MIPR NO: 92MM2588

TITLE: CHEMICAL AND STRUCTURAL CHARACTERIZATION OF NITROAROMATIC ADDUCTS WITH HEMOGLOBINS

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REPORT DATE: February 1, 1994

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research, Development, Acquisition and Logistics Command (Provisional), Fort Detrick, Frederick, Maryland 21702-5012

CONTRACTING OFFICER'S REPRESENTATIVE: Dr. Gunda Reddy

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	2/1/94	Final 7/15/92 - 9/30/93

4. TITLE AND SUBTITLE
Chemical and Structural Characterization of nitro-aromatic adducts with hemoglobin.

5. FUNDING NUMBERS
MIPR NO: 92MM2588

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER
USAMRD-01

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research, Development, Acquisition and Logistics Command (Provisional)
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The reactivity of dinitrobenzene (ortho-, para-, meta-DNB) with bovine hemoglobin has been studied in vitro using spectroscopic and chromatographic methods. Deconvolution of time-dependent VIS/UV spectra of mixtures of hemoglobin with DNB show that DNB reacts readily with deoxyhemoglobin to form methemoglobin without the need for metabolic activation that has been inferred from previous in vivo studies. No reaction is observed with oxy- or carboxy- hemoglobin. Column and HPLC chromatographic separations of hemoglobin treated with ^{14}C radiolabelled DNB did not produce evidence for adduct formation. Similar analyses of hemoglobin from radiolabelled DNB- and TNB-treated rats also showed no direct evidence of strong adducts.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Dinitrobenzene, Hemoglobin, Deoxyhemoglobin, Carboxyhemoglovin Nitroaromatic	55
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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A-1	

/Walter J. Stein/ 6/12/94

PI - Signature Date

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Chemical and Structural Characterization of Nitroaromatic Adducts with Hemoglobin

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Fort Detrick, MD 21702-5012

MIPR Number: 92MM2588

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I. INTRODUCTION

Covalent adducts of hemoglobin (Hb) with chemical mutagens and carcinogens have been proposed as quantitative biomarkers for exposure to these toxic compounds (Neumann, 1984; Albrecht & Neumann, 1985; Ehrenberg & Tornqvist, 1990; Tannenbaum, 1991; Bryant & Osterman-Golkar, 1991; Bechtold et al., 1992; Filser et al., 1992; Sabbioni, 1993). For many alkylating and arylating agents, the formation of Hb adducts correlates very closely with administered dose, and there is nearly a constant ratio between the reaction of these agents with tissue DNA and hemoglobin over a wide range of doses (Neumann, 1984). For example, recently, Hb adducts have been studied as markers for exposure to tobacco-specific carcinogens (Talaska et al., 1991; Foiles et al., 1992; Schlaffler et al., 1993; Hammond et al., 1993). The nitroaromatic compounds and their derivatives constitute a particularly important class of toxic substances because of their widespread use in the chemical and dye industries and their prevalence in the manufacture, use, and destruction of munitions (Hartter, 1984; Miller & Miller, 1983). Reliable biomarkers for nitroaromatics would find widespread use in situations where there is a high probability for exposure.

The toxic effects of nitroaromatic compounds and their metabolic intermediates has been well-documented (Linch et al., 1971), and includes the induction of cyanosis and methemoglobinemia in workers who have been exposed to them. Data from investigations of industrial exposure in humans and from controlled animal studies have documented the formation of methemoglobin (metHb[1]) upon inhalation, ingestion, or skin contact with nitroaromatic compounds:

$$\begin{bmatrix} Hb(Fe^{2+}) \\ deoxy\text{-}Hb \end{bmatrix} \text{ or } \begin{bmatrix} Hb(Fe^{2+})O_2 \\ oxy\text{-}Hb \end{bmatrix} \xrightarrow[n\ steps?]{\phi\text{-}(NO_2)_x} \begin{bmatrix} Hb(Fe^{3+})H_2O \\ met\text{-}Hb \end{bmatrix}$$

In humans, cyanosis resulting from inadequate tissue oxygenation becomes apparent when metHb in the blood (normally less than 1%

of total Hb species) is present at approximately 10% or more (Von Oettingen, 1941). Prior to development of effective prevention practices, metHb levels of greater than 30% were routinely observed in cyanotic plant workers (Linch et al., 1971). The reactivity of the nitroaromatics with hemoglobin encourages the isolation and identification of hemoglobin adducts as potential biomarkers for exposure to these compounds.

The objective of the current research project is to chemically and structurally characterize the formation of covalent adducts between Hb and toxic nitroaromatic compounds and their metabolic intermediates, and to find the relationship between metHb formation and adduct formation. The process of judging the potential usefulness of monitoring chemical adducts formed between Hb and nitroaromatic compounds as indicators of exposure will benefit from further knowledge of the fundamental chemistry that results in adduct formation. Knowledge of the molecular mechanism of formation of such adducts will lead to an understanding of the differences in Hb reactivity for different animal species, the relationship between the chemical composition of toxic species and their adduct-forming ability, and the dependence of adduct formation on the physical and chemical states of Hb. The reaction of nitrobenzenes and related metabolic products with Hb can depend on the source of Hb (human, rat, bovine, etc.), the isomeric form of the nitrobenzene (eg. ortho-, meta, or para-dinitrobenzene), and the chemical state of Hb (dimers, tetramers, oxy-, deoxy- carboxy-, etc.). An understanding of these dependencies and the molecular mechanism of adduct formation can be developed through the application of: 1) *in vitro* kinetic and chemical studies of adduct formation using purified Hb and (radiolabelled) nitroaromatics; 2) structural studies using protein crystallography to develop three-dimensional atomic models of the adducts; and 3) molecular modelling studies to develop a structural and electronic mechanism of adduct formation.

Ultimately, comparative studies on human, bovine, and rat

hemoglobin will reveal the chemical and mechanistic differences between species. For many Hb adduct-forming chemicals, cysteine residues on the globin have been shown to be the point of attack (Albrecht & Neumann, 1985). Consequently, differences in the number of cysteines in the Hb of various species can lead to dramatic differences in Hb reactivity (Sabbioni, 1992). For example, it has been observed that rat hemoglobin is much more reactive with nitrosobenzene than is hemoglobin from mouse (Neumann, 1988). This increased reactivity stems from two reactive α-chain cysteines in rat that do not exist in mouse. In human hemoglobin, one cysteine on the β-chain (cys-F9) has been implicated in adduct formation with various chemicals, so considerable differences may be expected in the reactivity of rat versus human hemoglobin. Most of the experiments reported here were carried out with bovine hemoglobin. In bovine Hb, there is only one cysteine, and it resides on the β-chain in a position homologous with the human Hb. Globin histidines have also been implicated in adduct formation (Beland & Poirier, 1993)and similar species differences for histidine reactivity can be expected.

The initial effort to chemically and structurally characterize adduct formation is focussed on the dinitrobenzenes (DNB), which are thought to form adducts of both the parent compounds and reduced metabolic intermediates with hemoglobin. The parent compounds (ortho-, meta-, and para-dinitrobenzene) are the first objects of investigation, since the formation of adducts with these species *in vivo* is not complicated by the action of cellular reductases. In the early phases of this research, the chemical and crystallographic studies are proceeding in parallel in order to establish a base of knowledge that will then be used to develop specific strategies for theoretical modeling and subsequent experimental verification of the mechanism of adduct formation.

As part of this project, a collaboration has been established with scientists in the laboratory of Dr. Enrico Bucci at the

University of Maryland Medical School in Baltimore. Dr. Bucci's group has extensive experience in the preparation and handling of hemoglobin *in vitro*. They have kindly assisted us in developing protocols and analyses, and have also provided us with fresh, highly-purified samples of bovine hemoglobin which have been used in most of the work that is described below.

II. BACKGROUND

A. Formation of methemoglobin *in vivo* and *in vitro*

There have been a number of studies of reactivity of nitrobenzenes and related compounds in animals, animal tissues, whole blood, and hemolysates. Oral administration of 67 mg/kg meta-dinitrobenzene (*m*-DNB) to rats (Senczuk et al.,1976) dramatically illustrated the metHb-forming properties of the compound. At this dose, a blood level of metHb over 50% occurred within 1 hr, and the total level reached a maximum of 80% at 4 hr. Additional oral studies using rats, monkeys, and mice have yielded similar results (Cossum & Rickert, 1987; Facchini & Griffiths, 1981). When Cossum and Rickert (1987) incubated washed erythrocytes from rats, monkeys, and humans with 100 μM concentrations of ortho-, meta-, and para-DNB (*o*-, *m*-, and *p*-DNB), they observed accelerated formation of metHb in every case, with a considerable degree of variation depending upon species and chemical isomer. Their observation that the *o*- and *p*-DNB isomers formed S-(nitrophenyl)-glutathione conjugates in the presence of erythrocytes, while *m*-DNB gave no such potentially urine-clearable conjugates may be of significance in explaining the relatively prolonged recovery period for chemical plant workers who experience cyanosis as a result of exposure to the DNB meta-isomer (Linch et al., 1971).

Several other *in vitro* studies using hemolysates and whole blood have demonstrated that incubation with arylnitro compounds

can lead to metHb formation. Kusumoto and Nakajima (1970) reported the formation of 15% metHb in rabbit hemolysates after 5 hr in the presence of 5mM nitrobenzene, which was about 10% above the background oxidation level for this time period. Similar results were obtained by Watanabe and coworkers (1976) using rat hemolysate in the presence of *o*-, *m*-, and *p*-dinitrobenzenes. Finally, Facchini and Griffiths (1981) observed dramatic levels (25-90% of total hemoglobin) of metHb formation upon incubation of whole rat blood with 2mM dinitro-benzenes.

While nitroaromatic compounds are have been shown to induce methemoglobinemia *in vivo*, the exact chemistry has yet to be established. Prior work with nitroaromatic compounds, specifically nitrobenzenes, showed a great propensity for inducing methemoglobinemia *in vivo*, though not *in vitro* (Filser, et al., 1992). These studies compared the activities of nitrobenzene and a variety of nitrobenzene derivatives. The addition of a second nitro group increased the effect, though adding a third nitro group showed no further increase in metHb formation. The addition of methyl groups were shown to decrease this activity. *In vivo* activity is complicated by metabolic enzymes (such as glutathione-S-transferase, GST) which reduce nitrobenzenes to amines through nitrosobenzene and phenylhydroxylamine intermediates (Sabbioni, 1993). Phenylhydroxylamines can directly oxidize oxyhemoglobin (HbO_2) to metHb and are reduced to nitrosobenzenes in the process. The nitrosobenzenes can in turn be enzymatically reduced back to the hydroxylamine form. So, the most probable hemoglobin-oxidizing forms of these nitrobenzene compounds would be their hydroxylamine derivatives. This could imply a necessary "activation" step before the nitrobenzene species can induce metHb *in vivo*, but, as will be demonstrated below, such activation has not been found necessary in the present work.

B. Formation of adducts with hemoglobin *in vivo* and *in vitro*

Using denaturing electrophoresis, Goldstein and Rickert (1984) demonstrated that 82% of the radioactivity bound to macromolecules following a single oral dose of [^{14}C]nitrobenzene to rats was associated with the polypeptide portion of hemoglobin. The finding of polypeptide-bound adducts is significant, since this rules out labile heme-associated Fe-nitroso- or Fe-hydroxylamine complexes as the candidate adducts (Jackson & Thompson, 1954; Gibson, 1960; Farnell & McMeekin, 1973). The authors conclude that "the specificity of binding of orally-administered nitrobenzene to hemoglobin may suggest that hemoglobin is catalyzing the generation of a reactive intermediate."

Jackson and Thompson (1954) explored the reactions of p-phenylhydroxylamine and radioiodinated iodonitrosobenzene with purified hemoglobin. They determined that, under certain conditions, combination between either of these compounds with hemoglobin may occur without an accompanying change in the visible absorption spectrum. In light of subsequent work, one interpretation of this observation is that non-heme covalent adducts are formed between the protein and these metabolite analogs.

Reddy and coworkers (1991), reported Hb adducts in rats treated with [^{14}C]-1,3,5-trinitrobenzene. To date, the nitrobenzenes have not been observed *in vitro* to induce formation of methemoglobin, so it remains to be shown whether the observed adduct formation is directly correlated with methemoglobin formation. These studies provide a strong rationale for carrying out the present *in vitro* experiments involving adduct formation between purified hemoglobin and nitroaromatic compounds and/or their reduced metabolites.

C. Special Considerations for *In Vitro* Studies

There are a number of properties of Hb which affect its

chemical reactivity. The rate of Hb oxidation and adduct formation can be affected by the physical state of the protein. Zhang, et al. (1991) recently showed that the rate of autoxidation of Hb is enhanced by dissociation into dimers, presumably because dissociation affects the conformation of the protein and the exposure of the heme to oxidizing species (Perutz, 1989) . In the current experiments, the Hb concentration was initially maintained above 1 mg/mL, which is sufficient to maintain the tetrameric form of the protein (Antonini & Brunori, 1971). Removal of O_2 to form dxHb further stabilizes the tetrameric form. However, dilution of the sample prior to the spectroscopic measurements could enhance dissociation to dimers, but it is difficult to see how this could impact the relative reactivity of dxHb, HbCO and HbO_2.

There are also naturally occurring Hb variants (e.g. $\alpha_2\delta_2$) which may have different reactivities with nitroaromatic compounds than Hb A, and which may introduce artifactual results. Therefore, clean, homogenous Hb A is needed for these experiments. Purification by ion-exchange chromatography maintains the integrity of the chains, though probably as dimers.

Hb has a tendency to autoxidize to metHb, so autoxidation has to be distinguished from oxidation due to the test compounds. This can be achieved by having a control sample of Hb with water in place of test compound, and monitoring the autoxidation of this control in parallel with the oxidation induced by the test compounds. Also, the amounts of metHb present in the initial Hb samples should be minimized.

D. Experimental Approach

Initial experiments were designed to determine the conditions under which metHb is formed from the reaction of DNB with bovine Hb. Pure samples of bovine Hb A were prepared, and the reactivity of various isomers of DNB with Hb in various states (i.e. oxyhemoglobin, HbO_2, carboxyhemoglobin, HbCO, and deoxyhemoglobin, dxHb) was tested to determine whether metHb was

formed in the process. The formation of metHb over time was monitored by uv-vis spectrophotometry. Previous studies have shown that the distinct differences in the absorption spectra of the different Hb types (i.e. metHb, dxHb, HbCO and HbO_2) allow the deconvolution of the spectra of hemoglobin mixtures and the detection of the time-dependent formation of metHb.

Adduct formation was monitored by utilizing radiolabeled DNB compounds, which, when bound, can be followed through a series of reverse-phase HPLC purifications on whole and proteolyzed hemoglobin chains. Ultimately, proteolyzed globin fragments can be identified and localized by amino acid analysis and N-terminal sequencing. The adducts can then be characterized by mass spectrometry and NMR spectrometry.

It is important to apply the same procedures used for detecting adducts *in vitro* to samples of Hb that have been subjected to DNB *in vivo*. Dr. Tirimuru V. Reddy (U.S. EPA, Cincinatti, OH) kindly supplied Hb samples from rats that were treated with radiolabelled 1,3-DNB and 1,3,5-trinitrobenzene (TNB). These samples were analyzed by the same chromatographic methods used for the *in vitro* samples of Hb with radiolabelled DNB.

In parallel with the spectroscopic and chromatographic studies, crystallization of rat Hb was initiated in preparation for X-ray crystallographic studies of adduct structures. Crystallographic characterization of rat Hb is particularly important, since the structural characterization has never been attempted, and differences between the rat and human proteins could have significant consequences for toxicological studies that use rat models.

III. MATERIALS AND METHODS

Bovine Hb. The purified bovine Hb used in the spectroscopic assays was generously provided by Dr. Clara Fronticelli

(University of Maryland at Baltimore, Department of Biochemistry). An ion-exchange procedure has been developed for the purification of bovine Hb using a 20 mM MES to 20 mM MES/ 150 mM NaCl gradient on a Millipore SP1000 column. Using this system, an almost baseline-resolution separation of Hb was achieved (Figures 1-4). Samples of metHb (Figure 2) and HbO_2 (Figure 3) were run separately and mixed (Figure 1) in order to determine their individual purities and their combined contributions to the mixture. Sodium dithionite ($Na_2S_2O_4$) was added to the HbO_2 to reduce any metHb formed by autoxidation (Figure 4). At this time, a small preparative scale purification was attempted (Figure 5), but dilution-induced dissociation of both the subunits and heme was observed.

Preparation of Specific Forms of Hb. The first step in each experiment was to convert HbO_2 to either dxHb or HbCO. A 50 mL round-bottom flask, containing 15-17 mg (86-98 μmol) sodium dithionite and a magnetic stir bar, was sealed with a rubber septum and purged with either nitrogen or carbon monoxide. One mL of 20 mM Tris buffer (pH 8.0), which was purged with the appropriate gas, was added to the dithionite. The gas, which was hydrated by bubbling it through a water trap, or in the case of carbon monoxide, through 0.2 M NaOH and then 0.2 M citrate, was allowed to flow over the dithionite solution for 60 min. An injection of 65 μL HbO_2 (71.6 mg/mL) was made into the dithionite solution, resulting in a 4.6 mg/mL solution of either dxHb or HbCO, depending upon the gas used. The resulting Hb solution was then allowed to gently stir under the hydrated gas flow for 60 min. The Hb solution was diluted to 25 mL by transferring 20 mM purged Tris (pH 8.0) into the flask by cannula. This solution was then stirred and bubbled with the appropriate gas for 60 min.

Para-, Meta, and Ortho-DNB. Dinitrobenzene was obtained from Aldrich Chemical Company: *p*-DNB: 10,236-9, Lot 12902TY; *o*-DNB: 30,206-6, Lot 03926HH; *m*-DNB: D19,425-d, Lot 01817TJ.

Preparation of Samples for Spectroscopic Study. Two cuvettes were sealed with rubber septa and flushed with the appropriate

gas for 10-15 min. Using cannula, 1.5 mL of the Hb solution was transferred into each cuvette, followed by the addition of either 0.5 mL 20 mM Tris, for the reference cell, or 0.5 mL saturated *p*-DNB (ca. 476 μM) in 20 mM Tris (pH 8.0), for the sample cell. The spectra of both the reference and sample were taken against deionized, distilled water at 20° C.

Spectroscopy. A Varian Cary-4 UV/VIS spectrophotometer[2] was used to monitor changes in the Hb spectra over time. The absorbance between 350 and 700 nm was monitored using a spectral band width of 2.00 nm, a 0.500 s signal averaging time, a 1.000 nm data interval and a scan rate of 120.000 nm/min. A temperature controller was used to maintain a 20° C temperature over the course of the experiment, except for overnight time points, where the cuvettes were kept at 4° C overnight and then raised to 20° C 5 min before reading the spectra. The quartz cuvettes had a 1 cm pathlength.

Deconvolution of Spectra. Extinction coefficients (provided by Dr. Clara Fronticelli, University of Maryland at Baltimore, Department of Biochemistry) of each of the Hb species (dxHb, HbO_2, metHb, and HbCO, at 1 nm intervals from 350 nm to 700 nm) were utilized in a least squares fit of the observed spectra at each time point. The fit was obtained by reducing the Beer's Law equations at each wavelength to k simultaneous equations, where k is the number of applicable Hb species (3 or 4), and solving for the species concentrations by minimizing

$$\sum_i g_i = \sum_i (f_i - g_i),$$

where g_i is the observed absorbance at each wavelength, i, and f_i is the composite Beer's Law absorbance at each wavelength,

$$f_i = \sum_k c_k x_{ik} l.$$

The concentrations of each Hb species, c_k, are the values being sought, l is the pathlength, and χ_{ik} is the extinction coefficient for each species, k, at the particular wavelength, i.

Radiolabeled DNB. A custom synthesis of [^{14}C]-1,3-DNB was

ordered from New England Nuclear (Lot #2800-109). The DNB had a radioactivity of 5.0 mCi and a specific activity of 3.0 mCi/mmol.

In Vitro reaction of Hb with [^{14}C]-m-DNB. Bovine dxHb was generated as in the spectroscopic studies by transferring 1.0 mL of 8.9 mM Hb into a sealed 50 mL flask with 15 mg dithionite and stirring the solution under nitrogen for 2 h. A 25 mL flask containing 3.0 mg [^{14}C]-*m*-DNB was purged with nitrogen for 2 h. The dxHb was transferred to the flask with the labeled DNB and allowed to stir at room temperature for 2 days.

Rat Hemoglobin Fractions. Dr. T.V. Reddy (U.S. EPA, Cincinatti, OH) prepared lyophilized heme and globin fractions of rats that had [^{14}C]-*m*-DNB introduced *in vivo*. He also provided a lyophilized globin fraction from rats with [^{14}C]-1,3,5-TNB introduced *in vivo*. A portion of the lyophilized heme material was dissolved in deionized, distilled water for separation by HPLC at both pH 2.0 and 7.0 conditions. Both DNB and TNB globin fractions were dissolved in 0.05% TFA/water for the pH 2.0 separations, and in deionized, distilled water for the pH 7.0 separations.

Heme Standard. An HPLC standard of heme was prepared by dissolving bovine hemin chloride (Sigma H-2250, Lot 33H0829) in deionized, distilled water and adding ammonium hydroxide dropwise until it dissolved.

Chromatographic Separations. A Sephadex G-25 (Pharmacia, Medium Grade, Lot NE04212) column (2 x 4.5 cm) was poured for the separation of excess [^{14}C]-*m*-DNB from bovine Hb. The HPLC system used in the analytic separations consisted of two Waters Model 510 pumps, a Waters U6K injector, a Waters 490E multiwavelength detector and a Berthold LB507A radioactivity monitor with a Z1000 flow cell. A Berthold LB5035 pump was used to mix scintillant with the eluate for detection of the labeled compounds. The detector outputs were connected through a Waters system interface module, which allowed for the control and acquisition of data on a PC running Waters Maxima software. Vydac reverse-phase C4 (1.0 x 25 cm) and C18 (0.46 x 25 cm) columns were used for the

separations.

The separations under the pH 2.0 conditions used 0.05% TFA/water as solvent A and 70% acetonitrile/0.05% TFA/water as solvent B. The separations under the pH 7.0 conditions used 0.1 M triethylamine titrated to pH 7.0 with acetic acid as solvent A and acetonitrile as solvent B. The HPLC procedure used for separating heme at pH 2.0, Heme1, is a 1.0 mL/min flow with a linear gradient from 45 to 100% solvent B over 14 min. The separation procedure for heme at pH 7.0, Heme2, is a 1.0 mL/min flow with two linear gradients of 0 to 10% solvent B over 6 min and 10 to 70% solvent B over 9 min. Globin separations at pH 2.0, Globin1, were done with a 6.0 mL/min flow using a series of linear gradients from 0 to 50% solvent B over 9 min, from 50 65% solvent B over 20 min, and from 65 to 100% solvent B ove min. The pH 7.0 globin separations, Globin2, used a 6.0 mL/min flow with a series of linear gradients from 0 to 3% solvent B over 9 min, 3 to 50% over 20 min, and 50 to 70% solvent B over 2 min.

Rat Hb for Crystallization Studies. The rat Hb was a lyophilized preparation from Sigma Chemical Co. (#H3883, Lot 70H9309).

Crystallization of Rat Hb. Growing crystals of dxHb requires a deoxygenated atmosphere. A glovebox with a constant nitrogen flow was constructed and fitted with a special microscope for monitoring crystal growth. The nitrogen was passed through a Matheson oxygen trap before allowing it to enter the glovebox.

The crystals were prepared using a protocol based on the batch method protocols of Perutz (1968). The crystals were grown using two buffers of 80% 4.0 M ammonium sulfate, 5% ammonium phosphate monobasic, and 15% ammonium phosphate dibasic, adjusted to either pH 6.5 or 7.5 with ammonium hydroxide. A 0.5 M solution of ferrous citrate was also prepared. All solutions were bubbled with nitrogen for at least 60 min after being transferred into the glovebox.

IV. RESULTS AND DISCUSSION

p-DNB with HbO_2. DxHb was generated prior to generating HbO_2 in order to reduce any metHb to the ferrous (Fe^{2+}) state. The *p*-DNB was added to the sample cuvette, the septum was removed, then air was introduced with a pipet to generate HbO_2. There was a gradual change in the spectra over time for both the sample and reference cells (Figures 6 and 7). Spectral deconvolutions showed a slow increase in the concentration of metHb in both the sample and reference solutions, which was accompanied by a proportional drop in HbO_2 over the 48 h experiment. More metHb was present at start in the sample cuvette, but the overall rate of increase of metHb was comparable to that of the reference cell.

The slow increase of metHb can be ascribed to autoxidation. The higher initial concentration of metHb in the sample cell is probably due to a rapid initial reaction with dxHb prior to achieving *equilibrium* with O_2. The samples were prepared by first treating with N_2 and dithionite to remove any oxygen and to reduce any metHb present. The *p*-DNB was added prior to exposing the solution to oxygen, and the conversion to metHb could be competitive with oxygen binding. It is also possible that residual dithionite in the cell converted HbO_2 back to dxHb, which then reacted with *p*-DNB. This initial increase in metHb concentration does not affect the rate of formation of metHb over the remaining course of the experiment.

p-DNB with HbCO. The spectra of HbCO showed no significant changes over the course of 70 h, with or without *p*-DNB, in both the Soret regions and between 450 and 700 nm (Figures 8 and 9). Comparison of the sample and reference spectra showed no significant differences, and the spectral deconvolutions were virtually identical. The Hb was primarily in the HbCO state, but there was a constant background concentration of about 25% metHb, most likely due to incomplete reduction during the initial

preparation using sodium dithionite. There was no detectible conversion of HbCO to another Hb form throughout the 70 h experiment.

From these results, it is clear that *p*-DNB does not react with the heme when the iron is strongly bound to another ligand. CO induces an allosteric change in the protein similar to that of O_2, but, since it has a tighter binding affinity for the heme, it should maintain the integrity of the tetrameric state better when the sample is diluted for spectroscopic studies. HbCO does not react with dithionite, so, once CO is bound to the heme iron, there is no way for it to be removed to allow reaction of the heme with DNB. This may explain why there is no early increase in the amount of metHb as was observed in the initial stages of the O_2 experiment.

p-DNB, o-DNB, and m-DNB with dxHb. The time course of the experiment began immediately after the DNB was added to the sample cell and the UV/VIS spectrum of the dxHb was acquired. For *p*-DNB there was a noticeable change in the Soret region of the sample, showing almost complete conversion of dxHb to metHb within the first 4 hours (Figures 10), with virtually undetectable amounts of HbO_2 present throughout the experiment. Examination of the region from 450 to 700 nm also confirmed the conversion. Isosbestic points can be identified at about 360, 410, 455, 520, 600 and 645 nm, implying that only two species contributed to the changes in the spectra.

The reference cell spectra (Figure 11) show a slow transition of the dxHb to HbO_2, which was virtually complete at 18 h. This is indicative of a slow leak of the cuvette seals. A small buildup of metHb (10%) was also observed in the reference cell, presumably due to auto-oxidation of HbO_2. Comparing the spectra of the reference and sample cells illustrates the significant differences between the spectra of HbO_2 and metHb. This data also demonstrates that the dxHb converted directly to metHb without going through the HbO_2 species.

The dxHb also showed reactivity towards both *o*-DNB and *m*-DNB.

The reaction proceeded with a rate similar to *p*-DNB, and the conversion to metHb was about 90% complete within 200 min.

Effect of Dithionite. The effect of sodium dithionite on the interaction of DNB with dxHb was tested by generating dxHb using only a nitrogen flow (120 min) to displace the oxygen from HbO_2. The *p*-DNB was then added, as before, and the spectra were taken over time (Figures 12 and 13). Without the use of dithionite there was a noticeable amount of metHb present at start, but a rapid increase in the amount of metHb was seen in the presence of *p*-DNB but not in the reference cuvette. The confirms the ability of DNB to induce metHb formation.

Consistency of the Spectroscopic Deconvolutions. The consistency of the spectral deconvolutions can be checked by summing the populations of Hb subspecies to obtain the total concentration of Hb. When this was done, the total Hb concentration was found to decrease as a function of time as illustrated in Figures 6-13. The decrease is not consistent with extinction coefficient errors, since there is no proportional correlation between the decrease in total Hb and either the formation of metHb or the decrease in concentration of another species (unpublished results). However, the metHb spectrum is sensitive to pH changes, and a slight contribution from this effect cannot be ruled out. A more likely possibility is that the Hb is denaturing, particularly when in the metHb form, and the dissociation of the heme causes an apparent loss of total Hb. However, this phenomenon does not alter the observations or interpretations of DNB reactions with Hb.

Search for In Vitro Adduct Formation. Bovine DxHb was generated and allowed to react with the radiolabeled DNB for 2 days. A 200 μL portion of the Hb solution was added to a Sephadex G-25 column (2 x 4.5 cm). The 1.0 mL fractions were collected and counted. The Hb eluted in fractions 2-4, but the label eluted in fractions 2-12 (Figure 14). Fractions 2-3 were pooled and re-applied to the G-25 column. The Hb eluted in fractions 1-3 and the label eluted in fractions 1-9 (Figure 15).

Fraction 2 was examined by HPLC on a C4 reverse-phase column using a linear gradient (Figure 16). The eluate was examined at 230, 280, and 540 nm, and screened for the radiolabel. The radiolabel eluted at about 2.0 min, while the first peak at 280 nm eluted at 3.0 min. A peak eluting at 22 min absorbed at 230, 280 and 540 nm. The two peaks at 24 and 28 min absorbed at 280 nm, but not 540 nm, and most likely correspond to the α and β chains.

There was no indication that the radiolabelled fractions are associated with the protein. However, there is still the possibility that an acid-labile adduct has been lost during the chromatographic steps. Acid labile adducts have been reported previously in an *in vivo* study of nitrobenzene (Albrecht & Neuman, 1985). It is also possible that the second nitro group in DNB destabilizes any adduct bond, but this has not been investigated.

Search for In Vivo Adducts with DNB. A) Heme-Associated Adducts. The heme sample from the DNB-treated rats was separated by HPLC on a C18, reverse-phase column at pH 2.0 conditions using the Heme1 gradient (Figure 17). The eluate was evaluated at 230, 280, and 540 nm and screened for the ^{14}C label. A peak eluting at about 13 min absorbed at all three frequencies, which is characteristic for heme. The elution time corresponds to heme standard. A separate ^{14}C labelled compound eluted sharply at 5 min with no absorbance at 540 nm. Thus, while the radiolabelled compound apparently separated jointly with the heme during the fractionation procedure, the HPLC results show that the label is not strongly associated with the heme.

Search for In Vivo Adducts with DNB. B) Globin-Associated Adducts. The globin sample from the DNB-treated rats was separated by HPLC on a C4 reverse-phase column at pH 2.0 conditions using the Globin1 gradient (Figure 18). The eluate was examined at 230, 280, and 540 nm, and screened for the radiolabel. The label eluted in a series of peaks from 1.5 to 2.8 min, with a little absorbance at 230 nm, but no corresponding

absorbance at 280 or 540 nm. The first protein absorbance at 280 nm was at 5.3 min with the major peaks, most likely corresponding to the α and β chains, eluting at 18 and 22 min. Due to concerns about acid-lability, a second globin fraction was separated under pH 7.0 conditions on the C4 column using the Globin2 gradient (Figure 19). The peaks were broader and resolution was lower than what was observed at pH 2.0. The first 280 nm protein absorbance was at 4.8 min while the radiolabel eluted separately between 2.0 and 4.2 min. There was a weak signal detected at 5.0 min from the radioactivity monitor, but it was barely above the noise level. The major 280 nm peaks begin eluting at 10.2 min., well after the radiolabel. Thus, there is no evidence for strong covalent adducts in this case.

Search for Globin-Associated Adducts with TNB. The globin sample from the TNB-treated rats was separated at pH 2.0 conditions on a C4 HPLC column using the Globin1 gradient (Figure 20). The bulk of the radiolabel eluted at 1.6 min, but the signal persisted until 4.0 min. The first 280 nm protein peak began eluting at 3.5 min, though upon close examination of this area of the chromatograph is apparent that the radiolabel peaks fall between the 280 nm protein peaks (Figure 21). The sample was examined at pH 7.0 conditions on the C4 column using the Globin2 gradient (Figure 22). The label eluted by 2 min, and the 280 nm absorbance eluted later. There did not appear to be any specific absorbance at 280 nm which eluted simultaneously with the label, again implying no strong covalent adducts.

Rat Hb Crystallization. Crystallization of human hemoglobin is being carried out in the laboratory as part of another project. The best human dxHb crystals grew in 48 h using 77.5% of the pH 6.5 buffer, 4.2% water, 1.7% 0.5 M ferrous citrate, and 16.7% of 70 mg/mL human Hb. Rat dxHb crystals for the current project were grown under the same conditions as the human crystals except that the Hb concentration was 50 mg/mL and the pH 7.5 buffer was used. The rat dxHb crystals grew within 48 h.

The rat HbO_2 crystals were grown using hanging-drop methods.

The well contained 0.775 μL of the pH 7.5 buffer solution and 42 μL water. The drop consisted of 5 μL 50 mg/mL Hb and 5 μL of the well buffer. Snowflake-shaped crystals formed within 48 h. The crystals were crushed and seeded into a 5 μL drop of 10 mg/mL Hb with 5 μL of 62% pH 7.5 buffer, over a well of 0.50 mL pH 7.5 buffer and 0.31 mL water. These crystals were more uniform and grew within 48 h (Figure 23). The crystals are not yet large enough for diffraction studies.

V. CONCLUSIONS

The data from these experiments support the conclusion that *p*-, *o*-, and *m*-DNB can oxidize dxHb to metHb directly, without the prior metabolic activation that has been inferred from *in vivo* studies. The effect of the three isomers appears to be identical. The exact mechanism of oxidation is not known, but the absence of DNB adducts or any reaction products bound to either the heme or the globin suggests that a direct redox reaction (electron transfer) occurs between the heme-bound iron and the DNB without the formation of a strong Hb/DNB complex. The negative results for HbCO show that DNB cannot displace the carbonmonoxide ligand, and that CO prevents oxidation of the heme iron, either by raising the oxidation potential relative to dxHb or by preventing the close approach of DNB to the iron. In the case of HbO_2, some iron oxidation was observed, but this can be explained by the activity of excess dithionite in the solution, or reaction with DNB prior to establishing an equilibrium with the added O_2.

The chromatographic separations following the reaction of DNB with dxHb gave no evidence for the formation of stabile adducts. The direct oxidation of dxHb by DNB without the accompanying formation of a stabile complex is consistent with the recent studies of Dickerson, et al. on the autoxidation of iron(II) cyclidene models of heme-bound iron (Dickerson, et al., 1993). In their analysis of sterically-restricted cyclidene models, the

O_2 that oxidizes the iron is not bound to the heme. O_2 binding is actually found to be competitive with the autoxidation process. This interpretation was shown to be consistent with the observed O_2 partial-pressure-dependence of the rate of autoxidation of Hb or myoglobin. The conclusion is that iron oxidation occurs through an electron transfer process to an unbound oxidizing molecule (DNB in this case), but only if there is no ligand (O_2 or CO in this case) bound to the iron.

The preliminary analysis of hemoglobin from *in vivo* rat experiments is consistent with the *in vitro* studies. There is currently no direct evidence that DNB or TNB forms adducts with Hb. In fact, examination of the work of Albrecht and Neumann (1985), shows only circumstantial evidence for a covalent adduct of nitrobenzene with Hb. They isolated a crude Hb preparation which contained their radiolabel. After separating the heme from the globin with HCl/acetone, they lost 50% of their label to the supernatant, and the remaining label precipitated with the globin. After treatment with 0.1 M aqueous HCl, the label was solubilized and they found that the labeled compound had aniline-like mobilities in gas chromatography and thin-layer chromatography. They never provided any structural evidence of a covalent bond between the adduct and the Hb.

Several additional experiments are needed before a final conclusion can be reached on the ability of DNB to form stable adducts with Hb. A thorough chemical characterization of Hb from DNB-treated rats should be done while maintaining pH as close as possible to 7.0. The spectroscopic and radiolabelled chromatographic procedures used in the current set of experiments should be repeated for a compound like phenylhydroxylamine, which has been shown to form adducts with Hb. The rat Hb crystallization studies should be continued so that the structural differences between rat and human Hb can be established. Crystal soaking experiments should be performed with various compounds in order to attempt a structural characterization of both covalent and non-covalent adducts.

Finally, a series of structural characterization experiments should be performed to define the differences in reactivity of rat, human and bovine Hb when exposed to various alkylating and arylating substances.

ACKNOWLEDGEMENTS

The authors are grateful for the contributions of Dr. Matthew Mauro and Dr. Edith Grabbe in the early stages of this work, and useful discussions and advice from Dr. Clara Fronticelli. The crystallization experiments were carried out by Maria Tordová.

FOOTNOTES

[1]Abbreviations: DNB, dinitrobenzene; *p*-DNB, para- or 1,4-dinitrobenzene; *m*-DNB, meta- or 1,3-dinitrobenzene; *o*-DNB, ortho- or 1,2-dinitrobenzene; TNB, trinitrobenzene; Hb, hemoglobin; metHb, methemoglobin; dxHb, deoxyhemoglobin, HbO_2; oxyhemoglobin; HbCO, carboxyhemoglobin.

[2]Certain commercial equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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FIGURE CAPTIONS

Figure 1: HPLC chromatograph of a mixture of met- and oxyhemoglobin. A 15-40% gradient elution of Buffer A, 20 mM Mes (pH 6.0), to Buffer B, 20 mM Mes with 150 mM NaCl (pH 6.0), over 38 min on a MemSep SP 1000 cartridge was applied at a 2.0 mL/min flow rate. The eluent absorbance was monitored at 280 nm.

Figure 2: HPLC chromatograph of methemoglobin. A 15-40% gradient elution of Buffer A, 20 mM Mes (pH 6.0), to Buffer B, 20 mM Mes with 150 mM NaCl (pH 6.0), over 38 min on a MemSep SP 1000 cartridge was applied at a 2.0 mL/min flow rate. The eluent absorbance was monitored at 280 nm.

Figure 3: HPLC chromatograph of oxyhemoglobin. A 15-40% gradient elution of Buffer A, 20 mM Mes (pH 6.0), to Buffer B, 20 mM Mes with 150 mM NaCl (pH 6.0), over 38 min on a MemSep SP 1000 cartridge was applied at a 2.0 mL/min flow rate. The eluent absorbance was monitored at 280 nm.

Figure 4: HPLC chromatograph of oxyhemoglobin after treatment with sodium dithionite ($Na_2S_2O_4$) to convert trace methemoglobin back to oxyhemoglobin. A 15-40% gradient elution of Buffer A, 20 mM Mes (pH 6.0), to Buffer B, 20 mM Mes with 150 mM NaCl (pH 6.0), over 38 min on a MemSep SP 1000 cartridge was applied at a 2.0 mL/min flow rate. The eluent absorbance was monitored at 280 nm.

Figure 5: Preparative HPLC chromatograph of bovine oxyhemoglobin. A gradient increasing from 0-10% Buffer B in 5 min, then 10-30% B over 17 min was used for the elution of the sample on a MemSep SP 1000 cartridge with a 2.0 mL/min flow rate. Buffer A was 20 mM Mes (pH 6.0) and Buffer B was 20 mM Mes with 150 mM NaCl (pH 6.0) The eluent absorbance was monitored at 280 nm.

Figure 6: Upper: Overlay of HbO_2 spectra in the presence of 476 μM 1,4-DNB in 20 mM Tris at pH 8.0 at 0 through 180 min at 30 min intervals, 18 h. Lower: Deconvolution of the above spectra from 0 through 18 h into dxHb (+), HbO_2 (□), and metHb (◊).

Figure 7: Upper: Overlay of HbO_2 control spectra without 1,4-DNB at 0 through 180 min at 30 min intervals, 18 h and 48 h. Lower: Deconvolution of the above spectra from 0 through 18 h into dxHb (+), HbO_2 (□), and metHb (◊).

Figure 8: Upper: Overlay of HbCO spectra in the presence of 476 μM 1,4-DNB in 20 mM Tris at pH 8.0 at 0 through 240 min at 30 min intervals, 70 h. Lower: Deconvolution of the above spectra from 0 through 24 h into HbO_2 (+), HbCO (□), and metHb (◊).

Figure 9: Upper: Overlay of HbCO control spectra without 1,4-DNB at 0 through 240 min at 30 min intervals, 70 h. Lower: Deconvolution of the above spectra from 0 through 24 h into HbO_2 (+), HbCO (□), and metHb (◊).

Figure 10: Upper: Overlay of dxHb spectra in the presence of 476 μM 1,4-DNB in 20 mM Tris at pH 8.0 at 0 through 240 min at 30 min intervals, 18 h. Lower: Deconvolution of the above spectra from 0 through 18 h into dxHb (+), HbO_2 (□), and metHb (◊).

Figure 11: Upper: Overlay of dxHb control spectra without 1,4-DNB at 0 through 240 min at 30 min intervals, 18 h. Lower: Deconvolution of the above spectra from 0 through 18 h into dxHb (+), HbO_2 (□), and metHb (◊).

Figure 12: Upper: Overlay of dxHb spectra without dithionite in the presence of 476 μM 1,4-DNB in 20 mM Tris at pH 8.0 at 0 through 180 min at 30 min intervals, 24 h. Lower: Deconvolution of the above spectra from 0 through 24 h into dxHb (+), HbO_2 (□), and metHb (◊).

Figure 13: **Upper: Overlay of dxHb control spectra without dithionite and without 1,4-DNB 0 through 180 min at 30 min intervals, 24 h. Lower: Deconvolution of the above spectra from 0 through 24 h into dxHb (+), HbO_2 (□), and metHb (◊).**

Figure 14: Elution profile on G-25 Sephadex column of in vitro labeling of bovine Hb with [^{14}C]-1,3-DNB. The 100 μL sample was eluted with deionized, distilled water and collected in 1.0 mL fractions. The Hb eluted in fractions 2-4, and the error bars show 2σ deviation of the counts per minute.

Figure 15: Elution profile on G-25 Sephadex column of in vitro labeling of bovine Hb with [^{14}C]-1,3-DNB. The sample was the pooled fractions 2 and 3 of the previous G-25 separation. The sample was eluted with 0.05 M NaCl and collected in 1.0 mL fractions. The Hb eluted in fractions 1-3, and the error bars show 2σ deviation of the counts per minute.

Figure 16: Elution profile of bovine *Hb* on a C4 reverse-phase HPLC column at pH 2.0 conditions, increasing solvent B from 55 to 100% over 14 min. The [^{14}C]-1,3-DNB was mixed DxHb form *in vitro*. The eluate was monitored at 280 and 540 nm and for ^{14}C label. All UV/VIS channels were set to 1.0 AUFS. Scintillant was pumped at 1.2 mL/min and the sensitivity of the radiation monitor was 5K/10K (fine/coarse).

Figure 17: Elution profile of rat heme fraction co-injected with heme standard at pH 2.0 conditions on a C18 reverse-phase HPLC column using gradient Heme1. The [^{14}C]-1,3-DNB was introduced into the rats *in vivo*. The eluate was monitored at 230, 280, and 540 nm, and monitored for ^{14}C radiolabel. All UV/VIS channels were set to 1.0 AUFS. Scintillant was pumped at 1.2 mL/min and the sensitivity of the radiation monitor was 5K/10K (fine/coarse).

Figure 18: Elution profile of rat globin fraction at pH 2.0 conditions on a C4 reverse-phase HPLC column using gradient Globin1. The [^{14}C]-1,3-DNB was introduced into the rats *in vivo*. The eluate was monitored at 230, 280, and 540 nm, and monitored for ^{14}C radiolabel. All UV/VIS channels were set to 1.0 AUFS. Scintillant was pumped at 1.2 mL/min and the sensitivity of the radiation monitor was 5K/10K (fine/coarse).

Figure 19: Elution profile of rat globin fraction at pH 7.0 conditions on a C4 reverse-phase HPLC column using gradient Globin2. The [^{14}C]-1,3-DNB was introduced into the rats *in vivo*. The eluate was monitored at 280 and 540 nm, and monitored for ^{14}C radiolabel. All UV/VIS channels were set to 1.0 AUFS. Scintillant was pumped at 1.2 mL/min and the sensitivity of the radiation monitor was 5K/10K (fine/coarse).

Figure 20: Elution profile of rat globin fraction at pH 2.0 conditions on a C4 reverse-phase HPLC column using gradient Globin1. The [^{14}C]-TNB was introduced into the rats *in vivo*. The eluate was monitored at 280 nm at 1.0 AUFS and monitored for ^{14}C radiolabel. Scintillant was pumped at 1.2 mL/min and the sensitivity of the radiation monitor was 5K/10K (fine/coarse).

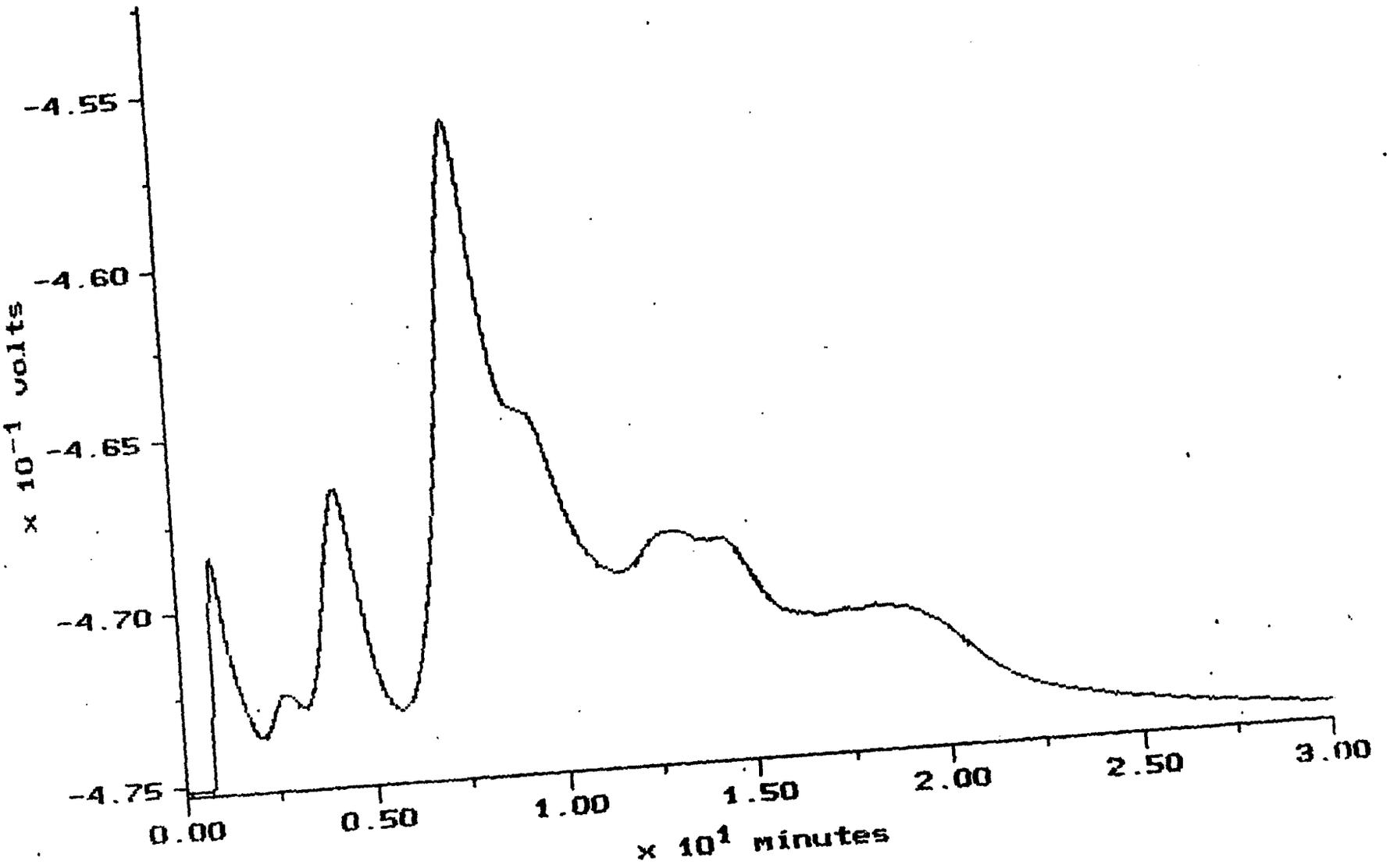
Figure 21: Close-up of elution profile of rat globin fraction at pH 2.0 conditions from 0.0 to 5.0 min on a C4 reverse-phase HPLC column using gradient Globin1. The [^{14}C]-TNB was introduced into the rats *in vivo*. The eluate was monitored at 280 nm at 0.25 AUFS and monitored for ^{14}C radiolabel. Scintillant was pumped at 1.2 mL/min and the sensitivity of the radiation monitor was 5K/10K (fine/coarse).

Figure 22: Elution profile of rat globin fraction at pH 7.0 conditions on a C4 reverse-phase HPLC column using gradient Globin2. The [^{14}C]-TNB was introduced into the rats *in vivo*. The eluate was monitored at 280 nm at 0.25 AUFS and monitored for

^{14}C radiolabel. Scintillant was pumped at 1.2 mL/min and the sensitivity of the radiation monitor was 5K/10K (fine/coarse).

Figure 23: Photomicrographs of rat oxy-hemoglobin crystals.

Figure 1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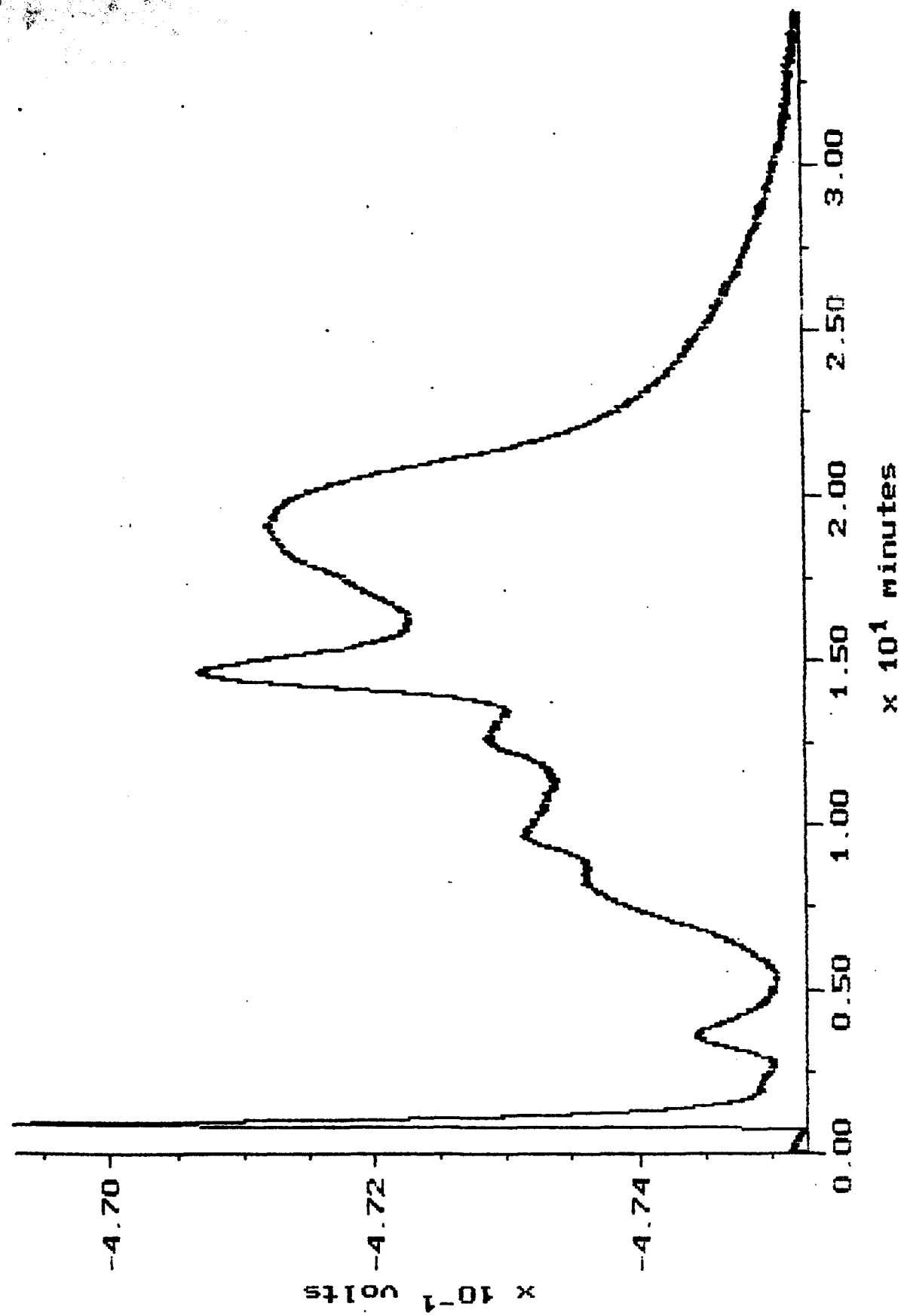
0.50
1.00
1.50
2.00
2.50
3.00
x 10^1 minutes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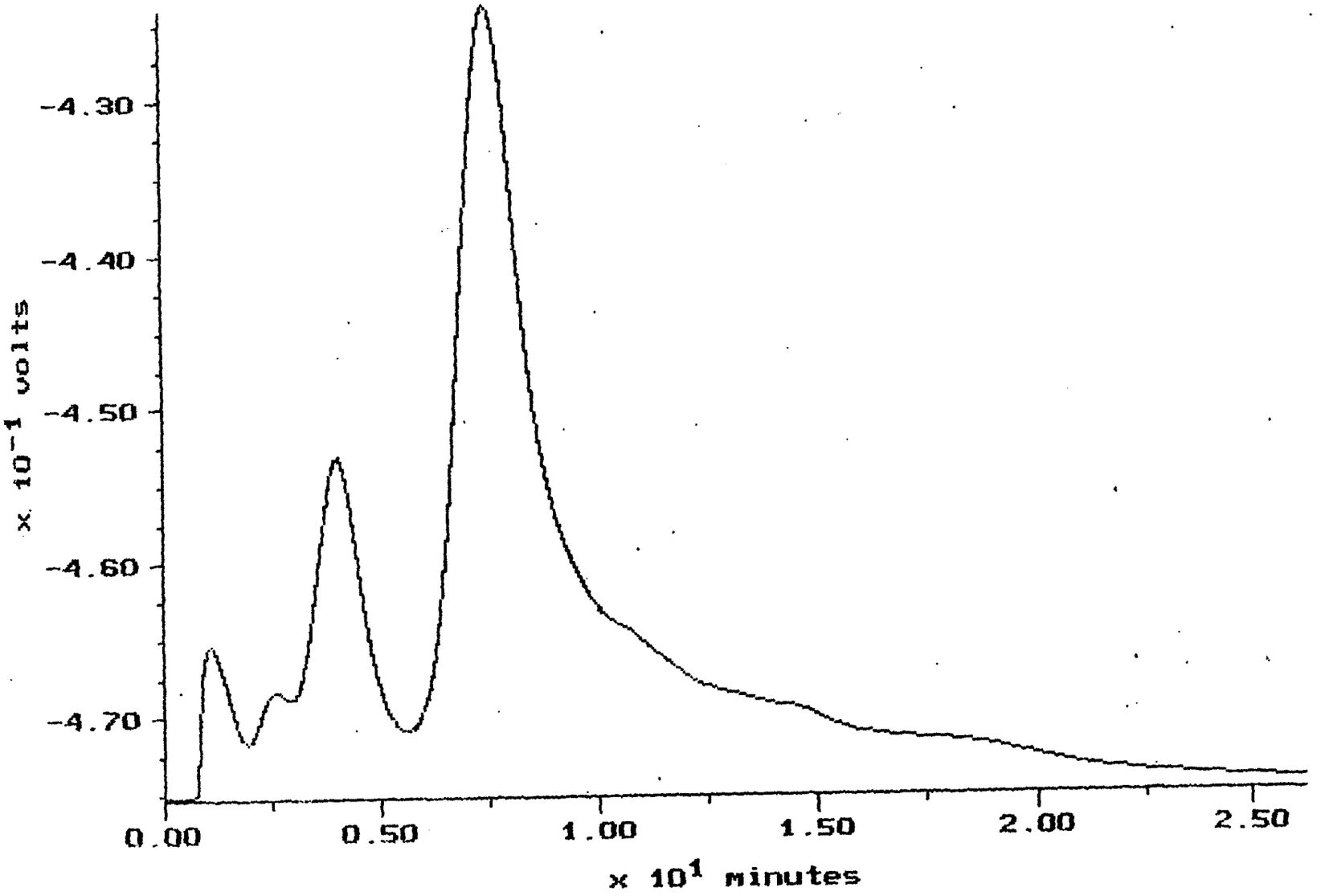
x 10^1 minutes

Figure 2

Figure 3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1.00
1.50
2.00
2.50
x 10¹ minutes

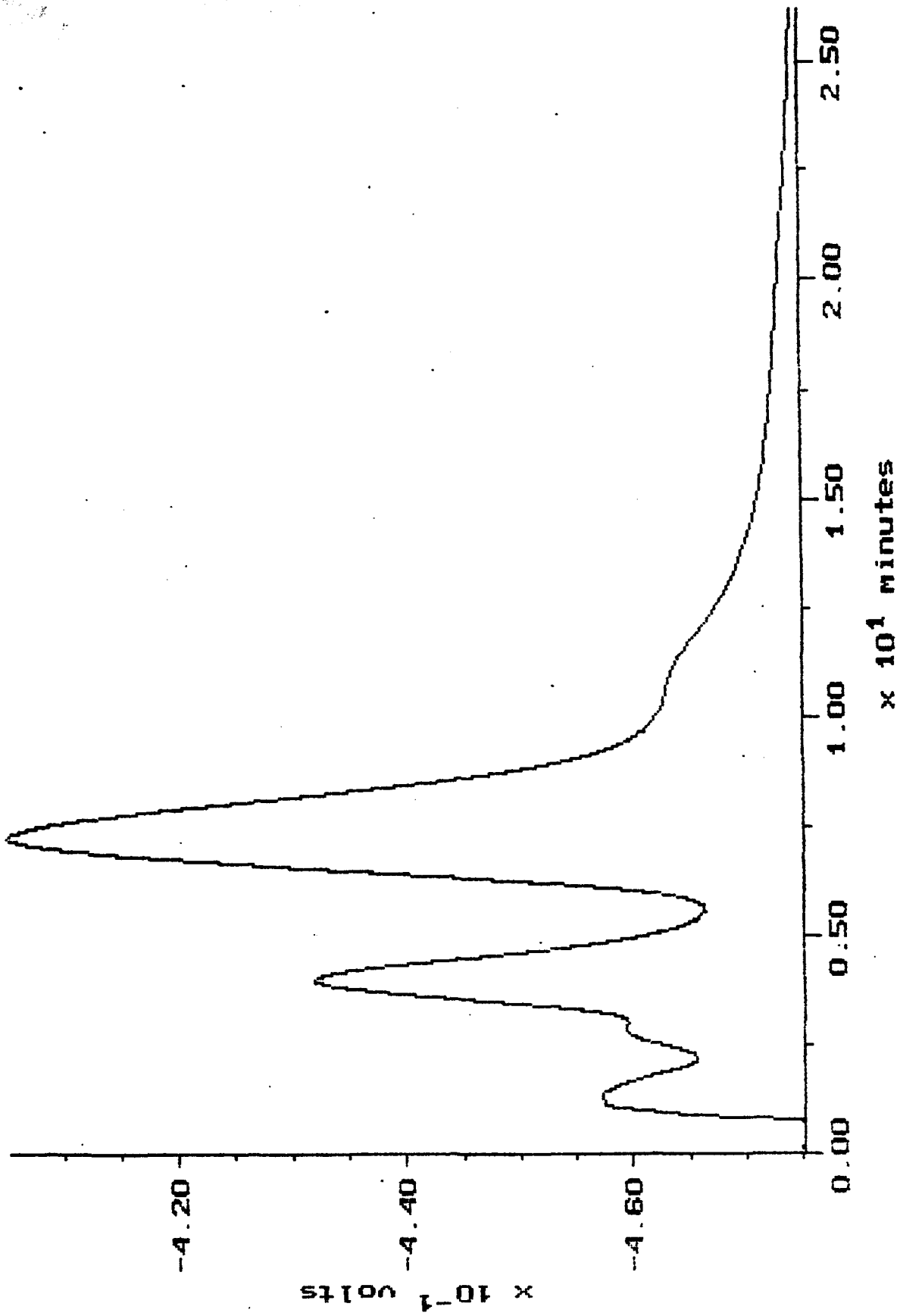
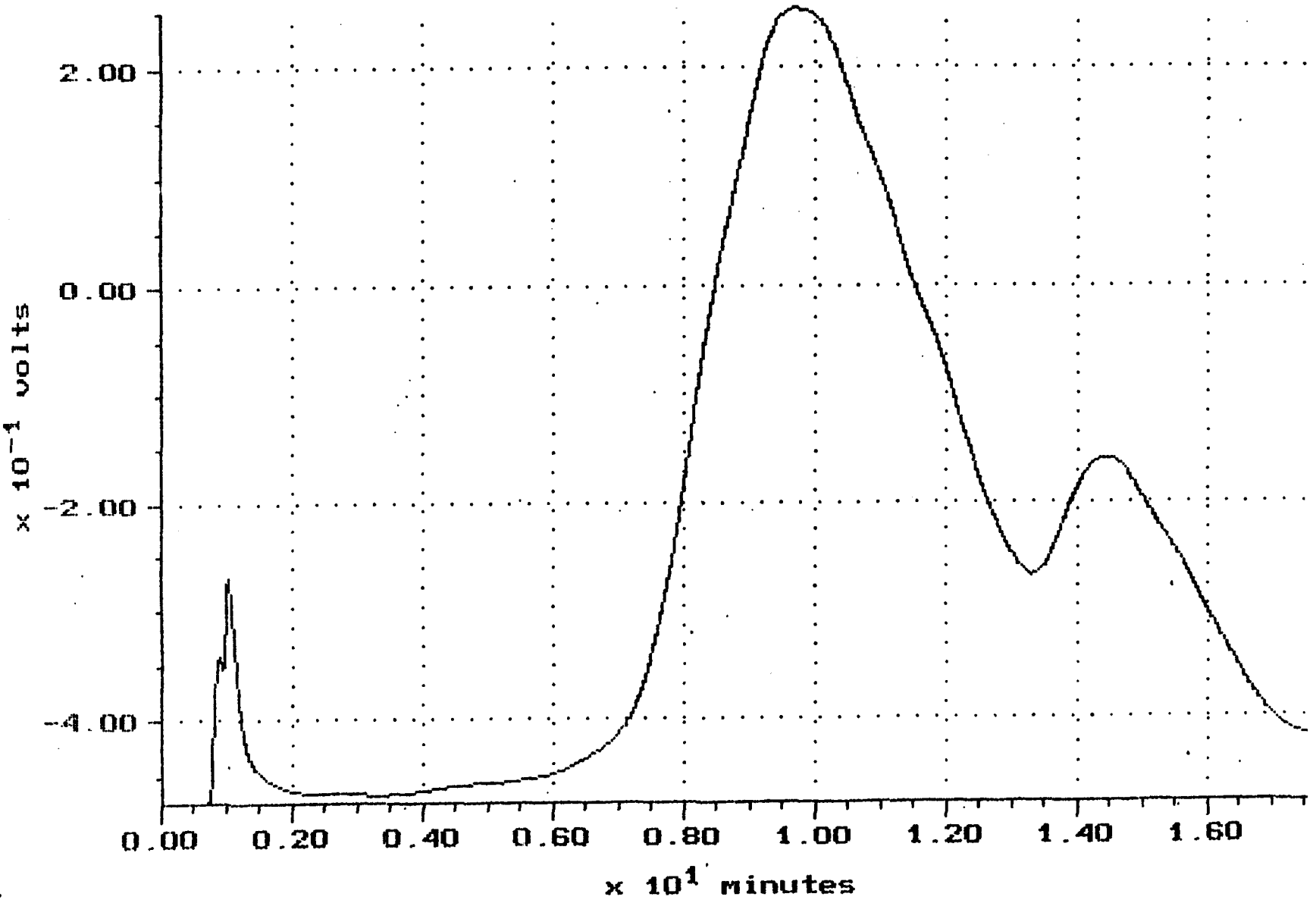


Figure 4

Figure 5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-4.00
x 10⁻¹ volts
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1.20
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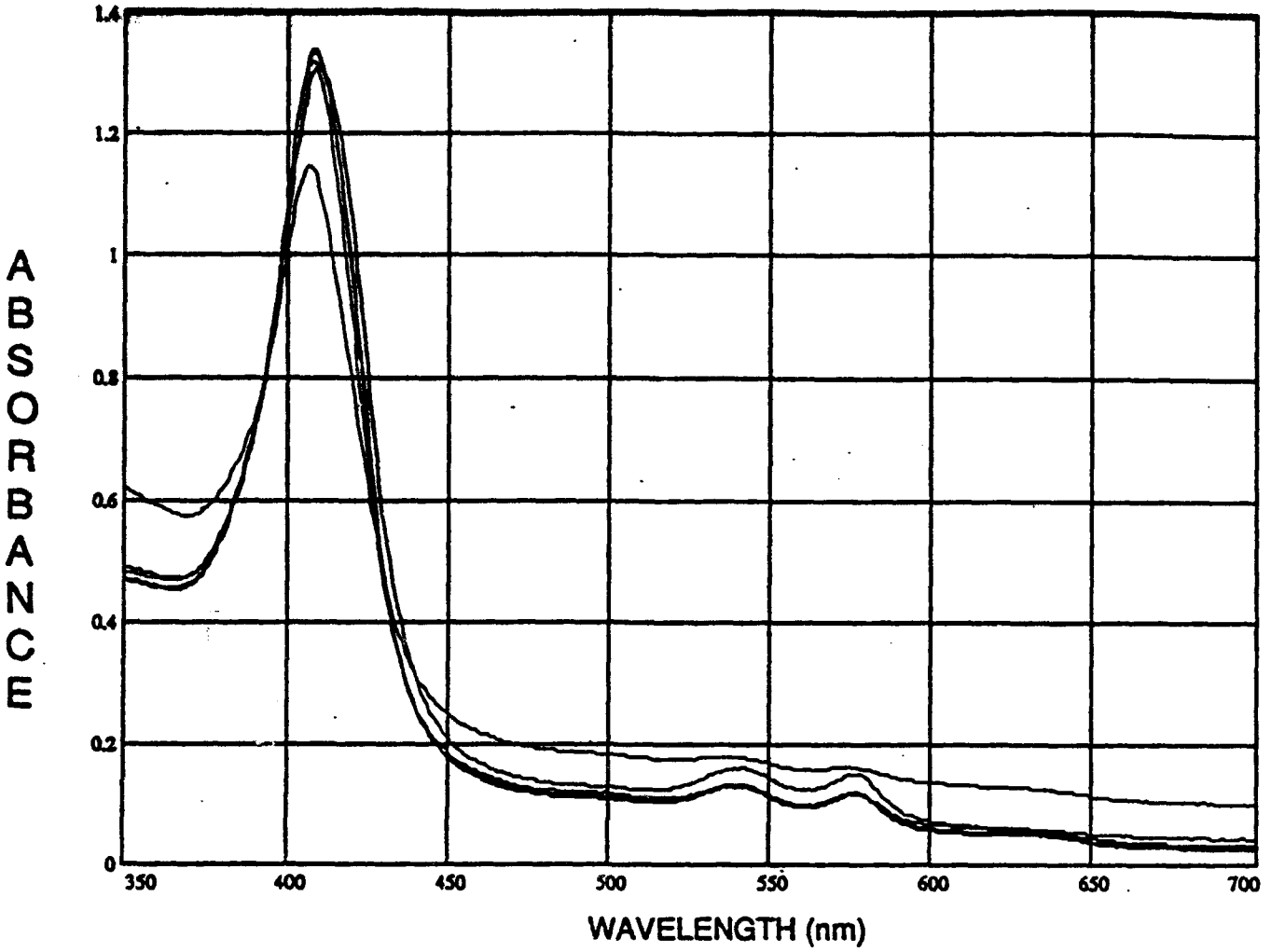
1.60
x 10¹ minutes

Figure 6


ABSORBANCE
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1.2
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0.2
0
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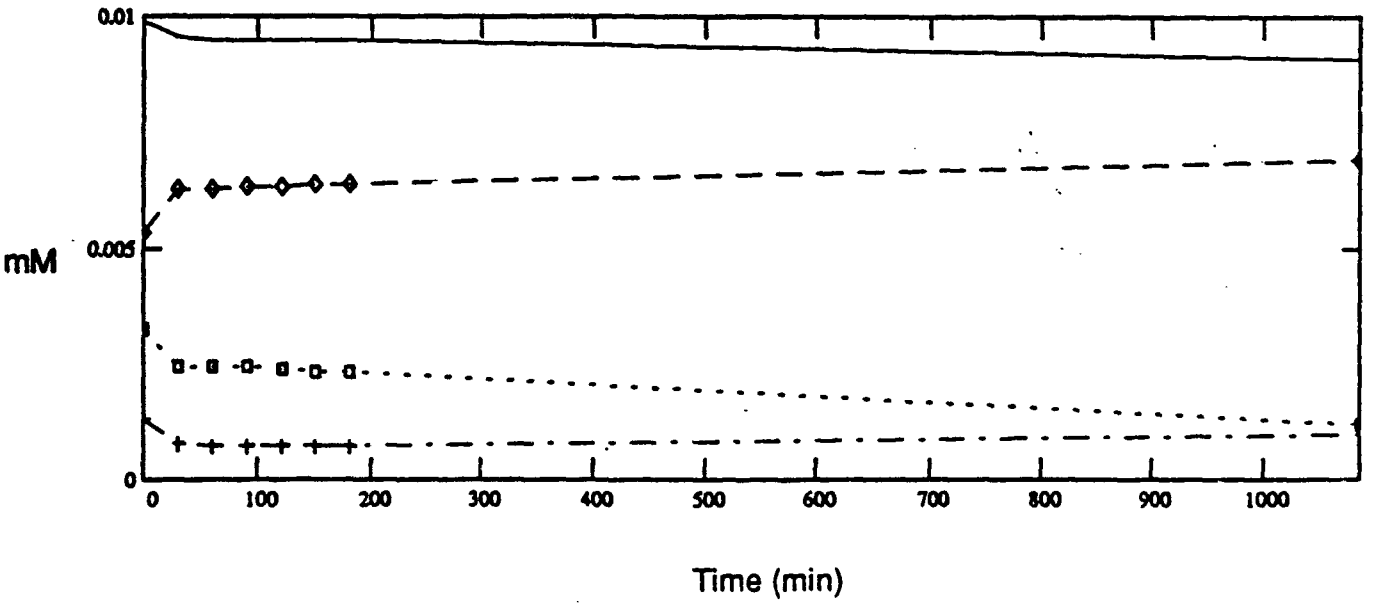
400
450
500
550
600
650
700
WAVELENGTH (nm)


mM
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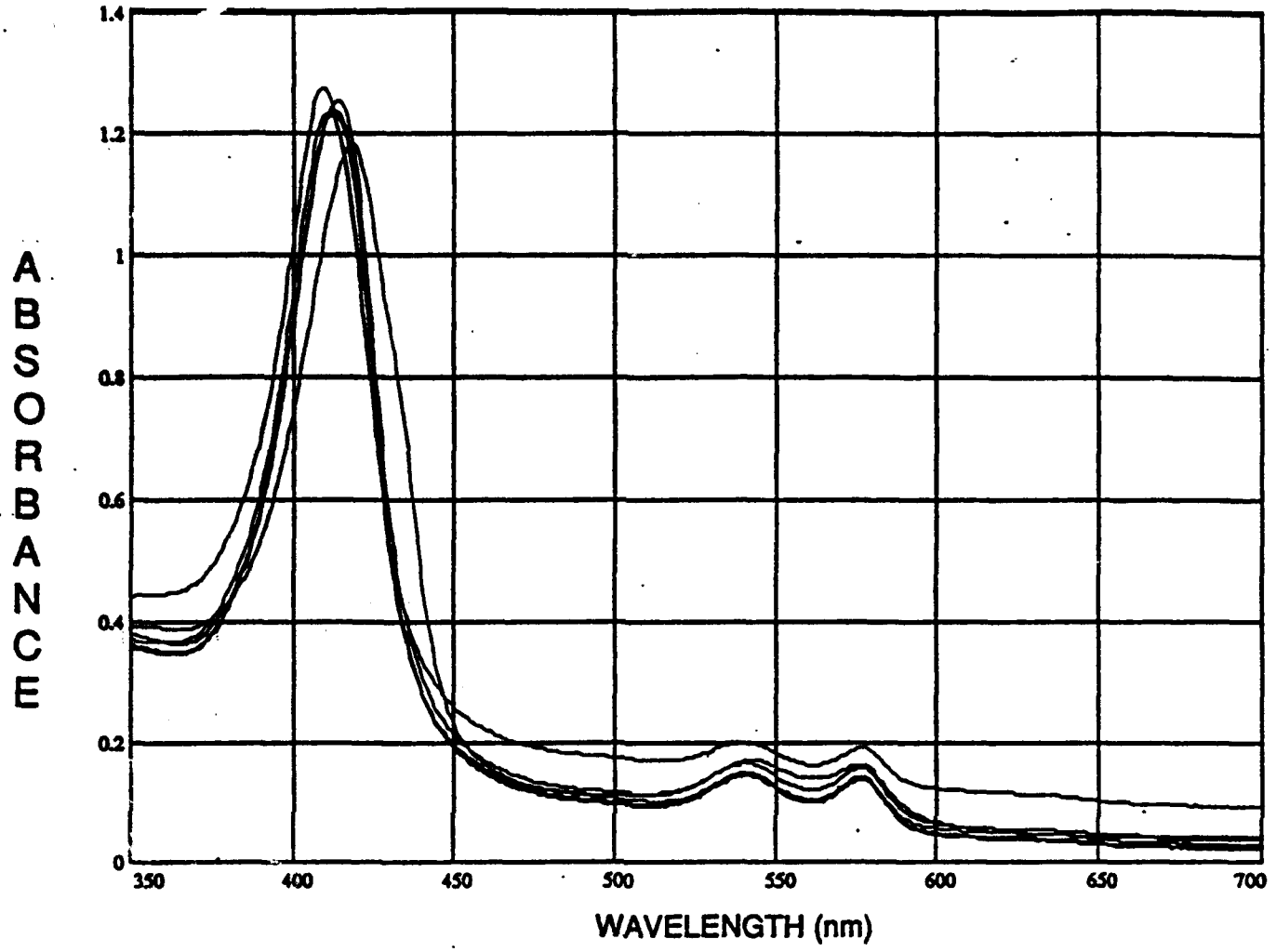
0
0
100
200
300
400
500
600
700
800
900
1000
Time (min)

Figure 7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1.4
1.2
1
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0.2
0
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400
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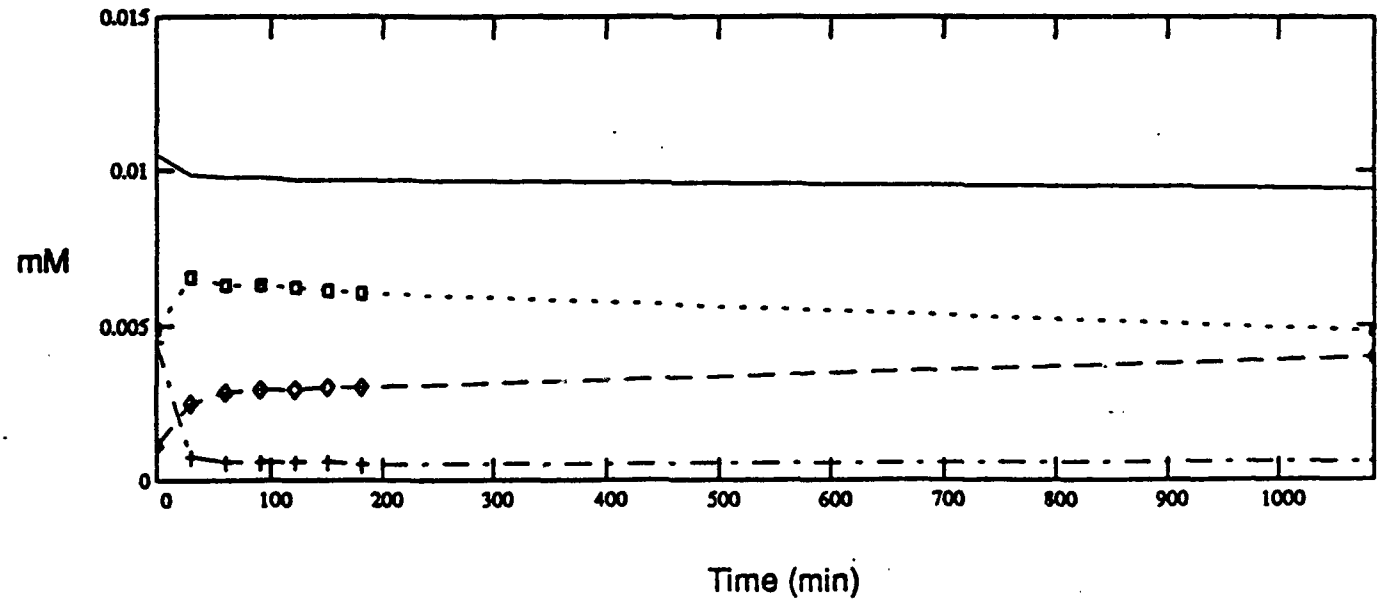
550
600
650
700
WAVELENGTH (nm)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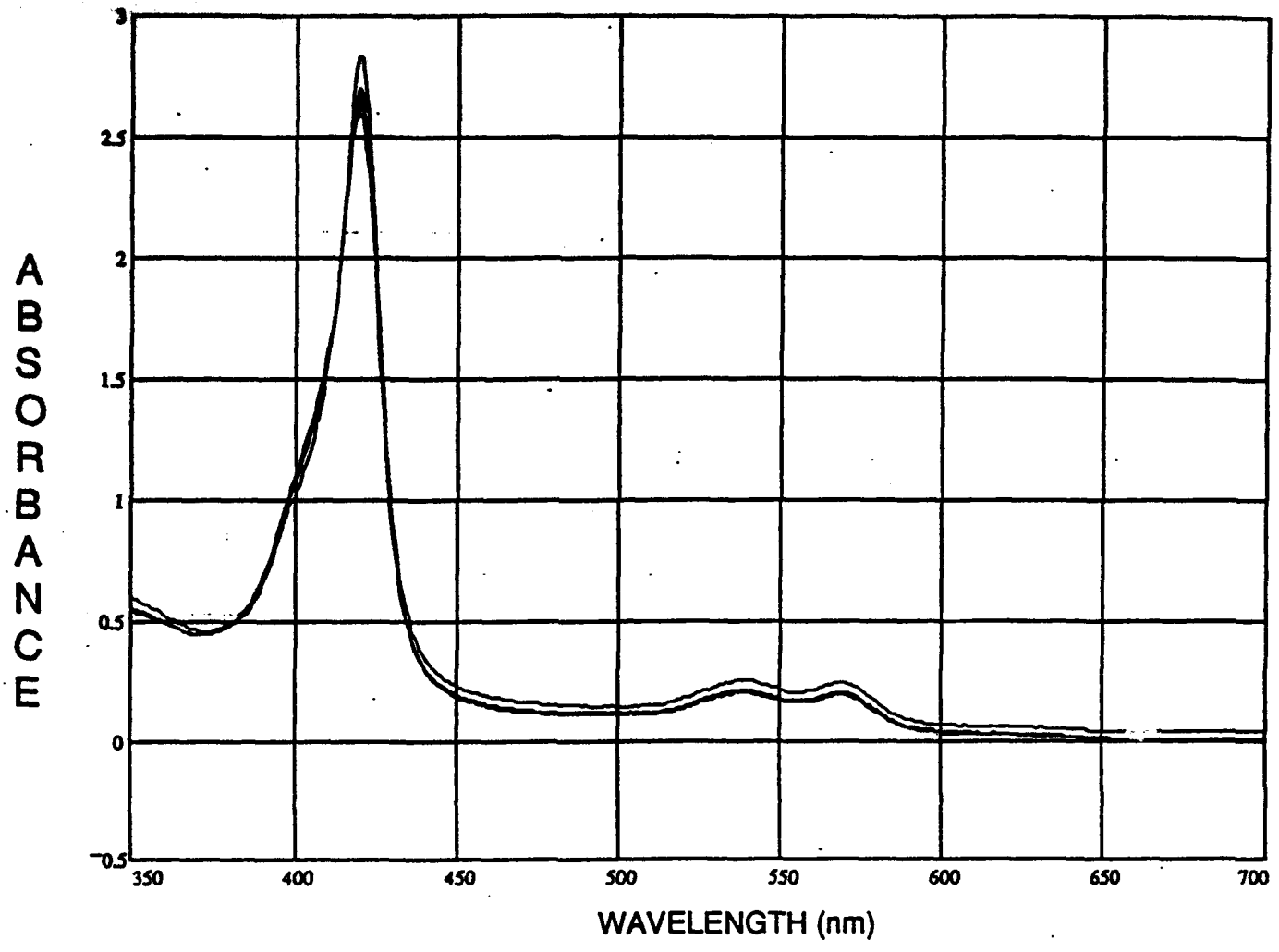
0
0
100
200
300
400
500
600
700
800
900
1000
Time (min)

Figure 8


ABSORBANCE
3
2.5
2
1.5
1
0.5
0
-0.5
350
400
450
500
550
600
650
700
WAVELENGTH (nm)

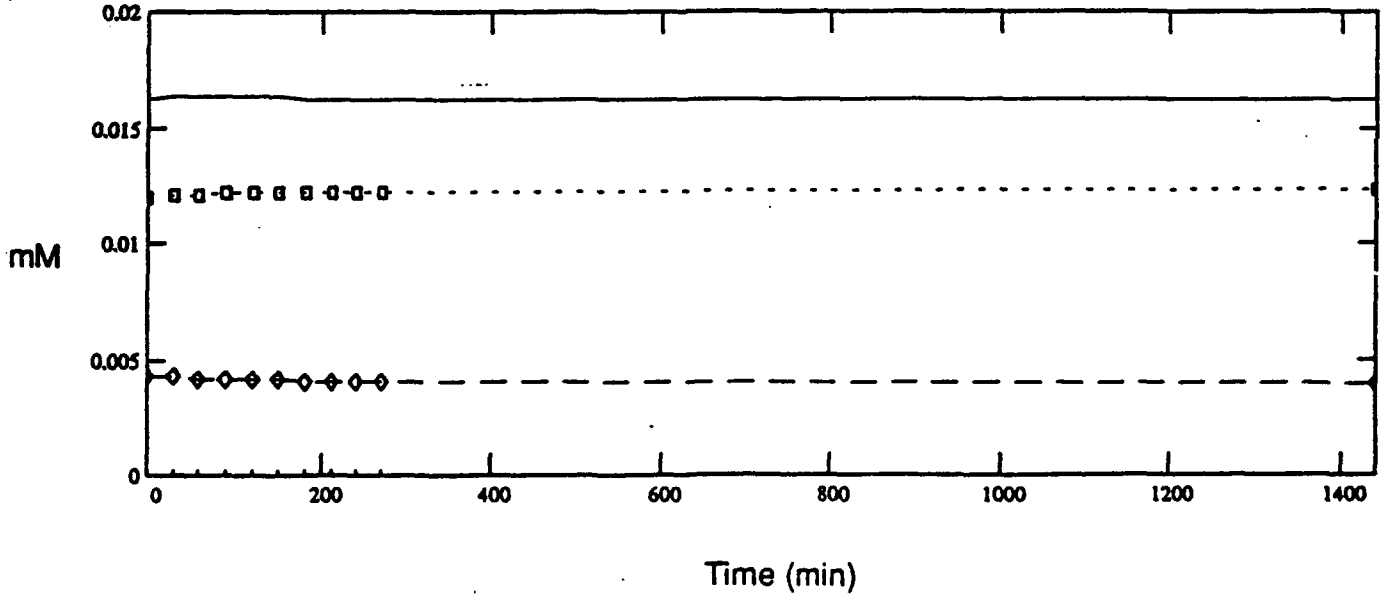
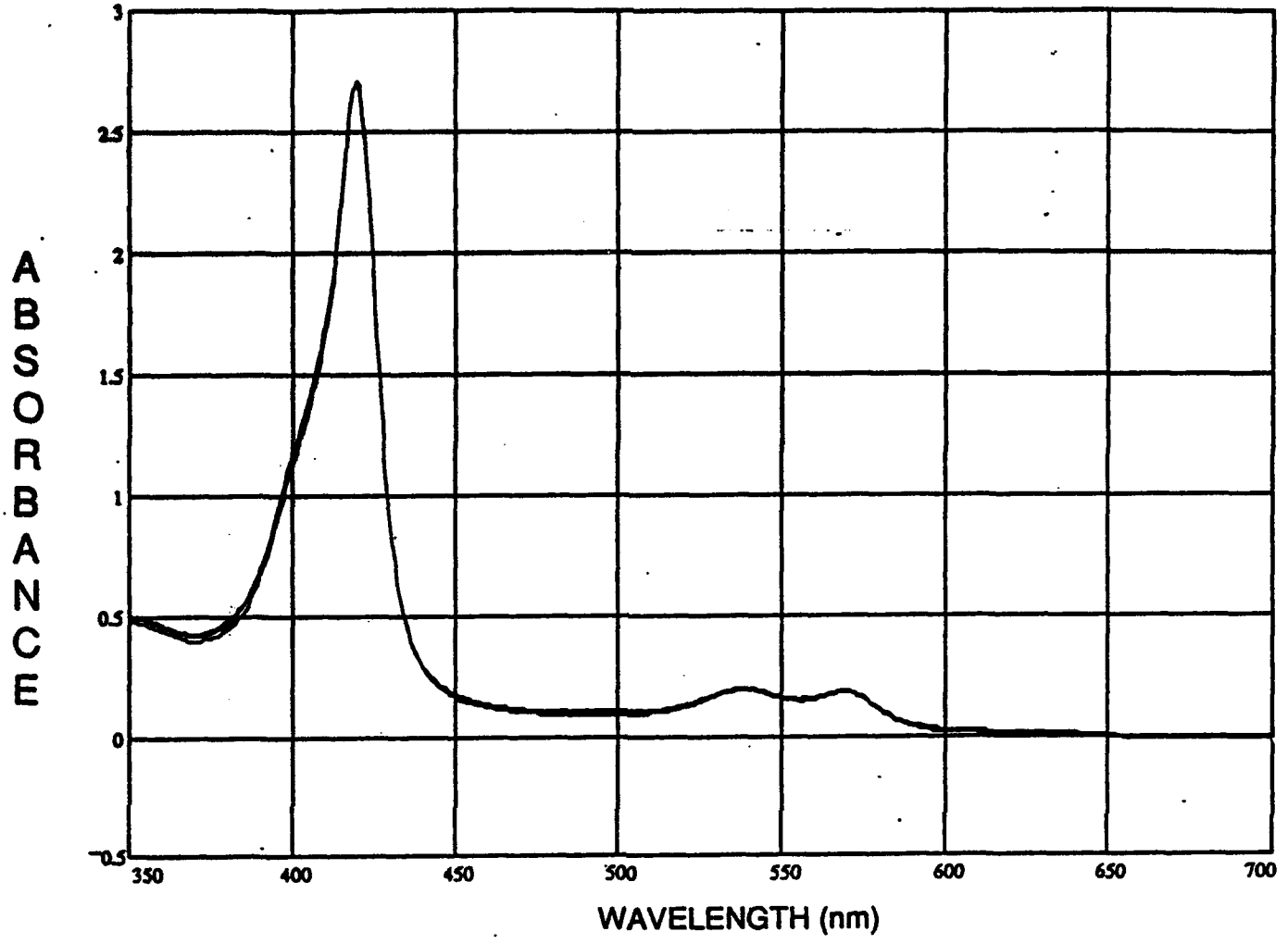
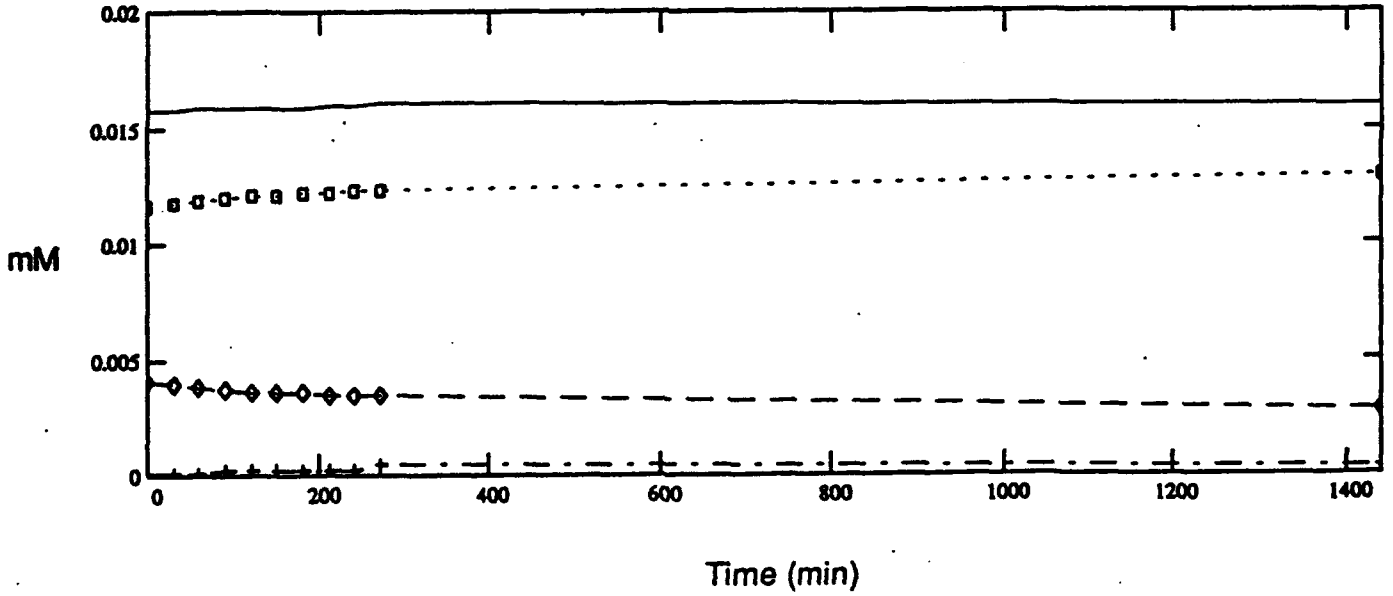

mM
0.02
0.015
0.01
0.005
0
0
200
400
600
800
1000
1200
1400
Time (min)

Figure 9


ABSORBANCE
3
2.5
2
1.5
1
0.5
0
-0.5
350
400
450
500
550
600
650
700
WAVELENGTH (nm)


mM
0.02
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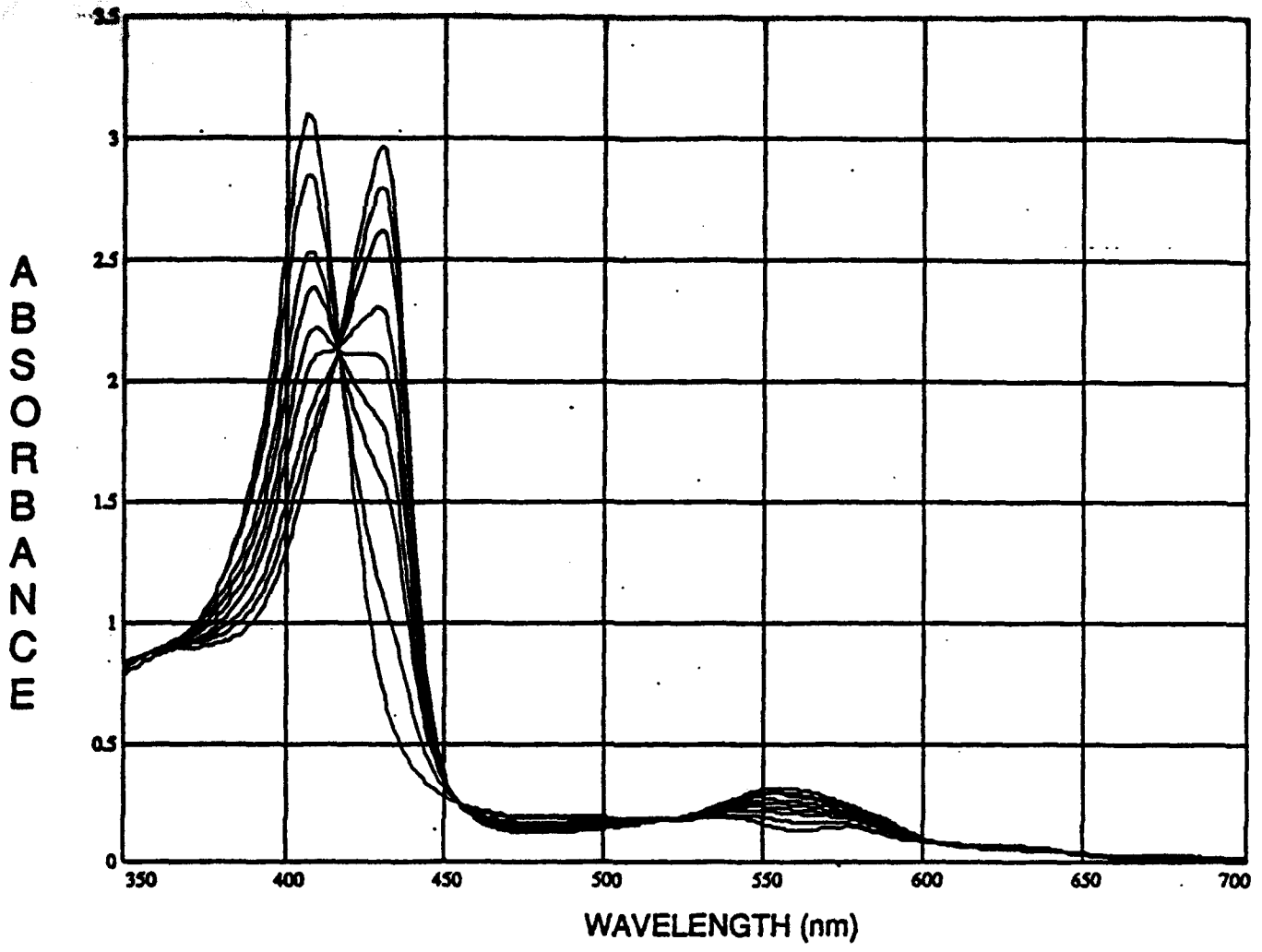
0.01
0.005
0
0
200
400
600
800
1000
1200
1400
Time (min)

Figure 10


ABSORBANCE
3.5
3
2.5
2
1.5
1
0.5
0
350
400
450
500
550
600
650
700
WAVELENGTH (nm)

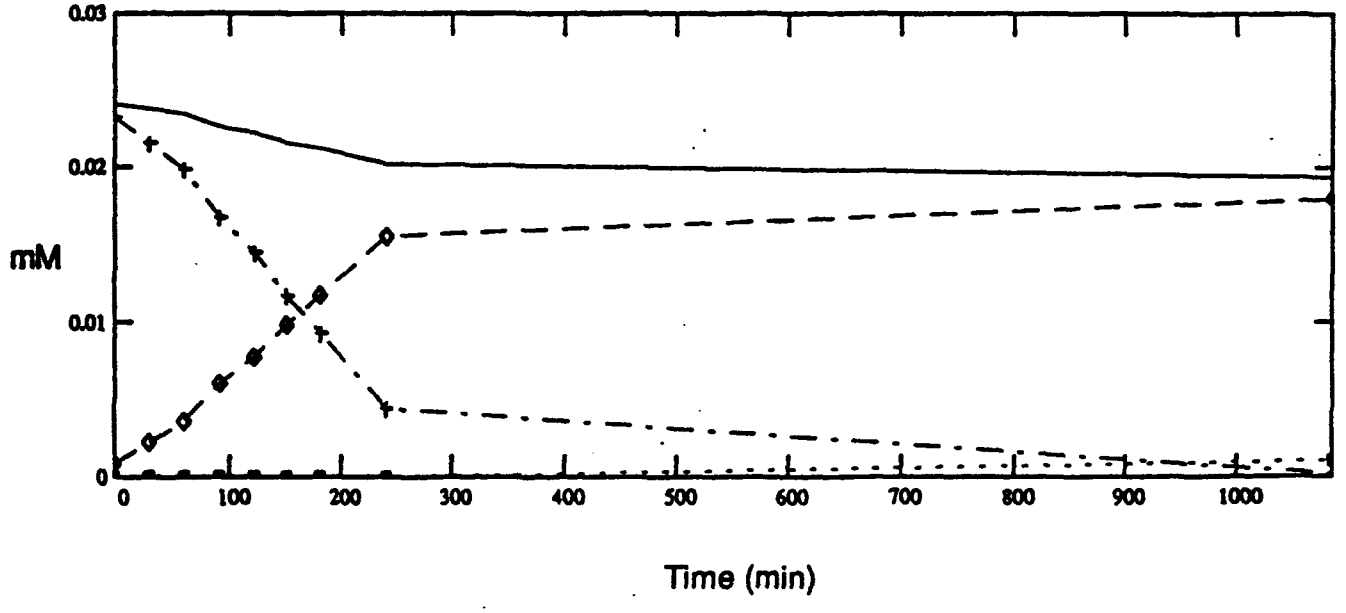
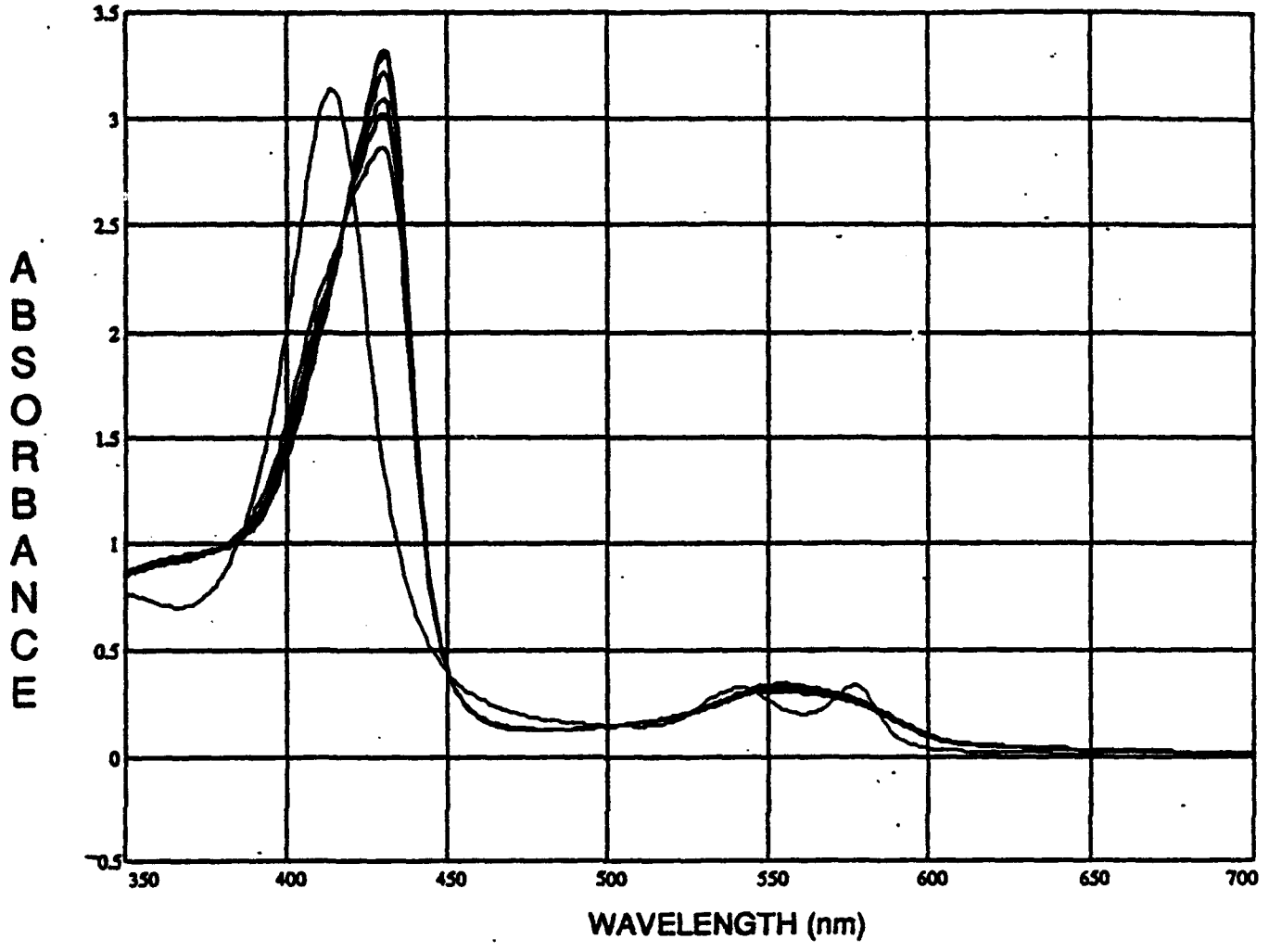

mM
0.03
0.02
0.01
0
0
100
200
300
400
500
600
700
800
900
1000
Time (min)

Figure 11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3.5
3
2.5
2
1.5
1
0.5
0
-0.5
350
400
450
500
550
600
650
700
WAVELENGTH (nm)

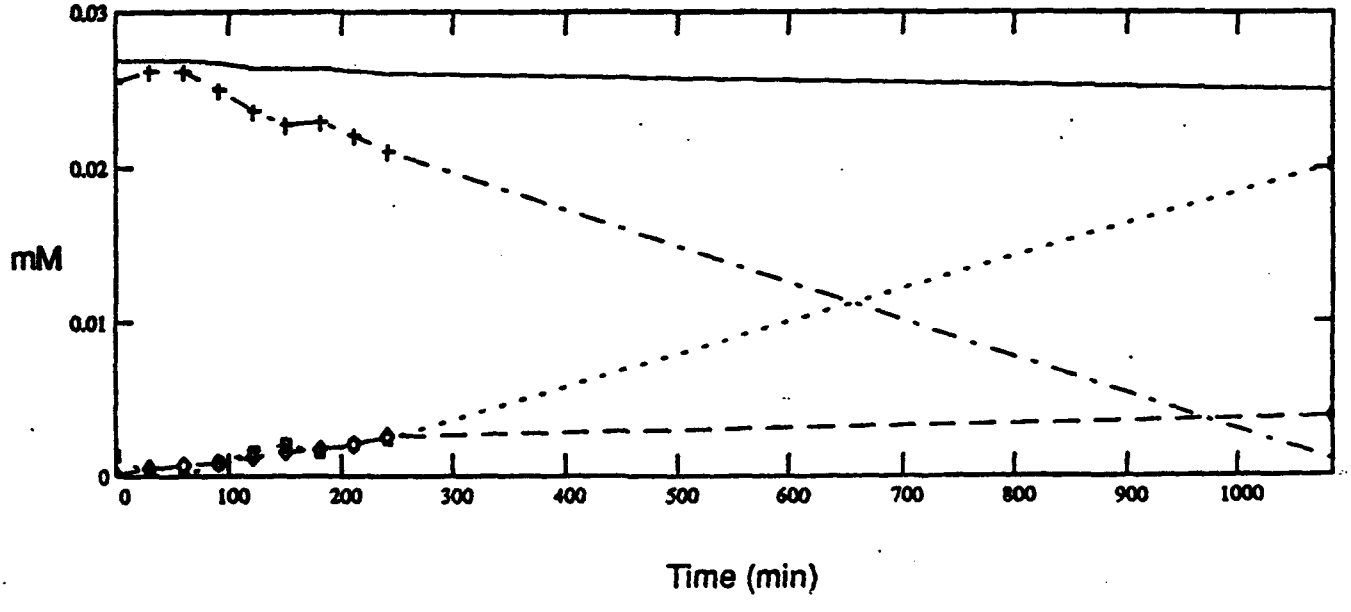
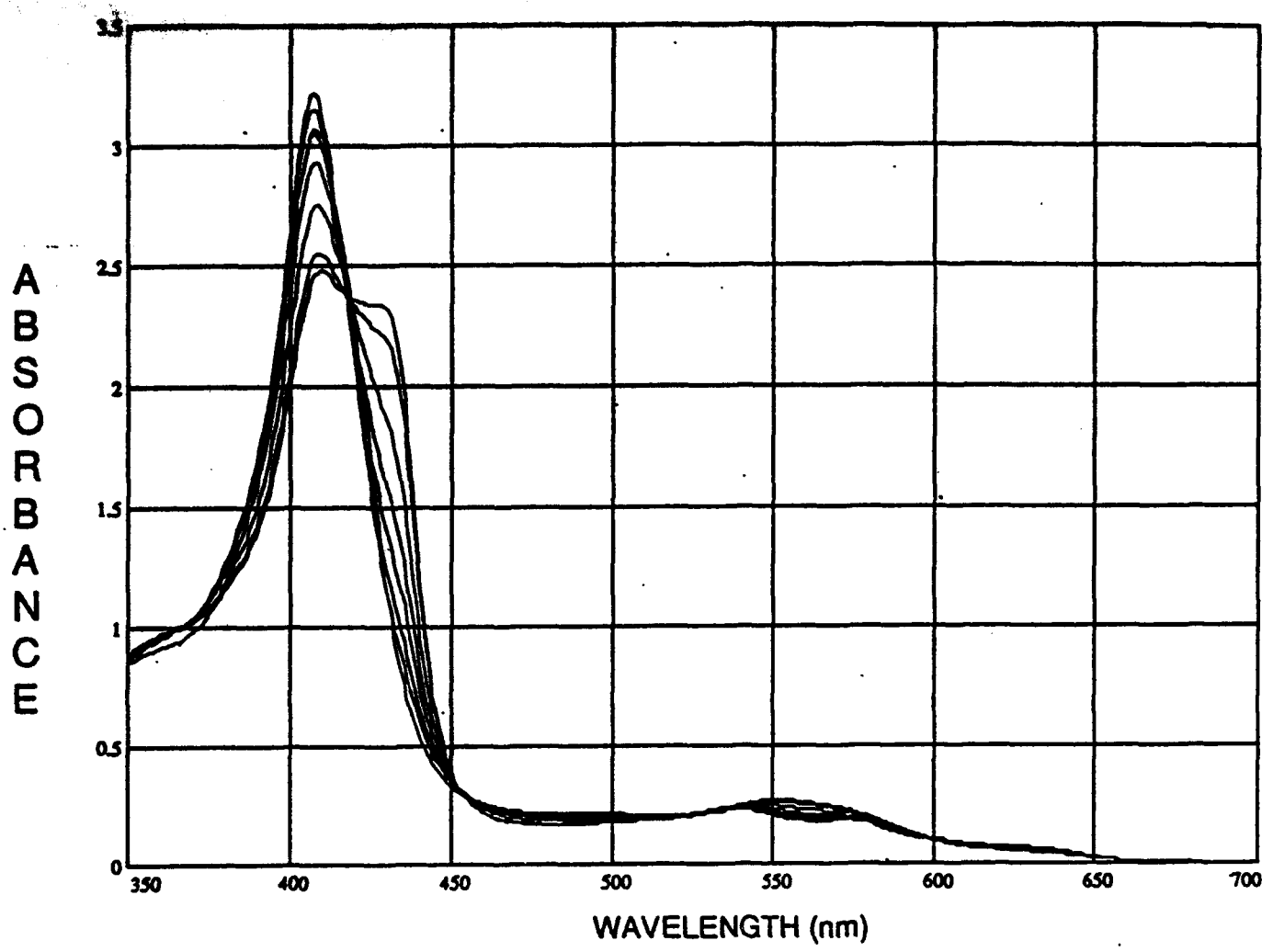
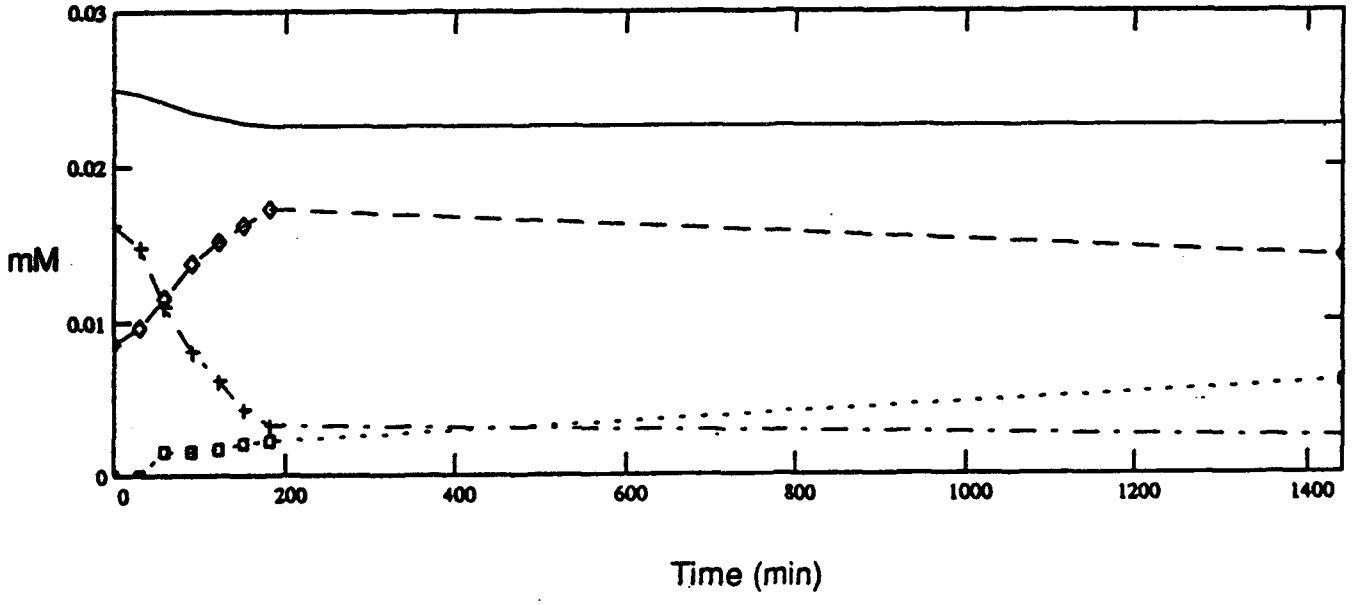

mM
0.03
0.02
0.01
0
0
100
200
300
400
500
600
700
800
900
1000
Time (min)

Figura 12


ABSORBANCE
3.5
3
2.5
2
1.5
1
0.5
0
350
400
450
500
550
600
650
700
WAVELENGTH (nm)


mM
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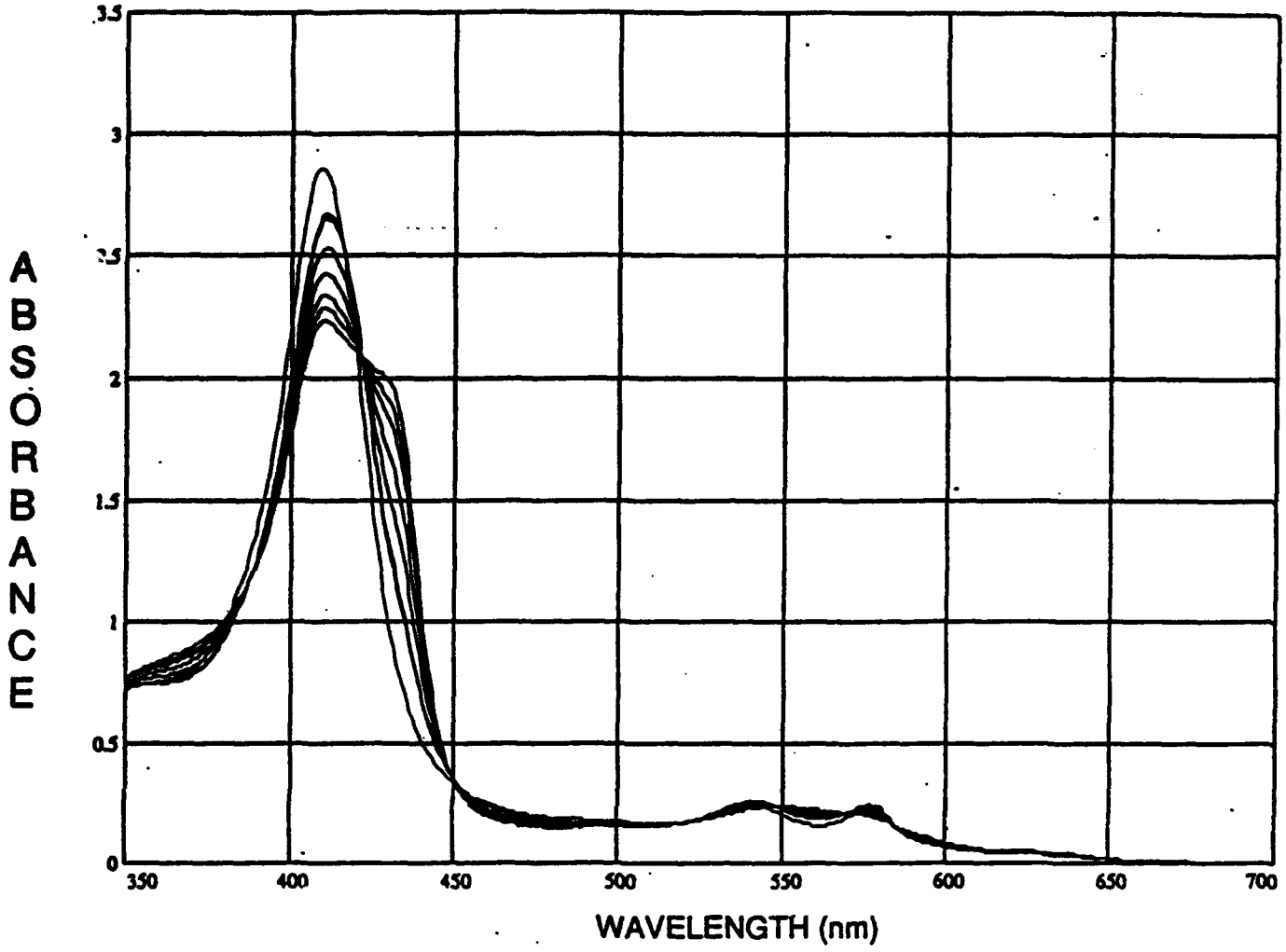
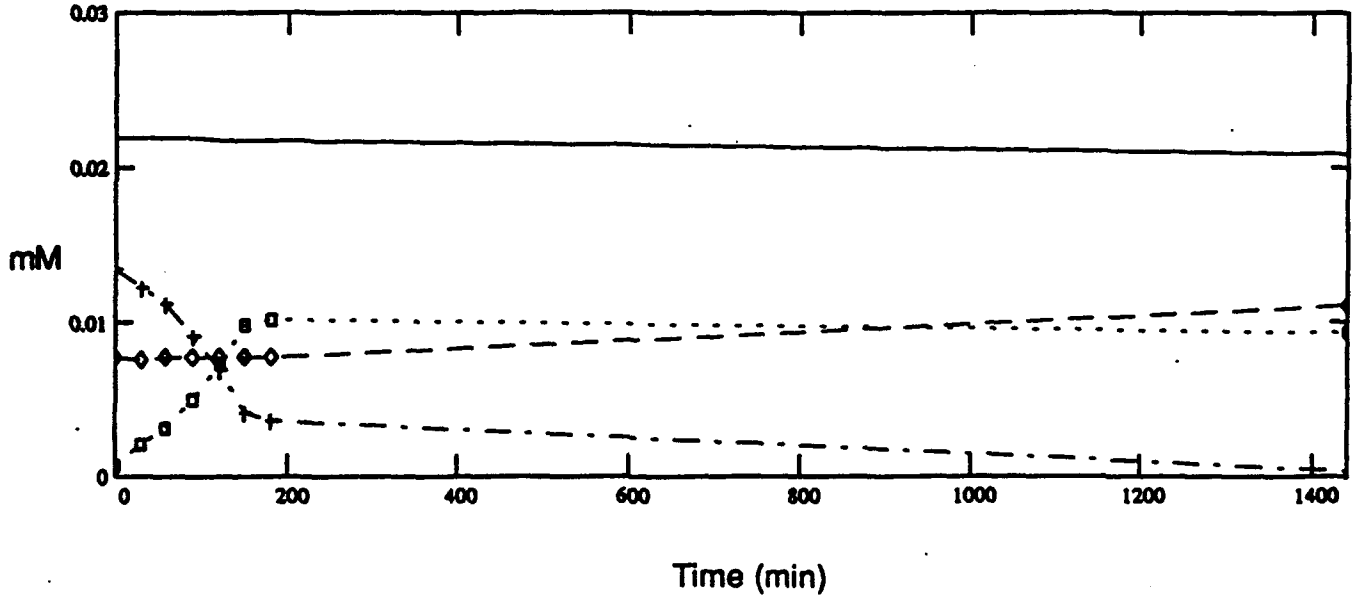
0.02
0.01
0
0
200
400
600
800
1000
1200
1400
Time (min)

Figure 13


ABSORBANCE
3.5
3
2.5
2
1.5
1
0.5
0
350
400
450
500
550
600
650
700
WAVELENGTH (nm)


mM
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0.02
0.01
0
0
200
400
600
800
1000
1200
1400
Time (min)

Figure 14

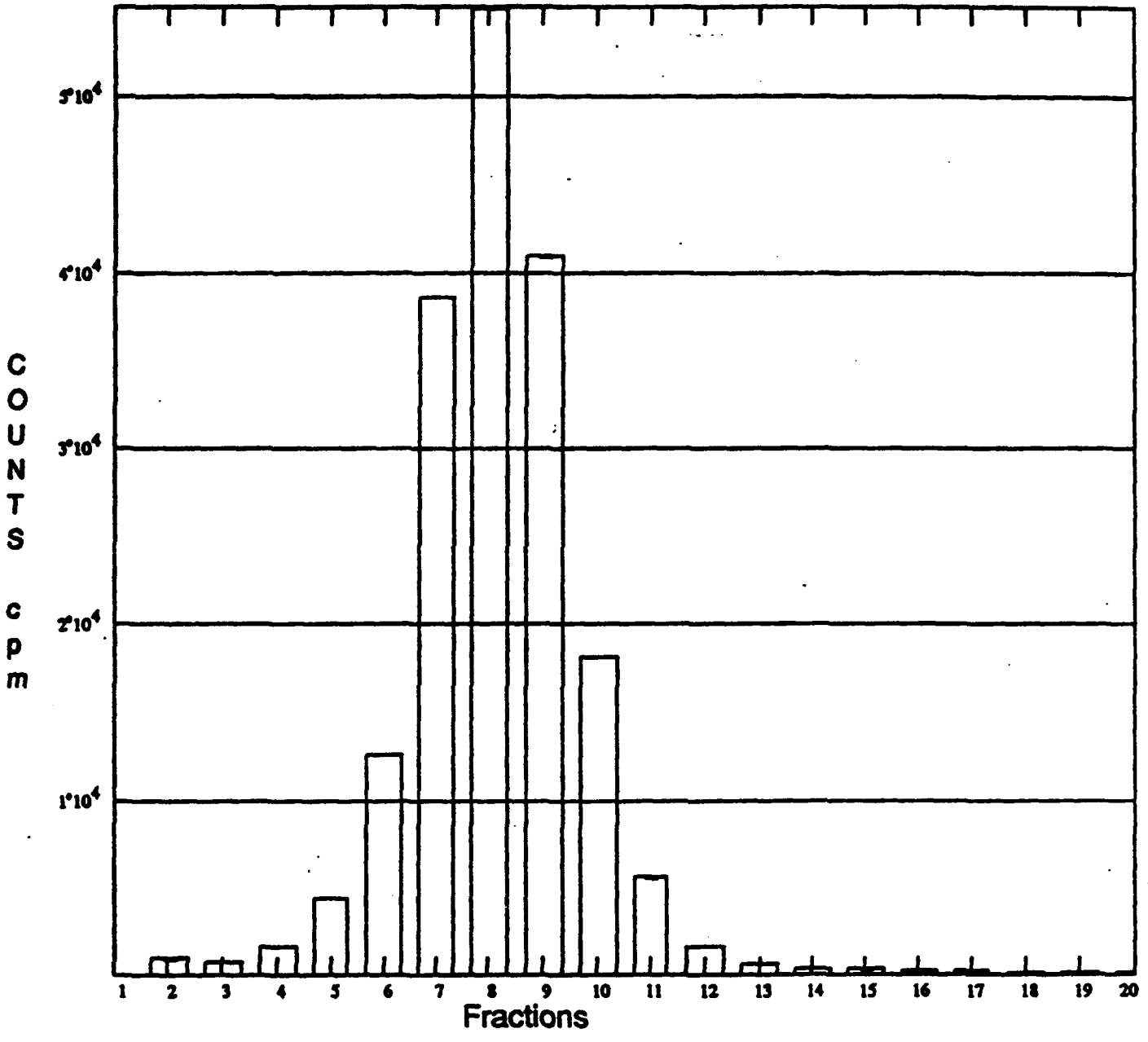
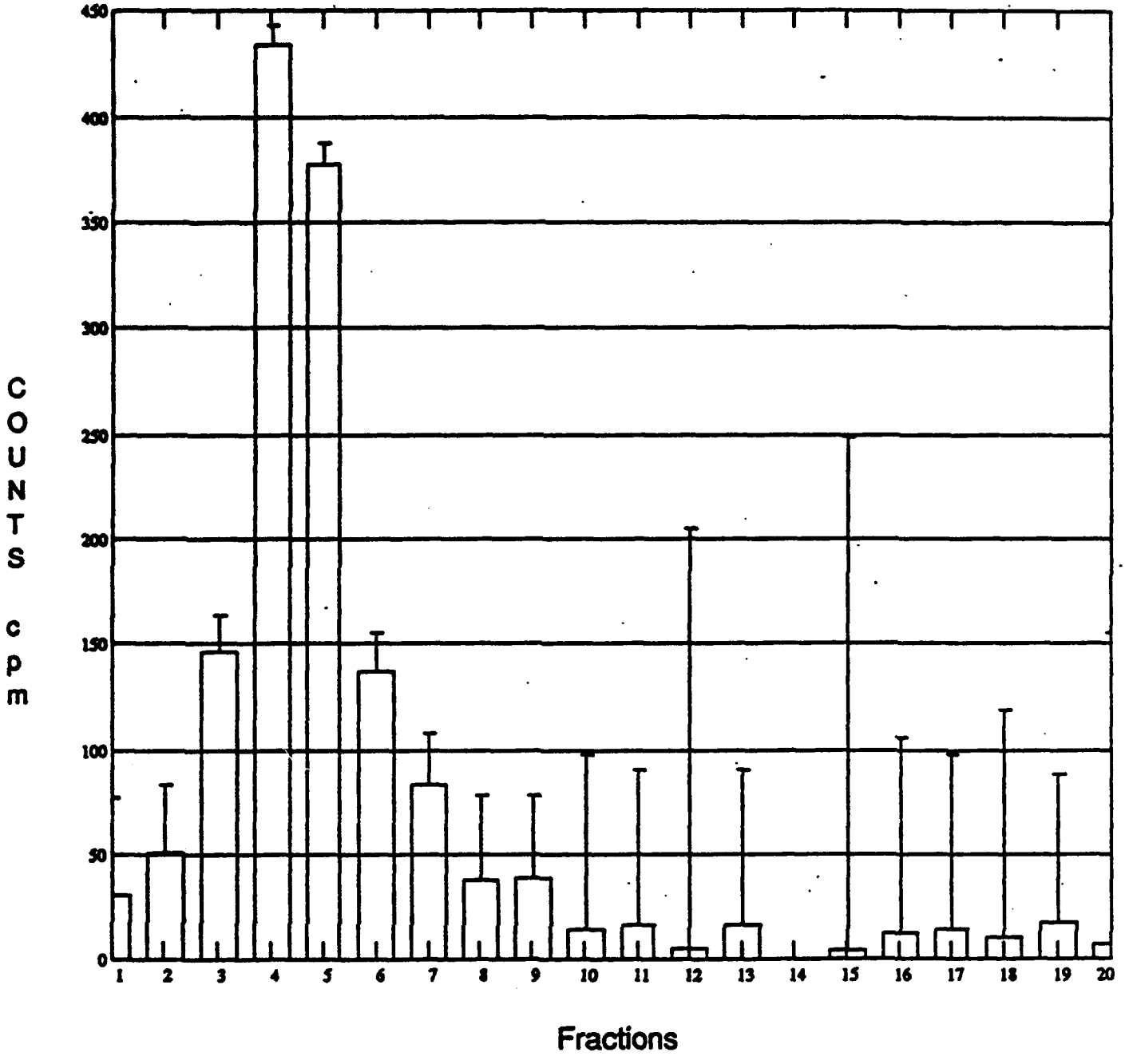

5*10⁴
4*10⁴
3*10⁴
2*10⁴
1*10⁴
COUNTS cpm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fractions

Figure 15


COUNTS cpm
450
400
350
300
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fractions

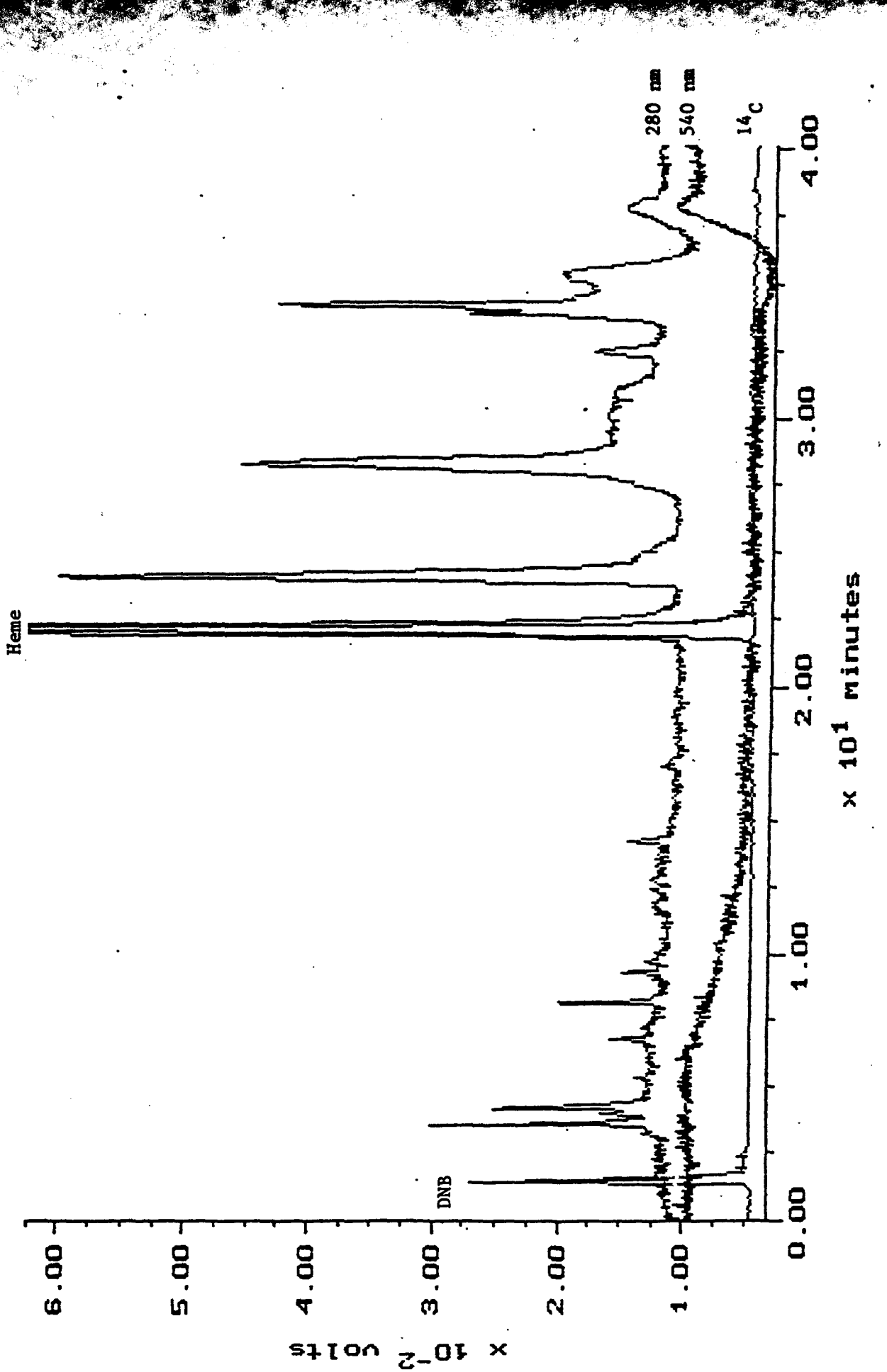

280 nm
540 nm
^{14}C
Heme
DNB
6.00
5.00
4.00
3.00
2.00
1.00
0.00
x 10^{-2} volts
1.00
2.00
3.00
4.00
x 10^{1} minutes

Figure 16

Figure 17

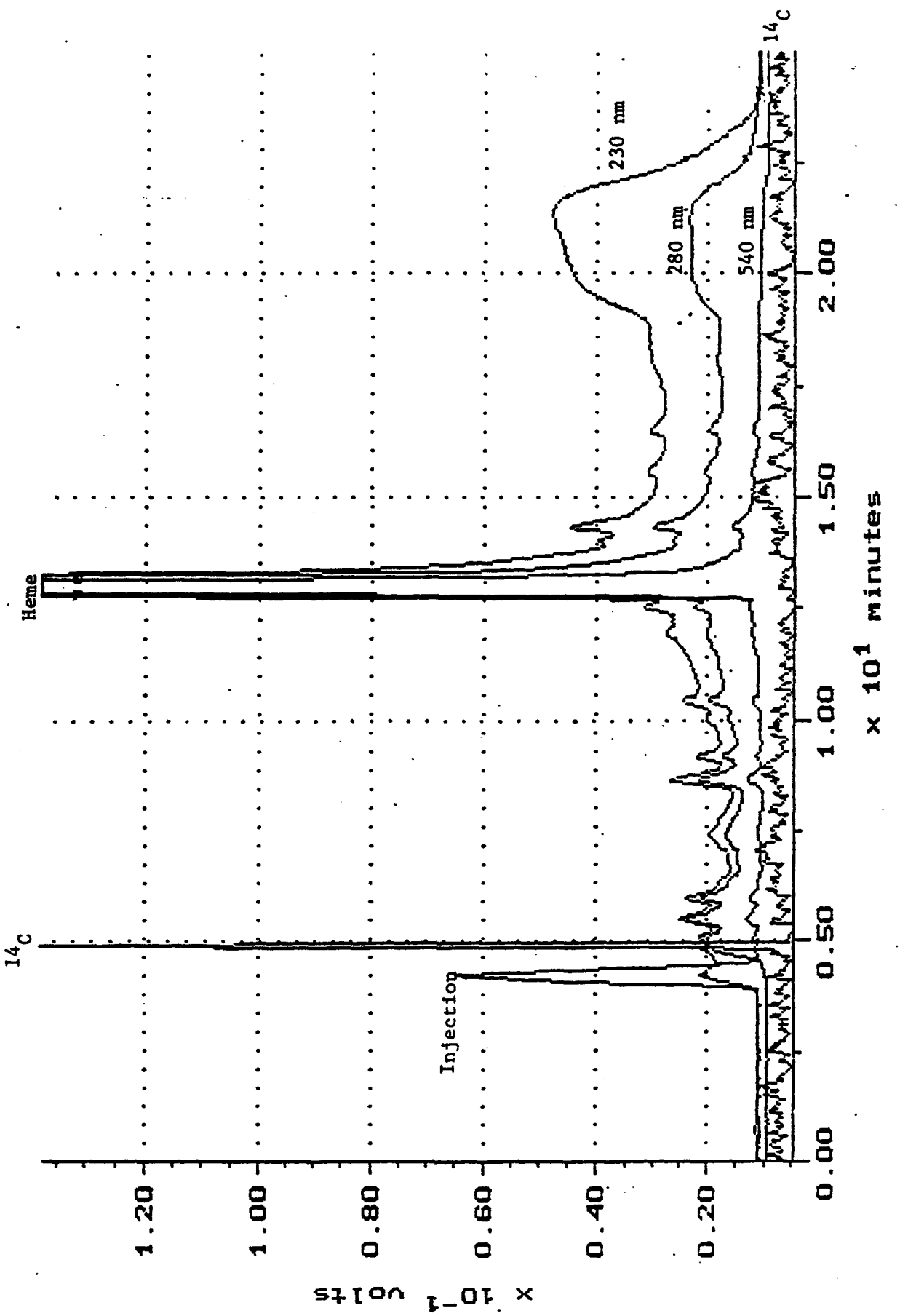

230 nm
280 nm
540 nm
Heme
^{14}C
Injection
x 10^{-1} volts
x 10^{1} minutes
0.00
0.20
0.40
0.60
0.80
1.00
1.20
0.50
1.00
1.50
2.00

Figure 18

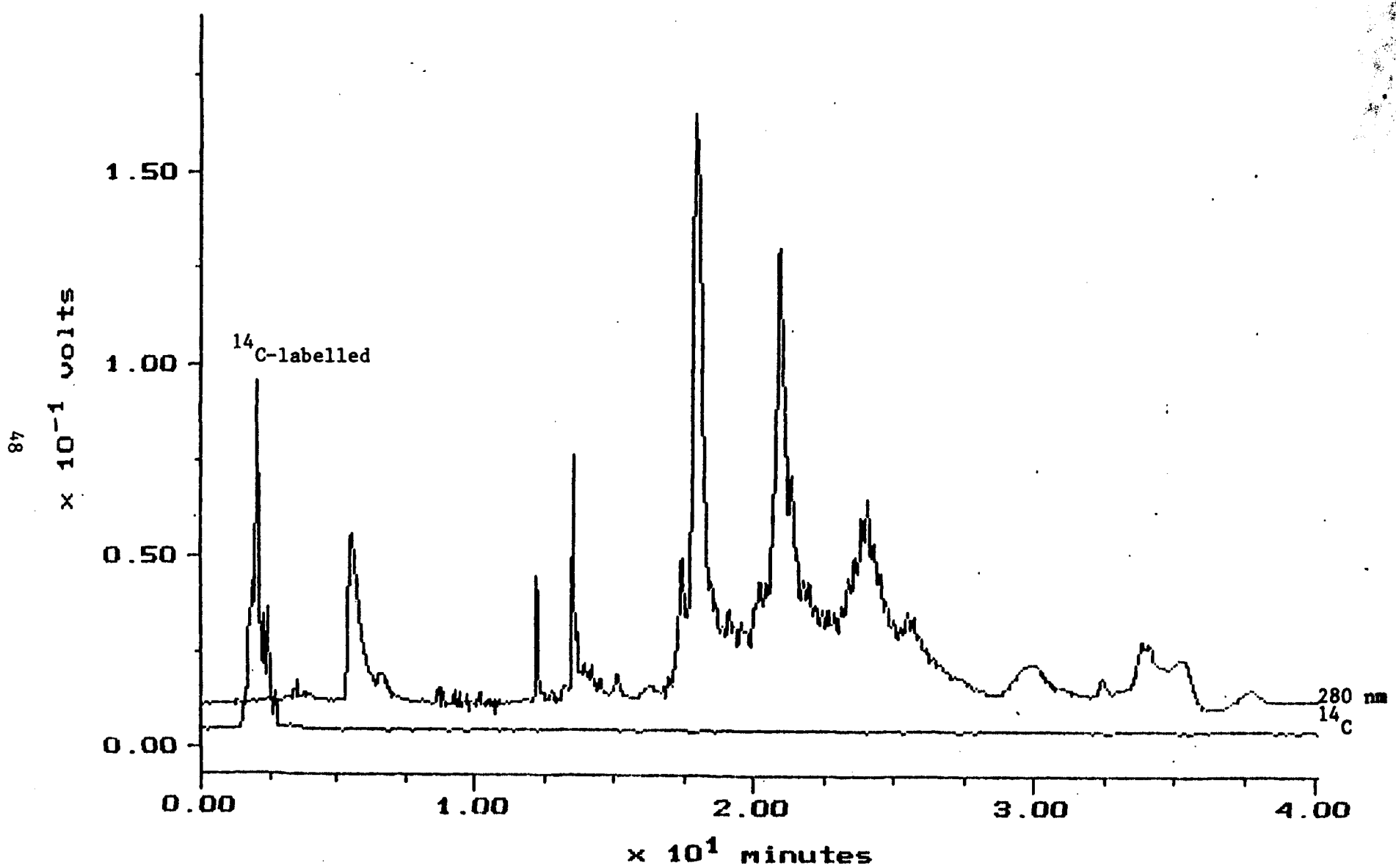

1.50
1.00
0.50
0.00
x 10⁻¹ volts
14C-labelled
280 nm
14C
0.00
1.00
2.00
3.00
4.00
x 10¹ minutes

Figure 19

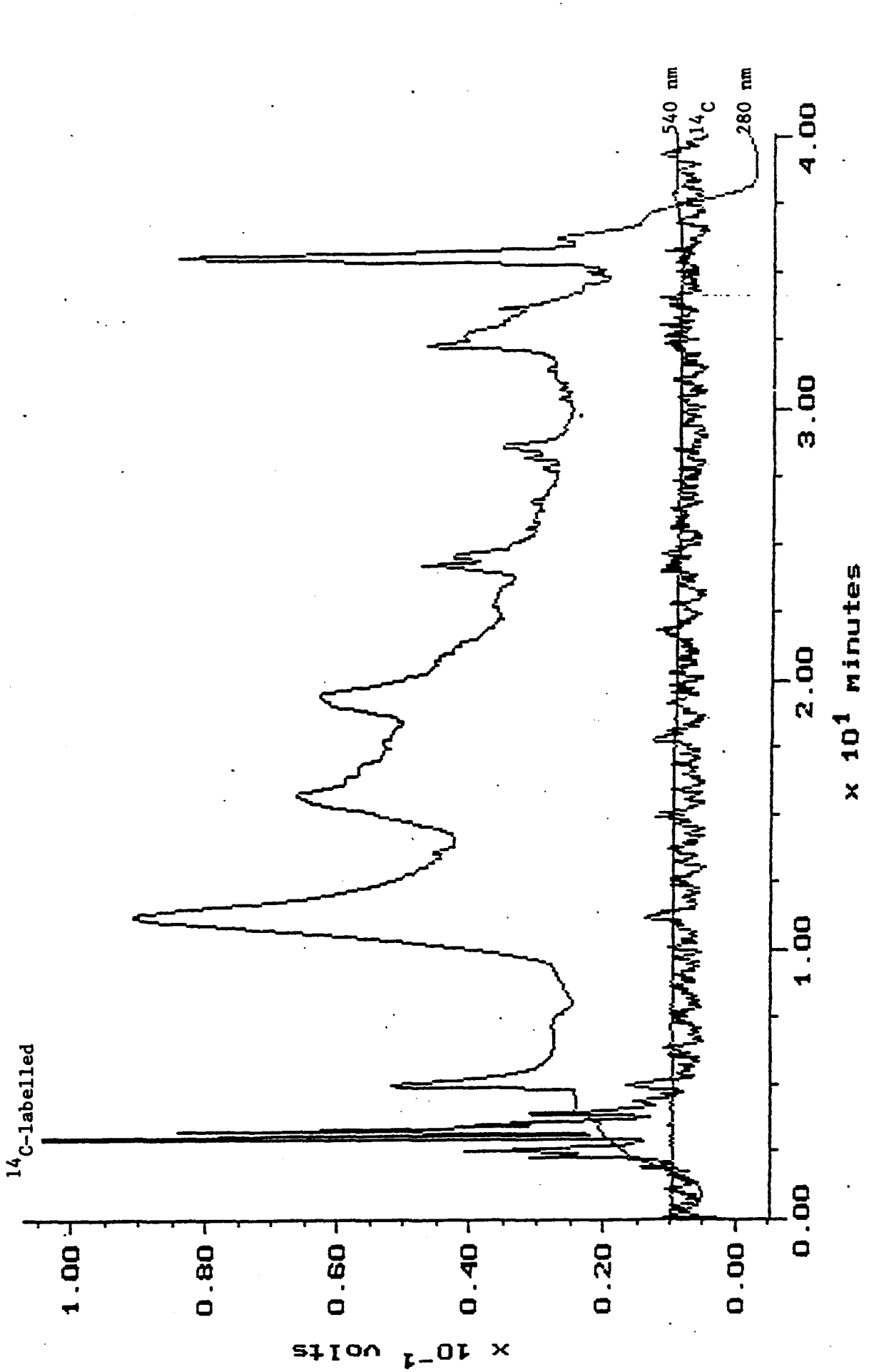

^{14}C-labelled
540 nm
λ_{14C}
280 nm
x 10^{-1} volts
1.00
0.80
0.60
0.40
0.20
0.00
0.00
1.00
2.00
3.00
4.00
x 10^{1} minutes

Figure 20

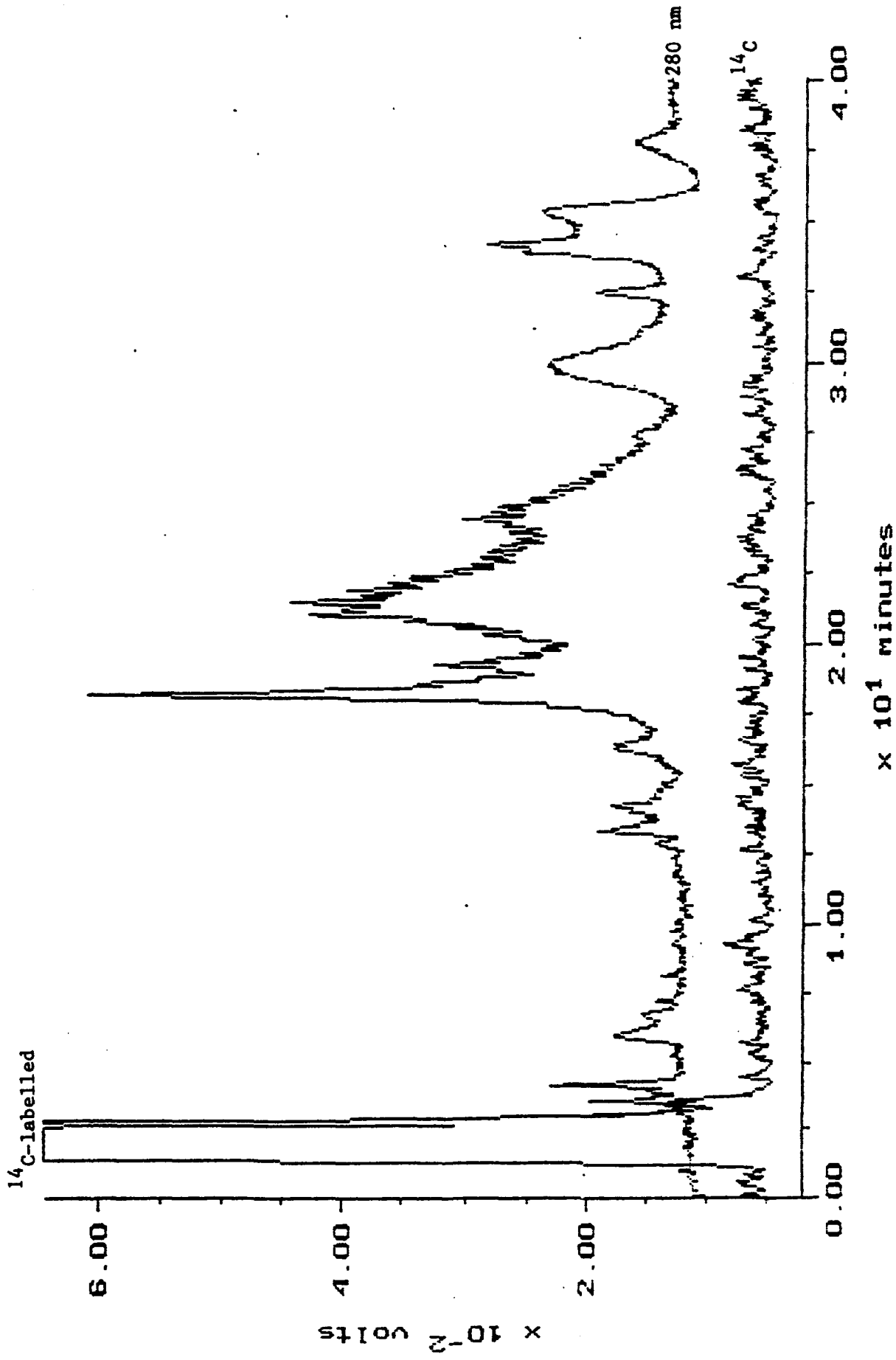

^{14}C-labelled
280 nm
^{14}C
6.00
4.00
2.00
x 10^{-2} volts
0.00
1.00
2.00
3.00
4.00
x 10^{1} minutes

Figure 21

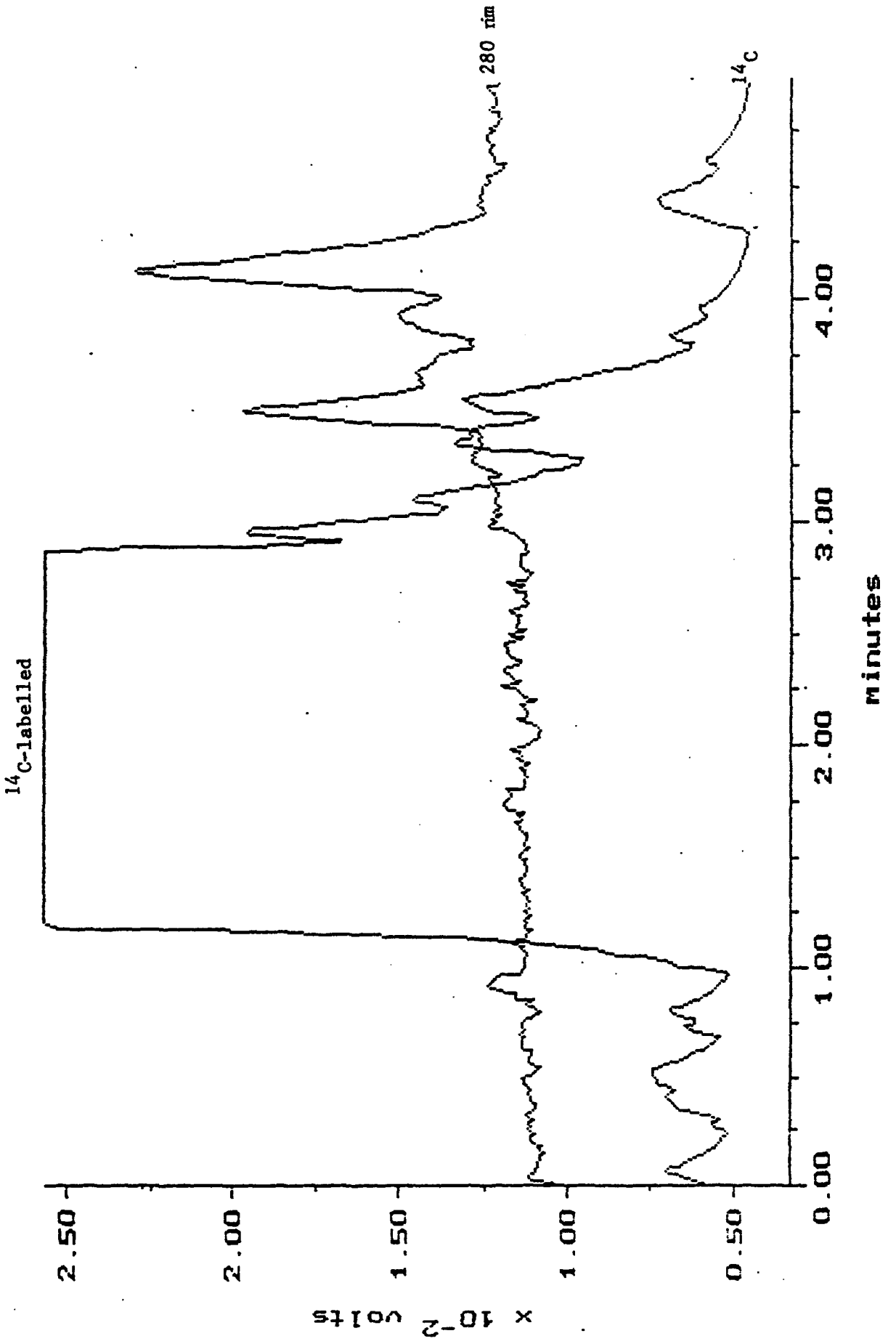

^{14}C-labelled
280 nm
^{14}C
x 10^{-2} volts
2.50
2.00
1.50
1.00
0.50
0.00
1.00
2.00
3.00
4.00
minutes

Figure 22

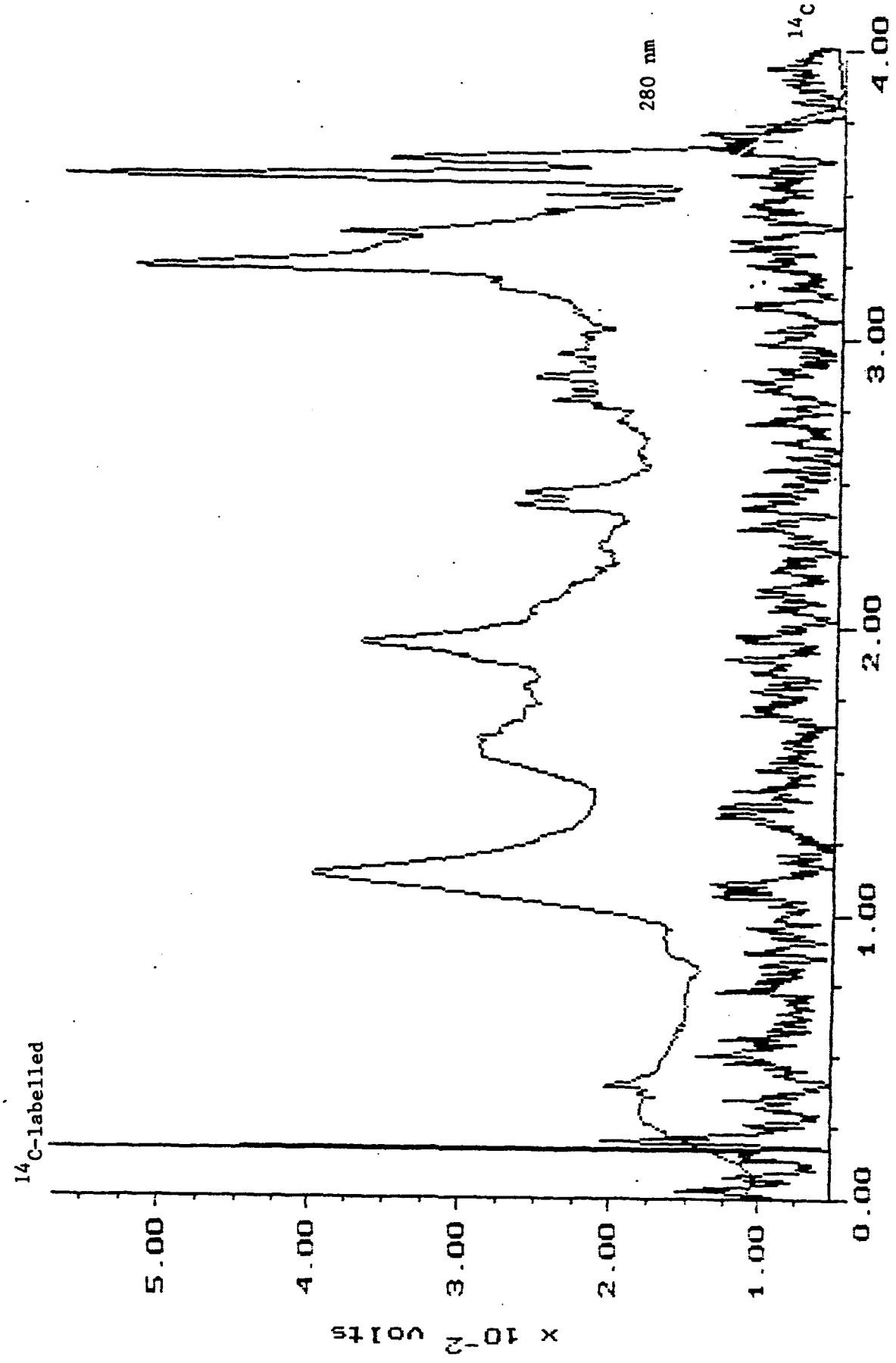
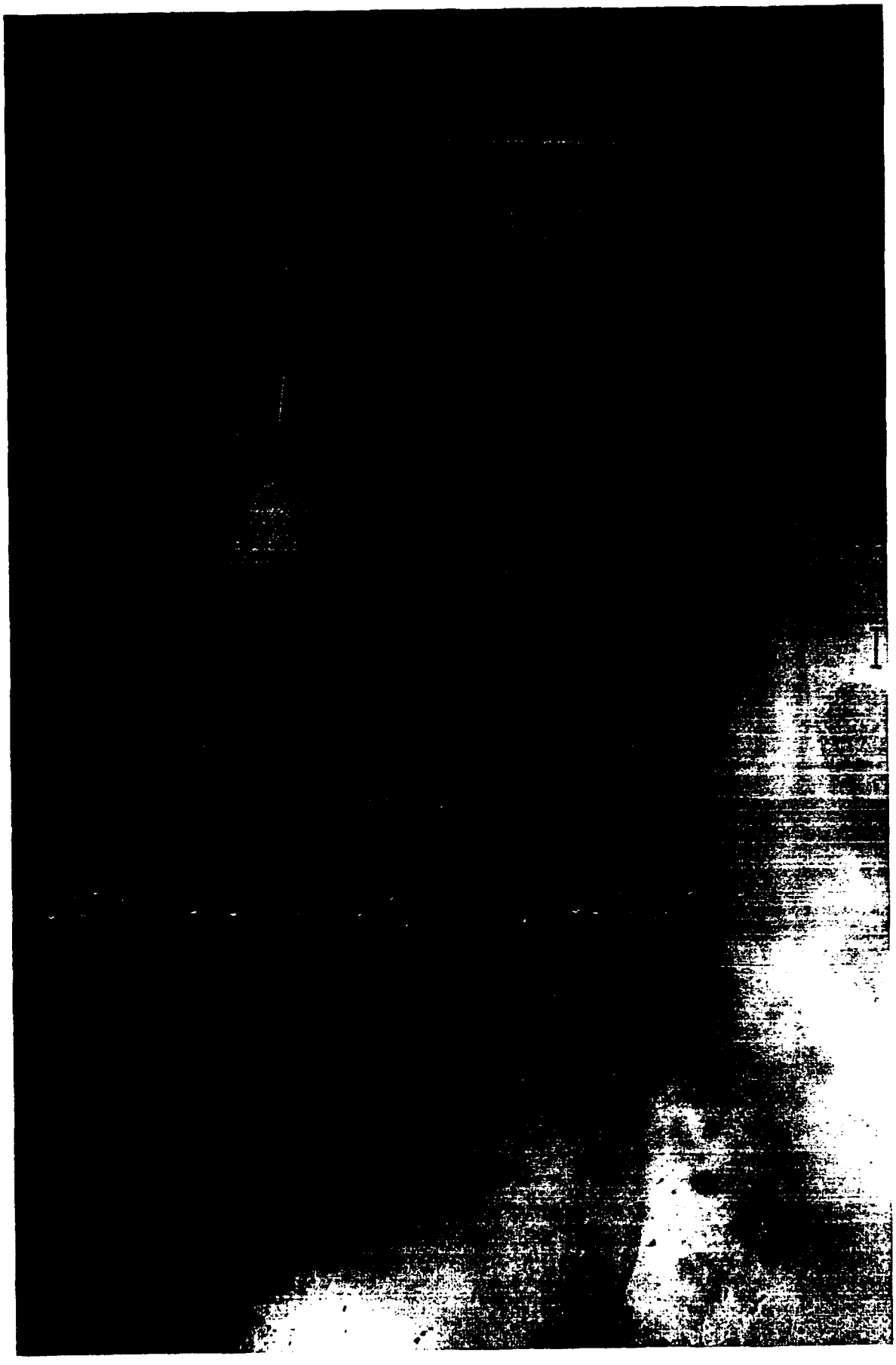

^{14}C-labelled
280 nm
^{14}C
x 10^{-2} volts
5.00
4.00
3.00
2.00
1.00
0.00
1.00
2.00
3.00
4.00
x 10^{1} minutes

Figure 23



APPENDIX A

Abstract submitted to the Society of Toxicology for a poster to be given at the Annual Meeting in Dallas, March, 1994.

FOR OFFICE USE ONLY
Fee $ _____
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Society of Toxicology 1994 Annual Meeting Abstract Form

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Nº 6045

Carefully read the abstract instructions (attached): Return this form by October 1, 1993, to: Program Committee, c/o Executive Secretary, Society of Toxicology, 1101 Fourteenth Street, N.W., Suite 1100, Washington, D.C. 20005-5601. Submit original abstract form, two copies of this page, and non-refundable abstract submission fee of $30 PER ABSTRACT. Abstract submitters are still required to register and pay the fee for the Annual Meeting. No cash or purchase orders will be accepted. *Submission Questions?* Please call Dawn Caruso at SOT Headquarters, (202) 371-1393; fax: (202) 371-1090.

ATTACH YOUR ABSTRACT TEXT HERE

STAY WITHIN BLUE LINES — CUT & PASTE IS ACCEPTABLE

INTERACTION OF DINITROBENZENES (DNB) WITH HEMOGLOBIN (Hb). G B Vasquez, W J Stevens and G Reddy[1], Center for Advanced Research in Biotech./National Institute of Standards and Technology, Rockville, MD. [1]U S Army Biomedical R&D Laboratory, Fort Detrick, MD.

DNBs are toxic by-products of the production of trinitrotoluene. Their interactions with Hb have caused cyanosis and methemoglobinemia in workers exposed to them. They form adducts with Hb that are associated with the globin chains. A molecular-level understanding of the reaction of Hb with DNBs and related metabolic products will enhance the development of toxicokinetic models that employ Hb adducts as biomarkers for exposure. The mechanism of metHb formation and the localization and characterization of the DNB adducts have been studied *in vitro* for the reaction of DNB tautomers with deoxy- (DxHb), oxy- (HbO2), and carbonmonoxy- (HbCO) hemoglobin. UV/VIS spectroscopic studies of the DNB/Hb reaction imply that DNB induces a direct conversion of DxHb to metHB, but little or no conversion occurs for either HbCO or HbO2. This implies that the reaction may require direct access of DNB to the heme, and/or that the reaction is initiated by oxidation of the heme, which occurs more readily in the deoxy state, accompanied by the formation of a reactive radical anion of DNB.

10.7 cm x 14.5 cm

ADDITIONAL FIRST AUTHOR INFORMATION:
Please type an X in the appropriate spaces.

1. Name and address of senior (first) author: Senior (first) authors can submit only one abstract for the meeting and are expected to present the abstract at the meeting.

Name: G.B. Vasquez
Organization: National Inst. Stds. and Tech.
Address: Ctr. for Adv. Res. in Biotech.
City: Rockville State: MD Zip: 20850
Country: USA
Telephone: (301) 738-6209
Fax: (301) 738-6255

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Platform ___ Poster X Either ___
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6. Minority authors: please indicate if you wish to have your abstract considered for presentation in a special minority poster session IN ADDITION to your other scientific presentation.
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1. 33 2. 64 3. 65 Topic not listed ___

8. Type three keywords that best describe the research presented in your paper. A keyword index will be prepared from the information you provide.

1. hemoglobin 2. dinitrobenzene 3. adduct