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Purification of Nucleoprotein Particles by Elution Preparative Gel Electrophoresis

Edward C. Uberbacher
Gerard J. Bunick

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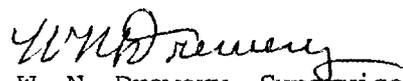
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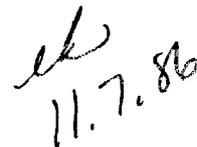
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PURIFICATION OF NUCLEOPROTEIN PARTICLES
BY ELUTION PREPARATIVE GEL ELECTROPHORESIS

Edward C. Uberbacher and Gerard J. Bunick

The University of Tennessee - Oak Ridge Graduate School of Biomedical
Sciences and Biology Division, Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37831

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ABSTRACT

A preparative elution gel electrophoresis technique is described for ultrapurification of nucleosomes, dimers, and other nucleoprotein macromolecules. The apparatus can accommodate 10–20 mg of sample and multiple runs can be obtained from one polyacrylamide gel. The eluate is recovered using an ordinary fraction collector and the sample distribution is measured by an in-line U.V. monitor. The apparatus has been used to further purify nucleosomes prepared by several different methods. Nucleosome core particles containing precise DNA lengths (146 ± 0.5 bp) have been obtained by this method for crystallization. Nucleosomes containing specific longer DNA lengths, such as 168 bp and 175 bp, or specific labels have also been obtained for biophysical studies. The ease of use and versatility of the system makes it an attractive method for obtaining the homogeneous samples required for reproducible biophysical studies of the nucleoprotein constituents of chromatin as well as other similar macromolecules.

INTRODUCTION

A common problem faced by investigators conducting biophysical studies on nucleosomes and related chromosomal particles is the difficulty in obtaining homogenous preparations of particles of a specific type and with tightly controlled DNA length. Most such particles are prepared from soluble or stripped chromatin or intact nuclei by nuclease digestion to trim the DNA to length. Unfortunately, in this type of procedure, the distribution of DNA lengths resulting is relatively wide, especially for particles other than 146 bp core nucleosomes where no natural stopping places exist for the nuclease. Even in core nucleosomes the distribution is no better than 146 ± 3 b.p. of DNA. Given a mixture of particles with varying DNA lengths and also differing protein composition, standard column chromatographic methods and sucrose gradient centrifugation are often unable to provide the degree of purification necessary to obtain reproducible results from biophysical studies.

As a result of the basic limitations of preparing these chromosomal particles, some uncertainty has arisen in the literature because of differing results from investigators using heterogeneous material for biophysical studies. Some unresolved areas include: (a) The presence or absence of a premelt in the thermal melting of nucleosome core particles [Weischet et al. (1), Simpson (2), Cowman et al. (3), Paton et al. (4)]; (b) The presence or absence of an ionic strength unfolding transition in 146 and 175 bp nucleosomes between 3 and 7 mM [Gordon et al. (5), Schlessinger et al. (6), Uberbacher et al. (7)]; (c) The structural properties of 146 and 175 bp nucleosomes below 1.5 mM ionic strength [Schlessinger et al. (6), Uberbacher et al. (7)]. A very important example

of the need for tight control of DNA length is the effect variations in nucleosome DNA length have on the crystallization properties of core particles. In our own crystallization work, we have found that in two crystal forms, nucleosome cores with DNA approximately 2 base pairs longer or shorter than optimum will not crystallize. A similar phenomenon has been reported by Finch et al. (8) where their nucleosome core particles crystallize with a b-axis dimension which varies depending on the precise DNA length present on the cores.

Reconstituted nucleosome particles of various types are also used for many biophysical studies. These include core particles with synthetic DNA such as poly(dA-dT)•poly(dA-dT) and also particles with modified or labelled histones, such as fluorescent labelled cysteines of H3. In reconstitution procedures a considerable fraction of the resulting particles may improperly reassemble, or be improperly labelled. It is often difficult to subfractionate these particles, since the molecular weight and physical properties are similar for both the improperly and correctly labelled or reconstituted products.

These examples demonstrate that there is certainly a need for improved methods of preparing core nucleosomes. Furthermore, as biophysical studies in the chromatin field seek to become more physiologically relevant, it would be desirable to work with homogeneous, well defined systems of larger sized particles, such as chromatosomes, dimers, and oligomers. It would also be advantageous to be able to purify material of these types with specific DNA or histone modifications, deficiencies in histones (such as H2A and H2B), or the presence of HMG proteins. It is only through the use of homogeneous material that it will be possible to evaluate the

effects of such modifications on the functional state of regions in the chromatin.

Preparative gel electrophoresis is not a new technique, but it seems to have been largely overlooked by investigators in the nucleosome and chromatin area, perhaps partly because the apparatus appears to be complicated. In actuality, the technique is extremely useful and versatile, and it is well worth the time and effort to make the apparatus work. The following sections describe the methods and procedures used to prepare several different types of material, along with specific examples illustrating the degree of separation that can be expected using the technique.

MATERIALS AND METHODS

The apparatus used was Buchler Model 200 preparative polyacrylamide gel electrophoresis instrument (Poly-Prep) modified for improved support of the gel. The modification consisted of increasing the internal space for the elution chamber from 1 mm (usual value) to approximately 3 mm and adding a uniform layer of 3 mm diameter siliconized glass beads over the membrane which forms the floor of the elution chamber. The instructions supplied by Buchler Instruments, for assembly and operation of the Poly-Prep were otherwise followed.

The gel electrophoresis system for the separation of nucleoprotein particles has been described in the literature (Sollner-Webb & Felsenfeld (9)). The polyacrylamide concentration is 5% and the buffer system is 0.1X Tris/Borate/EDTA which is diluted from a 5X stock solution containing 0.425 M Tris, (Sigma), 0.012 M Na₂EDTA (Mallinckrodt), and 0.445 M boric

acid (Mallinckrodt), pH 8.3. The acrylamide stock solution is 29:1 acrylamide: N,N^1 -Methylene-bis-acrylamide (Biorad Laboratories - electrophoresis purity). A 10% ammonium persulfate (Biorad) stock solution was prepared and stored frozen between use. For the 12 cm gels usually poured, 2.4 ml ammonium persulfate, 6.0 ml 5X TBE, and 50 ml acrylamide stock were diluted to 300 ml and degassed for 15 minutes. Polymerization was initiated by adding 100 μ l TEMED (N,N,N^1,N^1 -Tetramethylethylenediamine, Biorad). The gel solution was poured using a funnel attached to a short length of tygon tubing. The end of the tygon tubing was bevel cut and the tubing was placed in contact with the glass wall of the apparatus to minimize air bubble formation. The gel was immediately overlayersed 3-4 mm with 0.1X TBE using a peristaltic pump. The most uniform gel surface was obtained when the tubing contacted the side of the apparatus several millimeters above the gel surface being overlayersed. Cooling water (4°) was turned on as soon as the start of polymerization was observed. Polymerization was essentially complete after one hour. After removing the polyethylene gel cap, the lower surface of the gel was carefully checked for smoothness and any gel lodged within the elution capillary or at the perspex edge was removed. The modified elution chamber was filled with elution buffer to just cover the glass beads before the gel column assembly was attached. This operation was performed carefully so as not to disturb the glass beads in the elution chamber.

The buffer system was based on TBE. The upper electrode buffer solution was 0.1X TBE and both lower electrode buffer and the elution chamber buffer were 0.5X TBE. These buffers were made in 4 l quantities by dilution from a 5X TBE stock. The pH of these buffers must be maintained

at 8.30 for correct operation of the system. Dilution from a 10X TBE stock shifts the pH considerably, whereas the use of a 5X stock usually was satisfactory without further pH adjustment. The upper and lower electrode buffers were pumped by a peristaltic pump at approximately 1 ml/min. Effluents from these reservoirs were collected in separate containers. The elution buffer was drawn from the reservoir through the elution chamber then up the central elution capillary by a peristaltic pump connected between the central elution capillary output fitting and the fraction collector. An elution buffer flow rate of 1 ml/min was maintained. Newly poured gels were pre-electrophoresed to constant current (23mA) before samples were run. Water jacket temperatures were kept at 2-7°C. The lower electrode was positive. As pre-electrophoresis neared completion a sharp peak attributable to unreacted acrylamide products was observed by U.V. monitor.

Nucleosome samples were prepared in 0.1 × TBE, pH 8.3. The samples had volumes ranging from 2-3.5 mls and contained 10-20 mg of material. The density of the sample solution was increased by the addition of 1-2 mg crystalline sucrose (Mallinckrodt, analytical reagent grade). A small amount of bromophenol blue dye was also added. The standard teflon layering tube was used to underlayer the sample onto the gel surface in the upper buffer chamber. A 5 ml disposable syringe was used to apply the sample through the layering tube. The peristaltic pumps may be left operating during the sample loading. The Poly-Prep was run at constant power of 5 watts (200 V, 22-23mA). Fractions of 8 ml were collected using a LKB 2070 Ultrarac II fraction collector equipped with a LKB 8300 Uvicord II U.V. detector and an LKB 2210 single channel recorder.

Electrophoresis time of 10 hours was required for elution of monomer nucleosomes. As the sample and dye separated a clear, sharp refractive index boundary was almost always observed at the position in the gel where the nucleosomes migrated.

RESULTS

Ultrapurification of Nucleosome Core Particles

In order to obtain x-ray quality crystals of chicken erythrocyte nucleosome core particles, the cores obtained by micrococcal nuclease digestion of stripped soluble chromatin [Lutter (10); Uberbacher *et al.* (7)] were further purified by preparative gel electrophoresis. Before electrophoresis, the nucleosomes showed some heterogeneity in DNA length (146 ± 3 bp). Small quantities of subnucleosomal sized fragments and dimers were also present. Figure 1 shows the elution profile from preparative electrophoresis of a relatively homogeneous batch of nucleosome cores. The sharpness of the rising edge of the main peak (fractions 22-23) provides an indication of the resolution of the gel system. The trailing edge tails considerably, the width being primarily the result of DNA length heterogeneity. Of the nucleosome cores recovered (~90%), material from fractions 25-28 crystallizes well while material from fractions 22-24 and 29-33 does not. This is a typical result which demonstrates the excellent subfractionation obtainable from preparative (10-20 mg) electrophoresis on even the best nucleosome cores purified by standard methods. DNA gels from these cores are presented in reference 7. In addition to subfractionating the cores according to DNA length, the procedure removes subnucleosomal

F-1

fragments (3 small peaks before main peak) and dimers (peak near fraction 70).

Purification of KCl soluble core particles

Many investigators still prepare nucleosomes from micrococcal digests of intact nuclei. These nucleosomes usually contain some H1 histone and possess a rather heterogeneous distribution of DNA lengths. Dialysis against 100 mM KCl and 12 mM MgCl₂ [Uberbacher et al., (7)] precipitates the residual H1, contaminating dimers, and some nucleosomes containing long DNA. The resulting nucleosomes, although considered homogeneous by many investigators, are in fact quite heterogeneous; containing considerable amounts of subnucleosomes and nucleosomes with DNA ranging up to about 175 bp in size. Figure 2 shows the elution profile of KCl soluble nucleosomes subfractionated by preparative electrophoresis. The main peak (fractions 57-66) is considerably broader than the corresponding peak for material prepared from stripped soluble chromatin (Figure 1). It is evident, however, that by properly combining fractions, cores with reasonably homogeneous DNA length can be obtained from KCl soluble nucleosomes. The long trailing shoulder (Figure 2) represents nucleosomes with DNA lengths ranging between 150 and 175 bp with 165-168 bp cores at the point of maximum height. Nucleoprotein of subnucleosomal size is evident in fractions 50-56.

F-2

Purification of core particles containing 175 bp DNA

Nucleosome core particles with 175 bp DNA are prepared from a micrococcal nuclease digestion of soluble chromatin [Crothers et al. (11)]. The presence of H1 (H5) and the absence of a specific stopping point for

F-3 the nuclease leads to an extremely heterogeneous population of nucleosomes. Nucleoprotein fragments resulting from such a digestion include subnucleosomes, 146 bp cores, 168 bp cores, 175 bp cores, core particles with DNA as long as 220 bp, dimers, and other oligomers. Column chromatography (Sephacryl S-300 in this laboratory) removes most of these species, leaving only some subnucleosomes, 146 bp core nucleosomes, some 168 bp nucleosomes, 175 bp nucleosomes, and some nucleosomes with DNA up to about 220 bp. The elution profile from preparative gel electrophoresis of this type of material is shown in Figure 3. The two most prominent peaks represent 146 bp and 175 bp core particles, respectively, with 168 bp particles present on the leading side of the 175 bp peak. The long tail following the 175 bp peak (fractions 31-40) represents core particles with DNA from about 180 to 220 bp. The three small peaks ahead of the 146 bp peak are specific subnucleosomal species. Fractions 26 to 30 from the second main peak can be pooled to yield relatively pure 175 bp nucleosomes. See reference 7 for DNA and particle gels of this material.

N-(3-pyrene) maleimide - Labelled Nucleosomes

For biophysical studies it is often useful to reconstitute nucleosome core particles from labelled histones and native DNA, or prepare nucleosomes with synthetic polynucleotides, such as poly(dA-dT)·poly(dA-dT), or a cloned DNA fragment. Since reconstitution methods usually yield a significant fraction of improperly reassembled molecules or only partially labelled molecules, it is necessary to be able to isolate the properly reconstituted material.

F-4 Figure 4 shows the results of purifying nucleosome cores with N-(3-pyrene)maleimide (NPM) labelled H3 cysteines on the preparative gel

apparatus. These particles have been unfolded and labelled in 1.4 M KCl and then refolded by stepwise salt dialysis (12). It is clear from Figure 4 that the population of crude reconstituted nucleosomes was quite heterogeneous. The majority of material following the main peak consists of nucleosomes with DNA greater than 146 bp or with DNA which has not reassociated properly to the histone core. Also, these species show little excimer fluorescence. Low levels of excimer fluorescence are also observed for the material eluted prior to the main peak. These nucleoprotein fragments are subnucleosomal in size and probably are the result of improper reassembly of histones and/or DNA. The main peak (fractions 38-43) consists of nucleosomes with nominal DNA length and the highest level of excimer fluorescence.

DISCUSSION

Preparative gel electrophoresis provides an efficient and versatile method of preparing very pure samples of nucleosomes and related species. We have obtained excellent subfractionation of 10-20 mg samples of chromosomal nucleoproteins. This is in contrast to use of the Poly-Prep for protein isolation where apparently maximum loading is approximately 2 mg [Chrambach and Rodbard (13)]. We found that a particle gel is good for several runs of material, whereas protein gels with stacking regions can only be used once. These points suggest that the preparative gel system is much more useful for nucleoproteins than proteins.

Before the system worked as demonstrated, a number of problems had to be solved. At first, the most annoying problem was a tendency of the gel to slip downward and block the elution chamber. Polyacryamide gels

crosslinked with bis-acrylamide also swell with time. This swelling process contributes to eventual blockage of the elution chamber. The apparatus used is supplied with circular nylon screens which can be attached under the gel using adhesive strips. The purpose of the screen is to support the relatively soft gels above the elution chamber.

Unfortunately, this remedy was not totally successful. The best approach found was to fill the elution chamber with 3mm diameter siliconized glass beads (after adjusting the chamber to the correct height). The beads not only successfully support the gel, but serve to minimize the volume of the elution chamber. The resolution was not adversely affected by this modification, but in fact seemed to be somewhat enhanced.

As mentioned, the pH of the gel and buffer system is extremely critical. Diluting 10 × TBE stock solution to 0.1× or 0.5 × TBE results in a significant pH change. For this reason, 10 × TBE stock is not recommended. Even when buffers are prepared from a 5 × TBE stock, it is recommended that their pH be readjusted before use. If the pH of the system is incorrect, the nucleoproteins being separated will not resolve and a very broad peak will result.

Another difficulty encountered is that after a certain amount of time the gel shows signs of aging. Warning symptoms of gel aging include some loss of resolution and a slight separation of the gel from the glass, especially at the gel's upper surface. Sometimes the aging of the gel manifests itself catastrophically; the gel sags inward and pulls away from the glass walls, resulting in channel formation. Adhesion of the gel to the glass can be improved, thus reducing the risk of catastrophic failure, by treating the glass with methanolic KOH before pouring the gel.

Alternatively, an adhesion promoter such as Silane A 174 (Pharmacia Fine Chemicals) can be used. Usually it is possible to run 3-5 batches of material through a gel before replacement is necessary.

One must also consider that during the life of the gel, small amounts of free acrylamide and polyacrylamide are constantly leaching into samples migrating through the gel. This type of contamination is generally considered to be non-dialyzable. As far as we can ascertain, the level of this contamination seems to be quite small since it has not interfered with the growth of good nucleosome crystals; crystallization should be a very sensitive assay for contamination of this kind. The level of acrylamide contamination is certainly much lower using the Poly-Prep than if gel crushing is used to recover material. Depending on the application, it may be desirable to remove acrylamide contaminants by gel chromatography.

The particle gel formulation (5% acrylamide) is most useful for material sized between subnucleosomal fragments and 175 bp nucleosomes. Preparative gel electrophoresis used to prepare nucleosomal dimers, oligomers, short chromatin, or the DNA from such species must use an alternative gel formulation, since it is difficult to reduce the acrylamide concentration below approximately 3% and still obtain a gel which retains the mechanical strength necessary to support itself. The likely approach is to use polyacrylamide - agarose or agarose formulations. Composite gels with 0.5% agarose and 1.5% - 3% polyacrylamide have good mechanical strength and pore sizes useful for separating dimers and oligomers. The TBE (pH 8.3) buffer system can still be used in these formulations.

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FIGURE LEGENDS

FIG. 1. Preparative gel elution profile from a relatively homogeneous batch of nucleosome cores. The width of the trailing edge is primarily the result of DNA length heterogeneity. Only nucleosome cores from fractions 25-28 are suitable for crystallization.

FIG. 2. Preparative gel elution profile of KCl soluble nucleosomes. Fractions 57-66 represent the main monomer peak, while the trailing shoulder represents nucleosomes with longer DNA (from 150 to 175 bp). Fractions 50-56 are subnucleosomal.

FIG. 3. Preparative gel elution profile of a crude mixture of 175 bp nucleosomes. The two most prominent peaks represent 146 bp and 175 bp core particles, respectively. Fractions 31-40 represent core particles with DNA from about 180 to 220 bp.

FIG. 4. Preparative gel elution profile from a crude mixture of NPM-labelled nucleosome core particles (—). Normalized excimer fluorescence from fractions in the elution profile (---). The main peak (fractions 38-43) consists of nucleosomes with nominal DNA length and maximal excimer fluorescence.

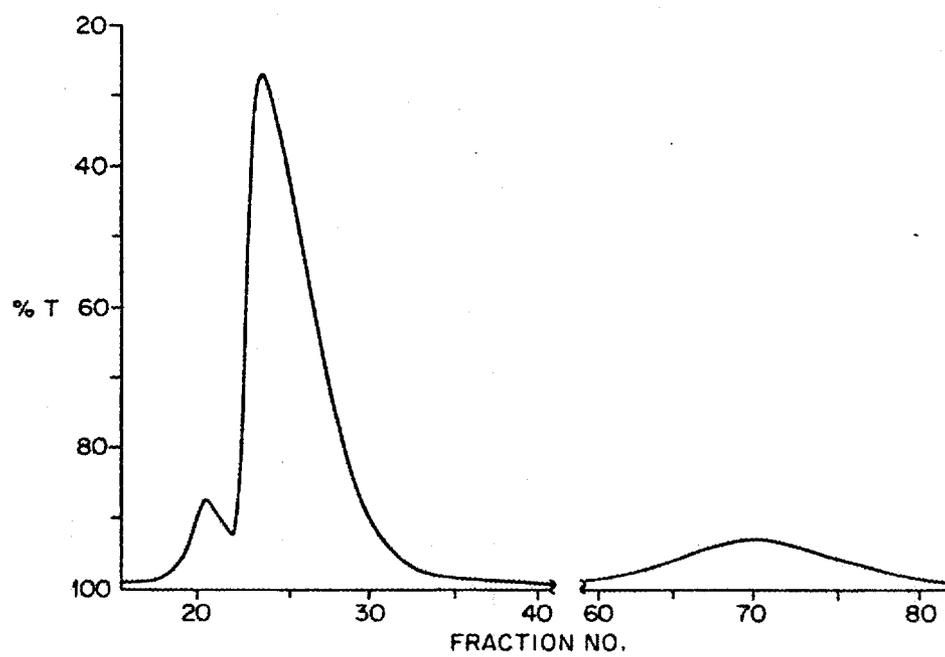


Figure 1

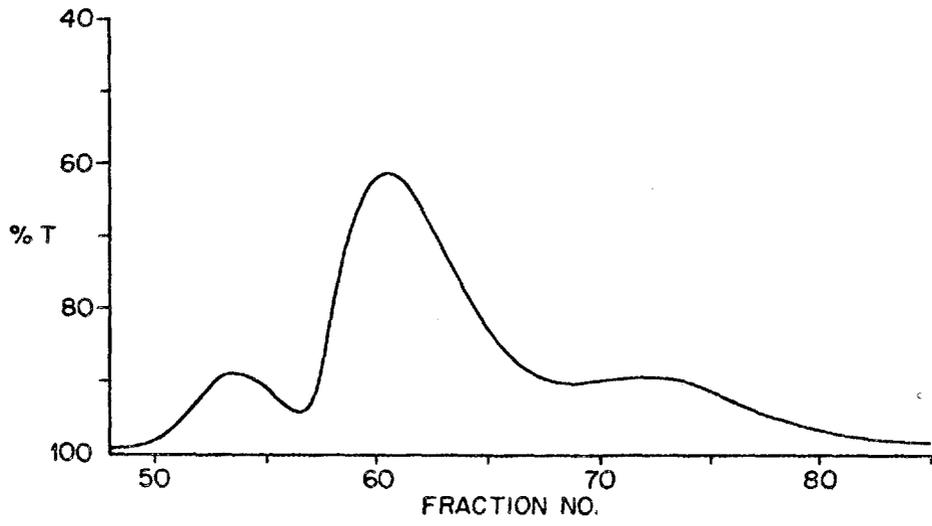


Figure 2

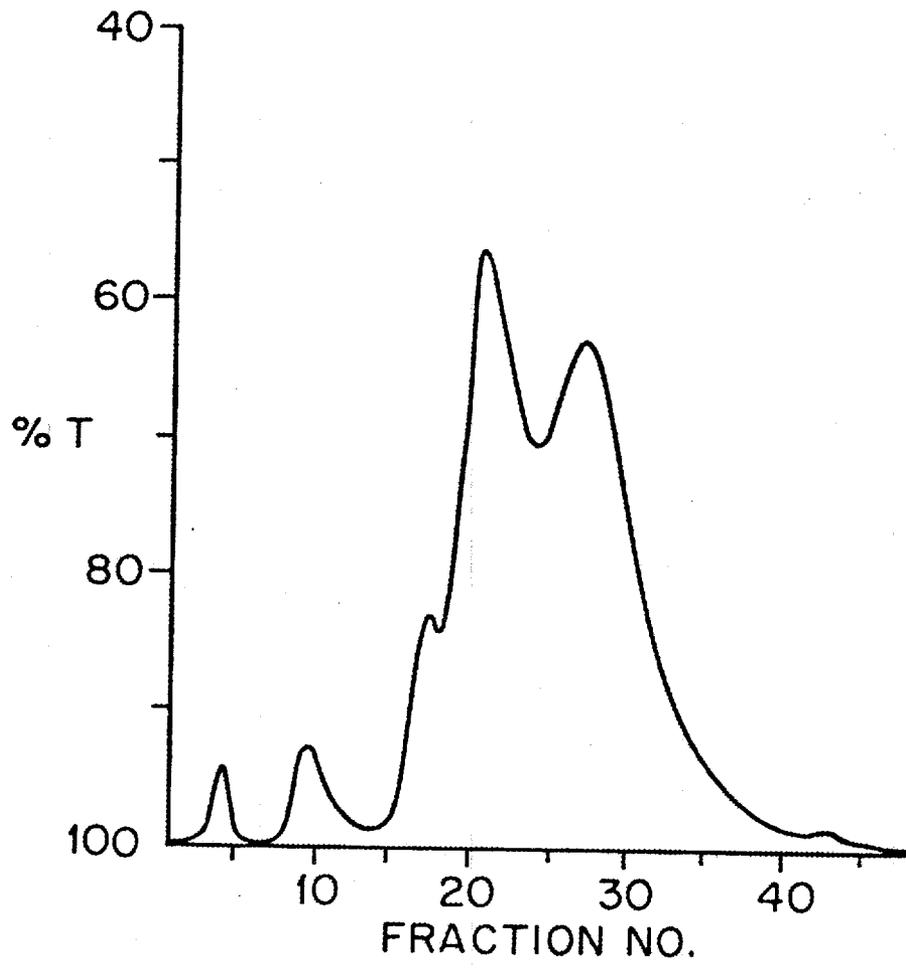


Figure 3

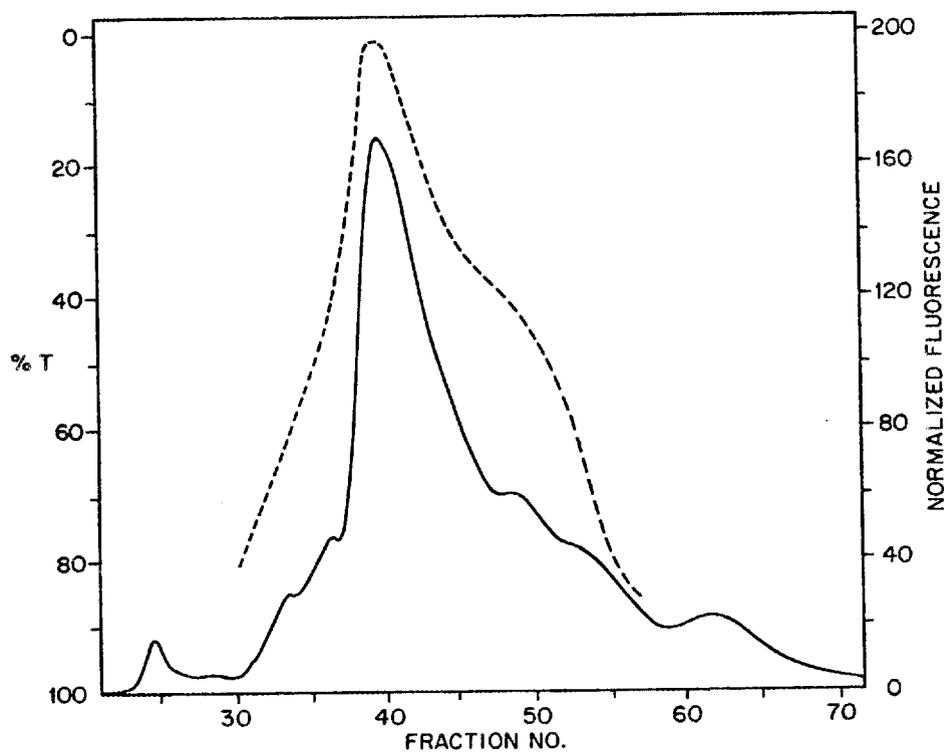


Figure 4

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