NUCLEIC ACID SYNTHESIS IN TYPES 4 AND 5 ADENOVIRUS–INFECTED HeLa CELLS*, ‡

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Adenoviruses instigate characteristic alterations in nuclei of infected cells (1–3).

Most striking of the changes are the development of prominent, central, basophilic masses and formation of basophilic crystals composed of a lattice array of viral particles (4–6). Cytochemical studies demonstrated that both of the newly formed basophilic structures contained deoxyribonucleic acid (DNA), and suggested that the deoxyribonucleic acid (DNA) content of the infected nuclei was increased (2). Biochemical investigation of the effect of adenovirus infection on the constituents of infected cells supported the contention that infection increased the DNA content (7). The DNA from infected cells was found to contain 2 different molecular species: one, extracted as a nucleoprotein, was soluble in water or 2 M NaCl but not in 0.15 M NaCl (termed water-soluble DNA); the second (termed saline-soluble DNA), when associated with protein was soluble in 0.15 M NaCl as well as water or 2 M NaCl (7). It was the so called saline-soluble DNA fraction which was markedly increased in virus-infected cells, whereas only very small quantities of DNA from uninfected cells could be extracted in 0.15 M NaCl. The most striking difference between the saline-soluble DNA and the water-soluble DNA was the significant difference in base compositions: whereas the water-soluble DNA had classical base pairing (adenine equal to thymine; guanine equal to cytosine), a similar structure did not obtain for the saline-soluble DNA in which adenine and thymine were not equal in amount, and guanine was strikingly increased (7).

Studying the biochemical changes in type 2 adenovirus–infected cells, Green confirmed the increase in DNA content of infected cells and reported that incorporation

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of P^{32} into deoxyguanylic acid and deoxycytidylic acid was increased (8). The finding of an increase in specific activity of the guanine and cytosine nucleotides of DNA from infected cells further supports the data previously published which suggested that adenovirus infection stimulates synthesis of a specialized molecular species of DNA (the saline-soluble DNA), and that this DNA has an increased quantity of guanine (7).

The study reported in this communication had as its objective the investigation of the synthesis of the "saline-soluble" and "water-soluble" deoxyribonucleic acids, as well as ribonucleic acid (RNA), in type 4 adenovirus-infected HeLa cells employing radioisotope techniques. The findings to be described indicate that synthesis of the saline-soluble, but not the water-soluble DNA, is increased in virus-infected cells; that the saline-soluble DNA is newly synthesized and derives its components from precursor pools of the cell as well as from the medium; that the synthesis of saline-soluble DNA begins before newly formed, infectious virus; and that synthesis of RNA is also increased, although not as markedly as the saline-soluble DNA.

Materials and Methods

Tissue Culture.—All experiments were done with HeLa cells propagated in continuous serial cultures using Eagle's basal medium supplemented with 10 per cent human serum. For infectivity titrations, cultures were prepared in screw-capped 14 × 150 mm. test tubes utilizing 40 per cent human serum in Hanks's balanced salt solution (BSS) for cell propagation. The tissue culture methods used were identical with those described previously (9, 10).

Viruses and Viral Infection.—Types 4 and 5 adenoviruses employed in earlier studies (2, 3, 7, 9, 10) were used. To prepare pools of virus, growth fluid containing human serum was removed from the cultures, the monolayers were washed twice with Hanks's BSS, a maintenance mixture composed of 67.5 per cent Scherer's amino acid–vitamin mixture, 25 per cent tryptose phosphate broth (Difco), and 7.5 per cent chicken serum was added, and approximately 1.2×10^3 tissue culture doses (TCD_{50}) of virus was inoculated into each culture (11). Infected cultures were incubated until cytopathic changes were complete (3 to 5 days). Cells and fluid were frozen and thawed 6 times to liberate virus (10), and then centrifuged to remove cell debris. Supernatant fluid containing virus was stored in flasks at −28°C. in an electrically operated deep freeze or in sealed glass ampules at −70°C. in a chest containing solid carbon dioxide. Infectivity titers were determined in tube cultures using 1:3.2 (*i.e.*, $10^{-0.5}$) serial dilutions (10). The endpoint was considered to be the highest dilution of virus effecting cytopathic changes in at least 1 of the 2 cultures inoculated per dilution after 10 to 12 days' incubation at 36°C. Maintenance mixture was not changed during the incubation period.

To obtain infection of all cells in the experiments to be described it was necessary to use relatively large volumes of undiluted virus or concentrated, partially purified virus. When a large volume of undiluted virus was employed, a cytotoxic effect, caused by the adenovirus-soluble "toxin" (2, 12–14) was observed. Virus heated at 56°C. for 15 minutes to inactivate viral infectivity but not cytopathic effect was employed as a control in some experiments (15). In other experiments, the viral suspension was incubated at 37°C. for 60 minutes with trypsin (0.12 mg. per ml. final concentration) to inactivate "toxin" (12–14). An equal quantity of purified soy bean trypsin inhibitor was added to neutralize trypsin activity before cultures were inoculated. Virus heated at 56°C. for 15 minutes and then treated successively with trypsin and soy bean trypsin inhibitor was used for controls. Experiments proved that com-

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parable differences in nucleic acid content and isotope incorporation between control and infected cultures were observed with both types of experiments.

Extraction and Separation of Nucleic Acid Components.—Cells from infected and control cultures were scraped from the glass into the culture fluid, mixed thoroughly, and an aliquot removed from each for viral infectivity titration and cell count by enumeration of nuclei (16). Cell suspensions were centrifuged at 750 R.P.M. for 30 minutes at 4°C. in a refrigerated International centrifuge. The supernatant fluid was discarded and the cells washed once with 200 ml. of 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate. Cells were next extracted serially with 10 ml. of 0.15 M NaCl for 24 hours at 4°C. followed by 10 ml. of 2 M NaCl or water at 4°C. for 2 days on a mechanical shaker as previously described. A modified Schmidt-Thannhauser fractionation (17) was carried out on each extract as detailed in a previous communication (7).

Chemical Determination.—The techniques previously employed were utilized in the experiments to be described (7). DNA was determined by the Burton modification of the diphenylamine reaction (18). RNA by the orcinol reaction (19), protein by the method described by Lowry *et al.* (20), and total phosphorous by a modification of the technique of Fiske and SubbaRow (21).

Radioisotopes and Techniques for Measurement.—Carrier-free P^{32}–inorganic phosphate of high specific activity (Oak Ridge National Laboratory) and sodium formate–C^{14} with specific activity of 2 *mc./m*M (New England Nuclear Corporation) were employed in individual experiments. Samples dried at infinite thinness were counted in a gas-flow counter (Chicago Nuclear) with window in place for P^{32} or windowless for C^{14} determinations. The number of disintegrations counted per sample was adjusted so that less than a 5 per cent error might occur.

EXPERIMENTAL

Incorporation of P^{32}–Inorganic Phosphate into Saline-Soluble and Water-Soluble DNA.—Previous studies indicated that DNA from HeLa cells was not homogenous, and that with type 4 adenovirus infection a marked increase occurred in DNA extracted in 0.15 M NaCl, but only a minimal increase was detectable in DNA extracted in water or 2 M NaCl (7). The significance of the relatively small increase in quantity of DNA extracted in water or hypertonic NaCl was not clear. With the use of radioactive isotopes experiments were planned to determine whether an increased synthesis of both or only one of the DNA fractions occurred in adenovirus-infected cells.

Two μc. P^{32}-labeled inorganic phosphate was introduced into each of 6 cultures containing 6 to 8 $\times$ 10^6 cells. Three of the cultures were infected with 5 ml. of undiluted type 4 adenovirus, so that an average of 3.5 TCD_{50} of virus per cell was present. An equal volume of heat-inactivated virus was added to each of the 3 remaining cultures which served as uninfected controls. Cultures were incubated at 36°C. for 36 hours, after which cell suspensions from each group of 3 cultures were made. Cell counts were done on each cell suspension, and infectivity titration carried out on the suspension of infected cells. Uninfected and infected cells were then extracted; chemical determinations and isotope content of each fraction were done as previously described.

The results of a representative experiment, summarized in Table I, demonstrate that the specific activity of the saline-soluble DNA was 2.7 times greater

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than that in uninfected cells. In striking contrast, the isotope incorporation was similar in the water-soluble DNA fraction from uninfected and infected cells. These data confirm findings previously reported and indicate that the major increase in DNA synthesis in adenovirus-infected cells occurred in the relatively small fraction soluble in 0.15 M NaCl; no significant increased synthesis of DNA extracted by 2 M NaCl or water could be measured (7). It is also important to note that the specific activity of saline-soluble DNA from infected cells was not only greater than that of the saline-soluble fraction from uninfected cells, but also that it was more than 2 times greater than the specific activity of the so called water-soluble DNA. These results lend strong support to the hypothesis that adenovirus infection of HeLa cells induces increased

TABLE I

Effect of Type 4 Adenovirus Infection on Quantity of Deoxyribonucleic Acid (DNA) and P^{32}–Inorganic Phosphate Incorporation into DNA Fractions from HeLa Cells

Fraction tested	Uninfected		Infected*		Per cent increase in infected cells‡	
	DNA content‡	Specific activity§	DNA content‡	Specific activity§	DNA	Sp. activity
	μg./10⁶ cells		*μg./10⁶ cells*			
0.15 M NaCl	1.8	3.36	4.86	9.15	170	172
2 M NaCl	23.8	4.90	34.80	4.10	46	0

* Cultures infected with average of 3.1 TCD_{50} of virus per cell.

‡ Determined 36 hours after viral inoculation.

§ C.P.M./μg. DNA/ml. 2 μc. P^{32} added to each culture at time of infection.

synthesis of only a small, perhaps specialized and unique fraction of the cellular DNA, and that the saline-soluble DNA is synthesized *de novo* and is not merely a degradation product of the host cell DNA (the water-soluble DNA) (7). The finding has been consistent that the increase in quantity of water-soluble DNA from infected cells was actually relatively slight, and that there was no increase in specific activity. Therefore, in presentation of data from subsequent experiments, only results obtained with so called saline-soluble DNA will be tabulated and discussed.

Effect of Time Isotope Was Added to Cultures on Incorporation into Saline-Soluble DNA of Infected Cells.—Questions were next posed concerning the origin and mode of synthesis of the saline-soluble DNA which increases in adenovirus-infected cells.

The basic design of the experiments done to investigate this question deviated from those already described only in the time at which radioisotope was added to the cultures and the

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period of time during which isotope was present in the culture fluids. In one series of cultures, 50 to 100 μc. of P^{32}-inorganic phosphate was added to the culture growth fluid 24 or 48 hours before viral infection. Immediately before inoculation of type 4 adenovirus, fluid containing P^{32} was removed and cultures were washed 4 times, each wash consisting of approximately 50 ml. of Hanks's BSS containing an excess of unlabeled phosphate ions. Virus and maintenance mixture were then added to one-half the cultures and heat-inactivated virus and maintenance mixture to the others. With another set of 3 cultures, 100 or 200 μc. P^{32} was added at time of viral infection; 3 cultures received radioisotope and heat-inactivated virus and served as controls. Another set of 6 cultures was divided into 3 infected and 3 control cultures and both groups incubated at 36°C. for 23 hours, at which time 100 or 200 μc. P^{32} was added to each culture. 24 hours after viral infection (1 hour after addition of isotope), all cultures were harvested, cultures from each group were pooled, aliquots removed for infectivity titrations and cell counts, and extractions carried out, followed by chemical and isotope content determinations. Other experiments were done exactly as described above except that 2 or 4 μc. of C^{14}-labeled sodium formate was substituted for P^{32}. When sodium formate–C^{14} was used *after* viral infection, 4 μc. was added 21 hours after virus.

TABLE II

Effect of Type 4 Adenovirus Infection on Specific Activity of Saline-Soluble DNA When HeLa Cells Were Labeled with P^{32}–Inorganic Phosphate or Sodium Formate–C^{14} 24 or 48 Hours Before Infection

Isotope	Time isotope added before infection*	Specific activity‡ DNA		Decrease DNA specific activity of infected cells
		Uninfected	Infected	
	hrs.	C.P.M./*μg. DNA*	C.P.M./*μg. DNA*	*per cent*
$P^{32}O_4$§	24	785.0	675.0	14
"	48	4100.0	3340.0	21
C^{14}-formate‖	48	64.5	51.7	20
" **	48	157.0	138.0	12

* At time of infection isotope removed, cells washed, and maintenance mixture without isotope added with virus.

‡ Determined 24 to 26 hours after infection with 3.0 to 4.5 TCD_{50} of virus per cell.

§ 200 μc. P^{32}–inorganic phosphate per culture.

‖ 2 μc. sodium formate–C^{14} per culture.

** 4 μc. sodium formate–C^{14} per culture.

The determinations of saline-soluble DNA from representative experiments in which cells were prelabeled with P^{32} orthophosphate or C^{14}-formate for 24 or 48 hours before viral infection are summarized in Table II. It is noted from these data that no increase in specific activity of saline-soluble DNA occurred under these circumstances. Indeed, a consistent, albeit small, *decrease* in specific activity of DNA from infected cells was measured. This is in sharp contrast to the results observed when isotope was added 21 or 23 hours after infection, just 1 to 3 hours before termination of the experiment (Table III), under which conditions a most impressive increase in P^{32} or C^{14} incorporation

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into saline-soluble DNA was obtained. A marked increase in specific activity of this DNA fraction was likewise demonstrated when isotope was added at the time of viral infection (these data are not presented in tabular form, as similar results are adequately shown in Tables I, IV, and V, and Fig. 1).

24 hours after viral infection saline-soluble DNA was increased approximately 100 per cent in infected cells (Fig. 1) (7). It should be emphasized again that when cells were labeled with isotope 24 to 48 hours before infection, and infected in the presence of media devoid of P^{32} or C^{14}, there was no increase in specific activity of saline-soluble DNA from infected cells; rather, a small but consistent *decrease* (12 to 21 per cent) in specific activity was noted. These

TABLE III

Incorporation of P^{32}–Inorganic Phosphate or Sodium Formate–C^{14} into Saline-Soluble DNA from Type 4 Adenovirus–Infected HeLa Cells to Which Isotope Was Added 21 or 23 Hours after Infection

Isotope	Time isotope added after infection	Specific activity* DNA		Increase DNA specific activity of infected cells
		Uninfected	Infected	
	hrs.	C.P.M./μg. *DNA*	C.P.M./μg. *DNA*	*per cent*
$P^{32}O_4$‡	23	5.00	35.00	600
" §	23	1.55	9.40	506
C^{14}-formate‖	21	1.10	6.75	514
" ‖	21	0.98	8.00	715

* Determined 24 hours after infection with 3.0 to 4.5 TCD_{50} of virus per cell.
‡ 200 μc. P^{32}–inorganic phosphate per culture.
§ 100 μc. P^{32}–inorganic phosphate per culture.
‖ 4 μc. sodium formate–C^{14} per culture.

results suggest that the major portion of the rapidly synthesized DNA which accumulated in infected cells was built from isotope-labeled materials in the host cell and was present before viral infection. That *de novo* synthesis of the saline-soluble DNA occurred and that some of the building blocks of the DNA were obtained from unlabeled precursors derived from the medium are indicated by the consistent decrease in specific activity of DNA from infected cells as a result of incorporation of unlabeled precursors from the medium which had diluted the host cell pool.

The addition of isotope to infected cultures during the incremental period of viral multiplication, 21 or 23 hours after infection (10), resulted in a marked increase in specific activity of saline-soluble DNA as compared to control cultures (Table III). This was striking despite the presence of isotope for only 1 or 3 hours before termination of the experiment. It may be concluded from these results that in infected cells saline-soluble DNA is being very rapidly

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synthesized during the period in which virus is also being most rapidly propagated (10).

Temporal Relationships of Increased Specific Activity, Accumulation of Saline-Soluble DNA, and Multiplication of Type 4 Adenovirus.—To estimate the significance of the finding that in type 4 adenovirus-infected cells a particular species of DNA was rapidly synthesized and accumulated in the cells, experiments were carried out to investigate the temporal relationship of the synthesis of the saline-soluble DNA to propagation of infectious virus.

Experiments were done exactly as described previously except that 18 or 24 cultures were employed so that 3 or 4 time intervals could be studied in each experiment (3 uninfected and 3 infected cultures per period). Eight separate experiments were carried out to determine DNA and RNA content and P^{32} or C^{14} incorporation into the nucleic acid fractions as well as the acid-soluble pool. With P^{32}, 200 μc. of labeled inorganic phosphate was added to each culture 1 hour before cells were harvested so that isotope incorporation occurred during the same period for all cultures, regardless of the period of total incubation. Experiments utilizing sodium formate–C^{14} were carried out in a manner similar to those with P^{32} orthophosphate except for the following modifications which were made to increase isotope incorporation: (*a*) 6 hours after viral infection monolayers were washed 2 times with Hanks's BSS and a maintenance mixture consisting of Eagle's basal medium (22) supplemented with 2 per cent calf serum was employed so that no purines and pyrimidines were present in the medium; and (*b*) 4 μc. per culture was added 6 hours after viral infection and was present throughout the remainder of the experimental period.

Two separate experiments were done to determine the rate of viral multiplication, and the geometric mean of the 2 infectivity titrations for each interval was calculated. Each of 20 cultures was infected with 2.6×10^7 TCD_{50} of trypsin-treated virus (4 TCD_{50} per cell) and incubated for 6 hours at 36°C. Infected fluid was removed from each culture, the cell sheet washed 3 times with 50 ml. Hanks's BSS containing 2 per cent chicken serum, and 10 ml. of fresh maintenance mixture added. At varying intervals after infection ranging from 6 to 24 hours, cells and fluid from 2 cultures were pooled, frozen, and thawed 6 times to disrupt cells (10), debris removed by centrifugation, and the supernatant fluid stored at −28°C. for infectivity titrations when all cultures had been harvested.

The results, presented as the geometric means of the individual experiments, are summarized graphically in Fig. 1. In confirmation of previous studies (10), the latent period of the multiplication cycle was 14 hours, followed by a rapid incremental phase of viral multiplication. Concomitant with the appearance of infectious virus, a gradual increase in saline-soluble DNA was detected. It is striking that approximately 4 hours before the increased DNA content of infected HeLa cells could be demonstrated, a significant increase in the specific activity of the saline-soluble DNA from infected cells was measured. Incorporation of P^{32} or C^{14}–formate into saline-soluble DNA continued to increase rapidly during the experimental period, and at a rate somewhat faster than the accumulation of saline-soluble DNA as measured by chemical techniques.

Measurement of an increase in specific activity of saline-soluble DNA

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significantly earlier than detection of an increased quantity of this DNA by chemical means suggests that the former technique is the more sensitive of the 2 methods for determination of increased synthesis of DNA or that a very rapid turnover of this DNA fraction occurs. The observation that saline-soluble DNA began to accumulate in adenovirus-infected cells at approximately the same time that newly propagated virus first could be detected

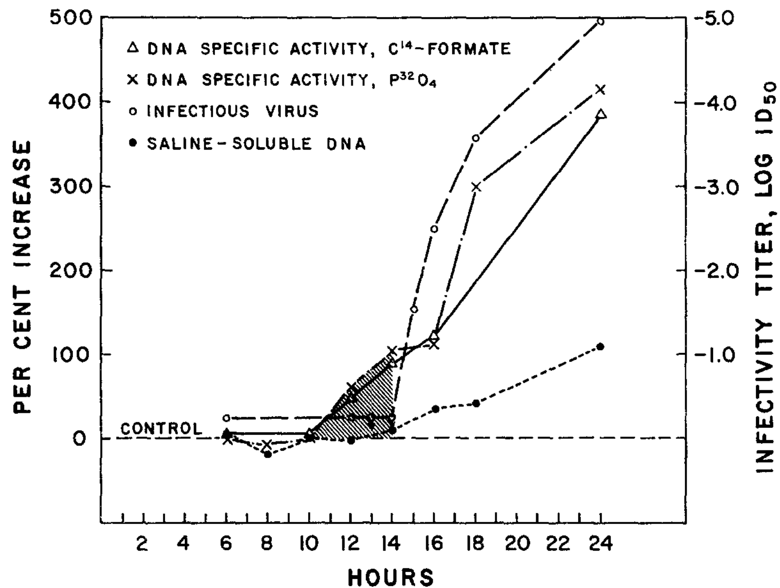

FIG. 1. Temporal relationships of type 4 adenovirus multiplication, increased content of saline-soluble DNA, and increased incorporation of P^{32}-inorganic phosphate or sodium formate-C^{14} into saline-soluble DNA of infected cells. Cells were infected with 2.7 to 4.5 TCD_{50} per cell: 200 μc. of P^{32} was added 1 hour before cells were harvested or 4μc. C^{14} was added 6 hours after viral infection. Each endpoint represents the geometric mean of at least 4 individual experiments for DNA content and isotope incorporation and 2 individual experiments for viral multiplication. The shaded area indicates the period of time between increased synthesis of saline-soluble DNA and detection of infectious virus.

suggests a relationship between the synthesis of virus and this particular DNA fraction. That synthesis of saline-soluble DNA as measured by P^{32} or C^{14}-formate incorporation commenced before formation of infectious virus could be detected, suggests that this DNA fragment may be a precursor of virus and that it may be incorporated into the mature viral particle during its final fabrication. It must be recognized that the temporal relationship between synthesis of saline-soluble DNA and infectious virus suggested by these data could be fortuitous, but recognition of the possible relationship merits further exploration.

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Incorporation of P^{32}–Inorganic Phosphate into Ribonucleic Acid (RNA) of Type 4 Adenovirus-Infected HeLa Cells.—Previous studies indicated that type 4 adenovirus infection did not affect RNA synthesis in HeLa cells to the same striking extent as DNA production (7). To determine whether viral infection might affect only a small fraction of RNA, as with certain bacteriophages (23), P^{32} incorporation into this nucleic acid was investigated.

Experiments similar to those described previously were carried out. Cultures were infected with 3 to 5 TCD_{50} of virus, and 200 μc. P^{32}-inorganic phosphate was added to each culture 1 hour before termination of the experiment. Intervals of 6 to 36 hours after viral infection were studied.

TABLE IV

Effect of Type 4 Adenovirus Infection on Content of and P^{32}–Inorganic Phosphate Incorporation into Nucleic Acids of HeLa Cells

Nucleic acid	Uninfected		Infected*		Increase in infected cells	
	Content	Specific activity‡	Content	Specific activity‡	Nucleic acid	Specific activity
	µg./10⁶ cells	C.P.M./*µg./ml.*	*µg./10⁶ cells*	C.P.M./*µg./ml.*	*per cent*	*per cent*
RNA§	4.55	43.00	6.35	59.0	39.6	37.2
DNA	2.98	3.86	10.50	20.8	252.0	438.0

* Cultures inoculated with 4.5 TCD_{50} of virus per cell.

‡ 200 μc P^{32} added 23 hours after viral infection and 1 hour before experiment was terminated.

§ Expressed as ribose.

The evidence obtained showed the specific activity of RNA from infected cultures to be 28 per cent greater than RNA from uninfected controls at 24 to 36 hours after infection (geometric mean of 11 experiments). The results of a representative experiment in which specific activity of the RNA was determined 24 hours after viral infection are presented in Table IV. For comparative purposes the data for the saline-soluble DNA are also summarized. In the experiment illustrated, the increase in RNA per cell and specific activity of RNA from infected cells as compared to controls were of the same order of magnitude, and were markedly less than values obtained for increase in DNA. It should be emphasized that no large increase in specific activity of RNA from infected cells was measured at any time from 6 to 36 hours after viral inoculation. These data confirm the findings previously reported, that type 4 adenovirus infection has no marked effect upon RNA synthesis (7).

Effect of Type 5 Adenovirus Infection on Synthesis of RNA and Saline-Soluble DNA in HeLa Cells.—A study of the biological and immunological characteristics of adenoviruses types 1 to 7 has indicated that these agents may be

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classified into 2 subgroups (2, 3, 10, 24–27). Types 1, 2, 5, and 6 have been shown to be very similar in the properties studied and to comprise one subgroup, whereas types 3, 4, and 7 are closely related and belong to the second subgroup. The consistent differences in the viruses in the two subgroups suggested that the effect on nucleic acid synthesis by an adenovirus other than a type closely related to type 4 should also be studied. Type 5 adenovirus was selected for this purpose, and experiments similar to those already described in detail were carried out using P^{32}-inorganic phosphate.

The results of a representative experiment summarized in Table V show a clear-cut difference from those obtained with type 4 infection (Table IV). As demonstrated by these data, although the quantity of RNA per infected cell

TABLE V

Effect of Type 5 Adenovirus Infection on Content of and P^{32}–Inorganic Phosphate Incorporation into Nucleic Acids of HeLa Cells

Nucleic acid	Uninfected		Infected*		Increase in infected cells	
	Content	Specific activity‡	Content	Specific activity‡	Nucleic acid	Specific activity
	μg./10⁶ cells	C.P.M./ *μg./ml.*	*μg./10⁶ cells*	C.P.M./ *μg./ml.*	*per cent*	*per cent*
RNA§	3.73	55.5	5.2	136.0	39.4	145
DNA	1.16	9.9	3.8	45.0	228.0	354

* Cultures inoculated with 8 TCD_{50} of virus per cell.

‡ 200 μc. P^{32} was added 23 hours after viral infection and 1 hour before experiment was terminated.

§ Expressed as ribose.

increased only 39 per cent, the specific activity of RNA increased 145 per cent, almost 2.5-fold. In other experiments an increase in RNA per cell of 75 to 100 per cent was occasionally seen with the increase in specific activity of the same order as that shown in Table V. The increase in saline-soluble DNA in type 5–infected HeLa cells was similar to that obtained with type 4 infection (Tables I, III, and IV).

It is not clear at present why the marked increase in specific activity of RNA is associated with type 5 but not type 4 adenovirus infections. A recent report of studies with type 2 adenovirus (8) showed similar findings which present further evidence of major differences of type 4 from types 2 and 5 adenoviruses. It should be noted that it is adenovirus types 1, 2, 5, and 6 which produce the greatest quantity of the toxin-like material associated with viral infection but separable from the viral particle (12–14). It is a possibility that the increased synthesis of RNA shown to be associated with types 2 and 5 infections may be intimately related to the production of the toxin-like substance which is largely protein in composition.

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Incorporation of P^{32}–inorganic Phosphate or Sodium Formate–C^{14} into Acid-Soluble Pool of Uninfected and Type 4 Infected Cells.—As part of the experiments previously described in which the incorporation of P^{32} orthophosphate or sodium formate–C^{14} into RNA (Table IV) and saline-soluble DNA (Table III and Fig. 1) was determined, incorporation of these isotopes into the acid-soluble pool was also studied. Radioactivity of the acid-soluble pool was determined by direct plating of 0.1 ml. of the supernate of the mixture from which the nucleic acids and protein had been precipitated by trichloracetic acid. Samples were dried with an infrared lamp. For convenience in the C^{14} experiments, the results (Tables VI and VII) are presented as the C.P.M. per 10^6 cells rather than as true specific activity (C.P.M. per μg. P) as in P^{32} experiments.

TABLE VI

Incorporation of P^{32}–Inorganic Phosphate and Sodium Formate–C^{14} into the Acid-Soluble Pool of Uninfected and Type 4 Adenovirus-Infected HeLa Cells

Isotope	Specific activity				Increase in C.P.M./10^6 infected cells
	Uninfected cells		Infected cells*		
	C.P.M./10^6 cells	*C.P.M./μg. P*	*C.P.M./10^6 cells*	*C.P.M./μg. P*	*per cent*
$P^{32}O_4$‡	30,200	11,450	38,200	11,900	3
"	24,700	7,030	33,900	8,370	37
C^{14}-formate§	12,500	—	28,000	—	124
"	14,400	—	38,200	—	165

* Infected with 3.5 to 5.0 TCD_{50} virus per cell.

‡ 200 μc. P^{32}–inorganic phosphate per culture added at time of viral infection. Experiment terminated 35 hours after infection.

§ 60 μc. sodium formate–C^{14} per culture added 6 hours after infection. Experiment terminated 40 hours after infection.

ments. When P^{32}–inorganic phosphate was utilized, no effect of viral infection on incorporation of isotope could be detected. However, when sodium formate–C^{14} was employed, the isotope content of the acid-soluble pool of infected cells was increased more than 2-fold. It is striking (Table VII) that the increased incorporation of C^{14} into the acid-soluble pool commenced at approximately 12 hours after viral infection, the same interval at which the specific activity of the saline-soluble DNA began to increase (Fig. 1). It should be pointed out that the specific activity of the acid-soluble pool, when studied with P^{32}–inorganic phosphate, did not increase at any time from 6 to 35 hours after infection.

The basis for the differences encountered with the use of P^{32} and C^{14}-formate is not readily apparent. It may be considered possible that with P^{32}–inorganic phosphate the components of the acid-soluble pool are so generally labeled that one could not detect an increase in specific activity of a minor segment. On

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the other hand, not only is C^{14}-formate incorporated into molecules more specifically, but also the formate not incorporated into larger molecules is volatilized and lost during drying. Therefore, an increased incorporation into a minor portion of the pool could be measured with sodium formate–C^{14}. The increased incorporation of sodium formate–C^{14} into the acid-soluble pool probably reflects incorporation of this precursor into amino acids such as serine and methionine as well as into nucleic acid bases, nucleosides and nucleotides.

TABLE VII

Time of Increased Incorporation of Sodium Formate–C^{14} into the Acid-Soluble Pool of Type 4 Adenovirus Infected HeLa Cells

Incubation period	"Specific activity"* acid-soluble pool		Per cent increase
	Uninfected	Infected‡	
hrs.	C.P.M./10^6 *cells*§	C.P.M./10^6 *cells*§	
10	13.7	10.0	0
12	12.3	23.2	89
14	13.3	38.7	191
16	11.0	26.1	137
24	16.7	58.2	249

* 4 μc. sodium formate–C^{14} per culture at time of viral infection.

‡ Infected with 2.8 TCD_{50} per cell.

§ Each value is the geometric mean of 2 identical experiments.

DISCUSSION

Experimental data presented in this communication demonstrate that type 4 adenovirus infection of HeLa cells induces an increased synthesis of DNA. The DNA that was synthesized preceding and accompanying viral synthesis is a fraction of nucleic acid which as a nucleoprotein is extracted from the infected cell with 0.15 M NaCl (termed the saline-soluble DNA). In contrast, the bulk of DNA, which is considered the normal host cell DNA, as a nucleoprotein complex is soluble in water or 2 M NaCl but not in 0.15 M NaCl, and is not increased in its synthesis during viral infection. Experiments utilizing P^{32}–inorganic phosphate or sodium formate–C^{14} confirmed the evidence previously reported that adenovirus-infected cells contained an increased quantity of DNA, and that viral infection effected increased production of the unusual molecular species of DNA, the saline-soluble DNA (7).

The discovery that saline-soluble DNA was the nucleic acid which accumulated in virus-infected cells implied that this DNA was synthesized *de novo* as an important stage in viral synthesis, produced concomitantly with viral synthesis but not directly related to it, or formed by a degradation of poly-

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merized host-cell DNA. Two lines of evidence obtained in this investigation bear directly upon these possibilities:

1. Studies in which host cell DNA was labeled before viral infection with either P^{32}–inorganic phosphate or sodium formate–C^{14} clearly indicate that saline-soluble DNA was synthesized from precursors in the medium as well as from materials in the cells before viral infection (Table II).

2. The specific activity of saline-soluble DNA was greatly increased over that which is probably normal host-cell DNA; *i.e.*, the water-soluble DNA, from uninfected or infected cells (Table I). Were the saline-soluble DNA derived directly from water-soluble DNA, the specific activity of both molecular species would be similar.

It may be concluded from these data that saline-soluble DNA is not formed merely by a breakdown of normal host-cell DNA, but rather is synthesized from small molecular building blocks derived from the host-cell pools and the nutrients of the medium. The experiments described do not distinguish between the hypotheses that saline-soluble DNA is synthesized as an essential step in viral propagation or that it occurs as a result of a non-specific stimulus.

The finding that synthesis of saline-soluble DNA precedes the first detectable production of infectious virus, however, and then accompanies viral synthesis at a similar rate, suggests that this DNA may be a precursor of virus and is incorporated with other substances to produce mature viral particles. It should be noted also that increased incorporation of sodium formate–C^{14} into the acid-soluble pool of infected cells occurs at the same time as the increased synthesis of saline-soluble DNA.

The alterations in DNA synthesis described are to date unique for an animal virus–infected cell. However, an increased synthesis of DNA has been reported to occur in cells infected with herpes simplex (28), vaccinia (29), equine abortion virus (30), or pseudorabies virus (31). With T_2 bacteriophage, as with adenoviruses, increased DNA synthesis commenced before newly synthesized infectious virus could be detected (32–34). The comparison of DNA synthesis in adenovirus-infected cells to that in *E. coli* infected with T_2 bacteriophage can be carried further with the similarity in production of an excess of DNA which then accumulates in infected cells and may be referred to as "surplus DNA" (34).

To clarify the picture completely as to the role of the saline-soluble DNA, it must be determined directly whether or not the DNA of the viral particle is identical with that which accumulates in infected cells. This approach will be aided by the finding that the saline-soluble DNA has a unique base composition: neither adenine and thymine nor guanine and cytosine are paired quantitatively, and guanine is markedly increased in amount (9). Experiments utilizing P^{32}–inorganic phosphate, sodium formate–C^{14}, or adenine-8–C^{14} all clearly demonstrate that synthesis of guanine in the saline-soluble DNA molecule is

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increased as compared to the other bases (35, 36). It remains to be determined whether the DNA of highly purified type 4 adenovirus has a base composition similar to that of the saline-soluble DNA. The relationships of the DNA of the virus and that which increased in infected cells can be tested further by determining whether the specific activity of the two are similar when synthesized in the presence of a radioisotope.

Total RNA was also shown to be increased in type 4 or 5 adenovirus–infected cells, but the increment was not nearly so great as for the saline-soluble DNA (7). RNA in type 4–infected cells was only approximately 28 per cent greater than the amount in control HeLa cells. RNA synthesis was increased to a greater extent in type 5 as well as in type 2 infection (8). It is the type 2 and 5 adenoviruses which have been shown to be chiefly associated with production of a soluble toxin (12–14), in addition to at least 2 soluble complement-fixing antigens and infectious virus (37, 38). The 3 newly synthesized soluble antigens are principally protein in nature (39). Although a similar toxin is synthesized in type 4–infected cells, it is found in much smaller quantities than in type 2 or 5 virus–infected cells (38). These data suggest that the increased RNA synthesis is associated with the marked increase in protein manufacture in adenovirus-infected cells. Furthermore, there are some findings which imply that propagation of a DNA-containing virus is dependent upon RNA synthesis (40).

One may conclude from the evidence described that adenoviruses have a profound effect upon the synthetic and metabolic apparatus of infected cells. The infected cell is stimulated to produce DNA and protein for fabrication of viral particles. RNA synthesis is probably increased in association with marked increase in viral sub-units. Not only do infected cells synthesize DNA and protein for formation of infectious viral particles, but the synthetic processes appear to manufacture specific viral precursors in great excess of that required for viral particles. Thus, large amounts of toxin and soluble complement-fixing antigens accumulate in infected cells free from viral particles. The saline-soluble DNA, which is postulated to be the precursor of viral DNA, also is made in great surplus and accumulates in nuclei of infected cells. The accumulations of DNA and protein form the distinctive and characteristic intranuclear inclusion bodies characteristic of adenovirus-infected cells (1–3). Thus, from the investigations described emerges a picture of some of the biochemical changes in nucleic acid and protein synthesis which are related to viral multiplication and form the basis for cell injury by a viral infection.

SUMMARY

Type 4 adenovirus infection of HeLa cells effected a marked increase in synthesis of the saline-soluble DNA fraction, but not the host-cell DNA (the water-soluble fraction). This was demonstrated by the marked increase in

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specific activity of saline-soluble DNA but not water-soluble DNA when P^{32}–inorganic phosphate or sodium formate–C^{14} was employed. When these isotopes were used to label cells before viral infection rather than during the process of viral propagation, the saline-soluble DNA from infected cells had a specific activity of 10 to 20 per cent less than that of uninfected cells, indicating that the saline-soluble DNA was synthesized both from prelabeled precursors of the cell pools and unlabeled materials from the medium. Saline-soluble DNA began to increase between 10 to 12 hours after viral infection and 3 to 4 hours before appearance of newly propagated infectious virus. The specific activity of the acid-soluble pool of infected cells also increased between 10 to 12 hours after viral inoculation when sodium formate–C^{14} was used as a radioisotope. When P^{32}–inorganic phosphate was utilized, the specific activity of infected-cell RNA was increased approximately the same relative amount as when total RNA was determined chemically; *i.e.*, 30 to 40 per cent. With type 5 adenovirus, not only did a 3- to 5-fold increase in saline-soluble DNA occur, but also an increase was measured in specific activity of RNA when P^{32}–inorganic phosphate was used.

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