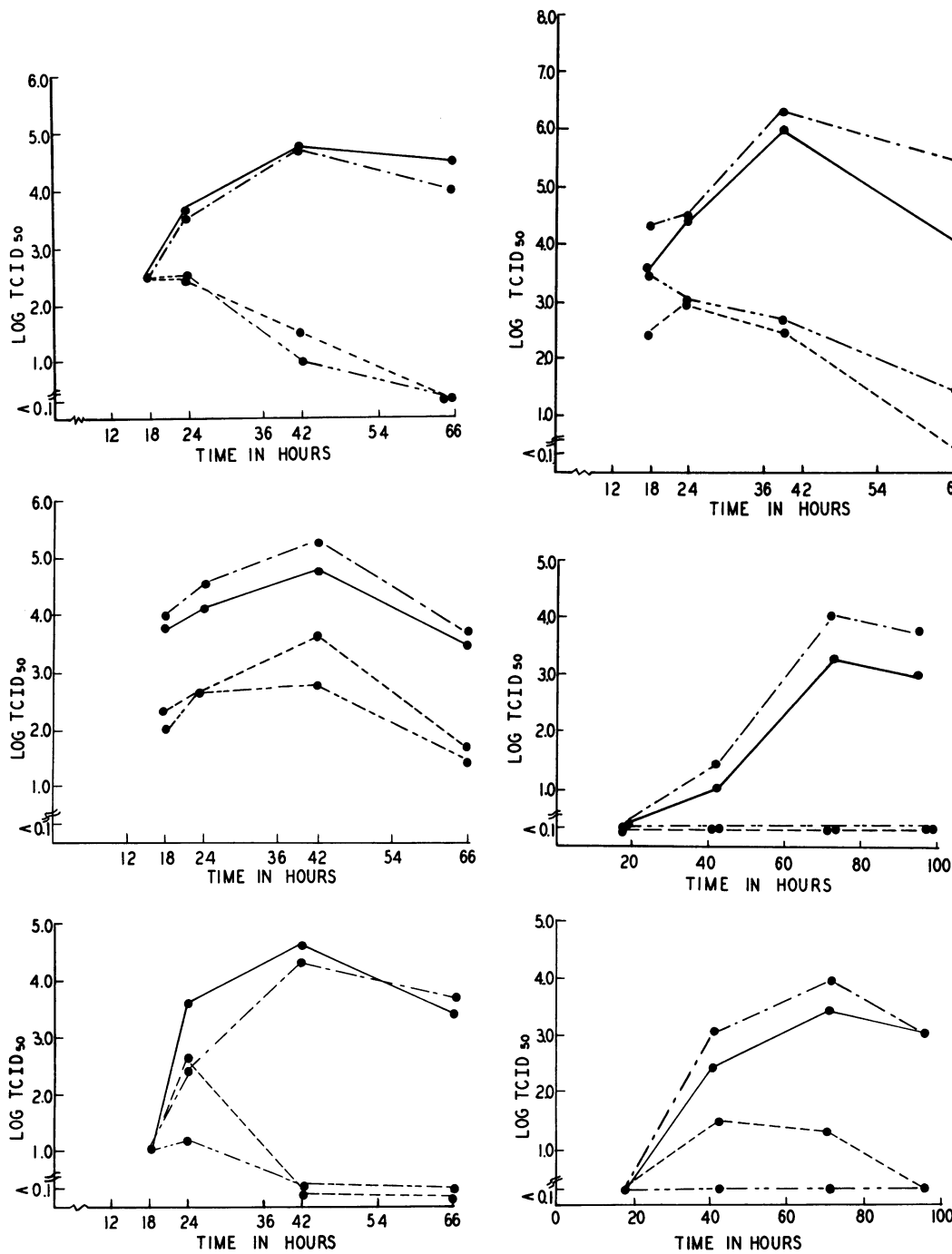
In a comparison of Coxsackie B3, poliovirus type II, and vaccinia virus production, it appears that in the rabbit kidney, chick embryo vascular endothelium, and HeLa cell cultures the dependency upon the amino acid group is not as marked in the case of poliovirus type II production as with the other two viruses. In particular, the production of poliovirus in HeLa cells reveals a near maximal yield in the balanced salt + glucose + glutamine medium. This is in agreement with the investigations of Darnell and Eagle (1958). Maximal poliovirus type II production in monkey heart cells, however, does require the addition of the amino acid group. It should be noted that the balanced salt solution + amino acid medium contains some glucose carried over with the virus inoculum.

Effect of amino acid group upon adsorption of Coxsackie B3 virus in monkey heart cells. Two groups of thoroughly washed monkey heart cell cultures were overlaid with balanced salt + glucose + glutamine medium and inoculated with 100 $TCID_{50}$ of Coxsackie B3 virus. After 2 hr incubation, the two groups of cultures were thoroughly washed with balanced salt solution to remove any unadsorbed virus. The last washings from both groups were retained. A balanced salt + glucose + glutamine medium was then added to one group of cells and a balanced salt + glucose + glutamine + amino acid medium was added to the second group of cells. The supporting media of both groups were harvested at 18, 24, 48, and 72 hr. The last washings from both groups of cells and the various media harvests were then titrated. The last washings of both groups were negative for virus. The titers of the various harvests from the two groups of cells revealed that the cells to which the amino acid group was added exhibited a normal pattern of virus production with a peak titer of 1×10^4 $TCID_{50}$ per 0.1 ml. The other group of cells in the absence of the amino acid group exhibited minimal virus production with a peak titer of $1 \times 10^{1.5}$ $TCID_{50}$ per 0.1 ml.

The absence of any titratable virus in the last washings from the two groups of cells indicates that any subsequent virus production would result from virus adsorbed prior to the washing procedure and before the addition of the amino acid group. Virus production in the cultures to which the amino acid group was added indicates that the amino acid group is not necessary for the initial adsorption of Coxsackie B3 virus in monkey heart cells.

Determination of specific amino acid requirements of Coxsackie B3 production in monkey heart cells. The 20 amino acids of medium 199 were divided into four groups as follows:

I. L-Arginine, L-histidine, L-lysine, L-tyrosine, DL-trytophan, and DL-phenylalanine.



Figures 1–6. Effect of various components of medium 199 upon virus production in various cell-virus systems. Balanced salt solution (BSS) + glucose + glutamine, ●- - - - - - - -●; BSS + glucose + glutamine + amino acids, ●- - - - -●; BSS + amino acids, ●-----●; Medium 199, ●——●.

Figure 1 (top, left). Coxsackie B3 virus production in monkey heart cells.

Figure 2 (center, left). Coxsackie B3 virus production in rabbit kidney cells.

Figure 3 (bottom, left). Coxsackie B3 virus production in chick embryo vascular endothelium cells.

Figure 4 (top, right). Coxsackie B3 virus production in HeLa cells.

Figure 5 (center, right). Vaccinia virus production in monkey heart cells.

Figure 6 (bottom, right). Vaccinia virus production in rabbit kidney cells.

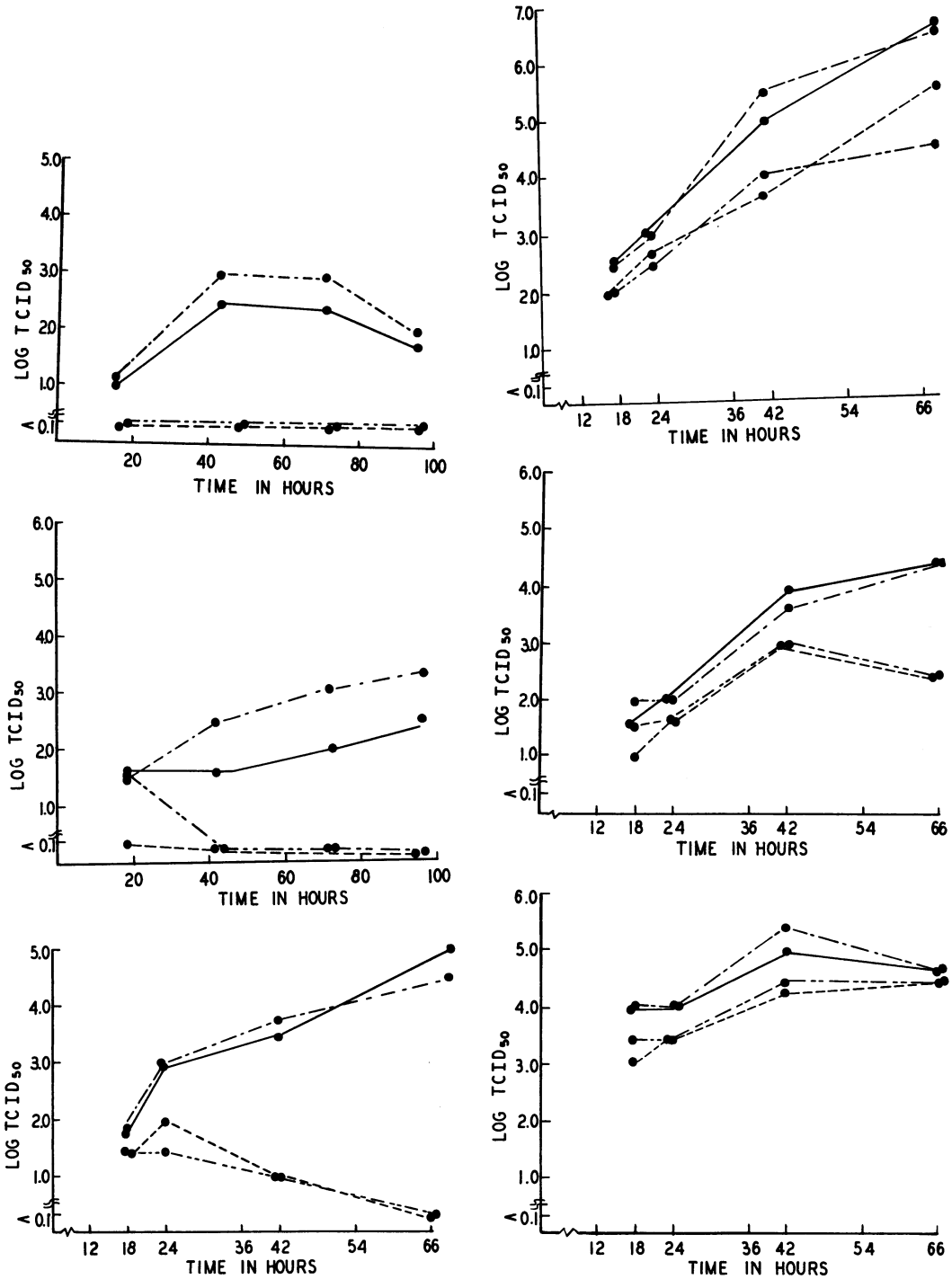


Figure 7–12. Effect of various components of medium 199 upon virus production in various cell-virus systems. Balanced salt solution (BSS) + glucose + glutamine, ●- - - - - - -●; BSS + glucose + glutamine + amino acids, ●- - - - -●; BSS + amino acids, ●- - - - -●; Medium 199, ●——●.

Figure 7 (top, left). Vaccinia virus production in chick embryo vascular endothelium cells.

Figure 8 (center, left). Vaccinia virus production in HeLa cells.

Figure 9 (bottom, left). Poliovirus II production in monkey heart cells.

Figure 10 (top, right). Poliovirus II production in rabbit kidney cells.

Figure 11 (center, right). Poliovirus II production in chick embryo vascular endothelium cells.

Figure 12 (bottom, right). Poliovirus II production in HeLa cells.

II. DL-Serine, DL-threonine, DL-leucine, DL-isoleucine, DL-valine, and DL-glutamic acid.

III. DL-Aspartic acid, DL-alanine, L-hydroxyproline, L-proline, and glycine.

IV. L-Cystine, L-methionine, and cysteine.

Each of the amino acid groups was dissolved in Hanks' balanced salt solution and tested for its ability to support Coxsackie B3 virus production in monkey heart cells. Three serial passages of the virus were made in each of the four media. Harvests of these passages were made 48 hr after inoculation of the virus. The titrations of these various harvests revealed that none of the virus passages in amino acid groups I, II, or III exhibited a titer greater than $1 \times 10^{1.5}$ $TCID_{50}$ per 0.1 ml. The titers of group IV passages, however, ranged from $1 \times 10^{3.5}$ to $1 \times 10^{4.2}$ $TCID_{50}$ per 0.1 ml. All uninoculated control cells were negative for virus.

It is evident from these data that the amino acid group IV possesses the effective amino acid or acids of the nineteen member amino acid group of medium 199.

Group IV consisted of the sulfur containing amino acids L-cystine, L-methionine, and cysteine. Three passages of Coxsackie B3 virus in monkey heart cells supported by a cystine-Hanks' balanced salt solution medium and a methionine-balanced salt solution medium were made in the same manner as with the four amino acid groups. The virus titers of these passages revealed a two log increase in titer in the harvests of the cystine-balanced salt solution medium as compared with the harvests of the methionine-balanced salt solution medium.

It is evident from the two preceding experiments that the amino acid L-cystine in balanced salt solution is capable of supporting the effective production of Coxsackie B3 virus in monkey heart cells.

Effect of various oxidation-reduction controllers upon Coxsackie B3 virus production in monkey heart cells. Solutions of 26 μM thioglycolate (oxidized), ascorbic acid, glutathione, cystine, and a 52 μM solution of cysteine dissolved in Hanks' balanced salt solution + glutamine were tested for their ability to support production of Coxsackie B3 virus in monkey heart cells.

Each of these test media as well as a balanced salt solution + glucose + glutamine medium and medium 199 was used as the supporting medium for cells inoculated with 100 $TCID_{50}$ of Coxsackie B3 virus. Harvests of these media were made 48 hr after inoculation of the virus. There was no evident cytopathogenic effect at the time of harvest in the cells overlaid with the thioglycolate, glutathione, ascorbic acid, and balanced salt solution + glucose + glutamine media, whereas marked degeneration was evident in the cells supported by the cystine, cysteine, and 199 media. The resultant titers obtained with the thioglycolate, glutathione, ascorbic acid, and balanced salt solution + glucose + glutamine media ranged from $1 \times 10^{2.0}$ to $1 \times 10^{3.0}$ $TCID_{50}$ per 0.1 ml. The titers obtained with the cystine, cysteine, and 199 media ranged from $1 \times 10^{5.3}$ to $1 \times 10^{5.7}$ $TCID_{50}$ per 0.1 ml. It is apparent that neither thioglycolate, ascorbic acid, nor glutathione is capable of replacing the cystine or cysteine in supporting maximum Coxsackie B3

TABLE 1

Poliovirus, type II; vaccinia, and Coxsackie B3 virus production in monkey heart cells

Virus	Test Media							
	I BSS* + glucose + glutamine		II BSS + glucose + glutamine + cyst(e)ine		III BSS + glucose + glutamine + 18 amino acids without cyst(e)ine		IV Medium 199	
	CPE†	Titer‡	CPE	Titer	CPE	Titer	CPE	Titer
Coxsackie B3	Negative	2.0	Positive	5.7	Doubtful	2.3	Positive	5.5
Poliovirus II	Negative	2.0	Positive	4.3	Positive	2.0	Positive	4.0
Vaccinia	Negative	1.5	Positive	3.5	Negative	1.0	Positive	3.3

* BSS = balanced salt solution.

† Cytopathogenic effect.

‡ Log $TCID_{50}$.

All uninoculated control cells were negative for virus.

virus production in monkey heart cells under these experimental conditions.

Effect of L-cystine on the 12 cell-virus systems. The following experiments were carried out to determine the effect of L-cystine in all 12 cell-virus systems. Each of the 12 different systems was tested for virus production in four different media. The compositions of these four media are as follows:

I. Balanced salt solution + glucose + L-glutamine.

II. Balanced salt solution + glucose + L-glutamine + L-cystine.

III. Balanced salt solution + glucose + L-glutamine + 18 amino acids of medium 199 excepting L-cystine and cysteine.

IV. Medium 199.

Four groups of each of the cell cultures were overlaid with the four media and inoculated with 100 $TCID_{50}$ of the appropriate virus. The Coxsackie B3 and poliovirus type II systems were harvested after 48 hr; the vaccinia systems were harvested after 72 hr. The cytopathogenic effect at the time of harvest and the resultant virus titers of the various systems are illustrated in tables 1 through 4.

The data in tables 1 through 4 reveal that the presence of L-cystine markedly stimulates the production of Coxsackie B3 and vaccinia viruses in the four cell cultures. It appears from these results that the effect of the amino acid group as previously shown in figures 1 through 12 is due solely to the presence of L-cyst(e)ine. The remaining combination of 18 amino acids seems to

TABLE 2

Poliovirus, type II; vaccinia, and Coxsackie B3 virus production in rabbit kidney cells

Virus	Test Media							
	I BSS* + glucose + glutamine		II BSS + glucose + glutamine + cyst(e)ine		III BSS + glucose + glutamine + 18 amino acids without cyst(e)ine		IV Medium 199	
	CPE†	Titer‡	CPE	Titer	CPE	Titer	CPE	Titer
Coxsackie B3.....	Negative	3.3	Positive	5.0	Negative	2.5	Positive	5.3
Poliovirus II.....	Positive	4.5	Positive	6.0	Positive	5.3	Positive	6.0
Vaccinia.....	Negative	1.3	Positive	4.0	Negative	1.0	Positive	3.7

* BSS = balanced salt solution.
† Cytopathogenic effect.
‡ Log $TCID_{50}$.
All uninoculated control cells were negative for virus.

TABLE 3

Poliovirus, type II; vaccinia, and Coxsackie B3 virus production in chick embryo cells

Virus	Test Media							
	I BSS* + glucose + glutamine		II BSS + glucose + glutamine + cyst(e)ine		III BSS + glucose + glutamine + 18 amino acids without cyst(e)ine		IV Medium 199	
	CPE†	Titer‡	CPE	Titer	CPE	Titer	CPE	Titer
Coxsackie B3.....	Negative	2.5	Positive	5.0	Negative	2.5	Positive	5.0
Poliovirus II.....	Doubtful	4.0	Positive	6.5	Positive	3.7	Positive	5.5
Vaccinia.....	Negative	2.5	Positive	3.7	Negative	2.0	Positive	4.0

* BSS = balanced salt solution.
† Cytopathogenic effect.
‡ Log $TCID_{50}$.
All uninoculated control cells were negative for virus.

TABLE 4

Poliovirus, type II; vaccinia, and Coxsackie B3 virus production in HeLa cells

Virus	Test Media							
	I BSS* + glucose + glutamine		II BSS + glucose + glutamine + cyst(e)ine		III BSS + glucose + glutamine + 18 amino acids without cyst(e)ine		IV Medium 199	
	CPE†	Titer‡	CPE	Titer	CPE	Titer	CPE	Titer
Coxsackie B3.....	Negative	4.3	Positive	5.3	Negative	4.3	Positive	5.3
Poliovirus II.....	Positive	5.5	Positive	6.5	Positive	5.3	Positive	6.0
Vaccinia.....	Negative	2.0	Positive	3.5	Negative	2.0	Positive	3.3

* BSS = balanced salt solution.

† Cytopathogenic effect.

‡ Log $TCID_{50}$.

All uninoculated control cells were negative for virus.

have little or no effect. Poliovirus type II production, particularly in rabbit kidney and HeLa cells, shows a cytopathogenic effect and a near maximal virus titer in the cell cultures overlaid with a balanced salt solution + glucose + glutamine medium in the absence of L-cyst(e)ine. This supports the results shown in figures 10 and 12. Maximum production of poliovirus type II by monkey heart cells, however, requires the presence of L-cyst(e)ine. Since the cells were ruptured by freezing and thawing at the time of harvest, it can be seen that the reduced virus yield from the cells infected in the absence of cyst(e)ine is not a result of any impediment to virus release.

DISCUSSION

Implication of amino acids in viral synthesis has been demonstrated by several investigators. Ackermann (1951) has demonstrated the requirement for L-methionine in the production of influenza A virus by chick embryo chorioallantoic membrane cultures. Ackermann, Rabson, and Kurtz (1954) have likewise demonstrated the reversal of fluorophenylalanine inhibition of poliovirus type III production by the addition of excess phenylalanine. The amino acids tyrosine, threonine, methionine, leucine, tryptophan, isoleucine, cyst(e)ine, phenylalanine, and valine have been shown by Bader and Morgan (1958) to be necessary for the activation of latent psittacosis infections of mouse fibroblast cell cultures. As previously mentioned, Dubes (1956) has shown a requirement for cystine in poliovirus production.

The importance of L-cystine in the production of Coxsackie B3, vaccinia, and to some extent, poliovirus type II in four continuous cell cultures has been illustrated in this investigation.

Since the L-cystine effect in this study is apparently not involved in the adsorption of the virus onto the host cell nor in the release of the virus from the cell, it would then appear to be operative during the actual replication of the new virus. Furthermore, the inability of glutathione, ascorbic acid, and thioglycolate to effectively replace cystine or cysteine indicates that cystine is probably not acting as a hydrogen acceptor or environmental stabilizer.

These observations may indicate that cystine is being utilized as an actual structural unit in the synthesis of new virus or in the synthesis of enzymes involved in the production of virus.

It is certainly not unlikely that a sulfur-containing amino acid could be involved in the synthesis of virus particles. It would be unreasonable, however, to assume that many of the other amino acids are not involved in virus production. Thus the requirement for only L-cystine may be an indication of an extensive amino acid pool in the infected cell. If this is the case it would seem that the L-cystine reserve is either lacking or at a minimal level in the various cell cultures, whereas the reserve of other amino acids involved in virus production is substantial enough so that addition of these amino acids to the supporting medium of the cell cultures is not required for support of virus production. It is of interest in this respect that studies by Piez and Eagle (1958) of the free

amino acid pools of three continuous cell cultures have revealed a minimal cellular reserve of cystine in all three cultures.

The fact that certain cell cultures require the presence of L-cystine for the effective production of Coxsackie B3 and vaccinia viruses but are capable of producing near maximal poliovirus type II yields in the absence of L-cystine may indicate an inherent difference in the actual structure or in the mode of synthesis of poliovirus type II as compared with Coxsackie B3 and vaccinia viruses. As previously mentioned, Chang (1959) has shown a difference in the production of Coxsackie B1 and vaccinia viruses as compared with poliovirus type I in bicarbonate depleted cell cultures. In these experiments poliovirus production was not as markedly inhibited by the bicarbonate depletion as was Coxsackie B1 and vaccinia virus production.

This study has also revealed a difference between the monkey heart cells in culture and the rabbit kidney and HeLa cell cultures. As shown in figure 9 and table 1, the monkey heart cells require the presence of L-cystine in the supporting medium for the maximal production of poliovirus type II. The rabbit kidney and HeLa cell cultures do not exhibit this marked dependency upon L-cystine for the support of poliovirus type II production. This again may be an indication of a quantitative difference between the L-cystine reserve of monkey heart cells and the rabbit kidney and HeLa cell cultures.

This investigation has thus demonstrated that under the experimental conditions employed, the nutritional requirements for the support of virus production in continuous cell cultures is dependent upon both the particular cell culture and the particular virus employed.

SUMMARY

The nutritional requirements, in terms of the components of medium 199, for the support of virus production in four continuous cell cultures were investigated.

The amino acid L-cystine was found to be necessary for the support of Coxsackie B3 and vaccinia viruses in rabbit kidney, chick embryo vascular endothelium, monkey heart, and to some extent in HeLa cell cultures. The support of poliovirus type II in monkey heart cells also requires L-cystine. Near maximal titers of poliovirus type II were produced by rabbit kidney and HeLa cell cultures in a balanced salt solution + glucose + glutamine medium in the absence of L-cystine.

Coxsackie B3 virus could be adsorbed onto monkey heart cells in the absence of L-cystine. Thioglycolate, ascorbic acid, and glutathione were incapable of effectively replacing cystine or cysteine in the support of Coxsackie B3 virus in monkey heart cells.

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