

DEOXYRIBONUCLEIC ACID (DNA) AND PROTEIN ALTERATIONS IN HeLa CELLS INFECTED WITH TYPE 4 ADENOVIRUS*

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Abundant evidence obtained by several different experimental approaches indicates that adenoviruses multiply within nuclei of infected HeLa cells (1-6) and that characteristic intranuclear structures containing deoxyribonucleic acid (DNA) form during viral infection (3). Indeed, certain of the structures containing deoxyribonucleic acid are composed of viral-like particles which in many instances are arranged in crystalline lattice arrays (4-6). The striking intranuclear changes encountered in the cytologic and cytochemical studies suggested that adenovirus infection of HeLa cells may serve as an excellent model in which to investigate the biochemical changes resulting from propagation of an animal virus within the nucleus of a cell. It is the purpose of this communication to present the results of studies which have demonstrated that a marked increase in deoxyribonucleic acid and protein occurs in HeLa cells infected with type 4 adenovirus, that the majority of the DNA is not incorporated into infectious viral particles, and that a large proportion of the accumulated DNA differs from normal host cell DNA in its solubility properties and relative nucleotide composition.

Materials and Methods

Tissue Culture.—Cultures of HeLa cells (Gey) were employed throughout. They were propagated either in 32 ounce duraglas prescription bottles with Eagle's basal medium containing 10 per cent human serum or in screw-capped 14 x 150 mm. test tubes with 40 per cent human serum in Hanks' balanced salt solution (BSS) by methods previously described in detail (7, 8).

Virus and Viral Infection.—Type 4 adenovirus, RI-67 strain, was used. Pools were prepared by inoculating approximately 1.2×10^8 tissue culture doses (TCD_{50}) of virus into bottle cultures containing 36 ml. of maintenance mixture composed of 67.5 per cent Scherer's amino

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acid-vitamin mixture, 25 per cent tryptose phosphate broth (Difco), and 7.5 per cent chicken serum (9). The cultures had previously been washed twice with 50 ml. of BSS per wash. Infected cultures were incubated at 36°C. until cytopathic effects were complete (3 to 4 days). Cells and fluid were then frozen and thawed 6 times, and cell debris removed by centrifugation at 2500 R.P.M. for 10 minutes in a model PR-1 International centrifuge (7). Supernatant fluid containing virus was stored in sealed glass ampules at -70°C. in a chest containing solid carbon dioxide, or at -28°C. in an electrically operated mechanical deep freeze.

Viral Infectivity Titrations.—Titrations were done in tube cultures, using 1:3.2 (*i.e.* $10^{-0.5}$) serial dilutions by methods previously described (8). Each of 2 cultures was inoculated with 0.1 ml. of a dilution and the endpoint was considered to be the highest viral dilution producing definite cytopathic effects in one of the 2 cultures inoculated. Titers are expressed as 50 per cent tissue culture doses (TCD₅₀) per ml.

Separation of Nucleic Acid Components.—To separate and measure deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein, either suspensions of washed cells or cell homogenates were employed. Homogenates were prepared by grinding cells in a mechanically driven, teflon homogenizer for 5 minutes in a measured volume of distilled water, using a small quantity of alundum. Fractionation and separation of nucleic acids and proteins were done by a modified Schmidt-Thannhauser procedure (10). Cold 50 per cent trichloroacetic acid (TCA) was added to the cell suspension or homogenate to make a 5 per cent solution of TCA by volume. The suspensions were chilled 20 minutes in crushed ice, and then centrifuged at 3000 R.P.M. for 30 minutes at 4°C. in a refrigerated International centrifuge. The supernatant liquid (designated acid-soluble fraction) was saved, and the sediment was washed with 40 ml. of 95 per cent ethanol to remove residual acid. The sediment was next extracted with alcohol:ether (3:1) twice for 30 minute periods at 50°C., and then washed with 25 ml. of cold 5 per cent TCA. To the sediment was added 2 to 5 ml. of 1 N KOH and the suspension incubated at 37°C. for 16 to 18 hours. The mixture was adjusted to pH 1.0-1.5 with 6 N HCl and kept at 4°C. for 30 minutes to precipitate the DNA and protein. The mixture was centrifuged at 4°C. and the supernate containing ribonucleotides was then decanted. The sediment containing DNA and protein was heated at 90°C. for 45 minutes in 4 ml. of 5 per cent trichloroacetic acid to solubilize the DNA. The protein residue was separated by centrifugation and dissolved in 2 to 4 ml. of 1 N NaOH.

Determinations for DNA, RNA, and protein.—DNA was determined by measuring deoxyribose with the Burton (11) or Seibert (12) modification of the diphenylamine reaction or the Stumpf modification (13) of the method utilizing concentrated H₂SO₄ and 1 per cent cysteine hydrochloride. Thymus DNA was used as the standard. RNA was determined by employing the orcinol reaction to measure ribose; D-ribose was utilized as the standard (14). The method of Lowry *et al.* (15) using the Folin-Ciocalteu phenol reagent was employed to determine protein concentration, with crystalline bovine albumin as the standard. Total phosphorus was measured by a modification of the method of Fiske and Subbarow (16) with KH₂PO₄ as standard.

Deoxyribotide Analysis.—DNA in uninfected and viral infected cell homogenates was separated by the modified Schmidt-Thannhauser (10) procedure described above. Following the degradation of RNA by 1 N KOH and precipitation of DNA and protein with 6 N HCl, the pH was adjusted to 6.5-7.0 with 0.1 N NaOH. Sodium versenate 0.05 M (sodium salt of ethylenediaminetetraacetic acid) (termed versene) was added to make a final concentration of 0.005 M and the solutions were dialyzed at 4°C. for 18 to 24 hours against 2 changes of water of 4 liters each. Versene and MgSO₄ were added to make final concentrations of approximately 0.003 and 0.005 M respectively. After the pH of each solution had been adjusted to pH 7.0-7.5, 2 to 3 mg. of 2 times crystallized deoxyribonuclease (DNAase) (Worthington) was added and the mixtures incubated at 37°C. for 2 hours. Completion of DNAase activity was determined by measuring precipitability of DNA with trichloroacetic acid. The solutions were then adjusted

to pH of 9.0–9.4 with 0.1 N NaOH and approximately 4 units¹ of purified snake venom phosphodiesterase (17) was added per mg. of DNA. The mixtures were incubated at 37°C. for 2 hours, and completion of the enzymatic reaction was tested by determining the precipitability of deoxyoligonucleotides with uranyl acetate–perchloric acid reagent (18). After completion of the enzymatic reaction, the enzyme was inactivated by incubating the mixture in a 100°C. water bath for 5 minutes.

The nucleotide mixtures thus obtained also contained protein and polysaccharides, which made subsequent chromatography on an ion exchange column difficult. To separate the nucleotides from these macromolecular contaminants the mixtures were dialyzed with stirring against 200 ml. of water for 24 hours at 4°C. Dialysis was repeated against 200 ml. of water for 48 hours. This procedure removed all detectable nucleotides from the dialysate. The dialyzing fluids containing the nucleotides were pooled and concentrated to approximately 25 ml. by evaporation.

Nucleotides were adsorbed on a Dowex 1-acetate column (12 cm. x 12.6 square mm.) and eluted by gradient elution (19). The elution was commenced with 300 ml. water in the mixer and 0.15 M ammonium acetate at pH 4.3 in the reservoir. After elution of thymidylic acid, 0.4 M ammonium acetate, pH 4.3, was placed in the reservoir. The flow rate was approximately 20 ml. per hour, and 5 ml. samples were collected.

EXPERIMENTAL

From the results of cytologic and cytochemical studies (3) it was anticipated that an increase in total DNA could be measured after infection with type 4 adenovirus, but no information existed as to whether alterations in total RNA and protein might occur. Experiments of the following type were therefore done.

Two cultures, each containing approximately 2×10^7 cells, were infected with $1.3 \times 10^{4.0}$ TCD of type 4 adenovirus and incubated 4 days at 36°C., at which time all cells had undergone cytopathic changes. Two uninfected control cultures were incubated in an identical manner. At the end of the incubation period cells were scraped from the glass into each culture fluid, and mixed thoroughly by pipetting. One ml. aliquots from the uninfected and infected cell suspensions were employed for cell enumerations in a standard hemocytometer, and for viral infectivity titrations. Cell suspensions from the infected cultures were pooled, centrifuged at 750 R.P.M. for 30 minutes, and washed one time with 30 ml. of 0.15 M NaCl. The uninfected control cultures were prepared identically. A modified Schmidt-Thannhauser fractionation was done with each cell pellet, and the total amount of DNA, RNA, and protein in each set of cultures determined.

The data presented in Table I represent the results obtained in 4 similar experiments. In each the differences indicated were of the same order of magnitude: a large and reproducible DNA increase occurred in viral infected cells. A concurrent but smaller increase in protein was observed, but little if any increase in RNA was detected.

These preliminary experiments indicated clearly that it should be possible to investigate by classical biochemical methods some of the alterations in nucleic acids and proteins which ensue following infection with type 4 adenovirus. The above experiments were done, however, under conditions which presented

¹ In the present studies 1 unit of diesterase converts 1 μ M of DNA phosphorus in 10 minutes at 37°C. to a form soluble in uranyl acetate–perchloric acid reagent (18).

a major disadvantage: only 1 in 100 cells approximately was infected by the initial viral inoculum. In all subsequent experiments to be described below the ratio of infectious units of virus to cells was of the order of 0.4 to 1.5:1, and the experiments were terminated after the initial cycle of multiplication; *i.e.*, 32 to 36 hours after viral infection (8). The requirement for a large viral inoculum presented certain problems because of the inherent "toxic" property of viral preparations when used in high concentration (3, 20). Careful measurement of the size of the viral inoculum, however, permitted this condition of virus-cell multiplicity to be met, but larger quantities of virus could not be employed.

Differential Extraction of DNA from Infected Cells.—In the previous experiments total nucleic acids and protein content of viral infected as well as unin-

TABLE I
Total Deoxyribonucleic Acid (DNA), Ribonucleic Acid (RNA), and Protein in Uninfected and Type 4 Adenovirus-Infected HeLa Cells
Fractionation by modified Schmidt-Thannhauser procedure.

| Component measured | Quantity | | Increase in infected cells <i>per cent</i> |
|--------------------|---|---|---|
| | Uninfected cells <i>μg./10⁶ cells</i> | Infected cells <i>μg./10⁶ cells</i> | |
| DNA | 6.4 | 17.9 | 180.0 |
| RNA* | 3.6 | 4.5 | 25.0 |
| Protein | 124.0 | 196.0 | 58.0 |

* Expressed as ribose.

infected cells were measured. It seemed desirable to attempt to separate the DNA formed as a result of viral infection from normal host nucleic acid present when infection was initiated, and many procedures were investigated in an effort to achieve this goal. Although the desired aim has not yet been attained completely, one of the experiments led to an unexpected and useful finding.

To separate the bulk of RNA and protein from DNA, washed cells were disintegrated in 5 to 10 ml. of 0.15 M NaCl and the homogenate stored at 4°C. for 20 to 24 hours, after which the mixture was centrifuged at 3000 R.P.M. for 60 minutes at 4°C. in a refrigerated International centrifuge. The sediment was washed once with 40 ml. of 0.15 M NaCl, and then resuspended in 10 ml. water and shaken at 4°C. for 2 days. After centrifugation at 3000 R.P.M. for 60 minutes at 4°C., the sediment was extracted a second time in water for 2 days. The 0.15 M NaCl and water extracts were further clarified by centrifugation at 10,300 *g* for 15 minutes in the number 40 rotor of a model L Spinco preparative centrifuge. Subsequent experiments have indicated that more complete extraction of DNA is accomplished when 10 per cent NaCl rather than water is employed for second extractions, but the qualitative results obtained were similar with both methods. Deoxyribonuclease activity could not be detected in homogenates of HeLa cells and therefore sodium citrate or similar agents which bind magnesium were not added to extracting fluids.

Deoxyribonucleoproteins in general are poorly soluble in 0.15 M NaCl, whereas ribonucleoproteins and other cell proteins readily go into solution in this fluid. The extraction procedure described above, carried out to accomplish this separation of DNA from RNA and protein, yielded unexpected results when infected cultures were employed. As illustrated by one of many experiments summarized in Table II, almost 6.6 times more DNA was extracted from infected than from uninfected cells by 0.15 M NaCl. This fraction will hereafter be referred to as the saline-soluble DNA. A much greater quantity of DNA was extracted from cells by water than by 0.15 M NaCl, and it is noteworthy that the total amount of DNA obtained in water was greater from infected than from

TABLE II
Effect of Type 4 Adenovirus Infection upon Deoxyribonucleic Acid Content of HeLa Cells Extracted Serially with 0.15 M NaCl and Water

| HeLa cell culture* | Fraction | Total DNA | DNA per 10 ⁶ cells | DNA increase |
|--------------------|-------------|----------------|-------------------------------|-----------------|
| | | $\mu\text{g.}$ | $\mu\text{g.}$ | <i>per cent</i> |
| Uninfected | 0.15 M NaCl | 470 | 0.73 | — |
| “ | Water | 8685 | 13.7 | — |
| Infected‡ | 0.15 M NaCl | 1485 | 4.85 | 560.0 |
| “ | Water | 7685 | 25.1 | 81.0 |

* When harvested uninfected cultures contained 644×10^6 cells and infected cultures contained 306×10^6 cells.

‡ Infected with 1.5×10^8 infectious doses of virus. Harvested 36 hours after infection.

control cells. It is striking, however, that the proportional increase in DNA from infected cells was considerably greater in the NaCl than in the water fraction. It must be pointed out that in all experiments the cells were harvested and counted 32 to 36 hours after viral inoculation, at which time there were of the order of 1.2 to 2.5 times more cells in uninfected than in infected cultures. The total nucleic acid in control cultures was occasionally equal to, or greater than, that from infected cultures, but as a consequence of the inhibition of cell division by viral infection, the quantity of DNA per infected cell was always much greater.

The results of 21 experiments are summarized in Table III, and are expressed as the geometric means of each component determined. These data further emphasize the results relative to DNA from the single experiment presented in Table II, and, in addition, indicate that there was also an increase in protein of infected cells. The protein increase was approximately the same in the 0.15 M NaCl and water fractions. The quantity of RNA is not considered to be increased in infected cells, because the orcinol reaction employed measures not only ribose but other sugars contained in polysaccharides present in the materi-

als studied. It should be pointed out that the material in the 0.15 M NaCl fraction measured as deoxyribose was considered to be DNA because 95 to 100 per cent was acid-precipitable and all was hydrolyzed by deoxyribonuclease (Worthington 2 X crystallized).

Association of Infectious Type 4 Adenovirus with 0.15 M NaCl and Water Fractions of Infected HeLa Cells.—The experiments described above clearly indicate an increase in DNA and protein in HeLa cells subsequent to adenovirus infection. An especially large relative increase in DNA was found in the material extracted from infected cells by 0.15 M NaCl. To obtain data on the significance of this finding, infectivity titrations were done on each fraction to determine

TABLE III
Deoxyribonucleic Acid (DNA), Ribonucleic Acid (RNA), and Protein in Uninfected and Type 4 Adenovirus-Infected HeLa Cells

| Component measured | 0.15 M NaCl extract | | | Water extract | | |
|--------------------|---------------------------|---------------------------|------------|---------------------------|---------------------------|------------|
| | Uninfected | Infected* | Difference | Uninfected | Infected* | Difference |
| | $\mu\text{g./}10^6$ cells | $\mu\text{g./}10^6$ cells | per cent | $\mu\text{g./}10^6$ cells | $\mu\text{g./}10^6$ cells | per cent |
| DNA† | 1.95 | 8.91 | 357.0 | 19.5 | 29.5 | 51.0 |
| RNA§ | 8.25 | 13.4 | 62.4 | 4.18 | 5.48 | 31.0 |
| Protein | 109.6 | 229.0 | 109.0 | 67.0 | 141.0 | 110.0 |

* Infected with $5 \times 10^{7.0}$ to $1.5 \times 10^{8.0}$ infectious doses of virus; a ratio of 0.7 to 3.0 infectious units of virus per cell.

† Geometric mean of 21 experiments.

§ Geometric mean of 13 experiments; expressed as ribose.

|| Geometric mean of 13 experiments.

whether infectious virus was associated chiefly with either the NaCl or water fraction or was randomly distributed in both.

The results of a typical experiment, presented in Table IV, indicate that although there was approximately 6.5 times more DNA in the water than in the 0.15 M NaCl fraction, 94.5 per cent, or 18 times more of the infectious virus was associated with the material soluble in 0.15 M NaCl. Similar results were obtained in each of 21 experiments in which concomitant determinations of DNA and infectious virus were made. The results cannot be explained by a greater lability of the infectious property of type 4 adenovirus in water than in 0.15 M NaCl, because previous experiments have clearly indicated the marked stability of this agent in water and in 0.15 M NaCl under the conditions of storage at 4°C. employed in these studies (21).

Determination of the Association of Infectious Viral Particles with DNA.—More than 98 per cent of the infectious virus was shown to be associated with the increased amount of saline-soluble DNA. These data raised the question

whether this DNA was incorporated into viral particles. Experiments were therefore designed to answer this query.

Water and 0.15 M NaCl fractions from infected as well as uninfected cells were centrifuged at 105,400 *g* for 60 minutes in the No. 40 rotor of a Spinco model L preparative centrifuge. After centrifugation, the upper 3 ml. of the supernate was carefully removed through the tube cap with a needle and syringe. The remainder of the supernatant fluid was then removed

TABLE IV
Association of Infectious Virus with 0.15 M NaCl or Water Extracts from Infected HeLa Cells

| Fraction | Quantity DNA | | Quantity infectious virus |
|-------------|--------------------|--|-------------------------------------|
| | $\mu\text{g./ml.}$ | | $\log \text{TCD}_{50}/\text{ml.}^*$ |
| 0.15 M NaCl | 53.5 | | 6.5 |
| Water | 346.0 | | 5.25 |

* 50 per cent tissue culture doses.

TABLE V
Comparison of the Effect of High Speed Centrifugation upon Deoxyribonucleic Acid (DNA) and Infectious Type 4 Adenovirus Particles

| Extract | Centrifugation* fraction | Quantity DNA | | Quantity infectious virus |
|-------------|-----------------------------|--------------------|--------------------|-----------------------------------|
| | | Uninfected† | Infected‡ | |
| | | $\mu\text{g./ml.}$ | $\mu\text{g./ml.}$ | $\log \text{TCD}_{50}/\text{ml.}$ |
| 0.15 M NaCl | Original | 17.5 | 23.0 | 5.25 |
| " " " | Supernate | 17.5 | 17.5 | <1.0 |
| Water | Original | 159.0 | 248.0 | 4.0 |
| " | Supernate | 176.0 | 260.0 | <1.0 |

* Centrifuged 105,400 *g* for 60 minutes.

† Cultures contained 183×10^6 cells.

‡ Cultures contained 98×10^6 cells.

with a pipette, and the sediment was resuspended to its original volume in 0.15 M NaCl or water. DNA, RNA, and protein determinations were done on all samples, and infectivity titrations were carried out on aliquots obtained from infected cells.

The results summarized in Table V indicate that high speed centrifugation of the 0.15 M NaCl or water extracts of infected cells sedimented more than 99.99 per cent of the infectious virus, but most of the DNA remained in the supernatant fluid. It should be pointed out that despite previous clarification the 0.15 M NaCl extracts were cloudy and contained flocculent material which formed a firm mucoid-like pellet upon high speed centrifugation. This pellet appeared to contain a large amount of cell protein and polysaccharide and was

difficult to disperse and redissolve. Therefore, quantitative recovery of materials from the sediment was not possible. High speed centrifugation produced changes in RNA and protein from uninfected and infected cells which were similar to those shown for DNA.

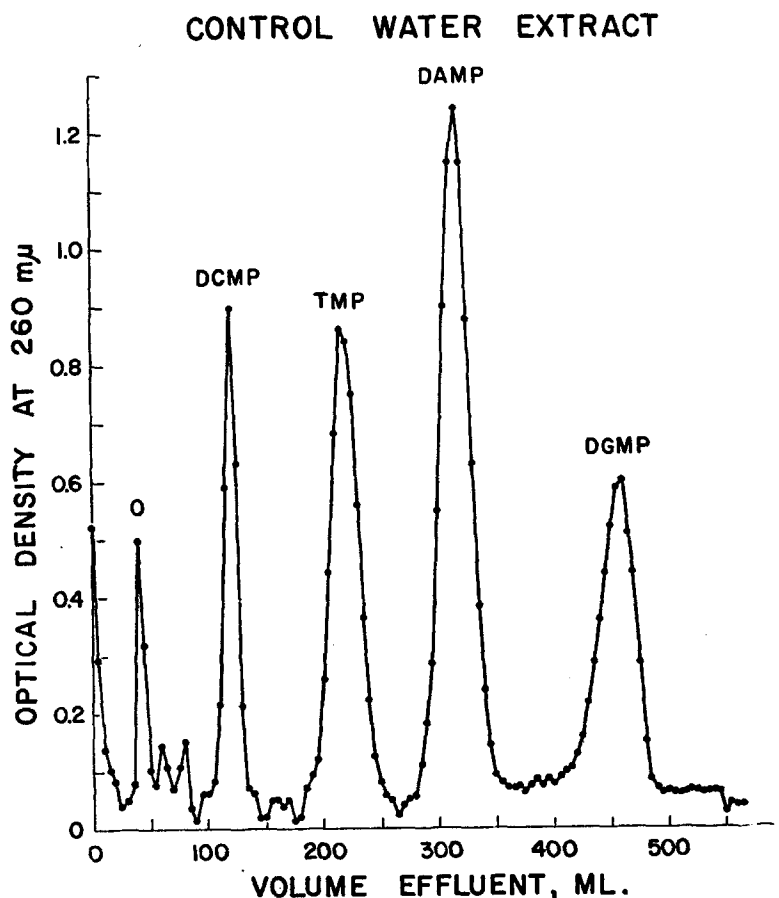


FIG. 1. Chromatogram of nucleotides from water-soluble DNA extracted from uninfected HeLa cells. Digest, prepared by successive treatment with DNAase and snake venom phosphodiesterase, was adsorbed to Dowex 1-acetate resin column (12 cm. x 12.6 square mm.) and eluted by gradient elution. Initially 0.15 M NH_4 acetate at pH 4.3 was added to 300 ml. water in the mixer until TMP was eluted; 0.4 M NH_4 acetate, pH 4.3, was then substituted for the 0.15 M NH_4 acetate in the reservoir. Flow rate was approximately 20 ml. per hour and 5 ml. samples were collected.

It can be inferred from the results of these experiments that although DNA, as well as protein, increases following infection, the bulk of these newly synthesized materials is not incorporated in infectious viral particles. Indeed, the

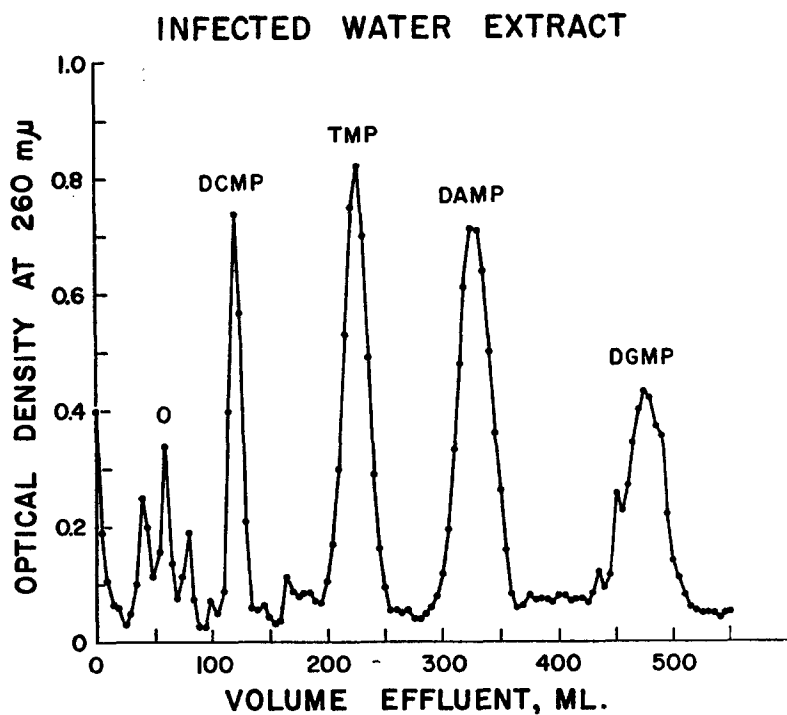


FIG. 2. Chromatogram of nucleotides from water-soluble DNA extracted from type 4 adenovirus-infected HeLa cells. Conditions for adsorption and elution of the enzymatic digest were exactly as described for Fig. 1.

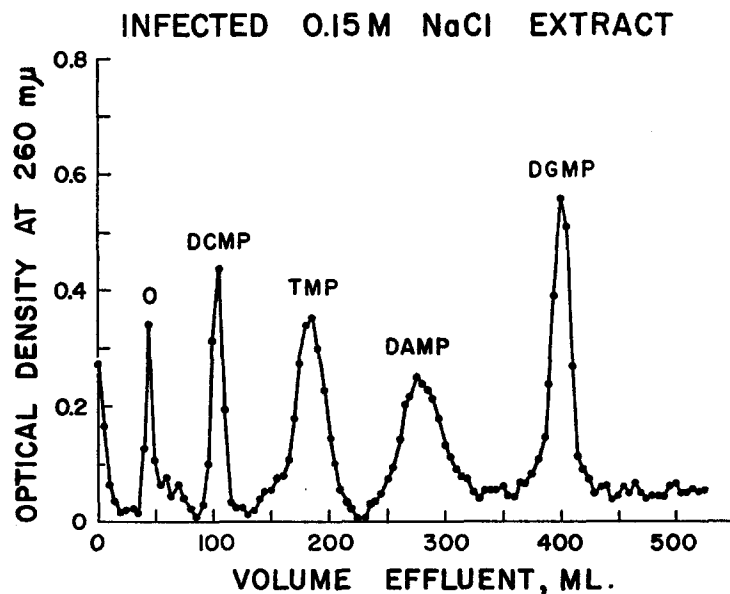


FIG. 3. Chromatogram of nucleotides from 0.15 M NaCl-soluble DNA extracted from type 4 adenovirus-infected HeLa cells. Conditions for adsorption and elution of the enzymatic digest were exactly as described for Fig. 1.

major portion of the relatively large quantity of DNA produced is not even formed into non-infectious viral particles which have physical characteristics similar to those of infectious virus. The exact relationship of the excess nucleic acids and protein to the viral materials cannot be stated as yet.

Analysis of Deoxyribotides² in 0.15 M NaCl and Water Extracts.—To characterize the DNA synthesized and accumulated in HeLa cells after infection, nucleotide analyses were carried out on each extract. To determine whether the excess DNA produced in viral infected cells differed in its nucleotides, either qualitatively or quantitatively, from host DNA concurrent analyses were done on extracts from uninfected cells.

These determinations had certain inherent difficulties because of the relatively small quantities of DNA present, and the interference caused by the relatively large amounts of protein, polysaccharides and possibly other acid-insoluble compounds present in the extracts. The first problem was partially met by pooling materials from 4 or 5 experiments (a total of 16 to 20 thirty-two ounce bottles for each group), so that extracts from approximately 5×10^8 cells were employed; however, this procedure still yielded a relatively small quantity of DNA in the 0.15 M NaCl extract from uninfected cells. The second problem was resolved by the simple expedient of dialyzing the nucleotides away from the protein and polysaccharides as described under Materials and Methods. Two complete analyses were carried out. Identification of each nucleotide was established by ultraviolet spectral analysis. The quantity of each nucleotide was calculated employing the maximum extinction coefficient at 260 $m\mu$ determined at pH 4.3.³

The patterns of nucleotide separation on Dowex 1 columns are presented in Figs. 1 to 3. Tables VI and VII summarize the quantitative relationship of the nucleotides to each other computed from the means of results of 2 experiments. Qualitatively there were no observable differences in nucleotides of DNA from uninfected or viral infected cells in either the 0.15 M NaCl or water extracts; that is, no unusual nucleotides were detectable in DNA from infected cells. Quantitatively, these experiments are more difficult to interpret because of minor variations in the relative quantity of a nucleotide from experiment to experiment, the relatively small amount of each nucleotide present in the uninfected 0.15 M NaCl extract, and the resultant incomplete separation of the nucleotides in the saline fraction. Because of the extremely small quantities of nucleotides obtained in the control 0.15 M NaCl fractions, the quantitative data based on these separations were considered unreliable and, therefore, are not presented. Results of the nucleotide analysis, however, do suggest that an excess of purines relative to pyrimidines does exist in this small DNA fraction

² The abbreviations to be employed are: DCMP, deoxycytidylic acid; TMP, thymidylic acid; DAMP, deoxyadenylic acid; and DGMP, deoxyguanylic acid.

³ These values were determined by Dr. Henry Z. Sable, Department of Biochemistry, Western Reserve University, School of Medicine, Cleveland.

from uninfected cells. Although relatively small amounts of nucleotides were also obtained by column chromatography of the 0.15 M NaCl extract from infected cells, the separations were distinct (Fig. 3) and the quantitative data sufficiently reproducible between the 2 experiments so that the molar ratios may be considered reliable. The first peak present in Figs. 1 to 3, designated 0, did

TABLE VI
Molar Ratios of Deoxyribonucleotides of 0.15 M NaCl and Water Extracts from Uninfected and Type 4 Adenovirus Infected HeLa Cells

| Nucleotide | Uninfected cells* Water | Infected cells* | |
|-------------------------------|----------------------------|-----------------|----------------|
| | | Water | 0.15 M NaCl |
| | <i>N/TMP</i> ‡ | <i>N/TMP</i> ‡ | <i>N/TMP</i> ‡ |
| Thymidylic acid | 1.00 | 1.00 | 1.00 |
| Deoxycytidylic acid | 0.58 | 0.70 | 0.67 |
| Deoxyadenylic " | 0.90 | 0.88 | 0.63 |
| Deoxyguanylic " | 0.48 | 0.68 | 0.80 |

* Mean of results from 2 analyses.

‡ Molar ratio of nucleotide to thymidylic acid.

TABLE VII
Molar Ratios of Deoxyribonucleotides of 0.15 M NaCl and Water Extracts from Uninfected and Type 4 Adenovirus-Infected HeLa Cells

| Nucleotide ratio | Uninfected cells* Water | Infected cells* | |
|-----------------------|----------------------------|-----------------|-------------|
| | | Water | 0.15 M NaCl |
| DAMP:TMP | 0.90 | 0.88 | 0.63 |
| DGMP:DCMP | 0.82 | 0.96 | 1.20 |
| <u>DAMP + TMP</u> | | | |
| DGMP + DCMP | 1.78 | 1.32 | 1.10 |
| <u>Purine</u> | | | |
| Pyrimidine | 0.88 | 0.91 | 0.85 |

* Mean of results from 2 analyses.

not contain detectable deoxyribose and therefore is not considered to contain deoxyribonucleotides.

It is clear from the data summarized in Table VI that HeLa cell DNA is a thymine-adenine predominant DNA similar to that in other animal cells studied (22). Within the limits of the methods employed, it may also be considered from the ratios expressed in Tables VI and VII that water-soluble DNA from both uninfected and infected cells had an equal pairing of adenine to thymine and guanine to cytosine. The nucleotide molar ratios, however, appeared altered in the saline-soluble DNA from infected cells. These altered ratios

are a reflection of a relative increase in DGMP and DCMP and a decrease in DAMP. Similar changes in DGMP and DCMP, although not as marked, appear to have occurred in the water-soluble DNA fraction from infected cells. The DAMP:TMP ratio was similar in water extracts from control and infected cells and in each case approached unity. It is striking, however, that this nucleotide ratio was markedly altered in the saline-soluble DNA from infected cells and was significantly less than 1 (0.63) as a result of the marked decrease of DAMP. The considerable increase in DGMP is reflected by a DGMP:DCMP ratio which was increased to 1.20 in the saline-soluble fraction. These relative changes in quantities of nucleotides are also readily apparent upon inspection of Figs. 1 to 3. Because of the present inability to compare the nucleotide composition of the saline-soluble DNA from infected cells with a similar fraction from control cells, it cannot be determined whether the saline-soluble DNA is an abnormal nucleic acid synthesized as a result of viral infection or a normal component usually present in very small amounts and considerably increased due to the infectious process. Further investigation of the structure and composition of HeLa cell DNA soluble in low ionic strength solutions (23, 24) is required for elucidation.

DISCUSSION

Characteristic morphologic alterations occur in adenovirus-infected cells (1-3). Studies employing cytochemical (3) and electron microscopic techniques (4-6) have demonstrated that most of the structures which form in infected cells contain DNA and are composed partially or entirely of virus-like particles. The quantity of infectious virus obtained from altered cells, as compared with the amount of DNA and the number of particles observed, suggests, however, that a large portion of the nucleic acid and virus-like particles is composed of non-infectious materials.

The evidence presented in the present study indicates that not only do the basophilic masses and inclusion bodies described (1-3) contain DNA, but also that the total DNA per infected cell is markedly increased. This finding implies active synthesis of DNA rather than mere rearrangement of the nucleic acid present before viral infection. An increase in protein accompanies the accumulation of DNA in cells infected with type 4 adenovirus. A relatively small increase in RNA was likewise noted, but its significance is not yet clear.

Of greater significance, perhaps, than the measurement of a total increase in DNA in virus-infected cells was the demonstration that a portion of the accumulated DNA had physical and chemical properties different from the bulk of normal host cell DNA. Within virus-infected cells, 20 to 25 per cent of the total DNA was soluble in 0.15 M NaCl, a solution in which DNA usually is insoluble. Indeed, 4.5 times more DNA was extracted by 0.15 M NaCl from infected than from uninfected cells; of the remaining DNA only approximately

1.5 times more was obtained from infected than from control cells when water or 10 per cent NaCl was employed. The saline-soluble DNA not only had unusual solubility properties, but also its chemical composition was distinctly different from that of the water-soluble DNA. Analyses of deoxyribotides from each DNA fraction did not reveal the presence of any unique nucleotide, but did demonstrate that changes in nucleotide molar ratios occur in DNA from infected cells, particularly in the saline-soluble fraction. The alterations noted were chiefly the result of a marked increase in the quantity of deoxyguanylic acid and an approximately proportionate decrease in deoxyadenylic acid (Tables VI and VII). Therefore, despite these changes the purine:pyrimidine ratio was not significantly altered.

It may be deduced from data obtained from the nucleotide analyses that the base composition of water-soluble DNA from control cells is consistent with the Watson-Crick model of the structure of DNA (25). Concurrently with type 4 adenovirus infection, however, the structure of DNA in the 0.15 M NaCl fraction is sufficiently altered so that it does not appear to fit this proposed model. A rough estimation of the purine:pyrimidine ratio in the saline-soluble DNA from uninfected cells suggests that this DNA also may not conform to the suggested structure.

It is not possible from the evidence described to determine whether the saline-soluble DNA from infected cells is a novel form of DNA produced as a response to infection, or a DNA which is present also in very small amounts in uninfected cells. That DNA from a single organ or species of cells may be heterogeneous has been amply demonstrated (24, 26). Moreover, the studies of Chargaff and coworkers on the composition of DNA molecules soluble in 0.4 M NaCl or higher electrolyte concentrations indicated that DNA at low salt molarity had a higher content of deoxyguanylic acid than nucleic acid soluble at higher ionic strength (23). These data of Chargaff *et al.* (23) suggest the possibility that saline-soluble DNA may be present in small quantity in normal HeLa cells, but is considerably increased as a result of synthetic processes stimulated by viral infection.

It is worthy of further emphasis that although 90 to 99.9 per cent of the total infectious virus measured was present in the 0.15 M NaCl fraction, only a small proportion of this DNA was incorporated into viral particles having the same size, shape, and specific gravity as the infectious viral particles. This finding is not surprising when it is noted that: (a) of the large number of particles observed by electron-microscopic study of thin sections of adenovirus-infected cells, relatively few can be measured as infectious particles; and (b) in addition to the structures containing virus-like particles and Feulgen-positive material, there are large basophilic masses which are Feulgen-positive and which by fluorescein-labelled antibody studies are shown to contain relatively small amounts of specific viral antigen (27).

It is not clear, therefore, what relationship the excess DNA which accumulates in infected cells has to the viral particle. Cytochemical studies suggest that the nucleic acid composition of adenoviruses probably consists largely of DNA (3, 6). Preliminary results of the chemical analysis of type 4 adenovirus, purified approximately 100-fold relative to protein content, indicated that DNA was the major nucleic acid present. A trace of RNA, approximately 2.5 to 5.0 per cent of the total nucleic acid, was measured, but it could not be determined whether this was a component of the viral particle or contaminating material (28). More highly purified virus, rigidly tested to determine purity, must be obtained before characterization of the chemical composition of type 4 adenovirus can be made with confidence. When this is accomplished it should be possible to ascertain whether the DNA of virus has a composition similar to the saline-soluble DNA extracted from infected cells. If this is the case, one may postulate that the accumulated DNA is a viral precursor which has been made in excess. It may be hypothesized further that as a result of viral infection, cellular control mechanisms have become deranged so that DNA as well as protein is synthesized in an amount greater than that required to form infectious viral particles. This hypothesis is strengthened by preliminary data from experiments employing radioisotopes. These experiments suggest that the saline-soluble DNA accumulated in viral infected cells is indeed newly synthesized DNA and not a degradation product from normal host nucleic acid (28). The altered solubility property of this DNA may be due to smaller polymer size, association with an unusual protein to form a nucleoprotein with different solubility characteristics, altered structure due to quantitative changes in nucleotide composition, or a combination of these factors.

Further investigation of the origin and nature of the accumulated DNA and protein should yield information concerning details of synthesis of specific viral materials, formation of viral particles, and development of the characteristic nuclear lesions.

SUMMARY

During a single cycle of multiplication of type 4 adenovirus in HeLa cells an average 2-fold increase in total DNA occurred over that measured in uninfected cells. Of the total DNA from infected cells about 23 per cent was extractable into 0.15 M NaCl, a quantity approximately four and a half times greater than that of the DNA obtained from normal cells in 0.15 M NaCl. Ninety to 99 per cent of infectious virus was also extracted into the 0.15 M NaCl fraction. Concurrently with the accumulation of DNA in virus-infected cells there occurred a 2-fold increase in total protein. The proportionate increases in protein were approximately equal in 0.15 M NaCl and water extracts of infected cells. High speed centrifugation indicated that the bulk of the DNA and protein was not directly associated with infectious viral particles. Although in virus-infected

cells a markedly increased synthesis occurred of a DNA which had solubility properties different from the major portion of normal host DNA, nucleotide analysis did not reveal any other qualitative differences. However, a marked change in nucleotide molar ratios was observed in the 0.15 M NaCl-soluble DNA from virus-infected cells.

It seems apparent from the findings that the biochemical alterations found in HeLa cells infected with type 4 adenovirus are intimately related to the infectious process.

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