INVESTIGATION AND SEPARATION OF NITROFURAZONE-DNA DERIVATIVES PRODUCED BY γ-RADIATION

By

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MASTER OF SCIENCE (1979) McMASTER UNIVERSITY
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ABSTRACT

Gamma irradiation (^{137}Cs) of aqueous solutions of DNA and ^{14}C-nitrofurazone (NF), labelled in the semicarbazone moiety, produced DNA to which NF was covalently bound. Maximum binding occurred under hypoxic conditions. The binding was first order with respect to DNA concentration and was independent of NF providing excess drug was present. Enzymatic hydrolysis of the purified labelled DNA to the nucleotide and nucleoside stages was achieved in volatile buffers. Chromatographic separation of the various components in nucleotide and nucleoside hydrolysates was examined on Sephadex G-50, G-25, G-10, DEAE A-25, and AG 50WX4 columns. The AG 50WX4 column provided good clean preliminary separation of the NF derivatives from normal nucleosides and separation of derivatives into four groups. The Sephadex DEAE A-25 column allowed a more complete separation of NF derivatives. Single stranded DNA exhibited enhanced binding of NF over double stranded DNA. Nucleoside hydrolysates gave different proportions of the NF derivatives from irradiated single stranded or double stranded DNA. Deoxyribohomopolymers were used to assess the origin of the NF derivatives. Two major derivatives were common to all deoxyribohomopolymers suggesting ribose or phosphate

derivatives. The other two groups of derivatives had UV absorption between 270 and 250 nm and appeared to be somewhat acidic in nature as assessed by their mobilities on ion exchange columns.

ACKNOWLEDGEMENTS

I foremost like to acknowledge Dr. D. R. McCalla for giving me his great patience, guidance and knowledge for the work leading up to and completion of this thesis. Also, I wish to acknowledge the efforts of Claudia Lu, Christel Kaiser, Byron Wentzell, Rick Stonard, Philip Zacarias, Marian Leeksma, and Douglas Bryant. Lastly, I would like to acknowledge my wife, Marie, for her untiring encouragement and aid in typing this thesis.

DEDICATION

To my parents Murray V. Rishea and Suzanne M. Rishea who helped me reach this goal through their love and guidance.

And, to Jesus Christ, my Lord and Saviour, from whom all wisdom comes.

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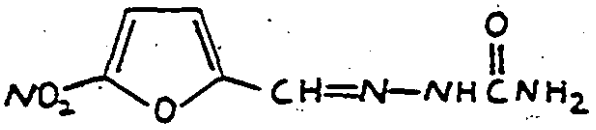
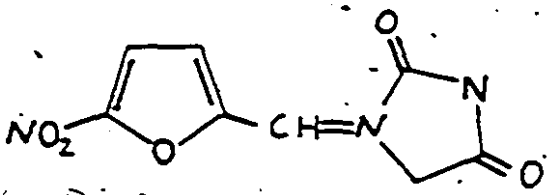
ABBREVIATIONS.

cpm	counts per minute
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
$\bar{e}$ aq	aqueous or solvated electrons
E.S.R.	electron spin resonance
H·	hydrogen radical
HO·	hydroxyl radical
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF	nitrofurazone
OD	optical density
POPOP	1,4-bis(2-(4-methyl-5-phenyloxazolyl))benzene
PPO	2,5-diphenyloxazole
poly dA	polydeoxyadenylic acid
poly dC	polydeoxycytidylic acid
poly dG	polydeoxyguanylic acid
poly dT	polythymidylic acid
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
ssDNA	single stranded deoxyribonucleic acid

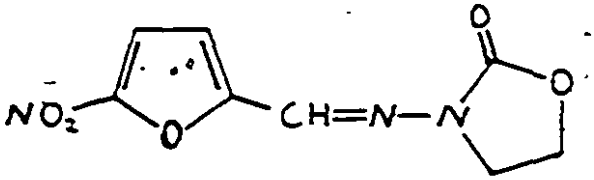
Structures



Nitrofurazone



Nitrofurantoin



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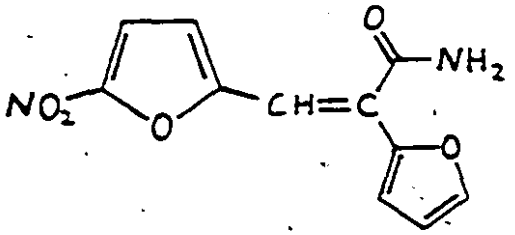

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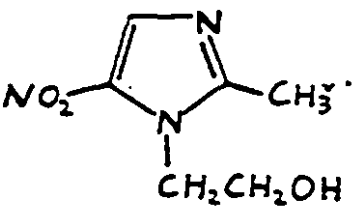

Metronidazole

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INTRODUCTION

Nitrofurans

Nitrofurans constitute a family of synthetic antibacterial agents which have had extensive clinical use. In North America, nitrofurazone has been employed in the treatment and prophylaxis of bacterial infections of skin and mucous membranes. Two other nitrofurans are also in clinical use, namely nitrofurantoin and furazolidone which are employed as urinary antibacterial and antidiarrheal agents, respectively (54). Humans have also been exposed to nitrofuran derivatives used as additives to livestock and poultry feeds and in Japan as a preservative for human food (89).

Initial pharmacological studies demonstrated that nitrofurans were readily metabolized by procaryotic and eucaryotic cells (13, 40, 105). Asnis showed that enzymatic reduction of nitrofurans produced compounds more toxic than nitrofurans themselves (13). He found evidence of two nitrofuran reductase enzymes, I and II, in Escherchia coli. Reductase II was strongly inhibited by oxygen and was maximally active under hypoxic conditions. In contrast, reductase I was fully active under aerobic conditions (12). Several mammalian enzymes have also been shown to reduce nitrofurans in hypoxia. These include xanthine oxidase (12, 39, 132),

microsomal NADPH cytochrome P-450 reductase (39, 140) and aldehyde oxidase (146). All these are flavoproteins and are relatively inactive towards nitrofurans in air saturated media. Unless enzymatic reduction occurs, the nitrofurans are relatively inactive and non-toxic (87). Specific E. coli reductase enzymes are currently being examined and purification and characterization attempted (86, 88).

Enzymatic studies showed that nitrofurazone interferes with glucose metabolism. Pyruvate dehydrogenase was inhibited as well as a number of other enzymes (83). Nitrofurans are thought to interfere with normal cell metabolism by producing an electron shunt, via free radical nitrofuran intermediates, between endogenous cellular reducing agents and oxygen. The free radical nitrofuran intermediates are reactive towards oxygen resulting in production of superoxide anion radicals which may themselves cause cytotoxic effects (19).

All nitrofurans tested proved to be mutagenic and many were carcinogenic. Only nitrofurantoin and 5-nitro-2-furamidoxime were mutagenic but not carcinogenic (31, 85, 133, 147). The nitro moiety proved to be essential for antibacterial activity, carcinogenicity and mutagenicity. Furan analogs were inactive (38). The nitro moiety was enzymatically reduced, accepting electrons from reducing agents such as NADPH and/or NADH. This enzymatic reduction

produced highly reactive intermediates whose exact nature has not yet been determined (86, 139). But since the amines, the end product of the enzymatic reduction, have proven to be biologically inactive (87), the active compound probably is intermediate in oxidation level between the nitro compound and the amine. However, this still leaves a large number of possible compounds.

The reduced nitrofurans bind to protein and to DNA(26). AF-2 (2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide) can be reduced using xanthine oxidase and NADH and the reduced-products bind to DNA (90). Nitrofurans also show DNA modifying activity by the repair test using E. coli rec+ / recA and Salmonella typhimurium uvr+ / uvrB (90). Breakage of DNA has been observed in bacterial (84) and mammalian cells (101) after incubation with nitrofurans.

Ionizing Radiation and Chemical Radiosensitizers

Ionizing radiations are known to produce mutagenic, carcinogenic, aging and other effects on living things. When a cell is exposed to ionizing radiation, one expects a complex interplay of biochemical effects stemming from a primary lesion in some vital cellular component which, if unrepaired, ultimately causes death. It is believed that death and mutations caused by ionizing radiation arise from indirect interactions of ionizing radiation with DNA (29).

It has been long recognized that oxygen was a potent radiosensitizer. Irradiation of procaryotic and eucaryotic cells in air saturated conditions (21% oxygen) demonstrated approximately three fold enhancement of cell death relative to that found under hypoxic conditions (8, 10, 26, 28, 44, 63). Oxygen has been shown to sensitize mammalian cells in every phase of the cell cycle (24, 76, 80). The additional damage induced by radiation in the presence of oxygen is believed to be due to enhanced damage of the DNA in cells. This enhanced damage was demonstrated by an increase in single strand breaks (36, 104), release of inorganic phosphate (21, 66, 72), base damage (30, 57) and by binding of oxygen itself to DNA (78, 120). Although many chemicals are capable of enhancing the sensitivity of hypoxic bacterial and mammalian cells to ionizing radiation, none have proven to be more effective than oxygen.

Survival curves of cultured mammalian cells subjected to radiation show a shoulder of varying size then an exponential decrease in survival. However, tumors in situ give survival curves showing two exponential components (Fig. 1). This is believed to represent cells of two sensitivities (107). The initial slope of this multicomponent curve is typical of the killing of well oxygenated cells. It was proposed that the second component represents survival of hypoxic

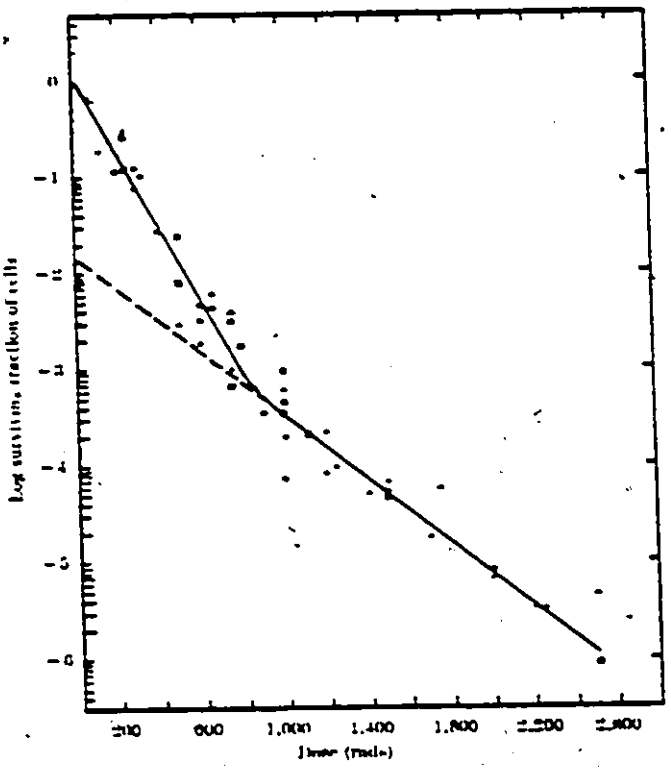


Figure 1. Multicomponent radiation survival curve.
Survival of 6C3HED mouse lymphosarcoma cells irradiated *in vivo*.

(Taken from Powers, W. E., and Tolmach, L. J., *Nature* 197, p.710, (1963)).

cells which were present in much smaller numbers. These hypoxic cells exhibit an increased survival. Further, radiation of cells in situ in mice killed by either cervical fracture or asphyxiation in nitrogen (all tissues of which are presumably hypoxic), and anemic animals showed an increase in the proportion of radiation-resistant cells (60, 62, 107).

This behaviour of cells in a tumor exposed to radiation has been rationalized on the basis that the outer cells of a poorly vascularized tumor consume much of the available oxygen (129, 131). As a result, some of the cells in the interior of the tumor become hypoxic enough to be radioresistant. In addition, direct measurements have established that cells in solid tumors are in regions of low oxygen concentration (94, 134). It is thought that these hypoxic cells in animal and human tumors limit radiocurability (45, 61, 103). Regrowth of the tumor at the primary site is thought to result from the hypoxic cells which have survived the lethal action of the most practical radiation treatment (29). Under the assumption that these hypoxic cells play an important role in limiting the radiocurability of tumors, a number of different approaches have been made to deal specifically with these radiation resistant cells. Basically, these involve attempts to make the cells in the interior of the tumor as sensitive as those towards the exterior.

The obvious approach of improving the effectiveness of oxygen in radiotherapy through the use of hyperbaric oxygen chambers has been disappointing (71). Another approach has been to search for chemicals which would specifically act as efficient radiosensitizers of hypoxic cells. These compounds, because they are not normal metabolites might be able to diffuse to the hypoxic region of the tumor and thus rendering them more sensitive to ionizing radiation.

Initially it was recognized that p-nitroacetophenone was a radiosensitizer (1, 24). However, it was much less effective than O_2. Studies performed with p-nitroacetophenone analogs demonstrated that the nitro moiety of the aromatic ring was essential for radiosensitization (25). Further, the extent of radiosensitization could be correlated with the electronegativity of nitrobenzene analogs (109). Examination of other nitro-bearing chemicals finally lead to the introduction of nitrofurans, which were more electronegative than the nitrobenzene derivatives (116). Nitrofurans had the additional attraction of having established clinical acceptability (106).

Nitrofurans have been found to have excellent radiosensitizing potential in mammalian cells <u>in vitro</u>(Fig. 2) (26, 112) and in model chemical systems which measured damage to nucleic acid bases (47, 48) and release of phosphate from phosphate esters (49, 110). The radiosensitization

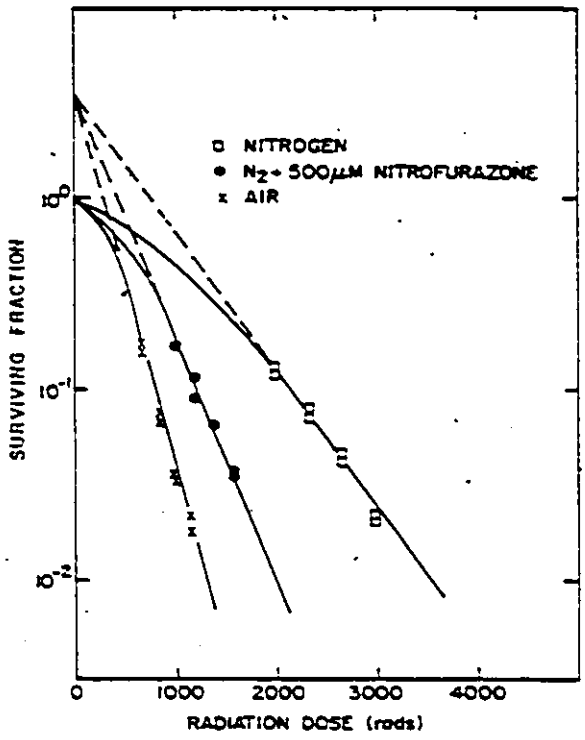


Figure 2. Nitrofurazone radiosensitization of mammalian cells. Survival curves for Chinese hamster cell line V79-379-A irradiated while contact inhibited in hypoxia, in hypoxia with 500 μM Nitrofurazone and in air-saturating conditions.

(Taken from Chapman, J. D., Reuvers, A. P., Borsa, J., Petkau, A., and McCalla, D. R., Cancer Research 32, p. 2630, (1972)).

was observed only under hypoxic conditions (26). In mammalian cells nitrofurans have been found to be dose modifying (Fig. 2) and independant of the cell cycle and culture growth phase (26). Radiation-induced single strand DNA breaks of Chinese hamster cells were enhanced by nitrofurans in hypoxia nearly to the extent observed for oxygen under air saturaing conditions (36).

Studies using ^{14}C-labelled nitrofurazone (NF) have demonstrated that ionizing radiation causes the nitrofuran to bind to DNA, polyribosehomopolymers, serum albumin and constituents of whole cells under hypoxic conditions. No binding was observed under air saturating conditions (26, 27).

Extensive studies have led to the following scheme to explain how radiation affects the integrity of DNA. Ionizing radiation, impinging on an aqueous solution, excites the water molecules along the track of the photon producing hydrogen (H·) and hydroxyl (HO·) radicals and solvated electrons ($\bar{e}$ aq.) (29, 42).

$$H_2O \xrightarrow{h\nu} \bar{e}\ aq. + H\cdot + HO\cdot$$

These are termed "primary radicals". The primary radicals then react with biological molecules via hydrogen abstraction, radical addition, substitution, addition to double bonds and bond cleavage producing "secondary radicals" (29).

It is the secondary radicals that constitute the potenially lethal or mutagenic events. This damage is referred to as an "indirect action" of radiation.

Reactions of primary radicals with biological molecules produce two basic types of secondary radicals. Reactions of solvated electrons produce radical anions, whereas reactions of H· and HO· produce neutral radicals. The former are most susceptable to electron transfer, the latter to adduct formation (27, 51). Various compounds can pick up the free electrons of the radical anions thus preventing charge recombination, and returning the secondary radical to its original state (2, 50). However, similar processes involving electron transfer from the neutral radicals could lead to radiosensitization. In this latter case the fixation of free radical damage in biological molecules is a manner analogous to spin-trapping can occur (22, 27).

The electronegative nitro moiety of nitrofurans has an electron withdrawing effect on the rest of the molecule. As a result it activates the rest of the molecule toward nucleophilic attack (93). Such an electronegative or electron affinic group would tend to stabilize the introduction of a free electron by becoming more negative itself. Thus, the nitro moiety allows the production of a relatively stable radical intermediate.

The electron transfer reactions of secondary radical

anions of pyrimidine and purine bases with nitrofurans has been examined (51). This electron transfer occurs at diffusion controlled rates ($k = 10^{10}\ M^{-1}\ sec^{-1}$), that is the only time factor involved is the time required to bring the reactants together. Nucleoside and nucleotide radical anions also transfer electrons to nitrofurans at rates which are close to diffusion controlled $\sim 3.4 \times 10^{9}\ M^{-1}\ sec^{-1}$. However, these reactions themselves lead to reconstituted molecules and can not be responsible for chemical radiosensitization unless some intramolecular reorganization has occurred. The rate at which the corresponding neutral radicals transfer electrons is an order of magnitude lower, but this process results in radical nitrofuran adduct formation. In irradiation of nitrofurans alone, primary radicals can react with nitrofurans at, or close to, diffusion controlled rates (27, 53).

Effect of Radiation on DNA and Its Constituents

The formation of radicals on DNA and subsequent reactions of the DNA with other molecules in believed to be responsible for the lethal effects of radiation if the damage can not be corrected. Damage to the bases of DNA is considered to be important in the killing of cells by ionizing radiation (23) and in radiation-induced mutagenesis and carcinogenesis (92). The details of the mechanism for

these reactions, which are due to the reactions of H·, HO· and hydrated electrons with DNA are therefore of considerable interest.

When purine and pyrimidine nuclectides in dilute aqueous solution are exposed to ionizing radiation, they are destroyed by two pathways (120). The major pathway involves attack on the base moiety (56). The minor pathway is by attack which results in release of the base from the phosphate-sugar moiety (120). It has been reported that bases and nucleosides were liberated from irradiated DNA (57, 136, 143). The amount of base released is approximately a quarter of the amount of bases destroyed. These studies indicated that one base in 10^4 was damaged per Krad. Oxygen present during irradiation increases the amount of base damage by a factor of 2.5 (57).

Adenine and cytosine were released in slightly greater amounts than guanine and thymine (57, 136). Of the four bases thymine was most susceptable to destruction followed by cytosine, guanine and adenine (57, 121). This same order applies to damage arising from exposure of powdered bases or nucleosides to radiation (65), but in this situation the damage was due to direct ionization of bases and nucleosides. Schnabl (118) proposed that this order may be related to the lone pairs of electrons on the oxygen of carbonyl groups and therefore damage increased with the number of such groups in the various

bases. Alternatively, Isaacson (65) suggested that the decreasing resonance energy per π electron was related to increasing damage by ionizing radiation. In general, purines appeared to be twice as stable to ionizing radiation as pyrimidines (121). However, the release of purines was not different from the release of pyrimidines when DNA was exposed to radiation under hypoxic conditions (57).

In addition to free bases, 2,6-diamino-5-formamido-4-hydroxylpyrimidine was found in DNA exposed to radiation (57). This arose from attack on the guanine moiety (56). Attack on an adenine moiety formed the corresponding formamidopyrimidine (58). These products have labile sugar linkages and the ultimate result was depurination.

Many studies employing E.S.R. (Electron Spin Resonance) have examined radicals in DNA or its constituents produced by radiation. However, many of these studies had been performed on frozen solutions, crystalized or powdered nucleic acid constituents at low temperatures. The radicals produced in this manner were a result of the direct action of radiation. Most radicals produced were ionic in nature caused by expulsion or addition of electrons. These charged radicals were largely immobile and unable to react. However, this approach had been adopted due to the ease of examining the resultant radicals by E.S.R. The radicals had much longer life times under these conditions than in aqueous solution. This approach served as a starting

point for more involved studies. Also, it was applicable to situations where an organism's DNA was in a dry state as in bacterial spores and viruses.

An alternative approach is the irradiation of DNA or its constituents in aqueous solution. In this approach the radicals produced are the result of indirect action of radiation. These radicals may be examined by E.S.R. (37, 114, 126). Recently, the technique of spin-trapping has been applied in E.S.R. studies with considerable success in the identification of radiation damage in solutions of DNA constituents (69, 72, 77, 113). It can be argued that the situation of DNA in the cell is very unlike that of DNA in solution. However, a better approximation of the effects of radiation on DNA is obtained with these studies than in studies with immobilized DNA constituents.

An examination of the radicals produced in aqueous solutions of DNA and its constituents allows a direct identification of the secondary radicals. Since it is the secondary radicals which are responsible for binding of NF to DNA (27) it is possible to predict in a limited fashion the extent to which the various bases should form adducts. Thus, knowledge about the main points of attack of H·, HO·, and hydrated electrons is indispensable for the interpretation of the chemical processes involved in radiation damage to dissolved substances.

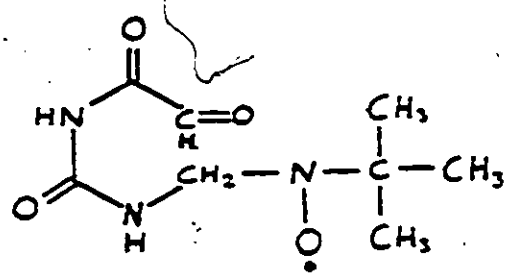
Radicals produced on pyrimidines and their nucleoside

and nucleotide derivatives have been successfully examined by E.S.R. and spin-trapping methods(37, 69, 72, 77, 113, 114, 126). However, experiments giving a similar insight into radical processes on purines are lacking. It appears that the absence of pronounced hyperfine structure in most of the E.S.R. spectra of these compounds is a serious difficulty for the unequivocal identification of purine radicals. In most cases the radical yields were below the detection level and in those cases where E.S.R. signals can be observed their intensity was too weak. This was despite the observation in pulse radiolysis studies that attack of primary radicals on purines was nearly diffusion controlled (123) and not much slower than the attack on pyrimidines (91, 123).

E.S.R. studies on pyrimidines exposed to radiation have shown that addition of HO· takes place predominately at the C_5-C_6 double bond of the base. HO· radicals were found to add mainly to the C_5 and to a lesser extent to C_6 (114). Radicals formed by H abstraction from the methyl group of thymine were also observed (114). In a study of uracil radiolysis products, H· and HO· radicals were found to add to the C_5-C_6 double bond (125, 126). There appeared to be little difference in the extent of damage in various pyrimidines (42).

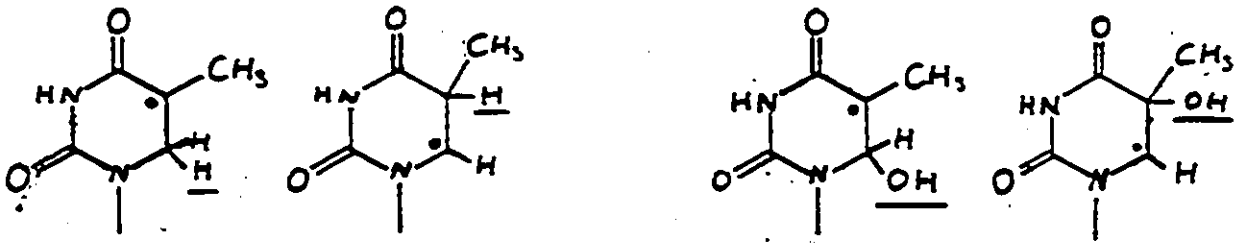
In the spin-trapping technique the short lived secondary radicals react with the spin trap R-N=O and are

converted into long lived nitroxide radicals which can be conveniently identified by E.S.R. (69, 72, 77, 113, 114). These studies support the earlier E.S.R. studies and demonstrated that additional secondary radicals were produced. For all pyrimidine derivatives, two or more radicals formed by the addition of H· and HO· to the C_5-C_6 double bond of pyrimidine derivatives were observed. Evidence for a radical on the methyl group of thymine was also obtained. Thymine, and its corresponding nucleoside and nucleotide also demonstrated a radical site generated by H abstraction at the N_3 position. Ring opening of uracil at the C_5-C_6 bond to produce a t-butylnitroxide spin label was observed (69):



Radicals of this nature may be present in cytosine and thymine as well (69). In addition, studies with nucleotides demonstrated the release of thymine and uracil with the production of an N_1 position spin label adduct (72, 113). This observation suggests attack causing release of a radical base from its corresponding nucleotide. Such a radical cleavage of the N-glycosyl bond could alternatively

result in a radical site on the remaining sugar moiety. The addition of H· and HO· to the C_5-C_6 double bond was found to be the most common reaction in pyrimidine derivatives (69, 72, 98, 100, 113, 114, 126).



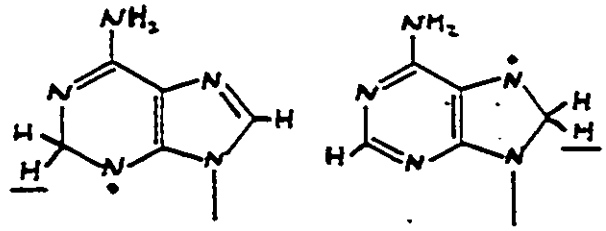
H· Adduct HO· Adduct

H· and HO· Additions to the C_5-C_6 Bond in Thymidine

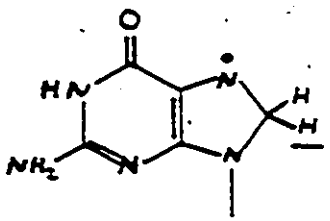
These secondary radicals in aqueous solution can also be generated by rapid-mixing techniques with Ti^{3+}, H_2O_2 systems (34, 96, 117) and in flash photolysis of solutions containing H_2O_2 (82, 114). These systems allow exclusive production of HO·, and the results demonstrated that the same secondary radicals were produced as with ionizing radiation. A small fraction of HO· were found to abstract H atoms from the methyl group of thymine and from the N_1 and N_3 positions of pyrimidines generating radicals at these points (114). An examination of dihydropyrimidine derivatives showed that the hydrogen atoms at

C_6 are most susceptable to abstraction by HO· and to a lesser extent those on the methyl group of a dihydrothymine (115).

As mentioned earlier the study of purine radicals produced by ionizing radiation was difficult. However, certain radicals have been identified and those originating from the addition of H· to the C_8 position of guanine and to C_2 and C_8 of adenine have been adequately characterized (6, 33, 35, 81). These have been demonstrated in the base and corresponding nucleosides, in low temperature studies.

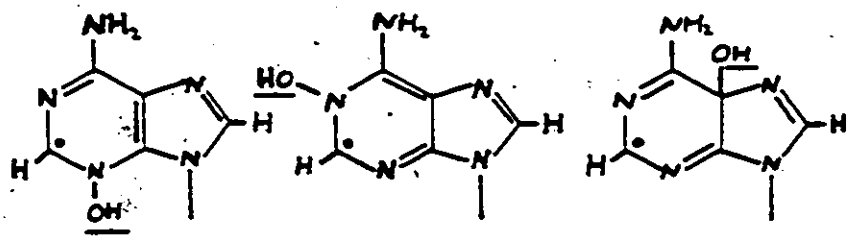


Adenosine Adducts

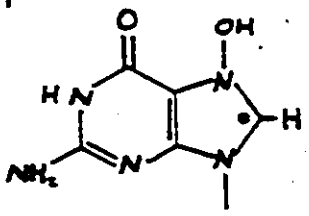


Guanosine Adducts

Schmidt and Borg (117) have been able to identify purine nucleoside radicals in solution by a fast flow technique using the Ti^{3+}, H_2O_2 system. They have identified possible radical sites at the C_8 position in guanine derivatives and at the C_2 position in adenine derivatives. They examined the bases and their corresponding ribose and deoxyribose nucleosides. These were believed to arise via HO· addition on the N's in the purine ring or on the C_5 position. An HO· adduct on the C_8 and C_2 position of caffeine has also been reported (34).



Adenosine Adducts

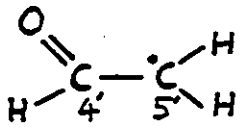


Guanosine Adducts

Radicals on the deoxyribose moiety of nucleosides and nucleotides have been observed in low temperature E.S.R. studies of dry powders or crystals of nucleosides and nucleotides exposed to ionizing radiation (7, 9, 16, 43, 55, 64, 75). However, aqueous studies were more difficult. Evidence of sugar radicals in nucleotides and nucleosides in aqueous solution have been found but spin-trapped sugar radicals were very unstable at room temperature (72). Deoxyribose subjected to radiation did give rise to radicals (69), but the spectra of deoxyribose pyranose sugars (six membered ring) appeared distinctly different from those of deoxyribose furanose (five membered ring) found in nucleotides and nucleosides(117). Free deoxyribose attains a six membered ring in solutions. Evidence of H abstraction followed by secondary reactions have been found in sugars (99).

In a study involving a crystal of deoxycytidine 5' phosphate monohydrate exposed to radiation, the radiation

induced cleavage of the phosphate ester bond was demonstrated (75). The radical formed by scission of the $C_{5'}$-$O_{5'}$ bond was localized at the $C_{5'}$ position of the deoxyribose moiety. Subsequent post-irradiation radical transformations occured, which were associated with fragmentation of the furanose ring. In fragmentation the $C_{3'}$-$C_{4'}$ bond was cleaved producing the following radical (59):



A radical situated at $C_{1'}$ position of the deoxyribose moiety was also indicated in this study. Radical sites produced in sugar moieties by H abstraction at $C_{5'}$ (7), at $C_{4'}$ (64) and at the $C_{1'}$ positions have been observed, as has liberation of the phosphate group which caused a $C_{5'}$ radical (mentioned above) or HO· abstraction from the $C_{5'}$ position in a nucleoside (137).

It was only recently that radicals centered on the phosphate group in DNA constituents were observed. Primary radicals react with phosphate at much lower rates than with the bases: $k_{\bar{e}\ aq} = 7.7 \times 10^6$, $k_{H\cdot} = < 5 \times 10^3$, and $k_{HO\cdot} = < 10^7\ M^{-1}\ sec^{-1}$ at pH 7 (51), whereas reaction rates with bases were of the order of 10^9-$10^{10}\ M^{-1}\ sec^{-1}$. In the reaction with $H_2PO_4^-$, hydrated electrons are converted into H· (68):

$$\bar{e}\ aq + H_2PO_4^- \longrightarrow H\cdot + HPO_4^{2-}$$

Black and Hayen proposed this mechanism for pulse radiolysis of phosphate anion in aqueous solution (20):

$$HO\cdot + H_2PO_4^- \longrightarrow HO^- + H_2PO_4\cdot$$

A low temperature E.S.R. study examining a number of crystalline deoxyribonucleotides exposed to radiation demonstrated that phosphate centered radicals accounted for about 0.1 to 5% of the total free radicals produced (17). Eighteen of the 19 dinucleotides examined showed evidence of this radical. It was proposed that the free electron was localized on one or both of the non-ester oxygens. Ionizing radiation had been shown to yield phosphate free radicals which involved the loss of one electron (79, 128, 130). A phosphate radical in an irradiated crystal of uridine 5'-phosphate has also been examined. This radical was formed by the scission of the $C_{5'}$-$O_{5'}$ bond (108). The same bond was found to be broken in deoxycytidine 5'-phosphate exposed to radiation (75). The only difference in the two molecules is that in the former the electron remained on the phosphate and in the latter on the sugar moiety. These phosphate radicals were unstable at temperatures above 100°K (17, 108) and it must be kept in mind that these phosphate radicals were produced by direct ionization.

Another low temperature E.S.R. study with deaerated wet DNA provided evidence for a phosphate-centered radical. The radical was observed upon warming of the sample (74). Disappearance of HO· radicals and an increase

in phosphate centered radicals was observed with increasing temperature. It seemed likely that the formation of the phosphate radicals was initiated by a small fraction of HO· located in the vicinity of the DNA. These HO· increase their mobility upon heating. If such was the case, then it would support the notion that phosphate radicals are formed on DNA in aqueous solution.

The biological significance of phosphate centered radicals depends on their decay or adduct products. Even if these radicals were low-probability events, their location near the critical phosphodiester bond may give rise to single strand breaks in polynucleotides. The release of inorganic phosphate from nucleotides in irradiated aqueous solutions was thought to be largely due to HO· attack on the deoxyribose moiety (141, 142). Reaction rates of HO· with sugar were close to diffusion controlled (10^9 M^{-1} sec^{-1}), but hydrated electrons and H· react much more slowly ($\sim 10^7$ M^{-1} sec^{-1}) (51). Hence, it is believed that single strand breaks in DNA arose mostly from deoxyribose radical formation rather than by phosphate centered radicals.

The radicals elucidated by these various methods are likely to constitute the secondary radicals. Thus, in summary, using RH to represent a "nucleic acid molecule", the steps in these diffusion controlled reactions are as follows:

Hydrogen abstraction:

$$RH + H^{\cdot} \longrightarrow R^{\cdot} + H_2$$

$$RH + HO^{\cdot} \longrightarrow R^{\cdot} + H_2O$$

Hydrogen addition at a double bond:

$$RH + H^{\cdot} \longrightarrow RH_2^{\cdot}$$

Hydroxyl addition at a double bond:

$$RH + HO^{\cdot} \longrightarrow RHOH^{\cdot}$$

In pyrimidines, adducts on the C_5-C_6 bond are common. As for purines no generalizations are yet possible. Phosphate and deoxyribose centered radicals are produced in diminished amounts relative to radicals in the bases.

Role of Hydroxyl Radicals in Nitrofuran Binding to DNA

Studies indicate that the secondary radicals produced by HO· were responsible for NF adduct formation (27). When t-butanol, which is an effective scavenger of HO·, was added very little binding of NF to DNA occurred upon irradiation. This suggests that HO· radicals were necessary for NF adduct formation. In contrast, nitrous oxide, which reacts with solvated electrons at diffusion rates to produce HO· (20, 27, 51):

$$\bar{e}\ aq + N_2O \longrightarrow N_2 + HO^{-} + HO^{\cdot}$$

enhanced the binding of NF to DNA by approximately 25% (27). Hence, it appears that HO· was the principal reactive

species which ultimately resulted in the binding of NF to DNA. In the presence of t-butanol and nitrous oxide, where the principal radical is H·, no binding of NF to DNA occurred upon irradiation.

No real difference in the rates of electron transfer with NF were apparent between purine and pyrimidine radicals nor among nucleotides and nucleosides. That suggested that the same species, namely the free radicals associated with the bases, were principally involved (50).

Nitrofurazone has been found to be an excellent radiosensitizer in experimental systems in vitro. However, subsequent attempts to demonstrate hypoxic cell radiosensitization by NF in vivo have met with little success (111). Several nitro compounds including nitrofurans, which were able to sensitize hypoxic cells at low cell concentrations in vitro were found to lose a large fraction of their ability to radiosensitize when the cell density was increased (5, 135). High cell concentrations would obviously be encountered in solid tumors in vivo. However, nitroimidazole derivatives particularly metronidazole (2-methyl-5-nitroimidazole-1-ethanol)sensitized cells in vitro at both high and low cell concentrations (135). Metronidazole has been shown to sensitize hypoxic tumor cells in vivo in a number of test systems (5, 15, 111, 127, 135) and nitroimidazoles are currently in clinical trial as radiosensitizers.

Nitrofurazone was employed in this study as it has been the subject of much investigation and can easily be synthesized with a ^{14}C in the semicarbazone moiety, and thus provided a means of examining the ionizing radiation-induced binding of NF to DNA. The ultimate objective of this work was to elucidate the mechanism by which nitrofurazone potentiates radiation induced damage to DNA. This thesis describes i) the effect of parameters such as nitrofurazone and DNA concentration and different doses of radiation on the binding of ^{14}C NF to DNA, ii) methods for the hydrolysis of labelled DNA in volatile buffers and the separation of labelled products from each other and from normal nucleosides, and, iii) through experiments with deoxyribohomopolymers provides an indication of the source of the various labelled DNA derivatives seen.

MATERIALS AND METHODS

^{14}C Nitrofuazone (5-nitro-2-furaldehyde semicarbazone; NF) labelled in the semicarbazone moiety (specific activity 7.0 Ci/mole) was synthesized by Dr. D. R. McCalla. Highly polymerized calf thymus DNA, RNA, deoxynucleotides, deoxynucleosides, deoxyribonuclease I (bovine pancreas) and snake venom phosphodiesterase (Crotalus adamanteus) were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Alkaline phosphatase (Escherichia coli) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Deoxyribohomopolymers (poly-dA, poly-dC, poly-dG, and poly-dT) were obtained from PL Biochemical Inc., Milwaukee, Wis., U.S.A. Buffer constituents, chloroform, isoamyl alcohol, xylene, ethanol, PPO and POPOP were purchased from Fisher Scientific Co., Toronto, Ont., Canada. Pure argon and nitrogen (<20ppm O_2) were obtained from Union Carbide of Canada Ltd., Oakville, Ont., Canada.

I. Liquid Scintillation Counting

One ml aqueous samples were counted for radioactivity in a Beckman Liquid Scintillation Counter after addition of 7ml of a mixture of 1 liter Triton X-144, 3 liter xylene,

0.8gm PPO and 12 gm POPOP.

II. Purity of ^{14}C Nitrofurazone (NF)

^{14}C-NF dissolved in distilled water was applied to a Sephadex G-25 column (60 X 0.9cm or 100 X 0.8cm) which was shielded from light with aluminum foil and eluted with water. The NF was located by its absorption at 254nm using an LKB UVICORD II. The fractions containing NF were then pooled, evaporated to a convenient volume, and used immediately.

Elutants were monitored for UV absorption at 254nm with the LKB UVICORD II and fractions collected in an LKB Ultrurac Fraction Collector. The columns were eluted using a peristaltic pump to maintain a constant flow rate.

III. Irradiation of Samples

DNA and ^{14}C-NF were dissolved in 0.01M NaCitrate 0.01M NaCl (pH 7.0), 0.01M Tris HCl 0.01M $MgCl_2$ (pH 7.0), or in distilled water. The solutions were rendered hypoxic by flushing them with water-saturated argon or nitrogen gas for one hour. The glass vessels were then sealed and irradiated with a ^{137}Cs source at a dose rate of $\sim$290 rad/min

as determined by Fricke dosimetry (139). All solutions were prepared from double glass distilled water. Additional details are presented in the figure captions.

IV. Purification of Nitrofurazone Treated DNA

A. Precipitation with Ethanol

The DNA in the radiolysis mixture was precipitated with three volumes of 95% ethanol and left overnight at -20°C to ensure complete precipitation. The precipitate was centrifuged at 2000 rpm, the supernatant poured off and the precipitate dried by passing air over it. The precipitate was redissolved in water, reprecipitated and dried as described above. The twice-precipitated DNA was redissolved in water and applied to a Sephadex G-25 column (40 X 2.5cm) and eluted with water. Fractions containing DNA were pooled, evaporated to a convenient volume and stored at -20°C until used.

B. Chromatography on Sephadex G-25 or G-50

The radiolysis mixture was sonicated three times for 20 seconds on a Branson Sonicator operated at full power, then applied to a Sephadex column (40 X 2.5cm). Sonication of the DNA prior to application to the columns was necessitated by the highly polymerized DNA that was used. Without sonication the DNA eluted over a much larger

volume. The fractions containing DNA were pooled, evaporated to approximately five ml and reapplied to the same column. The DNA from this second chromatographic step was pooled, evaporated to a convenient volume and stored at -20°C until used. Approximately 10mg DNA can be isolated this way.

C. Dialysis

The radiolysis mixture was placed in a dialysis bag which had been previously boiled to eliminate nuclease activity. The mixture was dialyzed against several changes of distilled water at 4°C for one day. The contents of the dialysis bag were then removed and stored at -20°C until used.

V. Analysis of Low Molecular Weight Radiolysis Products

Chromatography on a small Sephadex G-25 column (100 X 0.8cm), eluted with water, allowed the separation of the low molecular weight radiolysis products from DNA and from the unchanged NF. The relative amounts of the radiolysis products were estimated from their radioactivity. This method worked best for irradiations done in 0.01M NaCitrate 0.01M NaCl (pH 7.0).

VI. Ionizing Radiation-Induced Binding of Nitrofurazone to DNA

A. Effect of Different Radiation Doses

Half a ml of the radiolysis mixture was sonicated three times for 20 seconds and chromatographed on a small Sephadex G-25 column eluted with water. One ml fractions of the elutant were collected and assayed for radioactivity. The radioactivity eluting at the void volume of the column (20-30ml eluted) was used to assess the extent of binding of NF to DNA.

B. Effect of Different Nitrofurazone Concentrations

DNA (1.0 mg/ml) and ^{14}C-NF at two concentrations: 0.84 μg/ml and 4.96 μg/l, were irradiated with doses from 0 to 68.6 Krad, then treated as described in Section VI (A).

C. Effect of Different DNA Concentrations

Double stranded DNA at concentrations ranging from 0 to 6.0 mg/ml and ^{14}C-NF (8.4 μg/ml) were irradiated with a dose of 25.7 Krad. Then 0.5ml (or 0.5mg DNA, for samples containing more than 1 mg DNA/ml) was treated as described in Section VI (A), except that a Sephadex G-50 column (100 X 0.8cm) was used.

VII. Assessment of Nitrofurazone Destruction

A. Chromatography on Sephadex G-25

Chromatography on a Sephadex G-25 column (100 X 0.8cm) eluted with water separated NF from the radiolysis products. NF eluted at approximately 68 to 83ml. This allows an assessment of the amount of unchanged ^{14}C-NF still present.

B. Absorbance Measurement

This method consisted of measuring the absorbance at 375nm (λmax of NF) of the post-irradiated samples and comparing this value to that of unirradiated sample.

VIII. Enzymatic Hydrolysis of Labelled DNA

The enzymatic hydrolysis of DNA followed the procedure of Baird and Brookes (14). To ensure complete digestion to nucleosides each mg of DNA was dissolved in 1.0ml 0.01M Tris HCl 0.01M $MgCl_2$ (pH 7.0) and 260 Kunitz units of deoxyribonuclease I were added and the solution incubated for four hours at 37°C. Then, 0.1ml of 1.0M Tris (pH 9.0) was added to raise the pH to approximately 9 and 0.1 units of snake venom phosphodiesterase added and the solution incubated at 37°C for 24 hours. Next, 2.5 units of alkaline phosphatase were added and digestion continued for another 24 hours at 37°C. The hydrolysate

was stored at -20°C until used.

Alternatively, hydrolysis in volatile buffers was employed using 0.01M ammonium bicarbonate.0.01M $MgCl_2$ (pH 7.0) (pH adjusted with acetic acid) and 1.0M ammonium carbonate (pH 9.0) (pH adjusted with ammonium hydroxide). This was advantageous for large scale hydrolysis and subsequent purification of components where buffer salts would have been a hindrance. Both systems were equally effective and DNA concentrations of 1.0 mg/ml and higher were digested without difficulty.

The stages in the hydrolysis of the labelled DNA were followed by chromatography on a Sephadex G-25 column (100 X 0.8cm) eluted with water. After hydrolysis with deoxyribonuclease I and snake venom phosphodiesterase, which digested DNA to nucleotides, a sample was applied to the Sephadex G-25 column. Hydrolysis to nucleosides by further digestion with alkaline phosphatase was similarly analyzed on the Sephadex G-25 column. The UV and radioactivity profiles were examined to ensure that hydrolysis had occurred.

IX. Chromatography

All columns were equilibrated prior to use with the appropriate elutant and flushed overnight. Fresh buffers

were made immediately before applying the samples. In all cases the sample volume was 1-2% of the column volume. When applying samples to the ion-exchange columns (DEAE A-25 and AG 50WX4) they were adjusted to the pH of the initial elutant buffer or dissolved directly in the initial elutant buffer.

A. Sephadex G-10, G-25 and G-50 Columns

Labelled DNA, nucleotide hydrolysate, nucleoside hydrolysate and radiolysis mixtures were analyzed on Sephadex G-10, G-25 and G-50 (Fine) columns of various sizes. The conditions for elution of these columns are given in the captions to the figures. DNA and radiolysis mixtures were sonicated three times for 20 seconds each before being applied to the columns.

B. AG 50WX4 Columns

The resin, "minus 400 mesh", was purchased from Bio-Rad, Richmond, Ca., U.S.A. The resin was cleaned and charged according to Jumowicz and Spencer (70) then equilibrated with the appropriate buffer. This resin was employed in two fashions: 1) The column was eluted with 0.015M or 0.30M ammonium hydroxide the pH of which was adjusted to 9.20 or 8.9 with formic acid, and 2) initial elution with 0.015M ammonium hydroxide adjusted to pH 3.20 with formic acid. Then, after sufficient elution with this

buffer, the elutant was changed to 0.3M ammonium hydroxide adjusted to pH 9.20 with formic acid.

The columns were unpacked after each use, and the resin washed. In the first case, this was probably not necessary. However, in the second case, due to contraction of the column when the second buffer was applied, the resin had to be repacked before re-use.

C. Sephadex G-10: AG 50WX4 Mixed Column

This column was prepared by mixing equal volumes of Sephadex G-10 and AG 50WX4 equilibrated in 0.015M ammonium hydroxide adjusted to pH 3.20 with formic acid. The column (60 X 0.9cm) was flushed overnight with 0.015M ammonium hydroxide adjusted to pH 3.20 with formic acid prior to its use. This column was eluted following the second procedure outlined in Section IX (B).

D. Sephadex DEAE A-25 Column

Sephadex DEAE A-25 gel was washed with several volumes of 0.5M HCl and then repeatedly with 0.5M NH_4OH. The gel was then washed extensively with 0.5M ammonium bicarbonate(pH 8.00) and finally with 0.01M ammonium bicarbonate (pH 8.00). Columns were packed and flushed overnight prior to use. The swollen gel, if stored, was always washed with 0.01M ammonium bicarbonate (pH 8.00) prior to use. Samples were applied to the column and

a linear gradient started. Solutions consisting of 75ml 0.01M ammonium bicarbonate and 75ml 0.60M ammonium bicarbonate at pH 8.00 were mixed using a Buchler gradient maker. The reproductibility of the Sephadex DEAE A-25 column was fairly good. However, peak positions did shift from run to run due to the difficulty of maintaining identical concentration gradients. However, the order of elution of the peaks was maintained.

X. Comparison of the Labelled Products in DNA Purified By Chromatography or Ethanol Precipitation

DNA (1.0 mg/ml) and ^{14}C-NF (0.84 μg/ml) were irradiated with a dose of 25.7 Krad. The radiolysis mixture was then divided into two equal volumes. Half was purified by the ethanol precipitation method and the other half by chromatography on Sephadex G-25 as outlined in Section IV (A) and (B) respectively.

Labelled DNA isolated using the two methods was hydrolyzed to the nucleotide or nucleoside level and analyzed on Sephadex G-25 column. Nucleoside hydrolysates were also analyzed on the AG 50WX4 column following the second method in Section IX (B).

XI. Binding of Nitrofurazone to Double and Single Stranded DNA

^{14}C-NF (8.4 µg/ml) and double stranded or single stranded DNA (1.0 mg/ml) were irradiated with a dose of 25.7 Krad. The two radiolysis mixtures were purified by the "Chromatography on Sephadex G-25" method, Section IV (B). The labelled DNA from these two irradiations was hydrolyzed to nucleosides and analyzed on Sephadex G-25 and AG 50WX4 column. The AG 50WX4 column was eluted according to the second method in Section IX (B).

Single stranded DNA was prepared by heating commercial DNA in a boiling water bath for 10-15 minutes and then cooling it quickly in an ice bath. Alkali denaturation was used in Section IV (D). The single stranded DNA was then dialyzed in a bag previously boiled to eliminate nuclease activity. The material was dialyzed against several changes of distilled water at 4°C for one day.

XII. Separation of Nitrofurazone Derviatives from Normal Nucleosides

DNA was digested to nucleosides in volatile buffer, then lyophilized to remove the salt and stored dry at -20°C until used. A 90 X 1.5cm column was packed with AG 50WX4 resin equilibrated in 0.015M ammonium hydroxide adjusted

to pH 8.9 with formic acid. The lyophilized nucleoside digest was dissolved in 0.015M ammonium hydroxide adjusted to pH 8.9 with formic acid and applied to the column. The NF adducts eluted earlier than the "normal" nucleosides. Hydrolysates of 10 to 20mg DNA were applied to this column without overloading, and higher amounts could probably be chromatographed.

XIII. Binding Studies with Deoxyribohomopolymers

The deoxyribohomopolymers were used as supplied and dissolved in 1ml of distilled H_2O. Poly-dC was not soluble in distilled H_2O but dissolved in 0.01M NaCitrate 0.01M NaCl (pH 7.0). The deoxyribohomopolymers (5 OD units/ml) and ^{14}C-NF (1.68 μg/ml) were irradiated with a dose of 34.3 Krad. The radiolysis mixtures were then placed in dialysis bags which had been previously boiled to eliminate nuclease activity and dialyzed against several changes of 2mM ammonium carbonate (pH 9.0) at 4°C for one day. The contents of the dialysis bags were emptied and lyophilized. The residue was hydrolyzed to nucleosides in volatile buffers (Section VIII), then lyophilized again to remove volatile salts and stored at -20°C until used. Immediately prior to application to the Sephadex DEAE A-25 column the sample was dissolved in 0.01M ammonium bicarbonate (pH 8.00).

RESULTS

I. Purity of ^{14}C Nitrofurazone (NF)

Chromatography of the ^{14}C nitrofurazone (7.0 Ci/mole) on a Sephadex G-25 column revealed the presence of a small amount of impurities in a stock solution which was approximately one month old (Fig. 3). NF dissolved in distilled water or 0.01M NaCitrate 0.01M NaCl (pH 7.0) also contained minor impurities (less than 0.5%). Higher molecular weight impurities (Y and Z in Fig. 3), as assessed by Sephadex G-25 chromatography, built up as the solution aged. Solutions protected from light were more stable than illuminated ones. NF was purified by chromatography on Sephadex G-25 before each experiment to remove these impurities.

II. Purification of Nitrofurazone Treated DNA

Upon irradiation of NF in the presence of DNA, a number of radiolysis products were produced as the NF was destroyed. These included species which were bound to DNA. Four methods for the separation of this derivatized DNA from low molecular weight ^{14}C labelled material were examined. They were: ethanol precipitation, chromatography

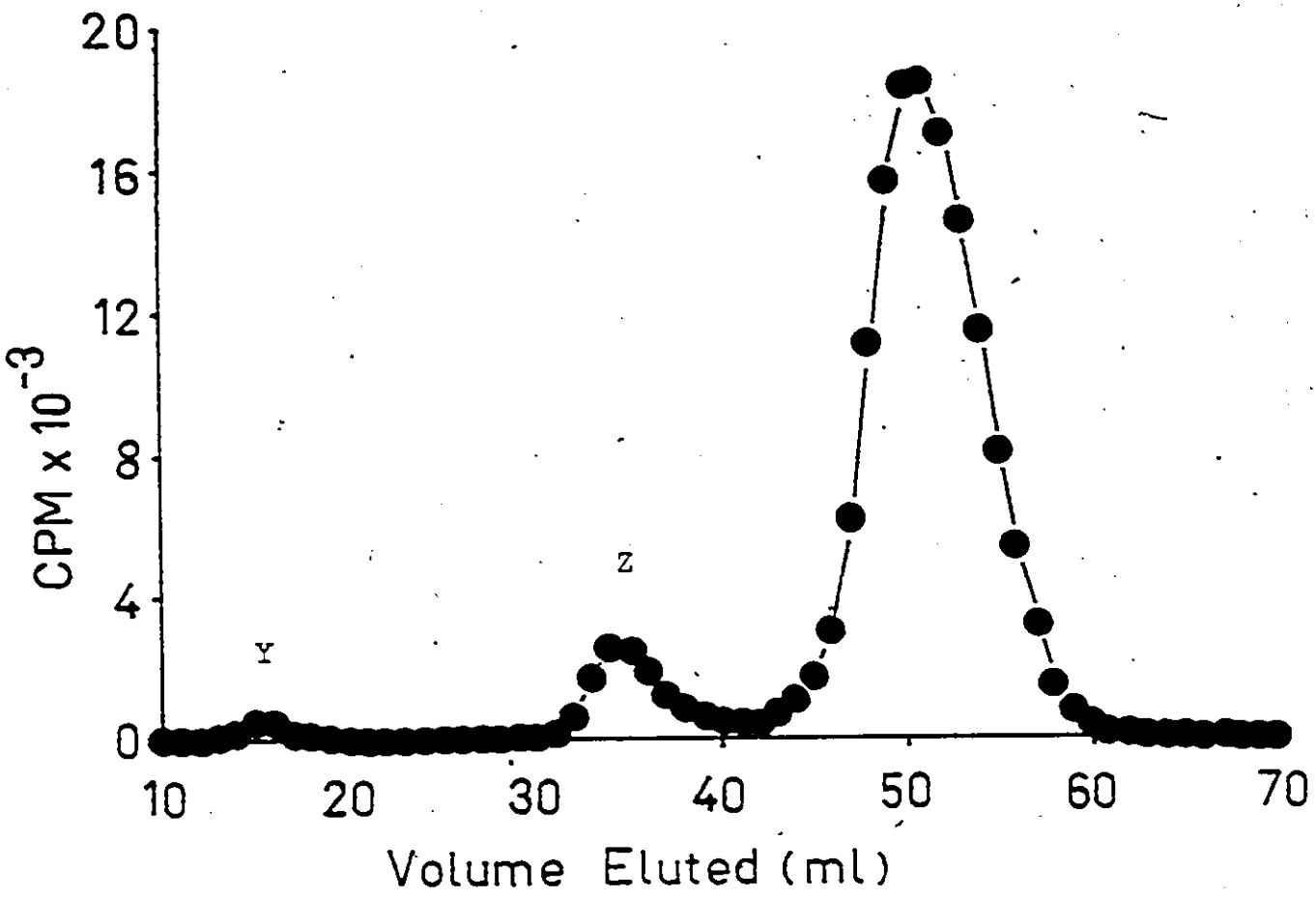


Figure 3. Chromatography of ^{14}C Nitrofurazone on a Sephadex G-25 (60 X 0.9cm) eluted with distilled water, 0.5 ml/min.

on Sephadex G-25 and G-50, and dialysis.

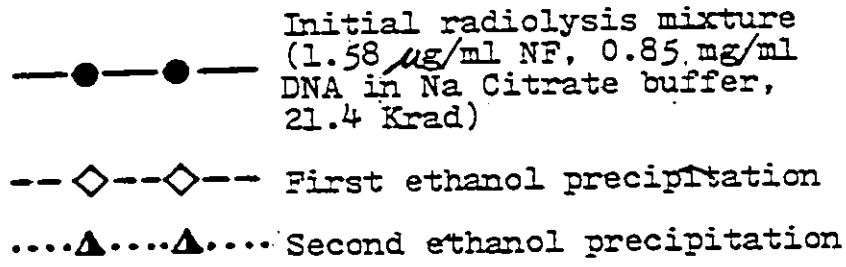
A. Precipitation with Ethanol

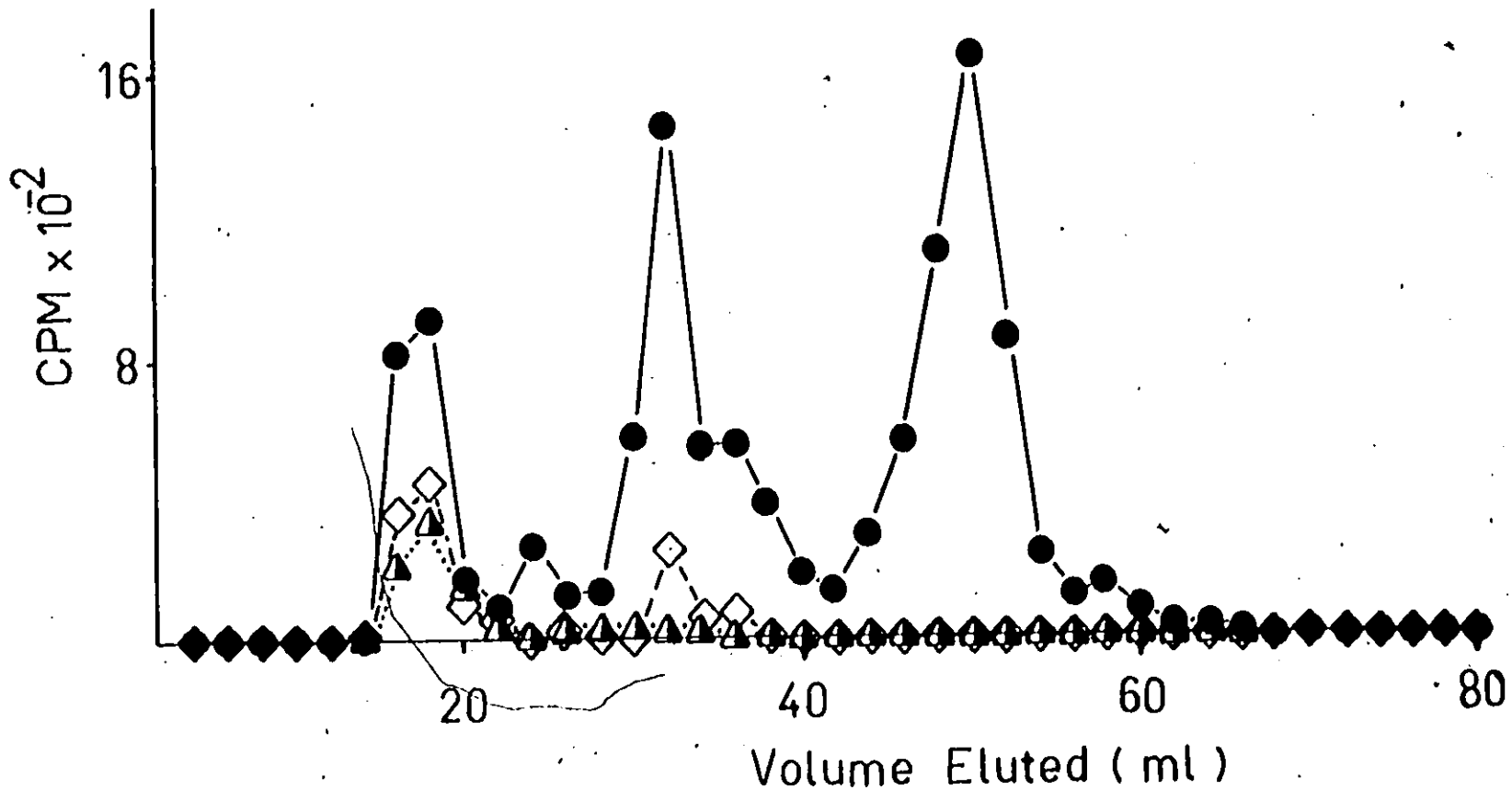
One precipitation of the labelled DNA with ethanol removed 96% of undestroyed NF and low molecular weight radiolysis products. After the second ethanol precipitation the resulting material still had 15% of the radioactivity in low molecular weight compounds (Fig. 4, third profile). Chromatography on Sephadex G-25 was used to remove this material. As can be seen in Figure 4, NF was removed much more effectively than its low molecular weight radiolysis products. The three profiles in Figure 4 demonstrated significant loss of label from the DNA peak. After the second ethanol precipitate only 43.4% of the label found in this peak of the initial radiolysis mixture remained.

B. Chromatography on Sephadex G-25

After the first Sephadex G-25 step, 14.1% of the low molecular weight products remained with the DNA (Fig. 5, second profile). However, after reapplication of this DNA to the column and recovery of fractions containing DNA, the labelled DNA was free from low molecular weight products (Fig. 5, third profile). The overall recovery of high molecular weight label was 37.6%. But this apparently low recovery was likely due to the overlapping of the low

Figure 4. Purification of Nitrofurazone treated DNA by precipitation with ethanol. Sephadex G-25 column (60 X 0.9cm) eluted with distilled water, 0.5 ml/min.




16
8
CPM x 10^{-2}
20
40
60
80
Volume Eluted (ml)

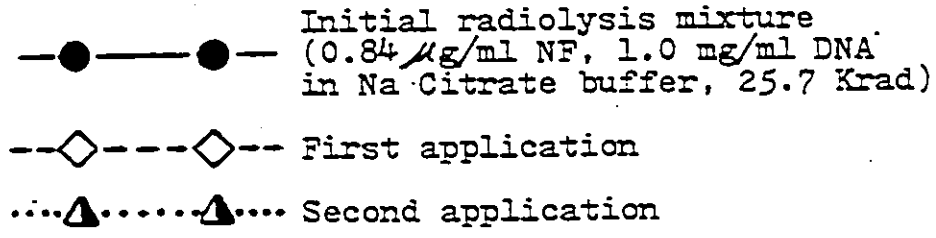
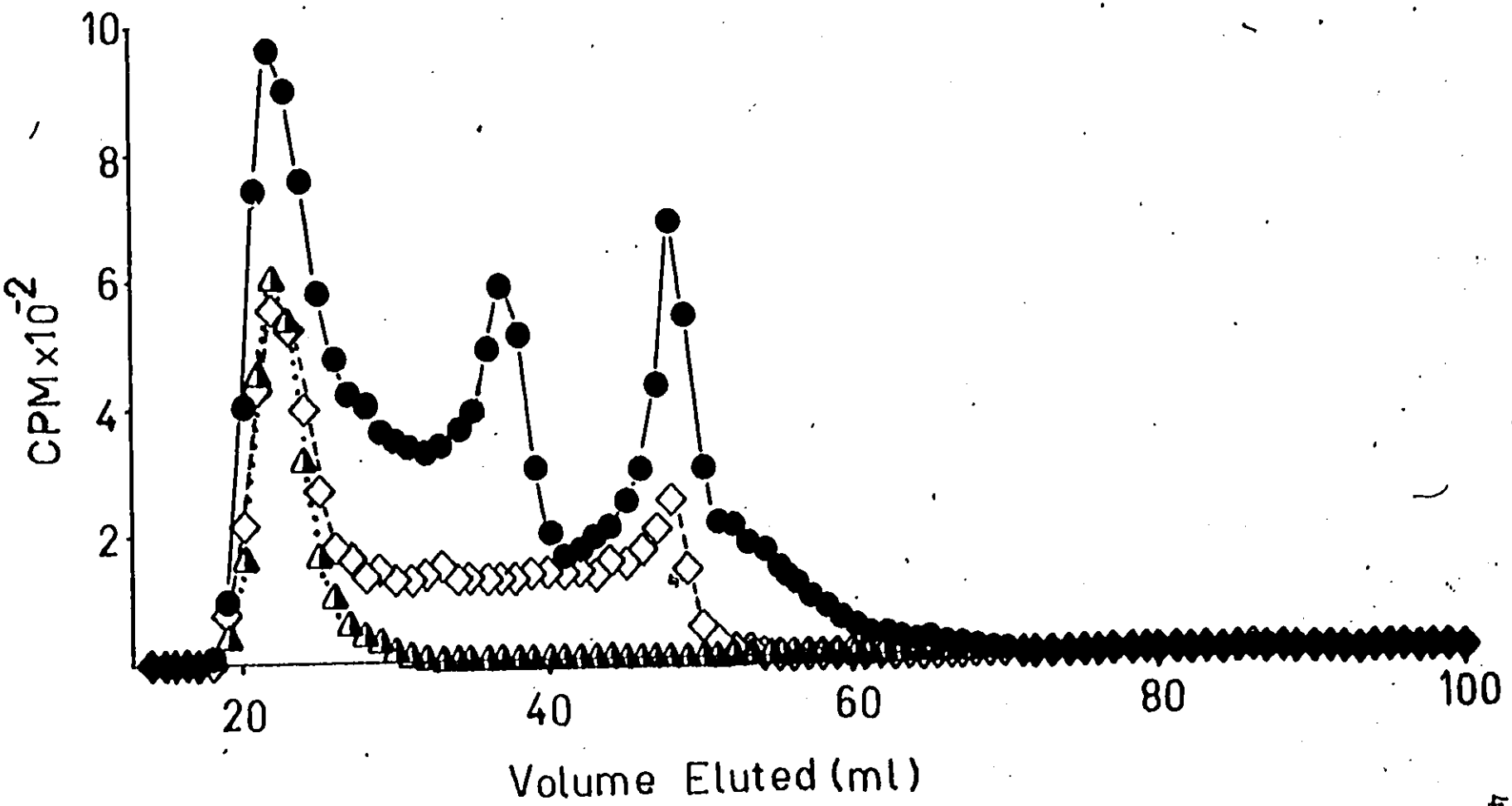


Figure 5. Purification of Nitrofurazone treated DNA by chromatography on Sephadex G-25. Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.


10
8
6
4
2
CPM x10⁻²
20
40
60
80
100
Volume Eluted (ml)

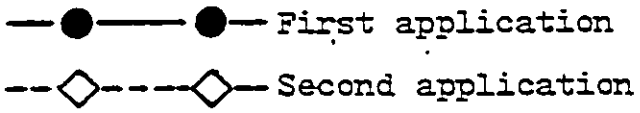
molecular weight radiolysis products with the labelled DNA (Fig. 5, first profile). The overlapping in this case was greater than seen in Figure 4 (first profile). There was very little further loss of high molecular weight label after the second chromatography step (Fig. 5, third profile relative to the second profile).

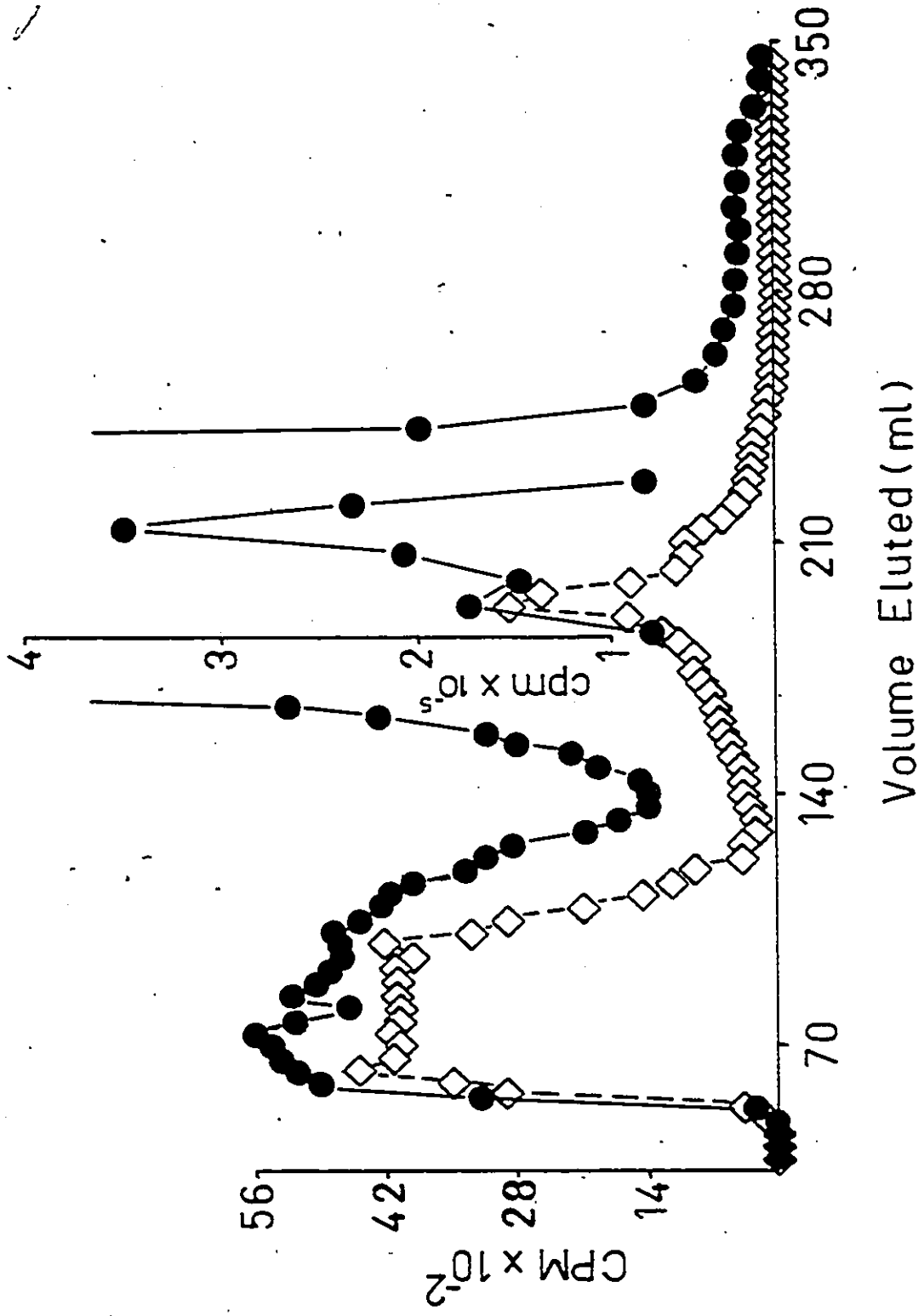
C. Chromatography on Sephadex G-50

Figure 4 demonstrates the course of removal of the low molecular weight material from the labelled DNA. The profiles shown in Figure 6 were those obtained with a large Sephadex G-50 column (40 X 2.5cm). The profiles of Figure 5 were obtained with aliquots set aside before and after the chromatography steps. On the Sephadex G-50 column the separation between DNA and low molecular weight material was much greater than on the Sephadex G-25 column. After the first application less than 1% of the low molecular weight material remained. After two preparative runs the DNA was completely free from low molecular weight material. The overall recovery of high molecular weight label was 61.9% of the label found in the DNA peak of the initial radiolysis mixture. This supports the notion that the overlap of low molecular weight products inflated the apparent amount of NF bound to DNA in the Sephadex G-25 chromatogram described above (Fig. 5, first profile).

The purification demonstrated in Figure 6 is

Figure 6. Purification of Nitrofurazone treated DNA by chromatography on Sephadex G-50 column (40 X 2.5cm) eluted with water, 0.87 ml/min.



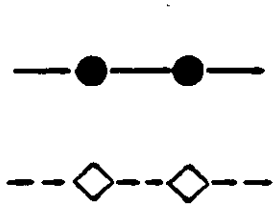

CPM x 10⁻²
56
42
28
14
cpm x 10⁻⁵
4
3
2
1
70
140
210
280
350
Volume Eluted (ml)

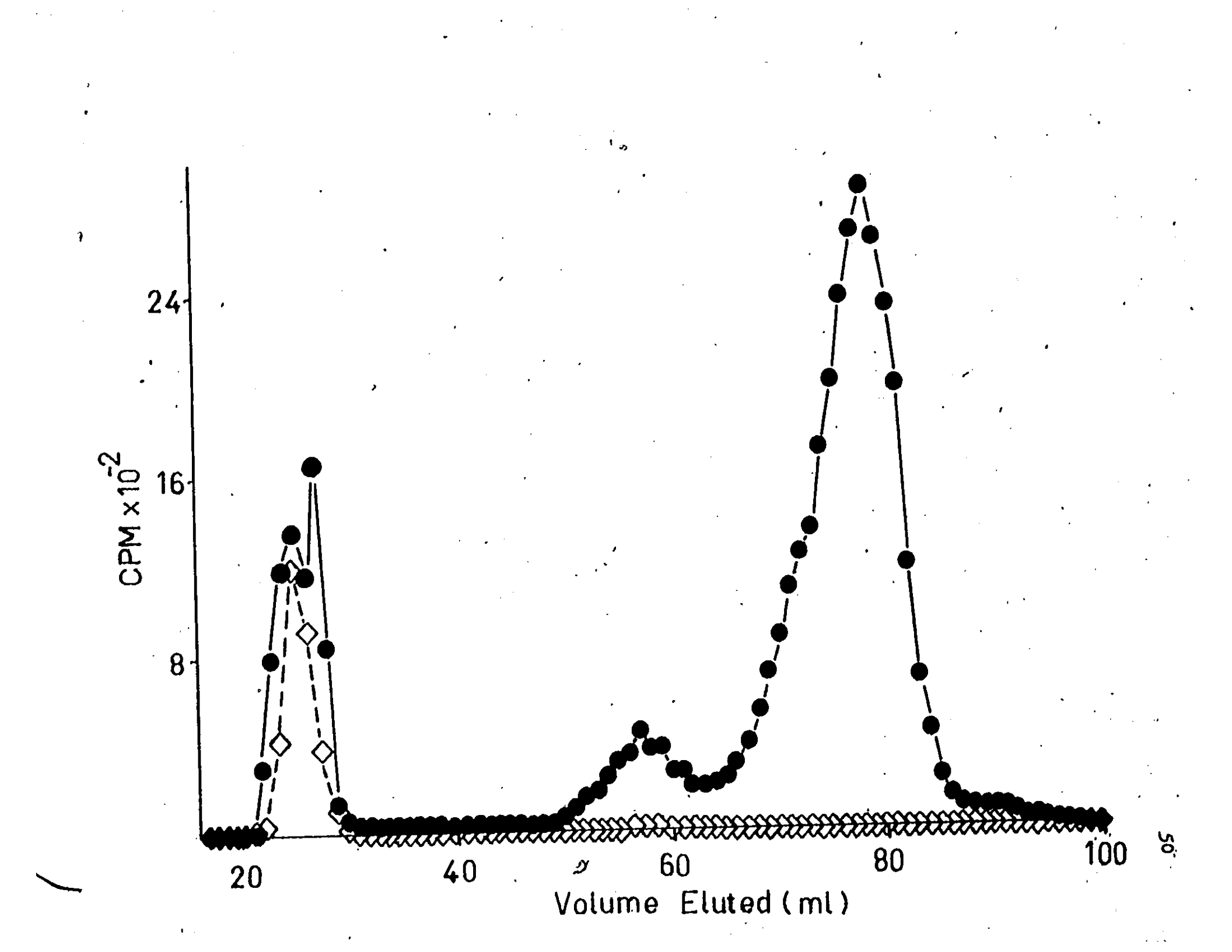
abnormal in respect to the massive amounts of low molecular weight material. The radiolysis mixture applied to this preparative Sephadex G-50 column was made up of the remainders of many small scale irradiations which were mixed and applied to the column. These small scale irradiations involved varying DNA and NF concentrations, and doses of ionizing radiation. Much of the low molecular weight material was unchanged NF. Also of note is that the DNA peak in Figure 6 is rather broad due to overloading of the column with DNA.

D. Dialysis

Dialysis of the radiolysis mixture against distilled water was the easiest and fastest of the purification methods tried. Figure 7 illustrates the effectiveness of dialysis. Almost complete removal of low molecular weight material was achieved after 20 hours with seven, two liter, changes of water. Note that the low molecular weight radiolysis products are different here than in Figure 4 and 5. In earlier experiments DNA and NF were irradiated in 0.01M NaCitrate 0.01M NaCl (pH 7.0) whereas in this experiment single stranded DNA and NF were dissolved in distilled water at approximately pH 6 (see Section III (B) which deals with the NF radiolysis products formed in different media). In this radiolysis mixture a product that eluted immediately after DNA was found (Fig. 7, 1st profile). Removal of this

Figure 7. Purification of Nitrofurazone treated DNA by Dialysis.
Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.




24
16
8
CPM x10^-2
20
40
60
80
100
Volume Eluted (ml)

product by Sephadex G-25 or G-50 preparative chromatography would have been difficult. Little if any loss of DNA out of the dialysis bag was observed as assessed by checking the absorbance at 260nm before (100.0%) and after (102.7%) dialysis.

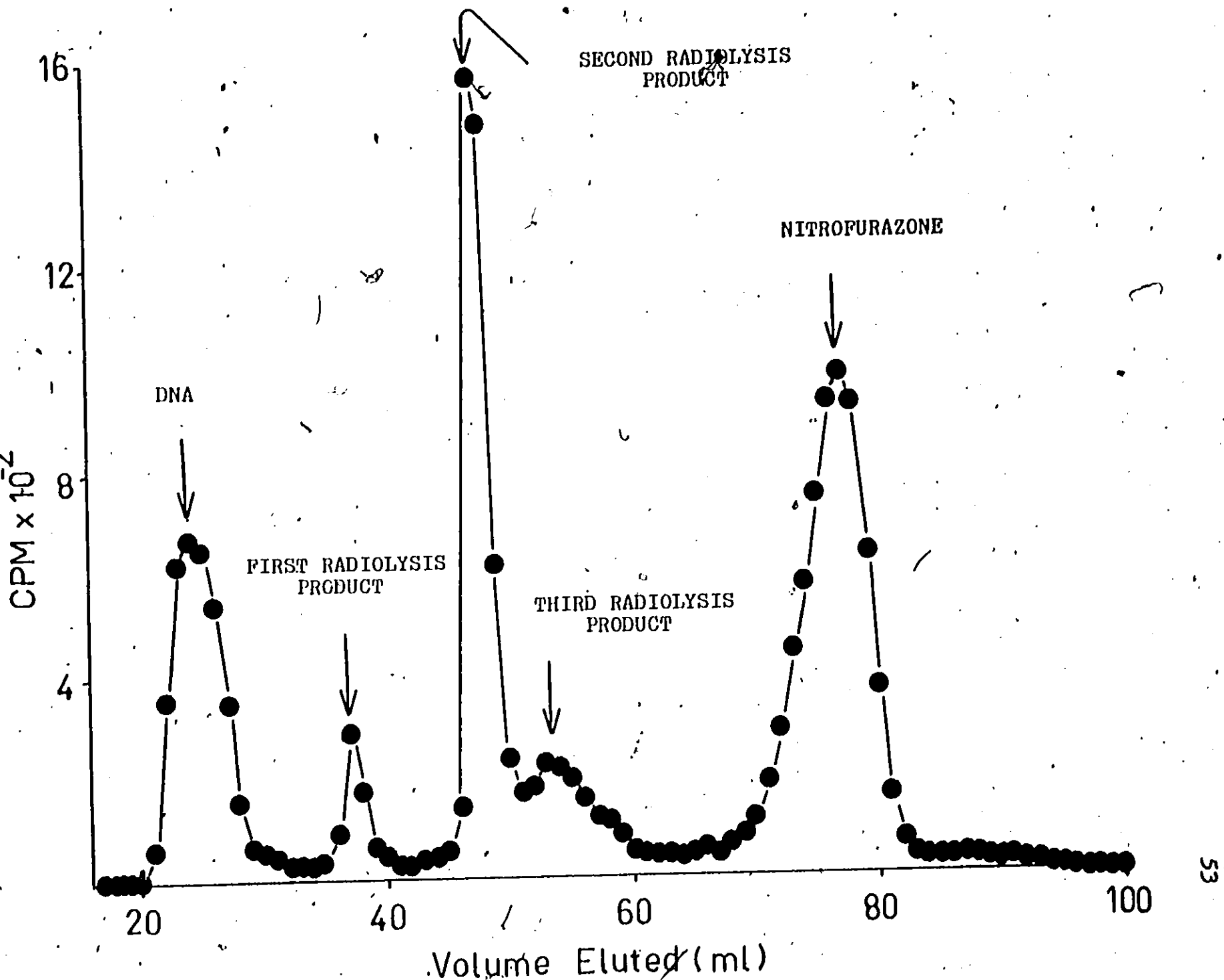
The labelled DNA obtained during these purification steps showed no loss of radioactivity when extracted with phenol or chloroform : isoamyl alcohol= 9:1 1% SDS, nor was label lost when the DNA was dialyzed against 5M NaCl.

III. Analysis of Low Molecular Weight Radiolysis Products

A. Analysis By Chromatography on Sephadex G-25 and G-50 Columns

Radiation of DNA and NF in 0.01M NaCitrate 0.01M NaCl (pH 7.0) and analysis on a Sephadex G-25 column (100 X 0.8cm) exhibited three major low molecular weight radiolysis products (Fig. 8). These radiolysis products eluted between the unchanged NF and the labelled DNA. Chromatography of the same radiolysis mixture on a Sephadex G-50 column (100 X 0.8cm) allowed much better separation of labelled DNA from the low molecular weight material but did not allow resolution of the low molecular weight materials. Both Sephadex G-25 and G-50 can be used to assess the binding of NF to DNA.

Figure 8. Radiolysis mixture of Nitrofurazone in the presence of DNA.
Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min, 0.84 μg/ml NF, 1 mg/ml DNA, 22.9 Krad in Na Citrate buffer.


SECOND RADIOLYSIS PRODUCT
NITROFURAZONE
DNA
FIRST RADIOLYSIS PRODUCT
THIRD RADIOLYSIS PRODUCT
CPM x 10-2
16
12
8
4
20
40
60
80
100
Volume Eluted (ml)

B. Effect of Various Media on the Formation of Low Molecular Weight Radiolysis Products

The low molecular weight radiolysis products formed depended upon the media used. DNA and NF were dissolved in 0.01M NaCitrate 0.01M NaCl (pH 7.0), in 0.01M TrisHCl 0.01M Mg_2Cl_2 (pH 7.0), or in distilled water. Different radiolysis products were formed under these three conditions (Fig. 9). These products were not further characterized.

IV. Ionizing Radiation-Induced Binding of Nitrofurazone to DNA

There was no binding of NF to DNA, in 0.01M NaCitrate 0.01M NaCl (pH 7.0) media, nor any destruction of NF in the absence of radiation. NF alone showed some destruction when irradiated (Fig. 10, second profile) but no high molecular weight radiolysis product was formed. However, in the presence of DNA, the rate of destruction of NF was greatly increased and a high molecular weight product which co-eluted with DNA on Sephadex G-25 columns was present (Fig. 10, first profile).

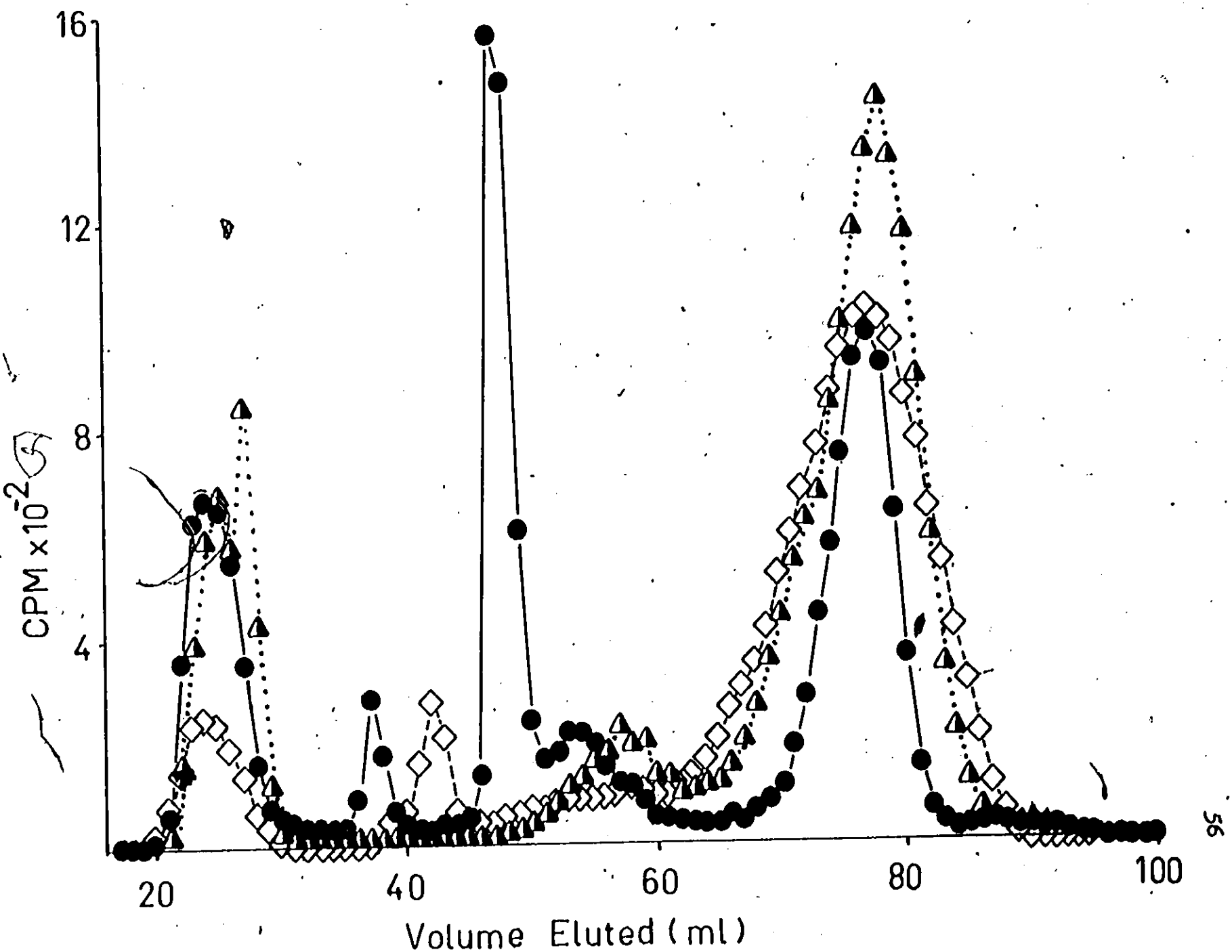
The radiolysis of NF was very much reduced in the presence of oxygen. Under conditions where all NF was destroyed in hypoxia (1 mg/ml DNA, 0.84 µg/ml NF, 25.7 Krad in NaCitrate media) less than 5% was destroyed when the solution was saturated with air. No binding to DNA occurred

Figure 9. Effect of various media on the formation of low molecular weight radiolysis products. Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.

—●—●— 0.01 M Na Citrate NaCl, pH 7.0

—◇—◇— 0.01 M Tris HCl $MgCl_2$, pH 7.0

...▲...▲... Distilled water


16
12
8
4
CPM x10-2
20
40
60
80
100
Volume Eluted (ml)

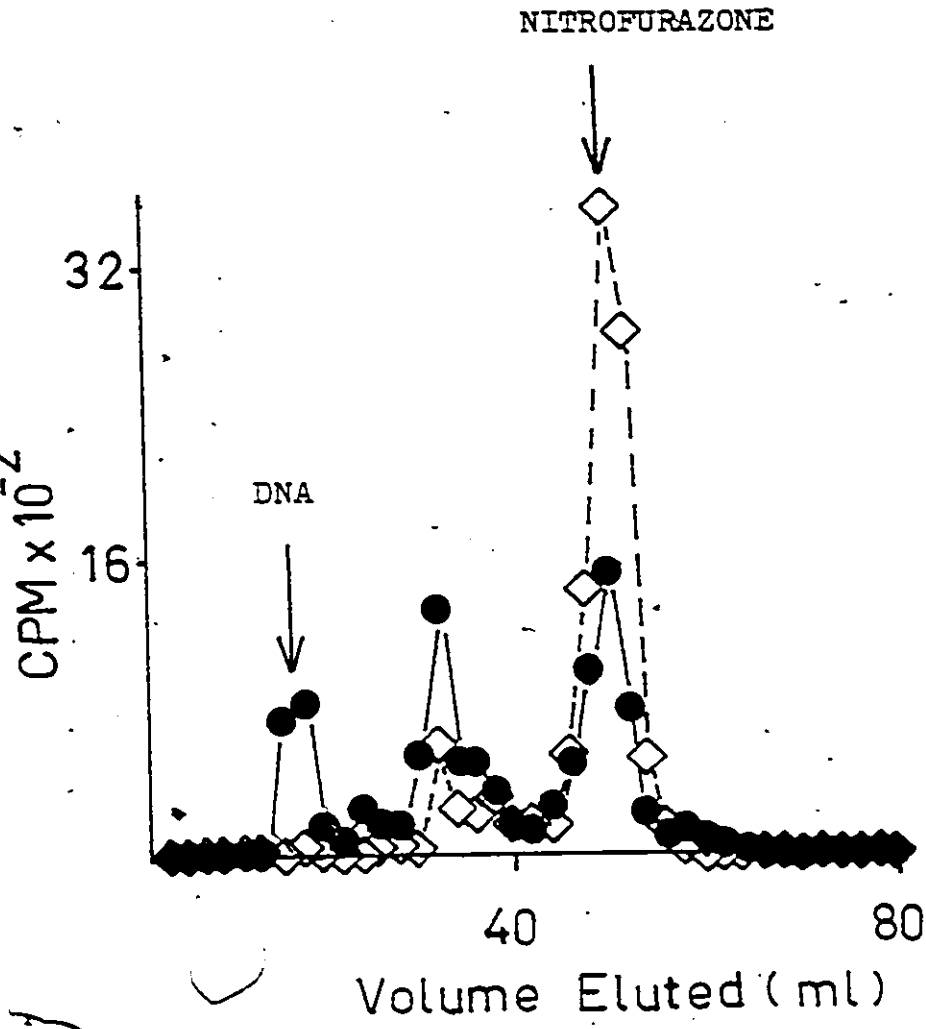


Figure 10. Radiation-induced destruction of Nitrofurazone in presence and absence of DNA.
Sephadex G-25 column(60 X 0.9cm) eluted with distilled water, 0.5 ml/min.

—●—●— 0.87 mg/ml DNA

--◇--◇-- 0.0 mg/ml DNA

in these air saturating conditions. Only the second and third radiolysis products were formed and these in diminished amounts. Binding of NF to DNA and the production of the first radiolysis product depend critically on the absence of oxygen.

A. Effect of Different Radiation Doses

The production of all radiolysis products increased linearly with increasing doses of radiation. A concomitant linear decrease of NF was also observed (Fig. 11 and 12). Note that the curves level off as the NF is exhausted. Of the NF destroyed in citrate media 36% was bound to DNA, 6% went into the production of the first radiolysis product, 24% to the second, and 13% to the third.

B. Effect of Different Nitrofurazone Concentrations

Varying the concentration of NF had little effect on the binding of NF to DNA. Neither the destruction of NF nor the production of low molecular weight radiolysis products were altered over a broad range of NF concentrations. Figure 13 illustrates that a six fold increase in NF concentration did not increase the radiation induced binding to DNA at doses up to about 70 Krad.

C. Effect of Different DNA Concentrations

Increasing DNA concentrations caused increased

Figure 11. A) Radiation-induced binding of Nitrofurazone to DNA.
Sephadex G-25 column (60 X 0.9cm) eluted with distilled water, 0.5 ml/min.

B) Radiation-induced destruction of Nitrofurazone in the presence of DNA.
Sephadex G-25 column (60 X 0.9cm) eluted with distilled water, 0.5 ml/min.

0.84 µg/ml NF, 1 mg/ml DNA in Na Citrate buffer

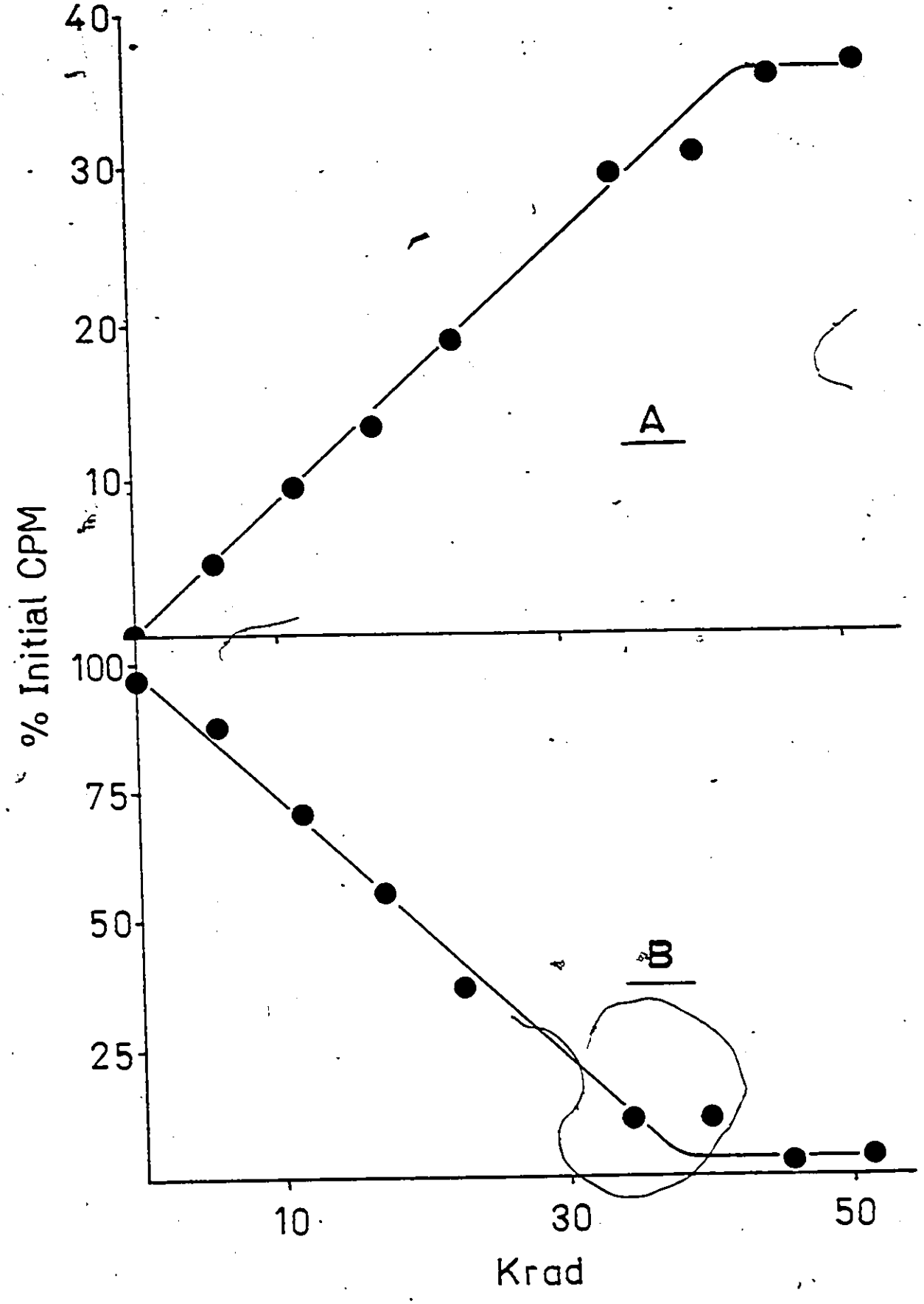

40
30
20
10
100
75
50
25
% Initial CPM
A
B
10
30
50
Krad

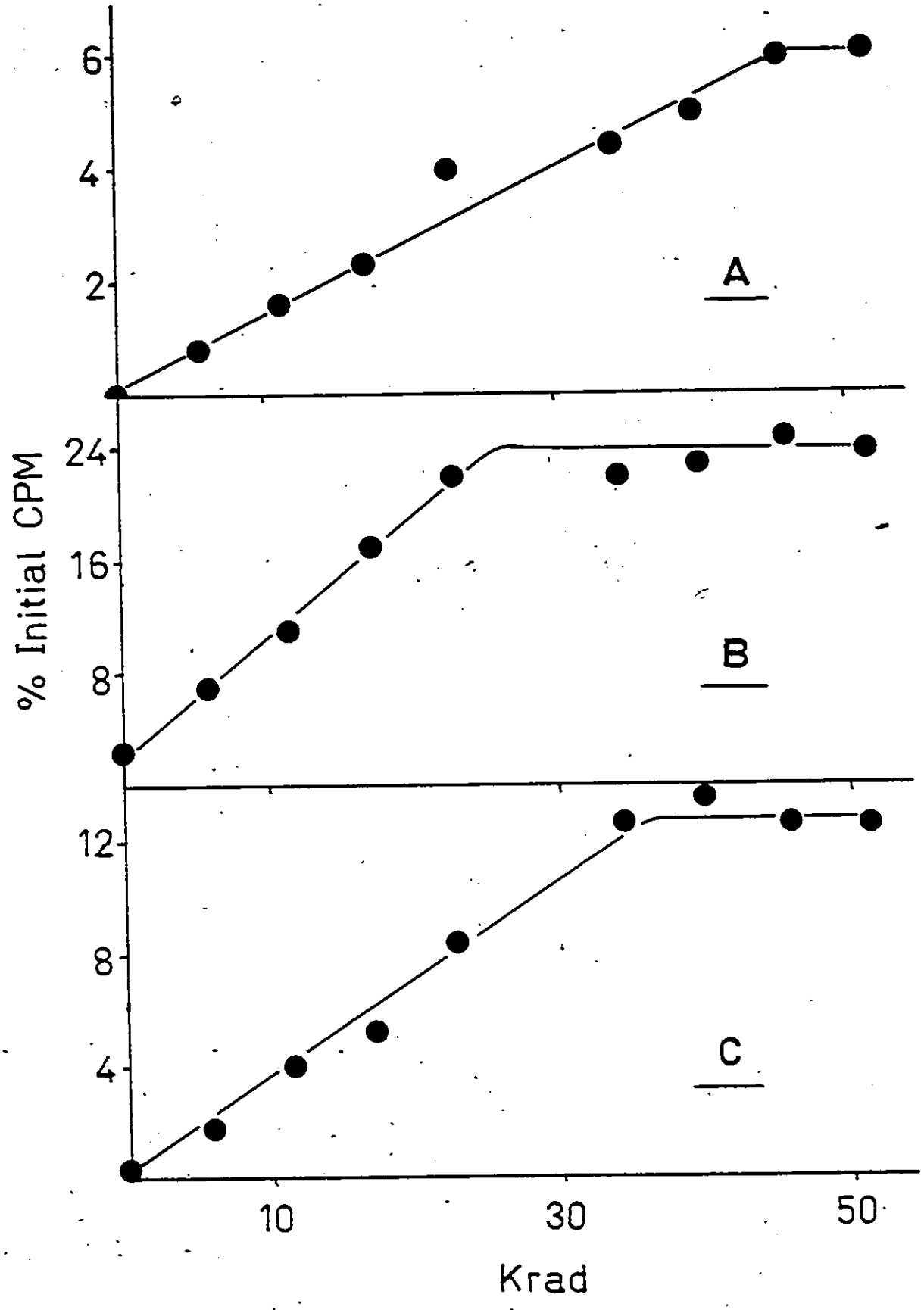
Figure 12. Production of low molecular weight Nitrofurazone radiolysis products. Sephadex G-25 column (60 X 0.9cm) eluted with distilled water, 0.5 ml/min.

A) First radiolysis product

B) Second radiolysis product

C) Third radiolysis product

0.84 µg/ml NF, 1 mg/ml DNA in Na Citrate buffer


6
4
2
A
24
16
8
B
% Initial CPM
12
8
4
C
10
30
50
Krad

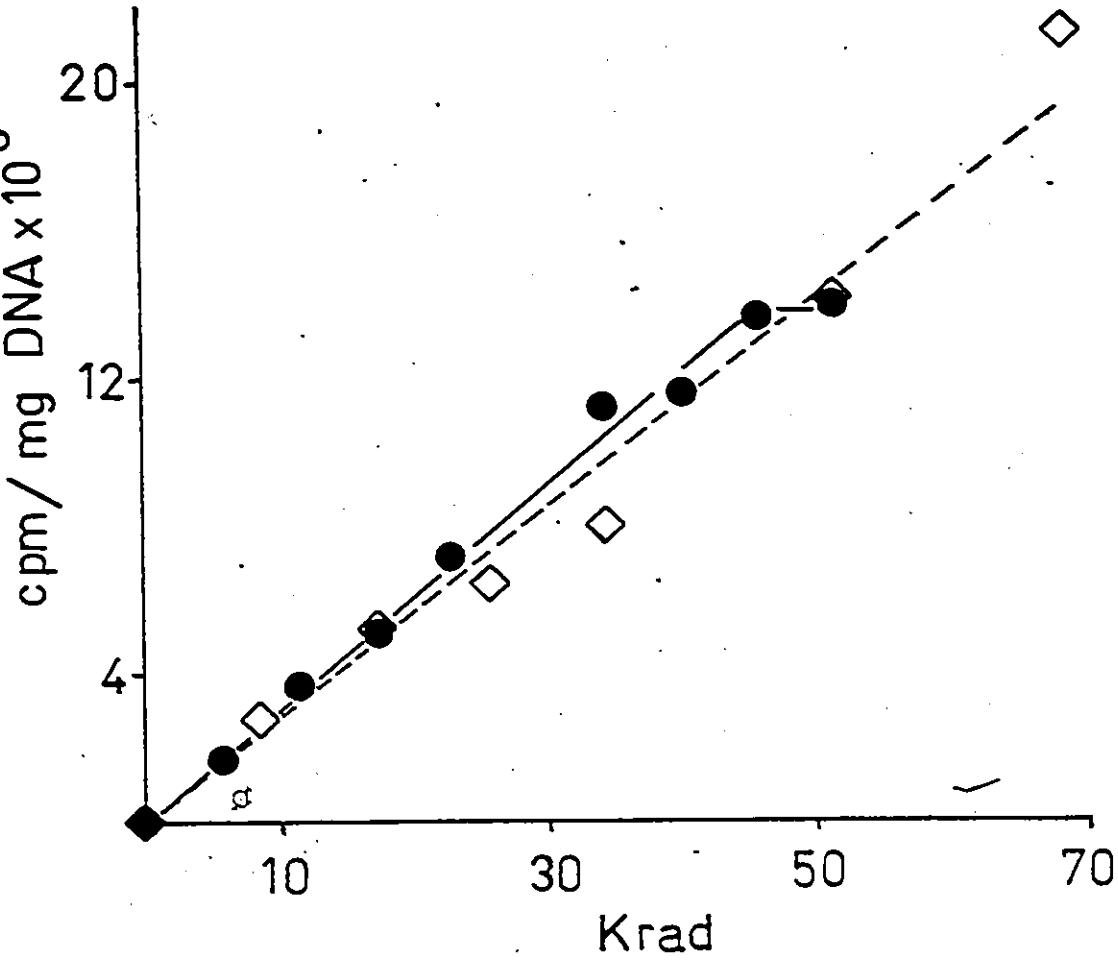


Figure 13. Effect of different Nitrofurazone concentration on radiation-induced binding to DNA. Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.

—●—●— 0.84 µg/ml NF

--◇--◇-- 4.96 µg/ml NF

1 mg/ml DNA in Na Citrate buffer

radiation-induced destruction of NF and increased the amount of NF bound to DNA, but the amount of NF bound per mg DNA remained constant. Figure 14 illustrates the linear relationship between total label bound to DNA and the concentration of DNA. Sonication of DNA prior to or after exposure to ionizing radiation did not alter the binding of NF to DNA or the radiolysis products formed.

V. Assessment of Nitrofurazone Destruction

A. Chromatography on Sephadex G-25

On a Sephadex G-25 column (100 X 0.8cm) NF eluted at approximately 77ml , the amount of ^{14}C radioactivity that eluted there served to indicate the amount of NF still present. As already mentioned (Fig. 11-B) the concentration of NF decreased linearly with increasing dose of ionizing radiation.

B. Absorbance Measurement

The rate of destruction of NF can be easily assessed by measuring the absorbance at 375nm (the λmax of NF) before and after exposure to ionizing radiation. The loss in absorbance at 375nm was linearly related to the dose of radiation applied, regardless of the concentration of NF. Similarly, binding to DNA was linearly related to the decrease in absorption at 375nm (Fig. 15). This method was applicable

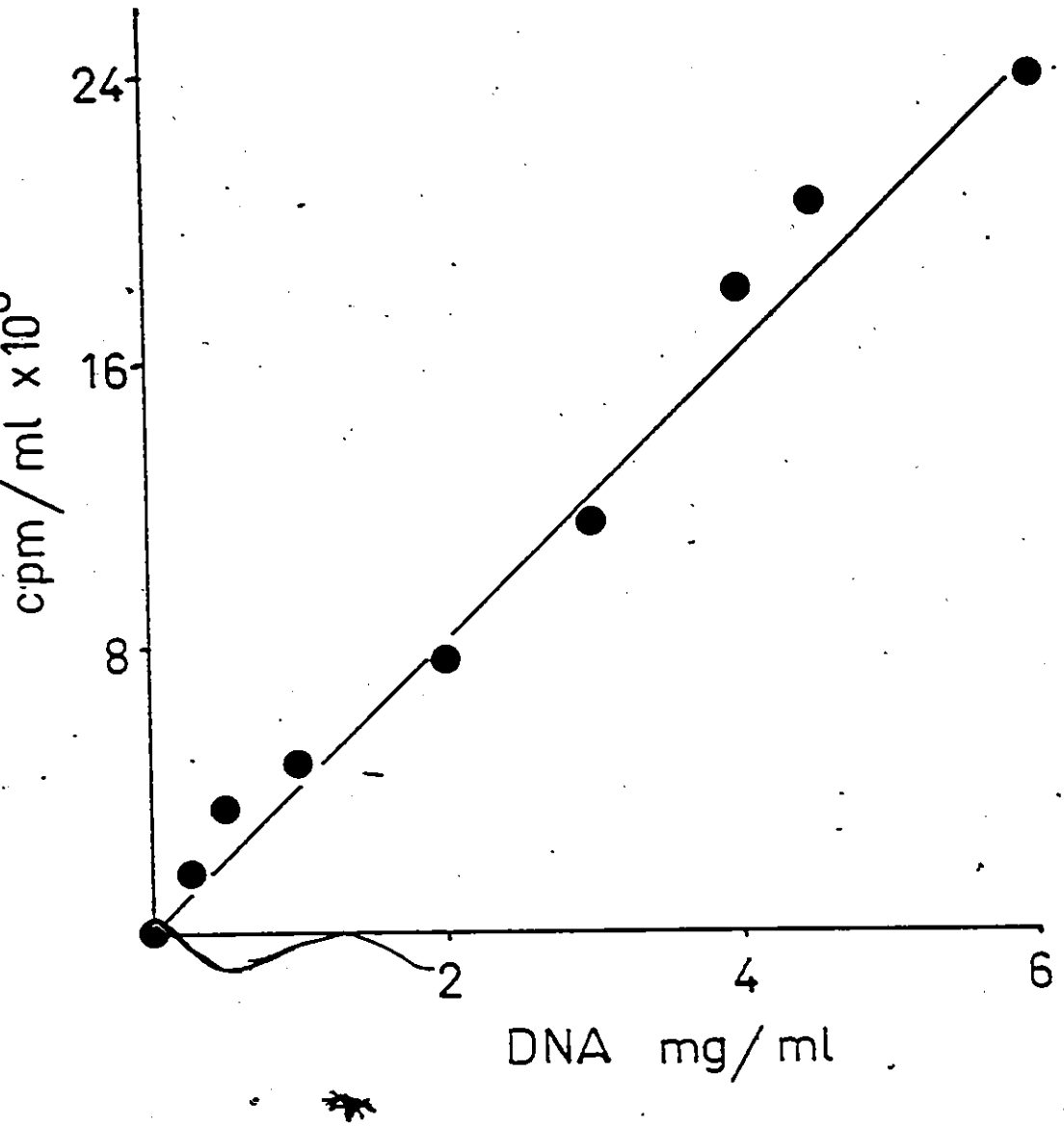
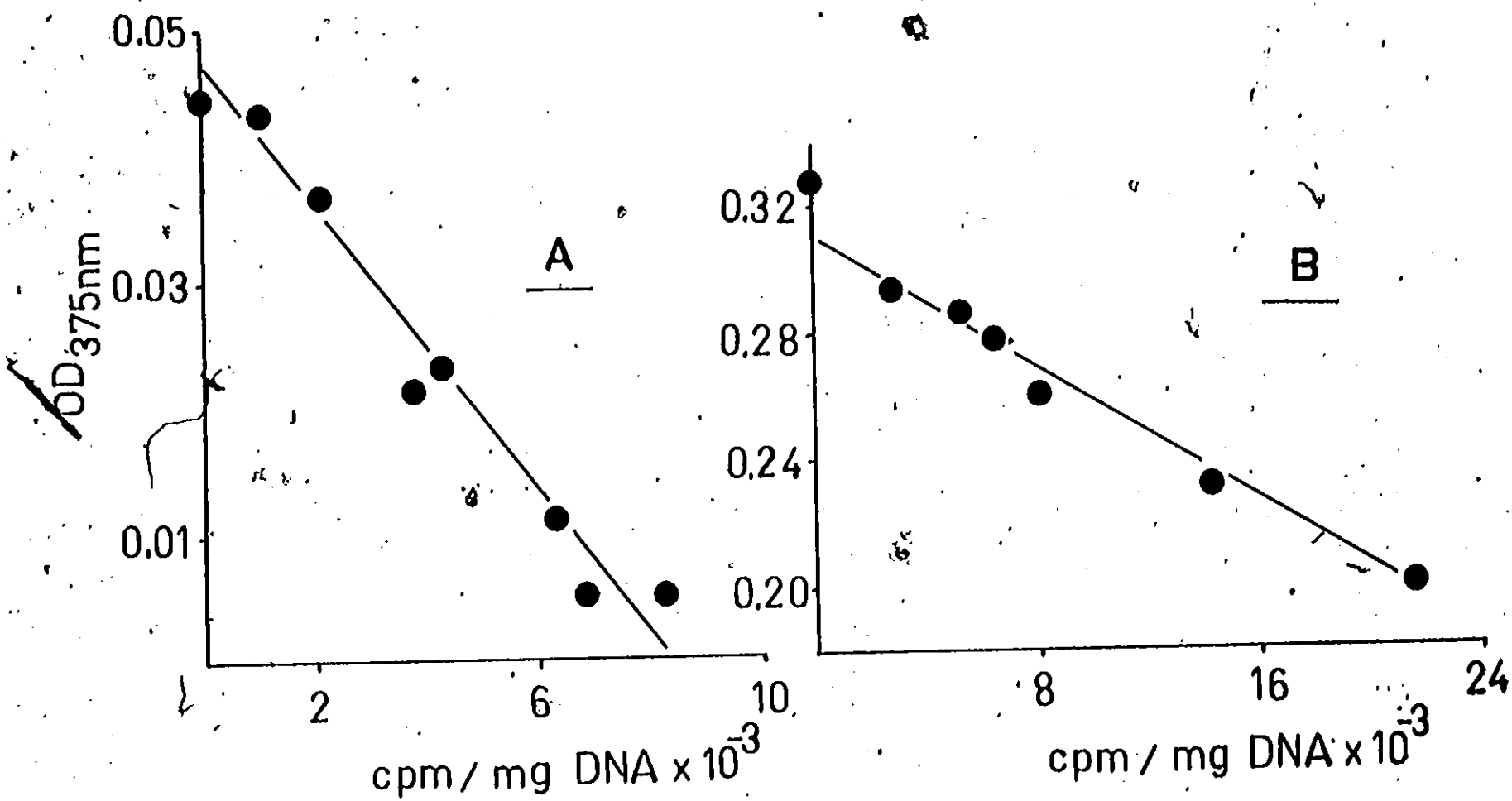


Figure 14. Effect of different DNA concentrations on radiation-induced binding of Nitrofurazone to DNA.
Sephadex G-50 column, (100 X 0.8cm) eluted with distilled water, 0.5 ml/min
8.4 µg/ml NF, 25.7 Krad in Na Citrate buffer

Figure 15. Radiation-induced loss of Nitrofurazone absorbance at 375 nm and its binding to DNA.
Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.

A) 0.84 µg/ml NF, 0.91 mg/ml DNA in Na Citrate buffer

B) 4.96 µg/ml NF, 0.91 mg/ml DNA in Na Citrate buffer


0.05
0.03
0.01
OD_{375nm}
A
2
6
10
cpm / mg DNA x 10^{-3}
0.32
0.28
0.24
0.20
B
8
16
24
cpm / mg DNA x 10^{-3}

to initial concentrations as low as 0.84μg/ml NF. However, accurate measurements of absorption due to NF became more difficult at these lower concentrations. This method was best suited for higher NF concentrations, whereas the above method (Section V-A) was useful for much lower NF concentrations.

VI. Enzymatic Hydrolysis of Labelled DNA

A. Analysis on Sephadex G-25

The labelled DNA can be digested to the nucleotide stage using deoxyribonuclease I and snake venom phosphodiesterase and further to the nucleoside stage with alkaline phosphatase (Fig. 16). Of note was the profile for the nucleotide digest where a significant amount of material eluted close to the void volume of the Sephadex G-25 column. This "Void peak" disappeared after the addition of alkaline phosphatase. To determine if this peak was undigested DNA, another sample was digested with deoxyribonuclease I and snake venom phosphodiesterase for an additional two days. The peak remained unchanged. When the nucleotide digest was chromatographed on a Sephadex G-50 column, none of the label eluted near the void volume (Fig. 17). In the nucleoside digest the radioactivity chromatographed earlier than most of the normal nucleosides.

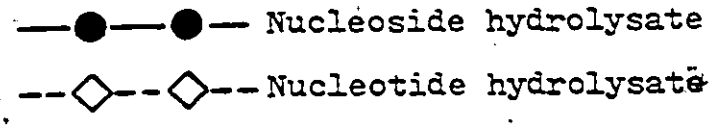


Figure 16. Enzymatic hydrolysis of labelled DNA to nucleotide and nucleoside stage. Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.

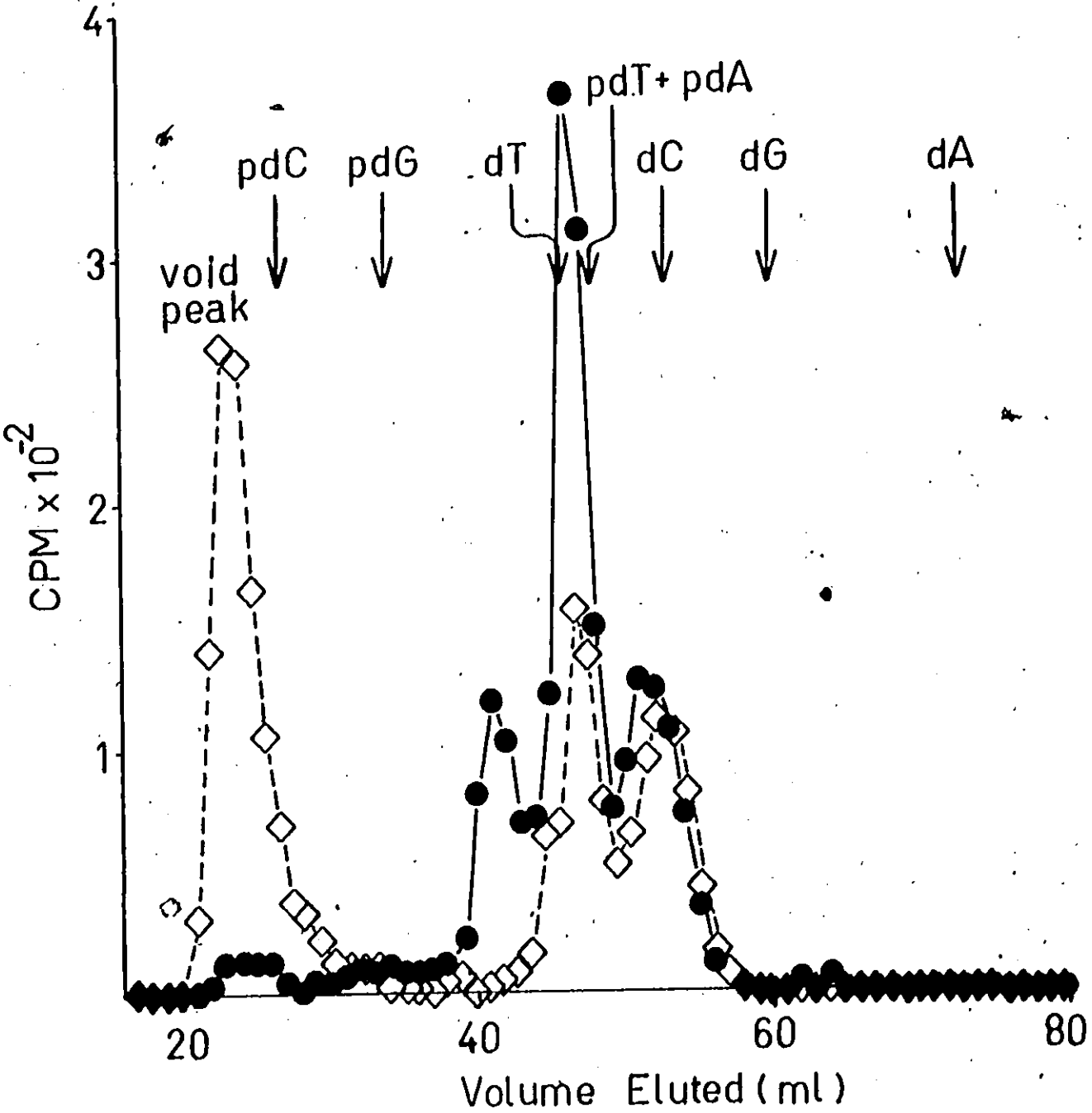
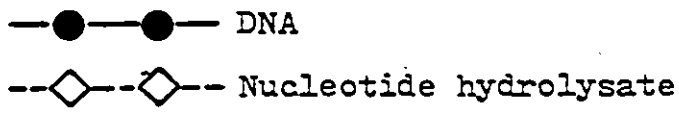
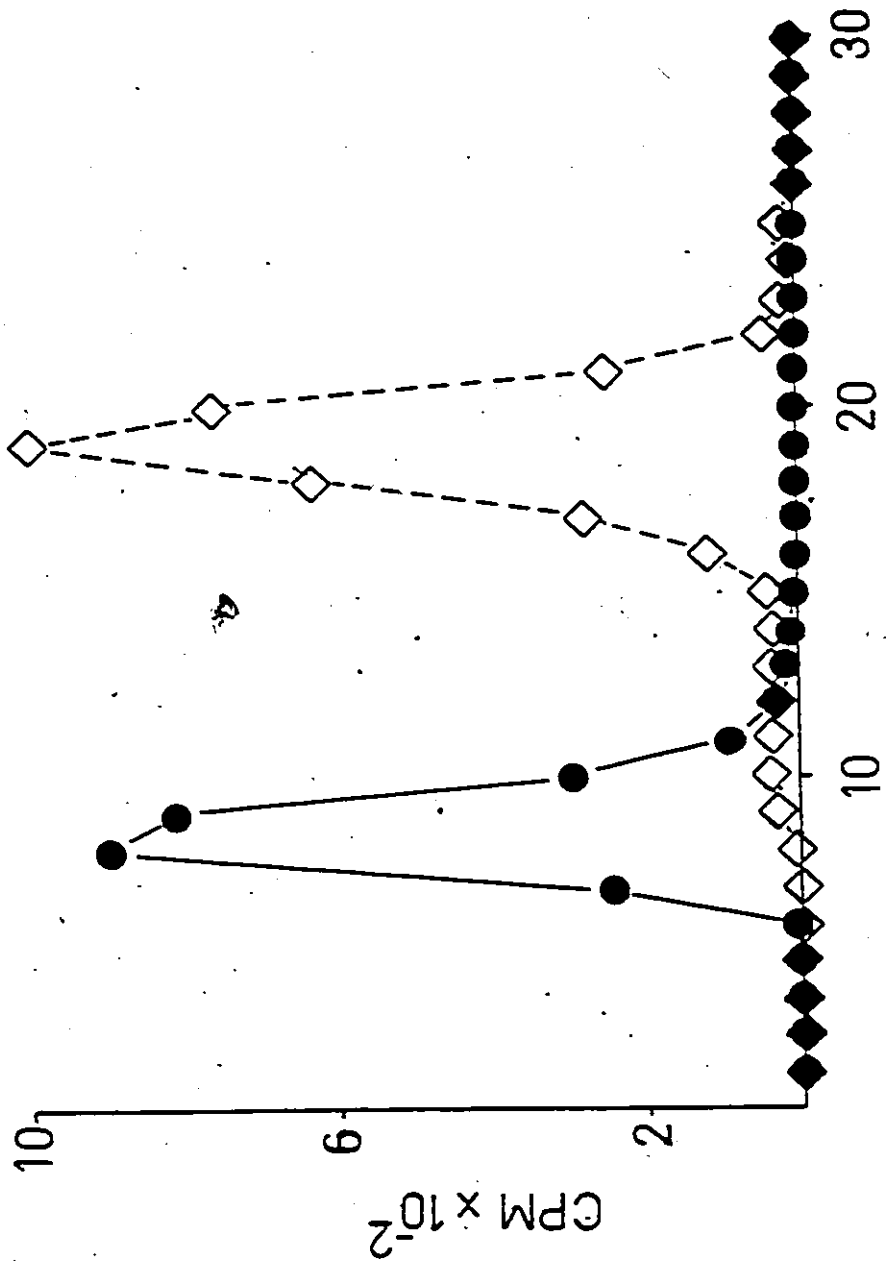

pdT+ pdA
pdC
pdG
dT
dC
dG
dA
void peak
CPM x 10^-2
4
3
2
1
20
40
60
80
Volume Eluted (ml)

Figure 17. Labelled DNA and enzymatic nucleotide hydrolysate on a Sephadex G-50 column (30 X 0.9cm) eluted with distilled water, 0.5 ml/min.




CPM x 10⁻²
10
6
2
10
20
30
Volume Eluted (ml)

B. Analysis on Sephadex G-10

Chromatography of the nucleoside digest on the Sephadex G-25 column (100 X 0.8cm) showed evidence for at least three major components (Fig. 16). Use of a Sephadex G-10 column (100 X 0.8cm) showed evidence of four to five components. The Sephadex G-10 columns were equilibrated under three conditions: acidic (pH 3.20), neutral (H_2O, pH approximately 6), and alkaline (pH 9.20). Different profiles were obtained in each case (Fig. 18). While correlation between the three profiles was difficult, it was apparent that one major component (the last) eluted at approximately the same position under all three conditions.

C. Analysis on Bio-Rad AG 50WX4

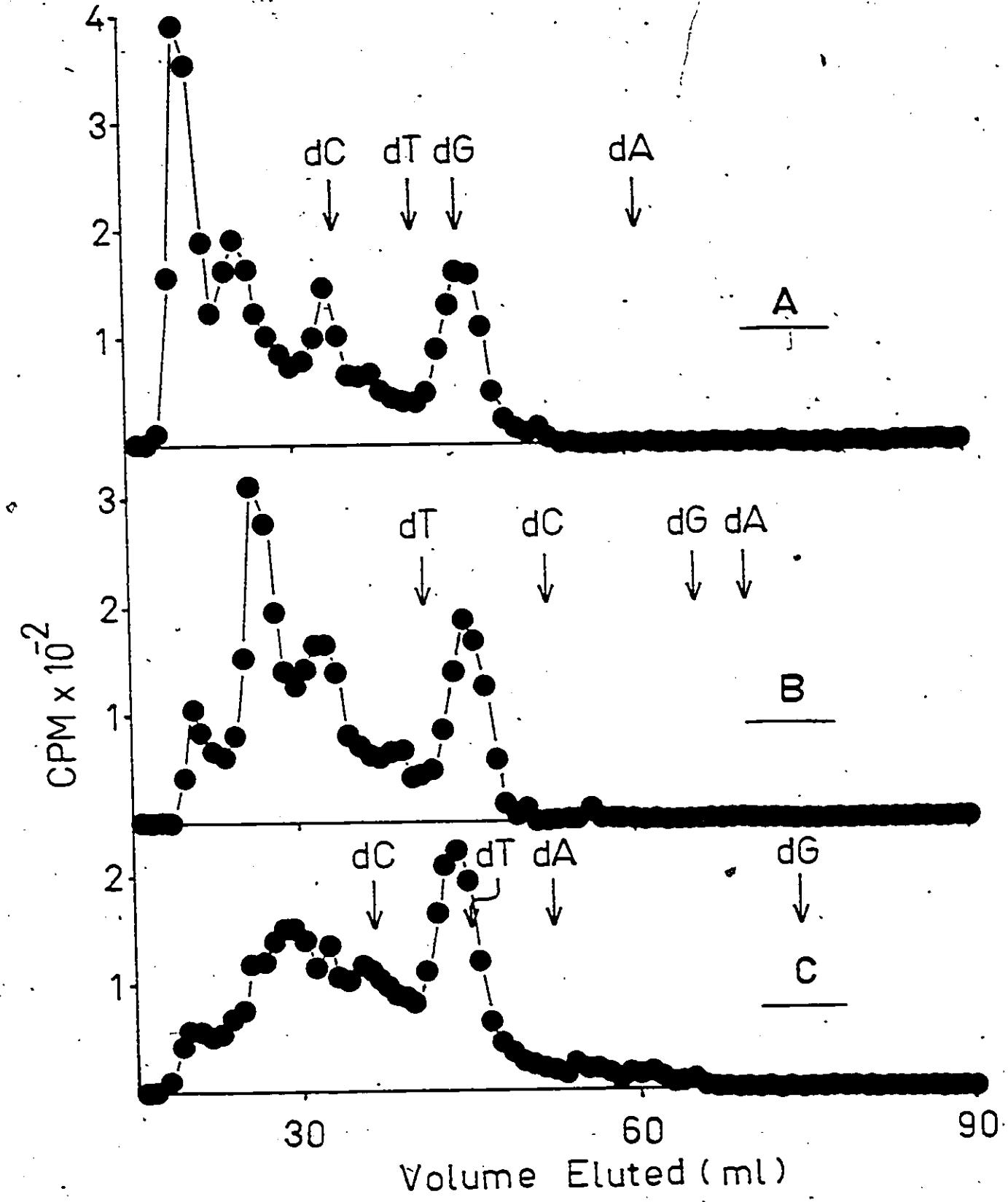
Nucleotides and nucleosides have been successfully separated with Bio-Rad Ag 50WX4 under acidic and alkaline conditions respectively (70). The chromatographic properties of nucleotide and nucleoside digests of the labelled DNA were examined using this cation exchange resin. Elution of the Ag 50WX4 column at 0.015M or 0.30M NH_4OH (pH 9.20) gave a single peak of radioactivity with a nucleotide digest. However, a nucleoside digest showed some separation (Fig. 19), giving three peaks: I, II, III. Initial elution of this sulfonated resin with 0.015M NH_4OH (pH 3.20) followed by 0.30M NH_4OH (pH 9.20) gave better resolution than was

Figure 18. Enzymatic nucleoside hydrolysate on a Sephadex G-10 column (100 X 0.8cm). Elutant:

A) 0.015M NH_4OH pH 9.20, pH adjusted with formic acid, 0.5 ml/min.

B) Distilled water, 0.5 ml/min.

C) 0.015M NH_4OH pH 3.20, pH adjusted with formic acid, 0.5 ml/min.


4
3
2
1
dC
dT
dG
dA
A
3
2
1
dT
dC
dG
dA
B
CPM x 10⁻²
2
1
dC
dT
dA
dG
C
30
60
90
Volume Eluted (ml)

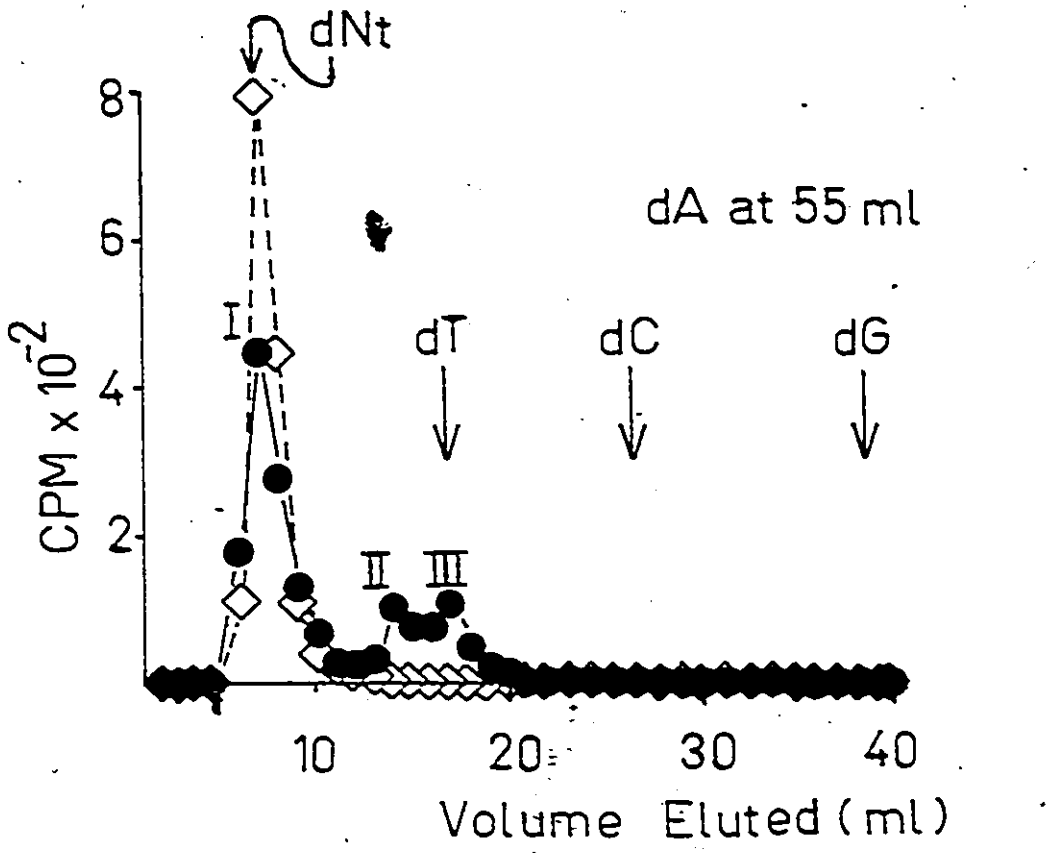


Figure 19. Enzymatic hydrolysis of labelled DNA to nucleotide and nucleoside stage.
AG 50WX4 column (30 X 0.9cm) eluted with 0.015M NH_4OH pH 9.20, pH adjusted with formic acid, 0.5 ml/min.

—●—●— Nucleoside hydrolysate

--◇--◇-- Nucleotide hydrolysate

obtained at pH 9.20 only. The nucleotide digest showed three peaks, whereas the nucleoside digest showed four peaks: A, B, C, and D (Fig. 20). One of the peaks found in both the nucleotide and nucleoside digest showed a strong affinity for the column at pH 3.20 and was not recovered even after prolonged elution of the column at the low pH but was readily removed upon changing the pH to 9.20. Measuring the pH of the fractions showed that this peak eluted at approximately pH 9.

Henceforth, for convenience, the AG 50WX4 column eluted at 0.015M NH_4OH (pH 9.20) will be referred to as the "AG 50WX4 pH 9.20 column". The AG 50WX4 column for which the pH of the eluting buffer was changed from 0.015M NH_4OH (pH 3.20) to 0.30M NH_4OH (pH 9.20) will be referred to as the "AG 50WX4 pH 3.20-9.20 column".

D. Analysis on a Sephadex G-10: AG 50WX4 Mixed Column

A column composed of half Sephadex G-10 gel and half AG 50WX4, by volume, was prepared in order to determine if the A and D peaks of the nucleoside digest on the "AG 50WX4 pH 3.20-pH 9.20 column" were multicomponent, since they eluted at the exclusion volume and the "change over" volume of the column. Figure 21 shows the profile obtained. The A and D peaks showed broadening and loss of symmetry suggesting that they contained more than one component. While the B

Figure 20. Enzymatic hydrolysis of labelled DNA to nucleotide and nucleoside stage. AG 50WX4 column (30 X 0.9cm) eluted with 0.015M NH_4OH pH 3.20 changed to 0.30M NH_4OH pH 9.20, pH adjusted with formic acid, 0.5 ml/min.

—●—●— Nucleoside hydrolysate

--◇--◇-- Nucleotide hydrolysate

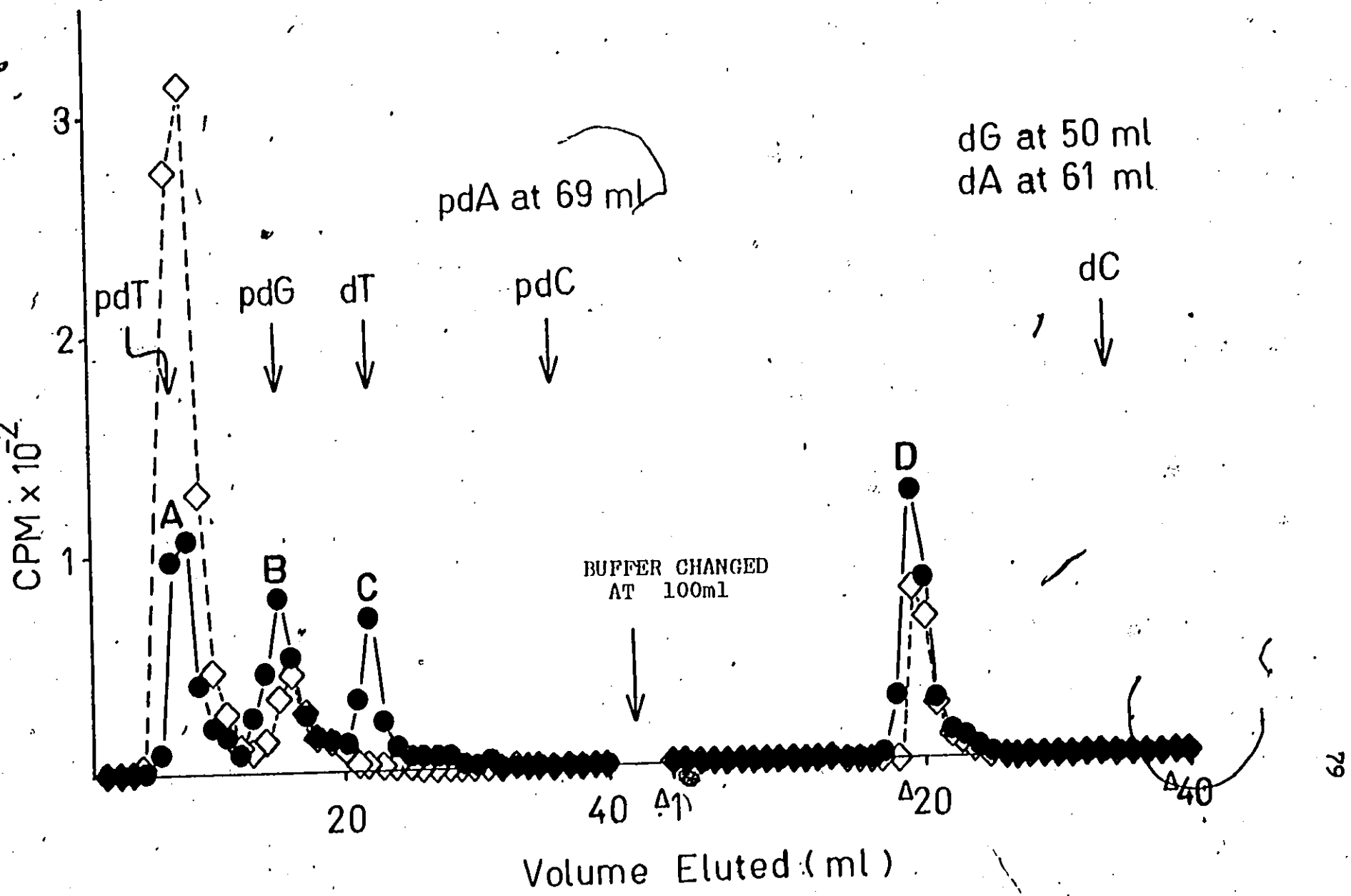
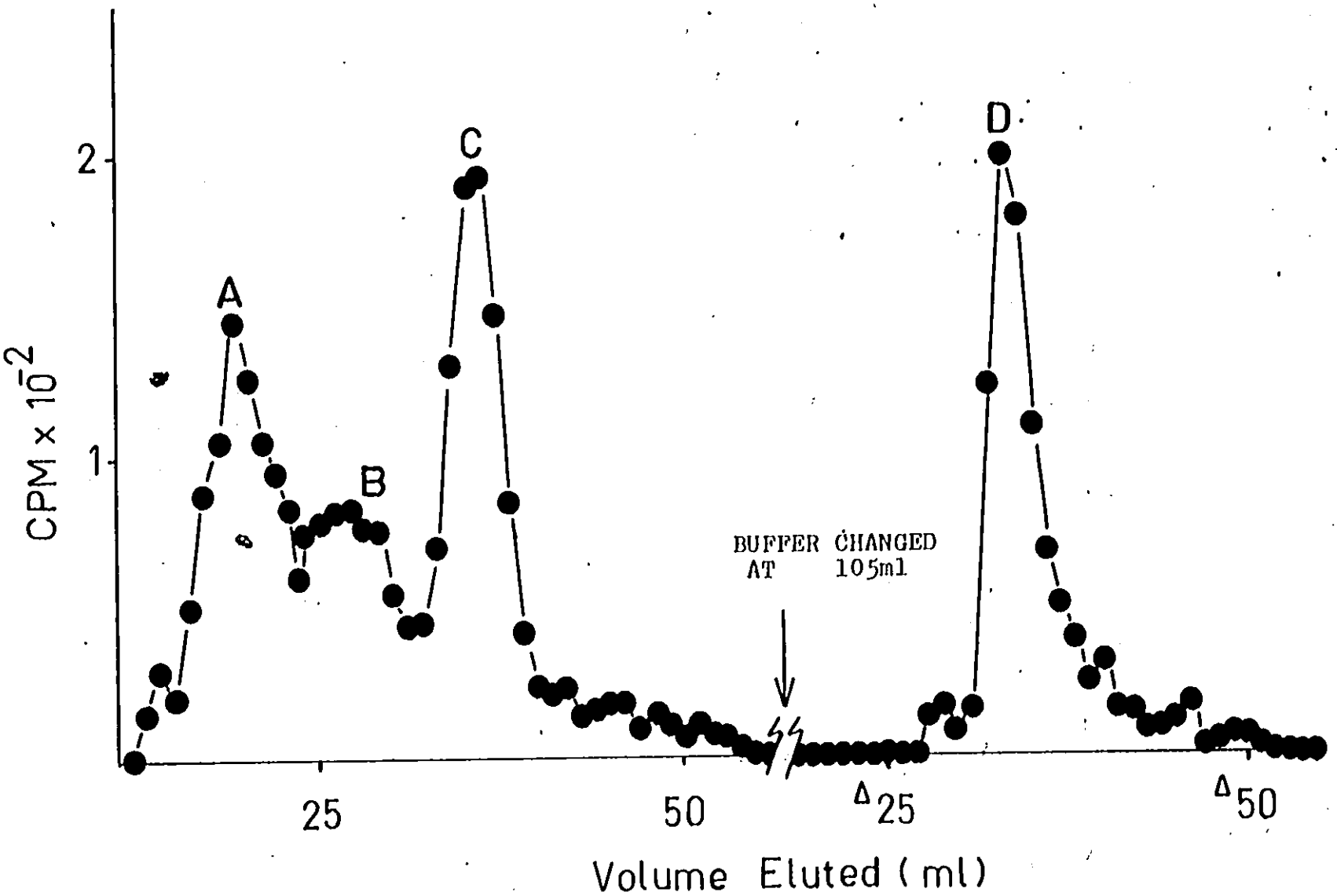

pdA at 69 ml
dG at 50 ml
dA at 61 ml
pdT
pdG
dT
pdC
dC
A
B
C
D
BUFFER CHANGED
AT 100ml
CPM x 10⁻²
3
2
1
20
40
Δ1
Δ20
Δ40
Volume Eluted (ml)

Figure 21. Enzymatic nucleoside hydrolysate on a Sephadex G-10: AG 50WX4 mixed column (60 X 0.9cm) eluted with 0.015M NH_4OH pH 3.20 changed to 0.30M NH_4OH pH 9.20, pH adjusted with formic acid, 0.5 ml/min.


CPM x 10^{-2}
2
1
A
B
C
D
BUFFER CHANGED
AT 105ml
25
50
Δ25
Δ50
Volume Eluted (ml)

peak was obscured by the broadening of the A peak, the C peak retained its symmetrical shape, suggesting that it was a single component.

VII. Comparison of the Labelled Products in DNA Purified by Chromatography or Ethanol Precipitation

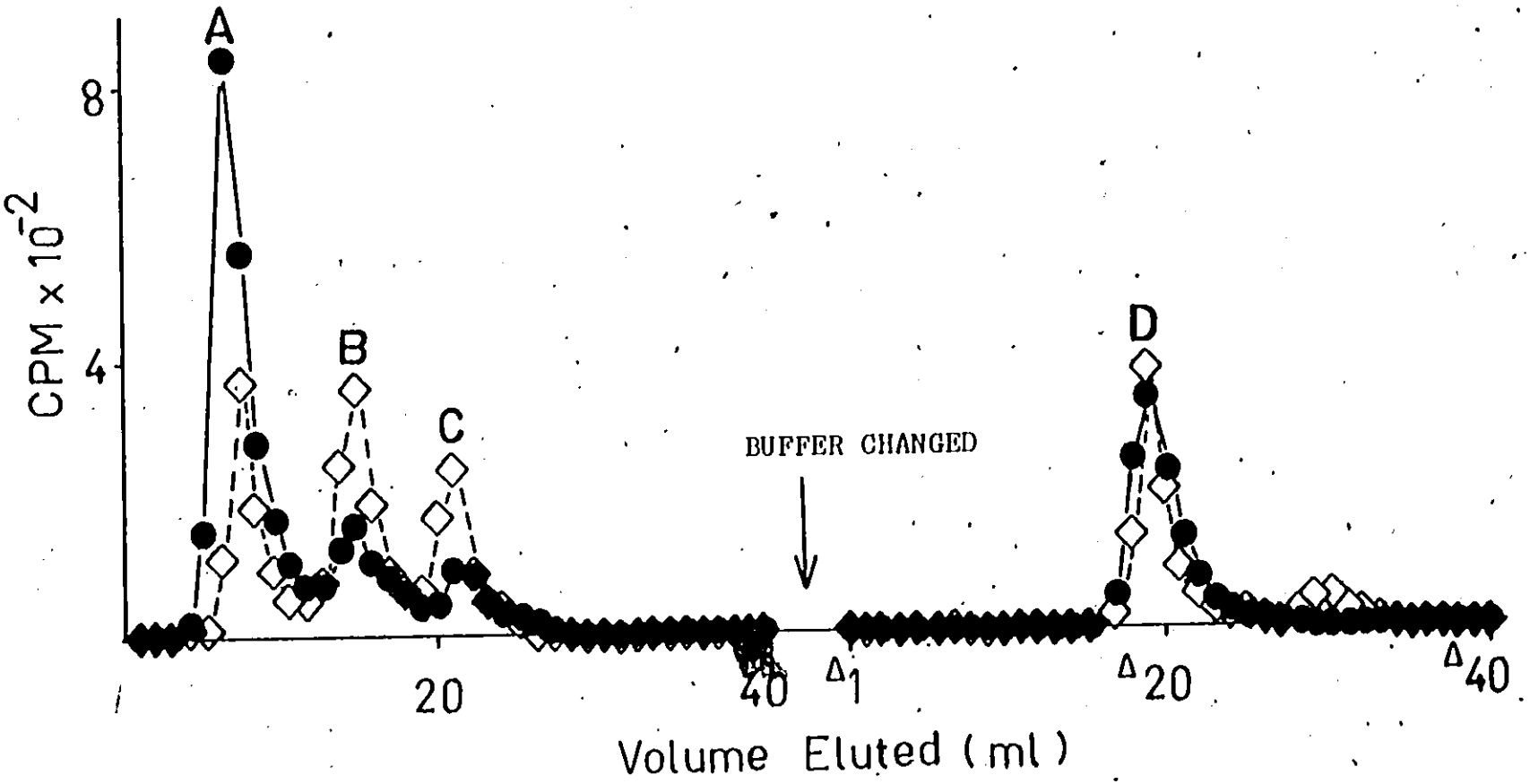
Labelled DNA purified by precipitation with ethanol yielded 2,800 cpm/mg DNA whereas the material purified by chromatography on Sephadex G-25 method gave 5,100 cpm/mg DNA. It was readily apparent from this that losses of DNA and/or of bound ^{14}C-NF from the DNA had occurred in the case of the ethanol purified DNA. This may also have been the case with the Sephadex G-25 purified DNA but the losses were obviously not so extreme as those suffered by ethanol purification. In order to gain more insight into these losses, the DNA purified by the two methods was hydrolyzed. Comparison of the various profiles indicated that the individual components were all present, but that their relative amounts were significantly different in the hydrolysates of the labelled DNA purified by the two methods.

Examination of the nucleoside digest on the "AG 50WX4 pH 3.20-9.20 column" showed that, relative to the DNA purified by Sephadex G-25 chromatography, the ethanol purified material experienced more loss of the A and D components than of the B and C components (Fig. 22).

Figure 22. Enzymatic nucleoside hydrolysate of labelled DNA purified by chromatography or ethanol precipitation. AG 50WX4 column (30 X 0.9cm) eluted with 0.015M NH_4OH pH 3.20 changed to 0.30M NH_4OH pH 9.20, pH adjusted with formic acid, 0.5 ml/min.

—●—●— Sephadex G-25 chromatography

--◇--◇-- Ethanol precipitation


A
B
C
D
8
4
CPM x 10^{-2}
BUFFER CHANGED
20
40
Δ_1
Δ_{20}
Δ_{40}
Volume Eluted (ml)

This suggested that the losses of label from DNA were not simply due to random loss of labelled DNA. Similar differential losses were seen in the Sephadex G-25 chromatography of nucleotide and nucleoside hydrolysates (Fig. 23 and 24).

VIII. Binding of Nitrofurazone to Double and Single Stranded DNA

There was 27% more binding of NF to single stranded DNA than to double stranded DNA. However, the proportions of the low molecular weight radiolysis products showed no significant differences.

Both labelled DNA's were hydrolyzed to nucleosides and the hydrolysates analyzed on the "AG 50WX4 pH 3.20-9.20 column" (30 X 0.9cm) (Fig. 25). The ssDNA appeared to be richer in components A and B but poorer in components C and D than dsDNA. Comparison of the nucleoside hydrolysate analyzed on Sephadex G-25 also showed significant differences in the relative amounts of the various components (Fig. 26). The initial peaks of the hydrolysate of ssDNA (19 to 44ml) were present in greater proportions than those in the dsDNA hydrolysate; whereas, the later peaks of the profile (45 to 59ml) were smaller in the ssDNA hydrolysate. The "Void peak" in the ssDNA nucleotide hydrolysate profile (data not presented here) was markedly larger than the "Void peak" seen in any dsDNA nucleotide

Figure 23. Enzymatic hydrolysis of labelled DNA, purified by ethanol precipitation, to nucleotide and nucleoside stage. Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.

—●—●— Nucleoside hydrolysate

--◇--◇-- Nucleotide hydrolysate

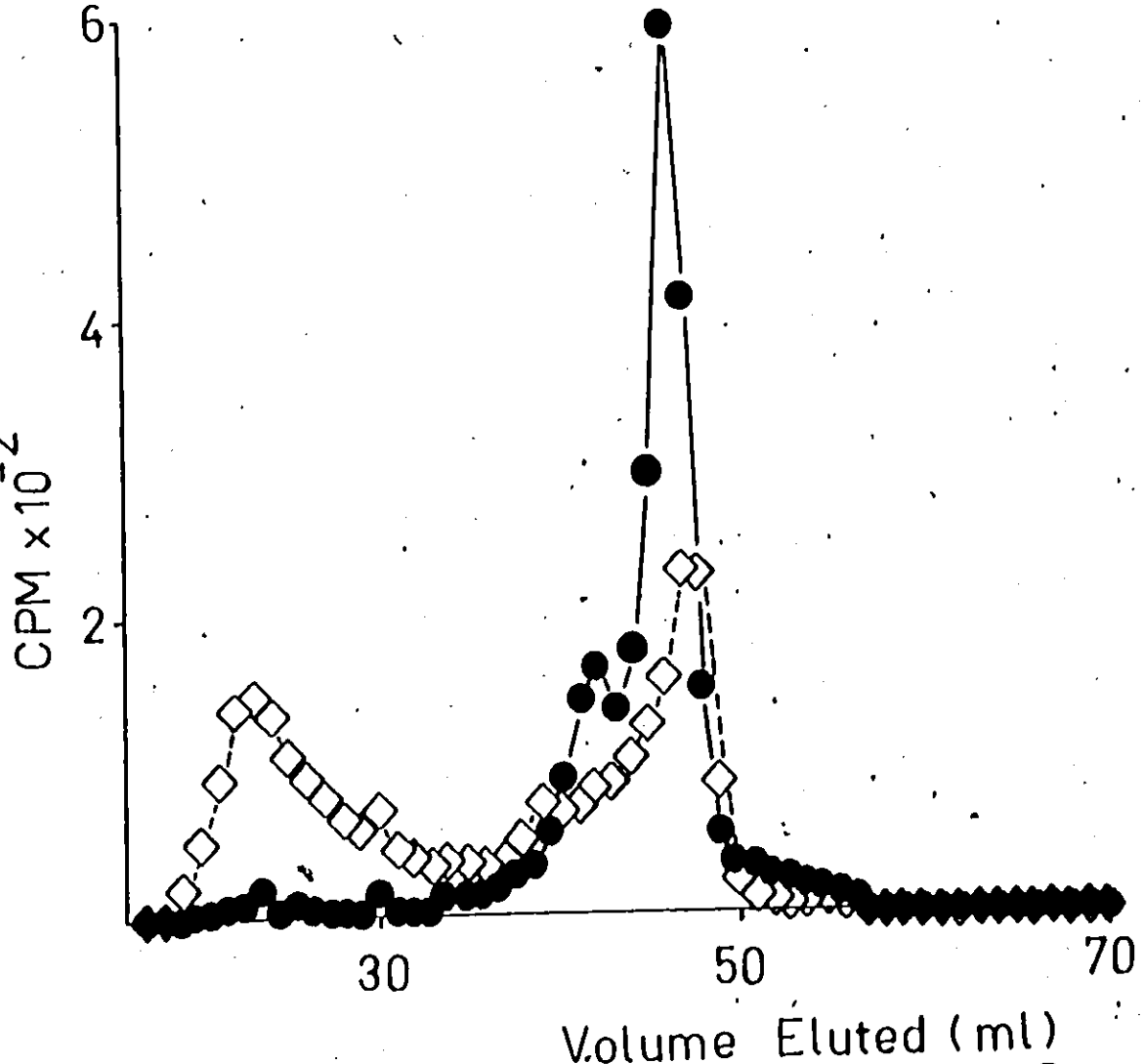
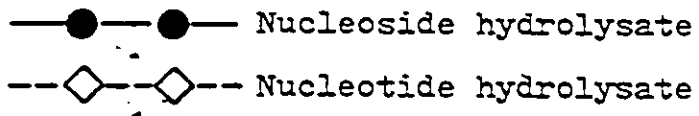

6
4
2
CPM x10^-2
30
50
70
Volume Eluted (ml)

Figure 24. Enzymatic hydrolysis of labelled DNA, purified by Sephadex G-25 chromatography, to nucleotide and nucleoside stage. Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.



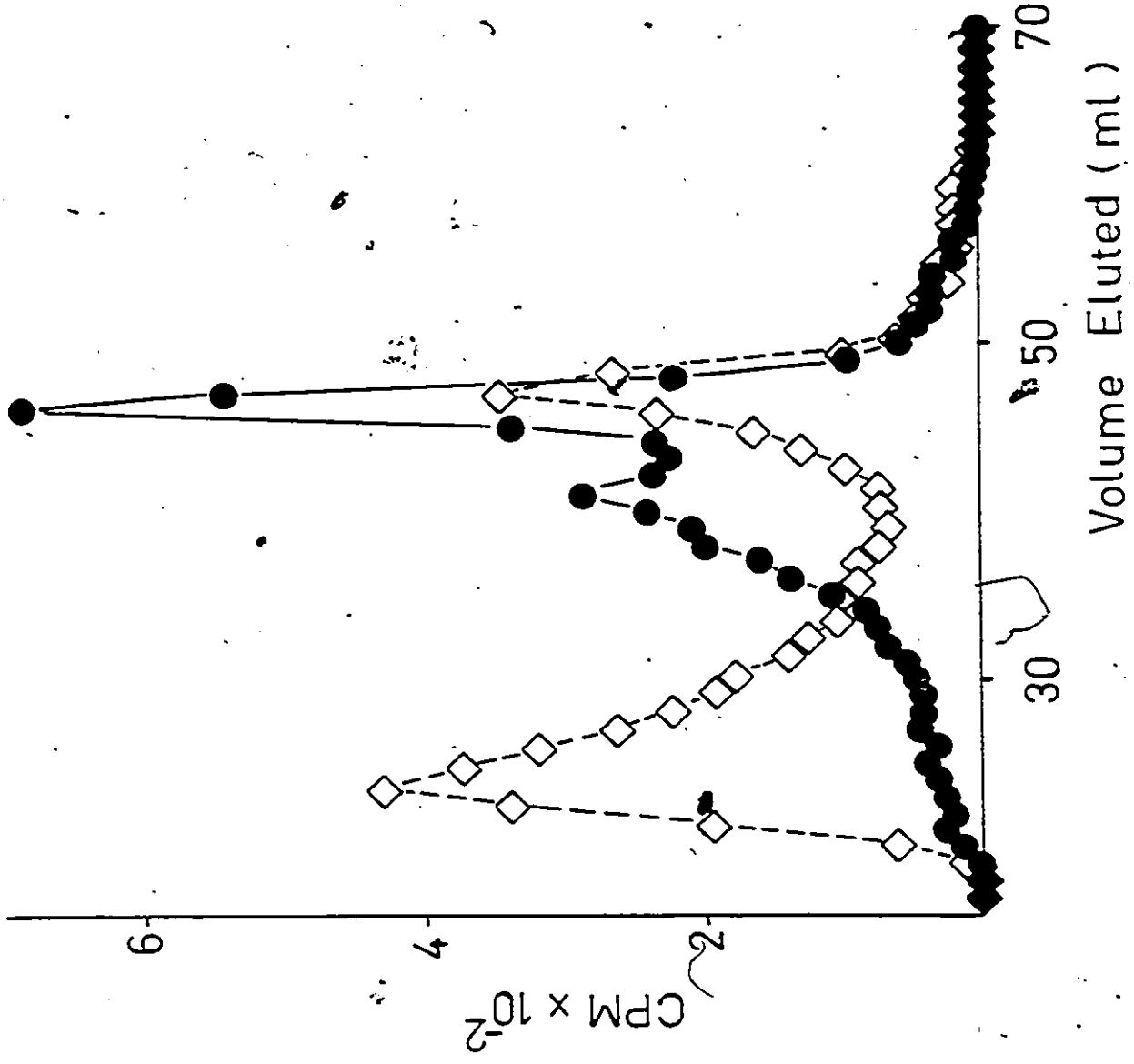

CPM x 10⁻²
6
4
2
30
50
70
Volume Eluted (ml)

Figure 25. Enzymatic hydrolysis of labelled double stranded and single stranded DNA to nucleoside stage.
AG 50WX4 column (30 X 0.9cm) eluted with 0.015M NH_4OH pH 3.20 changed to 0.30M NH_4OH pH 9.20, pH adjusted with formic acid, 0.5 ml/min.

—●—●— Double stranded DNA

--◇--◇-- Single stranded DNA

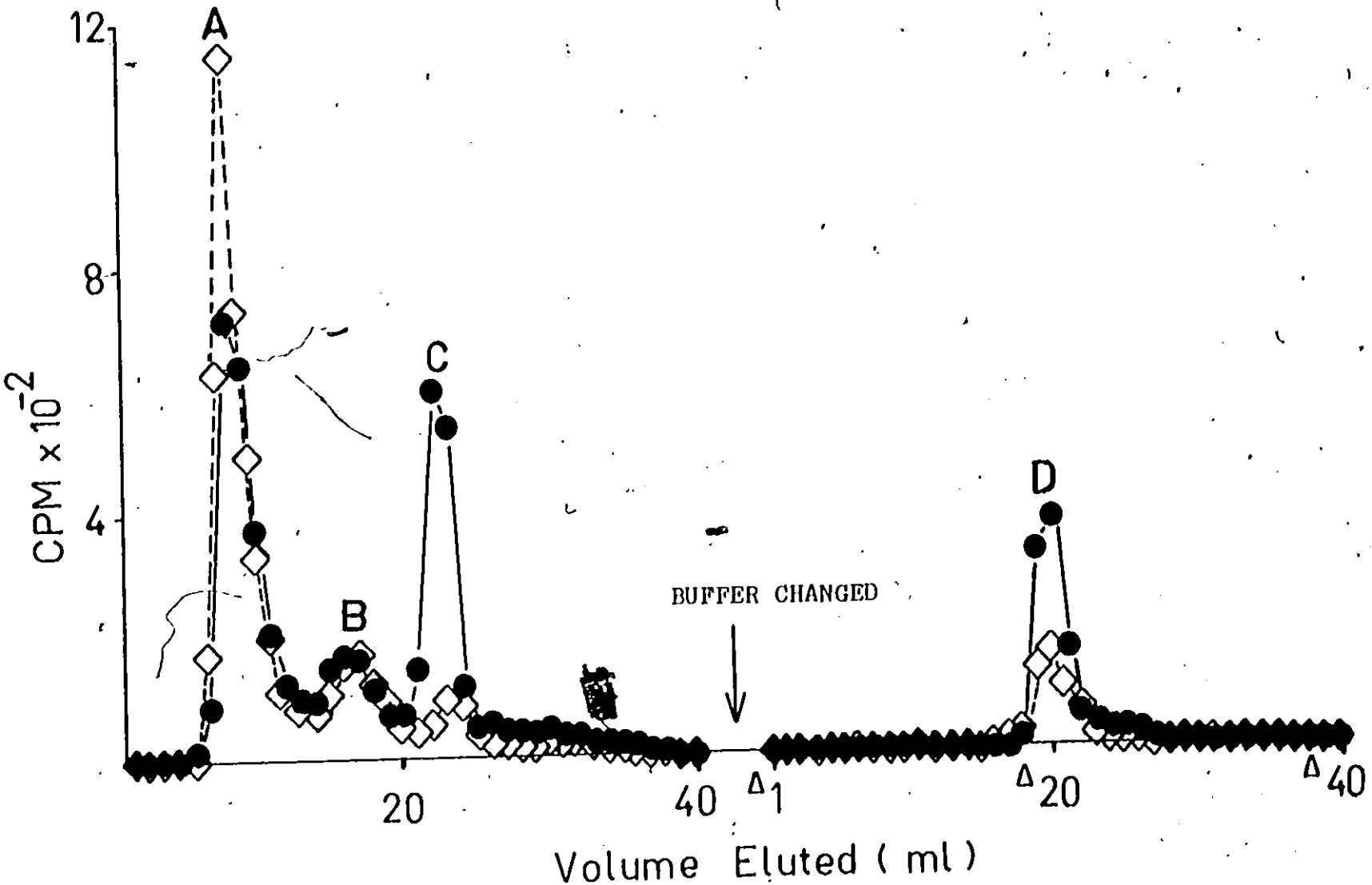
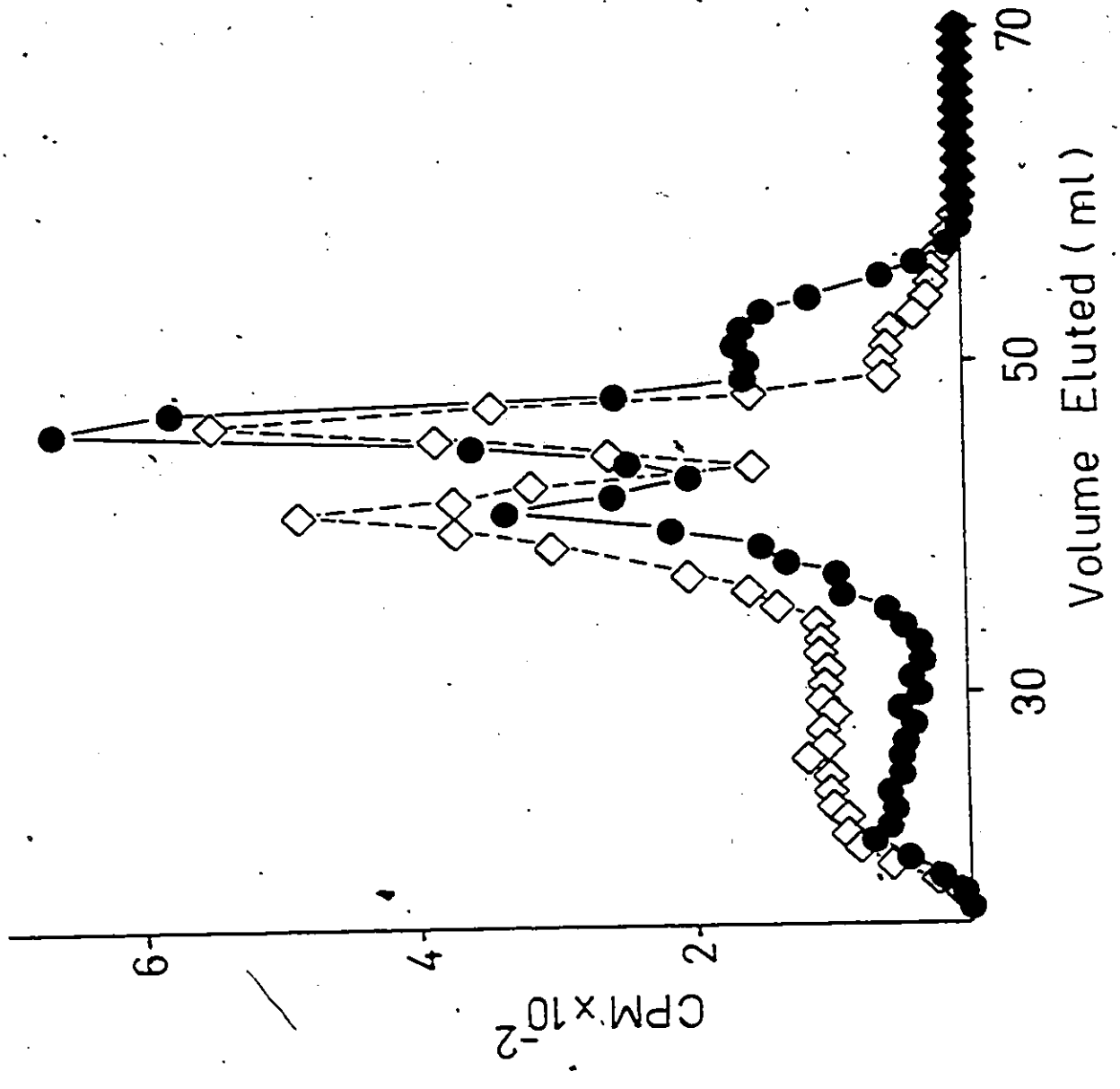

12
8
4
CPM x 10^-2
A
B
C
D
BUFFER CHANGED
20
40
Δ1
Δ20
Δ40
Volume Eluted (ml)

Figure 26. Enzymatic hydrolysis of labelled double and single stranded DNA to nucleoside stage. Sephadex G-25 column (100 X 0.8cm) eluted with distilled water, 0.5 ml/min.

—●—●— Double stranded DNA

--◇--◇-- Single Stranded DNA


CPM x 10⁻²
6
4
2
30
50
70
Volume Eluted (ml)

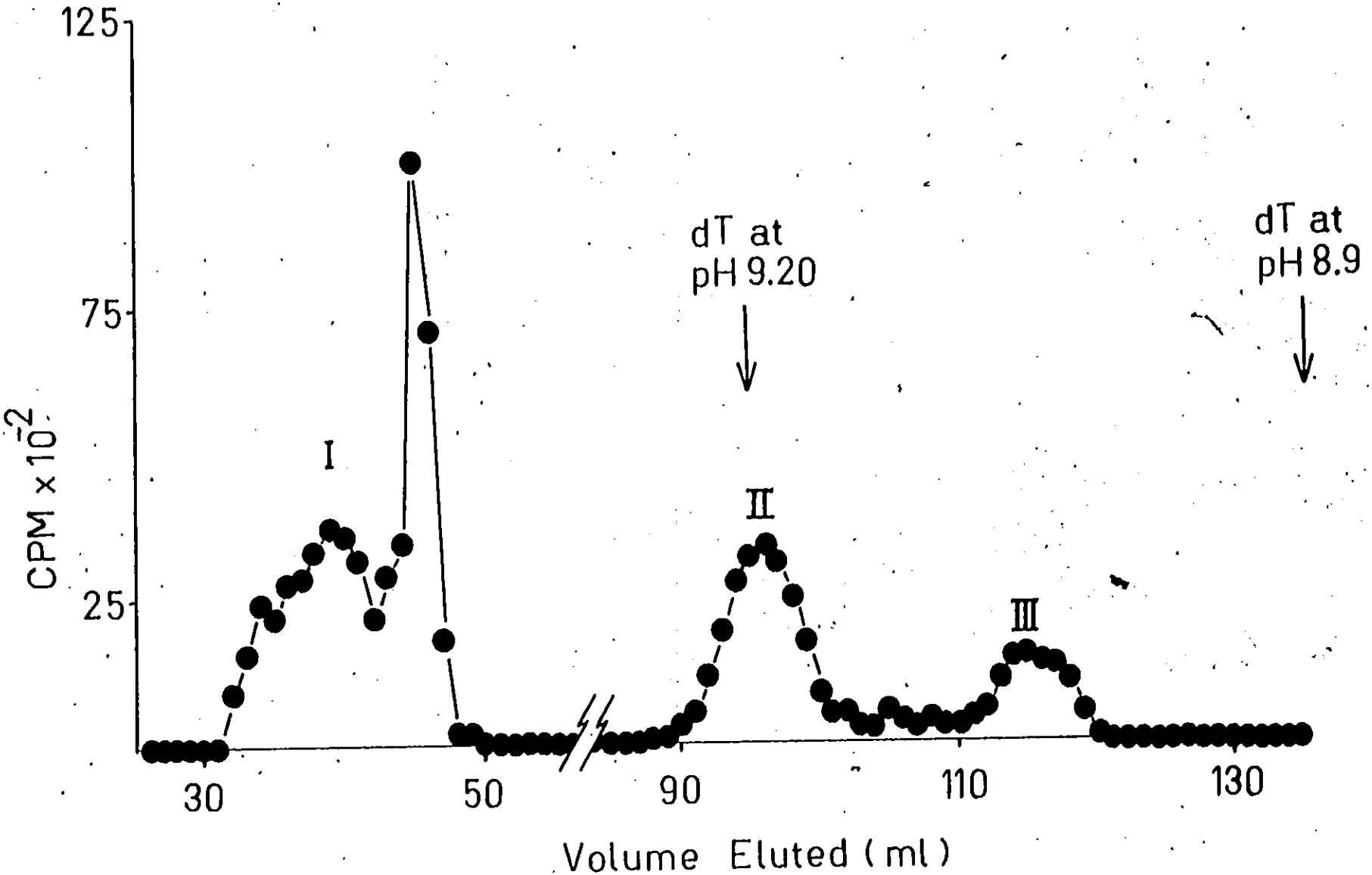
hydrolysates.

The binding of NF to RNA was also examined. RNA bound 35% less NF than dsDNA, but the amounts of the low molecular weight radiolysis products were significantly increased such that more NF was destroyed than in the case of dsDNA at the same dose of Krad.

IX. Separation of Nitrofurazone Derivatives from Normal Nucleosides

In order to remove the normal nucleosides from the NF derivatives the "AG 50WX4 pH 9.20 column" was chosen. This column demonstrated the best separation of labelled components from normal nucleosides (Fig. 19). A larger column was used to achieve this, 90 X 1.5cm (Fig. 27). The labelled NF derivatives came out early on this column. The profile showed a multicomponent peak (I) which eluted first, followed by a pair of peaks (II and III). No radioactivity was found after 120ml (to 500ml). The elution profile followed that seen on the smaller column, 30 X 0.9cm (Fig. 19). On this large column only thymidine posed a problem as it chromatographed with peak II. However, by changing the pH of the eluting buffer to pH 8.90 from pH 9.20 the chromatographic mobility of thymidine was altered, but the chromatographic mobilities of peak II and III remained unchanged, so that, peaks II and III, as well

Figure 27. Enzymatic hydrolysis of labelled DNA to nucleoside stage. AG 50WX4 column (90 X 1.5cm) eluted with 0.015M NH_4OH pH 9.20/8.9 (column eluted at pH 9.20 or 8.9 with no change in mobility of peaks I, II, and III), pH adjusted with for formic acid, 0.17 ml/min.


125
75
25
CPM x 10⁻²
I
II
III
dT at
pH 9.20
dT at
pH 8.9
30
50
90
110
130
Volume Eluted (ml)

as peak I eluted before thymidine (Fig. 27).

The multicomponent peak I was associated with absorbance at 260nm which followed the radioactivity profile (Fig. 28-A). The radioactivity and UV profiles showed evidence for at least four components. The λmax of each fraction was determined and this gave evidence of four to five components matching the positions suggested by absorbance at 260nm and the radioactivity profiles (Fig. 28-B). The ratio of cpm to absorbance 260nm units also provided evidence for five components (Fig. 28-C). Thus, four criteria provided support for at least five components in this multicomponent peak I.

Obviously the chromatographic procedures so far described do not have adequate resolving power. A search for better methods was made. The most successful of these is described below.

X. Analysis on Sephadex DEAE A-25

Chromatography of a nucleoside digest on a Sephadex DEAE A-25 column (60 X 0.9cm) using a linear concentration gradient of 0.01M to 0.60M ammonium bicarbonate (pH 8.00) provided clear evidence for at least 10 components (Fig. 29). In separate experiments it was discovered that normal nucleosides thymidine, deoxyadenosine, and deoxycytidine were eluted early and without need of a concentration gradient.

Figure 28. Peak I of "AG 50WX4 pH 9.20/8.9 column" (90 X 1.5cm)

A) OD 260nm and cpm

B) λ max

C) Ratio of cpm to OD 260nm

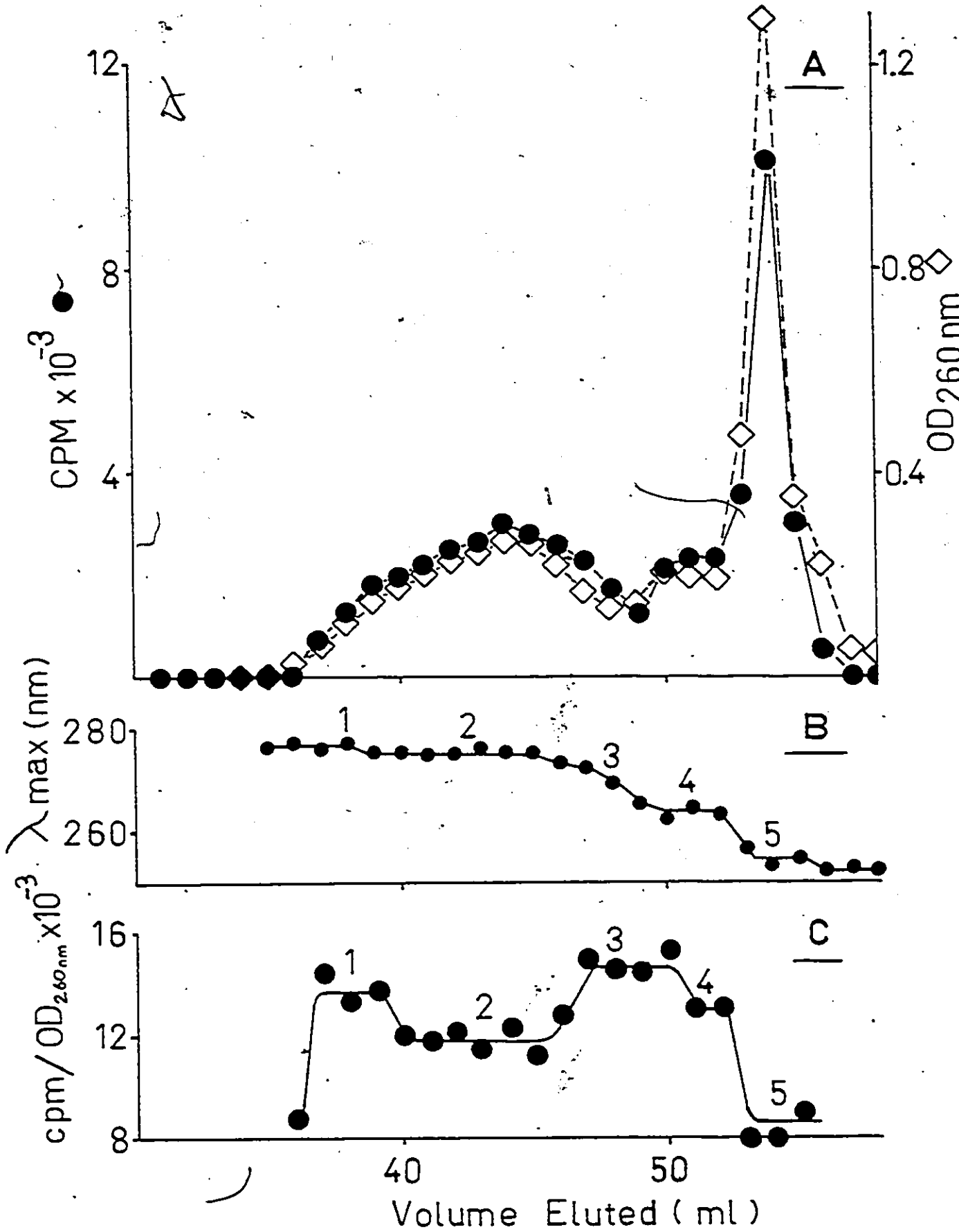
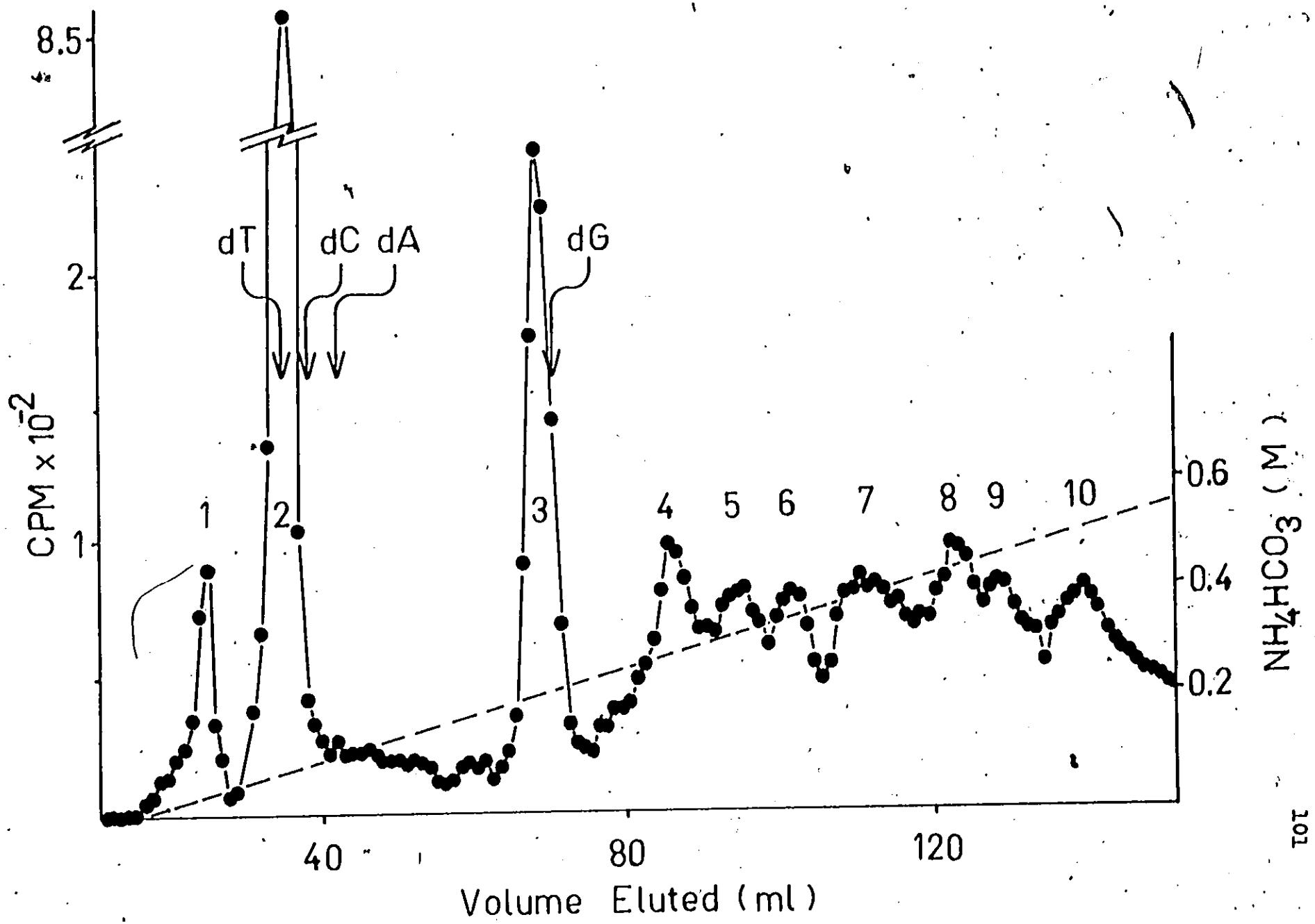

A
B
C
CPM x10^-3
OD260nm
λmax (nm)
cpm/OD260nm x10^-3
Volume Eluted (ml)
12
8
4
1.2
0.8
0.4
280
260
16
12
8
40
50
1
2
3
4
5

Figure 29. Enzymatic hydrolysis of labelled DNA to nucleoside stage. Sephadex DEAE A-25 column (60 X 0.9cm) eluted with 0.01M to 0.60M ammonium bicarbonate pH 8.00 gradient, 0.17 ml/min.


8.5
2
1
CPM x 10⁻²
dT
dC dA
dG
1
2
3
4
5
6
7
8
9
10
0.6
0.4
0.2
NH4HCO3 (M)
40
80
120
Volume Eluted (ml)

This was also true of peak #2 (the largest peak) of the radioactivity profile. All other components were retained on the column until the gradient was initiated.

XI. Binding Studies with Deoxyribohomopolymers

As noted above, the Sephadex DEAE A-25 column gave the best resolution of nucleoside hydrolysate products and revealed a complex mixture of NF derivatives. Experiments with deoxyribohomopolymers were therefore undertaken to determine the origin of the various components seen with this column. Table 1 shows the extent to which the four deoxyribohomopolymers bound NF. Poly-dT gave seven times more binding than ssDNA, poly-dC: 1.5 times, poly-dA: equivalent, and poly-dG: only 0.5 times as much. With poly-dT, the equivalent of one NF molecule was bound for every 224 nucleosides at the dose of 34.3 Krad.

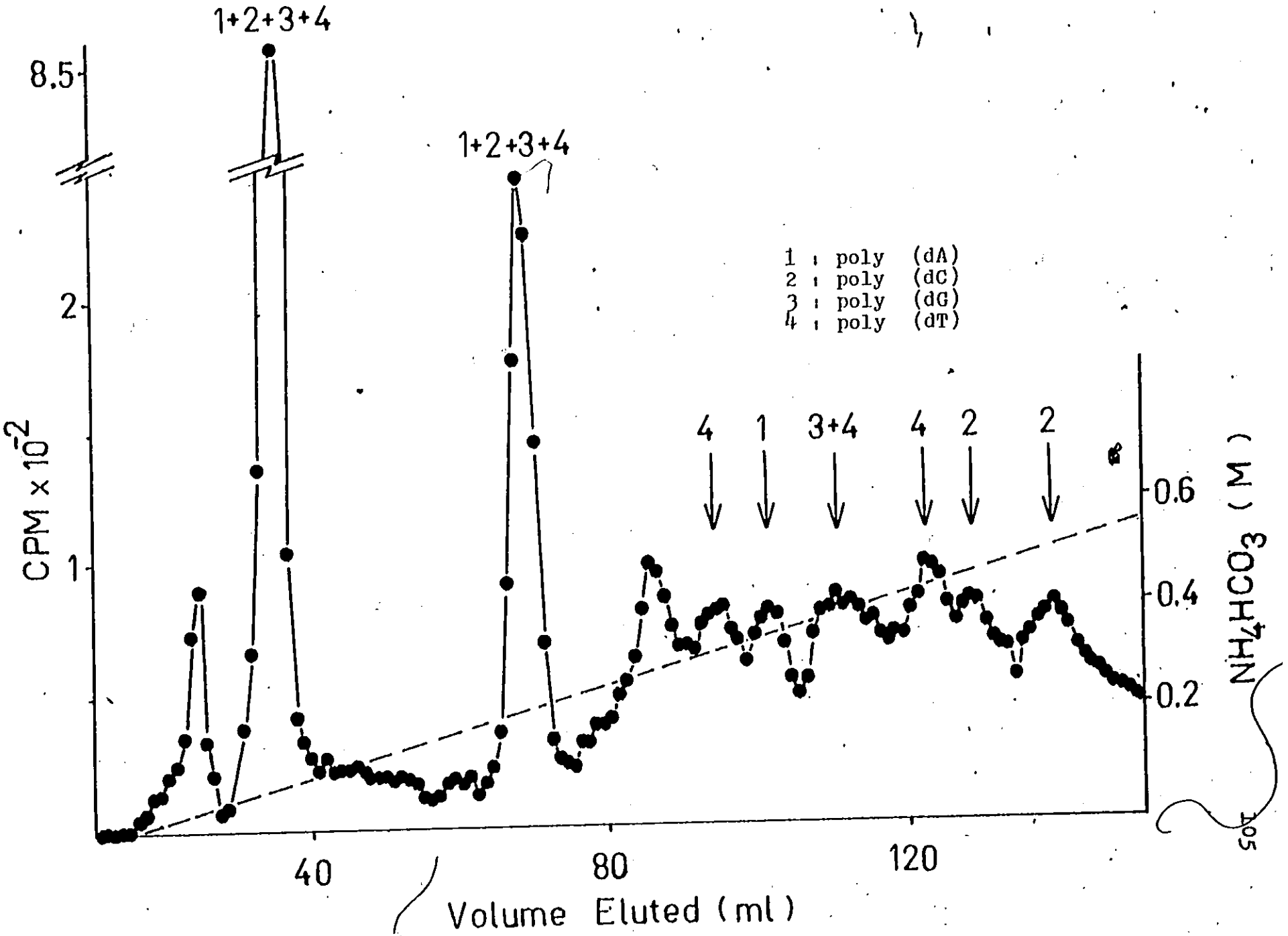
The labelled deoxyribohomopolymers were digested to nucleosides and applied to the DEAE A-25 column. The positions to which the major derivatives of each deoxyribohomopolymer eluted are indicated by arrows on Figure 30. Thus, the origin of most of the components derived from NF treated DNA were determined. Of interest is the fact that the peaks #2 and #3 were formed with all the deoxyribohomopolymers. Components #1 and #4 could not be unambiguiously assigned.

Table 1. Radiation induced binding of Nitrofurazone to deoxyribohomopolymers (Ns = Nucleoside).

1.68 g/ml NF, 34.3 Krads in distilled water

	cpm/mg	nmoles NF bound/mg	moles Ns/ moles NF	moles NF/ 1000 moles Ns
poly dT	111,111	13.89	224	4.10
poly dC	23,750	2.97	1,101	0.91
poly dA	16,667	2.08	1,457	0.69
poly dG	7,709	0.96	3,010	0.33
ss DNA	16,142	2.02	1,518	0.66

Figure 30. Assignment of the origin of compounds, in an enzymatic nucleoside hydrolysis of labelled DNA, on the Sephadex DEAE A-25 column (60 X 0.9cm). This is a replot of Fig. 29.


1+2+3+4
1+2+3+4
8.5
2
1
CPM x 10⁻²
1 : poly (dA)
2 : poly (dC)
3 : poly (dG)
4 : poly (dT)
4
1
3+4
4
2
2
0.6
0.4
0.2
NH4HCO3 (M)
40
80
120
Volume Eluted (ml)

XII. Re-chromatography

In order to compare the various components in the nucleoside hydrolysate separated on different columns some of the peaks were re-chromatographed.

A. "AG 50WX4 pH 9.20 Column" Peak I on "AG 50WX4 pH 3.20-9.20 Column"

Peak I of the "AG 50WX4 pH 9.20 column" (Fig. 27) was reapplied to an "AG 50WX4 pH 3.20-9.20 column" (Fig. 31). This multicomponent peak separated to give the A and D peaks.

B. "AG 50WX4 pH 9.20 Column" Peak I on Sephadex DEAE A-25 Column

Application of the multicomponent peak I (Figs. 27 and 28) to a Sephadex DEAE A-25 column (60 X 0.9cm) gave a radioactivity profile matching a nucleoside hydrolysate of labelled DNA except that it lacked peaks #2 and #3 (the two major peaks) which were common to all four deoxyribose homopolymers (Fig. 32).

C. "AG 50WX4 pH 9.20 Column" Peak II on Sephadex DEAE A-25 Column

Application of the peak II of Figure 27 to the Sephadex DEAE A-25 column (60 X 0.9cm) showed that it was the peak #2 and major peak of a nucleoside hydrolysate of

labelled DNA (Fig. 32).

Figure 31. Rechromatography of "AG 50WX4 pH 9.20 column" peak I on "AG 50WX4 pH 3.20 to 9.20 column" (90 X 1.5cm).

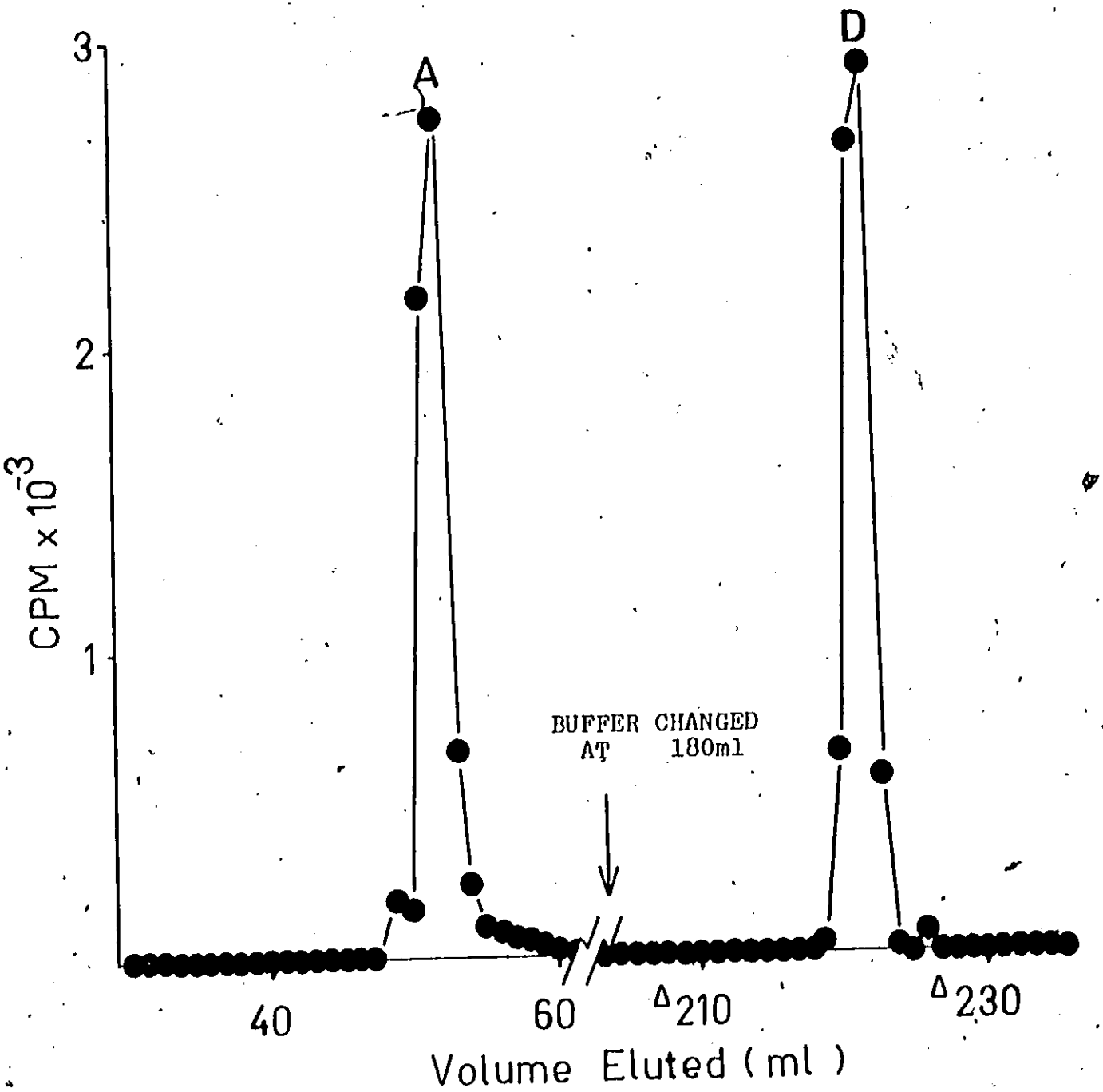
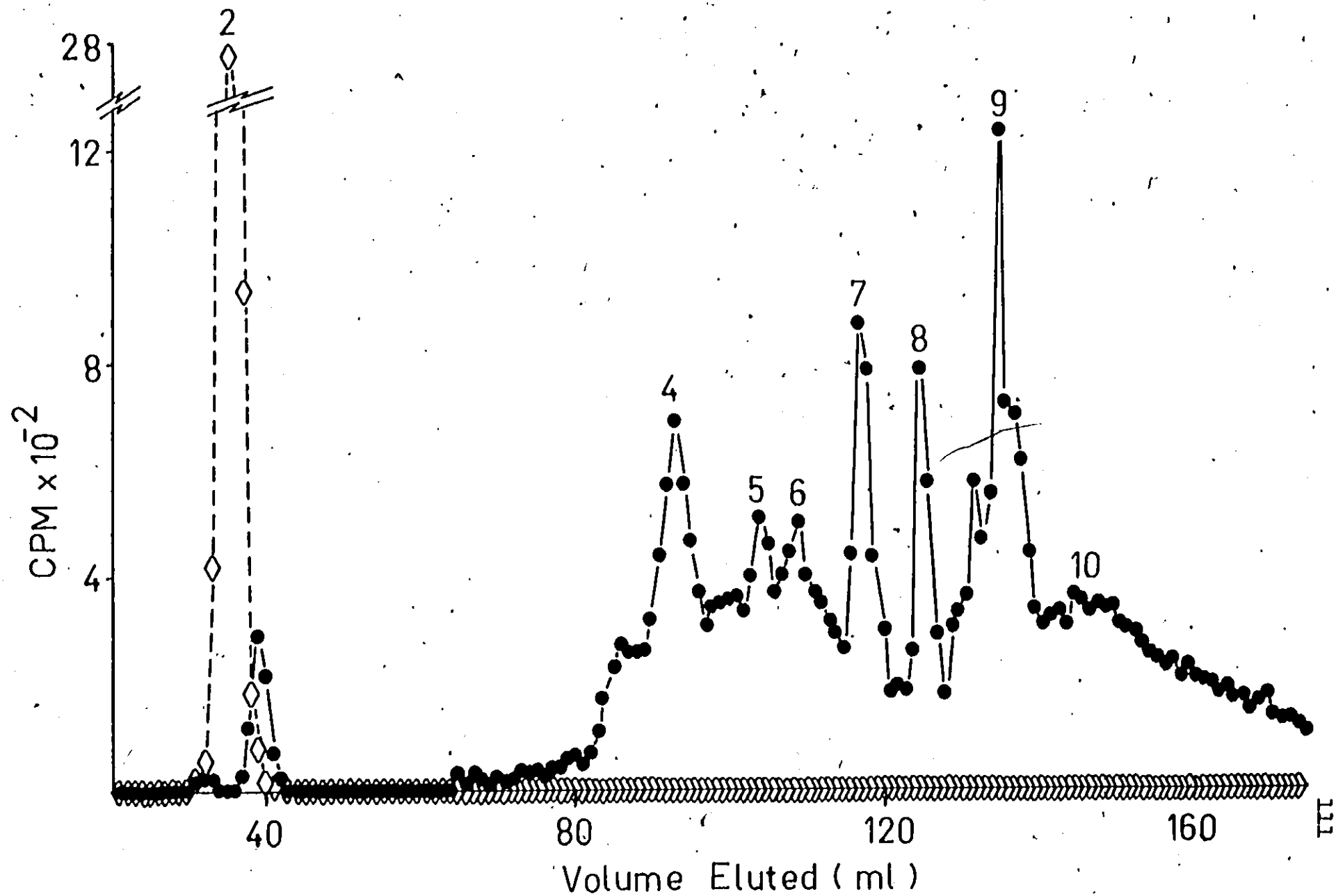

A
D
3
2
1
CPM x 10^-3
BUFFER CHANGED
AT 180ml
40
60
Δ210
Δ230
Volume Eluted (ml)

Figure 32. Rechromatography of "AG 50WX4 pH 9.20 column" peaks I and II on Sephadex DEAE A-25 column (60 X 0.9cm) eluted with 0.01M to 0.60M ammonium bicarbonate pH 8.00 gradient, 0.10ml/min.

—●—●— Peak I

--◇--◇-- Peak II


28
12
8
4
CPM x 10⁻²
2
4
5
6
7
8
9
10
40
80
120
160
Volume Eluted (ml)

DISCUSSION

Purification of Nitrofurazone (NF) Bound DNA

In order to separate the labelled DNA from the low molecular weight radiolysis products and the undestroyed NF, chromatography on Sephadex columns, precipitation by ethanol and dialysis were employed. All these approaches served to remove the unwanted contaminants, but dialysis proved to be the most efficient method. With dialysis, the total time required for removal of low molecular weight contaminants was reduced, there was less loss of labelled DNA, and larger amounts of labelled DNA could be purified than by chromatography on Sephadex columns. However, chromatography on Sephadex G-25 and G-50 provided excellent methods for assessing the extent of NF binding to DNA. Recovery of labelled DNA from ethanol precipitation was poor. This was not simply due to loss of labelled DNA, but to differential losses of the NF derivatives bound to the DNA.

Studies have shown that NF will bind to protein and whole cells as well as to nucleic acids (26). However, the observation that the label bound to DNA was not extractable by phenol or chloroform isoamyl alcohol provided evidence that the binding was not due to contaminating protein in

the DNA employed. The enzymatic hydrolysis of the labelled DNA to the nucleotide and nucleoside levels altered the chromatographic mobilities of the labelled material at each stage of hydrolysis;thus, providing further evidence that binding to protein was not involved (Fig. 16).

The dialysis of the labelled DNA against 5M NaCl caused no loss of label indicating that the binding of NF to DNA was unlikely to be due to ionic interactions. The precipitation with ethanol and extraction with phenol and chloroform suggested that non-covalent hydrophobic interactions of NF or NF radiolysis products to DNA did not occur.

Radiation-Induced Binding of NF to DNA

As outlined in the Introduction, NF binding to DNA is related to the production of secondary radicals on the DNA produced by ionizing radiation. Primary radicals (solvated electrons, $H^{\cdot}$ and $HO^{\cdot}$) can react with DNA, NF and the constituents of the medium producing secondary radicals. Both reactions of secondary radicals on DNA with NF and reactions of NF secondary radicals with DNA could produce NF adducts to DNA. However, the former results in most of the binding (27).

A comparison of the radiolysis products obtained using different media made it apparent that the nature of

the low molecular weight radiolysis products were determined by the media used (Fig. 9). This suggests that some of the radiolysis products seen could be solute adducts of NF. The reaction rates of various biomolecules with primary radicals are known (11). In studies examining the rate of radiolysis of NF in the presence of these biomolecules, it is evident that the radiolysis of NF is greatest in the presence of biomolecules which react fast with primary radicals. Here too, the secondary radicals produced by HO· are responsible for the bulk of the radiolysis of NF (27). Thus, the reactivity of the media constituents with primary radicals, particularly HO·, might affect the overall binding of NF to DNA via competetive reactions with the available NF.

In the presence of oxygen, very little, if any, binding of NF to DNA occurred. Oxygen, being more electron affinic, competes very efficiently with NF for secondary radical sites in DNA. This has the effect of protecting NF from the secondary radicals in DNA and thus decreasing NF destruction. Also, oxygen could quench any NF radical formed with the conversion of oxygen to superoxide (19), which could also decrease NF destruction.

The observation that increasing concentrations of NF did not enhance the labelling of DNA nor the appearance of NF radiolysis products is consistant with the proposal that the secondary radicals on DNA are largely responsible

for adduct formation. If the NF binding to DNA occurred via NF radicals then one would expect an increase in NF concentration to produce more NF radicals which would enhance binding to DNA and NF destruction. However, the same amount of NF was destroyed and bound to DNA regardless of the concentration of NF used (Fig. 13 and 15). If the secondary radicals on DNA are responsible for the binding of NF to DNA, an increase in DNA concentration should increase the number of secondary radical sites produced by ionizing radiation. Thus, one would expect in increased NF binding to DNA and radiolysis of NF. This is precisely what was observed (Fig. 14). The rate of binding of NF to DNA was first order with respect to the concentration of DNA. Hence, the rate of production of secondary radicals in DNA was likely also first order with respect to the concentration of DNA at any given dose of radiation.

Binding of NF to Double Stranded DNA Relative to Single Stranded DNA

Studies examining the binding of nitrofurans, "activated" via enzymatic reduction, to DNA have shown that there is greater binding to single stranded DNA (ssDNA) than to double stranded DNA (dsDNA) (144). Examination of radiation-induced binding of NF to DNA gave the same results.

This enhanced binding could be due to two possibilities: ssDNA might offer more sites for secondary radical formation on DNA and hence more sites for NF adduct formation, or, the less structured ssDNA could allow a freer access to the secondary radicals on DNA some of which have been in a sterically hindered area in dsDNA and thus would have been protected from NF attack. Ward and Kuo (143), in a comparison of radiation damage of double and single stranded DNA, suggest that bases in intact double stranded DNA are sheltered from radical attack by virtue of being in the center of the double helix.

Examination of the nucleoside hydrolysates of ssDNA and dsDNA showed very different distributions of the NF derivatives formed, some components being produced in greater amounts in dsDNA, while others were produced in greater amounts in ssDNA. This suggests that it was not just a matter of more secondary radicals being produced, but rather due to production of different secondary radicals or different reactivities of the secondary radicals produced in the two situations.

Nature and Separation of NF Derivatives

Of the various columns examined in search of efficient separation of NF derivatives in the nucleoside hydrolysate, the Sephadex DEAE A-25 and AG 50WX4 columns

were most successful. Combination of chromatography on the AG 50WX4 pH 8.9 column and the Sephadex DEAE column will provide an efficient means of separating the various NF derivatives in nucleoside hydrolysates of the labelled DNA. Results of these columns also provided preliminary information with respect to the nature of these NF derivatives.

Peak I on the "AG 50WX4 pH 9.20 column" was a multicomponent peak (Fig. 28). It showed evidence for at least five components. However, upon reapplication of this peak to the Sephadex DEAE A-25 column it became apparent that it contained many more than five components. Figure 32 provides evidence for a minimum of nine separate components. Peak I was also associated with UV absorbing material, with λ max's in the region of 276 to 253nm suggesting that it could contain NF-nucleoside derivatives. If such is the case, these modified nucleosides show distinct differences from normal nucleosides. On the "AG 50WX4 pH 9.20 column" peak I eluted at close to the void volume of the column suggesting that these components had no affinity for the resin of the column. They eluted much sooner than normal nucleosides. On the Sephadex DEAE A-25 column normal nucleosides eluted ahead of the peak I NF derivatives. In this column they were retained and released after a concentration gradient was applied. The only exception was a minor peak which eluted at 39ml (Fig. 32). These observations made with two oppositely charged

columns suggested that the peak I components are more acidic in nature than the normal nucleosides, possessing a net negative charge at pH 8-9.

When peak I of the "AG 50WX4 pH 9.20 column" was reapplied to the "AG 50WX4 pH 3.20 to 9.20 column" the components in peak I separated into two groups represented by peaks A and D of a nucleoside hydrolysate on the "AG 50WX4 pH 3.20 to 9.20 column". This observation supports the suggestion made in Results Sections VI-D that the peaks A and D were multicomponent. Also, this indicates that at pH 3.20 approximately half the NF derivatives of peak I were negatively charged and thus not retarded by the column, whereas, the other half were retained and not eluted until the buffer was changed to pH 9.20. This suggests that the NF derivatives in peak D of the "AG 50WX4 pH 3.20 to 9.20 column" were positively charged at pH 3.20 but became negatively charged or neutral at pH 9.20.

When peak II of the "AG 50WX4 pH 9.20 column" was re-chromatographed on the Sephadex DEAE A-25 column, it eluted exclusively in the position of the largest peak, #2 of a nucleoside hydrolysate on the Sephadex DEAE A-25 column (Fig. 32). This suggests strongly that this material represents a single compound since on the two oppositely charged columns, "AG 50WX4 pH 9.20 column" and Sephadex DEAE A-25 column, it eluted as a single symmetrical peak. Also, this compound did not appear to undergo any ionic

interaction with either column which suggests that at pH 8-9 it is neutral.

It became evident that peaks B and C on the "AG 50WX4 pH 3.20 to 9.20 column" may be the same as the peaks II and III on the "AG 50WX4 pH 3.20 to 9.20 column". From a consideration of the proportion of the radioactivity under these peaks, peak B is equivalent to peak III and and peak C is equivalent to peak II. Also, on the basis of the ratio of radioactivity under peaks II and III, the peak #3 on the Sephadex DEAE A-25 column is equivalent to peak III. Table 2 and Figure 33 summarize these conclusions.

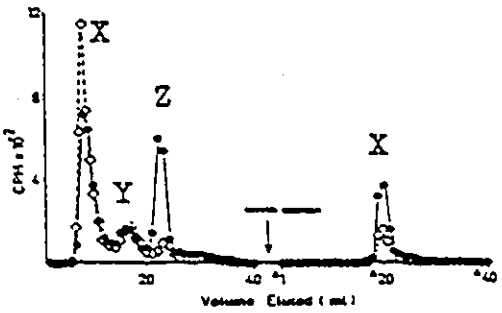
An additional word regarding peak III is that it appears to be somewhat similar in nature to peak II. Observations from the three previously mentioned columns suggest that these peaks represent single components present in relatively large amounts which are apparently neutral at both pH 3.20 and pH 9.20. However, at pH 8.00 on the Sephadex DEAE A-25 column peak #3 (peak III) appeared to be somewhat negatively charged as it was retained by the gel but eluted first on the concentration gradient.

Corroboration of the acidic nature of peak I of the "AG 50WX4 pH 9.20 column" can be seen on the Sephadex G-25 and G-10 columns to which nucleoside hydrolysates were applied. Sephadex gels have a slight cationic exchanger character due to a low content of carboxylic groups (148). Also, with the lower G value gels (G-10,

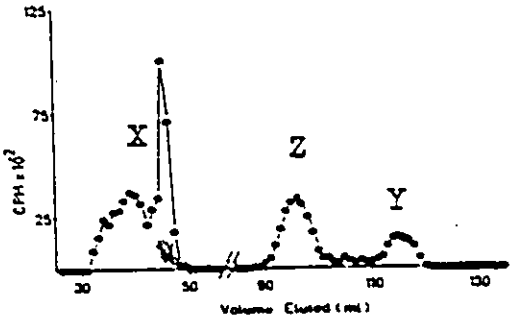
Table 2. Peak relationship between Sephadex DEAE A-25 and AG 50WX4 columns of nucleoside hydrolysates.

AG 50WX4 pH 3.20 to 9.20	AG 50WX4 pH 9.20	DEAE A-25
A	I	Remainder
B	III	Third
C	II	Second
D	I	Remainder

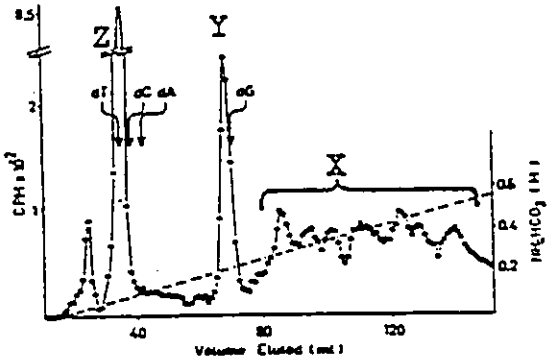
Figure 33. Correlation of the peaks of various chromatograms. X represents a group of Ns-NF adducts, whereas Y and Z are likely single compounds.



AG50WX4 pH 3.20-9.20 chromatogram



AG50WX4 pH 9.20 chromatogram



DEAE A-25 chromatogram

G-15, and G-25) adsorption of aromatic and heterocyclic compounds occurs (41). In this respect nucleosides and bases are adsorbed on the column, eluting later than the bed volume of the column. However, nucleotides elute much earlier. The NF derivatives elute ahead of most normal nucleosides on the Sephadex G-25 and G-10 columns suggesting that they are more acidic. Thus, the NF derivatives found in nucleoside hydrolysates appear, in general, to be more acidic and less hydrophobic than normal nucleosides.

Binding Studies With Deoxyribohomopolymers

Analysis of the treated deoxyribohomopolymers demonstrated that the pyrimidine homopolymers were more susceptable to attack by NF resulting in adduct formation than were the purine homopolymers. This implies that secondary radicals on pyrimidines were more reactive towards NF or, alternatively, were present in greater amounts than secondary radicals on purines. This agrees with the known rates of destruction of pyrimidines and purines by ionizing radiation (57, 121). Among the pyrimidines, thymine derivatives show significantly greater destruction by ionizing radiation than do cytidine derivatives (57). The NF binding to DNA agreed with this also, poly-dT bound 4.3 times more NF than poly-dC

(Table 1). The order of binding of NF to the deoxyribohomopolymers was: thymine, cytidine, adenine and guanine. This is in general agreement with the order of susceptability to destruction by ionizing radiation (57, 121).

The radioactivity profiles of the nucleoside hydrolysates of the deoxyribohomopolymers on Sephadex DEAE A-25 column showed a number of major and minor peaks. It is expected that a great variety of NF derivatives would result from even a single deoxyribohomopolymers, considering the number of possible secondary radicals that could be formed. However, in each nucleoside hydrolysate of the deoxyribohomopolymers there were present major components which corresponded to the peaks seen in a nucleoside hydrolysate of treated ssDNA. Poly-dT showed five major peaks, poly-dC exhibited four, poly-dA and poly-dG showed three each. Each of the deoxyribohomopolymers shared two common peaks, these being peak #2 and #3 of the nucleoside hydrolysate of treated ssDNA on the Sephadex DEAE A-25 column. In both the ssDNA and deoxyribohomopolymers nucleoside hydrolysate they were the largest peaks.

From a consideration of the various secondary radical sites in DNA by ionizing radiation some tentative predictions concerning the sites of NF adducts can be attempted. The rates for the reactions of solvated

electrons, H·, HO· with nucleic acid constituents have been extensively measured and all are close to diffusion controlled. For solvated electrons the rate constants are in the range of 0.5 to 2 X 10^{10} (46, 122, 124), for H· 1 to 5 X 10^{8} (95, 122) and for HO· 2 to 8 X 10^{9} M^{-1} sec^{-1} (46, 122). These reaction rates represent the mean rates for formation of all the various secondary radicals produced in DNA. Secondary radicals produced by solvated electrons are not believed to give rise to NF adducts (27). In this respect the number of secondary radicals produced by HO· are, in general, an order of magnitude higher than the number produced by H·. Thus, we would expect a predominence of secondary radicals produced by HO· and therefore a predominence of NF adducts to secondary radicals produced by HO·. This assumes that NF reacts at similar rates with all secondary radicals.

The sites of attachment of NF adducts in pyrimidines can be readily predicted. Secondary radicals are most common on the C_5-C_6 bond (69, 72, 98, 100, 113, 114, 126). Those produced by HO· appear to be in predominance. The HO· were found to add mainly to the C_5 position and to a lesser extent at the C_6 position of pyrimidines (114). Radical addition to the C_5 position would introduce the radical site at the C_6 position. Thus, in the case of thymidine and cytosine, a "5-hydroxy, 6-pyrimidine adduct" could be expected in major amounts.

Since the examination of purine secondary radicals is not as advanced as pyrimidines, no easy predictions as to the major NF adducts can be made. But NF adducts can be made. These adducts could occur on all secondary radical sites on purines elaborated in the Introduction.

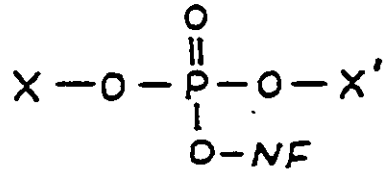
The nature of the adduct formed with NF is another matter. Presumably the adduct linkage is some type of reduced nitro moiety. Alternatively, the DNA secondary radicals could add to the carbon bearing the nitro moiety in NF resulting in loss of the nitro moiety as nitrous acid (HNO_2). This type of reaction does occur with HO· addition to nitrofurans (52, 53).

Analysis of the nucleoside hydrolysates of the deoxyribohomopolymers showed the two largest peaks, peaks #2 and #3 of the nucleoside hydrolysate of ssDNA on the Sephadex DEAE A-25 column, to be common to all four homopolymers. In light of the existence of secondary radicals on phosphate and deoxyribose moieties it is possible these peaks represent phosphate or deoxyribose adducts with NF.

The report that bases are released from DNA after exposure to ionizing radiation (57, 136, 143) suggests that NF might attack the deoxyribose moiety vacated by the base. Additional evidence shows that thymine and uracil bearing radical sites on the N_1 position are

released from their corresponding nucleotides (72, 113) indicates radical cleavage of the N-glycosidic bond. Such a cleavage could leave a deoxyribose radical at the C_1, position which might in turn react with NF. H abstraction from the C_1, positions has also been suggested, and would produce a radical in proximity to the N-glycosidic bond (59). If this is a case of cleavage of the N-glycosidic bond followed by NF adduct formation, it would produce a common derivative regardless of the original base. Hence, a 1'-deoxyribose adduct is a possibility. Alternatively, there could be a number of other deoxyribose-NF adducts with or without an attached base. Also NF adducts attached to fragments of the deoxyribose moiety could exist. In short, as with NF base adducts, a great variety of compounds are theoretically possible.

Recent evidence for phosphate centered radicals support the idea of NF-phosphate adducts (17, 20, 74, 79, 108, 128, 130). However, the common derivatives seen on the DEAE A-25 column do not appear to be appreciably acidic as would be expected of a phosphate derivative. However, a NF-phosphate adduct might have a sufficiently different pKa so as to behave quite differently from ordinary phosphate esters. One structure of this nature could be:



where X and X' are nucleosides. In this case, the acidic nature of the phosphate is masked and the NF adduct might prevent enzymatic hydrolysis of the phosphate-sugar bond. However, there are sixteen possible triester derivatives and due to the charges on the bases of the nucleoside moieties they should be easily separable on the ion exchange columns. NF-phosphate-deoxyribose adducts having an intact or fragmented deoxyribose moiety are also possible.

CONCLUSIONS AND PROSPECTS

It is apparent that a great number of NF adducts are formed when NF is irradiated with DNA. However, from the data in this thesis it is clear that a few of these appear in substantial amounts. The "AG 50WX4 pH 3.20 to 9.20 column" provides a good clean preliminary separation of the NF derivatives from normal nucleosides and allows an easy separation of the two major NF derivatives, peaks B and C. Also, it provides a preliminary separation of the rest of the NF derivatives into two groups, peaks A and D. Re-chromatography of these multicomponent peaks on the Sephadex DEAE A-25 column allows a more complete separation of the remaining NF derivatives. These two columns together provide a convenient means of separating of the NF derivatives (Fig. 34).

The two major derivatives, which are common to all the deoxyribohomopolymers, appear to hold the best promise for future identification. Their common nature suggests that they may be phosphate or sugar derivatives. Experiments could be scaled up to provide the greater amounts of these derivatives which would be necessary for their characterization. Using phosphate radiolabelled DNA the possibility that some of the derivatives contain phosphorous as well as ^{14}C from NF could be examined.

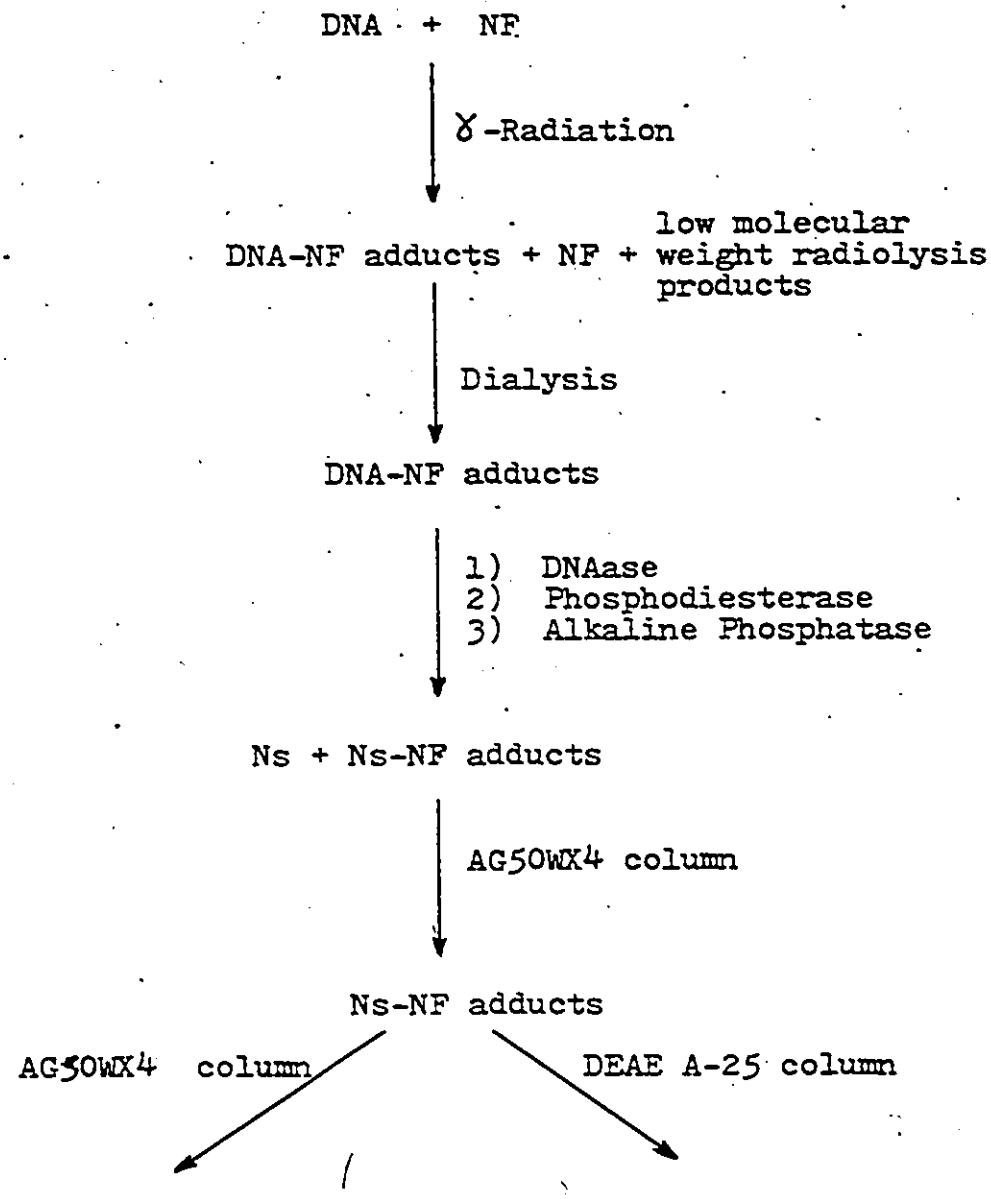


Figure 34. Procedure for the binding of NF to DNA, purification and hydrolysis of labelled DNA, and isolation of the Ns-NF adducts.

Further characterization of the peak I components from the "AG 50WX4 pH 9.20 column" is possible.. These NF derivatives demonstrated UV absorption. Better separation of these on the Sephadex DEAE A-25 column, used in conjunction with the "AG 50WX4 pH 3.20 to 9.20 column", could be accomplished using less steep gradients, longer columns or alterations in the pH of the eluting buffer.

APPENDIX

Correlation of Sephadex G-25 and "AG 50WX4 pH 3.20 to 9.20 Columns"

Comparison of Sephadex G-25 column profiles of labelled nucleotide and nucleoside hydrolysates with "AG 50WX4 pH 3.20 to 9.20 column" profiles for the same nucleoside hydrolysate allow some correlations to be made between the amount of radioactivity in peaks of the different columns' peaks. The difficulties with this approach are that the peaks A and D of the "AG 50WX4 pH 3.20 to 9.20 column" are multicomponent and resolution on the Sephadex G-25 columns was rather poor. Nevertheless, some generalizations are possible based on peak heights in the various profiles (Figs. 16, 22-26). For comparisons, the Sephadex G-25 column nucleotide hydrolysate profile was divided into two parts: 1) Void peak, 19-36mls eluted; and 2) Latter peak, 37-56ml eluted. The Sephadex G-25 column nucleoside hydrolysate profile was divided into three parts: 1) First peaks, 19-43ml eluted; 2) Middle peak, 44-48ml eluted; 3) Last peaks, 49-60ml eluted. The "AG 50WX4 pH 3.20 to 9.20 column" nucleoside hydrolysate profile was divided into peaks A, B, C, and D. Table 3 summarizes the correlations. Consistant agreement was

seen between the various sets of column profiles (Figs. 16, 22-26).

Table 3: Peak Correlations Between Sephadex G-25 and "AG 50WX4 pH 3.20 to 9.20 Columns"

"AG 50WX4 pH 3.20 to pH 3.20 Column" Nucleoside Hydrolysate	Sephadex G-25 Nucleoside Hydrolysate	Sephadex G-25 Nucleotide Hydrolysate
A	First peaks	Void peak
B	Middle peak	Latter peak
C	Middle peak	Both
D	Last peaks	Both

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