Mechanism of the Arginine Requirement for Adenovirus Synthesis

I. Synthesis of Structural Proteins

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The mechanism of the arginine requirement for adenovirus was studied in cultures of KB cells infected with adenovirus type 2. Macromolecular synthesis was found to be severely impaired in uninfected cells under complete arginine deprivation, whereas an arginine concentration of 50 μ M yielded a moderate and reversible inhibition of growth and nucleic acid synthesis. At this concentration, viral structural proteins were accumulated in excess although the virus yield was reduced more than 1,000-fold. The arginine-sensitive step appeared to occur early during the first 15 hr postinfection in the virus growth cycle. Virus-infected cells deprived of arginine to 50 μ M showed, when reversed, a 4- to 5-hr lag period before the increase in virus growth was observed. Analysis of the radioactive pattern of labeled virions synthesized after reversion showed that all polypeptides were synthesized after addition of arginine to the medium, and none of the virion-polypeptides which are revealed by gel electrophoresis appeared to be preferentially synthesized after arginine reversion. The excess pool of structural proteins formed during depletion appeared to a large extent to be unavailable for virus assembly.

Synthesis of adenovirus has been shown to be strongly dependent on the concentration of arginine in the growth medium (23; V. H. Bonifas and R. W. Schlesinger, *Fed. Proc.* 18:560, 1959). Synthesis of herpes simplex virus (1, 7), simian virus 40 (4), polyoma virus (35), and reovirus (10) also appears to require arginine for production of infectious progeny.

Adenoviruses contain an internal protein component associated with the viral deoxyribonucleic acid (DNA), which is exceptionally rich in arginine (23 mole per cent) (8, 21, 28). None of the outer capsid proteins or the remaining core proteins contains arginine to higher concentrations than 12 mole per cent (8, 17). It has therefore been suggested that the arginine requirement reflects a maturation process in which the synthesis of the arginine-rich protein is rate-limiting for assembly of mature progeny (26, 30). This hypothesis was based on kinetic studies of viral DNA synthesis and the presence of structural proteins at arginine depletion. Both viral DNA and structural proteins can be identified at depletion (23, 26), but it is not clear whether the preformed proteins are utilized for virus progeny at reversion.

The present study reports quantitative data on synthesis of virus and structural proteins at

different levels of arginine depletion and after reversion.

MATERIALS AND METHODS

Cells. KB cells were grown either in Spinner cultures in Eagle's Spinner medium (3) with 7% calf serum, penicillin, streptomycin, and kanamycin or in 32-oz (ca. 900 ml) prescription bottles with 30 ml of Eagle's minimal essential medium (MEM) and 15% calf serum.

Virus. The prototype strain of adenovirus type 2, originally obtained from R. J. Huebner, National Institutes of Health, Bethesda, Md., was used. Unlabeled purified pools were used for infection, and purification was performed by a modification of the procedure of Green and Piña (5, 19). Radioactively labeled virus was purified further by sedimenting partially purified preparations on 15 to 30% sucrose gradients formed on a cushion of CsCl ($\rho = 1.40$ g/cc) for 45 min at $150,000 \times g$. Virus materials from the sucrose gradients were then banded on self-generating CsCl gradients. Purified virus was dialyzed against 0.25 M sucrose containing 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4), 1 mM MgCl₂, and 0.5% *n*-butanol (18).

Virus infectivity. Adenovirus infectivity was assayed by fluorescent focus formation in KB cells grown in 35-mm plastic petri dishes with Eagle's MEM with 15% calf serum (19). The results are expressed in fluorescent focus units (FFU).

Virus infection of Spinner cultures. KB cells in Spinner cultures at cell densities of 10^5 to 4×10^6 cells/ml were harvested by centrifugation at room temperature for 10 min at $300 \times g$. The cells were washed twice in 10% of the original volume in Eagle's Spinner medium with 2% calf serum at 37 C. The cells were resuspended in Spinner medium without calf serum, and virus was added to a multiplicity of 100 FFU/cell at a cell density of 10^7 to 3×10^7 cells/ml. Adsorption was carried out in a water bath at 37 C for 30 min. After attachment, the cells were centrifuged and resuspended in Spinner medium with 7% calf serum at a density of about 3×10^6 cells/ml. In all arginine depletion experiments, washing of cells and virus adsorption were carried out in arginine-free media and dialyzed serum was used.

Virus infection of monolayer cultures. After washing twice with Eagle's MEM without calf serum, bottles were infected at a multiplicity of 30 FFU/cell in 2 ml of phosphate-buffered saline (PBS) for 60 min at 37 C with gentle shaking every 15 min. After adsorption, the cells were washed once with MEM containing 10% calf serum, and then 30 ml of the same medium was added. The 37 C environment was maintained during all steps.

Immunoassays. Quantitative determinations of hexon, penton base, and fiber antigens were carried out by the Mancini technique (16). Standard curves with pure preparations of antigens and monovalent antisera showed a linear relationship between the square of the precipitate ring diameter and antigen concentration. The penton base antigen was assayed with an anti-penton serum adsorbed with purified fiber antigen until no further fiber antibodies were detectable by immunodiffusion. The amount of penton base is given in terms of penton equivalents, since the assay was standardized with purified intact penton.

Interference of penton antigen on the assay of fiber antigen was studied separately, and it was found that the fiber content could be accurately estimated at a twofold or higher molar excess of fiber over penton antigen. This condition is fulfilled in the experiments reported.

Antisera were prepared by methods described previously (17). Complement fixation was carried out with the microassay described by Sever (32). Antigen titers were determined in a total volume of 0.125 ml with 4 units of antibody and 2 units of complement. All titers given represent the dilution before the addition of antiserum, complement, and hemolytic system.

Radioimmunoprecipitation. The procedure described by Scharff et al. (29) was followed. Hyperimmune antisera against hexon and heavy chains from immunoglobulin E (anti-epsilon) were used. Equivalent concentrations of the two rabbit antisera and goat anti-rabbit immunoglobulin G (IgG) (Microbiological Associates, Bethesda, Md.) were determined by immunodiffusion.

Unspecific precipitation was estimated by using uninfected ^{14}C -amino acid-labeled KB cells and an anti-hexon serum. Sufficient amount of anti-epsilon was included in each experiment in a separate tube to

give the same unspecific precipitation as anti-hexon serum.

Samples were incubated at 37 C for 1.5 hr with anti-hexon and anti-epsilon sera followed by 1.5 hr with anti-IgG, and the precipitates were washed three times in 0.14 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4) at 4 C. The precipitates were dissolved in 0.2 M NaOH before counting.

Arginase assay. Arginase was assayed by determining labeled urea from guanido-labeled arginine by thin-layer chromatography (TLC; reference 24) according to the reaction:

Uninfected or infected KB cells were withdrawn, washed twice in cold PBS, resuspended in 0.01 M maleate-NaOH (pH 7.0) with 0.01 M MnCl_2 , sonically treated for 30 sec in the cold, and incubated at 45 C for 2 hr to activate the enzyme. The extract was then diluted with an equal volume of 0.1 M glycine-NaOH (pH 9.5) and assayed for enzyme activity. Reaction products were separated by cellulose TLC (Whatman CC 41) with the solvent system ethylacetate-pyridine-acetic acid-water (5:5:1:3). Radioactivity was eluted from the TLC plates with ethanol. Arginase activity is expressed as micromoles of urea per microgram of protein per hour.

Assay of cellular pools of amino acids. Cells (10^7 to 2×10^7) were sedimented by centrifugation for 3 min at $1,500 \times g$ at 37 C, washed twice with 0.15 M NaCl, 0.01 M Tris-hydrochloride (pH 7.5), and resuspended in distilled water. Cold 10% trichloroacetic acid was added, and the supernatant fluid was extracted repeatedly with diethyl ether in the cold, lyophilized, hydrolyzed with 6 N HCl, and analyzed on a "Biochrom" automatic amino acid analyzer.

To detect small amounts of arginine during arginine depletion, an isotope dilution technique based on the following reaction was used:

Cells were washed as above, sonically treated for 15 sec in the cold, and centrifuged at $3,250 \times g$ for 15 min. The supernatant fluids were tested for free L-arginine. The assay conditions were as described in detail by Rubin and Goldstein (25), with the following exceptions. Instead of *Escherichia coli* "soluble" ribonucleic acid (RNA), a preparation of *Saccharomyces* sp. "soluble" RNA (Schwarz BioResearch) was used, and the aminoacyl-transfer RNA (tRNA) synthetase mixture was prepared from *Saccharomyces* sp.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Polypeptides were dissociated essentially as described by Maizel et al. (15). Newly made virus suspensions at a concentration of 200 to 2,000 μg of protein per ml were dialyzed against 0.05 M Tris-

hydrochloride (pH 8.0) and added in final concentrations of sodium dodecyl sulfate (SDS) to 2%, dithiothreitol to 0.2 M, and ethylenediaminetetraacetic acid (EDTA) to 0.005 M. The samples were then incubated at 95 C for 3 min and subsequently made 10% with respect to glycerol.

Acrylamide gels contained 10% acrylamide and 0.25% *N,N'*-methylene bisacrylamide both of which were recrystallized before use (9). The buffer contained 0.1% SDS (recrystallized from ethanol) and 0.005 M EDTA in 0.1 M Tris-hydrochloride (pH 8.0). Polymerization was catalyzed by 0.1% (v/v) tetraethylmethylenediamine and 0.04% (v/v) ammonium persulfate. The gels were made in 13-cm glass tubes with an internal diameter of 6 mm. After the gels had solidified, they were prerun at 8 v/cm for 1 hr (6 to 7 ma/cm).

The samples were layered on the gel surfaces and overlaid with the electrophoresis buffer 0.1 M Tris-hydrochloride (pH 8.0) with 0.1% SDS and 0.005 M EDTA. Electrophoresis was carried out for 15 min at 3 v/cm and then for 2.5 hr at 8 v/cm. The gels were stained for 18 hr in Coomassie Brilliant Blue R250 dissolved in 10% acetic acid-30% methanol. Destaining was performed in 10% acetic acid-30% methanol. Gels were cut in 1-mm slices, and radioactivity was extracted with 0.4 ml of 1 M NaOH at 37 C for 18 hr and counted.

Assay of trichloroacetic acid-insoluble radioactivity and protein. Cells were withdrawn from Spinner cultures, sedimented by centrifugation (for 10 min at $300 \times g$) at 4 C, and washed once in 2 ml of ice-cold PBS. The cell pellet was resuspended in 0.5 ml of ice-cold PBS and an equal volume of ice-cold 10% trichloroacetic acid was added. After thorough mixing, the material was incubated overnight at 4 C. The precipitate was sedimented, washed twice with 1 ml of ice-cold 5% trichloroacetic acid, and then extracted once with a mixture of ethanol and diethyl ether (3:1). After complete drying at 45 C, the precipitate was dissolved in 0.1 M NaOH and counted. Protein was measured by the method of Lowry et al. (12) with bovine serum albumin as standard.

Isotopes. L-Arginine-monohydrochloride-*guanido*- ^{14}C (4.6 mCi/mmol) and ^{14}C -L-arginine-monohydrochloride (uniformly labeled) (254 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Adenosine-*T-G* (500 mCi/mmol) and L-arginine-monohydrochloride-*5-T* (500 mCi/mmol) were obtained from Radiochemical Centre, Amersham. ^{14}C -L-reconstituted protein hydrolysates (1 mCi/mg) were obtained from International Chemical and Nuclear Corp., Irvine, Calif. Radioactive isotopes were counted in a Packard liquid scintillation spectrometer with a dioxane-based scintillation fluid (6) containing an equal dry volume of Cab-O-Sil (Cabot Corp., Boston, Mass.).

RESULTS

Synthesis of virus and structural proteins under normal growth conditions. To establish the kinetics of synthesis of both virus and structural proteins during one-step growth conditions, virus and antigens were quantitated both during the logarithmic

and the linear phase of virus production. Monolayers and Spinner cultures of KB cells were infected and cultivated at the normal arginine concentration of 1.2 mM. At different time intervals, approximately 1.5×10^7 cells were withdrawn, washed, and, after sonic treatment, assayed for virus infectivity and virus antigens by the complement fixation and radial immunodiffusion techniques. The time course for virus synthesis was the same in monolayers and Spinner cultures. The logarithmic increase in structural proteins was followed with the complement fixation test since it is about 10 times more sensitive than radial immunodiffusion. Late in infection during the linear increase, quantitative estimates were made of the excess antigens. As shown in Fig. 1, the four antigens hexon, fiber, penton base, and the major core protein (the alanine and arginine rich core protein, AAP; 21) appeared one after another at 1-hr intervals. The antigen first detectable was the hexon, followed by the fiber and penton base. The AAP was detected only 1 hr before the rise in virus infectivity. The quantitative data show that the fiber antigen was in excess over hexon and penton base up to 22 hr after infection, but at this time there was a shift and hexon antigen increased more rapidly than the other capsid antigens (Fig. 2). A correlation in time between the linear increase in the antigens and the infectivity at 20 to 35 hr after infection also appeared to exist.

FIG. 1. Logarithmic synthesis of infectious virus and structural proteins in Spinner cultures of KB cells under normal one-step growth conditions. Top: Cell-associated fluorescent focus units (FFU) at 0 to 64 hr postinfection. Bottom: Antigen synthesis measured by complement fixation at 5 to 24 hr postinfection. No antigens were detected between 5 and 9 hr postinfection when samples were analyzed at hourly intervals.

FIG. 2. Linear synthesis of infectious virus and structural proteins in Spinner cultures of KB cells under normal one-step growth conditions. Top: Cell-associated fluorescent focus units (FFU) in per cent of maximum yield at 10 to 64 hr postinfection. Bottom: Production of viral antigens measured as micrograms of protein per 10^6 cells. The antigens were measured with radial immunodiffusion by using specific antisera.

Incorporation of arginine into protein in control and virus-infected cells. To reveal differences in arginine incorporation into protein between infected and control cells, Spinner cultures of KB cells were infected at a multiplicity of 100 FFU/cell in normal medium. Control cultures were examined in parallel.

At intervals, 10^6 cells were withdrawn and pulse-labeled for 30 min at 37°C with ^3H -arginine at $1 \mu\text{Ci}/\text{ml}$. Cells were assayed for total protein, trichloroacetic acid-insoluble radioactivity, and virus infectivity. Figure 3 shows that the rate of arginine incorporation in control cells was constant over the period examined. On the other hand, there appeared to be a slight increase in the rate of arginine incorporation in infected cells up to 12 to 14 hr after infection, coinciding with the logarithmic rise in structural proteins. At this time of maximum arginine incorporation

the rate exceeded by 20% the rate in control cells; afterwards there was a continued decrease. The net increases in protein content of virus-infected and uninfected cells were similar, suggesting that the rates of protein synthesis are the same (not shown).

Cellular pool of arginine in normal and arginine-depleted KB cells. The pools of free arginine and other amino acids in normal and arginine-depleted cells were analyzed. The arginine concentration was slightly lower than that for all other amino acids analyzed and was sensitive to complete arginine depletion (Table 1).

The concentration of free arginine in the cell was determined at depletion and upon reversion. KB cells were maintained in normal medium (1.2 mM arginine) for 2 hr and then divided into

FIG. 3. ^3H -arginine incorporation in protein at intervals in a one-step growth curve of infected cells. Uninfected cells were included as a control. The percentages of the maximum rate of arginine incorporation in infected cells calculated as trichloroacetic acid-insoluble radioactivity per milligram of protein per 30-min pulse is plotted versus the time of incubation. Time zero is the time of infection.

TABLE 1. Pools of free amino acids in KB cells after incubation in complete medium (1.2 mM) and arginine-depleted medium (0 μM)^a

Amino acid	Normal medium ($\mu\text{moles}/10^6$ cells)	Medium without arginine ($\mu\text{moles}/10^6$ cells)
Arginine.....	0.136	0.037
Lysine.....	0.231	0.207
Proline.....	0.586	0.562
Glycine.....	0.966	0.901
Alanine.....	0.431	0.277
Leucine.....	0.207	0.217

^a Arginine-deprived cells were incubated for 5 hr before analysis.

two equal parts. One was transferred to arginine-depleted medium, and the other was kept in normal medium as a control. After 2 hr of additional incubation, the depleted culture was reversed to 1.2 mM arginine. Samples were analyzed by the isotope dilution method described above. By 15 min after removal of arginine, the pool was decreased by 80%, and at 15 min after reversion to normal arginine concentration the cellular arginine pool was completely restored (Fig. 4).

Growth of uninfected KB cells at different concentrations of arginine. Elimination of single amino acids from the medium of mammalian cells drastically influences the synthesis of macromolecules (14). Therefore, it was necessary to study the effect of arginine depletion on uninfected KB cells. Monolayers of KB cells were trypsinized, washed, and incubated on plates in medium with final concentrations of arginine ranging from 0 to 1,200 μM . At zero time and at 24 and 48 hr after plating, the cells were gently scraped off in their growth medium, washed in PBS, and resuspended in 0.1 M NaOH, and the total cell protein was determined. The amount of protein synthesized decreased when arginine was eliminated, but in the presence of 50 μM arginine cell protein remained constant (Fig. 5). Higher concentrations of arginine allowed an increase in

FIG. 4. Cellular pool of free arginine at arginine depletion (0 μM) and upon reversion to normal concentration (1.2 mM) expressed as per cent of the maximum concentration in micromoles per microgram of protein in control cells. The arrow A indicates the time point of depletion, and B indicates the time point of reversion. Symbols: ●, cells exposed to depletion and reversion; ○, control cells.

FIG. 5. Effect of arginine concentration on increase in cellular protein of uninfected KB cells. Total cell protein was determined in cell cultures at 0, 24, and 48 hr of incubation in Eagle's MEM with different concentrations of arginine, and the difference of protein content over 48 hr is plotted versus arginine concentration.

total cell protein, and therefore an arginine concentration of 50 μM was chosen for further arginine depletion experiments.

DNA and RNA synthesis in arginine-depleted KB cells. An arginine concentration of 50 μM which showed growth inhibition was chosen to establish that overall RNA and DNA synthesis was intact and reversible during arginine depletion in uninfected cells. KB cells were washed and incubated in Spinner cultures with 50 and 1,200 μM arginine at a cell density of about 3×10^5 cells/ml. After 48 hr of incubation, half of the depleted cells were reversed to the normal concentration of arginine (1.2 mM), and ^3H -adenosine was added to a final concentration of 0.03 $\mu\text{Ci/ml}$ to all three cultures (depleted, reversed, and control, respectively). At different time intervals, 2-ml samples were withdrawn, washed, resuspended in cold PBS, and precipitated with an equal volume of cold 10% trichloroacetic acid. ^3H -adenosine incorporated into RNA and DNA was determined according to Schmidt and Tannhauser (31). After 2 days of arginine depletion (50 μM), cells incorporated ^3H -adenosine into RNA and DNA to 50 and 30%, respectively, of the incorporation in normal cells (Fig. 6). After reversal to high concentrations of arginine, these values were increased to 80 and 100% of normal levels for RNA and DNA, respectively, demonstrating the reversibility of the changes observed after arginine depletion. After arginine reversion, the 30-min lag phase for DNA synthesis was reproducibly found, although no such delay was apparent for RNA synthesis.

Virus yield as a function of arginine concentration. To determine the relationship between virus yield and arginine concentration of the medium, monolayers of KB cells were infected and incubated with different concentrations of arginine

FIG. 6. Incorporation of ³H-adenosine into total RNA and DNA as determined by the Schmidt-Tannhauser technique. Control (1.2 mM arginine) and depleted (50 μM arginine) cultures of uninfected KB cells were kept for 2 days. Half of the depleted culture was then reversed to 1.2 mM arginine, and isotope was added at zero time to control, reversed, and depleted cells. Samples were taken at indicated times. (A) RNA synthesis; (B) DNA synthesis.

ranging from 0 to 1,200 μM. In this type of experiment, monolayers were used because of the more simple handling of cultures.

The cells were incubated at 37 C for 35 hr, and the samples were assayed for infectivity after sonic treatment. As shown in Fig. 7, virus yield increased continuously with an increase in arginine concentration of the medium. No threshold level was apparent. Repression of final virus yield never exceeded 4 log units and amounted to 99.95% at 50 μM arginine.

These results suggest that a concentration of 50 μM arginine could be used to study the effect of arginine depletion on virus synthesis, since it caused a more than 1,000-fold reduction in virus yield, and, as shown above, this concentration reversibly inhibited growth and RNA and DNA synthesis of uninfected cells.

Virus yield at delayed arginine depletion. To reveal the arginine-dependent period during adenovirus replication, a simple kinetic experiment was designed. Monolayers of KB cells were infected and cultivated in normal medium (1.2 mM arginine). At 2-hr intervals, cells were washed twice in 20 ml of Eagle's MEM without arginine and then further incubated with 30 ml of arginine-deprived medium. Since the cellular pool of free arginine has been shown to respond within 15 min upon arginine depletion and reversion (Fig. 4), it is assumed that a completely arginine-deprived environment is obtained very rapidly after washing the cells in arginine-free medium. All cell cultures were incubated for a total period of 35 hr postinfection. Cells were then harvested, washed, and assayed for infectivity. Table 2 shows that if arginine is removed from the medium

about 16 hr or later postinfection and incubation is continued at complete deprivation for a total period of 35 hr, no inhibition of virus yield is achieved. These results might imply that all arginine-dependent synthetic events take place during the first 15 hr of the virus growth cycle.

Synthesis of structural proteins at different concentrations of arginine. The amount of virion antigens present at different degrees of arginine depletion was determined next. Cells infected for 35 hr at different concentrations of arginine were sonically treated and centrifuged in 0.6-ml samples at 45,000 × g for 30 min. The supernatant fluids were assayed for the amount of antigen. The yields of antigens (micrograms/10⁷ cells) at different arginine concentrations of the medium are shown in Fig. 8. All the three anti-

FIG. 7. Virus yield as a function of arginine concentration of the growth medium. V_d = virus yield at various degrees of arginine depletion. V_c = virus yield of control at 1.2 mM arginine. Mean values of three separate experiments.

TABLE 2. Effect of delayed arginine depletion on adenovirus multiplication

Time period of cultivation under complete arginine depletion (hr)	Percentage virus yield ^a
0-35	0.07
2-35	0.04
4-35	0.05
6-35	0.2
8-35	3.5
10-35	2
12-35	11
14-35	27
16-35	100
18-35	100
21-35	100

^a Values are expressed as a percentage of the yield in a control maintained under normal conditions for 35 hr.

FIG. 8. Synthesis of structural proteins in monolayers of virus-infected KB cells cultivated at different concentrations of arginine. Viral antigens were measured as micrograms per 10^7 cells with radial immunodiffusion by using specific antisera. Mean values of three separate experiments.

gens which can be assayed quantitatively are synthesized in excess independent of the concentration of arginine. Thus, hexon, penton base, and fiber antigen are only reduced by 90, 70, and 60%, respectively, of maximum yield at complete arginine deprivation, although virus infectivity is reduced by 99.95% (Fig. 7). It was not possible to quantitate the penton base at complete depletion, although it was present as revealed by complement fixation. The amount of AAP measured by complement fixation ranged between 50 and 100% of the highest titer when the arginine concentration was varied from 10 to 1,200 μ M, indicating that the synthesis of AAP is also not significantly affected by arginine depletion.

At an arginine concentration of about 100 μ M, there was a shift in the amount of fiber and hexon synthesized; at concentrations below 100 μ M, the fiber was in excess over the hexon antigen, whereas at higher concentrations conditions were reversed. This shift was also evident in normal one-step growth curves at about 22 hr after infection (Fig. 2).

When the quantitative data are expressed in virus equivalents together with the number of virus particles formed, it is evident that all three outer capsid structural proteins are 10^8 to 10^5 times in excess over virions at complete arginine depletion; the excess over virions decreased to 2- to 100-fold when the arginine concentration was raised to 1.2 mM (Fig. 9). These results suggest that none of the four antigens examined has a rate-limiting function on virus assembly or synthesis during arginine starvation.

Synthesis of virus and structural proteins at

FIG. 9. Quantitative data on the synthesis of structural proteins in monolayers of virus-infected KB cells from Fig. 8 are expressed in virus equivalents by using molecular weights for the hexon, penton, and fiber of 360,000, 400,000, and 200,000 daltons, respectively (L. Philipson and U. Pettersson, *Progr. Exp. Tumor Res.*, in press). The corresponding yields of infectious virus are recalculated into number of virus particles by using a factor of 30 for the ratio of particles to fluorescent focus units (11).

reversion of arginine depletion: virus. The kinetics of virus synthesis after reversion were studied in detail. KB cells were infected in Spinner cultures with an arginine concentration of 50 μ M. At intervals, about 1.5×10^7 cells were withdrawn, washed in cold PBS, resuspended in a final volume of 1 ml of 0.01 M Tris-hydrochloride (pH 8.1), and assayed for virus infectivity and antigen content. At 30 hr after infection, the culture was divided and arginine was added to one half at a final concentration of 1.2 mM. Synthesis was then followed at intervals for another 30 hr.

Figure 10 shows the growth curves, and it is clear that the rise in virus infectivity was not apparent until 20 to 22 hr at 50 μ M arginine, whereas the eclipse period under normal growth conditions occurred at about 14 hr. Furthermore, the final virus yield was about 4 log units below the normal yield (see also Fig. 7). Upon reversion (to 1.2 mM arginine) after 30 hr of depletion, a lag period of 4 to 5 hr was observed before a rise in virus titer. This lag phase extended for 4 to 5 hr irrespective of the arginine concentration (0 to 150 μ M) during starvation. The final virus yield after reversion always was one order of magnitude below that in controls. These experiments suggest that synthetic events are required before virus synthesis, although hexon, penton, fiber, and AAP antigens are present in great excess at depletion (Fig. 8).

Synthesis of virus and structural proteins at reversion of arginine depletion: structural proteins. Since the preceding experiments showed a large accumulation of antigens even at low concentrations of arginine (Fig. 8), it was of interest to investigate whether the antigen levels were affected by arginine reversion. All samples assayed for infectivity in the growth curves after reversion (Fig. 10) were assayed quantitatively for virus antigens. As shown in Fig. 11, the amounts of cell-associated hexon and fiber antigens increased slightly under prolonged arginine depletion. The amount of penton base did not change under these conditions. After reversion, synthesis of fiber was unaffected but the penton base increased fivefold. The hexon antigen, on the other hand, showed a rapid change with a 10-fold increase over 33 hr and synthesis started by 1 hr

after addition of arginine. The amounts of hexon and fiber antigens after reversion never reached the values for control cells kept at high concentration of arginine (1.2 mM); the final values were 20 to 30% lower. On the other hand, penton base reached about normal levels after arginine reversion.

Synthesis of virus and structural proteins at reversion of arginine depletion: analysis of isotope labeled virions and virion polypeptides. The incorporation into virions of isotope-labeled antigens synthesized during arginine depletion and the fate and specific activity of labeled arginine in virions formed in control and in reversed arginine-depleted systems were determined next.

(i) Virus-infected KB cells were cultivated in the presence of ^3H -arginine (1 $\mu\text{Ci}/\text{ml}$, 2 μM) and unlabeled arginine at 500 μM from 0 to 40 hr. (The 500 μM concentration of unlabeled arginine was used to avoid an unnecessary dilution of the isotope.) Final virus yield was not affected as shown above (Fig. 7). After 40 hr, the cells were harvested, and virus was purified, assayed for infectivity, protein content, and radioactivity, and directly analyzed by polyacrylamide gel electrophoresis.

(ii) Virus synthesized after arginine reversion was prepared in parallel, and, after 30 hr of arginine depletion at 50 μM , ^3H -arginine (1 $\mu\text{Ci}/\text{ml}$) was added to equilibrate with cells 1 hr before the addition of unlabeled arginine in a final con-

FIG. 10. Growth curves of adenovirus in Spinner cultures of KB cells under one-step growth conditions in growth medium containing 1.2 mM and 50 μM arginine. The arrow indicates the time point of reversion when arginine was added to half of the depleted culture to give a final concentration of 1.2 mM arginine.

FIG. 11. Synthesis of viral structural proteins in Spinner cultures of KB cells under one-step growth conditions at 50 μM (B) arginine and upon reversion to 1.2 mM arginine (A). The arrow indicates the time point for reversion. The time axis gives total time of incubation after infection. Antigens were measured as micrograms per 10^6 cells with radial immunodiffusion by using specific antisera.

centration of 500 μM . The cells were further incubated for 30 hr after reversion.

(iii) Structural proteins synthesized during arginine depletion were labeled with ^3H -arginine (1 $\mu\text{Ci}/\text{ml}$) and 50 μM unlabeled arginine for 30 hr. After a thorough wash, the cells were reversed to 500 μM arginine and incubated for another 30 hr. Virus was purified as above.

Two separate experiments showed that the specific activity of virus synthesized after reversion (ii) was about 80% of that of control virus (i), which indicates that not more than 20% of the viral proteins are utilized from the preformed pool after reversion, assuming that the specific activities of the arginine pools of control and arginine-depleted cells are the same (Table 3). This result is confirmed by the fact that after reversion prelabeled virus antigens (iii) were only utilized at less than 10% of the rate of the analogue control virus.

The patterns of radioactivity for control virus (i) and virus synthesized after reversion (ii) are almost identical, and no significant proportions between any peaks are revealed (Fig. 12A and B), which implies that all virion polypeptides are synthesized upon arginine reversion. Since the polypeptide of the purified arginine-rich major core protein (AAP) bands as peak VII in this system (21) and the radioactivity of this peak reaches almost the same level for both virus preparations, it appears unlikely that the arginine-rich core protein is preferentially synthesized after reversion.

Degradation of arginine and hexon antigen during arginine depletion: arginase. To determine whether the arginase activity was decreased in arginine depletion, KB cells were incubated in arginine-depleted medium (0 μM) and control medium (1,200 μM), and samples were withdrawn and tested for arginase activity. Figure 13 shows a drastic decrease in enzyme activity during the first 12 hr, followed by a continuous decrease up to 85 hr after depletion.

TABLE 3. Specific activity of virions synthesized in the presence of ^3H -arginine during a normal growth cycle, during depletion, and after arginine reversion

Labeling time of ^3H -arginine	Specific activity of virus (counts per min per μg of protein) ^a
At 0-40 hr, whole cycle.....	468
At 30-60 hr, after reversion.....	372
At 0-30 hr, during depletion.....	32

^a Values are given as the average of two separate experiments.

FIG. 12. (A) Polyacrylamide gel electrophoresis of the polypeptides of ^3H -arginine-labeled control adenovirus type 2 grown in KB cells in the presence of ^3H -arginine (1 $\mu\text{Ci}/\text{ml}$) and unlabeled arginine at 500 μM from 0 to 40 hr. The virus was harvested and purified. The anode is on the right. (B) Adenovirus grown in KB cells at arginine depletion (50 μM) for 30 hr at which time the cells were reversed to high concentration of arginine (500 μM), supplied with ^3H -arginine (1 $\mu\text{Ci}/\text{ml}$), and incubated for another 30 hr. Adenovirus labeled with ^{14}C -amino acids at a high concentration of arginine (500 μM) from 0 to 40 hr was run in the same gel as a reference.

The same procedure was repeated for virus-infected and control cells at different times in the infectious cycle at an arginine concentration of 1.20 mM. As shown in Table 4, there was no difference in arginase activity between infected and control cells during 25 hr of incubation, indicating that the hydrolysis of arginine is unchanged during adenovirus replication.

Degradation of arginine and hexon antigen during arginine depletion: turnover of hexon antigen. Because of the drastic changes in hexon antigen concentration on arginine reversion (Fig. 11), the effect of arginine depletion on turnover of hexon protein was estimated.

KB cells in monolayers were washed and infected, and, after 8 hr of incubation, ^3H -labeled amino acids were added at 1 $\mu\text{Ci}/\text{ml}$. Incubation was continued for 12 to 16 hr in normal medium, and, after washing, half of the bottles were sup-

FIG. 13. Arginase activity expressed as micromoles per microgram of protein per hour in uninfected cells after incubation in normal medium (1.2 mM arginine) and arginine-deficient medium (0 μ M).

TABLE 4. Arginase activity in infected and uninfected KB cells^a

Time (hr)	Amt (μ moles) of urea per microgram of protein per hr	
	Control cells	Infected cells
0	1,365	1,365
10	1,255	1,365
14	1,185	1,170
18	1,065	1,065
25	1,000	1,150

^a Enzyme activity is expressed as micromoles of urea formed per microgram of protein per hour. Time refers to time after infection for infected and to time in culture for uninfected cells.

plied with normal medium and the other half were supplied with arginine-depleted medium. As shown earlier (Table 2), complete depletion at this time after infection and a continued incubation under arginine depletion for about 24 hr did not affect the final virus yield. Incubation was continued for another 24 to 28 hr; the cells were harvested, washed twice, and finally resuspended in 0.01 M Tris-hydrochloride (pH 8.1) at 10^7 cells/ml. After sonic treatment for two 30-sec periods and centrifugation at $45,000 \times g$ for 30 min, the supernatant fluids were assayed for hexon antigen by radial immunodiffusion and then diluted to contain the same amount of hexon. Hexon antigen was then precipitated by radioimmunoprecipitation, and the per cent radioactivity specifically precipitated as hexon antigen was determined.

Total activity added in separate experiments

ranged from 80,000 to 180,000 counts/min. The mean values from three separate experiments showed that about $2.9 \pm 0.7\%$ of the radioactivity was precipitable as hexon antigen in control cells, whereas about $2.2 \pm 0.9\%$ was precipitated in the depleted cells. Thus, there was no significant degradation of hexon antigen after a complete arginine deprivation for 24 to 28 hr.

DISCUSSION

It has been known for a long time that arginine depletion drastically affects the synthesis of adenoviruses (23, 26). Since the demonstration that the "late P-antigen" is sensitive to arginine depletion (26) and the finding of internal, arginine-rich virus proteins associated with the viral DNA (8, 22, 28), it has been suggested that these proteins may be of importance for virus assembly and may be particularly sensitive to arginine depletion.

A penetration of the arginine-sensitive step required a detailed study of the synthesis of the structural proteins under normal one-step growth conditions. The logarithmic synthesis of the three main structural units, hexon, penton base, and fiber, was studied at normal arginine concentration, and previous results were confirmed (27). The major core protein, AAP, is synthesized with a 1-hr lag after the penton base and thus precedes virus maturation by about 1 hr (Fig. 1). The AAP also appears to be produced in excess in infected cells. During the linear increase in structural proteins at 15 to 30 hr postinfection, the fiber antigen is in fourfold molar excess over the hexon antigen at the start of this period, as was also observed by White et al. (33). This is followed by a significant increase in hexon synthesis which results in a molar ratio of 0.5 to 1 between hexon and fiber antigen at about 30 hr after infection (Fig. 2).

The enhanced requirement for arginine in infected cells can be demonstrated by pulse-labeling at different times during the growth cycle at a normal arginine concentration. At 12 to 14 hr postinfection, the rate of arginine incorporation reaches a maximum (Fig. 3). This increase corresponds in time to the logarithmic increase of all structural proteins (Fig. 1). It is not very likely that the increase in arginine incorporation only reflects an enhanced protein synthesis, since infected and uninfected cells incorporate labeled phenylalanine, valine, and lysine at similar rates as reported by Polasa and Green (20). Therefore, it is suggested that the increased uptake of arginine may be specific.

Attention has recently been focused on the role of enhanced proteolytic activity in amino acid-

depleted bacteria (2) and mammalian cells. Cycloheximide (34) and amino acid deficiency (13, 14) affects ribosomal RNA synthesis in mammalian cells, suggesting that synthetic events which require continuous synthesis of proteins with high turnover will be sensitive to deprivation of essential amino acids. It is therefore important to define the depletion level which is reversible for the normal host cells to utilize reversion experiments to map the sensitive step in virus synthesis. KB cells are sensitive to complete arginine deprivation, but a concentration of 50 μM appears to cause a reversible inhibition of growth and overall nucleic acid synthesis (Fig. 5 and 6).

Arginine depletion studies on virus-infected KB cells at 50 μM arginine reveal that the virus yield is reduced more than 1,000-fold, although structural proteins (expressed in virion equivalents) are accumulated in 10^3 - to 10^5 -fold excess over virions (Fig. 7 and 9).

Furthermore, the molar ratio of excess fiber antigen to hexon antigen varies from 3 to 6 after 35 hr at arginine concentrations ranging from 0 to 75 μM (Fig. 8), a situation which resembles the early phase of linear increase in structural proteins, 15 to 20 hr postinfection in a normal one-step growth curve (Fig. 2). An experiment with delayed complete arginine depletion revealed that, when arginine is expelled from the growth medium as early as 14 hr after infection, only a 75% reduction of virus yield is achieved (Table 2). These findings might imply that the arginine block is an early event.

Kinetic studies of virus and antigen synthesis upon reversion after 30 hr of depletion (50 μM) reproducibly showed a 4- to 5-hr lag period until a rise in the infectious titer was apparent (Fig. 10), which further suggests that profound synthetic events are impaired during arginine depletion. This finding is not in agreement with the results obtained by Rouse and Schlesinger, who observed no lag period (23). Thus, the major effect of arginine depletion with regard to the outer capsid units is that the increase in the pool of hexon (which gives a shift in the hexon-fiber ratio) requires 150 μM or higher concentrations of arginine and that hexon synthesis increases sharply upon arginine reversion, although the other capsid proteins only show a moderate increase (Fig. 11). The mechanism by which the hexon antigen responds to high concentrations of arginine is unclear. It has furthermore been established that radioactive arginine furnished at reversion is incorporated into all polypeptides of the virion (Fig. 12), and the specific activity of virions made after arginine reversion expressed in radioactivity on a protein weight basis is about

80% of that for normal virions (Table 3). These results indicate that the majority of the proteins maturing into virus particles at arginine reversion are formed subsequent to the addition of high concentrations of arginine and that the considerable excess pool of structural units present at arginine depletion is not available for virus synthesis. No severe degradation of hexon was observed during complete arginine depletion.

Based on the combined data, we would like to propose the following hypothesis. Arginine deficiency in adenovirus infection may interfere with the synthesis of host or virus-derived protein(s) made early in the infectious cycle. On what macromolecular level this protein(s) functions cannot be stated on the basis of the experiments in this report. Yet it appears that lack of this protein allows the excess pool of structural units to accumulate in such a way that it not longer is available for virus assembly. Perhaps the *in vitro* assembly system of Winters and Russell (36) may be used to study directly whether the structural proteins of arginine-deprived cells will be incorporated into virions in the same way as DNA from these cells is utilized for virus assembly.

Furthermore, we have evidence that this early factor is involved in the replication of DNA and the subsequent transcription, since a blocking of these functions will not allow an increase in virus synthesis upon arginine reversion (*to be published*).

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