STUDIES ON THE ACTIVATION OF NITROFURANS

By

SHYAMA SHARON RAMCHARITAR, B.Sc.

A Thesis

Submitted to the School of Graduate Studies,

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

September 1980

STUDIES ON THE ACTIVATION OF NITROFURANS

MASTER OF SCIENCE (1980) McMASTER UNIVERSITY

(Biochemistry) Hamilton, Ontario

TITLE: Studies on the Activation of Nitrofurans

AUTHOR: Shyama Sharon Ramcharitar

SUPERVISOR: Professor D.R. McCalla

NUMBER OF PAGES: xvii; 161

ABSTRACT

Nitrofurans, initially developed in the 1940's as antibacterial agents, have recently been shown to have mutagenic and carcinogenic effects. Nitrofurans exert such effects after metabolism by bacterial and mammalian nitroreductases to produce short-lived, electrophilic intermediates which react with nucleophilic centres of protein and DNA. To characterise the putative intermediates of nitrofurazone, an *in vitro* activation system was used in which synthetic L-lysine peptides were used to trap the reactive species. Present studies indicate that a small amount of binding of 'activated' nitrofurazone to lysine does occur but the amount of adducts formed is insufficient to permit structural analysis.

The bacterial nitroreductases are of two types: type I nitroreductase is active under aerobic conditions while type II is active only under hypoxic conditions. Analysis of crude extracts of type I reductase of wild-type and mutant strains of *E. coli* K12 by chromatography on DEAE-cellulose has resulted in the separation of three distinct activities which catalyse the reduction of nitrofurans probably by different enzymatic pathways. Two of the activities were previously identified but the third

is apparently inactivated by $MnCl_2$ which has been used as a partial purification step in the past.

The mutagenic potencies of nitrofurans vary over a wide range: for the four nitrofurans, AF_2, ANFT, furazolidone and nitrofurazone, there is an 500-fold difference between the strongest and weakest mutagen. The overall rate of reduction and the kinetic constants of the type I nitroreductases with the four nitrofurans as substrates do not vary by more than a factor of three and thus cannot account for the observed differences in mutagenicity, however, the stronger mutagens do produce a larger yield of macromolecule-nitrofuran adducts than the weaker mutagens. Therefore, mutagenicity appears to be related to the stability and electrophilicity of the 'activated' nitrofurans.

ACKNOWLEDGEMENTS

The author wishes to express her gratitude to Dr. D.R. McCalla under whose supervision this research was carried out. Special thanks are also extended to Byron Wentzell, Douglas Bryant and Robert James for their advice throughout this project.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND COMMON NAMES

Nitrofurans

AF_2	2-(2-Furyl)-3-(5-nitro-2-furyl)-acrylamide
ANFT	2-Amino-4-(5-nitro-2-furyl)thiazole
FANFT	N-(4-(5-Nitro-2-furyl)-2-thiazolyl)-formamide
Furazolidone	N-(5-Nitro-2-furfurylidene)-3-amino-2-oxazolidin-2-one
Nitrofuroic acid	5-Nitro-2-furoic acid
Nitrofurantoin	1-((5-Nitrofurfurylidene)amino)-hydantoin
Nitrofurazone (NF)	5-Nitro-2-furaldehyde semicarbazone

Chemicals and Terminology

CM	carboxymethyl
cpm	counts per minute
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
E.S.R.	electron spin resonance
KCl	potassium chloride
$MnCl_2$	manganous chloride
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate

NFR	nitrofuran resistant
nfs	nitrofuran sensitivity
Sil 41	Silent 41
TCA	Trichloracetic acid

1. INTRODUCTION

I. Chemical Carcinogenesis

Chemical carcinogenesis is obviously a very important subject in this modern age when apart from existing chemical pollutants, hundreds of new chemicals are being introduced into our environment annually. Recent advances in the areas of epidemiology and geographic pathology have established that 60 to 90% of all human cancers have important environmental factors in their etiologies (65). Since oncogenic viruses are not highly contagious and since cosmic rays and ultraviolet light are fairly uniformly distributed, chemicals are presumed to be the predominant environmental carcinogens (2).

The large list of compounds that are proven carcinogens in man is gradually increasing and, unfortunately, many of these compounds were incriminated as human carcinogens as the tragic result of industrial exposures. This impressive variety of chemicals are made up of a number of vastly different chemical structures, and although no single molecular feature can be pinpointed as cancer producing, some common features of chemical reactivity can be identified (1). Therefore, knowledge of

the metabolic fate of these chemicals is necessary to provide clues as to the underlying mechanisms of chemical carcinogenesis and to assess the carcinogenic risk involved through exposure to such compounds.

Several observations have suggested that the metabolism of chemical carcinogens might be a key factor in their carcinogenic activities. An early clue to the possible metabolic activation of a carcinogen was the finding that a derivative of N,N-diethyl-4-aminobenzene binds covalently to the hepatic proteins of rats fed this dye (1). Subsequent studies indicated that certain proteins of the target tissue had marked specificities for binding of this carcinogen (2). Since that time the administration of all chemical carcinogens that have been adequately studied has yielded DNA-, RNA-, and protein-bound derivatives in the target tissues (4).

These studies led to the generalisation that the great majority of chemical carcinogens were active only after metabolism to 'ultimate' carcinogens, that is, the derivatives that actually initiate the neoplastic event (3). Further, a consideration of the literature on the known and postulated reactive forms suggested that most of the compounds are converted in vivo into potent electrophilic reactants. Thus, these ultimate carcinogens, containing relatively electron-deficient atoms, initiate the

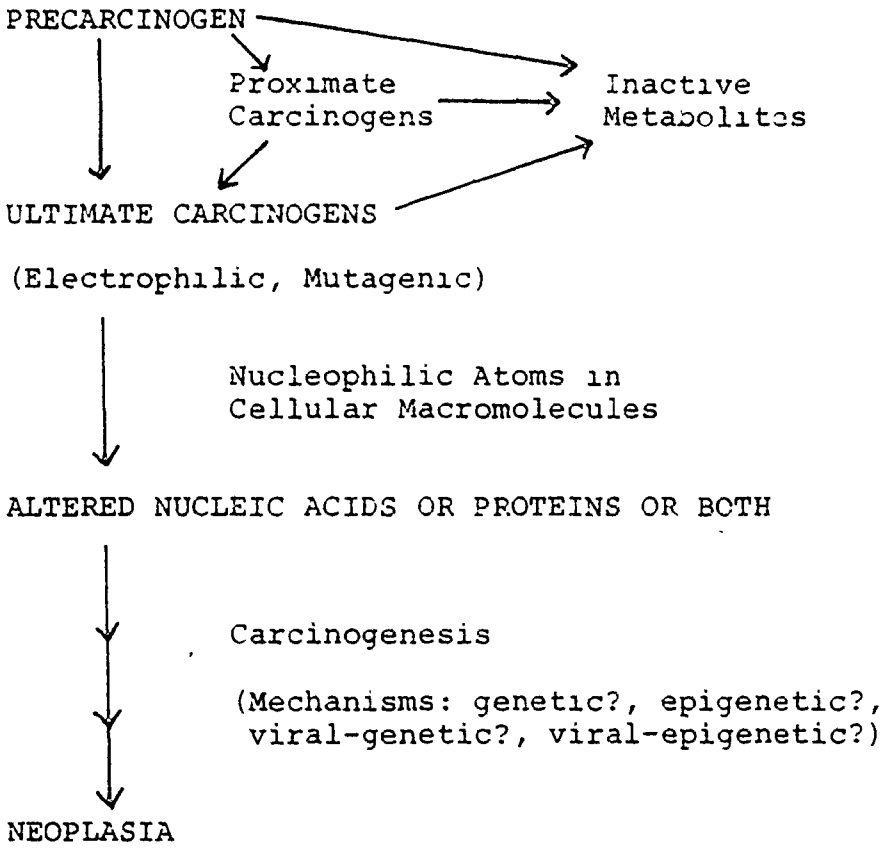
carcinogenic process through certain of their reactions with nucleophiles in crucial tissue components, such as the nucleic acids and proteins as shown in Figure 1 (5).

These strong electrophilic reactants differ in at least one important aspect from the normal cellular nucleophiles: the 'normal' electrophiles and nucleophiles are enzymatically controlled when they react whereas those derived from chemical carcinogens generally attack non-enzymatically and with relatively little discrimination. Thus a large proportion of the ultimate carcinogens, that is, the active form(s) probably reacts with relatively non-critical nucleophiles and only a small fraction perhaps is involved in the initiation of malignant transformation (3). Accordingly, basic problems of great importance today are to determine which of the macromolecule-bound products are important in carcinogenesis and the identification of the specific role of each adduct in the carcinogenic process.

The strong electrophilic nature of ultimate carcinogens is consistent with both genetic and epigenetic mechanisms of carcinogenesis (3). The epigenetic hypotheses have as their fundamental premise that the genomic information of tumour cells need not be altered from that of normal cells of the same organism; the genetic mechanisms assume that the change from normal to tumour cell is dependent on genomic alterations.

FIGURE 1

The overall metabolism of the majority of chemical carcinogens (3)



Complex organisms develop from single fertilised ova - the mechanism of this differentiation is not understood, however, it is a perpetuated process that does not involve a mutation of the DNA, but rather a 'programmed' alteration of gene expression. The theory that oncogenesis results from abnormal differentiation has evolved from this generally accepted fact. If normal differentiation is the consequence of epigenetic phenomena, similar epigenetic modifications of cellular transcription or translation or both may also be involved in the conversion of apparently normal cells to tumour cells with relatively stable phenotypes (4). From what is known about the controls for the expression or repression of information in bacterial genomes, models for the induction of tumours by chemicals through proliferation of cell lines with altered transcriptional controls have been proposed (5).

The epigenetic theory is supported by a variety of studies. The potential for the differentiation of malignant cells to non-malignant cells has been demonstrated for several types of tumours in mice, including cloned teratocarcinoma cells and stem cells from a transplantable squamous cell carcinoma (6); the terato-carcinoma cell genotype was transmittable to the second generation mice through the sperm. Fetal isozymes and other fetal oncodevelopmental antigens are present in a variety of neoplasms (31). This

finding is consistent with the concept that genes normally dormant in the adult tissues are activated during the neoplastic process due to alterations in the repressor mechanisms of the 'fetal' genes (32), that is, an arrest in the normal process of differentiation results in de-differentiation.

The genetic theory of carcinogenesis receives primary support from the fact that the potential of a cell is determined by the information coded in the genome, and genomic changes would be expected to occur most frequently as a result of direct modification of DNA by the carcinogen. Quite compelling evidence exists to show that alterations in genomic information results in the development of tumours (8) and to transformation in cell culture (9). The most direct evidence to support this theory is the studies that show that the extent of alkylation of O^6 of guanine by agents such as ethlymethanesulphonate and N-methyl-N'-nitro-N-nitrosoguanidine correlates well with carcinogenicity (33). Most carcinogens are mutagens, either directly or after metabolism, and the carcinogenesis is often ascribed to their mutagenic effects. Overall, alterations of cellular DNA is currently viewed by many investigators as the most attractive mechanism for the initiation of the carcinogenic process by chemicals. However, other mechanisms are also possible, for example,

changes in the structure of a DNA polymerase that resulted in a more error-prone enzyme could also lead to altered cellular DNAs.

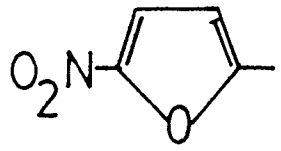
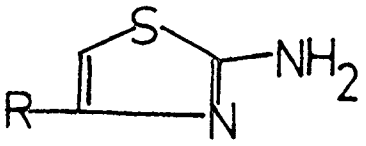
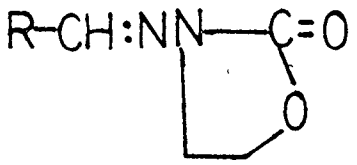
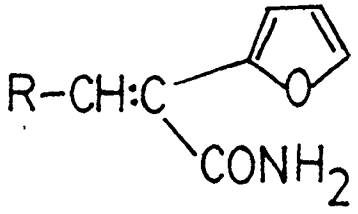
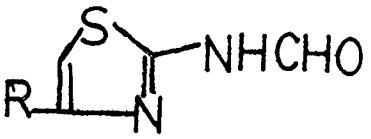
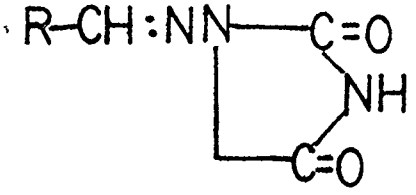
II The Nitrofurans

Uses

A vast number of 5-nitrofuran derivatives have been synthesised, and a variety commercially marketed, since the discovery in 1944 by Dodd and Stillman (10) that nitration of the furan nucleus at the 5-position resulted in a marked increase in antibacterial activity. The corresponding analogues, such as furaldehyde semicarbazone, lacking the nitro group on the furan ring showed a much reduced or absent antibacterial activity (26). Nitrofurans are not known to occur in nature. Representative members of these compounds are illustrated in Table 1 (27).

5-Nitrofurans have found widespread use as food preservatives, food additives for livestock and as topical and systemic antibacterial agents in both clinical and veterinary medicine. The most widely employed topical 5-nitrofuran is nitrofurazone which has been used for the treatment of infections affecting the skin, eyes, ears and gerito-urinary tract (28). Nitrofurantoin is still being administered most frequently for systemic human infections primarily those involving the urinary tract; it is estimated that approximately 5 million courses of this drug are given yearly in the United States and Canada (66). Clinical experience has shown that the primary side effect of

Table 1. The structural features of some representative members of the 5-nitrofuran series (69)

Common Name or Abbreviation	Structure
	R= 
Nitrofurazone	
ANFT	
Furazolidone	
AF_2	
FANFT	
Nitrofurantoin	

nitrofurantoin is lung toxicity which is probably mediated by the superoxide formed during its metabolism (66). Topical use of other nitrofurans can lead to a low incidence of dermatitis (60) while nausea and vomiting are common side effects of oral administration of these compounds (61). Toxic doses of nitrofurans cause testicular atropy and arrest spermatogenesis in the rat, mouse and dog at the primary spermatocyte stage (62).

Nitrofurazone was employed as a preservative in all Japanese meat and fish products from 1950 to 1965 (29). Peripheral neuritis and joint pain led to its replacement in 1965 with AF_2 which was presumed to be less toxic than nitrofurazone. In 1974, the use of AF_2 was discontinued when experimental oncogenicity was reported (67); AF_2 induced mammary tumours and tumours of the forestomach in rats and mice (67). Nitrofurazone has also been considered for use in radiation therapy since its inherent electron affinity gives rise to a radio-sensitising effect in hypoxic mammalian cells (30). However, nitrofurans may not be effective radio-sensitisers in vivo because their rapid metabolism and elimination would reduce the effective level within a solid tumour (68).

Mutagenic and Carcinogenic Properties

A controversy over the use of nitrofurans arose from studies indicating nitrofurazone to be mutagenic in

Escherichia coli in 1964 (34). Later work has revealed that many of the nitrofurans are potent mutagens in both bacterial and mammalian cells. McCalla and Voutsinos (35) used spot tests of a series of twenty-two 5-nitrofurans to detect revertants in E. coli WP_2 trp$^-$ or its uvr A$^-$ mutant. Most were found to be clearly mutagenic although a few were questionably or weakly active. Tonomura and Sasaki (36) reported that nitrofurylacrylamide, furypyrinol and AF_2 caused chromosome breaks, essentially of the chromatid type, in cultured human lymphocytes or fibroblasts. More recently, it has been shown that the mutagenicity and lethalithy of nine nitrofurans in E. coli varies over almost five orders of magnitude (57). Interestingly, nitrofurans induce both single base substitutions (35) and frame shifts.

Many of the studies done on the mutagenicity of the nitrofurans indicate that bacteria are much more sensitive to the mutagenic effects of these compounds than are mammalian cells. This could be due to the fact that the enzymes responsible for metabolism of the nitrofurans have a much higher specific activity in bacteria (12) than in animal cells (63). Also, two different enzyme systems are involved in the metabolism of nitrofurans: type I and type II nitroreductases yield different intermediates with bacterial type I reductase producing a higher degree of protein binding per unit of reduction (12).

A wide spectrum of tumours has been reported in rodents after feeding with nitrofurazone (37,38,39). FNT was non-specific in the production of neoplastic lesions (40) while nitrofurazone and NFT caused predominantly mammary tumours and FANFT was a bladder carcinogen (37). These and many other studies (28) clearly established that many of the nitrofurans, including some to which humans have been exposed, are carcinogenic. In addition, some nitrofurans such as nitrofurantoin are reported not to induce tumours (41). However, the methods used were not adequate and no firm conclusion can be made. Since this nitrofuran is still being used extensively in humans, its close chemical similarity to carcinogenic derivatives, and its possible carcinogenicity is a matter of some importance (69).

Mutation has long been suggested to be one of the mechanisms of carcinogenesis and the hypothesis that cancer is induced by alterations of the primary structure of DNA seems to be well-supported by the correlation of the mutagenic effects, determined by bacterial tests, and carcinogenic effects, within a series of nitrofuran compounds. Despite the wealth of literature on nitrofuran derivatives, the molecular basis of the carcinogenicity of these compounds is not known. However, it has been established that the 5-nitro group is required for anti-

bacterial activity (10) and mutagenic activity in bacteria (35) and metabolic reduction of that group is believed to be also required for carcinogenic activity (40). Variations in the substituent at the 2-position of the furan ring may not affect carcinogenic activity, but cause major differences in organ specificity (42). With few exceptions, the nitrofurans that are carcinogenic proved to be mutagenic, therefore an understanding of the relationship between these processes is of critical importance.

Mechanism of Action

Early investigators revealed that nitrofurans inhibit respiration of intact bacteria (43). Nitrofurazone disrupts glucose metabolism in both bacterial and mammalian systems by a mechanism which appears to involve pyruvate dehydrogenase and glutathione reductase, among other enzymes (43). This effect was observed in both sensitive and resistant bacteria under aerobic conditions. However, the activities of these enzymes in cell-free extract were not affected by nitrofurazone, hence the site of inhibition of glucose metabolism remains unknown. It is likely that other targets are more important in the antibacterial action of nitrofurazone (69). DNA, RNA and protein synthesis are affected by nitrofurans; FANFT inhibits DNA synthesis to a greater extent than protein or RNA synthesis (45).

Since the carcinogenic nitrofurans induce neoplasms at a variety of sites remote from the point of administration, there seems little doubt that they require metabolic activation. Examination of the distribution of nitrofurans in various body fluids has shown that these compounds are capable of binding to plasma proteins, with the extent of binding being a function of their ionic characteristics. In most cases, the nitrofurans have a relatively short serum half-life of 25 minutes (46), thus, low blood levels of these compounds have been related to rapid metabolism and renal clearance. To elucidate the site of degradation, intact nitrofurans in stomach, small intestine and large intestine of rats were quantitated (47). About 80% of AF_2 remained unchanged in the stomach; only traces of the intact nitrofuran remained in the intestines, indicating the latter to be a primary site of degradation. Subsequently, Tatsumi et al (47) found that the responsible enzyme of intestinal mucosa is xanthine oxidase with allopurinol, an inhibitor of this enzyme, markedly reducing the extent of degradation.

Early work established that whole bacterial cells and bacterial cell-free extracts could reduce nitrofurazone as indicated by the disappearance of a characteristic spectrophotometric absorption peak at 375nm and the concomitant appearance of a new peak at 335nm (48).

Although the reductive pathway of nitrofuran metabolism is not completely known, it has been established that the nitro group of nitrofurazone is reduced by pyridine nucleotide-dependent enzymes in animal tissue and bacteria (12,13). E. coli contains at least three nitrofuran reducing enzymes (12). Reductase I (molecular weight ~50,000) is active in the presence of oxygen and uses both NADPH and NADH as electron donors. Reductase IIa and reductase IIb have higher molecular weights and reduce nitrofurazone only under anaerobic conditions in the presence of NADH. Mutant bacteria lacking reductase I are resistant to nitrofurazone suggesting that the intermediates formed during the reduction are more toxic than the nitrofuran per se (13). However, the mutants are susceptible under anaerobic conditions probably as a result of activation of nitrofurazone by the higher molecular weight enzymes, reductase II (12). Mutants lacking reductase I also suffer markedly less damage to their DNA, induction of mutations, binding of nitrofuran to protein and damage to ribosomes than takes place in the wild-type strains (69).

The 'activated' intermediates formed as a result of the reductive metabolism of nitrofurazone are known to react (probably covalently) with a number of cellular nucleophiles (12). In wild-type strains of E. coli, nitrofurans caused alkali-labile damage to DNA, and the number of breaks caused by three quite different derivatives

was roughly correlated with the carcinogenicity of the compounds (45). Type I reductase also catalyses the formation of compounds that bind extensively and covalently to serum albumin (12) which serves as an efficient electrophilic trap. The reduced intermediates formed from nitrofurazone by type II reductase are much less reactive towards protein than those formed by reductase I (12).

In contrast to bacterial systems, where the predominant metabolic feature is the reduction of the nitro group, mammalian metabolism appears to be considerably more complex. When rats were fed nitrofurazone, a number of compounds, such as 5-nitrofurfuraldehyde, aminofurfuraldehyde semicarbazone and 4-cyano-2-oxobutyraldehyde, were found in the urine (53). Several mammalian enzymes are known to be involved in the reduction of nitrofurans including xanthine oxidase (35), aldehyde oxidase (45), and NADPH cytochrome c reductase (35). Maximum rates of reduction generally occur in the presence of NADPH and under anaerobic conditions. It has been estimated that the specific activity of mammalian nitroreductases is at least two hundred times lower than that of reductase I of wild-type E.coli (12).

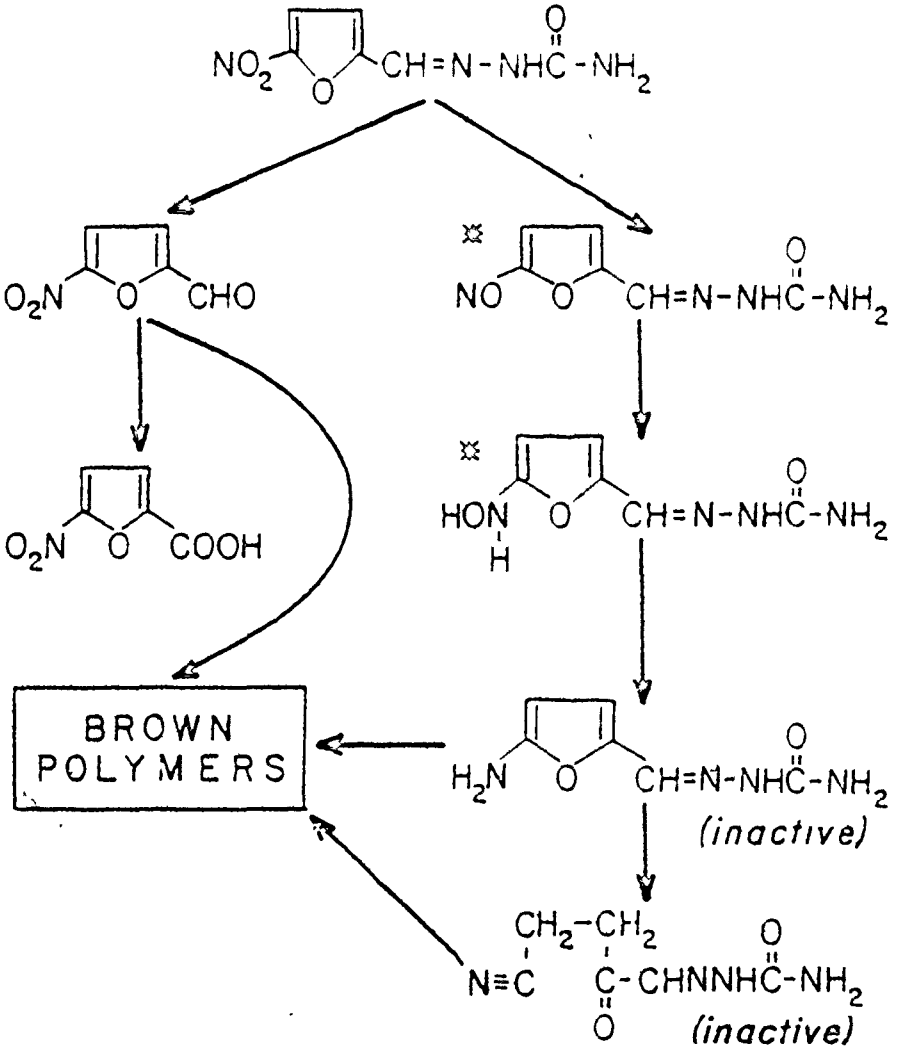
On the basis of spectrometric and polarographic measurements, Beckett and Robinson (23) suggested that the 5-nitro group of nitrofurazone was the primary site of

reduction, yielding the hydroxylamino- or amino- analogues as products of reduction. Furthermore, the end-product of reduction was found to be identical with 5-amino-2-furaldehyde semicarbazone, obtained by chemical reduction in that they had comparable ultraviolet spectra and polarographic characteristics (23). However, in studies with purified xanthine oxidase 5-hydroxyamino-2-furaldehyde semicarbazone (the 4-electron reduction product) seemed a more likely candidate as shown in Figure 2 (49). The proposed pathway is plausible since it is generally believed that the hydroxylamine is the reactive metabolite which is responsible for many of the biological effects of the nitro compounds. For example, the powerful mutagen 4-nitroquinoline oxide (4-NQO) is first reduced to 4-hydroxy-aminoquinoline-N-oxide (4-HAQO) which is then further activated by amino-acylation with serine to form seryl-4HAQO, a compound which, unlike 4-HQO, reacts primarily with guanine and adenine residues in nucleic acids (50). The putative intermediates between the nitro and amino derivatives of nitrofurazone (Figure 2) are based on indirect evidence and have never been isolated or synthesised, presumably because of their extremely short half-life.

Recently, Peterson, et al (51) have examined the reduction of nitrofurazone with type I and type II reductases using electron spin resonance (ESR) and visible

Figure 2. Possible pathways in the degradation of nitrofurazone by mammalian and bacterial enzymes (49).

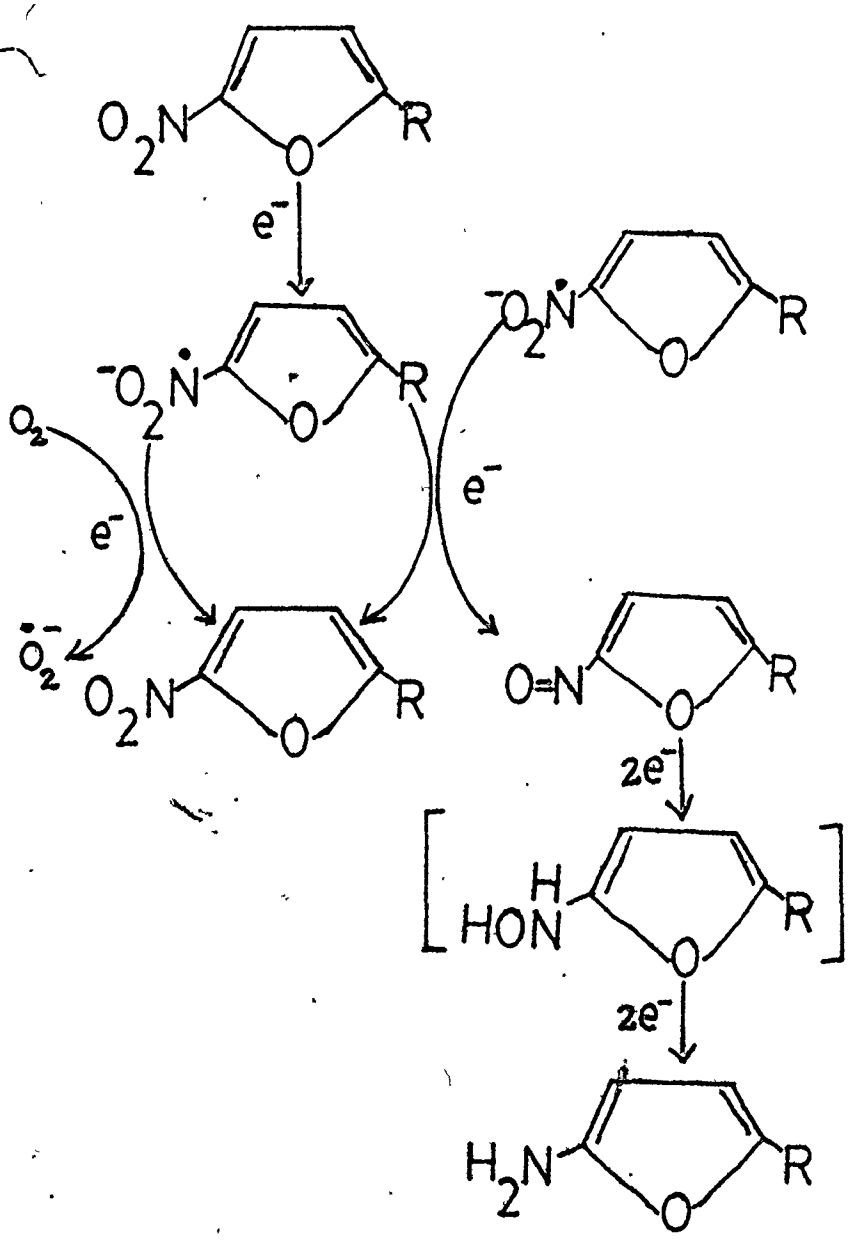
* Intermediates that have not been isolated and are only postulated


BROWN POLYMERS
(inactive)
(inactive)

spectroscopy. In the presence of oxygen, type II reductases of bacteria and mammalian tissues catalyse the addition of one electron to the nitro-group thus producing the nitro radical anion. This radical rapidly reacts with oxygen to form superoxide and regenerate the parent nitro compound, as shown in Figure 3. In this futile metabolism the nitro compound acts as a catalyst in the reduction of oxygen to superoxide and does not itself undergo a net reduction. This cycle also consumes the oxygen dissolved in the medium and will thus eventually produce hypoxic conditions. The radical anions are more stable under hypoxic conditions and their concentrations can build up thus allowing a disproportionation reaction to occur in which two molecules of the anion are converted to one molecule of the nitroso compound plus one molecule of the parent nitro compound (Figure 3). Nitroso compounds are quite reactive and can be reduced by NADPH or by the nitro anion free radical to form the amine. ESR and other studies indicate that the end-product of this anaerobic reduction is the amine (51,23), and the reactive intermediate must have an oxidation state between the nitro anion free radical and the amine (51). In the complex mammalian system, some confusion still exists as to which possible metabolic pathway contributes most to the toxic, mutagenic and carcinogenic effects of the nitrofurans because of the production of superoxide

FIGURE 3

Proposed mechanism of nitrofurazone reduction by E.coli Reductase II (51)

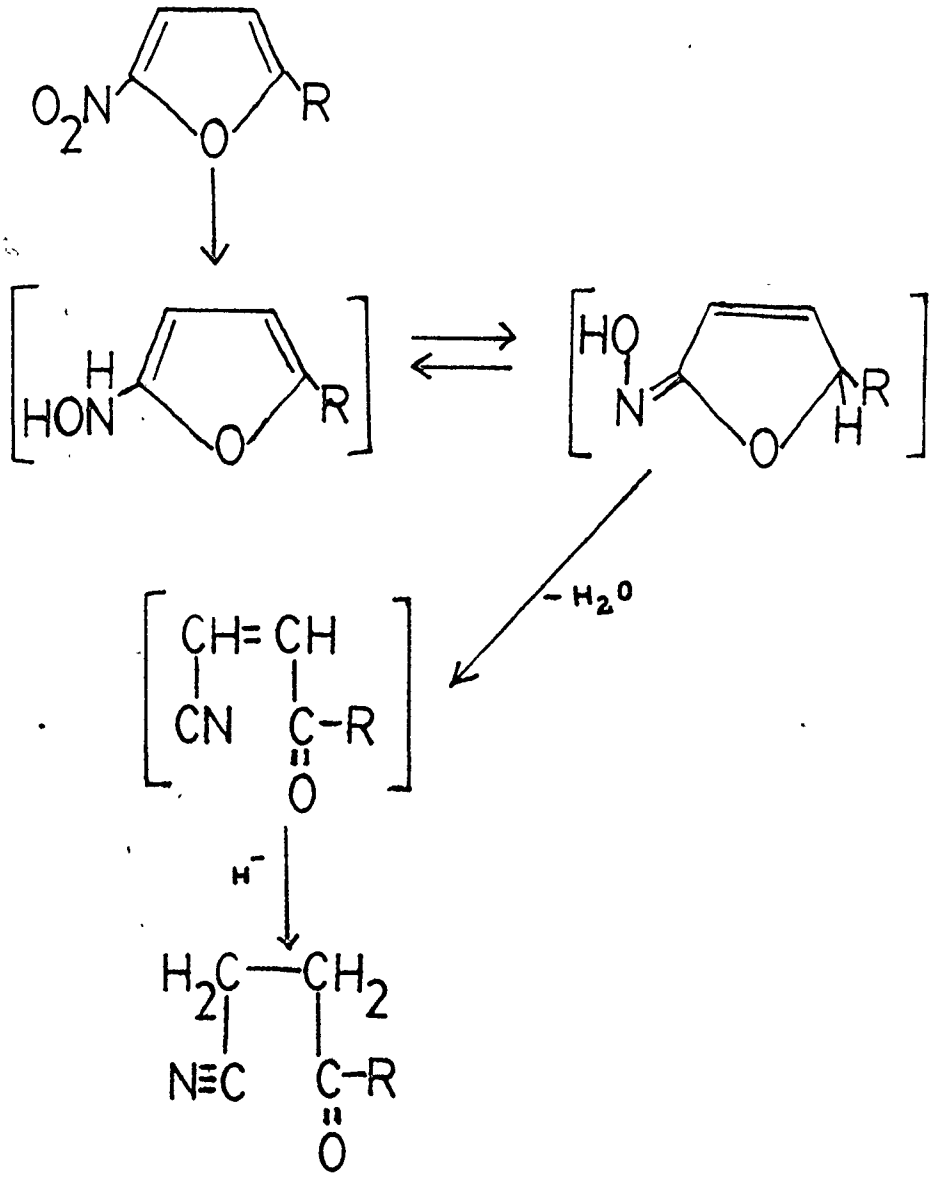


during the reduction process.

In contrast, oxygen-insensitive type I reductase does not form the nitro-anion free radical in detectable levels as determined by both ESR and visible spectroscopy. In addition, the oxygen consumption and superoxide formation in the presence of nitrofurazone is much less than with reductase II. The only product of nitroreduction that was detected was that of the open-chain nitrile (Figure 2). It has been suggested that this product could be formed without going through the amine and the pathway followed during reduction of this compound consists of a rearrangement of the hydroxylamine derivative to a trans-oxime followed by ring opening and the reduction of the aliphatic double bond, as shown in Figure 4 (51,52). If the nitroso intermediate is the first product of the reduction, then this enzyme catalyses a 2-electron transfer to nitrofurans. Whereas if the hydroxylamine is the first product, then the enzyme catalyses a 4-electron reduction without the formation of an enzyme-free intermediate species (51). Thus, it appears that the type I reductases catalyse the addition of two or more electrons to the enzyme-bound nitro compound with the open-chain nitrile as the end-product. However, the structure of the 'activated' intermediate remains elusive. It is conceivable that a large number of hydroxylamine tautomers, resonance forms,

FIGURE 4

Proposed mechanism of nitrofurazone reduction by E. coli Reductase I (51)



esters or conjugates could give rise to the ultimate active agent.

Reactions with Cellular Nucleophiles

Central to the problem of chemical carcinogenesis is the specific interaction between carcinogens and cellular 'receptors' which trigger the chain of biochemical events leading to tumour formation. It has been known for a long time that many classes of chemical carcinogens become covalently bound to DNA, RNA and proteins in the cells of target tissues. Despite much intensive research, the critical macromolecular target has not been unequivocally identified, but most recent studies have been heavily concentrated on interactions with DNA.

There has been very little work published that provides definitive evidence for the binding of nitrofurans to DNA. Nevertheless, the evidence in favour of damage to DNA of both bacterial and mammalian cells by activated nitrofurans is overwhelming. All the nitrofurans that have been tested caused breaks and/or alkali-labile lesions based on the sedimentation characteristics of DNA from treated cells in alkaline sucrose gradients (58). Moreover, the extent of damage to DNA correlated well with the mutagenicity and carcinogenicity of the nitrofurans (12,58). Potential nucleophilic sites in nucleic acids include

certain oxygen and nitrogen atoms, such as the O^6 and N7 of guanine and N3 of adenine. Although attack on the DNA itself is often considered to be the only means of achieving a heritable change which could lead to neoplastic growth, alteration in DNA : protein complexes is an equally likely mechanism (7).

Perhaps one of the earliest demonstrations of cellular nucleophile-nitrofuran interaction was the antagonistic action of a number of amino acids, including L-lysine, on the inhibition of bacterial growth by nitrofurazone (43). Subsequent studies have confirmed the binding of a wide variety of carcinogens to cellular proteins. Milk xanthine oxidase catalyses the binding of NFTA (43) and nitrofurazone (12) to protein. Electrolytic reduction (12) or gamma irradiation (30) of ^{14}C-nitrofurazone in the presence of serum albumin results in the binding of label to TCA-insoluble material. Incubation of nitrofurazone with a variety of animal tissues also resulted in the binding of label into TCA-insoluble material (54) with the degree of binding directly related to the reducing capabilities of the tissue. The addition of cysteine to <u>in vitro</u> reaction mixtures, containing reductase I, substantially reduced the amount of nitrofurazone that reacted with protein (12). More recently, mechanistic considerations of a metabolite of AF_2, isolated

from rabbit urine (55), indicated that the structure apparently arose from the interaction of the parent compound with methionine or possibly from a direct interaction with cysteine followed by methylation by S-adenosyl-methionine.

Examples of nucleophilic centres present in proteins which may be attacked by activated carcinogens are: the sulphur of methionine and cysteine, the N1 and N3 atoms of histidine, the epsilon amino group of lysine and the C3 and possibly O^4 atoms of tyrosine. Tryptophan is another possible site of attack (7). The most clearly characterised reaction product of a carcinogen with a cellular protein is the formation of methionine derivatives after azo-dye and acetylamino-fluorene administration in rats (3).

It is likely that the critical protein target, if it exists, would be present in close proximity to the genetic material, that is, proteins that control DNA function and template activity. In many cases the binding of chemical carcinogens to DNA in vivo is relatively small while other nuclear macromolecules are more extensively attacked. Recent advances in the elucidation of the role of nuclear proteins (histones and non-histone proteins) in nuclear metabolism and chromatin structure indicate that these proteins may play an important role in changes

leading to neoplasia (7). Moreover, the premise of the epigenetic theory of cancer (discussed earlier) is that perpetuated changes result from interactions of a carcinogen with a repressor protein.

Research on the mode of action of nitrofurans has so far yielded little detailed information on the structural and interactional nature of the components involved. If there are more than one targets, then the kinetics of formation and loss of the macromolecule-carcinogen adducts must be investigated and correlated with oncogenicity. Chemical elucidation of the conjugates formed with cellular nucleophiles is another pressing goal currently.

III Bacterial Nitroreductases

As discussed earlier, in 1957 Asnis (13) reported that at least two types of enzymes were involved in the reduction of nitrofurans in E. coli. Later, McCalla, et al (12) isolated three separate nitrofuran reductases in E. coli. Reductase I is active under aerobic conditions in the presence of NADH or NADPH and the end-product of the type I catalysed reaction with nitrofurazone is the open-chain nitrile, 4-cyano-2-oxobutyraldehyde semicarbazone (52). Reductase II is strongly inhibited by oxygen and consists of at least two distinct components, IIa (molecular weight 120,000) has IIb (molecular weight 700,000). Reductase II preparations activate nitrofurazone to yield derivatives that break DNA in vitro and are cytotoxic and mutagenic (54,58). Nitrofurazone is reduced by the type II reductases to the nitroso compounds which are further reduced to the level of the inactive amine which undergoes further rearrangement to the isomeric open-chain nitrile (Figure 2) (51).

More recently, genetic analysis has established that the type I reductase activity of E. coli strains B/r and K12 (AB1157) is due to at least two components controlled by different genes and which differ in their sensitivities to urea and in their chromatographic properties (59). The genes involved are designated as 'nitrofuran sensitivity

genes', and the alleles are termed nfs A and nfs B. Thus the wild-type strains, AB1157, are nfs A^+ and nfs B^+.

Resistance to nitrofurazone and to many other nitrofuran derivatives is acquired in two mutational steps (59). In the first step, nfs A^+ mutates to nfs A, as in strain NFR402. A major fraction of the oxygen-insensitive nitrofuran reductase activity (IA) is lost, in fact, only 20 to 30% of the wild-type activity remains. In the second step, another component (IB) is lost reducing the type I reductase activity to a very low level of less than 10% of wild-type activity. The genotype of this mutant is nfs A B, as in strain NFR 502. Another mutant was obtained from a cross of wild-type nfs A^+ B^+ and an nfs A B mutant of Hfr H. The genotype of this mutant is nfs A^+ B as in strain Sil 41 and it has essentially the same sensitivity to nitrofurazone as AB1157, although the IB component is missing. Further selection of the nfs A B mutant with nitrofurazone, under aerobic conditions, does not lead to the selection of strains having higher levels of resistance. A variety of crosses have established that these genes are both located close to the gal operon of the E. coli genome; the most probable sequence is lac nfs B gal nfs A (59).

The natural substrates and 'normal' function of nitrofuran reductases are not known. The type I reductases are dispensable under the growth conditions employed in

the laboratory and the mutant strains do not appear to have any new nutritional requirements (59). However, mutants of the oxygen-sensitive reductase II have not been isolated so far, perhaps indicating that their normal functions *in vivo* are vital to the survival of the cell.

It has not been conclusively determined whether the entire reduction sequence of nitrofurans requires only the known reductase I components or if additional reductases are active in intermediate steps of the sequence. Since it is necessary to characterise the 'active' nitrofuran metabolites, it would be desirable to eliminate unnecessary contaminants in reductase I extracts.

IV Objectives: Part 1

As mentioned above, the activated intermediates formed as a result of the reductive metabolism of nitrofurans are known to react with a number of cellular nucleophiles, including proteins. It is possible that these reactions contribute to the toxic and carcinogenic effects of these agents. However, detailed knowledge concerning the chemistry of these events have been difficult to obtain mainly because of the instability of the reactive forms of the nitrofurans. Thus, the principal objective of the first part of this work was to 'trap' the ultimate active agent formed by the enzymatic reduction of nitrofurazone, as a stable metabolite, by elucidating the structures of the derivatives formed upon reaction with an appropriate poly amino-acid.

Examples of nucleophilic centres present in proteins which may be attacked by activated carcinogens are stated above (page 21). One of the potential nucleophilic centres is the epsilon amino groups of lysines in the uncharged state. L-lysine was chosen as the electrophilic trap because it is present in relatively large amounts in many pure proteins. For example, equine cytochrome c contains 19% lysine residues, bovine β-lactoglobin and ribonuclease contains 9% and bovine α-casein contains 7% lysine residues (71). In addition, the relative frequency of lysine in

E. coli proteins is 54, if the frequency of alanine is considered to be 100 (71); the relative frequency of cysteine is only 14. There are relatively few histidine, tryptophan and methionine residues, all of which contain potential nucleophilic sites, in most proteins. Moreover, the epigenetic theory of cancer suggests that nuclear proteins may be involved in the oncogenic process, thus the abundant L-lysine residues present in histones may be important in the initiation of neoplasia through interactions with nitrofurans.

Homopolypeptides such as poly-L-lysine, although not found in nature, are exceedingly valuable model compounds. Poly-L-lysine seemed particularly useful for these experiments since polymerising lysine substantially lowers the pKa of the epsilon amino groups and the nucleophilicity of this homopolymer depends on the pKa of these side groups. In addition, the polymer is soluble in aqueous solutions at physiological pHs. Since lysine represents only one of several possible sites of attack in a protein, it is expected that only a limited number of derivatives would be formed. Previous preliminary experiments indicated that activated nitrofurazone reacts with poly-L-lysine to form fairly stable derivatives while cysteine-nitrofurazone adducts are relatively unstable.

Unfortunately, the activation systems used in these experiments did not yield an adequate amount of nitrofurazone-

lysine adduct to allow structural characterisation of the product. Experiments that surveyed the characteristics of the components of E. coli nitroreductase I were then initiated as described in Part II.

Objectives: Part II

Recently, the mutagenic activity in E. coli of nine nitrofurans was tested and the results indicated that the induced mutation frequency varies over five orders of magnitude as shown in Table 2. AF_2 was the most active mutagen and 5-nitrofuroic acid the weakest.

Since it is known that nitrofurans must be activated by nitroreductases before they damage DNA, one possible explanation for the differences in the mutagenicity of these compounds, may be that there are wide differences in the rate at which the ultimate mutagens are formed from the various agents. While it does not appear from preliminary results, obtained at a single concentration (57) that differences in the overall rates of reduction could account for the differences in the potencies of these compounds, there is no published data on the Km and Vmax for the bacterial nitroreductases. Therefore, the kinetics of the enzyme-substrate interaction were studied with four nitrofurans: AF_2, ANFT, furazolidone and nitrofurazone.

In addition, some insight into the pathway of reduction of each of the components of reductase I can be gained if the binding of the metabolites of the two nitrofurans, ANFT and nitrofurazone, to DNA and protein are studied. While these studies were in progress, our group showed that the E. coli nitroreductases actually contain three enzyme components, IA, IB_I and IB_{II}, which were the products of three different genes (70) (discussed later). If different types of DNA or protein adducts are formed, or if the biological effectiveness of the intermediates produced by the three reductase I components differ, then the yield of macromolecule-nitrofuran adducts would be expected to be different.

TABLE 2

Comparison of the Mutagenic Potencies of 5-Nitrofurans

5-Nitrofuran (Common Name)	Relative Mutagenic Potency toward *E.coli* WP2*
AF_2	100
ANFT	34
Furazolidone	16
Nitrofurazone	0.2
Nitrofuroic Acid	0.002

* Data taken from Lu *et al.* (57). Data normalised (AF_2 = 100) from the 'Induced Mutation Frequency' at a single concentration of nitrofuran. The differences observed were not due to differences in permeability of the nitrofurans.

2. MATERIALS AND METHODS

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone, NF) and ^{14}C-nitrofurazone labelled in the semicarbazide moiety were synthesised by Dr. D.R. McCalla (specific activity 7.0 μCi/ μmole) and B. Wentzell (specific activity 13.8 μCi/ μmole). Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidin-2-one) was a gift from Norwich Pharmical, Norwich, N.Y., U.S.A. ANFT(2-amino-4-(5-nitro-2-furly)-thiazole) was purchased from Abbot Laboratories, Chicago, Ill. AF_2 (2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide) was obtained through the courtesy of Dr. T. Sugimura, National Cancer Center, Tokyo, Japan. Highly polymerised poly-L-lysine-HBr, L-lysyl-L-lysine-HCl, glucose-6-phosphate, trypsin (bovine pancreas), flavin-mononucleotide, DEAE cellulose, calf thymus DNA and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. and were stored dessicated at -20°C. $NADP^+$ and glucose-6-phosphate dehydrogenase (Leuconostoc mesenteroides; 1100 Units/ml) were purchased from Boehringer Mannheim, Montreal, Canada. Whatman 3M chromatography paper, buffer constituents, n-butanol, acetic acid, pyridine, ninhydrin, triethyl-ammonium formate, potassium chloride, xylene, PPO, POPOP and other chemicals were purchased from Fisher Scientific

Company, Toronto, Ontario, Canada.

I. Bacteria

Escherichia coli K12, strain AB1157 was obtained from the collection of the Medical Research Council Cell Mutation Unit, Brighton, England. Strains Sil 41, 402 and 502 were developed by Dr. D.R. McCalla (11). Cells were grown for enzyme preparations in Penassay broth (Difco) at 37°C with constant aeration.

II. Preparation of Nitrofuran Reductase

A. Extraction (12)

Cells from 2 to 5 litres of a 20-hour culture of bacteria (late-log phase) were harvested at 10,000 g for 15 minutes. The pellet was washed twice with 0.067 M potassium phosphate buffer, pH 7.2 (hereafter referred to as K-phosphate buffer). The final pellet was suspended in about 0.05 volume of cold K-phosphate buffer and sonicated on ice for several 30 second periods with 80% power from a Fisher Model 300 sonic dismembrator. The soluble fraction was obtained by centrifugation at 100,000 g for 1 hour in an IEC Model B60 ultracentrifuge. This extract will be referred to as the crude extract.

In some experiments, 0.050 ml. of a 1.0M $MnCl_2$ solution was added to every ml of the extract to precipitate

nucleic acids (22). This step resulted in little loss of protein if the pH of the extract was maintained at neutrality while adding the $MnCl_2$. After stirring at 4°C for 30 minutes, the precipitate was removed by centrifugation at 25,000 g for 15 minutes. Additional details are presented in the figure captions. Solid ammonium sulphate was added to the supernatant to bring it to 35% saturation. After the precipitate was removed, the resulting supernatant was subsequently raised to a 65% saturation level with ammonium sulphate. The precipitate recovered after centrifugation at 25,000 g for 15 minutes was gently resuspended in a minimum volume of 0.015M K-phosphate buffer, pH7.2 and then dialysed extensively against the same buffer.

B. Chromatography on DEAE-cellulose

The preparation described above was loaded on to a DEAE 52-cellulose column (60 cm x 0.9 cm) previously equilibrated with 0.015M K-phosphate buffer, pH 7.2 at 4°C. The sample was successively eluted with 0.015M K-phosphate buffer, pH 7.2, followed by a buffered 0.1M NaCl solution and/or a linear gradient of 0.1M to 0.8M NaCl, pH 7.2. The conditions for elution for these columns are given in the captions to the figures. Fractions were collected at a rate of 0.25 ml per minute and assayed for absorbance at

280nm, to estimate the concentration of protein, and for nitroreductase activity using the standard enzyme assay of Section III. Fractions with the highest nitroreductase activity were pooled and the enzyme was precipitated by adding solid ammonium sulphate to a level of 70% saturation. The precipitate was re-dissolved in a minimum volume of 0.015 M K-phosphate buffer and dialysed against the same buffer plus 1 µM FMN for about 20 hours, then concentrated by dialysis against 50% glycerol in 0.015 M K-phosphate buffer, pH 7.2 (V/V). Enzyme prepared in this way can be stored frozen at -60°C and is referred to as DEAE-purified.

III Enzyme Assay

Type I nitroreductase activity was measured spectrophotometrically by following the decrease in absorbance of nitrofurazone at its absorption maximum of 375 nm in a 1 ml, 1 cm light path cuvette in a double beam spectrophotometer (Beckman, Model 25). The standard reaction mixture routinely employed contained the following constituents: nitrofurazone, 0.05 µmoles; glucose-6-phosphate, 0.72 µmoles; glucose-6-phosphate dehydrogenase, 0.7 units; $NADP^+$, 0.034 µmoles; flavinmononucleotide, 1 µM; nitrofuran reductase, and 0.067 M K-phosphate buffer, pH 7.2 to make a final volume of 1.0 ml. All reagents were prepared in 0.067 M K-phosphate buffer. The activity was calculated from the extinction coefficient of nitrofurazone

($\varepsilon^{\ell}_{mole}$ = 15,850 at 375 nm, (23)) and expressed as μmoles min^{-1} ml^{-1}.

Protein concentrations were determined by the Lowry modification of the Folin procedure (14). The absorbances were converted to milligrams of protein by reference to a standard curve prepared by using bovine serum albumin. Protein concentrations in column fractions were also estimated by measuring the absorbance at 280 nm.

IV. Liquid Scintillation Counting

One ml aqueous samples were counted for radioactivity in a Beckman Liquid Scintillation Counter (LS 230) after addition of 7 ml of a mixture of 1 litre Triton X-114, 3 litres xylene, 0.8 gm PPO and 12 gm POPOP. Samples on glass fibre filters were counted in a toluene-omnifluor mixture.

Errors resulting from quenching of the sample were corrected for by determining the efficiency of counting from a prepared standard quench curve using the external standard method.

V. Purity of Poly-L-lysine

Poly-L-lysine of molecular weights 3400 and 24,000 (approximately) were applied to a Sephadex G-25 column (60 cm x 0.9 cm) and eluted with distilled water. A Buchler polystaltic pump was used to maintain a constant

flow rate. Poly-L-lysine was detected in the column effluent by measuring the absorption at 220 nm.

VI. Preliminary Investigative Reaction of Nitrofurazone with Poly-L-lysine

The reaction mixtures used in preliminary experiments were identical to the standard enzyme assay mixture of Section 2. III, except for the replacement of 0.5 ml of K-phosphate buffer with 5 mg of poly-L-lysine-HBr (molecular weight 3400) in 0.5 ml of K-phosphate buffer, pH 7.2. DEAE-purified nitrofuran reductase from Sil 41 and ^{14}C-nitrofurazone were employed. The reaction was allowed to proceed aerobically at room temperature (approximately 22°C) until the colour change of nitrofurazone from yellow to clear occurred. The reduction was complete in less than 30 minutes.

VII. Purification of Nitrofurazone-Treated Poly-L-lysine

A. Chromatography on Sephadex G-25

The reduced nitrofurazone-poly-L-lysine mixture was applied to a Sephadex G-25 column (60 cm x 0.9 cm) which had been previously equilibrated with distilled water. The absorbancy at 220 nm and the radioactivity of each fraction were measured.

B. Enzymatic Hydrolysis of Labelled Poly-Lysine

Poly-L-lysine which has label bound to it as a result of the reaction with nitrofurazone metabolites will be referred to as labelled poly-L-lysine. The fractions, from the above chromatography, containing poly-lysine were pooled, evaporated *in vacuo* to a volume of 0.2 to 0.5 ml and then re-dissolved in 5 ml of 0.01M KCl, pH 6.8. The poly-lysine concentration was determined by a quantitative ninhydrin procedure (19). The enzymatic hydrolysis of poly-lysine followed the procedure of Waley and Watson (1953) (17). One µg trypsin was incubated with each mg poly-lysine in 0.01M KCl (final volume was 15 to 20 ml) in a closed reaction vessel at 37°C with gentle shaking for 10 to 12 hours. The stages in the hydrolysis of the labelled poly-lysine were followed by chromatography on thin-layer silica gel plates which were developed with butanol : acetic acid : water : pyridine, 30:6:24:20 (v/v/v/v) (17).

C. Chromatography on Carboxymethyl Sephadex

CM-Sephadex gel (C25) was purchased from Pharmacia Fine Chemicals, Dorval, Quebec. Canada. CM-Sephadex was initially prepared and continually regenerated by two washes

with 0.1 M KCl, pH 6.8, or with the appropriate eluant, before packing. The columns were unpacked after each use and the gel washed. This was necessary since contraction of the gel occurs with a salt gradient with the result that regeneration was not possible while the column was packed.

The labelled poly-L-lysine hydrolysate was applied to the CM-Sephadex column and elution was carried out with a linear gradient in KCl concentration. The conditions for elution and range of the gradients of these columns are given in the captions to the figures. The absorbance at 220 nm and the radioactivity of each fraction were determined.

Column calibration was performed with poly-L-lysine, lysyl-L-lysine, L-lysine and reduced nitrofurazone.

VIII. Reaction of ^{14}C-Nitrofurazone with L-lysyl-L-lysine

A. Purity of L-lysyl-L-lysine

The purity of L-lysyl-L-lysine (molecular weight 347.3) dissolved in water was determined by chromatography on Sephadex G-10/distilled water and on thin-layer silica gel plates. The solvent system used was the same as that of Section 2.VIIB. Chromatograms were sprayed with a solution of ninhydrin reagent and developed at 100°C for 10 minutes.

B. Investigative Small Scale Reaction of Nitrofurazone and L-lysyl-L-lysine

The reaction mixtures used in these experiments were identical to the standard enzyme assay mixture of Section 2.III, except for the replacement of 0.5 ml of K-phosphate buffer with 5 mg of L-lysyl-L-lysine in K-phosphate buffer, pH 7.2. ^{14}C-nitrofurazone and DEAE-purified nitrofuran reductase were employed. The reduction of nitrofurazone was allowed to occur at room temperature until the distinct colour change from yellow to clear was observed.

C. Chromatography on CM-Sephadex

L-lysyl-L-lysine that has reacted with nitrofurazone metabolites will be referred to as labelled L-lysyl-L-lysine. Labelled lysyl-L-lysine was separated from the other components of the reaction mixture by chromatography on CM-Sephadex as described in Section 2.VII C.

The presence of lysyl-L-lysine in the column effluent was determined by absorbance at 220 nm and by a qualitative ninhydrin reaction: 0.050 ml of each fraction (2.0 ml each) was spotted onto Whatman 3M chromatography paper, air-dired, sprayed with a solution of ninhydrin and developed at 100°C for 10 minutes. L-lysyl-L-lysine produces a purple colour with the intensity of the colour proportional to the concentration of lysyl-L-lysine.

Each fraction was also assayed for radioactivity as described above.

IX. Preparative Scale Reaction of ^{14}C-Nitrofurazone with L-lysyl-L-lysine

To derivatise a larger amount of L-lysyl-L-lysine, an excess of saturated nitrofurazone solution (specific activity 13.8 µCi/ µmole) was added with continuous stirring over a two-hour period to the reaction mixture shown in Table 3.

Reduction of nitrofurazone was judged to be complete when the yellow colour of the mixture changed to light brown. Separation of labelled lysyl-L-lysine from the other components of the reaction mixture was carried out on CM-Sephadex as described in Section 2.VIII C.

Other preparative large scale reaction mixtures used to derivatise a larger quantity of lysyl-L-lysine are shown in Tables 4 and 5.

TABLE 3

Preparative Scale Reaction Mixture for the Binding of ^{14}C-Nitrofurazone to L-Lysyl-L-lysine

Reagent	Volume (ml)	Final Concentration of Reagent
Glucose-6-Phosphate	0.125	3.46 µmoles
Glucose-6-Phosphate Dehydrogenase	0.015	1.40 Units
$NADP^+$	0.050	0.336 µmoles
L-lysyl-L-lysine	0.800	50 mg
^{14}C-Nitrofurazone*	0.250	4.90 µmoles (0.58 µCi/umole)
Nitroreductase I	0.050	250 µg protein
Total Volume	1.290 ml	

* Added slowly over a 2-hour period

TABLE 4

Preparative Scale Reaction Mixture for the Binding of ^{14}C-Nitrofurazone to L-lysyl-L-lysine*

Reagent	Volume (ml)	Final Concentration of Reagent
Glucose-6-Phosphate	0.125	7.2 µmoles
Glucose-6-Phosphate Dehydrogenase	0.070	3.5 Units
$NADP^+$	0.050	0.336 µmoles
L-lysyl-L-lysine	0.800	50 mg
^{14}C-Nitrofurazone	0.250	4.9 µmoles (0.58 µCi/umole)
Nitroreductase I	0.100	300 µg protein
Total Volume	1.395 ml	

* The concentration of the components of the NADPH regeneration system was increased

Reduction was allowed to occur for 12 hours with constant stirring

TABLE 5

Preparative Scale Reaction Mixture for the Binding of ^{14}C-Nitrofurazone to L-lysyl-L-lysine*

Reagent	Volume (ml)	Final Concentration of Reagent
Glucose-6-Phosphate	1.250	7.2 µmoles
Glucose-6-Phosphate Dehydrogenase	0.150	7.0 Units
$NADP^+$	0.250	0.336 µmoles
L-lysyl-L-lysine	2.000	50 mg
^{14}C-Nitrofurazone	0.700	0.50 µmoles (13.8 µCi/umole)
Nitroreductase I	0.100	200 µg protein
K-phosphate buffer	5.550	
Final Volume	10.000 ml	

* The concentration of each component and the final volume was 10 times that of the standard enzyme assay mixture

X. Desalting and Separation of Labelled Lysyl-L-lysine from Unchanged Lysyl-L-lysine

A. Chromatography on Sephadex LH-20

Chromatography on a Sephadex LH-20 column eluted with 80% isopropanol was used to separate the labelled lysyl-L-lysine from the underivatised dipeptide. Isopropanol is fairly volatile and therefore can be easily evaporated off without leaving any residual salt, thus allowing the labelled lysyl-L-lysine to be re-dissolved in an appropriate solvent.

B. Chromatography on CM-Sephadex

Separation of the labelled and unlabelled lysyl-L-lysine was also carried out on CM-Sephadex with a linear gradient of 0.6M to 1.0M triethylammonium formate, pH 6.8. Lysyl-L-lysine in this eluant can be recovered after lyophilisation to remove the buffer components, and stored dry at -20°C. Triethylammonium Formate was prepared according to the method of Alexander and Wildman, 1948 (16).

The presence of lysyl-L-lysine in the column effluent was detected by the qualitative ninhydrin method of Section 2.VIII C since triethylammonium formate absorbs strongly at 220 nm.

XI. pH of Optimal Activity of Reductase I

The reaction mixture used to measure the pH of a fixed number of units of a DEAE-purified IA component of reductase I of Sil 41 was the same as that used for the standard enzyme assay of Section 2.III. Each reagent was prepared in the buffer used for measurements at that specific pH.

The rates of reduction of nitrofurazone over the pH range 3.5 to 11.0 were measured. Since many assays were performed at one time, it was convenient to prepare mixtures containing all constituents except the substrate and reductase I.

XII. Enzyme Assays for Km, Vmax Determinations

A. Enzymes

Recent evidence (70) indicates that the type I nitroreductase present in E.coli K12 contains at least three components, IA, IB_I, IB_{II} (discussed later).

The Km (Michaelis constant) and Vmax (the maximal rate of the enzyme-substrate reaction) of the three components of E. coli nitroreductase I (IA from Sil 41, IB from 402, and IB_{II} from 502) with four different substrates (nitrofurans) were determined. Measurements were carried out at room temperature (22°C) and at 37°C.

B. Substrates

The four substrates used were: nitrofurazone, furazolidone, ANFT and AF_2. Their molar extinction coefficients and wavelengths of maximum absorption are listed in Table 6.

The reaction mixtures for Km and Vmax determinations were the same as the standard enzyme assay mixture of Section 2.III with varying concentrations of one of the nitrofurans. The decrease in absorbance at the wavelength of maximum absorption of the nitrofuran present in the reaction mixture was followed. Prior to addition of the enzyme to initiate the reaction, the assay sample was preincubated at the reaction temperature for several minutes to achieve thermal equilibration. A small aliquot cf enzyme (5 µℓ or 10 µℓ) was added to initiate the reaction.

Stock solutions of the substrates were prepared in dimethylsuplhoxide, protected from light, and stored at -20°C.

XIII. Binding of ^{14}C-Nitrofurazone and ^{14}C-ANFT to Protein

The binding of ^{14}C-nitrofurazone and ^{14}C-ANFT to bovine serum albumin during reduction by each of the three components of reductase I (IA, IB_I, IB_{II}) were measured.

TABLE 6

The Molar Extinction Coefficients and Wavelengths of Maximum Absorption of 4 Nitrofurans

Nitrofuran	Wavelengths of Maximum Absorption	Molar Extinction Coefficients
Nitrofurazone	375 nm	15,850 (23)
Furazolidone	365 nm	16,500 (15)
ANFT	400 nm	11,500 *
AF_2	395 nm	13,500 *

* Determined by measuring the absorbance of known concentrations of these nitrofurans

A. Reaction Mixture

Each reaction mixture contained 5 mg bovine serum albumin; 0.068 μmoles NADP; 1.44 μmoles glucose-6-phosphate; 1.4 Units glucose-6-phosphate dehydrogenase; 0.10 μmoles ^{14}C-Nitrofurazone or ANFT, and 0.10 Units of each component of reductase I, in a final volume of 1.0 ml. Each reagent was prepared in 0.1 M Tris buffer, pH 7.2.

Reduction proceeded at room temperature for about 1 hour.

B. Determination of Radioactivity (12)

To determine the incorporation of radioactivity into protein samples, ice-cold 5% TCA was added to 200 ml of each reaction mixture. After 15 minutes at 4°C, the sample was filtered on glass-fibre filters; washed twice with cold 5% TCA, twice with 95% ethanol or diethyl ether and dried at 60°C. The filters were counted in toluene-omnifluor scintillation fluid.

XIV. Binding of ^{14}C-Nitrofurazone and ^{14}C-ANFT to DNA

The binding of metabolites to DNA resulting from the reduction of ^{14}C-nitrofurazone and ^{14}C-ANFT by the IA, IB_I,IB_{II} components of nitroreductase I were investigated.

The reaction mixture used for each assay was the

same as that of Section 2.XIII A, except for the replacement of serum albumin with 3 μmoles of 10^6 nucleotides calf thymus DNA. The amount of enzyme protein added (reductase I) to each reaction mixture was kept constant at 40 μg per assay. The concentration of DNA was determined by the method of Tso (20).

DNA was extracted from the reaction mixtures by the following method (21): concentrated (100%) saline citrate (1.5M NaCl, 0.15M Na citrate, pH7.0), was added to the reduction mixture to make a final concentration of 10% (saline citrate); two volumes of cold 95% ethanol was added to precipitate the DNA which was then removed by spooling on a glass rod; the DNA was dissolved in 1 ml dilute saline citrate (0.015M NaCl, 0.0015M Na citrate), pH7.0; an equal volume of phenol, saturated with standard saline citrate (0.15M NaCl, 0.015M Na citrate). After the two phases separated the aqueous phase, which contained the DNA, was removed; two volumes of 95% ethanol was added to precipatate the DNA which was collected by spooling on a glass rod, washed with 70% cold ethanol, and dissolved in a known volume of dilute saline citrate, pH7.0.

Radioactivity was measured as described above.

3. RESULTS

PART I

I. Homogeneity of Poly-L-lysine

Chromatography of two batches of poly-L-lysine on Sephadex G-25 columns (60 cm x 0.9 cm) revealed the presence of L-lysine polymers of many lengths in one batch of poly-L-lysine (stated molecular weight 30,000; degree of polymerisation 135) as shown in Figure 5A. Poly-L-lysine of stated molecular weight 3400 and degree of polymerisation 16 was more homogenous although the molecular weights of the L-lysine polymers did vary over a substantial range as shown by the width of the peak obtained (Figure 5B). Poly-L-lysine from this latter batch was used in the experiments on binding with nitrofurazone.

II. Investigative Scale Reaction of Nitrofurazone and Poly-L-lysine

A. Recovery of Poly-L-lysine

Upon reduction of ^{14}C-nitrofurazone in the presence of poly-L-lysine, a number of reduction products were produced. These included species that were bound to

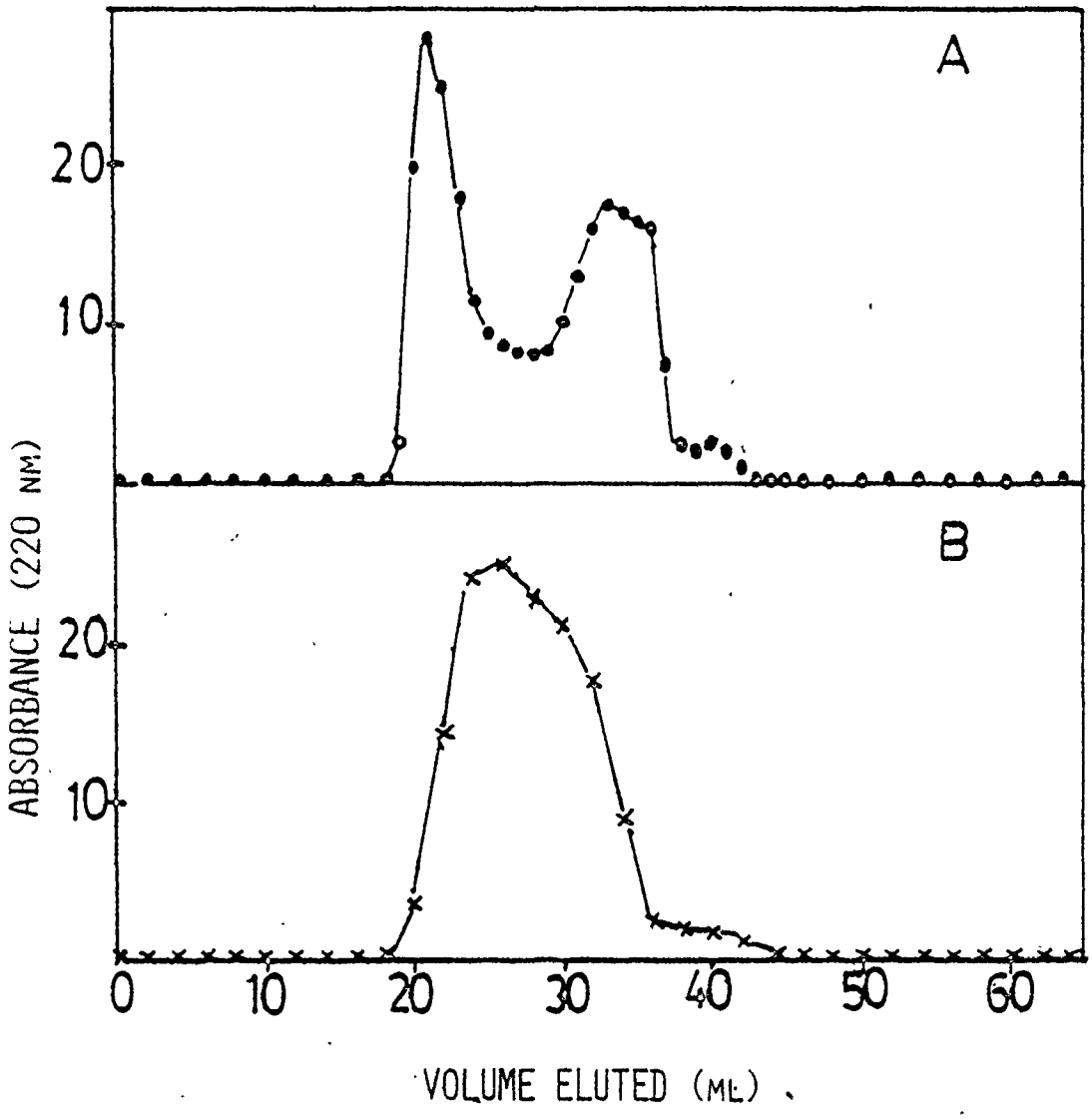


Figure 5.. Chromatography of poly-L-lysine on Sephadex G-25 (60 cm x 0.9 cm column). Eluted with distilled water at 1 ml/min.

A Molecular weight 30,000. Degree of polymerisation 135

B Molecular weight 3,400. Degree of polymerisation 16

poly-L-lysine as judged by the label associated with the polymer. In order to quantitate the interaction between nitrofurazone and poly-L-lysine, it was necessary to separate the labelled homopolymer from unreacted or reduced but unbound nitrofurazone. One method used to effect this separation was chromatography on Sephadex G-25.

B. Chromatography on Sephadex G-25

Figure 6 represents the chromatograms obtained upon fractionation of an investigative scale reaction mixture on Sephades G-25. Qualitative ninhydrin reaction, absorbance at 220 nm, and previous column calibration indicated the enzyme protein elutes in the 15 to 18 ml volume and poly-lysine elutes in the 19 to 30 ml volume. The first poly-L-lysine peak, peak 6B, elutes in the volume between 19 and 30 ml, but as can be seen from the radio-activity profile. label is associated with the volume between 21 and 30 ml only. This indicates that only part of the poly-L-lysine in the applied sample was derivatised and suggests that the labelled polypeptide eluted subsequent to the underivatised poly-L-lysine. Some of the reduction products of nitrofurazone also absorb at 220nm, as shown in Figure 7, and the large amount of label associated with the UV

Figure 6. Separation of ^{14}C-Nitrofurazone treated poly-L-lysine from other reduction products by chromatography on Sephadex G-25 (60 cm x 0.9 cm column). Eluted with distilled water.

O——O Absorbance at 220 nm

X— —X Radioactivity (cpm)

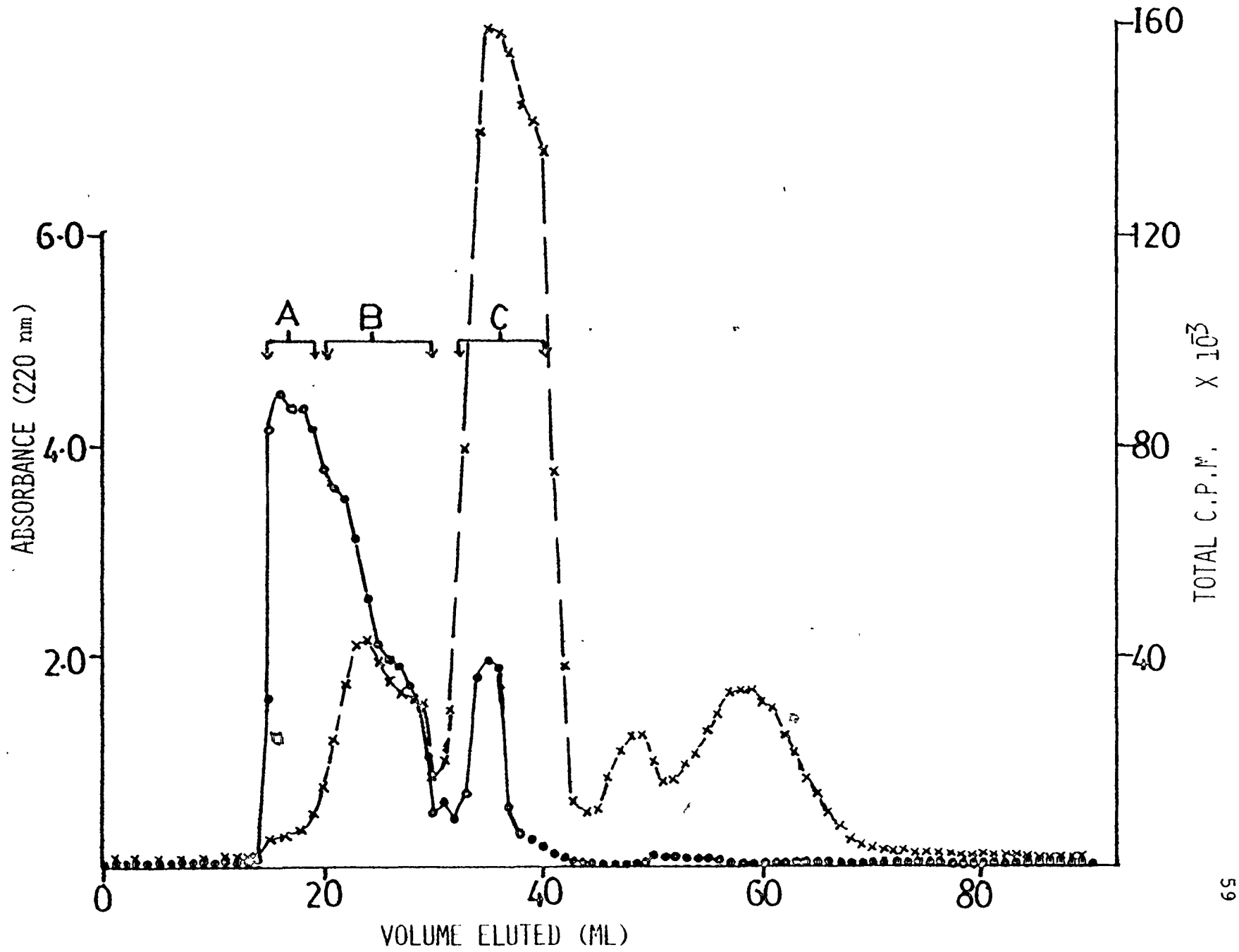
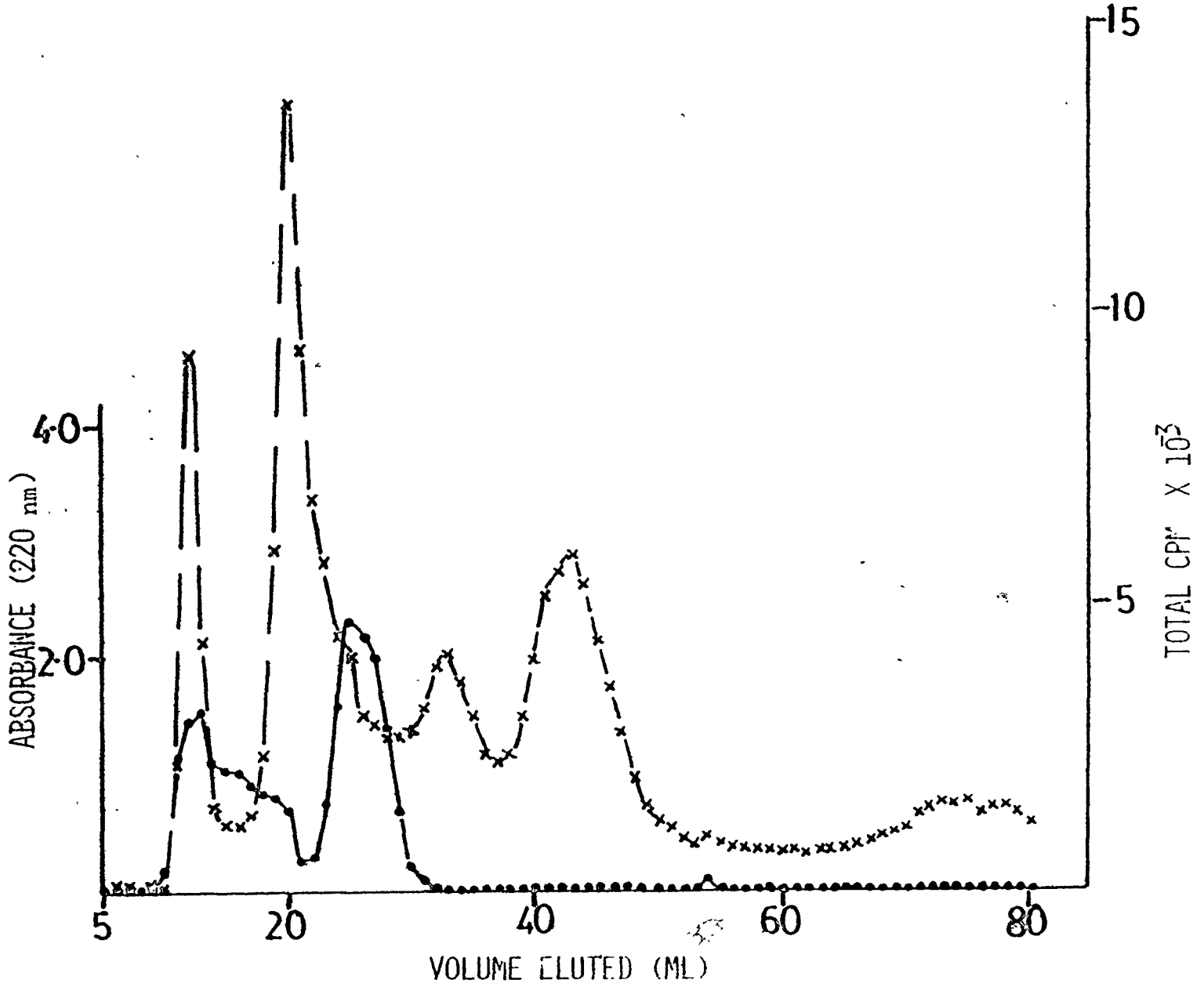

6·0
4·0
2·0
ABSORBANCE (220 nm)
A
B
C
160
120
80
40
TOTAL C.P.M. X 10⁻³
0
20
40
60
80
VOLUME ELUTED (ML)

Figure 7. Reduction of ^{14}C-Nitrofurazone in standard enzyme assay mixture.

Chromatography of the reduced nitrofurazone on Sephadex G-10 column (60 cm x 0.9 cm), with distilled water as eluant.

O——O Absorbance at 220 nm

X——X Radioactivity (cpm)


ABSORBANCE (220 nm)
4·0
2·0
TOTAL CPM X 10^{-3}
15
10
5
5
20
40
60
80
VOLUME ELUTED (ML)

T9

absorbing material between 32 and 42 ml (peak 6C) may be due to nitrofurazone and its reduced metabolites. Previous column calibration studies (18) indicated that nitrofurazone and its reduction products elute after 35 ml on a Sephadex G-25 (60 cm x 0.9 cm) column.

Obviously, this chromatographic procedure did not resolve the derivatised poly-L-lysine from the underivatised polymer. Perhaps, if the 20 ml to 37 ml region was pooled and re-chromatographed on Sephadex G-50, or dialysed before chromatography on Sephadex G-25, better resolution might have been obtained. However, for the investigative experiments, the 20 ml to 37 ml region was pooled and subjected to enzymatic hydrolysis. A 15 ml to 30 ml cut was not taken because the 30 to 37 ml region could also possibly represent fragments that have been cleaved from poly-L-lysine and overlaps with the region in which nitrofurazone elutes. This is not an unreasonable step because if that region contained the reduction products of nitrofurazone only, they would elute in the void volume of the next chromatographic step and would not interfere with the hydrolysis products of poly-L-lysine.

The radioactivity peak in the region of 15 to 37 ml contained 60% of the label applied to the column. The enzyme protein present eluted at the void volume between 15 and 18 ml (Figure 6, peak A) and the radioactivity profile

shows that there was essentially no label bound to that protein.

III. Enzymatic Hydrolysis of Poly-L-Lysine

Trpysin hydrolysis of poly-L-lysine provides a mild, non-acidic method of obtaining a series of L-lysine oligomers. Previous studies on the relative rates of trypsin-catalysed hydrolysis of various polymers of L-lysine (17) indicated that if the relative rate of hydrolysis of penta-lysine was 1000, then the relative rates for tetra- and tri-lysine were 100 and 1 respectively. L-lysine is not a product of this hydrolysis (17)

It was necessary to hydrolyse poly-lysine to oligomers to permit easier identification of the nitrofurazone-lysine adducts formed in the reaction. The various oligomers of L-lysine can be resolved on CM-Sephadex by elution with gradients of KCl, pH 6.8, of increasing ionic strength (18).

A. Column Calibration

The CM-Sephadex column (60 cm x 0.9 cm) was calibrated with a number of compounds, the results of which are listed in Table 7. Poly-L-lysine was hydrolysed with trypsin following the same procedure used for the samples present in the *in vitro* activation system and

TABLE 7

Molarities of KCl at which various compounds elute on CM-Sephadex[c]

Compound	M KCl pH 6.8
Reduced Nitrofurazone	0.1 - 0.25 M
L-lysine	0.5 M
L-lysyl-L-lysine	0.75 M
Poly-L-lysine trypsin hydrolysate	0.72 M[a]
	0.85 M[b]
	0.95 M[b]

[a] L-lysyl-L-lysine.

[b] Probably tri-lysine and tetra-lysine (see text).

[c] Column calibrations were done on CM-Sephadex columns (60 cm x 0.9 cm) with linear gradients of 0.1 M to 1.0 M KCl, pH 6.8.

chromatographed on CM-Sephadex. The hydrolysis products that elute at 0.85 M KCl and 0.92 M KCl (Figure 8) were assumed to be tri-lysine and tetra-lysine respectively by reference to the results of Waley and Watson (1953).

B. Analysis of Hydrolysis Products on CM-Sephadex

Figure 9 shows the elution pattern of a 10-hour trypsin hydrolysate of labelled poly-L-lysine on CM-Sephadex. Most of the radioactivity was eluted rapidly from the column. Label was not associated with the L-lysine oligomers, however, a small amount of label was close to but not coincident with the L-lysine residues. The radioactivity peaks which eluted between 0.1 M and 0.25 M KCl account for 80% of the label applied to the column and these peaks were present in the same volumes in which nitrofurazone and its metabolites would be expected to elute.

The peak at 0.75 M KCl of the profile was lysyl-L-lysine and the broad peak between 0.85 M and 0.95 M KCl represented tri-lysine overlapped by tetra-lysine, as judged from the column calibration studies of Table 7. The presence of these peaks in trypsin hydrolysates of material taken from the Sephadex G-25 column can be taken as conclusive evidence that the unhydrolysed material contained poly-L-lysine.

A

Figure 8. Hydrolysis of poly-L-lysine by trypsin.
Elution of the hydrolysate on CM-Sephadex with a linear gradient of 0.1 M to 1.0 M KCl, pH 6.8, at a rate of 0.25 ml/min.
Arrows indicate positions where various other compounds elute as determined by previous column calibration studies.
o——o Poly-L-lysine hydrolysate

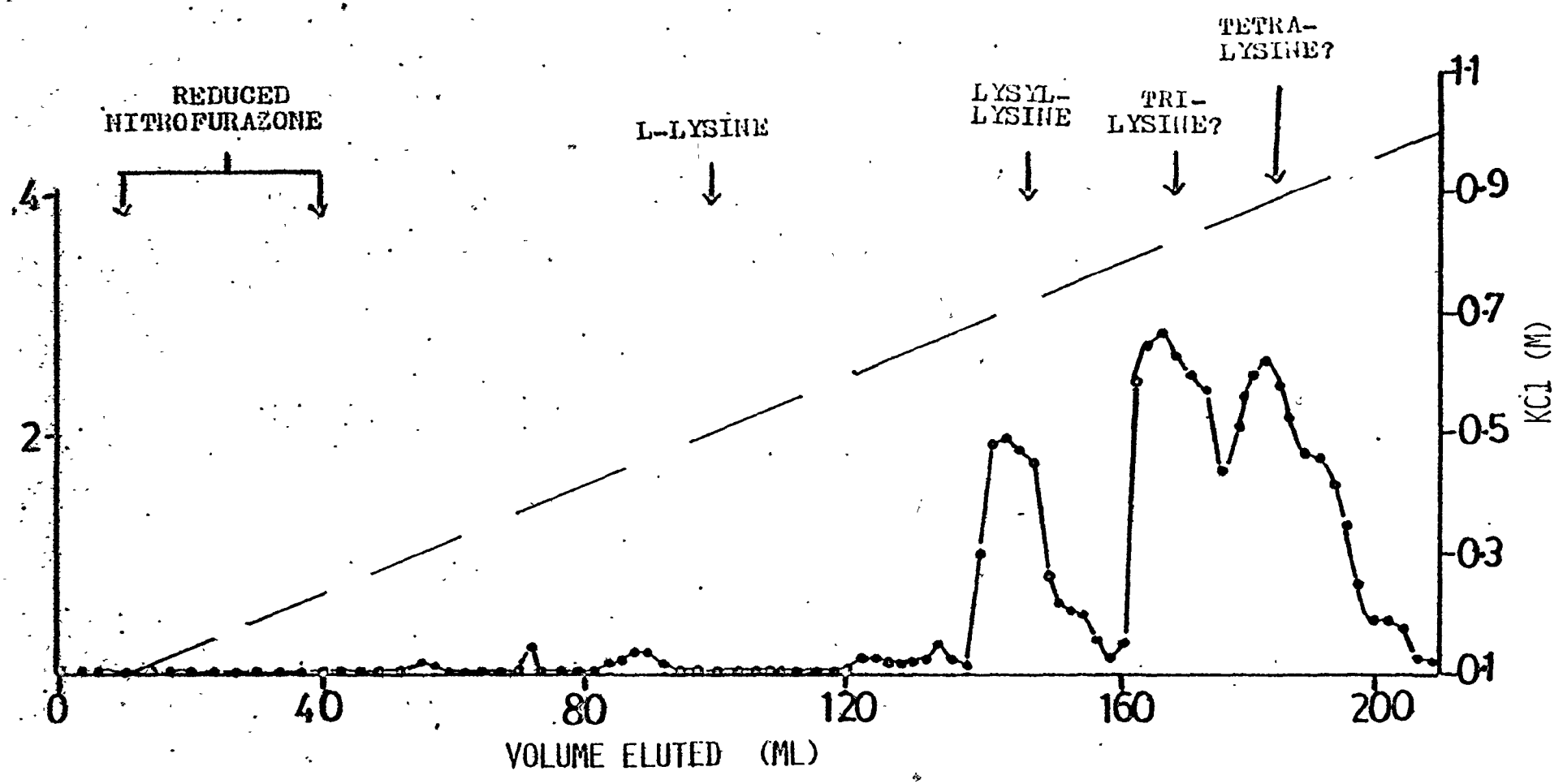
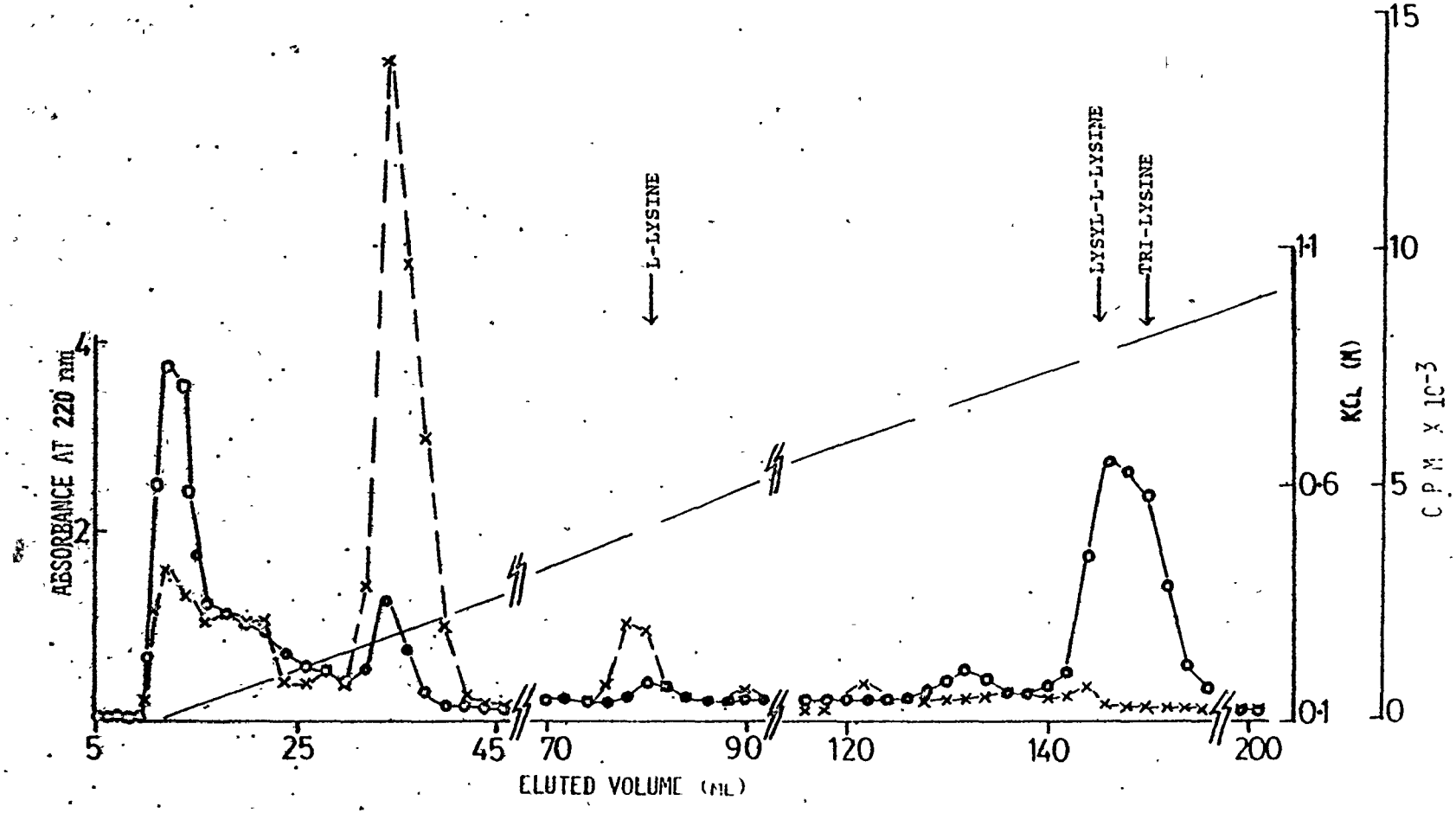

REDUCED
NITROFURAZONE
L-LYSINE
LYSYL-
LYSINE
TRI-
LYSINE?
TETRA-
LYSINE?
4
2
0
40
80
120
160
200
VOLUME ELUTED (ML)
1·1
0·9
0·7
0·5
0·3
0·1
KCl (M)

Figure 9. Chromatography of labelled poly-L-lysine/trypsin hydrolysate on CM-Sephadex with a linear gradient of 0.1 M to 1.0 M KCl, pH 6.8.

O——O Absorbance at 220 nm

X— —X Radioactivity (cpm)


L-LYSINE
LYSYL-L-LYSINE
TRI-LYSINE
ABSORBANCE AT 220 nm
KCL (M)
C.P.M. X 10-3
ELUTED VOLUME (ML)
4
2
1·1
0·6
0·1
15
10
5
0
5
25
45
70
90
120
140
200

The results obtained suggested that one of two possible events was occuring upon trypsin hydrolysis of labelled poly-L-lysine. The first was that a metabolite of nitrofurazone (NF) reacted with the lysine residues (the epsilon amino groups) of poly-L-lysine but the NF-lysine linkage was susceptible to trypsin hydrolysis or spontaneous cleavage. The result of this would be that the label that was bound to poly-L-lysine before hydrolysis would elute with nitrofurazone reduction products between 0.1 and 0.25 M KCl. Secondly, as a consequence of lysine derivatisation with nitrofurazone, the peptide bonds adjacent to the altered residue had been labilised towards trypsin attack with the result that 1:1 nitrofurazone-lysine derivatives were formed. The label eluting with L-lysine residues in Figure 9 was consistent with the latter event.

At this point, experiments that assess the reaction of lysyl-L-lysine dipeptides with nitrofurazone were undertaken thus eliminating the hydrolysis step needed to reduce poly-L-lysine to oligomers of L-lysine.

IV. Purity of L-lysyl-L-lysine

Chromotography of L-lysyl-L-lysine on thin-layer silica gel plates revealed the presence of a small amount of L-lysine.

In order to quantitate the amount of lysine residues present, 10 mg of lysyl-L-lysine was chromatographed on Sephadex G-10 and eluted with distilled water. L-lysyl-L-lysine was identified in the column effluent by a qualitative ninhydrin test and by absorbance at 220 nm. The other ninhydrin positive peak eluting after the lysyl-L-lysine peak was assumed to be lysine based on the TLC results (above). The fractions containing lysyl-L-lysine and L-lysine were pooled and assessed by a quantitative ninhydrin method (19). The stock lysyl-L-lysine contained about 1% lysine residues which is an insignificant amount for the binding studies and stock lysyl-L-lysine was therefore used without further purification.

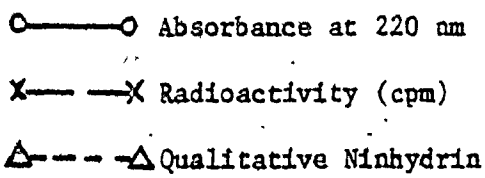
V. Investigative Small Scale Reaction of ^{14}C-Nitrofurazone and L-lysyl-L-lysine

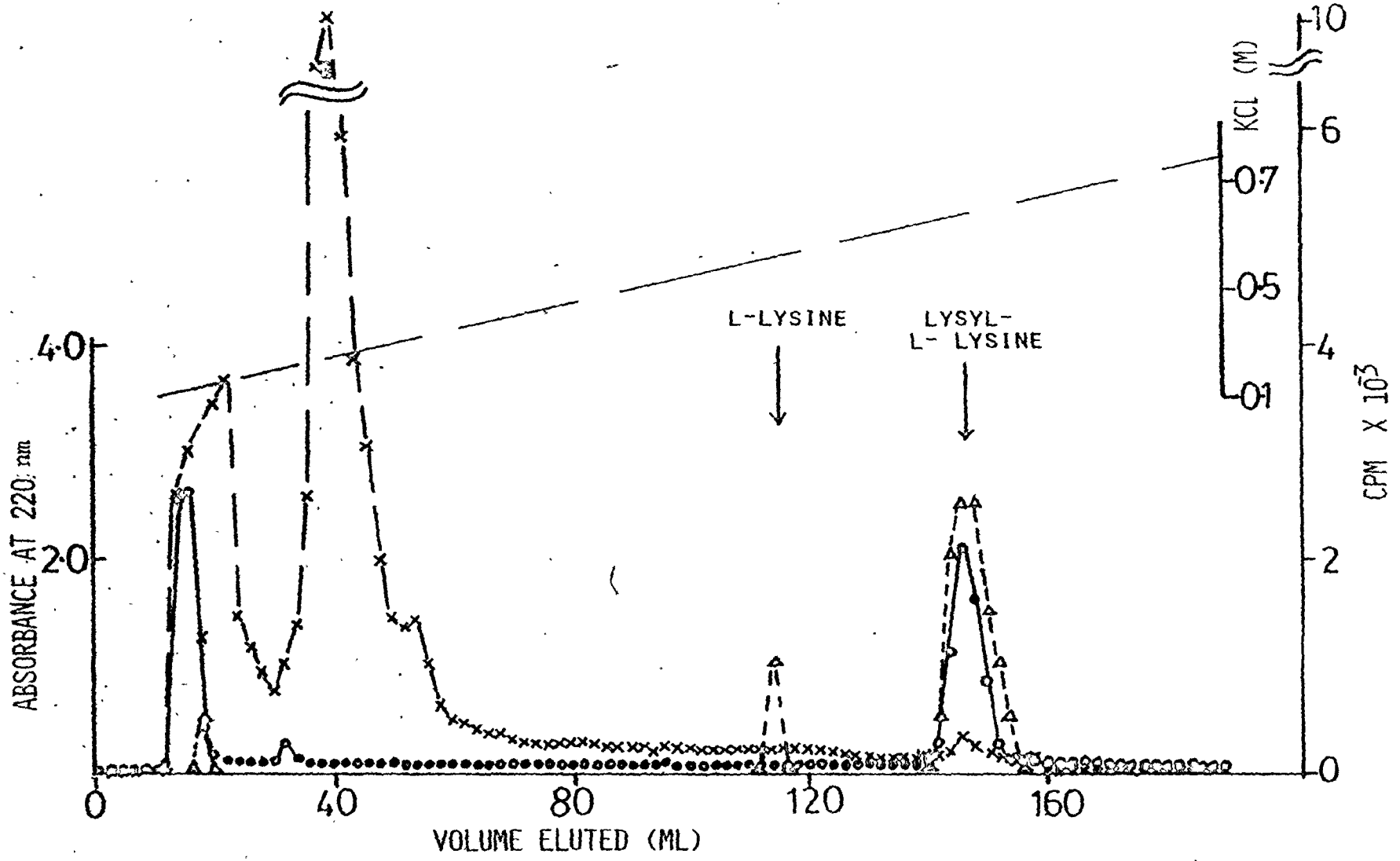
After the reduction of nitrofurazone in the presence of lysyl-L-lysine, labelled lysyl-L-lysine was separated from the other reduction products by ion-exchange chromatography.

A. Chromatography on CM-Sephadex

Figure 10 illustrates the effectiveness of the separation of labelled and unlabelled lysyl-L-lysine from the nitrofurazone metabolites on CM-Sephadex by elution

Figure 10. Investigative small scale reaction of ^{14}C-Nitrofurazone and L-lysyl-L-lysine. Chromatography on CM-Sephadex (60 cm x 0.9 cm column) with a linear gradient of 0.1 M to 1.1 M KCl, pH 6.8.




L-LYSINE
LYSYL-
L- LYSINE
KCl (M)
0·1
0·5
0·7
ABSORBANCE AT 220 nm
CPM X 10³
VOLUME ELUTED (ML)
0
40
80
120
160
2·0
4·0
2
4
6
10

with a linear gradient of 0.1 M to 1.1 M KCl, pH 6.8. Previous column calibration (Table 7) indicated that the peak at 0.72 M KCl was lysyl-L-lysine. This was the easiest and fastest of the purification methods tried.

The radioactivity peak which eluted from 142 to 150 ml accounted for only 2% of the label applied to the column. The major radioactivity peaks eluted with the nitrofurazone metabolites in the volume between 12 and 58 ml.

However, based on the specific activity of the starting compound, the eluted lysyl-L-lysine was calculated to have the equivalent of 5×10^{-4} μ mole of nitrofurazone bound to it. If it is assumed that only one of the epsilon amino groups of each lysyl-L-lysine residue was derivatised, then only 0.2 μ g or 0.004% of the original dipeptide was labelled. Moreover, these calculations are based on the assumption that the labelled material is pure lysyl-L-lysine thus the values obtained may be inflated if contaminants were present.

The preliminary experiments failed to provide sufficient material for the separation of the labelled from the unlabelled lysyl-L-lysine or for chemical analysis of the nitrofurazone-lysyl-L-lysine adducts. Therefore, to furnish a larger amount of the required material, the preparative scale reactions of Section 2.IX were performed.

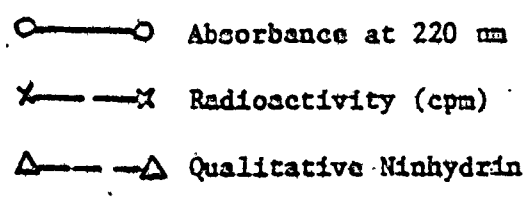
VI. Preparative Large Scale Binding Studies with Lysyl-L-lysine

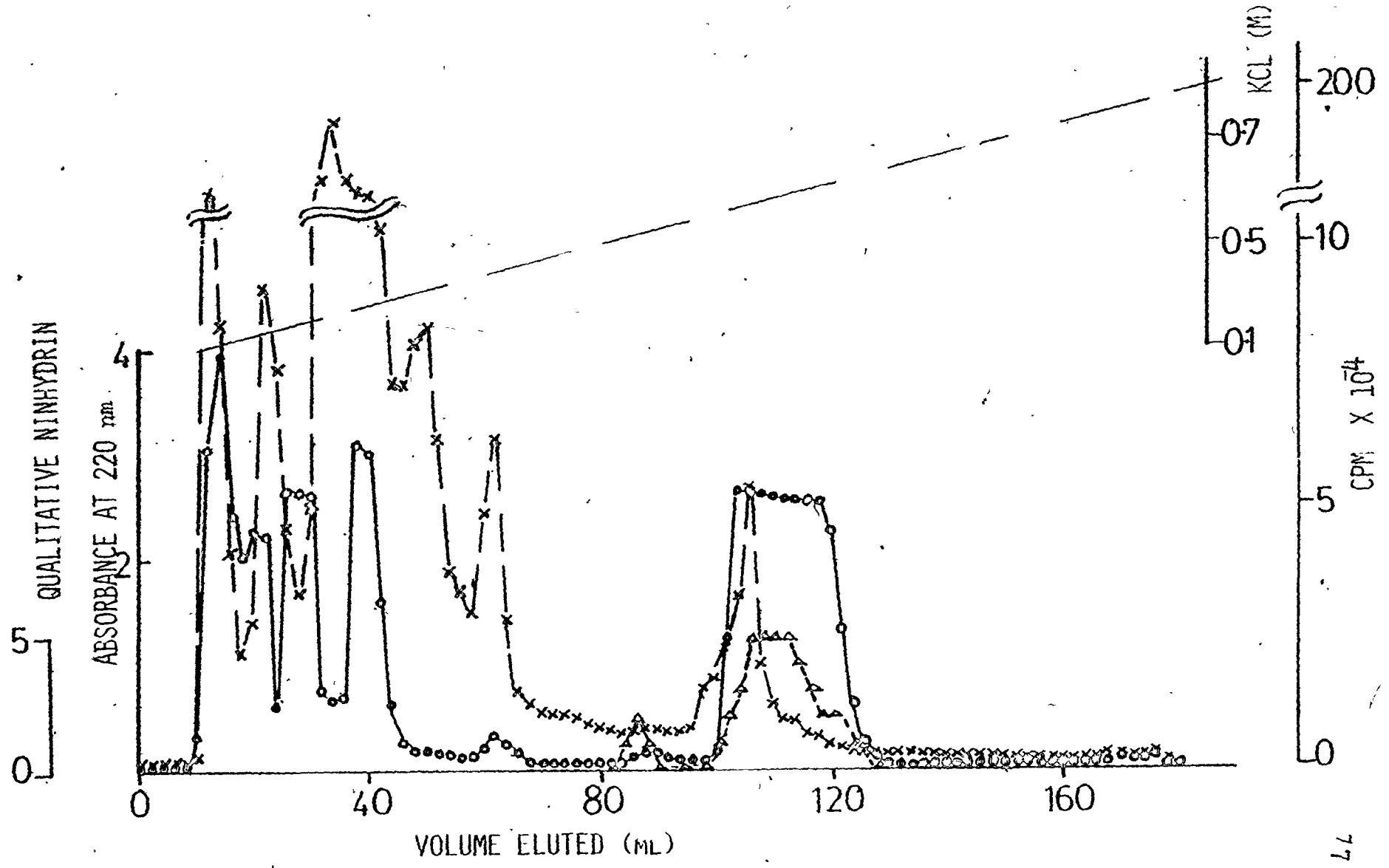
A. Chromatography on CM-Sephadex

Reaction mixtures containing a larger quantity of nitrofurazone and lysyl-L-lysine were used to assess the reaction of ^{14}C-nitrofurazone and lysyl-L-lysine during reduction by E. coli nitroreductase I (Table 3). When nitrofurazone was reduced, the colour of the reaction mixtures changed from yellow to brown, a colour that was perhaps due to the formation of polymers of nitrofurazone metabolites. That colour was observed only in the larger scale reaction mixtures because of the higher concentration of nitrofurazone present in those mixtures.

Figure 11 shows that the elution profile obtained by chromatography on CM-Sephadex with a 0.1 M to 1.2 M KCl gradient, pH 6.8, was essentially superimposable on that obtained with the investigative small scale reaction mixture, except that a larger amount of nitrofurazone reduction products was present. The radioactivity peak between the region of 96 ml and 110 ml, which was associated with lysyl-L-lysine, contained a larger percentage of label than the investigative small scale reaction. This label also eluted slightly ahead of the major absorbance peak of lysyl-L-lysine. This result was very consistent and it

Figure 11. Chromatography of a preparative, large scale, reaction mixture of ^{14}C-Nitrofurazone and L-lysyl-L-lysine on CM-Sephadex (60 cm x 0.9 cm column). Elution with a linear gradient of 0.1 M to 1.2 M KCl, pH 6.8.




QUALITATIVE NINHYDRIN
5
0
ABSORBANCE AT 220 nm
4
2
0
40
80
120
160
VOLUME ELUTED (ML)
KCL (M)
0·7
0·5
0·1
200
10
5
0
CPM X 10⁻⁴

was not surprising since lysyl-L-lysine that has been derivatised by nitrofurazone metabolites would probably have ionic properties that are different from those of unlabelled lysyl-L-lysine and would therefore be expected to behave differently on an ion-exchange column.

Based on the specific activity of the starting compound, the peak in the 96 ml to 110 ml volume was calculated to contain 0.12 μmole of nitrofurazone metabolite. The amount of lysyl-L-lysine labelled, assuming that only one of the two epsilon amino groups was labelled, was 33 μg or 0.066% of the starting material. Again, these calculations were based on the assumption that all the label eluting in the region of 96 to 110 ml was bound to pure lysyl-L-lysine. Nevertheless, this was still not enough material for instrumental analysis by gas chromatography or mass spectrometry. It therefore became necessary to carry out the binding studies on a much larger scale.

It was also necessary to remove the salt from the labelled lysyl-L-lysine and to separate it from the underivatised material for instrumental and chemical analyses.

VII. Separation of Labelled Lysyl-L-lysine from Unlabelled Lysyl-L-lysine

A. Chromatography on CM-Sephadex with a Shallow KCl Gradient

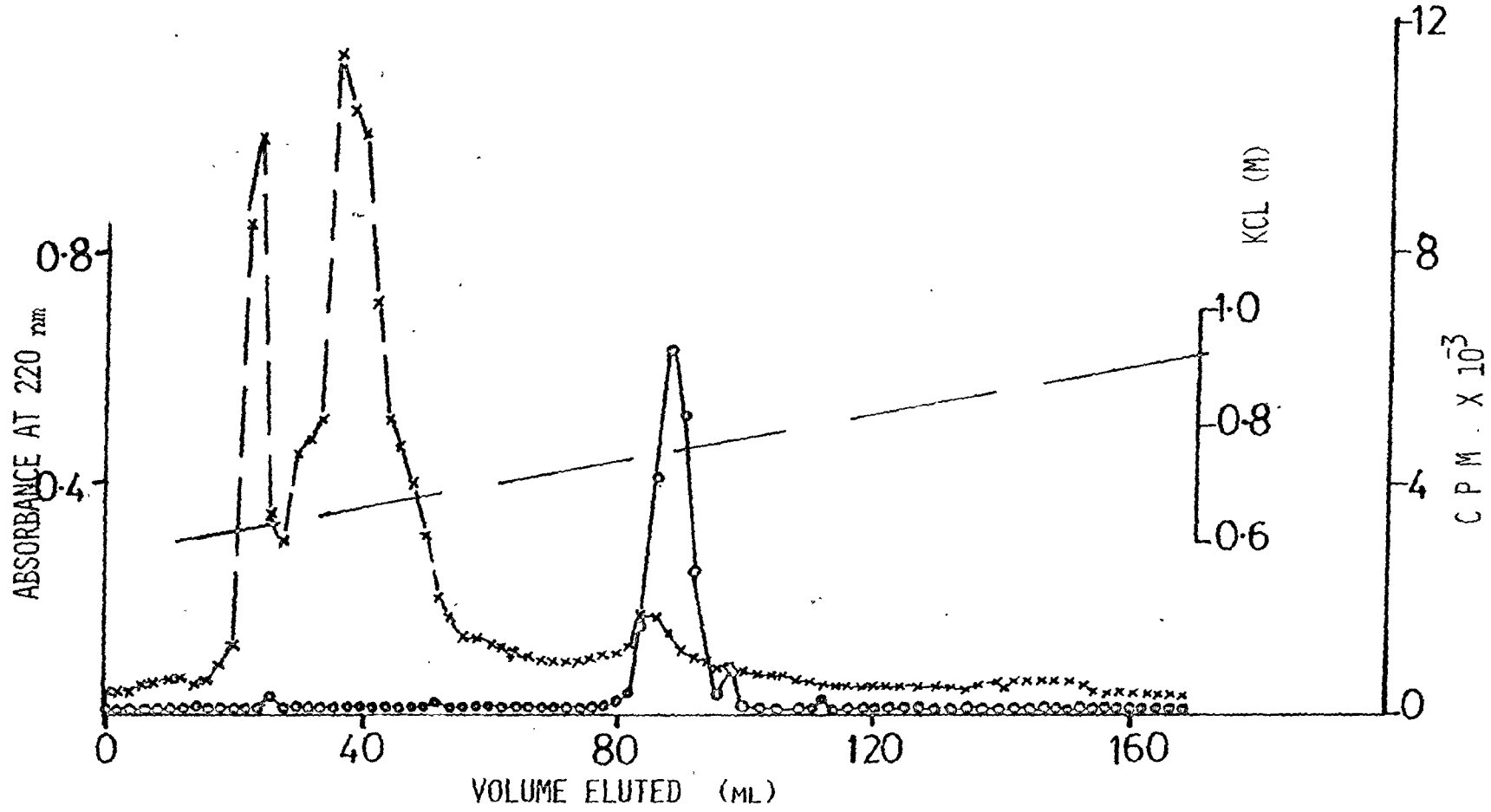
Chromatography of the major radioactivity peak eluting with lysly-L-lysine on CM-Sephadex with a shallow KCl gradient provided clear evidence of separation of the labelled from the unlabelled lysyl-L-lysine. The elution profile obtained, as shown in Figure 12, demonstrated that there were at least two major components, perhaps three, present in the labelled material.

Figure 12 also indicates that the material present in the 20 ml to 50 ml volume does not absorb significantly at 220 nm nor does it show a positive reaction with ninhydrin reagent. Based on previous calculations (Section 3.VI), the amount of labelled lysyl-lysine applied to the CM-Sephadex column was 20 µg. Assuming 100% recovery, the amount of lysly-lysine that was present in the 20 ml to 60 ml region was 0.5 µg per ml on average. To test for a reaction with ninhydrin reagent, 0.05 ml of each fraction, which was equivalent to 0.025 µg lysyl-lysine, was used - that amount was far below the detection limit of ninhydrin (19) and thus accounts for the negative results. A solution containing 1 mg pure lysyl-L-lysine per ml has an absorbance of approximately 1.9 at 220 nm, and therefore 0.5 µg per ml

Figure 12. Separation of labelled lysyl-L-lysine from unlabelled lysyl-L-lysine on CM-Sephadex with a shallow ionic gradient of 0.6 M to 1.0 M KCl, pH 6.8.

O——O Absorbance at 220 nm

X—— ——X Radioactivity (cpm)


ABSORBANCE AT 220 nm
0·8
0·4
0
40
80
120
160
VOLUME ELUTED (ML)
KCL (M)
1·0
0·8
0·6
C P M . X 10⁻³
12
8
4
0

lysyl-L-lysine would have an absorbance by 0.00095, which is insignificant and would not be detected by spectrophotometric means.

It is also possible that the reaction of activated nitrofurazone with lysyl-L-lysine could produce an adduct that has properties that are different from those of the original dipeptide. However, separation of the labelled lysyl-L-lysine from the unlabelled material by this method would still not prepare the sample for chemical and instrumental analyses because of the presence of relatively large amounts of KCl in each fraction.

B. Chromatography on Sephadex LH-20 and Isopropanol

80% isopropanol is volatile and would therefore be an ideal solvent to recover the labelled lysyl-L-lysine since it can be removed without leaving any residual salt.

As shown in Figure 13, this system did not resolve the two major components of the applied sample. A search for better methods was made; the most successful of these is described below.

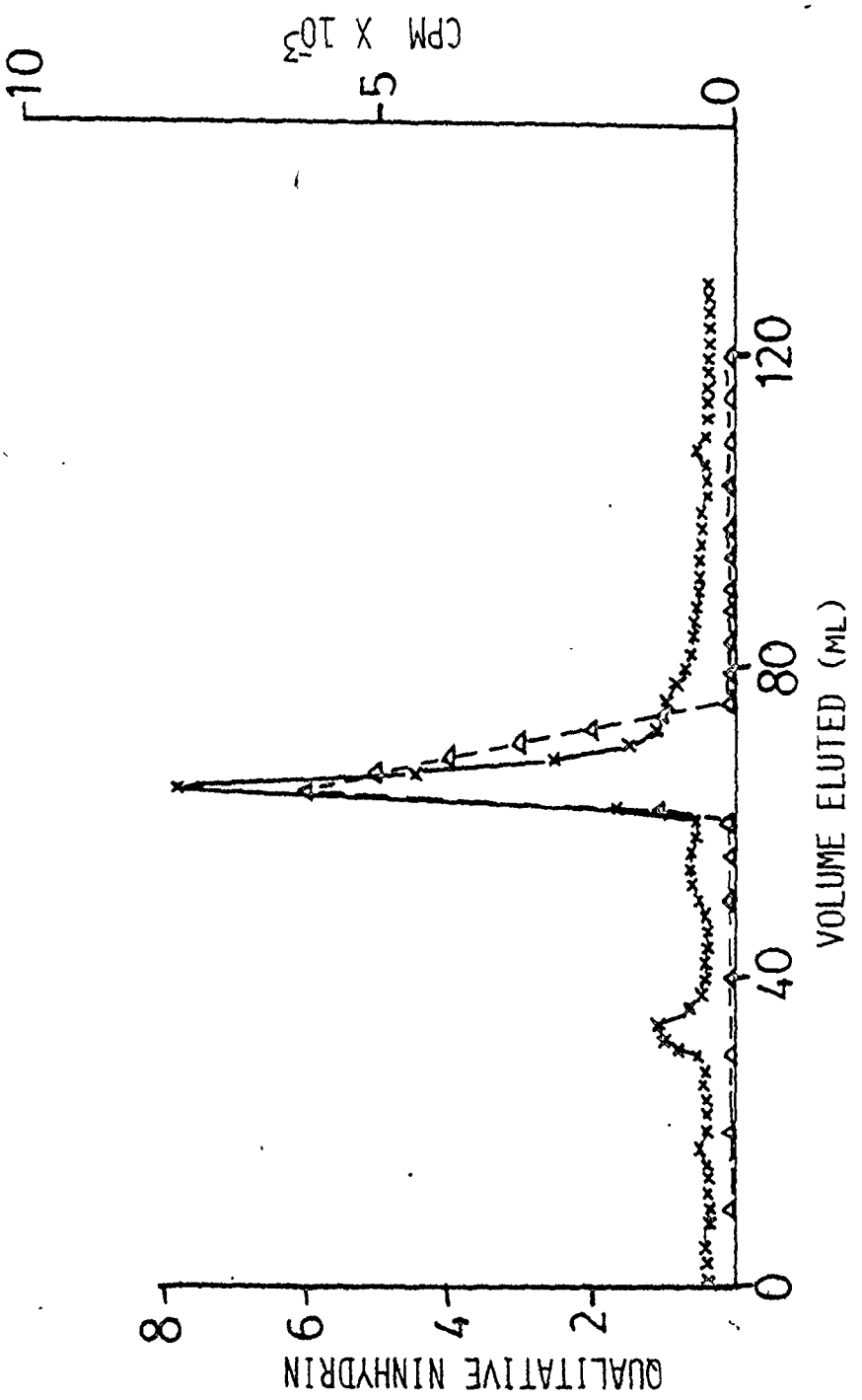
C. Chromatography on CM-Sephadex and Triethylammonium Formate

The linear gradient of triethylammonium formate used in this chromatography was of the same ionic strength

Figure 13. Separation of labelled lysyl-L-lysine from unlabelled lysyl-L-lysine by chromatography on Sephadex LH-20 with 80% isopropanol as eluant.

X——X Radioactivity (cpm)

Δ— — —Δ Qualitative Ninhydrin


CPM X 10^3
10
5
0
120
80
40
0
VOLUME ELUTED (ML)
8
6
4
2
0
QUALITATIVE NINHYDRIN

and pH as that of the KCl gradient used in Section 3.VII A. However, triethylammonium formate has the advantage that it is volatile and can be removed easily by lyophilisation. A solution of pure lysyl-L-lysine in this solvent can be lyophilised in about 15 hours leaving a white powder which is stable when stored at -20°C.

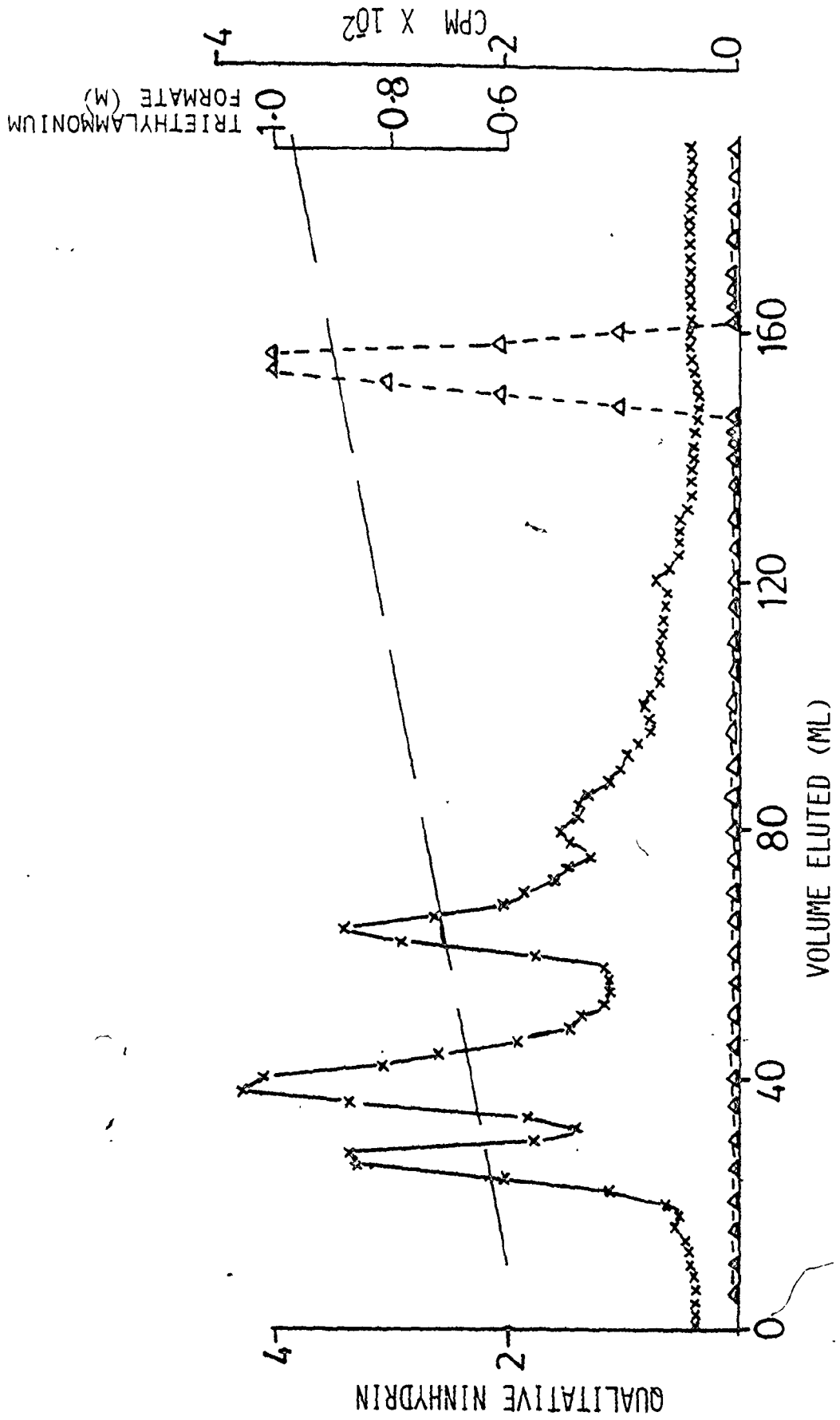
The resolution of labelled lysyl-L-lysine and unlabelled lysyl-L-lysine obtained using a shallow gradient of 0.6M to 1.0 M triethylammonium formate, pH 6.8, was quite adequate, as shown in Figure 14. The radioactivity profile provided evidence for four components in the labelled material. Of interest was the fact that no ninhydrin positive material matched the positions of the radioactivity peaks. This could perhaps be explained by the same reasons given for this result in Section 3.VII A since the same amount of labelled lysyl-L-lysine was applied to this column. Absorbance at 220 nm was not measured since triethylammonium formate absorbs strongly at that wavelength. The KCl present in the sample applied to the column eluted between 22 ml and 26 ml as determined by a qualitative reaction with silver nitrate.

Desalting by gel filtration on Sephadex G-10 was not successful since the KCl salt eluted in volumes that overlapped the radioactivity peak.

Figure 14. Separation of labelled lysyl-L-lysine from unlabelled lysyl-L-lysine on CM-Sephadex with a linear gradient of 0.6 M to 1.0 M triethylammonium formate, pH 6.8.

X——X Radioactivity (cpm)

△– – –△ Qualitative Ninhydrin


QUALITATIVE NINHYDRIN
4
2
0
40
80
120
160
VOLUME ELUTED (ML)
TRIETHYLAMMONIUM FORMATE (M)
1·0
0·8
0·6
CPM X 10²
4
2
0

VIII. Larger Scale Reaction Mixtures for Binding Studies with Lysyl-L-lysine

Several attempts were made to increase the scale of binding of ^{14}C-Nitrofurazone to lysyl-L-lysine during reduction by nitrofuran reductase I. The concentrations of various components of the reaction mixtures were increased and different methods to promote the binding reaction were used, some of which are listed in Tables 4 and 5.

Unfortunately, these experiments did not yield adequate amounts of labelled L-lysyl-Lysine; this may perhaps be due to a short half-life of the L-lysine adducts.

IX. Stability of L-lysine-Nitrofurazone Adducts

The stability of poly-L-lysine-nitrofurazone adducts was studied over a 7-day period. The reaction mixture of Section 2.VI, containing poly-L-lysine, was employed. After reduction was complete (as judged by the disappearance of the yellow colour of nitrofurazone) the solution was dialysed against distilled water at 3-5°C. Samples were taken periodically and poly-L-lysine concentration, as determined by the ninhydrin method, and radioactivity of TCA-insoluble material were determined.

As shown in Table 8, the labelled poly-lysine was fairly stable up to seven days after the start of the dialysis. The experiments on the binding studies with poly-lysine and lysyl-L-lysine (Sections 3.V to VII) were completed in less than four days. Less than 15% of the label was lost in 4 days as determined in this experiment, thus the small amounts of labelled L-lysine residues that were obtained in the experiments described above cannot be explained by a short half-life of the L-lysine-nitrofurazone adducts.

It was evident that the volume of the solution in the dialysis sac increased with time as the smaller molecules diffused out. Such volume change was corrected for somewhat by measuring both the protein concentration and radioactivity of a fixed volume of sample, and expressing the bound label in terms of protein concentration.

X. Binding of Activated Nitrofurazone to other Amino Acids

The reaction of ^{14}C-nitrofurazone metabolites with L-serine, L-histidine, L-arginine, L-cysteine, and L-lysine were studied by measuring the inhibition of the binding of the activated derivatives to serum albumin in the presence of the various amino acids. The reaction mixtures are described in Section 2.XIII; each contained 5 mg serum albumin, 7 µmoles amino acid and reductase IA.

TABLE 8

Stability of L-Lysine-Nitrofurazone Adducts

Time After Reduction (Hours)	CPM / 10 µg Poly-L-Lysine	
	Actual Values	Average Values
0	952 984	969
5.5	863 901	882
24	981 888	934
30	919 836	877
45	844 816	830
52	922 872	896
76	772 840	806
100	831 792	811
124	685 752	718
148	737 763	750
170	709 632	670

L-Cysteine inhabited the reaction of nitrofurazone metabolites with protein by over 90% while L-lysine produced only 28% inhibition, as shown in Table 9. This indicates that the nucleophilic sulphur groups of cysteine are much more reactive with the electrophilic metabolites and hence cysteine would perhaps be a better amino acid to trap the activated intermediates.

These results indicate that the amino acids in pure proteins that would be most readily derivatised are the cysteines. However, the relative amounts of each potential nucleophilic amino acid in proteins must be considered. Generally, the total number of cysteine residues in many pure proteins is less than that of lysine (71), and therefore it is possible that the total number of cysteine-nitrofuran adducts formed *in vivo* would be less than the number of lysine-nitrofuran adducts.

TABLE 9

Reaction of ^{14}C-Nitrofurazone with Protein[a] in the Presence of Various Amino Acids

Amino Acid[b]	Total CPM / 200 µl	Average Value	% Inhibition
None (Control)	31,364[c] 32,012	31,688	
Serine	27,496 28,196	27,847	12.1
Histidine	23,295 25,082	24,188	23.7
Arginine	26,481 25,899	26,190	17.4
Cysteine	2,407 2,746	2,576	91.9
Lysine	21,265 23,862	22,563	28.8

a Bovine serum albumin

b 7 µmoles amino acid present in each reaction mixture

c Values represent the mean for duplicate determinations

4. RESULTS

PART II

I. Type I Nitroreductases of Escherichia coli

As stated in the Introduction, it has been established that the genotype of wild-type E. coli K 12, strain AB1157, is nfs A^+B^+ while the genotypes of its mutants are as follows: NFR 402, nfs A B^+; NFR 502, nfs A B; and NFR Sil 41, nfs A^+B.

These strains, which were stored at -80°C, were all tested for their sensitivities to nitrofurazone, as described previously (24), to ensure that they were homogenous, that is, that they had not reverted or mutated further, before extractions of nitroreductase I were undertaken.

II. Chromatography of Nitroreductase Activity Present in E. coli AB 1157

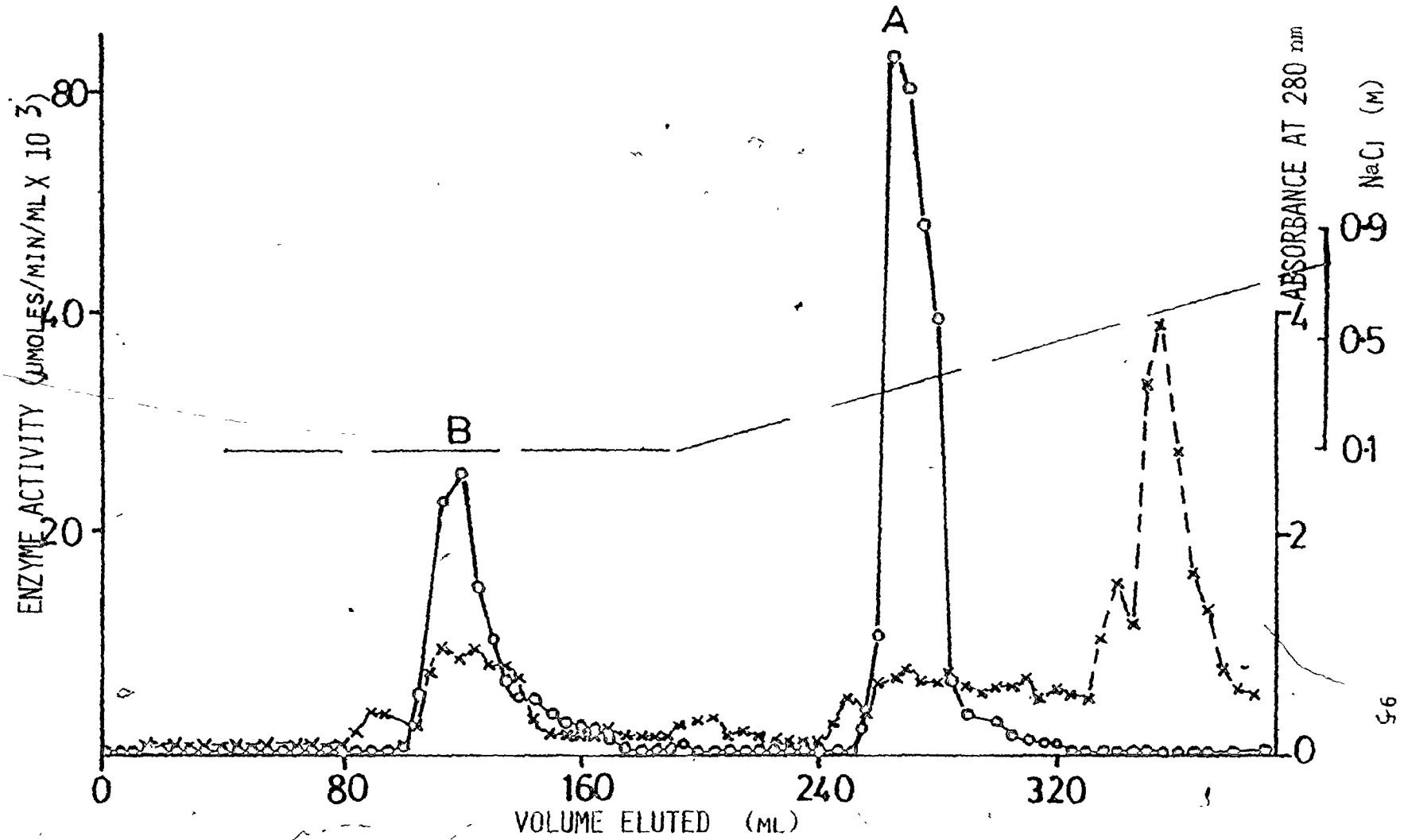
Figure 15 shows the elution profile of a crude extract of AB 1157, prepared without the use of $MnCl_2$, on DEAE-cellulose. NADPH-stimulated activity and absorbance at 280 nm indicated that the two components of activity expected in this wild-type strain (from previous work (24))

Figure 15. Type I nitroreductase activity present in wild-type E.coli AB1157 (nfs A^+ nfs B^+). This extract was prepared without the use of $MnCl_2$.

Chromatography on DEAE 52-cellulose (60 cm x 0.9 cm column). Eluted with 35 ml 0.015 M K-phosphate buffer, pH 7.2; 140 ml buffered 0.1 M NaCl followed by a linear gradient of 0.1 M to 0.8 M NaCl, pH 7.2.

O——O NADPH-stimulated Nitroreductase Activity (μmoles/min/ml)

X——X Absorbance at 280 nm.


A
B
ENZYME ACTIVITY (μMOLES/MIN/ML X 10 3)
80
40
20
0
80
160
240
320
VOLUME ELUTED (ML)
ABSORBANCE AT 280 nm
NaCl (M)
0·9
0·5
0·1
4
2
0

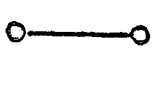
were present. Peak B, the product of the nfs B^+ gene, eluted with the 0.1 M NaCl wash while peak A, the product of the nfs A^+ gene, eluted with the salt gradient at about 0.3 M NaCl. The major UV-absorbing peak (280 nm) eluted after both activities at about 0.55 M NaCl.

III. Chromatography of Nitroreductase Activity Present in E.coli NFR Sil 41

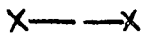
Figure 16 shows the elution profile of the mutant strain Sil 41 (nfs A^+ B) when the crude extract was prepared without the use of $MnCl_2$. Two peaks of activity whose positions of elution compared well with the parental strain AB1157 were obtained. That result was interesting as it revealed the presence of a B component of reductase I that was not expected in this mutant. Furthermore, the first peak of activity, B_{II}, cannot be ascribed to nfs B^+ revertants in the population since genetic tests of the Sil 41 culture inidcated that it was homogenous. The total activity present in peak B_{II}, was calculated to less than that of peak B of Figure 15 (AB1157) when the same amount of protein was loaded on the DEAE-cellulose column.

When a $MnCl_2$-treated extract was chromatographed on DEAE-cellulose, the elution profile revealed the presence of only one nitroreductase peak as shown in Figure 17. The peak that eluted at 0.3 M NaCl was

Figure 16. Type I nitroreductase activity of *E. coli* Sil 41 (*nfs* A^+ *B*). Crude extracts were prepared without the use of $MnCl_2$. Chromatography on DEAE-cellulose (60 cm x 0.9 cm column). Eluted with 0.015 M K-phosphate buffer, pH 7.2; 0.1 M NaCl, pH 7.2 plus a linear gradient of 0.1 M to 1.0 M NaCl, pH 7.2.



Nitroreductase Activity (μmoles/min/ml)



Absorbance at 280 nm

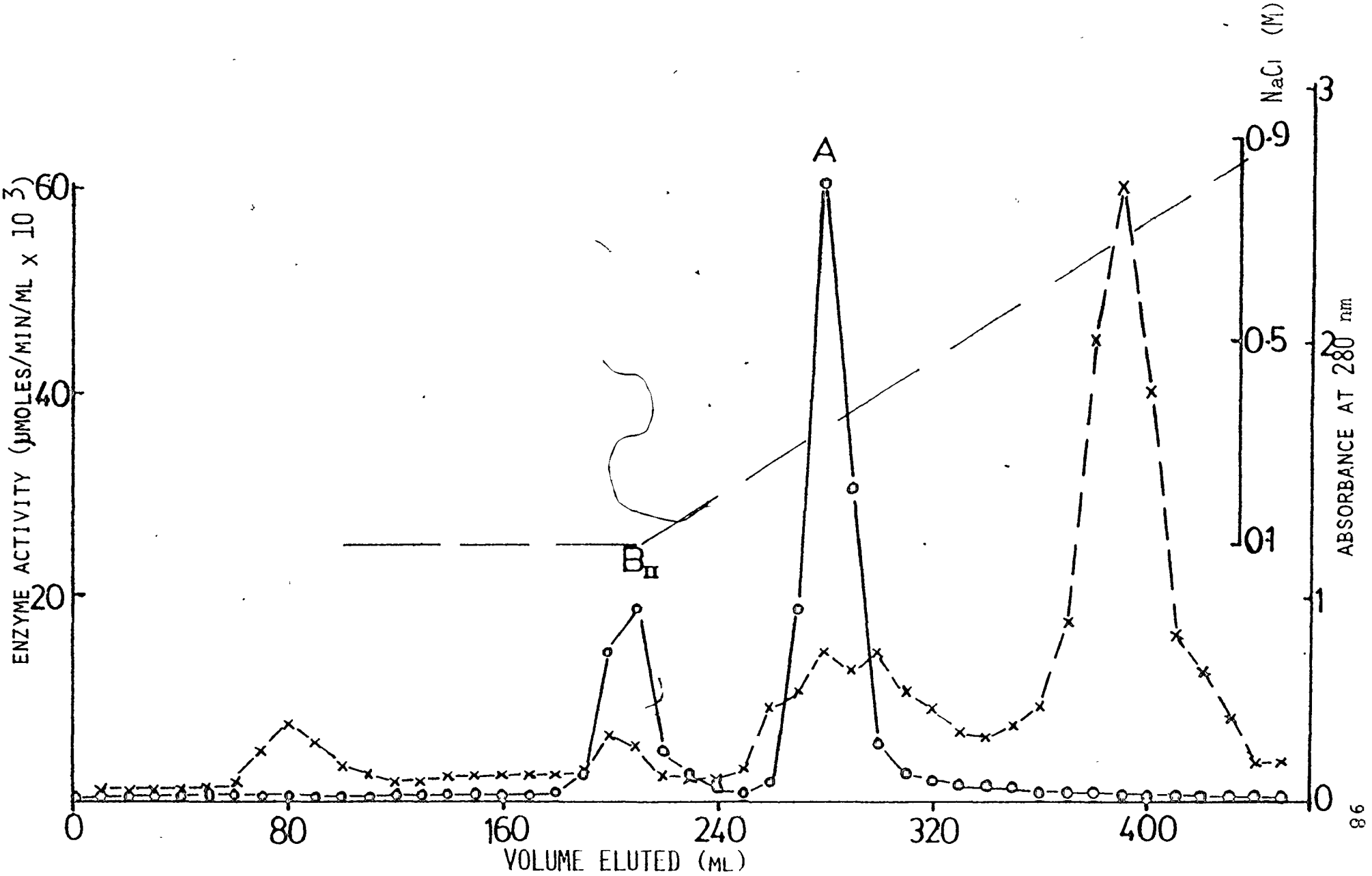
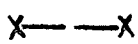
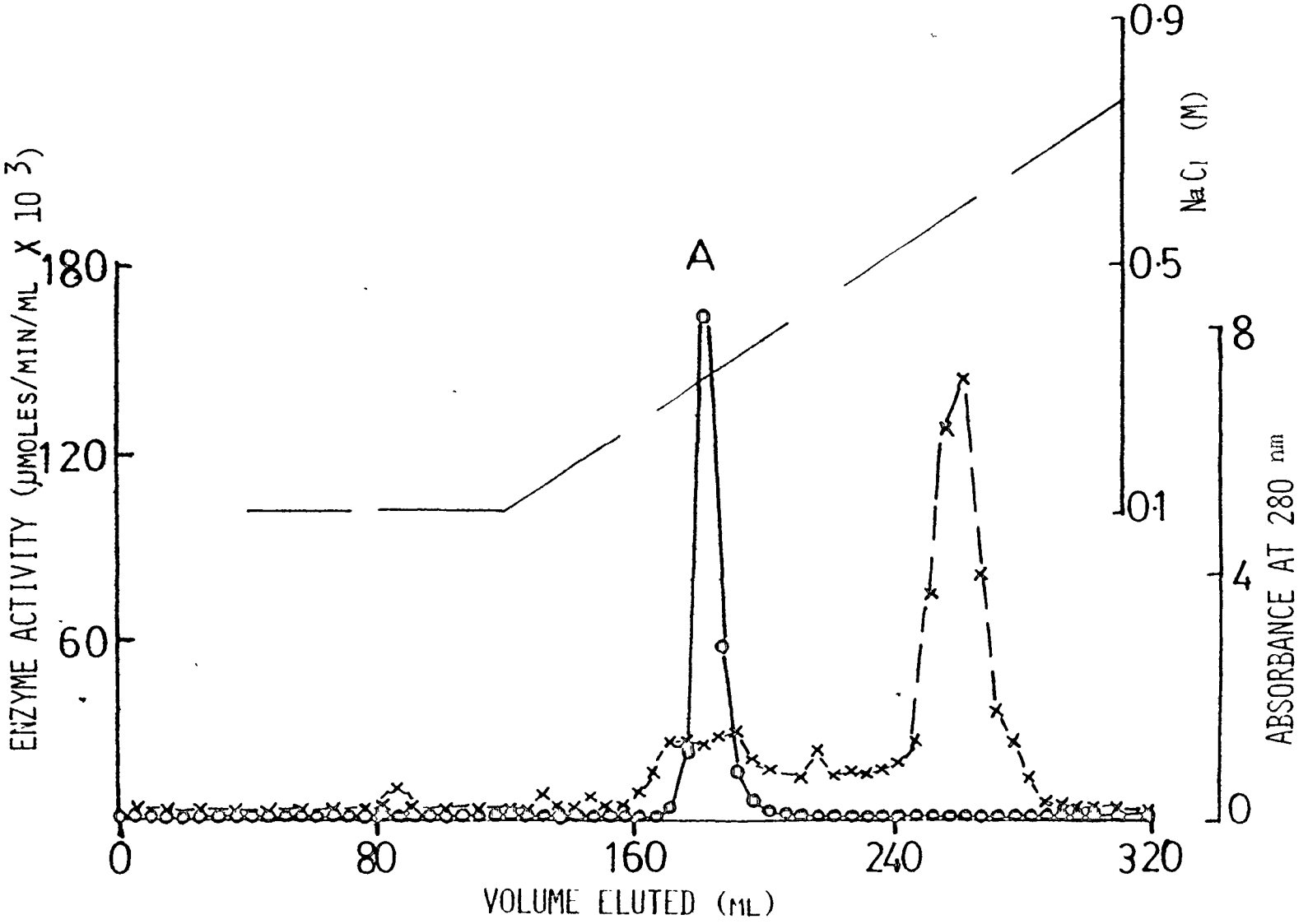

ENZYME ACTIVITY (μMOLES/MIN/ML x 10 3)
60
40
20
0
80
160
240
320
400
VOLUME ELUTED (ML)
A
B II
NaCl (M)
0·9
0·5
0·1
ABSORBANCE AT 280 nm
3
2
1
0

Figure 17. Nitroreductase activity present in the NFR mutant Sil 41.

Chromatography of a crude extract, prepared with the use of $MnCl_2$, on DEAE-cellulose. Eluted with 0.015 M K-phosphate buffer, pH 7.2; 0.1 M NaCl, pH 7.2 and a linear gradient of 0.1 M to 0.8 M NaCl, pH 7.2.


Nitroreductase Activity (μmoles/min/ml).


Absorbance at 280 nm


ENZYME ACTIVITY (μMOLES/MIN/ML X 10 3)
180
120
60
0
80
160
240
320
VOLUME ELUTED (ML)
A
0·9
0·5
0·1
NaCl (M)
8
4
0
ABSORBANCE AT 280 nm

equivalent to the 'A' component of wild-type AB1157. Therefore, it appeared that peak B_{II} of Sil 41 was inactivated by $MnCl_2$.

The major UV-absorbing peak (280 nm) eluted after 0.5 M NaCl.

IV. Chromatography of Nitroreductase Activity present in E. coli NFR 402

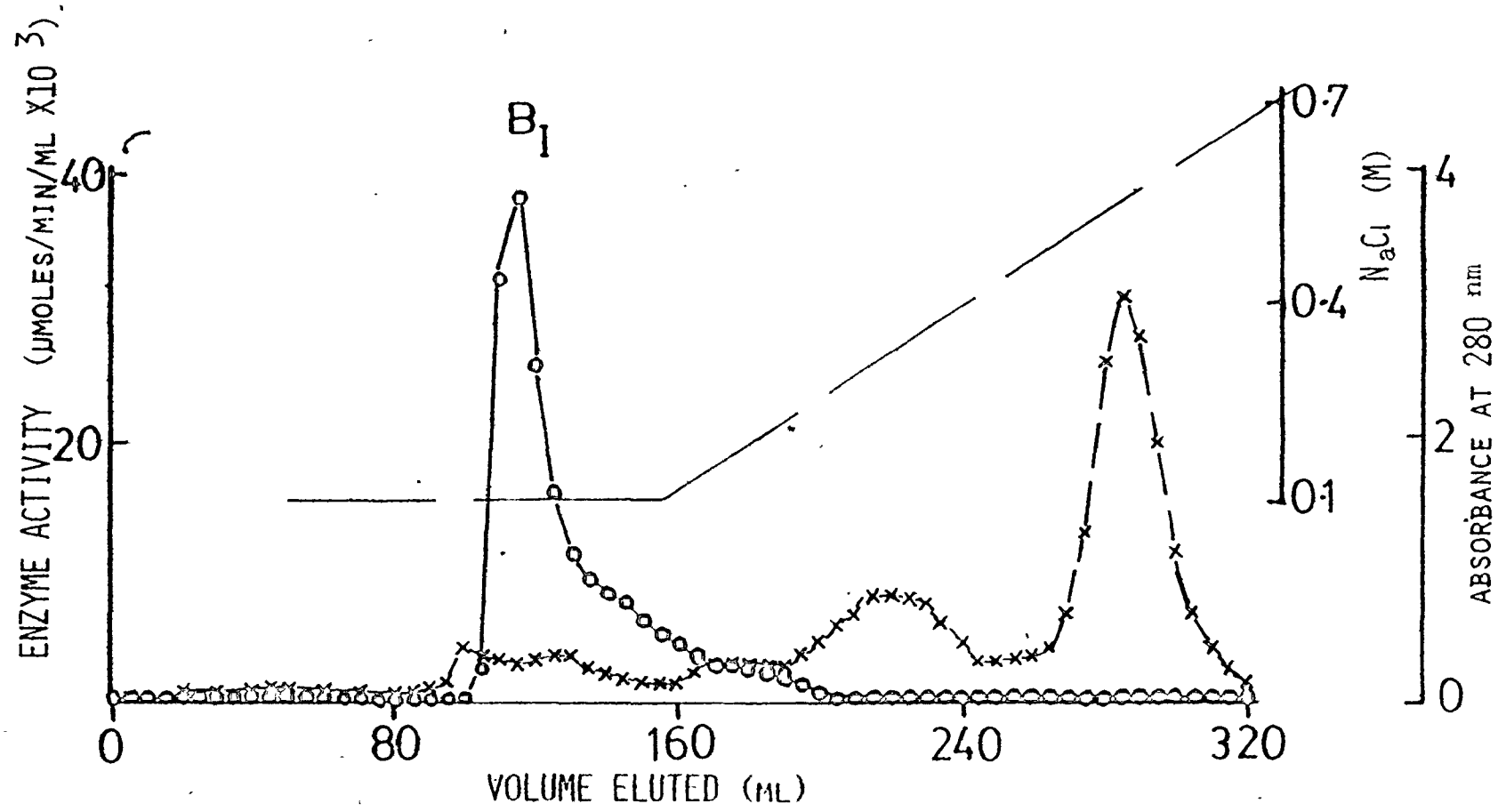
The elution profile of crude extracts of E. coli NFR 402 (nfs A^- B^+) prepared with the use of $MnCl_2$ is shown in Figure 18. There was only one major peak of NADPH-stimulated activity eluting in the position characteristic of the B component of AB1157, which agreed with the genetic characterisation of this mutant. Moreover, that component was not lost upon treatment with $MnCl_2$, although a small amount of the total activity was lost by the $MnCl_2$ step during the purification procedure. The major protein peak eluted at 0.55 M NaCl, pH 7.2

Thus, the reductase I component present in NFR 402 (the nfs B^+ gene product) appeared to be different from the B_{II} component of NFR Sil 41, although they were both eluted with 0.1 M NaCl on DEAE-cellulose. If the B_{II} component was present in this mutant, it would have been lost by the

Figure 18. Nitroreductase activity present in the NFR mutant, E.coli 402 (nfs A B^{+}). Chromatography of a crude extract, prepared with the use of $MnCl_2$, on DEAE-cellulose (60 cm x 0.9 cm column). Eluted with 0.015 M K-phosphate buffer, pH 7.2; 0.1 M NaCl, pH 7.2 followed by a linear gradient of 0.1 M to 0.8 M NaCl, pH 7.2.

O——O Nitroreductase Activity (μmoles/min/ml)

X— —X Absorbance at 280 nm


B1
ENZYME ACTIVITY (μMOLES/MIN/ML X10 3)
40
20
0
80
160
240
320
VOLUME ELUTED (ML)
0·7
0·4
0·1
NaCl (M)
4
2
0
ABSORBANCE AT 280 nm

$MnCl_2$ step during the extraction process.

V. Chromatography of Nitroreductase Activity Present in E. coli 502

Extracts of NFR 502 (nfs A B) were prepared without the use of $MnCl_2$ and chromatographed on DEAE-cellulose. Because of the small amount of nitroreductase activity remaining in this strain, a relatively large amount of protein was loaded on the column in order to detect significant activity in the column fractions.

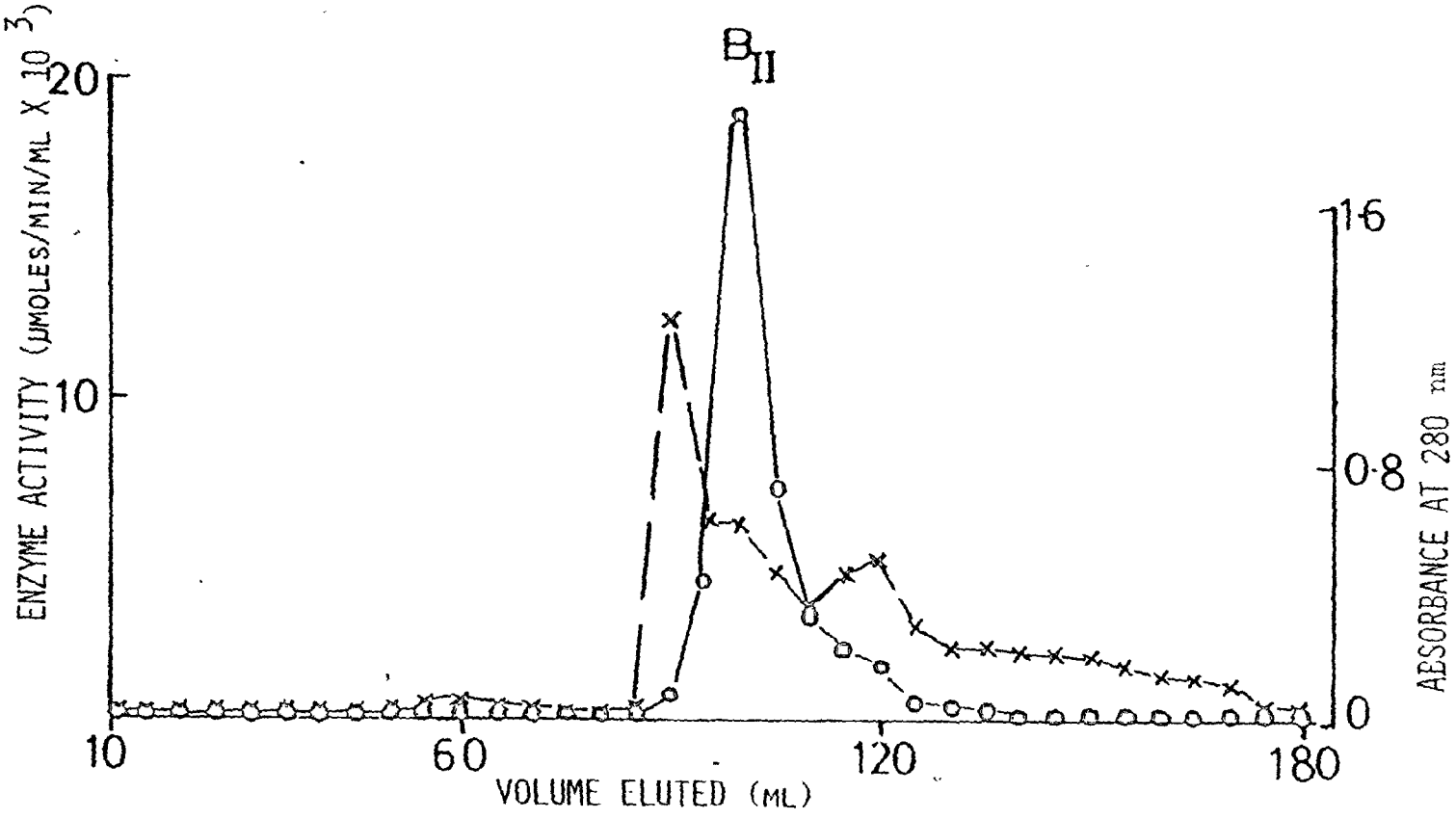
Figure 19 shows the activity and protein elution pattern with buffered 0.1 M NaCl as eluant. One peak of activity was obtained at a position characteristic of the B component of the parental strain. No nitroreductase activity eluted with the NaCl concentration gradient which served only to remove extraneous proteins bound to the ion-exchange matrix. Subsequently, it was found that the activity present in NFR 502 was sensitive to $MnCl_2$, in fact, the reduction of nitrofurazone by the 502 extract, in the presence of $MnCl_2$, decreased to an insignificant rate quite rapidly.

These results, together with those described above, suggested that the B peak that eluted in the 0.1 M NaCl wash of wild-type AB1157 and NFR 402 (without $MnCl_2$) might actually contain two different components of nitroreductase

Figure 19. Nitroreductase activity present in the NFR mutant, E.coli 502 (nfs A B).
Chromatography of a crude extract, prepared without the use of $MnCl_2$, on DEAE-cellulose (60 cm x 0.9 cm column). Eluted with 0.015 M K-phosphate buffer, pH 7.2; followed by 0.1 M NaCl, pH 7.2.

O——O NADPH-stimulated Nitroreductase Activity (μmoles/min/ml)

X— —X Absorbance at 280 nm


ENZYME ACTIVITY (μMOLES/MIN/ML X 10 3)
20
10
B II
1·6
0·8
0
ABSORBANCE AT 280 nm
10
60
120
180
VOLUME ELUTED (ML)

activity which eluted together on DEAE-cellulose. One of the components (peak B_{II}) was inactivated by $MnCl_2$ and was probably the single component present in NFR 502. Thus, it was reasoned that when crude extracts of type I reductase of Sil 41 are prepared with the use of $MnCl_2$, the nfs A^+ gene product (IA) can be purified on DEAE-cellulose. When NFR 402 extracts are prepared in the same manner, the nfs B^+ gene product (component IB_I) can be purified. When NFR 502 extracts are prepared without the use of $MnCl_2$ another component, IB_{II}, which is perhaps the product of another gene, can be purified on DEAE-cellulose.

Further investigation of this hypothesis was made by other workers in our laboratory. However, the type I reductase components were purified, as described above, and used in the experiments that follow.

VI. Factors Influencing Enzyme Reaction Velocity

The factors which determine the form of the progress curves of enzyme reactions are numerous and varied. The chief factors which determine the initial velocity of a particular reaction are the presence of activators or inhibitors, temperature, enzyme concentration, pH and substrate concentration (72). The latter three effects on the rate of type I nitroreductase activity were studied.

A. pH of Optimal Activity of the IA Component of Type I Reductase of E. coli Sil 41.

Figure 20 shows the pH activity profile of the IA component of type I reductase extracted from NFR Sil 41. The enzyme was quite active over a pH range of 6 to 8.5; the pH of optimal activity was 6.7. This value is in good agreement with the result of Asnis (13) who found the optimum pH of nitroreductase I to be 6.9.

The pH of the standard enzyme assay mixtures used throughout these experiments is 7.2. This pH falls within the optimum pH range of the type I reductases as shown in this experiment.

B. The Effect of Enzyme Concentration on Reaction Rate of Type I Reductase

When the effect of enzyme concentration on the activity of the IA component of reductase I from NFR Sil 41 was studied, it was found that the reaction velocity increased proportionally with enzyme concentration, as shown in Figure 21. No more than 10 µl of the extract (or 20 µg protein) was used in the standard incubation mixtures - the relationship of reaction velocity to enzyme concentration was certainly linear up to that concentration of enzyme. In addition, there were no toxic impurities present in the reaction mixtures as there was no lag in

Figure 20. The activity profile of the IA component of Type I reductase of E.coli NFR S11 41 at various pH's.
Nitroreductase activity measured in µmoles/min/ml.

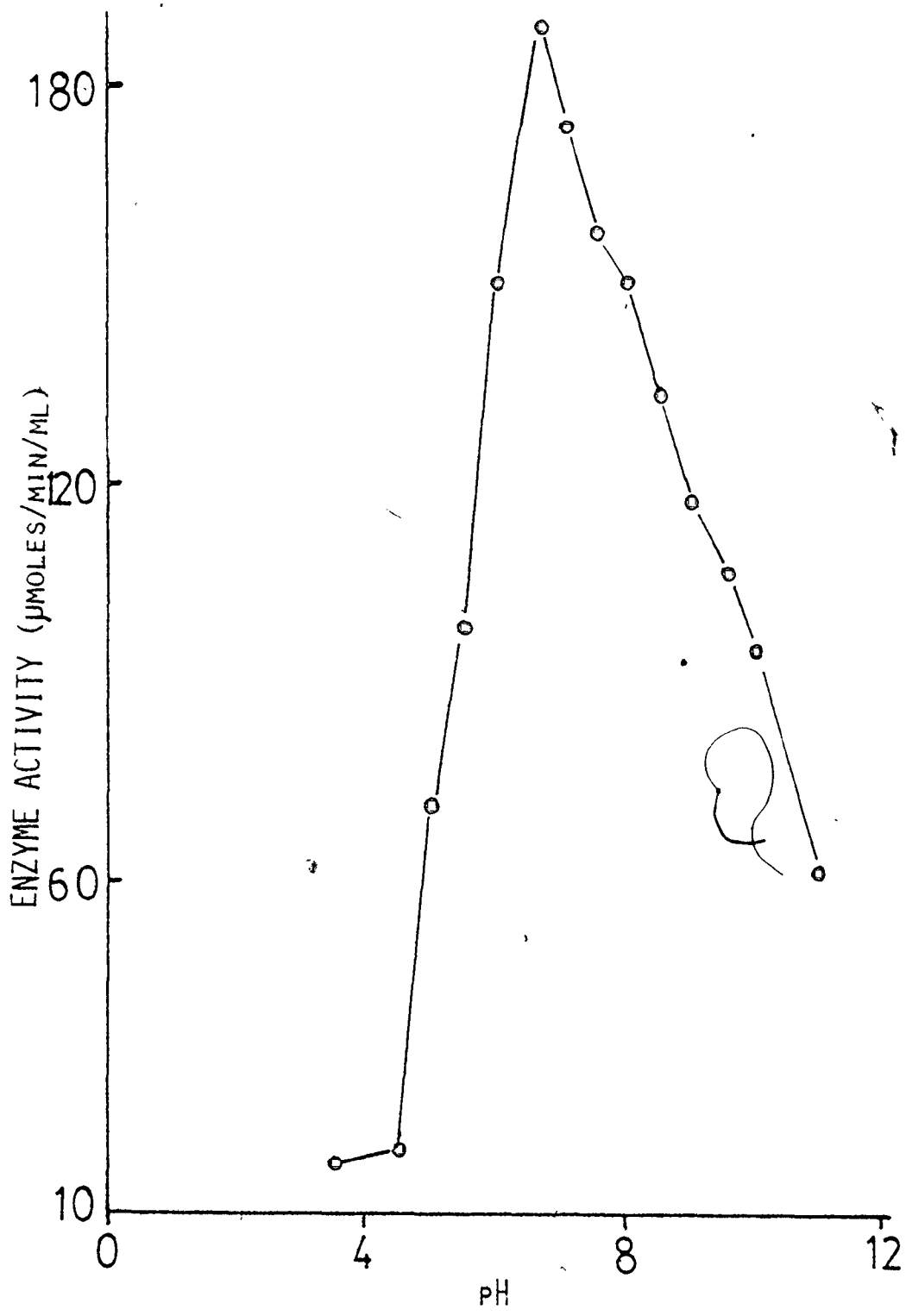
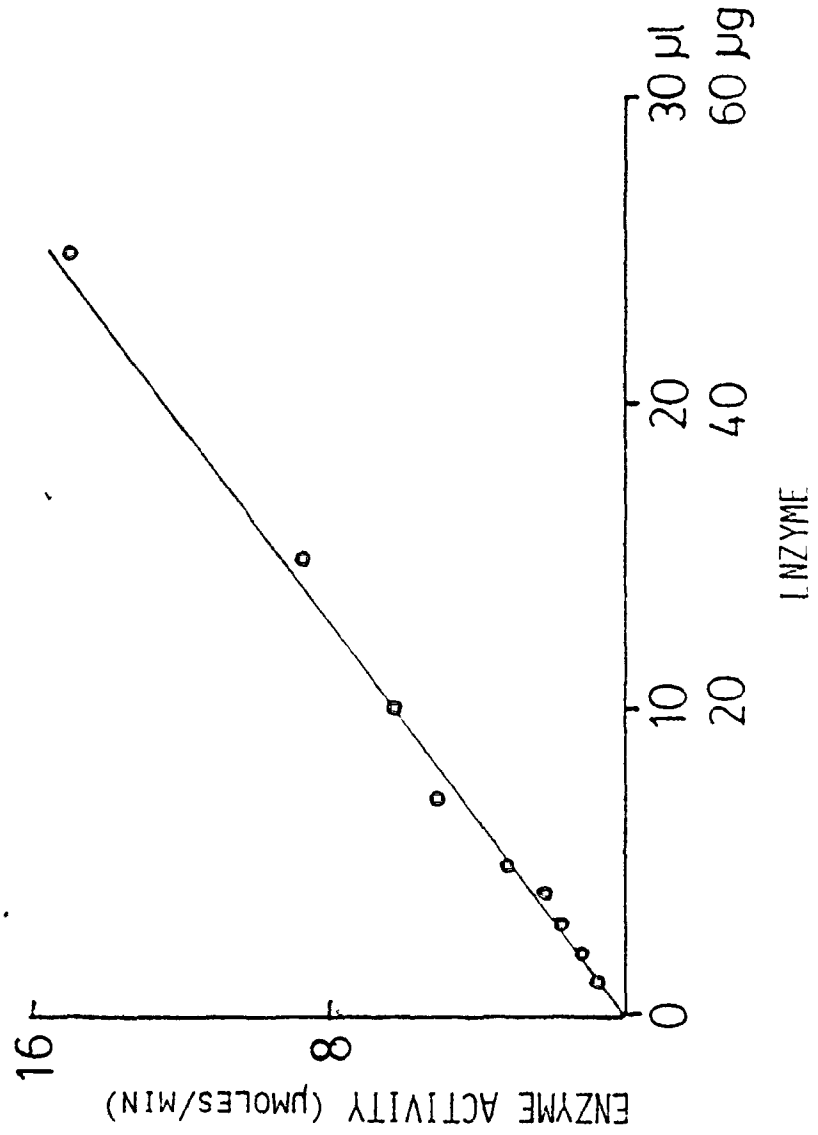

180
120
60
10
ENZYME ACTIVITY (µMOLES/MIN/ML)
0
4
8
12
pH

Figure 21. Dependence of activity (μmoles/min/ml) of Type I nitroreductase, from NFR Sil 41, on enzyme concentration (μl).


ENZYME ACTIVITY (μMOLES/MIN)
16
8
0
10
20
20
40
30 μl
60 μg
ENZYME

the reaction rates at low enzyme concentration.

VII. Km and Vmax Determinations

The Km and Vmax values of the three components of E. coli nitroreductase I with four nitrofurans were studied in order to determine if there were any correlations between the kinetic constants of the enzyme and different substrates and the mutagenic activity of those substrates, as described in Introduction. The three components of nitroreductase I (IA, IB_I and IB_{II}) were prepared as described above; the same number of units of activity were added to each enzyme assay mixture (2.5×10^{-3} units).

The initial rate of enzyme activity was obtained by drawing the tangent to the initial phase of the progress curves which resulted from continuous assays. Km and Vmax values were obtained from the Michaelis-Menten equation using the Lineweaver-Burk double reciprocal graphical method. Initially, concentration of substrate ranging from those required for minimal nitroreductase activity to those that gave maximum activity were used to calculate the Km and Vmax values. Subsequently, more accurate double reciprocal plots of initial rate of activity versus substrate concentration were done on an expanded scale using only substrate concentrations that were 50% above and below the calculated Km values. The best straight

lines through the points were estimated by linear regression statistical analysis.

VIII. Km, Vmax of IA Component of Reductase I of NFR Sil 41

The Km and Vmax values of the nfs A^+ gene product of NFR Sil 41 (component IA) were determined at room temperature (22°C) and at 37°C with nitrofurazone as substrate. Figure 22A shows the Lineveaver-Burk plot of a representative experiment at 22°C; Km was 12.5 μM and Vmax was 0.42 μmoles/min. When the same parameters were plotted on an expanded scale, more refined values were obtained since the activities at low substrate concentrations, at which low rates of change were difficult to measure with accuracy, were eliminated. As shown in Figure 22B, the Km value at 22°C was 10 μM which agrees quite well with the value obtained from the plot of Figure 22A; the reciprocal relationship of enzyme activity and substrate concentration was linear to as low as 400 mM^{-1} nitrofurazone. At 37°C, the Km of the enzume was 50 μM as determined by the double reeiprocal plot of Figure 23B (expanded scale) and the reciprocal relationship was linear up to 300 mM^{-1} nitrofurazone.

The Km and Vmax values of the IA component of Sil 41 with nitrofurazone, furazolidone, ANFT and AF_2 as

Figure 22. A. Double reciprocal plot of enzyme activity (V) versus substrate concentration (S) at 22°C.
Enzyme: IA component of nitroreductase I of NFR S11 41.
Substrate: Nitrofurazone
B. Same as above (A)
Data plotted on an expanded scale.

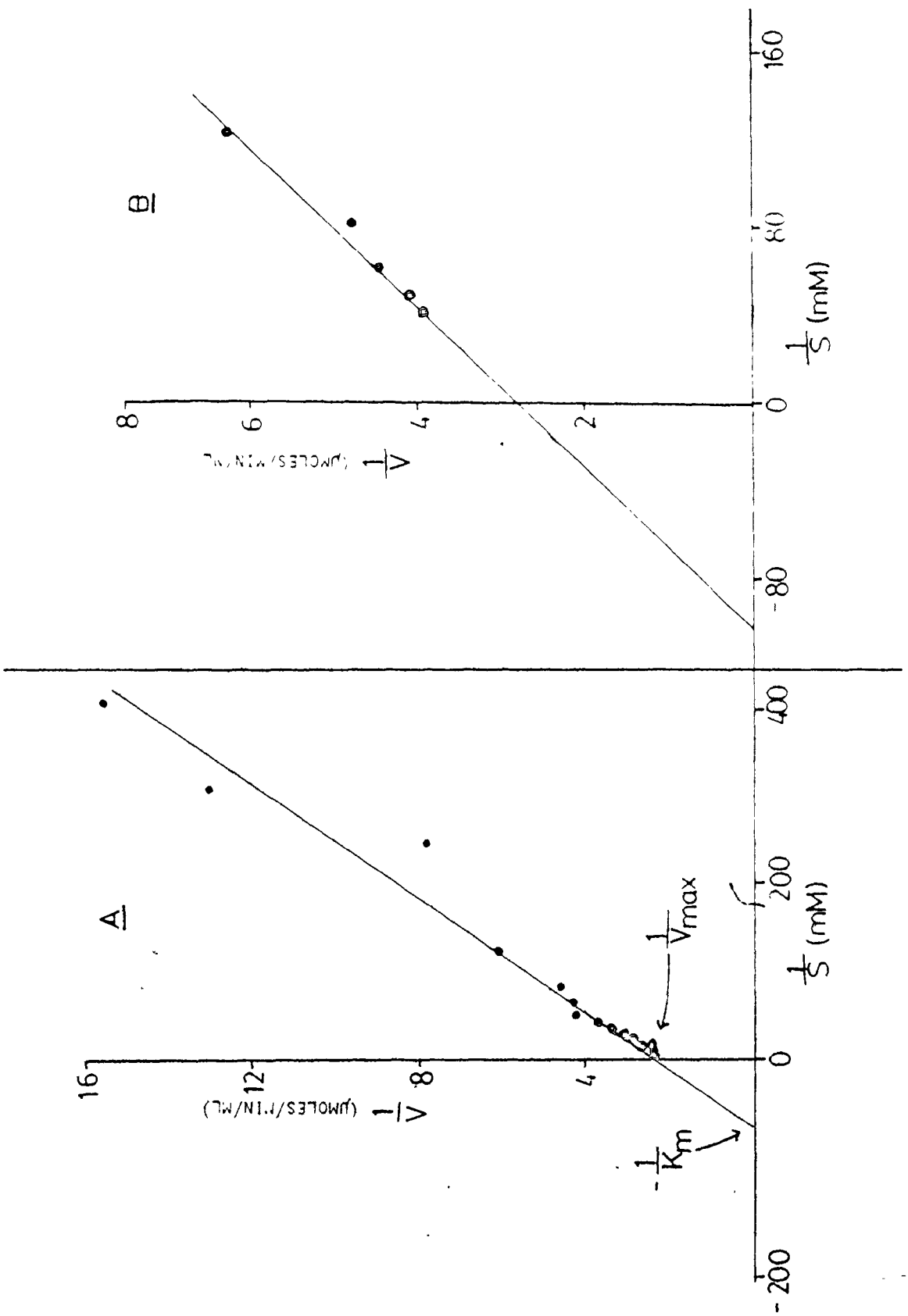

A
B
$\frac{1}{V}$ (μMOLES/MIN/ML)
$\frac{1}{S}$ (mM)
$\frac{1}{V_{max}}$
$-\frac{1}{K_m}$
16
12
8
4
0
-200
200
400
-80
80
160
2
6
8

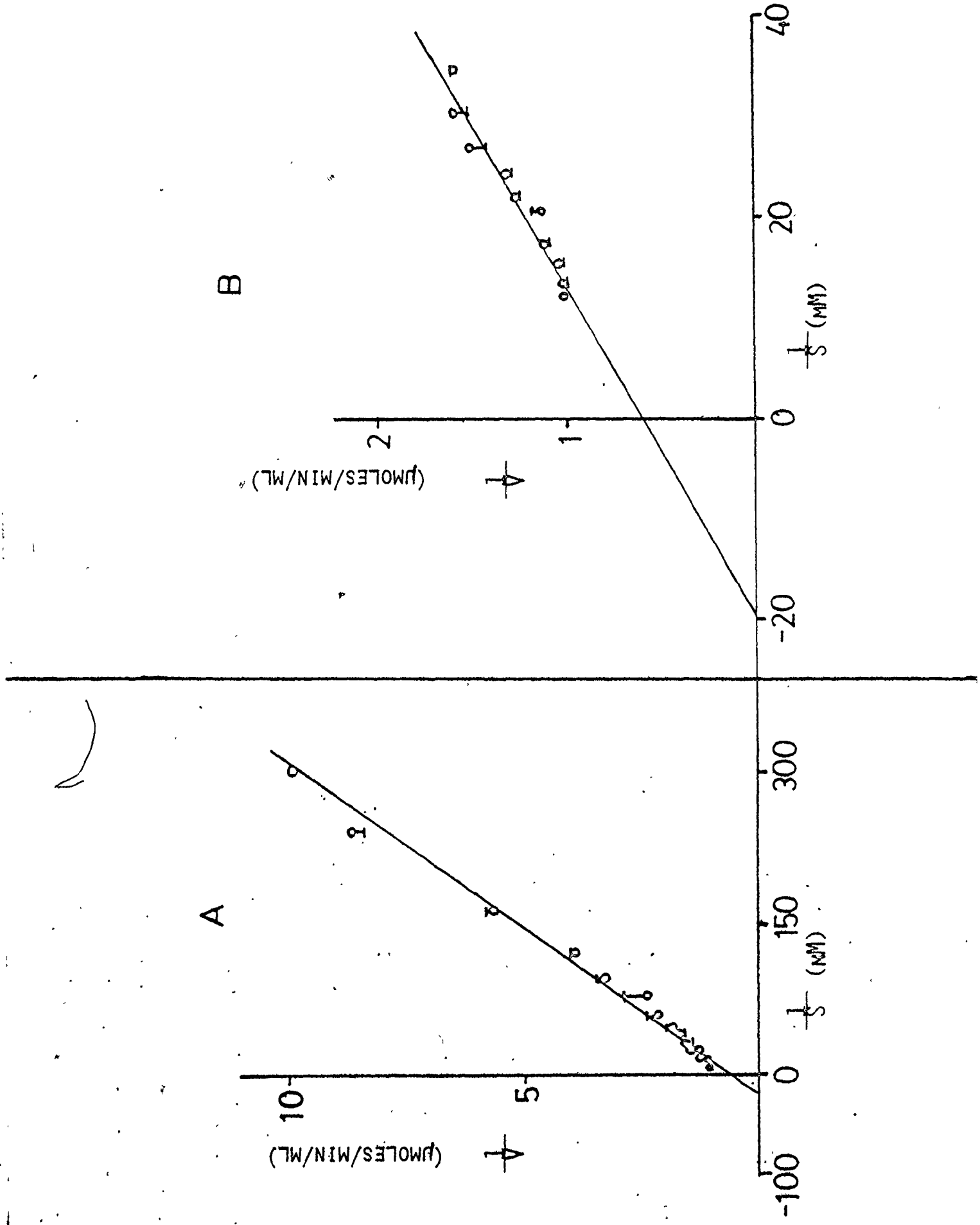
Figure 23. A. Double reciprocal plot of enzyme activity (V) versus substrate concentration (S) at 37°C.

Enzyme: IA component of NFR S1141

Substrate: Nitrofurazone

B. Same as above (A)

Data plotted on an expanded scale.


A
B
1/v (μMOLES/MIN/ML)
1/S (mM)
-100
0
150
300
5
10
-20
0
20
40
1
2

substrates at 23°C and 37°C are listed in Table 10. Representative double reciprocal plots with two substrates are shown in Figures 22 to 25 (A and B). The values of Km of the IA component of Sil 41 with the four substrates ranged from 13.3 μM to 19.7 μM at 22°C and 38.3 μM to 55.1 μM at 37°C. Thus, there is less than a two-fold difference between the highest and lowest values. However, the values of Km at 37°C was two to five times more than that at room temperature and perhaps indicate that the binding affinity of the enzyme for the substrate at 22°C was more than that at 37°C. The Vmax values at both temperatures changed proportionally with the Km values.

IX. Km, Vmax of IB_I Component of Reductase I of NFR 402

The Km and Vmax values of the IB_I component of NFR 402 with nitrofurazone, furazolidone, ANFT and AF_2 as substrates at room temperature and at 37°C are listed in Table 11. Representative Lineweaver-Burk double reciprocal plots with the substrates, nitrofurazone and AF_2, are shown in Figures 26 to 29.

As with the IA component of nitroreductase I, the difference between the highest and lowest values for Km was just slightly greater than two-fold at both temperatures. Although the Km values for the individual substrates were

TABLE 10

Km, Vmax Values of IA Component of Reductase I of Sil 41 with Nitrofurazone, Furazolidone, ANFT and AF_2 as Substrates

Substrate	Km (μM)		Vmax (μmoles/min/ml)		Double Reciprocal Plots
	22°C	37°C	22°C	37°C	
Nitrofurazone	10.5	55.6	0.45	1.85	See Figures 22, 23
	18.0	54.6	0.71	1.82	
	12.5		0.42		
	12.0		0.40		
(Average)	13.3 ±2.9	55.1 ±0.7	0.50 ±0.15	1.83 ±0.02	
Furazolidone	39.7	91.0	1.07	4.54	
	24.4	85.0	0.78	4.02	
	22.0		0.74		
	18.0		0.81		
(Average)	26.1 ±8.2	88.0 ±4.2	0.85 ±0.15	4.28 ±0.30	
ANFT	9.9	75.9	0.31	1.26	
	10.0	60.0	0.43	1.21	
	21.0		0.56		
	13.2		0.33		
(Average)	13.5 ±4.5	68.0 ±10	0.41 ±0.11	1.23 ±0.04	
AF_2	20.0	36.5	0.50	0.73	See Figures 24, 25
	20.3	40.0	0.42	0.80	
	17.9		0.44		
	20.5		0.41		
(Average)	19.7 ±1.0	38.3 ±2.6	0.44 ±0.04	0.77 ±0.04	

Figure 24. A. Double reciprocal plot of enzyme activity (V) versus substrate concentration (S) at 22°C.
Enzyme: IA component of NFR Sil 41
Substrate: AF_2

B. Same as above (A)
Data plotted on an expanded scale.

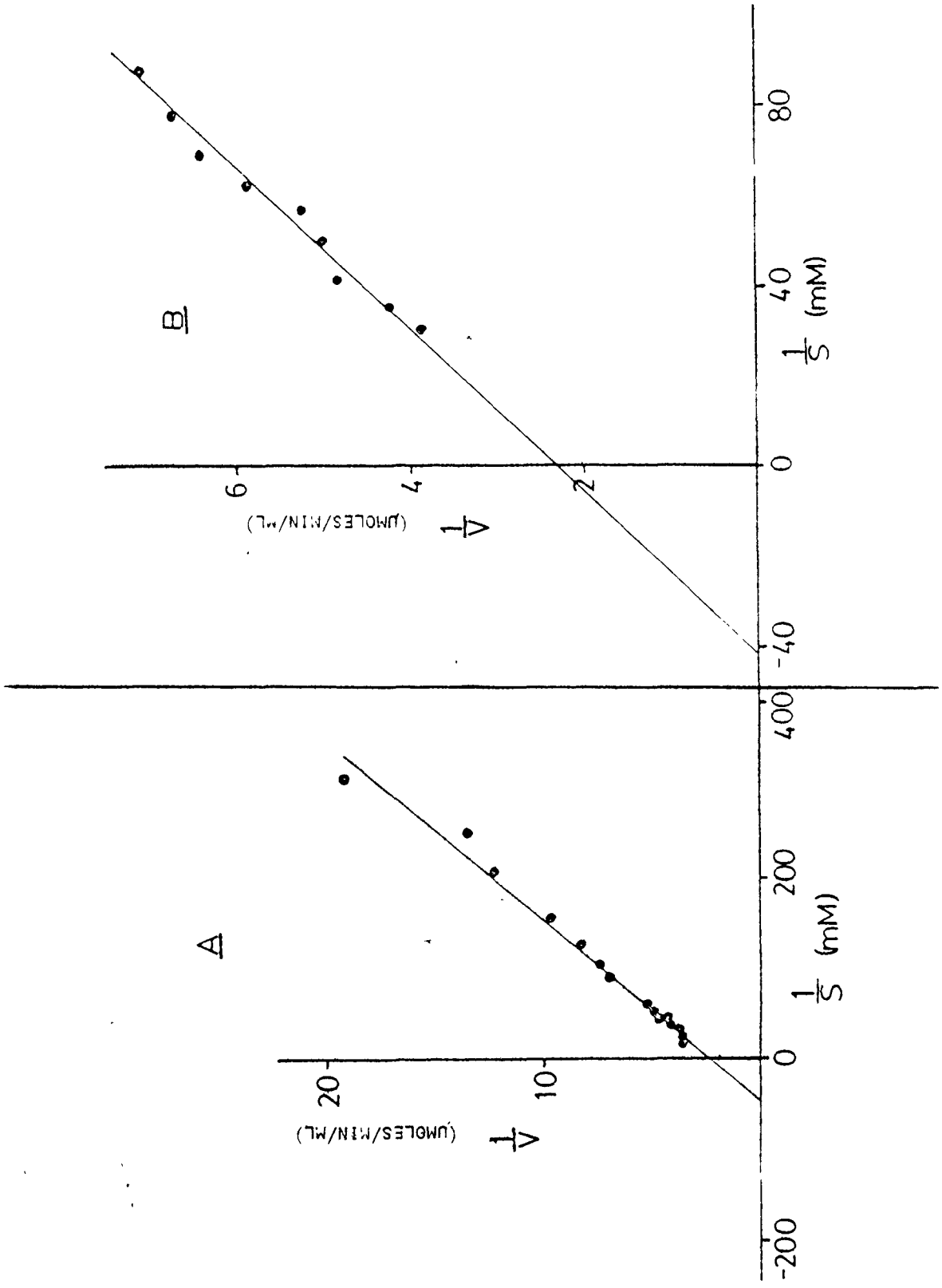

A
B
1/V (UMOLES/MIN/ML)
1/S (mM)
20
10
0
-200
200
400
6
4
2
0
-40
40
80

Figure 25. A. Double reciprocal plot of enzyme activity (V) versus substrate concentration (S) at 37°C.

Enzyme: IA component of NFR Sil 41

Substrate: AF_2

B. Same as above (A)

Data plotted on an expanded scale.

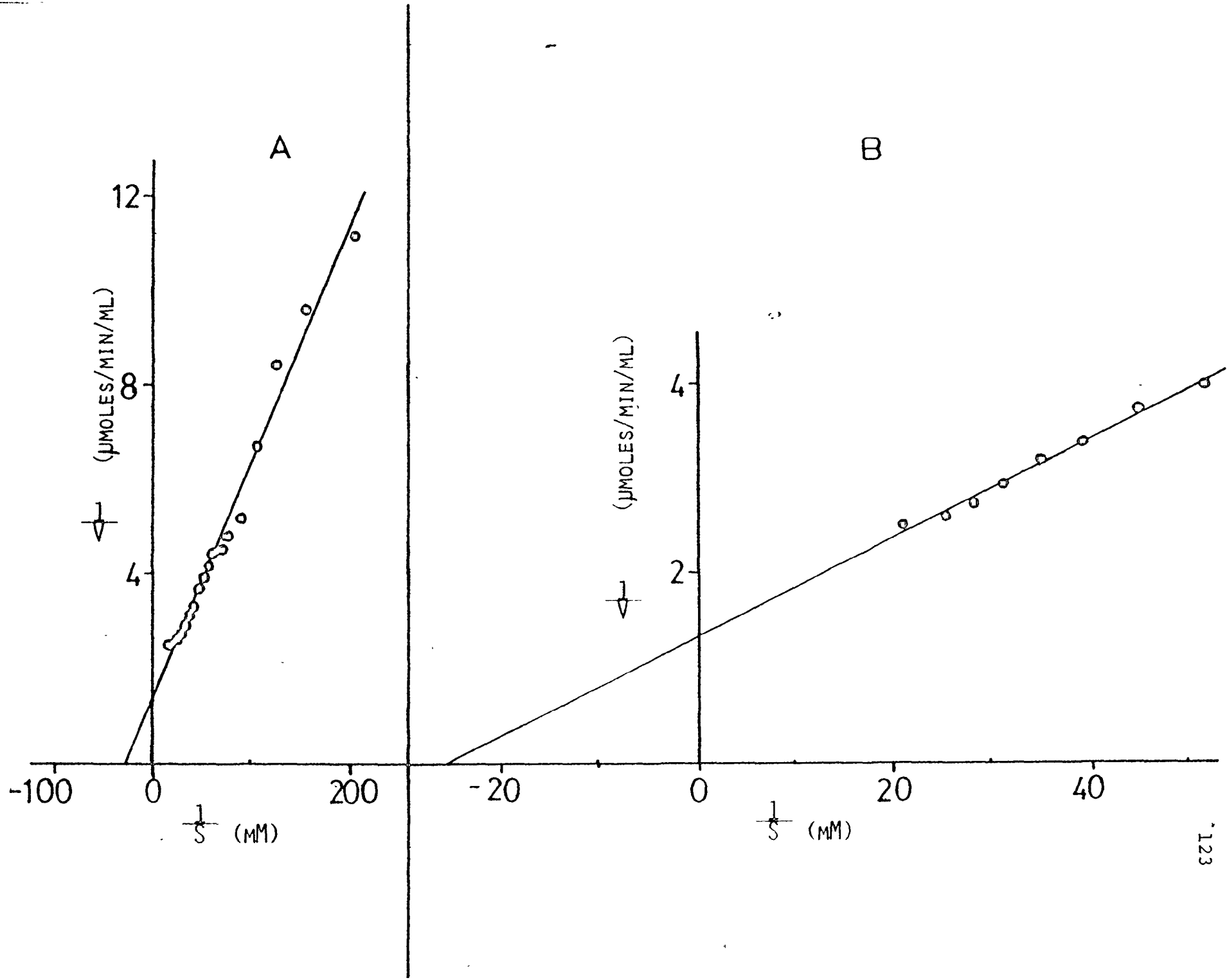

A
B
12
8
4
(μMOLES/MIN/ML)
1/V
-100
0
200
1/S (mM)
4
2
(μMOLES/MIN/ML)
1/V
-20
0
20
40
1/S (mM)

TABLE 11

Km, Vmax Values of IB_I Component of Reductase I of NFR 402 with Nitrofurazone, Furazolidone, ANFT and AF_2 as Substrates

Substrate	Km (μM)		Vmax (μmoles/min/ml)		Double Reciprocal Plots
	22°C	37°C	22°C	37°C	
Nitrofurazone	105	109.0	0.88	1.56	See Figures 26, 27
	95.3	137.0	0.79	1.72	
	125.0		0.82		
	129.0		1.02		
(Average)	113 ±16	113 ±19	0.88 ±0.1	1.64 ±0.11	
Furazolidone	77.8	117.0	1.11	2.04	
	62.5	82.0	1.00	1.79	
	63.0		0.90		
	78.0		1.50		
(Average)	70.3 ±8.7	99.5 ±24	1.13 ±0.11	1.91 ±0.18	
ANFT	41.7	55.6	0.60	0.93	
	30.0	70.0	0.38	1.16	
	44.4		0.44		
	46.7		0.59		
(Average)	40.7 ±7.2	62.8 ±10	0.50 ±0.10	1.04 ±0.98	
AF_2	54.0	50.5	0.68	1.01	See Figures 28, 29
	65.7	48.0	0.69	0.96	
	55.0		0.72		
	58.2		0.74		
(Average)	58.2 ±5.3	49.3 ±1.8	0.71 ±0.03	0.98 ±0.03	

Figure 26. A. Double reciprocal plot of enzyme activity (V) versus substrate concentration (S) at 22°C.
Enzyme: IB_I component of nitroreductase I of NFR 402
Substrate: Nitrofurazone

B. Same as above (A)
Data plotted on an expanded scale.

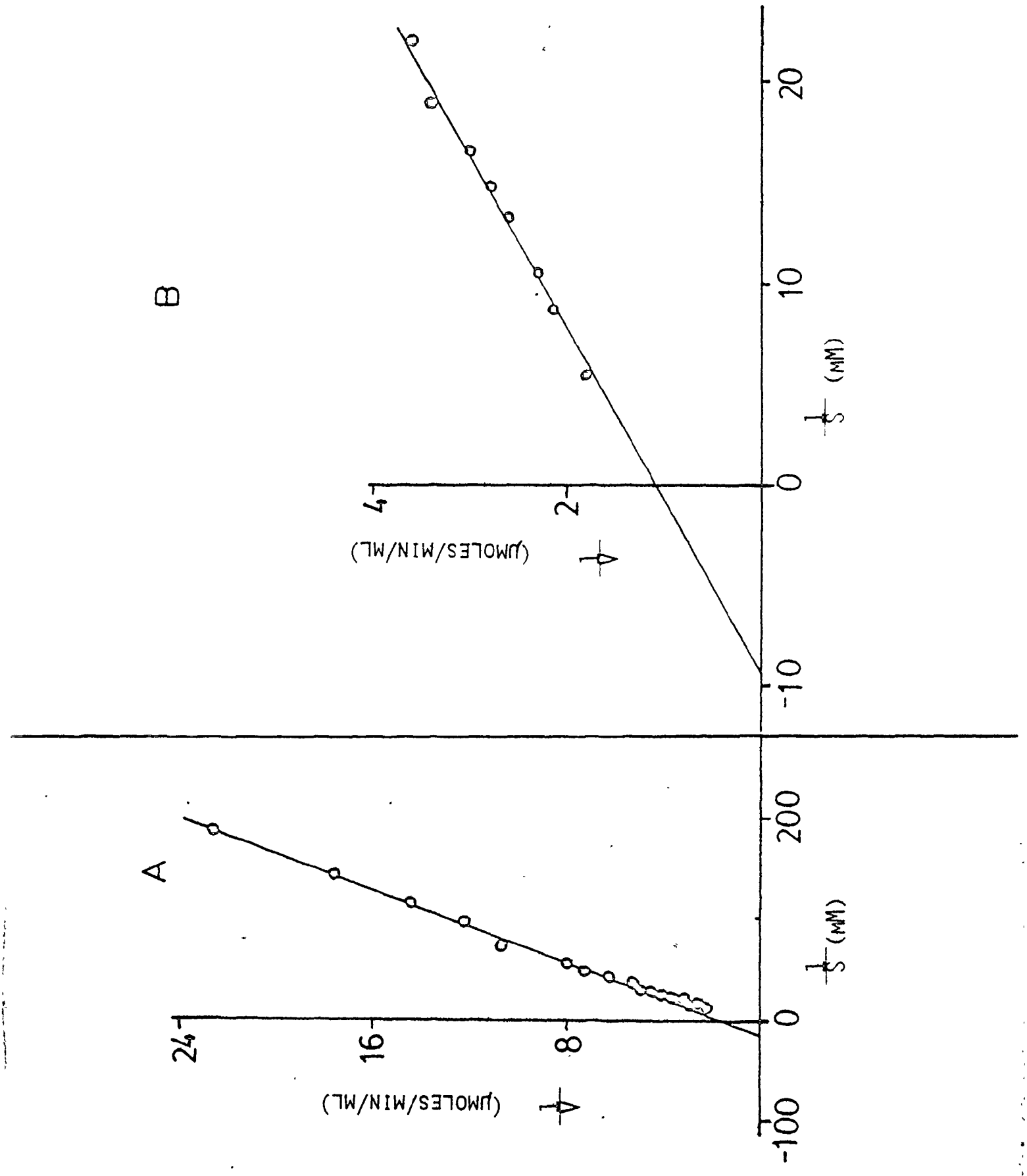

A
B
(μMOLES/MIN/ML)
1/S (mM)
24
16
8
4
2
200
0
-100
20
10
-10

Figure 27. A. Double reciprocal plot of enzyme activity (V) versus substrate concentration (S) at 37°C.
Enzyme: IB_I component of NFR 402
Substrate: Nitrofurazone

B. Same as above (A)
Data plotted on an expanded scale.

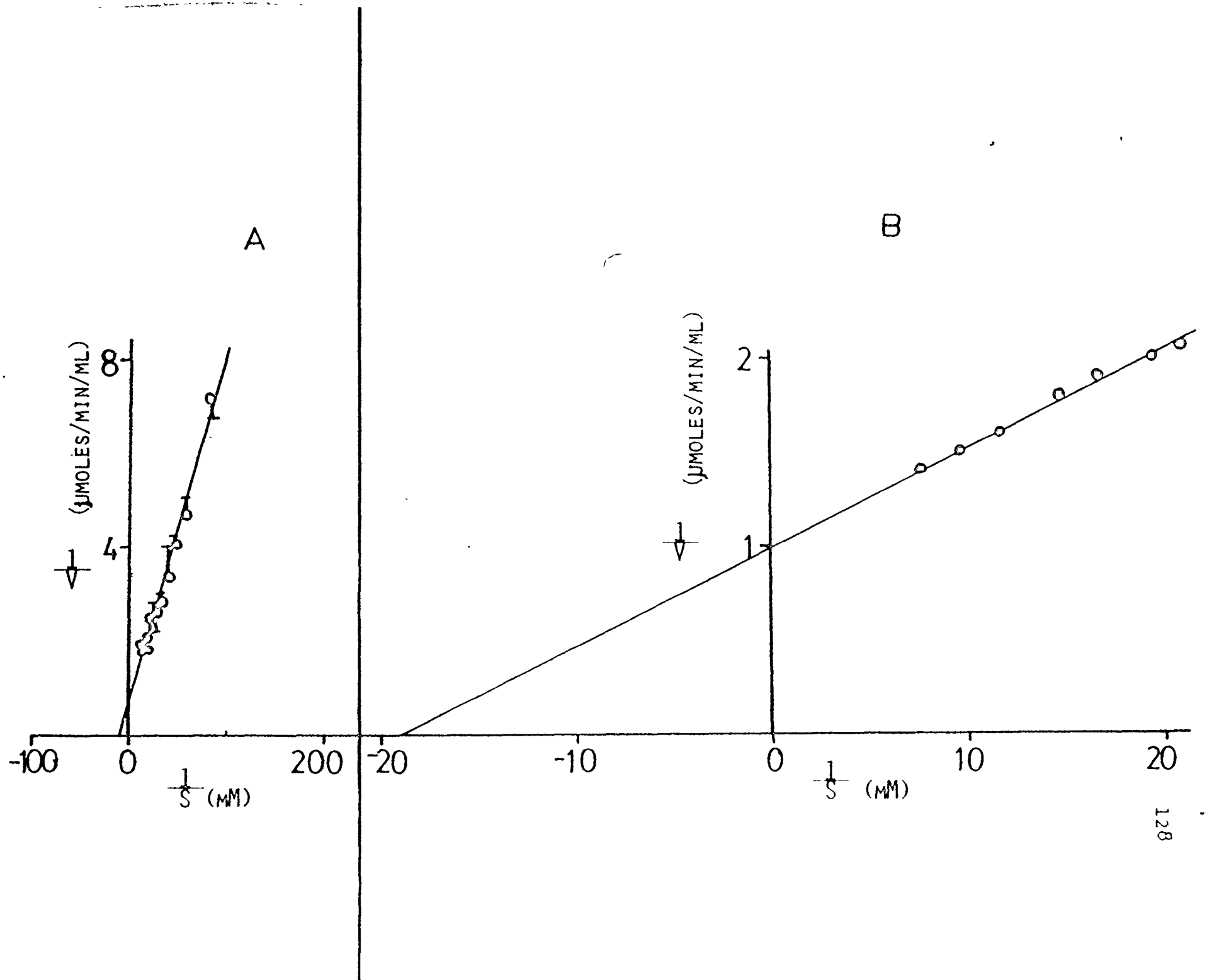

A
B
(µMOLES/MIN/ML)
8
4
2
1
-100
0
200
-20
-10
0
10
20
1/S (mM)

Figure 28. A. Double reciprocal plot of enzyme activity (V) versus substrate concentration (S) at 23°C.
Enzyme: IB_I component of NFR 402
Substrate: AF_2

B. Same as above (A)
Data plotted on an expanded scale.

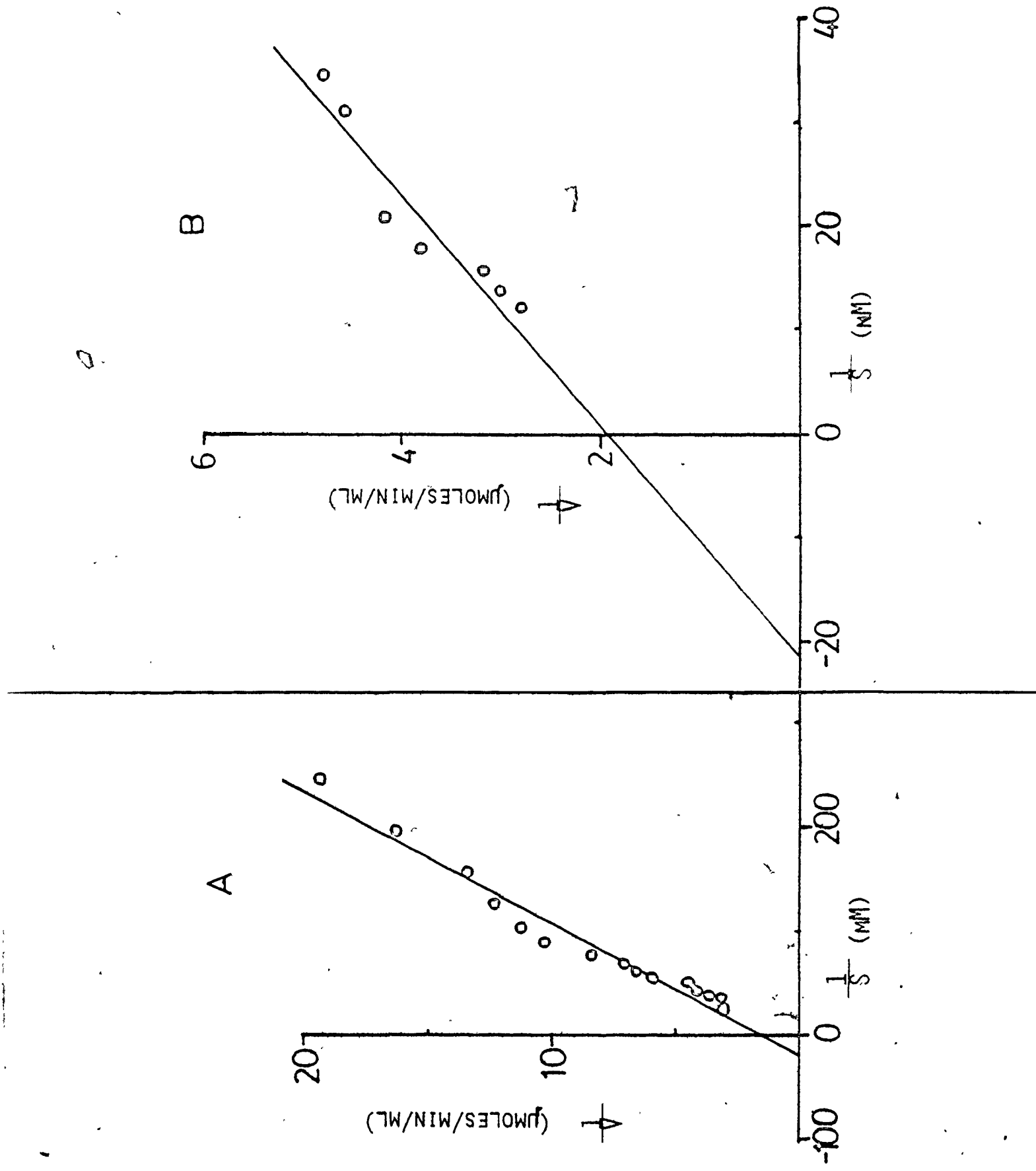

A
B
(μMOLES/MIN/ML)
1/S (MM)
20
10
0
-100
200
6
4
2
0
-20
40

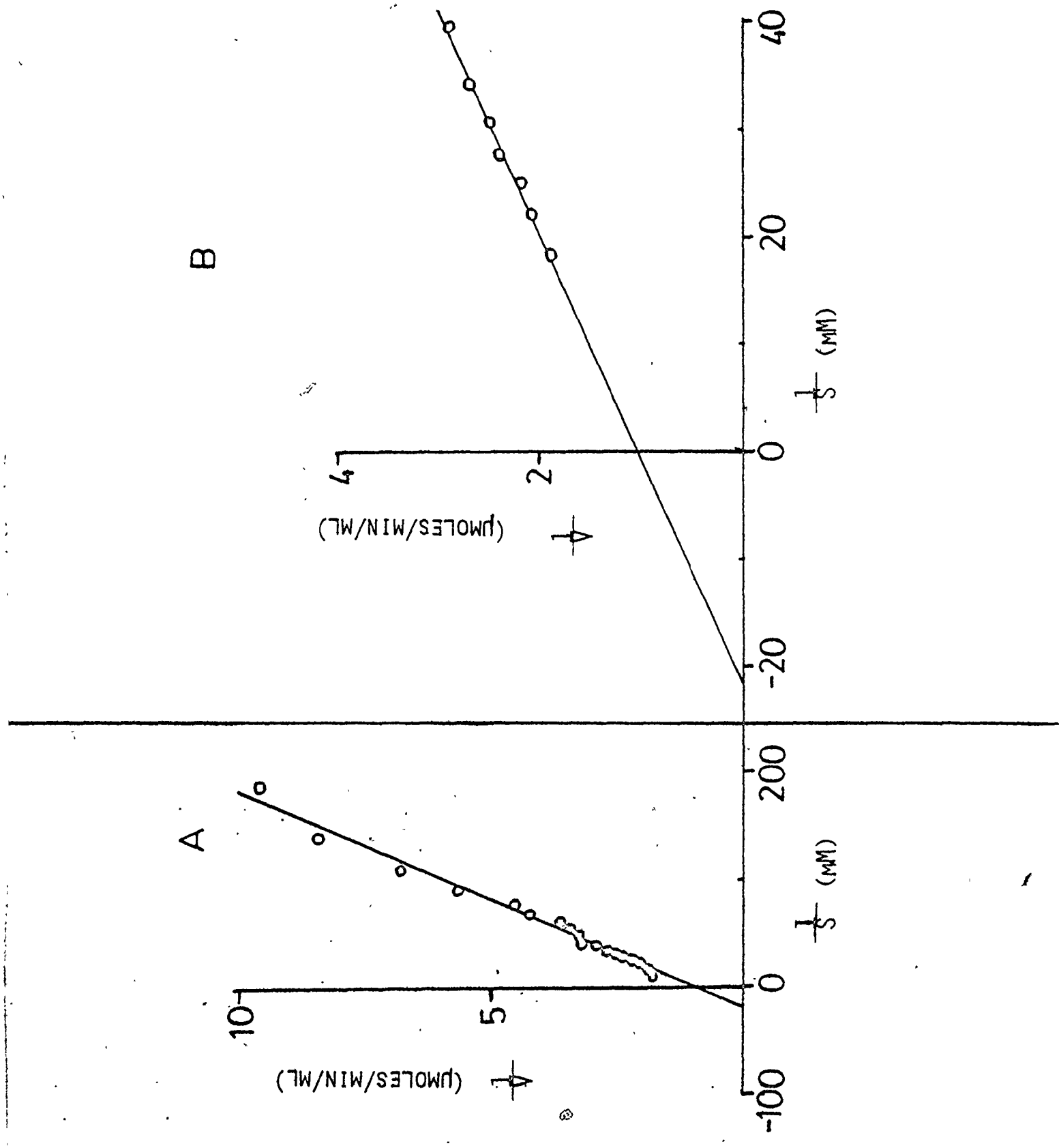
Figure 29. A. Double reciprocal plot of enzyme activity (V) versus substrate concentration (S) at 37°C.

Enzyme: IB_I component of NFR 402

Substrate: AF_2

B. Same as above (A).

Data plotted on an expanded scale.


B
40
20
0
-20
4
2
(μMOLES/MIN/ML)
1/S (mM)
A
200
0
-100
10
5
(μMOLES/MIN/ML)
1/S (mM)

slightly greater at 37°C than at 22°C, there was less than a two-fold difference between the values for each substrate. In fact, the greatest difference was seen with the substrate furazolidone; the Km value at 37°C was 99.5 μM while the value at 22°C was 70.3 μM. However, there was a significant difference (3 to 9-fold) between the Km values of the IB_I component and those of the IA component, at room temperature.

X. Km, Vmax of IB_{II} Component of Reductase I of NFR 502

The Km and Vmax values of the IB_{II} component of NFR 502 with nitrofurazone, furazolidone, ANFT and AF_2 as substrates, at room temperature, are stated in Table 12. Again, the Km values among the four substrates varied by less than three-fold; the Vmax values varied by less than two-fold. In addition, the Km values for the IB_{II} component with the individual substrates were greater than the values for the IB_I and IA components.

A comparison of the average Km and Vmax values of the components of nitroreductase I with the four nitrofurans is summarised in the Discussion in Table 15.

XI. Comparison of the Reaction Progress Curves of the Components of Reductase I

Comparison of the initial rate curves (absorbance change versus time) of the IA and IB_I components with

TABLE 12

Km, Vmax Values of IB_{II} Component of Reductase I of NFR 502 with Nitrofurazone, Furazolidone, ANFT and AF_2 as Substrates, at 22°C

Substrate	Km (μM)	Vmax (μmoles/min/ml)
Nitrofurazone	175.0	0.76
	148.0	0.74
(Average)	161 ±19	0.75 ±0.01
Furazolidone	60.0	0.64
	81.0	0.82
(Average)	70.5 ±14	0.73 ±0.12
ANFT	56.0	0.43
	63.0	0.50
(Average)	59.5 ±4.9	0.47 ±0.04
AF_2	90.0	0.83
	102.0	1.00
(Average)	96.0 ±84	0.92 ±0.12

nitrofurazone, ANFT and AF_2 at 22°C and 37°C indicates that the enzyme activity falls off more rapidly with time at 37°C as shown in Figures 30 (A to C). The rate of change of substrate over the first three minutes at 37°C was greater than or equal to the change that occured at room temperature, with a saturating concentration of substrate, however, the rate of change levelled off relatively quickly after then.

This type of change was observed with all the substrates except AF_2 and the IB_I component of NFR 402, at both temperatures. As shown in Figure 30 D, at a saturating concentration of AF_2, there was a very rapid initial decrease in absorbance at 395 nm followed by an abrupt termination of activity. Comparison of the absorption spectrum of the initial reaction mixture and that obtained after the termination of activity revealed a peak at 355 nm which is probably the absorption maximum of the reduction products of AF_2. This elusive behaviour with AF_2 could not be explained by the hypothesis that there may be contaminants present in the enzyme preparations that use NADPH more rapidly than NFR 402 reductase since incubation of the IB_I component with NADPH for 10 minutes prior to the addition of substrate produced a similar activity

Figure 30. Reaction progress curves of the IA component of reductase I of Sil 41 at 22°C (O——O) and at 37°C (X——X).

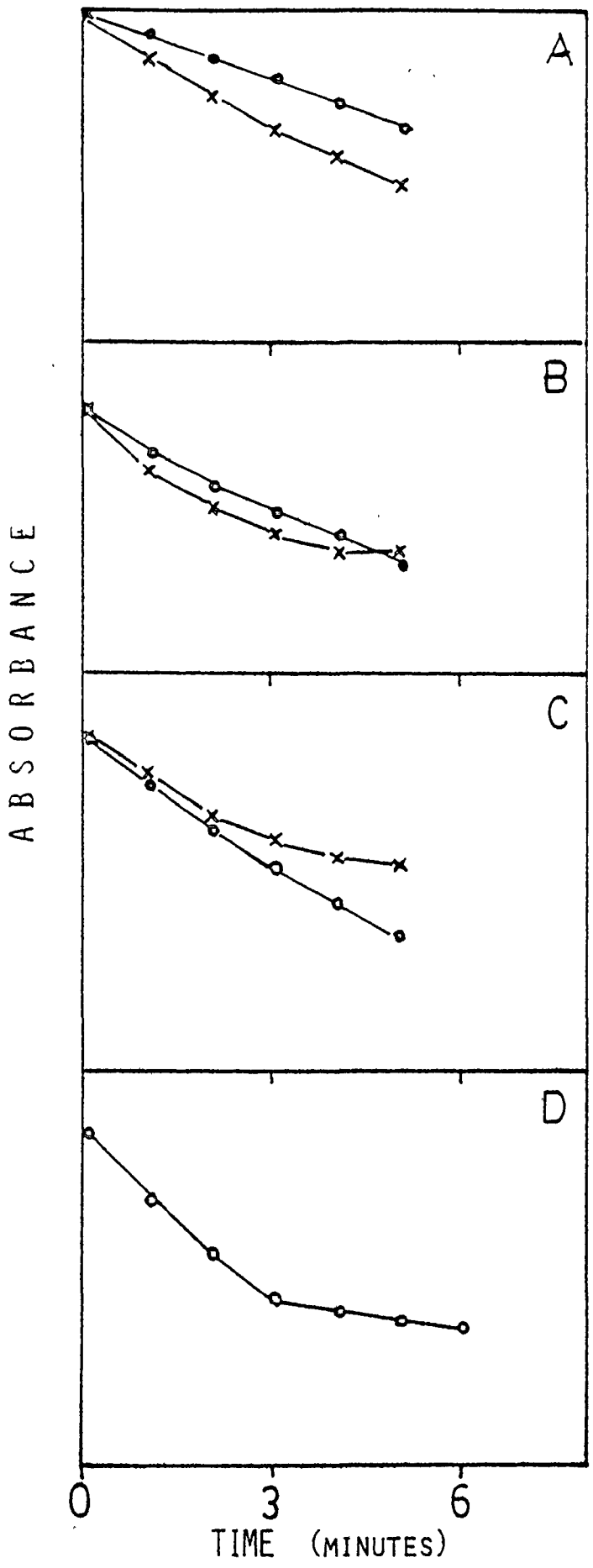
A. Substrate: Nitrofurazone

B. Substrate: ANFT

C. Substrate: AF_2

D. Progress curve of IB_I component of NFR 402 with AF_2 as substrate (22°C and 37°C).

This data was obtained in 6 successive experiments


A
B
C
D
ABSORBANCE
0
3
6
TIME (MINUTES)

profile. Furthermore, this type of progress curve was not observed with the other substrates and thus suggests that this behaviour was peculiar to the IB_I component of NFR 402 and the substrate AF_2.

XII. Binding of ^{14}C-Nitrofurazone and ^{14}C-ANFT Metabolites to Protein

If the binding of labelled nitrofurans to protein and DNA by the IA, IB_I and IB_{II} components of nitroreductase I are measured, then the results might indicate whether different amounts and/or different types of protein adducts are formed or whether the biological effectiveness of the intermediates produced by each component are different.

Table 13 shows the results of the reaction of ^{14}C-nitrofurazone and ^{14}C-ANFT with bovine serum albumin during reduction by the three components of nitroreductase I. The same number of units of enzyme activity was added to each reaction mixture, and since the specific activities of the enzymes were different, the protein content of the reaction mixtures were standardised by expressing the results in units of DPM per mg protein. With both nitrofurazone and ANFT, the IA component produced intermediates that reacted much more extensively with proteins, in fact, three times more label was found in protein after reduction by

TABLE 13

Binding of ^{14}C-Nitrofurazone and ^{14}C-ANFT to Protein During Reduction by Reductase I Components

Enzyme*	Experiment	DPM / mg Protein	
		^{14}C-Nitrofurazone	^{14}C-ANFT
IA (NFR Sil 41)	1	3066	3956
	2	3079	3987
		3072	3971
IB_I (NFR 402)	3	1521	2204
	4	1794	2403
		1657	2303
IB_{II} (NFR 502)	5	1036	1758
	6	988	1702
		1012	1730

*

IA: Specific Activity = 0.33 μmoles/min/mg

IB_I: Specific Activity = 0.32 μmoles/min/mg

IB_{II}: Specific Activity = 0.084 μmoles/min/mg

the IA reductase (Sil 41) than after reduction by the IB_{II} component (502).

XIII. Binding of ^{14}C-Nitrofurazone and ^{14}C-ANFT Metabolites to DNA

In the assay mixtures with DNA, a constant amount of enzyme protein (IA, IB_I components) was added to each reaction mixture. This was necessary in order to keep the DNA : protein ratio constant since the intermediates formed during the reduction of nitrofurazone bind much more readily with protein than with DNA (12).

The results of the experiments shown in Table 14 indicate that the metabolites produced when nitrofurazone and ANFT were reduced by the IA component of reductase I of Sil 41 react more readily with DNA than those formed during the reduction by the IB_I component of NFR 402. Also, the metabolites formed by the reduction of ANFT by both enzyme components were apparently much more reactive than those formed by the reduction of nitrofurazone since the yield of DNA adducts was greater with ANFT. This result was consistent with those obtained in the binding experiments with protein (Section 4. XIII).

TABLE 14

Binding of ^{14}C-Nitrofurazone and ^{14}C-ANFT to DNA During Reduction by Components of Reductase I*

Enzyme	Experiment	Molecules bound/10^6 Nucleotides	
		^{14}C-Nitrofurazone	^{14}C-ANFT
IA	1	100	120
(NFR Sil 41)	2	99	150
		100	135
IB_I	3	49	94
(NFR 402)	4	42	82
		46	88

* DNA:protein ratio kept constant by adding the same amount of enzyme protein to each reaction mixture.

DISCUSSION

I. Binding of ^{14}C-Nitrofurazone to Lysine

In bacteria and animal cells the reduction of nitrofurans produces electrophilic compounds that damage cellular constituents and appears to be a prerequisite for covalent binding to proteins and DNA. The implications of these labile electrophilic metabolites in carcinogenesis in animals and mutagenesis in bacteria and eukaryotic organisms has emphasised the importance of the elucidation of the ultimate active forms.

The problem of identifying the short-lived electrophiles of nitrofurazone was approached through the use of an *in vitro* activation system in which synthetic poly-L-lysine was added with the hope that it would trap the chemically reactive species. This study has shown that the aerobic reduction of nitrofurazone by partially purified *E. coli* reductase I results in a small amount of binding of metabolites to poly-lysine (referred to as 'labelled poly-L-lysine'). Binding appears to result in a covalent interaction as it does with serum albumin (12).

It is possible to resolve a series of L-lysine oligomers completely by CM-Sephadex chromatography using a linear gradient of KCl at pH 6.8. The exceptional

resolving power of this system suggests that it is extremely sensitive to minor variations in charge. Analysis of the L-lysine oligomers produced by hydrolysis of labelled poly-L-lysine by trypsin on CM-Sephadex (Figure 9) demonstrated that label was not associated with the oligomers. This could have resulted from two major factors: the nitrofurazone-lysine linkage was perhaps susceptible to trypsin hydrolysis, or secondly, as a consequence of lysine derivatisation with nitrofurazone, the peptide bonds adjacent to the altered residue had been labilised towards trypsin attack resulting in the production of nitrofurazone-lysine residues rather than oligomers. With the hope of resolving this problem a simpler nucleophile, L-lysyl-L-lysine, which can be studied without the need for enzymatic hydrolysis, was employed in the in vitro nitrofurazone activation system.

Lysyl-L-lysine-nitrofurazone adducts (referred to as 'labelled lysyl-L-lysine') produced as a result of enzymatic reduction of ^{14}C-nitrofurazone, and underivatised lysyl-L-lysine can be resolved from the other components of the reaction mixture quite adequately by chromatography on CM-Sephadex with a linear gradient of KCl (Figure 10). The labelled lysyl-L-lysine elutes slightly before the underivatised dipeptide probably because of subtle differences in charge between the two species. Of the various columns

examined in search of a system that would efficiently separate the labelled lysyl-L-lysine from the underivatised lysyl-L-lysine, the CM-Sephadex column with a shallow, linear concentration gradient of triethylammonium formate at pH 6.8 was the most successful. At the same time, this system resolved the labelled material into at least four distinct components, and had the added advantage that the buffer could be easily lyophilised without leaving any residual salt which is important for later instrumental and chemical analysis of the compounds.

The absence of ultraviolet absorption of the peptide bonds at 220 nm and lack of positive ninhydrin reactions in association with the isolated lysyl-L-lysine was perhaps due to the small amount of lysyl-L-lysine derivatives formed, that is, the amount was below the sensitivities of both detection systems. Alternatively, as a consequence of the binding reactions, the properties of labelled lysyl-L-lysine might be different from those of unchanged lysyl-L-lysine.

Unfortunately, it was not possible to derivatise enough of the dipeptide with the activation systems used in this study to permit elucidation of the structure and properties of the adducts. Nevertheless, the limited amount of nucleophilic epsilon amino groups of lysine that do become derivatised might reflect what indeed happens <u>in vivo</u>, in

other words, the properties of both the epsilon amino groups and the activated nitrofurans may be such that extensive reactions do not occur. The possibility also exists that the active electrophile is not an obligate intermediate but rather it is a minor equilibrium product or minor resonance form which is selectively reactive.

The small yield of labelled lysyl-L-lysine obtained was not due to a loss of adducts because of instability since it was shown that poly-L-lysine-nitrofurazone adducts are quite stable for at least six days. It is possible that the lysyl-L-lysine-nitrofurazone adducts might have a shorter half-life because of the possible de-stabilising effect of the α-carboxyl groups of the dipeptide which is present in close proximity to the bound nitrofurazone metabolites.

To date, histidine, cysteine, lysine, tyrosine, tryptophan and methionine are the amino acids that have been identified as having reacted with other types of carcinogens (7). Analysis of the reactivity of other amino acids with activated ^{14}C-nitrofurazone indicated that cysteine was the most efficient 'trap' for the electrophilic derivatives, as judged by competition experiments. Cysteine inhibited the binding of the metabolites to pure proteins by over 90% while lysine, at the same molar concentrations, caused only 28% inhibition (Table 10). However, the relative

amount of lysine in pure proteins is generally greater than cysteine, and although the number of exposed sites depends on the conformation of the proteins, this fact might compensate for the lower nucleophilicity of lysine and thus make the epsilon amino groups important sites of attack by activated nitrofurans in vivo.

It must be recognised, however, that the formation of derivatives with in vivo nucleophiles could correspond to detoxification process and, in this respect, cysteine has been implicated in a particularly facile pathway of inactivation. Such possible inhibition of chemical carcinogenesis and mutagenesis is based on the concept that antioxidants or reducing agents, such as cysteine and glutathione, will exert a scavenging effect on the reactive species of carcinogens or mutagens thus protecting other cellular constituents from attack (75). Recently, cysteine has been shown to protect against the carcinogenic effect of some agents and to decrease the mutagenic action of some 5-nitrofurans towards bacteria (76). However, these considerations apply to free cysteine and glutathione, mainly in the cytosol, and not to the amino acids present in proteins.

It is possible, however, that the electrophilic species of nitrofurans which conjugate with free cysteine or glutathione in the detoxification reactions are similar in nature to those that bind to critical nucleophilic targets.

Hence, characterisation of these conjugates might provide information on the nature of the biologically active intermediates involved in mutagenesis and carcinogenesis. Unfortunately, previous experiments have shown that cysteine-nitrofurazone adducts are quite unstable *in vitro* (McCalla, Yu, unpublished).

Other amino acids that might be reactive towards the activated nitrofurans are the tyrosine and tryptophan residues of proteins. These amino acids are only slightly soluble in aqueous solutions but they may nevertheless produce relatively large, stable yields of adducts depending on their nucleophilic strength at physiological pH's.

Although nitrofurans have been shown to interact with proteins *in vivo* and *in vitro*, details of the metabolic activation of these compounds and the chemistry of the reactions of the activated intermediates with proteins and other cellular constituents are virtually unknown and represent important areas for future research.

II. Type I Nitroreductases of E. coli

A. Components of Reductase I

As mentioned above, it is known that nitrofurans must be activated by nitroreductases before they damage damage cellular constituents. E. coli K12 contains two general types of nitroreductases: type I reductase is active under aerobic conditions and mutants lacking this enzyme are resistant to the effects of nitrofurans; type II reductases are active only at low oxygen tensions and are present in the reductase I deficient mutants. Published data suggest that reductase I activity of E. coli is due to at least two components controlled by two different genes (11).

Previous analysis of crude extracts of the mutant E. coli Sil 41 (nfs A^+ B) by DEAE-cellulose chromatography and gel electrophoresis resulted in profiles that were inconsistent: the profiles of some extracts indicated the presence of a major and a minor peak of activity while others showed only the expected major peak of activity, that is, the nfs A^+ gene product. This work showed that extracts of NFR Sil 41 do contain two type I reductase components and and that differences observed were due to loss of the minor component from extracts which had been treated with $MnCl_2$ to remove nucleic acids (Figures 15 to 19).

Further examination of the reductase I components

by D. Bryant and M. Leeksma, using a combination of DEAE-cellulose and CM-cellulose chromatography, has shown that there indeed three type I nitroreductase components in wild-type E. coli K12. The major component, A (80% total type I activity), is the product of the nfs A^+ gene; component B_I (12% of total type I activity) is the product of the nfs B^+ gene and the third component, B_{II} (less than 5% wild-type activity) which is apparently inactivated by $MnCl_2$, is perhaps the product of a third gene nfs C^+. Whereas the IB_I and IB_{II} activities coelute on DEAE-cellulose at pH 7.2, they can be differentiated by their behaviour on CM-cellulose eluted with a buffered NaCl gradient at pH 5.8 (70) as IB_{II} is the only one of three activities that binds to CM-cellulose.

The major component IA specifically requires NADPH for activity while both of the minor components, IB_I and IB_{II}, are active with either NADPH or NADH as cofactors. However, the gene locus nfs C for the IB_{II} component has not been mapped and its existence is inferred from biochemical results only; gene loci for nfs A and nfs B have been mapped previously (11).

B. Kinetic Properties of Reductase I Components

The rate of metabolism of nitrofurans by bacterial nitroreductases may help to determine the widely dispartate

mutagenic and cytotoxic activity of these agents in vivo. Table 15 summarises the average Km and Vmax values of the components of nitroreductase I with four nitrofurans which differ strikingly in their mutagenic potencies, as substrates.

Comparison of the Km values of the reductase I components indicates that the values for the IB_{II} component with each of the four substrates is greater than that of the IB_I component which, in turn, is greater than the values of the IA component. For instance, with nitrofurazone as substrate, the Km for the IA component was 13.3 μM; for the IB_{II} component, the Km value was 161 μM, more than twelve times greater. Clearly, the IA component has a greater affinity for each of the four nitrofurans tested and this perhaps contributes to the relatively greater specific activity of this component in vivo. The Vmax values do not differ by more than a factor of three and do not appear to follow a pattern although the values of the IB_I component with the four substrates were generally greater than those of the IA and IB_{II} components.

The wild-type activity of reductase I is represented by the predominant IA component. Thus, it appears that while the concentrations of nitrofurazone and AF_2 required to produce an equal frequency of induced mutations differ by 500-fold, the Km and Vmax values varied by less than a factor of two. Obviously, the overall rates of reduction

TABLE 15

Test of correlation Between the Kinetic Constants of Reductase I and the Mutagenicity of Four Nitrofurans

Nitrofuran (substrate)	Km (μM) *			Vmax (μmoles/min/ml) *			Relative Mutagenic Potency *
	IA	IB_I	IB_{II}	IA	IB_I	IB_{II}	
Nitrofurazone	13.2	113	161	0.50	0.88	0.75	0.8
Furazolidone	26.1	70.3	70.5	0.85	1.13	0.73	8
ANFT	13.5	40.7	59.5	0.41	0.50	0.47	20
AF_2	19.7	58.2	96.0	0.44	0.71	0.92	100

* Data taken from Tables 2, 10, 11, 12

and the affinities of reductase I for the nitrofurans cannot account for the differences in the mutagenicity of the compounds. Therefore, the basis for the differences in mutagenic activity may lie in such properties of the 'activated' nitrofurans as stability, reactivity towards cellular targets, or the ease of further metabolism to inactive products.

The results of Tables 13 and 14 suggest that the yields and biological effectiveness of DNA-nitrofuran and protein-nitrofuran adducts produced in vitro after reduction by the various forms of reductase I are different. While every effort was made to ensure that the same concentration of protein was used in the reaction mixtures with these different enzymes, it is still possible that the differences observed could be due to differences in the extent of binding with the specific proteins present in the partially purified reductase I components. However, this seems unlikely since the extent of binding to both DNA and protein catalysed by the IB_I and IB_{II} components was about three times less than that with IA with ^{14}C-nitrofurazone or ^{14}C-ANFT as substrates. If proteins in the preparations of the B components really bind less of a reactive species then more of that species should be left to react with DNA - an effect that was not observed. Thus it seems likely that more than one type of protein adducts and DNA adducts are formed by reductase I components. At the same time, the

specific activities of protein- and DNA-nitrofuran adducts were less than two-fold greater with the stronger mutagen ANFT than the weaker mutagen, nitrofurazone.

When the Km values of the reductase I components with the two nitrofurans are taken into account, the differences in the yields of adducts by each component may not be significant. However, if the differences are considered to be significant then these results might indicate that the reduction of nitrofurans by each reductase I component follows a different enzymatic pathway. This hypothesis predicts that in wild-type E. coli which contains all three components of reductase I, several different types of ultimate mutagens would be formed which, after reacting with nucleophilic targets, would produce different types of macromolecule-nitrofuran adducts. Indeed it has been shown recently (64) that at least two functionally and chemically distinct adducts were produced by the binding of ANFT to DNA in vivo.

In addition, the finding that the yield of adducts with the stronger mutagen ANFT was greater than with nitrofurazone is supported by in vivo studies. The stronger mutagen AF_2 binds to a greater extent to DNA of E. coli, as measured by daughter-strand gaps, and it produces a larger yield of DNA-nitrofuran adducts than the less potent mutagen ANFT (64). The weakest mutagen tested, nitrofurazone, became bound to DNA to a noticeably smaller extent and the yield of adducts was about 10-fold less than the yield obtained with ANFT. However, the yield of daughter-

strand gaps formed when DNA containing AF_2 induced adducts replicated was about 60-fold greater than that formed after nitrofurazone treatment and thus cannot account for the 500-fold difference in the mutagenic potencies of these two compounds.

From the available information, it appears that the binding affinities of the components of nitroreductase I with the four nitrofurans are different but they cannot account for the large differences in the mutagenic potencies of the compounds; the yield of adducts is greater with the stronger mutagen than with the weaker one, and finally, the three components of reductase I may follow different enzymatic pathways with the result that different types of electrophilic metabolites and thus different types of adducts may be formed by each component. Thus, it seems likely that the different potencies observed are due to a combination of many properties but perhaps to the greatest degree on the stability and electrophilicity of the active species.

C. The IB_I Component of NFR 402 and AF_2

The progress curves of NADPH-stimulated activity of the three components of nitroreductase I and the four nitrofurans followed the general form in which velocity fell gradually with time (Figure 30). However, the reaction of the IB_I component of NFR 402 with the substrate AF_2 produced

initial velocity curves that were biphasic in nature (Figure 30 D). One possible explanation for the latter profiles is that the disappearance of substrate was due to the simultaneous action of two or more enzymes. In other words, there may have been another enzyme present in the reductase I preparations that reacted with AF_2 and not with the other substrates.

The type II nitroreductases of bacterial and mammalian cells have been shown to catalyse the cis-trans isomerisation of vinyl derivatives of nitrofurans, in particular AF_2 (73, 74). Furthermore, the isomerising activity, unlike the nitroreductase activity of these enzymes, can occur in the presence of oxygen. Therefore, it is possible that the biphasic activity progress curves obtained with IB_I and AF_2 may be due to the presence of reductase II in the reductase IB_I preparations, as a contaminant. It can be postulated that the initial phase of the curve is due to nitroreduction of the cis isomer (λ max 395 nm) and when the radical anion or other ionic species are produced, isomerisation produces the trans isomer of AF_2 and results in an abrupt change to a lower velocity as reduction of the latter isomer occurs. Unfortunately, the trans isomer (λ max 402 nm) was not clearly detected in the absorption spectra of the reaction mixtures after reduction; the

presence of small amounts of other components were generally masked by the large absorbance of the reduction products at 350 nm, but a slight shoulder was seen on some spectra at about 400 nm.

It is also interesting to note that the E. coli mutants, nfs A B C^+ (502), are markedly resistant to nitrofurans and very few mutations are induced in aerated cultures with agents such as nitrofurazone. However, in the presence of AF_2 considerable mutation takes place (C.Lu, unpublished) indicating, perhaps, that a residual amount of type I reductase remains in these mutants that is somewhat specific for AF_2. The implications of these observations with AF_2 have yet to be fully resolved.

REFERENCES

1. Miller, E, Miller,J. (1947). Can. Res. 7: 468

2. Heidelberger, C. (1975). Ann. Rev. Biochem. 44: 79

3. Miller, J.A. (1970). Can. Res. 30: 559

4. Miller, E.C. (1978). Can. Res. 38: 1479

5. Pitot, H.C., Heidelberger, C. (1963). Can. Res. 23: 1694

6. Pierce, G.B., Wallace, C. (1971). Can. Res. 31: 127

7. Gronow, M. (1980). Chem. Biol. Interact. 29: 1

8. Cleaver, J.E., Bootsma, D. (1975). Ann. Rev. Genet. 9: 19

9. Bouck, N., DeMayorca, G. (1976). Nature. 264: 722

10. Dodd, M.C., Stillman, J. (1944). J. Pharmacol.& Exp. Thera. 82: 11

11. McCalla, D.R., Kaiser, C., Green, M. (1978). J. Bacteriol. 133: 10

12. McCalla, D.R., Reuvers, A., Kaiser, C. (1970). J Bacteriol. 104: 1126

13. Asnis, R.E. (1957). Arch. Biochem. Biophys. 66: 208

14. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R. (1957). J. Biol. Chem. 193: 265

15. Chapman, J., Reuvers, A., Borsa,J. (1973). Br. J. Radiol. 46: 623

16. Alexander, J., Wildman. (1948). J. Am. Chem. Soc. 70: 1187

17. Waley, S.G., Watson, J, (1953). Biochem. J. 55: 328

18. Stonard, R. (1977). Undergraduate Thesis, McMaster University, Biochemistry Dept., Hamilton, Ontario, Canada.

19. Plummer,D. (1971). In:'An Introduction to Practical Biochemistry' p. 145.McGraw Hill, London.

20. Ts'O, P., Helmkamp, G., Sander, C. (1962). Biochim. Biophys. Acta. 55: 584

21. Wentzell. B. Unpublished method

22. Asnis, R.E. (1955). J. Biol. Chem. 213: 77

23. Beckett, A.H., Robinson, A.E. (1959). J. Med. Pharm. Chem. 1: 135

24. McCalla, D.R., Kaiser, C., Green, M. (1978). J. Bacteriol. 133: 10

25. Lehninger, A.L. (1975). In: 'Biochemistry', p. 186. Worth Publishers, N.Y.

26. Gillette, J.R., Mitchell, J.R., Brodie, B.B. (1974). Ann. Rev. Pharmacol. 14: 271

27. Bryan, G.T. (1978). 'Occurence, Production and Uses of Nitrofurans, p. 1. In: ' Carcinogenisis', Vol. 4. Ed. G.T. Bryan. Raven Press, N.Y.

28. IARC (1974). IARC Monographs. 7:27 (143)

29. Matsuda, T. (1966). J. Ferment Technol. 44: 495

30. Chapman, J., Reuvers,A., Borsa, J., Petkau, A., McCalla, D.R.(1972). Can. Res. 32: 2630

31. Busch, H. (1976). Can. Res. 36: 4291

32. Malkin, A., Kellen, J., Caplan, B. (1978). Scand. J. Immunol. 7, Suppl. 8, part 7

33. Singer, B. (1979). J. Natl. Can. Inst. 62: 1329

34. Zampieri, A., Greenberg. J., (1964). Biochim. Biophys. Res. Comm. 14: 172

35. McCalla, D.R., Voutsinos, D. (1974). Mut. Res. 26:3

36. Tonomura, A., Sasaki, M. (1973). Jpn. J. Genet. 48: 291

37. Erturk. E., Cohen, W.M., Price, J., Bryan, G.T.(1969). Can. Res. 29: 2219

38. Jacobs, J.B., Arai, M., Cohen, S.M., Friedell, G.H. (1977). Can. Res. 37: 2817

39. Jacobs, J.B., Cohen, S.M., Fridell, G.H. (1978). Proc. Am. Assoc. Can. Res. 19:4

40. Erturk, E., Morris, J.L. Cohen, S.M. Vonesch, A.M., Corvetti, A.J., Bryan, G.T. (1971). J. Natl. Can. Inst. 47: 473

41. Stein, R.J., Yost, D., Petroliunas, F., Von Esch, A. (1961). Fed. Proc. 25: 291

42. Cohen, S.M., Erturk, E., Von Esch, A., Bryan, G.T. (1973). J. Natl. Can. Inst. 51: 403

43. Green, M.N. (1948). Arch. Biochem. 19: 397

44. Paul, M.F., Bryson, M.J., Harrington, C. (1956). J. Biol. Chem. 219: 463

45. McCalla, D.R., Reuvers, A., Kaiser, C. (1971). Can. Res. 31: 2184

46. Buzard, J.A., Conklin, J.D, Buller, R.H. (1961). Am. J. Physiol. 201: 492

47. Tatsumi, K., Ou, T, Yamaguchi, T., Yoshimura,H. (1973). Chem. Pharm. Bull. 21: 191

48. Huges, E.E., Acru, S.F. (1937). Ind. Eng. Chem. Anal. Ed. 9: 318.

49. Paul, H.E., Ells, V.R., Kopko, F., Bender, R.C. (1960). J. Med. Pharmac. Chem. 2:563

50. Tada, M., Tada, M. (1975). Nature. 255: 510

51. Peterson, F.J., Mason., Hosvepian, J., Holtzman, J. (1979). J. Biol. Chem. 254: 4009

52. Gavin, J.J., Ebetino, F.F., Freedman, R., Waterbury,W. (1966). Arch. Biochem. Biophys. 113: 399

53. Schellenberg, A., Hellerman, K. (1958). J. Biol. Chem. 231: 547

54. McCalla, D.R., Reuvers,A, Kaiser,C. (1971). Biochem. Pharmacol. 20: 3532

55. Ou, T., Tatsumi, K., Yoshimuro, H. (1971). Biochim. Biophys. Res. Commun. 75: 401

56. Sugimura, T., Sato, S., Nagao, M., Yahagi, T., Matsushima, T., Seino, Y., Takeuchi, M., Kawachi, T. (1976). In:'Fundamentals in Cancer Prevention', p. 191. Eds. P.N. Magee, et al. Univ. of Tokyo Press, Tokyo/Univ. Press, Baltimore, U.S.A.

57. Lu, C., McCalla, D.R., Bryant, D. (1979). Mut. Res. 67: 133

58. McCalla, D.R., Olive, P., Tu Yu, Fan, M.L. (1975). Can. J. Microbiol. 21: 1484

59. McCalla, D.R., Kaiser, C., Green, M. (1978). J. Bacteriol. 133: 10

60. Glascock, H.W., MacLeod, P.F., Davis, J. (1969). Rev. Allergy. 2: 52

61. Finegold, S.M., Ziement, I. (1970). In: 'Antimicrobial Therapy', p. 102. W.B. Saunders, Philadelphia, U.S.A.

62. Nissim, J.A. (1957). Lancet. 1: 304

63. Olive, P., McCalla, D.R. (1977). Can. Res. 35: 781

64. Wentzell, B., McCalla, D.R. (1980). Chem. Biol. Interact. 31: 133

65. Higginson, J., Muir, C.S. (1979). J. Natl. Can. Inst. 63: 1291

66. Tolman, K.G. (1980). Annals. Int. Med. 92: 119

67. De Serras, F.T. (1974). Mut. Res. 26: 1

68. Olive, P.L. (1979). In: 'Carcinogenesis' Vol.4, p.131. Ed. G.T. Bryan. Raven Press, N.Y.

69. McCalla, D.R. (1979). Nitrofurans. In: 'Antibiotics' Vol. 1. Ed. F.E. Hahn. Springer-Verlag, N.Y.

70. Bryant, D., McCalla, D.R., Leeksma, M. (1980). Submitted for publication.

71. Lehninger, A.L. (1975). 'Biochemistry', p. 101. Worth Publishers, N.Y.

72. Dixon, M., Webb,E.(1964).'Enzymes', Chap. 2. Academic Press Inc., N.Y.

73. Tomoeda, M., Kitamura, R. (1977). Biochim. Biophys. Acta. 480: 315

74. Tatsumi, K., Koga, N., Kitamura, S., Yoshimura, H., Wardman, P., Kato, Y. (1979). Biochim. Biophys. Acta. 567: 75

75. Wattenberg, L. (1975). In: 'Fundamentals in Cancer Prevention', p. 153. Eds. P.N. Magee, et al. Tokyo/ Univ. Press, Baltimore, U.S.A.

76. Rosin. M., Stitch, H. (1978). Mut. Res. 54: 73