A DNA-BINDING PROTEIN FROM HELA CELLS

WHICH BINDS PREFERENTIALLY TO

DNA DAMAGED WITH ULTRAVIOLET LIGHT

OR N-ACETOXY-N-ACETYL-2-AMINOFLUORENE

by

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Abstract

A DNA-binding protein, PIII, was partially purified from extracts of Hela cells by high-speed centrifugation, and chromatography on DEAE-cellulose, phosphocellulose and UV-irradiated DNA-cellulose columns. It eluted from the phosphocellulose column with 0.375 M potassium phosphate and from the UV-irradiated DNA-cellulose column between 0.5 M and 1 M NaCl. PIII binds preferentially to supercoiled PM2 DNA treated with ultraviolet light (UV-DNA) or N-acetoxy-N-acetyl-2-aminofluorene (AAAF-DNA) as compared to native supercoiled PM2 DNA. The binding is noncooperative.

A filter-binding assay utilizing GF/C glass fibre filters was used to detect PIII during the purification steps. Characterisation of PIII-DNA complex by glycerol gradient centrifugation indicates that the retention of the complex by the filters does not involve DNA precipitation, aggregration, or a conformational change of the DNA which results in a detectable change in the sedimentation coefficient of the DNA. The binding of PIII to DNA is reversible.

PIII is a protein as indicated by its sensitivity to proteinase K. The sedimentation coefficient of the protein estimated by glycerol gradient centrifugation is 2.0–2.5 S corresponding to a molecular weight of about 20–25,000 if the protein is spherical.

The binding between UV- or AAAF-DNA and PIII is optimal at around 100–200 mM NaCl and is relatively independent of temperature and pH. $MgCl_2$ and $MnCl_2$ at concentrations between 1 mM and 7 mM do not markedly affect the binding but it is inhibited by sucrose, ATP and caffeine.

Competition experiments indicate that PIII is a single protein which binds to AAAF-induced and UV-induced DNA binding sites with equal affinity. PIII also binds preferentially to supercoiled PM2 DNA treated with N-methyl-N'-nitro-nitrosoguanidine but has little or no preferential binding activity for methyl methanesulphonate-treated or depurinated PM2 DNA. It also possesses some binding activity to unit length, single-stranded PM2 DNA. Nicked or linear forms of PM2 DNA (damaged or untreated) are not efficient substrates for PIII, indicating a requirement of DNA supercoiling for the binding activity of PIII. The possible nature of the DNA-binding sites for PIII is discussed.

The biological significance of PIII remains to be determined. It does not possess significant glycosylase, endonuclease or exonuclease activities. The binding of PIII does not alter the susceptibility of UV-irradiated supercoiled PM2 DNA to the single-stranded endonuclease of *Neurospora crassa*. A DNA-binding protein similar to PIII was found to be present in extracts of a normal human fibroblast cell line and two xeroderma pigmentosum fibroblast cell lines (XP-cell lines). The concentration of this protein in the extracts of these cell lines was comparable to that of PIII in Hela cells. The two XP-cell lines were XP5EG and XP2NE. They belong to the A and D complementation groups of xeroderma pigmentosum, respectively. The cell line XP5EG appeared to be deficient in another DNA-binding protein, which eluted from the phosphocellulose column with 180-250 mM potassium phosphate.

The dissociation equilibrium constant for the binding reaction of

PIII to the UV- or AAAF-induced binding sites on DNA is estimated to be 7×10^{-11} M. The association rate constant and the dissociation rate constant are 4×10^{6} $M^{-1}sec^{-1}$ and $3 \times 10^{-4} sec^{-1}$, respectively. There are at least 10^{5} molecules of PIII per Hela cell.

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Abbreviations

AAAF	N-acetoxy-N-acetyl-2-aminofluorene
AAAF-DNA	PM2 DNA treated with AAAF
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
CLL	chronic lymphocytic leukemia
DEAE-cellulose	O-(diethylaminoethyl) cellulose
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	disodium ethylene diaminetetraacetate
MMS	methylmethanesulphonate
MMS-DNA	PM2 DNA treated with MMS
MNNG	N-methyl-N'-nitro-nitrosoguanidine
MNNG-DNA	PM2 DNA treated with MNNG
MNUA	N-methyl-N-nitrosourea
rpm	revolution per min
S	sedimentation coefficient
Tris	tris-(hydroxymethyl)-aminomethane
u-DNA	untreated native PM2 DNA
UV	ultraviolet light
UV-DNA	PM2 DNA UV-irradiated
UV-endonuclease	endonuclease which cleaves DNA adjacent to pyrimidine dimers
XP	xeroderma pigmentosum

<u>Introduction</u>

The structural and functional integrity of the DNA genome in a cell is sometimes altered by DNA damage which can arise either spontaneously (1) or by the action of chemical and physical agents (2-4). If the lesions are not corrected by DNA repair processes, normal DNA metabolism and gene regulation will be affected.

Several types of DNA repair processes have been proposed and reviewed (5, 6). Information concerning these processes has largely been obtained from studies with procaryotes. However, human cells probably also repair DNA damage by similar processes.

Several repair-deficient human genetic diseases have been identified (7, 8). In some of these diseases, the patients are cancer prone (9). Among them, xeroderma pigmentosum is probably the best characterised (10, 11). Patients with xeroderma pigmentosum (XP) are very sensitive to sunlight and all of them have the tendency to develop skin tumors.

Cell lines have been established from the skin fibroblasts of XP patients. Except for a group of XP cells called XP variant, the fibroblasts of these XP cell lines have been shown to be defective in the excision repair of UV-induced thymidine dimers. In normal cells, excision repair of the pyrimidine dimer is believed to be initiated by an incision on the DNA in the vicinity of a dimer. The incision is made either by a specific endonuclease activity (UV-endonuclease activity) or via a combination of a glycosylase activity and an apyrimidinic endonuclease activity (12). The DNA damage and adjacent nucleotides are then removed by an exonuclease. The gap thus created is then filled with a DNA polymerase activity and finally the

repair patch is joined to the remaining DNA by a ligase (5, 6). The excision repair deficiency in the XP cells seems to lie in the incision step of the process (10, 11, 13). Defects of XP cells in other DNA repair processes have also been reported (14-17). Cell hybridization studies indicate that the excision repair defect in XP cell lines falls into at least seven complementation groups (10). This finding suggests that the incision step of the excision repair pathway for pyrimidine dimers is a complex process.

Furthermore, those XP cells which repair the pyrimidine dimer deficiently are also defective in the repair of bulky DNA lesions caused by other "UV-like" DNA damaging agents such as AAAF and bromobenzanthracene (8, 11). These XP cells however can repair proficiently DNA lesions incurred by other damaging agents such as MMS and X-rays (8, 11). The DNA damaging agents of the latter group each elicits a short repair patch size of about 3-4 nucleotides in a cell; whereas with the former group of agents, the repair patch size may be as long as 120 nucleotides (6, 18). It is possible that the excision repair of DNA lesions introduced by UV and by the "UV-like" DNA damaging agents may share the same repair enzymes or some regulatory proteins.

Other studies have also suggested the existence of regulatory molecules in chromatin which might determine the removal of pyrimidine dimers from DNA. It was found that extracts of XP cells from the complementation groups A and D and the XP variant were capable of excising thymidine dimers from purified UV-irradiated DNA. In contrast, extracts from cells of the XP group A and the XP variant did not excise dimers from their endogenous chromatins under conditions where extracts

of normal cells and XP group D cells did (19, 20). However, these results contradict the repair capacity of intact cells, where the XP variant cells but not the XP group D cells exhibit normal excision repair (21). Nevertheless, it was suggested that the XP cells are not defective in the UV-endonuclease activity, which must act before the dimers are excised. Rather, there may be factors which affect the recognition of DNA damage in chromatin by the UV-endonuclease, and some XP cells may have a deficiency in one or more of these factors (19, 20).

An added complexity for DNA repair in human cells is imposed by the chromatin structure. Basically the chromatin structure is composed of repeating units of nucleosome core particles with the DNA wrapped around octamers of histones. These core particles are connected by the linker-DNA (22). DNA lesions in the nucleosome core are less accessible to DNA repair enzymes than the linker DNA (21, 23-27). For human cells irradiated with ultraviolet light, it has been calculated that the probability of repair synthesis per unit length of DNA in the linker regions is 15-fold greater than that in the core particles, while there is no predominance of induction of pyrimidine dimers in the linker regions (27). It has also been shown that both the UV-endonucleases of *Micrococcus luteus* and phage T4 have limited access to the dimer sites in permeable irradiated human cells (26). Additional sites became accessible when the cells were exposed to a high concentration of NaCl which presumably disrupts the chromatin structure. Thus, the incision step in the human excision repair process is carried out by an endonuclease and by factors which control the accessibility

of DNA damage to the putative repair endonuclease.

A protein which appears to influence the rate of DNA incision by a UV-endonuclease has been identified in human lymphocytes from patients with chronic lymphocytic leukemia (CLL) (28). This protein was purified by DNA-cellulose chromatography. It eluted from a UV-irradiated calf thymus DNA-cellulose column with 1 M NaCl and from a single-stranded DNA-cellulose column with 2 M MaCl. It had a molecular weight of 24,000. This protein can enhance the melting or unwinding of poly[d(A-T)] and UV-irradiated calf thymus DNA but not native calf thymus DNA. Interestingly, the rate of cleavage of UV-irradiated supercoiled ϕX-174 DNA by the UV-endonuclease activity of *Micrococcus luteus* was enhanced by this unwinding protein. Using an immunochemical procedure, this protein was not detected in lymphocyte extracts from normal individuals. The presence of this protein might explain the higher DNA repair capability of CLL cells compared with normal cells (28).

We and others have so far failed to purify a pyrimidine dimer specific endonuclease activity from human cells. These failures may be due to the small quantity or the lability of the endonuclease activity in crude extracts of human cells (19, 29). It is possible that the UV-endonuclease activity is a complex of several protein molecules, which dissociates upon chromatography leading to a loss of endonuclease activity.

An analogous situation exists for the UV-endonuclease activity coded by the *uvrA*, *B* and *C* genes of *Escherichia coli*. Of the three UV-endonuclease activities purified from procaryotes, the one coded

by the *uvrA, B* and *C* genes in *Escherichia coli* is probably the best model for the UV-endonuclease activity in human cells. Unlike the UV-endonucleases of *Micrococcus luteus* and phage T4 which are specific for pyrimidine dimers, the UV-endonuclease of *Escherichia coli* recognizes bulky DNA adducts. Such adducts are repaired less efficiently in XP cells. Mutations in the *uvrA, B or C* genes render *Escherichia coli* sensitive to both UV light and to agents which can produce bulky DNA adducts (6, 30, 31). It has been shown that each of the *uvrA, B or C* gene products does not have an appreciable endonuclease activity. They however can complement each other to yield an ATP-dependent endonuclease activity specific for UV-irradiated DNA (32). The *uvrA* protein apparently has a molecular weight of 100,000. It binds to UV-irradiated superhelical DNA and to a lesser extent to unirradiated superhelical DNA (33). Recently, a dimer specific endonuclease activity has been isolated from calf thymus (34). It is labile and is probably associated with a high molecular weight complex.

It is likely that proteins which bind strongly to DNA damaged by UV or other agents have a role in DNA repair. Thus, one approach to isolate proteins which function in DNA repair is to assay for their binding abilities to damaged DNA. The simplest assay involves incubation of the protein with DNA in a reaction mixture and subsequent filtration of the mixture through a nitrocellulose filter. The protein-DNA complex in the reaction mixture is retained by the filter. Such filter-binding assays have been shown to be useful in the purifications of several proteins which are involved or may be involved in DNA repair. These proteins included the UV-endonuclease activity from *Micrococcus luteus* (35), the T4 endonuclease V (36),

the *uvrA* protein (33), an ATP-independent UV-endonuclease from *Escherichia coli* (37), a DNA-binding protein which can insert purines into apurinic sites (38, 39) and the apurinic endonuclease activity from human fibroblasts (38). The filter-binding assays have also allowed the purification of two human placental DNA-binding proteins. Their biological functions remain to be determined. One of them binds to UV-irradiated DNA but recognizes DNA lesions other than pyrimidine dimers (40). It also binds to DNA treated with nitrous acid or sodium bisulfite (41). The other protein binds efficently to DNA treated with either AAAF, MMS or MNUA but has no affinity towards UV-irradiated DNA (42).

Recently, glass fibre filters have also been used in the filter-binding assays of three DNA-binding proteins. The three proteins are the DNA-terminal protein of adenovirus (43, 44), a protein from Hela cells which binds tightly to cellular DNA with an average spacing of about 50,000 base-pairs (44) and the poly(ADP-ribose) polymerase from bovine thymus which binds to DNA containing single- or double-stranded breaks (45). The last protein might have a role in DNA repair (46, 47).

In the hope that we may be able to isolate an activity which is involved in the incision step of excision repair of bulky DNA adducts, we have attempted to purify DNA-binding proteins from Hela cells which bind preferentially to UV-DNA and AAAF-DNA. In this thesis, the partial purification and characterisation of such a DNA-binding protein is reported. We have developed a filter-binding assay using GF/C glass fibre filters for the assay of this DNA-binding protein. In addition, fibroblasts from a normal human cell line and cell lines of XP group A and XP group D were screened for the presence of this DNA-binding protein.

Materials and Methods

1. Tissue culture

(a) Cell lines

Hela cells were purchased from Flow Laboratories, Inc., Rockville, Maryland. Cell line 207 was a gift from Dr. S. Wood, Department of Medical Genetics, University of British Columbia. It is derived from a skin biopsy from a 32 year old normal male Caucasian. XP cell lines were obtained from the Human Genetic Mutant Cell Repository, Institute of Medical Research, Camden, New Jersey. Cell line XP5EG belongs to the A complementation group of xeroderma pigmentosum and was derived from a skin biopsy of a 23 years old white female. Cell line XP2NE belongs to the D complementation group of xeroderma pigmentosum and was derived from a skin biopsy of a 4 year old white male of Egyptian background born of consanguineous parents.

(b) Culture media

Minimal essential Eagle's medium (Gibco) was supplemented routinely with 10% fetal calf serum (Gibco) and the following antibiotics: penicillin (100 µg/ml), streptomycin sulphate (30 µg/ml), kanamycin (100 µg/ml) and fungizone (2.5 µg/ml). The antibiotics were purchased from Gibco. The medium was adjusted to pH 7.0-7.5 with sodium bicarbonate. The culture medium was sterilized by filtration through a GSWP Millipore membrane filter with a pore size of 0.2 µm.

(c) Solution for harvesting cells

Trypsin-EDTA solution was prepared with 8.0 gm of NaCl, 0.2 gm of KH_2PO_4, 0.2 gm of KCl, 1.15 gm of Na_2HPO_4, 0.2 gm of EDTA and 0.5 gm of trypsin (Trypsin 1:250, Difco) and 1 liter of double distilled water. The solution was sterilized by filtration through

a GSWP Millipore filter. 1 liter of phosphate buffered saline (PBS, pH 7.1) contained 8 gm of NaCl, 0.2 gm of KCl, 1.15 gm of Na_2HPO_4 and 0.2 gm of KH_2PO_4 and was sterilized by autoclaving.

(d) Cell growth

Hela cells were maintained in 75-cm^2 tissue culture flasks (Falcon Plastics) with 15 ml of culture media. Four flasks of confluent cells were pooled and used to inoculate ten roller bottles (Bellco Biology Glassware). Each roller bottle has a surface area of 840 cm^2. 100-150 ml of culture medium was used in each bottle. The roller bottles were incubated at 37^oC and rotated at a speed of 0.1-0.2 rpm. After 4-6 days, the cells were harvested. The cell culture medium was decanted, and the cells were washed briefly with 10 ml of the trypsin-EDTA solution. The Hela cells then were detached from the surface of the bottles by incubation with another 10 ml of the trypsin-EDTA solution for 5-10 min at room temperature. During this period the roller bottles were rotated at a speed of 3 rpm. The cells were pelleted by centrifugation at 200-400 g for 6 min. The pellet was washed three times with 10 ml of PBS by repeated resuspension and pelleting. The final cell pellets were stored in liquid nitrogen. Ten bottles normally gave 0.5-1.0 x 10^9 cells.

Human fibroblasts were grown in plastic tissue culture flasks (Nunc Company) with a surface area of 174 cm^2. The volume of the culture medium was about 30 ml. Incubation was at 37^oC in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. Confluent cells were split 1:3. Cells were harvested near confluency in lots of 24 flasks with a yield of 0.5-1.0 x 10^8 cells. The cells culture medium was

decanted, and the cells were washed for 2-5 min with 5 ml of the

trypsin-EDTA solution. Cells were detached from the tissue culture

flask by incubating with another 5 ml of the trypsin-EDTA solution

for 5-10 min at 37°C. They were washed and pelleted with PBS as

described for Hela cells.

2. Preparation of ^{3}H-labeled PM2 DNA

The PM2 DNA was prepared as described previously (48) except that

the *Pseudomonas* Bal-31 bacteria were infected at a cell density of 3-5

x 10^8 cells/ml with a multiplicity of infection of 10 phage per bacterium

instead of 2-4 phage per bacterium, and that 2 mCi/liter of methyl-^{3}H-

thymidine (specific activity, 25 Ci/mmol, Amersham) was used to label

the PM2 DNA. The higher multiplicity of infection was found to increase

the yield of the PM2 phage. The PM2 DNA had a specific activity of

17-22,000 cpm/μg of DNA.

Unlabeled DNA was prepared in the same way as the ^{3}H-labeled DNA

except that no radioactive thymidine was added.

3. Preparation of modified DNA

UV-irradiation of DNA was carried out at a DNA nucleotide

concentration of 0.5 mM in 10 mM Tris-HCl, pH 7.5, using a petri dish on

ice and a 60 watt GE G15T8 germicidal lamp. The incident dose was

measured with a Blak-ray ultraviolet meter (Ultraviolet Products, Inc.).

The standard dose used was 1,200 J/m^2.

AAAF-DNA was prepared by incubating PM2 DNA at a DNA nucleotide

concentration of 0.5 mM at 37°C for 1 h with various concentrations of

AAAF (a gift from Dr. J. Scribner, Fred Hutchinson Cancer Research

Centre, Seattle) in 10 mM Tris-HCl, pH 7.5, and 10% DMSO. The AAAF-DNA

used in the standard DNA-binding assay was prepared with 0.01 mg/ml AAAF.

The DNA then was diluted to a nucleotide concentration of 0.2 mM and dialysed overnight against two changes of 500 ml of 10 mM Tris-HCl, pH 7.5.

Depurination of PM2 DNA was carried out by heating DNA at a nucleotide concentration of 0.5 mM at 70°C for 15 min in 10 mM Tris, 0.1 M NaCl and 0.01 M sodium citrate at a pH of 5.0 (adjusted with HCl). The treatment created about 1.5 apurinic sites/PM2 DNA molecule as determined by the nicking assay of Kuhnlein et al. (49).

MNNG-DNA and MMS-DNA were prepared by incubating PM2 DNA at a nucleotide concentration of 1 mM in 10 mM Tris-HCl, pH 7.5, with various concentrations of MNNG or MMS for 30 min at 37°C.

Supercoiled circular PM2 DNA was converted to a linear form by incubating PM2 DNA at a nucleotide concentration of 0.1 mM for 3 h with 32 units/ml of restriction endonuclease Msp I (New England Biolabs) in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 6 mM KCl and 100 µg/ml of acetylated BSA. In experiments where single-stranded DNA was used, the linear PM2 DNA was extracted first with an equal volume of chloroform-octanol (9:1) and then dialysed overnight against two changes of 400 ml of 10 mM Tris-HCl, pH 7.5. The linear PM2 DNA was denatured immediately before use by a 10-min incubation in a boiling water bath. Nicked PM2 DNA was prepared by treating the supercoiled circular PM2 DNA with bovine pancreatic DNase I (Sigma). Native PM2 DNA at a nucleotide concentration of 0.1 mM was incubated with 6.5 µg/ml of DNase I at 37°C for 1 h in a reaction mixture containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl and 100 µg/ml of acetylated BSA. After this treatment virtually all the DNA molecules were nicked.

4. DNA-binding assay

The standard DNA-binding assay mixture contained 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 139 fmol of ^{3}H-labeled PM2 DNA molecules (14,000-18,000 cpm), 175 mM NaCl and an aliquot of protein in a total volume of 300 μl in a borosilicate test tube. The mixture was incubated for 10 min on ice. The assay mixture then was diluted with 1.7 ml of ice cold 10 mM Tris-HCl, pH 7.5, and 100 mM NaCl (buffer G), and filtered immediately through a GF/C filter at a flow rate of 10-30 ml/min. The filtration speed was controlled by a Manostat Varistaltic pump. The reaction tube was rinsed once with 1.7 ml of buffer G, and the resulting solution was filtered. The filter funnel (Millipore) and the filter then were washed with another 1.7 ml of buffer G. Filters were dried under a heat lamp and the radioactivity was determined by liquid scintillation counting. A unit of DNA-binding activity is defined as the amount of protein which retains 1 fmol of PM2 DNA on the filter under the standard conditions.

5. Precycling and preparation of column resins

(a) DEAE-cellulose and phosphocellulose

The two kinds of resins were precycled in the same way. Routinely, 100 gm of resin was suspended in 2 liters of distilled water in a beaker. The resin was allowed to settle for about 1 h and the supernatant containing fine particles was decanted. The procedure was repeated three times, and the resin was resuspended in 1 liter of 0.5 M NaOH for 20 min. The suspension was filtered through a Whatman No. 1 filter paper. The resin then was washed with distilled water until the filtrate had a neutral pH. It was stirred with 2 liters of 10 mM potassium phosphate, pH 7.5, and left at 4°C overnight. The suspension

was filtered and washed with distilled water. Finally, the resin was resuspended in 10 mM potassium phosphate, pH 7.5, and stored at 4°C.

(b) UV-irradiated DNA-cellulose

Cellulose (Cellex 410, Bio-Rad) was precycled as described by Alberts and Herrick (50). 100 gm of cellulose was suspended in 1 liter of ethanol and incubated at 80°C for an hour. The cellulose was allowed to settle, and the ethanol was poured off. The procedure was repeated three times. The cellulose then was successively washed by suspending and filtering at room temperature with 500 ml each of 0.1 M NaOH, 1 mM EDTA and 10 mM HCl. After washing with H_2O until the pH of the effluent was neutral, the cellulose was lyophilized and stored at room temperature.

A solution containing 2 mg/ml of calf thymus DNA in 10 mM Tris-HCl pH 7.4, and 1 mM EDTA (buffer X) was prepared. 40 ml of the DNA solution in a polypropylene beaker with a diameter of 5 cm was UV-irradiated for 35 min with a G15T germicidal lamp. The incident UV-dose was 12 $J/m^2/s$. The DNA solution was mixed vigorously with a magnetic stirrer during the irradiation. 20 g of the lyophilized cellulose was added to the UV-irradiated DNA solution. The lumpy mixture was spread out on a glass dish with a glass rod. The dish was covered with gauze and air dried at 37°C overnight. Afterwards the DNA-cellulose was ground to a powder and lyophilized overnight to complete the drying procedure. The dry DNA-cellulose was resuspended in 20 ml of 95% ethanol and UV-irradiated at a dose rate of 10 $J/m^2/s$ for 20 min. The DNA-cellulose was air-dried again at 37°C overnight. The DNA-cellulose then was resuspended in 1 liter of buffer X and left at 4°C for a day. It was washed twice by resuspension and filtration with 2 liters of buffer X to

remove free DNA. Finally, the DNA-cellulose was resuspended in 100 ml

of buffer X plus 0.15 M NaCl and stored as a frozen slurry at $-20^{\circ}C$.

6. Purification of the DNA-binding protein, PIII

All operations were at $4^{\circ}C$. The columns were made from B-D plastic

syringes. Dialysis was carried out with Spectrapor I dialysis tubing

with a molecular weight cut off of 6,000-8,000. The column fractions

were collected in polypropylene or polyethylene tubes.

(a) Crude extract

About 2 x 10^9 Hela cells were used for the purification of PIII.

Hela cells were thawed and suspended in 35 ml of 50 mM Tris-HCl, pH 7.5,

1 mM EDTA and 1 mM DTT (buffer A). The Hela cells were disrupted by

sonication with six 20-sec pulses using a Biosonik III sonicator

(Bronwill Scientific). The sonication was performed at an intensity

setting of 30 using a 4 mm probe. The sonicate was centrifuged for

50 min at 50,000 rpm in a Beckman 50 Ti rotor. The supernatant was

centrifuged once more under identical conditions to insure complete

removal of all sedimentable material. The final supernatant (high

speed supernatant fraction) was retained for further purification.

(b) DEAE-cellulose chromatography

A column (3.8 cm^2 x 5.3 cm) with 20 ml of Whatman DE-22 DEAE

cellulose was prepared and equilibrated with 50 mM Tris-HCl, pH 7.5,

1 mM EDTA, 1 mM DTT, 10% glycerol and 0.4 N NaCl (buffer B). The

high speed supernatant fraction was brought to the same buffer content

and loaded onto the column at a flow rate of 0.25-0.5 ml/min. The

column then was washed with buffer B at the same flow rate. Fractions

of 10 ml were collected. A total of 10-12 fractions were collected

and assayed for DNA-binding activity. The fractions with activity were

pooled and dialysed overnight against two changes of 1 liter of 10 mM

potassium phosphate, pH 7.4, 10% glycerol, 1 mM DTT, 1 mM EDTA (buffer

C). The whitish precipitate (appearing after 1-2 h of dialysis) was

removed by centrifugation at 15,000 rpm for 15 min in a Beckman 50 Ti

rotor. The supernatant (DEAE-fraction), about 60 ml, was retained for

further purification.

(c) <u>Phosphocellulose chromatography</u>

The DEAE-fraction was applied to a 45-ml column (5.5 cm^2 x 8 cm)

of Whatman P-11 phosphocellulose previously equilibrated with buffer

C. The column then was washed with 30 ml of buffer C. Fractions of

10 ml were collected. The column subsequently was washed with 50 ml

of 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT and 10%

glycerol (buffer D). Afterwards the column was eluted with a 400-ml

linear gradient from 50 mM to 500 mM potassium phosphate buffer, pH

7.5, containing 1 mM EDTA, 1 mM DTT and 10% glycerol. Fractions of 6

ml were collected. The flow rate was 0.3-0.5 ml/min. The column

fractions were made 40% in glycerol and stored at -20°C. Fractions

containing DNA-binding activity eluting between 325-425 mM potassium

phosphate were pooled (phosphocellulose fraction) and subjected to

further chromatography.

(d) <u>UV-DNA cellulose chromatography</u>

A column (0.6 cm^2 x 5 cm) with 3 ml of UV-irradiated DNA-cellulose

was equilibrated with 10 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA

and 1 mM DTT (buffer E). A 13-ml aliquot of the phosphocellulose fraction

was made 100 µg/ml in β-lactoglobulin and dialysed against 1 liter of

buffer E for 3-4 h. The dialysed extract was applied to the DNA-

cellulose column at a flow rate of about 0.25-0.5 ml/min. The column

was washed with 3 ml of buffer E containing 100 µg/ml of β-lactoglobulin

(buffer F) followed successively by 10 ml each of buffer F containing

0.15 M NaCl, 0.5 M NaCl, 1 M NaCl or 2 M NaCl. Fractions were stored

in 55% glycerol and 100 µg/ml of β-lactoglobulin at -20°C.

7. Analysis of DNA-binding proteins from human fibroblasts

The purification procedures were modified from those for Hela

cells. Briefly, about 5-6 x 10^7 human fibroblasts were used in each

analysis. They were disrupted by sonication in 4 ml of buffer A as

described for Hela cells. The sonicate was centrifuged at 50,000 rpm

for 50 min in a Beckman 50 Ti rotor. The supernatant was passed

through a column (0.64 cm^2 x 3.3 cm) of 2 ml of DEAE-cellulose. The

DEAE-cellulose column then was washed with buffer B at a flow rate of

0.17 ml/min. Fractions of 1 ml were collected. DNA-binding activity

was detected in the first 7-8 fractions. These were combined and

dialysed overnight in a Spectrapor I dialysis tubing against two

changes of 500 ml of buffer C. The dialysed extract was centrifuged at

15,000 rpm for 15 min in a Beckman 50 Ti rotor to remove a precipitate

formed during the dialysis. The supernatant (DEAE-fraction) was

chromatographed on a 3.5-ml phosphocellulose column (1.1 cm^2 x 3.2 cm).

The column was washed with about 3 ml of buffer C and 3-5 ml of buffer

D. Fractions of 1 ml were collected. The column then was eluted with

a 30-ml linear gradient from 50 mM to 500 mM potassium phosphate buffer,

pH 7.5, containing 1 mM EDTA, 1 mM DTT and 10% glycerol at a flow rate

of about 0.17 ml/min. Fractions of 0.5 ml were collected and

β-lactoglobulin was added to a final concentration of 200 µg/ml. The

column fractions were assayed immediately for DNA-binding activity.

8. Glycerol gradient sedimentation of PIII

0.2 ml of a diluted aliquot of PIII was layered on a 4.8 ml, 10-30% linear glycerol gradient in a polyallomer centrifuge tube. The glycerol gradient was buffered with 10 mM Tris-HCl, pH 7.5, and contained 1 mM EDTA, 1 mM DTT, 0.15 M NaCl and 100 μg/ml bacitracin. Where indicated, the gradient solution contained 0.5 M NaCl instead of 0.15 M NaCl. Gradients were centrifuged at 49,000 rpm and 4°C for 27 h in a Beckman SW 50.1 rotor. Fractions were collected in polypropylene tubes from the bottom of the gradient. BSA (4.25 S), egg white ovalbumin (3.5 S), α-chymotrypsin (2.5 S), whale skeletal muscle myoglobin (2.0 S) and cytochrome C (1.7 S) from Sigma Chemical Company were used as marker proteins. The molecular weights of these proteins are 64,000, 45,000, 24,300, 18,000 and 12,400 respectively. The sedimentation profile of these marker proteins was monitored by measuring the absorbance at 280 μm and in the case of cytochrome C, also at 410 μm. A symmetrical peak of absorbance was obtained for each protein.

9. Glycerol gradient sedimentation of PIII-DNA complex

Unless otherwise stated, the DNA-binding reaction was performed under the standard conditions. A 200-μl aliquot of the assay mixture was layered on a 4.8 ml, 10-30% linear glycerol gradient containing 10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 1 mM EDTA and 100 μg/ml β-lactoglobulin. In some experiments, the gradient solutions contained 50 mM or 150 mM NaCl. Gradients were centrifuged at 49,000 rpm and 4°C in a Beckman SW 50.1 rotor for 2 or 3 h. Fractions were collected from the bottom into polypropylene tubes. A 50-μl aliquot from each fraction

was assayed for radioactivity. The remaining portions of the peak fractions of each gradients were filtered through GF/C filters as described for the standard DNA-binding assay. The amount of PIII-DNA complex was determined by the amount of radioactive PM2 DNA retained on the filters.

10. Sucrose gradient sedimentation of DNA

A 200-μl aliquot of the DNA solution was layered on a 4.8 ml 5-20% linear sucrose gradient containing 50 mM Tris-HCl, pH 7.5, and 0.25 M NaCl. Centrifugation was for 3 h at 50,000 rpm and 4°C in a Beckman SW 50.1 rotor. Fractions were collected from the bottom. A 50-μl aliquot from each fraction was assayed for radioactivity.

11. Enzyme assays

Endonuclease activity was assayed by measuring the conversion of supercoiled PM2 DNA to nicked circles. The standard assay for DNA nicking was according to the method of Kuhnlein et al. (16, 49). 50 μl of the reaction mixture was diluted with 150 μl of 0.01% of sodium dodecyl sulphate, 2.5 mM EDTA (adjusted to pH 7.0 with HCl). 200 μl of 0.3 M K_2HPO_4-KOH, pH 12.5, then was added. After 2 min at room temperature, the solution was neutralized with 100 μl of 1 M KH_2PO_4-HCl, pH 4.0. This treatment denatured nicked PM2 DNA, but not covalently closed circular PM2 DNA. 200 μl of 5 M NaCl and 5 ml of 50 mM Tris-HCl, pH 8.0, and 1 M NaCl then were added successively. The solution was filtered through a nitrocellulose membrane filter paper (Schleicher and Schuell type BA 85, 0.45 μm pore size) which selectively retained denatured DNA. The filter was washed with 5 ml of 0.3 M NaCl, 0.03 M sodium citrate and dried. The amount of DNA retained on the filter was determined by measuring the radioactivity on the filter by liquid

scintillation counting. The average number of nicks per DNA molecule (ω) was calculated from the equation $\omega = -\ln(1-X)$ (16), where X is the fraction of nicked PM2 DNA molecules.

Glycosylase activity was measured by determining the number of alkali-labile apurinic/apyrimidinic sites introduced into the DNA. The assay for alkali-labile sites was similar to the assay for DNA nicking except that the 2-min alkali-treatment was replaced by a 1 h incubation with 200 µl of 0.3 M K_2HPO_4-KOH, pH 12.5, and 50 mM L-lysine at 37°C. This procedure hydrolyses apurinic/apyrimidinic sites (16, 40).

Exonuclease activity was measured by determining the amount of radioactive DNA rendered soluble in 6% trichloroacetic acid.

For the assay of ATPase activity, aliquots of PIII were incubated in a reaction mixture containing 139 fmol of PM2 DNA molecules, 1 mM ATP, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and 0.8 µCi/ml of 2, 8-^{3}H-ATP (25 Ci/mmol, New England Nuclear). After incubation for 1 h at 37°C, the conversion of ATP to ADP was monitored by the method of Kornberg et al. (51). In this method aliquots of the reaction mixtures are analysed by thin layer chromatography on strips (0.6 cm x 6 cm) of polyethyleneimine cellulose (Brinkmann) with a solution of 1 M formic acid and 0.5 M LiCl at room temperature. The chromatography procedure separates ATP from ADP: ATP remains near the origin, and ADP migrates to the middle of the strip. The strips were cut into two portions to determine the amount of radioactive ATP and ADP in the reaction mixture.

12. Protein determination

Protein concentrations were determined by the method of Lowry et al. (52) or the method of Bradford (53). For the latter assay, the dye was purchased from Bio-Rad Laboratories. BSA (Sigma) was used as a protein standard in both methods.

13. Phosphate determination for the column fractions

The reagent solution for phosphate determination contained one volume of 10% ascorbic acid and six volumes of a solution containing 0.42% ammonium molybdate and 1 N H_2SO_4. For each assay, 1.4 ml of the reagent solution was mixed with 0.6 ml of the sample to be tested. After incubation for 20 min at $45^{\circ}C$, the mixture was cooled to room temperature and the absorbance at 660 μm was determined (54). Inorganic phosphate solutions were used as standards. The absorbance was linear between 10 and 100 nmol of phosphate.

14. Scintillation fluid

Filters were counted in toluene (BDH Chemicals) containing 4 gm/l of 2,5-diphenyloxazole (PPO) (Amersham) and 0.1 gm/l of 1,4-bis[2-(5-phenyloxyazolyl)]-benzene (POPOP) (Syndel Laboratory). For aqueous samples ACS scintillation liquid (Amersham) was used.

15. Miscellaneous

Proteinase K (20 mAnson units/mg) was purchased from E. Merck Biochemicals. Bovine pancreatic ribonuclease (Type IA, 76 Kunitz units/mg) and the single-stranded specific endonuclease of *Neurospora crassa* (535 units/mg) were purchased from Sigma.

BSA was acetylated with acetic anhydride (Fisher) as described previously (49).

Caffeine, $MnCl_2$, Tris (Trizma), ATP and ADP were obtained from Sigma. $MgCl_2$, sucrose and glycerol were purchased from Fisher.

Unless otherwise stated, all pH measurements were performed at room temperature.

Results

1. Purification of the DNA-binding protein, PIII

Details of the purification procedures are described in Materials
and Methods. The results of a typical purification are summarised in
Table I.

A high speed supernatant fraction was prepared from 2×10^9 Hela
cells and was filtered through a DEAE-cellulose column in buffer B
which contained 0.4 M NaCl. The DEAE-fraction had a higher DNA-binding
activity than the high speed supernatant fraction. This result might
reflect a removal of cellular DNA or another inhibitor of DNA-binding
activity by filtration through the DEAE-cellulose.

The DEAE-fraction was fractionated by phosphocellulose column
chromatography. Three major peaks of DNA-binding activity were
separated (Fig. 1). The activity which eluted at 325-425 mM potassium
phosphate bound preferentially to UV- or AAAF-DNA as compared to the
untreated DNA (u-DNA); with a ratio of 6:1. This peak of DNA-binding
activity was stable for at least a year when stored at -20°C in the
presence of 40% glycerol.

The two other peaks of DNA-binding activity eluted in the flow-
through fractions (data not shown) and with 180-250 mM potassium
phosphate. They did not show any binding specificity to UV- or
AAAF-DNA under our present assay conditions.

Fractions 59-71 of the phosphocellulose column were pooled
(phosphocellulose-fraction) and further fractionated by DNA-cellulose
chromatography. The UV-irradiated DNA-cellulose column was eluted
with a step gradient. The major species of DNA-binding protein,
designated arbitrarily as PIII, eluted with 1 M NaCl from the column

Table I. Purification of PIII from Hela cells.

Fraction	Protein mg	Volume ml	Activity, units x 10^{-3}		Units of activity x 10^{-3} per mg of protein	
			UV-DNA	u-DNA	UV-DNA	u-DNA
I. High speed supernatant	350	40	540	250	1.6	0.7
II. DEAE	300	60	750	400	2.5	1.3
III. Phospho-cellulose	2.6	117 [1]	270	45	104	17
IV. UV-DNA-cellulose	– [2]	54 [3]	95	14	–	–

[1] Volume of the fraction in 40% glycerol.

[2] The amount of protein was not determined.

[3] Volume of the fraction in 55% glycerol.
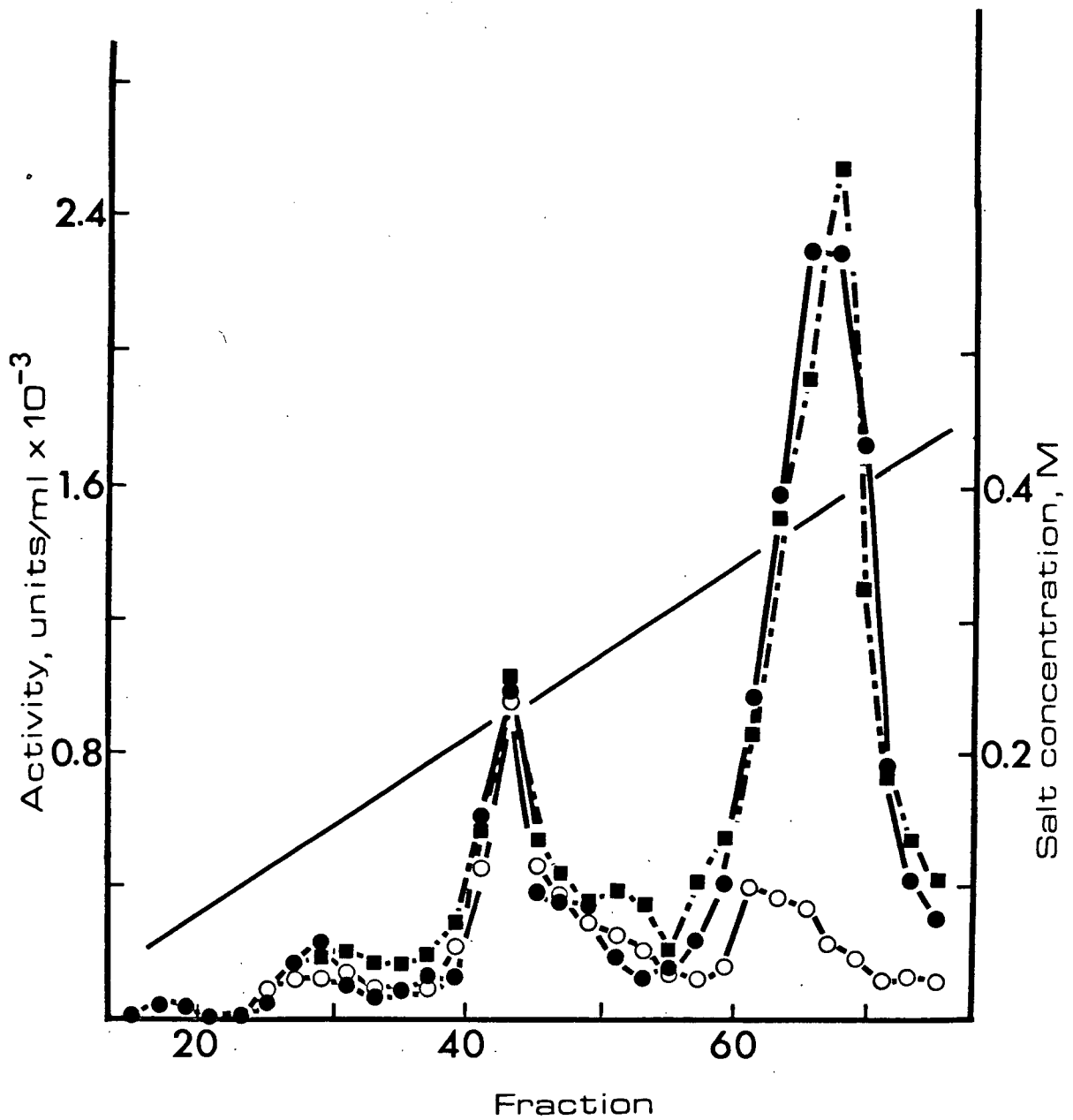
Fig. 1. Chromatography of DNA-binding proteins on phosphocellulose.
Assays were performed with UV-DNA (●), AAAF-DNA (■)
or u-DNA (○) as the binding substrates. Phosphate
concentration (————).

(Fig. 2). This purification step separated PIII from another DNA-binding activity which eluted from the column with 0.5 M NaCl. Peak fractions of PIII were stored in 55% glycerol and 100 μg/ml β-lactoglobulin with no loss in DNA-binding activity for at least half a year. Routinely, 100 μg/ml of β-lactoglobulin was included in the elution buffers for the DNA-cellulose chromatography of PIII.

It has been reported that carrier proteins such as lysozyme affect the elution of steroid receptors on DNA-cellulose chromatography (55). We have not observed any effect of β-lactoglobulin on the elution of PIII from the UV-irradiated DNA-cellulose column. A similar elution profile as that shown in Fig. 2 was obtained when the chromatography was carried out with elution buffers without β-lactoglobulin. However, if stored in the absence of β-lactoglobulin, PIII lost at least half of its activity in 16 h at 4°C. Other attempts which included the storage of PIII in the absence of carrier protein at -20°C or in liquid nitrogen with and without 55% glycerol failed to stabilize PIII. BSA or acetylated BSA can be used to stabilize PIII, but we have chosen arbitrarily β-lactoglobulin.

The purification of PIII after phosphocellulose chromatography was at least 40-fold. The ratio of AAAF- or UV-DNA-binding activity relative to u-DNA-binding activity increased from about 2:1 in the first two fractions to about 7:1 in the final fraction. This indicates an enrichment of the specific binding activity for AAAF-DNA and UV-DNA during the purification.
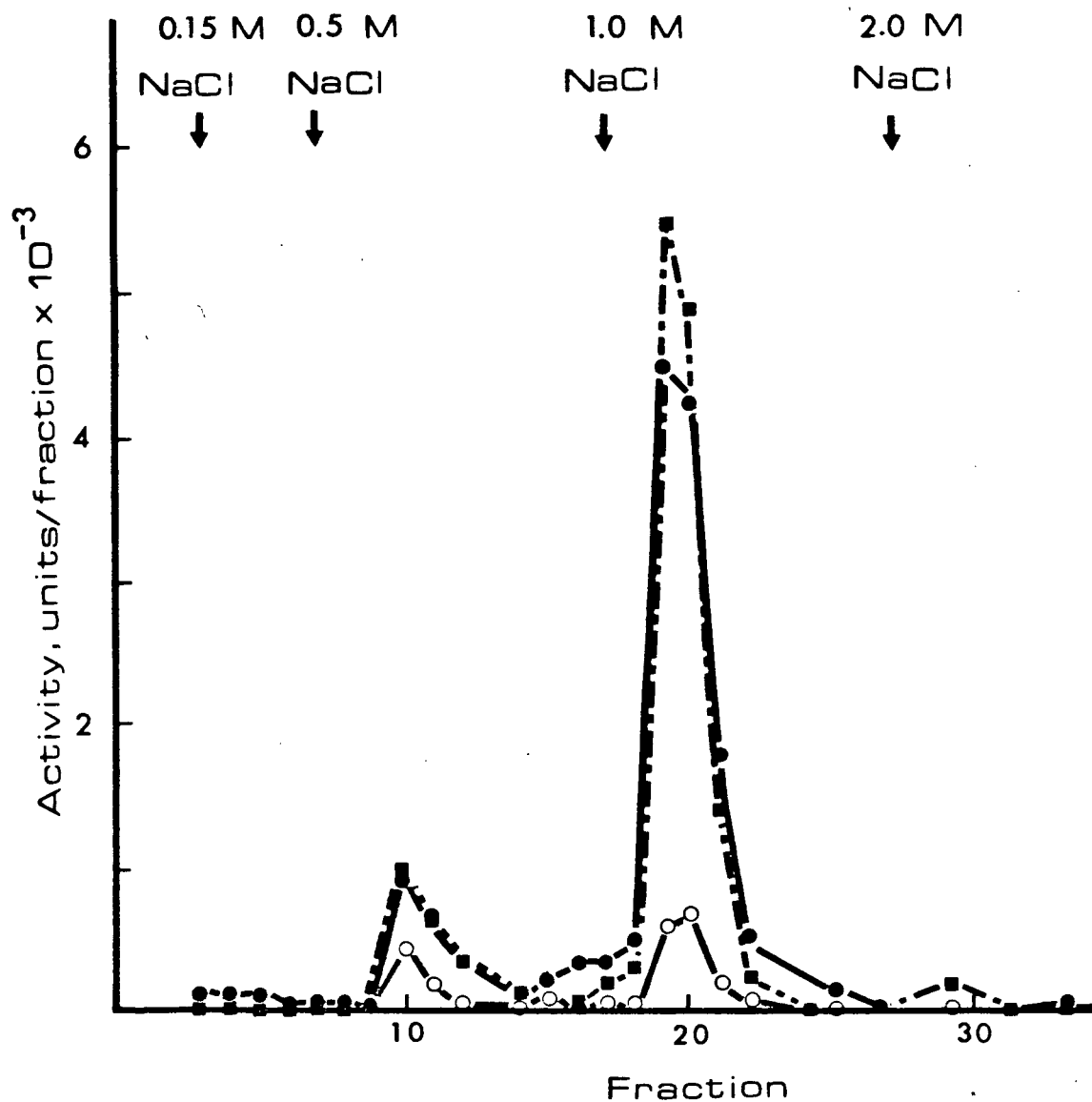
Fig. 2. UV-DNA cellulose chromatography of the phosphocellulose
fraction of PIII.
Assays were performed with UV-DNA (●), AAAF-DNA (■)
or u-DNA (○) as the binding substrates.

2. Properties of the DNA-binding assay

Basically, the DNA binding assay consisted of four steps: (1) incubation of PIII with DNA; (2) dilution of the assay mixture; (3) filtration of the mixture through GF/C glass fibre filters; (4) washing of the filter and filter funnel with filtration buffer.

The standard conditions for the DNA-binding assay were established by studying several parameters. First, the influence of NaCl concentration on the DNA-binding activity of PIII was investigated. Fig. 3 indicates that the binding of PIII to UV- or AAAF-DNA was optimal at 100-200 mM NaCl. Thus, PIII can bind optimally to UV- or AAAF-DNA at ionic strength near physiological conditions. A salt concentration of 175 mM NaCl was used in the standard assay mixture.

The binding of PIII to DNA was very fast and an incubation of two min at 0°C was sufficient for the establishment of an equilibrium (Fig. 4). Therefore, our standard incubation condition of 10 min was more than adequate.

The next condition we studied was the NaCl concentration of the dilution buffer used in the second step of the DNA-binding assay. It should be noted that the binding reaction could still occur in this second step. The result depicted in Fig. 5 indicated that the amount of UV-, AAAF- or u-DNA retained on the filter by PIII was maximal at 20-50 mM NaCl. Since we were interested primarily in the specific binding of PIII to UV-DNA and AAAF-DNA, a dilution buffer with 0.1 M NaCl was chosen to minimize the nonspecific binding of PIII to DNA and/or the retention by the filters of complex formed via the nonspecific binding of PIII to DNA (Fig. 5). The former was more likely since the GF/C filter can retain the adenovirus terminal
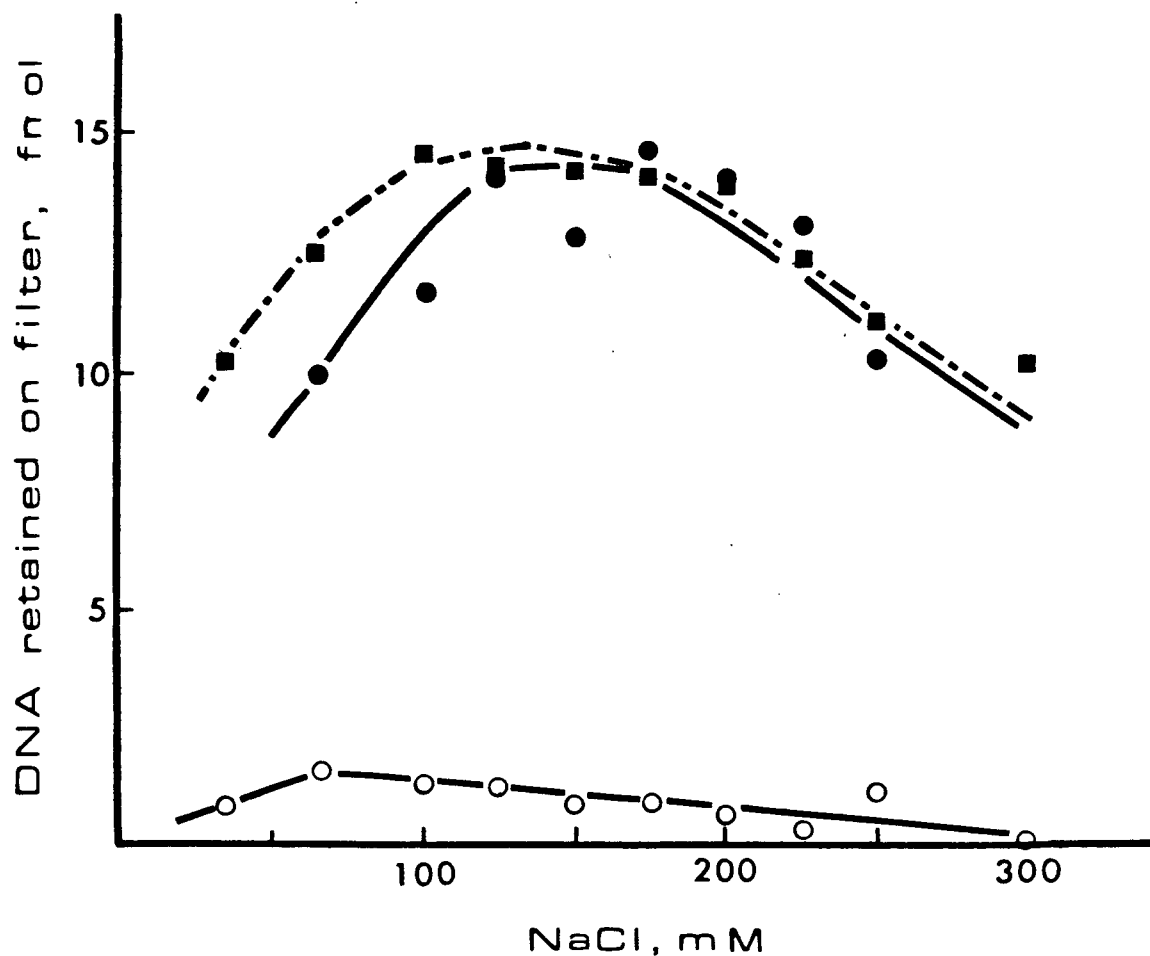
Fig. 3. Effect of NaCl concentration in the assay mixture on the
DNA-binding activity of PIII.
The DNA-binding assays were performed with the standard
assay mixture except that the NaCl concentration was varied
as indicated. After incubation at $4^{\circ}C$ for 10 min, the
assay mixtures were diluted with 1.7 ml of 10 mM Tris-HCl
buffer (pH 7.5) containing NaCl to give a final concentration
of 100 mM NaCl. The assays were then completed as described
for the standard DNA-binding assay. DNA-binding activity
was measured with UV-DNA (●), AAAF-DNA (■) or u-DNA (○).
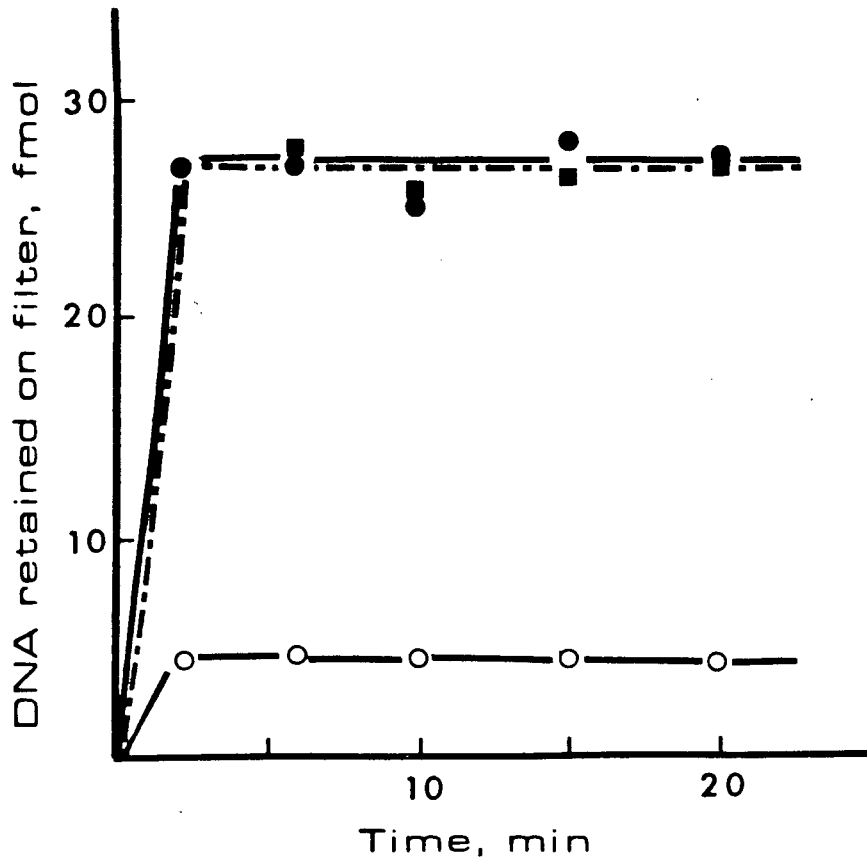
Fig. 4. Time course of DNA-binding by PIII.
DNA-binding assays were performed under the standard
conditions for various incubation times with UV-DNA
(●), AAAF-DNA (■) or u-DNA (○) as the binding
substrates.
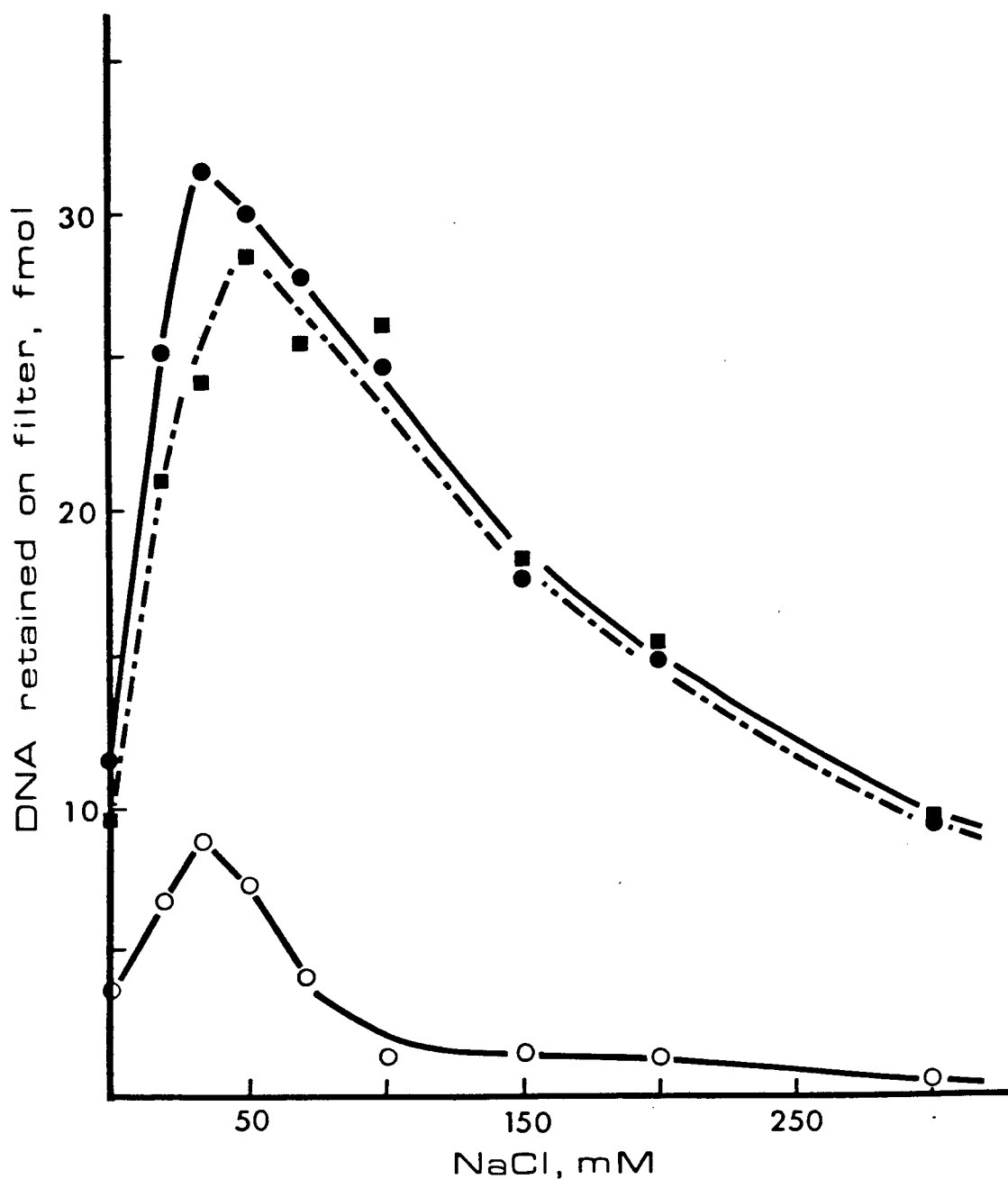
Fig. 5. Retention of PIII-DNA complex by the filters as a
function of the NaCl concentration of the dilution
buffer.
The DNA-binding assays were performed under the standard
conditions except that the NaCl concentration in the
dilution buffer was varied as indicated. DNA-binding
activity was assayed with UV-DNA (●), AAAF-DNA (■)
or u-DNA (○).

protein complex with an equal efficiency at various NaCl concentrations from 0.15 M to 4 M NaCl (43, 44). A similar phenomenon has been observed in the binding of bovine thymus poly(ADP-ribose) polymerase to nicked DNA. A salt concentration of 0.1 M NaCl also was used to inhibit the nonspecific binding of the polymerase to closed DNA (45).

We found that filtration speeds faster than 30 ml/min or slower than 5 ml/min resulted in a substantial loss of the amount of PIII-DNA complex retained on the filters (Fig. 6). Thus we usually filtered at a flow rate of 10-30 ml/min. It is well documented that a slow flow rate results in a more reproducible and greater retention of protein-DNA complex by nitrocellulose filters (56, 57). The reason for the loss of PIII-DNA complex with low filtration speeds remains to be determined.

There are five different kinds of Whatman GF grade glass fibre filters which differ in their thicknesses and pore sizes (Table II). The pore size of each filter is defined as the size of the particles that can be retained by the filter with an efficiency of 98% (Glass microfibre filters, Whatman Publication 824). Different kinds of filters were tested for their ability to retain PIII-DNA complex. Filters with pore sizes less than 1.5 μm retained the PIII-DNA complex efficiently (Table II).

Usually, assays were performed in conditions where 5-25% of the input UV-DNA or AAAF-DNA was retained by the filters. In experiments where the assays were carried out in duplicate, the duplicates usually agreed to within 10%.

In the absence of PIII, the amount of DNA retained on the filters
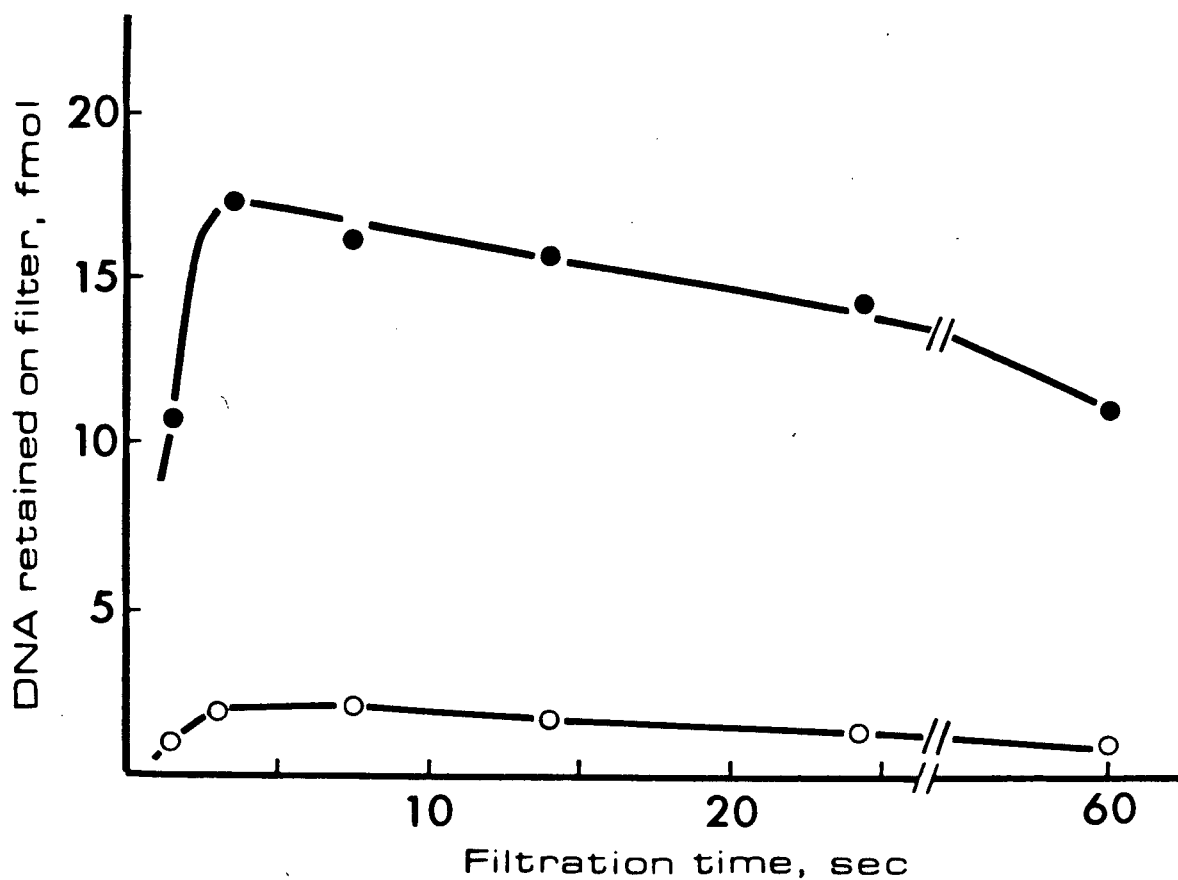
Fig. 6. Effect of the filtration speed on the retention of PIII-
DNA complex.
The binding-assays were performed with UV-DNA (●) or
u-DNA (O) at the various filtration speeds indicated.
Each points is the average of duplicate assays. The
filtration time was the time needed for a 2-ml assay
mixture to pass through a filter.

Table II. Retention of PIII-DNA complex by different types of
          Whatman glass microfibre filters

| Type | Thickness, mm | pore size, μm | DNA retained on filter, fmol | |
			UV-DNA	u-DNA
GF/A	0.25	1.6	11.5	1.9
GF/B	0.71	1.0	13.2	4.0
GF/C	0.25	1.2	12.0	2.9
GF/D	0.65	2.7	8.5	2.8
GF/F	0.44	0.7	12.2	2.4

The DNA-binding assays were performed under the standard conditions
with different types of Whatman glass fibre filters. The data
were the averages of duplicate assays.

(background) was less than 1.5% for the various kinds of duplex PM2 DNA

we have used. The background for the unit length single-stranded PM2

DNA was even lower and was 0.3-0.5% of the input DNA. Where appropriate,

the backgrounds were substracted from the amount of DNA retained on

the filters in the presence of PIII.

3. Formation of PIII-DNA complex as a function of the amount of
DNA damage and the concentration of PIII

An aliquot of PIII which contained 23.5 units of UV-DNA-binding

activity only retained 2.4 fmol of u-DNA on the filter. The amount

of PIII-DNA complex retained on the filter increased when the DNA

was treated with increasing dose of UV or AAAF (Fig. 7). The maximum

amount of PIII-DNA complex was retained when the UV-dose and the

AAAF-dose reached 1,200 J/m^2 and 0.01 mg/ml, respectively. The result

also suggested that DNA UV-irradiated with a dose of 1,200 J/m^2 was

equivalent to DNA treated with 0.01 mg/ml AAAF as a binding substrate

for PIII. Thus, UV-DNA and AAAF-DNA were prepared accordingly and

used in experiments where the binding of PIII to both DNA substrates

were compared.

Under the standard conditions, the retention of DNA was linearly

dependent on the amount of PIII (Fig. 8). However, at higher

concentrations of PIII, that is, when more than 40 fmol of PIII-DNA

complex was retained by the filters, the binding curves for UV-DNA

and AAAF-DNA started to level off. We have estimated the units of

DNA-binding activity of the various aliquots of PIII from the linear

portion of the binding curves.

The leveling off of the binding curves might be due to (1) a

limited binding capacity of the filters; (2) a low retention efficiency
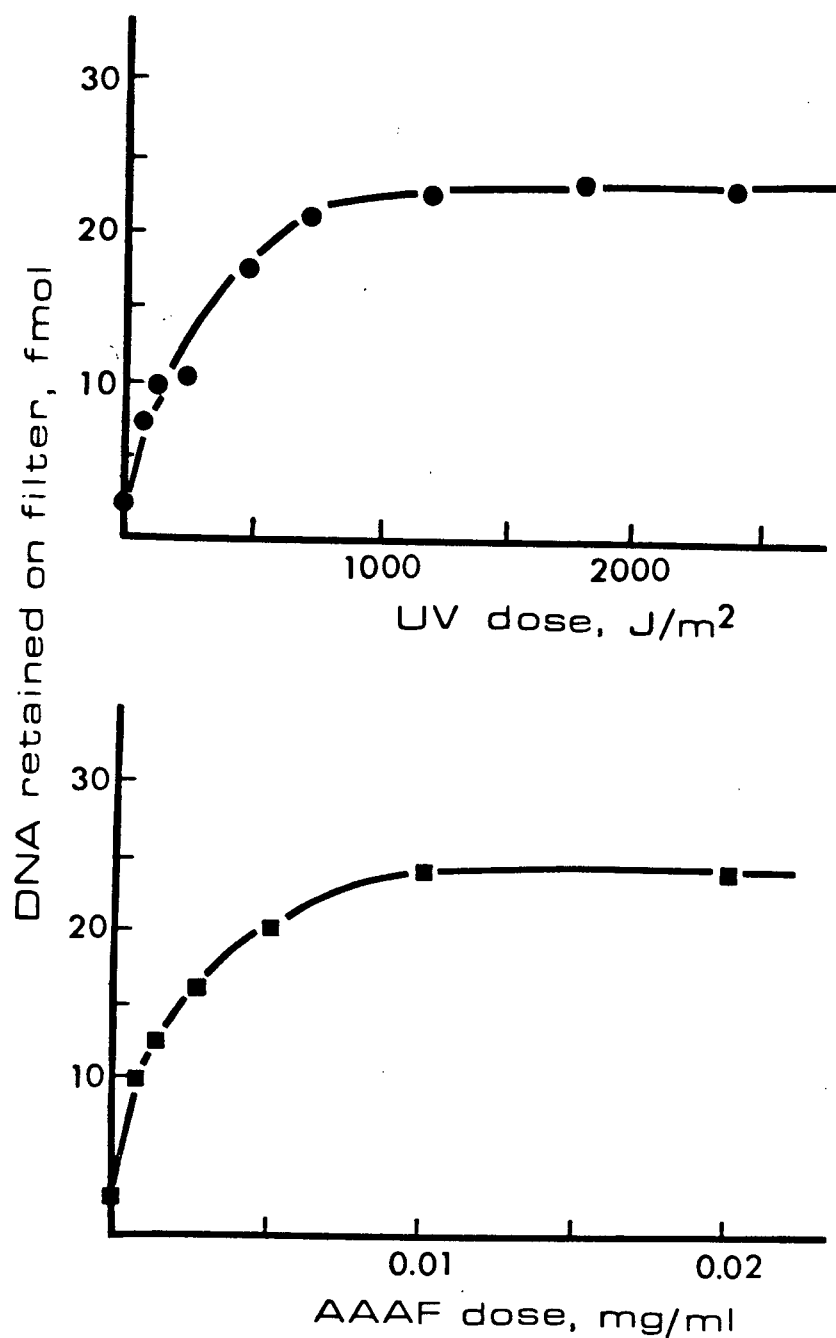
Fig. 7. DNA-binding of PIII as a function of UV-dose
and AAAF-dose.
The DNA-binding assays were performed under
the standard conditions with an aliquot of
PIII containing 23.5 units of UV-DNA-binding
activity.
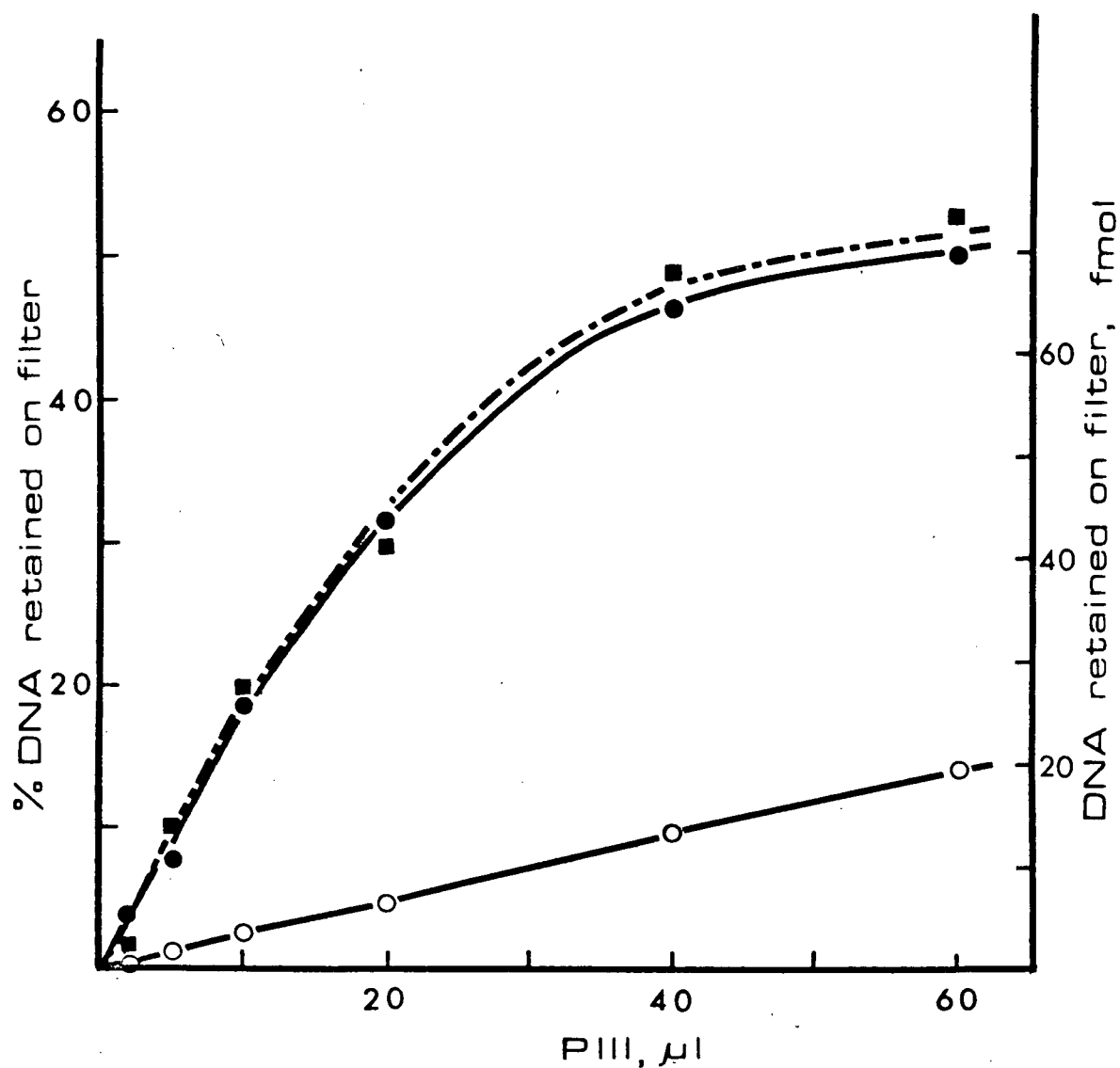
Fig. 8. DNA-binding as a function of the amount of PIII.
The DNA-binding assays were performed under the standard
conditions with various amounts of PIII. PIII had a
UV-DNA-binding activity of 2.5 units/μl. The assays
were performed with UV-DNA (●), AAAF-DNA (■) or
u-DNA (○) as the binding substrates. Each point is
the average of duplicate assays.

of the PIII-DNA complex; or (3) a limited number of DNA-binding sites.

In order to rule out possibility (1), two reaction mixtures containing PIII with 100 units of UV-DNA-binding activity were filtered through the same glass fibre filter. The amount of DNA retained was about 140 fmol of DNA, which was twice the amount retained when a single reaction mixture was filtered (Fig. 8). Thus the binding capacity of the filter was not limiting. The experiment also suggested that washing the filter with an additional 3.7 ml of filtration buffer (2 ml from the second assay mixture and 1.7 ml from washing the second reaction tube) did not result in a significant elution of the PIII-DNA complex retained during the first filtration.

The retention efficiency of the glass fibre filters was determined by filtering a reaction mixture through three filters stacked on top of each other. PIII-DNA complex was only detected on the first filter. and was not present in the filtrate which passed through the first filter (Table III). Assuming that interaction with the filter does not cause the dissociation of the PIII-DNA complex, the retention efficiency of the filter is close to 100%.

The formation of complex between PIII and UV- or AAAF-DNA was largely due to the binding of PIII to UV- or AAAF-induced sites on the DNA (specific binding of PIII to UV- or AAAF-DNA). However with a saturating amount of PIII, a significant amount of complex is expected to be formed as a result of the binding of PIII to sites that are not induced by DNA damage. To estimate the amount of specific binding to UV- or AAAF-DNA for a saturating amount of PIII, the data points in Fig. 8 were each corrected by using the equation:

Table III.   Efficiency of retention of PIII–DNA complexes
             by the GF/C filters.

| Experiment | no. of filter | DNA retained on filters, fmol | | | |
| | | UV–DNA | | u–DNA | |
		+PIII	–PIII	+PIII	–PIII
I	1st	33.0	1.0	3.5	1.4
	2nd	1.3	0.8	0.7	1.4
	3rd	0.6	0.7	0.6	0.8
II	1st	30.8	1.3	4.4	0.7

In experiment I, each DNA–binding assay mixture was filtered
through three filters stacked on top of each other.   In
experiment II, each assay mixture was filtered through one
filter.   The data are averages of duplicate assays.

$$y = \frac{A/0.8 - B/0.8}{100\% - B/0.8} \times 100\% \qquad (1)$$

where y is the % specific retention of supercoiled PM2 DNA that is

either UV-irradiated or AAAF-treated; and A and B are the % retention

of UV- or AAAF-DNA and u-DNA, respectively. The factor of 0.8

takes into account that only 80% of the PM2 DNA were supercoiled.

As will be discussed in a later section, PIII did not bind

efficiently to the nonsupercoiled form of UV- or AAAF-DNA. The

corrected binding curve is shown in Fig. 9. A plateau level of

DNA retention is approached when the amount of PIII in the

assay mixtures exceeds 100 units of UV-DNA-binding activity. The

plateau corresponds to a retention of about 55% of the supercoiled

UV- or AAAF-DNA. It should be noted that equation (1) is only valid

for a saturating amount of PIII. For a nonsaturating amount of PIII,

it leads to an overestimation of the amount of specific binding of

PIII to UV- or AAAF-DNA since the binding affinity of PIII to UV- or

AAAF-DNA is about 30-fold higher than u-DNA (see later section

entitled "Substrate specificity").

Fig. 10 shows the binding curves for PIII determined at a DNA

concentration 16-fold lower than that used in the standard assay

conditions. The DNA was UV-irradiated with a dose of 1,200 J/m^2 or

600 J/m^2. Again, the data points in Fig. 10 are corrected by using

equation (1) to plot the specific binding curves shown in Fig. 11. With

saturating amounts of PIII, the specific binding curve for DNA UV-

irradiated with a dose of 1,200 J/m^2 approaches a plateau which

corresponds to the retention of about 65% of the supercoiled PM2
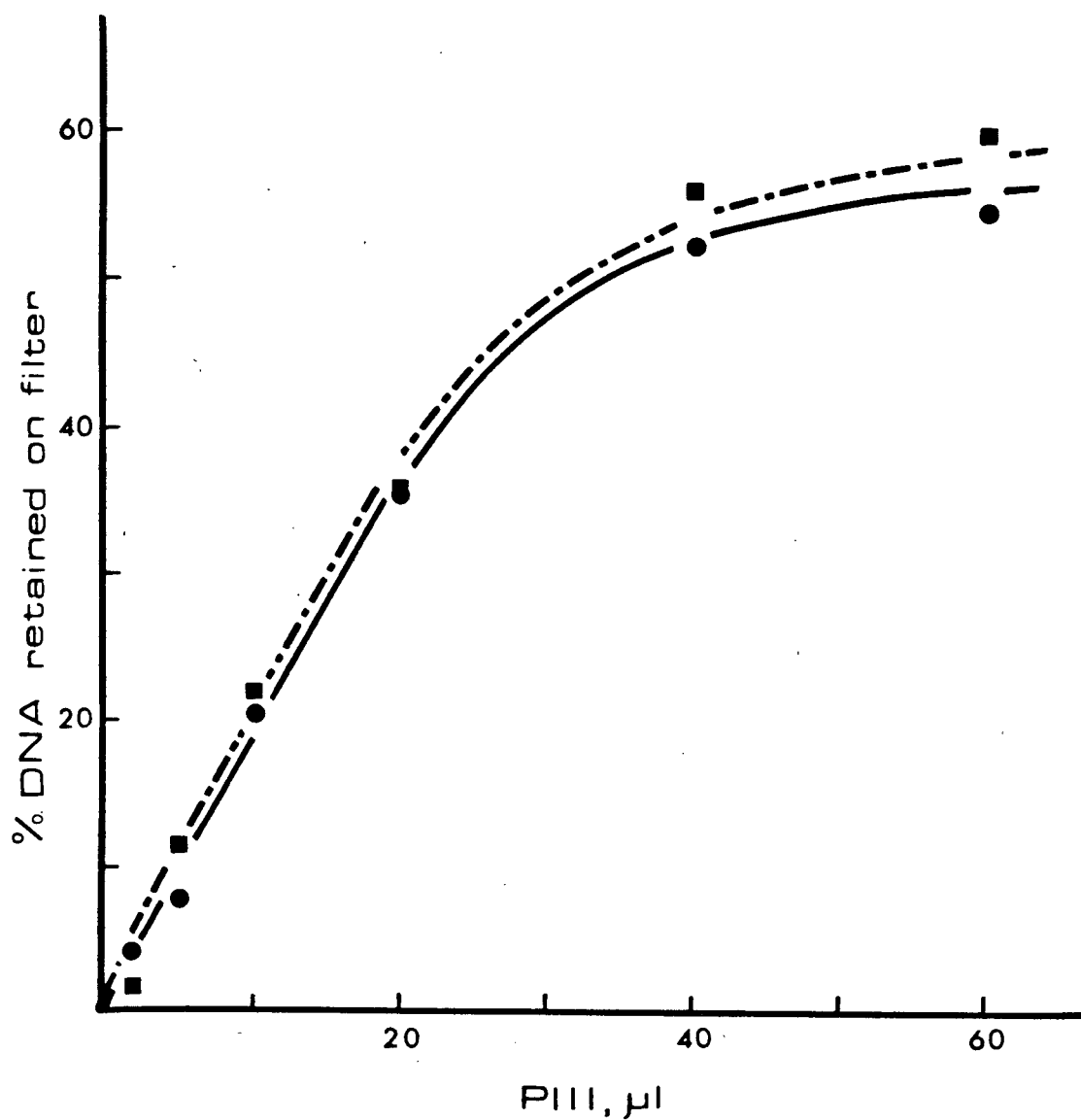
Fig. 9. Specific binding of UV-DNA and AAAF-DNA with various
amounts of PIII.
The data points for UV-DNA (●) or AAAF-DNA (■) were
calculated from the results of Fig. 8 as described in
text.
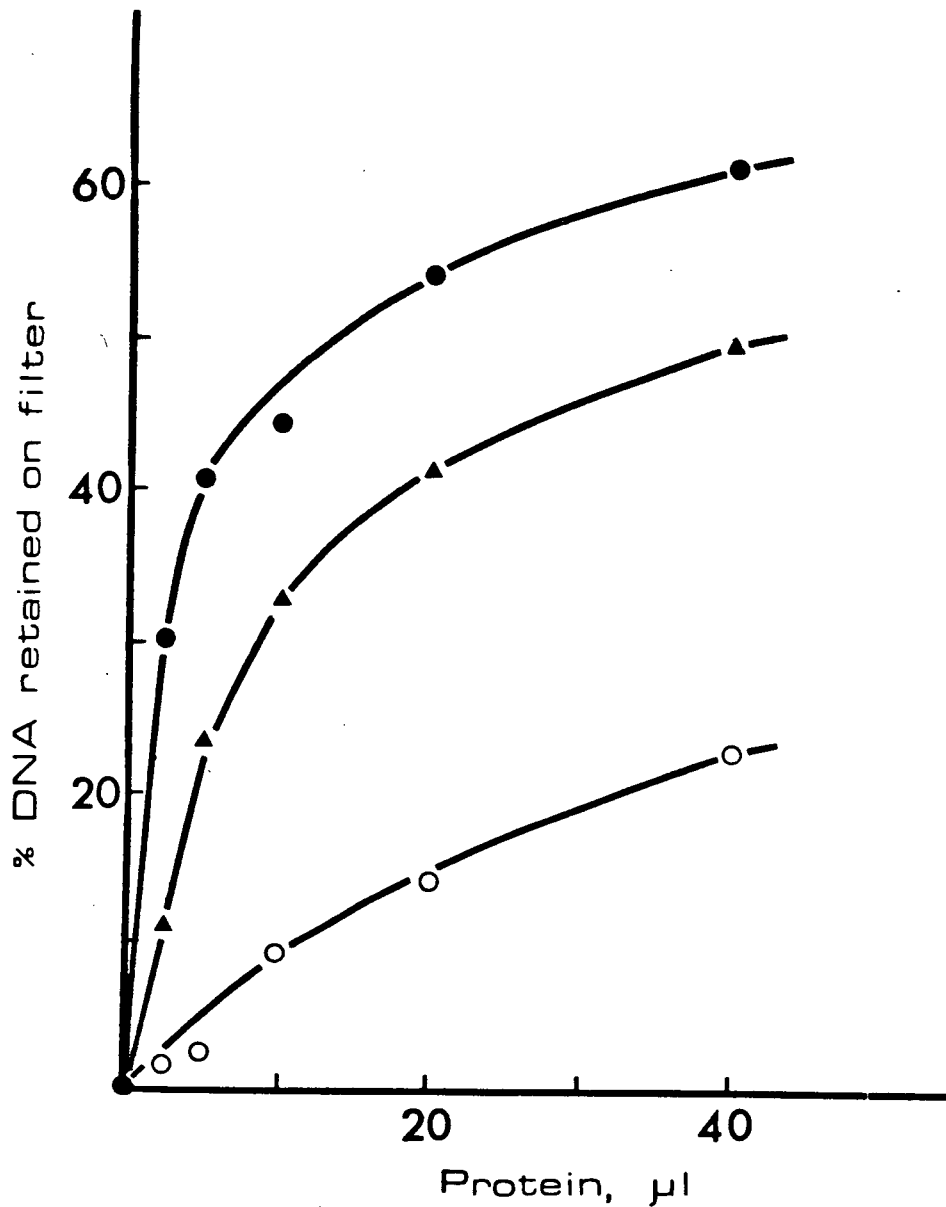
Fig. 10. DNA-binding as a function of the amount of PIII at a
low concentration of DNA substrates.

The DNA-binding assays were performed under the standard
conditions but with 8.7 fmol of PM2 DNA and with various
amounts of PIII as indicated. The UV-DNA-binding
activity of PIII used was 3.3 units/µl. DNA-binding
activity was assayed with PM2 DNA UV-irradiated at
1200 J/m^2 (●), PM2 DNA UV-irradiated at 600 J/m^2 (▲)
and u-DNA (O). Each point is the average of duplicate
assays.
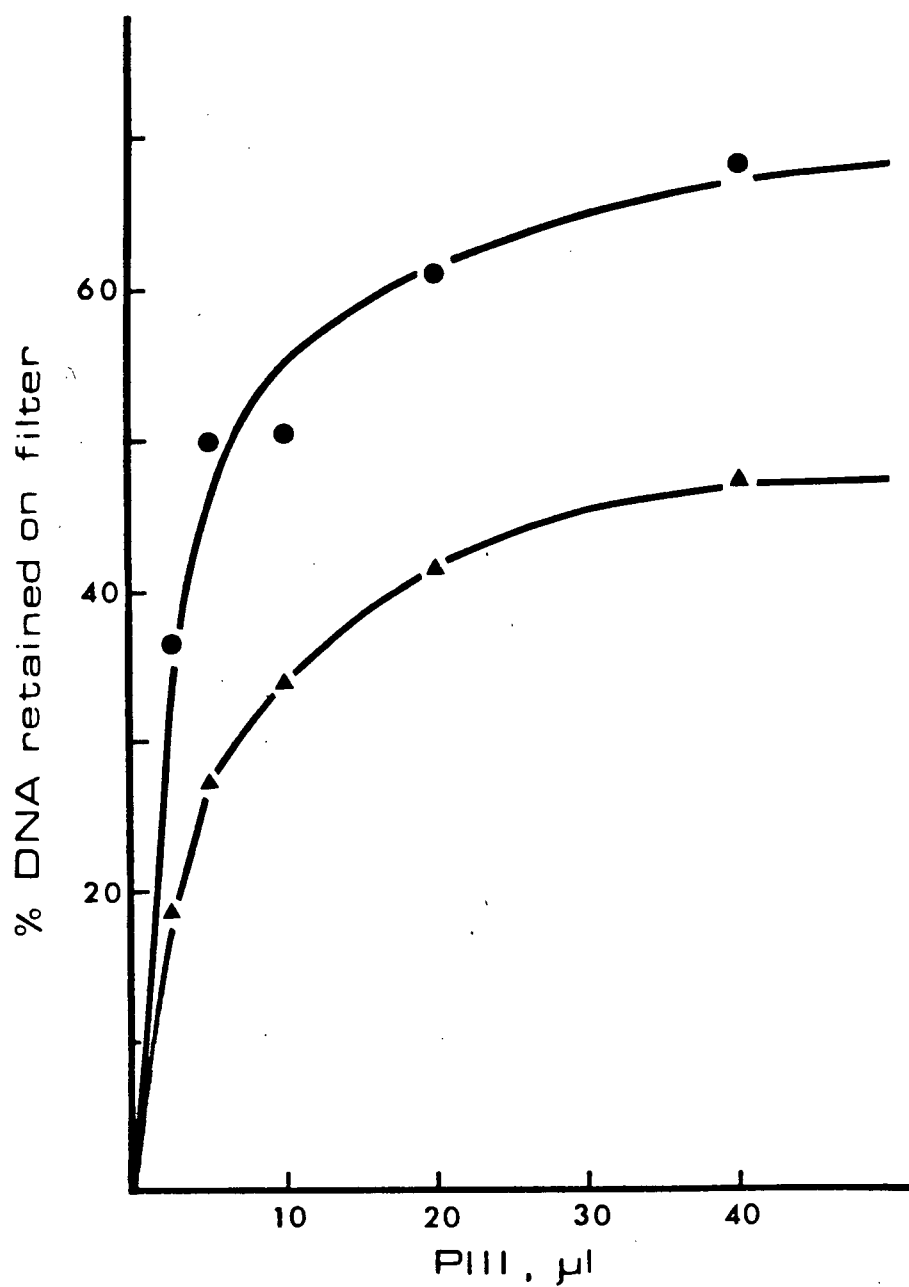
Fig. 11. Specific binding of UV–DNA with various amounts
of PIII at a low concentration of DNA substrates.
The data points for DNA UV-irradiated with 1,200 J/m^2
(●) or 600 J/m^2 (▲) were calculated from the results
shown in Fig. 10 as described in text.

DNA. For DNA UV-irradiated with a lower dose of 600 J/m^2, a lower

plateau value which corresponds to 45% of the supercoiled PM2 DNA is

obtained. These results can be interpreted as follows. After

irradiation of DNA at doses of 1,200 J/m^2 and 600 J/m^2, about 65%

and 45% of the supercoiled DNA contained at least one UV-induced binding

sites for PIII, respectively. This interpretation is only valid if every

PIII-DNA complex formed in the assay mixture was retained by the filter

and if the binding of one PIII molecule was enough to cause the

retention of a PM2 DNA molecule. The first assumption was already

discussed in the previous paragraphs. The second assumption seems to

be valid since DNA-binding is linear at low concentration of PIII,

where DNA is in excess (Fig. 12). If the retention of DNA required more

than one binding event, a sigmoidal binding curve would be expected (56).

The linearity of the binding curve also indicated that the binding of

PIII to DNA is noncooperative.

With higher UV-doses more PIII binding sites per DNA molecule can

be introduced. With a UV-dose of 3,600 J/m^2, about 70% of the total

DNA (or 90% of the supercoiled DNA) was retained on the filters

(Fig. 13). Thus, the leveling off of the binding curves depicted

in Fig. 8-11 was likely due to limited binding sites on the UV- or

AAAF-DNA molecules.

4. Substrate specificity

Fig. 14 illustrates a competition experiment where increasing

amounts of unlabeled UV- or u-DNA were added to the reaction mixture

to compete with a constant amount of labeled AAAF-DNA. With a 32-fold

excess of UV-DNA in the assay mixture, the binding of PIII to AAAF-DNA

was nearly eliminated. This suggested that PIII is a single protein
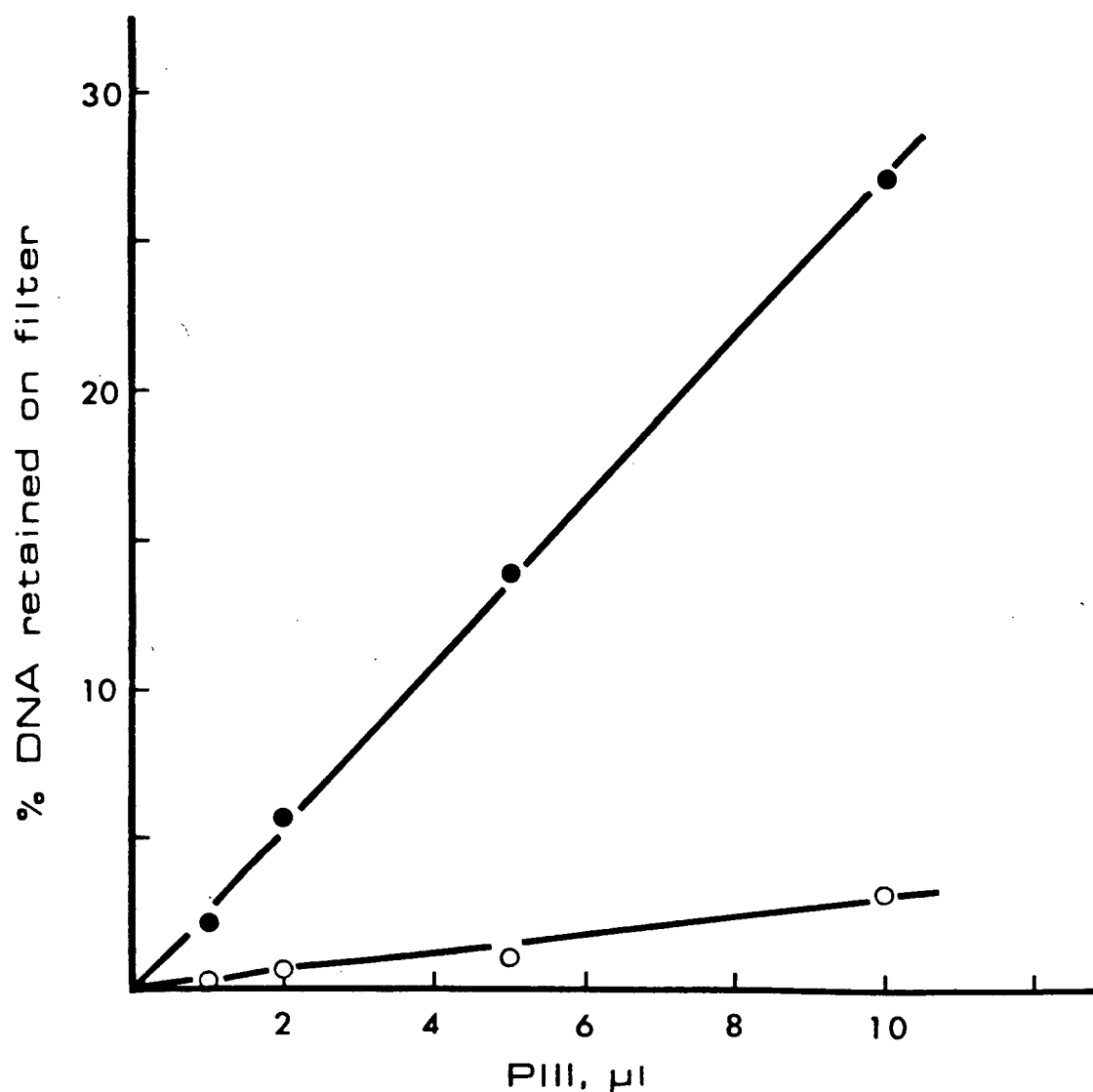
Fig. 12. DNA-binding curve at low concentration of PIII.
The DNA-binding assays were performed under the
standard conditions with various amounts of PIII.
PIII used has a UV-DNA-binding activity of 2.5
units/μl. The binding substrates were UV-DNA
(●) or u-DNA (O). Each point is the average
of duplicate assays.
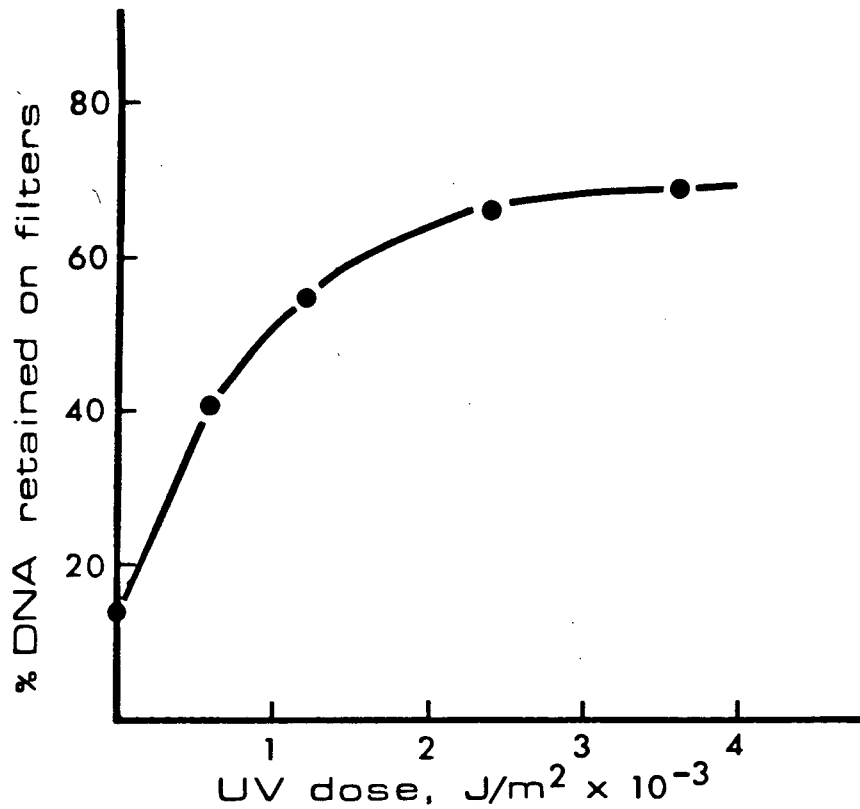
Fig. 13. Binding of PIII to DNA irradiated with high UV–doses .
The DNA-binding assays were performed under the standard
conditions with 8.7 fmol of UV-irradiated PM2 DNA. Each
assay was carried out with an aliquot of PIII containing
66 units of UV–DNA–binding activity.
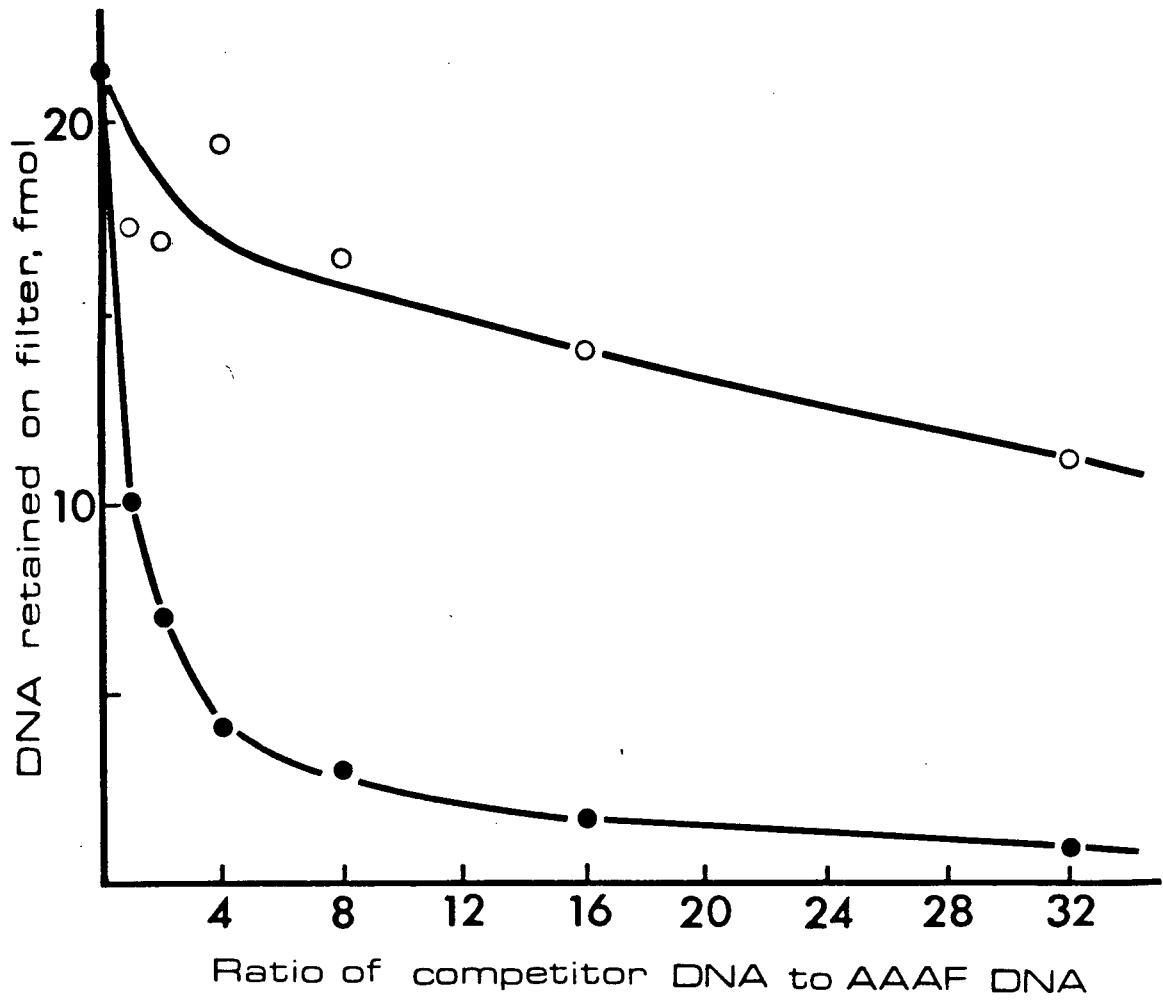
Fig. 14. AAAF-DNA-binding activity of PIII in the presence of
competitor DNA.
[3]H-AAAF-DNA binding activity of PIII was assayed
under the standard conditions in the presence of
various amounts of unlabeled UV-DNA (●) or u-DNA
(O).

which binds to both UV-DNA and AAAF-DNA. PIII binds less efficiently

to u-DNA since a 32-fold excess of u-DNA reduces the AAAF-DNA-binding

activity by 50%. A reciprocal plot of the data of the competition

experiment was made according to Spillman et al. (58) to determine the

relative binding affinity of PIII to the different DNA substrates

(Fig. 15). With UV-DNA as the competitor, the reciprocal plot yields

a straight line with a slope close to 1, indicating that PIII binds to

UV-DNA and AAAF-DNA with the same affinity. With u-DNA as the

competitor, the reciprocal plot yields a straight line with a slope

of about 0.03. Thus, PIII has about 30-fold less binding affinity

to u-DNA than UV- or AAAF-DNA. Since the average number of binding

sites per DNA molecule on the AAAF-DNA and UV-DNA are about equal

(Fig. 9), we can conclude that PIII has the same affinity towards the

AAAF-induced and UV-induced DNA-binding sites.

To investigate the effect of DNA conformation on the binding

activity of PIII, supercoiled AAAF-DNA, UV-DNA and u-DNA were nicked

with bovine pancreatic DNase I or converted to a linear form by

treatment with the restriction endonuclease Msp I. Msp I is an

isoschizomer of Hpa II. Both restriction enzymes recognize the sequence

CCGG. However, in contrast to Hpa II, Msp I cleaves the DNA even if

the internal cytosine is methylated (59, 60). Hpa II makes only one

double-stranded cut per PM2 DNA molecule (61). Sucrose gradient

analyses of PM2 DNA cleaved by Msp I indicated that most of the

circular form of PM2 DNA was converted to the linear form (Fig. 16).

The linear form of PM2 DNA sedimented slightly slower than the nicked

circular form of PM2 DNA. This is analogous to the observation that the

linear RF III form of ϕX174 phage DNA has a smaller S value than the
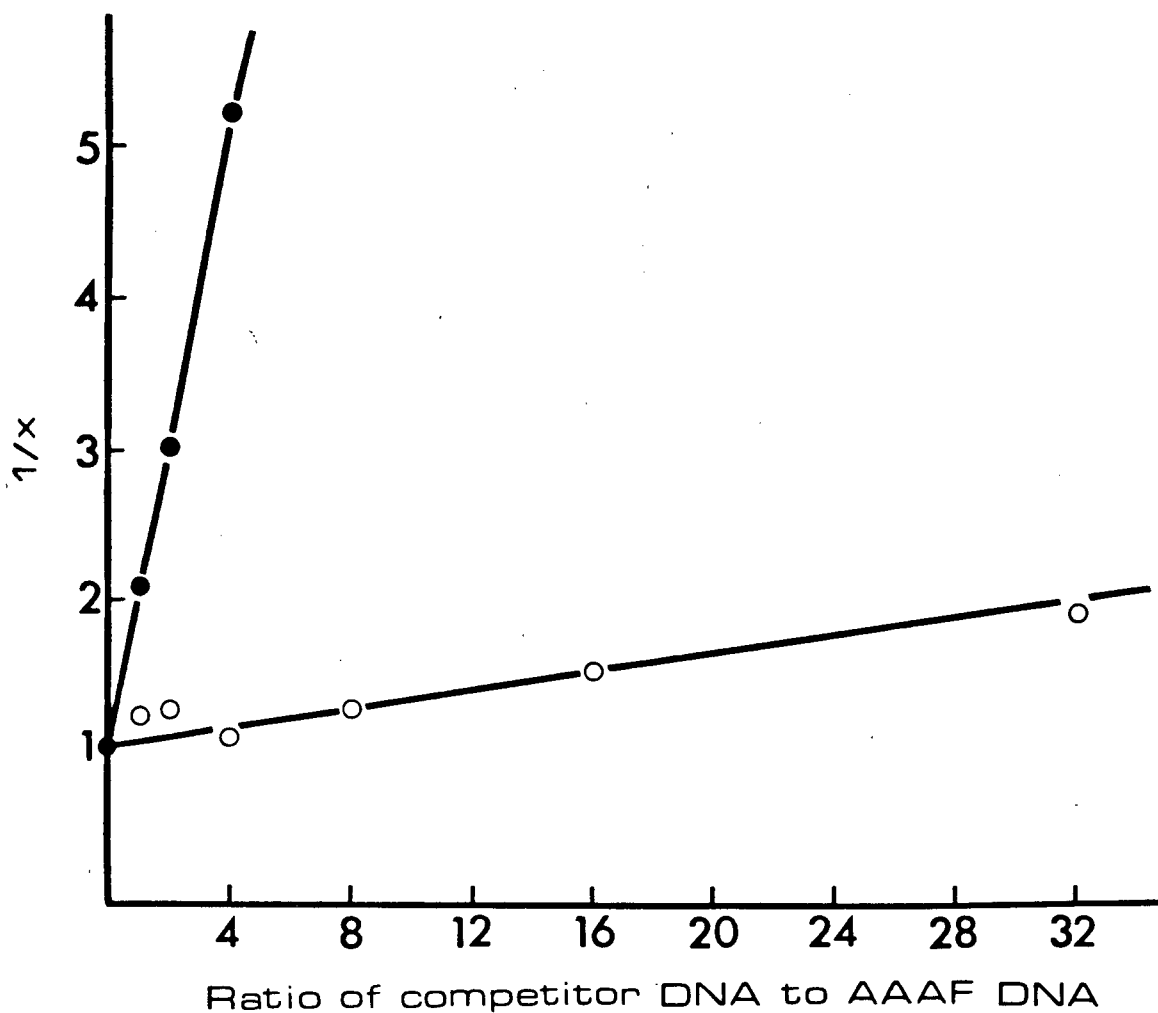
Fig. 15. A reciprocal plot of the data of the competition
experiment depicted in Fig. 14.
x is the ratio of the amount of AAAF-DNA retained
on a filter in the presence of competitor DNA to
the amount of AAAF-DNA retained in the absence of
competitor DNA. Unlabeled UV-DNA (●) or u-DNA
(○) was used as competitors.
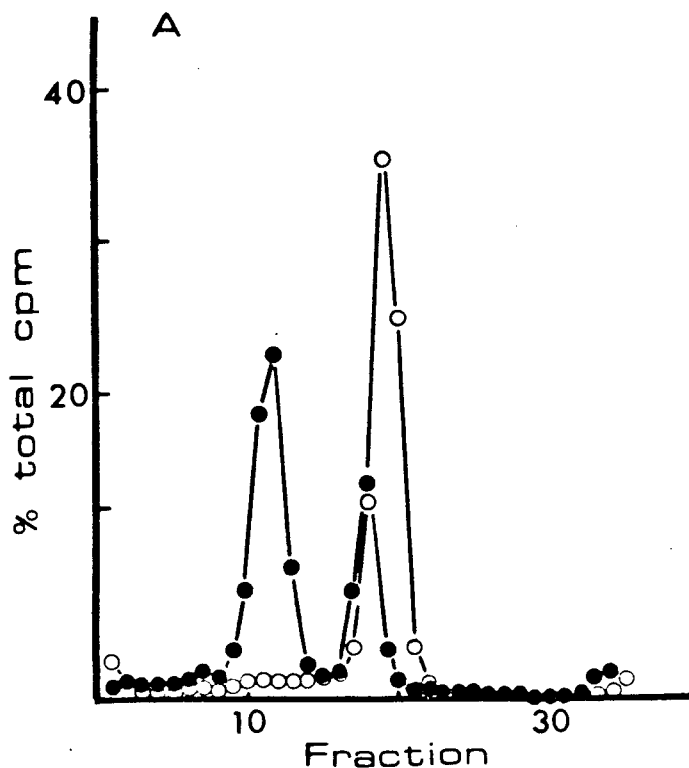
Fig. 16. Sucrose gradient sedimentation of Msp I-treated DNA.
PM2 DNA was treated with Msp I (open symbols) or
without Msp I (filled symbols). The DNA then was
subjected to sucrose gradient sedimentation as
described in Materials and Methods. (A) DNA which
was UV-irradiated with 1200 J/m^2 (●,○). (B) DNA
which was treated with 0.02 mg/ml of AAAF (■,□).
(C) untreated DNA (▲,△). The sedimentation was
from right to left.
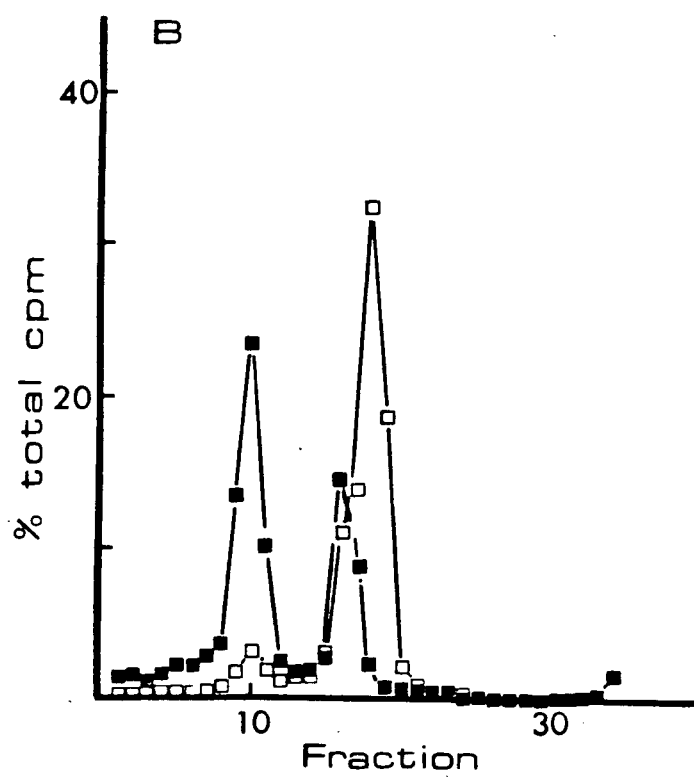
Fig. 16 (B).
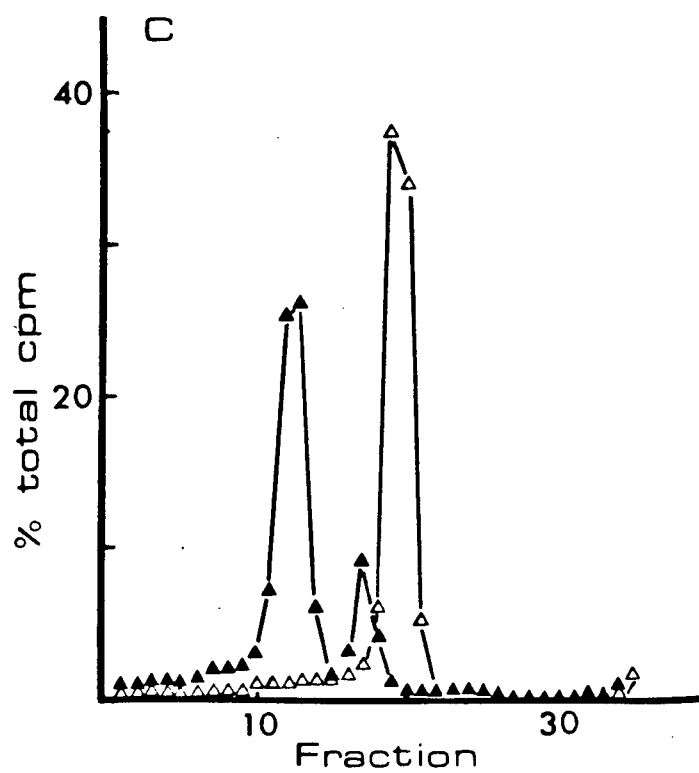
Fig. 16 (C).

nicked circular RF II form (62).

It is clear from Table IV that PIII prefers supercoiled DNA as the binding substrate. PIII binds to the linear or relaxed circular form of UV- or AAAF-DNA 5- to 10-fold less efficiently than to the supercoiled form of DNA.

We have also tested the binding activity of PIII towards DNA treated with two alkylating agents, MNNG and MMS. Using different types of filter-binding assays for DNA lesions, the extent of DNA alkylation can be estimated (49). The levels of alkylation in the various MMS-DNA preparations were similar to or greater than the two MNNG-DNA preparations (Table V). The binding activity of PIII assayed with DNA treated with 1 mM MNNG was about 40% of that assayed with UV- or AAAF-DNA. DNA treated with 5 mM MNNG was at least as efficient a substrate for PIII as UV- or AAAF-DNA. On the other hand, DNA treated with 10-100 mM MMS had little if any binding sites for PIII (Table VI).

PIII did not bind efficiently to depurinated DNA with about 1.5 apurinic sites. The binding activity with single-stranded DNA as a substrate was the same as with u-DNA. The latter result suggests that the single-strandness of the DNA alone does not account for the DNA-binding activity of PIII.

5. Other properties of PIII

DNA-binding activity of PIII was eliminated to a large extent after a treatment with 20 µg/ml of proteinase K at 37°C for 30 min. When incubation at 37°C was omitted, the DNA-binding reaction of PIII was not inhibited, indicating that proteinase K did not interfere with the formation of PIII-DNA complex (Experiment I and II, Table VII). We therefore concluded that the DNA-binding activity of PIII was due

Table IV. Effect of DNA conformation on the DNA-binding
activity of PIII.

Treatment	DNA retained on filter, fmol		
	AAAF–DNA	UV–DNA	u–DNA
−Msp I	15.4 (100%)	15.4 (100%)	1.4 (9%)
+Msp I	3.2 (21%)	1.7 (11%)	0.0 (0%)
−DNase I	—	14.9 (100%)	1.5 (10%)
+DNase I	—	3.5 (23%)	0.6 (4%)

The DNA was treated with UV or AAAF prior to cleavage by
Msp I or DNase I as described in Materials and Methods.
The values in parentheses were % DNA-binding activity
relative to that assayed with supercoiled UV–DNA.

Table V. Estimation of DNA damage on various DNA substrates.

DNA	Average number of nicks (or filter-binding sites) per DNA molecule			
	Assay I	Assay II	Assay III	Assay IV
MNNG–DNA (5 mM)	0.74	1.58	0.88	3.10
(1 mM)	0.40	0.67	0.43	1.12
MMS–DNA (100 mM)	1.23	>3.50	1.67	>3.50
(40 mM)	0.43	2.40	0.85	>3.50
(20 mM)	0.35	1.10	0.62	>3.50
(10 mM)	0.25	0.57	0.36	1.90
u–DNA	0.22	0.20	0.17	0.24
AAAF–DNA	0.37	0.36	0.25	0.48
UV–DNA	0.27	0.34	0.29	0.66

Assay I is the standard nicking assay as described in Materials and Methods. It measures mainly single- and double-stranded breaks in DNA and to a certain extent local distortions of DNA. Assay II is the standard nicking assay except that the DNA is incubated for 45 min at $37^{o}C$ after addition of the alkali denaturation buffer. Assays III and IV were the same as assays I and II, respectively, except that the DNA was incubated at $70^{o}C$ for 7.5 min before the nicking assays were performed. The amount of alkali-labile sites in the DNA can be calculated by subtracting the results of assay I from those of assay II. The amount of heat-induced alkali-labile sites can be estimated by subtracting the results of assay III and the amount of alkali-labile sites from those of assay IV. The amount of DNA breakage detected by the various assays generally increases with increasing level of DNA alkylation. For a discussion of the scope of the various types of DNA damage that can be measured with these assays and the limitations of the assays, see Kuhnlein et al. (49).

Table VI. Substrate specificity of PIII.

Substrate	% activity
UV-DNA	100
AAAF-DNA	100
u-DNA	12
MNNG-DNA (5 mM)	97
(1 mM)	39
MMS-DNA (100 mM)	10
(40 mM)	15
(20 mM)	12
(10 mM)	10
depurinated DNA	18
single-stranded DNA	10

DNA-binding activities were assayed under the standard conditions
with the various DNA substrates. 15-20 units (UV-DNA-binding
activity) of PIII were used in these experiments. The data for
UV-DNA, depurinated DNA and single-stranded DNA are the averages
of duplicate assays. The various DNA substrates were prepared as
described in Materials and Methods. The binding activity
towards single-stranded DNA has been multiplied by two since
binding to one double-stranded DNA molecule gives twice the
amount of radioactivity as the binding of one single-stranded
DNA molecule.

Table VII. Sensitivity of PIII to proteinase K and RNase A
treatments

Experiment	Treatment	Incubation at 37°C, min	DNA retained on filter, fmol	
			UV–DNA	u–DNA
I	–Proteinase K	30	14.2	2.8
	+Proteinase K	30	0.3	1.0
II	–Proteinase K	0	20.8	4.0
	+Proteinase K	0	20.1	3.5
III	–RNase A	30	7.6	1.1
	+RNase A	30	7.3	0.8

Proteinase K solutions (1 mg/ml) were prepared in a buffer
containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and 1 mM $CaCl_2$.
This solution was further diluted to 0.2 mg/ml with 10 mM
Tris-HCl, pH 7.5, and 0.1 M NaCl. Aliquots of PIII were
incubated with 20 µg/ml of proteinase K or 100 µg/ml of
RNase A at 37°C for various periods of time as indicated.
The treated aliquots of PIII were then assayed for DNA
binding activities under the standard conditions.

to protein molecules. In addition, RNA appears not to be involved in the DNA-binding by PIII, since PIII is not sensitive to RNase A (Experiment III, Table VII).

The presence of 1-7 mM $MgCl_2$ or $MnCl_2$ in the assay mixtures did not markedly affect the binding of PIII to UV-DNA and u-DNA. (Fig. 17).

The binding activity of PIII was also measured with the assay mixture buffered with either Tris-HCl or potassium phosphate at different pH values. PIII bound to UV- or u-DNA to a similar extent at pH values between 6 and 9 (Fig. 18). Variation of the incubation temperature of the reaction mixture between $0^{o}C$ and $37^{o}C$ did not affect the DNA-binding activity of PIII significantly (Table VIII).

The DNA-binding activity of PIII is quite resistant to freeze-thaw treatment. In two separate experiments, less than 25% of the UV-DNA-binding activity of PIII was lost by freezing and thawing several times (Table IX). PIII is also quite heat stable. When incubated for 50 min at $60^{o}C$ in a buffer containing 5 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.5 mM EDTA, 0.5 mM DTT, 55% glycerol and 100 μg/ml of β-lactoglobulin, PIII lost only 30-40% of its binding activity to UV-DNA (Fig. 19). The inactivation of the binding activity of PIII to u-DNA was faster, a loss of 70-80% of the binding activity was observed in 50 min at $60^{o}C$. This result suggests that the UV- or AAAF-DNA-binding activity of PIII may be contaminated with a protein (or proteins) which binds only to u-DNA. The latter may be purified from the former by glycerol gradient sedimentation (see later section and Fig. 23).

Three chemicals were found to inhibit the DNA-binding activity
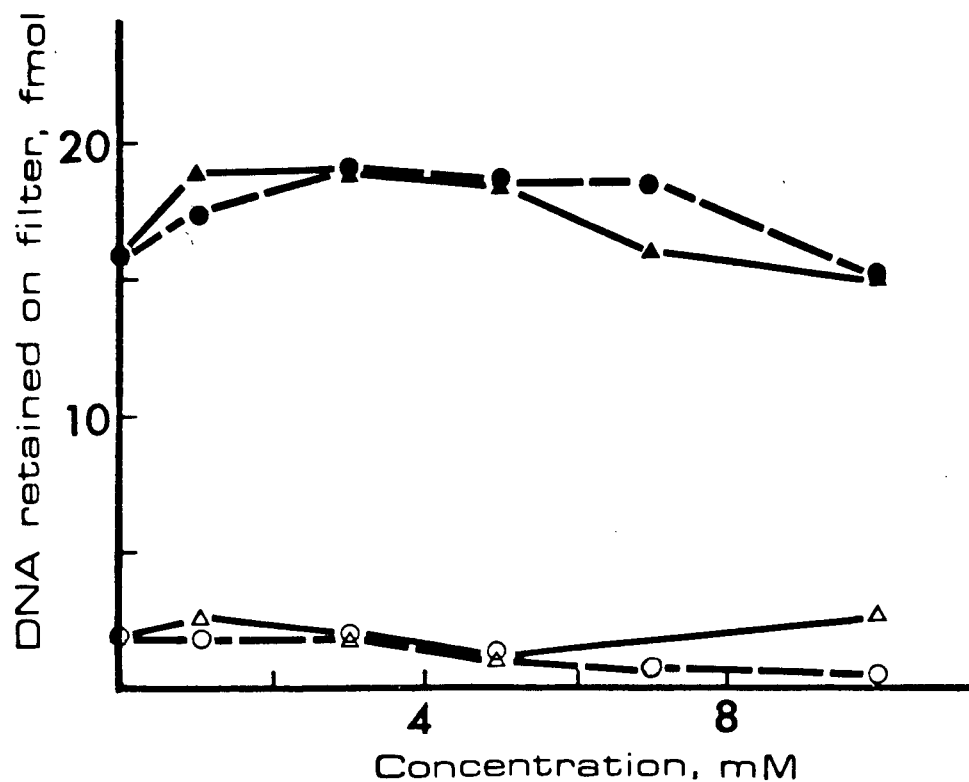
Fig. 17. Effect of $MgCl_2$ and $MnCl_2$ on the binding activity of
PIII.
The DNA-binding assays were performed under the standard
conditions in the presence of various concentrations of
$MgCl_2$ (●,○) or $MnCl_2$ (▲,△) in the assay mixtures.
DNA-binding activity was assayed with UV-DNA (●,▲) or
u-DNA (○,▲).
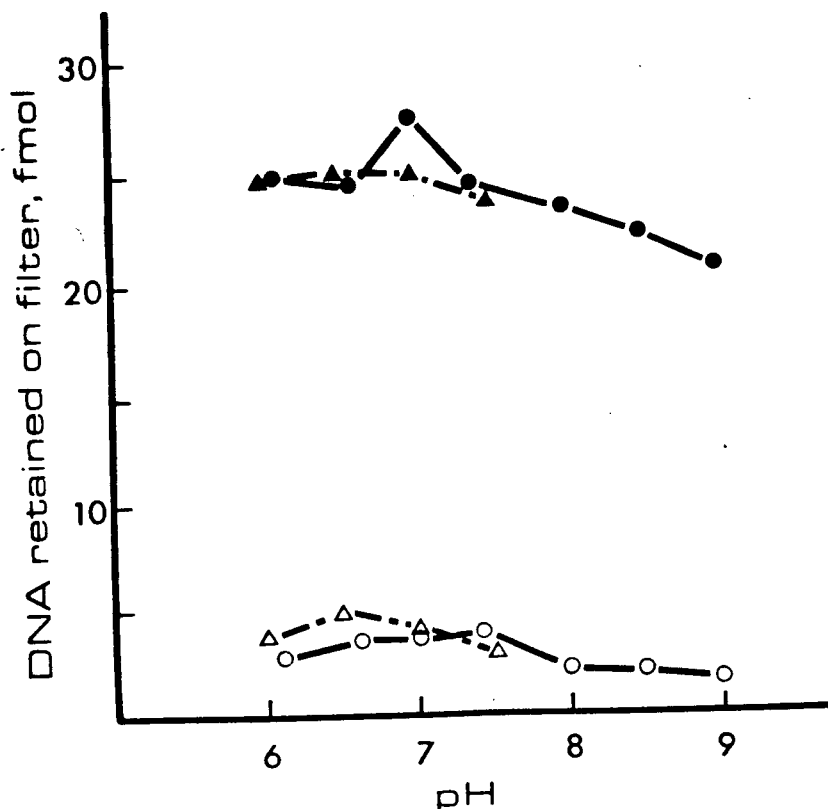
Fig. 18. DNA-binding activity of PIII: pH dependence.
DNA binding reactions were carried out under the standard
conditions except that the reaction mixtures were
buffered with 10 mM Tris-HCl (●,O) or potassium
phosphate (▲,△) at the indicated pH values. Each
reaction was performed with an aliquot of PIII
containing about 25 units of UV-DNA-binding activity.
After incubation for 10 min on ice, each assay mixture
was diluted with 1.7 ml of 100 mM NaCl and filtered
under the standard conditions. The binding substrates
were UV-DNA (●,▲) or (O,△). The pH values of the
buffers were measured at 50 mM salt concentration at
room temperature (22°C). The pH values of the Tris-HCl
buffers at 0°C will be about 0.7 higher (Trizma, Sigma
Technical Bulletin No. 106B).

Table VIII. Effect of temperature on the DNA-binding activity
           of PIII.

Temperature (^{o}C)	DNA retained on filter, fmol	
	UV-DNA	u-DNA
0	23.3	3.5
22	20.4	2.8
37	23.2	3.9

The DNA-binding assays were carried out under the standard
conditions except that the assay mixtures were incubated
at various temperatures as indicated. The data are averages
of duplicate assays.

Table IX. Freeze-thaw stability of PIII.

Experiment	Freeze-thaw treatment	DNA retained on filter, fmol	
		UV-DNA	u-DNA
I	−	25.4	2.8
	+	19.6	2.5
II	−	7.1	0.8
	+	5.4	0.8

Aliquots of PIII were frozen in liquid nitrogen and thawed with
cold running water. The freeze-thaw procedure was repeated five
times in experiment I and three times in experiment II. The
freeze-thaw treated aliquots and untreated aliquots of PIII were
then assayed for DNA-binding activity. The data are the averages
of duplicate assays.
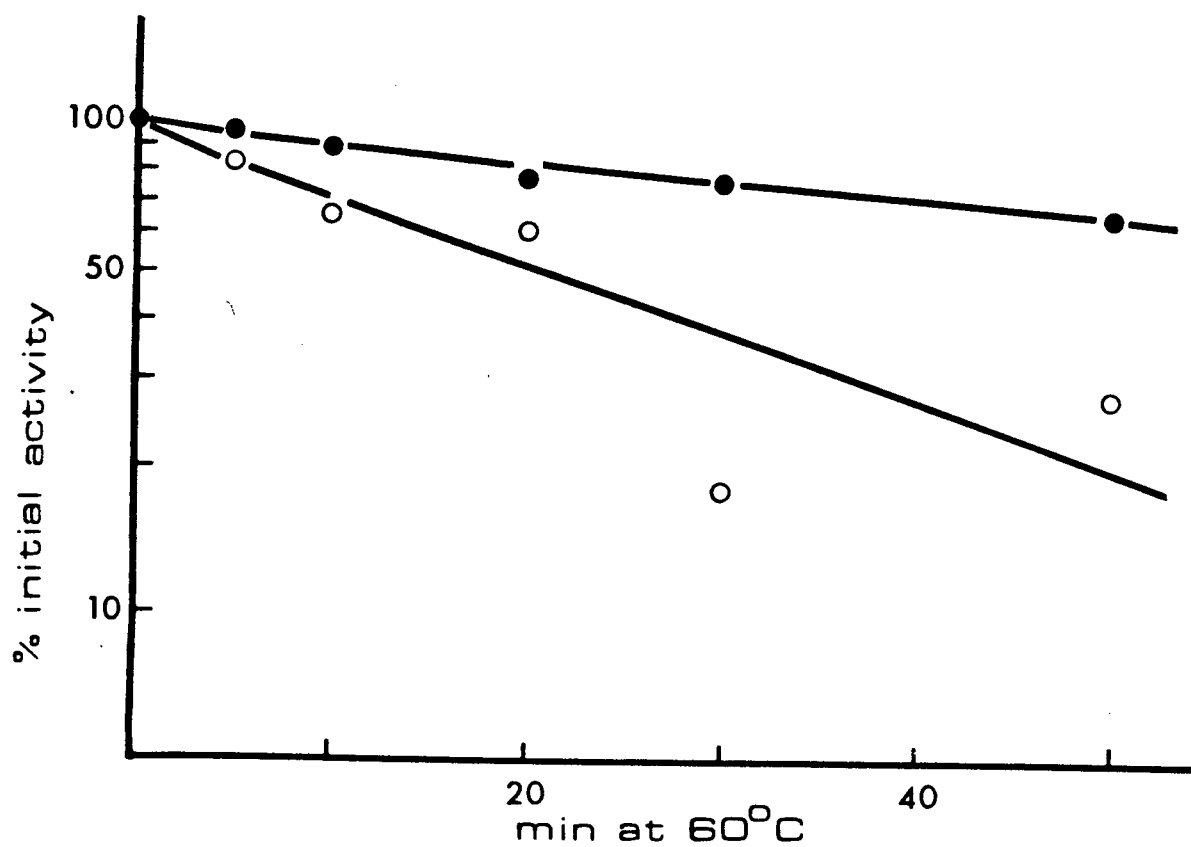
Fig. 19. Heat sensitivity of PIII.
Aliquots of PIII were heated at 60°C for various
periods of time and then assayed for DNA-binding
activity with UV-DNA (●) or u-DNA (O) as the
binding substrates. Initially, each aliquot of
PIII contained about 21 units of UV-DNA-binding
activity per reaction mixture.

of PIII to UV-DNA and u-DNA. They were ATP, caffeine and sucrose.

In the presence of 10 mM ATP, the binding of PIII to UV-DNA was reduced by 40% and the binding of PIII to u-DNA was reduced to nearly zero (Fig. 20). In this regard, PIII is different from the *uvrA* protein of *Escherichia coli* whose binding activity to UV-DNA is stimulated by the presence of ATP and GTP.

Caffeine, which is known to inhibit DNA repair and bind to single-stranded regions of DNA (63, 64), is an inhibitor of PIII binding (Fig. 21). The inhibitory effect of caffeine was greater with AAAF-DNA and u-DNA than with UV-DNA. 3 mM caffeine inhibited the UV-DNA-binding activity of PIII by about 35%, the AAAF-DNA-binding activity by nearly 70% and the u-DNA-binding activity by about 60%. In the experiment shown in Fig. 21, the DNA was preincubated with caffeine for 30 min before the addition of PIII. Similar results were obtained in experiments where neither PIII nor DNA were preincubated with caffeine or where PIII rather than the DNA was preincubated with caffeine for 30 min. It is likely that caffeine binds to DNA and thereby alters or masks the DNA-binding sites for PIII. This is suggested by the observation of a differential effect of caffeine on the binding of PIII to AAAF-DNA and UV-DNA.

The binding activity of PIII was also inhibited by sucrose. The presence of 5% sucrose in the assay mixture reduced the UV-DNA-binding activity by half and the u-DNA-binding activity to nearly zero. On the other hand, glycerol had little effect on the DNA-binding activity of PIII (Table X). Based on these observations, sedimentation experiments were performed with glycerol gradients rather than sucrose gradients.
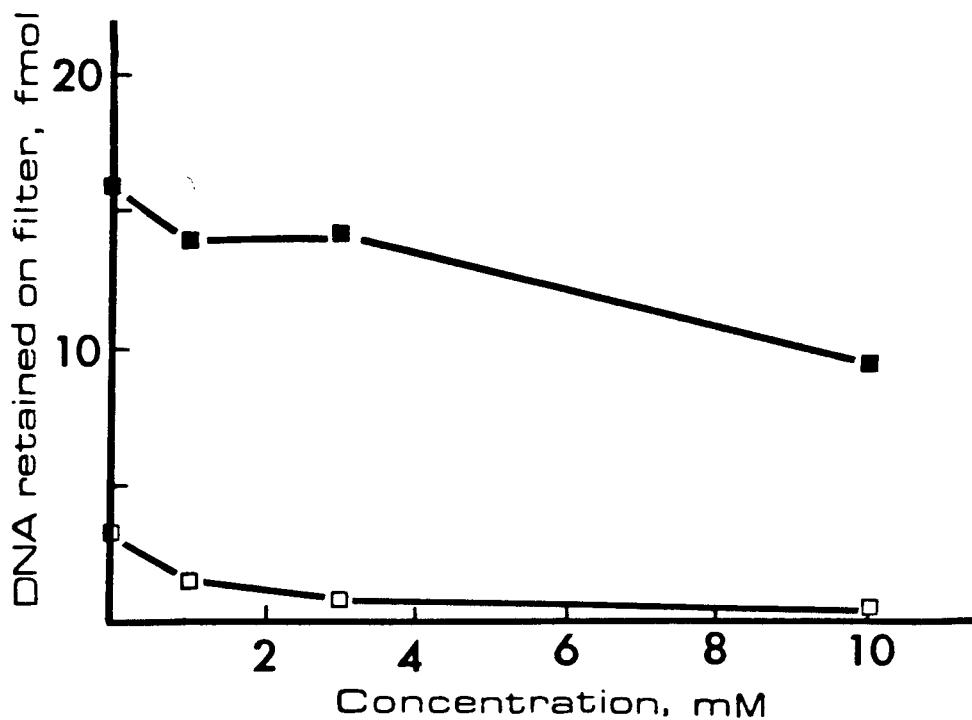
Fig. 20. Effect of ATP on the DNA-binding activity of PIII.
DNA-binding assays were carried out under the
standard conditions in the presence of various
concentrations of ATP. The binding substrates
were UV-DNA (●) or u-DNA (O). Each point is the
the average of duplicate assays.
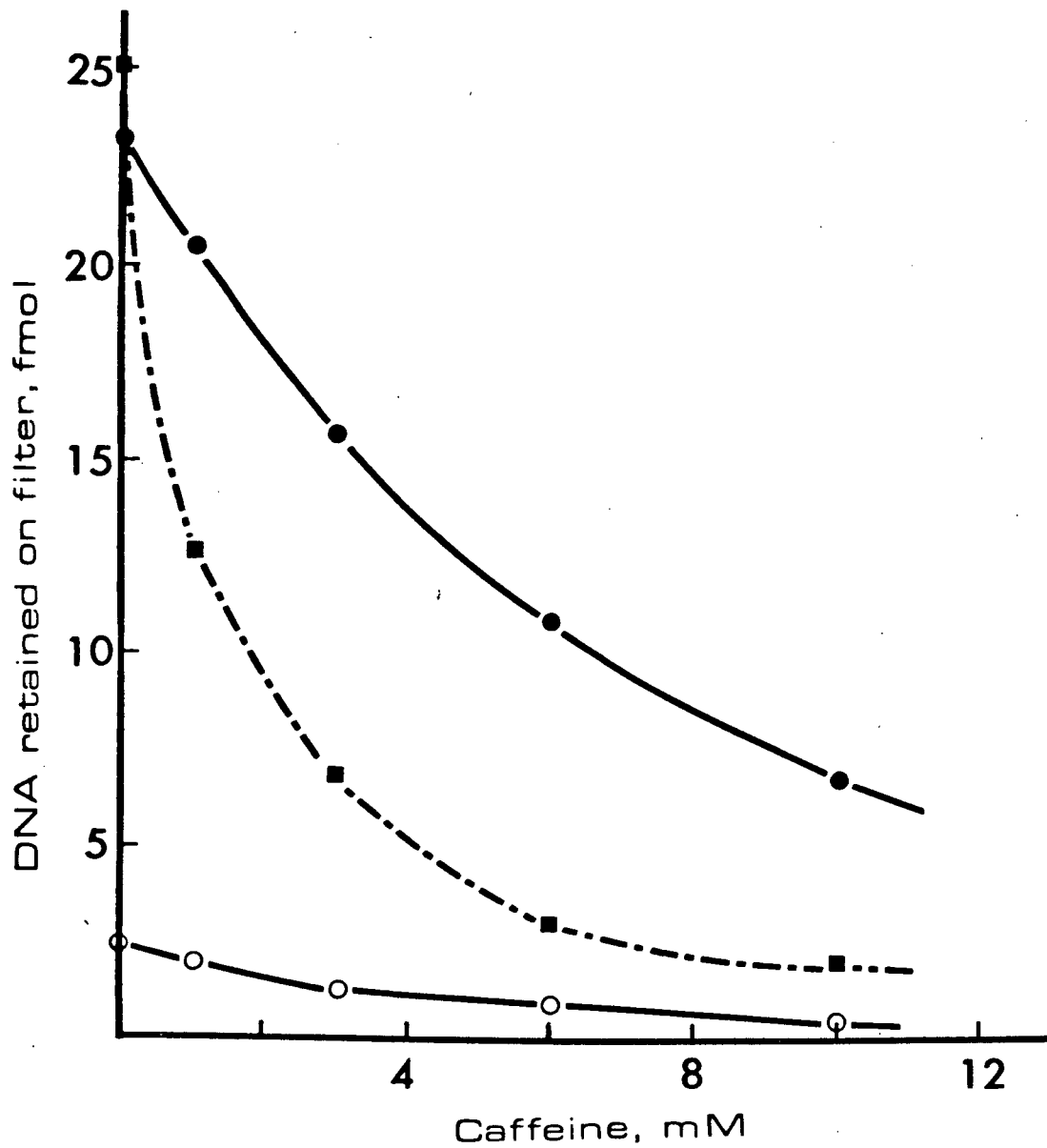
Fig. 21. Effect of caffeine on the binding activity of PIII. Caffeine at the indicated concentrations was added to the standard assay mixture and incubated with UV-DNA (●), AAAF-DNA (■) or u-DNA (○) for 30 min on ice. Afterwards, PIII was added and the DNA-binding assays were carried out under the standard conditions.

Table X.   Effects of sucrose and glycerol on the DNA-binding
activity of PIII.

		DNA retained on filter, fmol	
		UV–DNA	u–DNA
% sucrose	0	12.2	2.4
	5	6.9	0.3
	10	2.6	0.6
	20	0.7	0.8
% glycerol	10	13.6	1.4
	30	11.5	1.7

The binding assays were carried out under the standard
conditions except that the assay mixtures also contained
various amounts of sucrose or glycerol as indicated.

The experiments described above were carried out at a saturating level of DNA damage. At nonsaturating level of DNA damage, the dependence of the DNA-binding activity of PIII on NaCl, temperature, $MgCl_2$, $MnCl_2$ or pH was similar. However, the inhibitory effect of ATP on the binding activity of PIII to UV-DNA or u-DNA was more pronounced and amounted to a 60% inhibition at 3 mM ATP when the DNA-binding assays were carried out with DNA UV-irradiated with 580 J/m^2 and aliquots of PIII each containing 34 units of UV-DNA-binding activity.

6. Glycerol gradient sedimentation analysis of PIII

In order to get an estimate of the molecular weight of the DNA-binding protein, aliquots of PIII were analysed by sedimentation in 10-30% glycerol gradients. 100 µg/ml of bacitracin was included in the glycerol gradient solution to stabilize the DNA-binding activity of PIII during the sedimentation. Bacitracin is an antibiotic produced by *Bacillus licheniformis*. It consists of a mixture of closely related polypeptides. The major component is a cyclic dodecapeptide called bacitracin A with a molecular weight of about 1,500 (65). Bacitracin was chosen because of its low molecular weight. A preliminary experiment indicated that the presence of 100 µg/ml of bacitracin in PIII did not affect the DNA-binding activity of PIII.

Fig. 22 shows that PIII sedimented through a 10-30% glycerol gradient containing 0.15 M NaCl with a sedimentation coefficient of 2.0-2.5 S relative to the marker proteins. A similar sedimentation coefficient was obtained when the centrifugation was carried out in 0.5 M NaCl in the gradient (Fig. 23). The ratio of the AAAF- or UV-DNA-
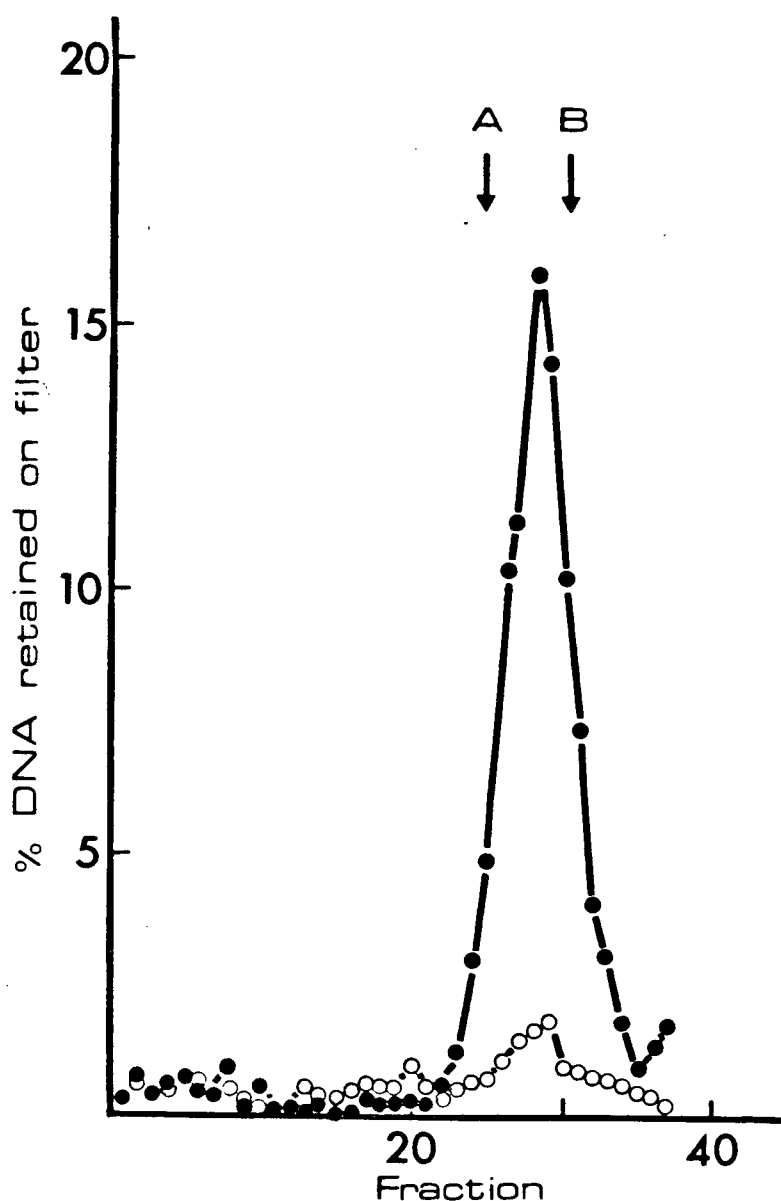
Fig. 22. Sedimentation velocity analysis of PIII in the presence
of 0.15 M NaCl.

A 200-µl aliquot of PIII in 10 mM Tris-HCl, pH 7.5,
1 mM EDTA, 1 mM DTT, 3.3% glycerol, 0.3 M NaCl and
100 µg/ml of β-lactoglobulin was sedimented for
17 h through a 10-30% glycerol gradient containing
0.15 M NaCl as described in Materials and Methods.
37 fractions were collected. A 50-µl aliquot from
each fraction was assayed for binding activity
towards UV-DNA (●) or u-DNA (O). Marker proteins
were: A, ovalbumin; B, cytochrome C. The
sedimentation was from right to left.
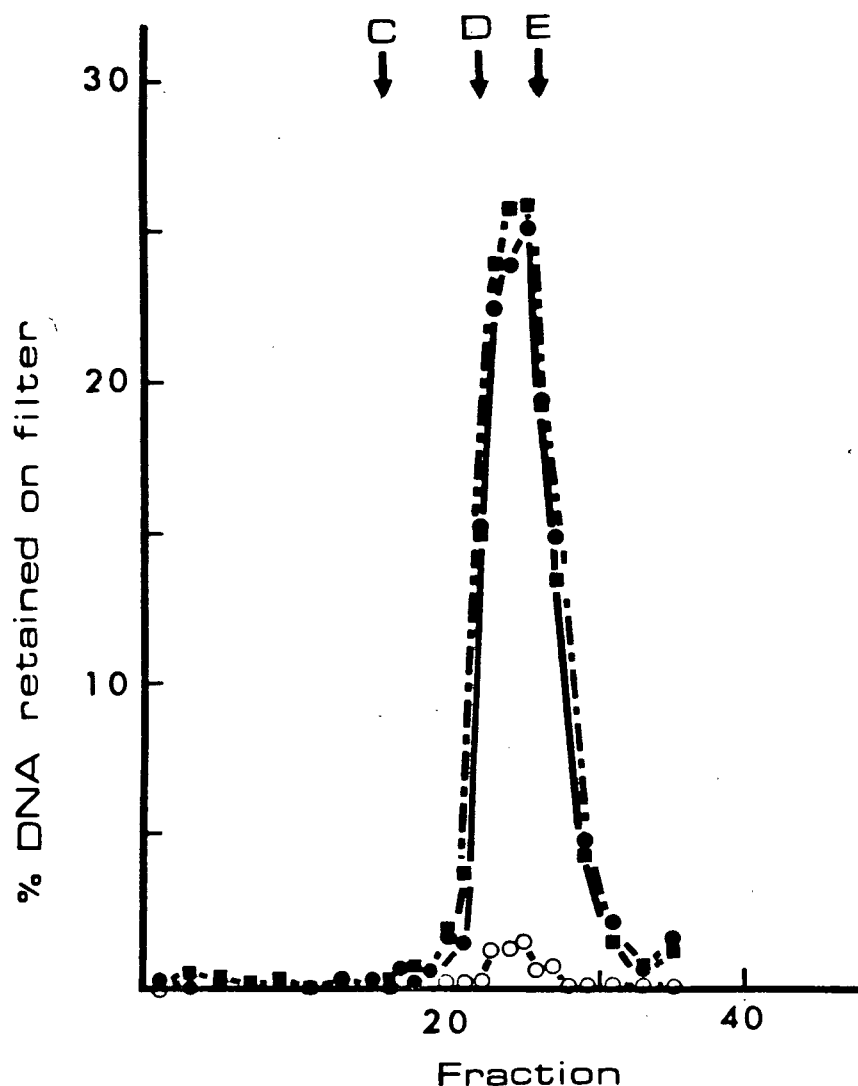
Fig. 23. Sedimentation velocity analysis of PIII in the presence
of 0.5 M NaCl.
A 200-µl aliquot of PIII in 10 mM Tris-HCl, pH 7.5,
1 mM EDTA, 1 mM DTT, 5% glycerol, 0.5 M NaCl
and 100 µg/ml of β-lactoglobulin was sedimented
for 27 h through a 10-30% linear glycerol gradient
containing 0.5 M NaCl as described in Materials
and Methods. 35 fractions were collected. A
30-µl aliquot from each fraction was assayed for
binding activity towards UV-DNA (●), AAAF-DNA
(■) or u-DNA (○). Marker proteins were: C,
bovine serum albumin; D, α-chymotrypsin; and E,
myoglobin. The sedimentation was from right to left.

binding activity to the u-DNA binding activity of PIII increased from 10:1 in the former experiment to 20:1 in the latter experiment, indicating a further purification of the specific binding activity for AAAF-DNA and UV-DNA. Assuming the protein is spherical, the molecular weight of PIII was estimated to be 20-25,000. The recovery of the DNA-binding activity from these glycerol gradients was greater than 90%.

7. Characterisation of the PIII-DNA complex

Glass fibre filters are commonly used to retain protein or DNA precipitates. We therefore investigated the possibility of DNA precipitation in our assays. An aliquot of PIII containing 60 units of UV-DNA-binding activity was incubated with UV-DNA under the standard conditions. The assay mixture was then centrifuged for 10 min at 10,000 g. It was found that all the DNA remained in solution.

PIII-DNA complex was also analysed by sedimentation through 10-30% glycerol gradients in the presence of 0 mM NaCl, 50 mM NaCl and 150 mM NaCl. Two major forms of PM2 DNA were resolved by velocity gradient sedimentation: the faster sedimenting covalently-closed circular supercoiled form and the nicked, relaxed circular form. Incubation with PIII did not induce any detectable changes in the sedimentation profiles of UV-DNA (Fig. 24-26). PIII bound primarily to the supercoiled form of UV-DNA but had much less binding activity towards the nicked form of PM2 DNA (Inserts, Fig. 24-26). In the absence of NaCl, there was very little if any dissociation of the bound PIII from the supercoiled DNA (Insert, Fig. 24). In the presence of 50 mM NaCl, half of the bound PIII molecules dissociated from the UV-DNA during the 2 h of sedimentation (Insert, Fig. 25); and in the presence

% UV-DNA retained on filter	
+ PIII	− PIII
56.6 (14–17)	0.4 (14–17)
5.4 (20–22)	1.9 (20–22)
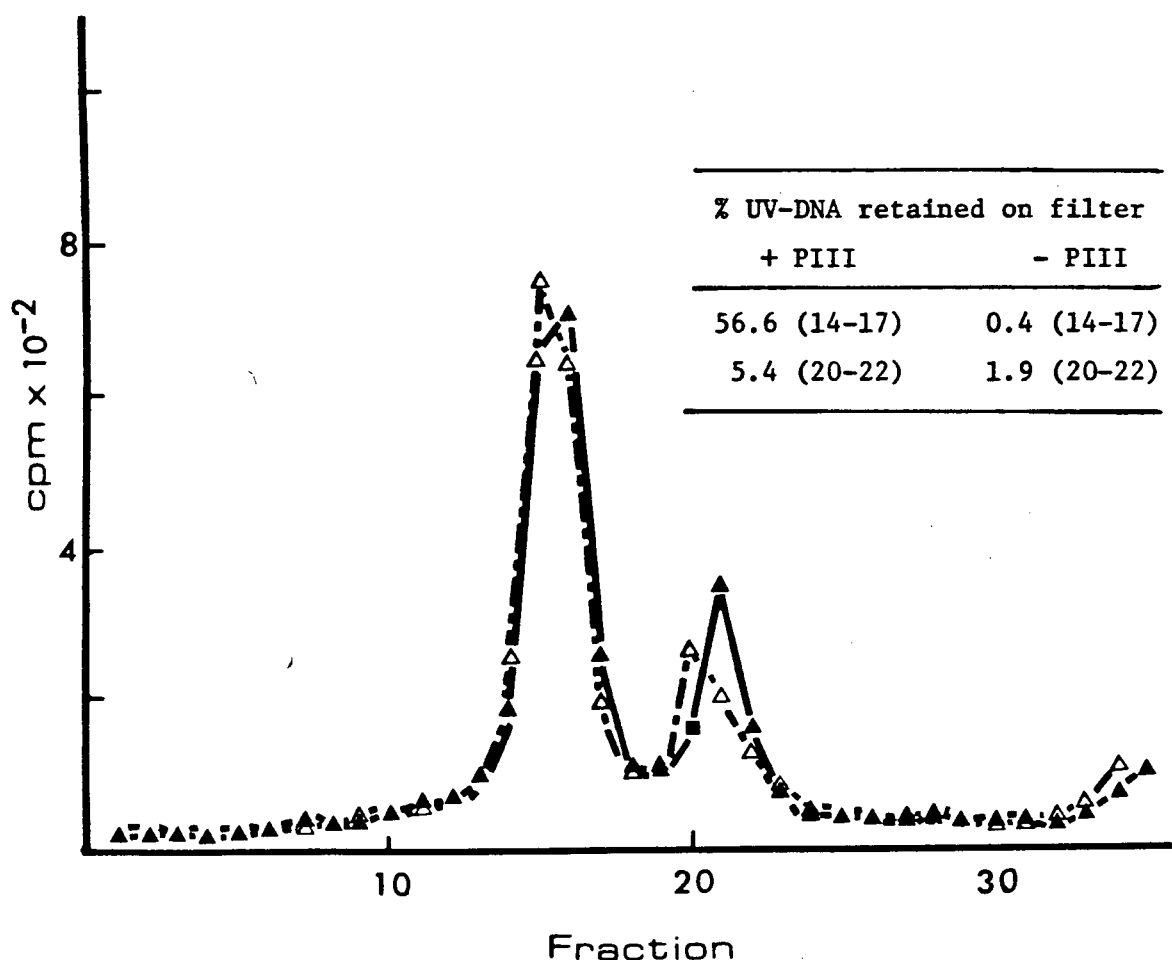
Fig. 24. Sedimentation of PIII-UV-DNA complex in 10-30% glycerol and 0 mM NaCl.

UV-DNA was incubated with an aliquot of PIII containing 80 units of UV-DNA-binding activity (▲) or without PIII (△) under the standard conditions. 200 μl of the assay mixture was sedimented through a 10-30% glycerol gradient in the absence of NaCl for 3 h as described in Materials and Methods. The sedimentation was from right to left. In the experiment where the UV-DNA was incubated with PIII, 35 fractions were collected. In the experiment where the UV-DNA was incubated without PIII, 34 fractions were collected. A 50-μl aliquot from each fraction was was assayed for radioactivity. The background (25 cpm) was not subtracted. The remaining portions of the peak fractions were filtered over GF/C filters to measure the amount of PIII-DNA complexes and the results were shown in the insert. Numbers in parentheses in the insert indicated the fraction numbers.

% UV-DNA retained on filter	
+ PIII	− PIII
19.6 (20-23)	−0.2 (20-23)
3.8 (24-26)	0.5 (24-26)
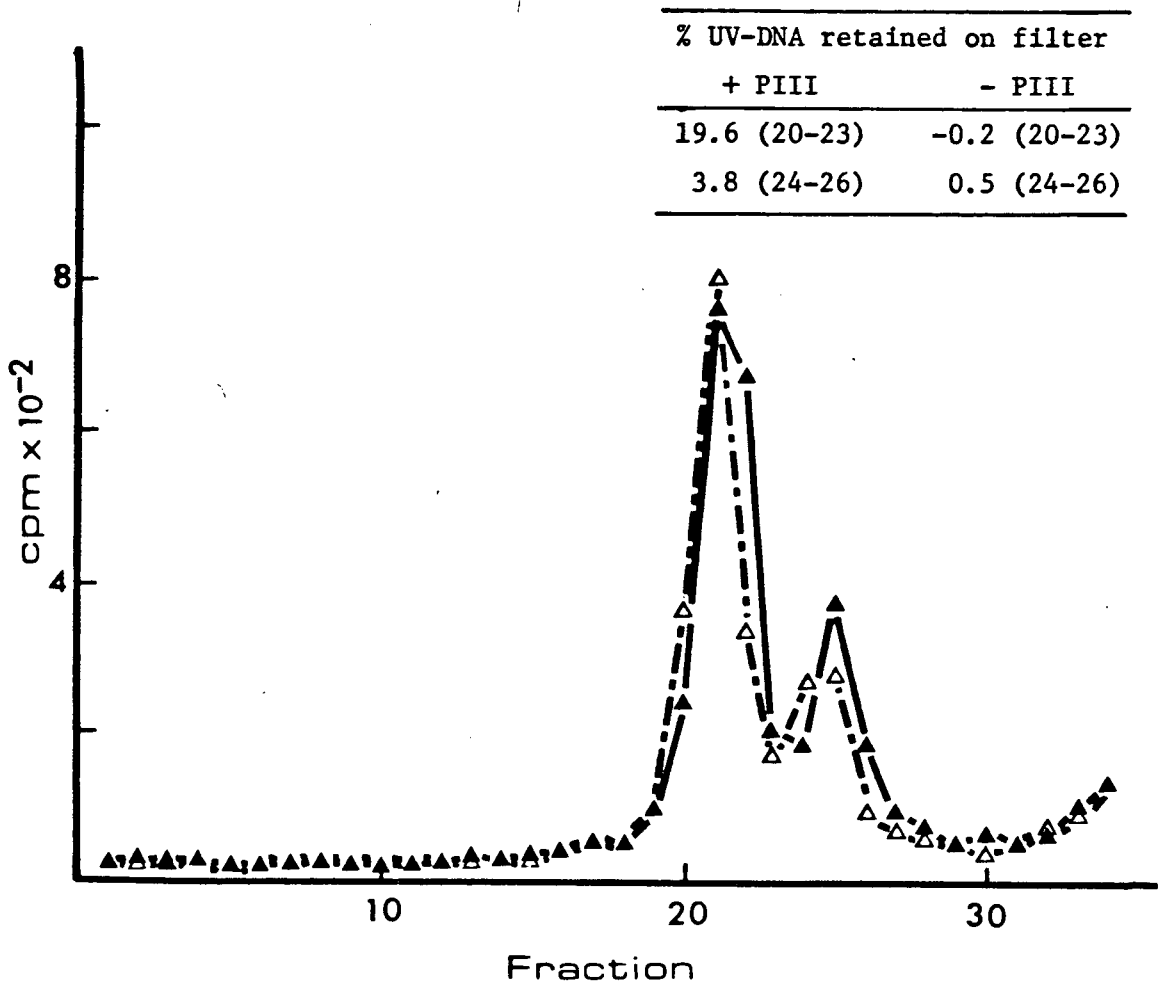
Fig. 25. Sedimentation of PIII-UV-DNA complex in 10-30% glycerol and 50 mM NaCl.
The experiment was performed as described in the legend to Fig. 24 except that the glycerol gradient solution contained 50 mM NaCl in addition to the other ingredients and the sedimentation was for 2 h. 34 fractions were collected for each sedimentation analysis.

% UV-DNA retained on filter	
+ PIII	− PIII
2.5 (18-21)	−0.2 (17-20)
1.6 (23-25)	2.2 (21-23)
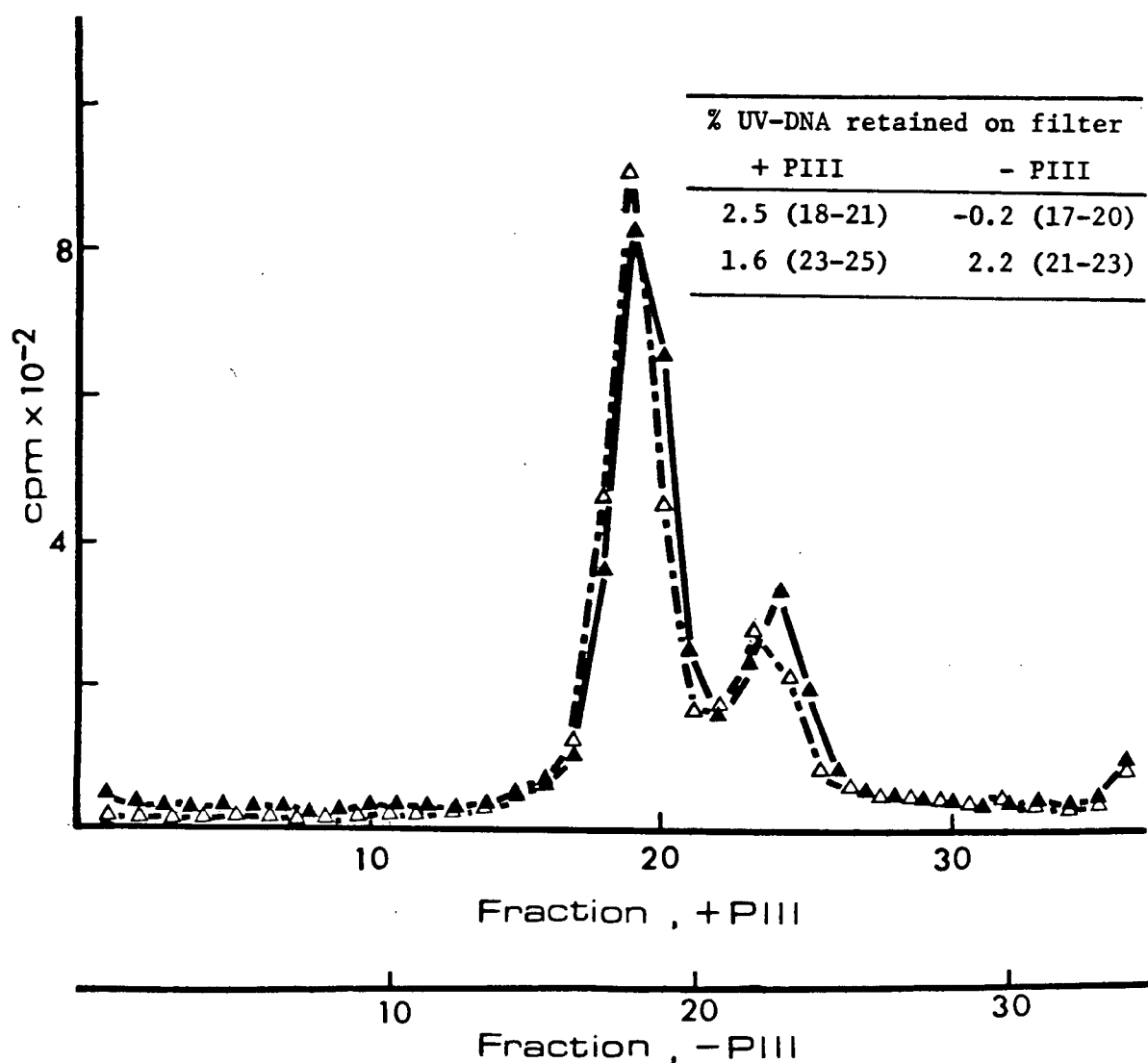
Fig. 26. Sedimentation of PIII-UV-DNA complex in 10-30% glycerol
and 150 mM NaCl.
The experiment was performed as described in the
legend to Fig. 24 except that the glycerol gradient
solution contained 150 mM NaCl in addition to the
other ingradients and the centrifugation was for
2 h. In the experiment where UV-DNA was incubated
with PIII (▲), 36 fractions were collected. In
the experiment where the UV-DNA was incubated
without PIII (△), 34 fractions were collected.
To faciliate comparison of the two sedimentation
profiles, the fraction numbers of the latter
experiment were plotted on a different scale.

of 150 mM NaCl, the dissociation was nearly complete in 2 h (Insert, Fig. 26). The effect of salt on the dissociation of PIII from nicked DNA was qualitatively similar. These results do not contradict the finding that the specific binding of PIII to UV-DNA was optimal between 100-200 mM NaCl as illustrated in Fig. 3. Sedimentation separated the free molecules of PIII from the DNA and thereby favored the dissociation of PIII-DNA complex. Thus the binding of PIII to UV-DNA was reversible and did not involve the formation of covalent bonds between PIII and DNA. Covalent bonds between proteins and DNA are formed by adenovirus terminal-protein (43, 44), *Escherichia coli* topoisomerase I (66) and the DNA untwisting enzyme of rat liver (67).

PIII-DNA complex was also analysed for undamaged DNA in the absence of NaCl. Again, the binding of PIII did not alter the sedimentation profile of the DNA (Fig. 27) and there was a strong binding preference for supercoiled DNA (Insert, Fig. 27). Sedimentation analyses for PIII-u-DNA complex in the presence of NaCl was not performed.

Incubation of UV-DNA with PIII for 45 min at 37°C in the presence of 3 mM ATP, 7.5 mM MgCl$_2$ and 1 mM DTT also did not induce any change in the sedimentation profile of the DNA (Fig. 28), nor did it render the complex more stable (Insert, Fig. 28).

The reversibility of the binding of PIII to UV-DNA was also evident from the competition experiment shown in Fig. 29. The results indicate that PIII dissociates from the UV-DNA very slowly after it is bound. The amount of the radioactive PIII-UV-DNA complex was only reduced by

| % u–DNA retained on filter	
+ PIII	–PIII
24.2 (13–17)	1.0 (13–17)
4.4 (19–21)	2.9 (19–21)
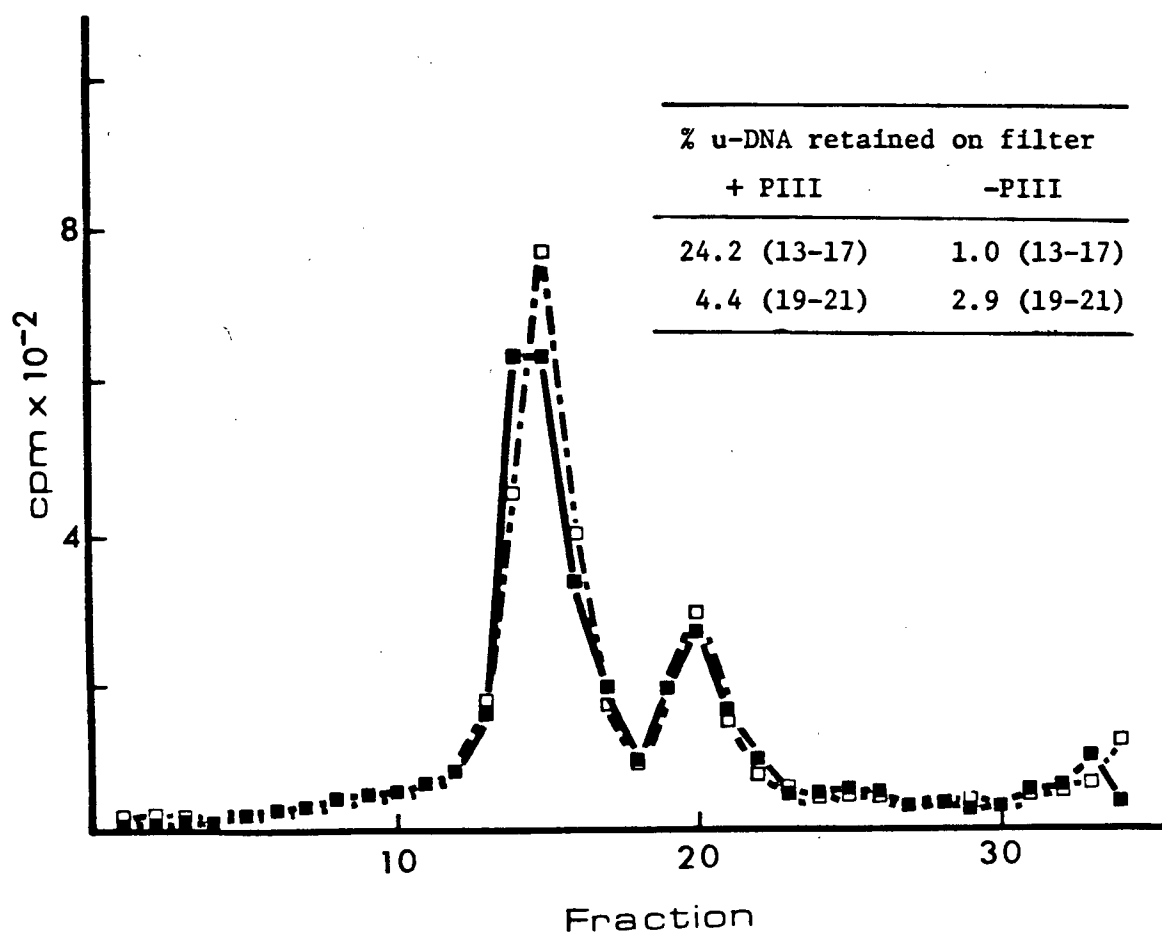
Fig. 27. Sedimentation of PIII–u–DNA complex in 10–30% glycerol and 0 mM NaCl.
The experiment was performed as described in the legend to Fig. 24 with u–DNA incubated with (■) or without PIII (□). 34 fractions were collected for each sedimentation analysis.

% UV-DNA retained on filter	
+ PIII	− PIII
18.3 (19–22)	0.1 (19–22)
4.2 (23–25)	2.5 (23–25)
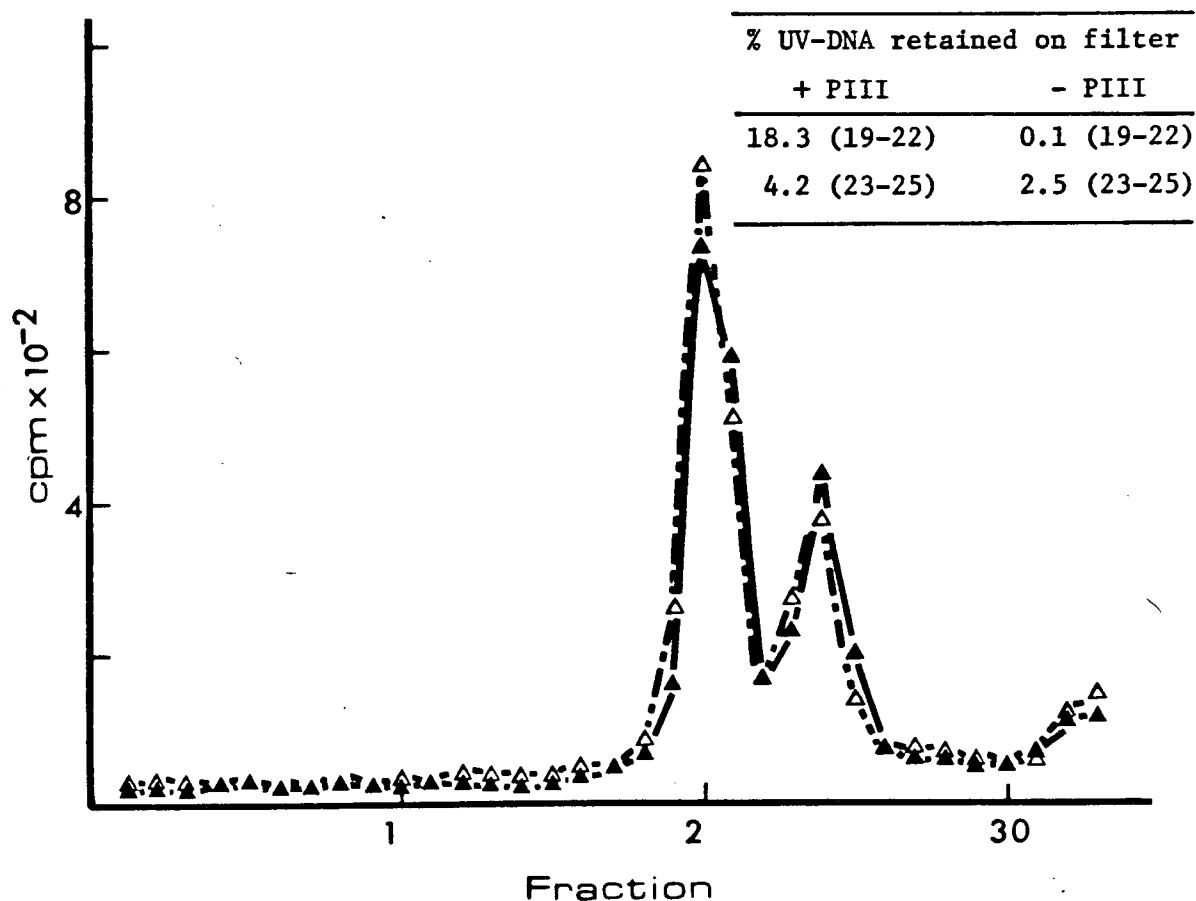
Fig. 28. Sedimentation of PIII-UV-DNA complex formed in the
presence of ATP and $MgCl_2$.
139 fmol fo UV-DNA was incubated with 80 units
(UV-DNA binding activity) of PIII (▲) or without
PIII (△) in 300-μl of buffer containing 10 mM
Tris-HCl (pH 7.5), 3 mM ATP, 7.5 mM $MgCl_2$ and
1 mM DTT. After incubation for 45 min at 37°C, a
200-μl aliqout was subjected to sedimentation
analysis as described in the legend to Fig. 25.
33 fractions were collected for each sedimentation
analyses.
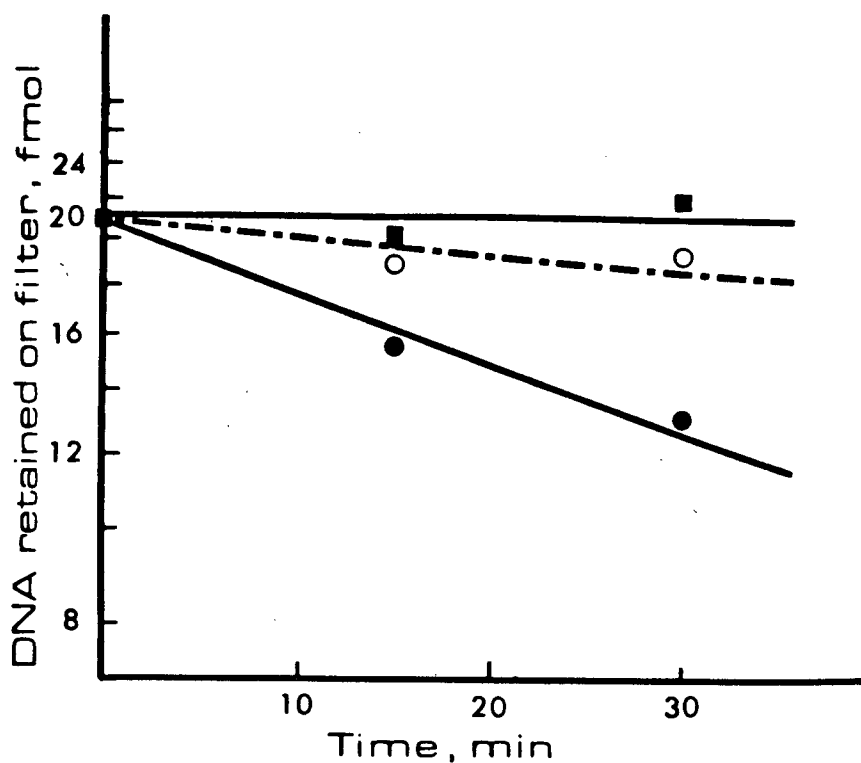
Fig. 29. Reversibility of the binding of PIII to UV-DNA.
Aliquots of PIII were incubated with labeled UV-DNA
under the standard conditions. Prior to filtration,
the assay mixtures were further incubated for various
periods of time with 5 μl of 10 mM Tris-HCl, pH 7.5,
(■) or 5 μl of 10 mM Tris-HCl, pH 7.5, containing
1.39 pmol of unlabeled PM2 DNA. The unlabeled DNA
was either unirradiated (O) or UV-irradiated with
1200 J/m² (●). Each point is the average of duplicate
assays.

40% when incubated with a 10-fold excess of unlabeled UV-DNA at 0°C for 30 min. Assuming first order kinetics, the dissociation can be described by the equation:

$$- \ln \frac{B}{B_o} = k_2 t \qquad (2)$$

where k_2 is the rate constant for the dissociation of the PIII-UV-DNA complex; t is the time of incubation; B_o is the amount of PIII-DNA complex at time 0; and B is the amount of complex at time t. The estimate for k_2 is about 3×10^{-4} sec^{-1}, which corresponds to a half-life of the complex of about 40 min.

8. Catalytic activity

No significant amount of endonuclease or glycosylase activity was detected in aliquots of PIII under the various conditions described in Tables XI and XII.

Incubation of UV- or u-DNA under the standard conditions with aliquots of PIII containing 80 units of UV-DNA-binding activity did not produce any material soluble in 6% trichloroacetic acid. UV-DNA, u-DNA or linear PM2 DNA which had been incubated with PIII for 45 min at 37°C in the presence of 10 mM Tris-HCl, pH 7.5, 33 mM NaCl and 5 mM $MgCl_2$ also was not soluble in 6% trichloroacetic acid. Thus, under these two conditions, PIII does not exhibit any exonuclease activity or behave like certain glycoproteins which solubilize DNA in dilute trichloroacetic acid without degrading the DNA (68, 69). No ATPase activity was detected with aliquots of PIII containing 50 units of UV-DNA-binding activity in the presence of UV-DNA, u-DNA or single-stranded PM2 DNA.

Table XI. Assay for DNA endonuclease activity.

	% DNA nicked	
	UV-DNA	u-DNA
+PIII	21.7	19.3
-PIII	19.7	18.8

Aliquots of 40 units (UV-DNA-binding activity) of PIII were incubated for 30 min at 37°C with 139 fmol of UV-DNA or u-DNA in a 300 μl of reaction mixture containing 10 mM Tris-HCl, pH 7.5, 33 mM NaCl and 5 mM $MgCl_2$. Afterwards, assays for DNA endonuclease activity were performed as described in Materials and Methods. The data are the averages of duplicate assays.

Table XII. Assays for UV-DNA endonuclease and glycosylase
activities under various conditions.

| MgCl$_2$ | DTT | ATP | % UV-DNA nicked | | | |
| | | | endonuclease assay | | glycosylase assay | |
	mM		+ PIII	- PIII	+ PIII	- PIII
0	0	0	28.2	28.9	47.9	48.3
0	0.1	0	30.5	29.9	43.1	44.0
2	0	0	30.7	30.1	42.1	42.3
2	0.1	0	30.6	30.0	41.4	40.3
5	0.1	0	30.7	29.5		
5	0.1	3	29.8	30.0		

Aliquots of PIII each containing 40 units of UV-DNA-binding activity
were incubated with 139 fmol of UV-DNA or u-DNA in 300-µl reaction
mixtures containing 10 mM Tris-HCl, pH 7.5, 33 mM NaCl and various
concentrations of MgCl$_2$, DTT and ATP as indicated. Incubations
were at 37°C for 30 min. Afterwards, 50-µl aliquots were assayed
for DNA nicking or alkali-labile sites as described in Materials
and Methods. The amounts of DNA nicking and alkali-labile sites
introduced by PIII were taken as a measure of the endonuclease
and glycosylase activities, respectively. The data are
the averages of duplicate assays.

It is possible that PIII may unwind the DNA double helix adjacent to the DNA binding site. We have therefore studied the effect of PIII on the susceptibility of UV-DNA and u-DNA to cleavage by the single-stranded specific endonuclease from *Neurospora crassa*. This endonuclease attacks supercoiled circular DNA, probably by recognizing some unpaired regions in the DNA (62, 70, 71). It preferentially nicked UV-irradiated DNA rather than unirradiated DNA (72). It has been shown that the destabilization of the DNA double helix by the *rep* protein of *Escherichia coli* faciliates cleavage of the DNA by the *Neurospora crassa* endonuclease (73). Fig. 30 shows that the binding of PIII did not alter the susceptibility of UV- or u-DNA to cleavage by the *Neurospora crassa* endonuclease. It also appears that the cleavage sites for the endonuclease are not masked by PIII. In our experiment, the rate of nicking of the UV-DNA by the endonuclease is 3-4 fold higher than that of u-DNA.

9. DNA-binding proteins in normal human and XP-fibroblasts

DNA-binding proteins were also analysed in extracts of a normal human fibroblast and two XP-fibroblast cell lines. DEAE-fractions were prepared from about 5-6 x 10^7 cells and contained 9-13 mg of protein (Table XIII).

When the DEAE-fraction of normal human fibroblasts was subjected to phosphocellulose chromatography, three major peaks of DNA-binding activity were eluted from the column as with Hela cells (Fig. 31). The peak which showed a binding preference for UV-DNA and AAAF-DNA rather than u-DNA was eluted at about 375 mM potassium phosphate. A similar elution profile was obtained with fibroblast extract of the XP-group D cell line, XP2NE. Fibroblasts of the XP-group A cell line,
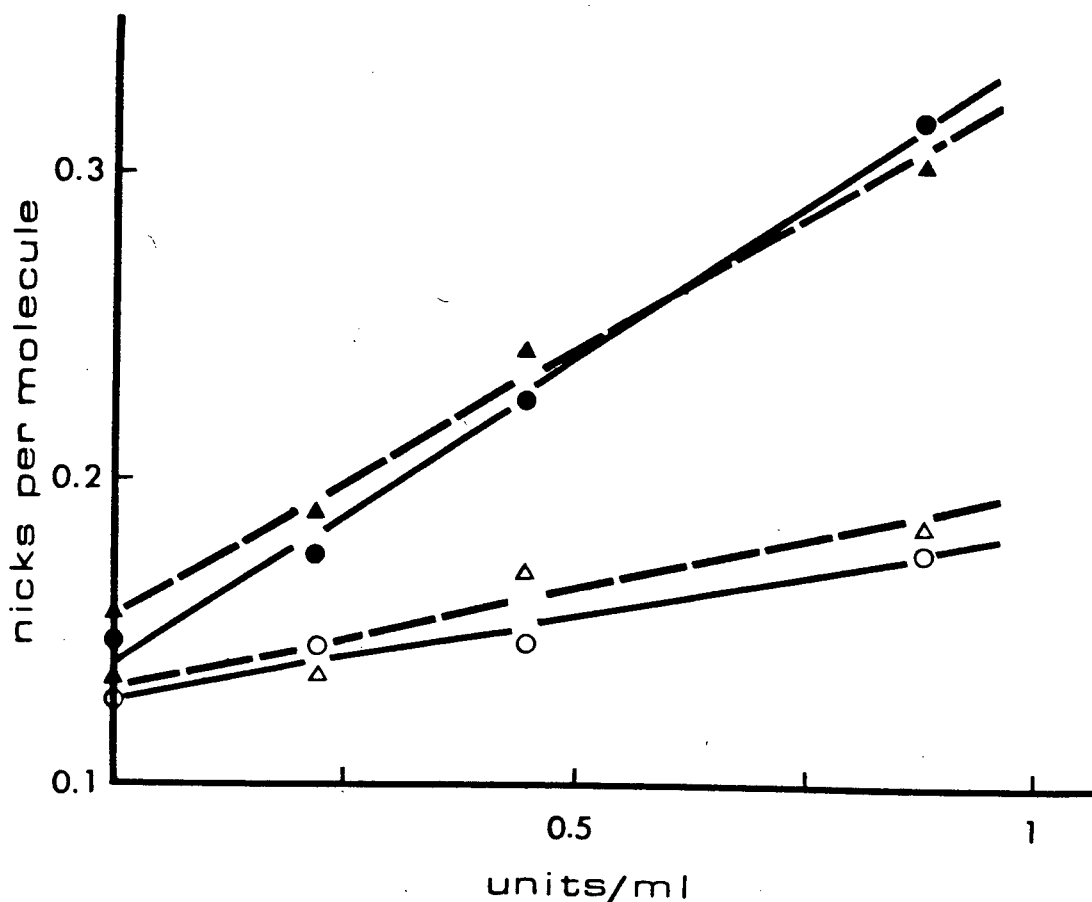
Fig. 30. Effect of PIII on the susceptibility of DNA to the
single-stranded specific endonuclease from *Neurospora*
crassa.

UV-DNA (filled symbols) or u-DNA (open symbols) were
incubated with 40 units (UV-DNA-binding activity) of
PIII (▲,△) or without PIII (●,○) under the standard
conditions except that the assay mixtures also contained
100 µg/ml of acetylated BSA. The assay mixtures were
then incubated for 30 min at 37°C with 5-µl aliquots
of various concentrations of the single-stranded
specific endonuclease from *Neurospora crassa.* The
final concentrations of the endonuclease were as
indicated. 50-µl aliquots of the reaction mixtures
were assayed for DNA nicking. Each point is the
average of duplicate assays. The *Neurospora crassa*
endonuclease had an activity of 535 units/mg and was
in 3.2 M $(NH_4)_2SO_4$, pH 6.0. It was diluted in 10 mM
Tris-HCl (pH 7.5) and 100 µg/ml of acetylated BSA
prior to experiment.

Table XIII.   Preparation of extracts used for the analyses of
             the DNA-binding proteins from human fibroblasts.

Cell line	Cell number	Volume of DEAE-fraction ml	Amount of protein in DEAE-fraction mg
207[1]	4.8×10^7	7.1	8.5
XP5EG[2]	5.1×10^7	7.1	9.2
XP2NE[3]	6.0×10^7	8.5	12.9
XP5EG(2)[4]	5.0×10^7	8.0	9.4

[1] A normal human cell line.

[2] A XP-group A cell line.

[3] A XP-group D cell line.

[4] A repeated analysis with the cell line XP5EG. Read legend of
   Fig. 31 for details.
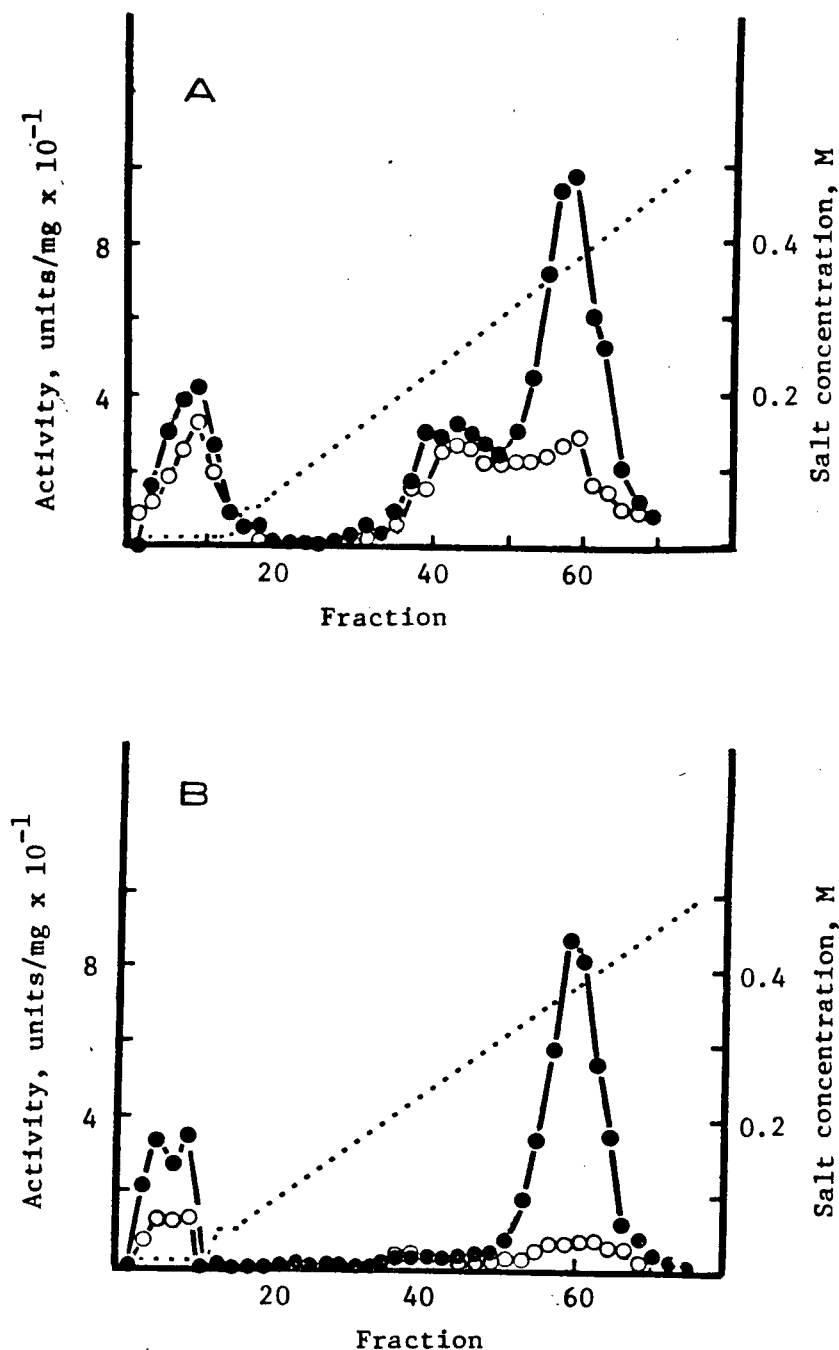
Fig. 31. Phosphocellulose chromatography of DNA-binding
proteins from human fibroblast extracts.
Fibroblast extracts of the cell lines (A) 207,
(B) XP5EG and (C) XP2NE were subjected to analyses.
The extracts were from cells harvested at the 10th
cell passage. (D) A repeated analysis for the
cell line XP5EG was performed with cells harvested
at the 13th passage. Each column fraction was
assayed with either UV-DNA (●) or u-DNA (○)
under the standard conditions. Phosphate
concentration (··········).

Fig. 31 (C) and (D).

Activity, units/mg x 10⁻¹

Activity, units/mg x 10⁻¹

Salt concentration, M

Salt concentration, M

84
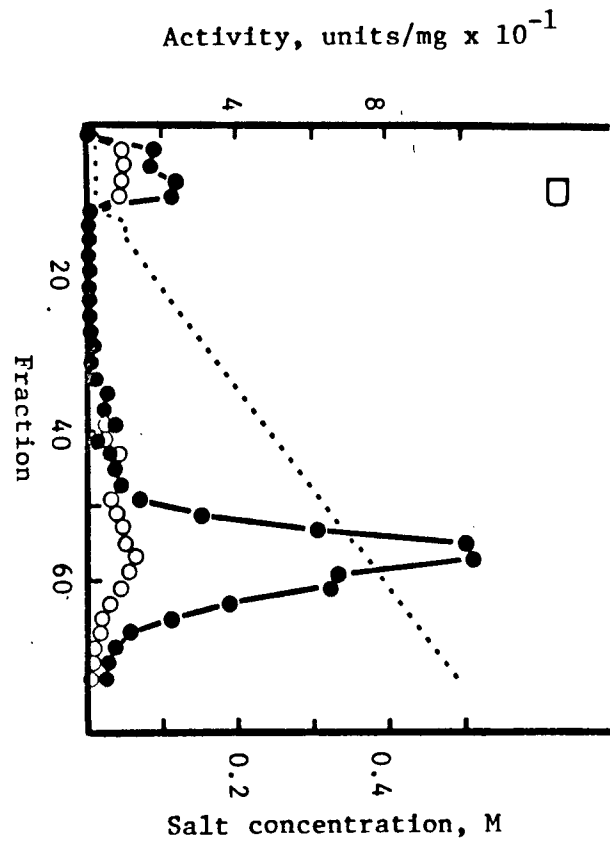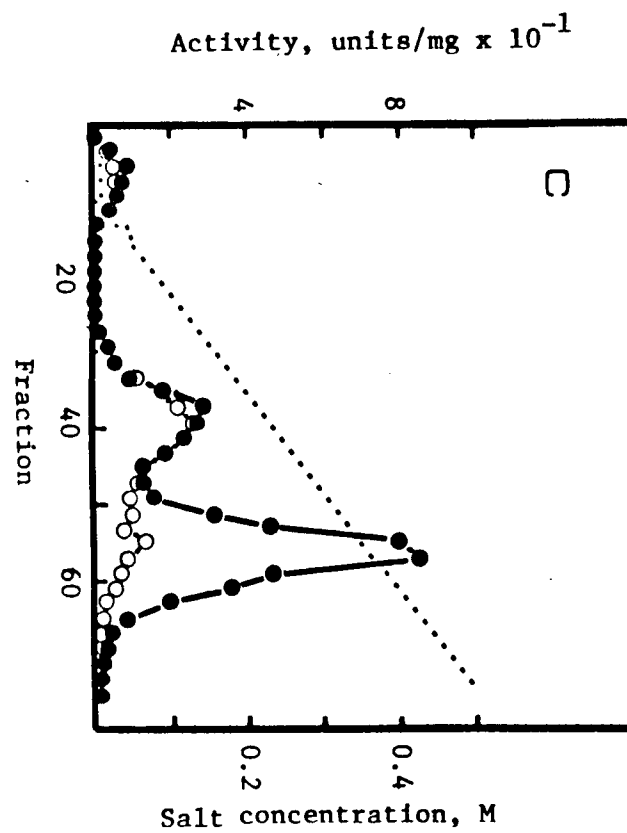
XP5EG, seemed to be deficient in a DNA-binding protein which eluted with 180-250 mM potassium phosphate from the column. A repeated analysis with an independent extract of XP5EG indicates that this deficiency was reproducible. The amounts of DNA-binding protein (expressed as units of DNA-binding activity per mg of protein in the DEAE-fraction) which eluted at 375 mM potassium phosphate in the normal human fibroblasts and XP fibroblasts were similar to that of the phosphocellulose fraction of PIII from Hela cells (Table XIV).

10. Estimation of the equilibrium constant of the binding reaction and the concentration of PIII

For the estimation of the equilibrium constant of the binding reaction and the concentration of PIII, we will assume that one PIII molecule is enough to cause the retention of a DNA molecule and that every PIII-DNA complex is retained by the filter. The validity of these two assumptions was already discussed in an earlier section. We will further assume that the binding reaction between PIII and the binding sites on the UV-DNA are as follows:

$$P + S \rightleftharpoons PS$$

The dissociation equilibrium constant of the reaction can be calculated according to the equation:

$$K_d = \frac{[P_f]\,[S_f]}{[PS]} \tag{3}$$

where P_f is the concentration of the free PIII molecules, S_f is the concentration of free binding sites on UV-DNA, and PS is the concentration of PIII-DNA complex.

From equation (3), it can be shown that

$$[S]_{1/2} = K_d + 1/2\,[P] \tag{4}$$

Table XIV. Summary of the analyses of a UV-DNA-binding protein
in human fibroblast extracts.

| Fraction | Cell line | Units of activity x 10^{-3} per mg of protein in the DEAE-fraction | |
		UV-DNA	u-DNA
DEAE	207	2.06	1.29
	XP2NE	2.12	1.38
	XP5EG	1.74	0.74
	XP5EG(2)	1.90	0.70
Phospho-cellulose[1]	207	0.96	0.21
	XP2NE	0.82	0.10
	XP5EG	0.88	0.10
	XP5EG(2)	1.02	0.14

See text and legend of Table XIII for details of the various
cell lines.

[1] Peak fractions of the DNA-binding activity which eluted between
350-400 mM phosphate were pooled and assayed in duplicates for
DNA-binding activity.

where $[S]_{1/2}$ is the concentration of binding sites on UV-DNA that will saturate one-half of the PIII molecules, and $[P]$ is the concentration of of PIII in the assay mixture (56). As shown in Fig. 7, $[S]_{1/2}$ occurs at an UV-dose of about 250 J/m^2. From Fig. 11, it can be inferred that 50% of the supercoiled PM2 DNA UV-irradiated with a dose of 600 J/m^2 has at least one binding site for PIII. Assuming a Poisson distribution of the binding sites on the supercoiled DNA and that the average number of binding sites per supercoiled DNA molecule increases linearly with an UV-dose up to 600 J/m^2, we calculate that the average number of binding sites per molecule of supercoiled DNA irradiated with an UV-dose of 250 J/m^2 is 0.29. With 3.7 x 10^{-10} M of supercoiled DNA molecule in the assay mixture, $[S]_{1/2}$ is therefore about 1.1 x 10^{-10} M.

From the plateau of the dose response curve shown in Fig. 7, one can estimate that the assay mixture contained about 23.5 fmol of PIII molecules. $[P]$ is therefore 7.8 x 10^{-11} M.

Using the values of $[S]_{1/2}$ and $[P]$, K_d is calculated from equation (4) to be about 7 x 10^{-11} M, which corresponds to a binding energy of 13 kcal/mol. Since the dissociation rate constant of the binding reaction is 2.9 x 10^{-4} sec^{-1} as has been estimated from Fig. 30, the association rate constant of the binding reaction can be calculated to be 4.1 x 10^6 M^{-1} sec^{-1}.

The K_d of the binding reaction of PIII is about 2-3 orders of magnitude higher than the K_d for the binding of *lac* repressor to operator which is in the order of 10^{-13} M (56). It is much lower than the K_d values for the binding of *lac* repressor to nonoperator DNA which is in the order of 10^{-5} M (74), the noncooperative binding of

gene 32 protein of phage T4 to duplex DNA which is in the order of 10^{-4} M (75) and the noncooperative binding of the gene D5 protein of phage T5 to single-stranded DNA which is 1.85×10^{-8} M (76). It is, however, comparable to the K_d values of the cooperative binding of gene 32 protein to single-stranded DNA which is in the order of 10^{-10} M (75) and the cooperative binding of the gene D5 protein to duplex DNA which is 6.27×10^{-10} M (76)

We had used 10 μl of PIII in the experiment depicted in Fig. 7. The results of the experiment suggests that there were 23.5 fmol of PIII molecules per 10 μl of PIII. The concentration of PIII was therefore 2.35×10^{-9} M. If our purification scheme had a 30% recovery for PIII, and if all PIII molecules were extracted from the Hela cells, the amount of PIII per Hela cell was in the order of 10^5 molecules.

Discussion

1. Advantages of using glass fibre filters in the filter-binding assay

We have used GF/C filters in the filter-binding assay of PIII. Conventionally, nitrocellulose membrane filters are used for the filter-binding assay of nucleic acid-binding protein (35-42, 56, 57, 77-81). For our present study, the nitrocellulose filters have the disadvantage that they also bind single-stranded DNA and to a certain extent DNA with helical distortions (49, 82). To reduce a high background due to the retention of these forms of DNA in the filter-binding assays, it is necessary to treat the nitrocellulose filters in an alkali solution followed by extensive washing and neutralization of the filters (42, 77, 80, 81). Glass fibre filter, however, retained less than 2% of the various kinds of DNA we have used in our present study, including the single-stranded DNA.

In comparison to the nitrocellulose filters, glass fibre filters are more convenient to use. The glass fibre filters can be used directly by wetting them briefly with the filtration buffer, whereas the nitrocellulose filters have to be presoaked in the filtration buffer for a period of time before use. A high filtration speed can be used in the filter binding assays with glass fibre filters. Generally, filter-binding assays were performed with filter speeds of less than 5 ml/min with the nitrocellulose filters (36, 57, 77). We have demonstrated that in our filter-binding assay, the retention of PIII-DNA complex is optimal at a filtration speed of 10-30 ml/min. A speed of 30-40 ml/min has been used with GF/F glass fibre filters for assaying the DNA-binding activity of the poly(ADP-ribose) polymerase of of bovine thymus (45).

In the cases where the recovery of protein-DNA complex from the filter is desired, Coombs et al. (43) have discussed another advantage of using glass fibre filters in the filter-binding assays. They reported that the elution of the adenovirus DNA-terminal protein complex with sodium dodecyl sulphate from the glass fibre filters was 50-fold more efficient compared with the nitrocellulose filters.

In conclusion, we think the filter-binding assay using GF/C filters is a simple, sensitive and reproducible assay for DNA binding proteins.

2. Mechanism of retention of PIII-DNA complex by the GF/C filters

We do not understand the mechanism by which the glass fibre filters retain the PIII-DNA complex.

We are aware that the glass fibre filters are commonly used to retain protein or DNA precipitated by trichloroacetic acid or other denaturants. However, the retention of the PIII-DNA complex was clearly not due to DNA precipitation or intermolecular DNA aggregration. This was concluded from the sedimentation analysis of the PIII-DNA complex through the glycerol gradients, since DNA complexed with PIII had a similar sedimentation coefficient as free DNA.

We think that the interaction between the filter and the protein component of the complex is most likely responsible for the retention of the complex. Basic proteins such as albumen, are strongly bound to the surface of the fibres (Glass microfibre filters, Whatman Publication No. 824). In fact, GF/C filters have been used to retain soluble antigen-antibody complexes through interactions between protein molecules and the filters (83). We have not yet determined whether

the protein molecules of PIII alone are retained by the GF/C filter.
However, Coombs et al. (43) have claimed that all adenovirus proteins
bind to GF/C filters.

We have also shown that glass fibre filters with pore sizes
greater than 1.5 µm are less efficient in retaining the PIII-DNA
complex. This result may indicate that the hydrodynamic diameter
of the PIII-DNA complex is less than 1.5 µm. It is however difficult
to envisage that the binding of one or a few molecules of PIII can bring
about a drastic change in the hydrodynamic diameter of a PM2 DNA
molecule without a shift in the sedimentation coefficient of the DNA.
Alternatively, GF/C filters with smaller pore sizes might be more
efficient because of a higher content of surface materials which
interact with the PIII molecules.

3. Comparison of PIII with other UV- or AAAF-DNA-binding proteins from human cells

PIII is likely to be different from an AAAF-DNA-binding protein
purified from human placenta by Moranelli and Lieberman (42). The
latter protein binds efficiently to linear duplex T7 DNA treated with
AAAF, MMS and MNUA but does not recognise UV-irradiated DNA. PIII,
on the other hand, binds efficiently to supercoiled PM2 DNA treated
with UV, AAAF, MNNG but not MMS.

PIII is also different from a UV-DNA-binding protein that has
been purified from human placenta by Feldberg and Grossman (40, 41).
The latter UV-DNA binding protein binds efficiently to linear DNA
treated with nitrous acid and sodium bisulphite. Whether it binds
to DNA treated with AAAF or MNNG has not been reported. The UV-DNA
binding protein elutes from the phosphocellulose column at around

0.175 M potassium phosphate, and has a molecular weight greater than
100,000. PIII, on the other hand, elutes from the phosphocellulose
column at around 0.375 M potassium phosphate and has a molecular weight
of 20-25,000.

There are some similarities between PIII and the UV-DNA unwinding
protein from CLL lymphocyte extracts (28). The molecular weight of
the unwinding protein is 24,000, which is about the same as PIII.
Both proteins are eluted from an UV-irradiated DNA-cellulose column at
1 M NaCl. The binding substrate specificity of the unwinding protein
has not been reported. However, the two proteins may differ in
their binding affinities to single-stranded DNA. The unwinding protein
binds tightly to single-stranded DNA-cellulose column and required 2 M
NaCl for elution. On the other hand, PIII appears to have a weaker
affinity for single-stranded DNA than UV-DNA.

In fact, to our knowledge, the only other naturally occuring
protein which binds to DNA treated with UV and AAAF is the gene 32
protein of phage T4 (cited in reference 84).

4. Biological significance of PIII

We infer from the abundance of PIII, which is probably 10^5
molecules per Hela cell, that it must be important in DNA metabolism.

Two observations suggest a possible role of PIII in DNA repair.
First, the binding activity of PIII is damage-dependent. Second,
the binding of PIII to UV- or AAAF-DNA has a small dissociation
equilibrium constant of 7×10^{-11} M which indicates a strong affinity
of PIII to UV- or AAAF-DNA damage. However, direct evidence
for a repair function of PIII is still lacking. A DNA-binding protein,
which is probably equivalent to PIII, appears to be present in the

fibroblasts of the repair deficient cell lines, XP2NE and XP5EG, at

similar levels as that in normal human fibroblasts. Further, PIII

does not possess any significant DNA endonuclease, glycosylase,

exonuclease or ATPase activity. An argument against a role of PIII

in the excision repair of pyrimidine dimers is the requirement of

high UV-dose for the creation of only a limited number of binding

sites for PIII on the DNA. With a UV-dose of 600 J/m^2, about 45%

of the DNA molecules have a binding site for PIII while more than

60 thymidine dimers are introduced per DNA molecules (85). There is

also the possibility that PIII might actually be a protein involved

in DNA replication or transcription. UV or other treatments could

creat unnatural DNA-binding sites for PIII.

Nevertheless, recalling that the *uvrA, B* and *C* proteins complement

each other to form a UV-endonuclease activity, it is possible that

PIII may only reveal its repair function in the presence of other

proteins.

It is also interesting that PIII binds efficiently to MNNG-DNA

but not MMS-DNA[1]. MNNG alkylates the DNA via a S_N1 reaction while

MMS alkylates the DNA primarily via a S_N2 reaction (86, 87). MNNG,

a N-nitroso alkylating agent, has a higher affinity for oxygen in

nucleic acids than MMS (87). One of the alkylated sites on the DNA

treated with MNNG is the O^6-position of the guanine residue (86). The

O^6-alkylguanine residue can base-pair with a thymine residue in the

DNA, and results in a GC to AT transition mutation (88). There is

---

[1]In this discussion, we assume that the protein which binds to

AAAF-DNA and UV-DNA also binds to MNNG-DNA and u-DNA.

a good correlation between the persistence of O^6-alkylguanine in brain tissue and the frequency of occurence of brain tumors in rats (89, 90). The removal of O^6-alkylguanine from the XP group A and XP group C cells was found to be deficient (6, 91). Thus, MNNG may be similar to UV and AAAF in its ability to induce some DNA lesions which are not repaired efficiently in XP cells. In other words, the repair process of certain DNA damage introduced by MNNG, AAAF or UV may be related. Furthermore, pretreatment with acetylaminofluorene was found to enhance the repair capacity of O^6-alkylguanine of the rat liver cells (92). The latter result suggests that the pretreatment may have induced or activated a process or a specific enzyme which is involved in the repair of O^6-alkylguanine and DNA damage induced by acetylaminofluorene.

The dependence of the binding activity of PIII on DNA supercoiling suggests that PIII might unwind the DNA helix. The higher free energy of a supercoiled DNA than an nonsupercoiled DNA favors the binding of proteins which can unwind the DNA double helix and thereby reduce the number of superhelical turns (61). The difference in the equilibrium constants for the binding of an unwinding protein to superhelical and nonsuperhelical DNA can be very large (93). However, we have failed to detect an unwinding effect of PIII with the single-stranded specific endonuclease from *Neurospora crassa*. It remains to be tested if PIII will enhance the UV-endonuclease activity of *Micrococcus luteus* in a similar way as the UV-DNA-unwinding protein of the CLL lymphocytes.

5. Nature of the binding site for PIII

A likely feature of the DNA binding site for PIII is single-

strandness which is suggested from the affinity of PIII to single-stranded DNA and the inhibition of PIII by caffeine. Local denatured regions are known to be present in DNA treated with UV- or AAAF (94-96). Native supercoiled PM2 DNA also contains regions with unpaired bases (at least transiently), particularly in the A+T-rich regions (97). A bulky nucleotide adduct may enhance the melting of such regions and thereby increase their affinity for PIII. Instead of A+T-rich regions, hairpin or cruciform structures (70, 98, 99) may be important for the formation of binding sites for PIII. It has been suggested that the base-pairing in a hairpin structure is preferentially disrupted by DNA damage (70). But single-strandness alone apparently is not enough for an efficient binding by PIII, since single-stranded DNA is not bound efficiently by PIII as compared with the supercoiled DNA damaged with UV, AAAF or MNNG. Thus, DNA supercoiling is required for an efficient binding of PIII to DNA damage.

The PM2 DNA isolated from natural sources is composed of a Boltzmann distribution of geometrical isomers which differ from each other by an integral number of superhelical turns (100, 101). There are on the average about 90 superhelical turns in the PM2 DNA (102) which has about 9,000-10,000 base-pairs. In eucaryotic cells, the DNA in chromatin is estimated to have one to two superhelical turns per nucleosome particle (103, 104). The superhelical density may influence the interaction of proteins with DNA in two ways. We have already discussed that the free energy associated with the superhelical turns favors the binding to DNA by an DNA-unwinding protein. Alternatively, DNA supercoiling might be necessary for the formation or stabilization of the DNA-recognition site for a protein. The

susceptibility of some supercoiled DNA to certain single-stranded

specific nucleases is known to be dependent on the degree of DNA

supercoiling (61, 70, 98, 99). For example, PM2 DNA molecules are

not sensitive to the *Neurospora crassa* endonuclease unless they have

superhelical densities higher than -0.029 (70). The nuclease-sensitive

sites on the supercoiled DNA may be specific and seem to lie in the

loops of potential cruciform structures (99). The cruciform structures

may be more stable in DNA with high supercoiled densities and in A+T-

rich regions (98, 99). It is thus possible that the damage-induced

binding sites of PIII might only be formed on those PM2 DNA molecules

with superhelical densities higher than a certain value. Studies of

the DNA-binding activity of PIII with DNA of different defined

superhelical densities should allow us to determine whether there

exists a minimal superhelical density above which the binding sites for

PIII are readily induced by DNA damage.

There is another interesting possibility for the nature of the

binding sites of PIII. Substitution of a bulky group at the 8-position

of the purine nucleotides can cause the purine residues to assume

the *syn* conformation instead of the usual *anti* conformation (105).

For DNA treated with AAAF, the major adduct is formed by a substitution

at the 8-position of the guanine residue (95). UV can also introduce

adduct to the 8-position of the purine residues in the DNA. A specific

endonuclease activity directed towards the UV-induced 8-alkylated

purines in the PM2 DNA was identified in extracts of *Micrococcus*

luteus, which probably recognised the *syn* conformation of the purine

residues (105). We plan to determine if PIII will recognise

the UV-induced 8-alkylated purine residues. Interestingly, the *syn* conformation of the guanine residues is preferred in the left-handed Z-DNA structure (106). A DNA structure which is probably assumed by poly(dG-dC)·poly(dG-dC) at salt concentration higher than 2.5 M NaCl (107). By already assuming a *syn* conformation, the alkylated purine residues favor the structural transition of the DNA from the right-handed B-form to the Z-from at lower salt concentration. Indeed, some residues of poly(dG-dC)·poly(dG-dC) modified with AAAF adopt the Z form even in 1 mM phosphate buffer (108). It is possible that PIII recognises the *syn* conformation of purines residues and that the free energy of the binding reaction is used to induce a structural transition of segments of the DNA from a B-form to a Z-form.

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