Adenovirus Proteins

II. N-Terminal Amino Acid Analysis

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Adenovirus types 2, 4, 5, 6, 18, 21 and 27, were analyzed for N-terminal amino acids by use of ³⁵S-labeled phenylisothiocyanate of high specific radioactivity. Two free N-terminal amino acids, alanine and glycine, were found in these viruses in the molar ratio (alanine-glycine) of 2.5:1, with the exception of type 2 where the ratio was 3.6:1 and type 5 where the ratio was 5.5:1. Adenovirus type 2 was disrupted by acetone treatment, and two protein fractions were obtained after sucrose gradient centrifugation. One of these fractions, which was associated with the viral deoxyribonucleic acid and comprised approximately 18% of the total protein of the virus, was greatly enriched with respect to N-terminal alanine and glycine.

Adenoviruses are composed only of deoxyribonucleic acid (DNA) and protein (6, 7). The DNA of a number of human adenoviruses was found to have a molecular weight of about 23×10^6 (Green et al., Proc. Natl. Acad. Sci. U.S., *in press*) and to comprise 13% of the dry weight of the virus (12). From these data, it may be calculated that particles of adenoviruses contain 154×10^6 daltons of protein.

The adenovirus virion has been shown to contain a number of different antigens. It was proposed (11, 14) that the capsid is composed of 240 nonvertex capsomeres carrying the group-specific complement-fixing antigen and 12 vertex capsomeres which are immunologically distinct and carry projections. Following the nomenclature of Ginsberg et al. (5), a distinction can be made between hexon (nonvertex capsomeres), penton (vertex capsomeres plus projections), and fiber antigen (vertex projections only).

These antigens are presumably protein in nature, but nothing is known about the size or number of polypeptide chains of which the antigens are composed.

Electrophoresis in polyacrylamide gels of adenoviruses disrupted by treatment with sodium dodecyl sulfate and 2-mercaptoethanol resulted in the separation of 10 different protein bands (10). However, it is not known whether these represent 10 different kinds of polypeptide chains.

In the present work, a search was made in adenoviruses for N-terminal amino acids, since

knowledge of the number of these will give some indication as to the number of different polypeptide chains occurring in the virus particle.

MATERIALS AND METHODS

Viruses. Adenovirus types 2, 4, 5, 6, 18, 21, and 27 were grown in suspension cultures of KB cells, and the viruses were purified by use of the methods described by Green and Piña (6, 7). After centrifugation in a cesium chloride density gradient, the viruses were freed from cesium chloride by dialysis against water at 4 C. The resulting virus preparations were usually well-dispersed and highly opalescent suspensions, but in some instances complete and apparently irreversible aggregation occurred during dialysis.

The preparations of adenovirus were examined in the electron microscope for the presence of small particles of adeno-associated virus (2).

Labeled virus. Adenovirus 2 with ³²P-labeled DNA and ³H-labeled protein was prepared by growing the virus in medium containing ³²P-inorganic phosphate and ³H-valine.

N-terminal amino acid analysis. Preparations of adenoviruses and of the isolated structural proteins were analyzed for N-terminal amino acids by a modification of the Edman method with the use of ³⁵S-labeled phenylisothiocyanate of high specific radioactivity (8). The ³⁵S-phenylthiohydantoin (PTH) derivatives of the N-terminal amino acids of the viral proteins were mixed with unlabeled carrier PTH derivatives of appropriate amino acids, and the mixtures were chromatographed on 2.5-cm wide strips of Whatman no. 1 filter paper in solvents D, E, and F of Edman and Sjoquist (3). After chromatography, the positions of the unlabeled PTH amino acids on the strips were located under ultraviolet light. The strips were then cut into 12-mm segments, and the radioactivity on each segment was counted in a Packard liquid scintillation spectrometer. The specific activity

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of the ^{35}S -phenylisothiocyanate used in each experiment was determined by diluting this 1:10,000 with nonradioactive phenylisothiocyanate and counting 1 μliter of the diluted material under the same conditions used for the unknown samples. The specific activity of the ^{35}S -phenylisothiocyanate was approximately 4×10^7 counts per min per μmole .

In an attempt to assess quantitatively the yields of PTH-amino acids from the viral proteins, known amounts of proteins, well characterized with respect to their content of N-terminal amino acids (bovine serum albumin and horse hemoglobin), were sometimes added to the viral proteins before analysis. It was assumed that the recovery of PTH-amino acids from these known proteins would be the same as the recovery from the unknown samples.

Disruption of adenovirus by acetone. Preparations of purified adenovirus particles, suspended in distilled water at a concentration of 2 to 3 mg/ml, were mixed with 4 volumes of acetone at room temperature. No precipitation of the virus occurred. The mixture was allowed to stand for 10 min at 20°C; then the virus was flocculated by adding a drop of 0.1 M NaCl and centrifuged ($1,500 \times g$, 5 min). The supernatant fluid was poured off, and the pellet was drained thoroughly and dissolved in water. Experiments with labeled virus showed that there was no loss of viral DNA or protein during this operation. Occasional preparations of adenovirus, when precipitated with acetone in this fashion, did not redissolve easily in water.

Protein assays. Protein was assayed by a micro-modification of the Lowry method (9) with crystalline ovalbumin as standard.

RESULTS

N-terminal amino acid analysis. N-terminal amino acid analysis of a number of different types of adenovirus showed that these possessed two free N-terminal amino acids, alanine and glycine (Fig. 1, Table 1). Some ^{35}S -labeled material was associated with PTH-threonine (which runs between PTH-glutamic acid and PTH-tyrosine) when the PTH amino acids were chromatographed in solvent F (representative chromatograms as shown in Fig. 1c and 1i). This material was eluted from the chromatogram and rerun in solvent E. It was clearly seen (Fig. 2) that the radioactivity was not associated with PTH-threonine, and most likely represents a by-product of the Edman reaction.

Some adenovirus preparations also possessed N-terminal methionine, but, since others (even of the same type) did not (Fig. 1d and 1e), it is assumed that the N-terminal methionine was associated with some contaminant rather than with a structural protein of the virus. In addition, activity was observed in the area of PTH-leucine when the chromatograms were run in solvent F. However, the peak of radioactivities did not coincide with the center of known PTH-leucine and was not observed when chromatographed in

solvent D. Most likely, this represents a degradation product of the Edman reaction.

Yields of PTH-alanine varied between 2.1 and 3.7 $\mu\text{moles/mg}$ of virus protein, and of PTH-glycine between 0.3 and 1.7 $\mu\text{moles/mg}$. The ratio of PTH-alanine to PTH-glycine was approximately 2.5 for adenoviruses 4, 6, 18, 21, and 27, but for adenovirus 2 the ratio of PTH-alanine to PTH-glycine was 3.6 and for adenovirus 5 it was 5.5.

Disruption of the virus particles and separation of some viral proteins. To concentrate preparations of adenoviruses prior to N-terminal amino acid analysis, the virus particles were precipitated by the addition of acetone. Although this was done at room temperature, the precipitates obtained usually dissolved easily and completely in water to give almost water-clear nonviscous solutions. These solutions were very much less opalescent than the original virus suspensions, suggesting that extensive disruption of the virus particles had occurred after acetone precipitation. That this was so was shown by means of sucrose gradient centrifugation of disrupted labeled virus and by sedimentation studies in an analytical ultracentrifuge.

The results of sucrose gradient centrifugation of acetone-disrupted adenovirus 2 having ^{32}P -labeled DNA and ^3H -labeled protein are shown in Fig. 3. Under the conditions of centrifugation chosen, where untreated particles of adenovirus sedimented completely to the bottom of the tube, the acetone-disrupted virus separated into two main fractions. One fraction (fraction A), which contained the viral DNA and 18% of the protein of the virus sedimented half way down the tube; the other (fraction B), containing protein only, remained near the top of the gradient. Some DNA and protein was recovered in a pellet at the bottom of the tube. This amounted to only 10% of the total applied and probably represented incompletely disrupted particles of the virus.

The two fractions (A and B) obtained by sucrose gradient centrifugation of acetone-disrupted adenovirus 2 were dialyzed against water to remove sucrose, and the protein in each was precipitated by adding 4 volumes of acetone and a few drops of 0.1 M NaCl. The precipitates were dissolved in water and analyzed for protein and for N-terminal amino acids.

N-terminal alanine was found to occur mainly in the protein associated with the DNA of the virus (fraction A, Fig. 3); much less occurred in the protein which remained at the top of the gradient (fraction B, Fig. 3 and 4). N-terminal glycine was also found in both fractions; in this case also, more was found to occur in the protein associated with the DNA than with the other

FIG. 1. Distribution of radioactivity on chromatograms (solvents F and D) of the ^{35}S -labeled N-terminal amino acid phenylthiohydantoins from a number of different adenovirus (Ad) types. The amount of protein analyzed in each case was as follows: a (Ad 2), 1.2 mg; b (Ad 4), 0.9 mg; c (Ad 5), 2.2 mg; d (Ad 5), 2.2 mg; e (Ad 5), 1.9 mg; f (Ad 6), 2.7 mg; g (Ad 18), 0.7 mg; h (Ad 21), 0.7 mg; i (Ad 27), 1.2 mg. The positions of unlabelled marker phenylthiohydantoins added to the reaction mixture are shown; the relative positions of other amino acid phenylthiohydantoins in solvents F and D are given by Edman and Sjoquist (3). With the exception of the Ad 2 analysis, the ^{35}S -phenylisothiocyanate used gave 3.93×10^7 counts per min per μmole when counted under the same conditions as used for the experimental strips. The ^{35}S -phenylisothiocyanate used in the Ad 2 analysis gave 1.88×10^6 counts per min per μmole .

TABLE 1. *N*-terminal amino acid analyses of adenovirus preparations

Adenovirus type	PTH-alanine recovered (solvent F) ^a	PTH-glycine recovered (solvent F) ^a	PTH-methionine recovered (solvent D) ^a	Ratio of PTH-alanine to PTH-glycine
2	1.3×10^{-3}	0.36×10^{-3}	None	3.6
4	3.45×10^{-3}	1.24×10^{-3}	None	2.8
5	2.51×10^{-3}	0.48×10^{-3}	None	5.2
5	2.15×10^{-3}	0.35×10^{-3}	0.50×10^{-3}	6.1
6	2.53×10^{-3}	0.99×10^{-3}	0.27×10^{-3}	2.5
18	2.13×10^{-3}	0.88×10^{-3}	None	2.4
21	2.51×10^{-3}	0.88×10^{-3}	None	2.8
27	3.70×10^{-3}	1.66×10^{-3}	None	2.2
Acetone-disrupted type 2				
Top fraction (B)	0.86×10^{-3}	0.28×10^{-3}		
Protein associated with DNA (A)	8.08×10^{-3}	1.53×10^{-3}		

^a Values given in micromoles per milligram of protein.

FIG. 2. Distribution of radioactivity on a chromatogram (solvent E) showing the absence of radioactivity in PTH-threonine. The material applied was eluted from the spot corresponding to PTH-threonine on a chromatogram (solvent F) of the ³⁵S-PTH amino acids from Ad 5 (Fig. 1c).

more slowly sedimenting protein fraction. It is unlikely that the low recovery of N-terminal alanine (and glycine) from the slowly sedimenting protein was due to losses during the Edman reaction and subsequent chromatography. Before the two protein fractions were reacted with ³⁵S-phenylisothiocyanate, an equal amount (260 μg) of bovine serum albumin (BSA) was added to each. Though vastly different amounts of N-terminal alanine were recovered from the two fractions, the yield of N-terminal aspartic acid (from the BSA) was the same in each case. The yield from 260 μg of BSA was 1.19×10^{-3} μmole of PTH-aspartic acid. This is 30% of the theoretical amount, assuming a molecular weight for BSA of 65,000 (13). If the yield of N-terminal alanine from the protein associated with adeno-

FIG. 3. Sucrose gradient centrifugation of acetone-disrupted adenovirus type 2 with ³²P-labeled DNA and ³H-labeled protein. The disrupted virus (0.2 ml) was layered onto a linear sucrose gradient [5 to 20% (w/v) sucrose in 0.001 M tris(hydroxymethyl)aminomethane buffer (pH 7.5)] and centrifuged in the Spinco SW 25.1 rotor at 22,500 rev/min for 1.5 hr at 10°C. Thirty fractions were collected through a hole in the bottom of the tube, and samples (0.5 ml) of each fraction were counted for ³²P and ³H (○ = ³²P; ■ = ³H). Fractions 7–17 (fraction A) and 22–30 (fraction B) were then pooled for analysis.

virus DNA was also 30% of theoretical, then it follows, from the amounts of PTH-alanine found, that the molecular weight of this protein is of the order of 35,000. However, N-terminal glycine was also found in this fraction, suggesting that more than one kind of polypeptide chain is present. Therefore, the molecular weight of the protein possessing an N-terminal alanine residue is likely to be less than 35,000. However, if this fraction is composed of more than one species of polypeptide with N-terminal alanine, then this estimate can be significantly in error.

Electrophoresis on cellulose acetate of adenovirus disrupted with acetone. A sample of adenovirus 2

FIG. 4. Distribution of radioactivity on chromatograms (solvent F) of the ^{35}S -labeled N-terminal amino acid phenylthiohydantoin from fractions A and B of acetone-disrupted type 2 adenovirus. In each case, 0.47 mg of protein was analyzed. A sample of crystalline bovine serum albumin (0.26 mg) was mixed with each fraction before analysis. The ^{35}S -phenylisothiocyanate gave 3.93×10^6 counts per min per μmole when counted under the same conditions as the experimental strips.

with ^{32}P -labeled DNA and ^3H -labeled protein was disrupted with acetone and electrophoresed on cellulose acetate strips in tris(hydroxymethyl)-aminomethane-boric acid-ethylenediaminetetraacetate buffer, pH 9.0 (2). The strips were then stained for protein with Procion brilliant blue (4), and one strip was cut into segments which were counted for ^{32}P and ^3H .

Two major protein bands, one of which remained at the origin, and a number of minor bands were obtained (Fig. 5). All of the ^{32}P remained at the origin.

The two fractions obtained after sucrose gradient centrifugation of acetone-disrupted adenovirus 2 were similarly electrophoresed. The fraction containing the DNA of the virus (fraction A,

FIG. 5. Drawings of cellulose acetate electrophoresis strips stained with Procion brilliant blue. (1) Acetone-disrupted adenovirus type 2. (2) Fraction A of acetone-disrupted adenovirus type 2. (3) Fraction B of acetone-disrupted adenovirus type 2. Electrophoresis at pH 9.0 was for 3 hr at 100 v/cm.

Fig. 3) formed a single band which remained at the origin, whereas the protein recovered at the top of the gradient (fraction B, Fig. 3) separated into two bands (Fig. 5).

DISCUSSION

Two free N-terminal amino acids, alanine and glycine, were found in the preparations of adenoviruses examined. Identification of these as the ^{35}S -PTH derivatives was achieved by paper chromatography in two different solvent systems. In each case, the radioactivity coincided exactly with authentic samples of unlabeled PTH-alanine and PTH-glycine run in the same lane on the chromatogram. It is unlikely that these viruses possess any other free N-terminal amino acids. In solvent F, the only PTH-amino acids which remain at the origin during chromatography are PTH-arginine and PTH-histidine. Because some radioactivity remained at the origin during chromatography of the ^{35}S -PTH-amino acids from adenoviruses, the presence of arginine or histidine as N-terminal amino acids in these viruses cannot be excluded completely. But in every case the amount of ^{35}S remaining at the origin was small, and was similar in amount to that found at the origin in this solvent when proteins known not to possess N-terminal arginine or histidine (e.g., BSA) were similarly analyzed.

N-terminal methionine was found in some adenovirus preparations, but in others (even of the same type) this N-terminal amino acid was not found, suggesting that N-terminal methionine is not associated with a structural protein of adenoviruses, but with some contaminant in the preparation. It did not appear to be associated

with the small adeno-associated virus described by Atchison et al. (2): two of the adenovirus preparations examined (types 4 and 21) were found to contain particles of adeno-associated virus, and neither of these preparations yielded N-terminal methionine (Fig. 1a and 1g).

When particles of adenovirus 2, suspended in water, were mixed with acetone at room temperature, extensive disruption of the virus particles occurred, and two protein fractions were isolated after sucrose gradient centrifugation of the disrupted virus. Fraction A (Fig. 3) was associated with the DNA of the virus, both during sedimentation through a sucrose gradient and during electrophoresis on cellulose acetate. This fraction yielded much more N-terminal alanine and glycine than did fraction B which remained at the top during sucrose gradient centrifugation and which showed a large amount of a rapidly migrating component during electrophoresis.

Small amounts of N-terminal alanine and glycine were found in fraction B, but it is likely that these belonged to small amounts of fraction A, present as a contaminant in the top fraction. If this is so, it follows that the protein in fraction B, which represents the bulk of the protein of the virus, has no free N-terminal amino acids: presumably these are masked in some way.

In the present work, these two protein fractions (A and B) have not been associated with any of the viral antigens, but it is possible that fraction A, which comprises approximately 18% of the total protein of the virus, is the inner protein component(s) suspected previously to occur in adenoviruses.

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LITERATURE CITED

1. ARONSSON, T., AND H. GRONWELL. 1957. Improved separation of serum proteins in paper electrophoresis—a new electrophoresis buffer. *Scand. J. Clin. Lab. Invest.* **9**:338–341.
2. ATCHISON, R. W., B. C. CASTO, AND W. MCD. HAMMON. 1965. Adenovirus-associated defective virus particles. *Science* **147**:754–756.
3. EDMAN, P., AND J. SJOQUIST. 1956. Identification and semiquantitative determination of 3-phenyl-2-thiohydantoins. *Acta Chem. Scand.* **10**:1507–1509.
4. FAZEKAS DE ST. GROTH, S., R. G. WEBSTER, AND A. DATYNER. 1963. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochim. Biophys. Acta* **71**:377–391.
5. GINSBERG, H. S., H. G. PEREIRA, R. C. VALENTINE, AND W. C. WILCOX. 1966. A proposed terminology for the adenovirus antigens and virion morphological subunits. *Virology* **28**:782–783.
6. GREEN, M., AND M. PIÑA. 1963. Biochemical studies on adenovirus multiplication. IV. Isolation, purification, and chemical analysis of adenovirus. *Virology* **20**:199–207.
7. GREEN, M., AND M. PIÑA. 1964. Biochemical studies on adenovirus multiplication. VI. Properties of highly purified tumorigenic human adenoviruses and their DNAs. *Proc. Natl. Acad. Sci. U.S.A.* **51**:1251–1259.
8. LAVER, W. G. 1961. A micromethod for the N-terminal amino acid analysis of proteins. *Biochim. Biophys. Acta* **53**:469–475.
9. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagents. *J. Biol. Chem.* **193**:265–275.
10. MAIZEL, J. V., JR. 1966. Acrylamide-gel electrophorograms by mechanical fractionation: radioactive adenovirus proteins. *Science* **151**:988–990.
11. NORRBY, E. 1966. The relationship between the soluble antigens and the virion of adenovirus type 3. I. Morphological characteristics. *Virology* **28**:236–248.
12. PIÑA, M., AND M. GREEN. 1965. Biochemical studies on adenovirus multiplication. IX. Chemical and base composition analysis of 28 human adenoviruses. *Proc. Natl. Acad. Sci. U.S.A.* **54**:547–551.
13. THOMPSON, E. O. P. 1954. The N-terminal sequence of serum albumins, observations on the thiohydantoin method. *J. Biol. Chem.* **208**:565–572.
14. VALENTINE, R. C., AND H. G. PEREIRA. 1965. Antigens and structure of the adenovirus. *J. Mol. Biol.* **13**:13–20.