# LATENT VIRAL INFECTION OF CELLS IN TISSUE CULTURE

## VII. Role of Water-Soluble Vitamins in Psittacosis Virus Propagation in L Cells*

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The multiplication cycle of psittacosis virus (6BC strain) in chick embryo cells (1) or mouse fibroblasts (L cells) (2) is interrupted if these cells are exposed to a nutritionally deficient medium prior to infection, and an experimental latent infection is established. Several hours after initial infection, virus can no longer be demonstrated in the cells, but the addition of a complete nutrient medium results in the reappearance of infectious virus. The importance of amino acids and vitamins for the completion of the infectious cycle was demonstrated through the use of a synthetic medium (3), and subsequently the specific amino acid requirements for the stimulation of psittacosis virus growth were defined (4). The specific vitamins required for the production of psittacosis virus in L cells are examined in these experiments.

### *Materials and Methods*

*Virus.*—The 6BC strain of psittacosis virus was maintained in this laboratory by yolk sac passage, and virus suspensions were prepared from infected chick embryos as previously described (1). For certain studies, stock virus suspensions were prepared from L cells infected with psittacosis virus and, in order to dilute vitamins in the infecting inoculum beyond an effective concentration, infected L cells were maintained on a medium which was free from the specific vitamin under examination. When cytopathic effects were first evident in these cultures, the fluids were removed and used as stock virus suspensions. A $10^{-3}$ dilution of virus suspension in Earle's balanced salt solution (BSS) or in vitamin-deficient medium was employed as the infecting inoculum.

*Virus Titrations.*—The single dilution method of Golub (5) was used to determine the amount of virus in the tissue culture fluids. 0.25 ml. of a $10^{-1}$ dilution of the culture fluid was inoculated into the yolk sac of each of a dozen 7-day-old chick embryos and virus titers were expressed as the $\log_{10}LD_{50}$.

*Test Media.*—The basic complete synthetic medium (CM) employed, containing only amino acids, water-soluble vitamins, glutamine, glucose, and inorganic salts, has been pre-

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viously described (4) and provides for survival but not multiplication of L cells. Each test medium was deficient in a single compound and/or its related derivative.

*L Cell Cultures.*—Cultures of L cells were grown in T-15 flasks[1] with a medium containing 20 per cent horse serum, 0.2 per cent lactalbumin hydrolysate, 0.07 per cent yeast extract, and Earle's BSS (2).

*Depletion Procedures.*—After a uniform layer of L cells had grown over the glass surface, the growth medium was replaced with BSS; BSS was added again the following day, and on the 3rd day, psittacosis virus was included in the replacement fluid to infect the cells. The fluids were changed with BSS after 24 hours and 2 days after infection the test media were added to the cultures and replaced each day for 2 more days. Virus titers of tissue culture fluids were determined daily.

In certain experiments, growth medium on the L cell cultures was replaced with a medium deficient in a single vitamin. The fluids were replaced on alternate days until the day of infection. Cultures were infected with psittacosis virus when morphological changes were noted in the cells which seemed to be a result of vitamin depletion or when a time considered sufficient for vitamin depletion had elapsed. One group of depleted cells was maintained on the deficient medium after infection, and the complete medium (CM) was added to another group. After infection, the fluids were replaced daily until the cultures had disintegrated and these fluids were titrated for infectious psittacosis virus.

*Cellular Activity.*—The hydrogen ion concentration of the test fluids covering the cells was estimated colorimetrically utilizing phenol red in the medium in comparison with phenol red color standards. Cell cultures were examined microscopically each day and the morphological appearances recorded.

## RESULTS

*Depletion with BSS.*—Metabolic depletion of L cells with BSS for 2 days rendered the cells incapable of supporting a complete cycle of psittacosis virus growth. The specific vitamin requirements for the propagation of psittacosis virus in this system were examined by adding a synthetic medium deficient in a single vitamin to the infected L cells. Of the vitamins and vitamin combinations which were examined, only thiamine was a specific requirement in the medium for psittacosis virus growth (Table I). Media deficient in other vitamins were fully capable of supporting the production of infectious virus in cells which had been depleted with BSS.

The pH of the complete medium covering infected cells was not altered significantly over the 24 hour interval between replacement of fluids, but was maintained at about 7.4. The pH of most of the vitamin-deficient media also remained constant, but culture fluids from cells exposed to a thiamine-free medium were consistently more acidic than control fluids.

The lack of stimulating activity of the thiamine-deficient medium for psittacosis virus production was associated with its failure to produce recovery of the cells from the morphological changes associated with the nutritional depletion. When L cell cultures were maintained on BSS for a total of 4 days, a decrease in cellular cytoplasm was observed microscopically. The addition of

[1] Obtained from Kontes Glass Co., Vineland, New Jersey.

the complete medium to these cultures restored the cells to a morphologically healthy condition, usually within 48 hours. Cells which were treated with a vitamin-deficient medium capable of stimulating virus growth also showed an increase in cytoplasm comparable to cells treated with the complete medium. Thiamine-deficient medium was capable of eliciting some cytoplasmic increase but the morphological recovery of the cells was not as complete as the recovery

TABLE I

*Effect of Vitamin-Deficient Media on the Growth of Psittacosis Virus*

| Culture medium | Virus growth | Recovery of cells following BSS treatment | pH of medium on 5th day after infection |
|---|---|---|---|
| Complete medium | + | + | 7.4 |
| BSS | 0 | 0 | 7.4 |
| Minus thiamine | 0 | ± | 6.8 |
| " pantothenate | + | + | 7.4 |
| " inositol | + | + | 7.4 |
| " choline | + | + | 7.4 |
| " biotin | + | + | 7.4 |
| " riboflavin | + | + | 7.4 |
| " PABA* | + | + | 7.4 |
| " folic acid | + | + | 7.4 |
| " PABA and folic acid | + | + | 7.4 |
| " niacin | + | + | 7.4 |
| " niacinamide | + | + | 7.4 |
| " niacin and niacinamide | + | + | 7.4 |
| " pyridoxal | + | + | 7.4 |
| " pyridoxine | + | + | 7.4 |
| " pyridoxal and pyridoxine | + | + | 7.4 |

L cells were treated with BSS for 2 days prior to infection with psittacosis virus and maintained for 2 additional days on BSS. Test media were added and culture fluids tested for virus growth. No virus was demonstrable in the cultures at the time of addition of test media.

* Para-aminobenzoic acid.

noted in cultures treated with complete medium or media deficient in other vitamins.

*Depletion of Single Vitamins.*—Since only thiamine appeared to be essential for psittacosis virus production in cells which had been treated with BSS, it seemed probable that a more extensive depletion period for other vitamins would be necessary for a fair estimate of the vitamins required for virus propagation. L cells will not survive longer than 8 or 9 days when maintained on BSS alone but the recovery of nutritionally depleted cells after treatment with certain vitamin-deficient media indicated that L cells could be maintained for longer periods on the vitamin-deficient media than on BSS alone. Therefore,

attempts to deplete L cell cultures of specific vitamins were made by maintaining the cells on a medium deficient in the vitamin under examination until the cells were infected with psittacosis virus. In these studies the virus inoculum was prepared from L cells which were maintained for a short period of time on the vitamin-deficient medium, as described in Materials and Methods.

*A. Thiamine Depletion.*—Depletion of thiamine from L cells and the dependence of psittacosis virus propagation on this vitamin was demonstrated again after a 5 day treatment of L cells with a thiamine-deficient medium (Fig. 1).

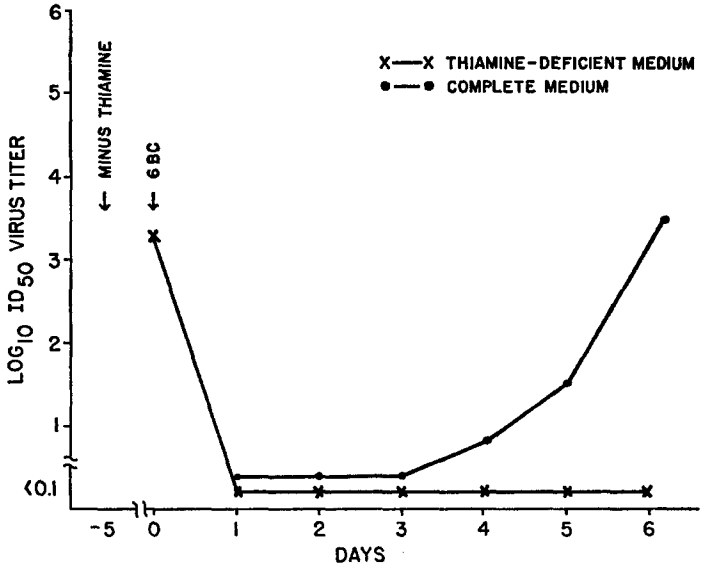


FIG. 1. L cell cultures were maintained on thiamine-deficient medium for 5 days before infection with 6BC psittacosis virus. After 24 hours complete medium was added to one group of cultures and thiamine-deficient medium was added to the other group.

No virus was demonstrable in the fluids of infected cells which had been treated with thiamine-deficient medium, but virus propagation did occur when complete medium was added after infection. Fluids of cultures containing the thiamine-deficient medium were more acidic than culture fluids from the controls, and cytopathic changes which were typical of virus action were seen in cultures containing complete medium before any cytopathology was noted in the thiamine-deficient cultures. The disintegration of L cell cultures containing the deficient medium was quite different from that usually seen after virus infection and could probably be attributed to the vitamin depletion.

*B. Pantothenate Depletion.*—A few days after the initial exposure of L cells to pantothenate-deficient medium, the culture fluids became acidic, and it was

necessary to replace fluids daily in order to keep the pH within the range of the phenol red indicator. The cultures were infected with psittacosis virus on the 12th day of pantothenate depletion. Control cultures to which the complete medium was added after infection did not become acidic, and the growth of psittacosis virus in these cultures reached high levels within a few days. Complete cytopathic degeneration occurred by the 7th day after infection in these

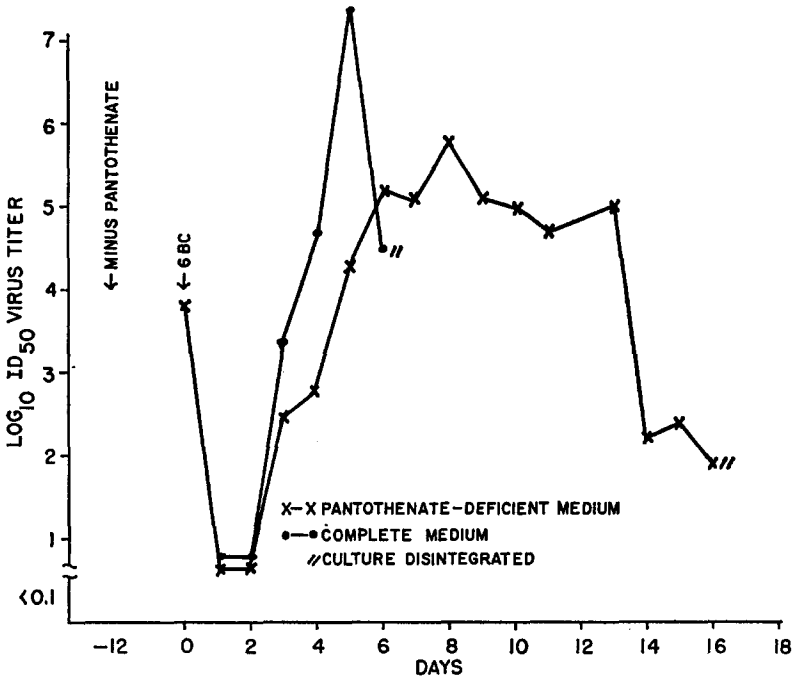


FIG. 2. L cell cultures were maintained on pantothenate-deficient medium for 12 days before infection with psittacosis virus. After 24 hours complete medium was added to one group of cultures and pantothenate-deficient medium was added to the other group.

cultures. The rate of virus production in the pantothenate-deficient cultures was retarded and the yield of virus never reached the magnitude of the control cultures (Fig. 2). Cytopathic effects were not apparent in the deficient cultures until long after the control cultures had disintegrated, and virus continued to be produced by the surviving cells.

*C. Niacin-Niacinamide Depletion.*—After maintenance of cells for 14 days on niacin-niacinamide–deficient medium the morphology of the cells began to change from the typical bipolar cells to cells with multiple cytoplasmic extensions. The number of cells exhibiting this change increased with continued niacin-niacinamide depletion, and after 18 days considerable vacuolization of

cells was noted. On the 24th day of depletion, ½ of the cultures were infected with psittacosis virus. The remaining cultures were maintained for 2 more weeks on the deficient medium and infected at this time. The cultures infected at 24 days and treated with complete medium demonstrated a pattern of psittacosis growth (Fig. 3) not significantly different from that usually found in L cell cultures. Cytopathic effects were observed after 6 days and the cultures had completely disintegrated by the 9th day after infection. Virus production

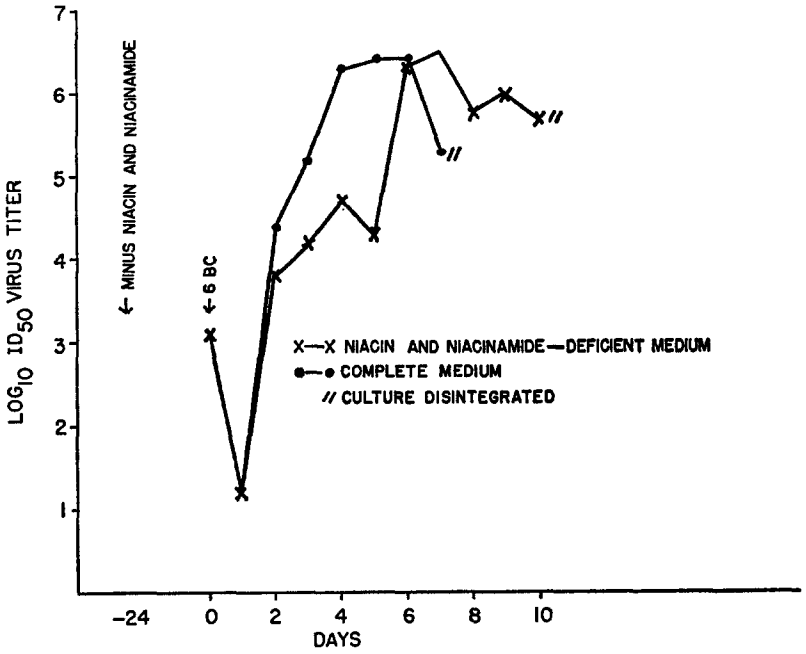


FIG. 3. L cell cultures were maintained on niacin- and niacinamide-deficient medium for 24 days before infection with 6BC psittacosis virus. After 24 hours complete medium was added to one group of cultures and the deficient medium was added to the other group.

was not as rapid in cultures which were treated with niacin-niacinamide-deficient medium, and the peak titers were not found until 2 days after the control cultures had reached maximum titers. Initial cytopathic effects were observed in the cultures fed with complete medium 2 days before the cultures on the deficient medium, and extensive degeneration of the cultures was also delayed in the cultures with medium deficient in niacin and niacinamide.

Similar results were noted in cultures which were infected on the 38th day after initiation of niacin-niacinamide depletion. Virus production was delayed in the deficient cultures and complete disintegration of cells did not occur until 4 days after the control cells had lysed. Some cytolysis due to vitamin deple-

tion alone was apparent at this time, since uninfected cultures which had been maintained on niacin-niacinamide-deficient medium had begun to disintegrate.

*D. Pyridoxal-Pyridoxine Depletion.*—Cultures which were maintained on a medium deficient in pyridoxal and pyridoxine showed no significant morphological changes over the first 3 weeks, but after this period the cells became slightly rounded, and a few cells began to disengage from the glass surface. After 24 or 38 days of depletion the cultures were infected with psittacosis

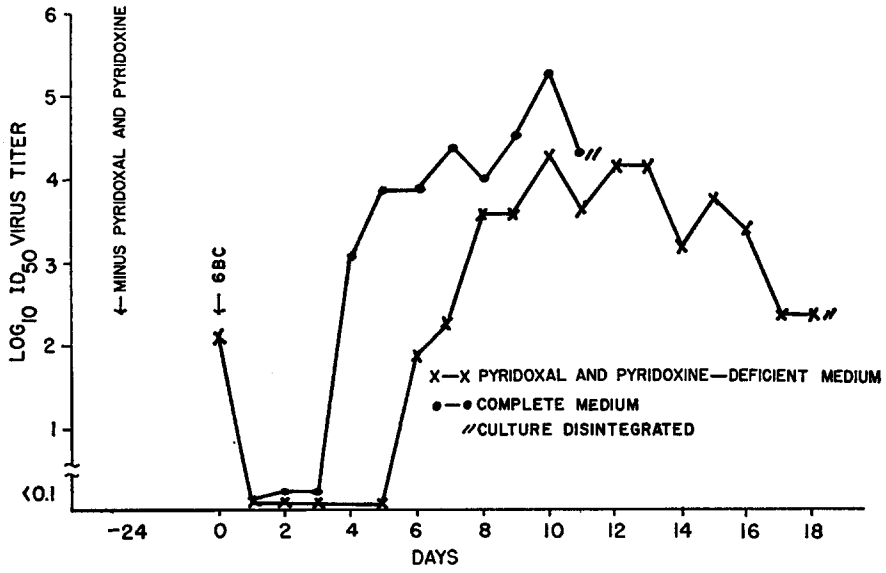


FIG. 4. L cell cultures were maintained on pyridoxal (pyridoxine)-deficient medium for 24 days before infection with 6BC psittacosis virus. After 24 hours, complete medium was added to one group of cultures and the deficient medium was added to the other group.

virus, and cultures which were maintained on pyridoxal-pyridoxone-deficient medium were compared with cultures to which complete medium was added after infection. Virus production after depletion for 24 days was considerably delayed in the cultures with the pyridoxal-pyridoxine-deficient medium (Fig. 4). Infectious virus did not appear in these cultures until 2 days after it had appeared in the controls fed with complete medium. Cytopathic effects were extensive in the control cultures by the 10th day after infection, but in the deficient cultures there was only a slow disintegration of cells until the 19th day after infection when lysis was practically complete.

A similar delay in the appearance of infectious virus was seen in the pyridoxal-pyridoxine-deficient cultures which were infected on the 38th day after initiation of the depletion procedure. No differences in the cytopathic effects could

be demonstrated, however, because of the extensive cellular degeneration which was noted during the experimental period in both infected and non-infected vitamin-deficient cultures.

*E. Choline Depletion.*—L cells were maintained on a choline-deficient medium for 24 days prior to infection. At this time the cells had changed only slightly in their morphological characteristics, although cells had begun to detach from the glass surface. Cultures containing complete medium produced higher levels

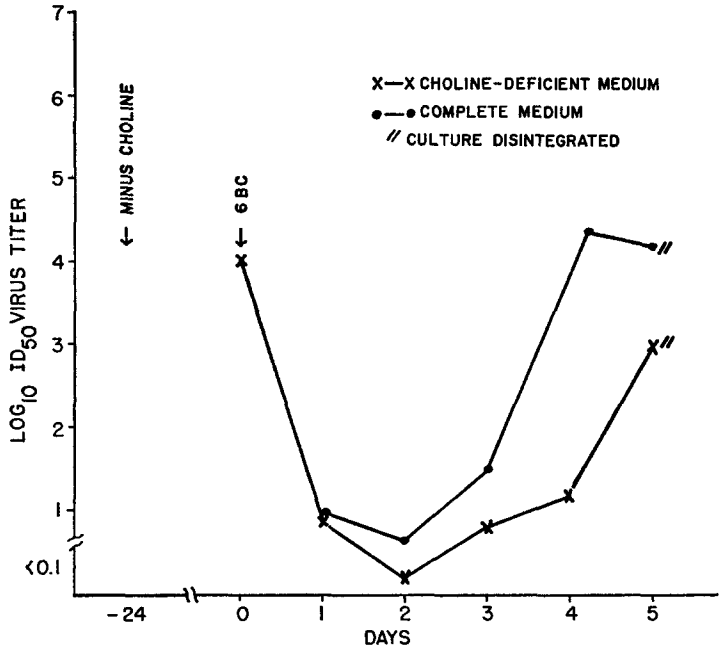


FIG. 5. L cell cultures were maintained on choline–deficient medium for 24 days before infection with 6BC psittacosis virus. After 24 hours, complete medium was added to 1 group of cultures and choline–deficient medium was added to the other group.

of psittacosis virus than the cultures without choline (Fig. 5). The large differences in titers could not be attributed to the loss of cells from deficient cultures during this period, but by the 6th day after infection cellular disintegration was extensive in both deficient and control cultures, and adequate determinations of vitamin essentiality were not possible after this time.

*F. Biotin, Inositol, and Para-aminobenzoic Acid (PABA) + Folic Acid Depletion.*—Depletion of L cell cultures with a biotin-deficient, inositol-deficient, or PABA and folic acid–deficient medium had little effect on the capacity of the cells to produce psittacosis virus. Cultures were maintained on biotin-deficient medium for 20 days before infection and subsequent viral titers of

cultures containing the deficient medium were not significantly different from titers of control cultures. Cultures were maintained for 45 days on inositol-deficient medium prior to infection, but the production of psittacosis virus did not appear to be affected, since complete medium controls and inositol-deficient cultures yielded similar amounts of virus. PABA and folic acid depletion of cultures for as long as 60 days had no apparent effect on the capacity of the cells to support the growth of psittacosis virus. Virus production proceeded at similar rates in control cultures and cultures containing the medium deficient in PABA and folic acid, and no differences were noted in the pattern of cytopathology exhibited by the test cultures.

*G. Riboflavin.*—After the 12th day of depletion with a riboflavin-deficient medium, cells became enlarged and degenerative changes restricted the period of experimentation. When cultures were infected after 7 days' depletion, no significant differences in psittacosis production could be demonstrated between the cultures containing riboflavin-deficient medium and those containing complete medium. The relatively short depletion period may not be sufficient to lower the effective concentration of riboflavin in the cells, and it is not felt that a riboflavin requirement for the growth of psittacosis virus has been eliminated.

## DISCUSSION

L cells which have been depleted of their nutritional reserves by maintenance on BSS for 48 hours prior to infection are incapable of supporting the growth of psittacosis virus (2), and the specific amino acids which are required in a medium capable of supporting psittacosis virus production have been delineated (4). In this system the deletion of specific vitamins from the complete synthetic medium had little effect on the potency of the medium for the support of virus growth, with one exception. Of the vitamins examined, only thiamine was demonstrated to be an important individual constituent of the medium. The acidity of the fluids from L cell cultures which had been treated with the thiamine-deficient medium is indicative of thiamine deficiency in the cells, since this vitamin is known to be an important factor in the metabolism of $\alpha$-keto acids and is probably necessary for the aerobic oxidation of carbohydrates. It has been observed that cultures containing a minimum of free oxygen are inhibited in their ability to support psittacosis virus production (6) and aerobic oxidation may be essential for the synthesis of psittacosis virus.

Considering the ease with which L cells can be depleted of their essential free amino acid constituents (4), it is apparent that most vitamins do not diffuse readily from the cell or are present in such concentration that many more changes of maintenance media are necessary to dilute the vitamin to a concentration which is ineffective for optimal metabolic activities. All of the vitamins examined have been shown to exist in combined forms with other metabolic compounds, and these vitamins may be bound in L cells, rendering

unlikely the depletion of the cells of a specific vitamin in such a short period of time.

The rapid depletion of thiamine from L cells and the dependence of psittacosis virus propagation on this vitamin was further shown by the failure of psittacosis virus to propagate in cells which were maintained on a medium deficient only in thiamine. L cells required much longer incubation periods with media deficient in other vitamins before any reduction in psittacosis virus growth was demonstrable.

The inability of cells to support high levels of psittacosis virus production after maintenance on a pantothenate-deficient medium may also be related to a dependence on the aerobic oxidation of carbohydrates and fats, since pantothenate is a constituent of coenzyme A, an important compound in the intermediary metabolism of these substances.

Darnell and Eagle (7) demonstrated that HeLa cells which had been depleted of pyridoxine could not support the growth of poliovirus if glucose, glutamine, and salts were subsequently used as a maintenance medium. However, if free amino acids were added the cells readily supported poliovirus growth, and these authors concluded that the available free amino acid pool was depleted in vitamin $B_6$–depleted cells. In the present studies, there is clearly indicated a dependence upon pyridoxine which goes beyond that of depletion of the free amino acids, since these latter substances were regular constituents of the pyridoxine-deficient medium.

The failure of the present studies to demonstrate a growth requirement of psittacosis virus for folic acid is consistent with other evidence which indicates that this virus is capable of bringing about a synthesis of folic acid in infected cells. L cells apparently lack the capacity for folic acid synthesis, since Eagle has demonstrated a requirement for this vitamin in the growth of L cells (8). Colon and Moulder (9) showed that folic acid was present in purified preparations of psittacosis virus, and Holtermann *et al.* (10) demonstrated an increase in the folic acid content of L cells infected with psittacosis virus. Also, the folic acid content of purified preparations of another member of the psittacosis group, mouse pneumonitis virus, was demonstrated to increase during *in vitro* incubation (11).

Eagle (8) was not able to demonstrate a biotin or inositol requirement for the growth of L cells and no requirement for these vitamins in the growth of psittacosis virus in L cells could be demonstrated in the present studies.

Although a number of specific vitamin requirements have been demonstrated for the maximum production of psittacosis virus in L cells, the exclusion of these vitamins, except thiamine, had little effect on the capacity of the medium for stimulating the production of infectious virus from a latent infection. Also, extended depletion of L cells of specific vitamins did not produce a latent infection *in vitro* as has been recently demonstrated with phenylalanine or isoleucine depletion (6).

SUMMARY

A study of the metabolic requirements for the growth of psittacosis virus in L cells has been extended to the water-soluble vitamins. In a system in which a balanced salt solution was used to deplete the cells of their vitamin constituents, only thiamine was essential for psittacosis virus production. Extended depletion of cells with media deficient in specific vitamins demonstrated that pantothenate, niacin (niacinamide), pyridoxine (pyridoxal), and choline, in addition to thiamine, were essential for maximal growth of psittacosis virus. No requirement for biotin, inositol, folic acid, or riboflavin was demonstrated, although the possibility of incomplete vitamin depletion of the cells has not been eliminated. In most cases in which a specific vitamin requirement was shown the decreased yield of virus was correlated with a delay in the cytopathic effects produced in the cell cultures by psittacosis virus.

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BIBLIOGRAPHY

1. Morgan, H. R., Latent viral infection of cells in tissue culture. I. Studies on latent infection of chick embryo tissues with psittacosis virus, *J. Exp. Med.*, 1956, **103**, 37.
2. Morgan, H. R., and Bader, J. P., Latent viral infection of cells in tissue culture. IV. Latent infection of L cells with psittacosis virus, *J. Exp. Med.*, 1957, **106**, 39.
3. Johnson, K. M., and Morgan, H. R., Latent viral infection of cells in tissue culture. II. Relationship of cell nutrition to initiation of growth of psittacosis virus, *J. Exp. Med.*, 1956, **103**, 765.
4. Bader, J. P., and Morgan, H. R., Latent viral infection of cells in tissue culture. VI. Role of amino acids, glutamine, and glucose in psittacosis virus propagation in L cells, *J. Exp. Med.*, 1958, **108**, 617.
5. Golub, O. J., A single dilution method for the estimation of $LD_{50}$ titers of the psittacosis-LGV group of viruses in chick embryos, *J. Immunol.*, 1948, **59**, 71.
6. Bader, J. P., and Morgan, H. R., unpublished observations.
7. Darnell, J. E., and Eagle, H., Nutritional requirements for poliovirus synthesis in HeLa cells, *Fed. Proc.*, 1958, **17**, 508.
8. Eagle, H., The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure, *J. Exp. Med.*, 1955, **102**, 595.
9. Colon, J. I., and Moulder, J. W., Folic acid in purified preparations of members of the psittacosis group of microorganisms, *J. Infect. Dis.*, 1959, **103**, 109.
10. Holtermann, O. A., Mergenhagen, S. E., and Morgan, H. R., Factors related to psittacosis virus (strain 6BC) growth. V. Folic acid–like factor in infected cells, *Proc. Soc. Exp. Biol. and Med.*, 1959, **100**, 370.
11. Colon, J. I., Formation of citrovorum factor in purified preparations of mouse pneumonitis virus, *Bact. Proc.* 1959, 85.