STRUCTURAL ALTERATION IN ISOLATED RAT LIVER NUCLEI AFTER REMOVAL OF TEMPLATE RESTRICTION BY POLYANIONS

EUGENE A. ARNOLD, DAVID H. YAWN, DAVID G. BROWN, ROBERT C. WYLLIE, and DONALD S. COFFEY

From the Department of Pathology and the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, and the James Buchanan Brady Urological Institute, The Johns Hopkins Hospital, Baltimore, Maryland 21205

ABSTRACT

Specific polyanions release DNA template restrictions for DNA synthesis in isolated rat liver nuclei. The degree to which DNA synthesis is enhanced can be correlated with a spectrum of changes in nuclear structure. Each polyanion which is effective in the release of template restriction produces a characteristic alteration in nuclear ultrastructure. Polyanions which have no effect on DNA synthesis do not appear to cause any change in nuclear organization or ultrastructure. Parallel measurements of nuclear DNA release and nuclear volume changes also indicate that template-activating polyanions cause remarkable changes in the structural organization of the treated nuclei. These results indicate that DNA template activation involves direct interactions between polyanions and nuclear constituents and suggest the possibility that naturally occurring polyanions might have a role in the control of gene activity.

INTRODUCTION

Anionic polyelectrolytes are capable of interacting with isolated mammalian nuclei or purified chromatin in a manner that alters their capacity to act as a template for the synthesis of DNA and RNA in vitro (1–8). For example, Allfrey and Mirsky (1) have shown that nuclei treated with DNase lose many of their biochemical functions but that the addition of polyanions restores many of these functions while polycations are ineffective or inhibitory. In addition, Frenster (2) has reported that the template activity of heterochromatin for RNA synthesis is enhanced in the presence of certain nuclear RNAs.

In more recent studies it has been shown that isolated rat liver nuclei or soluble chromatin do not serve as an effective template for DNA synthesis when incubated in the presence of E. coli DNA polymerase and the appropriate precursors (6–12). However, the addition of certain synthetic (7) and naturally occurring (8) polyribonucleotides, synthetic polyanion acids (6), or polysaccharides (6) causes a marked increase in the incorporation of (dTMP)3H into acid-insoluble DNA. It has also been shown that certain polyanions produce an increase in nuclear volume and changes in nuclear morphology (13, 14). Kraemer and Coffey (13) have reported that swelling and DNA solubilization occur in nuclei treated with heparin or dextran sulfate, and that heparin causes a loss of nucleoplasmic granularity and the appearance of dense spherical bodies on the inner nuclear membrane when examined by light microscopy.

A striking feature of these studies is the specific-
ity with which polyanions cause the release of DNA template restrictions and changes in nuclear morphology. Among the polyanions, heparin and dextran sulfate are the most active and generally cause the greatest alteration in any characteristic so far examined (1, 6, 12). On the other hand, hyaluronic acid, chondroitin sulfate, and polygalacturonic acid were completely ineffective. Similarly, a high degree of specificity was seen among the synthetic homopolyribonucleotides (7), with the polypurines producing a much greater release of DNA template restriction for DNA synthesis than the polypyrimidines. When naturally occurring RNAs were tested it was found that ribosomal RNA and bacteriophage RNA stimulated DNA synthesis whereas transfer RNA, total yeast RNA, and yeast "core" RNA were completely ineffective (8).

The properties of polyanions which determine their specificity in causing the release of DNA template restrictions and changes in nuclear morphology include molecular size, the ratio of the polyanion to DNA, and (for polyribonucleotides) the amount and stability of their secondary structure (7-13) (see Table IV). It is important to note, however, that while these different characteristics have been observed to play a role in determining the template activating ability of polyanions, the basic mechanism for DNA template activation is not understood.

In this paper we wish to report our observations concerning the alterations in nuclear structure produced by several types of polyanions. We have measured DNA template activity, nuclear DNA solubilization, absorbance changes at 600 nm of nuclear suspensions, and alterations in nuclear dimensions produced by heparin, polysulfonic acid, and several homopolyribonucleotides. Assays for binding of selected polyribonucleotides to the isolated nuclei are also reported. The results indicate that all template-activating polyanions cause changes in nuclear morphology and also that specific and easily identifiable ultrastructural changes are manifested by each of the polyanions tested. The results also indicate that the effects of polyanions and heparin on DNA synthesis and solubilization and on the optical properties and physical dimensions of nuclei have no simple relationship. Instead, these effects appear to represent different aspects of complex interactions between polyanions and nuclear constituents.

**MATERIALS AND METHODS**

**Animals and Preparation of Nuclei**

Random-bred male Sprague-Dawley rats weighing 200-250 g were maintained ad lib. on chow and water. The animals were killed by cervical dislocation and the liver nuclei were isolated by the method of Blobel and Potter (15). The nuclei were washed twice with TKM buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM MgCl₂ and 25 mM KC₁) and resuspended in TKM to give a DNA concentration of 1.0 mg per ml.

**Assay for DNA Template Activity**

DNA template activity was assayed by measuring DNA synthesis in the presence of E.coli DNA polymerase (DNA deoxynucleotidyl transferase EC 2.7.7.7). A 0.1 ml reaction system contained 10 μmoles of Tris-HCl, pH 7.4 at 37°C, 0.7 μmoles of MgCl₂, 0.1 μmoles of β-mercaptoethanol, 18.7 μmoles each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dTTP), and deoxythymidine triphosphate (dTTP); 20 μmoles of Tris base, 0.3 μm of E. coli DNA polymerase (SA 5000 units/kg, Biopolymers Laboratory, Chagrin Falls, Ohio; Fraction VII); nuclei equivalent to 10 μg of DNA; 10 μg of neutralized polyanion where indicated; and an adenine triphosphate (ATP) generating system containing 0.25 μmoles ATP, 0.50 μmoles of sodium phosphate(3H)pyruvate, and 0.4 μg of phospho(3H)pyruvate kinase (Sigma II). All polyanions tested were soluble in this system. The system was incubated at 37°C for 30 min and assayed by the disk filter paper procedure (16). After appropriate washing, the radioactivity on the disk was determined by scintillation counting. The synthetic polyribonucleotides (obtained from Miles Laboratories, Inc., Elkhart, Indiana) exhibited a molecular weight by gel filtration of > 100,000 and sedimentation coefficients (S₂₀) in 0.05 M NaH₂PO₄ pH 7.0, in the range of 5.4-5.6 and 10.7.

**Assay for Solubilized Nuclear DNA**

This assay measures the amount of DNA released from nuclei treated with polyanions and was performed in the above medium from which dTTP-3H and E. coli DNA polymerase were omitted (polymerase-free medium). Each sample contained 100 μg/ml nuclear DNA and 100 μg/ml of polyanion. After incubation at 37°C for 15 min, the system was centrifuged for 10 min at 10,000 g (0°C) and the supernatant was decanted for assay. 1.0 mg of bovine serum albumin was added to all fractions, and acid-insoluble material was precipitated in the presence of 0.2 M perchloric acid (PCA) for 10 min at 0-4°C. After centrifugation
for 5 min at 10,000 g the pellets were resuspended in 0.2 M PCA and recentrifuged. The washed pellets were resuspended in 10 ml of 0.8 M PCA and heated at 70°C for 20 min. The tubes were then cooled for 10 min at 0-4°C and centrifuged for 5 min at 10,000 g. Hydrolyzed DNA was assayed by Burton's modification of the diphenylamine method (17). In each analysis the sums of the DNA in the nuclear pellet and its supernatant indicated a recovery of over 90% of the original amount of DNA present. The data represent the averages of three separate experiments.

Precipitation of Histones by Polyanions

Unfractionated histones (type II-A, Sigma Chemical Co., St. Louis, Mo.) were dissolved at 1 mg/ml in 0.12 M NaCl, 0.01 M Na acetate, pH 5.0. Polyanions were dissolved in 0.1 M Tris pH 7.6, also at 1 mg/ml.

The ability of each polyanion to precipitate histones was tested by mixing 0.1 ml of the polyanion solution with 0.1 ml of the histone solution. The resulting pH was 6.5. After 15 min at 37°C the mixtures were centrifuged at 10,000 g for 10 min. The supernatant was decanted and assayed for protein by the Lowry method (18), using calf thymus histone as the standard and correcting for the presence of Tris in the assay medium.

The amount of histone precipitated by each polyanion was calculated as the difference between the total histone present initially and the amount remaining in the supernatant after treatment.

Changes in Optical Density

Changes in absorbance at 600 nm were measured on nuclear suspensions in the polymerase-free medium at 37°C in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a Gilford model 2000 automated recording unit (Gilford Instrument Company, Oberlin, Ohio). Temperature was maintained by a Lauda Ultra-thermostat, (Lauda Instruments, Inc., Westbury, N.Y.).

The volume, concentration (13), and order of addition of the components was polymerase-free medium, 10 ml, nuclei (1 mg DNA/ml) 30 μl, and polyanion activator as specified (1 mg/ml) 30 μl. Absorbance was measured before addition of the polyanion and 15 min after its addition (See reference 13).

Assay for the Binding of Polynucleotides to Isolated Nuclei

The assays were performed in 0.1 ml of the DNA polymerase assay medium from which dCTP-3H and DNA polymerase had been omitted. Each assay contained nuclei equivalent to 23.6 μg of DNA and 6-12 μg of the appropriate labeled polynucleotide prepared to contain 66 μCi/100 μg of polyguanylic acid (poly G)-3H, 61 μCi/100 μg of polyadenylic acid (poly A)-3H, 60 μCi/100 μg of polyribonucleic acid (poly U)-3H, or 20 μCi/100 μg of polyinosinic acid (poly I)-3H. The molecular weight of all polynucleotides used was > 50,000 as determined by gel filtration (Miles Laboratories, Inc.). After 15 min at 37°C the nuclei were collected by centrifugation at approximately 1500 g in a clinical centrifuge for 10 min and the supernatant (0.1 ml) was assayed for radioactivity in 10 ml of a dioxane-based scintillation fluid.

The nuclear pellets, which had interacted with the labeled polynucleotides, were washed in 0.1 ml of TKM buffer by resuspension and recentrifugation. This supernatant was discarded. The washed nuclei were hydrolyzed in 0.2 ml of 0.8 M PCA for 20 min. After cooling 10 min at 0-4°C, the reaction tubes were again centrifuged and the supernatant was assayed for radioactivity in 10 ml of dioxane-based scintillation fluid. The results were calculated from the dpm in each fraction after the appropriate correction for the counting efficiency of the various samples.

Phase Contrast Microscopy

Nuclei were incubated for 15 min at 23°C in the same reaction medium used for the optical density studies (polymerase-free medium). The suspensions were then promptly examined by phase contrast microscopy. The heparin-treated nuclei, however, were examined after 2-3 min due to the rapidity with which morphological alterations took place.

Microinterferometric Measurements

Nuclear dimensions were measured with a Carl Zeiss interference microscope with an Achromat Plan Int II 40/0.65 objective lens. The nuclear suspensions were diluted with TKM so that no more than one nucleus for a microscopic field could be observed. Each nucleus was photographed, the image was projected, and the area was determined by planimetry.

Electron Microscopy

Nuclei from DNA template assays were centrifuged into a pellet after 15 min incubation at 37°C. Nuclei from reaction media containing heparin were also pelleted after a 2 min incubation at 25°C. The pellets were fixed for 2.5 hr in 1% glutaraldehyde buffered with sodium phosphate. After rinsing in phosphate buffer, they were postfixed in 1% OsO4 in phosphate buffer. All material was embedded in Araldite and ultrathin sections were prepared with the Porter-Blum ultramicrotome. The thin sections were stained for 4 min with lead citrate (except for heparin-treated nuclei incubated at 25°C for 2 min which were stained...
TABLE I

Effect of Polyanions on DNA Synthesis, DNA Solubilization, Absorbance Changes and Dimensional Alterations of Isolated Rat Liver Nuclei (See Materials and Methods)

| Polyion      | DNA synthesis* | DNA solubilization | Optical Clearance§ (600 nm) | Surface Area | Volume |
|--------------|----------------|--------------------|----------------------------|--------------|--------|
| None         | 30             | 2                  | 2 ± 167 ± 6                | 208 ± 8      |        |
| Heparin      | 370            | 16                 | 477 ± 24                  | 999 ± 69     |        |
| Poly X       | 350            | 46                 | NA                        | NA           |        |
| Poly I       | 265            | 42                 | 209 ± 14                  | 281 ± 20     |        |
| Poly Asp     | 245            | 44                 | 297 ± 2                   | 483 ± 28     |        |
| Poly G       | 180            | 10                 | 387 ± 22                  | 733 ± 60     |        |
| Poly U       | 70             | 10                 | 156 ± 7                   | 261 ± 15     |        |
| Poly A       | 50             | 4                  | NA                        | NA           |        |
| Poly C       | 40             | 5                  | 292 ± 16                  | 284 ± 31     |        |

* Picomoles of dTMP-3H incorporated into acid-insoluble product in 30 min at 37°C.
† Per cent of total nuclear DNA in supernatant after sedimentation of nuclei.
§ Per cent decrease in absorbance of nuclear suspensions after 15 min at 37°C. Initial absorbance equals 0.280.
∥ Volumes and surface area determined on 20-60 nuclei for each value as described in the Methods ± standard error of the mean.
NA = Not analyzed.

for 7 min). The sections were examined with an RCEMUS electron microscope.

RESULTS

The results of the experiments in which we examined the effect of polyanions on isolated rat liver nuclei with regard to release of nuclear DNA template restrictions, solubilization of DNA from nuclei, optical clearing of nuclear suspensions, and changes in surface area and volume are all summarized in Table I. The differential results expressed in Table I are not simply explained by rate differences or by concentration effects. For example, neither large increases in poly C concentrations (10–300 mg/ml) nor extensions in the time period of these reactions will mimic the effects seen with poly I at 100 μg/ml (7, 13). Fig. 1 is a frequency distribution diagram of the nuclear radii after polyanion treatment.

The synthesis of DNA is expressed as picomoles of dTMP-3H incorporated into acid-insoluble DNA in 30 min at 37°C. Earlier studies have shown that this incorporation is linear with respect to time for up to 45 min and requires the presence of all four nucleotides (7). The product can be solubilized by beef pancreatic deoxyribonuclease but not by ribonuclease or by 0.3 M KOH at 37°C for 1 hr. Incorporation of labeled precursor in the absence of nuclear template DNA is less than 2 picomoles.

Intact, isolated rat liver nuclei do not function as an effective template for DNA synthesis in the presence of exogenous DNA polymerase and the appropriate cofactors. However, the addition of specific polyanions results in an increase in the rate of incorporation of dTMP-3H into acid-insoluble DNA. The most effective release of DNA template restrictions is mediated by heparin which produces a 10–15-fold increase in the amount of label incorporated. Among the polyribonucleotides, the most effective are the polypurines; polyxanthyllic acid (poly X), poly G, or poly I produces an approximate 5–10-fold increase in the incorporation of dTMP-3H when compared to the control value. An exception to this is poly A which stimulates DNA synthesis to a level which is very small compared to that of the other polypurines tested. The polypyrimidines are relatively ineffective in stimulating DNA synthesis, with poly
Figure 1  Size distribution of control and polyanion-treated rat liver nuclei as determined by interference microscopy. Polyanion:DNA = 1.

U producing only a small increase and poly C showing no significant increase over control levels. Finally, the presence of polyaspartic acid (mol wt = 28,000) stimulates DNA synthesis to a level comparable to that produced by the polypurines. When rat liver soluble chromatin is used as the DNA template source, essentially identical results are obtained (8, 11, 12). This result indicates that the ability of the polyanions to activate template DNA is not limited by their ability to pass through the nuclear membrane.

Solubilization of Nuclear DNA by Polyanions

When nuclei were treated with certain polyanions a portion of the DNA could not be sedimented by the centrifugal forces utilized in our standard method (10,000 g, 10 min). We have used the term "solubilized" here in this restricted sense, since we have little data concerning the physical state of this nonsedimentable DNA. We do know, however, that it is acid precipitable and has a size sim-
ilar to that of bulk nuclear DNA when analyzed on an alkaline sucrose density gradient.

After nuclei are incubated at 37°C for 30 min in the absence of polyanions, 98% of the total DNA sediments with the nuclear pellet. Addition of poly C, poly A, or poly U produces no significant change in this amount of sedimentable DNA. On the other hand, incubation of nuclei with poly I, poly t-asparagin (poly Asp), or poly X results in a marked solubilization of nuclear DNA.

It can be seen from Table I that the ability of the polynucleotides to solubilize nuclear DNA can be correlated with their ability to activate template DNA. This correlation is not linear, however, since poly X solubilizes two to three times the amount of DNA that poly I or poly Asp solubilizes while producing only a 30–40% difference in the incorporation of dTMP-^3H. Poly G and heparin also exhibit anomalous behavior since solubilized DNA levels are low for these polyanions compared to their effectiveness in releasing the template restrictions on nuclear DNA. Although the reasons for this anomalous effect of poly X, poly G, and heparin are not known, we have made some observations on the morphological appearance or the physical state of the treated nuclei which may provide a partial explanation. Poly X consistently produces a striking ultrastructural alteration in nuclei which is characterized by a dense rim of condensed fibrillar material either within or on the nuclear membrane (see Fig. 13). This appearance suggests that poly X alters either the nuclear membrane or the physical conformation of chromatin, or both, in such a manner that the DNA remains confined to the nucleus and is, therefore, sedimentable. In the case of heparin, we have observed a marked increase in the volume of the sedimentable fraction with an alteration of the nuclear pellet to a gel-like consistency (see also reference 19). The low level of supernatant DNA may be due to entrapment of DNA in this gel-like pellet. Since heparin-treated nuclei lyse and both condensed and fibrillar chromatin are seen in extranuclear spaces (see Figs. 16 and 22), it seems unlikely that the retention of DNA in the pellet can be ascribed to the maintenance of chromatin structure or nuclear integrity. Poly X displays the same type of ultrastructural changes seen in heparin-treated nuclei (see Figs. 14 and 24). With poly X, however, these alterations in nuclear structure do not produce gels, so that entrapment of released DNA does not seem likely.

**Distribution of Available DNA Template and DNA Product after Treatment with Polyanions**

In an effort to corroborate the observations concerning the solubilization of nuclear DNA, we examined the localization and distribution of the DNA template and the product of the polymerase reaction after treating the nuclei with the various polyanions. This was done in three parallel experiments. In the first experiment, DNA template activity was measured exactly as described above. In the second experiment, nuclei were incubated with the appropriate polynucleotide in complete DNA polymerase medium except that the polymerase enzyme was omitted. After 30 min at 37°C, the samples were centrifuged at 1500 g for 5 min and the supernatant was decanted into a separate set of reaction vials. DNA polymerase was then added to this supernatant, and the template activity was measured as described previously. The pellets from each sample were resuspended in DNA polymerase medium and were also assayed in the usual manner for template activity. No additional polyanion was added to either fraction. In the third experiment, the DNA polymerase enzyme reactions were performed as usual. After 30 min at 37°C, the reaction vials were centrifuged at 1500 g for 5 min. Each supernatant and each pellet (resuspended in 0.1 ml of TKM) was then spotted for the filter paper assay.

Table II shows that when nuclei are treated with template-activating polyanions, much of the template and most of the product are found in the supernatant fractions. This is particularly apparent for poly X. For heparin, poly I, and poly asparagin acid the majority of the product appears in both fractions. A prominent exception to this trend occurs in the case of poly G where approximately 75% of both template and product remain in the nuclear pellet.

These results are entirely consistent with the DNA solubilization data shown in Table I. Poly X solubilizes the most DNA and does not produce gel-like pellets. On the other hand, poly I, poly Asp, and heparin release less DNA, and heparin, in particular, releases little DNA and similarly releases little template or product.
Distribution of Assayable DNA Template and DNA Product after Treatment of Isolated Liver Nuclei with Polyanions

| Polyanion | DNA synthesis in total | Location of template activity | Location of labeled product |
|-----------|------------------------|------------------------------|----------------------------|
|           | wet supernatant | pellet | supernatant | pellet |
| None      | 29 | 5 | 29 | 6 | 30 |
| Heparin   | 391 | 256 | 230 | 226 | 162 |
| PolyX     | 380 | 372 | 76 | 416 | 5 |
| Poly I    | 251 | 158 | 174 | 156 | 32 |
| Poly Asp  | 253 | 125 | 122 | 224 | 30 |
| Poly G    | 168 | 34 | 119 | 52 | 140 |
| Poly U    | 39 | 0 | 44 | 10 | 50 |
| Poly A    | 38 | 10 | 14 | 6 | 34 |
| Poly C    | 35 | 0 | 30 | 10 | 36 |

* Results are expressed as pmoles of dTMP-αH incorporated into acid-insoluble DNA in 30 min at 37°C.

Precipitation of Histones by Polyanions

All of the polyanions display the ability to precipitate unfractionated histones. In general, the polyribonucleotides are equally able to interact with and precipitate histones. In these cases 100 µg of polyanion will precipitate 70–90 µg of histone. Heparin and polyaspartic acid are also able to precipitate histones; however, these polyanions are less effective and only about 50 µg of histone are precipitated by 100 µg of polymer. Further experiments are in progress to determine whether specific histone fractions are precipitated by certain polyanions.

Effect of Polyanions on the Optical Properties of Nuclear Suspensions

The absorbance at 600 nm by nuclear suspensions in the presence of polyanions has been studied by Kraemer and Coffey (13). In these studies the absorbance changes appeared to be related to swelling of the nuclei in a manner analogous to the swelling of mitochondria, however, our data now indicate that these optical changes are probably due to several factors. Measurements of volume changes of polyanion-treated nuclei do not correlate well with the absorbance decreases observed for similarly treated nuclear suspensions. For example, poly Asp-treated nuclei have a volume almost twice that of poly I-treated nuclei, however, the absorbance of their suspensions differs by only 12%. A comparison between poly I and poly U shows that although the nuclear volumes are the same, these nuclear suspensions show an absorbance difference of nearly 25%. It is also apparent that although nuclei treated with poly G undergo a marked volume change (mean value of 789 µm²) and display significant structural alterations (Fig. 13), the absorbance data show only a slight decrease in optical density. Consideration of these spectrophotometric data in relation to the dimensional measurements and the ultrastructural alterations indicates that the changes in absorbance probably reflect a complex interaction of volume changes associated with alterations in the intranuclear organization of chromatin and nucleoli as well as differential losses of nuclear dry mass. Because of this, we think that changes in absorbance behavior should be designated by the term "optical clearing" rather than "nuclear swelling".

Radius, Surface Area, and Volume Changes

A bar graph of the frequency distribution of nuclear radii is shown in Fig 1. The data for surface area and volume calculated from the measurement of radius are presented in Table I. Control nuclei display a bimodal distribution of radius which correlates with a diploid and tetraploid population of nuclei. The mean radius of the 2N population was measured as 3.58 µm whereas the 4N population had a mean radius of 4.50 µm. Incubation of nuclei with poly C, poly U, or poly I produced a change in the mean value of the measured and calculated nuclear diameters which assume a position between the 2N and 4N distribution of the untreated controls. The dimensional changes are significant (p < 0.05) but are not striking, especially in the case of poly I which produces a marked increase in DNA synthesis and significant optical clearing. Interaction of nuclei with poly Asp, poly G, or heparin produces striking dimensional alterations with shifts of the mean values beyond the 4N mean for the untreated controls.

Binding of Polyribonucleotides to Isolated Nuclei

The results of the assays for polyribonucleotide binding to isolated nuclei (Table III) indicate that the extent of binding of these polyanions can be directly correlated with their ability to activate template for DNA-dependent DNA polymerase directed synthesis of DNA. Poly A, poly U, and

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TABLE III

| Polynucleotides | Total dpm | Supernatant dpm | Nuclear pellet dpm | Per cent bound |
|-----------------|-----------|-----------------|--------------------|---------------|
| Poly G [3H]     | 7.6 µg    | 22500           | 3780               | 21600         | 94            |
| Poly I [4C]     | 6.6 µg    | 3950            | 1120               | 2560          | 65            |
| Poly G [3H]     | 8.1 µg    | 15800           | 12600              | 1000          | 6.2           |
| Poly U [3H]     | 11.3 µg   | 19200           | 14700              | 400           | 3.2           |
| Poly A [3H]     | 8.2 µg    | 27800           | 20300              | 400           | 1.4           |

* Nuclei equivalent to 23.6 µg DNA were incubated at 37°C for 15 min. The nuclear pellet was obtained by centrifugation at 1500 g for 10 min. Per cent of total radioactivity bound to nuclei.

poly C are bound very weakly or not at all and are inactive in removing template restrictions for DNA synthesis. However, the polypurines, poly G and poly I, are bound to a highly significant degree and also produce a marked release of template restriction.

Further experiments were performed in which poly I-[14C]-treated nuclei were differentially washed according to the method of Chung and Coffey (20) or by increasing the concentration of sodium chloride in a stepwise fashion.

With the method of Chung and Coffey, approximately 30% of the poly I-[14C] was removed in the 0.14 M NaCl wash (soluble fraction S1; reference 20), while nearly 50% appeared in the 0.1 N NaOH wash (acidic fraction A; reference 20), and about 20% remained in the residue. No poly I-[14C] could be detected in the 0.1 M Tris wash (soluble fraction S2), in the histone fraction, or in the membrane protein fraction.

With the other method, nuclei were progressively washed with 0.14 M, 0.5 M, and 1.0 M sodium chloride. As above, 0.14 M NaCl removed about 30% of the poly I-[14C]; however, only 7% could be recovered in the 0.5 M NaCl wash and only 13% in the 1.0 M NaCl wash. About 50% of the poly I-[14C] again remained in the residue. Further analysis of the salt washes showed that they contained more than 95% of the nuclear DNA and more than 80% of the nuclear proteins. Therefore, the poly I-[14C] appears to be very tightly bound to nuclei in such a way that it cannot be removed by the simple washing procedures usually used to extract nuclear components.

Phase Contrast Microscopy

Photomicrographs of fresh smears of nuclei studied by phase microscopy are presented in Figs. 2-6. A control nucleus is illustrated in Fig. 2. These nuclei are spherical and have intact membranes. A single nucleolus is easily discernible.

Fig. 3 is a photomicrograph of a nucleus which has been treated with poly G for 15 min at 37°C. The nuclear diameter is larger than that of the control nucleus, and a peripheral rim of clumped material is easily seen (arrow). It can also be seen that an area of perinucleolar translucency is present (arrow) which corresponds to the perinucleolar halos seen in these nuclei on electron micrographs.

A nucleus from a preparation treated with poly X is illustrated in Fig. 4. This nucleus shows a marked increase in diameter when compared to the controls. Nucleoli (N) are prominent and appear to be enlarged. The nucleoplasm is evenly dispersed and small spherical bodies are present at the nuclear periphery (arrow).

Fig. 5 shows two nuclei from a suspension examined within 2 min after the addition of the heparin. The marked resemblance of these nuclei to those treated with poly X is apparent and corresponds to their appearance when examined in the electron microscope.

Fig. 6 shows several nuclei from a preparation exposed to heparin for a longer period of 15 min. These nuclei are very enlarged and do not show any normal internal structure. Large defects can be seen in the nuclear membranes, and prominent rounded spheres are present both inside and outside of the nuclear outlines.

These illustrative phase contrast micrographs indicate that the features described in the electron micrographs are present in unfixed, freshly prepared nuclear suspensions and are not an artifact of fixation or staining. In addition, they illustrate the dimensional alterations measured by interference microscopy.

Nuclear Ultrastructure

Fig. 7 is an electron micrograph of a nucleus from an untreated control preparation. The nuclear membranes are intact. Chromatin shows a condensed pattern and is distributed around the...
nuclear periphery with small foci in the nucleoplasm. A chromatin pedicle extends from the perinucleolar chromatin to the internal nuclear membrane. The nucleolus has a fibrillar and a granular nucleolouema with a prominent pars amorpha. Perichromatin granules are easily discernible and interchromatin granules are present throughout the nucleoplasm. No significant alter-
Figure 7  Representative control rat liver nucleus processed simultaneously with the polyanion-treated nuclei. Perichromatin granules are inconspicuous. Note pedicle of chromatin in continuity with the perinucleolar chromatin (double arrow). Electron micrograph. × 9000.

Figure 8  Poly C-treated nucleus showing no significant structural alteration. Electron micrograph. × 10,000.

Figure 9  Poly I-treated nucleus. The chromatin is dispersed and fibrillar with localized condensations (circle). Microspherules are prominent along the outer nuclear membrane, and some appear to be passing through the nuclear membrane (single arrow). Note perinucleolar halo (H). One nucleolus is almost completely dispersed (double arrow). Electron micrograph. × 9000.

Figure 10  Poly aspartate-treated nucleus with chromatin dispersion and prominent microspherules (arrows). Electron micrograph. × 9000.
FIGURE 11 Poly A–treated nucleus. Electron micrograph. × 9200.

FIGURE 12 Poly U–treated nucleus. Note multiple nuclear membrane breaks (single arrows) and dispersion of chromatin. Electron micrograph. × 10,000.

FIGURE 13 Poly G–treated nucleus showing characteristic accumulation of fibrillar material in nuclear membrane, chromatin dispersion, and perinucleolar halo (H). Electron micrograph. × 9200.

FIGURE 14 Poly X–treated nucleus showing remnants of dispersed nucleolus (double arrows) and prominent microspherules within and outside the nucleus (single arrow). Electron micrograph. × 9200.
The features of the control nuclei can be seen in preparations obtained from nuclei exposed to poly C (Fig. 8). Nuclei which have been incubated with poly A (Figs. 11 and 17) retain most of the features of the control preparations. A consistent alteration of nucleolar structure is found, however, which consists of a loss of fibrillar nucleolonema with a resultant prominence of the granular elements. Beginning formation of a perinucleolar halo is also noted which appears to be due to a separation of the perinucleolar chromatin from the nucleolonema.

The ultrastructure of poly U-treated nuclei is markedly different from that of the control preparations (Figs. 12 and 18). Multiple areas of loss of nuclear membrane can be seen (arrows, Fig. 12). Chromatin is evenly dispersed throughout the nucleus, and interchromatin granules appear as prominent aggregations. The nucleolus shows a condensation of the fibrillar nucleolonema with a prominent granular component (Fig. 18). The perinucleolar chromatin has a matted fibrillar appearance. A perinucleolar halo is not seen.

Fig. 9 is an electron micrograph of a nucleus which has been incubated with poly I. Electron-opaque microspheres appear to be attached to the external nuclear membrane. The nuclear membrane is intact except for sites where the electron-opaque spheres seem to be passing through (single arrow). The chromatin is partly dispersed, but localized regions of condensation are seen (circle). Nucleolar changes are present, consisting of loss of the fibrillar nucleolonema, a prominent granular component, and the presence of a perinucleolar halo (H). In Fig. 9, one nucleolus (double arrow) is almost completely dispersed, with only a remnant of the granular component remaining. A widening of the space between the inner and the outer nuclear membrane is seen (Fig. 19), with electron-opaque spheres both within and on the nuclear membrane. The microspheres (Fig. 19) are not membrane bound, are somewhat irregular in shape, and have a finely fibrillar periphery. A wide perinucleolar halo is present and the granular nucleolonema is prominent.

The ultrastructural alterations seen in poly Asp-treated nuclei are illustrated in Figs. 10 and 21. A moderate dispersion of chromatin has occurred and the interchromatin granules are prominent. Electron-opaque microspheres are present within the nucleoplasm (Fig. 21, double arrow) and have an appearance similar to those seen in the poly I-treated nuclei (Fig. 9). Areas of lysis of the nuclear membrane can be seen (Fig. 21, single arrow).

Virtually all nuclei incubated with poly G display a peculiar “bull’s-eye” appearance (Fig. 13). Electron-opaque fibrillar material is present both in and on the nuclear membrane completely around the periphery of the nuclei, and small amounts can also be seen in extranuclear spaces. Chromatin is dispersed and the interchromatin granules are prominent. Nucleoli (Fig. 20) are surrounded by a perinucleolar halo and show condensation of the fibrillar nucleolonema.

Poly X-treated nuclei (Figs. 14 and 24) show wrinkling of the nuclear membrane. Chromatin is markedly dispersed and large numbers of microspherules are present in a centrifugal distribution and in extranuclear spaces. Nucleoli are dispersed, with remnants of condensed fibrillar nucleolonema (Fig. 14, double arrows) being the only identifiable structures. Higher magnification (Fig. 24) shows microspherules in the nucleoplasm and extranuclear spaces, with some passing through the nuclear membrane (double arrow). These electron-opaque spheres have a prominent halo of fibrils, giving them a “hair-ball” appearance. Long strands of dispersed chromatin, chromatin fibrils, can be seen in the nucleoplasm and extranuclear spaces. These fibrils are thin, frequently have branch points, and have a beaded appearance (single arrow).

Nuclei incubated with heparin for 2 min at 25°C (Figs. 15 and 23) are similar in appearance to the poly X nuclei. Wrinkling of the nuclear membrane, centrifugally distributed microspherules, nuclear dispersion, and lysis of the nuclear membrane are seen. Localized condensation of chromatin can be seen (Fig. 15, arrow; Fig. 23, double arrow). Interchromatin granules are enlarged (Fig. 23, circle) and are localized as prominent islands in the dispersed chromatin. The appearance of the dispersed chromatin strands is similar to that of the poly X-treated nuclei.

When heparin-treated nuclei are incubated for 15 min at 37°C (Figs. 16 and 22), there is an almost total loss of identifiable nuclear structure. The nucleus is wrinkled and lobulated, and lysis of the nuclear membrane is prominent (Fig. 22, single small arrows). Nucleoli have disappeared and the nucleoplasm has been replaced by large numbers of microspherules and highly dispersed chromatin fibrils (Fig. 22, large single arrow). The microspherules again have a fibrillar peripheral halo (Fig. 22, double arrows).
FIGURE 15  Nucleus treated with heparin for 2 min at 35° C. Note marked chromatin dispersion, increased prominence of interchromatin granules, focal chromatin condensations (arrow), and numerous microspherules. Electron micrograph. × 9200.

FIGURE 16  Characteristic morphology of a nucleus treated with heparin for 15 min at 37° C. There is generalized disruption with increased prominence of the microspherules. Electron micrograph. × 9200.

FIGURE 17  Higher resolution electron micrograph of poly A-treated nucleus showing loss of fibrillar component of nucleolus. × 31,000.

FIGURE 18  Nucleolus of poly U-treated nucleus showing slight decrease of fibrillar component (F) and relatively increased prominence of the granular component (G). Electron micrograph × 31,000.
**FIGURE 19** Poly I-treated nucleus with well defined perinucleolar halo (H) and fibrillar material on and within nuclear membrane (arrows). Electron micrograph. × 31,000

**FIGURE 20** Higher resolution of poly G-treated nucleus showing perinucleolar halo (H). Electron micrograph × 27,000.

**FIGURE 21** Poly aspartate-treated nucleus with occasional nuclear membrane breaks (single arrow) and intranuclear microspherules (double arrow). Electron micrograph. × 81,000.

**FIGURE 22** Nucleus treated with heparin for 15 min at 37°C. Note long, branched chromatin fibrils (large arrow) and the prominent microspherules which appear to be aggregates of the chromatin fibrils (double arrow). Numerous membrane breaks are present (single arrows). Electron micrograph × 40,000.
Nucleus treated with heparin for 2 min at 25°C. Note localized ring-shaped nuclear condensation (large arrow), microspherules (double arrow), and membrane breaks (single arrows). The chromatin is dispersed and fibrillar and perichromatin granules are prominent (circle). Electron micrograph. × 53,000.

Poly X-treated nucleus. Note chromatin fibrils within and outside (single arrow) the confines of the nuclear membrane. One of the microspherules appears to be passing through the nuclear membrane (double arrow). Note prominent interchromatin granules. Electron micrograph. × 53,000
When isolated rat liver nuclei are treated with naturally occurring template-activating RNAs such as rat liver ribosomal RNA (9), nuclear ultrastructure again is altered. The changes include the dispersion of perinuclear chromatin, an increase in the number and size of interchromatin granules, and a decrease in the fibrillar component of the nucleolus with an accompanying prominence of the granular component (Brown, Yawn, Arnold, and Coffey, unpublished results).

**DISCUSSION**

The primary goals of the experiments in the present study were: (a) to determine if polyanions which demonstrate a differential specificity in their ability to release template restrictions on liver nuclear DNA for DNA synthesis also produce a differential effect upon nuclear structures, and (b) to quantify dimension alterations in polyanion-treated nuclei and correlate these with DNA synthesis, DNA solubilization, absorbance changes ("optical clearing"), and ultrastructural alterations. The polynucleotides show a wide range of effectiveness with a high degree of specificity (7). In this regard the polypurines are generally much more effective in the release of template restrictions than are the polypyrimidines. Heparin and polyaspartic acid represent two other classes of molecules, and both have been shown to be highly effective in the stimulation of DNA synthesis in isolated hepatic nuclei (6).

For each polyanion examined, a mass ratio of 1 for polyanion:DNA was used and the molecular weight of the polyanion was sufficiently large to produce maximal rates of DNA synthesis (see Table IV).

The prime criterion we have used is the ability of the polyanion to effect the incorporation of labeled dTMP into acid-insoluble DNA. From the data in Table I, it can be seen that heparin produces a remarkable increase (12-fold) in the incorporation of label when compared to the control values. The polynucleotide results confirm the specificity reported earlier (7). Polyaspartic acid is also a highly effective polyanion, producing incorporation levels similar to those of the polypurines.

An interesting point about the distribution of the incorporation values is that four separate levels of activity seem to occur: class I, controls and poly C with very low incorporation; class II, poly A and poly U, which show only slight but significant increase over the control level; class III, poly I, poly Asp, and poly G with about a 5-10-fold increase; and class IV, poly X and heparin which produce an approximate 10-15-fold increase. Examination of the electron micrographs, however, reveals that each polyanion produces its own specific alterations in the appearance of the nuclei. These changes were found to be reproducible in sections obtained from several different preparations and were present in virtually all the nuclei examined in each section. Comparing the polyanions, the ultrastructural alterations in the nuclei follow a spectrum of changes which are associated primarily with the nucleoli, the packing of chromatin, the appearance of electron-opaque microspherules which are fibrillar, and the appearance of defects in the nuclear membrane. If these types of change are considered together with the degree of alteration, then an approximate association of morphology with effectiveness for the release of template restriction can be made. Poly C is ineffective, and the nuclei are indistinguishable from control nuclei. Poly A is slightly effective and produces suggestive nucleolar alterations without an apparent effect on chromatin packing. Poly U, which is more effective than poly A but less effective than the other polypurines, produces chromatin dispersion, condensation of the fibrillar nucleolonema, and small defects in the nuclear membrane, but no microspherules appear. The polypurines I and G and poly Asp produce dispersion of chromatin, and microspherules are seen. Poly I and poly G differ from poly Asp, however, in that they produce a centrifugal distribution of the microspherules and rather marked nucleolar alterations. In addition, the polypurines produce nuclear membrane defects which are much less prominent in poly Asp-treated nuclei. Both poly X and heparin characteristically produce marked chromatin dispersion with the appearance of single strands of chromatin, large numbers of microspherules with a centrifugal distribution, marked or complete nucleolar dispersion, and lysis of nuclear membranes. From these observations, it appears that the fundamental alteration that can be associated with release of template restriction for DNA synthesis is dispersion of chromatin and that increasing degrees of effectiveness are associated with the appearance of electron-opaque microspherules. The polypurines and heparin also appear to have a more marked effect on nucleolar structure than either poly Asp or the polypyrimidines. It should be noted here also that these changes can be seen in nuclei examined by phase microscopy. In the
### Table IV

Properties of Polyanions which Determine Specificity

| Type of polyanion | Activation | No activation |
|-------------------|------------|--------------|
| Carbohydrate      | Heparin    | Hyaluronic acid |
|                   | Dextran sulfate | Chondroitin Sulfate |
|                   | Polyacrylic acid | Polygalacturonic acid |
| Protein           | Polyanaspartic acid | Phosvitin |
|                   | Polyglutamic acid | Casein |
| Ribonucleic acid  | Poly X     | Poly C |
|                   | Poly C     | Poly U |
|                   | Poly I     | Poly I |
|                   | Certain copolymers (purine) | Certain copolymers (pyrimidine or mixed) |
|                   | Ribosomal RNA | Transfer RNA |
|                   | Bacteriophage RNA | Whole yeast RNA |
| Molecular weight  | Activation | No activation |
| Protein           | Polyanaspartic acid, mol wt >20,000 | Polyanaspartic acid, mol wt = 5600 |
|                   | Polyglutamic acid, mol wt >20,000 | Polyglutamic acid, mol wt = 5600 |
| Ribonucleic acid  | Poly I     | Inosinic acid oligomers, mol wt = 600-1900 |
|                   | S20,w > 4  | |
| Ratio of polyanion to DNA | Activation | Inhibition |
| Carbohydrate      | Heparin ≤ 1:1 | Heparin ≥ 1:1 |
|                   | Dextran sulfate ≤0.2:1 | Dextran sulfate ≥0.2:1 |
| Ribonucleic acid  | Poly G ≤ 1:5:1 | Poly G ≥ 1:5:1 |
| Secondary structure | Activation | No activation |
|                   | Poly I     | Poly I |
|                   | Poly (I, G) | Poly (I, U) |
|                   | Poly G     | Poly methyl-I |

Interactions of polyanions with histone–DNA complex

The template activity of purified DNA can be inhibited by purified histones in vitro, and this complex can be reactivated by polyanions with the same specificity as stated above. In contrast, polyanions show no specificity in their ability to interact with histones alone.

The data for the solubilization of DNA can be correlated qualitatively with the release of template restriction as measured by DNA synthesis, however, this is not a linear relation. For example, the amounts of non-sedimentable DNA obtained after heparin or poly G treatment do not correlate with the template activity of the DNA.
The packing of nuclear material and its swelling in response to various polyanions were examined. The volume of a nucleus can be calculated using the formula $V = \frac{4}{3} \pi r^3$, where $V$ is the volume and $r$ is the radius of the nucleus. The swelling of the polyanion. The basis for the volume change is unknown, however. One possible explanation is that the swelling is a result of the dispersion and changes in packing conformation of the chromatin under the influence of polyanions in a semi-enclosed compartment.

The data on polynucleotide binding to the isolated nuclei indicate that the polyanions which are highly effective in removing template restrictions for DNA synthesis (poly G, poly I) are also bound quite efficiently. Poly U, poly A, and poly C, which are nearly or completely ineffective for stimulation, bind much less efficiently. Comparisons between the binding figures for the five polynucleotides tested and their efficiency in removing DNA template restrictions show that a linear correlation cannot be drawn; however, a qualitative factor does exist since only those which bind strongly are capable of activating nuclei.

Preliminary data on the localization and distribution of poly 1-14C in nuclei indicate that a small fraction may be located with the nuclear soluble proteins or loosely bound to the nuclear membrane. However, the majority of the poly I cannot be recovered from nuclei by procedures designed to extract soluble protein, histones, membrane proteins, or DNA. The majority of the poly I is recovered in the acidic protein fraction (0.1 M NaOH) and in the residual material remaining after the various extractions. The significance of these data is not yet clear, however, recent evidence suggests that both nuclear acidic proteins (21, 22) and nuclear membrane constituents (23) may play a critical role in the control of nuclear DNA template activity and the process of DNA synthesis. Further experiments are in progress to try to better define the localization and distribution of the binding sites for polyanions in nuclei. Also, it is not clear how the interaction of the polyanions might change the standard extraction of nuclear proteins.

The ultrastructural data we have obtained in this study are compatible with the hypothesis that polyanions may stimulate template activity for DNA synthesis by dissociating histones so that DNA is uncovered for replication (2, 6, 11, and 24). In fact, recent in vitro studies (11) show that the template properties of purified DNA can be inhibited by purified histones and then reactivated specifically by polyanions as used here. In this model a direct correlation exists between the ability of the various polyanions to activate nuclei or chromatin and to release the histone inhibition of purified DNA.
sibly result in conformational alteration of the structure of interphase chromatin so that dispersion would be expected to occur. In this regard it should be noted that DuPraw and Bahr (25) and Ris (26) have shown that interphase chromatin is largely in the form of a 200-250 A thick fiber. DuPraw and Bahr interpret this structure as a supercoiled supercoil which is maintained by histones (see also Zubay and Doty, 27). Ris (26) interprets the 250 A fiber as a 100 A fiber folded back on itself. Georgiev et al. (28) have suggested that the structural folding model of Ris may be due to interfiber linkages between histones so that the histones function not only as repressors of gene function, but as structural protein as well. Treatment of 250 A thick chromatin with 0.6 M salt which partially removes histones results in chromatin fibers of 100 A diameter and increased template activity for RNA synthesis (28). The nuclear ultrastructure which is observed with effective release of template restrictions suggests that such a process may be occurring in the polyanion-activated nuclei where individual thin chromatin fibers can be easily seen. The morphological changes also suggest that chromatin dispersion due to loss of histones as structural protein may play a significant role in nuclear volume change and possibly in absorbance changes.

The mechanisms which result in DNA template activation and changes in nuclear morphology are not completely understood. This is best exemplified by the fact that all of the polyanions were able to interact with and precipitate histones, yet only certain specific polyanions are able to activate nuclei, chromatin, or histone-inhibited purified DNA (11). Similarly, the basis for specificity and relative effectiveness of various polyanions is not known. One possibility, however, is that this specificity is a function of the molecular size and the secondary structure of the polyanion. For example, polyaspartic acid of molecular weight 5600 is ineffective whereas larger molecules of polyaspartic acid (16,800-35,000) are good template activators (6). Similarly, the ability of poly I to activate template DNA is dependent on its size (9-11).

Among the polyribonucleotides there is a close correspondence between the amount and stability of their secondary structure (poly G > poly I > poly U > poly A > poly C) and their relative template activating ability (8-29). Under the conditions of incubation, poly X, poly G, and poly I exist as multistranded complexes with a considerably stable helical structure, whereas poly A, poly G, and poly U are single-stranded molecules whose structure is essentially that of a random coil (20).

Analysis of the basis for the types of morphological alterations which we have observed in these experiments is difficult since the mechanisms by which polyanions remove the template restrictions for DNA synthesis are not firmly established. The microspherules which are seen in the more potent activating systems are probably aggregates of chromatin fibrils as suggested by the similarity of appearance of the fibrillar mass to the strands of dispersed chromatin. In the heparin-activated nuclei, the difference in the appearance of the nuclear chromatin at 2 min and at 15 min of incubation further suggests that the alterations in chromatin packing follow a biphasic development; dispersion of chromatin to the branched fibrillar form is followed by the formation of the spherical mass. It seems unlikely that the microspherules arise from the nucleus since no structure of this type was seen in nuclei of those nuclei in which the nucleus could be recognized. The type of interfibrillar bonding that would produce secondary chromatin condensation is unknown; however, recent findings concerning the amount and distribution of highly repetitious, rapidly renaturable DNA among chromosomes led us to the possibility that such chromatin condensation might involve, in part, heterochromatin or satellite DNA (31, 32, 33). We are now investigating this possibility.

Nucleolar changes seen in these nuclei do not correspond to the types of alteration which have been reported after the interaction of other chemical agents with nuclei (see review by Simard (34)). Nucleolar segregation, degranulation, and nucleolar microspherule formation were not seen in some aspects the nuclei have the appearance of the hypertrophied nuclei seen after thioacetamide administration. Nucleoli seen in thioacetamide-treated nuclei, however, have equal amounts of fibrillar and granular components, whereas the nuclei in the polyanion-activated nuclei show a less and condensation of the fibrillar component. Since the fibrillar component is the structural entity associated with the synthesis of 45 S RNA (35) and since the granular component is thought to be a transient structural complex without any known synthetic function (34), the changes seen in the polyanion-treated nuclei may be a reflection of an effect on the kinetics of synthesis of ribosomal precursor RNA. Such an interpretation is speculative; however, this possibility should be considered.
Changes in the nuclear membrane might represent either an alteration in nuclear pore structure or lysis of areas of the nuclear membrane at sites other than the pore annulus. In the systems where the number of membrane defects were small, normal nuclear pores were not seen. This suggests that the polyions in these cases may have interacted directly with some component of the pore structure. In cases such as poly X or heparin, the large number of defects, the wrinkling of the membrane, and the total collapse of structure indicate that other factors may be involved. The appearance of these nuclei suggests indiscriminate lysis of the nuclear membrane or loss of transport and diffusion controls, with rupture occurring subsequent to an increased intranuclear osmotic pressure and expanding chromatin volume.

Consideration of the effects of the polyions on DNA template activity and nuclear ultrastructure indicates that the release of template restrictions is accompanied by marked structural reorganization within the nucleus and that the type and/or degree of these changes vary for each of several polyions tested.

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