IN VITRO METABOLISM OF 2,4-DINITROPHENOL BY RAT LIVER HOMOGENATES

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
JULIE LOUISE EISEMAN
1971

LIBRARY
Michigan State
University

BINDING BY
HOAG & SONS'
BOOK BINDERY INC.

ABSTRACT

IN VITRO METABOLISM OF 2,4-DINITROPHENOL

BY RAT LIVER HOMOGENATES

By

Julie Louise Eiseman

The *in vitro* metabolism of C^{14}-2,4-dinitrophenol (DNP) was examined in rat liver (300-500 g, males) and DNP, 4-amino, 2-nitrophenol (4A2NP) and 2-amino, 4-nitrophenol (2A4NP) were separated. The extraction procedure for metabolite separation was developed on the basis of pH dependent aqueous-organic partitioning. When cofactors and pH were optimized *in vitro*, the anaerobic enzymatic disappearance of DNP was first order for 30 minutes. The pH optimum for metabolite formation *in vitro* was 6.5 (37°C). During a 30 minute incubation of DNP, whole liver homogenate, 81 ± 4% of DNP was metabolized: 75 ± 4% to 2A4NP, 23 ± 2% to 4A2NP, and 1% to 2,4 diaminophenol (DAP).

Most enzyme activity was located in the 105,000 *g* supernatant. Oxygen did not alter the distribution of enzyme activity, but did decrease metabolite formation in the whole homogenate after 10 minutes.

Michaelis-Menton kinetics for DNP disappearance and metabolite formation were examined and the apparent Km in the whole homogenate preparation was 1.8×10^{-4} M. The apparent Vmax was 1.13 nmoles/mg/min. o-Nitrophenol (ONP),

p-nitrophenol (PNP), p-nitrobenzoic acid (PNBA), and 2,4-dinitro, 6-sec butyl phenol (DNBP) were examined as inhibitors of DNP metabolism *in vitro*. ONP was a competitive inhibitor with an apparent Ki of 5.1×10^{-4} M. PNP and DNBP were non-competitive inhibitors, their apparent Ki's were 7.1×10^{-4} M and 1.3×10^{-4} M, respectively. PNBA had no effect on the reduction of DNP.

Mo^{+4} appeared to stimulate while Cu^{+2} appeared to depress DNP metabolism. Mo^{+4} blocked the depression produced by Cu^{+2}.

Initial heat denaturation studies were undertaken in an attempt to separate two enzymes or two steric sites. There was no evidence for more than one enzyme involved in the metabolism of DNP.

It was concluded that DNP was enzymatically metabolized by rat liver to 2A4NP and 4A2NP. The primary activity was located in the 105,000 *g* soluble fraction. Oxygen did not alter the intracellular distribution of enzyme activity but did depress metabolite formation indicating it was competing with 2,4-dinitrophenol for reduced cofactors. ONP, PNP, and DNBP, but not PNBA, were inhibitors of the DNP nitro reduction. Cu^{+2} and Mo^{+4} appear to inhibit and stimulate respectively, the DNP nitro reduction system. Only one enzyme system appears to be involved in DNP nitro reduction.

IN VITRO METABOLISM OF 2,4-DINITROPHENOL BY RAT LIVER HOMOGENATES

By

Julie Louise Eiseman

A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Pharmacology

1971

ACKNOWLEDGMENTS

The author wishes to thank the graduate committee members, Dr. Theodore M. Brody, Dr. David A. Reinke, and especially Dr. James E. Gibson and Dr. Perry J. Gehring, for their assistance and guidance throughout the various phases of this project.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
METHODS	7
Chemicals	7
Cell Fraction Preparation	7
Protein Determination	8
Incubation Procedure	9
Extraction Procedure	10
Chromatography	12
Test for Amino Group	14
Heat Denaturation	15
Statistical Analysis	15
RESULTS	16
Optimizing Cofactors	16
Optimization of pH	16
Intracellular Location of Enzyme Activity	19
Effect of Oxygen on Apparent Cellular Distribution of Enzyme Activity	23
Oxygen as an Inhibitor of DNP Metabolism	23
Effects of Copper and Molybdenum on DNP Nitro Reductase Activity	26
Heat Denaturation of Enzyme(s) Involved in DNP Reduction	26
Enzyme Kinetics	29
DISCUSSION	34
Optimization of Cofactors	34
pH Optimum	36
Location of Enzyme Activity	37
Oxygen Inhibition	39
Copper and Molybdenum, Inhibition and Stimulation	39
Heat Denaturation	40
Enzyme Kinetics	41
SUMMARY	44
BIBLIOGRAPHY	46

LIST OF TABLES

Table		Page
1	Verification of the Extraction Procedure Using Non-Radioactive Compounds	13
2	Optimization of NADP Concentration	17
3	Optimization of Glucose-6-Phosphate and Magnesium Sulfate	18
4	Effect of Cu^{+2} and Mo^{+4} on DNP Nitro Reductase Activity of Whole Rat Liver Homogenates	27
5	Heat Denaturation of Homogenate Preparation	28
6	Kinetic Data for DNP Enzymatic Reduction	32

LIST OF FIGURES

Figure		Page
1	Determination of pH optimum for the metabolism of DNP and the formation of 4A2NP and 2A4NP	20
2	Anaerobic, *in vitro* metabolism for DNP under optimum conditions (pH 6.5, 37°C)	21
3	Distribution of activity in intracellular fractions	22
4	Distribution of activity in intracellular fractions and the effect of oxygen on the apparent intracellular distribution of enzyme activity	24
5	Oxygen inhibition of DNP enzymatic reduction	25
6	Inhibition of DNP nitro reduction	30
7	Hofstee Plot of DNP nitro reduction. . . .	33
8	Proposed Mechanism of reduction of DNP . .	35

INTRODUCTION

Aromatic nitro compounds may be metabolically reduced to their corresponding amines by mammalian enzyme systems. Channon *et al.* (1944) proposed that the nitro groups of 2,4,6-trinitrotoluene (TNT) were reduced *in vivo* through the steps: $-NO_2 \xrightarrow{2H} -NO \xrightarrow{2H} NHOH \xrightarrow{2H} -NH_2$, a chemical process involving the transfer of 6 hydrogen atoms. Bueding *et al.* (1946) provided experimental evidence that demonstrated TNT nitro-reduction to the corresponding amines via nitroso and hydroxyl amine intermediates which required xanthine oxidase.

This enzyme system has been termed the "nitro reductase system." Reduced pyridine nucleotides act as hydrogen donors for the nitro reduction and several workers have demonstrated the involvement of flavin nucleotides and heavy metals in the reduction mechanism. Westerfeld *et al.* (1957) postulated the essential role of molybdenum. In addition, copper and molybdenum antagonism was investigated in bacterial nitro reduction (Saz, 1954; McElroy and Glass, 1956; Mackler *et al.*, 1954). Otsuka (1961) showed the presence of this ion activity in the mammalian system as well.

Fouts and Brodie (1957) located the nitro reductase for p-nitrobenzoic acid (PNBA) and chloramphenicol in microsomes and soluble fractions of liver and kidney. NADPH or NADH served as hydrogen donors and excess flavins accelerated the reaction. Oxygen but not aureomycin completely inhibited the reaction. Previously, Westfall (1943) reported similar nitro reduction rates under aerobic and anaerobic conditions.

Species variability appeared to be an important characteristic of the nitro reductase system. Mouse or guinea pig liver was three times more active than rabbit liver which was ten times more active than rat. Rat liver was found to be fifteen times more active than dog liver with respect to nitro reductase (Fouts and Brodie, 1957).

Otsuka (1961) separated two protein fractions from swine liver by ion exchange chromatography (DEAE cellulose) which were involved in the nitro reduction of p-nitrophenol (PNP). Two nitro reductase enzymes were postulated for the reduction of the nitro and nitroso groups respectively. Both enzymes were active at pH 7.8 and both required NADH as hydrogen donor. Nitro reductase but not nitroso reductase was inhibited by oxygen. Both enzymes were inhibited by chelating agents suggesting metallo-protein involvement.

Kamm and Gillette (1963) also studied flavin stimulation of nitro reductase using chloramphenicol, PNBA, and

nitrobenzene as substrates. Their results supported the view that the anaerobic reduction of nitro compounds and the aerobic oxidation of NADPH were catalyzed by the same enzyme system. Gillette and Sasame (1965) found that carbon monoxide and SJF 525-A inhibited nitro reductase, implying involvement of cytochrome P450. Gillette and Kamm (1968) linked an NADPH-dependent enzyme of liver microsomes, presumably cytochrome P450, with the reduction of p-nitrobenzoate.

Kato *et al.* (1969) studied the reduction of PNBA by rat liver and noted two enzyme systems, one present in the soluble fraction and one in the microsomal fraction. These two systems displayed different properties with respect to stimulation by phenobarbital and inhibition by oxygen. The enzyme system of the microsomal fraction appears to be NADPH-linked while that in the soluble fraction appeared to be NADH-linked. Kato's results were in agreement with Kamm and Gillette (1963) who observed that flavin-adenine dinucleotide (FAD) was reduced by purified NADPH-cytochrome c reductase and that FADH could reduce p-nitrobenzoate non-enzymatically. Purified p-nitrophenol reductase from swine liver homogenate reduced FAD to flavin mononucleotide (FMN) but only FADH could reduce PNP; thus only FAD would stimulate the PNP reductase. Purified p-nitrophenol reductase in addition was NADH dependent and only negligible activity was observed with NADPH.

The differing results of Otsuka (1961) and Kamm and Gillette (1963) suggest the involvement of a different enzyme system for nitrophenolic reduction.

Juchau (1970) investigated nitro reductase in liver and placental tissues of man and rats (microsomal fractions) and described a model system for the reduction of p-nitrobenzoic acid. The system included: NADPH or NADH as initial hydrogen donors, flavins (FAD, FMN, or riboflavin), a sulfhydryl donor (glutathione or cysteine) and a salt ($MnCl_2$). Carbon monoxide inhibited activity in boiled placental fractions but not in model systems, suggesting a chemical interaction with an electron carrier to inhibit non-enzymatic election transfer.
Carlson and DuBois (1970) described two nitro reductase systems in rat liver. One resided in the soluble fraction and was not dependent upon the addition of NADP. A smaller portion of the nitro reductase activity was found in the microsomal fraction which required NADPH for maximal activity. This microsomal system was inducible by phenobarbital and DDT but the induction of the system *in vivo* did not enhance total nitro reduction of PNBA. DuBois *et al.* (1970) therefore concluded that the toxicity of drugs detoxified by nitro reduction was not reduced by inducing agents.

Few studies have considered the enzymatic nitro reduction of 2,4-dinitrophenol. Saz and Slie (1954)

reported that DNP was unique among all the compounds tested in their bacterial (*E. coli*) system since it was resistant to significant enzymatic reduction. DNP (3×10^{-3}M) inhibited the reduction of various other nitro compounds such as chloramphenicol, 3,5-dinitrobenzoate and m-dinitrobenzene. Fouts and Brodie (1957) found the nitrophenols, DNP, PNP, and m-nitrophenol, resistant to rapid metabolism by the mammalian nitro reductase systems. Instead DNP and p-nitrophenol antagonized the reduction of PNBA.

In previous studies, Greville and Stern (1935) reported DNP metabolism by a bacterial succinic dehydrogenase system (*E. coli*). 2A4NP was found as the major metabolite. In addition, Magne *et al.* (1932) found 2A4NP to be the primary metabolite in the urine of man and dogs. On the other hand, Parker (1952) using rat liver homogenates found 4A2NP as the major metabolite while 2A4NP and DAP were minor metabolites.

The biological effects of DNP are well known. DNP increases the rate of tissue metabolism in many species including man, and the heat production associated with the increased metabolic rates exceeds the physiological capacity for heat dissipation, and fatal hyperthermia can result. This response is a result of the ability of DNP to uncouple oxidative phosphorylation. DNP was used clinically for treatment of obesity because of its ability to

stimulate fat metabolism. Its use, however, was discarded because of hyperthermia. Horner (1942) reported that prolonged intake of DNP produced cataracts in 1% of the individuals. However, the cataractogenic species of DNP in unknown. Robbins (1944) was able to experimentally produce DNP cataracts in baby ducks and chicks and Ogino and Yasukura (1957) extended this work to include guinea pigs and rabbits. The cataractogenic agent in DNP cataract formation was concluded to be 2-amino-p-quinonimine. Gehring and Buerge (1967) explored the cataractogenic properties of DNP in ducklings and rabbits.

The present investigation was undertaken to characterize the *in vitro* metabolism of DNP by rat liver homogenates. The metabolic reduction products were identified. Optimum experimental conditions were established with respect to cofactor requirements and pH. In addition the intracellular distribution of DNP nitro reductase was determined and the effects of varying concentrations of oxygen were investigated. The effects of PNBA, PNP, o-nitrophenol (ONP) and 2,4-dinitro,6-sec-butyl phenol (DNBP) on the kinetics of DNP metabolism were determined.

METHODS

Chemicals

^{14}C-2,4-dinitrophenol (0.50 millicuries; 80.5 milligrams, lot no. 277-227) was obtained from New England Nuclear Corp., Boston, Mass. and diluted to a final volume of 50 ml with distilled water. This solution was then diluted and combined with 2,4-dinitrophenol (Eastman Organic Chemicals, Rochester, New York) to make up experimental concentrations of 1×10^{-5}M, 5×10^{-5}M, 1×10^{-4}M, 5×10^{-4}M, and 2.5×10^{-3}M, with 0.05 μc ^{14}C-DNP/ml.

Cell Fraction Preparation

Male Sprague-Dawley rats weighing 300-500 g (Spartan Research Animals, Haslett, Michigan) were housed in stainless steel cages and allowed standard lab block diet and water *ad libitum* until use. Animals were sacrificed by decapitation and their livers were perfused with 10 ml of cold 0.05 M tris-histidine buffer (pH 6.5). The liver was excised, blotted, minced with a scissors, weighed and homogenized in two volumes of buffer using a teflon-glass Potter-Elvejhem homogenizer.

Nuclei and cell debri were obtained from the homogenate fraction by centrifugation at 900 *g* for 10 minutes

using an International Refrigerated Centrifuge, Model B-20. The supernatant fraction was readjusted with buffer to the initial volume of the homogenate and recentrifuged at 9000 *g* for 20 minutes to obtain the mitochondrial fraction. The mitochondrial fraction was washed and resuspended in buffer to the initial volume. The 9000 *g* supernatant was also readjusted to the initial volume. Microsomes were separated from the soluble fraction by centrifugation of the 9000 *g* supernatant fraction for 90 minutes at 105,000 *g* on a Beckman Model L ultracentrifuge. The microsomes were washed with buffer and recentrifuged for 60 minutes. After washing, the microsomes were resuspended in buffer to the initial volume of the homogenate. The volume of the 105,000 *g* supernatant containing the soluble cell fluids was also readjusted to the initial volume with buffer. All fractions were maintained at 4°C.

Protein Determination

The protein content of the homogenate and each cell fraction was determined by the method of Lowry *et al.* (1951). The values for a 1 to 100 or 1 to 10 dilution of the cell fraction were compared with a standard bovine serum albumen curve read at 500 or 750 mμ on a Beckman DB spectrophotometer.

Incubation Procedure

In the various experiments ^{14}C-DNP was incubated with the different cell fractions in 25 ml beakers in Dubnoff metabolic incubators (90-100 rpm, 37°C) in an atmosphere of nitrogen and/or oxygen. Under optimized conditions, each beaker contained 250 mmoles ^{14}C-DNP (0.025 μc); 0.5 ml of .25 M tris-histidine buffer (0.25 M tris (tris [hydroxy methyl] aminomethane) and 0.25 M L-histidine, pH 6.5, 37°C); 0.5 ml of cell fraction, 12.5 μmoles glucose-6-phosphate (G-6-PO_4), 12.5 μmoles magnesium sulfate ($MgSO_4$) and 1 μmole nicotinamide adenine dinucleitide phosphate (NADP) in a final folume of 2.5 ml. A marble was added to each beaker to insure adequate mixing. Aerobic samples were flushed with 7%, 21%, or 100% oxygen for 15 minutes prior to the addition of substrate and during the timed incubation. Carbon dioxide was removed from the gas mixture by passing it over a soda lime (Soda Sorb, Ohio Chemical, Madison, Wisconsin) column. The pH of samples was checked before and after incubation to make certain the pH remained constant. Anaerobic samples were flushed with 100% N_2 in the same manner. The enzymatic reaction was terminated by the addition of 1.5 ml of 20% trichloroacetic acid (TCA). Blanks without substrate, standards containing the ^{14}C-DNP but no cell fraction, and samples containing both ^{14}C-DNP and TCA precipitated cell fraction were incubated and served as controls.

Extraction Procedure

DNP and its metabolites were separated on the basis of their pH dependent aqueous organic partitioning. After terminating the enzymatic reaction, all samples were transferred to 15 ml test tubes. The beakers were rinsed to remove residual radioactivity. The 15 ml tubes were then centrifuged for 5 minutes to sediment precipitated protein. The supernatant as well as 2 rinses of the precipitated protein, and 1 ml of 10^{-5} M DNP, 4A2NP, 2A4NP, and DAP (carrier) were transferred to 50 ml centrifuge tubes. Samples were adjusted to pH 3.0 and DNP was extracted into 10 ml of reagent grade chloroform by shaking. The chloroform layer was transferred to 15 ml screw top test tubes and DNP was reextracted into 2 ml of 0.2 M sodium hydroxide (NaOH). One ml of aqueous phase was transferred to a counting vial. This fraction was considered to represent DNP. Ten ml of modified Bray's solution (100 g naphthalene, 6 g diphenyloxazole (PPO) in one liter reagent grade dioxane) was added to each vial. One drop of 5 M hydrochloric acid (HCl) and 0.5 ml distilled water were added to each vial to clear the solution. The radioactivity present in each sample was assayed by liquid scintillation counting using external or internal standardization to determine disintegrations per minute from the count rate.

The aqueous fraction remaining after DNP extraction was adjusted to pH 10-11 with 20% NADH to dissociate

metabolic complexes. After 20 minutes, these samples were adjusted to pH 6.5 and the extraction procedure was repeated with 10 ml of chloroform. At pH 6.5, 4A2NP was readily extracted into chloroform while 2A4NP and DAP remained in the aqueous phase. The chloroform layer was transferred to 15 ml screw-top test tubes and the 4A2NP was reextracted into 2 ml of 0.2 M NaOH. One ml of the aqueous fraction was counted in the manner described for DNP.

2A4NP and DAP were separated at pH 7.4 using a cellulose cation exchange column (Cellex C-M, Biorad Laboratories, Richmond, California). 2A4NP remained in the aqueous phase and DAP adsorbed to the column. DAP was eluted as a reddish-pink band with 0.1 M NH_4OH. DAP accounted for less than 1% of all the metabolites in the initial experiments. Therefore for most experiments, this separation was discontinued. 2A4NP (and DAP) were quantified by counting a 2 ml aliquot of the aqueous phase. In the case of 2A4NP, a drop of 5 M HCl and 0.5 ml of distilled water were not necessary to clear the solution. One drop of 5 M HCl was added to the DAP samples. The aqueous samples were then counted in the manner previously described.

The extraction procedure was confirmed using non-radioactive DNP, 4A2NP, 2A4NP, and DAP in concentrations of 10^{-3} to 10^{-5} M and the partition coefficient was determined for 4A2NP and 2A4NP extraction at pH 6.5 using the equation:

$$CN = CO \left(\frac{KV_1}{KV_1 + V_2}\right)^n$$

where: CN = concentration of the amount remaining after mixing

CO = concentration of the initial solution

K = partition coefficient when the volumes are equal, in this case 9 for the extraction of 2A4NP from an aqueous layer (pH 6.5)

V_1 = volume of the aqueous layer

V_2 = volume of the solvent (chloroform)

n = number of times the extraction is carried out.

DNP was measured at 360 mμ in K_2HPO_4 buffer at pH 7.4;

2A4NP was measured at 460 mμ and

4A2NP was measured at 480 mμ in base or 420 mμ at pH 6.5.

The partition coefficient at pH 6.5 for 4A2NP was 6.805 ± .321 (approximately 87% of total 4A2NP was extracted with one 10 ml extraction) and for 2A4NP 0.146 ± .010 (5-15% of the 2A4NP was extracted at pH 6.5) (Table 1).

Chromatography

Thin layer chromatography (cellulose) was used to identify the parent compound and metabolites using a basic solvent system (95% ethanol; n-butanol; NH_4OH; benzene; 4:2:2:2). Rf values for DNP, 2A4NP, 4A2NP, and DAP respectively were: 0.75-0.80, 0.55-0.65, 0.25-0.45 and 0.0-0.1. Similar separations were obtained with an acidic solvent system (benzene; 95% ethanol; acetic acid; 4:2:2).

Table 1. Verification of the Extraction Procedure Using Non-Radioactive Compounds

Compound	Conc	OD Before pH 3 Extraction	OD After pH 3 Extraction	Approximate[4] % Extracted	OD Before pH 6.5 Extraction	OD After pH 6.5 Extraction	Approximate[4] % Extracted
DNP[1]	(10^{-5} M)	.042±.001[5]	.005±.000	97.4%	- - - - -	- - - - -	- - - -
	(10^{-4} M)	.416±.002	.005±.002	96.5%	- - - - -	- - - - -	- - - -
	(10^{-3} M)	.418±.010	.007±.001	97.0%	- - - - -	- - - - -	- - - -
4A2NP[2]	(10^{-5} M)	.027±.005	.023±.005	0.0%	.023±.005	.002±.001	93 %
	(10^{-4} M)	.232±.010	.220±.010	~5.0%	.220±.010	.034±.010	86 %
	(10^{-3} M)	2.25	2.22	~3 %			
	(1→10 dil)	(.225±.010)	.222±.012	3 %	.222±.012	.025±.010	94.5%
2A4NP[3]	(10^{-5} M)	.007±.001	.007±.001	- - -	.007±.001	.005±.001	13.0%
	(10^{-4} M)	.048±.005	.047±.005	- - -	.047±.005	.041±.010	7.0%
	(10^{-3} M)	.438±.010	.438±.010	- - -	.438±.010	.379±.018	14.0%

1 DNP solns were measured at 360 mμ.

2 4A2NP solutions were measured at 420 mμ.

3 2A4NP solutions were measured at 450 mμ.

4 The % extracted were determined using a Beer's law plot by substracting the extrapolated value remaining after extraction from the total before extraction and dividing by standard value before extraction x 100%.

5 Mean ± standard error.

Rf values for DNP, 2A4NP, 4A2NP, and DAP in this system, respectively were: 0.65-0.75, 0.50-0.55, 0.45-0.50, 0.02-0.10. Silica gel plates were run in a solvent system composed of petroleum ether; ether, formic acid (8:1:1). Rf values in this system were: DNP, 0.87-0.95; 4A2NP, 0.75-0.80; 2A4NP, 0.70-0.75; and DAP, 0.65-0.70. Standard solutions of DNP, 4A2NP, 2A4NP, and DAP were run with each sample. Rf values for non-radioactive compounds were determined by inspection under ultraviolet light (ChromatoVue, Model CC-22, Ultra Violet Prod., San Gabriel, Cal.)

For radioactive samples the chromatograms were cut into two x one centimeter strips (0.5 cm preceding the origin to the point of solvent front), and the strips placed in consecutively numbered counting vials. One half ml of distilled H_2O was added to the counting vial to dissolve the radioactive compounds. Ten ml of modified Bray's solution was added to each vial and the samples were counted in the manner previously described. Rf's calculated for the radioactive compounds using the peak location were in agreement with Rf values of standard compounds determined by inspection.

Test for Amino Group

The procedure outlined by Bratten and Marshall (1939) was modified slightly and used to verify the presence of primary amines of DNP metabolites. Two ml of 1 N TCA and 1 ml of 0.1% $NaNO_2$ were added to one ml of the unknown.

The sample was stored on ice for ten minutes. One ml of 0.5% ammonium sulfamate was added. After 3 minutes, one ml of Marshall reagent was added. The reaction was allowed to proceed for 30 minutes in the dark. A deep purple or brown color indicated a positive test for amino groups.

Heat Denaturation

Heat denaturation of the whole homogenate was performed by incubating the homogenate before incubation with substrate at a constant temperature for a previously determined time. This procedure was used in an attempt to specifically denature one enzyme or active site responsible for metabolism of DNP.

Statistical Analysis

Statistical analysis of data was by Students t test. The level of significance was chosen at $P < 0.05$. Linear regressions were calculated by the method of least squares (Steele and Torrie, 1960). Data presented are the mean from at least 3 experiments with duplicate determinations.

RESULTS

Optimizing Cofactors

The *in vitro* metabolism of DNP was measured in two ways: 1) by ^{14}C-DNP substrate disappearance and 2) by the appearance of the metabolic products, 2A4NP and 4A2NP.

The concentration of cofactors, G-6-PO_4 and $MgSO_4$, and NADP were optimized by altering individual cofactors. The *in vitro* incubation preparation consisted of 250 mmoles ^{14}C-DNP (0.25 µc), 0.5 ml 33% liver homogenate, 0.5 ml of 0.25 M tris-histidine buffer, 0.5 ml of G-6-PO_4 and $MgSO_4$ (variable concentration), NADPH (variable concentration) and distilled water to bring the final volume to 2.5 ml. The optimum concentrations of NADPH, G-6-PO_4 and $MgSO_4$ were 1 µmole, 12.5 µmoles and 12.5 µmoles respectively (Tables 2, 3).

Optimization of pH

DNP disappearance and metabolite formation was determined *in vitro* over the pH range of 3.5-9.5 using five buffer systems (0.1 M sodium acetate, pH 3.5 to 5.5; 0.1 M and 0.05 M tris-histidine, pH 5.5 to 9.5; 0.1 M K_2HPO_4, pH 5.5 to 9.5; and 0.1 M tris, pH 6.5-9.5 and a 30 minute incubation. The overlap points did not differ significantly

Table 2. Optimization of NADP Concentration

NADP Conc.	Substrate or Products	Substrate remaining or product formed (nmoles/mg protein) Time of Incubation in Minutes 3	5	10	15	30
.33 µmole	DNP[1]	6.428±.536[2]	6.427±.444	4.994±.426	4.365±.121	3.692±.186
	4A2NP	.495±.033	.529±.060	.806±.074	1.097±.151	1.253±.138
	2A4NP	1.367±.205	1.864±.192	2.678±.321	3.357±.341	3.831±.251
.66 µmoles	DNP	5.862±.460	5.282±.353	4.131±.240	3.560±.179	2.966±.316
	4A2NP	.664±.073	.775±.028	.904±.085	1.294±.213	1.409±.216
	2A4NP	2.220±.205	2.889±.305	3.545±.455	3.753±.436	4.309±.499
1 µmole	DNP	5.665±.373	4.849±.359	3.423±.167	2.668±.127	1.632±.194
	4A2NP	.698±.039	.855±.041	1.189±.083	1.220±.124	1.677±.208
	2A4NP	2.795±.192	3.409±.305	4.485±.413	4.806±.606	5.588±.620
2 µmoles	DNP	5.965±.398	5.659±.386	4.095±.141	3.174±.242	1.557±.157
	4A2NP	.527±.046	.675±.087	.949±.132	1.218±.145	1.672±.192
	2A4NP	1.303±.191	2.409±.250	3.679±.335	4.428±.557	5.749±.565
3 µmoles	DNP	6.834±.487	6.099±.509	4.276±.228	3.253±.141	1.886±.076
	4A2NP	.460±.109	.625±.064	..967±.125	1.148±.198	1.808±.190
	2A4NP	1.753±.159	2.074±.204	2.888±.490	3.847±.511	5.352±.328

1 DNP concentration used was 1×10^{-4} M.

2 Mean ± Standard error.

Table 3. Optimization of Glucose-6-Phosphate and Magnesium Sulfate Concentrations.

Conc G-6-PO_4 $MgSO_4$ (μmoles)	DNP[1] unmetabolized (nmoles/mg protein)	metabolites formed (nmoles/mg protein)	
		4A2NP	2A4NP
6.25	1.263±.154[2]	1.211±.072	5.096±.175
12.5	1.451±.220	1.160±.131	5.161±.331
25.0	1.469±.200	1.135±.097	4.303±.235

1 DNP concentration used was 1 x 10^{-4} M. Incubation time was 30 minutes.

2 Mean ± standard error.

between buffer systems indicating that the buffer system did not interfere with the reaction (Figure 1).

The optimum pH for DNP disappearance was 5.5 to 6.5, but the optimum for metabolite formation was 6.5. Over the entire pH range, 2A4NP was the major metabolite. 4A2NP was formed in lesser amounts. Both monoamines were formed more readily than DAP.

Using optimized conditions DNP disappearance was linear and first order for 30 minutes. No enzyme or co-factors limited the reaction. The linear regression line by least squares analysis had a correlation coefficient of 0.97 (Figure 2).

Intracellular Location of Enzyme Activity

Homogenateswere divided into various cell fractions (nuclei and cell debri, mitochondria, mitochondria-free supernatant, microsomes, and 105,000 g supernatant) and incubated with ^{14}C-DNP for 30 minutes. Metabolism was expressed as the amount of metabolites formed (Figure 3). Maximum enzyme activity for the reduction of DNP was found in the soluble fraction. This correlates with the location of other nitro reductase enzymes (Parker, 1952; Fouts and Brodie, 1957; Kamm and Gillette, 1963; Kato *et al.*, 1969; Juchau *et al.*, 1970). The high activity in the nuclear fraction may be caused by the sedimentation of whole cells since this fraction was a crude preparation. Absence of activity in the washed microsomal fraction may

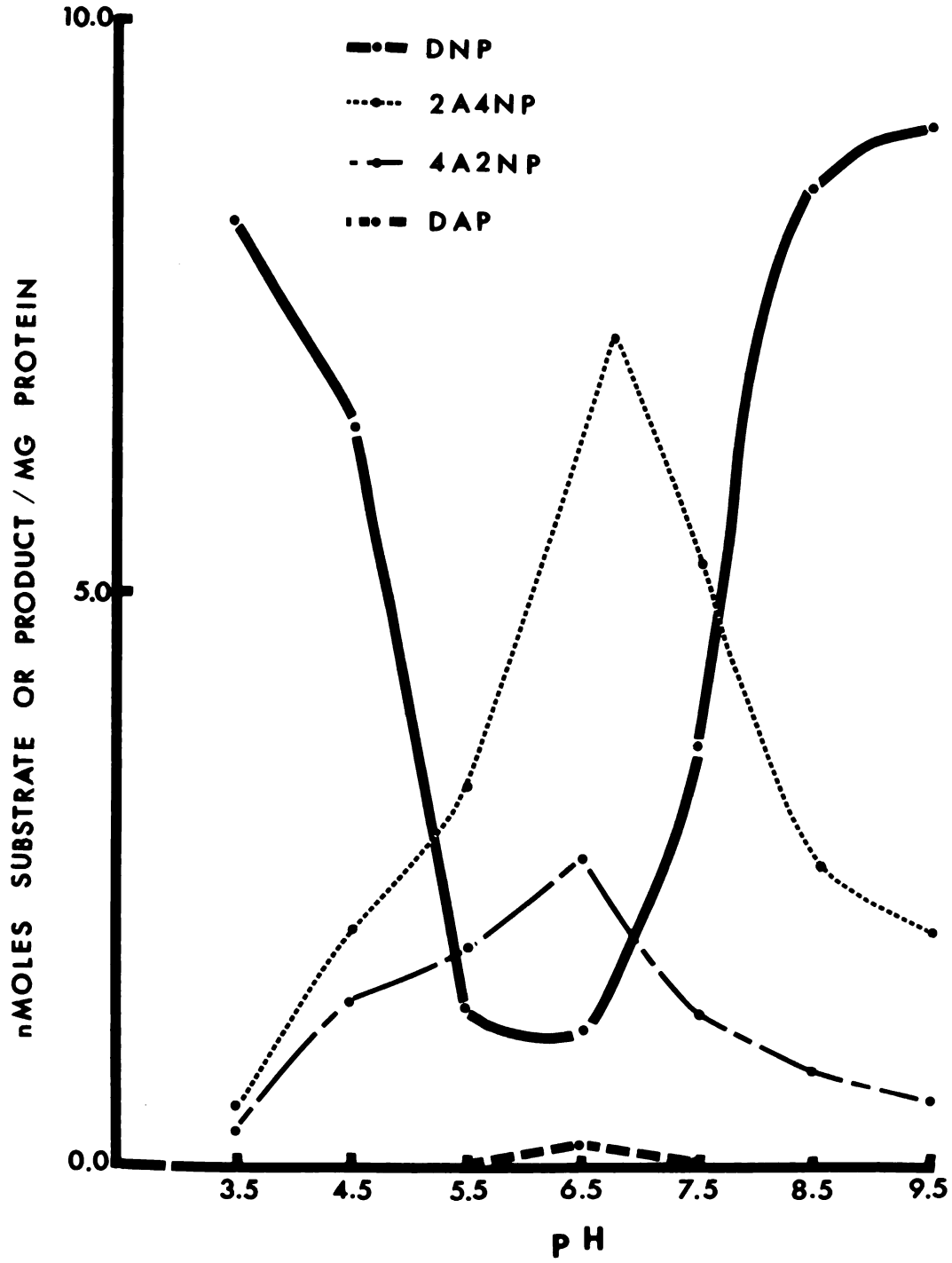

Figure 1. Determination of pH optimum for the metabolism of DNP and the formation of 4A2NP and 2A4NP. Values are the mean for 30 minute incubations under nitrogen at 37°. Five buffers were used over the pH range: pH 3.5 to 5.5, 0.1 M acetate; pH 5.5 to 9.5, 0.1 M and 0.05 M tris-histidine; pH 5.5 to 9.5, 0.1 M K_2HPO_4; pH 6.5 to 9.5, 0.1 M tris.

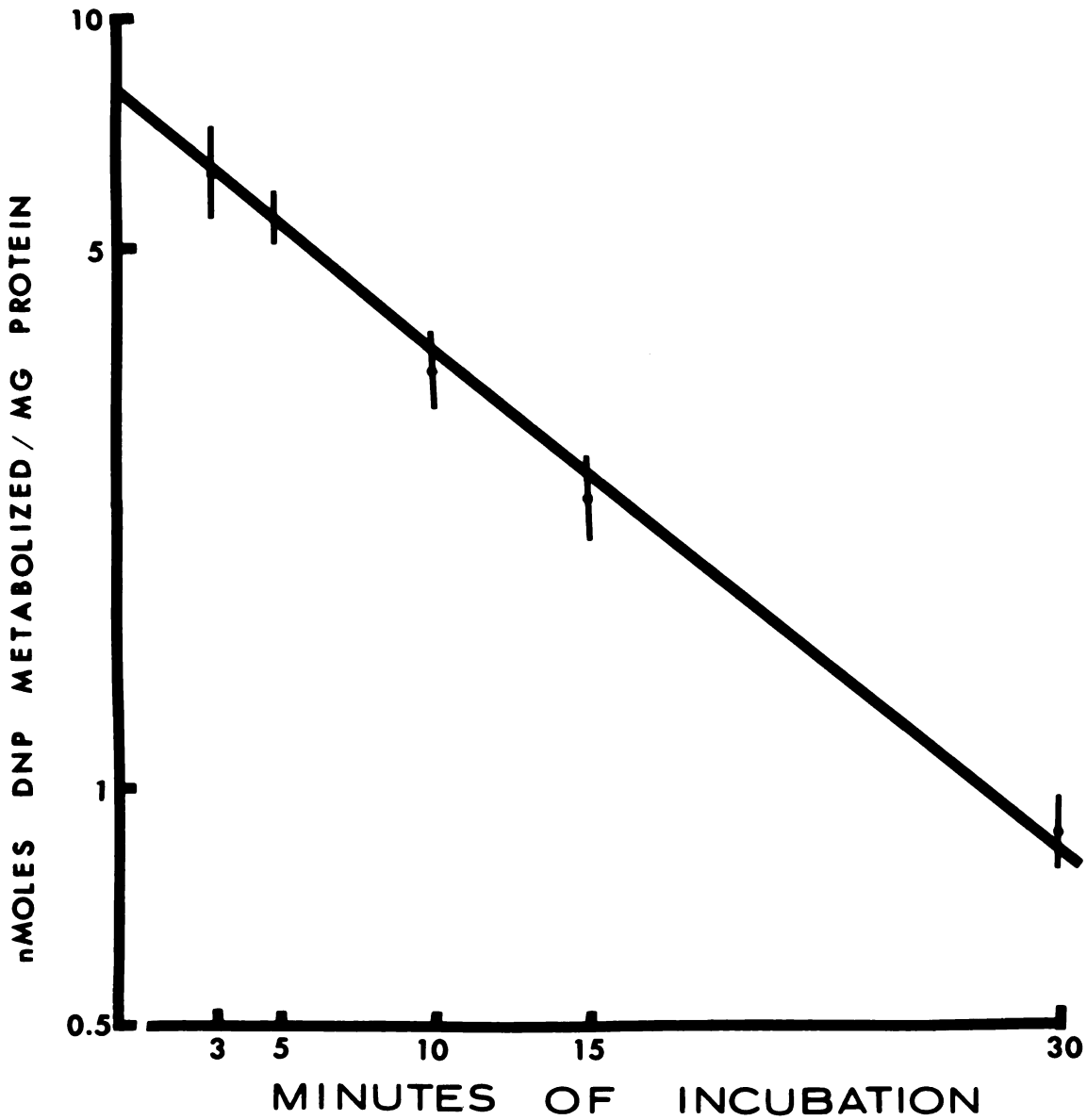

Figure 2. Anaerobic, *in vitro* metabolism for DNP under optimum conditions (pH 6.5, 37°C). The reaction medium contained: 0.5 ml of rat liver homogenate, 1 mmole NADP, 12.5 µmoles G-6-PO_4, 12.5 µmoles $MgSO_4$, 250 mmoles C^{14}-DNP, and 0.5 ml of .25 M tris-histidine buffer (pH 6.5) in a final volume of 2.5 ml. The reaction was linear for 30 minutes and the linear regression line by least squares analysis had a correlation coefficient of 0.97.

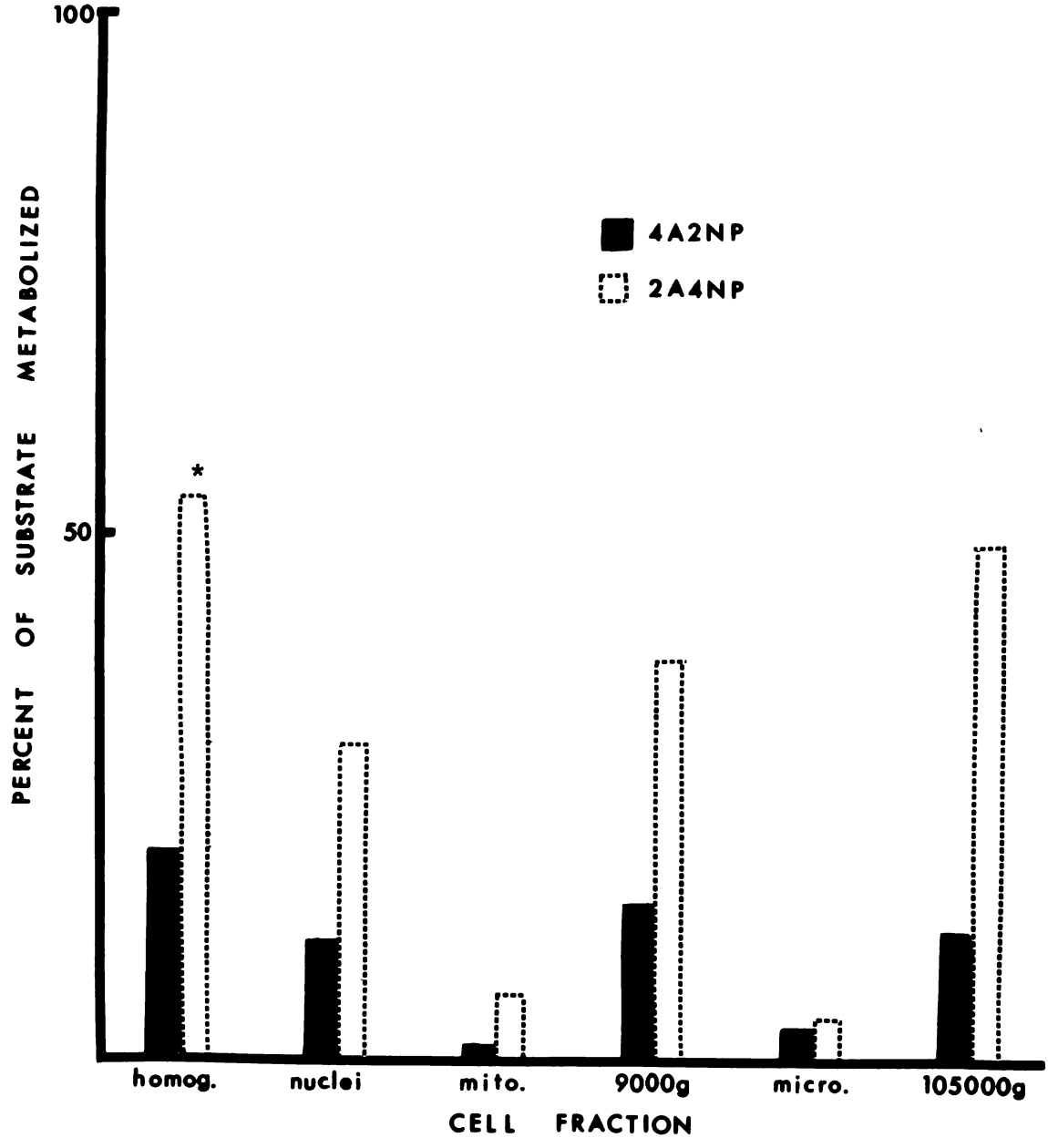

Figure 3. Distribution of activity in intracellular fractions. The amounts of 4A2NP and 2A4NP formed during a 30 minute anaerobic incubation under optimum conditions are expressed as percent of total DNP substrate. The specific cell fraction was substituted for the rat liver homogenate in the incubation medium.

*2A4NP formed by incubation with homogenate should read 67% instead of 55%.

be explained by the deficiency of soluble enzymes such as glucose-6-phosphate dehydrogenase which are necessary for NADPH generation. The 9000 *g* supernatant fraction did not possess more activity than the combined microsomes and soluble fraction (Figure 3).

Effect of Oxygen on Apparent Cellular Distribution of Enzyme Activity

When samples were incubated under an atmosphere of 21% oxygen for 30 minutes, oxygen did not alter the apparent cellular distribution of enzyme activity since the soluble fraction maintained the greatest activity. However, the formation of metabolites was depressed in all fractions (Figure 4).

Oxygen as an Inhibitor of DNP Metabolism

The temporal aspects of oxygen inhibition of DNP reduction were examined using whole homogenates (Figure 5). The disappearance of DNP was followed for 30 minutes under an atmosphere of 100% N_2, 7% O_2, 21% O_2, or 100% O_2. Gas mixtures used were: 95% O_2 and 5% CO_2, air from an air valve; and 7% O_2, 5% CO_2 and 88% N_2. All the gas mixtures were passed over a "Sodasorb" column to remove CO_2. The pH of select samples was measured at the end of the incubation and there was no change in pH greater than 0.2 pH.units. Oxygen, in the concentrations examined, did not alter the initial

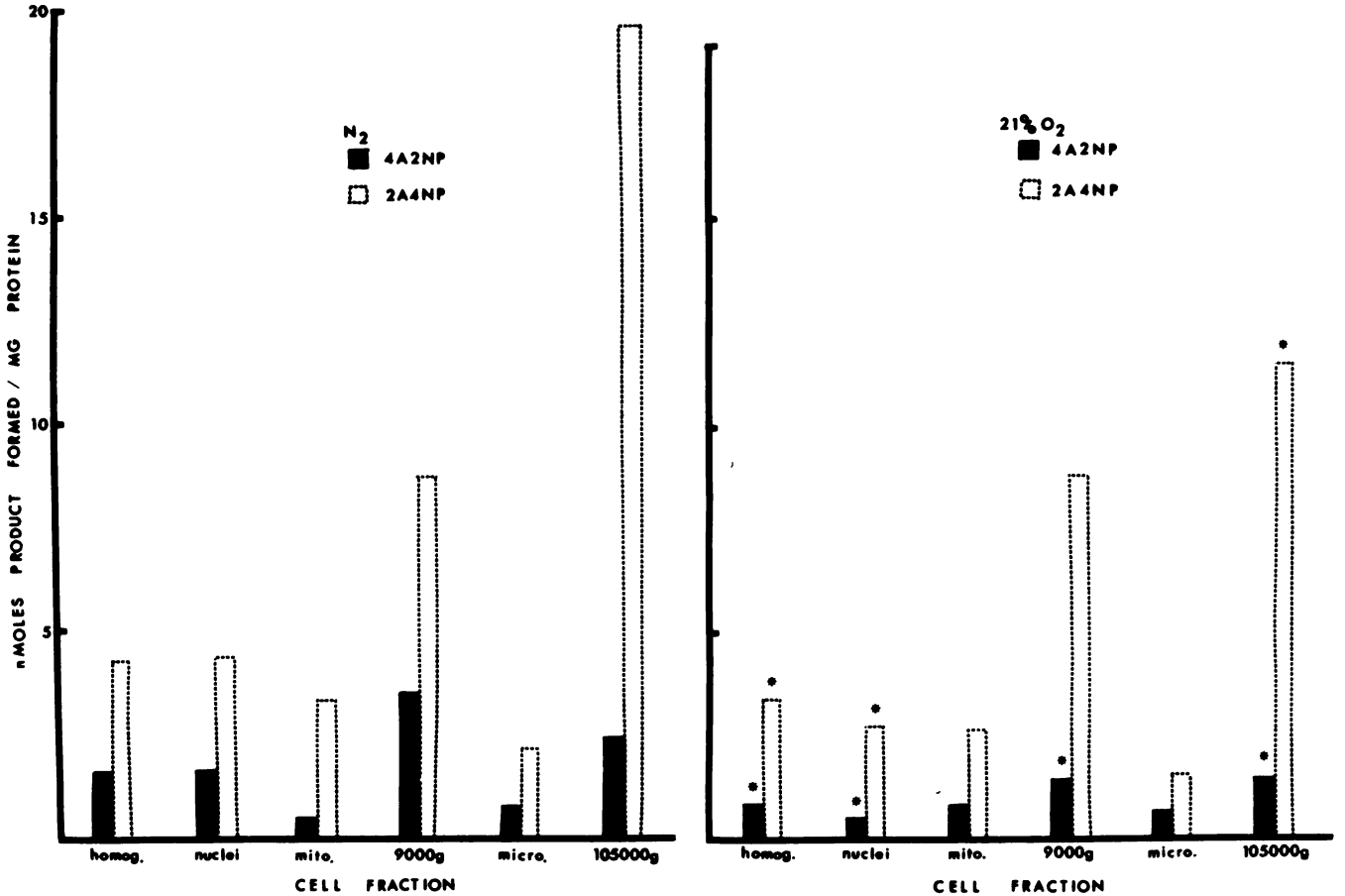

Figure 4. Distribution of activity in intracellular fractions and the effect of oxygen on the apparent intracellular distribution of enzyme activity. The relative amounts of 4A2NP and 2A4NP formed during a 30 minute anaerobic incubation under optimum conditions and during a 30 minute incubation period under 21% oxygen are shown. 21% oxygen depressed the amounts of 2A4NP and 4A2NP formed during a 30 minute incubation at 37°C. Asterisk (*) indicates significant difference at $P < 0.05$ from the 30 minute anaerobic incubation. The specific cell fraction was substituted for the rat liver homogenate in the incubation medium.

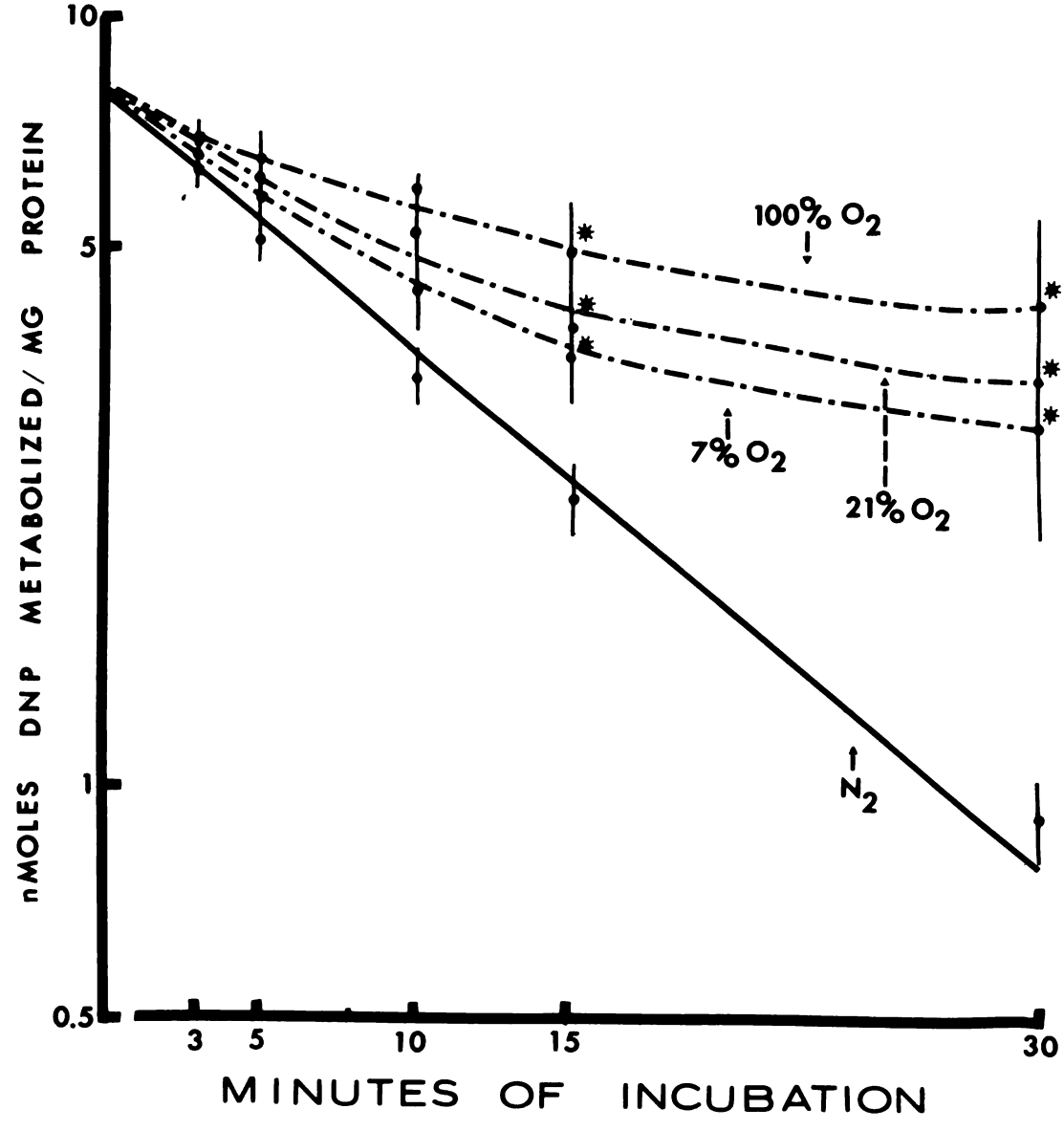

Figure 5. Oxygen inhibition of DNP enzymatic reduction. 7%, 21% and 100% oxygen significantly depressed the metabolism of DNP after 10 minutes. Asterisk (*) indicates significance at $P < 0.05$.

rate of DNP metabolism; but did significantly depress metabolite formation beyond ten minutes. 100% oxygen, however, did not completely inhibit the metabolism of DNP. This suggests that the nitro reductase(s) involved in DNP metabolism are not as sensitive to oxygen as was the nitro reductase responsible for metabolizing p-nitrobenzoic acid.

Effects of Copper and Molybdenum on DNP Nitro Reductase Activity

Copper in final concentration of 5×10^{-4} M inhibited DNP metabolism below control values in a 30 minute incubation. Molybdenum in final concentrations of 5×10^{-3} M and 5×10^{-4} M stimulated the metabolism of DNP. The addition of both Cu (5×10^{-4} M) and Mo (5×10^{-3} M) did not alter metabolism from control levels (Table 4). These data suggest that the DNP nitro reductase system, like bacterial nitro reductase systems, can be altered by metal cations in the media.

Heat Denaturation of Enzyme(s) Involved in DNP Reduction

A preliminary attempt was made to separate enzymes forming 4A2NP and 2A4NP by selective heat denaturation. Two initial experiments monitoring DNP disappearance suggested that incubation at 50°C for 10 minutes prior to incubation with substrate could decrease DNP metabolism by as much as 50%. This heating period was then selected and the metabolite formation ratio was examined (Table 5).

Table 4. Effect of Cu^{+2} and Mo^{+4} on DNP Nitro Reductase Activity of Whole Rat Liver Homogenates

	DNP unmetabolized (nmoles/mg protein)	Metabolites formed (nmoles/mg protein)	
		4A2NP	2A4NP
Control[1]	1.422 .212[2]	1.276 .010	4.747 .014
Na_2MoO_4 (5×10^{-5} M)	1.048 .072	1.366 .008	4.936 .017
Na_2MoO_4 (5×10^{-4} M)	.897 .020[3]	1.492 .011	5.178 .001
Na_2MoO_4 (5×10^{-3} M)	.752 .037[3]	1.714 .000	5.186 .010
$CuSO_4$ (5×10^{-5} M)	1.368 .153	1.162 .015	4.824 .028
$CuSO_4$ (5×10^{-4} M)	2.188 .210[3]	1.083 .011	4.448 .100
$CuSO_4$ (5×10^{-4} M) + Na_2MoO_4 (5×10^{-3} M)	1.204 .061	1.446 .012	4.873 .000

1 Incubation medium is the same as described in Methods. In treated samples, the solution of either $CuSO_4$ or $NaMoO_4$ replaced 0.5 ml distilled water. Incubation time was 30 minutes.

2 Mean ± standard error.

3 Significant difference from control $P < .05$.

Table 5. Heat Denaturation of Homogenate Preparation

	Time of Incubation (minutes)	DNP unmetabolized (nmoles/mg protein)	2A4NP/4A2NP formed
Control	5	5.745±.374[1]	3.658±.213
	10	4.081±.469	4.191±.249
	30	1.721±.045	4.041±.137
Treated[2]	5	7.893±.290	3.281±.141
	10	5.860±.106	3.660±.156
	30	3.490±.710	3.772±.328

1 Mean ± S.E. of 2 experiments with duplicate determinations at each time.

2 Heat denaturation at 50°C for 10 minutes prior to incubation with substrate.

For normal incubation procedures, the metabolite ratio (2A4NP : 4A2NP) is approximately 3:1. The ratio of metabolites was not altered although the amount of DNP was markedly reduced. This data suggest that there may be only one enzyme system responsible for reduction of DNP. The preference for reduction of the nitro group in the 2 position may be explained by its closer proximity to the phenolic hydroxyl (OH) group.

Enzyme Kinetics

Kinetic analysis of DNP nitro reductase activity over five substrate concentrations (2×10^{-6} M, 10^{-5} M, 2×10^{-5} M, 10^{-4} M, 5×10^{-4} M) revealed an apparent Km of 1.8×10^{-4} M and an apparent Vmax of 1.13 nmoles/mg protein/min. Incubations were carried out under optimized conditions for 3, 5, and 10 minutes and the velocity determined by measuring total product formed (mmoles) per minutes in each time interval.

PNBA, DNP, PNP, and DNBP were examined as inhibitors of the DNP nitro reductase. Incubations were carried out for 3, 5, and 10 minutes at optimum conditions with five concentrations of substrate and 1 concentration of inhibitor (2×10^{-4} M). PNBA had no effect, while ONP, PNP and DNBP significantly inhibited DNP reduction (Figure 6). Kinetic analysis utilizing the Hofstee plot (Dowd and Ross,1965) and linear regression analysis showed ONP to be a competitive inhibitor of DNP reduction while

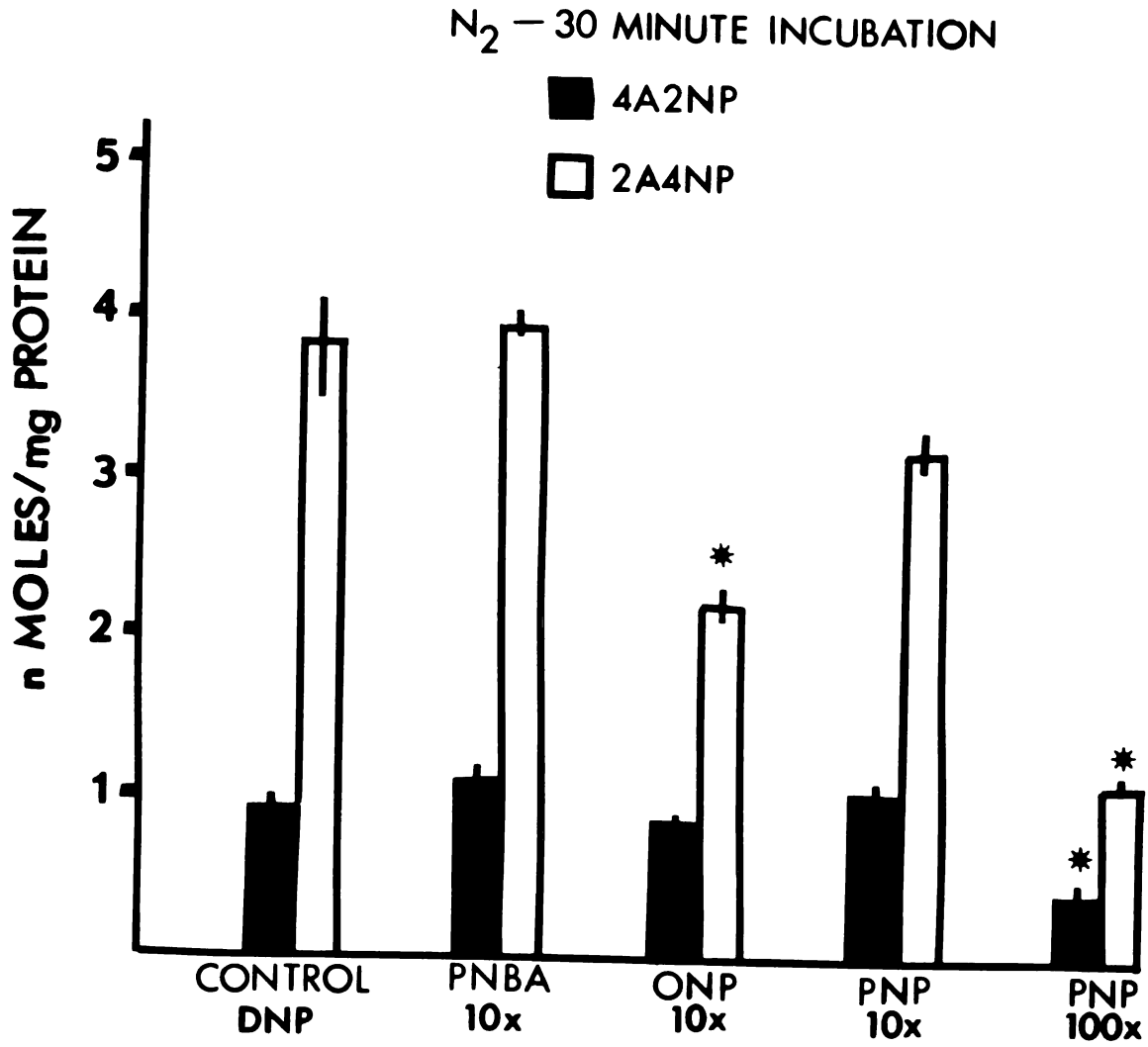

Figure 6. Inhibition of DNP nitro reduction. DNP concentration was 10^{-4} M. PNBA (10^{-3} M) had no effect on DNP reduction. ONP (10^{-3} M) and PNP (10^{-2} M) significantly depressed the formation of metabolies during the 30 minute incubation. Asterisk (*) indicates significance at $P < 0.05$.

PNP and DNBP were non-competitive inhibitors (Figure 7). The respective K_i's calculated from the regression lines were: 5.1×10^{-4} M, 7.1×10^{-4} M, and 1.3×10^{-4} M (Table 6).

Table 6. Kinetic Data for DNP Enzymatic Reduction

Constant	Apparent Value
Vmax	1.13 nmoles/min/mg protein
K_M [1]	1.8×10^{-4} M
K_I ONP [2]	5.1×10^{-4} M
K_I PNP [3]	7.1×10^{-4} M
K_I DNBP [4]	1.3×10^{-4} M

1 DNP concentrations used were: 2×10^{-6} M, 1×10^{-5} M, 2×10^{-5} M, 1×10^{-4} M, and 5×10^{-4} M. Incubations were run for 3, 5, and 10 minutes.

2 ONP concentration was 2×10^{-4} M.

3 PNP concentration was 2×10^{-4} M.

4 DNBP concentration was 2×10^{-4} M.

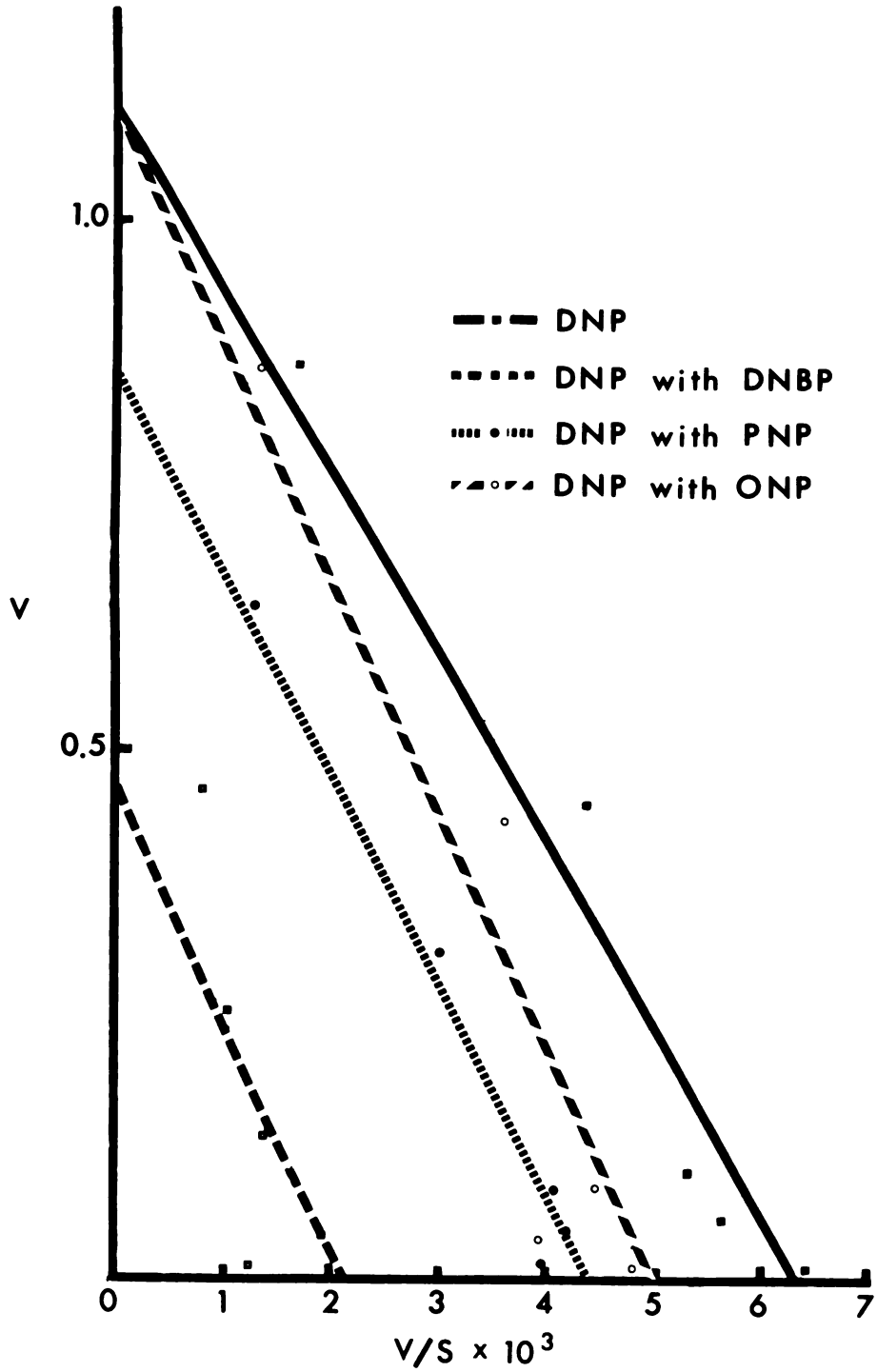

Figure 7. Hofstee Plot of DNP Nitro Reduction. Each point is the mean of at least 3 experiments with duplicate determinations at incubation times of 5 and 10 minutes. The lines are the linear regression lines. Concentrations of DNP substrate(s) used were: 2×10^{-6} M, 1×10^{-5} M, 2×10^{-5} M, 1×10^{-4} M and 5×10^{-4} M. The concentration of each inhibitor was 2×10^{-4} M. Velocity (V) and V/S are expressed as nmoles/mg protein/minute and nmoles / M/mg protein/minute respectively. ONP was a competitive inhibitor; PNP and DNBP were non-competitive.

DISCUSSION

The *in vitro* reduction of DNP to 2A4NP and 4A2NP by rat liver homogenates was confirmed (Figure 8). The presence of only trace amounts of DAP indicates its role as a minor reduction product as Parker (1952) suggested. The extent of formation of other metabolites, however, differed markedly from Parker's results. In the present study, 2A4NP was the major metabolite contributing 75 ± 4% of total amine while 4A2NP contributed only 23 ± 2% of total amine. 4A2NP was the least stable of the two and could be oxidized to a non-extractable product. Conceivably spontaneous oxidation of 4A2NP could have contributed to the difference in results but when the metabolites were extracted from the sample within one hour after incubation, the proportional relationship between the amines remained. The proportion of metabolites formed by the liver preparation seems to parallel the activity of the bacterial succinic dehydrogenase system reported by Greville and Stern (1935).

Optimization of Cofactors

The DNP nitro reductase cofactor requirement was similar to that described by Fouts and Brodie (1957). The addition of G-6-PO_4, $MgSO_4$, and NADP increased the activity

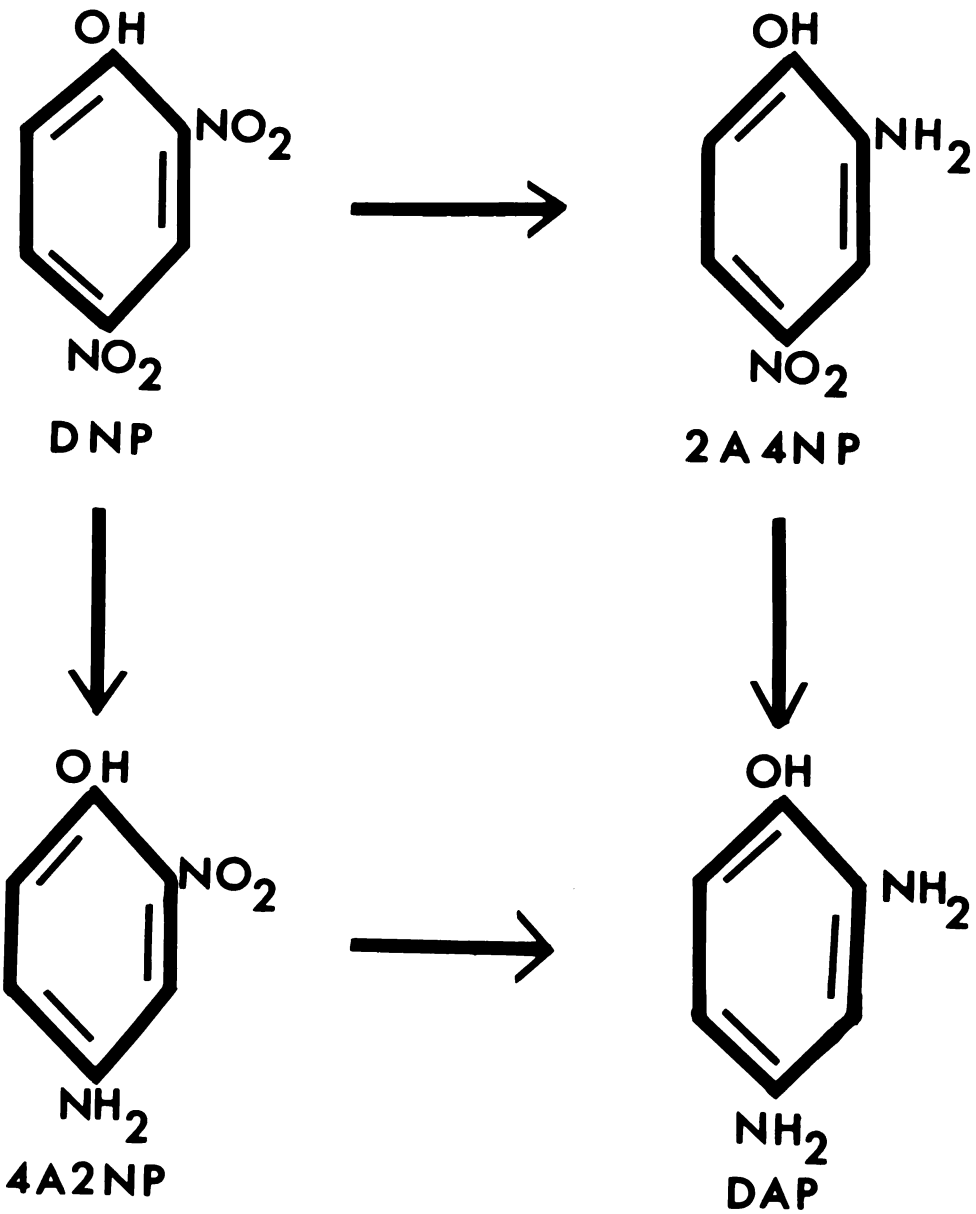

Figure 8. Proposed Mechanism of Reduction of DNP.

in the homogenate. NADP apparently was reduced to act as the hydrogen donor for the nitro group reduction. G-6-PO_4 and NADP, together with glucose-6-phosphate dehydrogenase from the soluble fraction of the cell presumably served to generate NADPH.

pH Optimum

The pH optimum (6.5, 37°C) for the formation of both metabolites was more acidic than the pH of 7.4 used by Parker (1952) or the pH of 7.2 used by Fouts and Brodie (1957). In addition it was more acidic than the pH of 7.8 used by Otsuka in his study of p-nitrophenol reduction. However, the pH optimum of the present system was in agreement with that reported by Juchau *et al.* (1970) and close to the pH optimum of 7.0 used by Kato and Takahashi (1969). The lower pH optimum may be indicative of a different enzyme system for nitrophenolic compounds than the system which reduces PNBA. Fouts and Brodie (1957) found nitrophenols resistant to rapid reduction by rat liver slices but in the present study DNP was rapidly metabolized. Moreover, DNP and p-nitrophenol were found to antagonize the reduction of PNBA *in vitro*.

One pH optimum for DNP reduction suggested the involvement of only one enzyme system in the metabolism of DNP to 2A4NP and 4A2NP. The enzyme system, however, appears to reduce the ortho nitro group 3 times more readily then the para nitro groups. The enzyme therefore may

possess steric specificity for the 2 position. The OH group in such close proximity may serve to position the molecule on the enzyme.

Location of Enzyme Activity

Enzyme activity appears to be located primarily in the soluble fraction. Most other nitro reductases are located in both the microsomes and the soluble fraction. Fouts and Brodie (1957) found 60% of the enzyme activity in the soluble fraction while the microsomes possessed the remaining 40%. However, they could only demonstrate activity in the microsomal fraction by adding a trace of the soluble fraction. Presumably soluble enzymes such as glucose-6-phosphate dehydrogenase were necessary for the generation of reducing equivalents.

More recently, Kato *et al.* (1969) showed two separate enzyme systems for the reduction of PNBA. The enzyme located in the soluble fraction required NADH as a hydrogen donor while the enzyme in the microsomes was specific for NADPH. Using PNBA and the carcinogenic agent, 4-nitroquinoline N-oxide (4-NQO), Kato and Takahashi (1969) defined separate enzyme systems. PNBA was reduced mainly by microsomes while 95% of 4NQO reduction activity was found in the supernatant. Both systems, PNBA and 4NQO, required reducing equivalents furnished by an NADPH generating system.

Carlson and DuBois (1970) performed quantitative assays of nitro reductase activity in liver fractions and confirmed that the soluble fraction plays the major role in total nitro reductase activity of the liver. The activity of the soluble fraction per unit of tissue was unaffected by changes in the amount of tissue, NADP concentration, pretreatment with DDT or phenobarbital, cobra venom (hydrolyzes NADPH) or by SKF 525-A, a microsomal enzyme inhibitor. Cyanide, however, inhibited the soluble enzyme to a greater extent than the microsomal reductase. Apparently the induction of microsomal nitro reductase was not as important for detoxification reactions as the soluble enzyme system.

The enzyme system metabolizing DNP appears to be more closely related to the 4NQO system. That system utilizes NADP as a cofactor and activity in both the homogenate and soluble fraction was increased by the addition of G-6-PO_4 and NADP. The ability of the microsomes to metabolize DNP was much lower than that of the soluble fraction. The 9000 *g* cell fraction, containing both microsomes and cell fluids, did not possess greater total activity than that of the soluble fluids or microsomes alone. Furthermore, the microsomal enzyme system was not as active in the metabolism of DNP as in the metabolism of p-nitrobenzoic acid. Therefore, the primary system for DNP metabolism appears to be located in the soluble fraction.

Oxygen Inhibition

The location of the major DNP nitro reductase activity was not altered by oxygen, but the formation of the metabolites after ten minutes was depressed. The amount of depression appeared to be concentration dependent. The incomplete inhibition of the DNP nitro reductase by 100% oxygen suggested that the nitrophenolic system was different from the PNBA system which was completely inhibited by oxygen (Fouts and Brodie, 1957). The DNP reduction system may be similar to certain bacterial systems (*E. coli*) where the reaction was not markedly inhibited by oxygen (Saz and Slie, 1954). Both systems may require a cofactor or cofactors which are not as sensitive to oxygen as the cofactors involved in the mammalian reduction of PNBA. Kato and Takahashi (1969) defined the soluble nitro reductase for 4NQO and found that it was only slightly inhibited by air (21% O_2). The DNP reductase system therefore, may be more closely related to the mammalian 4NQO system.

Copper and Molybdenum, Inhibition and Stimulation

Westerfeld *et al.* (1957) postulated molybdenum as an essential component of nitro reductase since the enzyme level in rat liver was significantly lower in molybdenum deficient animals. The copper inhibition and molybdenum stimulation of enzyme activity postulated for mammalian systems was shown to function in bacterial systems (Saz, 1954; Mackler, 1954) and in nitrate reduction (Fridovich

and Handler, 1956). The two elements are known to be mutually antagonistic on metabolic and physiologic events (Hochster and Quastel, 1963). The present findings confirm the antagonistic properties of Mo^{+4} and Cu^{+2} and extend the evidence to include crude mammalian nitro reductase preparations. Cu^{+2} inhibited the metabolism of DNP and Mo^{+4} stimulated the reduction. However, a combination of a inhibitory concentration of Cu^{+2} and a stimulatory concentration of Mo^{+4} added together to the *in vitro* preparations had no effect on nitro reductase activity. The role of Cu^{+2} and Mo^{+4} in the nitro reductase system is not known. However, Mo^{+4} is required for normal activity of xanthine oxidase. Xanthine oxidase may be the rate limiting step as indicated by the effects of copper and molybdenum.

Heat Denaturation

Heat denaturation at 50°C for 10 minutes did not alter the DNP metabolite ratio although DNP metabolism was decreased by 30%-50%. If different enzymes or active sites were involved in the selective metabolism of DNP to 2A4NP and DNP to 4A2NP, selective alteration by heat denaturation may have been expected to shift metabolite ratios during the step-wise denaturation. However, no change was found in the metabolite ratios indicating the involvement of only one enzyme. Heat denaturation at

50°C, however, may not have been selective enough to effect only one enzyme. Evidence, however, from pH optima, oxygen inhibition and heat denaturation support the conclusion that only one enzyme system is involved.

Selective reduction of the DNP ortho nitro group, while not explained by a different enzyme, may be explained by the electrophilic properties of the substituted groups and by their orientation on the benzene ring. The OH in DNP is strongly activating and releases electrons making the ring more negative. Substituent groups attached to neighboring carbon atoms would be influenced extensively. Both NO_2 groups on the other hand are deactivating and electron withdrawing. The close proximity, however, of the NO_2 group in the ortho position to the electron releasing OH group exerts a stronger withdrawing power over the π electron cloud of that group than that of the para NO_2. The increased electronegativity attracts H^+ reducing equivalents. Therefore, the ortho NO_2 is more subject to reduction than the para NO_2.

Enzyme Kinetics

The kinetics of DNP nitro reduction were determined using the Hofstee plot where v was plotted against v/s. The Hofstee plot was chosen over the Lineweaver-Burk plot since the latter distributes weight inappropriately among observations. Plots of the calculated linear regression lines yielded a Km of 1.8×10^{-4} M and a Vmax

of 1.13 nmoles/mg protein/minute. Parker (1952) determined Michaelis constants for DNP nitro reductase from the slope of a Lineweaver-Burk plot and found Km to be 8.5×10^{-5} M DNP. The value of Km determined in this study was about twice that reported by Parker, but this was acceptable since the conditions varied between the two systems.

Compounds structurally related to DNP, PNBA, ONP, PNP, and DNBP, were examined as inhibitors of the nitro reductase system. PNBA did not effect DNP nitro reduction, suggesting separate metabolic pathways for the two compounds. These data may explain the observation of Fouts and Brodie (1957) and Saz and Slie (1954) where DNP was found resistant to reduction in their systems. Furthermore the present data explain the observed absence of DNP antagonism of PNBA by the above authors. In contrast to the PNBA results, ONP was found to be a competitive inhibitor of DNP nitro reduction. ONP presumably was competing for the same active site as DNP. Although it was not demonstrated here ONP was probably reduced to o-amino phenol since Smith and Williams (1951) found that ONP was reduced *in vivo* to o-amino phenol.

PNP and DNBP, unlike ONP, were found to be non-competitive inhibitors of DNP nitro reduction. Apparently, PNP and DNBP bind at different site(s) on the enzyme(s) than DNP. Smith and Williams (1951) showed PNP metabolism to p-amino phenol *in vivo* and Otsuka (1961) showed PNP

metabolism by a swine liver nitro reductase preparation. In other studies, DNBP was shown to be reduced to 4 amino-2-nitro-6 sec butyl phenol and to 2-amino, 4-nitro, 6-sec butyl phenol in rat liver homogenates (Ernst, 1964). Apparently, however, the 6-alkyl group of DNBP hinders its attachment to the enzyme in the same manner as DNP.

The interpretation of the present data are complicated by the fact that one substrate is reduced to two products. Furthermore the system was an impure preparation. One cannot predict, therefore, whether there are two active sites on the DNP nitro reductase enzyme, or whether there is one specific enzyme for each nitro group. Taken as a whole, however, it was concluded from the present data that only one enzyme is involved in DNP reduction.

SUMMARY

DNP was reduced enzymatically by rat liver homogenates to 2A4NP, 4A2NP and DAP. 2A4NP was the major metabolite found in amounts three to four times greater than 4A2NP. DAP was present in trace amounts.

DNP disappearance and product formation showed a pH optimum of 6.5 which was more acidic than the optima for many nitro reductases.

NADPH, G-6-PO_4, and $MgSO_4$ were required for optimum *in vitro* activity of DNP nitro reductase.

The major enzyme activity was found in the 105,000 g soluble fraction of liver cells and this distribution was not changed in the presence of oxygen. Oxygen, however, depressed the amount of metabolites formed in all cell fractions. Presumably oxygen competed with DNP for reducing equivalents.

DNP nitro reduction was responsive to Mo^{+4} stimulation and Cu^{+2} inhibition indicating cation influence on the nitro reductase enzyme(s).

Heat denaturation reduced DNP reductase activity 30-50% but had no effect on the product ratio.

Kinetic studies showed an apparent Km of 1.8×10^{-4} M and an apparent Vmax of 1.13 mmoles/mg protein/minute for

the DNP nitro reductase. PNBA, a non phenolic nitro compound did not inhibit the enzyme system. ONP, however, was a competitive inhibitor with an apparent Ki of 5×10^{-4} M. PNP and DNBP were non-competitive inhibitors and had Ki's of 7×10^{-4} M and 1×10^{-4} M respectively. The kinetic data suggested more than one active site on the DNP nitro reductase enzyme.

It was concluded that a single soluble hepatic enzyme from rats may reduce DNP to 2A4NP, 4A2NP and DAP.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adamson, R. H., Dixon, R. L., Frances, F. L. and Rall, D.P.: Comparative biochemistry of drug metabolism by azo and nitro reductase. Biochemistry, Vol. 54, 1386-1391, 1965.

Bratton, A. and Marshall, E.: A new coupling component for sulfanilamide determination. J. Biol. Chem. 128: 537-550, 1939.

Bueding, E. and Jolliffe, N.: Metabolism of trinitrotoluene (TNT) *in vitro*. J. Pharmacol. Exp. Ther. 88: 300-312, 1946.

Carlson, G. P. and DuBois, K. P.: Studies on the induction of nitro reductase activity by DDT and phenobarbital. Toxicol. and Appl. Pharmacol. 17:203-216, 1970.

Channon, H. J., Mills, G. T. and Williams, R. T.: The metabolism of 2:4:6-trinitrotoluene (α-T.N.T.) Biochem. J. 38:70-85, 1944.

Clifford, D. R., Fieldgate, D. M. and Watkins, D. A. M.: Chromatography of dinitrophenols. J. of Chromatography 43:110-119, 1969.

Dowd, J. E. and Riggs, D. S.: A comparison of estimates of Michaelis-Menton kinetic constants from various linear transformations. J. Biol. Chem. 240:863-869, 1965.

Ernst, W. and Baer, F.: The conversion of 2,4-dinitro-6-sec.-butylphenol and its esters in the animal organism. Arzneimittel-Forsch. 14:81-84, 1964.

Fouts, J. R. and Brodie, B. B.: The enzymatic reduction of chloramphenicol, p-nitrobenzoic acid and other aromatic nitro compounds in mammals. J. Pharmacol. Exp. Ther. 119:197-207, 1957.

Gehring, P. J. and Buerge, J. F.: The cataractogenic activity of 2,4-dinitrophenol in ducks and rabbits. Toxicol. Appl. Pharmacol. 14:475-486, 1969.

Gillette, J. R., Brodie, B. B. and LaDu, B. N.: The oxidation of drugs by liver microsomes: On the role of TPNH and oxygen. J. Biol. Chem. 216:532-539, 1957.

Gillette, J. R., Kamm, J. J. and Sasame, H. R.: Mechanism of p-nitrobenzoate reduction in liver: Possible role of cytochrome P-450 in liver microsomes. Mol. Pharmacol. 4:541-548, 1968.

Greville, G. D. and Stern, K. G.: Reduction of dinitrophenols by Redox indicators and enzymes. Biochem. J. 29:487-499, 1935.

Hitchcock, M. and Murphy, S. D.: Enzymatic reduction of 0,0-(4-nitrophenyl) phosphorothioate, 0,0-diethyl 0-(4-nitrophenyl) phosphate, and 0-ethyl 0-(4-nitrophenyl) benzene triophosphonate by tissues from mammals, birds, and fishes. Biochem. Pharmacol. 16: 1801-1811, 1967.

Hochster, R. M. and Quastel, J. H.: Metabolic Inhibitors. New York, New York: Academic Press, Vol. 2, 1963.

Horner, W. E.: Dinitrophenol and its relation to the formation of cataract. Arch. Ophth. 27:1097-1121, 1942.

Juchau, M. R., Krasner, J. and Yaffe, S. G.: Model systems for aromatic nitro group reduction - Relationships to tissue catalyzed reagents. Biochem. Pharmacol. 19:443-455, 1970.

Kamm, J. J. and Gillette, J. R.: Mechanism of stimulation of mammalian nitro reductase by flavins. Life Sci. 4:254-260, 1963.

Kato, R., Oshima, T. and Takanaka, A.: Studies on the metabolism of nitro reduction by rat liver. Mol. Pharmacol. 5:487-498, 1969.

Kato, R. and Takanaka, A.: Metabolism of drugs in old rats (1) Activities of NADPH-linked electron transport and drug-metabolizing enzyme systems in liver microsomes of old rats. The Jap. J. of Pharmacol. 18:381-388, 1968.

Kato, R. and Takahashi, A.: Characteristics of nitro reduction of the carcinogenic agent, 4-nitroquinoline N-oxide. Biochem. Pharmacol. 19:45-55, 1970.

Keilley, R. K.: Purification of liver xanthine oxidase. J. Biol. Chem. 216:405-412, 1955.

Lowry, O. H., Rosebrough, N. I., Farr, A. L. and Randall, A. J.: Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265-275, 1951.

Mackler, B., Mahler, H. R. and Green, D. E.: Studies on metalloflavoproteins. I. Xanthine oxidase a molybdoflavoprotein. J. Biol. Chem. 210:149-163, 1954.

Magne, M., Mayer, A. and Pantefoe, L.: Action pharmacodynamique des phenols nitris. Un agent augmentant les oxidations cellulaires. Annls. Physiol. Physicochem. biol. 32:650-652, 1932.

Mc Elroy, M. and Glass, B.: Inorganic Nitrogen Metabolism. Baltimore, Maryland: Johns Hopkins Press, 1956, pp. 539-551.

Ogino, S. and Yasukura, K.: Production of cataracts in guinea pigs with dinitrophenol. Am. J. Ophthal. 43:936-946, 1957.

Otsuka, S.: Studies on nitro-reducing enzymes of swine liver. J. Biochem. (Tokyo) 50:85-94, 1961.

Parke, D. V.: The determination of 2,4-diaminophenol and its salts. Biochem. J. 56:12-15, 1961.

Parke, D. V.: The metabolism of [^{14}C] nitrobenzene in the rabbit and guinea pig. Biochem. J. 62:339-346, 1956.

Parker, V. H.: Enzymatic reduction of 2,4-dinitrophenol by rat tissue homogenates. Biochem. J. 51:363-370, 1952.

Robbins, B. E.: Dinitrophenol cataract: Production in experimental animal. J. Pharmacol. Exp. Ther. 80: 264-268, 1944.

Robbins, B. E.: Dinitrophenol cataracts in the chick: Effect of age. J. Pharmacol. Exp. Ther. 82:301-302, 1944.

Robinson, D., Smith, J. N. and Williams, R. T.: Nitro compounds. (a) The metabolism of 0-,m-, and p-nitrophenols in the rabbit. (b) The glucuronides of the mononitrophenols and observations on the anomalous optical rotations of triacetyl β-σ-nitrophenol-glucuronide and its methyl ester. Biochem. J. 50: 221-227, 1951.

Sasame, H. A. and Gillette, J. R.: Studies on the relationship between the effects of various substances on absorption spectra of cytochrome P-450 and the reduction of p-nitrobenzoate by mouse liver microsomes. Mol. Pharmacol. 5:123-130, 1969.

Saz, A. K. and Slie, R. B.: The inhibition of organic nitro reduction by aureomycin in cell free extracts. II. Cofactor requirements for nitro reductase enzyme complex. Arch. Biochem. Biophys. 51:5-16, 1954.

Smith, J. N., Smithies, R. H. and Williams, R. T.: Urinary metabolites of 4,6-dinitro-0-cresol in the rabbit. Biochem. J. 54:225-230, 1953.

Steel, R. G. and Torrie, J. H.: Principles and Procedures of Statistics. New York: McGraw-Hill Book Company, 1960.

Westfall, B. B.: The reduction of 2,4,6-trinitrotoluene by animal tissue *in vitro*. J. Pharmacol. Exp. Ther. 78:386-393, 1943.

Westerfeld, W. W., Richert, D. A. and Kiggens, S.: The metabolic reduction of organic nitro groups. J. Biol. Chem. 227:379-391, 1957.

Webb, J. L.: Enzyme and Metabolic Inhibitors, Volume 1, New York, New York: Academic Press, 1963.

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 03071 2198