STUDIES ON THE CHEMICAL INTERACTIONS OF NITROFURAZONE WITH NUCLEIC ACIDS DURING γ-IRRADIATION

By

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TITLE: Studies on the Chemical Interactions of Nitrofurazone with Nucleic Acids during γ-Irradiation

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ABSTRACT

Nitrofurazone (NF) alters the radiation chemistry of nucleic acids in hypoxic aqueous solution. (^{14}C-NF), labelled in the semicarbazone moiety, was found to bind in a stable, covalent manner to DNA, maximum binding occurring under hypoxic conditions. The nuclease plus alkaline phosphatase hydrolysate of the products formed with ^{14}C-NF and DNA, t-RNA, poly C, and poly U were analysed on a cation exchange column (AG 50WX4) and on an anion exchange column (Sephadex DEAE A25). The mobilities of the adducts on these columns suggested that they are neutral and acidic compounds. Analysis of the nucleoside digest of (^{14}C-NF)-poly U adducts on DEAE anion exchange paper (with and without borate) indicated that these products lack an intact ribose moiety. Analysis of the nucleoside and nucleotide digests of (^{14}C-NF)-poly U adducts by paper chromatography indicated nucleotide-like products(according to its chromatographic behaviour), and acidic products which appeared to be different from bases, nucleosides or nucleotides. Similar analysis of uracil, uridine, and 5'UMP after exposure to γ-rays in the presence of ^{14}C-NF,suggested nucleotide-like products with the NF molecule bound to the uracil base.

Experiments with (^{3}H-Me)thymidine-DNA and (^{3}H-Me) thymidine showed the existance of thymine derivatives or break-down products which did not contain radioactivity

from the ^{14}C-NF. Paper chromatograpy of (2-^{14}C)uracil radiolysis products formed with and without NF showed NF to enhance the yield of a compound tentatively identified as uracil glycol. It is concluded that hydrolysates of the products formed when ^{14}C-NF reacts with irradiated polynucleotides consist of a large number of compounds which contain ^{14}C and are therefore likely to be adducts. These can readily be separated from the normal nucleosides which are also present but not from radiolysis products. Thus the analytical problem of isolating pure adduct remains a formidable one.

The ability of NF to increase single-strand break (SSB) production in γ-irradiated λDNA was demonstrated and compared to NF binding to λDNA. Values of 0.64-1.08 were found for the ratio of SSB / NF molecule bound.

ACKNOWLEDGEMENT

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ABBREVIATIONS

(^{14}C-NF)-DNA	^{14}C-NF bound to DNA
(^{14}C-NF)-poly C	^{14}C-NF bound to poly C
(^{14}C-NF)-poly U	^{14}C-NF bound to poly U
(^{14}C-NF)-RNA	^{14}C-NF bound to RNA
CPM	counts per minute
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dT	thymidine
$\bar{e}$ aq	aqueous electrons
ESR	electron spin resonance
GMP	guanosine monophosphate
H$\cdot$	hydrogen radical
HO$\cdot$	hydroxyl radical
NF	nitrofurazone
P_i	inorganic phosphate
poly C	polycytidylic acid
poly U	polyuridylic acid
PNAP	p-nitroacetophenone
POPOP	1,4-bis(2-(4-methyl-5phenyloxazolyl))benzene
PPO	2,5-diphenyloxazole
SSB	single-strand breaks
TAN	triacetoneamine-N-oxyl
t-RNA	transfer ribonucleic acid
UMP	uridine monophosphate
U	uridine

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INTRODUCTION

Hypoxic Cell Radiosensitizers in Radiotherapy

Cancer therapy is a multidisciplinary problem which includes radiotherapy as one of the major modes of treatment. Although radiotherapy is of little help in controlling widely disseminated disease, it can be used for curing localized neoplasms and eradicating microscopic metastases. However, the success of radiotherapy may be limited by extremely radioresistant neoplasms or by a tumour invading a very radiosensitive normal tissue. In such cases it is impossible to deliver a curative dose of radiation to the tumour without causing unacceptable damage to normal tissue. Therefore, many radiotherapy techniques are aimed at increasing the vulnerability difference between tumour and normal tissue.

The advent of dose fractionation and the great advances made in the potentiation of radiation beams have greatly improved radiotherapy. However, the radioresistance of a tumour may still prove to be a problem. Though the different radiosensitivities of tumours mainly depend on the radiosensitivities of their cells of origin, many other factors come into play. Finding ways of sensitizing irradiated tissues has therefore become an important part of

radiotherapy research (1,2).

The most potent modifier of radiation response known is the degree of oxygenation of the tissue at the time of irradiation. In fact, cells in a solid tumour are inactivated by ionizing radiation according to a multi-component survival curve which is related to the variation in oxygen concentration within the tumour (3). Cells irradiated under well-oxygenated conditions are about three times more sensitive than hypoxic cells (see Fig. 1). Such hypoxic cells rarely exist in well-organized normal tissues because the vascular system is well enough developed to supply all cells with ample amounts of oxygen (in fact, more than is needed for sensitization). However, in tumours the rate of production of tumour cells may exceed the maximum rate of production of new blood vessels. The tumour cells push the capillaries apart, and intercapillary distances become too great for maintenance of an adequate supply of nutrients. Necrosis begins to develop about 150 microns from the capillaries, and as the tumour grows so does the anoxic center. Even very small tumours may contain hypoxic cells and the presence of even a small proportion of hypoxic cells dramatically increases the radiation dose needed to cure tumours (2,4). Therefore, the presence of hypoxic cells in tumours greatly limits the success of radiotherapy treatments, and the search has been on for years on how to overcome this problem.

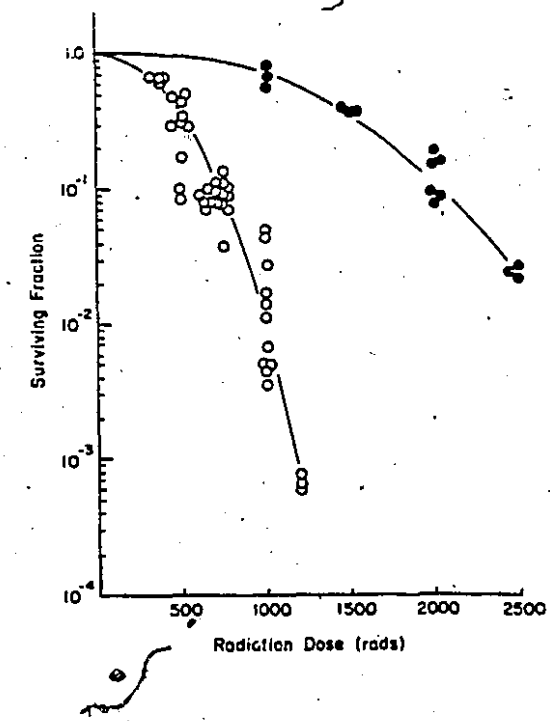


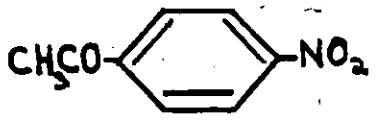
Fig. 1. Survival of EMT6 tumor cells irradiated in vitro under normal aeration (o), and severe hypoxia (•).

(Taken from K.A.Kennedy, B.A.Teicher, S. Rockwell, A.C.Sartorelli: Biochem. Pharmacol. 29, 1 (1980).)

The use of dose fractionation has itself reduced part of the hypoxia problem because re-oxygenation may occur between treatments since there remains less oxygen-consuming cells. However, patterns of re-oxygenation in experimental animals vary within the tumour line, and with the dose and conditions of irradiation so that optimum timing of subsequent doses of radiation is complex and critical. More importantly, in some cases (i.e. for rapidly growing sarcomas and other slowly shrinking tumours) re-oxygenation is very slow and hypoxic cells continue to be a problem even during fractional therapy. In overcoming this hypoxia problem several different approaches are currently in study: 1) Hyperbaric oxygen is used in the hope of increasing oxygen concentration at the tumour site (an unsuccessful approach to date); 2) With densely ionizing radiation (fast neutrons and pi mesons) it is found that the presence of anoxic cells is not so critical since they provide lower oxygen enhancement ratios (an expensive and impractical aproach); 3) Chemical radiosensitizers are used to sensitize hypoxic cells (1,2). The third approach seems the most promising at present and includes many different classes of chemicals. By far the largest and most important group of chemical radiosensitizers is that of the "electron-affinic radiosensitizers" which are part of a group of oxygen mimics. They are so named because their effectiveness as radiosensitizers appears to be related directly to their electron

affinities (5,6). Radiosensitizers function by diffusing out of the tumour blood supply to be absorbed by and sensitize distant hypoxic cells. They mimic the sensitizing effect of oxygen only in hypoxia and so do not increase the radiation response of well-oxygenated cells in the normal tissue irradiated during therapy. None are more efficient than oxygen, but unlike oxygen some of them have long metabolic lifetimes *in vivo* thus providing enough time for their diffusion from the tumour blood supply to the distant hypoxic cells. Therefore, although they are less efficient than oxygen, they are more effective, making them promising as potential oxygen substitutes (1).

The nitrobenzene derivative, paranitroacetophenone (PNAP) (I) was the first real electron-affinic radiosensitizer to show appreciable radiosensitization of hypoxic mammalian cells *in vitro* (7).

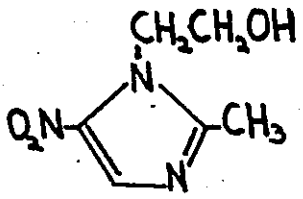
I 

Other more water soluble nitrobenzenes showed sensitization both *in vitro* and *in vivo*.

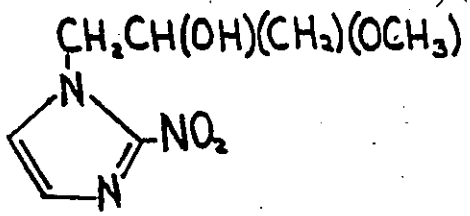
After the sensitizing ability of nitrobenzenes became evident a search began for other nitro compounds which might have similar sensitizing abilities. The nitrofurans were found to be potent hypoxic cell sensitizers *in vitro* and were generally more active than the nitrobenzenes

because of their greater electron affinities (8,9). This was very much of practical interest because several nitrofurans were already in clinical use as anti-bacterials with considerable toxicological and pharmacological information available concerning them. However, *in vivo* studies with nitrofurans have since proved disappointing, mostly because of their rapid metabolism and the toxicity of the high doses necessary for sensitization (1).

While studies with nitrofurans were under way, another group of nitro compounds began to show promise as hypoxic cell radiosensitizers (10). These compounds, the nitroimidazoles, have been shown to be relatively less toxic than other nitro sensitizers, and have a high degree of metabolic stability. The nitroimidazoles being most studied at this time are metronidazole(II) and misonidazole(III) both of which are effective radiosensitizers *in vitro* and *in vivo*.



II



III

These are presently undergoing clinical evaluations and preliminary data indicates their effectiveness in radiotherapy, though neurotoxicity can be a problem at high doses (11).

Effects of Ionizing Radiation on DNA and its Constituents

As sensitizers are believed to interact with DNA radicals during irradiation as part of their mechanisms of action, knowledge of the radiochemistry of DNA and its components is essential for the study of the actions of radiosensitizers. Discussions of the effects of ionizing radiation on DNA therefore becomes relevant at this point in view of later discussion of sensitizer mechanisms of action.

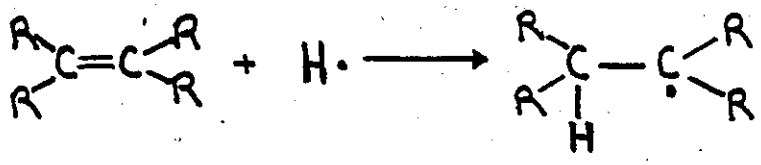
The main cellular lethal and mutagenic damage caused by ionizing radiation has been shown to be on DNA or DNA-membrane complexes.This damage manifests itself as unpaired electrons (radical centers) at various sites in the macromolecule as a result of either direct or indirect action of irradiation. Most lesions are caused indirectly (as much as 70 percent (6)), being brought about by the reactions of radiation-induced primary radicals with DNA. These form secondary radicals on the macromolecule. In aqueous systems (as found at the cellular level) these primary radicals mainly originate from water molecules which yield ·H, ·OH (hydroxyl radicals), $\bar{e}$ aq (hydrated electrons), H_2, H_2O_2, and H_3O^+ (1). Hydroxyl radicals, hydrogen atoms, and hydrated electrons can all react with DNA, though in oxygen ·H and $\bar{e}$ aq react with oxygen to form unreactive species (12). Measurable DNA lesions which have been observed include double-strand breaks, single-strand breaks, base damage, and cross linking of

DNA to DNA or other molecules (1).

ESR (electron spin resonance) spectroscopy has been found useful in determining the structure of radicals resulting from the direct and indirect effects of radicals, and in determining their relative concentrations. Pulse radiolysis experiments have been used to monitor radical changes (U.V. or visible) during radiolysis reactions. Since it is difficult to identify changes in nucleic acids upon irradiation with ionizing radiation, most experimental work has been done on the radiation chemistry of nucleic acid constituents in the hope of being able to extrapolate results to larger molecules. However, the sum of the ESR spectra of irradiated DNA nucleotides is not the same as the spectrum of irradiated DNA. This suggests that the radical localization in nucleic acids involves interactions that are not present in the constituents (13).

Hydrogen radicals react with organic molecules to give organic free radicals by abstraction of a hydrogen atom from saturated carbon atoms, and by addition to unsaturated bonds:

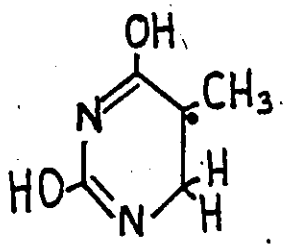
$$R_3CH + H\cdot \longrightarrow R_3C\cdot + H_2$$



In DNA, this results in altered bases due to the addition of H to bases, and chain breaks or loss of bases due to

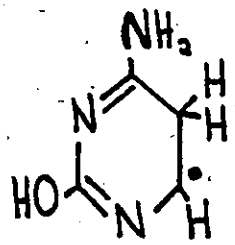
hydrogen-abstraction from the deoxyribose moiety(13).

In pyrimidines the 5,6 double bond is the most reactive site for radical formation. With thymine H adds almost exclusively to C_6, resulting in the 5-yl radical (14,15).



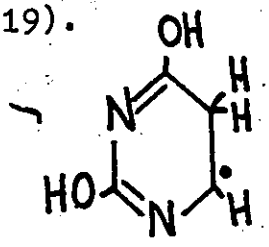
5,6-dihydrothymin-5-yl

For cytosine, 5,6-dihydrocytosin-6-yl appears to be the main product of H-addition, though the 5-yl radical has not yet been excluded (13,16).



5,6-dihydrocytosin-6-yl

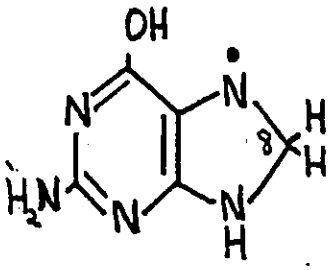
The hydrogen atom also adds to C_5 or C_6 in uracil, though from experiments with poly U the 6-yl radical seems most likely (13, 17, 18, 19).



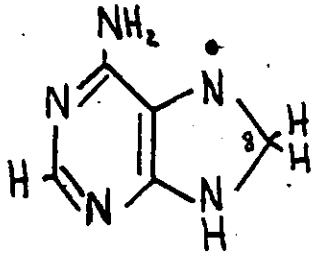
5,6-dihydrouracil-6-yl

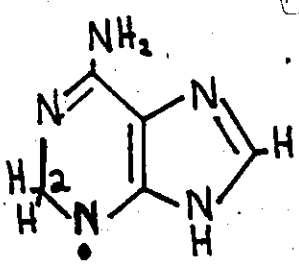
As for purines, guanine H-adducts have been identified

at C_8:

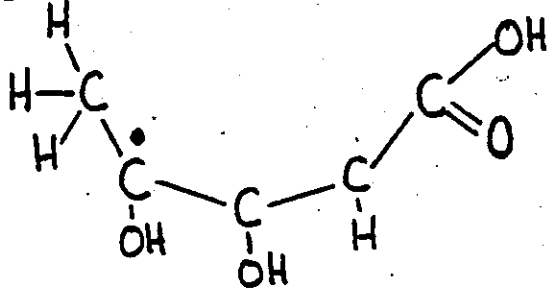


Adenine H-adducts have also been found at C_8, as well as at C_2 (20):





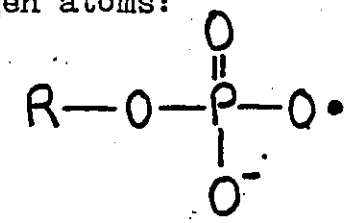
Many radicals have been observed for deoxyribose exposed to H, but few have been identified. The predominant radical, resulting from H-abstraction at C_4, has the following proposed structure (21):



Work on the radiation chemistry of nucleosides and nucleotides has shown the production of altered bases due to hydrogen atoms which are the same as for those produced when bases alone are irradiated. Other radicals were also observed and thought to be located on the sugar moiety (13). The contribution of dihydrothymin-5-yl to the ESR spectrum

of irradiated thymidylic acid has been found to be 30% (22).

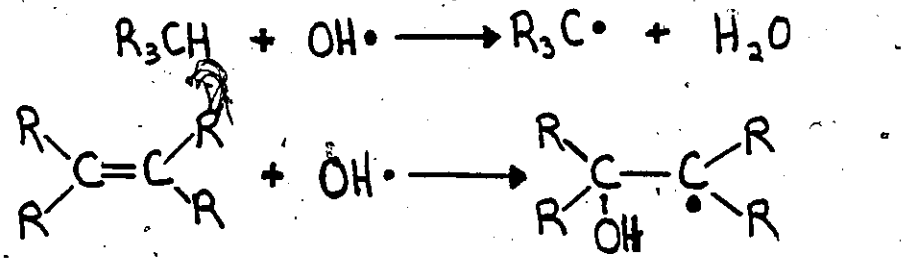
Cytidylic, deoxycytidylic, and uridylic acid exposed to hydrogen atoms have shown ESR lines attributable to an unpaired spin on the phosphate group (23). Therefore, the phosphate moiety of nucleic acids may also be prone to damage by hydrogen atoms:



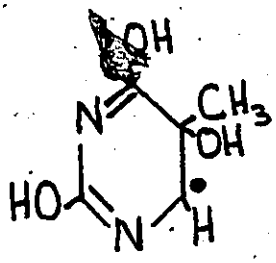
The study of the radiation chemistry of DNA and RNA has been difficult due to the fact that different nucleic acids from different sources, and nucleic acids treated in different ways have different ESR spectra after reacting with hydrogen atoms. Computer simulation analysis of salmon sperm DNA has assigned the following proportions to H-induced radicals: 9% deoxyribose radicals giving a triplet; 9% deoxyribose radicals giving a doublet; 23% 5,6-dihydrothymin-5-yl radical; 49% radicals from addition of H to guanine and adenine; and 10% dihydrocytosin-yl radicals. That is, 18% of the radicals were attributed to the deoxyribose moiety, and 82% to the base moiety with the radicals being distributed approximately in proportion to base abundance (24).

All model studies indicate that ·OH is the principal reactive species responsible for target radical formation. Hydroxyl radicals react with organic compounds

by abstraction of a hydrogen atom from saturated carbon atoms, and by addition to unsaturated bonds (13):

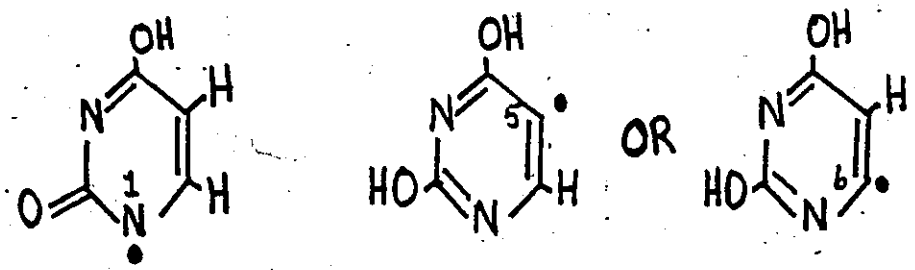


Hydroxyl radicals react mainly with pyrimidines by addition across the C_5-C_6 double bond with a predominating tendency to form the C_5 adduct, though the C_6 adduct as well as other radicals are also present (17,25, 26,27,28).

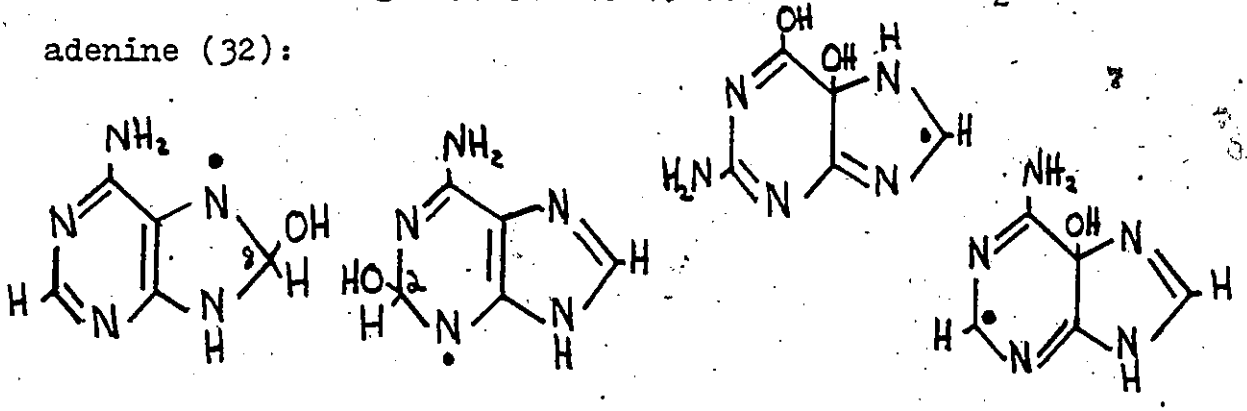


5,6-dihydro-5-hydroxythymin-6-yl

It has also been observed that some hydroxyl radicals abstract hydrogen atoms from the methyl group of thymine (27). Similarly hyrogen abstraction has been observed for uracil at N_1 and at C_5 or C_6 (18):



Sites of attack of ·OH on adenine and guanine are not known exactly, but most evidence indicates that much of the reaction occurs at the central C_4-C_5 double bond. There is also evidence for OH-addition at the C_8 of the imidazole ring (13,25,29,30,31), and to the C_2 in adenine (32):



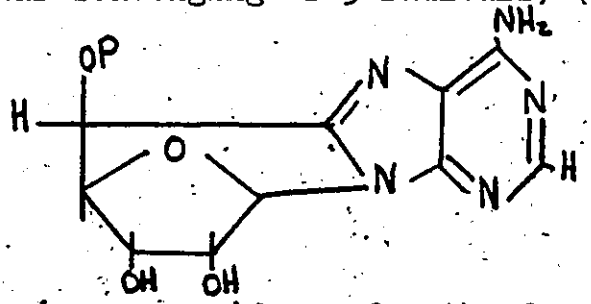
Thymine, uracil, cytosine, and adenine, as well as nucleosides and nucleotides have approximately the same reactivities towards ·OH. Attack on each base in nucleic acids is about proportional to the relative amount of each (13).

Hydroxyl radicals also cause the abstraction of hydrogen atoms from the sugar moiety. One ESR study with γ-irradiated wet DNA indicated a correlation during thermal annealing between the disappearance of ·OH radicals and the appearance of a signal which could be assigned to either a phosphate-associated radical or a formyl radical (sugar origin). It was thought probable that the signal was associated with damage to the sugar moiety (breakage of C_3·-C_4· bond) (32,33).

A proposed reaction scheme of a γ-irradiated

de-oxygenated aqueous solution of 2-deoxy-D-ribose indicated that approximately 50% of attacking ·OH and ·H abstract hydrogen atoms from C_1· and C_2·, 20% from C_3·, 20% from C_4·, and 10% from C_5· (34).

Through identification of stable products of irradiated DNA and its constituents there is evidence to support the production of ·OH-induced radicals at C_1· (35), C_3· (36,37), C_4· (38,39), and C_5· (26,36,40,41). A cyclic nucleotide has been isolated from 5'AMP irradiated in an N_2-saturated solution, which not only supports the existance of the 5'radical but also shows the type of interaction possible between the 5'radical and the nucleotide base (intramoleoular scavenging of 5'radicals) (36).

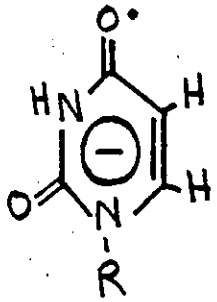


There is some evidence for the formation of phosphate radicals upon exposure to ·OH radicals (32,42, 43). Pulse radiolysis of phosphate anions gave optical absorptions which suggested the possibility of electron transfer processes or hydrogen abstraction (42):

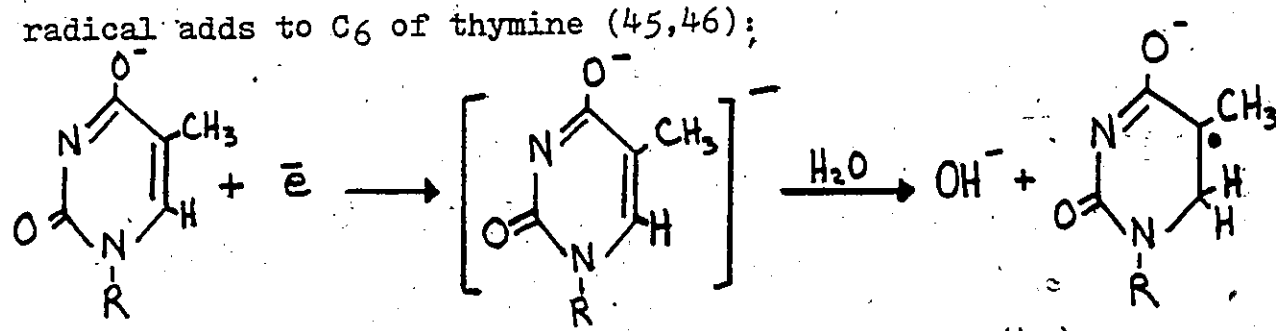
$$\cdot OH + H_2PO_4^- \longrightarrow H_2\dot{P}O_4 + OH^-$$

Pulse radiolysis of dinucleotides gives an ESR signal attributable to the corresponding radical in nucleotides, $(RO)_2PO_2$ (43).

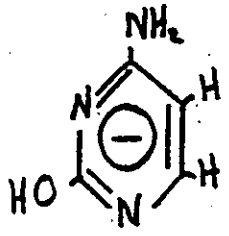
For the reactions of hydrated electrons one finds that the main reduction product of uridine 5'-phosphate is the electron adduct (anion) radical of the base (17,19, 24,44):



Similar anion radicals are found with other pyrimidines. Proton transfer to this anion radical occurs for thymidine giving a radical which is the same as that when a hydrogen radical adds to C_6 of thymine (45,46):

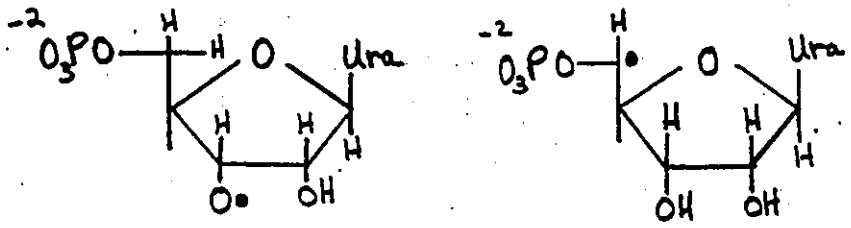


Cytosine and deoxycytidine react in a similar way (47).



The C_6 H-adduct radical has also been seen after 5'UMP reduction with the anion radical believed to be the

precursor. Though the C_5 hydrogen adduct has also been observed, its precursor has not been identified. Two other radicals seen for 5'UMP result from deprotonation of O_3^- with a primary cation radical as intermediate, and from H-abstraction from C_5· of the ribose (19).



Hydrated electrons may also cause the formation of radicals on the phosphate moiety since they can react with phosphate anions (48):

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

In nucleosides and nucleotides, the bases are the main site of attack, reacting with 70-85% of the available radicals. Most of the remaining radicals attack the sugar moiety. Therefore, very little attack on the phosphate moiety can actually occur (49).

Radicals formed from the direct absorption of ionizing radiation are unlike radicals formed by ·OH and ·H radicals, though they may decay to similar structures. Radicals formed in nucleic acids appear different in ESR spectra from those formed by irradiation of constituents (13).

From EPR studies of γ-irradiated solid DNA it was found that the radicals produced are composed of a mixture of ionic free radicals. Direct ionization in target molecules produces positive ions and electrons,

leaving the molecules with charges and holes. Secondary reactions which may occur include charge recombination amongst radicals, and the formation of secondary radicals (29,50):

nucleotides ⟶ cations + anions

cations + H_2O ⟶ OH-addition radicals + H^+

anions + H_2O ⟶ H-addition radicals + OH^-

No ionic radicals have been found for the sugar moiety.

Pyrimidines form anion radicals (electron adducts) (50,51,52,53). Though cation radicals have been seen at pH > 12 for pyrimidines, this is not biologically significant (54).

Cation radicals are produced on purines by ionizing radiation-induced electron ejection (guanine seems particularly prone). Though generally purines are more resistant toward ionizing radiation, they may play an important role in DNA such as transfering energy to pyrimidines via the triplet state or higher excited states (most efficient within a base pair) (55,56).

Breakage of bonds is also a direct effect of ionizing radiation. Scission of the $C_{5'}$-$O_{5'}$ bond during irradiation forms a PO_4^{-2} radical in deoxythymidine 5'-phosphate (57), while scission of the same bond in deoxycytidine 5'-phosphate results in a radical on the sugar moiety (58). This difference is probably due to different charge distributions in different nucleotides (57).

Other phosphate free radicals may be produced by ionizing radiation. Free radicals have been seen as a result of the loss of one electron by phosphate anions:

$$PO_4^{-3} \longrightarrow PO_4^{-2}$$

$$(OH)_2PO_2^- \longrightarrow (OH)_2PO_2$$

There is also evidence for an addition event (though of low probability) (43):

$$PO_4^{-3} \longrightarrow PO_4^{-4}$$

The chemical modifications of irradiated DNA, which follow radical formation, are not fully understood. Small molecules are known to be released, such as bases, uncharacterized nucleosides, modified sugars, tritiated water from tritiated DNA, and P_i (5,33,35,45,59,60).

The amount of bases released from irradiated DNA has been found to be about one-fourth of the total bases destroyed (59), and is probably caused by H-abstraction from the sugar moiety (37,60,61).

Different types of reactions are known to release P_i. There are immediate breaks due to damage on the base or sugar((a) and (b)); there are breaks caused by the decay of labile sugar-phosphate bonds at pH 7 (due to sugar or base damage) ((a) and (b)); and there are alkali labile sites which are due to base damage ((c) and (d)) (61):

(a) $B\text{-}S^* \begin{matrix} P \\ < \\ P \end{matrix} \longrightarrow BS^* + 2P_i$

(b) $B^*\text{-}S\begin{matrix}P\\P\end{matrix} \longrightarrow B^*\text{-}S + 2P_i$

(c) $B^*\text{-}S\begin{matrix}P\\P\end{matrix} \xrightarrow{\text{alkali}} B^*\text{-}S + 2P_i$

(d) Base damage leading to loss of 2 carbons $\longrightarrow S^*\begin{matrix}P\\P\end{matrix} \xrightarrow{\text{alkali}} 2P_i$

H-abstraction from the sugar moiety is the most likely route to the release of P_i (from both C'_5 and C'_3) (34,36,38,61). Of the aqueous radical species only ·OH has been found to react with deoxynucleotides to release P_i (61):

$$RCH_2OPO_3^{-2} + {}^{\cdot}OH \longrightarrow \dot{R}CHOPO_3^{-2} \xrightarrow{H_2O} P_i$$

The yields of radiation-induced phosphate release have been found to be similar for all deoxynucleotides and independent of the presence of oxygen (62).

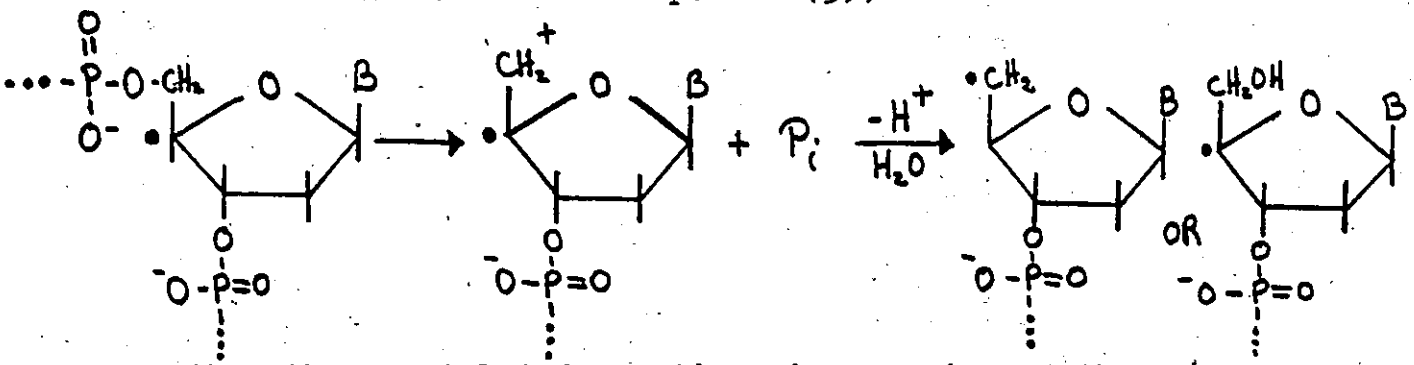
Strand breakage in DNA is a major form of damage produced by ionizing radiation in living cells. The chemical steps which induce this are not well understood. Through the use of radical scavengers, an assessment has been made of the relative contributions of the direct and indirect actions of ionizing radiation in producing single strand breaks in mammalian DNA. It was estimated that about 70% of single strand breaks were caused by the indirect action of radiation (65). Specifically hydroxyl radicals were responsible for the indirect effect (63,64).

In aqueous solutions breakage of the phosphate

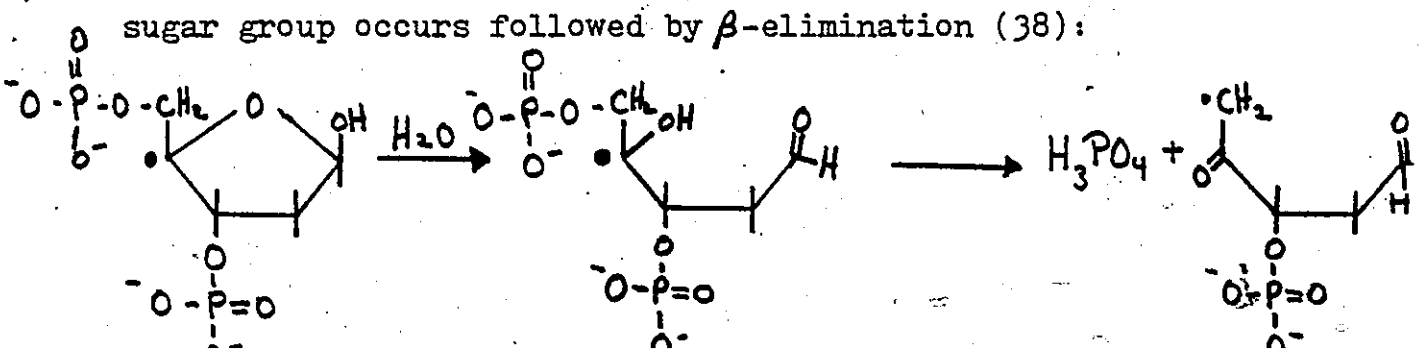
ester linkages is more likely to cause most of the strand breaks, rather than the scission of a C-C bond of the deoxyribose moiety (38).

One study suggests that 12% of radiation-induced chain breakage in DNA is due to the liberation of an intact nucleoside, 18% to the release of an altered nucleoside, 30% to attack on $C_{3'}$ (splitting of phosphate bond), 10% to hydrolysis on $C_{5'}$, and 30% after alkali treatment (18% via liberation of base, and 12% opening of furanose ring or oxidation on $C_{2'}$) (61). Loss of the $C_{3'}$ phosphate group predominates over that of $C_{5'}$ (60,65).

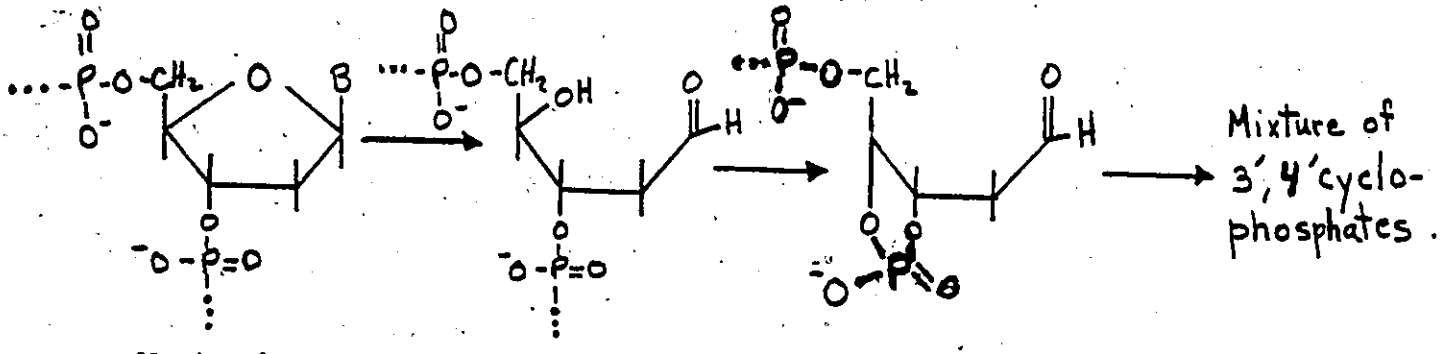
There are two routes postulated for strand breakage from the $C_{4'}$ H-abstraction radical (resulting in $C_{3'}$-phosphate ends). In one reaction the radical eliminates a phosphate anion via a carbocation intermediate which adds to water with the loss of a proton (39):



In the other postulated reaction, ring opening of the sugar group occurs followed by β-elimination (38):



Subsequent breakage in alkali following base release and destruction of the furanose configuration is mostly by β-elimination (cleaving 3'-O linkings). Alkali breakage may also be by formation of 3'-4' cyclophosphates following base release (65,66):



Mechanisms of Action of Electron-Affinic Radiosensitizers

Electron-affinic radiosensitizers are effective only when present during irradiation, and in the absence of oxygen. The molecular mechanisms involved in the action by which they increase the lethal properties of radiation in hypoxic living cells is not yet clearly established, though it is generally agreed that fast free-radical reactions are involved. Like oxygen they interact by oxidation or addition with the free-radical centers on DNA thereby inhibiting radical recombination processes that would otherwise lead to repair (1) :

$$RH \xrightarrow{\gamma} \cdot R \text{ (biological damage by radiation)}$$

$$\cdot R + O_2 \longrightarrow \cdot RO_2 \text{ (lethal, lesion fixation)}$$

$$\cdot R + XSH \longrightarrow RH \text{ (non-lethal, lesion repaired)}$$

$$\cdot R + \cdot R \longrightarrow RH + \cdot R \text{ (partially lethal)}$$

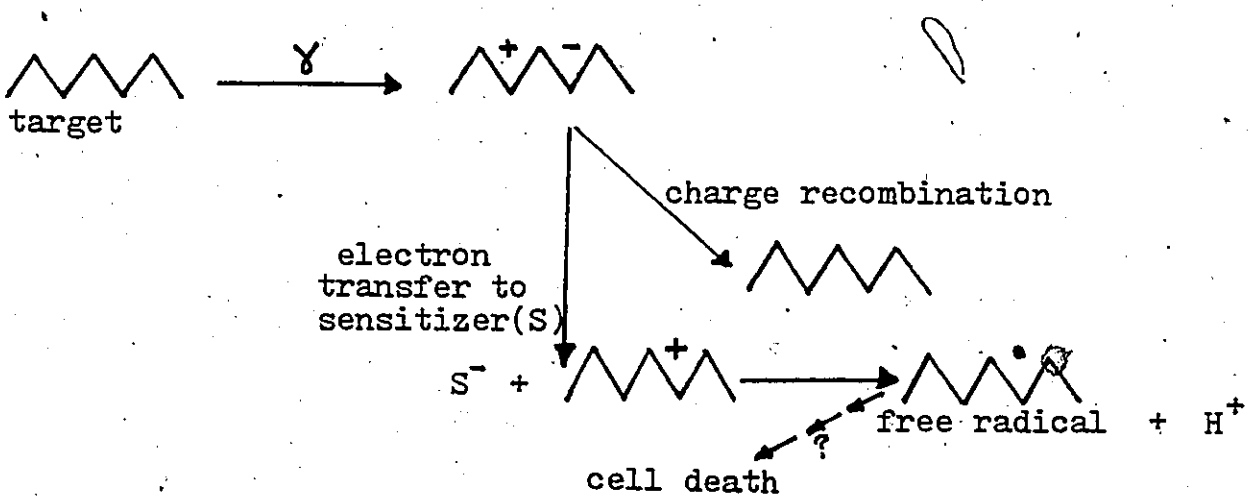
$$\cdot R^+ + \cdot R^- \longrightarrow R + R \text{ (charge recombination)}$$

$$\cdot R + S \text{ (sensitizer)} \longrightarrow \cdot R^+ + \cdot S^- \text{ (oxidation)}$$

$$\cdot R + S \longrightarrow RS \text{ (addition)}$$

Molecular studies have shown that the electron affinity of these radiosensitizers is by far the dominant property affecting sensitization efficiency (6,67,68,69). It is this property which influences the interaction of the sensitizer with radical centers, and they probably act at least in part by the same processes involved in sensitization by oxygen (70).

There are several models which have been proposed to explain the mechanism of action of electron-affinic radiosensitizers. The "direct action model" postulates that following direct ionization in a target molecule, electrons migrate from the ionized molecule to the electron-affinic radiosensitizers. This irreversible electron transfer lowers the probability of self repair by charge recombination , and favours the decay of the cation radicals to neutral free radicals (5):



Electron adducts of DNA bases have been shown by pulse radiolysis to undergo rapid electron transfer to various sensitizers (71,72,73). Other evidence to support the direct action model includes the ESR observation that in the presence of the sensitizer, misonidazole, irradiated solid DNA shows an increase in the number of unrecombined positive charges (i.e. of (+) guanines) (51). Misonidazole has also been shown to prevent the formation of thymine and adenine H-addition radicals whose precursors are anion radicals (51,71,74).

In contrast, the "electron sequestration model" postulates that the role of the sensitizer is to scavenge hydrated electrons and consequently prevent them from reacting with hydroxyl radicals (which can be biologically damaging) (75). Little of sensitizer activity seems attributable to such reactions.

One other model for the mechanism of action of electron-affinic radiosensitizers involves a two-step process where hydroxyl radicals first react with a biological target to leave a radical site. The sensitizer molecule then reacts with this site, either by oxidation or addition (adduct formation) (76):

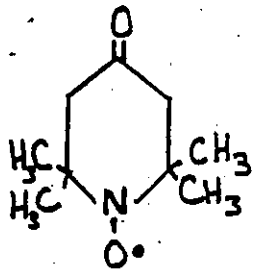
$$B + \cdot OH \longrightarrow BOH\cdot$$

$$BOH\cdot + S \longrightarrow BOH^{+} + S^{-} \quad \text{(oxidation charge transfer)}$$

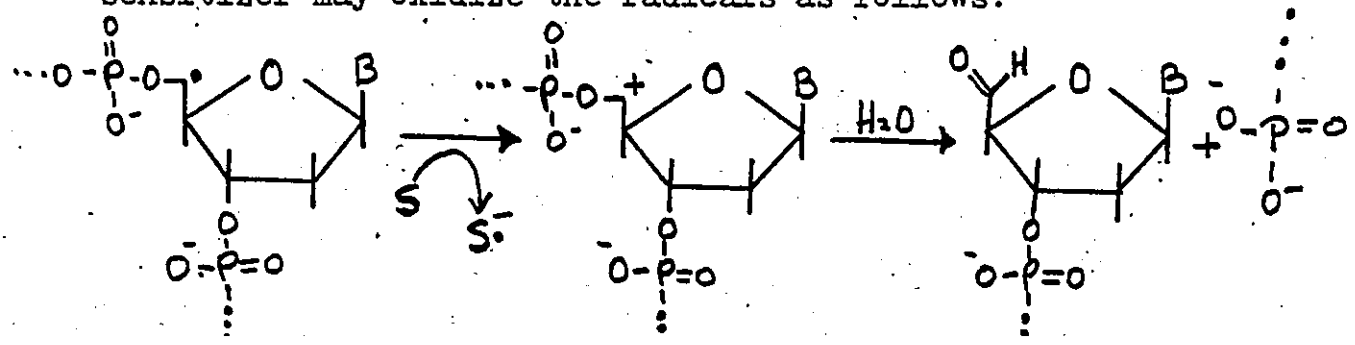
$$BOH\cdot + S \longrightarrow (BOH)S \quad \text{(addition)}$$

Scavenging experiments have shown that ·OH is the active radical species involved in the action of electron-affinic sensitizers. However, it is still not known to what extent OH-induced DNA radicals are involved in the sensitization process (71). For instance, it has been shown that hydroxyl radical scavengers (e.g. t-butanol) reduce but do not totally eliminate the sensitizing effect of PNAP. The "non-OH" component is probably attributable to the direct action model for sensitization (77).

Several sensitizers have been shown to bind to the target molecule, such as N-oxyls (e.g. TAN(IV)) which favour binding (73) and nitrofurans (which are also efficient radical oxidants) (78). The amount bound is usually found to increase with dose.

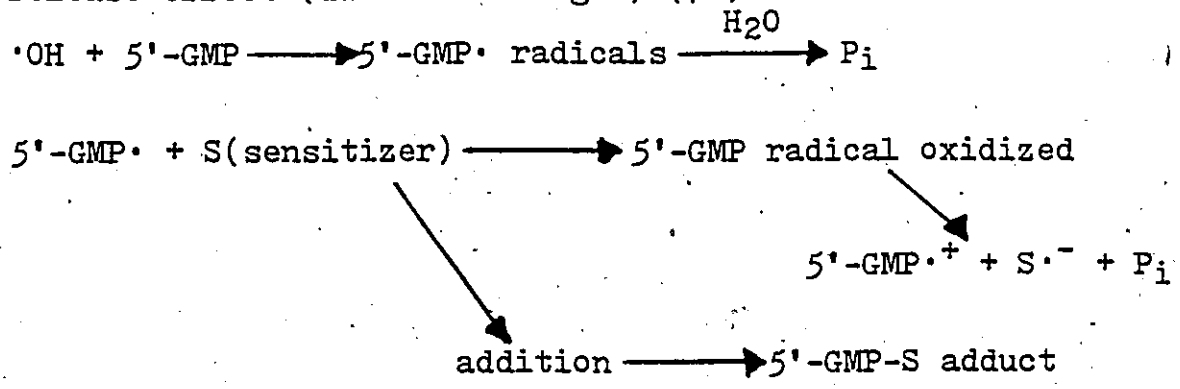
IV 

Electron transfer oxidation has been reported for neutral base radicals but the yields of transfer are low (40,71). A small amount of damage has also been observed on the deoxyribose moiety which is efficiently and rapidly oxidized (71,78). For example, at $C_{5'}$ the sensitizer may oxidize the radicals as follows:



This would cause an increase in strand breakage, as would oxidation of a $C_{3'}$ radical. Oxidation of a $C_{1'}$ radical might cause base loss (40).

The radiosensitization of mammalian cell killing by some sensitizers has been directly correlated with their ability to sensitize radiation-induced phosphate release (79). PNAP and nitrofurans have been shown to increase the release of phosphate from irradiated 5'-GMP, while the N-oxyl TAN protects from this radiation-induced release effect (due to binding?) (76):



Study of the effect of nitrobenzenes on the release of inorganic phosphate in aqueous solutions of 3' and 5' nucleotides under hypoxia has shown that 5' purine nucleotides are sensitized, as are 5' pyrimidine nucleotides though they depend on sensitizer electron affinity and concentration. However, 3' purine and pyrimidine nucleotides were protected indicating that the binding of 3' ribose radicals by nitrobenzenes in a secondary reaction may be the basis for protection. Nitrobenzenes (and nitrofurans) are known

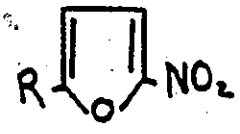
to form adducts with nucleophilic alkyl radicals (36).

Along with the release of inorganic phosphate, most radiosensitizers have been shown to increase the number of single strand breaks produced in DNA during irradiation. In vivo studies have indicated a correlation between the amount of radiosensitized cell killing and the extent of single-strand breaks in cellular DNA. Enhancement ratios for survival plotted against the corresponding enhancement ratio for strand breakage shows two levels of radiosensitization (see Fig. 2). The value for air differs from the expected one by six standard deviations, suggesting that nitro chemical sensitizers have a common mechanism of action which differs at least in part from that of oxygen (80).

Strand breakage may therefore be the route by which chemical sensitizers cause cell killing. Phosphate ester cleavage also occurs on phospholipids and so damage to membranes may also be involved in cell killing (40). Binding of sensitizers to DNA may also be involved since such adducts might lead to interuptions in the synthesis of daughter strands, or cause breaks (81).

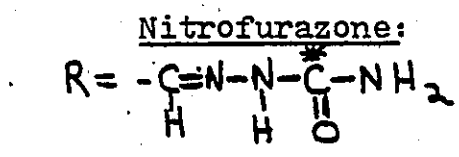
The Nitrofuran Group of Electron-Affinic Radiosensitizers

The 5-nitrofurans of the general formula:



* ^{14}C in ^{14}C-NF

Nitrofurazone:



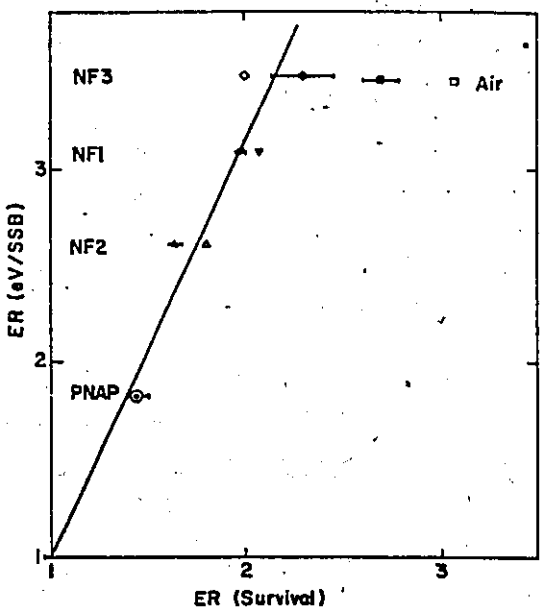


Fig. 2. Correlation between enhancement ratios for survival and DNA strand-breakage for a series of radiosensitizers. (Closed symbols - 330 rads/min; open symbols - 2100 rads/min. ●, ○ - N_2 + 1mM PNAP; ▲, △ - N_2 + 500 μM NF2; ▼, ▽ - N_2 + 500μM NF1; ◆, ◇ - N_2 + 500μM NF3; ■, □ - N_2.

(Taken from D.L.Duglè, J.D.Chapman, C.J. Gillespie, J.Borsa, R.G.Webb, B.E.Meeker, A.P.Reuvers: Int. J. Radiat. Biol. 22, 545 (1972).)

are a synthetic group of compounds which have been widely used as anti-bacterials in human and veterinary medecine, animal food additives, and until recently as food preservatives (82). The nitrofurans have a broad antibacterial spectrum *in vivo* that makes them useful in a variety of clinical situations (83). However, since most nitrofurans (including nitrofurazone) have been found to be oncogenic, and all are mutagenic (caused by their interaction with DNA), their use is now considered unwarranted for many situations (84). It is relevant that the structural properties believed essential for carcinogenicity (such as the presence of a nitro group) are also those needed for anti-microbial efficacy. The two effects have yet to be separated in terms of structure-activity relationships (82).

The biotransformation of nitrofurans includes mainly reduction of the 5-nitro group, hydroxylation of the furan ring, and side chain modifications (i.e. oxidation, deacylation). Quantitatively, the most important metabolic process is the reduction of the 5-nitro group which is greater under anaerobic conditions in mammalian systems (82). It has been shown that this bioreduction is necessary for the anti-bacterial (85), mutagenic and probably also for the carcinogenic (82) effects of nitrofurans, though which intermediate metabolite is the active compound is not known. As well as being toxic to cells, intermediates formed in the reduction of nitrofurans have

been shown to bind irreversibly to macromolecules (85), and to cause single-strand breaks in bacterial DNA (86). They also alter the radiation response of tissue by preferential cytotoxicity in hypoxic tissue (the reducing enzymes function under hypoxic conditions mostly, and produce toxic intermediates); or by altering cellular respiration thus changing the size of the hypoxic population; or by recombining with SH groups (which might otherwise repair DNA lesions). Therefore, the metabolism of nitrofurans may also play an important role in their effectiveness as radiosensitizers in vivo (82,87).

Aerobic toxicity in mammalian systems is lower than anaerobic toxicity and is generally believed not to involve toxic reduction intermediates but rather the electron-affinic compound alters the position of the electron-transport equilibria or acts as an electron "sink". They might also interfere with the function of enzymes which are electron acceptors (1). Some minor reduction of nitrofurans does also occur under aerobic conditions, and as such can subject the cells to further toxic effects, including mutations.

5'-Nitrofurans have been shown to be excellent in vitro radiosensitizers. They are able to achieve almost the full extent of the oxygen effect in radiosensitizing mammalian cells in vitro, as can be seen from the survival curves in Fig. 3. This effect involves a dose-

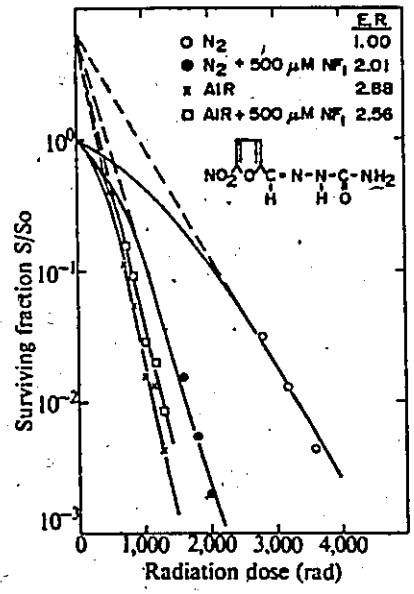


Fig. 3. Survival curves for Chinese hamster cells irradiated with 250 kVp X-rays in air-saturated conditions (x), in air saturated conditions with 500 μM nitrofurazone (□), in hypoxic conditions (○), and in hypoxic conditions with 500 μM nitrofurazone (●).

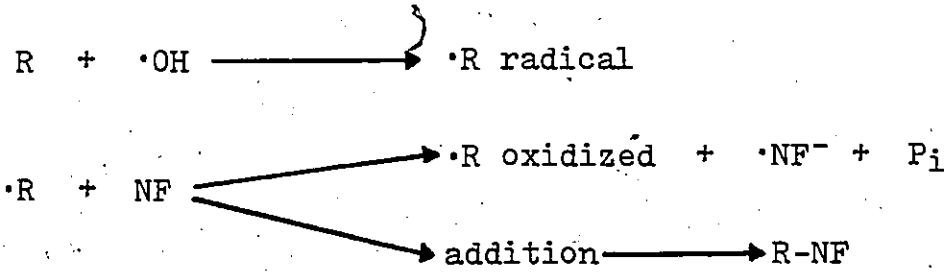
(Taken from A.P.Reuvers, J.D.Chapman, J. Borsa: Nature 237, 402 (1972).)

modifying mechanism which, like oxygen, is effective in every phase of the cell cycle (90). The radiosensitizing abilities of nitrofurans is consistent with findings that they are highly electron affinic and have high rate constants for electron transfer from electron adducts and free radicals (91). Although nitrofurans themselves can form radicals during irradiation (react with electrons and ·OH), it is not nitrofuran radicals which interact with DNA to cause radiosensitization, as seen from rapid mix experiments (8). Unchanged nitrofuran reacts with DNA radicals.

Both types of sensitizer interaction with target molecules (addition and oxidation) have been observed for nitrofurans with DNA. That is, radiation-induced binding of nitrofurazone and other nitrofurans to DNA has been reported (72,90,92), and their ability for electron transfer oxidation has been demonstrated (72,92). Through the use of ·OH scavengers (e.g. t-butanol) it has been estimated that 60% of the reaction of nitrofurans with free radicals in DNA leads to binding , and the remainder may involve electron transfer oxidation (92). The biological significance of this has not yet been determined.

Nitrofurans have also been shown to cause the radiation-induced release of inorganic phosphate from mononucleotides (76), and the production of single-strand breaks (80), which is consistent with their elec-

tron-transfer oxidative properties and the involvement of ·OH:



The biological significance of the reactions of nitrofurans with DNA radicals is not known, and since both addition and oxidation may occur the sensitizing mechanism of action may involve binding, strand breaks, or other damage.

Although nitrofurans show little promise as *in vivo* radiosensitizers, they can be used to elucidate the mechanism of action of electron-affinic radiosensitizers. Nitrofurazone which can easily be labelled in its semicarbazone moiety with ^{14}C is found particularly useful in the study of the reaction it undergoes with DNA during irradiation (i.e. binding). Enzyme-catalysed binding of ^{14}C-NF to DNA (via reduction of NF) also occurs and can be used for comparison with radiation-induced binding. Nitrofurazone can also serve to examine the production of DNA single-strand breaks in its presence during irradiation.

Previous work on the study of nitrofurazone binding to DNA under hypoxia during γ-irradiation has con-

firmed that binding is directly proportional to radiation dose and to DNA concentration (though NF bound / mg DNA remains constant). Binding was however found independent of sensitizer concentration provided it was present in excess (i.e. at least 0.84 μg NF per mg DNA per ml). This study also provided the means of separation by column chromatography (cation exchange Bio-Rad AG 50WX4, and anion exchange Sephadex DEAE A25) of nucleoside hydrolysates of NF-labelled DNA where the normal nucleosides were separated from each other and from the NF-labelled derivatives (93). These principles and techniques are described in the methods section and applied in this present study.

In this study the radiation-induced binding of ^{14}C-NF to several nucleic acids was examined using various chromatographic techniques. This was done in the hope of achieving some degree of understanding as to the chemical interactions of DNA with NF during γ-irradiation which leads these two molecules to form an adduct. At the very least, one hoped to determine the nature of the NF derivatives obtained with simple molecules (e.g. nucleotides) or with nucleic acids (hydrolysed before chromatography). By discovering where exactly NF binds on the DNA molecule, we would advance one step further in understanding the radical process which initiates the final adduct formation. Furthermore, such information

would be indispensable in the study of radiosensitizer mechanism of action, should adduct formation prove to be a major route of sensitization for nitrofurans.

In looking at the oxidative abilities of NF, the radiolysis products of uracil were examined in the absence and presence of NF, to determine whether NF would increase the production of any product. Such a finding would suggest oxidation of DNA radicals as a potentially important route of sensitization for nitrofurans. In the same vein, it was also thought pertinent to look at the production of single-strand breaks in DNA caused by NF over and above those formed by radiation itself. Binding, relative to such breaks, was also determined so as to estimate the relative importance of binding as opposed to breaks. Should there be a great deal more breaks than bound molecules, one would suspect that the production of single-strand breaks via oxidation might be of relevant importance in the mechanism of action of NF as a radiosensitizer.

MATERIALS AND METHODS

^{14}C-nitrofurazone (5-nitro-2-furaldehyde semicarbazone; NF) labelled in the semicarbazone moiety was synthetized by Dr. D.R.McCalla (7 C_i/mole) and by B.Wentzell (13.8 C_i/mole). (^{3}H-Me)thymidine (48 C_i/mmole) was from Amersham/Searle (Arlington Heights, Ill.). (U-^{14}C)5'-uridine monophosphate (280 mC_i/mM) was obtained from ICN (Montreal, P.Q.). (2-^{14}C)uracil (46.1 mC_i/mmole) as well as the Omnifluor came from New England Nuclear (Boston, Mass.). The following chemicals were obtained from the Sigma Chemical Co. (St. Louis, Mo.): highly polymerized calf thymus DNA, polyuridylic acid, uracil, uridine, thymine, thymidine, calf intestine alkaline phosphatase (type VII), micrococcal nuclease (Staph. aureus), snake venom phosphodiesterase I (Crotalus adamanteus), bovine pancreatic deoxyribonuclease I (type II), bovine pancreactic ribonuclease A (type IIA). Uridine 5' monophosphate, t-RNA (E.coli), and ultra pure sucrose were from Schwarz Bioresearch,Inc. (Orangeburg, N.Y.). Pronase (Streptomyces griseus) came from Boehringer Mannheim (W.Germany). PPO (2,5-diphenyloxazole) and POPOP (1,4-bis (2-(4-methyl-5-phenyloxazolyl)) benzene) were obtained from the Fisher Scientific Co. (Toronto, Ont.). λ DNA and (^{3}H) λ viral DNA (3 $\mu C_i/\mu$mole) were from Miles Biochemicals (Elkhart, Ind.).

The pure argon gas came from Union Carbide of Canada (Oakville, Ont.).

I - Irradiation of Samples

The samples to be irradiated (1-5 mg/ml) were dissolved in 0.01 M Tris HCl, 0.01 M $MgCl_2$ (pH 7.0). Hypoxia was achieved by flushing the solutions in sealed glass tubes with argon. The samples were irradiated with a ^{137}Cs source at a dose rate of 558 rad/min (10 cm from the shield) as determined by Fricke dosimetry by Marian Leeksma.

II - Purification of Irradiated Polynucleotides

After irradiation, polynucleotides were separated from small molecular weight material by dialysis. That is, the solutions were placed in dialysis bags (previously boiled to destroy nucleases) and dialysed against several changes of distilled water at 4° C for one day. An alternate method of purifying adducts (though perhaps not as efficient) involved chromatography on Sephadex G-50, eluted with water(93).

III - Assessment of Stability of DNA-Nitrofurazone Adducts

One mg DNA + ^{14}C-NF in 1 ml 0.01 M Tris HCl, 0.01 M

$MgCl_2$ (pH 7) was irradiated and dialysed as previously described. Dialysis was then continued for 12 days (with 0.2 % sodium azide in the water to prevent microbial growth). Samples of 0.1ml were taken every second day to determine radioactivity.

IV - Enzymatic Hydrolysis of Polynucleotides to Nucleosides

A - DNA

Each mg (max. 1ml/mg) of DNA was incubated for 4 hours at 37°C with 260 Kunitz units of deoxyribonuclease I. Then, after the addition of 0.1 ml/ml of ~~1.0~~ M Tris (pH 9) (to raise the pH to 9) and 0.1 units of snake venom phosphodiesterase the solution was incubated for 24 hours at 37°C. Following this, 2.5 units of alkaline phosphatase were added and the digestion continued for another 24 hours at 37°C. In later experiments the pH of the hydrolysate was re-adjusted to 7 with 1.0 M HCl, and the mixture re-incubated at 37°C overnight (to ensure that deoxythymidine was in a single form). The final digest was stored at -20°C until used.

B - RNA

Each mg RNA (max. volume of 1ml/mg) was digested by adding 0.1 units of venom phosphodiesterase and 2.5 units of alkaline phosphatase, and incubating at 37°C for 24 hours

at pH 9.0. Re-adjusting the pH to 7 and incubating overnight at 37°C left uridine in one main form.

C - Poly C

Each mg of poly C was digested at 25°C three times for three hours, and once overnight with a mixture of 10 units micrococcal nuclease and 0.8 units alkaline phosphatase.

D - Poly U

Each mg of poly U was digested three times for 2 hours at 25°C with a mixture of 10 units ribonuclease A and 0.8 units alkaline phosphatase, along with an equal volume of 1.0 M Tris pH 9. After the digestion was completed the pH was re-adjusted to 7.

V - Detection Methods

A - Liquid Scintillation Counting

To 1 ml aqueous samples was added 7 ml of a mixture of 1 liter Triton X-144, 3 liters xylene, 0.8 gm POPOP, and 12 gm PPO. These were then counted for radioactivity in a Beckman liquid scintillation counter.

To detect radioactive areas in paper chromatograms, 1 cm strips were cut and eluted in 1 ml water and thus forth treated as aqueous samples.

An alternate method of detecting radioactive areas in paper chromatograms (e.g. in paper anion exchange) was to cut strips and place them in 3 ml of 0.4% Omnifluor in toluene.

B - Detection by Ultraviolet Absorption

In column chromatography fractions were collected in an LKB Ultrorac Fraction Collector. Absorbance at 254 nm was monitored by an LKB UVICORD II before fraction collection, or fractions were individually analysed at 260 nm on a Beckman spectrophotometer.

UV absorbing areas on paper chromatograms were detected using a short wave UV lamp.

In paper anion exchange the UV detectable samples were analysed by cutting 1 cm strips, eluting in 0.1 N HCl for two hours, and reading OD_{260} on a Beckman spectrophotometer.

V - Chromatography

A - Bio-Rad AG 50WX4 Cation Exchange Column (93)

The resin (minus 400 mesh) was obtained from Bio-Rad, Richmond, Ca.. The resin was washed twice with 2.5 volumes of 1 N HCl, followed by a wash with 2.5 volumes of deionized water, followed by three washes with 2.5 volumes of a 0.2 dilution of concentrated NH_4OH, and finally washed

four times with 2.5 volumes of the elutant buffer, 0.015 M NH_4OH pH adjusted to 8.9 with formic acid. Once the column was packed it could be re-used provided it was well washed with the elutant buffer before sample application.

B - Sephadex DEAE A25 Anion Exchange Column (93)

Before packing the column, the Sephadex DEAE A25 gel was pre-washed with the following solutions: once with 2 volumes 0.5 M HCl; three times with 2 volumes o.5 M NH_4OH; four times with 2 volumes 0.5 M NH_4HCO_3, pH 8; and finally twice with 2 volumes 0.01 M NH_4HCO_3, pH 8 (in which the gel was stored). Equilibration of the column was achieved by extensive washing with 0.01 M NH_4HCO_3, pH 8. Once the sample was applied to the column, a linear gradient was started whose components consisted of equal volumes of 0.01 M NH_4HCO_3, pH 8, and 0.6 M NH_4HCO_3, pH 8, mixed in a Buchler gradient maker. The columns were unpacked and the resin re-washed after each use.

C - Whatman DE-81 Anion Exchange Paper

In the "DEAE system" the paper was pre-soaked with 0.1 M sodium acetate, pH 7.5, and dried. Development was by the ascending technique with 0.1 M sodium acetate, pH 4.5.

In the "DEAE-borate system", the paper was pre-soaked in 0.2 M sodium borate, pH 6.5 (made by adjusting

0.2 M boric acid to pH 6.5 with 0.1 M sodium borate). Development was with the same borate solution.

D - Paper Chromatography

Samples were spotted on Whatman No. 3MM sheets and chromatographed by the descending technique. Various experiments were done using different solvent systems including 1-butanol: glacial acetic acid: water (120:30:50; v/v/v), 1-butanol: water (86:14; v/v), and 1-propanol: concentrated NH_4OH: water (55:20:25; v/v/v).

E - High Pressure Liquid Chromatography

These analyses were performed on a Model 332 Beckman instrument using an Ultrasphere-ODS C_{18} reverse phase column (25cm). Samples to be applied were concentrated in the smallest possible volume, and 20µl of each sample was injected. Absorbance at 254 nm was monitored during each run with the mobile phase being 12% methanol in 0.01 M $(NH_4)H_2PO_4$ (pH 5.1).

VII - (^{3}H-Me)Thymidine-Labelling of DNA

Ten ml of an overnight culture of E.coli KL188 cells (K.B.Low strain: thi-1, pyr D34, his-68, trp-45, thy A25, mtl-2, xyl-7, mal A1, gal K35, str A118)(from E.coli Genetic Stock Center #4211) was used to inoculate one liter

of Davis-Mingioli medium which consisted of: 750 ml water; 250 ml salts (i.e. a solution of 2.8% K_2HPO_4, 0.8% KH_2PO_4, 0.4% $(NH_4)_2SO_4$, 0.1% tri-sodium citrate, and 0.04% $MgSO_4 \cdot 7H_2O$); 20 ml 20% glucose; and 25 ml 1% casamino acids. In addition KL188 has growth requirements including thiamine (5μg/ml), histidine (20μg/ml), tryptophan (20μg/ml), thymidine (4μg/ml), cytidine (10μg/ml), and uridine (10μg/ml). To label the DNA of the cells, 0.5 mCi (^{3}H-Me)thymidine (48 Ci/mmole) was also added to the medium. After overnight growth, the DNA was extracted from the KL188 cells according to a phenol extraction method (94). The final purified DNA was re-dissolved in 1 ml 0.01 M trisHCl 0.01 M $MgCl_2$ pH 7.

VIII - Detection of λDNA Single-Strand Breaks (SSB)

A - Sedimentation

DNA (less than 0.1 ml of less than 0.5 mg/ml) were applied to the tops of 5 ml 5-20% (w/v)(0.1 M NaOH) alkaline sucrose gradients, and centrifuged 3 to 6 hours at 45,000 rpm in a SW50.1 rotor in a Beckman Model L ultracentrifuge (95). Fractions (0.25 ml) were collected from the top of the gradients onto strips of Whatman No. 17 filter paper which were washed twice with 5% trichloroacetic acid and then with 95% ethanol, dried and counted in 4 ml 0.4% Omnifluor in toluene (96).

B - Calculation of Molecular Weight

From the relationships (97):

(1) (weight average molecular weight) $M_w = \left(\frac{\beta}{w^2 ta}\right)^k \frac{\Sigma C_i (d_i)^k}{\Sigma C_i}$

(2)(number average molecular weight) $M_n = 0.5\ M_w$ (for more than 5 breaks)

(3) $d_i = d_f \times (F_i - 0.5)$ = distance (cm) to middle of i^{th} fraction.

the number average molecular weight can be calculated according to (since the results of this study gave more than 5 breaks) (96,97):

(4) $$M_n = 0.5\left(\frac{\beta d_f}{w^2 ta}\right)^k \frac{\Sigma C_i\ (F_i - 0.5)^k}{\Sigma C_i}$$

where d_f is the distance per fraction(0.2125cm), w the angular velocity (rpm), t the time (hr) of sedimentation, C_i the fraction of total counts in the i^{th} fraction, and F_i the i^{th} fraction. k and a are constants which have been found to be 2.50 and 0.0528 respectively in this system (98), while β is a constant calculated from sedimentation experiments with native λDNA as follows:

$$\beta = \frac{sw^2 t}{d}$$

where s is the sedimentation coefficient of λDNA in alkaline sucrose gradients (found to be 40.1 (98)), and d is the distance from the origin to the middle of the peak fraction.

The number of SSB can be calculated from (96):

$$N = \frac{M_n \text{ control DNA}}{M_n \text{ fragmented DNA}} - 1$$

The number of breaks per 10^5 nucleotides can then be calculated (where molecular weight of 10^5 nucleotides is taken to be 300×10^5):

$$300 \times 10^5 \times \frac{N}{M_n \text{ control DNA}}$$

RESULTS

I - DNA-(^{14}C-NF) Adduct Formation in Hypoxia

Irradiation of DNA with ^{14}C-NF with or without oxygen, and chromatography on Sephadex G-50 (see Fig. 4) confirmed that radiation-induced DNA-NF adduct formation can only occur in the absence of oxygen. DNA and DNA-adducts ran well apart from free ^{14}C-NF on the Sephadex G50 column. The nitrofuran molecule is small enough that if bound to DNA, the whole adduct would elute in a position characteristic of DNA itself (at the void volume). It should be mentioned at this point that any discussion of so-called "adducts" is based on an assumption. That is, we have no analytical chemical proof for the existance of true adducts, but rather their presence is inferred from the fact that ^{14}C counts seen associated with DNA at the void volume are not found in the absence of DNA (i.e. when ^{14}C-NF alone is irradiated). Also, the counts remained associated with the DNA even after dialysis (which would remove small molecular weight products).

II - Importance of DNA and ^{14}C-NF Presence during Irradiation

This experiment was carried out to determine

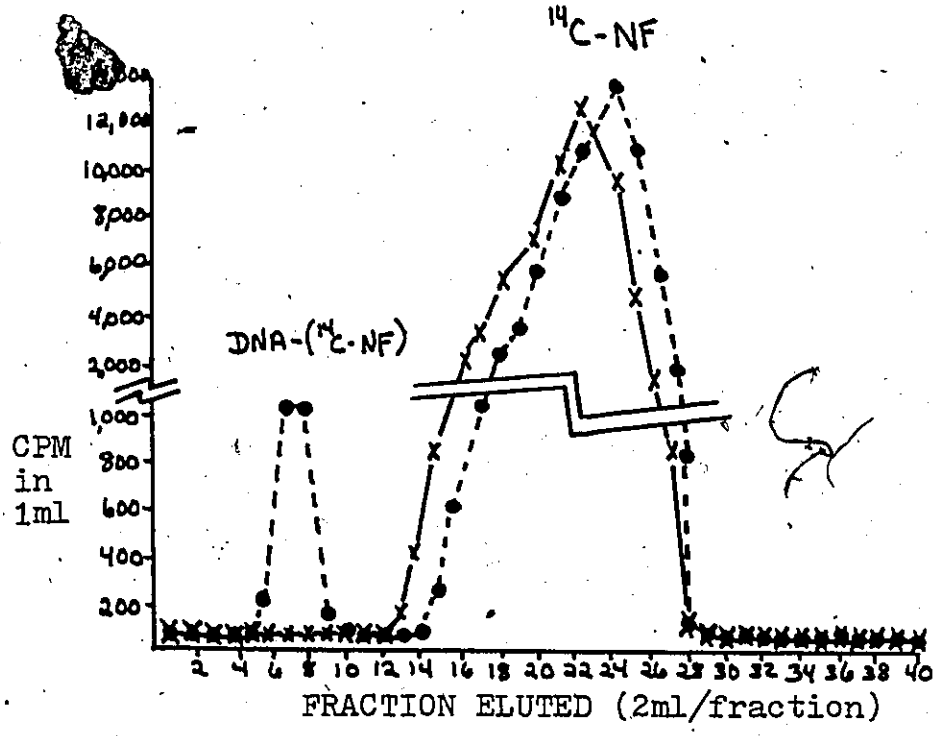


Fig. 4. DNA + ^{14}C-NF irradiated with 7 krads and run on Sephadex G-50 column (0.9 x 60cm) eluted with distilled water.

—X— Irradiated with oxygen

---●--- Irradiated without oxygen (flushed with argon)

whether both components (DNA and NF) must be present at the same time during γ-irradiation for binding to occur. Table 1 shows the three possible situations and the results after the components were incubated, dialysed, and counted to estimate radioactive adducts. No adducts were apparent in any of the cases, or at least no counts were distinguishable above background (50 CPM). These results confirmed that the forms in which the components react are short-lived, occur during irradiation and probably involve a free-radical process. Neither the stable radiolysis products of DNA nor of ^{14}C-NF seemed able to combine with the other component to cause binding. There was therefore no interference from stable radiolysis products in the binding process. Although previous work has shown radicals on DNA to be the essential element, the results of this experiment do not establish which component is required to undergo a short-lived change before binding, or even if both must change. Very rapid mixing would have been needed to acquire such information.

III - Assessment of Stability of DNA-(^{14}C-NF) Adducts

The procedure for assessing the stability of these adducts previously described in Methods (section III) involved the use of dialysis for purification of adducts (removal of small molecular weight products). The results in Table 2 indicate that if there is loss of adducts, it

Incubated after irradiation under hypoxia for 45 min.	Labelled adducts (CPM)
Irradiated DNA + Non-irradiated ^{14}C-NF	NONE *
Non-irradiated DNA + Irradiated ^{14}C-NF	NONE
Irradiated DNA + Irradiated ^{14}C-NF	NONE
DNA + ^{14}C-NF irradiated together under hypoxia.	2,000

TABLE 1. Importance of DNA and ^{14}C-NF presence during irradiation.

* NONE = No counts over background (~50CPM).

DAY	CPM in 0.1ml
1	395
2	330
4	308
6	341
8	274
10	285
12	283

TABLE 2. Determination of stability of (^{14}C-NF)-DNA adducts in a dialysis system.

is a very slow process and should not be a significant factor during the usual length of time required to complete an experiment.

IV - Analysis of DNA-(^{14}C-NF) Nucleoside Adducts by Chromatography

After γ-irradiation , the mixture (DNA with ^{14}C-NF) was dialysed, hydrolysed, and applied to a Bio-Rad AG 50WX4 cation exchange column in an attempt to purify the NF-bound derivatives. The radioactive material appeared to be successfully separated from the normal nucleosides (see Fig.5) provided that the mixture was re-incubated at pH 7 after hydrolysis before chromatography. That is, if left at the pH(9) existing under the conditions of hydrolysis, thymidine is found in two or more forms which run as two distinct peaks on AG 50WX4. If the pH is re-adjusted to 7, thymidine runs as a single peak. In fact, it has been shown that incubation at pH 9 of pure dT and U samples have caused these nucleosides to run as 2 peaks on AG 50WX4, while re-incubation at pH 7 resulted in their running as a single peak. The ^{14}C-NF bound derivatives can therefore clearly be separated from the normal nucleosides (which are much more strongly retarded). It must be kept in mind when discussing the character of adducts that separation on this type of column is not totally on the basis of charge but may also involve non-ionic interactions with the resin, so that although

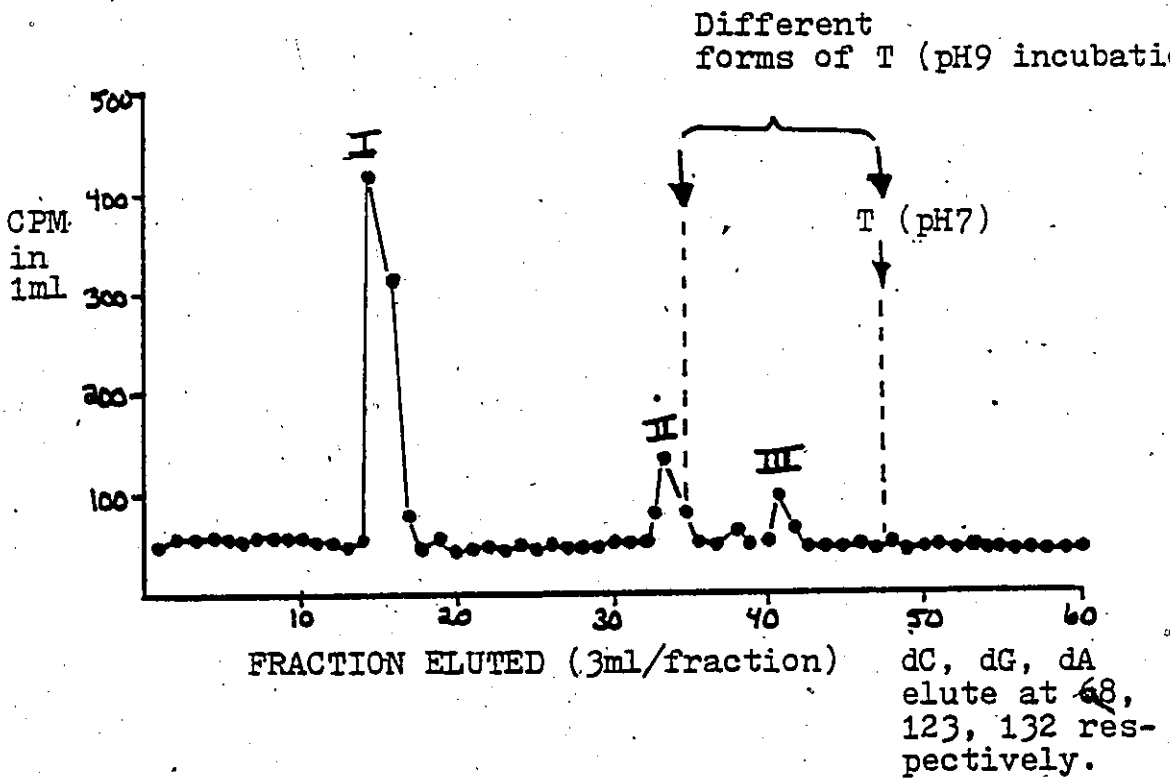


Fig. 5. (^{14}C-NF)-labelled DNA (1mg with dose of 25 krads) hydrolysed to nucleosides and chromatographed on AG 50WX4 column (1.5 x 90cm) eluted with 0.015M NH_4HCOO^- pH 8.9.

adenine and cytidine are neutral at pH 8.9, they are none the less substantially retarded by the column (A in fact running last of all the nucleosides). However, polynucleotides and nucleotides are totally unretarded. In a preliminary fashion the adducts were separated from each other on this column, running in at least three distinct peaks. One major component(s) (peak I) was totally unretarded by the column, suggesting an acidic nature. Two other minor components (peaks II and III) were slightly retarded by the column, suggesting they are neutral or slightly acidic. In a previous study re-chromatography of peak I on Sephadex DEAE A25 has demonstrated the mixture of components it contains (93). In contrast, re-chromatography here of peak II on Sephadex DEAE A25 (Fig. 6) showed that this component still ran as a single peak and was totally unretarded by the column (indicating it may be of a neutral nature since it also showed little interaction with the cation exchange resin). At least for the component(s) of peak I the radioactive material may not be completely separated from the DNA radiolysis products on the AG 50WX4 column since UV-absorbing material was seen at that position (void volume) both when DNA alone or DNA with ^{14}C-NF was irradiated. Also, calculated amounts of adducts present (1.4×10^{-7} mmole in total 3ml fractions of peak I in Fig. 5) were too small to be expected to show UV absorbance.

Fig. 7 shows an experiment which attempted to see

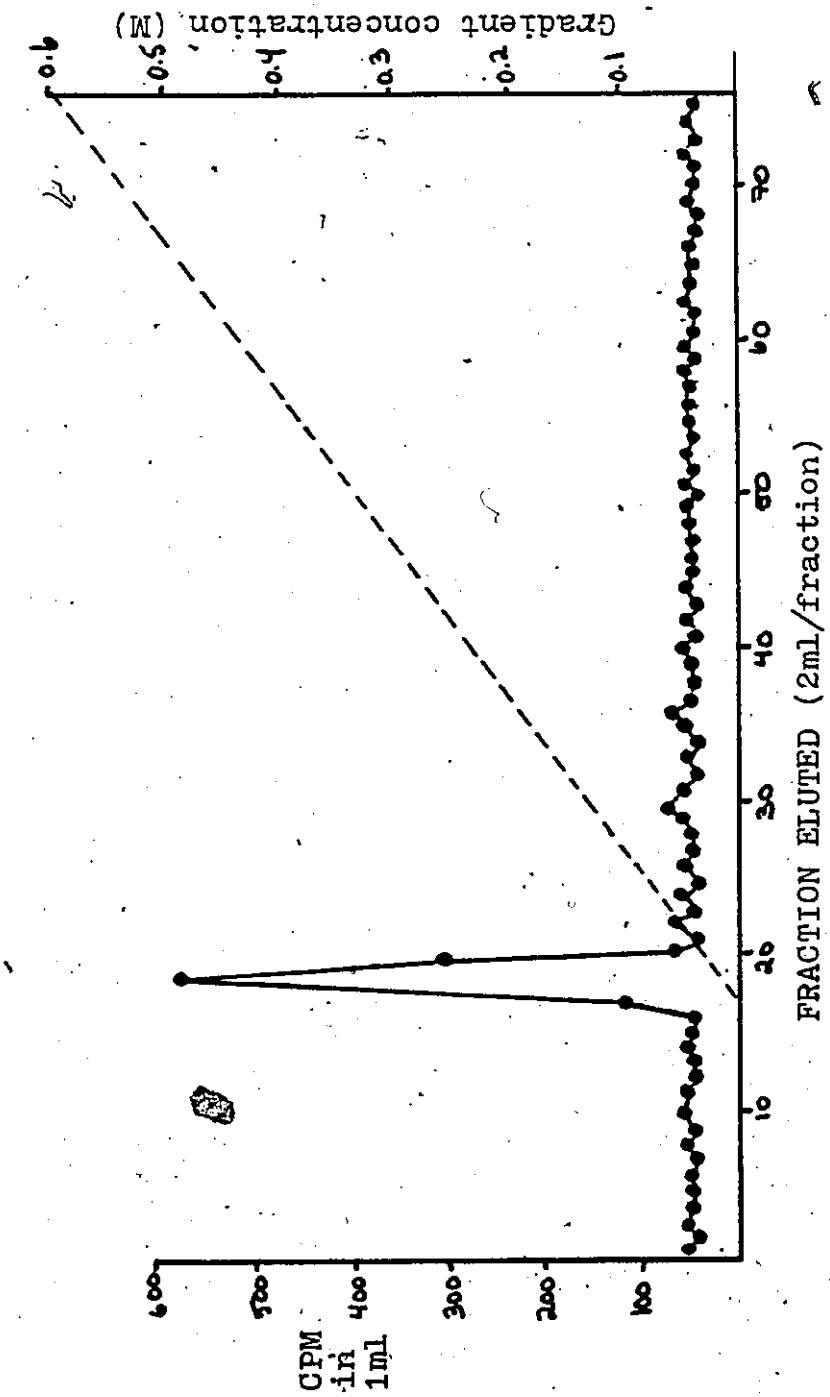


Fig. 6. Re-chromatography AG 50WX4 peak II of (^{14}C-NF)-DNA on Sephadex DEAE A25 column (0.9 x 60cm) eluted with 0.01M to 0.6M ammonium bicarbonate pH8 gradient, 0.28ml/min.

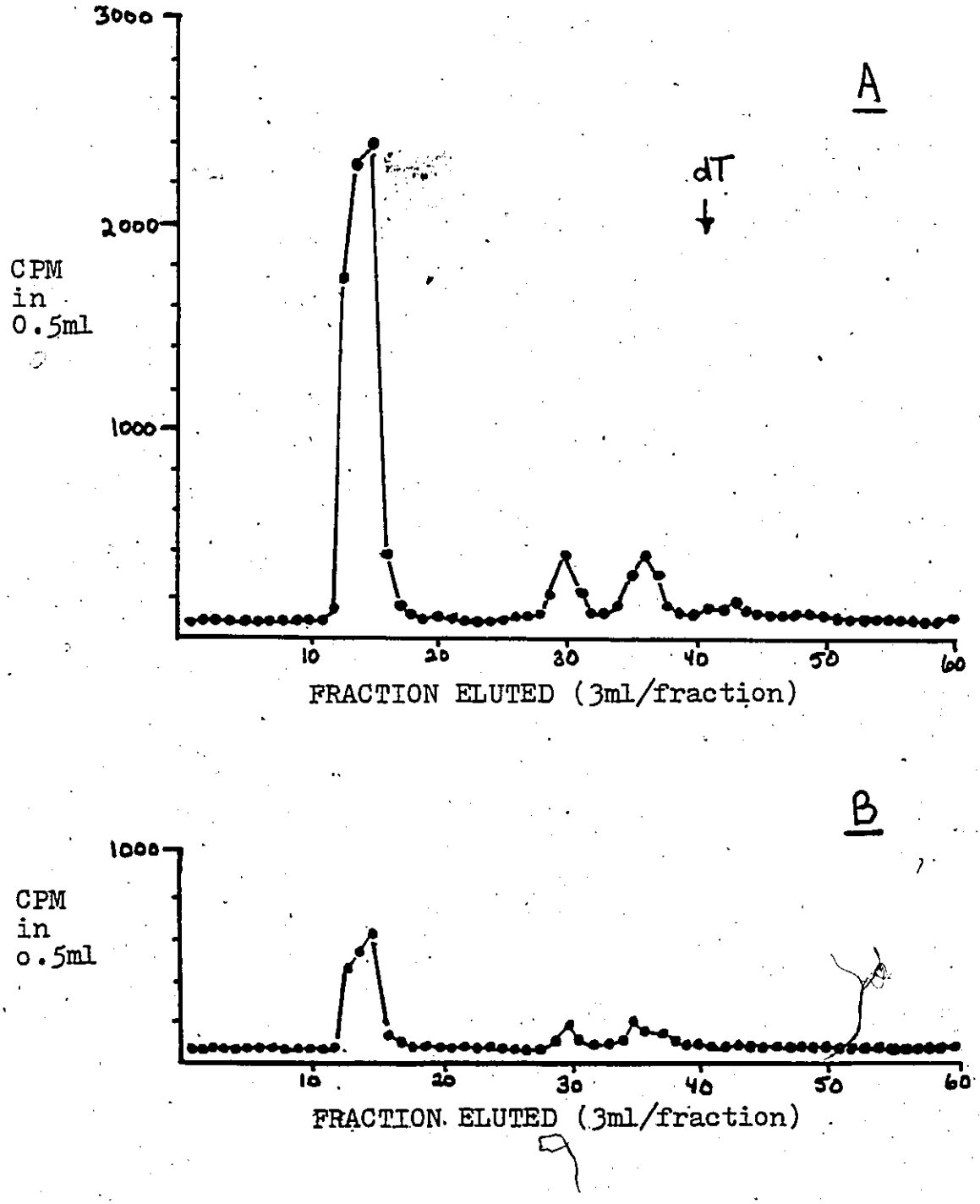


Fig. 7. (^{14}C-NF)-labelled DNA hydrolysed to nucleosides and chromatographed on AG 50WX4 column (1.5 x 60cm) eluted with 0.015M NH_4HCOO^- pH8.9.

A - 10mg DNA with dose of 34.5 krads.

B - 10mg DNA with dose of 9.5 krads.

whether there were variations in adduct proportions using a large dose (34 krads) and a much smaller dose (9.5 krads). There appeared to be no obvious difference in effects, both profiles showing the characteristic large unretarded peak and two smaller peaks just before thymidine was eluted. Therefore, although an increase in radiation dose caused an increase in total production of adducts, the relative proportions of the different adducts appeared to remain constant.

V - γ-Radiation-Induced Binding of NF to (^{3}H-Me)Thymidine-Labelled DNA

The use of DNA containing radioactive thymidine labelled in its base, makes it possible to attempt to determine if any thymidine bases are present in the radiation -induced NF derivatives. (^{3}H-Me)thymidine-labelled DNA (2 mg in 1 ml) was γ-irradiated with and without cold NF (4 μg/ml). After dialysis and hydrolysis the samples were applied to a Bio-Rad AG 50WX4 column. The results (Fig. 8) indicated that for the control and the DNA irradiated with NF there appeared to be no significant difference in counts at the positions where adducts are known to run (from Fig. 5). The counts seen at the void volume were no doubt due to radiolysis products of DNA, so either the increase of products due to NF-adducts which would run there is too small to be detected, or else NF-DNA adducts do not involve thymine bases. An increase in products due to NF

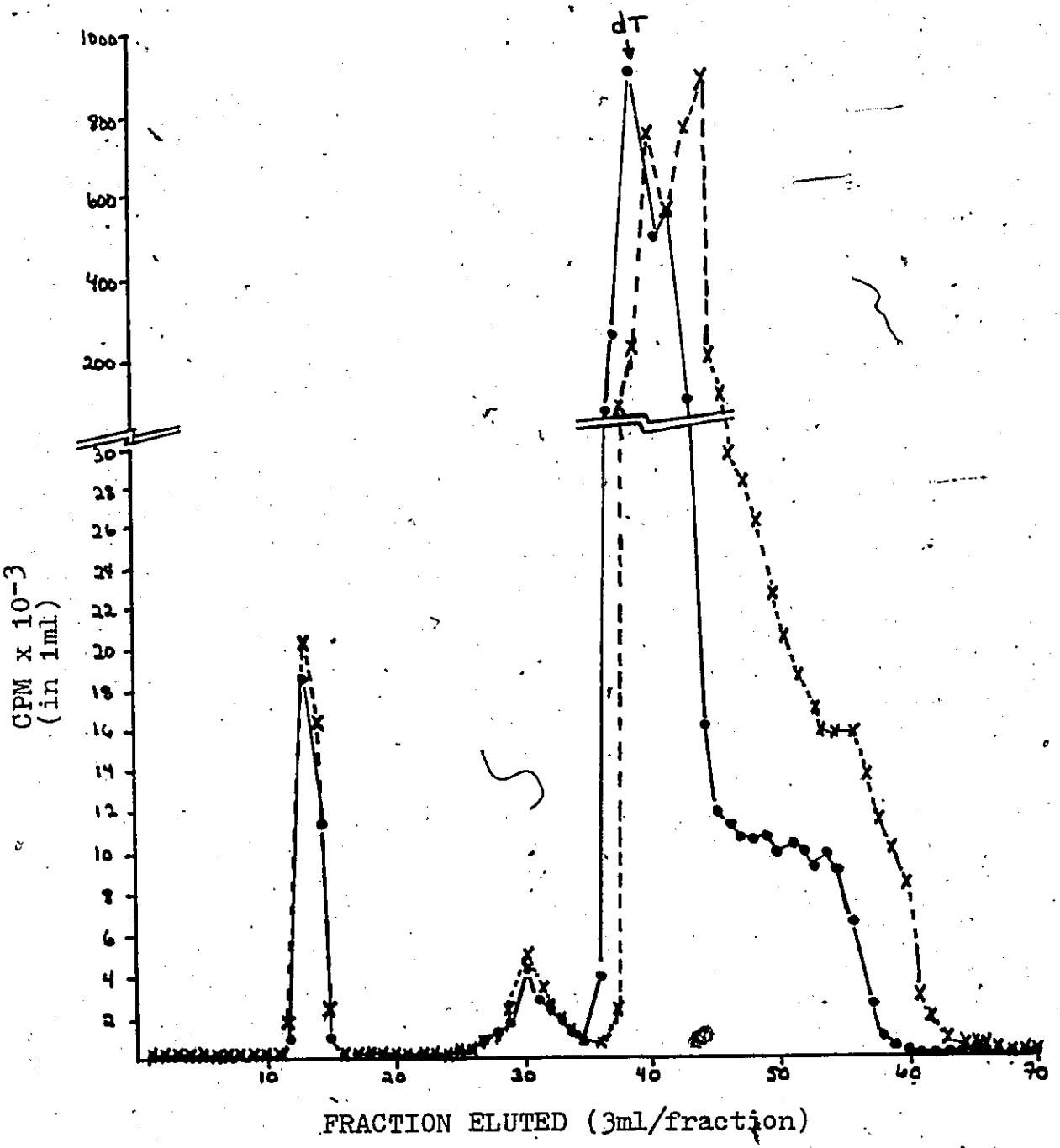


Fig. 8. (^{3}H-Me)thymidine-labelled DNA ± cold NF (2mg DNA with dose of 34 krad) hydrolysed to nucleosides and chromatographed on AG 50WX4 column (1.5 x 90cm) eluted with 0.015M NH_4HCOO^-, pH8.9.

—●—(^{3}H-Me)thymidine-DNA

---×---(^{3}H-Me)thymidine-DNA + NF

could however be seen in the area where thymidine runs. This may indicate some form of damage, caused by the presence of NF, which does not involve stable NF binding (since no ^{14}C-NF adducts were seen in this area; see Fig. 5)

VI - Analysis of (^{3}H-Me)Thymidine-(^{14}C-NF) Adducts

The use of simple nucleosides was examined as a possible approach for further simplification of the NF-derivatives. In one experiment, (^{3}H-Me)thymidine was used in an attempt to determine if the thymine base is present in radiation-induced thymidine-NF products. Thymidine was chosen in particular so as to be comparable with the (^{3}H-Me) thymidine-labelled DNA experiment in section V. Dual labelling with ^{14}C-NF was done to determine if adducts do occur. (^{3}H-Me)thymidine (1 mg/ml) with or without ^{14}C-NF (2 µg/ml) were γ-irradiated and applied to a Bio-Rad AG 50WX4 column. The results (Fig. 9) indicated the presence of label mainly eluting at the "void volume" (seen from ^{14}C profile in Fig. 15-B). The excess of ^{3}H counts (representing thymine) in Fig. 9-B over ^{14}C counts, which may represent NF-adducts, exceeded ^{3}H counts seen when (^{3}H-Me)thymidine alone was irradiated (Fig. 9-A). This may indicate the presence of the thymine base in the adducts, and/or of some other base damage caused by NF. In the (^{3}H-Me)thymidine-DNA experiment (Fig. 8) new ^{3}H-labelled products due to NF were seen to

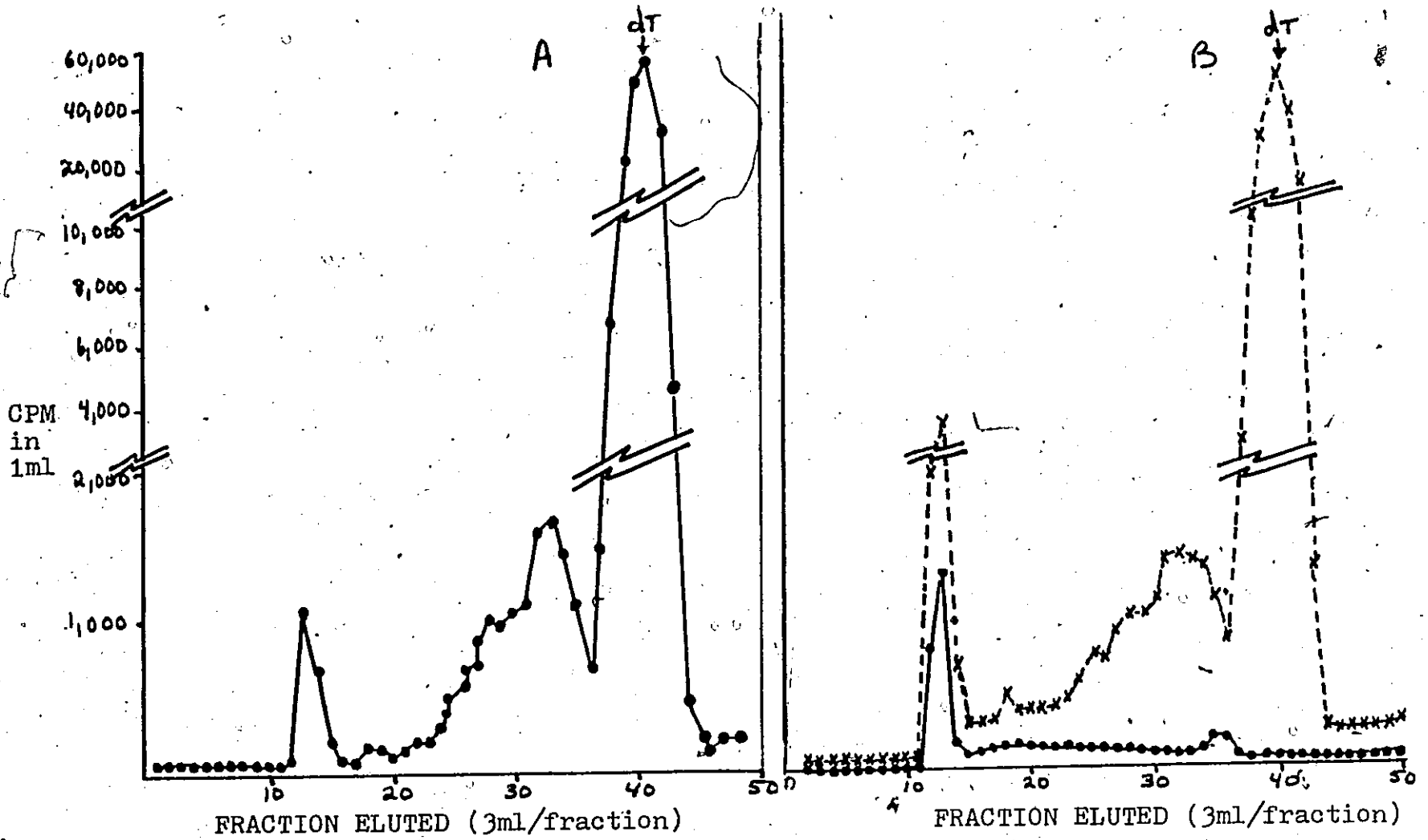


Fig. 9. γ-irradiated (34 krads) (^{3}H-Me)thymidine ± (^{14}C-NF) chromatographed on AG 50WX4 column (1.5 x 90 cm), eluted with 0.015M NH_4HCOO^- pH8.9.

A - (^{3}H-Me)thymidine (1mg/ml) —●— ^{3}H counts

B - (^{3}H-Me)thymidine (1mg/ml) + (^{14}C-NF)(2.0 μg/ml) : --x-- ^{3}H + ^{14}C counts
—●— ^{14}C counts only

elute in the same area as dT. Such products were not seen in this experiment with (^{3}H-Me)thymidine as a sharp peak was evident for dT both with and without NF. Such results may be related to the use of a nucleic acid as opposed to a nucleoside.

Chromatography of irradiated ^{14}C-NF alone on the Bio-Rad column has led to a modified interpretation of the previous results. Irradiated ^{14}C-NF ran at the "void volume" (see Fig. 10) so as to interfere with any adducts that might run there. Unirradiated NF stayed on the column indefinitely. Therefore, although we could not be certain of any NF-thymidine adducts at the "void volume" (since free NF was not separated from other material), the excess of ^{3}H counts still indicated the presence of some NF-induced products, be they adducts or otherwise.

To gain further information on the"void volume" peaks of Fig. 9, they were re-chromatographed by HPLC (see Fig. 11). No difference in the OD_{254} profiles was apparent with or without NF, the UV absorbing material being the radiolysis products of thymidine. Therefore, if there were adducts or other damage there was very little UV absorbing material associated with them, or the adducts were not resolved or eluted with this particular solvent.

VII - <u>Analysis of (^{14}C-NF)-RNA Nucleoside Adducts on a Bio-Rad AG 50WX4 Column</u>

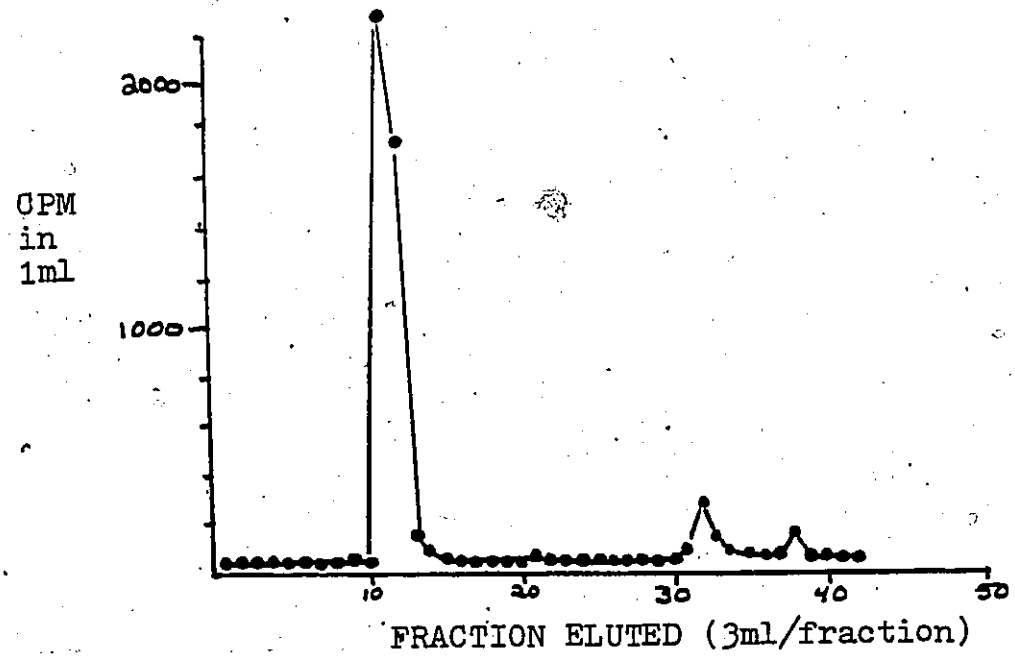
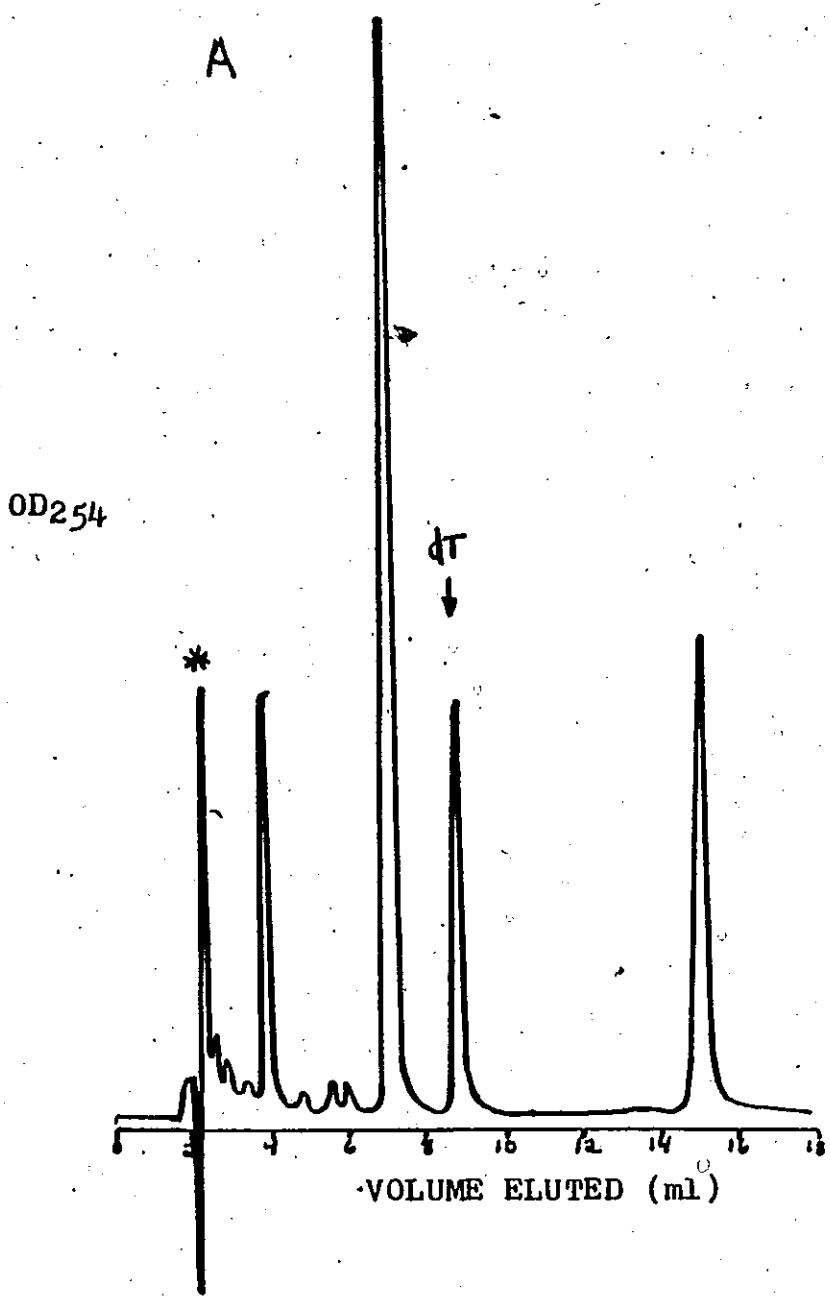


Fig. 10. ~15,000 CPM (^{14}C-NF) irradiated with 34 krads γ-rays and chromatographed on AG 50WX4 column (1.5 x 90 cm) eluted with 0.015M NH_4HCOO^- pH8.9.



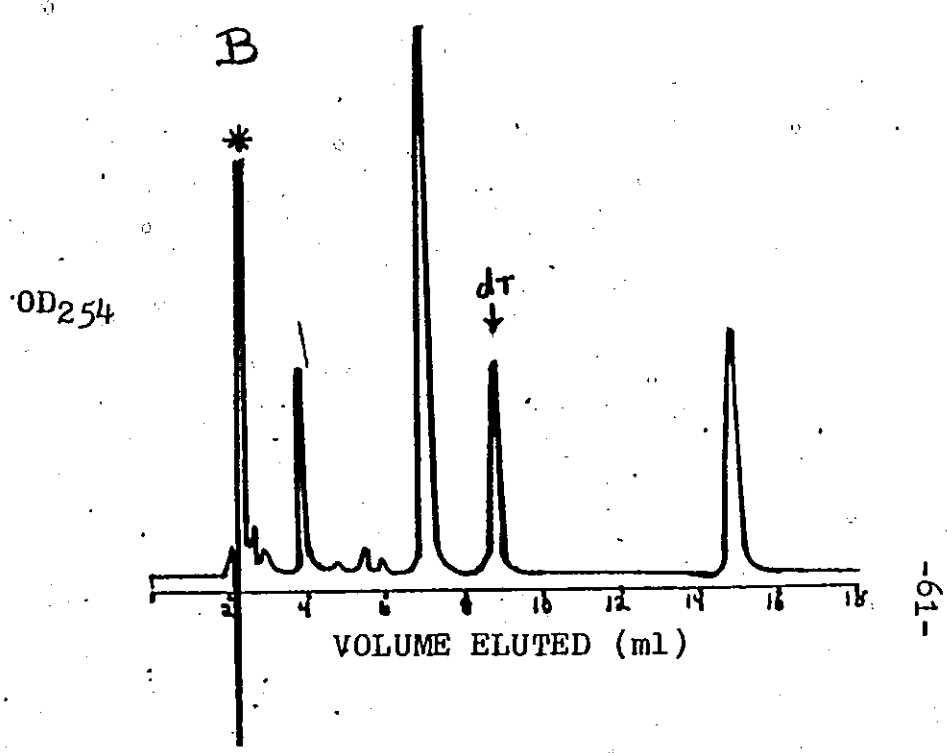


Fig. 11. (^{3}H-Me)thymidine ± (^{14}C-NF) γ-irradiated, run on AG 50WX4, and "void peak" re-chromatographed by HPLC, eluted with 12% MeOH in 0.01M $(NH_4)H_2PO_4$, pH5.1.

A - (^{3}H-Me)thymidine sample

B - (^{3}H-Me)thymidine + (^{14}C-NF) sample

* Solvent front disturbance

Though DNA is the nucleic acid which is biologically relevant in terms of sensitizer binding, RNA was also examined as a prelude to homopolymer studies (ribopolymers having been chosen because they are inexpensive). After E.coli t-RNA with (^{14}C-NF) was γ-irradiated, dialysed, and hydrolysed to nucleosides, the hydrolysate was applied to a Bio-Rad AG 50WX4 column. Fig. 12 shows that adducts were also formed between t-RNA and NF, but appeared to be only about one half of that seen with the same concentration of DNA at the same dose. The (^{14}C-NF)RNA adducts ran as one broad peak at the "void volume", well away from uridine (the closest nucleoside). Unlike DNA it therefore appeared that all the adducts have a negative character, causing them to be unretarded by the column. Since UV-absorbing material was found at the void volume, this may make it difficult to separate different adducts from each other and from RNA radiolysis products.

VIII- Analysis of (^{14}C-NF)-poly C Nucleoside Adducts by Chromatography

As the complexities associated with NF-adducts of DNA became evident, we turned to a simpler system through the use of polynucleotides. In one experiment a sample of poly C with ^{14}C -NF was γ-irradiated, dialysed, hydrolysed and chromatographed on a Bio-Rad AG 50WX4 column. Fig.13 shows that, like RNA, the radioactive adduct(s) ran as a

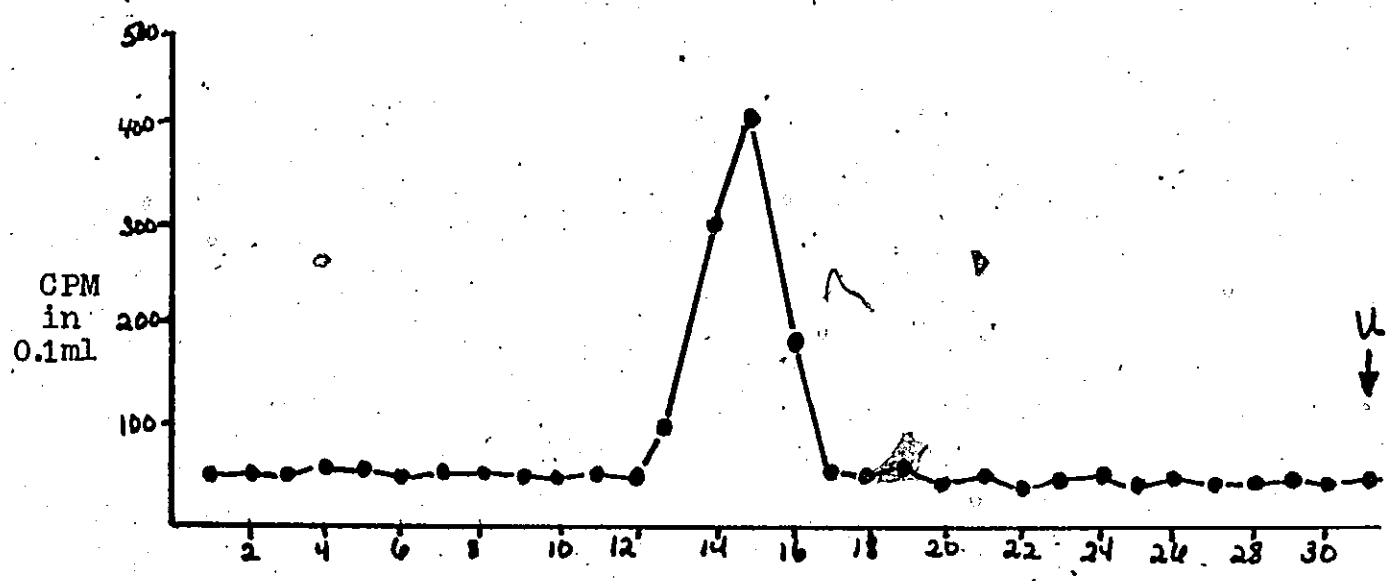


Fig. 12. (^{14}C-NF)-labelled t-RNA (10mg with dose of 34 krad) hydrolysed to nucleosides and chromatographed on AG 50WX4 column (1.5 x 90cm) eluted with 0.015M NH_4HCOO^-, pH8.9.

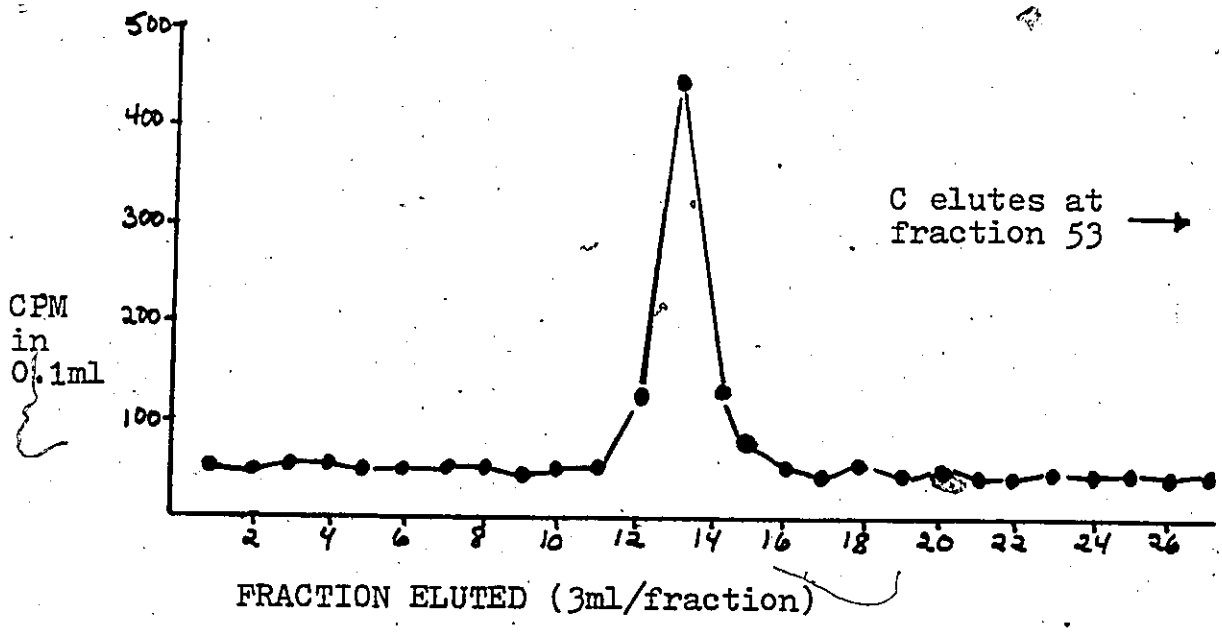


Fig. 13. (^{14}C-NF)-labelled poly C (10mg at dose of 50 krad) hydrolysed to nucleosides and chromatographed on AG 50WX4 column (1.5 x 90 cm) eluted with 0.015M NH_4HCOO^-, pH8.9.

single acidic peak, away from the normal nucleoside, cytidine. The extent of binding was calculated to be about one-half that of DNA.

To estimate the complexity of the adduct peak, it was re-chromatographed on a Sephadex DEAE A25 column. The results (Fig. 14) indicated that the peak is multicomponent in nature, reflecting a variety of different adducts. The radioactivity peak at the "void volume" is probably a mixture of neutral products, while the other peaks eluting with the gradient probably have a net negative charge (since they were unretarded by a cation exchange column, but were retarded by an anion exchange column). The amount of UV-absorbing material was too small to construct a profile, but such material is probably related to the radiolysis products of poly C.

IX - Analysis of (^{14}C-NF)-poly U Nucleoside Adducts by Chromatography

In these experiments the NF-binding properties of another polynucleotide was examined. The purified (^{14}C-NF)-poly U nucleoside adducts were applied to a Bio-Rad AG 50WX4 column. As for poly C and t-RNA, only one peak at the void volume (reflecting acidic products) was apparent in the radioactivity profile (see Fig. 15). The extent of binding of NF to poly U was estimated to be equivalent to that for DNA.

The Bio-Rad AG 50WX4 radioactivity peak, reflecting

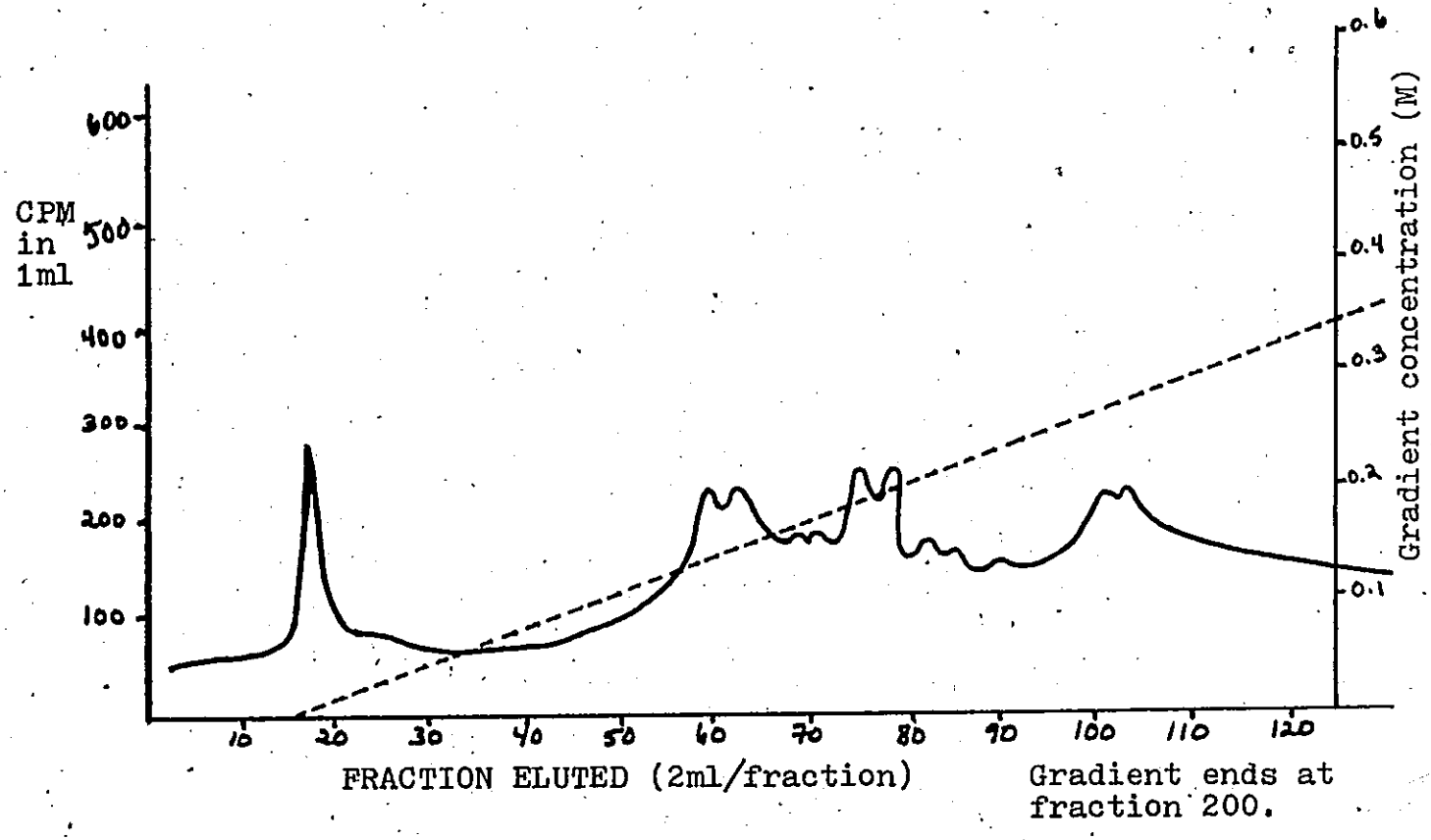


Fig. 14. Re-chromatography of AG 50WX4 peak for (^{14}C-NF)-poly C on Sephadex DEAE A25 column (0.9 x 60cm) eluted with 0.01M to 0.6M ammonium bicarbonate pH8 gradient, 0.1ml/min.

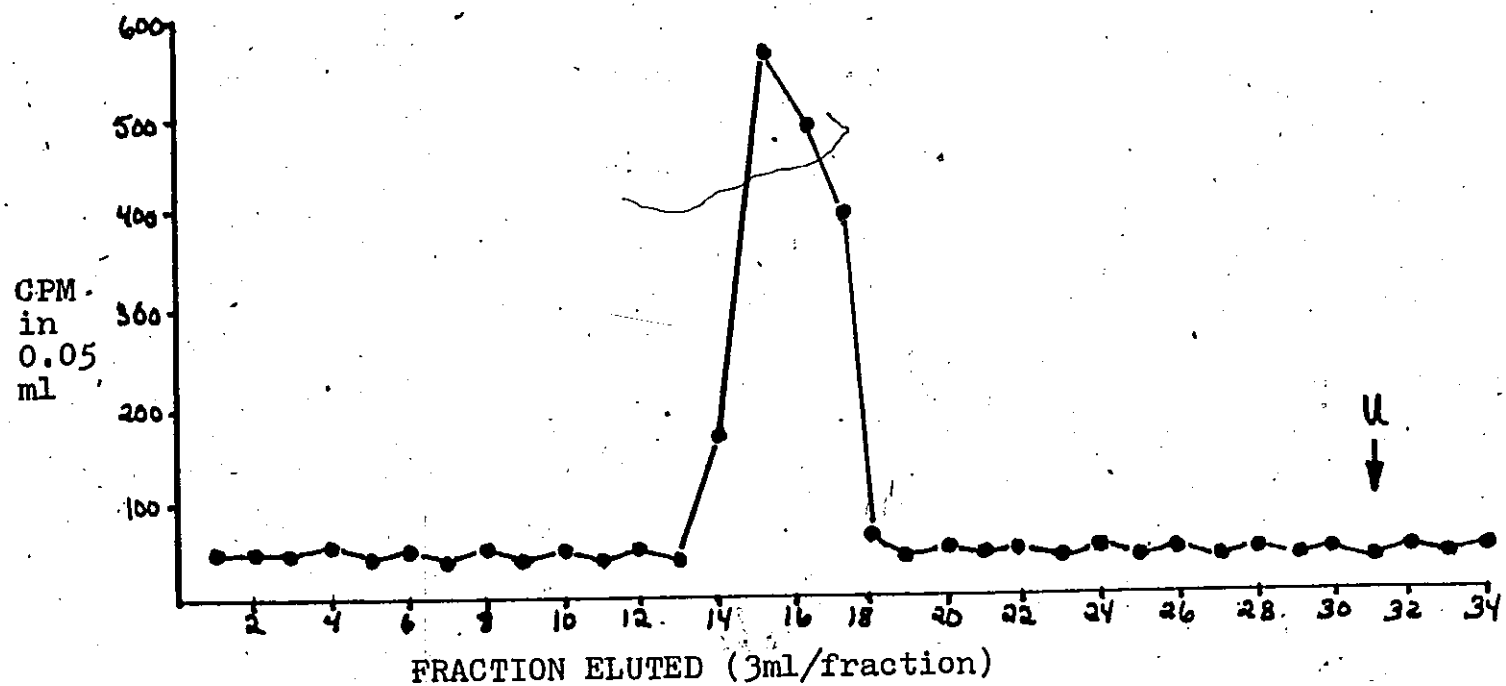


Fig. 15. (^{14}C-NF)-labelled poly U (10mg with dose of 25 krad) hydrolysed to nucleosides and chromatographed on AG 50WX4 column (1.5 x 90cm) eluted with 0.015M NH_4HCOO^-, pH8.9.

(^{14}C-NF)-poly U nucleoside adducts, was re-chromatographed on a Sephadex DEAE A25 column for further separation. From Fig. 16 it can be seen that a mixture of adducts was present, with perhaps two main ones at peaks B and E. Peak B could well contain a mixture of neutral products, while peak E (~one-tenth of total counts) may be a more purified negatively charged adduct. Little or no radioactivity corresponded to any UV-absorbing material. Therefore, separation of the adducts from each other and from the radiolysis products of poly U seems to be possible under these conditons. Increasing the amounts of material used in the experiment (100mg poly U) may however prove difficult as preliminary experiments along these lines led to problems of inconsistancies and high background counts.

Peaks B and E of the Sephadex DEAE A25 profile (from Fig 16) were re-chromatographed on Whatman DE-81 anion exchange paper (paper impregnated with DEAE cellulose). The purpose of this experiment was to determine if the ribose moiety is intact in the adducts. This was determined by running identical samples simultaneously in two different solvent systems: acetate and borate (described in Methods). Polynucleotides and nucleotides remain at the origin in both systems. Ribonucleosides are retarded in the borate as compared with the acetate system (due to borate complexing with cis-OH groups of ribose), while deoxyribonucleosides and the free bases show no such difference. Fig. 17 shows the pro-

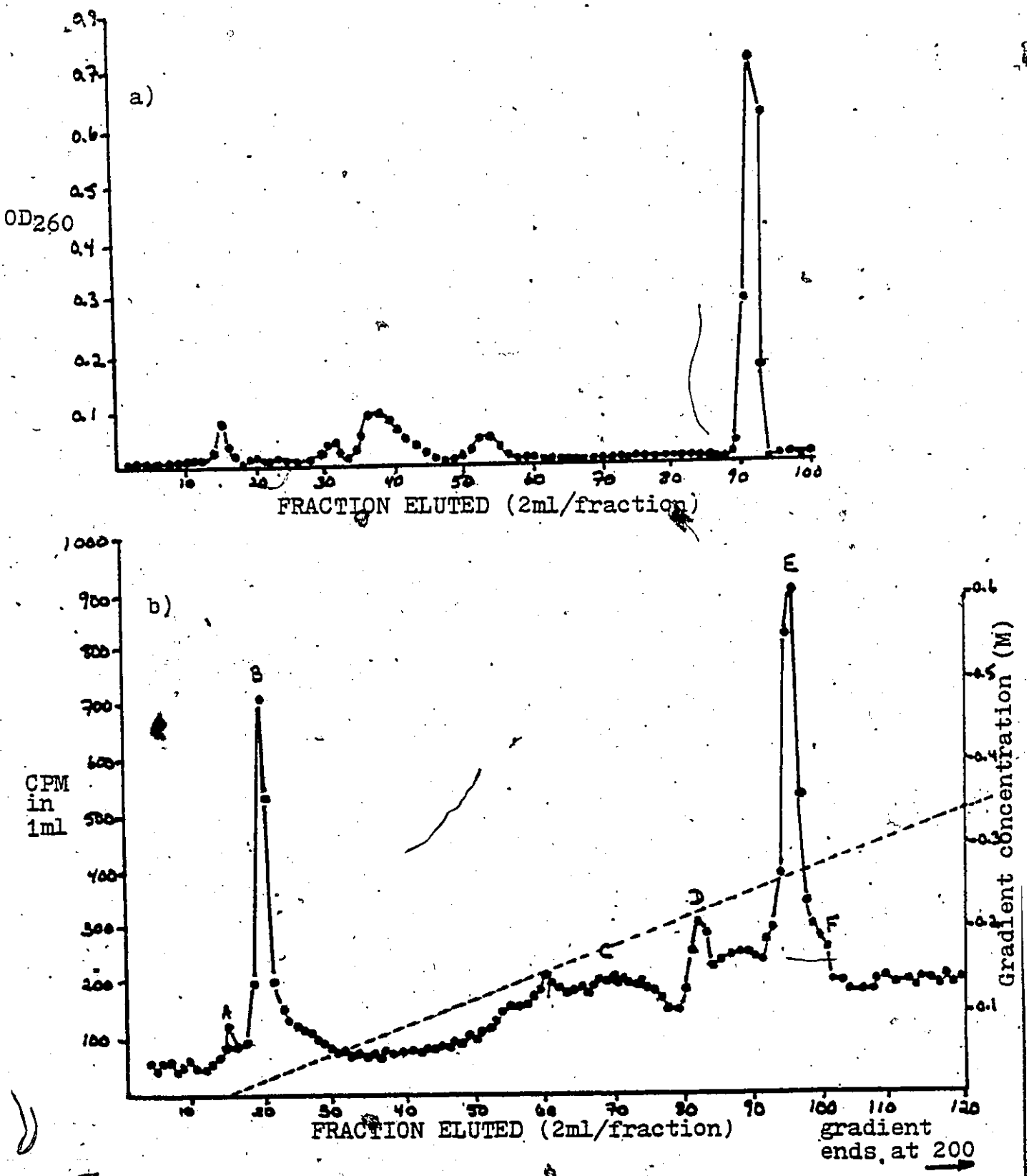


Fig. 16. Re-chromatography of "AG 50WX4 column " of (^{14}C-NF)-poly U peak I on Sephadex DEAE A25 column (0.9 x 60cm) eluted with 0.01 to 0.6M ammonium bicarbonate pH8 gradient.

a) - O.D.260 profile

b) - Radioactivity profile

Peak B

Peak E

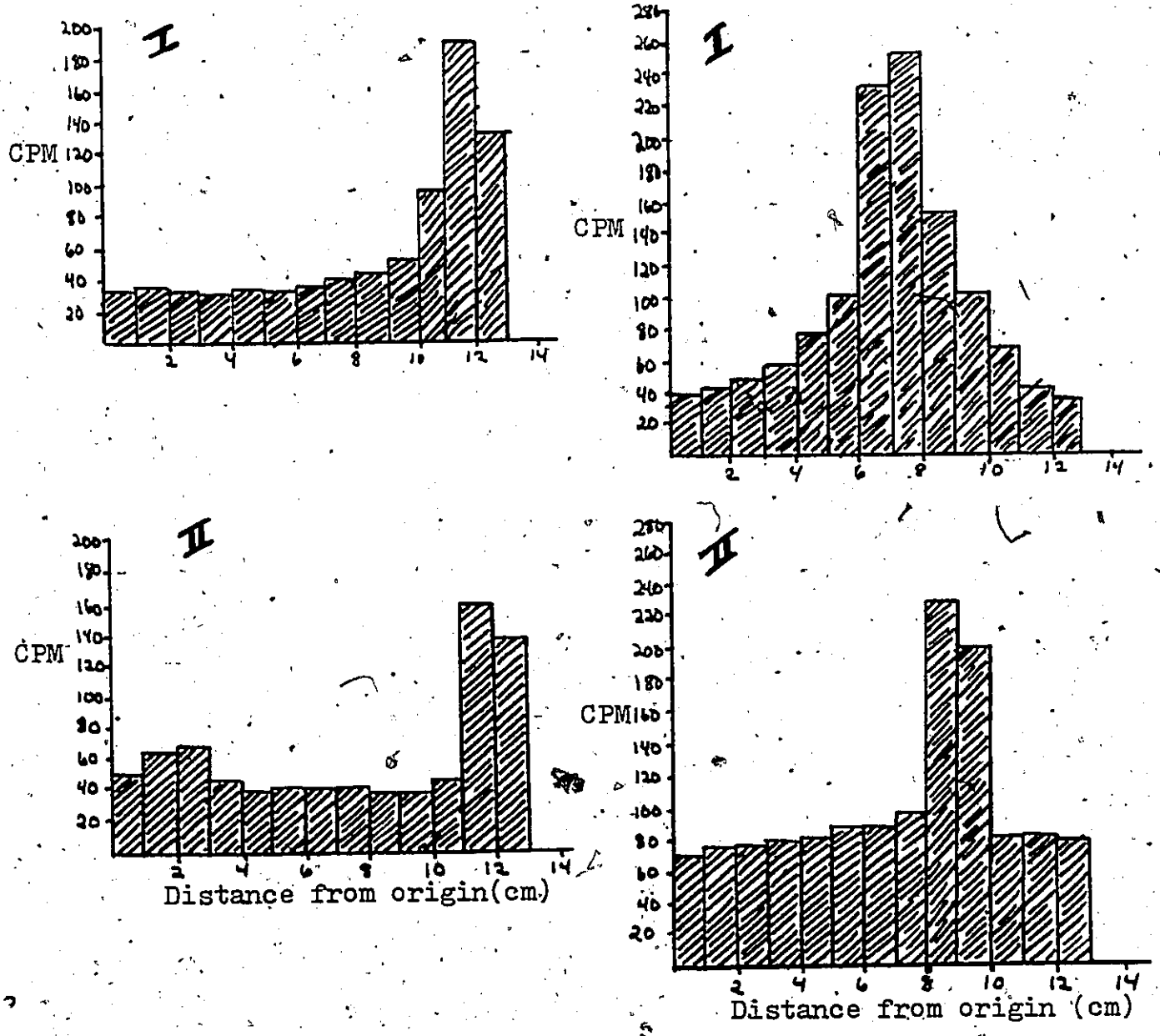


Fig. 17. Re-chromatography of Sephadex DEAE A25 column of (^{14}C-NF)-poly U peaks on Whatman DE-81 anion exchange paper eluted with: I - 0.1M sodium acetate, pH4.5; II - 0.1M sodium borate, pH6.5

Peak B - Peak B of DEAE A25 CPM profile for (^{14}C-NF)-poly U

Peak E - Peak E of DEAE A25 CPM profile for (^{14}C-NF)-poly U

files for DEAE peaks B and E in the two development systems. For peak B we found one main component which migrated close to the solvent front (R_f=0.89 compared to acetate R_f for uridine = 0.73) in both solvent systems, while only a minor and possibly insignificant component(s) was retarded by the borate system (R_f = 0.15) as expected for ribose compounds (borate R_f for uridine = 0.08). Therefore, it would seem that most or all of peak B lacked the normal ribose moiety suggesting that ribose was altogether missing, or that it had undergone a change at position $C_{2'}$ or $C_{3'}$. Similarly, for peak E, a main component(s) migrated at R_f = 0.62 in both solvent systems, while a minor component(s) may have been retarded in the borate system. Therefore, most or all adducts in peak E lacked an intact ribose moiety. For both peaks B and E it is significant that no counts remained at the origin in either solvent systems. This means that the isolated adducts do not have a nucleotide or oligonucleotide structure (i.e. they may lack phosphate groups), a situation that could have resulted if the digesting enzymes were unable to hydrolyse the adduct-containing DNA to nucleosides.

(^{14}C-NF)-poly U adducts were also studied by paper chromatography in order to obtain more specific information as to the nature of the adducts. When these nucleoside adducts were run on Whatman No. 3MM paper with n-butanol: water (86:14), all the counts remained at the origin (see Fig. 18-A).

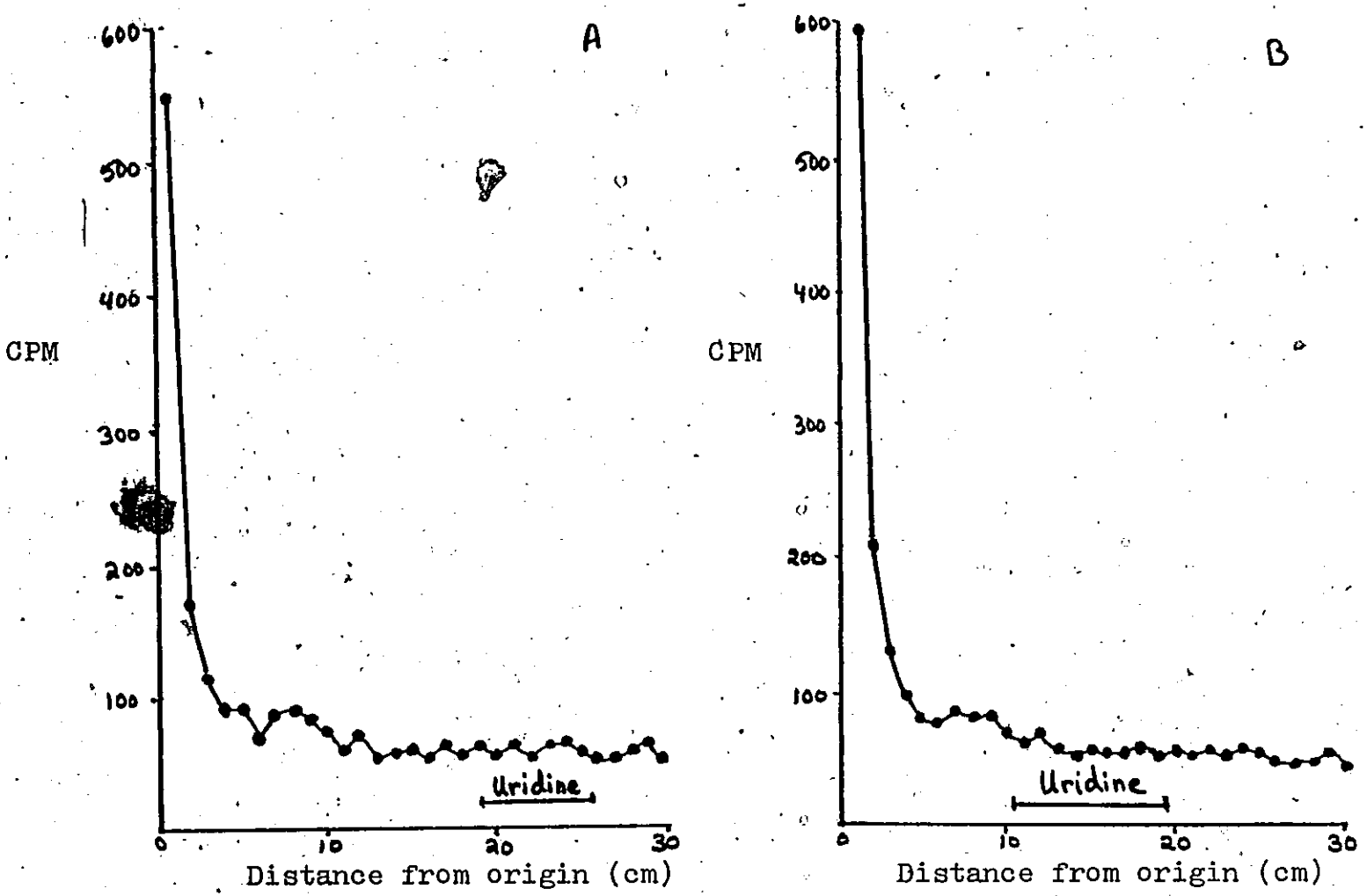


Fig. 18. (^{14}C-NF)-poly U nucleoside digest chromatographed on Whatman No. 3MM paper.

A - n-butanol : water (86:14).

B - n-butanol : acetic acid : water (60:15:25).

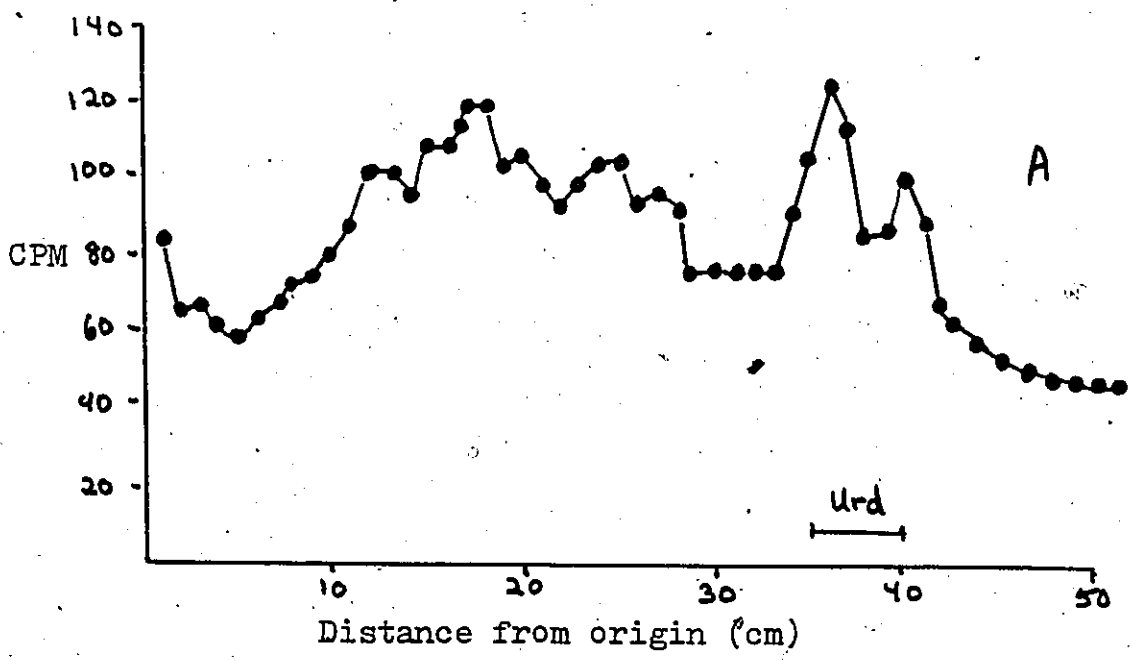
Since in this solvent system nucleosides migrate but nucleotides do not, these results indicated a nucleotide, oligonucleotide, or phosphate-bound product at the origin; or else it might be that the nitrofuran group has a negative charge (due to side-chain breakdown leaving a carboxylic acid) causing the whole adduct to remain at the origin. In order to eliminate the possibility that the adduct ran at the origin solely because of a negative charge on a derivative of NF, the same sample was run in n-butanol:acetic acid: water (60: 15: 25) in which any carboxylic acid groups but not the phosphate group should be protonated. Fig. 18-B shows that all the counts still ran at the origin even under these conditions, implying that the presence of carboxylic acid groupswas not the reason for the overall negative charge on the adducts. In fact, a phosphate group was probably responsible.

The same (^{14}C-NF)-poly U nucleoside digest was also run on paper with 1-propanol: concentrated ammonium hydroxide: water (55: 20: 25), a solvent system in which nucleotides (UMP R_f = 0.38), nucleosides (uridine R_f = 0.66), and bases (uracil R_f = 0.68) migrate while inorganic phosphate remains at the origin. The stability of adducts in this solvent was verified by incubation of (^{14}C-NF)-poly U in it, followed by dialysis and counting for radioactivity. No breakdown was apparent. Therefore, in trying to separate possible phosphate-containing or other adducts from nucleotide-adducts

the nucleoside digest of (^{14}C-NF)-poly U was chromatographed in this solvent (Fig. 19-A). Most of the counts no longer stayed at the origin but rather a group of peaks was found between R_f = 0.20-0.26 and R_f = 0.64-0.82 (where uridine runs). These may represent a nucleotide-like adduct (due to the inability of the enzyme to remove the phosphate) and a nucleoside-type or base-type adduct (less likely because dialysis would remove free bases). On the other hand, the nucleotide digest of (^{14}C-NF)poly U in the same solvent system did show most counts at the origin (see Fig. 19-B) with perhaps a group of peaks further on. The nucleotide-type products seen with the nucleoside digest may also be present here but in addition, a phosphate-bound adduct seems possible. The reason why this adduct was not seen with the nucleoside digest may be due to the additional enzyme (alkaline phosphatase) used to remove the phosphate groups, which may also have hydrolysed the phosphate-bound adduct.

X - Analysis of Uracil, Uridine, or UMP (^{14}C-NF)-Adducts by Paper Chromatography

Paper chromatography experiments were done using separate constituents of poly U (i.e. uracil, uridine and uridine 5' monophosphate) in an attempt to narrow down the location of the NF bound to the poly U molecule. Separate samples of uracil (2mg/ml), uridine (10mg/ml), and UMP (10mg/ml) were γ-irradiated with ^{14}C-nitrofurazone and run on Whatman No. 3MM paper with 1-propanol: concentrated NH_4OH:



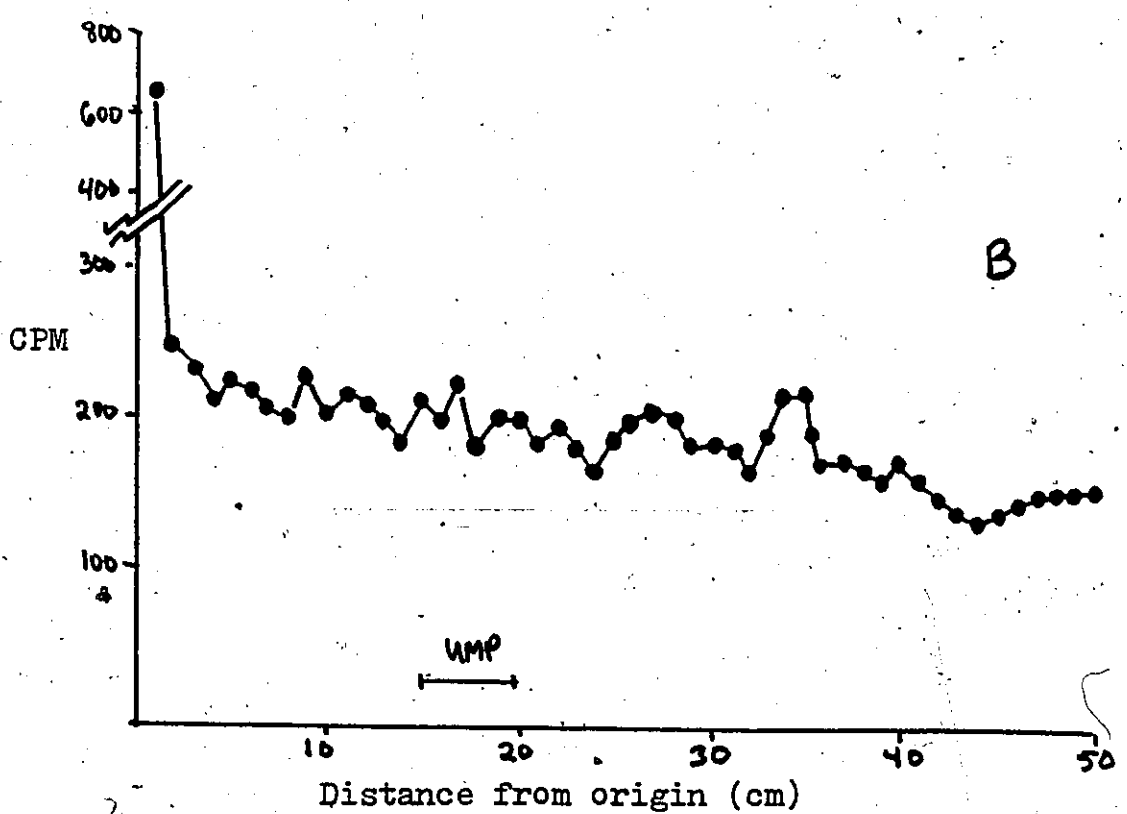


Fig. 19. Digests of irradiated poly U (10mg/ml for 34 krads) + (^{14}C-NF), applied to Whatman No. 3MM paper eluted with 1-propanol : concentrated ammonium hydroxide : water (55:20:25).
A - Nucleoside digest.
B - Nucleotide digest.

water (55: 20: 25). In this solvent system irradiated and non-irradiated ^{14}C-NF ran above R_f=0.61 and therefore interfered with any adducts that migrated there. However, one could still see, for uracil, a group of peaks (R_f = 0.48) possibly due to adducts; and a similar group for uridine (R_f = 0.32) and UMP (R_f = 0.27) (see Fig. 20 and 21). In going from uracil to uridine to UMP one found this group of peaks getting closer to the origin in the same order as uracil, uridine and UMP themselves ran. The profiles for uridine (Fig. 21-A) and UMP (Fig. 21-B) resembled those obtained when (^{14}C-NF)-poly U was digested to nucleosides and nucleotides respectively (Fig. 19). The profile for UMP adducts showed a large peak at the origin as was seen for the (^{14}C-NF)-poly U nucleotide digest. These results suggest that similar adducts are obtained with the components of poly U as with poly U itself. Furthermore, this experiment again supports the existence of a phosphate-containing adduct (appearing with the use of UMP). A uracil-bound adduct also seems probable since the use of the uracil base yielded NF adducts (i.e. the group of peaks described earlier which also appeared with uridine and UMP).

To determine if the UMP adduct(s) running at the origin contained the uracil base, (U-^{14}C) 5' UMP (10mg/ml) was irradiated with or without nitrofurazone. When run on paper in the same solvent previously described (1-propanol: concentrated NH4OH: water), these samples showed no apparent

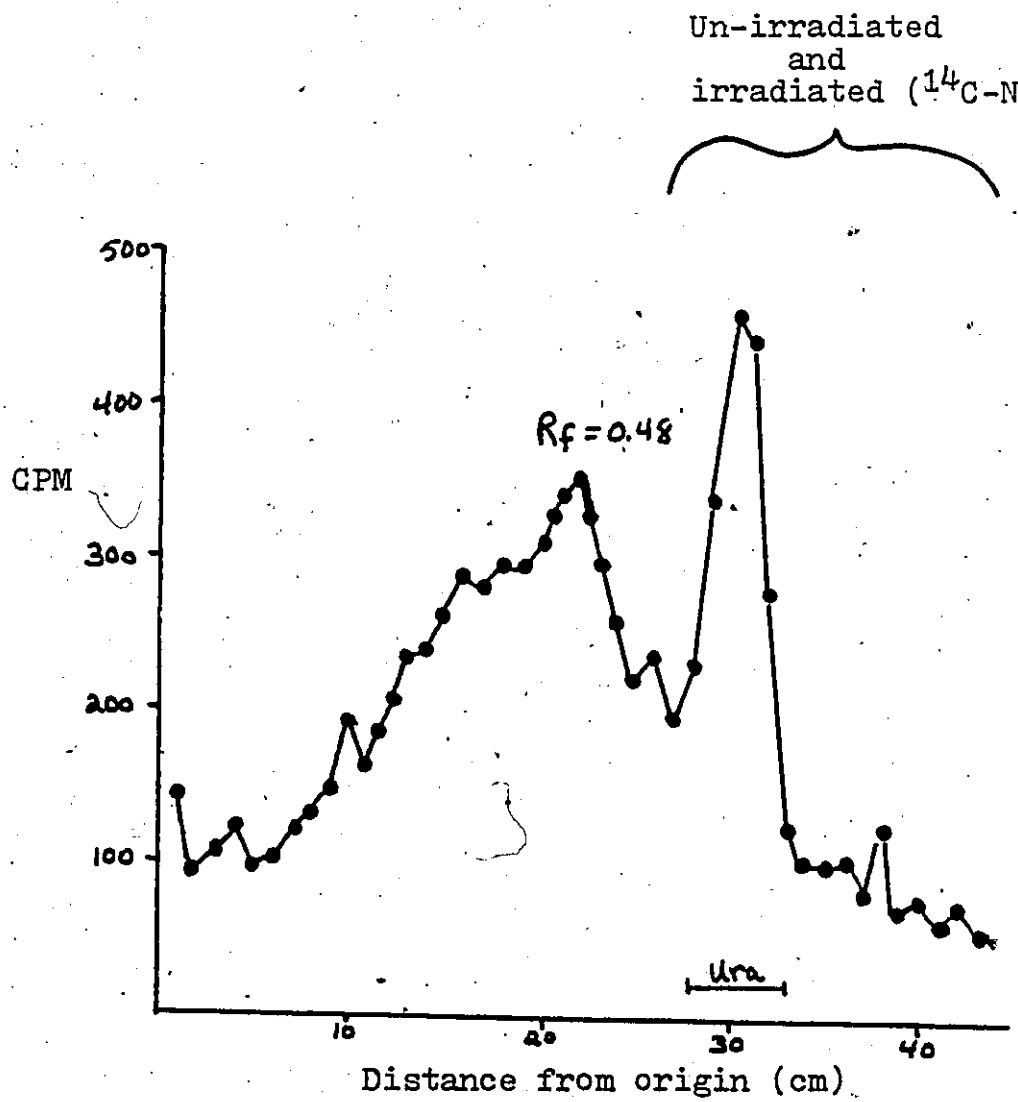
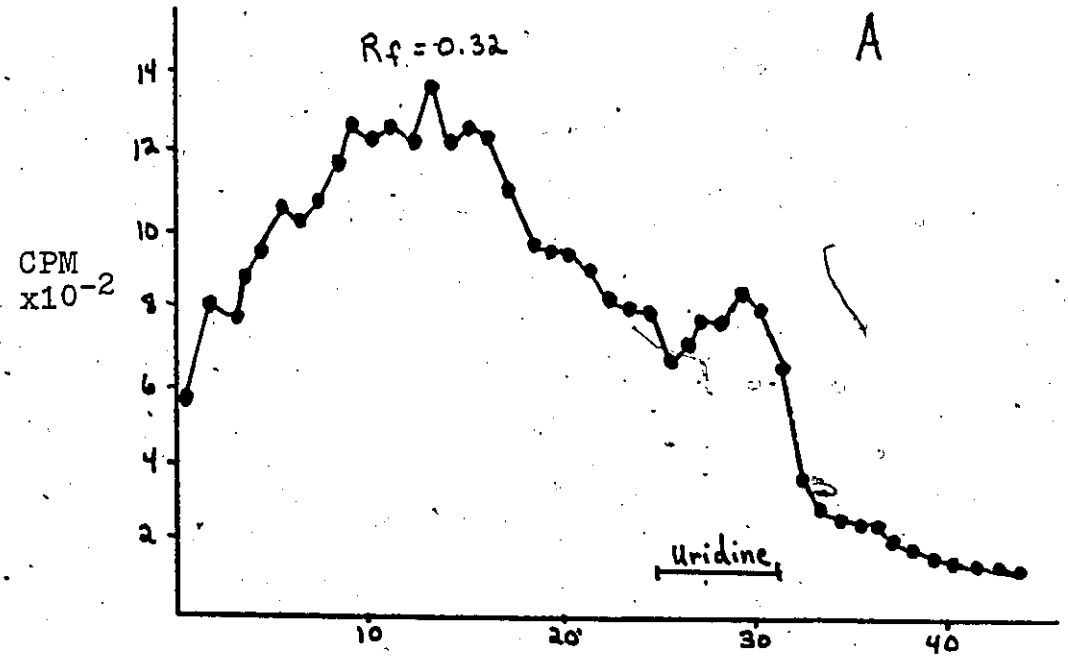


Fig. 20. Uracil (2mg/ml) + (^{14}C-NF), irradiated (34 krads) and applied to Whatman No. 3MM paper eluted with 1-propanol : concentrated ammonium hydroxide : water (55:20:25).



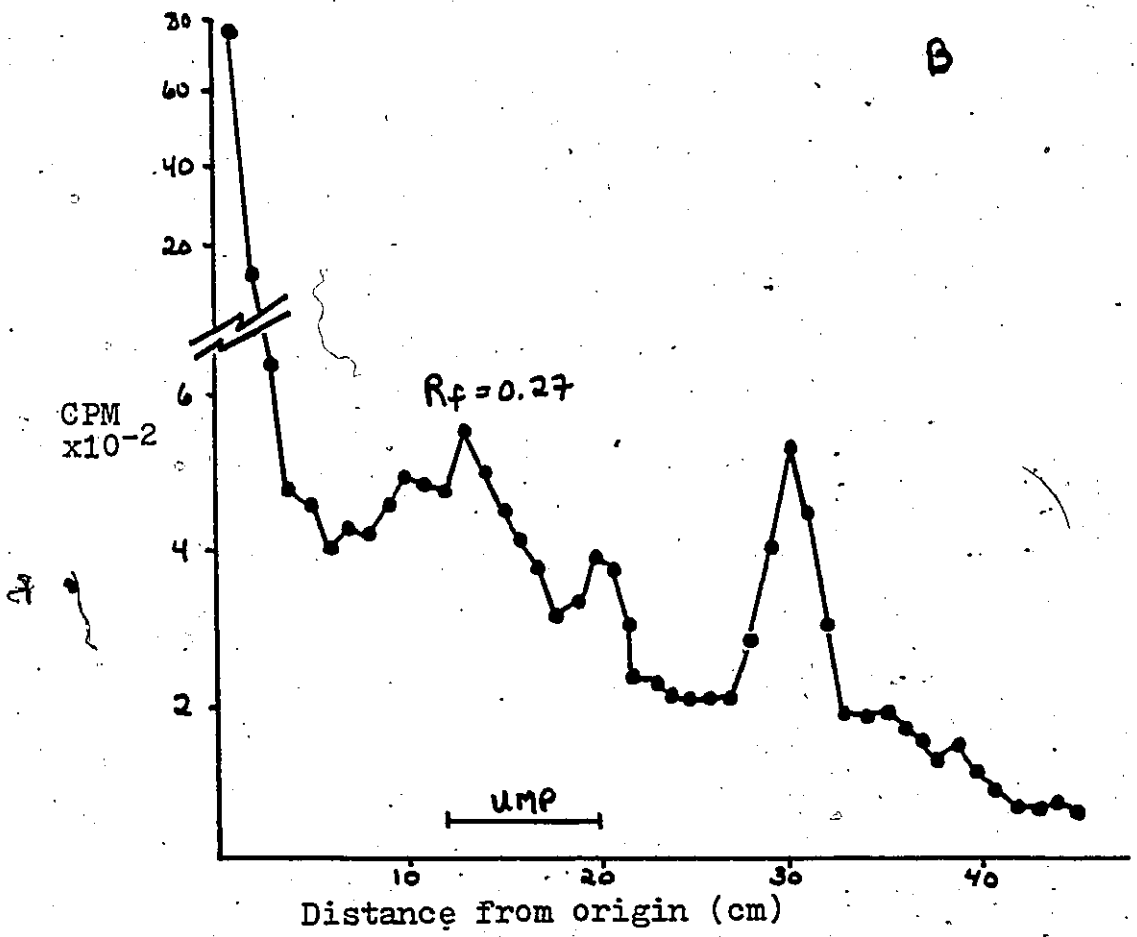


Fig. 21. Irradiated samples (34 krads) applied to Whatman No. 3MM paper and eluted with 1-propanol : conc. NH_4OH : water (55:20:25).
A - Uridine (10mg/ml) + (^{14}C-NF)
B - UMP (10mg/ml) + (^{14}C-NF)

differences in their radioactivity profiles (Fig. 22 and 23). This either indicates that the base was not present in these adducts, or that the number of radioactively-labelled adducts was too small to be visible (this is in fact very likely possible even if the base was present).

XI - Analysis of (2-^{14}C)Uracil-NF Adducts by Paper Chromatography

In a previous study where (2-^{14}C)uracil was irradiated under hypoxia, it was shown that various radiolysis products of uracil could be separated by paper chromatography with n-butanol: water (86: 14) (see Fig 24a). When the sensitizer, p-nitroacetophenone (PNAP), was present during irradiation the amount of one of the radiolysis products (uracil glycol) was increased, while the dimeric product (of which uracil glycol is a precursor) was decreased (28).

To see the effects with NF we first repeated with PNAP the experiment just mentioned to determine what could be observed under our conditions. Because of impurities in the (2-^{14}C)uracil, it was first purified by application on paper eluted with n-butanol: water (86: 14), and the pure compound isolated and irradiated with uracil (10mg/ml) with or without PNAP (4μg/ml). Though no separation of radiolysis products was visible (see Fig. 25), a small increase in radioactivity could be seen at the R_u expected for uracil glycol (R_u = 0.25), when PNAP was present. The

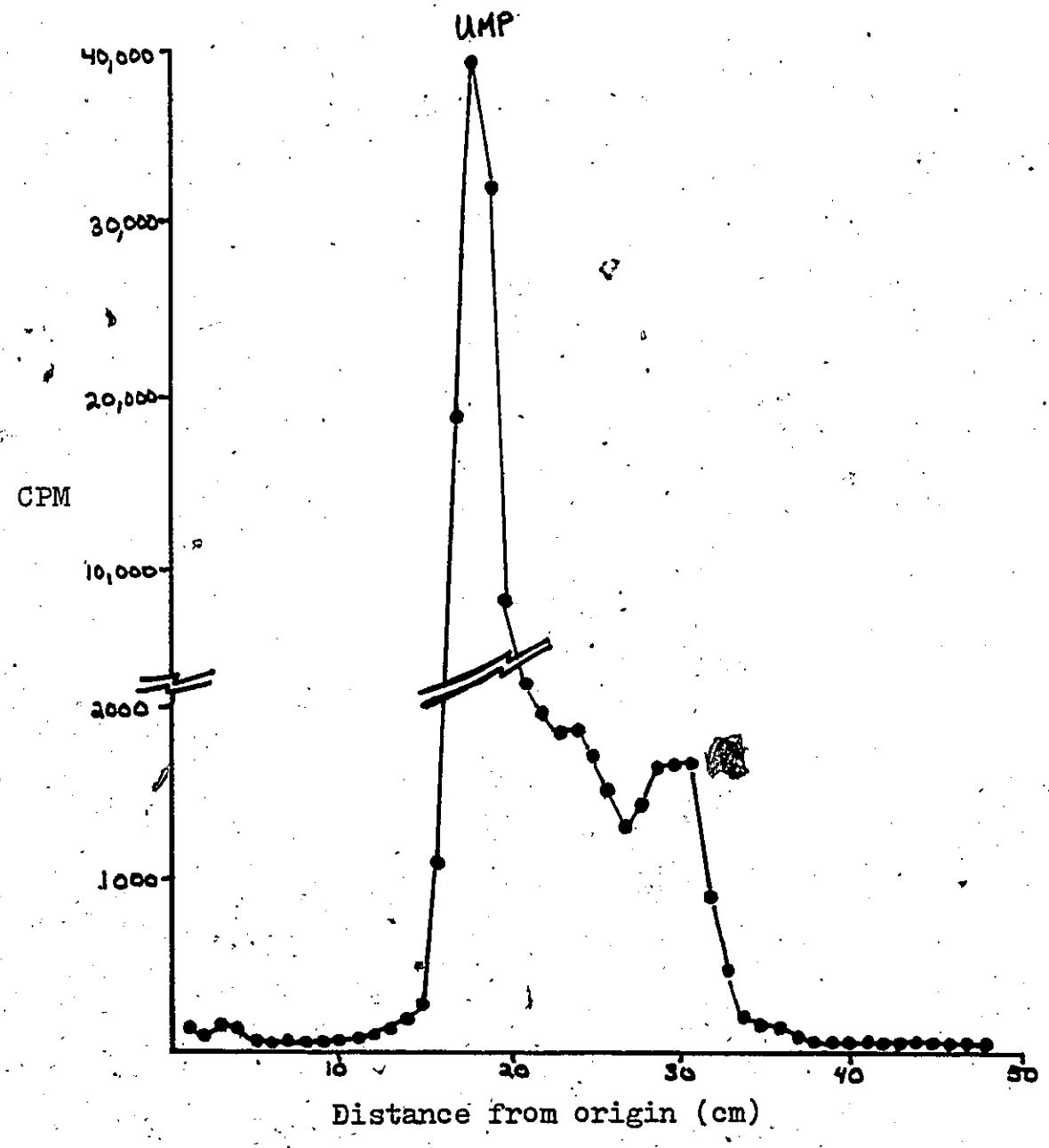


Fig. 22. ($U^{14}C$)-5'-UMP (10mg/ml) irradiated with 34 krads, applied to Whatman No. 3MM paper and eluted with 1-propanol : concentrated ammonium hydroxide : water (55:20:25).

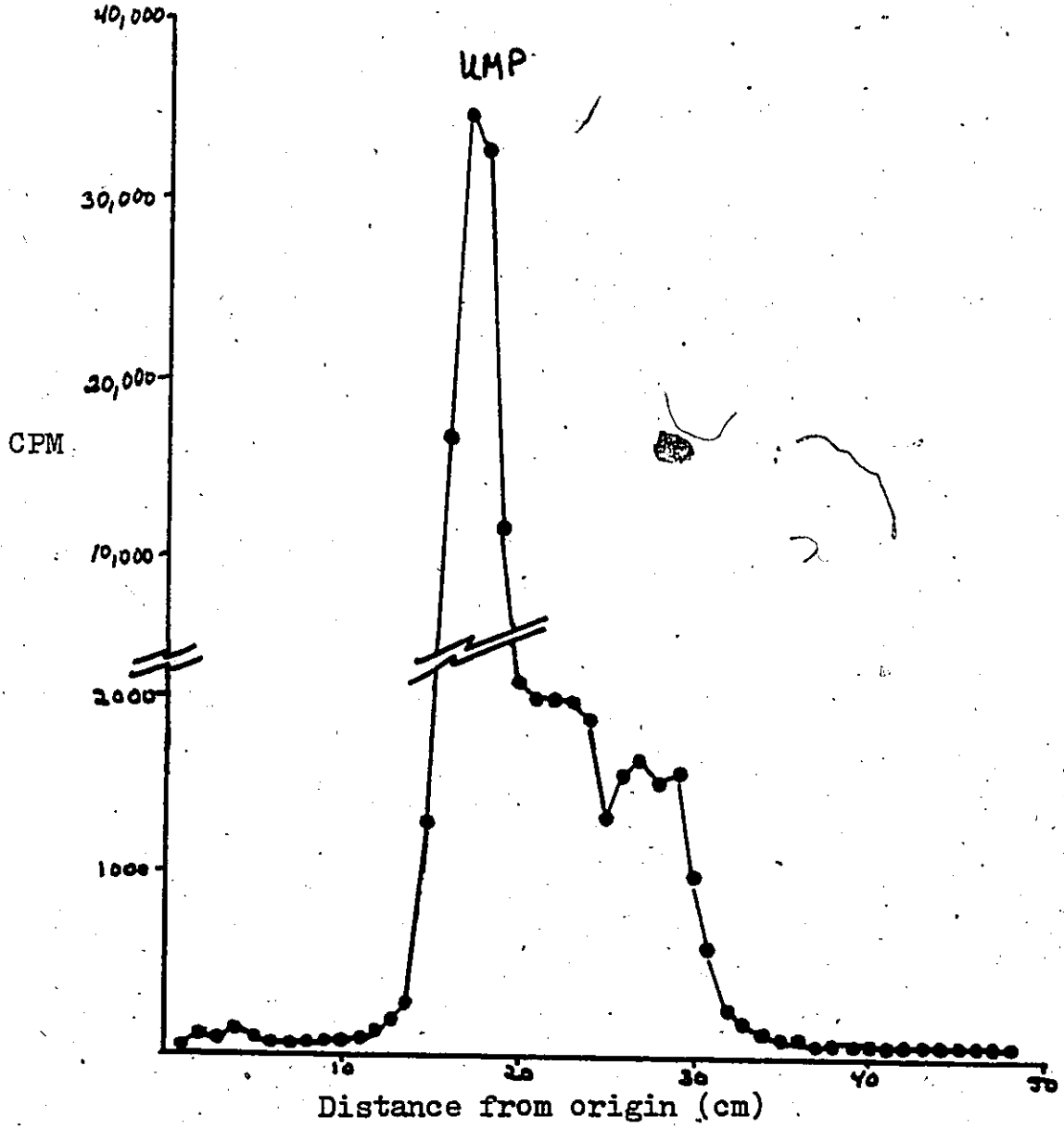


Fig. 23. (U-^{14}C)-5'-UMP + NF(20 µg/ml) irradiated with 34 krads, applied to Whatman No. 3MM paper and eluted with 1-propanol : concentrated ammonium hydroxide : water (55:20:25).

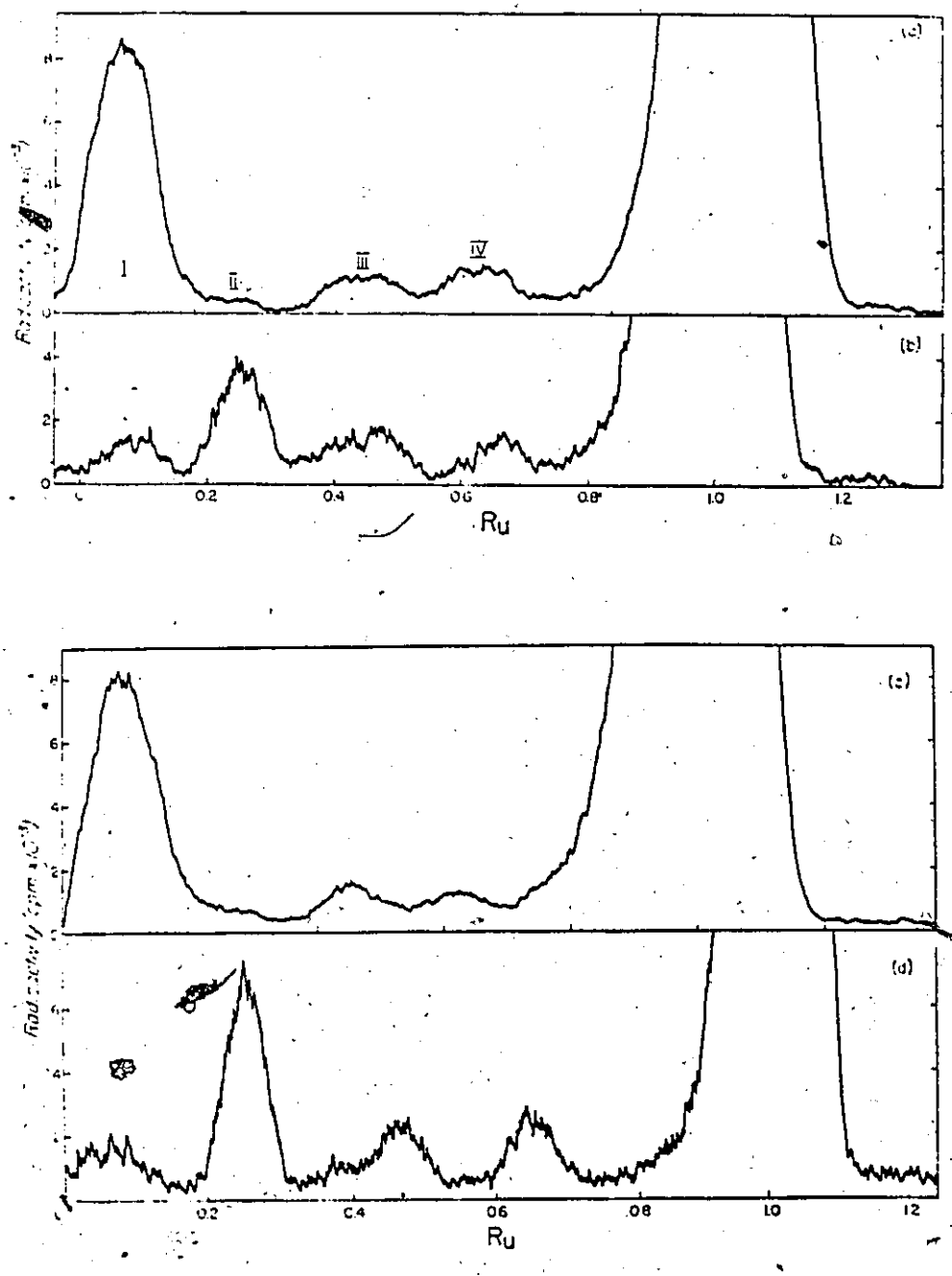


Figure 24 Relative distribution of radioactivity on paper chromatograms developed in *n*-butanol : water (86 : 14) of uracil (2×10^{-3} M) exposed to ionizing radiation (75 krad) in aqueous solution at pH 5. (*a*) in N_2, (*b*) in N_2 in the presence of PNAP (5×10^{-4} M), (*c*) in N_2O, (*d*) in N_2O in the presence of PNAP (5×10^{-4} M). The chromatograms were scanned using a Nuclear Chicago strip scanner. R_u represents the relative R_f values with respect to that of uracil. The major peaks (I, II, III and IV were identified as due to, the dimer-type product, uracil *cis* glycol, the *trans* glycol and 6-hydroxy-5,6-dihydro uracil respectively. The radioactive area with R_u of 1 corresponds to uracil.

(Taken from A.J.Varghese: Int. J. Radiat. Biol. 28, 477 (1975).)

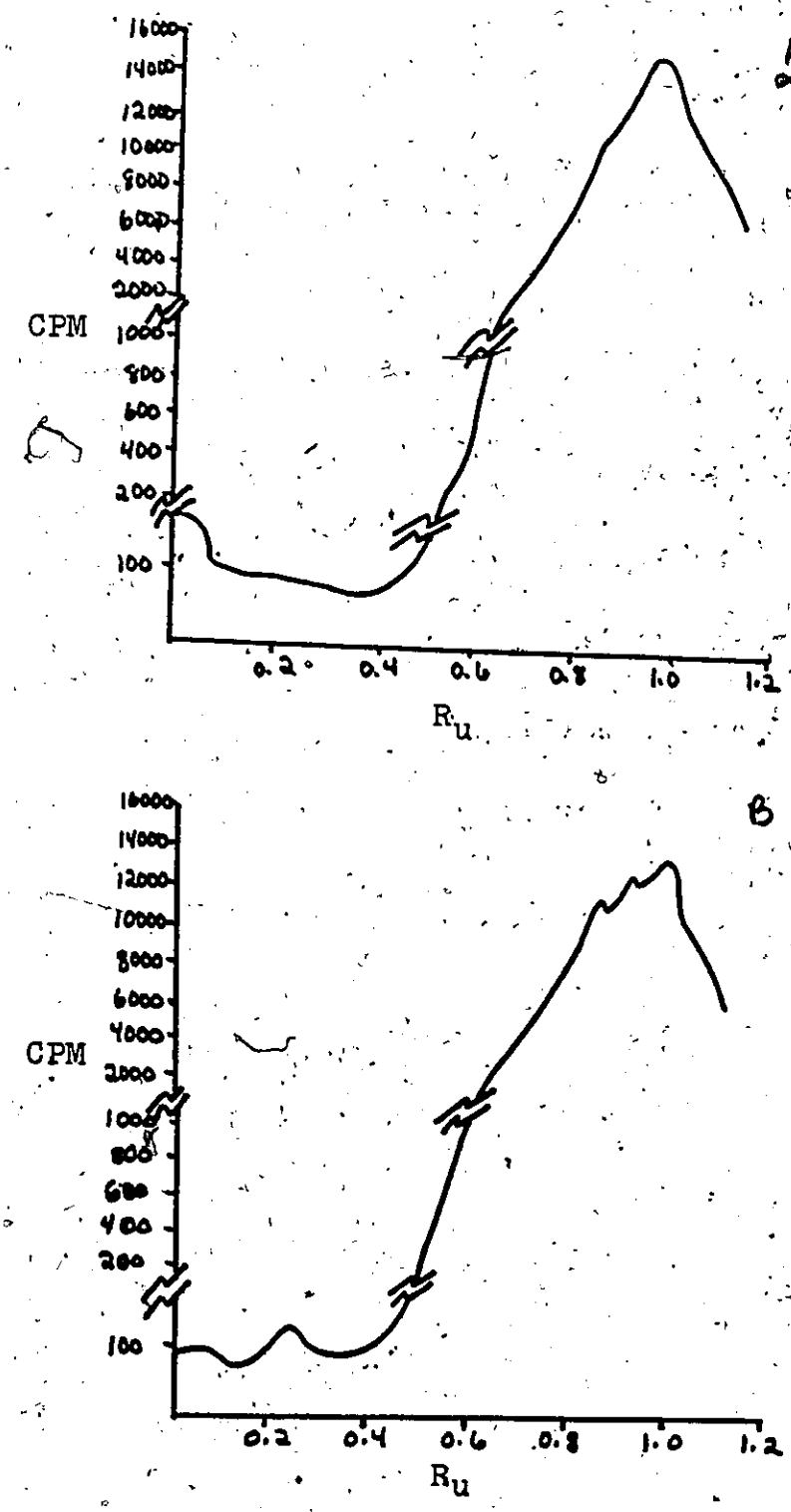


Fig. 25. (2-^{14}C)uracil ± PNAP Y-irradiated, applied to Whatman No. 3MM paper and eluted with n-butanol : water (86:14).

A - (2-^{14}C)uracil (2mg/ml).

B - (2-^{14}C)uracil (2mg/ml) + PNAP (4µg/ml)

R_u = R_f values relative to uracil.

results for the same experiment with NF are shown in Fig. 26. Again, a small increase at the expected R_u is observed with the sensitizer. Therefore, NF may induce an increase in uracil glycol production as does PNAP.

XII - NF-Induced Single-Strand Breaks (SSB) and NF-Binding to DNA

In this experiment the determination of NF-induced SSB in excess of NF binding was considered a possible approach in the examination of NF-induced damage of DNA during irradiation, other than adduct formation. λDNA was the DNA of choice because it is a short, well-defined, double-stranded molecule which is commercially available both in unlabelled and ^3H-labelled form. Samples (0.26 ml) of λDNA with (^{14}C-NF); (^3H)λDNA; and (^3H)λDNA with NF were γ-irradiated, and the (^{14}C-NF) mixture dialysed and counted to estimate (^{14}C-NF) binding. The other two samples were sedimented on alkaline sucrose gradients (5-20%) for estimation of breaks. Fig. 27 shows typical alkaline sucrose gradient profiles with such samples. It appears that at the given radiation dose (5.7 krads) radiation-induced breaks caused the λDNA to sediment much more slowly than native unirradiated λDNA, while NF-treated irradiated λDNA ran slightly more slowly than irradiated λDNA indicating the presence of additional, NF-induced breaks. The λDNA may not be totally homogeneous at the start of the experiment (as can

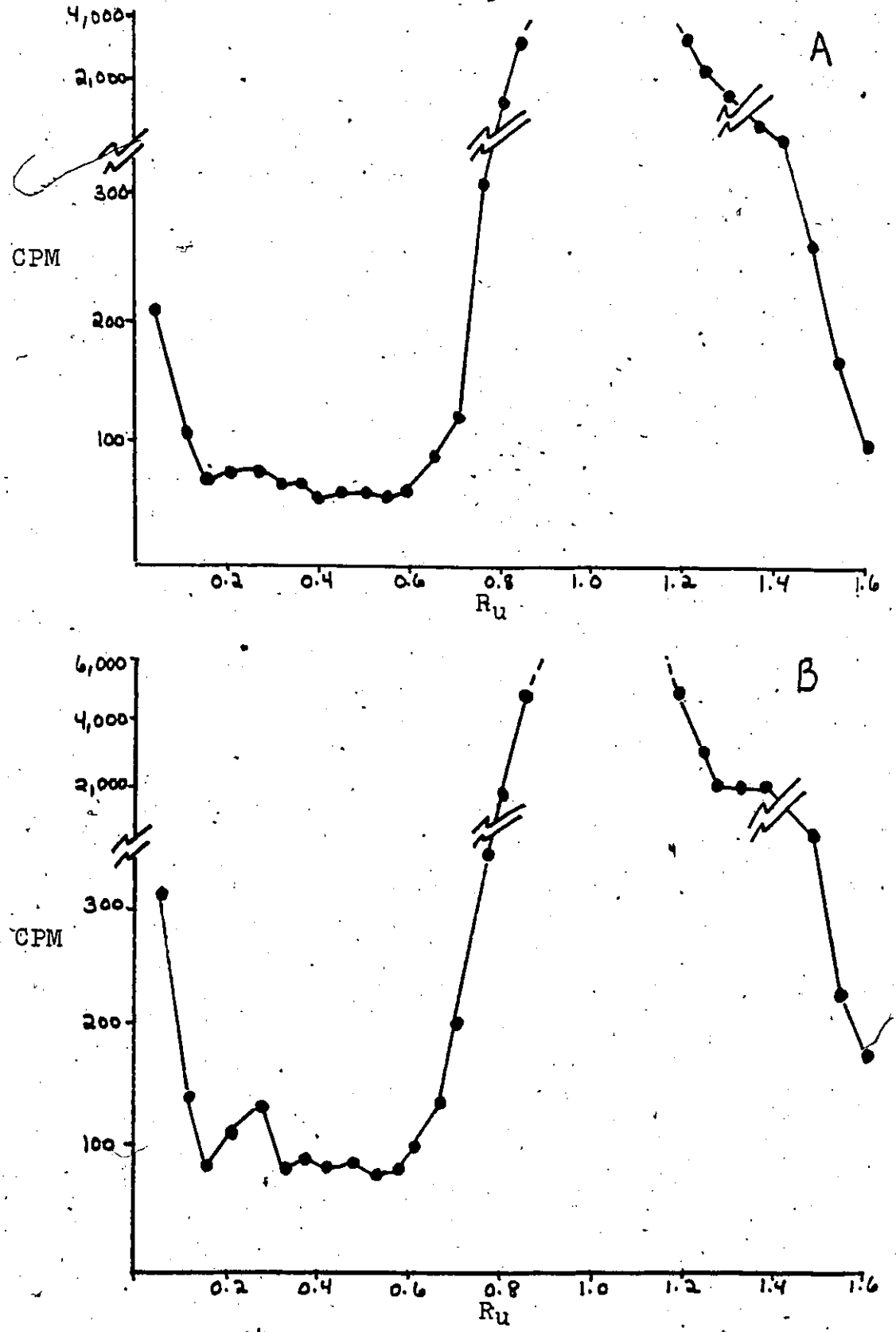


Fig. 26. (2-^{14}C)uracil ± NF γ-irradiated, applied to Whatman No. 3MM paper and eluted with n-butanol : water (86:14) ; R_u = R_f values relative to uracil.
A - (2-^{14}C)uracil (2mg/ml).
B - (2-^{14}C)uracil (2mg/ml) + NF (4μg/ml).

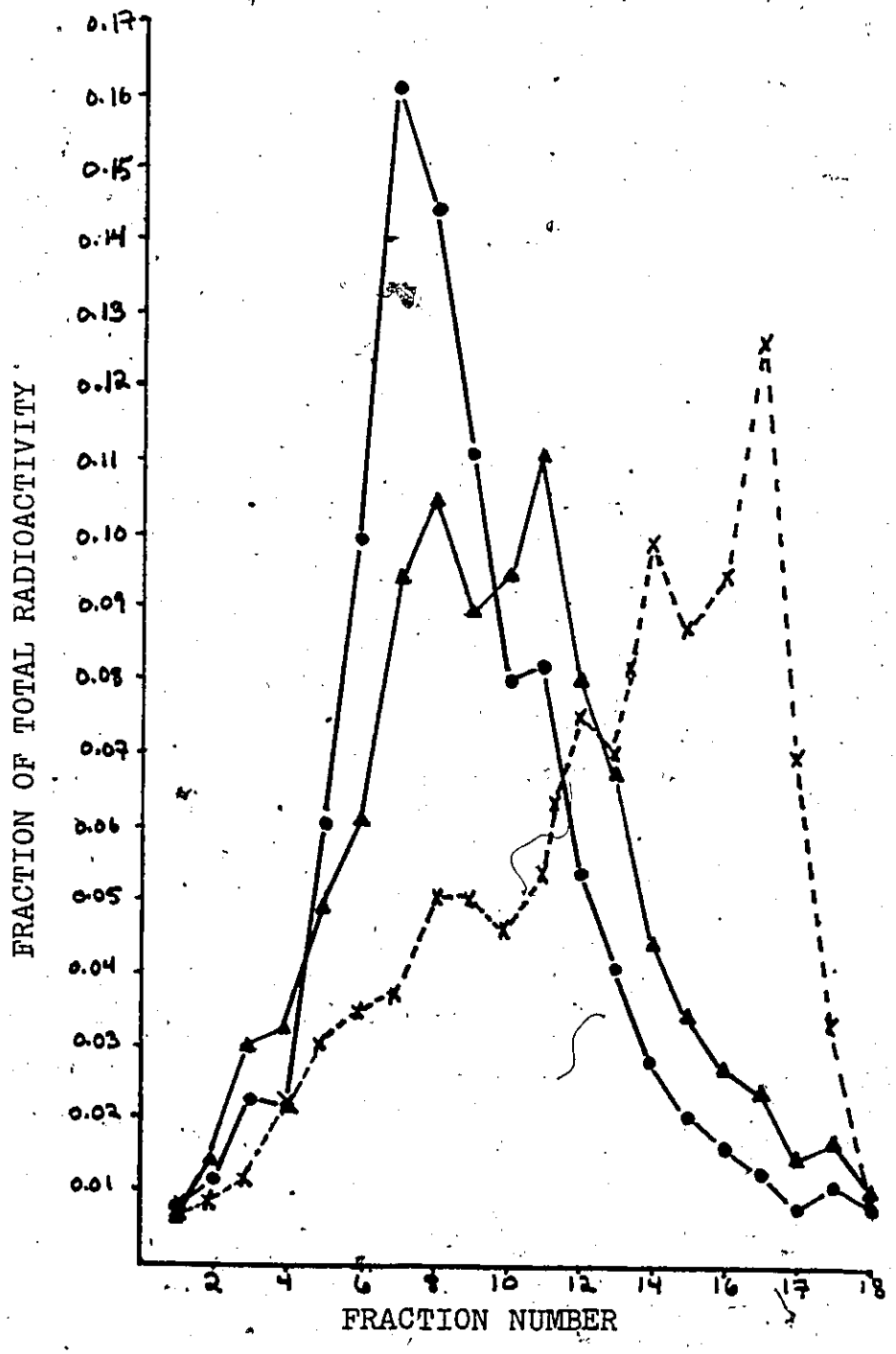


Fig. 27. Alkaline sucrose gradient sedimentation of irradiated λDNA (0.2mg/ml, 5.7krads) with and without nitrofurazone.

—▲—λDNA irradiated.
—●—λDNA + NF (8μg/ml)(0.04μmole/ml)
--×--Un-irradiated λDNA control.

be seen from the tailing end of the control λDNA in Fig. 27). In the presence of nitrofurazone there was consistently an an increase in the production of radiation-induced SSB (Table 3) (i.e. 2.7-4.9 NF-induced SSB / 10^5 nucleotides, or 11.8-21.6% increase of SSB). The ratio of NF-induced SSB to NF molecules bound (0.64-1.08) appeared to increase with dose, indicating that NF-induced SSB may be more sensitive to increases in dose than NF-binding, or that the margin of error resulted in similar values. When considering these results one must keep in mind that the calculations are subject to a considerable margin of error since the NF-induced SSB were obtained by subtraction of the γ-induced SSB (e.g. 9.8) from the γ- and NF-induced SSB (e.g. 12.5). The results indicated that the number of SSB produced by NF is less than or equal to the amount of NF molecules bound.

DNA conc. (mg/ml)	Total dose. (krads)	DPM bound / 0.05mg DNA.	(^{14}C-NF) molecules bound / 10^5 nucleotides.	γ- & NF-induced SSB / 10^5 nucleotides.	γ-induced SSB / 10^5 nucleotides.	NF-induced SSB / 10^5 nucleotides.	% increase of SSB due to NF.	NF-induced SSB / NF molecule bound.	Ave. M_n x 10^6	
									γ	γ + NF
0.19	5.7	215	4.2	12.5	9.8	2.7	21.6	0.64	2.57	2.09
0.19	8.5	215	4.2	13.6	10.9	2.7	19.8	0.64	2.35	1.94
0.38	34	226	4.5	41.7	36.8	4.9	11.8	1.08	0.82	0.69

TABLE 3. Formation of DNA-adducts and induction of single-strand breaks (SSB) by nitrofurazone (NF) when γ-irradiated with λDNA. M_n of denatured DNA is 16 x 10^6.

Specific activity of (^{14}C-NF) = 13.8 C_i / μmole.

DISCUSSION

In studies of nitrofurans as radiosensitizers, radiation-induced binding of nitrofurans to DNA has been found to occur in the absence of oxygen (90,92). In this study, 0.7 molecules nitrofurazone (NF) were bound per krad per 10^5 nucleotides, which is comparable to another study's findings of 1.0 molecule NF per krad per 10^5 nucleotides (90). It was also confirmed in this study that, in the presence of oxygen, no observable binding of NF to DNA occurs. The highly electron-affinic oxygen molecules apparently compete much more efficiently than nitrofurans for binding sites on DNA. Therefore, effective radiation-induced binding of NF to DNA occurs only under hypoxia.

The experiment which demonstrated the need for both components (NF and DNA) to be present during irradiation for binding to occur (Table 2), supports the belief that the reactions involved in the binding of NF to DNA are of a fast free-radical nature. Indeed, NF-binding to DNA has been shown to involve principally secondary radicals on the DNA itself, which are formed from HO· originating from the radiolysis of water (92). In support of the radicals involved being on DNA, is the fact that the extent of binding has been found to be independent of NF concentration while directly proportional to DNA concentration (93).

Through the use of dialysis, and ethanol precipitation

and extraction with phenol of DNA-NF adducts , previous work has eliminated the possibilities of ionic or hydrophobic interactions as being responsible for the binding in DNA-NF adducts. Along with this, the relative stability of the adducts which has been demonstrated in this study indicates the newly formed bond is probably a covalent one. In fact, a covalent bond is likely in the case of binding between an electron-affinic molecule and a free-radical.

As noted in the Introduction, one of the objectives of this study was to isolate and characterize one or more of the radioactive adducts which are produced when DNA is γ-irradiated in the presence of ^{14}C-NF. This objective has not been achieved and indeed the bulk of the results presented above illustrates the complexity of the products which are formed in such situations. Some of the complexities had been observed in an earlier study where a method was worked out for separating the ^{14}C-labelled adducts, found in enzymatic hydrolysates of DNA, from the four normal nucleosides (93). However, this separation is now shown to be inadequate for insolation of adducts since radiolysis products of DNA also elute in the same fractions as the adducts. This was seen from the radiolysis products of the DNA containing ^{3}H-thymidine (Fig. 8).

Analysis of (^{14}C-NF)-DNA nucleoside digests on a cation exchange column (Fig. 5) has shown that one major NF-containing component(s) (peak I) did not interact with

the resin, while two minor components (peak II and III) were slightly retarded. By analysis on Sephadex DEAE A25, peak I has been shown to be a mixture of acidic compounds (i.e. they were retarded on this anion exchange column)(93). The multicomponent nature of this peak is not surprising since various products not retarded by the cation exchange column would elute together at the "void volume". Though separated from the normal nucleosides the adducts were not successfully separated from the DNA radiolysis products at this point. Structural similarities may in fact exist between the NF-derivatives and the DNA radiolysis products so as to make separation very difficult. On the other hand, both peaks II and III seem to represent single products unassociated with UV absorbing material nor apparently with radiolysis products. Peak II is probably a neutral compound since it is only slightly retarded by the cation exchange column (may involve non-ionic interactions), but is totally unretarded by the anion exchange column (Fig 6). Peak III may be slightly acidic since it was only slightly retarded by the cation exchange column but was also retarded by the anion exchange column, being eluted soon after the gradient was begun (93).

Experiments with DNA radiolabelled with (^{3}H-Me) thymidine (Fig. 8) have not succeeded in showing any adducts containing the thymine base. Considering the wide variety of adducts present, some must contain the ^{3}H-thymine

base, however the yield of adducts is probably too small relative to thymine radiolysis products to be distinguishable. However, the experiment did hint at NF-induced products containing thymine which does not involve stable NF binding.

Experiments with the nucleoside (^{3}H-Me)thymidine (Fig. 9) gave similar results in that they did not demonstrate the presence of thymine in adducts, though there was an excess of thymine ^{3}H counts which might indicate thymine-containing adducts or some other type of NF-induced products. The other NF-induced products unrelated to adducts (i.e. which lack ^{14}C counts hence are not adducts), which were seen to run close to dT in the (^{3}H-Me)thymidine-DNA experiment (Fig. 8), were not seen with the radiolabelled nucleoside. This may be due to the use of a nucleoside as opposed to a nucleic acid. Different radiolysis products are obtained with DNA than with its constituents (13). Also, the phosphate group might be part of these NF-induced products seen with DNA. The phosphate group might be just a structural part of the product although this is unlikely because the compound is not highly negatively charged, or the phosphate group might play a direct role in the interaction with NF and so be altered (and the negative charge masked)

Chromatography of irradiated and digested samples of ^{14}C-NF with RNA, poly C, and poly U gave a single AG 50WX4 peak at the void volume for all three nucleic acids. For poly U, poly C and to a small extent t-RNA(which is only 30% single-stranded) the lack of minor peaks of a less acidic

nature as seen with DNA could be due to their being single stranded rather than to their having a ribose instead of a deoxyribose moiety. It has been shown that as well as there being 27 % greater γ-induced binding of NF to single-stranded DNA than to double-stranded DNA, the relative amounts of radioactivity in the AG 50WX4 peaks for adducts differed. In fact, the single-stranded DNA showed a greater proportion of peak I (with very little of the other adducts) (93), which is consistent with our results with single-stranded nucleic acids. A single-strand molecule might lead to the greater production and exposure of certain radical sites that might compete more effectively for NF binding than those sites which result in adducts found in peaks II and III. On the other hand, the fact that total binding with these single-stranded nucleic acids was not greater than with double-stranded DNA may be related to the presence of the ribose moiety or absence of deoxyribose. Although the ribose and deoxyribose compounds are not expected to behave very differently on an ion exchange column, a certain adduct(s) formed with NF may be possible with the ribose moiety (e.g. binding at a radical on C_2 of deoxyribose which might not occur in ribose). Of course, peak I for DNA and ribopolymers may contain totally different adducts since little information can be obtained from unretarded peaks. Different ribose products might also explain the lack of smaller, less acidic peaks for t-RNA.

Re-chromatography of hydrolysates of poly U and

poly C NF-derivatives has shown that a variety of neutral to acidic adducts are present. Re-chromatography on DEAE A25 of the (^{14}C-NF)-poly U nucleoside adducts (Fig. 16) has shown two main products: peak B at the void volume and peak E at the gradient concentration 0.25M. Peak E appeared to have very similar structural properies to the major radiolysis product(s) of poly U in that their mobilities were similar. Re-chromatography of peak B and E on DE 81 paper (Fig. 17) has shown that the bulk of the products lack an intact ribose moiety. It is unlikely that the sugar is altogether missing due to an adduct to a free base since dialysis of the polymer before hydrolysis would have removed free bases. However, an adduct on a phosphate group alone might be possible, especially after digestion with nuclease and alkaline phosphatase. It could also be that only part of the sugar is present with C2'-OH and/or C3'-OH missing; or that the hydroxyl group of C2' or C3' has been altered.

Paper chromatography of (^{14}C-NF)-poly U nucleoside adducts (Fig. 18) has confirmed the acidic nature of these adducts because they were found to remain at the origin while the nucleoside migrated. Furthermore, it seems most likely that the negative charge is on the nucleoside part of the adduct rather than on the NF part. This might indicate the presence of a phosphate group in the major adduct(s) due either to the inability of the digesting enzymes to remove the phosphate group near the adduct

leaving nucleotide-like products; or to phosphate-containing NF-derivatives freed from the sugar moiety after digestion. The presence of nucleotide-like products seems more probable in this experiment since they ran like nucleotides in one solvent system (Fig 19A) where nucleotides migrate but inorganic phosphate remains at the origin. However, a nucleotide digest (Fig. 19B) eluted with the same solvent system showed a much greater proportion of counts at the origin. One could speculate that if an NF-derivative on the phosphate group resulted in 5'-phosphate ends, then digestion with ribonuclease A (a 5' endonuclease), might cause the release of free phosphate-containing adducts. These adducts may not be seen in the nucleoside digest because the other enzyme (alkaline phosphatase) used to further the digestion, might also hydrolyse the phosphate-NF bond. A second product(s) (nucleotide-type) could still also be present in both the nucleoside and the nucleotide digest. This gives an idea of the many types of reactions possible during irradiation and the experimental treatments which follow.

Paper chromatography experiments with constituents of poly U (uracil, uridine and UMP)(Fig. 20 and 21) confirmed the results obtained with poly U, giving similar profiles for uridine and 5'-UMP as were obtained with the nucleoside and nucleotide digests respectively. Incidently, the fact that the UMP had 5' phosphate ends still does not

contradict the previously discussed idea of free phosphate-containing NF adducts, as the binding of NF may also be able to cause 5' bond-breaks. In fact, nitrofurans have been shown to increase the amount of radiation-induced release of inorganic phosphate from 5' nucleotides (76). The experiment with uracil (Fig. 20) strongly indicated that there is a base-bound NF adduct(s), which may also be present when uridine or UMP is used.

In retrospect the high degree of complexity of the NF adducts formed with nucleic acids might have been expected because of the wide variety of secondary radicals which are known to be formed when primary radicals interact with DNA. Radiation-induced binding of NF to DNA has been shown to be predominantly due to the sensitizer's interaction with DNA radicals produced by the indirect action of HO· (92). Radicals due to HO· have been found with all the constituents of DNA. That is, there is evidence to support HO·-induced radical sites on the base and sugar moieties (13), as well as possible phosphate radicals (42,43). Therefore, theoretically NF-derivatives may form on any part of the molecule. With pyrimidine nucleotides, HO· mainly reacts by addition across the C_5-C_6 double bond of the base with the C_5 OH-adduct predominating. This supports the suggestion for an NF-uracil adduct seen in this study, and in other studies which have also detected binding of sensitizers to pyrimidines (78). NF could therefore con-

ceivably bind to the C_6 radical site, with this being quantitatively a major adduct produced by NF interaction with DNA. Nevertheless, adducts are no doubt formed on purine bases, and may also be formed on the sugar and phosphate moieties. Two Sephadex DEAE A25 peaks were found common to the four deoxyhomopolymers irradiated with ^{14}C-NF, which suggests a sugar or phosphate adduct (93), as do paper chromatography experiments in this study where products migrated in a way unlike bases, nucleosides or nucleotides. Such adducts (or their precursors; e.g. C_1' radicals) may have led to the release of inorganic phosphate or bases, so that a wide variety of resulting adduct compounds is possible. Further evidence for sugar-bound adducts includes the protection afforded by some sensitizers to the radiation-induced release of inorganic phosphate from nucleotides (76,36); and the fact that nitrofurans have been shown to form stable adducts with nucleophilic alkyl radicals (36).

γ-Irradiation of ^3H-λDNA(32 x 10^6 daltons) under hypoxia gave 1.1-1.7 SSB per krad per 10^5 nucleotides. These values are within the range obtained in other studies including calf thymus DNA irradiated in air (8.5 SSB/krad/10^5 nucleotides)(99); bacteriophage B3 DNA (21 x 10^6 daltons) irradiated in air (9.9 SSB/krad/10^5nucleotides); and PM2 DNA (6 x 10^6 daltons) irradiated in air (0.62 SSB/krad/10^5 nucleotides)(101). It is not surprising that the production

of SSB is greater in air, since oxygen is known to increase radiation-induced SSB production in DNA (with an enhancement ratio of 2.9)(97), and although SSB values may vary amongst different experiments they appear to be contained within a limited range. The small amount of SSB observed is probably reasonable in the light of what we know about SSB verses other damage (including base degradation which predominates) (99).

In the presence of NF we consistently found an increase in the production of radiation-induced SSB in DNA (see Table 3). This indicates that at the concentration used (4×10^{-5}M) NF does not protect against DNA breakage as do the N-oxyl sensitizers which at concentrations as high as 1×10^{-3}M do not increase radiation-induced SSB and, in fact, decrease SSB at higher doses (102). Since N-oxyls are known to interact with DNA during irradiation almost entirely by covalent binding (73), and since they do not cause SSB, this suggests that in addition to binding, NF interactions with DNA may involve oxidative electron-transfer which in turn may lead to SSB. Furthermore, our results showed NF-induced SSB to be 0.64 to 1.08 times the amount of bound NF molecules. Assuming the validity of these values (although they approach a one to one ratio), this may indicate binding of NF to different sites of the DNA with only certain sites leading to SSB and therefore resulting in more binding than SSB. However, it is generally

believed that binding of a sensitizer would protect from rather than cause SSB, this preventing the radical from further deterioration that might lead to SSB (76). On the other hand, a radiation-induced interaction of NF with DNA, unrelated to binding (which may prevail), and involving electron-transfer oxidation may be occurring and leading to SSB. In fact, the ability of nitrofurans for electron-transfer oxidation is well known (72,92); and consistent with this they have been shown to cause the radiation-induced release of inorganic phosphate from mononucleotides (76), as well as to produce SSB in DNA(80). Other experiments in this study suggest that NF may oxidize DNA radicals in DNA, including the ^{3}H-thymidine-DNA experiment which showed an increase in NF-induced products other than adducts (Fig. 8). These products contained the radioactivity from the thymine base, so the oxidizing reaction that took place could not have been one which caused the release of the base. However, other oxidizing reactions could have taken place, such as those causing strand breaks (e.g. oxidation of radicals at C_3' or C_5'; or radicals resulting in ring opening).

The (2-^{14}C)uracil experiment (Fig. 26) in which various radiolysis products of uracil produced with and without NF were chromatographed on paper, indicated that the presence of NF caused an increase in the production of one of the radiolysis products, uracil glycol (keeping in mind that the identification of this product was somewhat

tentative). One proposed mechanism for the higher yield of glycol in the presence of a sensitizer involved the transfer of an electron from a pyrimidine HO· radical to the sensitizer, thereby creating a carbonium ion intermediate which is hydrolysed to the glycol compound (28). This was proposed for PNAP, but from our evidence the same may hold for NF:

$$\cdot PyrOH + NF \longrightarrow PyrOH^{+} + NF^{-}$$

$$PyrOH^{+} + H_2O \longrightarrow Pyr(OH)_2 + H^{+}$$

This may be further evidence to involve oxidative electron transfer as one of the chemical interactions NF undergoes with nucleic acids under γ-irradiation.

The types of DNA damage (i.e. NF-binding and NF-induced SSB) we detected with radiation also occurs when NF is enzymatically "activated". Though binding is proportional to NF concentration, at 30μM the extent of binding of activated NF to DNA (2.5 NF molecules/10^5 nucleotides) was similar to our results (4.2 NF molecules/10^5 nucleotides). While the amount of SSB at that concentration was not calculated, it was estimated to be less than 0.095 per 10^5 nucleotides, thus giving a ratio greater than 25.9 molecules bound per SSB (96). Since in my system this ratio is 1 to 1.6, the extent of SSB seems to be greater in the radiation-induced rather than the enzymatically activated system. The chemical interactions in the two systems are different in that the enzymatically-activated NF yields a reactive

NF-derivative which reacts with normal DNA (probably entirely by binding), whereas in the radiolysis system unchanged NF reacts with radical sites in DNA where binding or other reactions may occur. The enzyme-activated system is in fact a much simpler system of adduct formation since guanine appears to be the principle site of reaction, and the adduct pattern is a simpler one showing two major peaks which appear pure on HPLC (103). In contrast, the complexity of the products which are formed in our system requires more effective means of separation than have been used to date.

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