The Effect of Heat on the Anatomy of the Adenovirus

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SUMMARY

When purified preparations of adenovirus type 5 were heated the icosahedral capsid of the virus was ruptured at the twelve vertices, allowing the viral DNA to become accessible to the action of DNAse. The viral DNA could then be substantially separated from protein by digestion with trypsin or pronase. An analysis of the distribution of viral DNA by radioactivity and the various capsid complement-fixing antigens after density gradient centrifugation of heated virus suggested the presence of some other hitherto undescribed protein within the capsid.

INTRODUCTION

The icosahedral nature of the capsid of the adenovirus was established by the classical experiments of Horne et al. (1959) using the negative staining technique. The fine structure of the virus was further elucidated by Valentine & Pereira (1965) who also correlated the 'soluble' antigens found in extracts of cells infected with human adenovirus type 5 with various morphological features of the virus. These antigens, designated hexons, pentons and fibres (Ginsberg et al. 1966) and their relationship to the virus were described in the previous communication (Russell et al. 1967). These investigations have defined the external features of the virus but there still remains the problem of what lies within the capsid. On morphological considerations alone only about 60% of the volume of the virion is accounted for by the capsomeres and the viral deoxyribonucleic acid (Valentine & Pereira, 1965). Since the virus is reported by Green (1962) and Green, Piña & Kimes (1967) to contain only protein and deoxyribonucleic acid it can be presumed that either the nucleic acid is very loosely packed into the available space or that there is a protein within the capsid in more intimate contact with the viral genome.

In this communication the effect of heat on purified preparations of adenovirus type 5 is reported. By using serological and electron microscopical techniques the fates of the various components of the virion on heating are determined and an attempt is made to ascertain if there is a hitherto undescribed internal protein.

METHODS

Virus. The virus (type 5, strain AD75) was propagated in HeLa cells and assayed as described previously (Pereira, Allison & Balfour, 1959). Purified virus was obtained by homogenization of infected cells in fluorocarbon and then layering the extract (or concentrate of the extract) on a preformed double layer of caesium chloride (in 0.05 M-HCl buffer, pH 7.4) consisting of 0.8 ml. (density = 1.45 g./cm.3) and a top layer of
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2·2 ml. (density = 1·32 g./cm.³) in a 5 ml. plastic centrifuge tube. After centrifugation at 100,000 g for 1½ hr, the virus was separated in an opalescent band: the 'soluble' antigens could be recovered from the solution above the band. The virus band was removed through a puncture at the bottom of the tube and the virus further purified by equilibrium density gradient centrifugation in caesium chloride (100,000 g, 18 hr) by placing 0·25 ml. of virus on a preformed double layer of caesium chloride consisting of 2·2 ml. (density = 1·32 g./cm.³) on top of a layer of 1·5 ml. (density = 1·45 g./cm.³). A visible band of virus was produced and removed by puncturing the tube. The purified virus preparations were generally stored at 4 ° in the concentrated caesium chloride solutions and dialysed against a suitable buffer when required. In these preparations electron microscopic examination revealed only the characteristic particles of adenovirus; there was no evidence of satellite viruses. As a further criterion of purity it was noted that such purified preparations of virus when submitted to acrylamide-gel electrophoresis did not show any migration of protein components thus indicating that there were no appreciable amounts of free capsid antigens or small cellular debris in the preparations (unpublished observations).

Preparations of tritium labelled virus. Virus labelled with tritium was prepared by infecting cells in the normal way but with the addition of [3H]thymidine (1 µc/ml.: 22,000 mc/m-mole). The virus was extracted from the infected cells by homogenization in fluorocarbon and the extract concentrated to about 1 ml. by dialysis against polyethylene glycol. Most of the smaller radioactive molecules were removed by gel filtration on Sephadex G-25 (Pharmacia) using ferritin as a marker to facilitate recognition of the viral fraction which eluted with the ferritin. Overnight equilibrium density gradient centrifugation in caesium chloride (100,000 g, 18 hr) gave a discrete band of virus corresponding to the radioactivity. The radioactivity was determined by spotting a sample of the test solution on to a square of filter paper, drying and placing in 10 ml. of toluene scintillating fluid and counting.

From three 20 oz bottles of HeLa cells, the yield of virus was generally about 0·5 ml. of a preparation containing about 1 to 5 x 10¹⁸ particles/ml. and 3 to 10 x 10⁵ counts/min./ml. All preparations had more than 90 ° of the radioactivity acid-insoluble and not accessible to deoxyribonuclease (i.e. the label was in the viral DNA). In one experiment the virus was labelled with 14C by using [14C]thymidine (0·05 µc/ml.: 224mc/m-mole).

Preparation of purified antigens. The capsid antigens were purified by chromatography on columns of DEAE Sephadex as described in the previous communication (Russell et al. 1967).

Sensitivity to deoxyribonuclease. The sensitivity of the labelled virus to deoxyribonuclease (DNase) was determined by incubating the virus in phosphate buffered saline with deoxyribonuclease 1 (Sigma crystalline grade 50 µg./ml., pH 7·2, Mg²⁺ 0·05 M) at 37° for 30 min. Cold trichloracetic acid was added to the digest to a concentration of 10 ° and in the presence of carrier protein (100 µg./ml.), allowed to stand at 4° for 30 min. and then centrifuged (500 g, 15 min., 4°). The amount of acid-soluble radioactivity was measured by removing a sample of the supernatant fluid into 10 ml. of dioxan scintillation fluid and counting.

Treatment with trypsin and pronase. Heated labelled virus was digested with these enzymes by incubating at pH 7·2 at 37° for 15 min. Trypsin was crystalline grade from Armour Chemicals and was used at a concentration of 0·1 %. Pronase, used at a
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similar concentration, was obtained from Calbiochem Inc. Both these enzymes were DNase-free.

*Scintillating fluids and counting.* Ten g. of 2,5-diphenyloxazole, 0.25 g. of bis-2(4-methyl-5-phenyloxazolyl)-benzene, 180 g. of naphthalene in 1 l. of dioxan or 5 g. of 2,5-diphenyloxazole, 0.50 g. of bis-2(4-methyl-5-phenyloxazolyl)-benzene in 1 l. of toluene. Counting was done in a Packard ‘Tricarb’ liquid scintillating counter. When $^{14}$C and $^{3}$H were counted together the channel settings were such that negligible counts from $^{3}$H appeared in the $^{14}$C channel and corrections were made for $^{14}$C counts in the $^{3}$H channel.

*Density gradients* were determined by measuring the refractive indices of individual drops at various points in the gradient with an Abbe refractometer. The relationship between density ($\rho$) and refractive index ($\eta$), $\rho D = 10.866\eta D - 13.4974$ derived by Ifft, Voet & Vinograd (1961) was used. Very consistent density gradients were obtained in equilibrium centrifugation by the use of the preformed double-layer technique.

*Complement-fixation tests* were done as described in the preceding communication (Russell et al. 1967).

*Protein* determinations were made using the method of Lowry et al. (1951) with bovine serum albumin as standard; 40 mm. microcells were used with the microcell housing of the Unicam 500 spectrophotometer.

*Electron microscopy.* Negative-staining techniques employing sodium silicatungstate were as described by Valentine & Pereira (1965).

**RESULTS**

*Effect of heating on virus infectivity*

Purified preparations of virus in phosphate buffered saline (Dulbecco & Vogt, 1954) were heated in stoppered 1 oz bottles at 56° for various times. The incubation was stopped by plunging the bottle into an ice bath and adding cold maintenance medium to a dilution of one in ten. Infectivity was then assayed in test-tube cultures of HeLa cells. In a virus preparation containing initially $2 \times 10^8$ TCD 50/ml. three logs of infectivity were lost within 1 min. at 56°. No infectivity could be detected after 5 min. at 56°.

*Effect of heating on morphology of capsid antigens*

Purified preparations of hexon, fibre and penton antigens were heated at 56° for 10 min. and then examined in the electron microscope. Hexons and fibres retained their characteristic morphology whilst pentons lost their bases and thus became indistinguishable from fibres.

*Effect of heating on morphology of virus*

When purified virus was heated for 10 min. at temperatures from 50 to 56° in different conditions of ionic strength, examination in the electron microscope revealed large holes at the twelve corners of the icosahedral capsid, apparently produced by the loss of the pentons and their neighbouring five hexons (Pl. 1, fig. 1). The resultant hexon shell consisting of 180 hexons was stable and retained its characteristic morphology after prolonged heating at 56°.
Effect of heating on sensitivity of tritium labelled virus to deoxyribonuclease

When tritium-labelled virus was heated at 56° most of the radioactive label became accessible to the action of DNase within 1 min. Thereafter, there appeared to be a small decrease in the accessibility of the label. At 50° the label was not accessible to DNase after 1 min. but was accessible after 5 min. (Fig. 1).

![Graph](image)

Fig. 1. Effect of heat on sensitivity of labelled virus to deoxyribonuclease.

- ×--×, 56°;
- ○--○, 50°.

Equilibrium density gradient centrifugation of heated virus in caesium chloride

An attempt was made to separate viral nucleic acid from the partially degraded capsid by heating tritium labelled virus at 56° for 10 min. and then centrifuging to equilibrium (for 42 hr) in caesium chloride at a density of 1.7 g./cm.³. Under these conditions it was expected that DNA (which should have a density of approximately 1.70 g./cm.³) would separate as a discrete band of radioactivity. The failure to achieve this (Fig. 2A) demonstrated that the major part of the nucleic acid was still firmly attached to protein. However, on treatment of the heated virus with trypsin or pronase the radioactivity did separate as a sharp band (Fig. 2B, C). Digestion of unheated virus with trypsin did not produce a similar separation and since trypsin has no effect on the morphology and antigenicity of purified hexons (Pereira, to be published), it must be assumed that the viral DNA is attached to the hexon shells or to some other
The effect of heat on adenovirus protein by some trypsin-sensitive bond. To demonstrate that this DNA was substantially the same as that obtained by the standard phenol extraction of papain- and detergent-digested virus (Green & Piña, 1964), adenovirus DNA labelled with $^{14}C$, was prepared by this method and added to heated and trypsinized virus labelled with $^3H$ and centrifuged to equilibrium in caesium chloride. Fig. 3 shows that the $[^{14}C]$DNA banded within two drops of the material labelled with $^3H$, thus indicating that the radioactive band obtained from the heated and trypsinized virus was associated with the viral DNA.

Since no product of the heated virus banded at a density of 1.7 g./cm.$^3$ further
investigations were made by centrifuging to equilibrium at a lower average density of caesium chloride. In these experiments the centrifugation was continued for 18 hr using the preformed double layer technique. Longer times of centrifugation did not significantly alter the results. Labelled virus, when heated in m-caesium chloride at 56° for 10 min. banded at a density of 1.394 g./cm.³ in contrast to the unheated virus which equilibrated at a density of 1.348 g./cm.³ (Fig. 4). To ascertain if the ionic environment in which the virus was heated had any effect on the final density at which the virus banded, the virus was heated in 0.01 M-phosphate buffer, in phosphate buffered saline (with calcium and magnesium) and in phosphate buffered saline with 0.005 m-EDTA. Under these conditions the radioactive virus banded at densities of 1.400, 1.410 and 1.420 g./cm.³ respectively; moreover, in the latter case the radioactive profile suggested the presence of two components (Fig. 4).

Serological analysis of heated virus after density gradient centrifugation

The distribution of the capsid antigens after density gradient centrifugation of (unlabelled) virus heated in phosphate buffered saline was determined by complement fixation on five drop fractions from the gradient. To facilitate detection of the antigens considerably more virus was used in these experiments than in the radioactive experiments described above. With unheated virus, a single opalescent band was obtained at a density of 1.350 g./cm.³; with this band was associated all the complement-fixing activity and protein in the gradient. With the heated virus two visible bands were obtained, one very sharp near the top of the tube and another, much lower, not so
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sharp, but flaky, in appearance. The distribution of hexons, fibres and protein was determined (Fig. 5). The antigens and most of the protein banded in two areas corresponding to densities of 1.305 and 1.382 g./cm.³. This latter band is significantly less dense than the band of radioactivity obtained on centrifuging the preparations of

![Equilibrium density gradient centrifugation of heated labelled virus in caesium chloride of average density 1.35 g./cm.³. A, heated in M-CsCl, 56°, 10 min.; B, unheated virus; C, heated in PBS + 0.005M-EDTA, 56°, 10 min.](image)

heated labelled virus. To ascertain the distribution of radioactivity in a similar situation, virus labelled with ³H was added to sufficient unlabelled virus for complement-fixation determination, and the virus heated at 56° for 10 min. and centrifuged to equilibrium. At a density of 1.385 g./cm.³ the peaks of antigenicity and radioactivity coincided (Fig. 6).

**Morphological examination of density gradient fractions**

Virus antigens and most of the protein were distributed in two areas of densities approximately 1.40 and 1.30 g./cm.³ (Fig. 5, 6). Electron microscopic examination of
Fig. 5. Equilibrium density gradient centrifugation of heated virus in caesium chloride of average density 1.35 g./cm$^3$. ○—○, hexons; ■—■, fibres; △—△, protein.

Fig. 6. Analysis of radioactivity and complement fixing antigens after density gradient centrifugation of heated virus. ○—○, radioactivity; △—△, hexon antigen.
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the less dense area revealed the presence of free fibres and hexons, hexon shells and
groups of nine hexons in a characteristic formation (Pl. 1, fig. 2) (cf. Smith, Gehle &
Trousdale, 1965). At the higher densities, examination was rendered difficult by exces-
sive clumping especially where larger quantities of virus were centrifuged. However,
clumping was not so marked in the experiments using radioactive virus and it was
possible to identify three morphological entities in these regions, namely:

(i) Groups of nine hexons.
(ii) Hexon shells in various configurations (Pl. 1, fig. 3).
(iii) Entities not recognizable as any of the capsid antigens (Pl. 2, fig. 4).

These were evident in the fraction of density 1.420 g./cm.³ obtained on heating the
virus in phosphate buffered saline containing versene (see Fig. 4). No capsid antigens
could be detected in this fraction.

Refractive index of CsCl

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<th>Density (g./cm.³)</th>
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Fig. 7. Calculated densities of a nucleoprotein containing 13 parts of DNA of density 1.70
g./cm.³ and varying amounts of protein of density 1.30 g./cm.³.

DISCUSSION

These investigations have shown that on heating adenovirus type 5 at 56° the ico-
sahedral capsid is ruptured probably as the result of the specific disintegration of the
thermolabile penton bases followed by the liberation of the surrounding five hexons.
Smith, Gehle & Trausdale (1965) who studied the effect of a detergent on the
architecture of the virus noted the characteristic grouping of nine hexons and suggested
that the points of weakest bonding between the capsomeres appeared to be along the
edges and at the vertices of the triangular facets.

A notable feature of our experiments is the apparent stability of the hexon shell
arrangement of 180 hexons. It should be noted that this arrangement of hexons allows
easy access of quite large molecules to the viral DNA and it is tempting to speculate
that a similar situation may occur in the infected cell. Thus, the virus, on infecting a
cell, may need only to lose its corner pentons and perhaps the surrounding five
hexons to allow transcription of the viral DNA to begin.
Serological analysis of the density gradient fractions after centrifugation of heated virus showed that hexon and fibre antigens could be detected in the band at a density of 1.30 g./cm³. This was confirmed by the electron microscope examination which showed free fibres and hexons as well as groups of nine hexons and hexon shells at this density. A similar correlation of the morphological and serological findings occurred at the higher densities, hexon antigen and hexon shells being apparent at densities of 1.38 to 1.41 g./cm³. At these densities viral DNA could also be detected by radioactivity. Although the unheated virus consistently banded at a density of 1.35 g./cm³, the heated virus equilibrated at much higher densities but with considerable variation and, in general, banded at relatively lower densities (i.e. 1.38 to 1.39 g./cm³), with clumping, when larger amounts of virus were centrifuged and at higher densities (1.40 to 1.42 g./cm³) when smaller amounts of virus were centrifuged.

If it is assumed that the virus contains 87 parts of protein of density 1.30 g./cm³ and 13 parts of DNA of density 1.70 g./cm³ (Green, 1962; Green et al. 1967), then the resulting density of the virus in cesium chloride can be calculated to be about 1.35 g./cm³, in good agreement with the experimental findings. If viral protein is progressively removed it can also be calculated that the resulting nucleoprotein will so increase in density that when one-third of the protein is removed the density will be 1.375 g./cm³. As more protein is removed the density of the nucleoprotein will rapidly increase (Fig. 7). Thus, in the region of density 1.40 g./cm³ relatively small differences in the amount of protein binding to the DNA will make considerable differences to the density of the resulting nucleoprotein. It thus seems reasonable to assume that the apparently considerable variations in the final density attained in equilibrium runs noted in different experiments on heated virus, were the result of relatively small differences in protein content of the viral nucleoprotein, accentuated by the fact that clumping, presumably caused by some 'sticky' protein, tended to make the band even less dense.

If it is assumed that the virion consists only of the capsid antigens and DNA and since the number and size of the various components of the capsid are known (Valentine & Pereira, 1965), removal of the pentons and their neighbouring six hexons will be equivalent to removing, at the maximum, one-third of the viral protein. Reference to Fig. 7 shows that the expected density of such a nucleoprotein would be 1.375 g./cm³. The densities at which the hexon shells are seen in the electron microscope are significantly higher than this (viz. 1.382 to 1.410 g./cm³). Groups of nine hexons also are frequently seen at these densities, but these are always associated with the hexon shells and are presumably breakdown products of the latter. (The hexon shell consists of 180 hexons and can be assumed to be composed of twenty groups of nine hexons.) Of further significance are the objects which are seen (Pl. 2, fig. 4) in the apparent absence of capsid antigens. In some preparations of heated virus, similar entities, not, however, so well defined, could be discerned. These were seen in close association with ruptured capsids (see Pl. 2, fig. 5) suggesting that they are internal structural components. If the 'thread-like' objects seen in the density gradient fractions are related to these components, then they could be a viral nucleoprotein and the denatured product of a more ordered structure.

A feature of the morphological analyses is the fact that significant quantities of hexon shells were detected in the released-antigen band at a density of 1.30 g./cm³. These hexon shells must have, at one stage, enclosed the viral DNA and since the
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experiments with the heated labelled virus showed that no significant quantity of the radioactive label banded at densities greater than 1.42 g./cm.³, it can be assumed that some other protein must be responsible for retaining the DNA at the lower densities. This is consistent with the finding that viral DNA as detected by radioactivity can be found in the apparent absence of capsid antigens.

From the above considerations it will be seen that the results of the morphological, serological and protein analyses of the fractions from the density gradient centrifugation of heated virus are incompatible with the presence only of the capsid antigens and viral DNA. The results are explicable, however, if it is assumed that there is some other protein (or proteins) within the virus which may comprise as much as 20 to 30% of the total viral protein.

Inner proteins have been described in the T2 and T4 bacteriophages (Hershey, 1955; Levine, Barlow & Van Vunakis, 1958; Minagawa, 1961) and consist of about 7% of the total protein. These proteins have been shown to be polybasic in nature and are associated with polyamines such as spermidine and putrescine which probably serve to neutralize the charge on the DNA. Other workers (Chaproniere-Rickenberg, Mahler & Fraser, 1964) have concluded that the internal proteins were unlikely to be responsible for the folding of the DNA during maturation and its function is unknown.

Electron microscope studies on the pox viruses (e.g. Easterbrook, 1966) have shown that they are composed of a number of layers enclosing the so-called lateral bodies and a brick-shaped internal core. However, the detailed structure, composition and function of these components are unknown. The herpes group of viruses occupy an intermediate position between the complex pox viruses and the adenoviruses. In this case the evidence that is available on the number and size of the capsomeres and on the viral DNA (Wildy, Russell & Horne, 1960; Russell, Watson & Wildy, 1963; Russell & Crawford, 1964) also suggests that there is more space within the capsid than can be accounted for by the viral nucleic acid alone, and this may be occupied by an internal protein.

Although there is clear evidence of the presence of internal proteins in the T bacteriophages and evidence (sometimes presumptive) of their presence in the larger animal viruses, their structure and function are entirely unknown. Since the various components of the capsid of the adenovirus have been well characterized (Valentine & Pereira, 1965; Russell et al. 1967) it may be that a more intensive study of this particular virus will provide some indication of the function of these viral structural proteins. If the presumed inner protein of adenovirus is specified by the virus genome it is not unlikely that in the virus growth cycle it would be made and complexed with the viral nucleic acid before the capsid antigens are produced. In this connexion, it is relevant that in the previous communication (Russell et al. 1967) a heat labile antigen, designated the P antigen, could be detected in 'early' extracts of infected cells. The possibility that this antigen is related to the suggested internal protein is examined in the following paper.

The co-operation of Dr K. Hayashi in a number of the experiments is gratefully acknowledged. Mrs Barbara Knight and Mr Robert Newman contributed expert technical assistance.
REFERENCES


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EXPLANATION OF PLATES

(see overleaf)
EXPLANATION OF PLATES

Electron micrographs of negatively stained preparations of heated virus and caesium chloride density gradient fractions. The bar indicates 500 Å.

**PLATE 1**

Fig. 1. Typical hexon shell arrangement seen in purified preparations of virus heated at 56°.

Fig. 2. Fraction at a density of 1.30 g./cm.³: groups of nine hexons, free fibres and hexons are seen in this field.

Fig. 3. Fraction at a density of 1.40 g./cm.³: hexon shells.

**PLATE 2**

Fig. 4. Fraction at a density of 1.42 g./cm.³ (drop nos 15-19 from gradient after heating virus in phosphate buffered saline containing EDTA—see Fig. 4 in text): ‘thread-like’ objects are possibly a viral nucleoprotein.

Fig. 5. Purified virus heated at 56° for 10 min.: possible inner structural components; free fibres, hexons and groups of 9 hexons are also evident.