### PARTIAL PURIFICATION AND CHARACTERISATION OF

### APURINIC ENDONUCLEASE ACTIVITY FROM

HELA CELLS.

by

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### ABSTRACT

Apurinic endonuclease activity in human fibroblasts had been previously resolved into a flow-through and a high-salt-eluate species by phosphocellulose chromatography (Kuhnlein, U. <u>et al.</u>, Nucl. Acid. Res. 5: 951-960, 1978). Enzyme activity in the flow-through species amounted to 20-30% that of the high-salt eluate species. The flow-through enzyme species was not found in cell lines of xeroderma pigmentosum complementation group D.

In this thesis, apurinic endonuclease activity was analysed in Hela cells. Specific enzyme activity in crude extracts of Hela cells was in the range of 400-800 units/mg protein, similar to that of , human fibroblasts which was between 380-680 units/mg protein. Three species of endonuclease activity for apurinic DNA were resolved by phosphocellulose chromatography. They were designated as Peak I, Peak II, and Peak III. Peak I did not adsorb to the phosphocellulose column at 10 mM KPO<sub>A</sub> (pH 7.4) (flow-through activity), Peak II eluted from the column at about 210 mM KPO\_4 (pH 7.4) and Peak III at 260 mM  $KPO_4$  (pH 7.4). Based on their affinity to phosphocellulose, we presumed Peak I and Peak III corresponded to the flow-through and highsalt eluate species in human fibroblasts respectively. Under our experimental conditions, the flow-through enzyme activity in both Hela cells and normal human fibroblasts was only 2-4% of the activity of high-salt eluate species. We suspect that tissue culture conditions may affect the cellular level of the flow-through species of apurinic endonuclease.

Peaks I-III were optimally active at pH-7.5-8.0 and 5-10 mM MgCl<sub>2</sub>.

They were inhibited by increasing concentrations of KCI and NaCI except Peak III which was slightly stimulated by 20-40 mM KCI. The three species were distinguished by their thermosensitivities in a 50 mM KPO $_{\it A}$  buffer. Peak I was stable at 45 $^{
m O}$ C. Peak III was heat-labile, having a half-life of 2-3 min at 45°C. Peak 11 seemed to contain two components, one with a half-life of 2-3 min at 45<sup>0</sup>C, and the other with a half-life of 25 min. In human fibroblasts, both the flow-through and high-salt eluate species of apurinic endonuclease were reported to be stimulated to 2.5-fold by 10 mM KCl. They had a half-life of 6 min at  $45^{\circ}$ C in a 230 mM KPO<sub>4</sub> (pH 7.4) buffer. Thus, Peaks I-III and enzyme species from human fibroblasts had a similar pH optimum, and Mg<sup>2+</sup> requirement. but they differed in their thermosensitivities and inhibition by higher salt concentration. We do not know as yet whether these differences reflect the neoplastic nature of Hela cells or the different tissue origins of Hela cells and human fibroblasts.

When either Peak I or Peak III was rechromatographed on the phosphocellulose column, activity was recovered in both the flowthrough and high-salt eluate fractions. The result suggested an interconversion phenomenon between the flow-through and high-salt eluate species of apurinic endonuclease. This was further supported by molecular weight determinations of the apurinic endonucleases in Peaks I-III. Apurinic endonuclease activity in Peak III and Peak II had a molecular weight of 35,000-40,000 and 22,000-25,000 respective-Iy. Peak I had two components with molecular weights similar to those of Peak II and Peak III. An understanding of the conversion

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between the different apurinic endonuclease species may help in elucidating the molecular defects of xeroderma pigmentosum complementation group D.

Apurinic endonuclease activity in Peaks I-III was found to be associated with a high molecular weight complex. The complex could be dissociated by high salt treatment. The possible biological significance of the high molecular weight complex is discussed.

We also found that apurinic endonuclease could adsorb to the Sephadex gel. The adsorption would lead to an aberrant estimation of molecular weight of the protein. The problem was solved with an elution buffer of high ionic strength.

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## ABBREVIATIONS

| AMV            | avian myeloblastosis virus  |
|----------------|---|
| BSA            | bovine serum albumin  |
| Ci             | Curie, one Curie = the quantity of a radioactive                      |
|                | isotope undergoing 3.7×10 <sup>10</sup> disintegrations per sec.      |
| CsCI           | cesium chloride   |
| DEAE-cellulose | O-(diethylaminoethyl) cellulose                                       |
| DTT            | dithiothreitol  |
| DNA            | deoxyribonucleic acid   |
| EDTA           | ethylenediaminetetracetate  |
| KP04           | potassium phosphate buffer, made up of dibasic potassium              |
|                | phosphate( $K_2$ HPO <sub>4</sub> ) and monobasic potassium phosphate |
|                | (KH <sub>2</sub> PO <sub>4</sub> )                                    |
| MOL            | multiplicity of infection   |
| 0.D.           | optical density or absorbance   |
| POPOP          | 1,4-bis (2-(5-phenyloxyazolyl))-benzene                               |
| POP            | 2,5, diphenyloxazole  |
| SDS .          | sodium dodecylsulphate  |
| Tris           | tris(hydroxymethyl) aminomethane                                      |
| UV light       | ultraviolet light   |

xIII.

### INTRODUCTION

## 1. Apurinic/apyrimidinic sites as common DNA lesions :

Perhaps one of the most common forms of DNA damage is the loss of purine and pyrimidine bases from the DNA. These processes, depurination and depyrimidination, involve breakage of the glycosidic bond between the purine or pyrimidine bases and the deoxyribose moieties of the DNA.

Purine and pyrimidine bases have been demonstrated to be released im detectable quantities from DNA at neutral pH and  $70^{\circ}C$ (1). The initial rate constant of depurination is  $2.4 \times 10^{-7} \text{sec}^{-1}$ . Depyrimidination is about 10-20 times slower than depurination (2). At physiological conditions of  $37^{\circ}C$  and pH 7.4, the <u>in vivo</u> rate constant of depurination has been estimated to be in the order of  $2 \times 10^{-9} \text{min}^{-1}$ .

DNA modified by some chemical or physical agents has a much higher depurination/depyrimidination rate (3). An example is alkylated DNA. Alkylation of DNA results in the formation of purine and pyrimidine derivatives with labile glycosidic bonds (4,5). Some modified bases such as 3-methyladenine and  $0^6$ -methylguanine can also be removed by specific DNA-glycosidases (6,7). The <u>in vivo</u> depurination rate constants of 7-methylguanine and 3-methyladenine are of the order of 1 x  $10^{-4}$ min<sup>-1</sup> and 4 x  $10^{-3}$ min<sup>-1</sup> respectively (8).

Finally, apyrimidinic sites are formed during the process of removal of uracil residues from the DNA. Uracil residues are

introduced into the DNA as a result of deamination of cytosine residues (9,10). Uracil can also be incorporated into DNA in place of thymine during replication (11,12). The enzyme that is thought to be involved in the removal of uracil residues in DNA has been purified from extracts of <u>E</u>. <u>coli</u> and human fibroblasts. It is called uracil glycosidase (13,14).

It can be concluded from the above argument that depurination/ depyrimidination of DNA occurs to a significant extent <u>in vivo</u>. Considering spontaneous depurination alone, a growing mammalian cell may loose 2,000-10,000 purines and a few hundred pyrimidine residues from its DNA during a cell generation time of 20 hours (1).

## 2. Possible cellular effects of apurinic/apyrimidinic sites :

Besides a direct loss of genetic information, the presence of apurinic/apyrimidinic sites on the DNA has several other consequences. During replication, the position opposite to the apurinic/ apyrimidinic sites in the newly formed complementary strand may be filled at random. Or, the replication mechanism may simply skip the lesions and deletions result. <u>In vitro</u>, the fidelity of DNA synthesis by AMV DNA polymerase was found to decrease with a depurinated poly d(A-T) template (15). Apurinic/apyrimidinic sites also lead to chain breakages (16), interstrand crosslink formation (17) in the DNA, and a destabilization of the DNA double helix (18). In T7 coliphage, one out of seven to eight depurination events was reported to be an inactivation hit (19,20).

One can therefore envisage that apurinic/apyrimidinic sites, if

unrepaired, will impose mutagenic and toxic effects on a cell. DNA repair mechanisms must have evolved to safeguard the cell from depurination and depyrimidination.

### 3. DNA repair mechanisms :

Several DNA repair mechanisms have been proposed to function in both procaryotes and eucaryotes for the repair of various DNA lesions (21-22). They are summarised in Figures 1 and 2. Most of the pathways illustrated are examplified by the repair of pyrimidine dimers, from which our present concepts of the various DNA repair mechanisms are largely derived. The formation of this DNA lesion involves a covalent linking of adjacent pyrimidines in a DNA strand and is induced by ultraviolet light.

The simplest mode of DNA repair is a direct reversion of the damaged DNA back to the undamaged form. To date, the only well established example is the enzymatic photoreactivation of pyrimidine dimers. In this process, an enzyme called photolyase or photoreactivating enzyme is able to monomerize the UV-induced pyrimidine dimers in the presence of light with a wavelength of 320-370 nm. Recently, it was suggested that  $0^6$ -methylguanine of alky-lated DNA could also be reverted back directly to the undamaged form via an enzyme-mediated dealkylation process. Thus, a specific enzyme called demethylase was isolated from rat liver which removed the  $0^6$ -methyl group from the altered guanine base (23). In the case of apurinic/apyrimidinic DNA, repair can be accomplished by insertase activities which simply place correct bases back into



the repair of intrastrand pyrimidine dimers in DNA.

For details, see references cited in text.



excision, repair replication



Figure

 Base excision DNA repair mechanism for the repair of partly deaminated DNA.
 The repair process takes place on double stranded DNA, but the complementary strand has been omitted for simplicity.

the lesion sites. Insertase activity for apurinic DNA has been found in human fibroblasts (24,25).

Another mode of DNA repair involves the excision of the damaged bases or nucleotides from the DNA and is thus termed excision repair. As shown in Figure 1, repair of pyrimidine dimer is initiated by a so-called UV-endonuclease which incises the DNA adiacent to the lesion. The damaged nucleotides and adjacent nucleotides are then excised by an exonuclease activity. Finally, the gap created during the excision step is filled and sealed by the concerted action of DNA polymerase and ligase. This is called "classical nucleotide excision repair". A similar mechanism is thought to operate for the repair of apurinic/apyrimidinic sites. A specific endonuclease for these DNA lesions has been purified and characterised from sources as divergent as E. coli (26,27), calf thymus (28), calf liver (29), human placenta (30) and plant embryo of Phaseolus multiflorus (31). The enzyme is generally known as apurinic endonuclease. So far, no separate endonuclease activities have been found for apurinic or apyrimidinic sites. In vitro, repair of apurinic sites has been demonstrated by incubating depurinated DNA with apurinic endonuclease, DNA polymerase I, the four deoxyribonucleoside triphosphates, polynucleotide ligase and its coenzyme (32). The repair role of apurinic endonuclease is further supported by mutants of E. coli defective in this enzyme activity. These mutants are more sensitive to methyl methanesulphonate (an alkylating agent) than the wild type (33,34).

Recently, a base excision repair mode has been proposed. In this

process, the first step is the removal of the damaged base by an N-glycosidase while the backbone of the DNA strand remains intact. The resulting apurinic or apyrimidinic site is then removed as described earlier. This kind of DNA repair is believed to be responsible for the removal of uracil or alkylated bases from the DNA molecule (35,36) (Fig. 2).

The remaining DNA repair pathways can be grouped together in a class termed daughter strand repair (25). With this mode of DNA repair the lesions are not removed from the DNA, but are merely diluted out as a result of DNA replication. These repair mechanisms are by far the least understood in term of the enzymes or proteins involved. In the process of postreplication recombination, the normal replication mechanism apparently bypasses the damage and leaves a gap in the daughter strand opposite the damaged region. This gap is then filled via a recombination event with the undamaged parental DNA as illustrated in Figure 1. This repair process is Another error-prone repair model called bypass reerror-prone. plication has been proposed a few years ago (37). According to this model, the position opposite the lesion in the newly formed complementary strand is filled at random. There is also an error-free bypass replication (38). This process involves a branch migration in which the two daughter strands anneal to one another. Then the gap corresponding to the lesion site in one daughter strand is filled by using the other daughter strand as a template.

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#### 4. Apurinic endonuclease in human cell lines :

Several human genetic diseases are associated with a DNA repair deficiency (39-41). Apurinic endonuclease activity has been analysed in crude extracts of fibroblasts derived from patients with these diseases, and is within the normal range in the cases of ataxia telangiectasia, Fanconi's anemia, Bloom's syndrome, Cockayne's syndrome and progeria (42,43). However, in some cell lines of xeroderma pigmentosum, apurinic endonuclease activity was shown to be defective (44,45).

### 5. Inherited DNA repair defects in xeroderma pigmentosum :

Xeroderma pigmentosum (XP) is an autosomal recessive disease, patients are extremely sensitive to sunlight and have a high incidence of skin cancer. When XP cells were treated with UV light or chemicals such as 4-nitroquinoline-1-oxide, bromobenz(a)anthracene or acetylaminofluorene, it was found that excision repair was deficient in most XP cell lines, but normal in others (46-48). The latter group of XP cells is called XP variants. Using Sendai virus, fibroblasts from different XP cell lines can be fused. The resulting heterokaryons may or may not have a normal level of DNA repair synthesis to UV damage. These experiments led to the assignment of various excision repair-deficient XP cell lines into five complementation groups. They are designated as groups A, B, C, D, E (49). Recently, the possible existence of three more complementation groups has been suggested (47). XP variants will complement with all other cell strains.

It is generally agreed that XP group A-E cells have a defect in the incision step of the nucleotide excision repair pathway for pyrimidine dimers (46,47), and cells of XP variants are defective in postreplication repair (50). Recent data indicate they may be defective in other modes of DNA repair as well. Thus, XP cells were found to have a lower level of photoreactivating enzyme than normal cells (51,52). A partial defect in postreplication repair was also observed in XP group A-D cells (53).

Kuhnlein et al. (44) reported that fibroblasts from XP group D cell lines had about one-sixth of the normal apurinic endonuclease activity. The apparent Michaelis constants ( $K_{M}$ ) of the apurinic endonuclease activity in extracts from XP group A and D cells were higher than those of normal cells. Interestingly, only patients of XP group A and D show neurological complications (54). Whether abnormal apurinic endonuclease activity has any etiological role in the neurological symptoms in the XP patients remains a question. Apurinic endonuclease activity in the crude extracts of normal human fibroblasts was resolved by phosphocellulose column chromatography into two species : an activity (flow-through activity) that did not adsorb to the column at 10 mM  $\mathrm{KPO}_4$  concentration and another activity (high-salt eluate) that eluted from the column at about 240 mM KPO $_{\mathtt{A}}$  concentration. The flow-through activity had a higher sedimentation coefficient of 3.3 S and an apparent K<sub>M</sub> of 5 nM apurinic sites, while the corresponding values for high-salt eluate activity were 2.8 S and 44 nM respectively (45)

Flow-through activity was not detected in fibroblast extracts from XP-D cell lines. In fibroblasts of XP variants, the level of uracil DNA N-glycosidase activity was claimed to be roughly half of that of normal fibroblasts (55). No abnormality was revealed in other XP cells. These observations suggest the base excision repair mechanism is defective in at least some XP cells. Thus, when cells from an XP group A cell line were exposed to an alkylating agent, such as ethylnitrosourea, the frequency of sister chromatid exchanges was severalfold higher than that of normal cells similarly treated (56). A slower removal rate for  $0^6$ -alkylguanine was also reported in this XP group A cell line (57).

#### 6. Objectives :

From the above discussion, one can infer that whatever the primary biochemical defect in XP cells is, it has a pleiotropic effect on different DNA repair mechanisms. One possibility to explain the genetic heterogeneity and multiple enzymatic deficiencies of XP is that the different repair enzymes share common regulatory components. One or more of these components may be defective or produced in reduced levels in XP cells. There is as yet no documentated evidence for the existence of repair enzyme complexes except perhaps the controversial endonuclease II activity from <u>E. coli</u> (6). This enzyme contains apurinic endonuclease activity and is also capable of releasing  $0^6$ -methylguanine and 3-methyladenine from methylated DNA. Endonuclease II may thus be a preparation containing several different enzymes (6, 7, 26, 34, 35). A stable dimer of "endonuclease II" and apurinic endonuclease can also be formed (58).

An interesting phenomenon was revealed when flow-through apurinic endonuclease activity from human fibroblasts was reapplied to the phosphocellulose column. The column was eluted first with a 10 mM and then a 0.3 M KPO<sub>4</sub> buffer. It was reported that about 80% of the recovered activity was again found in the flow-through fractions while the remaining activity now only eluted with 0.3 M KPO<sub>4</sub> buffer. This result suggests a possible conversion of the flow-through enzyme species to the highsalt eluate form. As discussed earlier, the flow-through enzyme species has a lower  $K_{M}$  and possibly a higher molecular weight than the high-salt eluate species. An accessory molecule may

complex with the high-salt eluate apurinic endonuclease to form the flow-through species. To pursue this problem further, a large amount of cell extract is needed. An alternative source of material therefore seems more preferable than human fibroblasts. A possible candidate is a human cell line called Hela cells, cells of which can grow in suspension culture. Before going into any large scale study, it is necessary to establish if Hela cells have also a flow-through and a high-salt eluate species of apurinic endonuclease as human fibroblasts. In this thesis, I shall report the partial purification and characterisation of apurinic endonucleases from Hela cells. The purification method was according to Kuhnlein <u>et al</u>. (45).

The analysis of apurinic endonuclease activity from Hela cells in itself is interesting since this cell line is neoplastic in origin (59). Research in the past decade has indicated a relationship between proficiency of DNA repair mechanism and susceptibility of an individual to cancer (39,60). Crude extract from Hela cells was reported to have a higher endonuclease activity for UV-irradiated DNA (61), yet its apurinic endonuclease activity was similar to those of normal human fibroblasts (42). It was pointed out that <u>in vitro</u> measurement of composite activity of multiple endonucleases in crude extract might not reveal a deficiency of the actual repair enzyme. An example was the finding that in <u>E. coli</u> UV endonuclease activity was resolved by the demonstration of two UV endonucleases, one of which

was absent in the mutants (62).

Therefore, one of my objectives was to see if I could show any difference in apurinic endonuclease activity between Hela cells and human fibroblasts. The different species of apurinic endonuclease from Hela cells were characterised with respect to their optimal requirements for MgCl<sub>2</sub>, pH optimums, heat sensitivities, salt concentration dependences and molecular weights. To provide further evidence for a conversion of the flow-through species to the high-salt eluate species of apurinic endonuclease, the preliminary experiment of of phosphocellulose column rechromatography as described earlier in this section, was repeated in more details. A linear gradient of KPO<sub>4</sub> solution was used as elution buffer in place of 300 mM KPO<sub>4</sub> solution. The objective was to show that enzyme activity derived from the flow-through species of apurinic endonuclease was also eluted from the phosphocellulose column at around 240 mM KPO<sub>4</sub> concentration.

#### MATERIALS AND METHODS

#### 1. Tissue culture :

(a) <u>Cell lines</u> : Hela cells were a gift from Dr. J.B. Hudson of Microbiology Department, University of British Columbia. Peggy cells, were human fibroblasts grown from a skin punch biopsy from a normal Caucasian female.

(b) <u>Culture media</u> : Dulbecco's modified Eagle's medium (Gibco) was routinely supplemented with 10% of fetal calf serum (Gibco) and the following antibiotics : Penicillin (80 units/ml, final concentration), streptomycin sulphate (23.7 µg/ml), Kanamycin (100 µg/ml) and Fungizone (2.5 µg/ml). The antibiotics were all purchased from Gibco. The medium was adjusted to pH 7.0-7.5 with 7.5% sodium bicarbonate solution. The culture medium was sterilized by filtering through a Sartorius membrane paper with a pore size of 0.2 µm. (c) <u>Cell growth</u> : Cells were grown in 32 ounce prescription bottles (Brockway Glass Co. Inc.) with 50 ml of culture media. Incubation was at  $37^{\circ}$ C in a humidified incubator with 5% CO<sub>2</sub> and 95% air. Confluent cells were split 1:4 after treatment with trypsin solution (Gibco).

(d) <u>Cell harvesting</u> : Cells were harvested in batches of 6-12 bottles when they were near confluency. The cell culture media were poured off. Cells were washed twice with 10 ml of phosphatebuffered saline (25 mM KPO<sub>4</sub> (pH 7.0)/0.15 M NaCl/0.015 M sodium citrate) and suspended in 40 ml of phosphate buffer saline by scraping from the bottle surface. Alternatively, the cells were re-

leased from the bottle surface after incubation with 5 ml of trypsin at 37<sup>o</sup>C for 10 min. The cells were then washed twice in phosphate buffer saline by centrifugation and resuspension. Cell pellets were the stored either directly in liquid nitrogen or they were first resuspend in 2 ml of 50 mM Tris-HCI(pH 7.5) before storage.

## 2. Preparation of PM2 phage DNA :

Methods for the culturings of <u>Pseudomonas</u> Ba1-31 bacteria and PM2 phage were modified from those of Espejo and Canelo (63). (a) <u>Ba1-broth</u> : 1 1. of sterilized solution contained 10 mM Tris-HCl (pH 7.5), 12 gm of magnesium sulphate (MgSO<sub>4</sub>.7H<sub>2</sub>O, Sigma), 26 gm of sodium chloride (Fisher),8 gm of bactonutrient broth (Difco), 0.01 M of calcium chloride, 3.5 ml of 20% potassium chloride. (b) <u>Ba1-top agar</u> : 5 gm of bacto-agar (difco) was dissolved in 1 1. of Ba1-broth and sterlized by autoclaving. It was liquified by heating in a water bath at 50<sup>O</sup>C before use.

(c) <u>Bal-plate</u> : 23 gm of bacto-agar (Difco) was dissolved in 1 l. of Bal-broth and sterlized by autoclaving, The agar solution was delivered in aliquots on plastic dishes (87.5 mm x 15 mm Canlab), and allowed to solidify at room temperature.

(d) <u>Plaque assay for phage titer</u> : <u>Pseudomonas</u> Bal-31 bacteria were grown in an aerated test tube with about 10 ml Bal-broth at 28<sup>o</sup>C overnight. For phage assay, 3 ml of Bal-top agar was added to 0.1 ml of overnight bacteria culture with C.1 ml of phage aliquot. The solution was mixed and poured on the Bal-plates and incubated overnight at room temperature. The phage titer was estimated by counting the number of plaques appeared.

(e) <u>Preparation of phage stock</u> : <u>Pseudomonas</u> Bai-31 bacteria were grown in 200 to 500 ml of Bal-broth at  $28^{\circ}$ C to a density of 2 x  $10^{7}$ /ml and infected with PM2 phage at an MOI of  $10^{-3}$  phage per bacterium. The culture was incubated overnight. The bacteria and cell debris were pelleted by centrifugation at 10,000 rpm for 15 min with a Beckman Type 21 rotor. The suspernatant was used as the phage stock and usually had a titer of 5 x  $10^{10}$ /ml.

(f) Preparation of <sup>3</sup>H-labelled PM2 DNA : Bacteria <u>Pseudomonas</u> Bal-31 were grown in a 1.5 |. Bal-broth in a 3 |. erlenemeyer flask at 28<sup>0</sup>C. Aeration was created by stirring the culture vigorously with a magnetic stirrer. When the bacteria reached a density of  $3 \times 10^8$ /m1 (titered with a Petroff-Houser bacteria counter), 0.15 gm of deoxyadenine (Sigma) was added to the medium. Five min later, the cells were infected with  $1-2 \times 10^{12}$  PM2 virus. After 5 min, 1.5 mCi of methyl-<sup>3</sup>H thymidine (specific activity 50 Ci/mmole, New England Nuclear) was added and the culture was incubated overnight. Bacteria and cell debris were removed by centrifugation for 15 min.at 10,000 rpm with a Beckman Type 21 rotor. The supernatant was centrifuged for 3 hours at 20,000 rpm with the same rotor. The pellet which contained the phage particles was resuspended in about 15 ml of RB buffer containing 20 mM Tris-HCI (pH 8.0)/1 M NaCl and centrifuged for 10,000rpm for 15 min in a Beckman Type 50 Ti rotor. The pellet was discarded, the supernatant was centrifuged for 50 min at 50,000 rpm in the same rotor. The pelletowas resuspended in 15 ml of RB buffer and centrifuged for another

10,000 rpm for 15 min. The final supernatant was made to have a density of about 1.28 gm/cc with CsCl. The phage particles were banded by centrifugation in a polyallomer tube with a Beckman Type 50 Ti rotor at 40,000 rpm for 24 hours at 20<sup>0</sup>C.

After the CsCl density gradient centrifugation, one major band of phage particles was evident in the middle of the polyallomer tube (Fig. 3). A minor band of material was also evident below the major band. The material in this minor band was not analysed and was discarded. The material in the major band was collected and dialysed against 1 I. of 0.02 M Tris-HCl (pH 7.5)/0.1 M NaCl/ 1 mM EDTA for 3 hours or more. The phage was lysed by 10% SDS added dropwise until the solution was cleared. The phage DNA was extracted in the aqueous phase by phenol extraction as described by Espejo <u>et al.</u>(64). The PM2 DNA was then dialysed extensively against 0.01 M Tris (pH 7.5).

PM2 DNA concentration was determined by measuring the absorbance at 260 nm. The molar extinction coefficient  $E_{1cm}^{1M}$  260 nm of PM2 DNA was assumed to be 6.5 x 10<sup>3</sup>. A typical result where the PM2 DNA had been diluted 10-fold and gave an 0.D.<sub>260nm</sub> of 0.32 was as shown in Figure 4. The concentration of the DNA was calculated as 0.49 mM. Typical yields for a 1.5 I. culture were 10-15 µmoles nucleotide of DNA with a radioactivity of 6,000-8,000 cpm/nmole.

3. Enzyme purification :

All purification procedures were carried out at 2°C.
 (a) <u>Preparation of cell extract</u>: Frozen cells were thawed and resus-



Figure 3. Isolation of PM2 phage particles by cesium chloride density-gradient equilibrium centrifugation. The upper whitish band contained the phage particles (arrowed).





PM2 DNA

----- 10 mM Tris-HCI (pH 7.5)

pended in 2 ml of 50 mM Tris-HCl (pH 7.5)/0.1 mM DTT. The cells were disrupted with sonic irradiation 6 times for 15 sec each. The sonicate was centrifuged for 50 min at 50,000 rpm in a Beckman Type 50 Ti rotor and the pellet discarded. The supernatant fluid (high speed supernatant) was subjected to further purification. 10 bottles normally yielded 8-10 mg of soluble protein. (b) <u>DEAE-cellulose chromatography</u> : A column of Whatman DE-22 DEAEcellulose (5 mm x 35 mm) was prepared and equilibrated with buffer A (50mmM Tris-HCl (pH 7.5)/0.4 M NaCl/10% glycerol/0.1 mM DTT). The high speed supernatant was adjusted to have the same buffer content as buffer A and loaded onto the column at a flow rate of 0.125 ml/min. The column was then washed with buffer A at the same flow rate. Fractions of 1 ml were collected, usually the first four fractions containing most of the activity were pooled and dialysed overnight against two 400 ml aliquots of buffer B containing 10 mM KPO $_{4}$ (pH 7.4)/ 10% glycerol/0.1 mM DTT. The final dialysate (DEAE pool) was retained.

(c) <u>Phosphocellulose chromatography</u> : A column of Whatman P-11 phosphocellulose (1.1 cm x 3.5 cm) was prepared and equilibrated by washing with buffer B. The DEAE pool was applied to the column at a flow rate of 0.04 ml/min. The column was then eluted with 7 to 9 ml of buffer B, then 3 to 5 ml of 50 mM KPO<sub>4</sub>(pH 7.4)/10% glycerol/0.1 mM DTT and a 46 ml linear gradient of 50 to 400 mM KPO<sub>4</sub>(pH 7.4)/10% glycerol/0.1 mM DTT. Fractions of 1 ml were collected in plastic tubes. 10 µl of 10 mg/ml acetylated BSA was added to each fraction to stabilize the enzyme activity. The fractions

with most enzyme activity were made to 35% glycerol and stored at  $-20^{\circ}$ C.

### (4) PM2 DNA depurination :

Depurination buffer was made up of 1 M NaCl, 0.1 M sodium citrate and adjusted to pH 4.0 with HCl. The depurination buffer was then diluted 10-fold with PM2 DNA which was in 10 mM Tris-HCl (pH 7.5). Thus, depurination was carried out at 70<sup>O</sup>C for 15 min with 0.5 mM DNA in 9 mM Tris-HCl, 0.1 M NaCl, and 0.01 M sodium citrate. The final pH of the solution was 4.6.

### (5) Filter-binding assay :

0.15 ml of 0.01% SDS/0.25 mM EDTA (pH 7.0) was added to the DNA reaction mixture followed by 0.2 ml of 0.3 M K<sub>2</sub>HPO<sub>4</sub>-KOH (pH 12.4). After 2 min at room temperature, the solution was neutralized with 0.1 ml of 1 M KH<sub>2</sub>PO<sub>4</sub>-HCl (pH 4.0). This treatment was found to denature nicked PM2 DNA, but not covalently closed molecules. However, when the pH of the 0.3 M K<sub>2</sub>HPO<sub>4</sub>-KOH added was above pH 12.8, unnicked PM2 DNA also became denatured (Fig. 5). 0.2 ml of 5 M NaCl and 5 ml 50 mM Tris-HCl (pH 8.0)/1 M NaCl were then added successively. The solution was filtered through a nitrocellulose membrane filter paper (Schleicher and Schnell type BA 85, 0.45  $\mu$ m pore size) which selectively retained denatured DNA (65). The filter was washed with 5 ml of 0.3 M NaCl/0.03 M sodium citrate, dried and counted in a liquid scintillation counter (Searle, Delta 300, liquid scintillation system) with 5 ml of scintillation fluid.



Figure

5. Standardization of filter-binding assay.

The filter-binding assay was carried out as described in Materials and Methods, except the 0.3 M  $K_2HPO_4$ -KOH added was of various pHs as indicated. Untreated DNA was used in this experiment. It contained less than 0.2 nicks/molecule.
Scintillation fluid was made up of 3.8 l. scintillation grade toluene (Fisher) with 15.2 gm PPO (Syndel Lab. Ltd.) and 0.38 gm of POPOP (Syndel Lab. Ltd.).

Total DNA presented in a reaction mixture was estimated by measuring the radioactivity of an aliquot spotted on a blank filter paper. The proportion of nicked PM2 DNA (X) retained on the filter paper was then calculated. The average number of nicks/. molecule in the DNA ( $\omega$ ) was obtained from the equation  $\omega = -\ln(1-X)$ . The equation was derived by assuming a Poisson distribution of the target sites among the DNA molecules (44). Assuming the PM2 DNA had 18,000 nucleotides, the amount of PM2 circles in a 50  $\mu$ l reaction containing 0.05 mM PM2 DNA nucleotide was 138.8 fmoles. Thus, the total nicks in a reaction mixture was estimated.

## 6. <u>Apurinic endonuclease assay</u> :

Endonuclease activity was assayed by monitoring the conversion of superhelical PM2 DNA to nicked circles. Unless otherwise stated, a standard reaction mixture (0.05 ml) contained 0.05 mM depurinated PM2  ${}^{3}$ H-DNA nucleotide, 50 mM Tris-HCI (pH 8.0), 10 mM MgCl<sub>2</sub>, 10 mM KCl, 10 mg/ml of acetylated BSA, a 100-fold dilution of the depurination buffer introduced with the depurinated DNA and an appropriate amount of enzyme. After an incubation for 10 min at 37°C, the reactions were chilled and the filter-binding assay carried out as described earlier. Assays were corrected for nicks occuring in the DNA preparation and during depurination. The blank was 0.2-0.4 nicks/molecules.

A unit of endonuclease activity catalysed the production of

1 pmole of nicks per min. Under our conditions, the assay gave a linear response with enzyme added up to a level which produced approximately one incision per molecule. The lowest level of nicking we used to calculate enzyme activity was at least 10% greater than the blank.

### 7. <u>Sephadex G-100 column chromatography</u> :

A column (0.9 cm x 27.5 cm) of Sephadex G-100 (particle size 40-120 µm,Sigma) was prepared and equilibrated with two different buffers : buffer X of 50 mM Tris-HCl (pH 7.5)/50 mM KCl/0.1 mM DTT/ 10% glycerol and buffer Y of (50 mM Tris-HCl (pH 7.5)/1 M KCl/0.1 mM DTT/10% glycerol.

To avoid stirring up of the gel during sample loading, a piece of Whatman no.1 filter paper was placed over the gel surface, and a layer of elution buffer was put on top of the column. The sample (0.5 ml) was made to 35% glycerol and layered onto the column carefully with a pasteur pipette. The column was eluted with buffer A or B at a rate of 0.056 ml/min. Fractions of 0.5 ml were collected. The following standard proteins were used for calibration and were purchased from Sigma : BSA, ovalbumin (egg white), β-lactoglobulin A and B (milk), myoglobin (whale skeletal muscle, Type II), cytochrome C (horse heart, Type VI). The molecular weights of these proteins are 64,000, 45,000, 35,000, 18,000, 12,384,respectively. The void volume (Vo) of the column was measured with blue dextran (average molecular weight 2,000,000, Sigma), the total volume (Vt) was determined with bromocresol purple (molecular weight 540.2, Sigma).

The absorbance at 660 nm was determined for each column fraction. The elution volume (Ve) for each standard protein was determined by the absorbance at 280 nm. The elution constant (Kav) was calculated by the equation Kav = (Ve-Vo)/(Vt-Vo).

### 8. Sucrose gradient centrifugation :

4.4 ml of a linear gradient of sucrose (5-20%) was layered above a 0.25 ml cushion of 60% sucrose in a polyallomer tube (Beckman). The sucrose solutions used to form the gradient contained 50 mM Tris-HCl (pH 7.5)/0.1 mM DTT and for some experiments, 1 M KCl also. The enzyme sample to be analysed was first dialysed overnight against two changes of 500 ml of 50 mM Tris-HCl (pH 7.5). To minimize disturbance of the gradient, 0.25 ml of the enzyme sample was layered on top of the gradient from a micropipette attached to a 1 ml syringe. Centrifugation was for 27 hours at 50,000 rpm in a Beckman SW 50.1 rotor at 2°C. Fractions of 0.2 ml were collected for enzyme assays. BSA (4.25 S), β-lactoglobulin (2.85 S), myoglobin (2.0 S) were used as marker proteins.

### 9. Salt treatment of enzyme aliquots :

The enzyme aliquots were made to 2 M KCl or 2 M NaCl and incubated on ice for an hour. They were then subjected to analysis by either Sephadex column chromatography or sucrose gradient centrifugation. In some experiment, the aliquots were centrifuged in a table-top Eppendorf table centrifuge for 5 min. No difference was observed between the experiments with or without the

### centrifugation step.

### 10. <u>Preparation of acetylated BSA</u> :

1 gm of BSA (Sigma) was dissolved in 25 ml of saturated sodium acetate and 25 ml of 0.2 N sodium phosphate (dibasic) and cooled to  $0^{\circ}$ C on ice. While stirring on ice, 50 µl of actetic anhydride was added to the BSA solution every 30 min for a total of six additions. The solution was stirred for a further 45 min and then dialysed extensively against distilled water. The solution was neutralized to pH 7 with 5 N NaOH, and stored at -20°C. This treatment sho ld destroy various contaminating enzyme activities as well as greatly reduce the affinity of BSA for various small molecules.

## 11. Protein determinations :

Protein concentration was measured by the method of Lowry  $\underline{et}$ al. (66) using BSA as a standard.

#### RESULTS

# 1. <u>Quantification of number of apurinic/apyrimidinic sites in de-</u> <u>purinated DNA</u>:

Apurinic sites in the DNA are susceptible to alkali hydrolysis. The number of alkali-labile sites in the depurinated DNA is in good agreement with the number of sites susceptible to the apurinic endonuclease activity (16,45). To achieve alkali hydrolysis, the filter-binding assay was modified by leaving the reaction mixture in alkali condition for a prolonged period of time at  $37^{\circ}$ C. Alkali hydrolysis of depurinated DNA was found to be complete in 40-60 min at  $37^{\circ}$ C (Fig. 6). The number of apurinic sites in the depurinated DNA for our enzyme assays was thus estimated to be about 3.1 nicks/ molecule (Fig. 7).

## 2. Purification of apurinic endonuclease activity from Hela cells :

The results of a typical purification are summarised in Table 1.

(a) <u>High-speed centrifugation</u>: This purification step is necessary to remove a non-specific endonuclease activity (enzyme activity that nicks native DNA) from the cell lysate. This activity was detected in the flow-through fractions (eluted from the column at 10 mM KPO<sub>4</sub> concentration) from the phosphocellulose column when the centrifugation step was done at a lower speed of 10,000 rpm. The presence of this nonspecific nicking activity would mask the detection of an apurinic endonuclease species.



Figure 6. Time course of alkali hydrolysis of apurinic PM2 DNA. PM2 DNA was depurinated for 0 min (o), 3 min (•) and 6 min (•). The DNA was then subjected to the normal filter-binding assay except that the DNA was left in the alkali condition for various times at 37<sup>o</sup>C.



Figure

7. Time course of depurination of FM2 DNA at 70°C. DNA was depurinated for various time as indicated and subjected to the filter-binding assay as described in Figure 6. The DNA was incubated in alkali condition for 45 min at 37°C before neutralization.

Table I. Purification of apurinic endonuclease activity from Hela cells

| Fraction                   | Volume<br>(ml) | Protein<br>(mg) | Activity<br>(units) | Specific activity<br>(units/mg) | % yield    |
|----------------------------|----------------|-----------------|---------------------|---------------------------------|------------|
| High speed<br>supernatant  | 1.7            | 10.2            | 8460                | 830                             | *********  |
| DEAE pool                  | 3.8            | 6.84            | 6700                | 980                             | 80         |
| Phospho- I<br>cellulose II | 3              | 3.3<br>0.124    | 30<br>80            | 8.2<br>630                      | 0.4<br>1.0 |
| 111                        | 6              | 0.18            | 1650                | 9200                            | 19.5       |

(b) <u>DEAE-cellulose chromatography</u>: This column was used to remove nucleic acids from the cell extract (67). About 80-90% of apurinic endonuclease activity and 70-80% of the total protein was recovered from the DEAE-cellulose column.

(c) Phosphocellulose chromatography : Three peaks of apurinic endonuclease activity were obtained from the phosphocellulose column. Fractions with the most activity were pooled (Fig. 8) and used in subsequent analysis. They are hereby designated as enzyme species Peaks I, II and III. Peak I did not adsorb to the phosphocellulose column at 10 mM KPO, Peak II came out from the column at about 210 mM KPO\_4 and Peak III at 260 mM KPO\_4. Based on their affinity to phosphocellulose, Peak I and Peak III presumably correspond to the flow-through and the high-salt eluate apurinic endonuclease activity of human fibroblasts, respectively (45). Peak III was the major species of apurinic endonuclease activity in Hela cells. Peak I and Peak II were relatively minor species, each amounted to only 2-5% the activity of Peak III. Peaks I and II did not seem to be artifacts resulting from overloading of the column. We obtained similar distribution of the three enzyme activities in several experiments where the amount of protein put on the phosphocellulose column ranged from 0.47 mg to 8 mg.

Recovery of apurinic endonuclease activity from the phosphocellulose column was generally about 30% of the total activity put onto the column.

With the assay conditions for apurinic endonuclease (see Methods and Materials), no significant amount of nonspecific endonuclease



Figure

8. Phosphocellulose chromatography of apurinic endonuclease activity from a Hela cells extract. The inserted diagram shows the flow-through activity in

a larger scale.



activity (activity that nicks native PM2 DNA) was found in the eluate of the phosphocellulose column except in the flow-through fract- † ions. However, when the enzyme assay was performed in a condition of 10 mM Tris-HCI (pH 7.5), two more peaks of nonspecific endonuclease activity were detected : One eluted from the column at about 180 mM  $\text{KPO}_4$  and another at about 300 mM  $\text{KPO}_4$ . We are now in the progress of determining whether they have any preference for other DNA le-Endonucleases that incised native DNA were also found in E. sions. Some were shown to be more active on DNA treated with UV coli. light and osmium tetroxide. One of the endonucleases, endonuclease V, was highly active on uracil-containing DNA. This suggested a nucleotide excision repair mechanism for removal of uracil residues from the DNA besides a base excision repair mode ultilizing uracil-DNA N-glycosidase (69).

Table I also indicates that at this stage of purification, Peaks I-III were still in a very crude state. This was particularly true for Peak I, since the bulk of the protein also eluted in the flowthrough fractions. The maximum purification factor achieved (calculated relative to the high speed supernatant) was about 10 for Peak III.

## 3. <u>Phosphocellulose rechromatography of apurinic endonuclease</u> <u>activity of Hela cells</u> :

Peak I and Peak III pools were each dialysed against two changes of 500 ml of a solution of 10 mM KPO<sub>4</sub> (pH 7.4) overnight and subjected to phosphocellulose chromatography again.

When Peak I was rechromatographed, 50-60% of the recovered activity again eluted in the flow-through fractions. A peak of activity at around 260 mM KPO4 was also evident. The rest of the activity eluted between 170-210 mM KPO, (Fig. 9). In the case of Peak III, about 96% of the recovered activity eluted from the column at 260 mM  ${\rm KPO}_{\rm A}.$  However, 4% of the recovered activity was now recovered in the flow-through fractions (Fig. 10). The result suggested that for a yet undetermined reason, there is an interconversion between the flow-through and the high-salt eluate species of apurinic endonuclease of Hela cells. To provide further evidence for this phenomenon, it would be of interest to rechromatograph the flow-through activity for a second time on the phosphocellulose column, and to see if there will be again a 50 : 50 distribution of enzyme activity in the flow-through and the high-salt eluate fractions. A larger amount of enzyme extract would be needed for such experiments.

4. <u>General properties of apurinic endonuclease activity in Hela cells</u> : (a) <u>Requirement of magnesium ions</u> : All three enzymes species had some residual activities in the absence of divalent cation, and were strongly stimulated by the presence of MgCl<sub>2</sub>. They were optimally active at around 5-10 mM MgCl<sub>2</sub> (Fig. 11). MgCl<sub>2</sub> concentrations above 15 mM were inhibitory.

(b) <u>pH optimum</u>: Peak I and Peak II had a pH optimum around 8 (Fig.
12). In both cases approximately 60 to 70% of the activity at optimum
timal pH was manifested at pH 7.3 and 8.6. Peak III had an optimum



Figure 9. Phosphocellulose

rechromatography of Peak I.



Figure 10. Phosphocellulose rechromatography of Peak III.





Hela cells.

(a) Peak I, (b) Peak II and (c) Peak III.

Enzyme assays were performed as described in Materials and Methods with the concentrations of MgCl<sub>2</sub> indicated.

▲ \_\_\_\_\_ ▲ apurinic DNA, △ \_\_\_\_\_ △ native DNA.



Figure 12. Effect of pH on apurinic endonuclease activity of Hela cells. (a) Peak 1, (b) Peak 11 and (c) Peak 111. The final pH of the standard reaction mixture was varied between 6.1 and 8.6.

at around pH 7.5. No significant nonspecific endonuclease activity was detected in Peaks II and III over the range of pH tested. However, nonspecific endonuclease activity in Peak I was greatly stimulated at pH below 7 (Fig. 12a). A similar finding was also reported with the human lymphoblastic cell line CCRF-CEM (71).

(c) <u>Effects of NaCl and KCl concentration</u> : Increasing concentrations of NaCl seemed to have an inhibitory effect on all three enzyme species. At 40 mM NaCl, all three enzyme activities were inhibited to about 70% of the activities in the absence of NaCl (Fig. 13). Peaks I and II were also inhibited by increasing concentrations of KCl, while the enzyme activity of Peak III was slightly stimulated by the presence of 20-40 mM KCl and was not inhibited by KCl concentrations up to 100 mM (Fig. 14).

(d) <u>Heat inactivation</u> : Aliquots of Peaks I-III were made 50 mM KPO<sub>4</sub> (pH 7.4)/10% glycerol/0.01 mM DTT/0.1 mg/ml acetylated BSA and heated at  $45^{\circ}$ C for various time. The results are summarised in Figure 15. Peak I was quite stable to prolonged heating at  $45^{\circ}$ C, while the other two species were heat-labile. Peak III was most heat-labile, it had a half-life of 2 to 3 min. Enzyme activity in Peak II was inactivated initially with a half-life of 2 to 3 min as Peak III, the remaining 40-45% enzyme activity was more heat-stable with a half-life of about 25 min. The result suggested the presence of two forms of apurinic endonuclease in Peak II with markedly different heat sensitivities.

5. Molecular weight determinations of Peaks [-1]:





The concentration of NaCl in the reaction mixture was varied between 0 and 0.1 M. Activity at 0 M NaCl was taken as 100%.

39.



Figure 14. Relative activity of (a) Peak I, (b) Peak II and (c) Peak III at different KCI concentrations.

The concentration of KCI in the reaction mixture was varied between 0 and 0.1 M. Activity at 0 M KCI was taken as 100%.



Figure 15. Heat inactivation curve of apurinic endonuclease activity in Peaks I-III.

Aliquots of Peaks I-III were made 50 mM KPO<sub>4</sub> (pH 7.4) by adding 1 M KPO<sub>4</sub> in the case of Peak I or by diluting with destilled water in the cases of Peaks II and III. The aliquots were heated at  $45^{\circ}$ C for 0-25 min. The residual apurinic endonuclease activity was determined as described in Materials and Methods. The results, as percentages of initial activities, were plotted on a semi-logarithmic scale.

41.

E

A Sephadex G-100 column was used to analyse the molecular weights of the three forms of apurinic endonuclease activity. Initially, Sephadex buffer X was used to elute the enzyme. However, when Peak 1 of a particular experiment was run on the Sephadex G-100 column, most of the recovered apurinic endonuclease activity eluted in the exclusion volume of the Sephadex G-100 column (Fig. 16). This would imply that the apurinic endonuclease activity was associated with a complex of a molecular weight greater than 100,000. The remaining activity was distributed in the fractions corresponding to molecular weights of 60,000-25,000. Similar results were obtained with Peaks II and III. But in addition, another peak of enzyme activity was obtained in the fractions corresponding to a molecular weight of 8,000-6,000 (Fig. 17). When a DEAE pool of Hela cells extract was run on the column, the distribution of apurinic endonuclease activity resembled that of a run of Peak I. These findings suggest the "low molecular weight" form of apurinic endonuclease activity dissociated from the high molecular weight complex due to the relative high ionic strength (1.2-1.56) of the 200 mM to 260 mM KPO, in the pools of Peaks II and III.

We then experimented with the conditions to dissociate apurinic endonuclease from the high molecular weight complex. For these experiments, DEAE pools of Hela cells extracts were used. It was found that the high molecular weight complexes dissociated with increasing salt concentrations. Incubation of the DEAE pool for an hour at  $0^{\circ}$ C in 2 M NaCl or KCl was sufficient to dissociate most of the high molecular weight complex. The apurinic endonuclease activity would



Figure 16. Sephadex G-100 chromatography of Peak I.

The column was eluted as described in Materials and Methods with Sephadex elution buffer X containing 50 mM Tris-HCI (pH7.5)/50 mM KCI/0.1 mM DTT/10% glycerol. apurinic DNA





44.

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then appear as having a low molecular weight of 6,000-8,000 (Fig. 18). Since apurinic endonucleases from other sources were reported to have a monomeric molecular weight of 40,000-28,000 (26-31, 45), our results were unexpected. To check if these results were peculiar properties of Hela cells or artifacts of the Sephadex column chromatography, the following experiments were devised.

We repeated the above experiments with flow-through activity obtained from normal human fibroblasts of Peggy cells. Apurinic endonuclease was also found to associate with a high molecular weight complex (Fig. 19), and a "low molecular weight" form of apurinic endonuclease appeared when the enzyme pool was treated with 2 M KCI or NaCI.

A pool of the "low molecular weight " form of Hela apurinic endonuclease was then rechromatographed on the Sephadex G-100 column. A smear of apurinic endonuclease activity was obtained all along the column. Recovery of enzyme activity was about 1-2%. But if the pool was first incubated with 2 M KCl before the rechromatography, a peak of "low molecular weight" apurinic endonuclease activity would again be detected (Fig. 20). The result could be explained if the "low molecular weight species of apurinic endonuclease had a tendency to aggregate. Alternatively, apurinic endonuclease could adsorb to the Sephadex G-100 column and only eluted from the column in the presence of higher KCl concentrations.

When a DEAE pool of Hela cell extract was analysed by sucrose gradient centrifugation, most of the apurinic endonuclease activity sedimented in the bottom of the centrifuge tube; with an S value much bigger than BSA (4.25 S) (Fig. 21). This agreed with the result of the Sephadex G-100 column chromatography. However in an experiment



Figure 18. Sephadex G-100 chromatography of a Hela DEAE pool. The DEAE pool was made to 2 M NaCl and 35% glycerol. It was then incubated at 0°C for 1 hour before put onto the column. The column was eluted with Sephadex buffer X. The markers A-E were dextran blue, BSA, β+lactoglobulin,

cytochrome C and bromocresol purple, respectively.



Figure 19. Sephadex G-100 chromatography of flow-through apurinic endonuclease activity purified from Peggy cells. The column was eluted with buffer X. The enzyme pool was applied onto the column without salt-treatment.

▲ apurinic DNA

△ \_\_\_\_ Anative DNA



Figure 20. Sephadex G-100 chromatography of "low molecular weight" form of Hela apurinic endonuclease. A peak of "low molecular weight" apurinic endonuclease activity was obtained from Sephadex G-100 chromatography of a Hela DEAE pool as shown in Figure 18. The fractions comprising this peak were pooled and subjected to Sephadex G-100 chromatography again either directly (D-----D) or after incubation with 2 M KCl (E------B) for an hour at 0<sup>O</sup>C.





where the DEAE pool was salt-treated with 2 M KCl prior to centri-.fugation, apurinic endonuclease activity sedimented in region corresponding to a molecular weight of 45,000-35,000 (Fig. 22). No low molecular weight species was detected. In this experiment, in order to prevent any reaggregration, the sucrose gradient was made 1 M KCl. The peak activity (fractions number 10-13) from this gradient run was pooled and chromatographed on the Sephadex G-100 column after a 2 M KCI salt-treatment. Half of the recovered apurinic endonuclease activity was again eluted in the low molecular weight fractions (Fig. 23). It was therefore concluded the "low molecular weight" species of apurinic endonuclease was an artifact due to adsorption of the enzyme to Sephadex. We found that this adsorption could be eliminated by an elution buffer with a high ionic strenght. Thus, elution buffer Y containing 1 M KCI was used in subsequent experiments. Ljungquist and Lindahl (72) had also used an elution buffer with a high ionic strength (1 M NaCl) to determine the molecular weight of apurinic endonuclease from E. <u>coli</u> by Sephadex G-75 column chromatography. A calibration of the Sephadex G-100 column eluted with buffer Y was shown in Figure 24.

To determine the molecular weight of apurinic endonuclease activity freed from the complex, Peaks I-III were salt-treated with 2 M KCI. They were then put onto a Sephadex G-100 column which was eluted with buffer Y.  $\beta$ -lactoglobulin (2.5 mg) was also added to each of the three enzyme pools and served as an internal marker. It did not have any significant effect on the endonuclease assay. The result of a run of Peak III was shown in Figure 25. The major peak of apurinic endo-



Figure 22. Sucrose gradient centrifugation of a Hela DEAE pool which had been salt-treated with 2 M KCl before centrifugation.

The sucrose gradient contained 50 mM Tris-HCI (pH 7.5) /1 M KCI/0.1 mM DTT.

Markers A, B and C were BSA,  $\beta$ -lactoglobulin and myoglobin, respectively.



Fig. 23. Sephadex G-100 chromatography of the peak fractions of apurinic endonuclease activity obtained in the experiment described in Figure 22. The peak fractions (number 10-20) of the sucrose gradient analysis depicted in Figure 22 were pooled and chromatographed on the Sephadex G-100 column after incubation in 2 M KCl for an hour at 0<sup>o</sup>C. The column was eluted with buffer X.



Figure 24. Calibration of Sephadex G-100 column.

The column was eluted with buffer Y containing 50 mM Tris-HCI (pH 7.5)/1 M KCI/0.1 mM DTT/10% glycerol. Markers A-E were BSA, ovalbumin,  $\beta$ -lactoglobulin, myoglobin and cytochrome C, respectively.



Figure 25. Sephadex G-100 chromatography of salt-treated Peak III with elution buffer Y. Peak III was incubated for 1 hour with 2 M KCl on ice prior to chromatography. The column was eluted with buffer Y. β-lactoglobulin was chromatographed together with the enzyme pool and served as an internal size marker.

nuclease activity was found to elute slightly before  $\beta$ -lactoglobulin. A molecular weight of 35,000-40,000 was estimated for this species assuming the enzyme was a globular protein. Two other minor peaks of activity were also detected. One eluted at fractions corresponding to a molecular weight of 70,000-75,000, possibly a dimer of apurinic endonuclease or an enzyme complex of other entity. Another activity eluted in the vicinity of cytochrome C. Interestingly, UV endonucleases of <u>M</u>. <u>luteus</u> were reported to have a molecular weight of 10,000-15,000 (67).

For Peak II, most of the apurinic endonuclease activity recovered eluted at fractions corresponding to a molecular weightoof 22,-000-25,000 (Fig. 26).

In the case of Peak I, the presence of KCI in the column fractions created a problem. Because of the low level of enzyme activity in this pool, 5-10 µl of aliquots of each column fraction was needed for the endonuclease assay. 5 µl of a column fraction would introduce 100 mM KCI into the assay mixture with a final volume of 50 µl. As discussed earlier, high concentrations of KCI were inhibitory to the apurinic endonuclease activity in Peak I. Thus, the column fractions were first dialysed in 1 l. of 50 mM Tris-HCI (pH 7.5)/0.1 mM DTT/ 10 % glycerol for 2-3 hours before they were used for enzyme assays. With this modification, apurinic endonuclease activity from Peak I was found to elute as a broad peak (Fig. 27). It was inferred apurinic endonuclease with a molecular weight of 45,000-50,000 was eluted in the first half of the broad peak. The other half of the broad peak was composed of enzyme species with a molecular weight similar to





Peak II with elution buffer Y.

Peak II was prepared and chromatographed as Peak III in Figure 25.



Figure 27. Sephadex G-100 chromatography of salt-treated

Peak I with elution buffer Y. Peak I was prepared and chromatographed as

Peak III in Figure 25.

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### DISCUSSION

## 1. <u>Comparison of apurinic endonuclease activity in Hela cells and</u> human fibroblasts :

(a) <u>General properties</u> : In agreement with another report (42), we found that activity of apurinic endonuclease in crude extracts (high-speed supernatant) of Hela cells was similar to that of normal human fibroblasts. The specific activity of apurinic endonuclease in crude extracts of Hela cells was in the range of 400-800 units/ $\mu$ g of protein. The value reported for normal human fibroblasts was between 380-670 units/ $\mu$ g proteins (44).

The apurinic endonuclease activity in Hela cells was then resolved into three peaks of activity by phosphocellulose column chromatography. They were designated as Peaks I, II, III. They had a similar pH optimum and Mg<sup>2+</sup> requirement as the enzyme species of human fibroblasts.

The enzyme species of human fibroblasts were further reported to be stimulated to 2.5-fold by 10 mM KCl. They had a half-life of 6 min at  $45^{\circ}$ C in 230 mM KPO<sub>4</sub> (pH 7.4). But in Hela cells, all three enzyme species were inhibited by increasing concentrations of KCl and NaCl, except Peak III which was only slightly stimulated by 20-40 mM KCl (fig.15). Peaks I-III of Hela cells were different in their thermosensitivities. Peak III was most heat-labile, its halflife ( $t_{1/2}$ ) at  $45^{\circ}$ C in 50 mM KPO<sub>4</sub> (pH 7.4) was only 2 to 3 min. Little loss of enzyme activity was observed for Peak I under these conditions. Preliminary experiments indicated that both species were more heat-sensitive in 250 mM KPO<sub>4</sub> (pH 7.4); at  $45^{\circ}$ C, their halflives were less than 1.5 min. In <u>E</u>. <u>coli</u>, a minor species of apurinic endonuclease, endonuclease IV, was found to be stable at  $45^{\circ}$ C (27). Endonuclease IV however had no Mg<sup>2+</sup> requirement and was fully active in the presence of EDTA. Peak II of Hela cells seemed to consist of a heat-labile ( $t_{1/2}$ = 2 min) and a more heat-stable ( $t_{1/2}$ = 25 min) components.

Thus, apurinic endonuclease activity of human fibroblasts and Hela cells differ in their thermosensitivities and inhibition by increasing salt concentrations. Whether these differences in properties reflect the neoplastic nature of Hela cells remains a question. |+ is however not uncommon that isoenzymes purified from different tissues or organs have different properties. For example, while the apurinic endonuclease in calf-liver has a pH optimum of 9.5 and an optimal  $Mg^{2+}$  concentration of 0.01-0.05 mM, the corresponding values for the calf-thymus enzymes are 8.5 and 0.5-3 mM respectively. Furthermore, the activity of the calf-thymus enzyme is stimulated by 0.04 M NaCl and that of calf-liver is inhibited to 50% by 0.025 M NaCl (29). This is also evident when apurinic endonuclease isolated from human fibroblasts and placenta are compared. For example, the placental enzymes are optimally active at around 3 mM MgCla and those of human fibroblasts have an optimum of 10 mM MgCl2. (b) Relative proportion of flow-through and high-salt eluate species of apurinic endonuclease activity : In normal human fibroblasts, activity of the flow-through species was about 20-30% that of high-salt eluate species. In Hela cells, activity of Peak I (flow-

through activity of Hela cells) was only 2-4% the activity of Peak III (high-salt eluate activity of Hela cells). To see if this represented a peculiar condition of Hela cells, we had purified apurinic ednonuclease activity from a supposedly normal cell line of human fibroblast (Peggy cell). The relative proportion of flowthrough and high-salt eluate activities was similar to that in Hela cells. We do not know the reason for this discrepancy. Perhaps the flow-through activity is subjected to cellular metabolic regulation which may be affected by tissue culture conditions. The cellular level of another DNA repair enzyme, photoreactivating enzyme, was claimed to be affected by composition of the tissue culture medium (73). It was found that human fibroblasts grown in Eagle's minimal essential medium contained very low levels of photoreactivating enzyme compared to cells grown in Dulbecco's modified Eagle's minimal medium. We had routinely supplemented our tissue culture medium with several antibiotics while such was not a practice in the earlier studies with human fibroblasts (44,45).

# 2. <u>Interconversion of flow-through and high-salt eluate species of</u> <u>apurinic endonuclease from Hela cells</u> :

The result of the phosphocellulose rechromatography experiment suggested that the flow-through and the high-salt eluate species of apurinic endonuclease in Hela cells are interconvertible. It will be of interest to identify the factors governing such interconversion. The study may ultimately lead to an understanding of the repair defect in XP-D cells, since they are deficient in the flow-through

species of apurinic endonuclease. One can postulate that adsorption of high-salt eluate activity to the phosphocellulose column is inhibited by a factor E. Flow-through activity therefore is a complex of E and the high-salt eluate species of apurinic endonuclease. This complex will dissociate upon phosphocellulose chromatography. An analogy is the sigma factor ( $\sigma$ ) in <u>E. coli</u> which usually forms a complex with RNA polymerase. It is required for the initiation of RNA synthesis. The sigma factor can be separated from the enzyme by chromatography on phosphocellulose (74,75).

Once factor E is isolated from human fibroblasts, we shall test whether addition of this factor to the high-salt eluate apurinic endonuclease activity from XP-D cells will result in the formation of any flow-through activity. This kind of experiment will determine whether in XP-D cells there is a defect in factor E or in its prodduction, or whether there is a defect in apurinic endonuclease which prevents the association of the enzyme with factor E.

### 3. Molecular weight determinations of Peaks 1-111 :

When Peaks I-III were analysed by Sephadex G-100 column chromatography, a major part of each of the three enzyme activities was found to be associated with a high molecular weight complex. This result was confirmed independently by the sucrose gradient sedimentation analysis. Preliminary experiments with Sephadex G-200 column chromatography indicated that the complexes are less than 150,000 in molecular weight. This again does not seem to be a peculiar property of Hela cells. We obtained similar result from fibroblasts of a

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normal human cell line, Peggy cells. Also, during the earlier stages of enzyme purification from the human lymphoblastic cell line CCRF-CEM (76) and <u>E. coli</u> (26), apurinic endonuclease activity was reported to be associated with high molecular weight complexes. However, with the same purification procedures as ours, Kuhnlein <u>et al</u>. (45) reported that flow-through apurinic endonuclease activity had a S value of 3.3, slightly larger than the high-salt eluate species which had a S value of 2.8. The two S values correspond to a molecular weight of around 40,000 and 35,000 respectively, if one assumes that apurinic endonuclease is a globular protein. No high molecular weight complex was detected. In these experiments, the two enzyme species were stored for a period of more than 1-2 months before analysis (U. Kuhnlein, personal communication). Presumably, over this length of time, the apurinic endonucleases had dissociated from the high molecular weight complexes.

Subsequently, we found that apurinic endonuclease activity could be dissociated from the high molecular weight complex by making the enzyme solution 2 M KCI or 2 M NaCI. The major apurinic endonuclease in Peak III had a molecular weight of 35,000-40,000. Those of Peak II were smaller with a molecular weight of 22,000-25,000. Peak I seemed to contain 2 kinds of apurinic endonuclease, one with a molecular weight of 45,000-50,000 and the other with a molecular weight similar to those of Peak II. Limited by the resolution of the Sephadex G-100 column, we could not conclude whether the enzyme species in Peak I were larger than the corresponding high-salt eluate species.

Another question which remained unanswered is whether the assoc-

iation of apurinic endonuclease with a high molecular weight complex has any biological significance or is merely adventitious. In this regard, it is of interest to note that apurinic endonuclease purified from the plant embryo <u>Phaseolus multiflorus</u> is a nonhistone protein of chromatin (31). The association of apurinic endonuclease with other accessory proteins may be important for its in vivo function. The high molecular weight complex may be part of a repair machinery or represent a storage form of apurinic endonuclease in the cytoplasm.

### 4. Conclusion :

Three species of apurinic endonuclease activity were found in Hela cells, including a flow-through species. For some yet unidentified reason, we got very little flow-through apurinic endonuclease activity from either Hela cells or normal human fibroblasts. Apurinic endonuclease activities from Hela cells differed in some respects, such as thermosensitivities, from those of human fibroblasts. Aside from these differences, we think Hela cells will provide enough enzyme material for further studies of the following problems: (1) the inter-relationships between the different species of apurinic endonucleases, (2) the biological significance for the association of apurinic endonuclease with a high molecular weight complex.

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