1968

An Investigation of the Relationship Between the Sulfhydryl Reactivity and the Inhibition of *In Vitro* Oxygen Uptake By Certain Molluscicidal B-Nitrostyrene Derivatives

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MASTER OF SCIENCE THESIS

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UNIVERSITY OF RHODE ISLAND

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AN INVESTIGATION OF THE RELATIONSHIP BETWEEN THE SULFHYDRYL REACTIVITY AND THE INHIBITION OF IN VITRO OXYGEN UPTAKE BY CERTAIN MOLLUSCICIDAL β-NITROSTYRENE DERIVATIVES

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PHARMACOLOGY

UNIVERSITY OF RHODE ISLAND

1968

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to his wife, Camille, and his parents, Mr. and Mrs. Joseph Louis-Ferdinand, for their inspiration, generosity and understanding. The guidance provided by Dr. George C. Fuller during the course of these studies is acknowledged and is sincerely appreciated.

The author also wishes to convey his thanks to his fellow students for the many helpful suggestions offered during these studies. The generosity of Dr. B. H. Pringle, who provided the snails used in this investigation, is gratefully acknowedged.

Financial support for this investigation was provided by the U. S. Department of Interior, Office of Water Resources Research, under P. L. 88-379.

ABSTRACT

Louis-Ferdinand, Robert T. MS. University of Rhode Island, 1968. An investigation of the relationship between the sulfhydryl reactivity and the inhibition of *in vitro* oxygen uptake by certain molluscicidal β-nitrostyrene derivatives. Major Professor: Dr. George C. Fuller.

Eleven derivatives of β-nitrostyrene were found to inhibit the oxygen uptake of whole tissue homogenates of the snail *Australorbis glabratus*. The inhibition of oxygen uptake by β-nitrostyrene was diminished in the presence of excess and equimolar concentrations of cysteine in the incubation media. The addition of cysteine resulted in changes in the ultraviolet spectra of β-nitrostyrene solutions. Similar addition of serine to β-nitrostyrene solutions had no discernible effect on the ultraviolet spectra. β-nitrostyrenes were found to delay the development of color which normally results from the reaction between cysteine and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The ability of the nitrostyrenes to delay the progress of the reaction between cysteine and DTNB was compared with the inhibitory effects of the same nitroolefins on the oxygen uptake of homogenates. A positive correlation between the effects of the nitroolefins on oxygen uptake and the effects on the cysteine-DTNB reaction was found. These data indicate that the sulfhydryl reactivity of the compounds studied may be responsible for the molluscicidal activity of the nitrostyrene series studied.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

One approach in the worldwide attempt to control schistosome dermatitis has been erradication of the intermediate hosts through the use of molluscicides. Since several species of fresh water snails have been implicated as schistosome transmitters in the western hemisphere (Cort, 1928) a variety of substances have been proposed and evaluated for molluscicidal activity. These include organo-metallic agents (Bond and Nolan, 1954; Frick and Jiminez, 1964), N-tritylmorpholine (Boyce, 1967), sodium pentachlorophenate, and Bayluscide, (Seiffer and Schoof, 1967).

The paucity of evidence regarding the biological activity of these agents justifies the pharmacological evaluation of these molluscicides. Research in this area would not only provide a theoretical basis for the screening of potential molluscicides but would also suggest the degree of specificity to be expected from chemicals proposed for molluscicidal use. Evidence published to date suggests at least two general classes of molluscicides. Certain chemicals viz. disubstituted amides, are postulated to act by a physical interaction with membrances or interfaces within the snail (de Villiers and Rossouw, 1967). Others are believed to interfere with enzymatic processes of the target organism by interaction with functional groups of vital enzymes (Mackworth, 1948).

The demonstration of the potent fungistatic properties of β-nitrostyrenes by Bocobo et al.,(1954) suggested that an investigation of the molluscicidal properties of β-nitrostyrenes could be warranted. Inspection of the β-nitrostyrene structure indicated that polarization of the α-β double bond could provide a chemical basis for the known toxicity of this group of compounds. It was felt that these compounds would be

capable of reaction with organic thiol groups and that such an interaction might well provide a rational basis for an in vitro screening procedure for similar compounds.

The studies to be described were designed to determine: whether nitrostyrene derivatives could be shown to inhibit vital enzymatic processes; whether a suitable model could be found which would reflect the ability of the test compounds to react with thiol groups of biological substances and finally, whether any correlation could be demonstrated between these properties.

II. SURVEY OF THE LITERATURE

Basis for Sulfhydryl Reactivity

The potential biological toxicity of nitroethylenic substances was indicated by Bousquet (1943) who proposed that such substances might be useful as pesticides. A chemical basis for the biological toxicity of chemical warfare agents similar to nitroethylenic compounds, was suggested by Dixon and Needham (1946). These authors attributed the polarization of olefinic double bonds by adjacent electron-withdrawing groups to the enhancement of the reactivity of such olefins toward nucleophillic reagents.

Nitroethylenic substances have been reported to react with sulfhydryl compounds *in vitro*. For example, Cason and Wanser (1951) were able to react aromatic thiols with nitrostyrenes in the presence of catalytic amounts of piperidine. Tweit *et al.*, (1965) described the pyrollidine-catalyzed addition of nitrostyrenes and 2-aminoethanol. Cavins and Friedman (1968) studied the reaction between acrylic acid derivatives and protein sulfhydryl groups. In this study the authors presented evidence which indicated that the acrylic acid derivatives were capable of reacting with bovine serum albumin sulfhydryl groups at physiological pH.

Determinants of Molluscicidal Activity

Evidence has been published which indicates that certain non olefinic substances are also capable of altering sulfhydryl levels of exposed snails. Devilliers (1963) reported that exposure of snails to nitrophenacyl chloride resulted in acid-soluble sulfhydryl levels which were 17% of those of control unexposed snails. However, exposure to Bayluscide could not be shown to alter acid-soluble sulfhydryl levels. These results

were interpreted to suggest an enzyme-protective function for acid-soluble thiols. Decreases in thiol levels were proposed to adversely affect certain enzyme activities. Therefore the molluscicidal properties of Bayluscide could not be attributed to altered thiol levels which suggested other determinants of molluscicidal activity.

Studies by Mackworth (1948) and Dixon (1946; 1948) indicated that enzymatic inhibition resulting from exposure to certain chemical warfare agents and molluscicides, could be mitigated in the presence of cysteine and glutathione. These data were taken to suggest that the toxicity of these agents was due to the inhibition of vital enzyme systems which required intact sulfhydryl groups for normal activity. However deVilliers (1967) found that maximum snail lethality could be obtained when snails were exposed to disubstituted amides with substituent acyl chain lengths of 12-13 carbon atoms. In addition, exposed moribund snails could be revived after repeated washing. Devilliers concluded that physical interactions of the molluscicides with snail membranes played a dominant role in the molluscicidal activity of these compounds.

Von Brand (1949) observed that 4-nitrostilbenes inhibited 99% of the oxygen uptake of Australorbis glabratus. Overnight washing of the exposed snails in aerated water did not return the oxygen uptake to levels greater than 38% of the pre-exposure values. The absence of recovery indicated some differences between the reversibility of the effects of the disubstituted amides and 4-nitrostilbene.

In Vitro Evaluation of Potential Pesticides

Yuki et al. (1966) attempted to relate the chemical reactivity of antiviral agents to the virulence observed by comparing thiol levels before and after incubation of the compounds with methyl N-acetyl

cysteinate (MAC). It was found that compounds which were capable of inactivating Adenovirus type 5, were also capable of reacting with sulfhydryl groups of MAC. Inactive compounds did not react with MAC. However, all MAC-reactive substances did not possess antiviral properties.

Nabih and Elwasimi (1968) determined the influence of Bayluscide on the biotransformation of solutions of N-N-dimethyl-p-phenylenediamine or hydrogen peroxide by intact snails. The reduction of color development or peroxide decomposition in the presence of the molluscicide was used to measure interference with catalase or peroxidase activity.

Tweit et al. ,(1965) found that while β-nitrostyrenes were potent antibacterial agents in vitro the in vivo activities were less than expected. This discrepancy was attributed to the irreversible binding of the β-nitrostyrene to thiol or amine groups of blood. These results were in accord with those of Evans et al., (1956) in which preincubation of fungi with β-nitrostyrene was found to protect mice. However, administration of β-nitrostyrenes afforded little protection to mice which had been pretreated with fungi.

The influence of β-nitrostyrene derivatives on oxidative phosphorylation in rat liver mitochondria was studied by Bovell et al., (1964). β-Nitrostyrene derivatives were found to uncouple oxidative phosphorylation and inhibit energy-dependent processes. However, at higher concentrations these agents were found capable of inhibiting respiration.

The Sensitivity of Bioenergetic Processes to Sulfhydryl Reagents.

Evidence has accumulated which suggests that bioenergetic processes are susceptible to sulfhydryl-reactive agents. Conceivably, oxidation of free protein groups on the same or on different peptide chains could result in alteration of the original protein structure (West et al., (1966).

Accordingly enzyme activity and/or membrane properties could be affected.

Penefsky (1967) has shown that decreases in the detectable sulfhydryl group content accompanies loss of ATPase activity. Lam (1968) has indicated that dithiols can reduce the extent of reagent-induced or storage-induced loss of energy-linked pyridine nucleotide reductase activity. Dithiols have also been shown to reverse arsenophenyl-associated inhibition of oxidative phosphorylation (Fluharty and Sanadi, 1963). Iodoacetamide and p-chloromercuribenzoate have been shown to inhibit the respiration of tissue minces prepared from Australorbis glabratus (Weinbach, (1953). These findings have been interpreted by the author to suggest the presence of sulfhydryl enzyme systems.

Thus the vulnerability of living systems to sulfhydryl-reactive substances is well established. The reactivity of certain α,β unsaturated compounds with protein sulfhydryl groups indicates that the nitrostyrenes may exert their toxic effects by interaction with biological systems which are susceptible to thiol-reactive agents. This study has been designed to determine the ability of certain nitroolefins to interfere with the reaction between cysteine and 5,5'-dithiobis (2-nitrobenzoic acid) and to determine the extent of correlation with the ability of the same nitro-olefins to inhibit oxygen uptake.

III. EXPERIMENTAL

Reagents

Reagents used were analytical grade or equivalent. Cofactors, substrates and amino acids used were obtained from Calbiochem of Los Angeles, California. 5,5'-Dithiobis (2-nitrobenzoic acid)(DTNB) was used as obtained from Aldrich Chemicals of Milwaukee, Wisconsin. The molluscicidal agents were prepared by the Pharmaceutical Chemistry department at the University of Rhode Island.

Animals

Laboratory reared Australorbis glabratus were provided by Dr. B. H. Pringle of the Northeast Marine Health Sciences Laboratories, Narragansett, R. I. Once obtained, the snails were maintained on an ad libitum diet of lettuce in glass beakers which contained 65.3 mg sodium thiosulfate pentahydrate per liter of tap water.

Analytical Procedures:

Spectral Studies

The effects of two amino acids (cysteine and serine) on the ultraviolet spectra of B-nitrostyrene solutions (0.1uM/ml in 0.1M phosphate buffer pH7.3) were determined. After blanking the instrument with phosphate buffer, the 1-cm cuvettes which contained 2 ml of the B-nitrostyrene solution were placed in a 30°C thermostat-controlled chamber of the Beckman DBG spectrophotometer. Once the initial B-nitrostyrene spectrum had been recorded, successive 0.1 ml aliquots of 1uM/ml solutions of the amino acid were added to the sample and reference cuvettes. A final addition of amino acid (0.1 ml of a 0.02mM/ml solution) was used. The resulting spectrum was recorded following each addition for comparison

with the initial β-nitrostyrene spectrum.

Sulfhydryl Reactivity

Sulfhydryl reactivity was determined by following the rate of the color-forming reaction between cysteine and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in the presence and absence of the suspected molluscicidal agent. The incubation media contained 7 ml cysteine (0.25mM), 10 ml ethylenediaminetetraacetate (EDTA) (0.5M) adjusted to pH7.3 with potassium hydroxide, 5ml deionized water and 6 ml (pH7.3) 0.1M phosphate buffer. Successive additions of solutions of the nitroolefins in ethanol were incubated with the test mixtures. Equal volumes of ethanol were added to controls and the mixtures were incubated in a Dubnoff incubator for 20 minutes at 30° C. Two ml aliquots were then pipetted into two 1cm light path spectrophotometer cuvettes which in turn were placed in a 30°C thermostat-controlled cuvette chamber of a Beckman DBG spectrophotometer. Twenty-five microliters of DTNB (4mg/ml) were prepared fresh daily in 0.1M phosphate buffer pH7.3 and were pipetted into the sample cuvette to start the color development. Five seconds later the development of color was monitored by measurement of the absorbance at 412mu. An accessory Beckman 10 inch linear-log recorder was used in these studies. The pH of the final incubation medium was measured at the end of the assay period. The rate of absorbance increase was measured from straight line portions of the recordings. Values used were the arithmetic means of at least two determinations. The reciprocal slopes calculated from the spectrophotometric determinations were taken to represent sulfhydryl reactivity. The addition of DTNB to aliquots removed from a similar incubation medium from which cysteine had been excluded resulted in negligible absorption at 412mu.

In Vitro Oxygen Uptake

Oxygen uptake was measured manometrically. Volumes of homogenates ranged from 1.0-0.2 ml. Preincubation was allowed to proceed at 30° C for twenty minutes in 16-30cc warburg flasks which contained the incubation medium unless otherwise noted. Incubation medium 'A' was used in initial studies and contained (in a final volume of 3.2 ml) 6 umoles potassium fumarate, 6 umoles potassium pyruvate, 12 umoles magnesium chloride, 3 umoles adenosine triphosphate, 585 umoles sucrose and 0.3ml (pH 7.3) 0.1M phosphate buffer. Sufficient distilled water was included to attain the desired final volume following the addition of the homogenate. Incubation medium 'B' contained (in a final volume of 2.7ml) 5 µmoles adenosine 5' diphosphate, 12.5 umoles succinic acid, 1.3 umoles magnesium sulfate, 625 umoles sucrose, 0.375 ml of 0.1M phosphate pH 7.3 buffer and 0.4 ml of homogenate.

Solvents used were either absolute ethanol or water-methanol 91:9 (v:v). An equal volume of the corresponding solvent was added to the final control incubation medium. Two tenths ml of 20% aqueous potassium hydroxide was added to the center well of each flask. The cofactor solution was tipped in from a side arm at the commencement of the measurement period. Following the measurement of the oxygen uptake in the presence of the nitroolefins, the contents of a group of flasks were pooled and the pH was measured. Aliquots of boiled homogenates were incubated with both media in the presence of nitroolefin and the apparent oxygen uptake measured were negligible. All readings were corrected for changes in barometric pressure through the use of a thermobarometer.

Estimation of Protein

The protein content of homogenates was estimated by the colorimetric

procedure of Lowry et al., (1951) with minor modifications. A 0.2ml aliquot of homogenate was added to 15ml test tubes containing 10 ml of 1N sodium hydroxide, instead of potassium hydroxide. The use of sodium hydroxide eliminated the formation of precipitates in the final solution. The tubes were heated in a water bath until dissolution was complete. Standard solutions of cystalline bovine serum albumin and a blank consisting of 0.25M sucrose were carried through the entire procedure. When the tissue was completely dissolved a 0.2 ml aliquot was removed from each tube and was placed into a second tube containing 1 ml of 0.5N sodium hydroxide solution. Five ml of reagent 'A' (prepared by the addition of 1.0 ml of 1% cupric sulfate solution plus 1.0 ml of 2.7% potassium tartrate, to 100 ml of 2% sodium carbonate solution) was added to each tube, mixed and the tubes allowed to stand at room temperature for twenty minutes. At this time 0.5 ml of reagent 'B' (prepared by diluting commercial Folin-phenol reagent with an equal volume of distilled water) was added to each sample with rapid mixing. The samples were allowed to stand at room temperature for 40 minutes for color development. The absorbance of the solutions was determined against the blank in a model DBG Beckman spectrophotometer.

Statistical Evaluation

The Student's 't' statistic (Fryer, 1966) was used to determine whether statistical differences existed between means.

The equation used was: $t = \frac{X_1 - X_2}{\delta \sqrt{1/N_1 + 1/N_2}}$ where $\delta = \sqrt{\frac{N_1 S_1^2 + N_2 S_2^2}{N_1 + N_2 - 2}}$

X_1 and X_2 = sample means

N_1 and N_2 = sample sizes

S_1^2 and S_2^2 = sample variances

The calculated 't' statistic was then compared with critical values obtained from the 't' distribution tables. Hypotheses of identity were rejected if the calculated statistic exceeded the bounds of the critical values. ($p<0.05$)

Spearman's formula (Spiegel, 1961) was used to determine sample correlation coefficients. Nitroolefins were ranked according to potency as molluscicides, inhibitors of oxygen uptake and sulfhydryl reactivity. The equation used was:

$$r = 1 - \frac{6 \sum D^2}{N(N^2-1)}$$

r = sample coefficient of rank correlation

$\sum D^2$ = sum of the squares of the differences between ranks of compared vales

N = number of pairs of values.

The 't' statistic was used to test the hypothesis that the population coefficient of correlation, denoted by ρ was equal to zero. The equation used was:

$$t = \frac{r\sqrt{N-2}}{\sqrt{1-r^2}}$$

r = sample coefficient of rank correlation

N = number of pairs of values compared

t = 't' statistic

The calculated 't' statistic was then compared with critical values for the 't' distribution. Hypotheses of equality between ρ and zero were rejected if the calculated statistic fell outside the bounds of the critical values ($p < 0.05$).

IV. RESULTS

Effect of Cysteine on the Ultraviolet Spectrum of β-Nitrostyrene

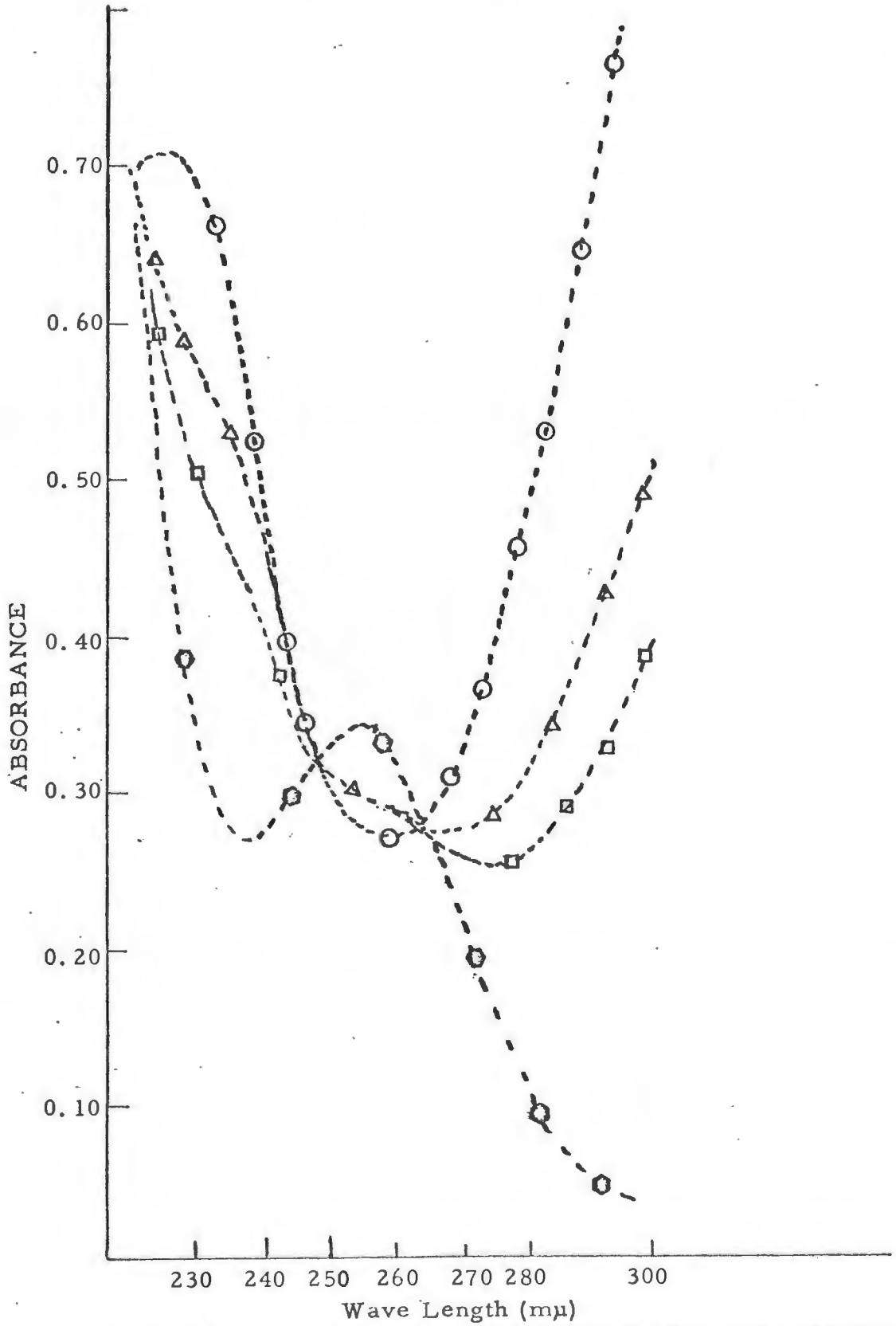
The effect of the addition of cysteine solutions on the ultraviolet spectrum of β-nitrostyrene is shown in Figure 1. The alterations observed include a decrease in the absorption at 300 mu, an increase in the absorption at 250 mu and a progressive shift in the absorption maximum at 230 mu to shorter wavelengths. Isosbestic points in the family of spectra can be seen near 263 mu and 250 mu. The results of similar additions of serine to β-nitrostyrene solutions are presented in Figure 2. No qualitative spectrum changes are discernible. The quantitative alterations seen are no greater than those to be expected from dilution of β-nitrostyrene solutions. The absorbance of β-nitrostyrene follows Beer's law through a concentration range of 0.05 to 0.15 μM/ml.

Sulfhydryl Assay

The effect of ethylenediaminetetraacetate (EDTA) on the stability of color formed following the addition of different concentrations of DTNB to cysteine solutions is shown in Figure 3. The decrease in absorbance proceeds at the same rate in the presence of either 0.025mMolar or 0.084 mMolar DTNB. The use of 0.08 molar EDTA results in color stability for at least 30 minutes. Comparison of the absorbance spectra obtained with and without EDTA (Figure 4) indicates that EDTA does not alter the absorbance spectrum of the color formed following the reaction between cysteine and DTNB.

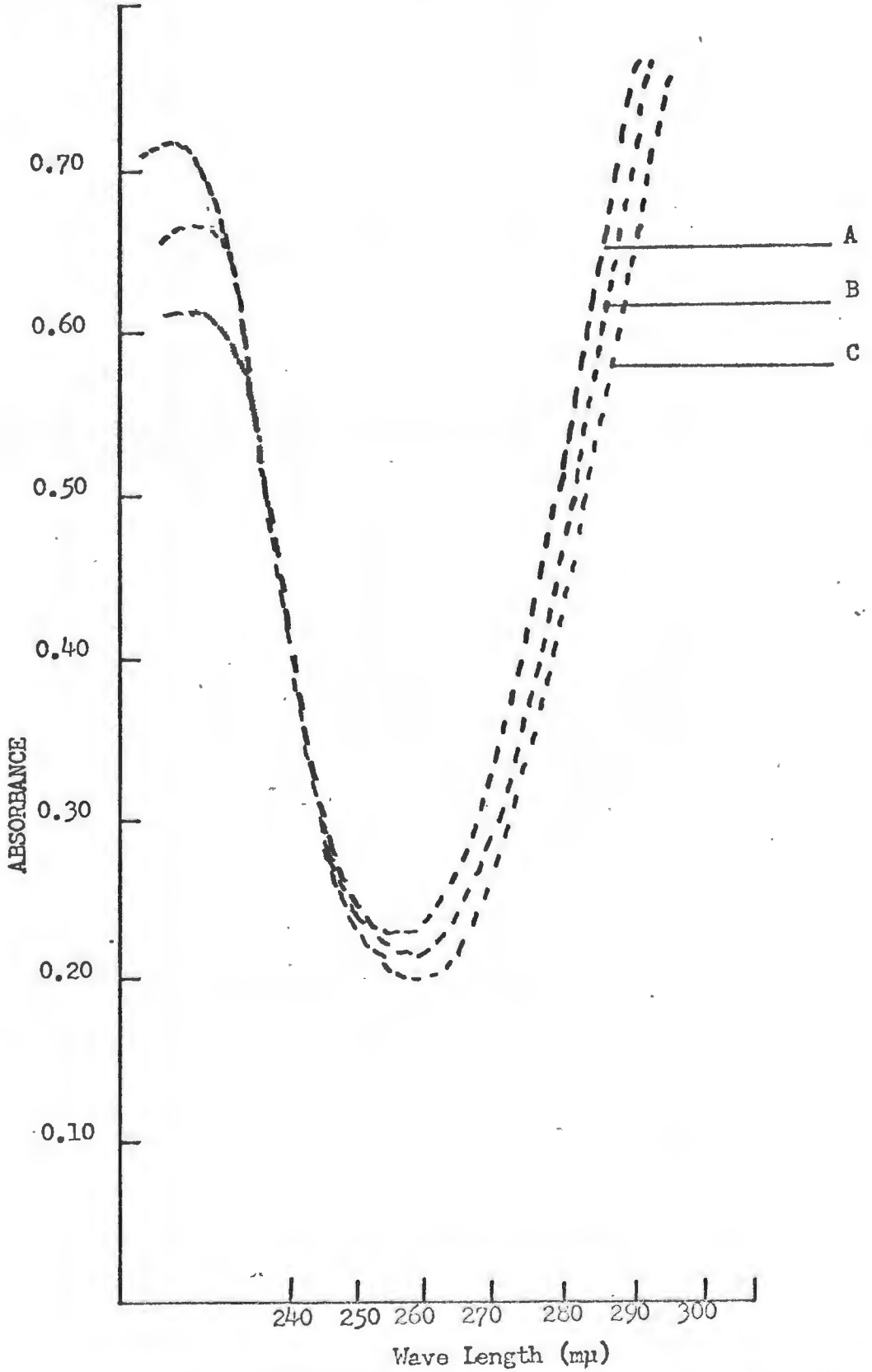
The presence of EDTA was also found to enhance the stability of cysteine solutions (Figure 5). The estimated half time for reactivity of cysteine with DTNB in the absence of EDTA ranged from 8 to 4 minutes.

FIG. 1. EFFECT OF CYSTEINE ON THE ULTRAVIOLET SPECTRA OF β-NITROSTYRENE



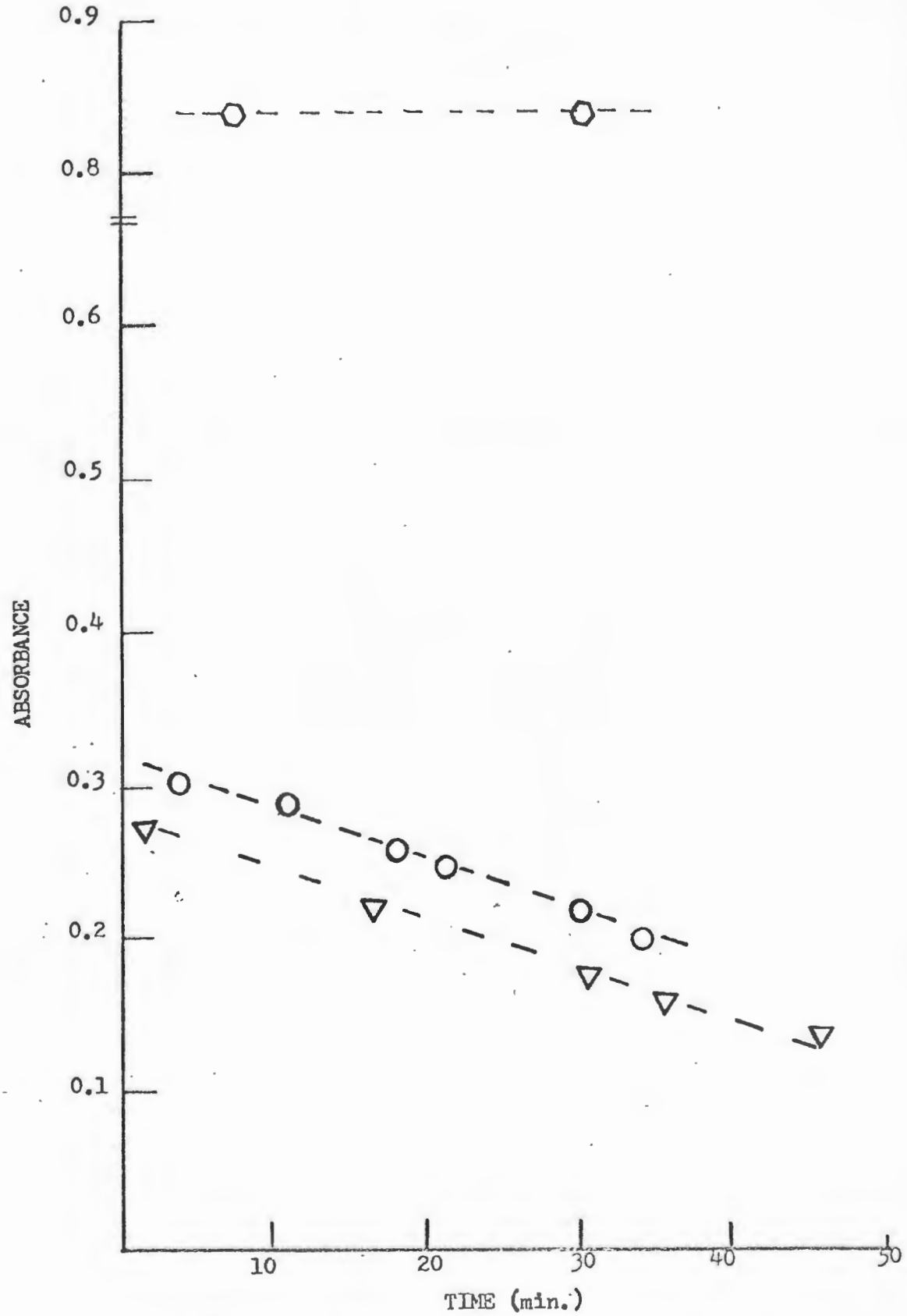
(O-O) β-Nitrostyrene 0.1 μm/ml; (Δ-Δ) 0.10 μmole cysteine plus β-nitrostyrene; (□-□) 0.20 μmole cysteine plus β-nitrostyrene; (●-●) 2.2 μmole cysteine plus β-nitrostyrene

FIG . 2. EFFECT OF SERINE ON THE ULTRAVIOLET SPECTRA OF β-NITROSTYRENE



A: β-Nitrostyrene 0.1 μm/ml; B: A plus 0.1 μmole serine;
C: A plus 2.2 μmoles serine.

FIG. 3. EFFECT OF 5,5'DITHIOBIS (2-NITROBENZOIC ACID) CONCENTRATION AND ETHYLENEDIAMINETETRAACETIC ACID ON COLOR STABILITY OF THE PRODUCT OF THE REACTION BETWEEN CYSTEINE AND 5,5'-DITHIOBIS (2-NITROBENZOIC ACID)



(-○--○-) 0.08 mMolar DTNB plus 0.083 molar EDTA
(-○--○-) 0.025 mMolar DTNB; (-▽--▽-) 0.08 mMolar DTNB

FIG. 4. EFFECT OF ETHYLENEDIAMINETETRAACETIC ACID ON THE ABSORBANCE SPECTRA OF THE PRODUCT OF THE REACTION BETWEEN 5,5'-DITHIOBIS (2-NITROBENZOIC ACID) AND CYSTEINE

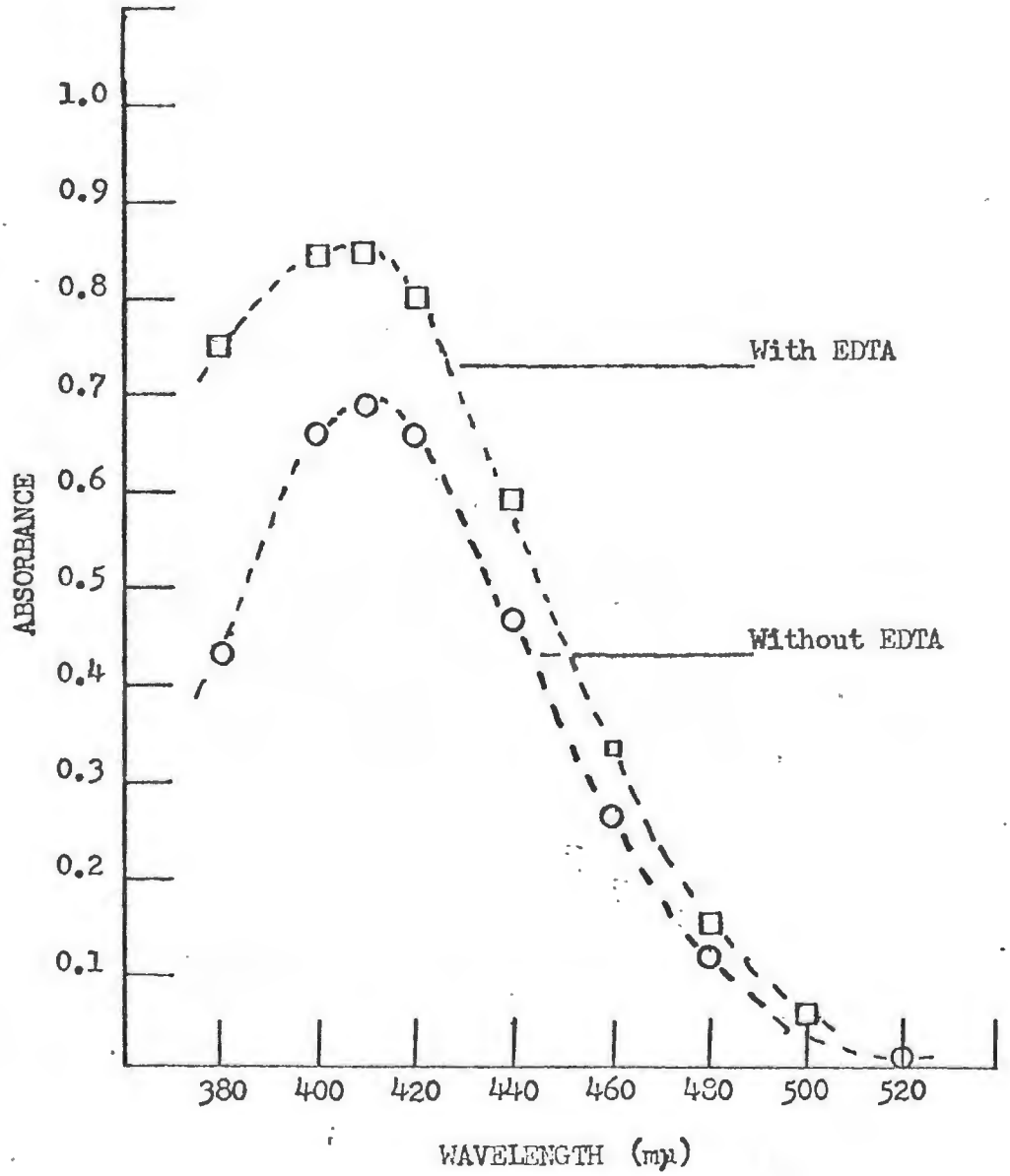
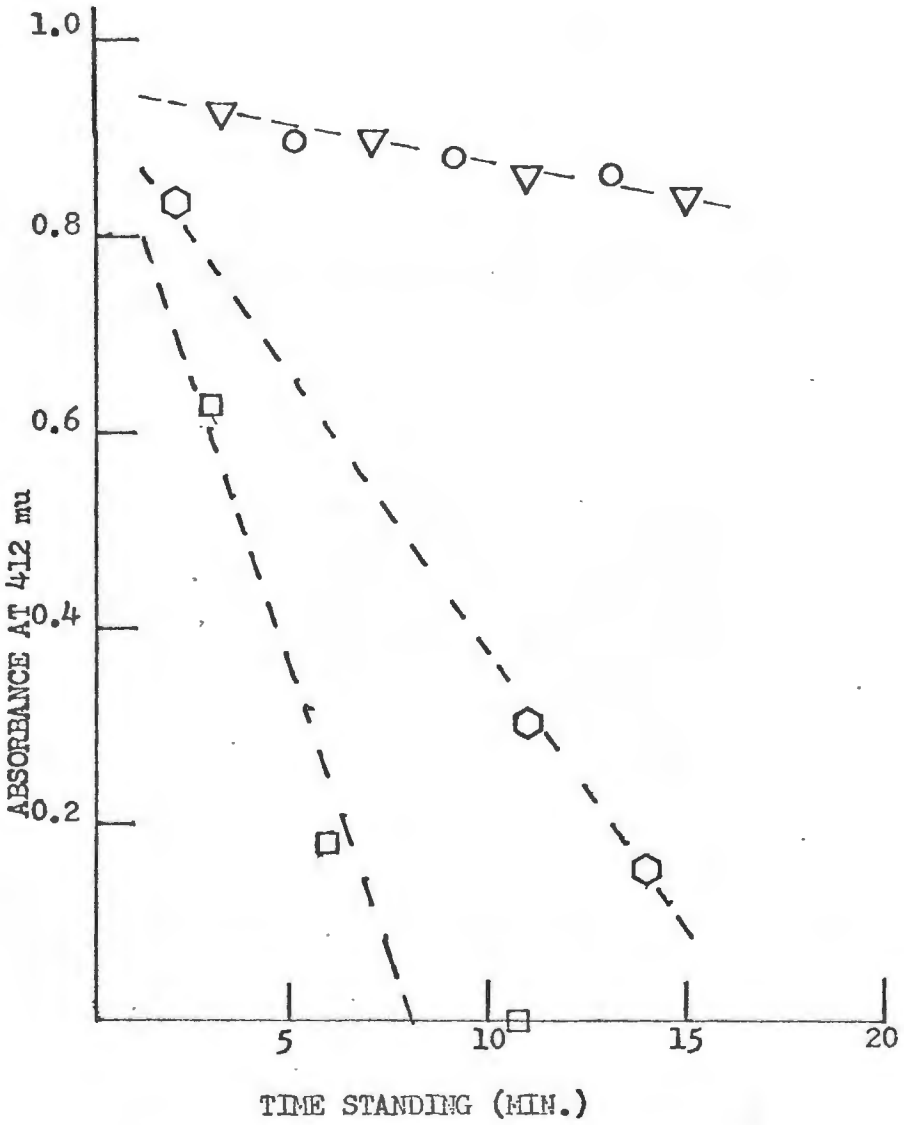


FIG. 5. EFFECT OF TIME AND ETHYLENEDIAMINETETRAACETIC ACID ON THE REACTIVITY OF CYSTEINE WITH 5,5'-DITHIOBIS (2-NITROBENZOIC ACID)



(-□-□-) 75 μmolar Cysteine pH8.0 at Room Temperature
(-○-○-) 75 μmolar Cysteine pH7.0 at Room Temperature
(-▽-▽-) 75 μmolar Cysteine pH8.2, 0.08molar EDTA at Room Temperature
(-○-○-) 63 μmolar Cysteine pH7.3, 0.15molar EDTA at 30°C.

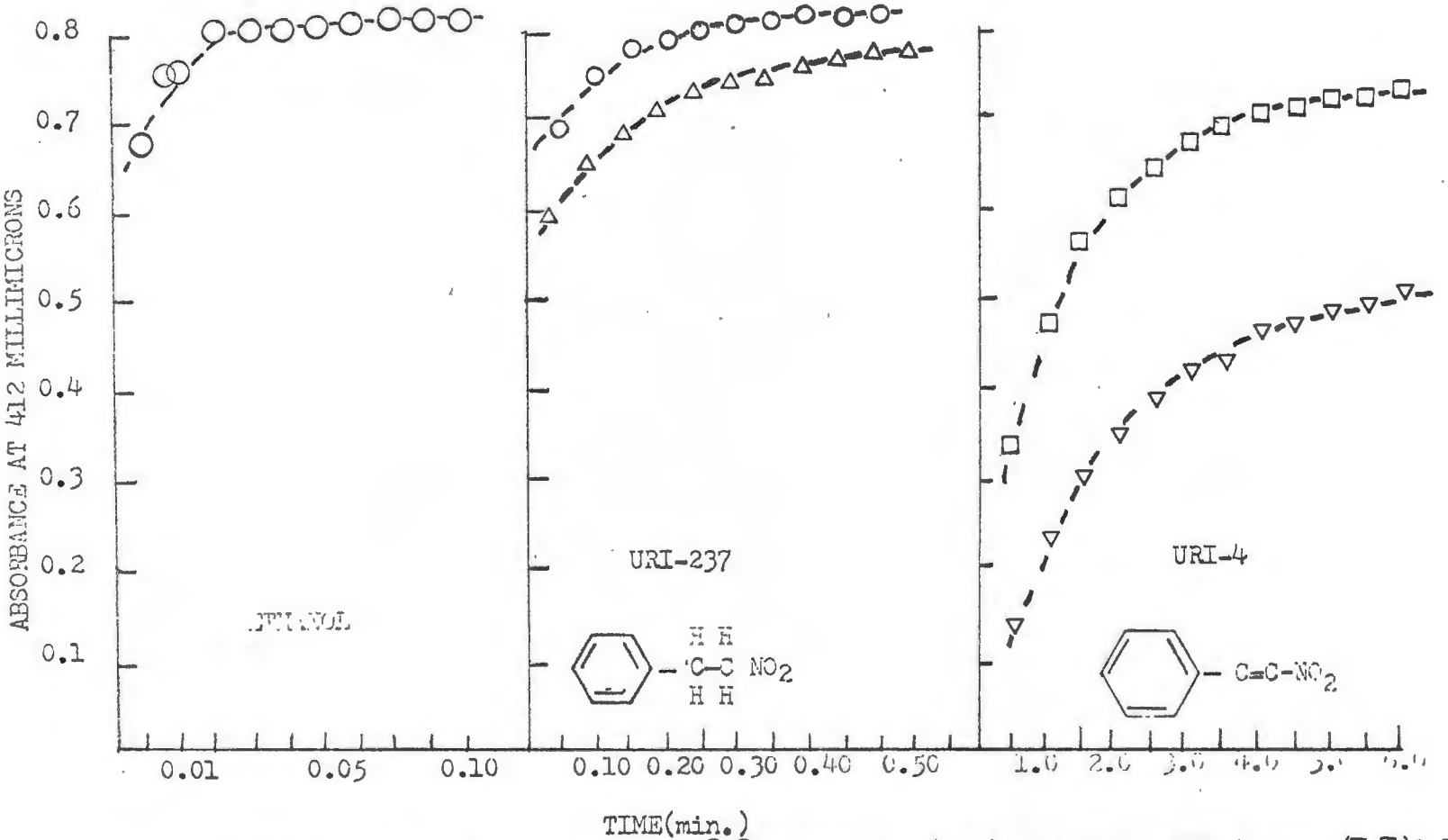
In the presence of 0.08 Molar EDTA, the absorbance obtained on the addition of DTNB to a cysteine solution which had been kept at room temperature for 15 minutes in pH 8.2 phosphate buffer was 88% of the extrapolated zero time value. It was estimated from the decay plot that the absorbance at 412 mu for cysteine solutions (incubated for 15 minutes at 30°C in 20mMolar pH 7.3 phosphate buffer) in the presence of 0.18 Molar EDTA was 93% of the initial values.

The absorbance obtained following the reaction of DTNB with cysteine in the presence of 0.18Molar EDTA was found to be linearly related to concentration in the concentration range used. The absorbance of a 1 molar cysteine solution in a 1 cm cuvette under the conditions of this assay was determined to be 13,400 absorbance units.

The development of absorption at 412 mu, following the addition of DTNB to a solution of cysteine is shown in Figure 6. The absorption approaches a maximum value within 0.03 minutes after commencement of the measurement period. In the presence of 0.13 mMolar β-nitrostyrene, the absorbance increases more gradually. An excess of DTNB does not increase the rate at which the absorbance changes in the presence of β-nitrostyrene (Figure 7). When cysteine is omitted from the reaction medium, the addition of DTNB results in an absorption of 0.015 absorbance units with 0.26mM β-nitrostyrene in the incubation medium with no further change in the absorbance. The interference with the progress of the reaction increases with increasing β-nitrostyrene concentration. In the presence of an excess of cysteine, viz. 3mMolar, the effect of β-nitrostyrene concentrations up to 0.49mMolar are not discernible.

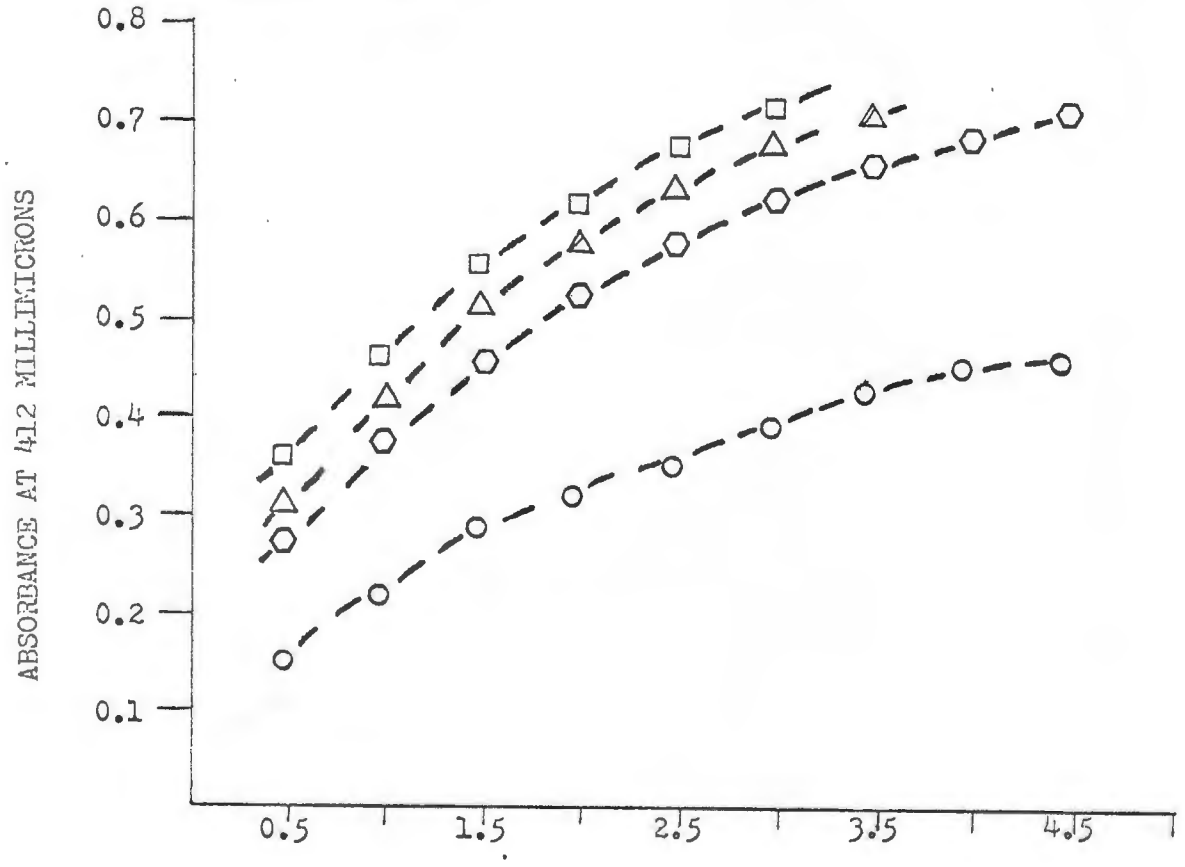
The effect of certain nitroolefin derivatives and phenylmercuric chloride on the reactivity of cysteine solutions with DTNB is shown in

FIG.6 THE EFFECT OF β-NITROSTYRENE DERIVATIVES ON COLOR DEVELOPMENT DUE TO THE REACTION OF CYSTEINE WITH 5,5'-DITHIOBIS(2-NITROBENZOIC)ACID



Cysteine conc.0.075mMolar;URI-237 conc.(○-○)0.24mMolar;(△-△-) 0.53mMolar;URI-4 conc.(□-□-)0.13mMolar (▽-▽-)0.36mMolar;(○-○-)Ethanol0.2ml/ml reaction medium.

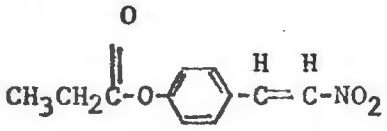
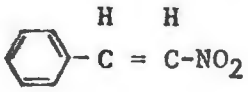
FIG. 7. THE EFFECT OF 5,5'-DITHIOBIS(2-NITROBENZOIC ACID) CONCENTRATION ON COLOR DEVELOPMENT WITH CYSTEINE SOLUTIONS IN THE PRESENCE OF β-NITROSTYRENE



Cysteine conc.0.075mMolar;β-Nitrostyrene conc.0.13mMolar;5,5'-Dithiobis(2-nitrobenzoic acid) conc.; (O-O)0.028mMolar; (⬡-⬡)0.084mMolar; (△-△)0.33mMolar; (□-□)0.66mMolar.

TABLE 1

COMPARISON OF THE EFFECT OF CERTAIN NITROOLEFINS AND PHENYLMERCURIC CHLORIDE ON THE DEVELOPMENT OF COLOR DUE TO THE REACTION BETWEEN CYSTEINE AND 5,5-DITHIOBIS (2-NITROBENZOIC ACID)

AGENT	CONCENTRATION PPM	CONCENTRATION mMolar	I/Slope(min./OD)
URI-110	30	0.13	10.1
	98	0.43	17.9
URI - 4	26	0.17	12.3
	67	0.42	23.8
	137	0.88	45.5
Phenylmercuric Chloride	3.6	0.011	143.0

Cysteine 0.017 mMolar . Phosphate pH 7.3 buffer, 0.026 M; nitroolefins added in absolute ethanol. Phenylmercuric chloride added in deionized water. 5,5-dithiobis (2-nitrobenzoic acid) 0.084 mMolar.

TABLE 2

EFFECT OF SERINE ON THE REACTION BETWEEN CYSTEINE AND 5,5'-DITHIOBIS (2-NITROBENZOIC ACID) IN THE PRESENCE OF β-NITROSTYRENE

REACTION SYSTEM	MEAN SLOPE (OD/min) [1]
Cysteine (0.086 mMolar) β-Nitrostyrene (0.083 mMolar) Phosphate buffer pH 7.3 (0.026 Molar) EDTA (0.22 Molar)	0.360
Cysteine (0.086 mMolar) β-Nitrostyrene (0.083 mMolar) Serine (0.086 mMolar) Phosphate buffer pH 7.3 (0.026 Molar) EDTA (0.22 Molar)	0.366
Serine (0.086 mMolar) β-Nitrostyrene (0.083 mMolar) Phosphate buffer pH 7.3 (0.026 Molar) EDTA (0.22 Molar)	------ [2]

[1] Values represent the mean of duplicate assays.

[2] On the addition of DTNB, the absorbance increased to 0.02 with no further change.

TABLE 3

EFFECT OF CERTAIN NITROOLEFINS ON THE RATE OF REACTION BETWEEN CYSTEINE AND 5,5'-DITHIOBIS (2-NITROBENZOIC ACID)

AGENT[1]	NITROOLEFIN FLASK CONCENTRATION	1/SLOPE (min/OD)[2]
URI-4	0.25 mMolar	3.46
URI-5	0.25 mMolar	3.61
URI-7	0.21 mMolar	3.18
URI-8	0.26 mMolar	1.66
URI-97	0.27 mMolar	1.19
URI-99	0.27 mMolar	5.56
URI-119	0.24 mMolar	2.46
URI-120	0.20 mMolar	2.54
URI-129	0.21 mMolar	5.24
URI-130	0.27 mMolar	3.50
URI-237	0.22 mMolar	----[3]

[1]Structural formulas of nitroolefins are presented in Table 7.

[2]Values represent the mean of duplicate assays.

[3]Maximum absorbance was reached within ten seconds.

Table 1. At this concentration of cysteine (0.017mMolar) the initial slope of the absorbance-time recording decreases with increasing β-nitrostyrene concentration. The presence of 3.6 parts per million phenylmercuric chloride results in a greater degree of interference with the development of absorbance at 412 mu than would be obtained with β-nitrostyrene. To simplify comparison of substances according to the ability to delay the progress of the DTNB-cysteine reaction, the reciprocal slope has been used. From Table 1 it is seen that the reciprocal slope in the presence of phenylmercuric chloride is approximately 10 times greater than that in the presence of β-nitrostyrene. Saturation of the α-β double bond of β-nitrostyrene results in a product in which the ability to interfere with the DTNB-cysteine reaction is considerably diminished (Figure 6).

The interference by β-nitrostyrene with the sulfhydryl assay was determined in the presence and absence of serine in order to estimate the specificity of β-nitrostyrene for thiol compounds under the conditions of this assay. From Table 2 it can be seen that the rate of color development resulting from the reaction between cysteine and DTNB was essentially the same in the presence or absence of serine. The effect of the nitroolefins on the sulfhydryl assay is shown in Table 3. The concentrations of nitroolefins used in oxygen uptake studies were 1.47 times greater than the concentrations used in the sulfhydryl assay.

Oxygen Uptake Studies

The uptake of oxygen by homogenates (incubated in medium 'A') in the presence of β-nitrostyrene decreases with increase in nitroolefin concentration (Table 4). The concentration of β-nitrostyrene at which the oxygen uptake approached 50% of control levels was determined from inspection of the dose-response plot to be 0.44 mg%. β-nitrostyrene was found

TABLE 4

OXYGEN UPTAKE OF WHOLE SNAIL[1] HOMOGENATES IN THE PRESENCE OF β-NITROSTYRENE AT 30°C

MEDIUM 'A'

CONC. NITROSTYRENE		60 MINUTE O_2 UPTAKE	% CONTROL
mg%	mMolar	ul	
---	------	44.0	100
0.17	0.011	41.0	93.2
0.34	0.023	25.1	57.1
0.84	0.056	10.7	24.4
1.12	0.075	3.6	8.2
1.68	0.113	7.5	17.0

MEDIUM 'B'

CONC. NITROSTYRENE		40 MINUTE O_2 UPTAKE	% CONTROL
mg%	mMolar	ul	
---	-----	36.9	100
2.4	0.15	28.3	76.7
4.8	0.30	25.4	68.8
9.6	0.60	16.9	45.8
19.2	1.20	10.5	28.5

[1] Australorbis glabratus

[2] Medium 'A' contained in a final volume of 3.2 ml per warburg flask: 6 umoles potassium fumarate, 6 umoles potassium pyruvate, 12 umoles $MgCl_2$, 3 umoles ATP, 585 umoles sucrose, 0.3 ml 0.1 Molar phosphate buffer pH 7.3 and 0.6 ml of 50 % whole snail homogenate, containing 8.4 mg/ml protein. Medium 'B' contained (in a final volume of 2.7 ml) 5 umoles ADP, 12.5 umoles succinic acid, 1.3 umoles $MgSO_4$, 625 umoles sucrose, 0.375 ml 0.1M phosphate (pH 7.3) buffer and 0.4 ml of homogenate (29.2 mg/ml Protein).

TABLE 5

OXYGEN UPTAKE[1] OF WHOLE SNAIL[2] HOMOGENATES IN THE PRESENCE OF β-NITROSTYRENE AND CYSTEINE

FLASK CONCENTRATION			FORTY MINUTE O_2 UPTAKE	% CONTROL
NITROSTYRENE		CYSTEINE		
(mg%)	(mMolar)	(mMolar)	(ul)	
-----	-------	---------	36.9± 1.7[4]	100
-----	-------	1.2	39.8± 1.9[4]	108
4.8	0.3	1.2	30.1± 1.9	81.6
19.2	1.2	1.2	21.7± 1.2	58.8
19.2	1.2	-----	10.5± 0.3	28.5

[1]Incubation medium 'B' was used in this study. The medium contained (in a final volume of 2.7 ml) 5 umoles ADP, 12.5 umoles succinic acid, 1.3 umoles $MgSO_4$, 625 umoles sucrose, 0.375 ml 0.1 Molar phosphate pH 7.3 buffer and 0.4ml of whole snail homogenate. Protein concentration was 29.2 mg protein/ml homogenate.

[2]Australorbis glabratus

[3]Values shown are the means± standard error for groups of at least three determinations.

[4]Statistically identical ($p < 0.05$)

to affect the uptake of oxygen by homogenates incubated in medium 'B' to a lesser degree. The flask concentration at which the oxygen uptake was 50% of control values was determined to be approximately 16 times that concentration of β-nitrostyrene producing the same degree of inhibition when tissue was incubated in medium 'A', viz. 7.8mg% (Table 4). In view of the reduced levels of oxygen uptake reported by Weinbach (1953) when pyruvate was used in similar studies, medium 'B' was used in subsequent studies.

When cysteine was added to the incubation medium prior to the addition of β-nitrostyrene, the levels of oxygen uptake were greater than that of homogenates in the presence of β-nitrostyrene alone. Tables 4 and 5 show that incubation of equimolar concentrations of cysteine and β-nitrostyrene resulted in mean oxygen levels which were approximately twice those of an aliquot of the same preparation in the presence of β-nitrostyrene alone. Accordingly the mean oxygen uptake of homogenates in the presence of an excess of cysteine was 10% greater than that of homogenates exposed to the same nitroolefin concentration. The mean oxygen uptake in the presence of cysteine alone was not statistically ($p < 0.05$) different from controls.

Inhibition of snail homogenate oxygen uptake was observed at nitroolefin concentrations ranging from 0.30 mMoles/ml to 0.44mMoles ml (Table 6). The oxygen uptake determined in the presence of the β-nitrostyrene derivatives was 46 to 80 percent of control values. The mean pH of pooled control media (measured at the termination of oxygen uptake determinations) was 7.3 ± 0.2 while that of media which contained nitroolefins was 7.4 ± 0.01. Ranking of the nitroolefins according to the ability to inhibit the color development of the sulfhydryl assay, and

TABLE 6

EFFECT OF CERTAIN NITROOLEFINS ON THE OXYGEN UPTAKE[1] OF SNAIL[2] HOMOGENATES

AGENT[3]	FIASK CONCENTRATION (mM)	FORTY MINUTE O_2 UPTAKE[4]
URI-4	0.37	21.0 ± 2.0
URI-5	0.37	17.7 ± 0.6
URI-7	0.30	23.5 ± 4.4
URI-8	0.38	29.1 ± 0.3
URI-97	0.40	19.2 ± 0.6
URI-99	0.39	16.8 ± 1.6
URI-119	0.36	27.1 ± 2.5
URI-120	0.30	18.0 ± 1.2
URI-129	0.32	21.0 ± 0.9
URI-130	0.40	18.9 ± 2.1
URI-237	0.33	28.9 ± 1.3
Control	——	36.4 ± 1.0

[1]Incubation medium 'B' was used in this study. The medium contained (in a final volume of 2.7 ml) 5 umoles ADP, 12.5 umoles succinic acid, 1.3 umoles $MgSO_4$, 625 umoles sucrose, 0.375 ml 0.1 Molar phosphate pH 7.3 buffer, and 0.4 ml of whole snail 50% homogenate. Protein concentration ranged from 32.8-29.2 mg protein/ml homogenate.

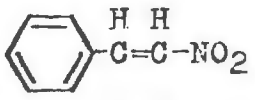
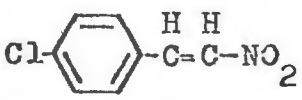
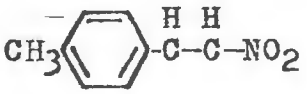
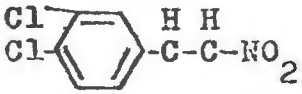
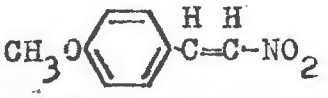
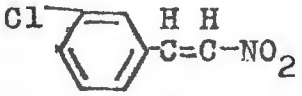
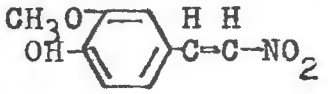
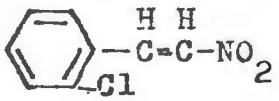
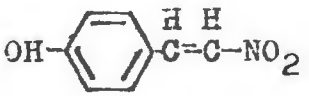
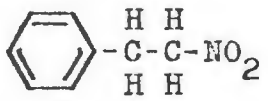
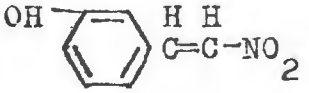
[2]Australorbis glabratus

[3]Structural formulas of nitroolefins are presented in Table 7

[4]Values represent means ± standard error for groups of at least four determinations.

TABLE 7

STRUCTURAL FORMULAS OF THE NITROOLEFINS USED

URI#	FORMULA	URI#	FORMULA
4		119	
5		120	
7		129	
8		130	
97		237	
99			

inhibit oxygen uptake indicates a positive correlation. A positive sample coefficient of correlation (0.645) was calculated when the two ranks were compared. The population coefficient of correlation was determined to be statistically different from zero at the 0.05 level of significance.

V. DISCUSSION

These data show that β-nitrostyrenes inhibit oxygen uptake of whole snail homogenates at the concentration used in these studies (300-440 μMolar). Evidence has been presented by Bovell et al., (1964) which indicates that certain β-nitrostyrenes are capable of stimulating respiration and inhibit energy dependent processes of rat liver mitochondria at concentrations up to 60 μMolar. At higher concentrations the β-nitrostyrenes inhibited respiration as well.

The inability of serine to alter the efficiency of β-nitrostyrene as an inhibitor of the DTNB-cysteine reaction indicates that the presence of equal concentrations of similar compounds possessing -OH and $-NH_2$ groups does not reduce the vulnerability of sulfhydryl compounds to interaction with β-nitrostyrene. The absence of ultraviolet spectral changes following the addition of excess serine to solutions of β-nitrostyrene provides indirect evidence that β-nitrostyrene does not interact with serine to the same extent as with cysteine.

Equimolar concentrations of cysteine have been shown to antagonize the effects of β-nitrostyrene on the uptake of oxygen by whole snail homogenates. The changes observed in the ultraviolet spectrum of β-nitrostyrene after the addition of cysteine but not observed with serine suggest that the antagonism may be due to reaction of cysteine sulfhydryl groups with β-nitrostyrene. The evidence suggests that β-nitrostyrenes inhibit the uptake of oxygen by interacting with enzymes which require intact sulfhydryl groups for normal functions.

The absorption of a 1 molar solution of cysteine following the addition of DTNB, determined under the conditions of the sulfhydryl assay (13,400), agrees with the value (13,600) published by Ellman (1959).

The data show that the inclusion of EDTA in the sufhydryl reaction medium enhances the stability of cysteine in solution at room temperature and stabilizes the color formed following the reaction of cysteine and DTNB. EDTA has not been found to produce any qualitative changes in the visible spectrum of the color developed. Ellman (1967) has reported a similar effect of EDTA on the stability of sulfhydryl compounds in solution.

Since an excess of cysteine abolishes the effect of β-nitrostyrene on the color development and excess DTNB is without effect, it is apparent that the concentration of cysteine is the limiting factor in the progress of the reaction. The development of color is progressively delayed with increasing nitrostyrene concentration. The results suggest that the delay in the development of color following the addition of DTNB to cysteine solutions in the presence of β-nitrostyrene is due to the interaction of β-nitrostyrene with cysteine. Similar effects on the DTNB-cysteine reaction have been described by Gabay et al., (1968) who has shown that the color development of solutions containing non-protein sulfhydryl material (following addition of DTNB) is delayed in the presence of protein sufhydryl groups.

A positive correlation (r=0.645) between the degree of inhibition of snail homogenate oxygen uptake, by the nitroolefins compared and the effect of these derivatives on the DTNB-cysteine reaction has been found. Thus the rate assay procedure described for the estimation of sulfhydryl reactivity appears to be a useful tool for the evaluation of sulfhydryl reactive compounds.

Comparison of molluscicidal activity (Bond, 1968) with oxygen inhibition and sulfhydryl reactivity data indicates less correlation between molluscicidal activity and the in vitro assay data. This may be due to

differences in the rate of penetration of membrane barriers, biotransformation, distribution and excretion of the β-nitrostyrenes which may be independent of the sulfhydryl reactivity of these compounds. However, in several instances some relation between the in vitro evaluation and the molluscicidal activity of the compounds is discernible. For example: the concentration of β-nitrostyrene found to be lethal to 50% of the exposed snails (LC_{50}) was determined to be 1.4 parts per million (PPM), while the LC_{50} for 4-propionoxy β-nitrostyrene (URI-110) was determined to be 15 PPM. (Bond, 1968) Phenylmercuric chloride was lethal to 30% of the exposed snails at concentrations of 0.3PPM. (Bond and Nolan, 1954) The saturated derivative of β-nitrostyrene, 1-phenyl 2-nitroethane (URI-237) was found to produce 40% lethality in snails tested at 300PPM (Bond, 1968). The agreement between the molluscicide data cited and the sulfhydryl assay values for these agents presented in Table 1 is evident.

VI. SUMMARY AND CONCLUSIONS

1) An interaction between cysteine and β-nitrostyrene was observed as evidenced by alterations in the ultraviolet spectra of β-nitrostyrene solutions following the addition of cysteine.
2) A method was devised to permit the use of the color-forming reaction between DTNB and cysteine as a rate assay for the determination of sulfhydryl reactivity.
3) The β-nitrostyrenes studied, were observed to have degrees of sulfhydryl activity when assayed under the conditions of this assay.
4) β-Nitrostyrene derivatives studied inhibit the oxygen uptake of homogenates prepared from the snail Australorbis glabratus at the concentrations used in these studies.
5) The inhibition of oxygen uptake resulting from incubation of the homogenates in the presence of β-nitrostyrene was mitigated in the presence of cysteine.
6) A positive correlation was found to exist between the effects of certain β-nitrostyrenes on oxygen uptake of snail homogenates and the effect of these compounds on sulfhydryl reactivity as determined by the ability of the β-nitrostyrene derivatives to inhibit the reaction between DTNB and cysteine.

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VITA

VITA

Robert T. Louis-Ferdinand was born in New York City on August 3, 1937, where he received his preliminary high school and college education. Following his graduation from college he served in the special service corps of the U.S. Army as a medical laboratory specialist with the Medical Research Laboratories, Ft. Knox, Kentucky and the Research Institute of Environmental Medicine, Natick, Massachusetts. Upon his separation from active duty, he accepted a position as a research biochemist at the Research Institute of Environmental Medicine until September, 1966, when he began full time graduate studies at the University of Rhode Island. He is married to the former Camille Pugliese of New York City. They have four children. He is a member of Phi Sigma, and Rho Chi.