Polyamines in Type 5 Adenovirus-Infected Cells and Virions

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The incorporation of L-[^{14}C]ornithine and [^{14}C]putrescine into putrescine, spermidine, and spermine in type 5 adenovirus-infected KB cells was identical to that in uninfected control cells early in infection, but incorporation into putrescine stopped after 8 to 12 h and the rate of incorporation into spermidine was reduced between 12 and 20 h after infection. The amount of polyamines found associated with purified virus could neutralize a maximum of 3 to 4% of the virus DNA, but the small quantities of polyamines detected could not be distinguished from nonspecific binding of polyamines to virions. These data suggest that polyamines are probably not integral components of adenovirus particles.

Although the polyamines putrescine, spermidine, and spermine occur in some form in all living cells examined and are required for growth of some cells (14), their physiological role remains obscure. Polyamines have a high affinity for polynucleotides and have been shown to be structural components of ribosomes (2). These observations have led to the postulate that polyamines function as naturally occurring counter-ions to neutralize a portion of the negative charges on RNA and DNA. Certainly the large amount of polyamines found in the heads of T-even bacteriophages, sufficient to neutralize 40 to 60% of the DNA phosphate (1), would support this view. In the study to be described, we have undertaken to determine whether polyamines may serve a regulatory role in adenovirus replication and whether they can be found in sufficient amounts in purified adenovirus virions to neutralize a significant portion of the virus DNA.

Preparation of stocks of type 5 adenovirus and determination of infectious titers, expressed as PFU per milliliter, have been described previously (7). Spinner cultures of KB cells were propagated in Eagle minimal essential medium supplemented with 10% calf serum (3). The cells were then infected with an input multiplicity of 100 PFU/cell. The medium was supplemented with 5% dialyzed horse serum instead of calf serum, since horse serum was found to be free from spermine oxidase activity.

To determine whether polyamines can be efficiently labeled with radioactive precursors, incorporation of L-[^{14}C]ornithine (8) and [^{14}C]putrescine (9) into polyamines was followed. Immediately after infection, L-[^{14}C]ornithine (172 mCi/mmol) or [^{14}C]putrescine (5 mCi/mmol) was added to infected and uninfected KB cell cultures at 0.02 and 0.01 μCi/ml, respectively. Samples (50 ml) were withdrawn at various times, and the intracellular polyamines were isolated by a modification of the procedure of Raina (11). The cells were collected by centrifugation and suspended in 5 ml of 0.1 N HCl, and 5 ml of 10% trichloroacetic acid was then added. After 1 h at 4 C, the insoluble material was removed by centrifugation and the supernatant was extracted three times with ether to remove the trichloroacetic acid. Traces of ether were removed by heating to 40 C under vacuum. The polyamines were then extracted into n-butanol at pH 13 by shaking for 30 min at 37 C. The butanol phase containing the polyamines was evaporated under vacuum, and the residue was dissolved in 0.1 N HCl. The polyamines were separated by paper electrophoresis on Whatman no. 1 strips in 0.07 M citric acid buffer (pH 3.4) for 2 h at 10 V/cm (12). The papers were dried and dipped in ninhydrin solution (ninhydrin, 1 g; cadmium acetate, 0.1 g; acetone, 100 ml; glacial acetic acid, 5 ml; water, 10 ml). After 10 min at room temperature, the color was developed for 90 min at 75 C. The colored bands were excised and eluted in 5 ml of a solution consisting of: glacial

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acetic acid, 500 ml; absolute ethanol, 400 ml; water, 100 ml; and cadmium acetate, 20 g. The polyamines were quantitated by optical density at 505 nm using the extinction coefficients reported by Raina and Cohen (12). Radioactivity was determined by cutting the stained bands from the strips, immersing them in 1 liter of toluene containing 6 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis-(5-phenyloxazolyl)-benzene (Packard Instrument Co., Downer's Grove, Ill.), and counting in a Packard Tri-Carb liquid scintillation spectrometer.

In the infected cells, incorporation of L-[^{14}C]ornithine into putrescine ceased after 8 h (between 8 and 20 h), but continued for 20 h in the control cells (Fig. 1a). The shutoff was not due to depletion of L-[^{14}C]ornithine in the medium since its incorporation into trichloroacetic acid-insoluble material continued at a constant rate for at least 30 h. The incorporation of radioactivity into spermidine and spermine indicated that these compounds were also synthesized in uninfected and infected KB cells.

For a more precise determination of how long spermidine and spermine synthesis continued in infected cells, the incorporation of [^{14}C]putrescine into the specific polyamines was measured (Fig. 1b). The specific activity of both spermidine and spermine increased at the same rate in the infected and uninfected cells for about 12 h after infection. After this time, the rate of incorporation into infected cell polyamines decreased with respect to the control cells, which remained constant. However, when [^{14}C]spermidine (5 mCi/mmol) was added to infected cultures at 0.01 μCi/ml, there was not any difference in the rate of spermine synthesis between infected and control cultures, and therefore the decreased rate of spermine synthesis observed in Fig. 1b was due to the decreased rate of synthesis of its precursor, spermidine. Incorporation of [^{14}C]spermine (5 mCi/mmol at 0.01 μCi/ml) showed that the rate of spermine degradation to spermidine was not altered by infection. In addition, no radioactivity from either [^{14}C]spermidine or [^{14}C]spermine was observed in putrescine in either infected or control cells.

Having established that polyamine biosynthesis continued for 8 to 12 h after infection with type 5 adenovirus, we examined the polyamine content of adenovirus particles to determine whether polyamines were components of the virions. Analysis of the polyamine content of purified adenovirus (7), according to the extraction and quantitation procedures described above, indicated that there was less than 0.004
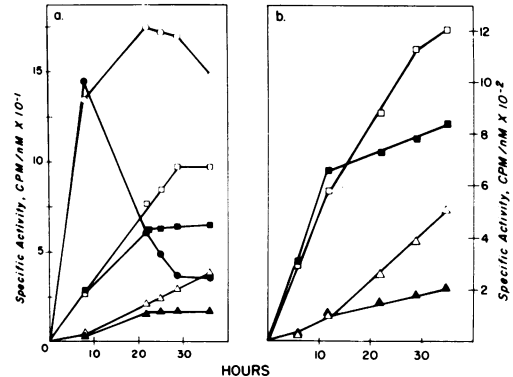

FIG. 1. Incorporation of L-[^{14}C]ornithine and [^{14}C]putrescine into polyamines in infected and uninfected KB cells. Suspension cultures of KB cells containing 1.5×10^5 cells/ml were infected with 100 PFU of type 5 adenovirus per cell. To infected or uninfected cultures either 0.02 μCi of L-[^{14}C]ornithine per ml (a) or 0.01 μCi of [^{14}C]putrescine per ml (b) was added at time zero. The specific radioactivity of intracellular polyamines was determined in 50-ml samples taken at the times indicated. Putrescine: ●, infected; ○, uninfected. Spermidine: ■, infected; □, uninfected. Spermine: ▲, infected; △, uninfected.

mol of each polyamine per mol of DNA phosphate. This meant that a maximum of 3.6% of the DNA could be neutralized by these compounds, based on the nitrogen content of each polyamine.

The possibility that endogenous polyamines were displaced in the purified virions could not be eliminated, however, since virus purification involved fluorocarbon extraction and several centrifugations in CsCl gradients. To avoid exposure to high salt concentrations, the incorporation of radioactive polyamines into adenovirus isolated without equilibrium sedimentation in CsCl was examined. The procedure employed was a modification of the sucrose density gradient procedure described previously (7). Cells were infected, and [^{3}H]thymidine (0.1 mCi/ml; 2 Ci/mmol) was added at 12 h to label virus DNA. The culture was divided into three portions, and 0.01 μCi of [^{14}C]putrescine, [^{14}C]spermidine or [^{14}C]spermine per ml was added. At 36 h after infection, the cells were harvested and treated, and the sonic extract was centrifuged into a linear 15 to 30% sucrose gradient, as described in the legend of Fig. 2.

Only a small fraction of the intracellular labeled polyamines cosedimented with the [^{3}H]thymidine-labeled virus, which coincided with the visible virus band (Fig. 2a, b, c). The percentage of total ^{14}C radioactivity in the virus band in each gradient was: putrescine, 0.14% (Fig. 2a); spermidine, 0.31% (Fig. 2b) sper-
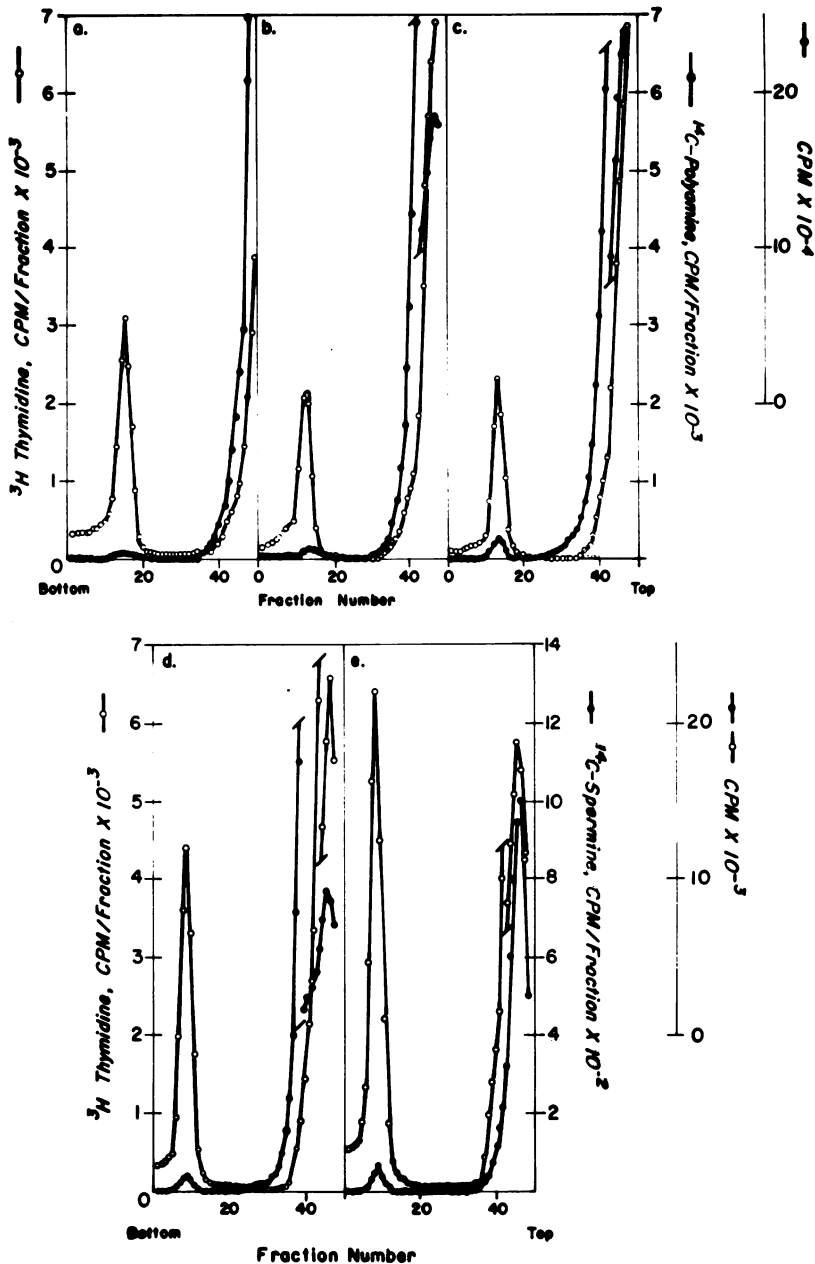
FIG. 2. *Sucrose gradient sedimentation of [¹⁴C]polyamine-labeled virus. Cells were infected in complete medium. At 12 h after infection 0.1 μCi of [³H]thymidine was added, the culture was divided into three 100-ml portions, and 0.01 μCi of [¹⁴C]putrescine (a), [¹⁴C]spermidine (b), or [¹⁴C]spermine (c) was added. At 36 h after infection, the cells were harvested and suspended in 2 ml of 0.01 M phosphate, pH 7.2. The cell suspension was treated with 0.5% deoxycholate and 10 μg of RNase per ml at 37 C for 30 min and sonically treated for 20 s, and 0.4 ml was layered on top of 4.6 ml of a linear 15 to 30% sucrose gradient in 0.01 M Tris-hydrochloride (pH 7.2). After centrifugation for 10 min at 35,000 rpm in an SW39 rotor, fractions were collected through a hole in the bottom of the tube and the radioactivity was counted. Experiments summarized in (d) and (e) were performed as for (a), (b), and (c), except that radioactive polyamines were not added after infection. After the cells from each culture were suspended in 1 ml of buffer, [¹⁴C]spermine was added to each of two 0.5-ml portions at (d) 0.03 μCi/ml and (e) 0.3 μCi/ml, and the suspension was treated as above. Symbols: O, [³H]thymidine; ●, [¹⁴C]putrescine, [¹⁴C]spermidine, or [¹⁴H]spermine.*

mine, 0.55% (Fig. 2c). Hence, the amount of polyamine associated with the virions could be as much as 0.0005 mol of putrescine, 0.001 mol of spermidine, and 0.001 mol of spermine per mol of DNA phosphate, a quantity of polyamine sufficient to neutralize about 1 to 2% of the DNA phosphate.

Since these polyamine levels were very low, the possibility of nonspecific binding of the polyamines to virions was examined. Cells were infected as in the previous experiment except that the labeled polyamines were omitted. The cells were harvested, washed, and suspended in 1 ml of buffer, and [^{14}C]spermine was added to a final concentration of 0.03 or 0.3 μCi/ml to equal samples. The extract was prepared and the virions were purified as described in the previous experiment. The amount of [^{14}C]spermine bound was similar at both concentrations of added isotope (Fig. 2d, e). The ratio of [^{14}C]spermine/[^{3}H]thymidine was 0.0087 and 0.0093 at [^{14}C]spermine concentrations of 0.03 and 0.3 μCi/ml, respectively.

These data suggest that the polyamines found associated with adenovirus particles may not have been integral components of virions, since similar quantities of exogenous polyamines readily became associated with the virus. Shortridge and Stevens (13) reported the presence of sufficient spermine and spermidine in purified adenovirus to neutralize 10% of the virus DNA. Although this amount is higher than that reported here, these investigators did not make any attempt to assess the purity of their virus preparations or to exclude nonspecific binding of cellular polyamines (13). In fact, these authors were unable to show incorporation of isotopically labeled methionine into virion polyamines (13), although we have demonstrated some polyamine synthesis during a major portion of the virus multiplication cycle.

Herpes simplex virus is the only mammalian virus in which polyamines have been demonstrated in purified virions; the virions contain sufficient spermine in the nucleocapsid fraction to neutralize 40% of the DNA phosphate. Spermidine was found only in the envelope (4). The morphogenetic processes of herpesvirus, however, may result in the trapping of significant amounts of cellular components.

The neutralization of DNA phosphate in adenovirus virions may be accomplished primarily by the arginine-rich internal polypeptide associated with the virus DNA (6, 10). This polypeptide, which appears to be a late viral protein and dependent upon viral DNA synthesis, is present in the virion in sufficient amounts to neutralize 60% of the adenovirus DNA phosphate (10). Other viruses which contain little or

no polyamines, such as Shope papilloma (5), might also be expected to contain basic proteins associated with the nucleic acid.

We conclude from these studies that polyamines probably do not play a major regulatory role during viral replication, that the quantity of polyamine associated with type 5 adenovirus virion is not sufficient to neutralize a significant fraction of the DNA, and that the polyamines detected in the virion are probably present owing to nonspecific adsorption.

(This work was presented in part at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2 to 7 May 1971.)

LITERATURE CITED

1. Ames, B. N., D. T. Dubin, and S. M. Rosenthal. 1958. Presence of polyamines in certain bacterial viruses. Science 127:814–816.
2. Cohen, S. S., and J. Lichtenstein. 1960. Polyamines and ribosome structure. J. Biol. Chem. 235:2112–2116.
3. Eagle, H. 1955. Propagation in a fluid medium of a human epidermoid carcinoma, strain KB. Proc. Soc. Exp. Biol. Med. 89:362–364.
4. Gibson, W., and B. Roizman. 1971. Compartmentalization of spermine and spermidine in the herpes simplex virion. Proc. Natl. Acad. Sci. U.S.A. 68:2818–2821.
5. Kass, S. J., and C. A. Knight. 1965. Purification and chemical analysis of Shope papilloma virus. Virology 27:273–281.
6. Laver, W. G. 1970. Isolation of an arginine-rich protein from particles of adenovirus type 2. Virology 41:488–500.
7. Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. J. Virol. 1:851–867.
8. Pegg, A. E., and H. G. Williams-Ashman. 1968. Biosynthesis of putrescine in the prostate gland of the rat. Biochem. J. 108:533–539.
9. Pegg, A. E., and H. G. Williams-Ashman. 1970. Enzymic synthesis of spermine in rat prostate. Arch. Biochem. Biophys. 137:156–165.
10. Prage, L., and U. Pettersson. 1971. Structural proteins of adenoviruses. VII. Purification and properties of an arginine-rich core protein from adenovirus type 2 and type 3. Virology 45:364–373.
11. Raina, A. 1963. Studies on the determination of spermidine and spermine and their metabolism in the developing chick embryo. Acta Physiol. Scand. 60(Suppl.):218.
12. Raina, A., and S. S. Cohen. 1966. Polyamines and RNA synthesis in a polyauxotrophic strain of E. coli. Proc. Natl. Acad. Sci. U.S.A. 55:1587–1593.
13. Shortridge, K. F., and L. Stevens. 1973. Spermine and spermidine-polyamine components of human type 5 adenovirions. Microbios 7:61–68.
14. Tabor, H., and C. W. Tabor. 1964. Spermidine, spermine and related amines. Pharm. Rev. 16:245–300.