# AEROBIC REDUCTIVE "ACTIVATION" OF 5-NITRO-2-FURALDEHYDE SEMICARBAZONE BY RAT LIVER XANTHINE DEHYDROGENASE

# AEROBIC REDUCTIVE "ACTIVATION" OF 5-NITRO-2-FURALDEHYDE SEMICARBAZONE BY RAT LIVER XANTHINE DEHYDROGENASE

By

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## ABBREVIATIONS

AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide

allopurinol: 4-hydroxypyrazole(3,4-d)-pyrimidine

BSA: bovine serum albumin

bis-tris: bis-(2-hydroxyethyl)imino-tris(hydroxy-methyl)methane

CM: carboxymethyl

D: dehydrogenase

D/O: dehydrogenase-oxidase

ENTQ: 4-(2-hydroxyethylamino)-2-(5-nitro-2-thienyl)quinazoline

FANFT: N-(4-(5-nitro-2-furyl)-2-thiazolyl)formamide

furazolidone: 3-((5-nitrofurfurylidene)amino)-2-oxazolidinone

GSH: glutathione

HMN: 3-hydroxymethyl-1-((3-(5-nitro-2-furyl)-allydidene)amino) hydrantoin

I: 2-(5-nitro-2-furyl)-4-(thiomorpholino-iminomethyl)thiazole-1',1'-dioxide

K-P: 67 mM potassium phosphate buffer, pH 7.2

LDH: lactate dehydrogenase

pNAP: P-nitroacetophenone

pNBA: P-nitrobenzoate

NFT: 4-(5-nitro-2-furyl)-thiazole

NFTA: N-(4-(5-nitro-2-furyl)-2-thiazolyl)acetamide

4NQO: 4-nitroquinoline-1-oxide

niridazole: 1-(5-nitro-2-thiazolyl)-2-imidazolidinone

nitrofurantoin: 1-((5-nitrofurfurylidene)amino)-hydantoin

nitrofurazone: 5-nitro-2-furaldehyde semicarbazone

O: oxidase

PNTQ: 4-(2,3-dihydroxypropylamino)-2-(5-nitro-2-thienyl)-quinazoline

paraquat: 1,1'-dimethyl-4,4'-bipyridylium

S9: homogenate 9,000xg supernatant

S105: homogenate 105,000xg supernatant

SOD: superoxide dismutase

SQ 18,506: 5-amino-3-(2-(5-nitro-2-furyl)vinyl)-1,2,4-oxadiazole

TNT: trinitrotoluene

# I. ABSTRACT

## I. ABSTRACT

5-Nitrofurans are synthetic antibacterial agents. In general, nitrofurans have been shown to be toxic and mutagenic to cultured mammalian cells and carcinogenic in rodents. The possibility that human exposure to nitrofurans may be causing genetic damage or cancer has stimulated research directed towards elucidating the metabolism and mechanism of action of these compounds. A comprehensive understanding of the molecular basis of nitrofuran action may also be useful for comprehending the mechanism of action of other aryl and heterocyclic nitro compounds.

It is known that enzymatic reduction of nitrofurans to reactive but uncharacterized metabolites that damage DNA constitutes an important "activation" step in both bacteria and hypoxic mammalian cells. However, since the known mammalian enzymes having nitroreductase activity are reported to be strongly inhibited by molecular oxygen, the relation of reductive activation to the DNA-damaging effects of nitrofurans in intact animals or in aerobic cultured cells is unclear.

In rodents the liver is a major site of nitrofuran reduction *in vivo*. Net reduction of 5-nitro-2-furaldehyde semicarbazone (nitrofurazone) by rat liver homogenate was found to be relatively insensitive to oxygen when compared to net nitroreduction by milk xanthine oxidase. Intermediates generated in the aerobic nitroreduction bound tightly and probably covalently to protein. The nitroreductase in the rat liver preparation was identified as xanthine oxidoreductase by its apparent MW, substrate specificity and inhibition by allopurinol.

Xanthine oxidoreductase is known to function *in vivo* as xanthine

dehydrogenase (D form) which is converted to xanthine oxidase (O form) during purification and storage. The O form is considered to be the major cytosolic nitroreductase and its activity is strongly inhibited by oxygen in vitro. Net nitroreduction by the D form has not been studied previously.

In the rat liver preparation the bulk of the aerobic nitroreductase activity was associated with the D form of xanthine oxidoreductase during chromatography on CM cellulose, heat conversion of D form to O form and chemical interconversion of D form to O form and back to D form. Thus, net reduction of nitrofurazone by xanthine dehydrogenase is considerably less sensitive to inhibition by oxygen than is net nitroreduction by rat liver or milk xanthine oxidase.

The ability of xanthine dehydrogenase to reduce nitrofurazone aerobically to highly reactive species in vitro suggests that this enzyme may play a role in a nitroreductive process which contributes to the mutagenic and carcinogenic action of nitrofurans and other nitroheterocyclic and nitroaromatic compounds in vivo. On the other hand, the nitroreductase activity of xanthine dehydrogenase in non-target tissues may, in some cases, decrease the amount of nitrocompound available in target tissues and hence play a "protective" role.

# II. INTRODUCTION

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## A. Nitrofurans

### i. Production and Uses

5-Nitrofurans are not known to occur in nature (Bryan, 1978). However, an estimated 2,000 nitrofuran derivatives have been synthesized (McCalla, 1980) following the report, in 1944, that the presence of the 5-nitro group greatly enhances the antibacterial activity of 2-substituted furans (see Bryan, 1978).

Nitrofurans act on a wide spectrum of anaerobic and aerobic bacteria as well as on other pathogenic organisms including fungi, protozoa and schistosomes (McCalla, 1979). Nitrofurans also enhance damage caused by physical agents such as heat and radiation; they are currently being evaluated for clinical use in cancer therapy as hypoxic cell radiosensitizers (see Olive, 1978).

Only a few nitrofurans are in wide commercial use (Bryan, 1978). Direct human exposures primarily result from uses in food preservation and in topical and systemic medicinal administration. A number of derivatives have been found to be carcinogenic and this has led to some curtailment in their usage (see McCalla, 1979). And, while further restrictions have been proposed, new derivatives and uses continue to be developed at a rapid pace (particularly in eastern Europe) (see McCalla, 1979).

### ii. Mutagenicity and Carcinogenicity

Few nitrofurans have been subjected to comprehensive analysis for genotoxicity in a number of test systems (Klemencic and Wang, 1978). However, nitrofurans, in general, are mutagenic and toxic to cultured

mammalian cells (see Olive, 1978) and essentially all nitrofurans are mutagenic to Escherichia coli and the strains of Salmonella typhimurium that contain the pKM 101 plasmid (Tazima et. al., 1975; McCann et. al., 1975). Nitrofurans are "direct acting" mutagens in the sense that they induce mutations without the addition of exogenous "activating" systems such as rat liver S9 (McCalla, 1983).

There is a strong correlation between mutagenicity and carcinogenicity of organic chemicals (McCann and Ames, 1976; see also Klemencic and Wang, 1978; McCalla, 1980). Thus, it is not surprising that, following the first reports of carcinogenic activity in animals sixteen years ago (see Cohen, 1978), a rapid succession of papers has demonstrated carcinogenic activity in 28 of 36 nitrofurans tested (see Klemencic and Wang, 1978; McCalla, 1979). Some of the apparently "noncarcinogenic" nitrofurans have received only limited testing (see Cohen, 1978).

The nitro group of nitrofurans is a basic structural requirement for mutagenicity and carcinogenicity since analogues lacking the nitro group are essentially lacking in activity (see McCalla, 1979). It is presumed (see sections II.A.iii. and II.A.vi.) that transient electrophilic intermediates formed during nitroreduction mediate the mutagenic and carcinogenic effects via interactions with cellular macromolecules. The 2-substituent modifies the chemical and biological properties of the 5-nitrofuran, thus markedly affecting the carcinogenicity and organ specificity (Cohen, 1978).

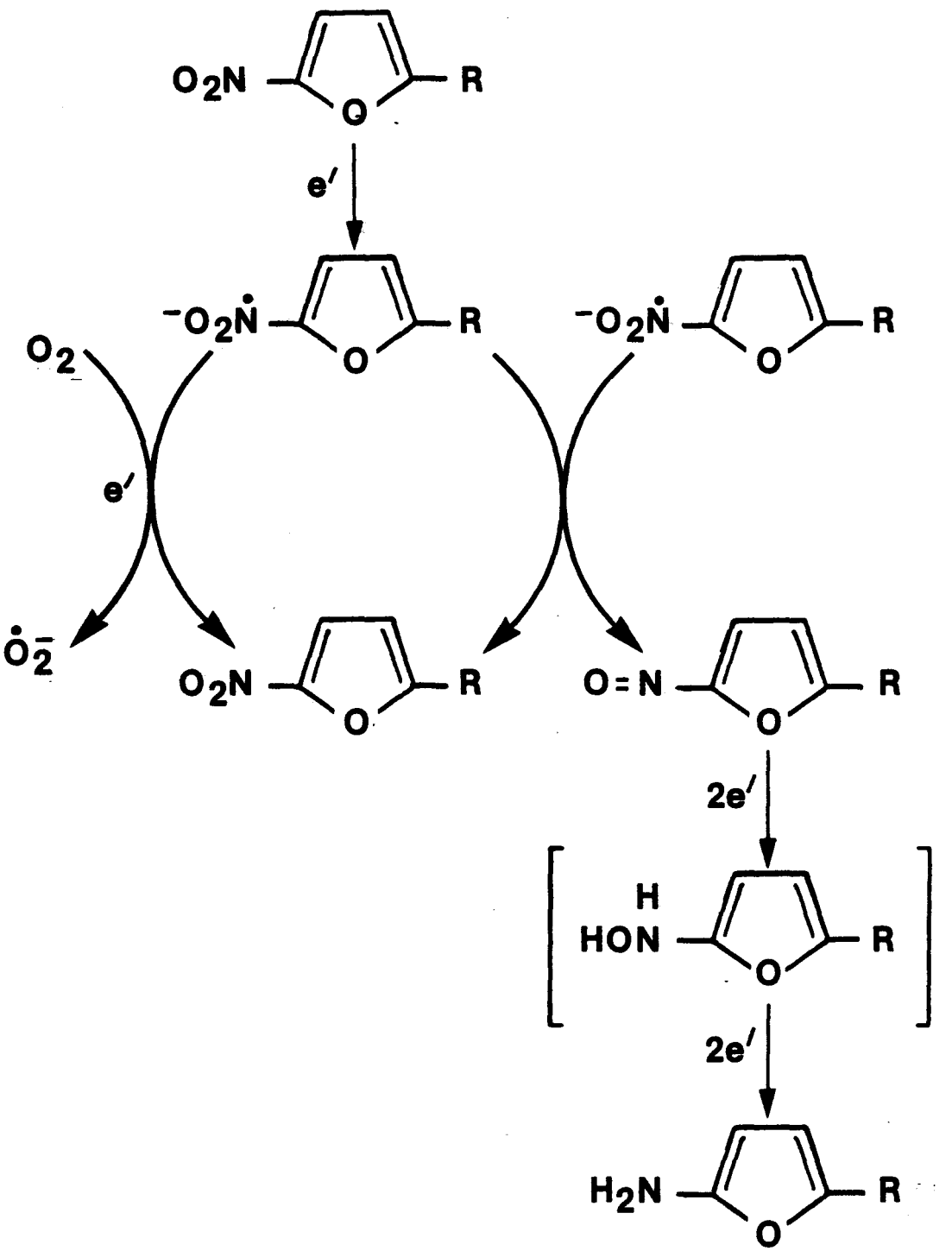
The bacterial mutagenicity data probably exaggerate the mutagenic and hence the carcinogenic potential of nitrofurans to mammals. This is

probably due to a combination of the much higher specific activities of the bacterial enzymes which "activate" nitrofurans along with the presence of quantitatively significant "detoxification" reactions in mammals (McCalla, 1983). Although there are difficulties in extrapolating animal test data to humans, carcinogenicity in animals is generally regarded as an indicator of potential carcinogenicity in humans (see McCalla, 1980).

iii. Nitroreductive "Activation" in Bacteria

Nitrofurans per se do not damage DNA or induce mutations. Rather, they are reductively metabolized to reactive species by endogenous bacterial enzymes (see McCalla, 1979). The "nitroreductases" can be divided into two types based on their activity in the presence and absence of oxygen (Asnis, 1957). With the type II nitroreductases the initial reduction product is the nitro radical anion (Fig. I) (Peterson et. al., 1979). In hypoxia, these radical anions disproportionate in a nonenzymatic reaction and produce the corresponding nitroso and nitro species (Mason and Holtzman, 1975a;b). The nitroso derivatives are further reduced to the amines but the processes involved have not been studied (Bryant et. al., 1981). However, in the presence of oxygen, the nitro radical anion is reoxidized to the original nitro compound with concomitant formation of superoxide anion (Peterson et. al., 1979). The relationship between the rates of reoxidation and disproportionation as a function of nitroradical anion and oxygen concentrations have not been well studied (Wardman and Clarke, 1976; Holtzman et. al., 1981). Under most experimental conditions, no net reduction occurs in the presence of oxygen.

Fig. I. Proposed mechanism of nitrofuran reduction by bacterial Type II nitroreductase (Peterson et. al., 1979).


O2N
R
O
e′
−O2Ṅ
O2
e′
O2−
O2N
O=N
2e′
H
HON
2e′
H2N

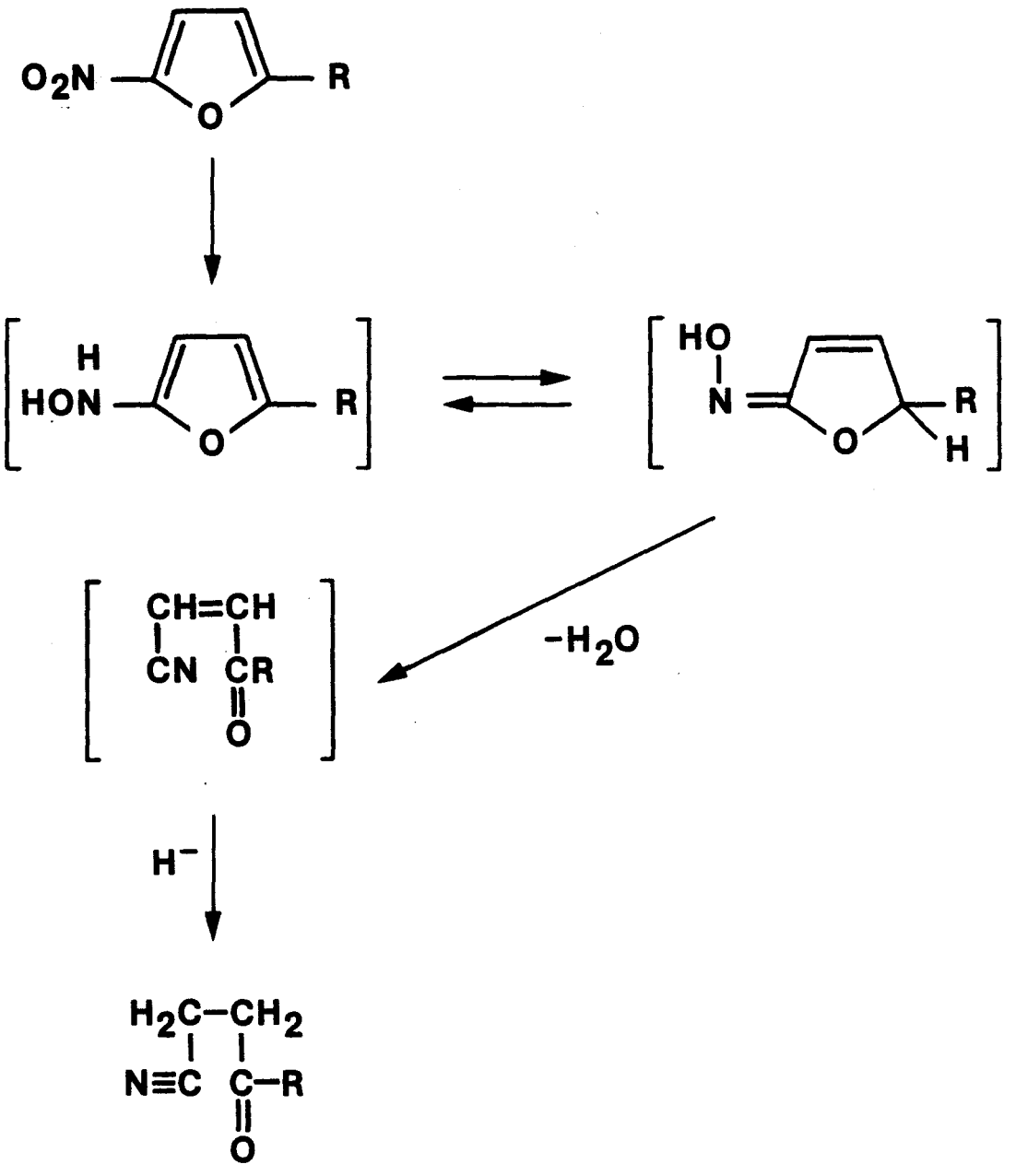
In contrast, type I nitroreductases bypass the oxygen-sensitive nitroradical anion species by initially catalyzing the transfer of two (or more) electrons to the enzyme-bound nitro compounds (Fig. II) (Peterson et. al., 1979). The end products appear to be open chain isomers of the aminofurans (see McCalla, 1979).

The aminofuran and nitrile end products of bacterial nitroreduction are not mutagenic or toxic. The ultimate mutagen(s) appears to be a transient intermediate in the reduction process but has so far eluded characterization (McCalla, 1983).

The critical role that DNA damage plays in the actions of nitrofurans is suggested by the observed correlation between their mutagenic and bactericidal activities; this is confirmed by the finding that mutants which lack excision or postreplicative repair capabilities are much more sensitive to the toxic action of these compounds than are their wild-type counterparts (see Olive, 1978; McCalla, 1979).

The activated nitrofuran intermediates give at least two chemically and functionally distinct adducts in Escherichia coli (Wentzell and McCalla, 1980) but the structures are, as yet, unknown. When E. coli DNA containing these adducts is replicated daughter strand gaps are formed; these gaps might act as predegradative structures and activate error-prone ('SOS') repair, thus resulting in mutation (Bryant and McCalla, 1980). Indeed, in many respects the processes involved in nitrofuran mutagenesis are similar to what is observed after UV irradiation, suggesting that at least some of the nitrofuran induced lesions are handled by the cells in the same general way as are pyrimidine dimers (McCalla, 1983).

Fig. II. Proposed mechanism of nitrofuran reduction by bacterial Type I nitroreductase (Peterson et. al., 1979).


O2N
R
O
H
HON
R
O
HO
N
R
H
O
CH=CH
CN
CR
O
-H2O
H−
H2C−CH2
N≡C
C−R
O

The potency of various nitrofurans varies over a 10,000-fold range (McCalla, 1980). Since a series of nitrofuran derivatives whose mutagenic potency covers a 500-fold range are all reduced at nearly the same rate (Lu et. al., 1979; Ramcharitar and McCalla, unpublished) it appears that the differences in potency reflect differences in the extent to which their active metabolites bind to DNA (Wentzell and McCalla, 1980) and the subsequent "repair" processes.

iv. Mammalian Metabolism

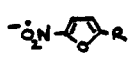
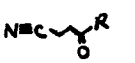
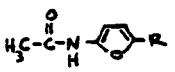
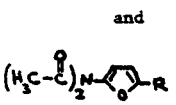
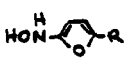
In general, nitrofurans do not accumulate in mammalian tissues due to their rapid absorption, distribution, biotransformation and excretion (Paul et. al., 1949; Swaminathan and Lower, 1978). Their biotransformations may be divided into "activating" and "detoxifying" pathways.

Nitroreduction is, quantitatively, the most important metabolic transformation in vivo (Swaminathan and Lower, 1978; Tatsumi et. al., 1981). At present, nitroreduction by endogenous nitroreductases (see section II.A.v.) is believed to be important in "activating" these chemicals (see section II.A.vi.). A number of different products of nitroreduction have been identified (Table I). They are believed to arise, in part, from reactions of partially reduced intermediates and, in part, from ring opening and further metabolism of the fully reduced nitrofurans (Fig. III).

Another metabolic transformation of nitrofurans has recently been described with preparations of rabbit kidney microsomes. Both FANFT and HMN are oxidatively metabolized by the prostaglandin hydroperoxidase activity of prostaglandin endoperoxide synthetase to uncharacterized

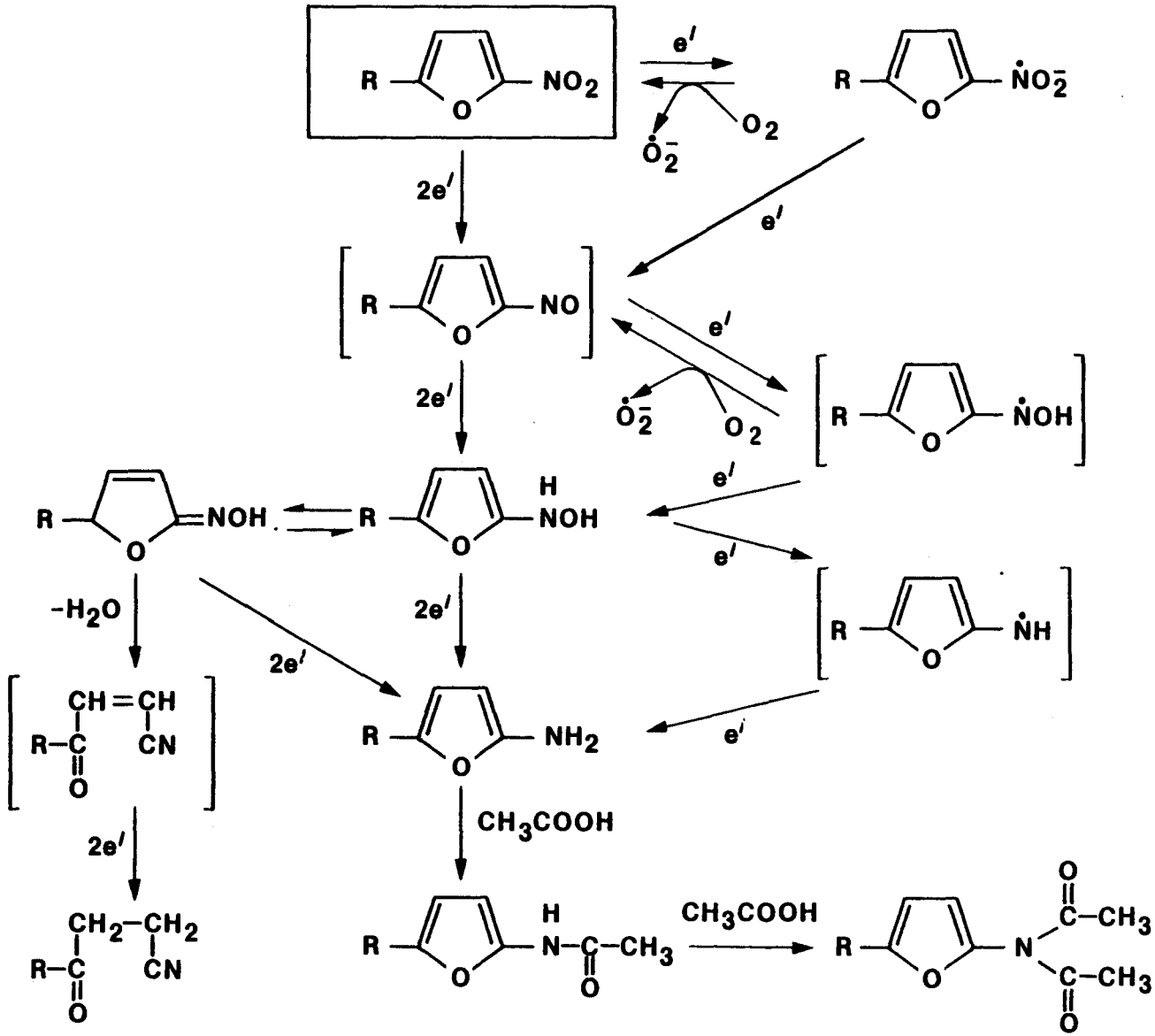
TABLE I

Examples of nitroreduction products formed *in vivo* or with mammalian "nitroreductases" *in vitro*.

| Nitroreduction Product | Example of Nitroheterocycle | Comments |
|---|---|---|
|  | nitrofurantoin[a]; | - observed anaerobically in rat liver microsomes by ESR |
| | nitrofurazone[b] | |
| dimers | TNT[c]; p-nitrobenzene sulfonated[d] | - azoxy dimers isolated after anaerobic reduction with rat liver xanthine oxidase |
| | furazolidone[e] | - isolated following anaerobic reduction with rat liver S9 and with milk xanthine oxidase |
|  | AF-2[f]; nitrofurazone[f] | - isolated following anaerobic reduction with rat liver microsomes, rat liver xanthine oxidase and milk xanthine oxidase |
| | furazolidone[e] | - isolated following anaerobic reduction with rat liver S9 and with milk xanthine oxidase |
| | nitrofurantoin[g] | - isolated following *in vivo* administration in the human |
|  | nitrofurazone[f] | - isolated following anaerobic reduction by milk xanthine oxidase |
| | nitrofurantoin[h] | - isolated following anaerobic reduction by various rat tissues and following aerobic reduction in the isolated, perfused rat liver |
| | I[i]; NFT[j] | - isolated following *in vivo* administration in the rat |
|  | 5-nitro-2-furaldehyde acetylhydrazone[k] | - isolated after *in vivo* administration in the rabbit |
| and  | | |
|  | methyl 5-nitro-2-furoate[l] | - identified by chemical trapping during anaerobic reduction with milk xanthine oxidase |

(a) Mason & Holtzman, 1975b
(b) Tatsumi *et. al.*, 1978
(c) Hoener & Patterson, 1981
(d) Swaminathan *et. al.*, 1981
(e) Tatsumi *et. al.*, 1981
(f) Tatsumi *et. al.*, 1976
(h) Aufrere *et. al.*, 1978
(i) Chatfield, 1976
(k) Olivard *et. al.*, 1962
(l) Yamada *et. al.*, 1982
(b) Peterson *et. al.*, 1979
(c) Beuding & Holliffe, 1946

Fig. III. Postulated metabolic pathways of nitrofuran reduction in mammalian cells. The mechanism(s) of dimer formation (not shown) have not been investigated. Adapted from Paul et. al., 1960; Swaminathan and Lower, 1978; McCalla, 1979; and personal communication with P.J. O'Brien, 1980.


R
NO2
O
e′
O2
O2−
R
NO2−
2e′
e′
R
NO
2e′
e′
O2−
O2
R
NOH
H
NOH
e′
e′
R
NOH
R
H
NOH
-H2O
2e′
2e′
R
NH
e′
CH
CH
R–C
CN
O
R
NH2
CH3COOH
2e′
CH2
CH2
R–C
CN
O
R
H
N–C–CH3
O
CH3COOH
R
N
C–CH3
C–CH3
O
O

products that bind to DNA, RNA and protein (Zenser et. al., 1980; Zenser et. al., 1981). It is not clear whether this represents an "activation" or a "detoxification".

A variety of other metabolic transformations may be important in the inactivation of certain nitrofurans (see McCalla, 1979) (Fig. IV).

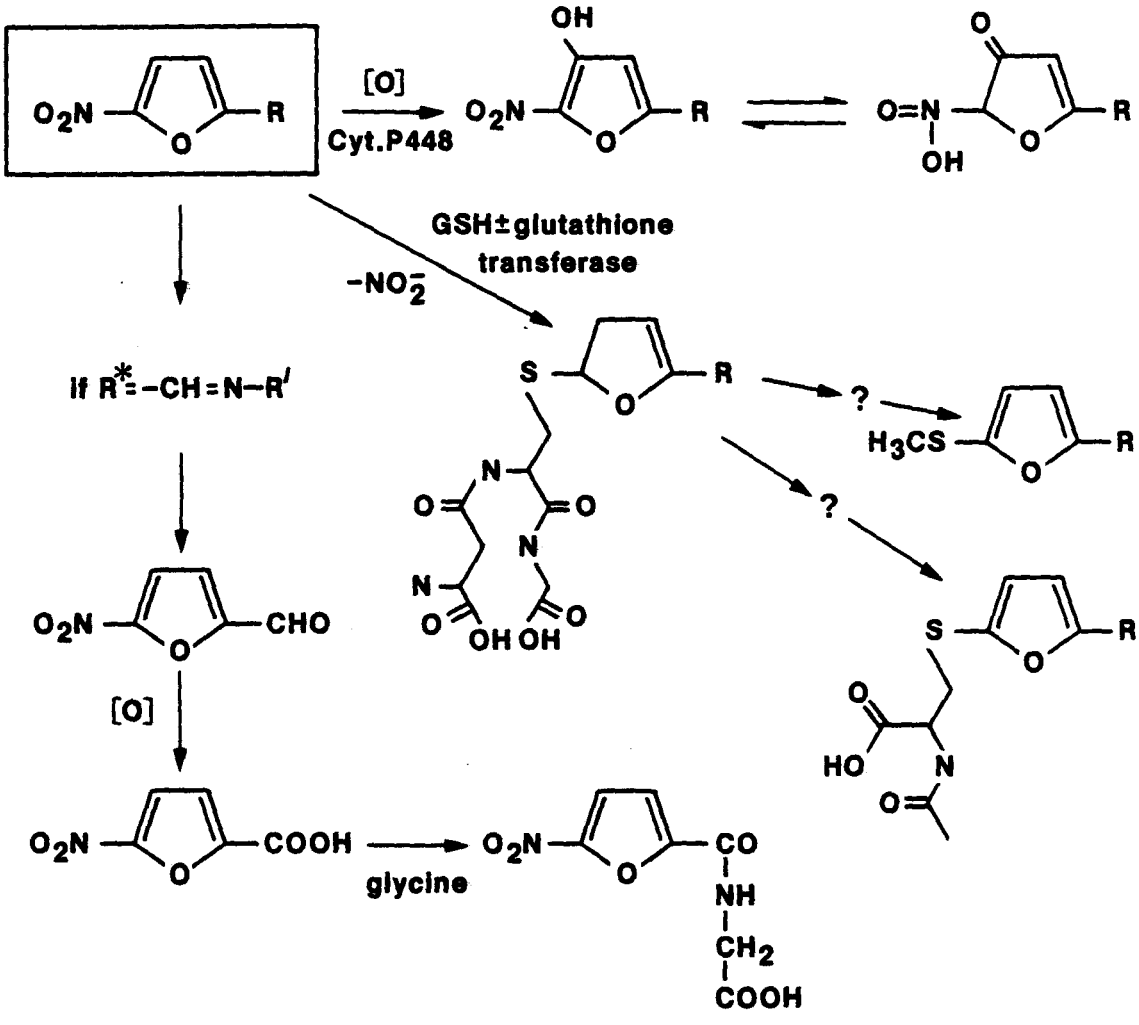
v. Mammalian "Nitroreductase"

A number of different mammalian enzymes are capable of catalyzing net nitroreduction. Their activities in vitro are affected both by the structure of the aromatic nitro compound and by the concentration of oxygen.

The earliest studies on mammalian "nitroreductase" were stimulated by the observation of toxic effects in humans occupationally exposed to trinitrotoluene (TNT). TNT was found to be enzymatically reduced by slices and homogenates of tissues from several animal species. The reduction, which was augmented by anaerobic conditions and largely inhibited by oxygen, resulted in 4-amino 2,6-dinitrotoluene via the 4-hydroxylamino 2,6 dinitrotoluene intermediate (Westerfall, 1943a; Beuding and Jolliffe, 1946). Several partially purified enzymes reduced TNT under anaerobic conditions. Succinic dehydrogenase gave the 4-amino product (Westfall, 1943b), xanthine oxidase gave the 4-hydroxylamino product and lipoyl dehydrogenase apparently gave the 4-nitroso species (Beuding and Jolliffe, 1946).

Subsequent studies have examined the reduction of various nitroheterocyclic and nitroaromatic compounds. The more electronegative nitroaromatics such as nitrobenzene, p-nitrobenzoate (pNBA) and chloramphenicol are rapidly reduced under anaerobic conditions by enzyme

Fig. IV. Non-reductive pathways of nitrofuran metabolism in mammalian cells. Adapted from Boyland et. al., 1961; Olivard et. al., 1962; McCalla, 1979; and Johen et. al., 1980).




* and other reactions of R which are characteristic of their structural classes eg. oxidations, reductions, deacetylations, deformylations, etc. ....

activities present in both the microsomal and soluble fractions of rabbit, mouse and guinea pig liver and kidney (with lower activity in rats and dogs) (Fouts and Brodie, 1957). In rats, the cytosolic and microsomal nitroreductase activities with pNBA were distinguished as separate enzyme systems based on differential activities both with NADH vs NADPH and in the presence vs. absence of oxygen (Kato et al., 1969). The anaerobic reduction of pNBA by mouse, rat and rabbit microsomes has been attributed to the action of cytochrome P450 reductase (Gillette et. al., 1968). Several nitroaromatics, including pNBA, are not reduced anaerobically by the cytoplasmic enzymes xanthine oxidase (Fouts and Brodie, 1957; Morita et. al., 1971) or aldehyde oxidase (Wolpert et. al., 1973) and thus the identity of cytosolic enzyme which reduces the more electronegative nitrocompounds remains to be established.

The more electropositive nitrocompounds are also reduced anaerobically by rat liver microsomal and cytoplasmic fractions. Nitroreduction in rat liver microsomes of niridazole (Feller et. al., 1971a;b), the nitrothiophenes PNTQ AND ENTQ (Wang et. al., 1975b) and the nitrofurans SQ 18506, furazolidone (Feller et. al., 1971a), nitrofurazone (McCalla et. al., 1971; Feller et. al., 1971a; Tatsumi et. al., 1976), nitrofurantoin (Boyd et. al., 1979b) and AF-2 (Tatsumi et. al., 1976) is inhibited by oxygen in vitro and is attributed to the action of NADPH cytochrome P-450(c)reductase. Nitroreduction in rat liver cytosol of niridazole (Feller et. al., 1971b; Morita et. al., 1971) ENTQ and PNTQ (Wang et. al., 1975b) and the nitrofurans SQ 18,506, furazolidone (Feller et. al., 1971a), nitrofurazone (Taylor et. al., 1951; McCalla et. al., 1971; Feller et. al., 1971a), nitrofurantoin (Boyd et. al., 1979b) and

NFTA (Wang et. al., 1974) is inhibited by oxygen in vitro and is attributed to the action of xanthine oxidase.

Aldehyde oxidase, a cytoplasmic enzyme which is evolutionarily related to xanthine oxidase (Krenitsky, 1978; see also section V.C.), has also been examined for nitroreductase activity in vitro (Wolpert et. al., 1973). It has a different range of nitro acceptor activity from that of xanthine oxidase-from which it can be distinguished by its lack of sensitivity to inhibition by allopurinol and by its inability to utilize reduced nicotinamide adenine dinucleotide as an electron donor (Wolpert et. al., 1973).

Studies with the nitrofurans nitrofurazone (McCalla et. al., 1971) and nitrofurantoin (Boyd et. al., 1979b) and with the nitrothiophenes PNTQ and ENTQ (Wang et. al., 1975) have shown that anaerobically, with both rat liver microsomal and cytosolic fractions, these compounds are reductively activated to species capable of binding to tissue macromolecules. The reduction of PNTQ and ENTQ was higher in the small intestine and liver than in the kidney and stomach, with the cytosol being more active than the microsomes.

Nitroreduction of 4-nitroquinoline-1-oxide (4NQO) differs from that of the nitrocompounds described above. 4NQO nitroreduction by rat liver preparations is only marginally affected by oxygen in vitro and is attributed to the action of DT diaphorase (Sigimura et. al., 1966; Kato et. al., 1970) and perhaps aldehyde oxidase as well (Wolpert et. al., 1973).

The functional relevance of NADPH cytochrome P-450(c) reductase and/or xanthine oxidase to in vivo nitrofuran reduction is not clear.

The mechanism of nitroreduction by cytochrome P-450(c) reductase is similar to that of the bacterial type II nitroreductase (see section II.A.iii.). This involves an initial one-electron transfer to the nitrofuran, generating the oxygen-reactive nitroaromatic anion free radical (Mason and Holtzman, 1975a;b; Biaglow et. al., 1976; Sealy et. al., 1978; Peterson et. al., 1979). If this enzyme is able to generate relatively high local concentrations of these anions then net nitroreduction might become competitive with reoxidation (Holtzman et. al., 1981). However, pretreatment of rats with phenobarbitol, which results in increased NADPH cytochrome P-450(c) reductase and anaerobic nitroreductase activities in vitro (Feller et. al., 1971b; Wang et. al., 1974; Boyd et. al., 1979b), does not affect the rate of nitroreduction in vivo (Johen and Kaufmann, 1980; Johen et. al., 1980).

The effect of oxygen in vitro on xanthine oxidase mediated nitroreduction suggests that it also generates the oxygen-reactive nitroaromatic anion free radical. Thus, it appears unlikely that this enzyme would be able to activate nitrofurans in vivo. However, xanthine oxidase was shown to metabolize nitrofurans in vivo during absorption through the mucosa of rat small intestine (Tatsumi et. al., 1973b; b; 1975). Also, the subcellular distribution of radioactivity within rat liver and kidney tissues following in vivo administration of $^{14}$C-labelled NFTA (which is metabolized by xanthine oxidase (Wang et. al., 1974)) suggested that activation occurred primarily in the cytosol (Cohen et. al., 1973; Wang et. al., 1975a). Finally, early work showed that nitrofurans were reduced aerobically in fresh liver homogenate (Akao et. al., 1971) and that nitrofurazone reduction in tissue slices correlated

with both the xanthine oxidase content and activity (Bender and Paul, 1951). The native form of xanthine oxidase, xanthine dehydrogenase (which is usually converted to the oxidase form during purification)(see section V.C.), has not been examined for nitroreductase activity in vitro.

In summary, mammalian tissues contain several enzyme systems which can catalyze the reduction of nitroheterocyclic or nitroaromatic compounds under anaerobic conditions in vitro. The relative contribution of each system can vary between species and amongst the various tissues of a particular species. A limited (and, so far, poorly defined) range of nitro compounds can act as electron acceptors with each system, with the physiochemical properties of individual nitro compounds determining their reactivity in a given system. However, in general, under anaerobic conditions in vitro NADPH cytochrome P450(c) reductase is the most important microsomal enzyme with all nitro compounds and xanthine oxidase (and possibly aldehyde oxidase, when present) is the most important cytosolic enzyme for the more electropositive nitro compounds. The cytosolic enzyme for the more electronegative nitro compounds remains to be identified. It is not clear whether enzymes such as NADPH cytochrome P450(c) reductase and xanthine oxidase are involved in the net nitroreduction of nitrofurans which occurs in vivo. If so, then they must produce a sufficient concentration of their one electron reduced product so that disproportionation becomes competitive with reoxidation by molecular oxygen. If not, then perhaps other, as yet uncharacterized enzymes (which can donate two or more electrons and bypass the oxygen-sensitive reoxidation), might be of greater importance in vivo.

vi. "Activation" in Mammals: Nitroreduction and Superoxide Formation

Nitrofurans appear to exert some of their toxic effects on mammals and on mammalian cells as a result of interactions with DNA (see Olive, 1978; McCalla, 1979). For instance, some of these compounds are mutagenic and toxic to cultured cells in the presence of oxygen, cause increased turnover of DNA within tissues of mice, induce chromosome alterations in rat bone marrow cells and in human lymphocytes (which are manifested as gaps, breaks and sister chromatid exchanges) and induce cancer in several animal species. However, the mechanism(s) by which the nitrofurans damage DNA is not yet completely understood. Nitroreduction intermediates and/or activated oxygen species (see below) are considered as the most likely mediators of this damage.

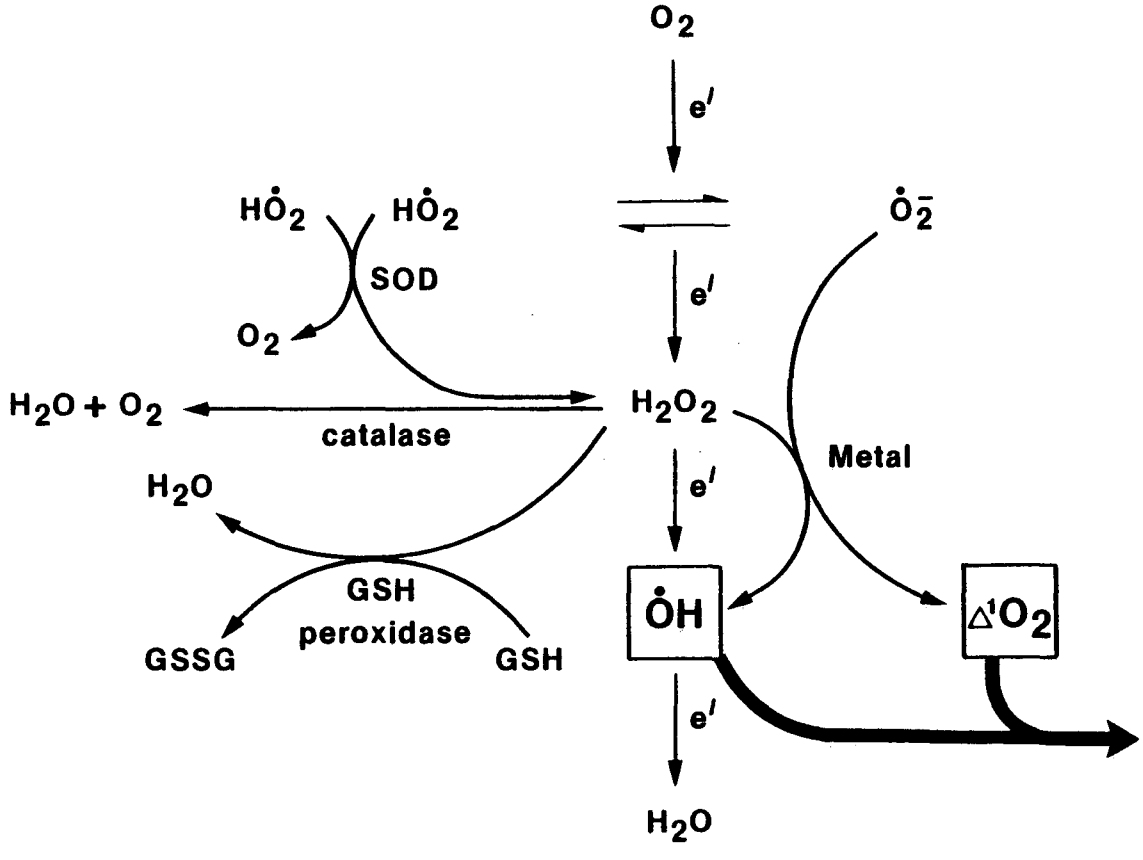
Nitroreduction intermediates are clearly capable of damaging DNA because when cultured mammalian cells are exposed to nitrofurans and the oxygen tension is reduced, an increase in both the extent of net nitroreduction and the magnitude of the lethal (Mohindra and Rauth, 1976), DNA breaking (Olive and McCalla, 1977) and mutagenic (McCalla et. al., 1978) effects of nitrofurans is seen. But, since extremely low levels of oxygen must be achieved before this "hypoxic increase" in toxicity takes place (Mohindra and Rauth, 1976) it is not clear whether the mutagenic and toxic effects seen with cultured mammalian cells in the presence of oxygen (Blijleven et. al., 1977; Nishi et. al., 1977; Hirsch-Kaufman et. al., 1978; McCalla, 1979) are due to actions of the nitrofurans per se, to the nitroradical anions, to the generation of a limited amount of further reduced species such as the nitroso or

hydroxylamino compounds and/or to the consequences of over production of superoxide ion, via reoxidation of the nitroradical anion.

This latter possibility, namely that superoxide ion and its products are involved in some of the DNA damaging effects observed with nitrofurans under oxygenated conditions, is currently being investigated with great interest (Adams et. al., 1976; Olive, 1978; Peerson et. al., 1979; Docampo et. al., 1981a,b). Indeed, acute pulmonary injury, an occasional side effect of nitrofurantoin therapy of urinary tract infections, may be mediated via the generation of superoxide ions at the high oxygen tensions found in the lung (Boyd et. al., 1979a; Sasame and Boyd, 1979; Boyd, 1980).

That reduced oxygen species, which are produced as a normal consequence of oxidative metabolism, can damage DNA is now well established. The monovalent pathway of oxygen reduction produces superoxide anion and hydrogen peroxide - which may generate the highly reactive hydroxyl radical and singlet oxygen species (Fig. V) (see Fridovich, 1978; Badwey and Karnovsky, 1980; Klebanoff, 1980). While precise mechanisms are difficult to define, it appears that the hydroxyl radical and singlet oxygen species mediate the effects of oxygen toxicity, which include lipid peroxidation, cell lysis, DNA damage and mutations, and bacterial and mammalian cell killing (see Fridovich, 1978; Kelly and Boden, 1980; Denke and Fanburg, 1980; Badway and Karnovsky, 1980; Braun and Fridovich, 1981). Indeed, the "normal" metabolic rate of univalent oxygen reduction has recently been implicated in the induction of "spontaneous" human cancers (Totter, 1980). The biological effects of "normal" levels of reduced oxygen species are minimized by the presence

Fig. V. Reductive "activation" of molecular oxygen and intracellular defence mechanisms. Adapted from Klebanoff, 1980; Denke and Fanburg, 1980; and McCord, 1979.




**FREE RADICAL REACTIONS**

- DNA damage
- lipid peroxidation*
- sulfhydryl enzyme inactivation

*a "back-up" system to the "front line" enzymatic defences (SOD, catalase, GSH peroxidase) is provided by antioxidants (ascorbic acid, tocopherols and glutathione) which prevent the propagation of lipid peroxidation

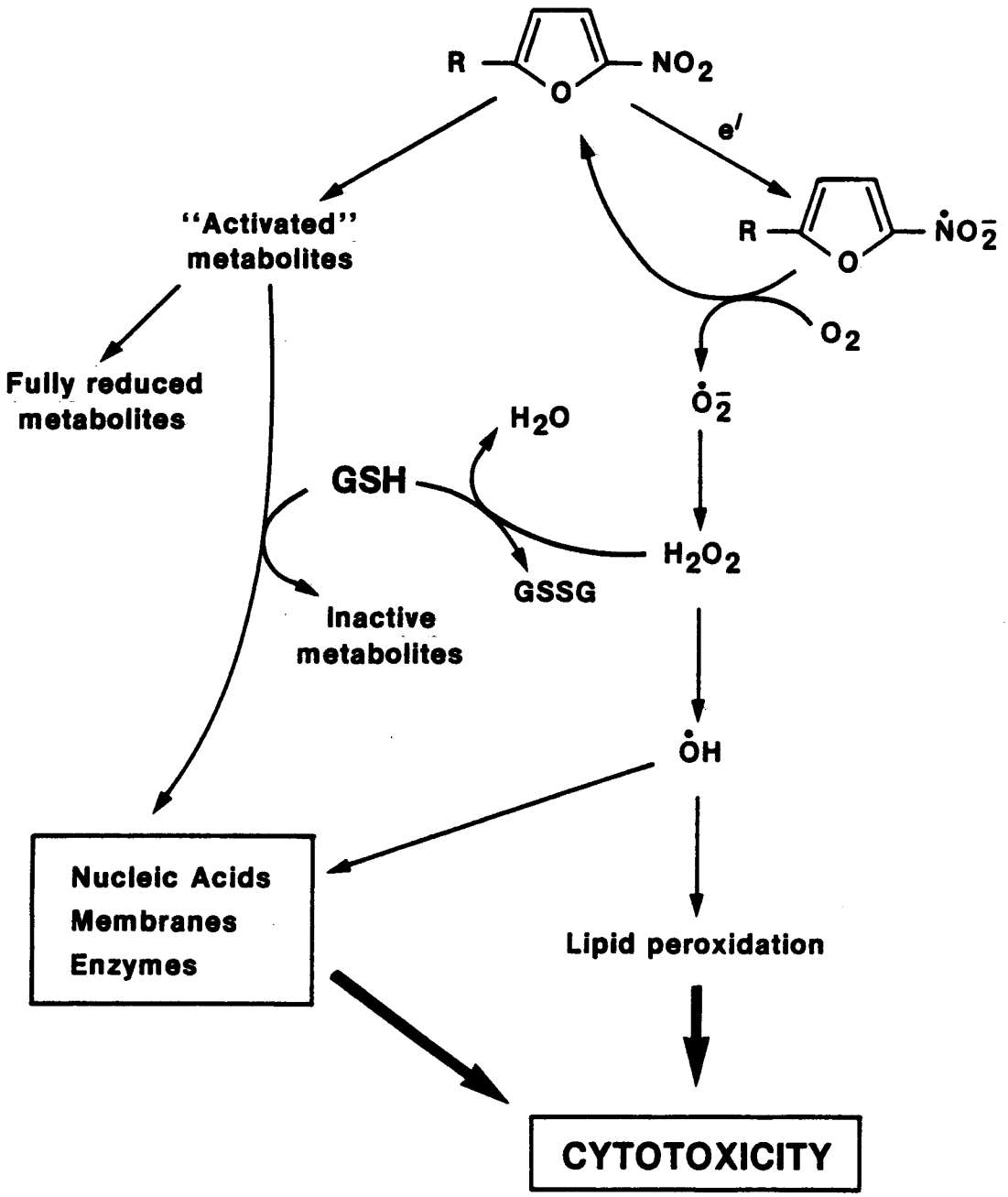
of intracellular scavenging enzymes such as superoxide dismutases, catalases and peroxidases (Fig. V) which are "backed-up" by antioxidants, such as Vitamins C and E, that protect against the propagation of free radical reactions (see McCord and Fridovich, 1978; McCord, 1979; Flohé, 1979). However, it is now evident that these defence mechanisms can be easily overwhelmed (Fridovich, 1979). Indeed, the mechanism of toxicity of chemicals like paraquat, alloxan and streptonigrin (and possibly bleomycin, the anthracyclines (e.g. adriamycin, daunorubicin) and the nitrofurans) involves them becoming oxygen-reactive after accepting one electron enzymatically. This catalytic production of superoxide generates active oxygen species which are believed to be responsible for toxic effects of these chemicals (Lown and Sim, 1977; McCord and Fridovich, 1978; Hassan and Fridovich, 1979; Fridovich and Hassan, 1979; Boyd et. al., 1979; Bus and Gibson, 1979; Mason, 1979; Smith et. al., 1979; Denke and Fanburg, 1980).

The importance of these reduced oxygen species to the actions of nitrofurans under oxygenated conditions in mammals and with cultured mammalian cells is not yet known. However, given that the active oxygen species also oxidize glutathione, thereby impairing an important protective mechanism which both scavenges radicals and reacts with and inactivates the reduced intermediates of nitrofurans, it seems likely that the effects of nitrofurans under oxygenated conditions are due to various combinations of these interdependent mechanisms (Fig. VI) (Holtzman, 1981; McCalla, 1983).

vii. Risk to Humans

Nitrofuran derivatives, when used for medicinal purposes, produce

Fig. VI. Postulated interdependence of "activated" nitroreduction intermediates and "activated" oxygen species in nitrofuran toxicity. Adapted from personal communication with P.J. O'Brien, 1980.


R
O
NO2
e/
"Activated" metabolites
R
O
ṄO2−
O2
Fully reduced metabolites
Ȯ2−
H2O
GSH
H2O2
GSSG
Inactive metabolites
ȮH
Nucleic Acids
Membranes
Enzymes
Lipid peroxidation
CYTOTOXICITY

a number of undesirable acute side effects in a small percentage of patients (see Cohen, 1978). These may be the result of formation of highly reactive metabolites that bind to cell macromolecules (Boyd et. al., 1979b) leading directly to cell toxicity or acting as haptens to secondary immunologic responses (Spielberg and Gordon, 1981). The generation of reduced oxygen species may also contribute to these side effects (see section II.A.vi.).

Approximately 5 million courses of the nitrofuran nitrofurantoin are given yearly in the U.S.A. and Canada for the treatment of urinary tract infections (Tolman, 1980). A number of patients are exposed to a relatively high cumulative dosage of this drug since nitrofurantoin is a drug of choice for chemoprophylaxis in women prone to recurrent infections (Brumfitt et. al., 1981; Nyren et. al., 1981; Ronald and Harding, 1981). A high risk of adverse reactions to this antibiotic has caused a great deal of concern and led to a call for re-evaluation of its use (Tolman, 1980; Holmberg et. al., 1980; Yiannikas et. al., 1981; Hainer and White, 1981; Willcox et. al., 1982). Indeed, adverse reactions to nitrofurantoin account for 10-20% of all incoming reports to the Swedish Adverse Drug Reaction Committee - by far outpacing those of any other drug (Holmberg et. al., 1980).

While these short-term risks of nitrofurantoin therapy are surely of concern, it is perhaps more significant that the long-term (i.e. mutagenic and carcinogenic) hazards to users of this agent are largely unknown. Only low doses of nitrofurantoin have been tested for carcinogenicity in rodents, with the four trials involving a total of only 70 treated animals (see McCalla, 1979) hence, as several authors

have indicated (McCalla and Voutsinos, 1974; Yahagi et. al., 1974; Wang and Lee, 1976; Rosenkranz and Speck, 1976; Boyd et. al., 1979: Shira and Wang, 1980; Russo et. al., 1982), the conclusion that nitrofurantoin is non-carcinogenic is premature. Indeed, nitrofurantoin is similar to other carcinogenic nitrofurans in terms of its metabolism (Boyd et. al., 1979), in its ability to cause "UV-type" damage to DNA of aerobically cultured human cells (Hirsh-Kauffman, 1978), in its ability to cause liver DNA damage after administration to rats (Russo et. al., 1982), and in its slow rate of detoxification in vivo (Klemencic and Wang, 1980). Now, it may be that this particular nitrofuran is not hazardous to people with normal renal function since it is excreted rapidly in the urine and serum levels remain low (McCalla, 1979). However, in a preliminary report of an ongoing prospective study examining cancer incidences following the use of 95 medicinal drugs, nitrofurantoin is associated with a statistically significant excess of cancers of the uterine corpus and female genitals (however, no firm conclusions can be drawn since the duration of follow-up is only, at most, 7 1/2 years) (Friedman and Ury, 1980).

This data on one of the more commonly used nitrofurans, nitrofurantoin, illustrates the uncertainty in establishing guidelines for the continuing exposure of humans to these DNA-damaging antibiotics. The short-term risks and benefits can be evaluated by comparison to other antibacterial drugs, however, the available data are not complete enough to permit a realistic assessment of the long-term risks, which would permit the formulation of reasonable regulations (see McCalla, 1980). Indeed, for a chemical to be recognized as a human carcinogen it must

cause either an unusual clustering of a common tumor or an increased incidence of an unusual type of tumor (Goldman, 1980). Hence, if a nitrofuran induced a low frequency of a common tumor it could well contribute significantly to the incidence of cancer if administered to a large population without this effect being recognized (see McCalla, 1980). Thus, it seems prudent that adequate protection be ensured for those engaged in the manufacture and handling of nitrofurans and of products containing these agents. Furthermore, their uses as antibacterial agents should be reserved for serious situations for which alternative antibiotics are not satisfactory and their wide-spread uses in some countries (e.g. in food, drink, soap, deodorant, etc.) (see McCalla, 1979) should be eliminated.

viii. Unresolved Issues

For a comprehensive understanding of the molecular basis of nitrofuran action the details of the chemical and biochemical processes which take place *in vivo* must be correlated to the biological endpoints of toxicity, mutagenicity and carcinogenicity.

In bacteria, it is established that mutagenesis results from error-prone repair of nitrofuran-DNA adducts, which are formed when nitroreduction of nitrofurans by endogenous nitroreductases produces electrophilic intermediates that attack nucleophilic sites in macromolecules (see section II.A.iii.). In anaerobically cultured mammalian cells nitroreduced intermediates also mediate the DNA-damaging effects of nitrofurans but it is not known whether the DNA-damaging effects seen in aerobically cultured mammalian cells and with mammals *in vivo* are mediated by the same type of nitroreduced species, by the

effects of "activated" oxygen species, by various combinations of these two interdependent mechanisms (see section II.A.vi.), or by other mechanisms.

Nitrofurans induce tumors at sites remote from the point of administration, indicating the need for metabolic activation (Swaminathan and Lower, 1978). FANFT administration to rats leads to pre-neoplastic foci in the bladder which evolve into tumors either with the continuing administration of FANFT or with the administration of promoting agents such as saccharin or DL-tryptophan (Cohen, 1978; Cohen et. al., 1978). Thus, nitrofurans appear to act as "initiating" agents in a multistep process of tumor induction (see Miller and Miller, 1981). Most workers believe that the four electron reduced metabolite of nitrofurans, the hydroxylamine, is the proximate carcinogen (Wang et. al., 1974; 1975a; Swaminathan and Lower, 1978; McCalla, 1979; Peterson et. al., 1979; Boyd et. al., 1979b), as has been proposed for the ultimate carcinogen of arylamines (see Miller and Miller, 1981). However, given that net reduction of nitrofurans by the known mammalian nitroreductases is strongly inhibited by oxygen (see section II.A.v.) the identity of the in vivo "activating" enzyme remains to be established. Furthermore, the determination of the tissue distributions, activities and specificities of all the enzymes which metabolize these compounds (see section II.A.iv.) will be necessary to provide an explanation for the target specificity of nitrofuran carcinogenesis (Cohen, 1978; Swaminathan and Lower, 1978). Also, the structures of the derivatives formed after reaction with cellular nucleophiles, the rates of carcinogen-macromolecular adduct formation and the details of the repair

of the DNA lesions need to be established. Finally, the process by which the "initiated", but dormant, tumor cell (see Trosko and Chang, 1979) is epigenetically "promoted" (facilitating the expression of the transformed phenotype and enhancing the outgrowth of the initiated cell (see Diamond et. al., 1980)), to a cancer cell, will need to be studied.

Solutions to these various problems should yield a comprehensive molecular understanding of nitrofuran carcinogenesis. The information gained from the study of this class of chemical carcinogens will likely be useful for comprehending the mechanism of action of other aryl and heterocyclic nitro compounds.

B. Experimental Design

As outlined in the previous sections, the mechanisms by which nitrofurans exert their effects on mammals and on aerobically cultured mammalian cells is not yet completely understood. In particular, the identity of the *in vivo* "activating" enzyme(s) has not been established. Mammalian nitroreductase activity leads to the formation of potentially toxic "activated" oxygen species (see section II.A.vi.) but it is not known if it also generates "activated" metabolites of nitrofurans since net nitroreduction by the known mammalian nitroreductases is extremely sensitive to oxygen *in vitro* (see section II.A.v.).

However, a good deal of indirect evidence suggests that "activated" metabolites of nitrofurans are produced *in vivo*. For instance, nitroreduction is the major pathway of nitrofuran metabolism in rats (Swaminathan and Lower, 1978), nitrofurans bind to components of animal cells following *in vivo* administration (Olive, 1978; Boyd *et. al.*, 1979b) and nitroreduction does occur in humans (Hoener and Patterson, 1981) and under oxygenated conditions in the isolated perfused rat liver (Aufrère *et. al.*, 1978). Furthermore, aerobically cultured fibroblasts from patients with xeroderma pigmentosum are 6-10 times more sensitive to the nitrofuran AF-2 than are fibroblasts from normal controls and from patients with ataxia telangactasia, even though the rates of nitroreduction are similar (Maher *et. al.*, 1976; McCalla *et. al.*, 1978). This implies that some of the toxic effects of AF-2 with human fibroblasts are mediated by the addition of a bulky substituent to DNA which is repaired by the "UV" but not the "X-ray" repair systems (see McCalla, 1979). And finally, the frequency of sister chromatid exchanges

(Shirai and Wang, 1980), the number of chromosome abberations (Tonomura and Sasaki, 1973; Goodman et. al., 1977), the extent of DNA repair synthesis (Tomomura and Sasaki, 1973), the aerobic cytotoxicity (Adams et. al., 1976) and the carcinogenic potencies (Klemencic and Wang, 1978) of several of these agents parallels their bacterial mutagenicities, suggesting that nitroreduction intermediates (having an oxidation state between that of the nitroaromatic anion free radical and the fully reduced product) mediate these DNA-damaging effects which occur in the presence of oxygen.

On the other hand, there is no evidence, as yet, to implicate "activated" oxygen species as intermediaries in the toxic effect of nitrofurans (see section II.A.vi.) except with the acute pulmonary injury seen in rats administered nitrofurantoin (Boyd et. al., 1979a).

Hence, on balance, current information favours the involvement of reduction intermediates in most of the actions of nitrofurans on mammals. This implies that either nitro anion free radicals, produced by mammalian nitroreductase-catalysed one electron reduction, disproportionate at the oxygen tensions found in vivo and/or that mammalian cells contain a nitroreductase which can bypass the oxygen-sensitive nitro anion free radical intermediates.

In rats, the liver is the major site of nitrofuran metabolism (Swaminathan and Lower, 1978) thus we decided to search, in this organ, for a nitroreductase which is active in the presence of oxygen.

# III. MATERIALS AND METHODS

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## A. Materials

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) was a gift from Norwich Pharmacal (Norwich, N.Y.). $^{14}$C-Nitrofurazone (13.8 mCi/mmole) was prepared by Byron Wentzell by condensing nitrofuraldehyde with $^{14}$C-labelled semicarbazide hydrochloride (Intl. Chem. and Nuclear Corp., Plainview, N.Y.), using a standard procedure (Shriner et. al., 1956). Allopurinol (4-hydroxypyrazole(3,4-d)-pyrimidine), hypoxanthine, xanthine, dithiothreitol, $N^1$-methylnicotinamide chloride, bis-tris (bis-(2-hydroxyethyl)imino-tris(hydroxy-methyl)methane), potassium pyruvate and milk xanthine oxidase (0.9 U/mg protein) were purchased from Sigma Chemical Co., St. Louis, Mo. Glucose-6-phosphate dehydrogenase from Leucognostoc mesenteroides (500 U/mg protein), lactate dehydrogenase (1050 U/mg protein), $NADP^+$ and $NAD^+$ were purchased from Boehringer Mannheim (Ville St. Laurent, Que). Omnifluor was purchased from New England Nuclear (Lachine, Que.) and p-hydroxymercuribenzoate from Aldrich Chem. Co. (Milwaukee, WI). All other chemicals were reagent grade. Argon (<0.002% $O_2$) was purchased from Canada Liquid Air Ltd. (Hamilton, Ont.).

All procedures, except the enzyme assays, were carried out at 0 to 4°C. All buffers contained 0.02% $NaN_3$ to prevent bacterial growth and 2 mM EDTA to prevent the acceleration of nitroreduction by Fe(II) salts (Clarke et. al., 1980). "K-P" buffer designates 67 mM potassium phosphate buffer, pH 7.2.

B. Methods

i. Preparation of Rat Liver S105 Supernatant

Male or female Sprague-Dawley (Can. breeding, Montreal, Que) or Wistar (Woodlyn Farms, Guelph, Ont.) rats, weighting 150-250 g, were killed by cervical dislocation. Livers were removed, washed and homogenized in a Potter-Elvehjem apparatus in three volumes of sterile K-P buffer. The homogenate was centrifuged at 9,000 g for 15 min, the supernatant (S9) centrifuged at 105,000 g for 60 min, and the final supernatant decanted. If not used immediately, 15 ml portions were fast frozen in liquid $N_2$ and stored at -75°C for up to two weeks.

ii. Preparation of a Partially Purified Rat Liver Nitroreductase

The "liver nitroreductase" was prepared by fractionation of the S105 supernatant on a gel filtration column. A 15 ml portion of the S105 supernatant was applied to a Biogel A1.5 (200-400 mesh) column (3.5 x 85 cm) and eluted with K-P buffer. The void volume (approx. 130 mls) was discarded and eighty 6.5 ml fractions collected at a flow rate of 30 ml per hour. Fractions exhibiting nitroreductase activity were pooled and, if not used immediately, 2 ml portions were fast frozen in liquid $N_2$ and stored at -75°C for up to a month.

The molecular weight of the major NADH-dependent nitroreductase activity was estimated by calibrating the column with horse spleen ferritin (MW 440,000), bovine serum albumin (MW 68,000), ovalbumin (MW 43,000), trypsin inhibitor (MW 21,500) and cytochrome c reductase (MW 12,380) as molecular weight standards.

iii. Nitroreductase Assay

Nitroreduction was monitored spectrophotometrically at 24°C by

following the decrease in absorbance at 375 nm (the absorbance maximum of nitrofurazone); this was converted to nmol nitrofurazone reduced using the relationship that a $\Delta A_{375}$ of 0.05 corresponds to the reduction of 0.01 µmol of nitrofurazone (McCalla et. al., 1970). Each assay was performed in a final volume of 600 µl K-P buffer and consisted of 300 µl liver nitroreductase preparation, 300 µl liver S9, or commercial xanthine oxidase (0.01 or 0.14 units), 0.02 µmol nitrofurazone, and either 1.5 µmol $N^1$methylnicotinamide, 1.5 µmol hypoxanthine, 1.5 µmol xanthine, an NADH generating system consisting of 0.02 µmol $NAD^+$, 2.5 µmol glucose-6-phosphate and 0.6 units of glucose-6-phosphate dehydrogenase or an NADPH generating system consisting of 0.02 µmol $NADP^+$, 2.5 µmol glucose-6-phosphate and 0.6 units of glucose-6-phosphate dehydrogenase. In some experiments the liver nitroreductase was pre-incubated for 5 min with 6.0 nmol allopurinol. Air-equilibrated solutions were used for aerobic assays. For anaerobic assays deoxygenated enzyme and substrate solutions (treated with argon gas in 25 ml Erlenmyer flasks for one min each) were decanted under argon into cuvettes which were then sealed with parafilm. A Thunberg cuvette (for example, see Tatsumi et. al., 1978) was used to validate this procedure. The ratios of aerobic to anaerobic nitroreductase activity varied somewhat from preparation to preparation (e.g. Tables II and III).

iv. Xanthine Oxidase (O) and Dehydrogenase (D) Assays

The xanthine oxidase and dehydrogenase activities were determined spectrophotometrically at 24°C by following the conversion of hypoxanthine to uric acid at 290 nm without $NAD^+$ ("oxidase" activity alone) or in the presence of $NAD^+$ ("oxidase plus dehydrogenase"

activities). "Dehydrogenase" activity was calculated by subtraction. The absorbance changes were converted to nmol uric acid formed using a molar absorptivity of $1.22 \times 10^4$ $cm^{-1}$ (Westerfeld et. al., 1959). Sample and reference cells contained 300 µl liver nitroreductase or 0.14 units of xanthine oxidase, with or without 1 mg $NAD^+$, in a final volume of 1.6 ml K-P buffer. Water (1 ml) was added to the reference cells. The reaction was started by the addition of 1 ml 0.6 mM hypoxanthine to the sample cell. The ratios of oxidase to dehydrogenase activity also varied somewhat from preparation to preparation.

v. Chromatography of the Rat Liver Nitroreductase on CM Cellulose

The liver nitroreductase preparation (13 ml) was dialyzed for three successive one hour periods against 1 l of 5 mM bis-tris, pH 5.8 and concentrated to about 3 ml by dialysis against 50% glycerol: 50 mM bis-tris. The concentrate was applied to a 0.9 x 58 cm CM cellulose column and eluted with 5 mM bis-tris, pH 5.8. Fifty 2 ml fractions were collected at a flow rate of 1.5 ml per hr.

vi. Chemical Interconversion of the D and O Forms of Xanthine Oxidoreductase

The rat liver nitroreductase preparation was incubated for 15 min at 37°C with 30 µM p-hydroxymercuribenzoate (which reacts specifically with free sulfhydryl groups to form mercaptides (Simmons and Walter, 1980)) and the xanthine oxidase and dehydrogenase activities plus the aerobic and anaerobic NADH-dependent nitroreductase activities were measured, as described in sections III.B.iv. and III.B.iii., respectively. Dithiothreitol was then added to the treated preparation to a final concentration of 10 mM and incubated for 15 min at 37°C to

reverse the effect of the p-hydroxymercuribenzoate.

vii. Assay of the Rat Liver Nitroreductase for Xanthine Oxidoreductase Intermediate (D/O) Form

Measurement of uric acid production at 302 nm under various conditions coupled with measurement of NADH production at 340 nm (by the method of Kaminski and Jezewska, 1979) permits determination of the individual activities of three forms (D, O & D/O) of xanthine oxidoreductase. If it is assumed, as others have done, that the intermediate (D/O) form acts as an oxidase in the absence of $NAD^+$ but as a dehydrogenase when $NAD^+$ is saturating and that the oxidase activity of the D/O form is equal to its dehydrogenase activity in the presence of saturating concentrations of the relevant electron acceptor (Kaminski and Jezewski, 1979) then (I) measurement of the increase in $A_{302}$ in the presence of $O_2$, $NAD^+$, lactate dehydrogenase (LDH) and pyruvate gives the total dehydrogenase activity of the D and D/O forms plus the oxidase activity of the O form (note that the LDH and pyruvate are added to prevent the accumulation of NADH which would otherwise partially inhibit the dehydrogenase reaction), (II) measurement of the increase in $A_{302}$ in the presence of $O_2$ alone gives the oxidase activities of the O and D/O forms, (III) measurement of the increase in $A_{302}$ in the presence of $O_2$ and $NAD^+$ gives the dehydrogenase activity (partially inhibited by accumulating NADH) of the D and D/O forms plus the oxidase activity of the O form and (IV) measurement of the increase in $A_{340}$ in the presence of $O_2$ and $NAD^+$ (in the same cuvette as in (III)) gives a measure of the dehydrogenase activity (partially inhibited by accumulating NADH) of the D and D/O forms. The activities of the various forms can be calculated

from these values (Kaminski and Jezewska, 1979): the dehydrogenase activity of form D is given by (I)-(II); the oxidase activity of form O by (III)-(IV); the oxidase activity of form D/O by (III)-((III)-(IV)); and the total dehydrogenase activity (the sum of that of forms D and D/O) by (I)-((III)-(IV)).

viii. Rat Liver Nitroreductase-Catalysed "Activation" of $^{14}$C-Nitrofurazone to Protein-Bound Species

The reaction mixture consisted of 4 ml liver nitroreductase, 10 mg bovine serum albumin, approximately 2 x $10^6$ CPM $^{14}$C-nitrofurazone and an NADH generating system (0.15µmol $NAD^+$, 20 µmol glucose-6-phosphate, 3.6 units glucose-6-phosphate dehydrogenase) in a final volume of 6 ml K-P buffer. Incubation was for one hour at 24°C in a 125 ml Erlenmyer flask, after which the mixture was fractionated in a Biogel A 1.5 column (as described in section III.B.ii.). The absorbance of each fraction was measured at 280 nm and 50 µl aliquots were pipetted onto small glass fibre discs. The discs were dried and counted in a Beckman LS-230 scintillation counter with a fluor consisting of 4 g Omniflour per litre of toluene.

The albumin-containing fractions were pooled, dialysed against 4 l K-P buffer at 4°C and the radioactivity measured, as described above, at 8 hr intervals. In another experiment, these fractions were dialysed successively for 8 hr against 1 l of 8 M urea, 4 l fresh K-P buffer, 2 l 5 M NaCl and, finally, 4 l fresh K-P buffer and the radioactivity was measured at each change.

# IV. RESULTS

# IV. RESULTS

## A. Identification of a Rat Liver Nitroreductase with Unusual Activity in the Presence of Oxygen

Net reduction of nitrofurazone by the 9000xg supernatant of rat liver extract (S9) was dependent on both the nature of the electron donor and on the concentration of the molecular oxygen. Aerobic nitroreduction in the presence of NADPH was increased by about 5x by the absence of oxygen whereas aerobic nitroreduction in the presence of NADH was only marginally increased by the absence of oxygen (Fig. VII). The NADH-dependent reductase activity, but not the NADPH-dependent activity, was found in the 105,000xg supernatant.

When rat liver S105 supernatant was fractionated on a large gel filtration column and each fraction analyzed for protein content (by $A_{280}$) and aerobic nitroreductase activity the profile in Fig. VIII was obtained. The major NADH-dependent nitroreductase activity eluted in fractions 15 to 21, which correspond to the position expected for a protein with molecular weight of about 280,000 (Fig. IX).

Both xanthine oxidase and aldehyde oxidase are known to be soluble enzymes with molecular weights of about 280,000 (Bray, 1975) and capable of reducing nitroheterocyclic compounds (see section II.A.v.). Rat liver is known to contain primarily xanthine oxidase (Wolpert et. al., 1973).

On gel filration of rat liver S105 the nitroreductase activity co-eluted with the rat liver xanthine oxidase activity (Fig. VIII). However, in fresh liver nitroreductase preparations (but not in aged preparations), net reduction of nitrofurazone was much less affected by

Fig. VII. Net reduction of nitrofurazone by rat liver S9: ■—■ , aerobic with NADPH; □—□ , anaerobic with NADPH; ●—● , aerobic with NADH, ○—○ ; anaerobic with NADH. See section III.B.iii. for experimental details.

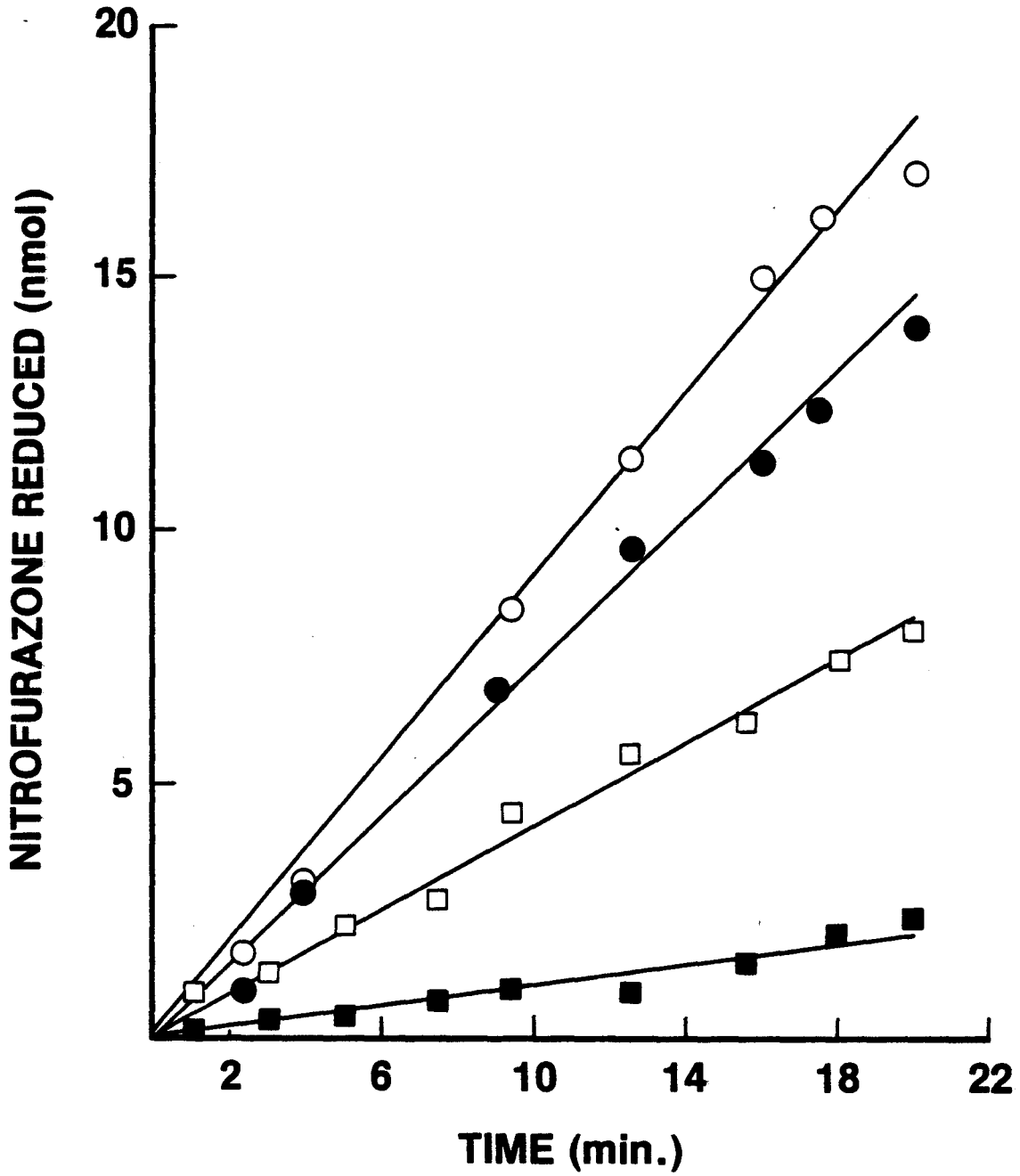

NITROFURAZONE REDUCED (nmol)
20
15
10
5
2
6
10
14
18
22
TIME (min.)

Fig. VIII. Fractionation of rat liver S105 preparation on Biogel A1.5: ——, absorbance at 280 nm; ——, nitrofurazone (absorbance at 375 nm) remaining following incubation with an NADH regenerating system for 30 min; •••• , uric acid (absorbance at 290 nm) formed from hypoxanthine in 5 min. See sections III.B.ii., iii. and iv. for experimental details.

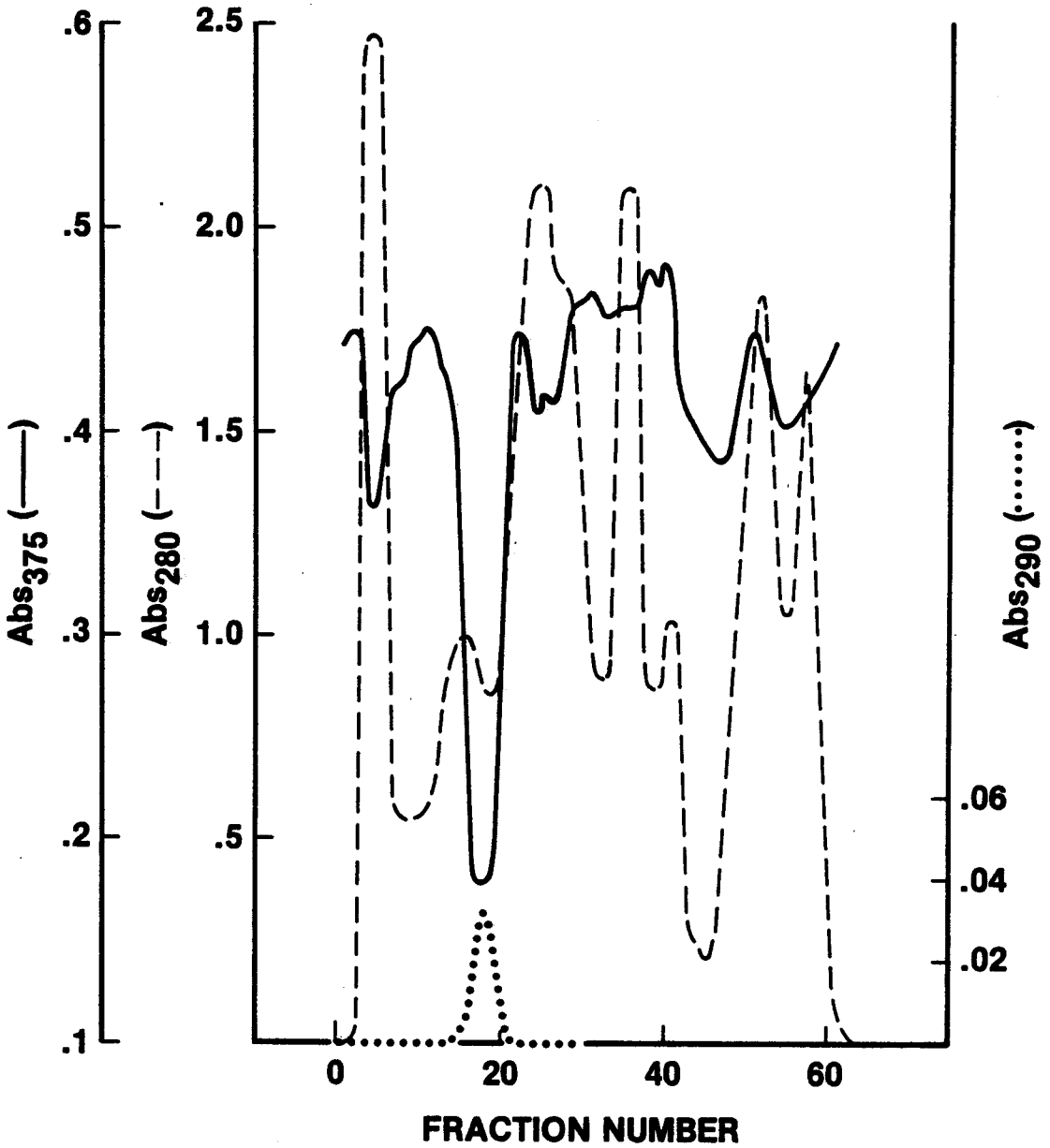
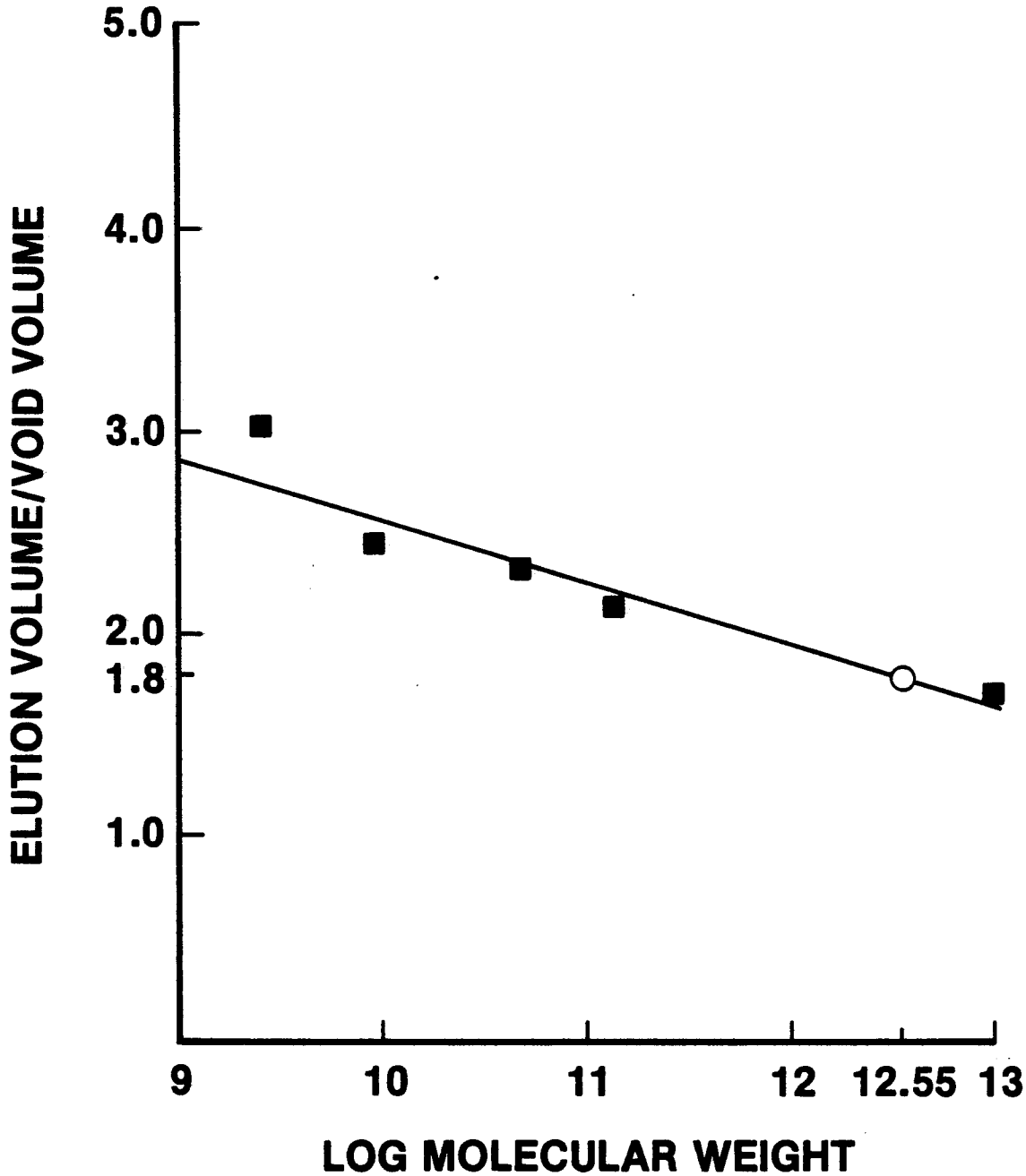

.6
.5
.4
.3
.2
.1
Abs375 (——)
2.5
2.0
1.5
1.0
.5
Abs280 (– – –)
.06
.04
.02
Abs290 (·····)
0
20
40
60
FRACTION NUMBER

Fig. IX. Molecular weight estimation for "liver nitroreductase": Calibration of Biogel A1.5 column with horse spleen ferritin, bovine serum albumin, ovalbumin, trypsin inhibitor and cytochrome c reductase. See section III.B.ii. for experimental details.


5.0
4.0
3.0
2.0
1.8
1.0
ELUTION VOLUME/VOID VOLUME
9
10
11
12
12.55
13
LOG MOLECULAR WEIGHT

oxygen than was the corresponding activity with commercial xanthine oxidase (Fig. X). This is a novel observation, since all reports on mammalian nitroreductases have shown that oxygen strongly inhibits net nitroreduction in vitro (see section II.A.v.).

B. "Activation" of Nitrofurazone by Rat Liver Nitroreductase

The nitroreductase of fresh liver preparations also catalysed the binding of radioactivity from $^{14}$C-nitrofurazone to protein under aerobic conditions. Figure XIA shows the results of an experiment in which the enzyme preparation was incubated for 1 hr with $^{14}$C-nitrofurazone, the requisite co-factors and added bovine serum albumin (BSA) followed by chromatography of the reaction mixture on a Biogel A1.5 column. Radioactivity was found in unchanged nitrofurazone (peak I; $\lambda$ max 375 nm), in the low molecular weight reduction product(s) (peak II; $\lambda$ max 330 nm) and in association with protein (peaks III & IV). Peak III contained label associated with the added BSA while peak IV contained label associated with the higher molecular weight proteins of the enzyme preparation itself. These protein zones contained radioactivity equivalent to about 25% of the nitrofurazone reduced. In control reaction mixtures, from which $NAD^+$ was omitted, nitrofurazone was not metabolized and no protein-binding was observed (Fig. XIB).

When the fractions containing labelled peak III ($^{14}$C bound to BSA) from the complete incubation mixture were combined and dialysed against 67 mM phosphate buffer pH 7.2 there was an initial loss of about 10% of the radioactivity followed by slower exponential loss having a half-time of several days. Since successive dialysis against 8 M urea, buffer, 5 M NaCl and more buffer all resulted in loss of activity at

Fig. X. Inhibition by oxygen of the net reduction of nitrofurazone -○-○- , liver nitroreductase, anaerobic with NADH; -●-●- , liver nitroreductase, aerobic with NADH; -□-□- , milk xanthine oxidase, anaerobic with hypoxanthine; -■-■- , milk xanthine oxidase, aerobic with hypoxanthine. See section III.B.iii. for experimental details.

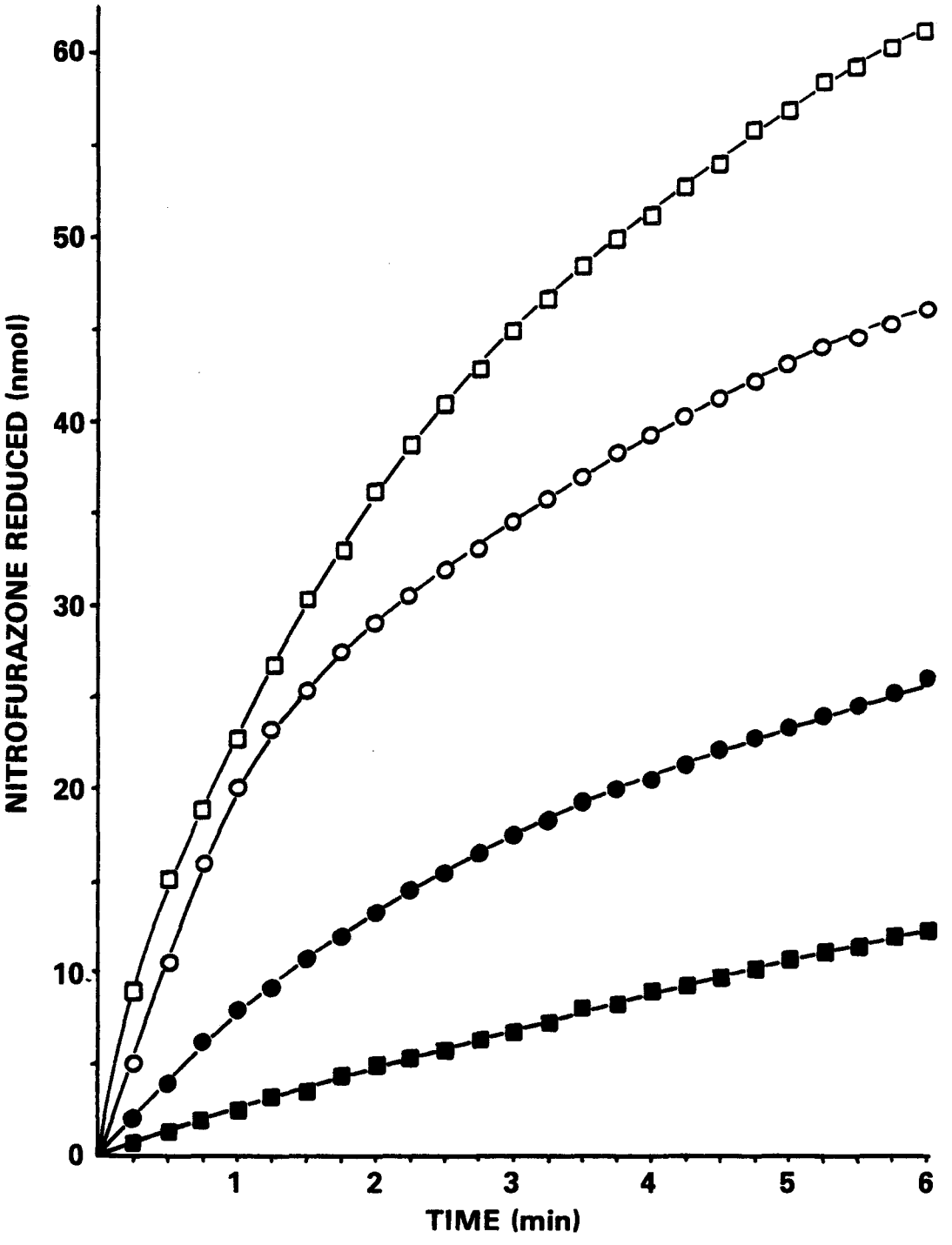
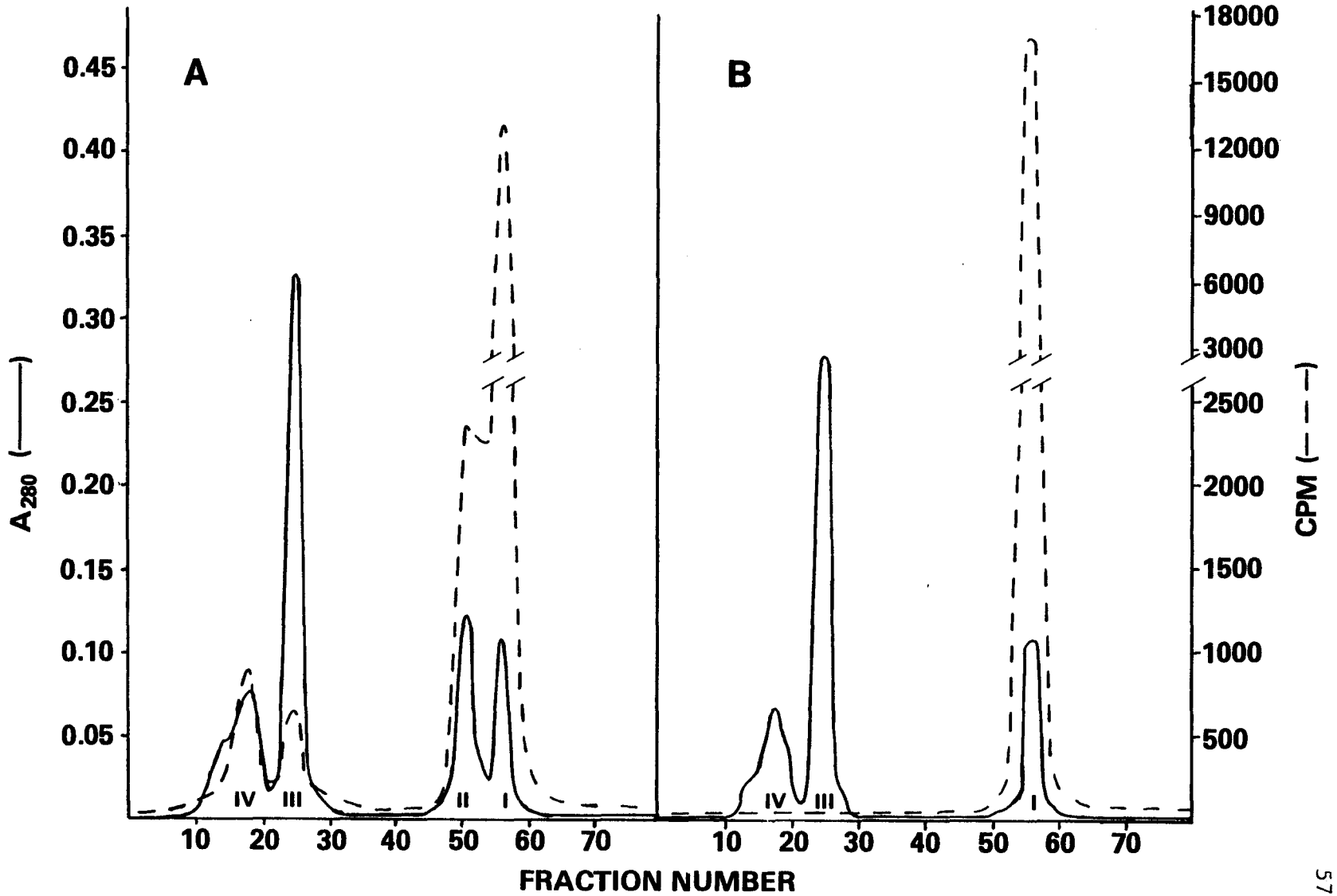

NITROFURAZONE REDUCED (nmol)
0
10
20
30
40
50
60
1
2
3
4
5
6
TIME (min)

Fig. XI. (A). Aerobic reduction of $^{14}C$-nitrofurazone by the liver nitroreductase preparation and binding of $^{14}C$ to proteins. The reaction mixture which contained bovine serum albumin and an NADH generating system in addition to the nitroreductase preparation and labeled nitrofurazone was incubated for 1 hr and then chromatographed on a Biogel A1.5 column. Peak I: $^{14}C$-nitrofurazone; peak II: reduced metabolite(s) of nitrofurazone; peak III: bovine serum albumin; peak IV: proteins of the liver nitroreductase preparation. See section III.B.viii. for experimental details.

(B). Control experiment, identical to that shown in A except that $NAD^+$ was omitted. See Caption for (A) and section III.B.viii. for experimental details.


A
B
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
$A_{280}$ (——)
18000
15000
12000
9000
6000
3000
2500
2000
1500
1000
500
CPM (— — —)
IV
III
II
I
10
20
30
40
50
60
70
FRACTION NUMBER

nearly the same rate (Fig. XII) it appears that the radioactivity was initially tightly (and probably covalently) bound.

The identification of a mammalian enzyme which reduces nitrofurazone almost as fast under aerobic conditions as in hypoxia (see section IV.A.) to intermediates which bind to protein is potentially important in relation to the DNA-damaging effects of nitrofurans which occur in vivo and in aerobically cultured mammalian cells.

C. Characterization of the Aerobic Rat Liver Nitroreductase as Xanthine Dehydrogenase

To investigate further the possible contributions of aldehyde and xanthine oxidoreductases to the nitroreductase activity of the liver preparation, allopurinol, a selective inhibitor of xanthine oxidase (Bray, 1975), and $N^1$-methylnicotinamide, which serves as an electron donor for aldehyde oxidase but not for xanthine oxidase (Wolpert et. al., 1973), were employed. The results (Table II) indicated that the bulk of the activity was due to xanthine oxidase and that aldehyde oxidase contributed, at most, a minor amount of activity. However, the characteristics of nitrofurazone reduction by liver nitroreductase and purified milk xanthine oxidase were quite different in two respects. First, as is also indicated in Fig. X, net nitroreduction by the liver nitroreductase was not as sensitive to inhibition by molecular oxygen as net reduction by milk xanthine oxidase and, second, only the former enzyme used NADH at a rate comparable to that obtained with the purines. This ability of the liver enzyme to readily accept electrons from NADH is particularly evident when concentrations of liver nitroreductase and milk xanthine oxidase which gives equal rates of anaerobic NADH-dependent net

Fig. XII. Loss of protein-associated radioactivity during successive 8 hr periods of dialysis of peak III, Fig. XI (A) against 8 M urea, K-P buffer, 5 M NaCl and more K-P buffer. Note that the vertical axis is a log scale. See section III.B.viii. for experimental details.

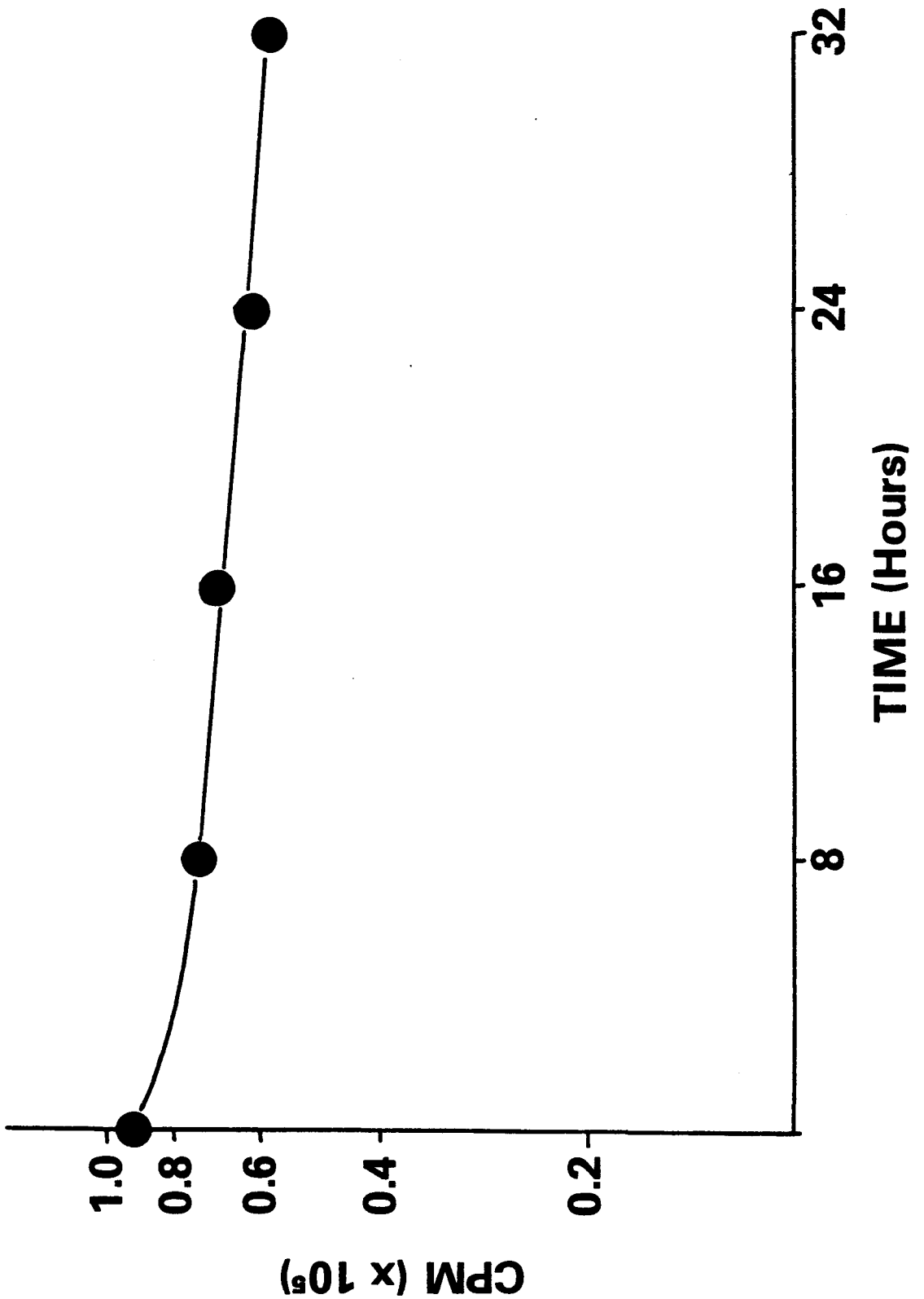

CPM (x 10⁵)
1.0
0.8
0.6
0.4
0.2
8
16
24
32
TIME (Hours)

TABLE II

Electron donor and enzyme inhibitor specificities for net nitrofurazone reduction by liver nitroreductase and milk xanthine oxidase.* See Section III.B.iii. for experimental details.

| Enzyme | Substrates and/or Inhibitors | nmol nitrofurazone reduced per min, (aerobic) | nmol nitrofurazone reduced per min, (anaerobic) |
|---|---|---|---|
| rat liver nitroreductase (0.64 mg protein per ml.) | hypoxanthine | 3.8 | 12.0 |
| | hypoxanthine + allopurinol | 0 | 0 |
| | xanthine | 2.0 | 4.8 |
| | xanthine + allopurinol | 0 | 0 |
| | NADH | 3.2 | 10.4 |
| | NADH + allopurinol | 0.2 | 0.8 |
| | NADPH | 0 | 0 |
| | $N^1$methyl-nicotinamide | 0 | 0 |
| milk xanthine (.01U) | NADH | 0 | 0 |
| | hypoxanthine | 2.4 | 24.0 |
| milk xanthine oxidase (0.14U) | NADH | 1.5 | 17.5 |
| | hypoxanthine | 57.6 | N.D.** |

*Hypoxanthine, xanthine and NADH are substrates for xanthine oxidase, $N^1$methylnicotinamide for aldehyde oxidase and NADPH for neither enzyme. Allopurinol is an inhibitor of xanthine oxidase.

nitrofurazone reduction are compared for their xanthine oxidase activities (Fig. XIII).

The ability of the freshly prepared liver nitroreductase to accept electrons readily from NADH suggested that the enzyme xanthine dehydrogenase, which is considered to be the native, intracellular form of xanthine oxidase (see section V.C.), might be responsible for the aerobic nitroreductase activity of the liver preparation. It has been shown that xanthine oxidase is only one form of an enzyme which has been renamed xanthine oxidoreductase. When rat liver supernatant is prepared with the minimum of manipulation and assayed promptly, most of the xanthine oxidoreductase activity appears as a dehydrogenase (D form) which is rapidly converted to the more stable oxidase (O form) under most conditions (see section V.C.). The O form and D form have similar Km's for xanthine but only the D form is able to use NADH at a rate comparable to that obtained with xanthine (Waud and Rajagopalan, 1976a). Indeed, xanthine dehydrogenase activity was present in the fresh liver nitroreductase preparation (see Table III, line I).

The relation between the xanthine dehydrogenase and aerobic nitroreductase activities of the preparation was examined in several ways. Firstly, the xanthine oxidase and dehydrogenase activities were partially separated by CM cellulose chromatography (Fig. XIV) and the aerobic nitroreductase activity followed the xanthine dehydrogenase activity.

Secondly, as shown in Fig. XV, heating of the liver preparation at 65$^{o}$C converted xanthine dehydrogenase to the oxidase form with essentially no loss in total dehydrogenase plus oxidase activity during

Fig. XIII. Oxidation of hypoxanthine to uric acid by concentrations of liver nitroreductase -O-O- and xanthine oxidase -□-□- which gave equal rates of anaerobic, NADH-dependent net nitrofurazone reduction. See section III.B.iv. for experimental details.

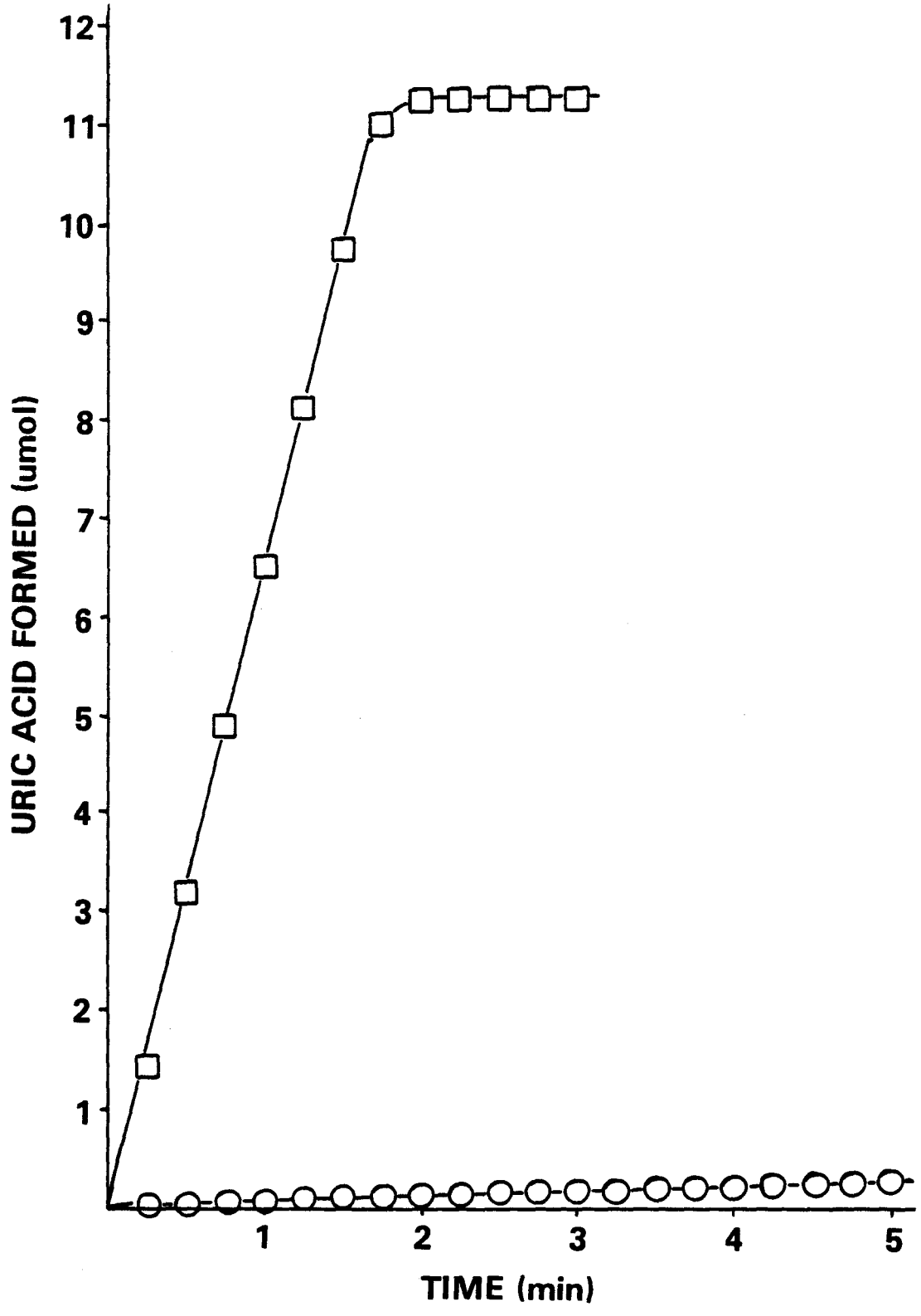

URIC ACID FORMED (umol)
12
11
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
TIME (min)

Fig. XIV. Chromatography of liver nitroreductase preparation on CM cellulose: -O-O- xanthine oxidase activity; -□-□- xanthine oxidase + xanthine dehydrogenase activities; -●-●- aerobic nitroreductase activity. Note that the xanthine dehydrogenase activity is the difference between the total activity (-□-□-) and oxidase activity (-O-O-). See section III.B.v. for experimental details.

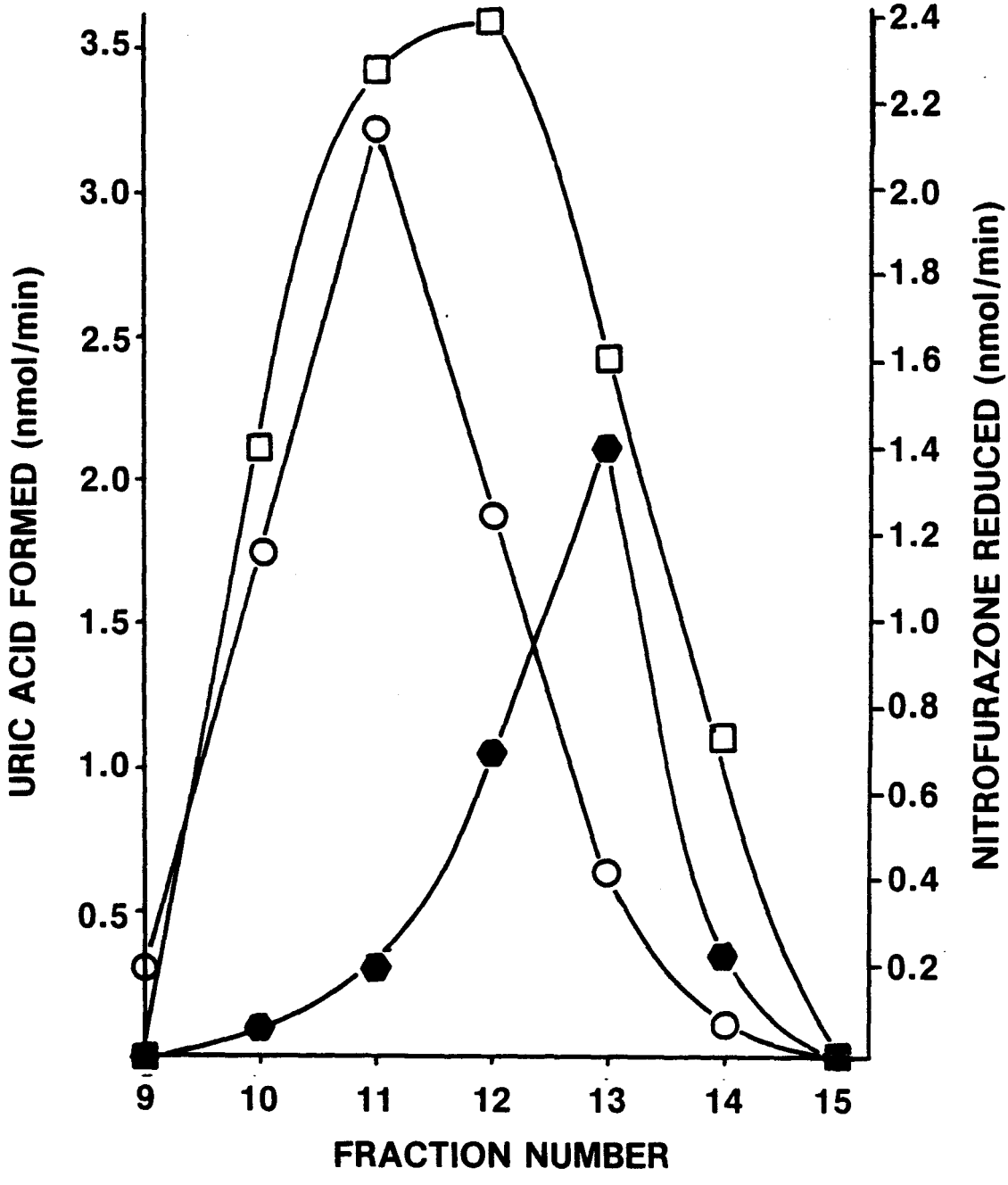
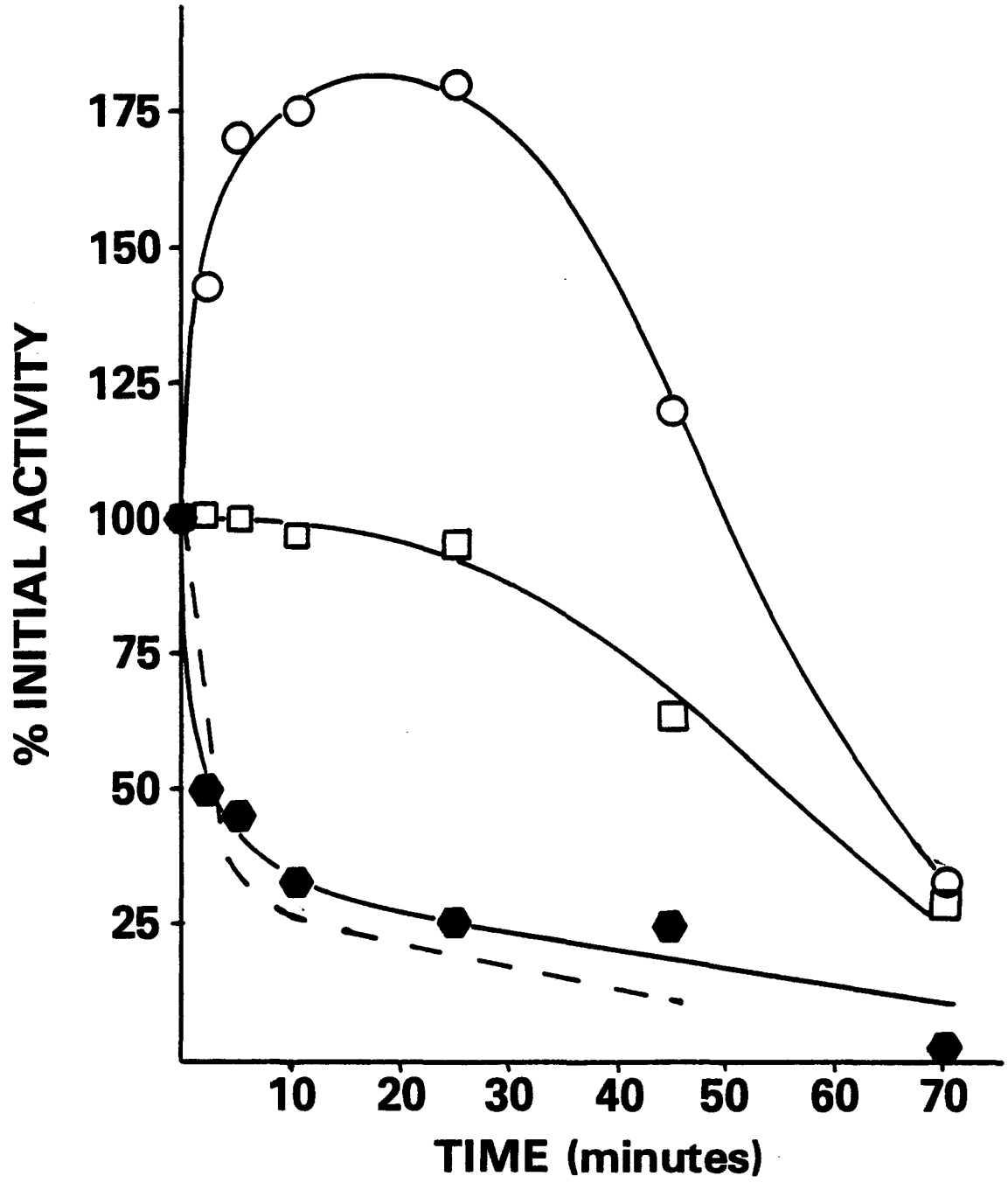

URIC ACID FORMED (nmol/min)
NITROFURAZONE REDUCED (nmol/min)
FRACTION NUMBER
3.5
3.0
2.5
2.0
1.5
1.0
0.5
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
9
10
11
12
13
14
15

Fig. XV. Effect of conversion of xanthine dehydrogenase to the oxidase by incubation at 65°C on the aerobic nitroreductase activity of the rat liver preparation. Symbols: -□-□-, total oxidase plus dehydrogenase activity; -○-○-, oxidase activity; ———, calculated dehydrogenase activity (-□-□- minus -○-○-); -●-●-, aerobic nitroreductase activity. See sections III.B.iii. and III.B.iv. for experimental details.


% INITIAL ACTIVITY
175
150
125
100
75
50
25
10
20
30
40
50
60
70
TIME (minutes)

the initial 25 min. Loss of xanthine dehydrogenase activity (which is at a similar rate to the conversion rate reported by Waud and Rajagopalan, 1976b) closely paralleled the loss of aerobic nitroreductase activity. Indeed, the xanthine dehydrogenase and the aerobic nitroreductase activities are somewhat unstable even in the cold (Waud and Rajagopalan, 1976b; data not shown).

Finally, as shown in Table III, about 87% of the dehydrogenase activity of a particularly active preparation was converted to oxidase activity by treatment with p-hydroxymercuribenzoate. Upon addition of dithiothreitol, the oxidase activity was converted to the dehydrogenase form (with 80% of the original dehydrogenase activity being recovered). Aerobic nitroreductase activity was decreased substantially when the dehydrogenase was converted to oxidase and largely restored (to 85% of original activity) when dithiothreitol was added. These treatments had little, if any, effect on the anaerobic nitroreductase activity or on the total xanthine oxidase plus dehydrogenase activities. The aerobic nitroreductase activity remaining after the p-hydroxymercuribenzoate treatment could be due to a combination of residual xanthine dehydrogenase plus a contribution from the oxidase which, as shown for milk xanthine oxidase in Table II (see also Holtzman et. al., 1981), had a small amount of aerobic nitroreductase activity. The observation that the NADH-dependent aerobic nitroreductase activity of milk xanthine oxidase was undetectable when the assay contained 0.01 units of enzyme but was easily seen with 0.14 units of enzyme (Table II) may be a consequence of the higher steady stage level of the radical anion and resulting disproportionation (see section II.A.v.).

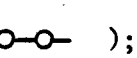
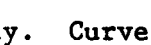
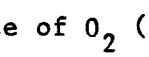
TABLE III

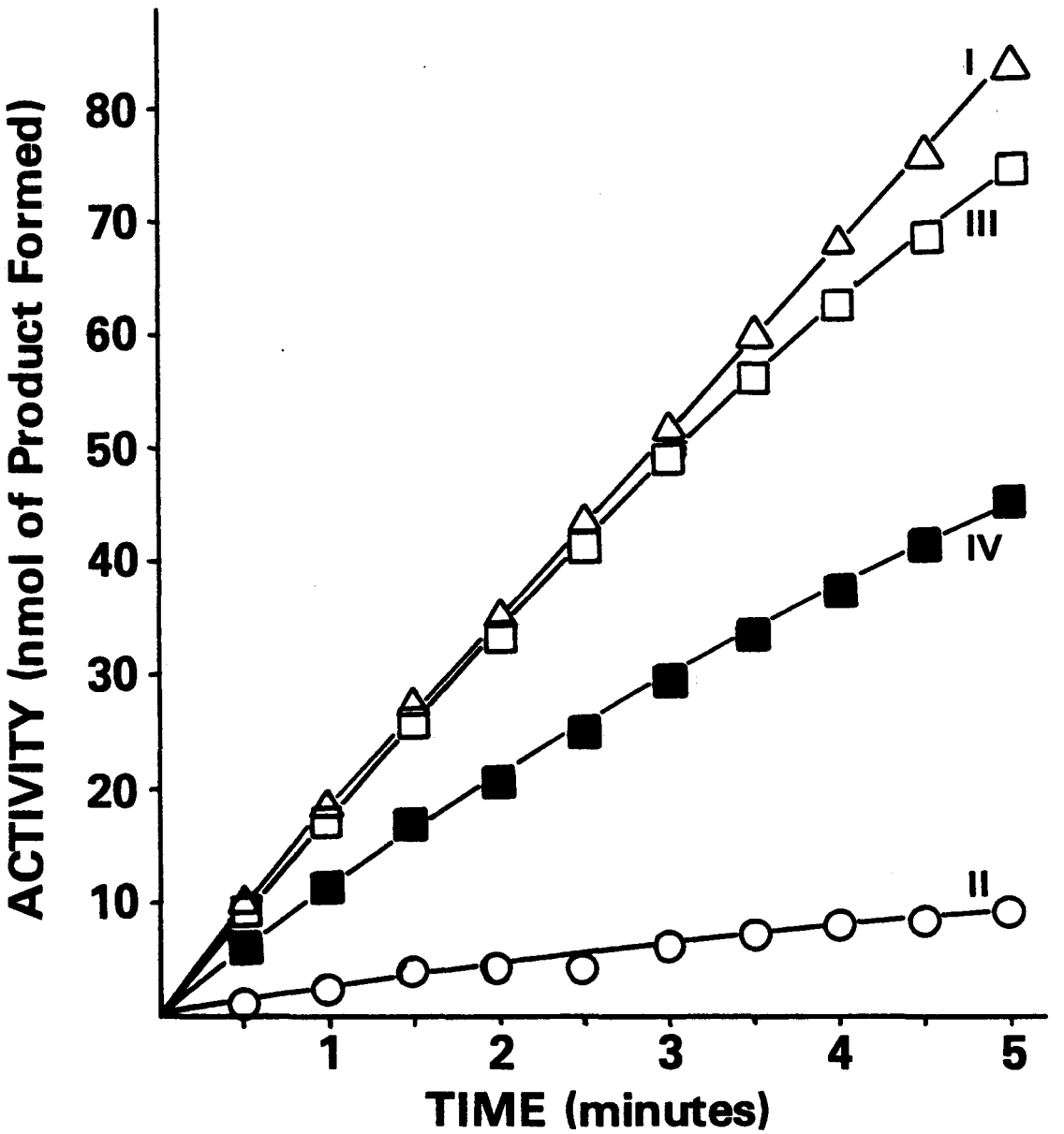
Reversible decrease in aerobic rat liver nitroreductase activity when xanthine dehydrogenase is converted to xanthine oxidase with p-hydroxymercuribenzoate and reconverted to xanthine dehydrogenase with dithiothreitol. See section III.B.vi. for experimental details.

| Treatment | Xanthine oxidase (uric acid formed (nmol per min.)) (A) | Xanthine oxidase + dehydrogenase activity (uric acid formed in the presence of $NAD^+$ (nmol per min.)) (B) | Xanthine dehydrogenase activity (B-A) | Nitroreductase activity | |
|---|---|---|---|---|---|
| | | | | Aerobic (nitrofurazone reduced (nmol per min.)) | Anaerobic (nitrofurazone reduced (nmol per min.)) |
| I. None | 0.9 | 4.1 | 3.2 | 8.4 | 15.4 |
| II. p-Hydroxy-mercuri-benzoate | 3.7 | 4.1 | 0.4 | 3.0 | 16.4 |
| III. p-Hydroxy-mercuri-benzoate followed by dithio-threitol | 1.3 | 3.9 | 2.6 | 7.2 | 16.9 |

Recently, Kaminski and Jezewska, 1979, have described a dehydrogenase-oxidase (D/O) form of xanthine oxidoreductase which they believe to be an intermediate in the conversion of the D form to the O form. By applying their spectrophotometric method to a sample of the liver nitroreductase preparation an estimate of the activities of the three forms of xanthine oxidoreductase was made (Fig. XVI). The oxidase activity of form O (III-IV) was approximately 6 nmol/min, the dehydrogenase activity of form D (I-II) was approximately 12 nmol/min, the oxidase activity of form D/O (II-(III-IV)) was approximately -1 nmol/min and the dehydrogenase activity of form D/O (I-(III-IV)-(I-II)) was approximately -1 nmol/min. The D/O form activity calculated at -1 nmol/min represents the absence of detectable D/O form in the liver preparation, within the experimental limits of the spectrophotometric method. Therefore, in this rat liver preparation about 2/3 of xanthine oxidoreductase was in the D form, 1/3 in the O form and a negligible amount in the D/O form.

In the absence of any detectable D/O form, the chromatography on CM cellulose, heat conversion and chemical interconversion experiments establish that net reduction of nitrofurazone by xanthine dehydrogenase is considerably less sensitive to inhibition by oxygen than is net nitroreduction by rat liver or milk xanthine oxidase. The nitroreductase activity of xanthine dehydrogenase accounts for the bulk of the NADH-dependent aerobic nitroreductase activity of the rat liver supernatant preparation.

Fig. XVI. Data for determination of the activities of the three forms of xanthine oxidoreductase in the liver nitroreductase preparation. Curves I to III, oxidation of xanthine in the presence of: $O_2$, $NAD^+$ and a NADH re-oxidizing system (  ); $O_2$ only (  ); $O_2$ and $NAD^+$ (  ) respectively. Curve IV, reduction of $NAD^+$ in the presence of $O_2$ (  ). See section III.B.vii. for details.


ACTIVITY (nmol of Product Formed)
80
70
60
50
40
30
20
10
1
2
3
4
5
TIME (minutes)
I
III
IV
II

# V. DISCUSSION

# V. DISCUSSION

## A. The Search for a Mammalian Nitroreductase with In Vivo Nitrofuran "Activating" Properties

Nitroreduction intermediates of 5-nitrofurans are clearly implicated as the ultimate mutagenic species in both bacteria and anaerobically cultured mammalian cells. On the balance, the current data also favours their involvement in the mutagenic and carcinogenic actions of these chemicals in intact animals (see section II.B.).

The apparent inability of the known mammalian nitroreductases to reduce nitrofurans past the oxygen-sensitive nitroaromatic anion free radical (see section II.A.v.) suggests that, in vivo, nitroreduction may occur either by the generation of high concentrations of these radicals (which would allow for disproportionation to compete effectively with reoxidation by molecular oxygen) or by the actions of nitroreductases which bypass this oxygen-sensitive one electron reduced species (see section II.B.). This led me to search for a mammalian nitroreductase activity which is able to "activate" nitrofurazone in the presence of oxygen.

## B. Partial Purification of a Rat Liver Nitroreductase which "Activates" Nitrofurazone in the Presence of Oxygen

Freshly prepared rat liver S9 supernatant was associated with an NADH-dependent aerobic nitroreductase activity which catalyzed the disappearance of nitrofurazone almost as quickly in the presence, as in the absence, of oxygen (see section IV.A.). This activity was partially purified by fractionation of rat liver S105 supernatant on a Biogel A 1.5 gel filtration column. This nitroreductase preparation catalyzed the

tight (and probably covalent) binding of $^{14}$C-nitrofurazone to protein (see section IV.B.).

This nitroreductase activity co-eluted with rat liver xanthine oxidase from gel filtration columns and was similar to the nitroreductase activity of purified milk xanthine oxidase both in its use of xanthine and hypoxanthine as electron donors and in its sensitivity to the xanthine oxidase inhibitor allopurinol. However, in contrast to the nitroreductase activity of purified milk xanthine oxidase, the liver preparation was able to accept electrons from NADH as efficiently as from purines and its catalysis of net nitroreduction was relatively insensitive to oxygen (see section IV.C.).

C. Xanthine Oxidoreductase

Xanthine oxidoreductase is one member of a class of related enzymes, the molybdenum hydroxylases. Within this class are various xanthine dehydrogenases (E.C. 1.2.1.37), xanthine oxidases (E.C. 1.2.3.2.) and aldehyde oxidases (E.C. 1.2.3.1.) (see Massey, 1973; Bray, 1975; Coughlan, 1980). The molybdenum hydroxylases are structurally similar. Each has a particle weight of about 300,000 daltons and is comprised of two equivalent and independent subunits. Each subunit contains four redox active prosthetic groups: one atom of molybdenum; one molecule of FAD; and two distinct iron-sulfur clusters.

Catalysis by the various molybdenum hydroxylases is mechanistically similar to that of the best-studied member of the group, bovine milk xanthine oxidase (Bray, 1975). Each of the redox active prosthetic groups interacts with specific substrates (Coughlan and NíFhaoláin, 1979). The substrate specificity amongst the molybdenum

hydroxylases is a result of structural differences in their binding sites (Bray, 1975). Substrate binding is dictated solely by the structural specificity of the active sites and is not influenced by the redox state of the enzyme (Coughlan and Rajagopalan, 1980). During catalysis each subunit can act as an electron transport chain between reducing and oxidizing substrates (Olson et. al., 1974; Bray et. al., 1979; Coughlan, 1980; Porras et. al., 1981). The electron distribution in each subunit is very rapid (i.e., not rate limiting) and is governed by the thermodynamic properties of the redox centers (Olson et. al., 1974; Coughlan, 1980; Hille et. al., 1981).

It is known that xanthine oxidases of human liver (Della Corte et. al., 1969), bovine milk (Battelli et. al., 1973) and rat heart, liver, small intestine, spleen, pancreas, lung and kidney (Battelli et. al., 1972) function in vivo as xanthine dehydrogenases (D form) which are usually converted into xanthine oxidases (O form) during purification. Sometimes during purification of the D form, a small amount of intermediate (D/O form) in the transformation to the O form can be detected (Kaminski and Jezewska, 1979). Rapid purification is necessary to preserve the D form because during purification and storage many of the free sulfydryl groups of the D form are converted into disulfides of the O form (Stirpe and Della Corte, 1969; Waud and Rajagopalan, 1976a,b). In vitro the D form of xanthine oxidoreductase can be converted to the O form by heat treatment or by treatment with a number of agents which are known to modify sulfhydryl groups; some of the modifications can be reversed by subsequent treatment with thiol compounds (Della Corte and Stirpe, 1972). Treatment of the D form with trypsin irreversibly

converts the enzyme into an O form by removing a thiol-rich peptide of approximately 20,000 daltons (Waud and Rajagopalan, 1976b). The change in acceptor specificity, from $NAD^+$ in the D form to oxygen in the O form, is believed to reflect a structural change in the vicinity of the flavin (Della Corte and Stirpe, 1972; Bray, 1975). This is schematically illustrated in Fig. XVII (see Stirpe and Della Corte, 1969; Bray, 1975; Waud and Rajagopalan, 1976a,b; Barber et. al., 1981).

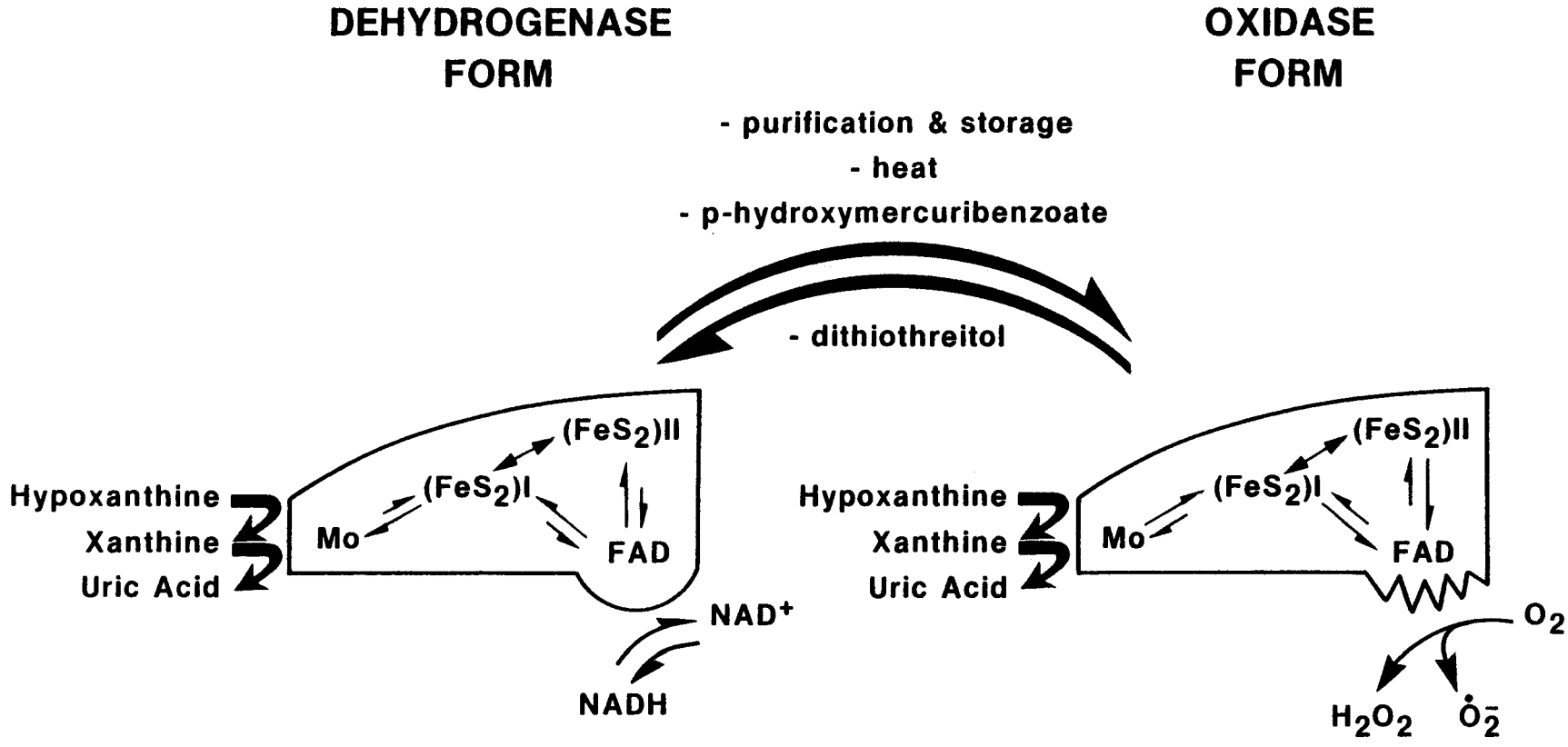
D. Characteristics of Nitrofurazone Reduction by the D and O Forms of Xanthine Oxidoreductase

The D form of xanthine oxidoreductase differs from the O form both in its ability to utilize $NAD^+$ as an electron acceptor when oxidizing purines and in its ability to catalyze efficient oxidation of NADH with any of several electron acceptors (Waud and Rajagopalan, 1976b). The ability of the rat liver nitroreductase preparation to accept electrons from NADH as efficiently as from purines (see section V.B.) suggests that it may contain a significant proportion of the D form of xanthine oxidoreductase. Indeed, the fresh rat liver preparation was found to contain approximately 2/3 D form and 1/3 O form, with no detectable D/O form.

The nitroreductase activity of xanthine dehydrogenase has not previously been studied (see section II.A.v.). The relative insensitivity of net nitroreduction to oxygen with the fresh liver preparation could be due to the actions of xanthine dehydrogenase if the D form of xanthine oxidoreductase (unlike the O form) can catalyse net nitroreduction efficiently in the presence of oxygen. Several lines of evidence confirm that the process of net nitroreduction of nitrofurazone

Fig. XVII. Schematic representation of a xanthine oxidoreductase subunit: Interconversion between dehydrogenase and oxidase forms.

# XANTHINE OXIDOREDUCTASE



by the D form is considerably less sensitive to inhibition by oxygen than is nitroreduction catalysed by the O form (see section IV.C.). Firstly, the dehydrogenase and oxidase activities were partially separated using CM cellulose chromatography and the aerobic nitroreductase activity followed the activity of the D form. Secondly, with heat-induced transformation of D form into O form, the loss in aerobic nitroreductase activity closely paralleled the loss of dehydrogenase activity. Finally, during chemical interconversion between the D and O forms, the aerobic nitroreductase activity followed the dehydrogenase activity, while the anaerobic nitroreductase activity was unchanged.

The mechanistic basis for the difference in oxygen sensitivities with the two forms of xanthine oxidoreductase is not known. With the bacterial Type II nitroreductases and with NADPH:Cyt P450(c)reductase inhibition of net reduction of nitrofurans by oxygen is entirely accounted for by reoxidation of the nitroradical anion (Peterson et. al., 1979; see section II.A.v.). Unpublished studies (R. Mason, private communication to D.R. McCalla, 1983) have demonstrated that the nitroradical anion is also formed during the anaerobic reduction of nitrofurans by milk xanthine oxidase but it is not seen in the presence of oxygen. Thus, it is possible that re-oxidation of the anion may contribute to the strong inhibition of the nitroreductase activity of xanthine oxidase by oxygen. If so, one might postulate that the dehydrogenase (like the bacterial Type I nitroreductases) may transfer more than one electron yielding an initial product which resists oxidation. Indeed, xanthine oxidoreductase is an unusual enzyme in that it can catalyze the transfer of either one or more electrons (see Bray,

1975; Coughlan, 1980; Hille and Massey, 1981). The chemical basis for the multiple-electron transfer to nitrofurazone by the D form may be a result of the structural difference between the D and O forms of xanthine oxidoreductase (see section V.C.; this structural alteration may explain the separation of the two forms on CM cellulose chromatography in Fig. XVI). It is known that the structural change alters the electron distribution amongst the various redox centres (Waud and Rajagopalan, 1976b; Barber et. al., 1977; Coughlan, 1980; Palmer and Olson, 1980). For instance, a subunit in the catalytically significant 4 electron reduced stage (Coughlan, 1980) has a much greater electron density on the flavin in the O form and on the molybdenum in the D form (Barber et. al., 1977; Coughlan, 1980). Thus one might hypothesize that, if nitroreduction occurs principally at the molybdenum site, then these thermodynamic considerations may allow for a greater degree of multiple-electron transfer when the enzyme is in the D form. Indeed, the ability of excess allopurinol (which binds to the molybdenum site (Massey, 1973; Bray, 1975)) to inhibit purine-dependent nitroreduction completely but NADH-dependent nitroreduction by over 10-fold (Table II) suggests that the majority of nitrofurazone reduction does occur at the molybdenum site.

Alternatively, given the strong oxidase activity of xanthine oxidase (Olson et. al., 1974; Bray, 1975), the mechanistic basis for the difference in oxygen sensitivities with the two forms of xanthine oxidoreductase may occur at the level of the enzyme itself, with reduction of oxygen competing preferentially with reduction of nitrofurazone when the enzyme is in the O form.

## E. Possible Role of Xanthine Oxidoreductase in Nitrofuran Carcinogenesis

The data reported here establish that the bulk of the aerobic nitroreductase activity in rat liver supernatant preparations is associated with xanthine dehydrogenase. The dehydrogenase-catalyzed aerobic reduction of nitrofurazone produces intermediates which bind to proteins. The structure(s) of these derivatives was not investigated. Further work is required to explore the reactivity of the reduced species with cellular constituents (especially DNA).

Thus, it is possible that aerobic reduction of nitrofurans and other nitroheterocyclic and nitroaromatic compounds by xanthine dehydrogenase may constitute a significant "activation" process which contributes to the toxic action of such agents. One attempt has been made to ascertain the relevance of xanthine oxidoreductase in the induction of tumors (Wang et. al., 1976). Rats were fed the bladder carcinogen FANFT with or without the xanthine oxidoreductase inhibitor allopurinol. Fewer tumors were induced in the rats fed FANFT alone. One may postulate that in the allopurinol-treated animals the rate of metabolism of FANFT was decreased so that the bladder was exposed to larger amounts of FANFT which would enhance the toxic pathway(s), whatever its nature (see section II.A.vi.). This implies that xanthine oxidoreductase is more important in detoxifying FANFT by metabolizing it in non-target tissues than in activating FANFT in the bladder tissue. Indeed, allopurinol administration leads to higher serum levels and prolonged lifetimes of a nitroimidazole derivative, metronidazole (Raleigh et. al., 1980) which may reflect inhibition of xanthine

oxidoreductase in organs such as the small intestine (see Tatsumi et. al., 1973a;b; 1975) and liver and/or to inhibition of renal clearance (Workman and White, 1982).

Further work is required to determine whether or not nitroreduction by xanthine dehydrogenase represents a biologically relevant pathway for the reduction of nitroheterocyclic compounds in vivo and, if so, whether, on the balance, xanthine oxidoreductase is more important in "activating" these compunds in particular target tissues or in decreasing the amount of carcinogen available in target tissues by metabolizing these compounds in non-target tissues.

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