Seven cases of leukemia were discovered among the spouses of the 876 index cases while five cases of leukemia were found among matched controls of the spouses. This difference is not statistically significant (Table 1; 7).

For the controls of the spouses, death certificates were matched on the following criteria: age within 5 years; sex; race; marital status; township of residence; date of death within 4 weeks. Since the death certificates used were bound in books of 500 by county of residence and month of death, selection of a control death certificate from the same or adjacent death book as that of the spouse automatically matched for county of residence and month of death. Conscious matching for marital status, age, sex, and race was done by first examining the death certificate of the spouse to be matched and looking at adjacent certificates alternately toward the front and back of the death book until a certificate which fulfilled the matching criteria was located.

Table 2 shows information obtained from death certificates of the seven couples, both members of which died of leukemia. No trend is evident for the interval between death, and in three of the seven couples, death was attributed to different types of leukemia.

Table 3 shows that the distribution of stated causes of death in mates of index cases is not significantly different from that of matched controls of the mates.

In summary, husbands and wives of individuals who die of leukemia do not have a demonstrably increased risk of dying of leukemia. This is consistent with the hypothesis that adult leukemia is not contagious in the usual sense.

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7. See W. G. Cochran, *Biometrika* **37**, 256 (1950) for details of this test.
8. This study would have been impossible without the dedicated death certificate searching of Mrs. Mary Postmayer and Miss Madeleine Igoe.

24 December 1964

Cyclic Structure of Adenovirus DNA

Abstract. *The deoxyribonucleic acids of adenovirus types 2, 7, and 12 were extracted and examined in the electron microscope. Cyclic forms of the molecules were found to exist in each viral type. The lengths of cyclic strands extracted from the most dense fraction of purified particles of adenovirus type 2 were highly variable, averaging about 2.5 microns. This corresponds to a molecular weight of about 5 million.*

Fiers and Sinsheimer (*1*) showed that the DNA molecule of ϕ174 bacteriophage is circular, and Dulbecco (*2*) obtained similar evidence for polyoma virus DNA. Kleinschmidt and Zahn devised a method for preparing nucleic acid molecules so that they can be studied in the electron microscope (*3*). With this technique, Weil and Vinograd confirmed that polyoma virus DNA exists in a cyclic form (*4*). We used a different preparative method in which DNA molecules were recovered from the surface of agar and made electron opaque by reaction with uranyl acetate. The details of this technique and others applied in our study have been described (*5*). The droplet rather than the centrifugation method was used for placing samples on agar.

In the course of studying artificially disrupted adenovirus particles, long, circular strands of small diameter were obtained from adenoviruses types 2, 7, and 12 after treatment with sodium lauryl sulfate (SLS). In this report we present some of the characteristics of these strands and the evidence for the conclusion that they are viral DNA molecules.

In order to compare the results obtained by Kleinschmidt and Zahn's method with those obtained in this study, polyoma virus purified by cesium chloride density gradient was disrupted with SLS, and the degradation products were prepared for microscopy by the agar method. Large numbers of circular strands were produced after treatment with SLS. Electron micrographs were made of typical fields and the lengths of the strands were determined with a map-measuring device. Magnification of the electron micrographs was checked against a carbon replica of a standard grating having an average dimension of 0.883 μ between lines. The lengths of the circular strands of polyoma virus ranged from 1.22 μ to 1.90 μ, and averaged 1.64 μ. This average is in agreement with the value of 1.58 μ previously reported by Weil and Vinograd for circular polyoma virus DNA (*4*) and thus confirms their determination by a different method. Noncyclic (linear) strands were also seen and were about the same length. Treatment of viral suspensions containing more than $10^{9.5}$ particles per milliliter produced masses of tangled strands which were difficult to interpret and measure.

Efforts were made to use very mild conditions for disruption of the particles. Experiments were first designed to determine the lowest concentration of SLS which would disrupt a given number of particles. Serial dilutions were made from a 1-percent stock solution of SLS in 0.15*M* saline, then constant amounts (about $10^{9.5}$ particles per milliliter) of purified adenovirus type 2 were added in equal volumes to each dilution of SLS. The purified virus suspensions used in our first experiments were pools containing particles of density 1.30 to 1.35 g/cm^3; they were obtained from cesium chloride density gradients. Mixtures were allowed to stand at room temperature (23°C) for 5 to 60 minutes. Samples were prepared at intervals and examined in the electron microscope. Disruption of at least 99 percent of the particles was achieved within 5 minutes after addition of SLS (final concentration, 0.12 mg/ml).

Suspensions of disrupted particles contained many circular strands and other viral components readily observable in the electron microscope. Figure 1 shows a group of purified particles of adenovirus type 2 before disruption. The DNA cores are intensely stained with uranyl acetate, whereas the protein coats are relatively unstained. Figure 2*A* shows an electron micrograph (low magnification) of a typical circular strand obtained by disruption of purified adenovirus type 2. Figure 2*B* shows a micrograph (higher magnification) of a portion of this same circular strand. Aggregates of viral protein subunits (capsomeres) can be seen in the background (see arrows); some of these capsomeres remained loosely attached to the circular strand. Circular strands were of various lengths, measured about 30 Å in diameter (although this small dimension was difficult to measure), and stained more intensely with uranyl acetate than did the protein capsomeres. A micrograph

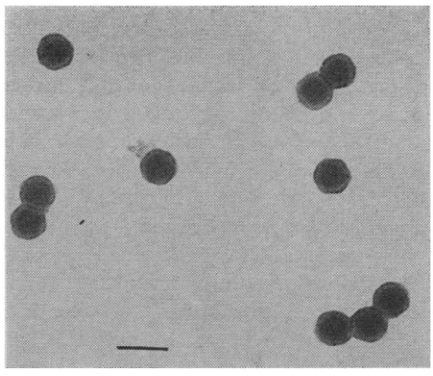

Fig. 1. Density-gradient purified adenovirus type 2 stained with uranyl acetate; bar, 100 mμ.

(higher magnification) of a segment from a circular strand is shown in Fig. 2*C*. These strands, after removal of SLS by brief dialysis against water, were disrupted by brief treatment (30 minutes at 37°C) with pancreatic deoxyribonuclease (100 μg/ml, recrystallized twice) dissolved in Eagle's basal medium. Neither pronase, trypsin, nor Δ-chymotrypsin, each at 250 μg/ml, had any observable effect upon

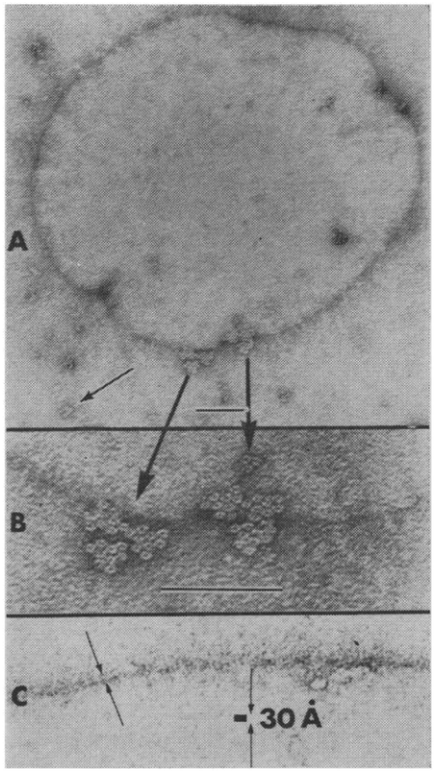

Fig. 2. *A* and *B*, A single circular strand of DNA released from adenovirus type 2 by treatment with sodium lauryl sulfate; bar, 100 mμ. *C*, An electron micrograph (higher magnification) of a section from a circular DNA strand; width of bar, 30 Å.

the strands when they were incubated for 30 minutes at 37°C. Pronase eventually digested the viral capsomeres without having any noticeable effect upon the strands. Circular forms were somewhat unstable, being converted to linear forms and tangled masses after storage for a few hours either at room temperature or at 4°C.

Density gradients of adenovirus type 2 were fractionated in order to obtain samples containing virus particles of fairly uniform densities (± 0.01). Presumably denser particles would contain greater amounts of DNA and might be expected to have longer DNA strands. To test this assumption, selected fractions were treated with SLS and the lengths of circular strands from each fraction were measured. Particles of density 1.357 contained cyclic strands averaging 2.1 μ in length (37 measurements) (Fig. 3). Lengths ranged from 0.9 to 4.0 μ, with a standard deviation of ± 0.70μ. The average length of cyclic strands in the fraction with a density of 1.319 was only 1.4 μ (40 measurements). Measurements ranged from 0.5 to 3.5 μ and the standard deviation was ± 0.74 μ. Thus denser particles contained, on the average, longer circular strands than did the less dense particles. However, there was a surprisingly large amount of overlap and variation in lengths.

Another passage of adenovirus type 2 was fractionated in the same way, treated with SLS, and the strand lengths from various fractions were measured (Table 1). Particles of greater density contained, on the average, longer circular strands than did the less dense particles. There was, again, great variation in lengths (0.75 to 6.1 μ). This was in sharp contrast to the relative uniformity of the lengths of polyoma virus cyclic strands (range, from 1.22 to 1.90 μ).

Adenovirus types 7 and 12 (both tumorigenic, *6*) were similarly extracted, and they contained circular strands. The lengths of strands from these types were as variable as those of adenovirus type 2 (nontumorigenic, *7*). Apparently both tumorigenic and nontumorigenic adenoviruses contain circular DNA strands.

The identification of these circular strands as DNA molecules is based upon the following observations:

1) The average length of circular strands from polyoma virus, when prepared by the agar method, was almost identical to that previously reported for polyoma virus DNA. Except for differences in average length, these strands could not be distinguished microscopically from those obtained from adenovirus types 2, 7, and 12.

2) Circular strands were disrupted by treatment with pancreatic deoxyribonuclease but were resistant to potent proteolytic enzymes.

3) Circular strands were very small in diameter—about 30 Å, which is the approximate diameter of DNA molecules.

4) Circular strands stained intensely with uranyl acetate, a well-known stain for DNA, whereas protein viral subunits in the same preparation did not.

5) Particles of higher density contained, on the average, longer circular strands than did particles of lower density (as might be expected if the strands were DNA).

One explanation for the wide range of strand lengths, even from particles of fairly uniform density, is that single particles may contain more than one strand. The most dense fractions of particles of adenovirus type 12 (density 1.34 to 1.35) contain the highest

Table 1. Length of cyclic DNA strands from disrupted purified adenovirus type 2.

Density	Number of rings measured	Average length of circular strands (μ)
1.300	10	1.6
1.332	5	2.3
1.358	26	2.9
1.300–1.349 (pool)	20	1.9

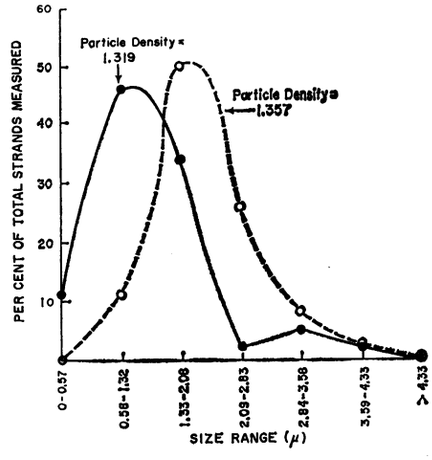

Fig. 3. Length distribution of circular DNA strands extracted from adenovirus type 2 particles by sodium lauryl sulfate treatment.

proportion of infectious particles (*5*), but only about 1 in 6 to 8 of these particles are infectious. These data suggest that only particles containing the largest amount of DNA are infectious, but there has been no good explanation for the fact that 83 to 88 percent of even the densest particles are noninfectious. Incorporation of two short DNA strands (both presumably incompletely coded and therefore noninfectious genomes) into a single particle might account for a particle's being dense without being infectious. This might explain the earlier observations (*5*). A second explanation for variation in strand lengths from particles of uniform density would be a lack of uniformity in the density of individual strands.

Green and Pina, in studying the chemical composition of adenovirus type 2 DNA, obtained sedimentation data which are compatible with a cyclic structure (*7*). The data presented here strengthens this interpretation. If a value of 2.5 μ for the average length of DNA from the densest particles (1.357 to 1.358) is applied and if the mass per unit length is the same as in strands of the sodium salt of DNA (*8*), this length corresponds to a molecular weight of about 5×10^6.

The possibility of disrupting viruses gently and measuring the lengths of their DNA strands now permits quantitative studies of the relation between DNA strand lengths within virus particles and the infectivity of the particles. In the case of tumorigenic adenoviruses, lengths of DNA strands within particles might also be correlated with subsequent production of malignant transformations of cells either in vivo or in vitro. This physical approach might also be usefully applied to the study of a recently recognized phenomenon: integration of two virus types within the protein coat of a single particle (*9*).

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Indole Compounds: Isolation from Pineal Tissue

Abstract. *Five indole compounds have been isolated from bovine pineal tissue and characterized as 5-methoxytryptophol, N-acetyl-5-methoxytryptamine (melatonin), 5-hydroxytryptophol, 5-methoxyindole-3-acetic acid, and 5-hydroxyindole-3-acetic acid. Pineal hydroxyindole-O-methyltransferase, with S-adenosylmethionine, converts 5-hydroxytryptophol to 5-methoxytryptophol.*

The presence of 5-hydroxyindole-3-acetic acid, 5-methoxyindole-3-acetic acid, and *N*-acetyl-5-methoxytryptamine (melatonin) in pineal tissue has been reported (*1*). Another compound, or compounds, which gave an atypical green color on paper when sprayed with Ehrlich's reagent and which antagonized the myotropic action of serotonin has also been noted (*2*). Because some β-carbolines have these properties (*3*) and can be derived from corresponding tryptamines (*4*), it was tentatively suggested that a tetrahydro-β-carboline might be present in pineal tissue (*2*). Bioassay by antagonism to serotonin indicated that pineal tissue was a particularly rich source of the unknown material.

Pineal glands removed from cattle immediately after slaughter were quick-frozen in an airtight container surrounded by dry ice. Tissue was always shipped and stored under the same conditions, namely at −10°C, with light and air excluded. Extracts were prepared and chromatographed in an atmosphere of nitrogen; the extracts were stored in the dark at −10°C. Approximately 50 kg of pineal tissue was extracted in batches of 1 kg.

Tissue (1 kg) was homogenized in 2 liters of redistilled ethyl acetate and filtered. Ethyl acetate in the filtrate was removed at reduced pressure and at 40°C with a rotary evaporator; the residue was partitioned between hexane and water to remove cholesterol. The aqueous phase was extracted twice with equal volumes of ethyl acetate; the extracts were combined, dried, and freed of solvent. The residue was dissolved in a minimum amount of ethanol for chromatographic separation. In silica-gel thin-layer chromatography (chloroform:methanol, 9:1; solvent A), five xanthydrol-positive compounds consistently appeared. These had R_F values of 0.70, 0.60, 0.35, 0.10, and 0.05 (Table 1). Two other xanthydrol-positive compounds were often seen: unknown No. 1 with R_F 0.95, and unknown No. 3A with R_F of 0.58.

Fifty derivatives of serotonin were used as reference compounds for the preliminary comparative identification of the indoles in pineal extracts, and it was necessary to synthesize some new compounds (*5*). Chromatography, electrophoresis, and ultraviolet spectra were used to characterize the unknowns.

Fractions were eluted from chromatoplates, and ultraviolet spectra were obtained. Evaporation of the eluates to dryness yielded gums. Several milligrams of unknowns, Nos. 5 and 6, were obtained, and recrystallization from toluene yielded 0.5 mg of each in crystalline form sufficiently pure for melting-point determination. Though each compound had a range of 10°C, the melting point of 5-methoxyindole-3-acetic acid when mixed with No. 5 was not depressed, nor did mixture with No. 6 depress the melting point of 5-hydroxyindole-3-acetic acid (Table 1).

The gum containing unknown No. 3 was dissolved in 0.2 ml of chloroform, and 0.5 mg of picric acid in chloroform was added. Standing for 24 hours at 4°C produced 0.5 mg of crystalline material which was removed by centrifugation; the material had the same melting point as *N*-acetyl-5-methoxytryptamine picrate and gave matching infrared spectra.

A neutral picrate of unknown No. 4 was obtained in the same manner; although it amounted to only 0.2 mg, its low solubility in chloroform permitted its isolation. It was identical with synthetic 5-hydroxytryptophol picrate.

We failed to identify unknown No. 3A, although some similarities were