L-CYSTINE REQUIREMENT FOR PRODUCTION OF COXSACKIE B3
VIRUS IN CULTURED MONKEY HEART CELLS

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ABSTRACT

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L-Cystine requirement for production of Coxsackie B3 virus in cultured monkey heart cells.
J. Bacteriol. 85:1339-1345. 1963.—The requirement for L-cystine in both the production of Coxsackie B3 virus in cultured monkey heart cells and in prolonging the survival of the cultured monkey heart cells is demonstrated. D-Cystine, DL-homocystine, DL-allo-cystathionine, DL-homocysteine thiolactone, 2-mercaptoethamine, allylglycine, 3,3'-dithiopropionic acid, and DL-ethionine could not replace L-cystine in either supporting Coxsackie B3 virus production or prolonging monkey heart cell culture survival time. By their ineffectiveness, these compounds suggest the specificity of the L-cystine requirement. Allylglycine, 3,3'-dithiopropionic acid, and DL-ethionine were likewise incapable of inhibiting the L-cystine effect in supporting both virus production and cell survival. Prolonged starvation of cell cultures prior to virus inoculation failed to reveal any additional marked nutritional requirements, but rather tended to accentuate the L-cystine requirement for virus production. Increased cell starvation did, however, lead to the establishment of a latent Coxsackie B3 virus infection of cultured monkey heart cells, the activation of which is L-cystine-dependent.

The requirement for the amino acid L-cystine (L-cystine) in a variety of cell-virus systems has been previously demonstrated (Dubes, 1956; Rappaport, 1956; Tyndall and Ludwig, 1960).

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The present investigation was undertaken to study more thoroughly the cystine requirement in the production of Coxsackie B3 virus by cultured monkey heart cells in terms of its specificity, possible inhibition, optimal concentration, and most importantly the consequences of its absence. These aspects of the L-cystine requirement were also studied in regard to their effect upon supporting the survival of monkey heart cell cultures.

MATERIALS AND METHODS

Cell culture. The monkey heart cell culture employed in this study was originally obtained from the Virus Research Laboratory, University of Pittsburgh. The culture was routinely propagated in growth medium of the following composition: 10% lactalbumin hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio), 5 ml; bovine serum, 10 ml; Hanks balanced salts solution (BSS), 85 ml; penicillin G, 10,000 units; dihydrostreptomycin, 10,000 µg; polymyxin B, 2,500 units; and nystatin, 5,000 units. All experiments and titrations in this study employed tube cultures prepared with approximately 10⁴ cells. Groups of four tubes were routinely used for each test system and each titration dilution.

Virus. The Coxsackie B3 virus pools were prepared by inoculating monkey heart cell cultures overlaid with 10 ml of Medium 199. The resultant virus pools were titrated and the number of 50% infectious doses for tissue culture per 0.1 ml (TCID₅₀) was determined by the method of Reed and Muench (1938). The virus inocula used in the different experiments were then prepared by the appropriate dilution of the virus pool in BSS.

Medium 199. Medium 199 diluted to one part in three with BSS was used as a maintenance medium for all virus titrations, as the supporting medium in the preparation of virus pools, and in some test systems.
**Balanced salts-glutamine medium (BSS + G).** BSS + G medium was prepared by the addition of 33 mg of L-glutamine to 1 liter of BSS.

**Cystine medium.** The cystine test medium was prepared by the addition of 7.0 mg of L-cystine to 1 liter of BSS + G medium.

**L-Cystine inhibitors and substitutes.** The stereoisomers D-cystine, the homologue DL-homocystine, and the possible metabolic intermediates DL-α-cystathionine, DL-homocysteine thiolactone, and 2-mercaptoethylamine were tested for their ability to support either Coxsackie B3 virus production or the survival of monkey heart cell cultures. The possible cystine inhibitors allylglycine, 3,3′-dithiopropionic acid, and DL-ethionine were tested for their ability to inhibit or support both Coxsackie B3 virus production and the survival of the monkey heart cell cultures.

To test these various compounds, 13 groups of monkey heart cell cultures were prepared. Each group consisted of seven tubes of 48-hr cultures of monkey heart cells. After a thorough washing and a 4-hr period of starvation in BSS, the various groups of cultures were overlaid with BSS + G medium containing the following test compounds: (1) D-cystine, (2) DL-homocystine, (3) DL-α-cystathionine, (4) DL-homocysteine thiolactone, (5) 2-mercaptoethylamine, (6) allylglycine, (7) 3,3′-dithiopropionic acid, (8) DL-ethionine, (9) allylglycine + L-cystine, (10) 3,3′-dithiopropionic acid + L-cystine, (11) DL-ethionine + L-cystine, (12) L-cystine. Group 13 served as a BSS + G control.

After addition of the test compounds, four cultures in each group were inoculated with approximately 300 TCID₅₀ of Coxsackie B3 virus. After 48 hr, these cultures were observed for evidence of cytopathic effect (CPE), harvested, and titrated for virus. The remaining three tubes per group were observed daily, and the average survival time was determined. The test medium on the uninfected control cells were changed every 3 days. The criterion for cell survival was the presence of unrounded cells adhering to the sides of the culture tubes which, upon addition of growth medium, still retained the ability to multiply.

**Cystine concentration studies.** Experiments designed to determine the optimal L-cystine concentration for maximal Coxsackie B3 virus production in monkey heart cells were carried out as follows. Six groups of six 48-hr monkey heart cell tube cultures were starved in BSS for a period of 4 hr. After starvation, each culture was overlaid with 1.0 ml of BSS + G medium. The first group of cultures received no cystine. Each of the five remaining groups received increasing amounts of cystine. The concentrations in milligrams of cystine per liter of medium were as follows: group 2, 0.05; group 3, 0.2; group 4, 0.6; group 5, 1.0; and group 6, 2.0.

After the addition of cystine, four cultures of six per group were inoculated with approximately 300 TCID₅₀ of Coxsackie B3 virus. The remaining two cultures in each group served as cell controls. The test cells were observed for the degree of CPE 48 hr after inoculation, after which the fluids and cells were frozen and thawed, pooled, and titrated for virus. The effect of the various concentrations of cystine on the degree of degeneration of the uninoculated control cells was also observed.

**Cell starvation studies.** An earlier study demonstrating the requirement for L-cystine in the production of Coxsackie B3 virus employed cell starvation times prior to virus inoculation of from 4 to 6 hr (Tyndall and Ludwig, 1960). It was possible, therefore, that additional nutritional requirements might be revealed by increasing the cell starvation time. The following experiment was performed to test this premise.

Three groups of cell cultures were starved in BSS for 4, 48, and 96 hr, respectively. Three test media were subsequently used in each group: BSS + G medium, cystine medium, and Medium 199. Following the appropriate starvation period, the cultures in each group were overlaid with one of the test media and inoculated with approximately 100 TCID₅₀ of Coxsackie B3 virus. Harvests of the cells with supporting medium were made in each case 48 hr after virus inoculation and the cells were titrated for virus.

**Establishment of a latent infection.** It had previously been demonstrated that Coxsackie B3 virus would be adsorbed by starved monkey heart cells in a cystine-free medium (Tyndall and Ludwig, 1960). Therefore, the following experiments were devised to determine whether, in the absence of cystine, inoculation of Coxsackie B3 virus might result in establishing a masked or latent infection.

Four groups of thoroughly washed monkey heart cell cultures were starved for 24 hr in BSS and then overlaid with BSS + G medium and
inoculated with approximately 300 TCID₅₀ of Coxsackie B3 virus. One group of these test cultures was retained as a BSS + G control group. A second group of test cultures was overlaid with cystine medium at the time of virus inoculation. At 48- and 96-hr intervals after virus inoculation, the third and fourth groups of test cultures were thoroughly washed, then overlaid with fresh cysteine medium. Both supernatant fluids and cells were harvested from each test group 48 hr after the initial addition of cysteine medium. All harvests were then frozen and thawed and titrated for virus. Inoculated control cultures (48 and 96 hr), overlaid with BSS + G medium, were likewise thoroughly washed, frozen and thawed, and titrated for residual virus. Supernatant harvests (24 hr) from the inoculated control groups were also titrated for virus.

**RESULTS**

**L-Cystine inhibitors and substitutes.** It is evident from the data in Table 1 that D-cystine, DL-homocystine, DL-allo-cystathionine, DL-homo-cysteine thiolactone, and 2-mercaptoethylamine support neither maximal Coxsackie B3 virus production nor, with the possible exception of allo-cystathionine, the survival of the monkey heart cell cultures. It is also evident that allylglycine, 3,3'-dithiopropionic acid, and DL-ethionine (used in the concentrations shown in Table 1) neither inhibit nor support Coxsackie B3 virus production or monkey heart cell culture survival.

**Cystine concentration studies.** The appearance of the control cells as well as the CPE and virus titers of the inoculated cells of a representative experiment are shown in Table 2. Figure 1 presents the data from four experiments. The optimal concentration of cystine in the supporting medium in terms of virus production was found to be approximately 1.0 mg per liter, or 0.0042 μmole/ml.

It is also interesting to note that the degree of degeneration of the control cultures showed a graded response similar to that for virus production, in that cell degeneration was minimal at a cystine concentration of approximately 1 mg per liter of medium.

| TABLE 1. Effect of various cystine inhibitors and substitutes on Coxsackie B3 virus production and monkey heart cell survival |
|---|---|---|
| Test compound | Conc | Virus titer* |
| | moles/ml | days |
| (1) D-Cystine | 0.03 | 2.5 | 8 |
| (2) DL-Homocystine | 0.06 | 2.3 | 7 |
| (3) DL-Allo-cystathionine | 0.06 | 3.0 | 10 |
| (4) DL-Homo-cysteine thiolactone | 0.06 | 2.0 | 7 |
| (5) 2-Mercaptoethylamine | 0.03 | 3.0 | 6 |
| (6) Allylglycine | 0.06 | 2.0 | 7 |
| (7) 3,3'-Dithiopropionic acid | 0.03 | 2.0 | 7 |
| (8) DL-Ethionine | 0.06 | 2.3 | 6 |
| (9) Allylglycine | 0.07-2.0 | 5.7 | 12 |
| + L-cystine | 0.03 |  |  |
| (10) 3,3'-Dithiopropionic acid | 0.07-0.7 | 5.7 | 13 |
| + L-cystine | 0.03 |  |  |
| (11) DL-Ethionine | 0.07-0.7 | 5.3 | 12 |
| + L-cystine | 0.03 |  |  |
| (12) L-Cystine | 0.03 | 5.5 | 13 |
| (13) BSS+G† | — | 2.0 | 7 |

*Expressed as the logarithm of the number of TCID₅₀ per 0.1 ml.
†Hanks basal salts solution plus glutamine.

| TABLE 2. Effect of various cystine concentrations on Coxsackie B3 virus production and appearance of monkey heart cells |
|---|---|---|---|
| Cystine conc | Coxsackie B3 inoculated cultures | Uninoculated control cultures* |
| mg/liter | CPE | Titer† | |
| 0.0 | — | 2.3 | + |
| 0.05 | + | 3.0 | + |
| 0.2 | ++ | 3.7 | + |
| 0.6 | +++ | 4.7 | ± |
| 1.0 | ++++ | 5.3 | 0 |
| 2.0 | ++++ | 5.5 | 0 |

*Degree of degeneration.
†Expressed as the logarithm of the number TCID₅₀ per 0.1 ml.

**Cell starvation studies.** As shown in Fig. 2, an increase in the cell culture starvation time prior to virus inoculation resulted in decreasing virus yields in all three test media. Part of this de-
crease was probably due to a noticeable loss of cells, particularly after 96 hr of starvation.

The 96-hr starvation period revealed some need for other nutrients (in addition to L-cystine) to obtain virus yields comparable with those obtained with Medium 199. This may merely represent requirements for prolonging cell survival which in turn would result in higher virus yields. However, after 48 hr of starvation, cystine medium could support nearly maximal virus yields. These experiments thus tended to accentuate the L-cystine requirement, as well as raise the question of the fate of virus inoculated into starved cells devoid of exogenous cystine.

Establishment of a latent infection. That the establishment of a latent Coxsackie B3 virus infection results from inoculating starved cells with Coxsackie B3 virus is illustrated in Fig. 3. Little or no virus could be detected in the washed test cultures 2 to 4 days after virus inoculation. Upon addition of cystine to these cells, however, a marked rise in virus titer was observed. It is interesting to note that the addition of cystine to the 48- and 96-hr latently infected cells, which contained minimal demonstrable virus, resulted in a rise in virus titer at a rate comparable with that of cells given cystine at the time of inoculation with approximately 300 TCID50 of Coxsackie B3 virus.

**DISCUSSION**

In the production of Coxsackie B3 virus by monkey heart cell cultures, the contribution of the virus to the replication of its kind is in its unique incitement of the infected host cells. This incitement redirects the synthetic and energy-yielding reactions of the host cell to the task of reproducing the invading virus. Thus, the study of the cystine requirement in the production of Coxsackie B3 virus by monkey heart cells should likewise consider the effect of the cystine upon the cellular portion of the cell-virus complex.

The importance of L-cystine in the production
of Coxsackie B3 virus in the monkey heart cell cultures is illustrated in Table 1, which reveals that addition of this compound to the BSS + G medium results in a significant increase in virus production. The addition of cystine to the BSS + G medium also has an effect upon the cell cultures per se in that the average culture survival time is increased from 7 to 13 days. A similar effect of L-cystine upon cultured chick embryo fibroblasts was reported by Morgan and Morton (1955). Table 1 likewise reveals that, with the possible exception of allo-cystathionine, all the compounds tested for their ability to replace L-cystine in supporting cell survival proved ineffective. Moreover, none of these compounds could support Coxsackie B3 virus production. Furthermore, DL-ethionine, allylglycine, and 3,3'-dithiopropionic acid failed to inhibit, under these experimental conditions, the cystine effect upon both virus production and cell survival.

In the inability of any of these test compounds effectively to support Coxsackie B3 virus production, the high degree of specificity of L-cystine becomes apparent. The requirement for the amino group of the L-cystine molecule is evident from the inability of 3,3'-dithiopropionic acid to support virus production. Not only is the presence of the amino group required, but likewise its spacial orientation is important, as suggested by the inability of d-cystine to replace L-cystine. The requirement for L-cystine rather than D-cystine also emphasizes the biological rather than physical nature of the cystine requirement. Both d-cystine and DL-homocystine contain a sulfur-sulfur bridge. The ineffectiveness of these two compounds in replacing L-cystine supports a previous conclusion (Tyndall and Ludwig, 1960) that the cystine requirement is not that of an external hydrogen acceptor. Further, the inability of homocystine to replace L-cystine demonstrates the importance of the three-carbon chain. The necessity for the three-carbon chain, and in particular the carboxyl group, is likewise evident in the lack of Coxsackie B3 virus production in cell cultures overlaid with 2-mercaptoethylamine medium in lieu of cystine medium. The importance of the sulfur atom of cystine was previously shown by the ineffectiveness of L-alanine in replacing L-cystine in the support of virus production (Dubes, 1956; Tyndall and Ludwig, 1960).

Thus, it appears that the presence and spacial orientation of the amino group, the presence of the sulfur atom and carboxyl group, and also the three-carbon chain are all specific requirements for a molecule to support the production of Coxsackie B3 virus by cultured monkey heart cells. Of the compounds tested, all four of these requirements can be met only by L-cystine.

It must again be pointed out that 3,3'-dithiopropionic acid, d-cystine, DL-homocystine, and 2-mercaptoethylamine were also ineffective in replacing L-cystine in support of monkey heart cell survival. Thus, the specificity for L-cystine applies to both cell survival and virus production.

It should also be noted that allylglycine was synthesized specifically to inhibit the divalent sulfur atom as found in cysteine (Dittmer et al., 1948). Assuming allylglycine enters the cells, its inability to inhibit L-cystine in support of virus production and cell survival may indicate that cystine rather than cysteine is the active form of the amino acid in these processes.

Inspection of the data in Fig. 1 reveals several noteworthy features. First, the amount of cystine required for maximal virus production is in great excess as compared with the number of infectious virus units produced. There are probably several reasons for the discrepancy between the cystine required and the infectious virus produced. Part of the cystine is undoubtedly required extracellulary to maintain a sufficient intracellular pool, as recently demonstrated by Eagle, Piez, and Levy (1961). Cystine may also be used in the production of noninfectious virus particles and in unassembled viral subunits. Perhaps the most plausible explanation for the large cystine requirement, however, may be the need for this sulfur amino acid to maintain the integrity of certain cellular structures or functions necessary for the replication of the invading virus. Table 1 shows the effect of L-cystine in supporting both monkey heart cell survival and virus production. The lack of support or inhibition of both cell survival and virus production by the various cystine substitutes and inhibitors is also evident in Table 1. Table 2 reveals that both virus production and cell survival respond from minimal to maximal values within the same rather narrow range of cystine concentrations. Thus, although recent studies by Dubes and Chapin (1958) and McBride (1962) have shown that the requirement for cystine in poliovirus production is governed by the virus strain em-
ployed, the present study suggests that the cystine requirement for Coxsackie B3 virus production may also reflect a basic requirement of the host cells for this sulfur amino acid.

This and previous studies (Tyndall and Ludwig, 1960) have demonstrated that cystine medium could support Coxsackie B3 virus production in monkey heart cell cultures to levels comparable with those obtained with Medium 199 and approximately 1,000-fold greater than with BSS + G medium. It was not anticipated, however, that addition of only L-cystine to the minimal BSS + G medium would continue to support near-maximal virus production after the host cell cultures had been starved for 48 hr prior to infection (Fig. 2). Although increased cell starvation before virus infection did not reveal any additional marked nutritional requirements for virus production, it did suggest the possibility of establishing a latent virus infection by inoculating starved cells.

Several noteworthy features of the latent Coxsackie B3 virus infection established by the inoculation of starved monkey heart cells devoid of exogenous cystine are shown in Fig. 3. In the particular experiment illustrated, no virus could be detected in the 48- and 96-hr latently infected cultures. The titer of detectable virus obtained from similarly latently infected cultures in five such experiments ranged from 0 to 30 TCID₅₀ per culture tube. Yet, when the media of such cell cultures were supplemented with L-cystine, virus production proceeded at a rate paralleling that of normal cultures inoculated with 300 TCID₅₀ of Coxsackie B3 virus. Although a negative virus titration does not necessarily rule out the presence of any virus, it would seem improbable that such an obviously low level of infective virus present in the culture could account for the response noted in the latently infected cultures as shown in Fig. 3. It would appear more likely that some virus persists in the cells in a form other than complete virus particles, perhaps as viral ribonucleic acid.

It should also be noted that with increasing starvation periods the maximal obtainable virus titer decreases. This resembles the effect of cell starvation upon virus yield when the virus was inoculated after starvation (Fig. 2). Thus, the effect of decreasing the virus yields by increasing cell starvation time seems to be independent of the presence or absence of the virus particle during the starvation period.

The establishment of the cystine-dependent latent Coxsackie B3 infection resembles the establishment of latent psittacosis virus infection as described by Morgan and Bader (1957), in that both systems were established by infection of nutritionally depleted cells. The activation of the latent psittacosis virus infection, however, required the addition of nine amino acids and water-soluble vitamins, whereas activation of the latent Coxsackie B3 virus infection required the addition of only L-cystine.

The fermentation patterns, virus susceptibility, and other characteristics of normal and latently infected monkey heart cell cultures are presently under investigation in an attempt to discern alterations in the latently infected cultures attributable to the presence of Coxsackie B3 particles.

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LITERATURE CITED


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