Submitted to Archives of Biochemistry
and Biophysics

UCRL-19496
Preprint

c2

DI-TERTIARYBUTYLNITROXIDE, A HILL REAGENT

G. A. Corker, M. P. Klein, D. La Font, and Melvin Calvin

January 1970

RECEIVED
LAWRENCE
RADIATION LABORATORY
APR 6 1970
LIBRARY AND
DOCUMENTS SECTION

AEC Contract No. W-7405-eng-48

TWO-WEEK LOAN COPY

This is a Library Circulating Copy which may be borrowed for two weeks. For a personal retention copy, call Tech. Info. Division, Ext. 5545

UCRL-19496
cy 2

LAWRENCE RADIATION LABORATORY
UNIVERSITY of CALIFORNIA BERKELEY

46

DI-TERTIARYBUTYLNITROXIDE, A HILL REAGENT

Gerald A. Corker*, Melvin P. Klein, Didier La Font†, and Melvin Calvin

Laboratory of Chemical Biodynamics, Department of Chemistry and Lawrence Radiation Laboratory, University of California

Berkeley, California 94720

*Present address: IBM Watson Laboratories, 612 West 115th St., New York City, New York

†Present address: University of Montpellier, Montpellier, France

DI-TERTIARYBUTYLNITROXIDE, A PARAMAGNETIC HILL REAGENT

ABSTRACT

Di-tertiarybutylnitroxide (DTBN), which we have tried to use as a trapping agent to identify the species giving rise to the photo-induced EPR signals in photosynthetic materials, functions as a Hill reagent with spinach chloroplasts. Evidence is presented which indicates that the reduction of DTBN is effected by photosystem II of the electron transport system of spinach chloroplasts. The reduced form of DTBN, the hydroxylamine, undergoes a photo-oxidation with spinach chloroplasts. Possible explanations of this apparent inconsistency are presented. A product which could be ascribed to a chemical coupling reaction between the nitroxide and the radical species giving rise to the photo-induced EPR signals in spinach chloroplasts was not detected, even using radioactive tracer methods.

INTRODUCTION

Since components essential for the reduction of carbon dioxide to the level of carbohydrate are easily removed from photosynthetic organisms, it is necessary to accept other criteria for the demonstration and study of the photosynthetic activity of sub-cellular particles isolated from these organisms. One common probe is the "Hill reaction" which involves supplying an exogenous oxidant to a suspension of such particles and monitoring either the photo-reduction of the oxidant or the photo-production of oxygen. Quantitative studies are difficult because of the methods used to monitor these changes. The photo-reduction is normally followed by spectrophotometric techniques which are complicated by the light scattering and absorption by the suspended particles. The production of oxygen is followed by manometric or electrolytic techniques both of which are complicated by oxygen uptake and by the requirement of a rapid exchange between the suspension and another medium.

This paper demonstrates the existence of a Hill oxidant whose reduction can be monitored by electron paramagnetic resonance (EPR). With this method the optical and diffusional properties of the system cause no interference with the measurements.

In an earlier communication (1), it was shown that di-tertiarybutylnitroxide (DTBN), a stable free radical, and its reduced form, di-tertiarybutylhydroxylamine (DTBNH), both react photochemically with spinach chloroplasts. Since the reaction of DTBNH was a photochemical

oxidation to the nitroxide as evident by EPR and since the oxidation products of DTBN could not be detected in the reaction mixtures following the destruction of DTBN, it was tentatively concluded that DTBN coupled chemically with photo-generated radicals in chloroplasts. Further investigation of the DTBN reaction in chloroplasts proves the preliminary conclusion incorrect. In this paper evidence is given which shows that DTBN is reduced to DTBNH photosynthetically by light system II of the chloroplasts, a conclusion drawn by Weaver (2) from observation of the disappearance of EPR signal of a different nitroxide radical with whole Chlorella.

Since the completion of this work, additional studies with other nitroxide radicals and biradicals indicates that the behavior described above for DTBN is not general for this class of radicals. Preliminary data indicate that under some conditions certain of these radicals do not act as Hill oxidants.

MATERIALS AND METHODS

DTBN and di-tertiarybutylhydroxylamine (DTBNH) were prepared according to the procedures of Hoffman (3). The starting material, 2-methyl-2-nitropropane, was prepared according to Kornblum (3) from tertiarybutylamine supplied by Matheson, Coleman and Bell. Radioactive DTBN was synthesized following the procedures of Ritter and Minieri (5), Kornblum (4), and Hoffman (3), from ^{14}C-tertiarybutyl alcohol supplied by New England Nuclear Corporation. E. I. du Pont de Nemours and Company and K & K Laboratories supplied the

3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), and 2,6-dichlorophenolindophenol (DCPIP), respectively. Salicylaldoxime was prepared and purified according to reference 6.

Chloroplasts were isolated from commercial spinach leaves according to the method of Park and Pon (7). Following their isolations, the chloroplasts were treated in one of the following ways: 1) washed three times with buffer (phosphate buffer, pH-6.8 in 0.5 M sucrose) solutions; 2) allowed to age in a cold room (0°C) for 3 days; or 3) ruptured either in distilled water or in 0.05 M sucrose buffered at pH 6.8. Chloroplasts treated in one of these three ways will be referred to as treated chloroplasts in this paper.

The chlorophyll (a + b) content of each chloroplast suspension was determined according to a method of Vernon (8, equations 7 and 8). The concentrations varied between 0.5 mg to 1.5 mg per ml of suspension. DTBN concentrations were determined from optical absorption spectra having assumed the extinction coefficient of the nitroxide in our solutions to be the same as it is in water (ε = 9.8 at 420 nm).

In attempting to ascertain the effect of a particular perturbation or chemical upon the nitroxide-chloroplast reaction, a control which showed the destruction of the nitroxide with the chloroplast or fragment preparation in question was compared with an identical sample which had been subject to the perturbation of interest or which contained the chemical of interest. This was required because the quantitative activity of the chloroplasts varied from preparation to preparation. Each set of experiments was repeated several times.

The EPR data were obtained with an x-band spectrometer (9.5 GHz). The microwave power was non-saturating for the concentrations of nitroxide studied. The modulation amplitude was 0.31 gauss, approximately 1/3 the line width of DTBN at the concentrations employed in these experiments.

A 1000 watt, 115 volt G.E. projection lamp (tungsten filament) was used to illuminate the EPR samples. The light from this source after having passed through a water bath 1-1/2 inches long, which contained flowing water and an infrared filter, was passed through additional filters (Corning 1-69 and 3-67) and focused onto the samples through slits in the EPR cavity. This filter combination essentially removes light of wavelength less than 5400 Å (intensity = 7.0×10^5 ergs/cm^2 sec).

Measurements of oxygen evolution were made with a Warburg apparatus. Flask constants were calculated having assumed the solubility of oxygen in the sucrose buffer equal to the solubility of oxygen in water at the proper temperature (0.31 ml O_2/ml liquid at 20°C) for the conversion of cm pressure to micromoles of O_2.

Product analyses were performed on reaction mixtures containing substrate amounts of DTBN. These mixtures, some of which were exposed to light and some kept in darkness, were separated into chloroplast pellets and water supernant by centrifugation. The pellets were extracted with cold methanol (0°C) in the dark in a cold room.

The water supernant (or an ether extract) and the methanol extract were analyzed by thin layer chromatography (TLC) using silica gel G supplied by Warner Chilcott Laboratories. Diethylether:

petroleum ether:tertiarybutyl alcohol in a ratio of 160:100:5, freshly prepared, was the solvent system. This system was modified by the addition of 1 ml of acetic acid to 265 ml of the mixture. The silica thickness was 5 mm spread from a slurry containing x grams to 2x ml of acetone. Radioautography and zonal mapping were employed when ^{14}C-labelled DTBN was used. A Packard Tri-Carb, liquid scintillation spectrometer, Model 3375, was employed to assay radioactivity.

The gas phases of these reaction mixtures were analyzed by vapour phase chromatography (VPC) on a molecular sieve 5-A column at 55°C with a helium flow rate of 12 ml/min. The temperature of injector and detector were 180°C and 250°C, respectively.

RESULTS

As was reported earlier (1), a photo-destruction of DTBN as monitored by EPR is effected by fresh or aged chloroplasts or chloroplast fragments. When mixtures containing freshly isolated chloroplasts or fragments (fresh or aged) and substrate amounts of DTBN are examined in a Warburg apparatus, the pressures in the systems increase. Typical results are shown in Figures 1 and 2. Included in Figure 1 are the pressure variations observed in samples containing chloroplasts with only buffer and with $K_3Fe(CN)_6$, a known Hill oxidant. The samples containing DTBN or $K_3Fe(CN)_6$ result in increases of molecular oxygen as evidenced by VPC (see Figure 3); the sample

with buffered chloroplasts, a decrease in oxygen. The oxidation products of DTBN, isobutylene and 2-nitroso-2-methylpropane, are not detected in the atmosphere of samples containing DTBN.

(Insert Figures 1 - 3 here)

As shown in Figures 1 and 2, after approximately 100 to 200 minutes of light exposure, oxygen production in these systems stops. When chloroplast suspensions are exposed to white light prior to the addition of DTBN, the initial rate and the maximum amount of O_2 produced decrease; the extent of decrease depending upon the length of the pre-illumination period. When the preillumination is 100 to 200 minutes, no oxygen production is observed.

When the initial concentration of nitroxide is varied, the initial rate of oxygen production, the amount of nitroxide destroyed and the total amount of oxygen produced also vary and in a linear manner to the initial DTBN concentration. Figure 4 shows the variations of the amount of nitroxide destroyed in the light (dashed line) and the amount of oxygen produced as a function of the initial concentration of DTBN. The ratio of the slopes of these two lines - the ratio of nitroxide destroyed photochemically to the moles of O_2 produced - is 4.6. No correction of the data shown in Figure 4 was made to account for the uptake of oxygen by the chloroplasts (see Figure 1).

(Insert Figure 4 here)

DCMU poisons the photo-production of oxygen and the photo-destruction of DTBN. However, it has no effect upon a dark destruction of DTBN which occurs in these systems. These effects are shown in Table I. Also presented in this table are data obtained with DTBNH included in the suspensions. DTBNH does not act as a Hill reagent, as evidenced by the lack of oxygen production from DTBNH-chloroplast mixtures. DTBNH does not alter the DTBN-chloroplast reaction. However, the photo-oxidation of DTBNH does occur in samples containing both DTBN and DTBNH (c. 7.5×10^{-6} moles in data presented in Table II). This photo-oxidation is also inhibited by DCMU.

(Insert Table I here)

Figure 2 shows a comparison of the oxygen production observed using DTBN with freshly isolated chloroplasts, fresh fragments and aged fragments. The amounts of nitroxide destroyed in these samples in the dark and during illumination and the quantity of photo-produced oxygen are tabulated in Table II. As can be seen in this table, the amount of DTBN which reacts in the dark decreases when the fragments or chloroplasts are aged in a cold room. This effect can also be obtained by repeated buffer washings. The quantities of oxygen produced given in this table are not corrected for oxygen uptake which increases with age of the chloroplast or fragments. The rate of oxygen production in the sample containing aged fragments just compensates for rate of oxygen uptake.

(Insert Table II here)

Product analyses of DTBN-chloroplast reaction mixtures using radioactive DTBN and thin layer chromatography yield radioautograms and zonal maps showing the presence of only two radioactive compounds. This result is obtained whether fresh chloroplasts, fresh fragments or aged fragments are used. Typical results are shown in Figures 5 and 6.

(Insert Figures 5 and 6 here)

One of these radioactive compounds is ^{14}C-DTBN, the other ^{14}C-DTBNH. These identifications were obtained by co-chromatography of the water phases of the reaction mixtures with ^{14}C-DTBN and non-radioactive DTBNH. The latter was detected by phosphomolybdic acid in ethanol. Co-chromatography was performed using two different solvent systems to develop the chromatograms. The unknown product in the reaction mixture exhibited an R_f identical to the hydroxylamine (DTBNH) in both systems.

Small amounts of DTBNH are also detected in DTBN-chloroplast samples kept in the dark, indicating that the dark reaction is also a reduction of DTBN. DTBN is reduced *in vitro* by sodium ascorbate, which is probably the reducing agent in the chloroplasts which effects the dark reaction of the nitroxide.

Shown in Table III are typical effects upon the photo-induced decay of DTBN by the addition of various chemicals to DTBN-chloroplast mixtures. Only those data enclosed in dashed lines can be compared, for they contain identical chloroplast preparations. As can be seen

(Insert Table III here)

in this table, DCMU, which truncates electron flow from H_2O, totally inhibits the DTBN reduction. However, the addition of reduced DCPIP, which is known to contribute electrons to photosystem I of DCMU poisoned chloroplasts, does not lift the DCMU inhibition of DTBN destruction. This indicates that DTBN is reduced by a component oxidized by photosystem I and reduced by photosystem II of the chloroplasts. The DCPIPH used was obtained by KBH_4 reduction of DCPIP since DTBN reacts with the reducing agent normally used, sodium ascorbate, but does not react with KBH_4. The partial inhibitory effects of the Hill agents, DCPIP and $K_3Fe(CN)_6$, are due to competition for electrons supplied by H_2O or to a direct reaction with the reduced form of DTBN resulting in a net decrease in the rate of DTBN reduction.

The two other treatments which cause an inhibition of the photodestruction of DTBN by chloroplasts are the extraction of the chloroplasts with heptane and heating the chloroplasts at 100°C for 15 minutes. Heating the chloroplasts for 15 minutes at 60° has no effect upon the photo-reduction of DTBN.

A product which could be ascribed to a coupling reaction between the nitroxide and the radical species which gives rise to the photoinduced EPR signal in spinach chloroplasts was not detected. Unfortunately, the most sensitive methods used for the detection of products (zonal mapping and radioautography) were not calibrated to yield quantitative data. However, in samples containing ca. 7% of the original ^{14}C-DTBN, an intense darkening of an X-ray film caused by this DTBN was observed in 58 hours. If only 1% of the original

^{14}C-DTBN had been involved in a coupling reaction, the product of this reaction should have caused a detectable darkening of the film, especially when three-week exposure times were employed.

DISCUSSION

The results reported in this paper show that chloroplasts sensitize a photo-reduction of di-tertiarybutylnitroxide to the corresponding hydroxylamine. Oxygen is produced concurrently in a ratio of approximately 1 mole of oxygen evolved to 4 moles of nitroxide reduced. The results are consistent with an interpretation that DTBN reduction is coupled with photosynthetic electron transport and that the oxygen evolved originates from water which is oxidized photosynthetically.

That the reduction is effected by photosystem II is suggested by the following observations: 1) DCMU which truncates photosynthetic electron flow between water and photosystem I, inhibits the photoreduction of DTBN. 2) Plastoquinones are thought to be involved in the chain which couples the two photosystems; when these are partially extracted from chloroplasts, the rate of destruction of DTBN is decreased. 3) KBH_4 and the reduced form of DCPIP which reduce plastoquinones cause a reduction in the destruction rate. 4) The reduced form of DCPIP, which is known to effect the photosynthetic reduction of NADP via photosystem I in DCMU-poisoned chloroplasts, will not lift the inhibition caused by DCMU upon the photo-destruction of DTBN.

The photochemical reduction of DTBN is accompanied by a dark reaction which also involves the reduction of DTBN. The species

within the chloroplasts which effects the dark reaction of DTBN can be removed from the chloroplasts by washing, aging, or rupturing them. The rate of the photochemical reaction sensitized by a particular chloroplast preparation is also affected by these treatments. However, the same photochemical reaction occurs whether fresh, aged, washed, or fragmented chloroplasts are used.

Observations which appear inconsistent with the above interpretation are the following: 1) Heating chloroplasts at 60° for 15 minutes, which normally destroys the oxygen evolving capacity of chloroplasts, causes no inhibition of the photo-destruction of DTBN. 2) The reaction products from the photo-reduction of DTBN, namely oxygen and the hydroxylamine (DTBNH), interact in the dark *in vitro* to produce DTBN. 3) The reduced form of DTBN, the hydroxylamine, is photo-oxidized by the chloroplasts to DTBN.

Although heating chloroplasts at 60°C uncouples the water to oxygen mechanism, it has not been shown that such treatment disrupts photosystem II. Thus, DTBN is probably reduced by photosystem II but another component acts as the electron source in heated chloroplasts.

The non-occurrence of the reaction between O_2 and DTBNH in the presence of chloroplasts is probably due to a competitive uptake of O_2 by the chloroplasts.

The observation of a DCMU sensitive, short-term photo-oxidation of DTBNH is more difficult to explain. There are two possible explanations for this: 1) The photo-generated oxidant is not an essential constituent of photosynthetic electron transport, is available in a limited quantity and is irreversibly expended. 2) The

photo-generated oxidant is an essential constituent of photosynthetic electron transport, is produced by photosystem II, and is normally involved in the oxidation of water. When the system of DTBNH-chloroplasts is initially illuminated, DTBNH competes with H_2O in supplying electrons to photosystem II. However, with increasing time of illumination, several factors become operative to effectively inhibit the photo-oxidation of DTBNH: 1) The rate of oxidant production by photosystem II becomes limited by the rate at which the reductants formed from this system become oxidized. 2) The sequence of constituents which couple the oxidation of water with photosystem II reaches a steady state so that water becomes more efficient in supplying electrons than DTBNH. 3) The reduction of DTBN to DTBNH, which is effected by electrons abstracted from both H_2O and DTBNH, eliminates the observation of the oxidation of DTBNH.

Although further investigation of this reaction is required before one could state which of these is the correct interpretation, the studies and interpretation of Izawa, Heath and Hing (9), who have observed the photo-oxidation of hydroxylamine itself, suggest that number two is the more likely. These investigators worked with EDTA-treated chloroplasts, systems in which the water splitting mechanism is uncoupled so that sustained oxidation of hydroxylamine occurs.

If our interpretation of the site of reduction of DTBN is correct and if we can show that DTBNH can replace water as an electron source for photosystem II, the system DTBN-DTBNH could be an excellent probe with which to investigate the properties of

photosystem II, since the reduction of DTBN or the oxidation of DTBNH can be followed by EPR.

ACKNOWLEDGEMENTS

This work was supported in part by the U. S. Atomic Energy Commission.

REFERENCES

1. CORKER, G. A., KLEIN, M. P. AND CALVIN, M., Proc. Nat. Acad. Sci. U.S. 56, 1365 (1966).
2. WEAVER, E. C. AND CHON, H. P., Science 153, 301 (1966).
3. HOFFMAN, K., FELDMAN, A. M., GELBLUM, E. AND HODGSON, W. C., J. Am. Chem. Soc. 86, 639 (1964).
4. KORNBLUM, N., CLUTTER, R. J. AND JONES, W. J., J. Am. Chem. Soc. 78, 4003 (1965).
5. RITTER, J. J. AND MINIERI, P. O., J. Am. Chem. Soc. 70, 4048 (1948).
6. HUNTRESS, H. E. AND MULLIKEN, S. P., in "Identification of Pure Organic Compounds," p. 63. John Wiley and Sons, New York (1941).
7. PARK, R. B. AND PON, N. G., J. Mol. Biol. 6, 105 (1963).
8. VERNON, L. P., Anal. Chem. 32, 1144 (1960).
9. IZAWA, S., HEATH, R. L. AND HIND, G., Biochim. Biophys. Acta 180, 388 (1969).

FIGURE CAPTIONS

Fig. 1 Pressure changes above fresh chloroplast suspensions containing 1.10 mg chlorophyll (a + b) with 0.01 M $K_3Fe(CN)_6$ (open triangles), 0.009 M DTBN (closed circles), and buffer (open circles). Illuminated with white light.

Fig. 2 Pressure changes above solutions of 0.011 M DTBN with fresh chloroplasts (open circles) containing 1.3 mg of chlorophyll (a + b), with fresh fragments (closed triangles) containing 1.3 mg chlorophyll (a + b), and with aged (6 days) fragments (closed circles) containing 1.7 mg of chlorophyll (a + b). Sample volume equals 2.5 ml.

Fig. 3 VPC traces of a sample of the atmosphere above a chloroplast suspension containing 0.92 mg chlorophyll (a + b) and 0.003 M DTBN before (a) and after (b) illumination of the sample for 1 hour with white light. The vessel was purged initially with helium. The column injector and detector were 55°, 180°, and 250°, respectively. Helium flow was 12 ml/min.

Fig. 4 Oxygen production and amount of DTBN photo-destroyed by spinach chloroplasts as a function of the initial concentration of DTBN.

Fig. 5 X-ray film exposed for 58 hours to a thin-layer chromatogram containing ^{14}C-DTBN-chloroplast reaction mixture kept in the dark (E_d) and one exposed to light (E_ℓ) and the methanol

FIGURE CAPTIONS (continued)

extract of chloroplasts from the same ^{14}C-DTBN-chloroplast reaction mixtures kept in the dark (M_d) and exposed to light (M_ℓ). Small dark spots on right due to radioactive ink used to align developed film with TLC plate.

Fig. 6 . Zonal maps of chromatograms of ether extracts of water phases of ^{14}C-DTBN-chloroplast reaction mixtures. Solid line mixture was exposed to light; dashed line mixture kept in darkness. "a" and "b" refer to chromatogram fronts, dark and light, respectively.

TABLE I

THE EFFECT OF DCMU AND DTBNH UPON THE PRODUCTION OF OXYGEN FROM THE DTBN-CHLOROPLAST SYSTEM

Initial Amounts					
DTBN	DTBNH	DCMU	Condition	Oxygen Produced	DTBN Destroyed
3.8×10^{-5}	0	0	Dark	0	1.44×10^{-5}
"	0	0	Light	2.4×10^{-6}	2.82×10^{-5}
"	0	3.0×10^{-7}	"	0	1.34×10^{-5}
"	3.9×10^{-5}	0	"	2.4×10^{-6}	2.07×10^{-5}
4.5×10^{-5}	0	0	Light*	2.9×10^{-6}	1.10×10^{-5}
0	5.9×10^{-5}	0	Light*	0	?

All quantities in moles. The sample volumes were 3 ml containing 1.21 mg of chlorophyll (a + b) except the ones marked *, which were 2.5 ml containing 0.92 mg of chlorophyll (a + b). All samples were 0.5 M in sucrose buffered at pH 6.8 with phosphate.

TABLE II

OXYGEN PRODUCTION FROM DIFFERENT CHLOROPLAST PREPARATIONS USING DTBN

Photosynthetic Material	Initial DTBN	DTBN Destroyed Dark	DTBN Destroyed Light	Oxygen Produced
Fresh chloroplasts*	2.7×10^{-5}	0.49×10^{-5}	1.9×10^{-5}	4.8×10^{-6}
Fresh fragments*	"	0.27×10^{-5}	1.0×10^{-5}	1.6×10^{-6}
Aged (6 days) fragments**	"	0.02×10^{-5}	0.76×10^{-5}	(?)

All quantities expressed in moles.

* Chlorophyll content (a + b) was 1.3 mg.

** Chlorophyll content (a + b) was 1.7 mg.

All samples were 2.5 ml which were 0.5 M in sucrose buffered at pH 6.8 with phosphate.

TABLE III

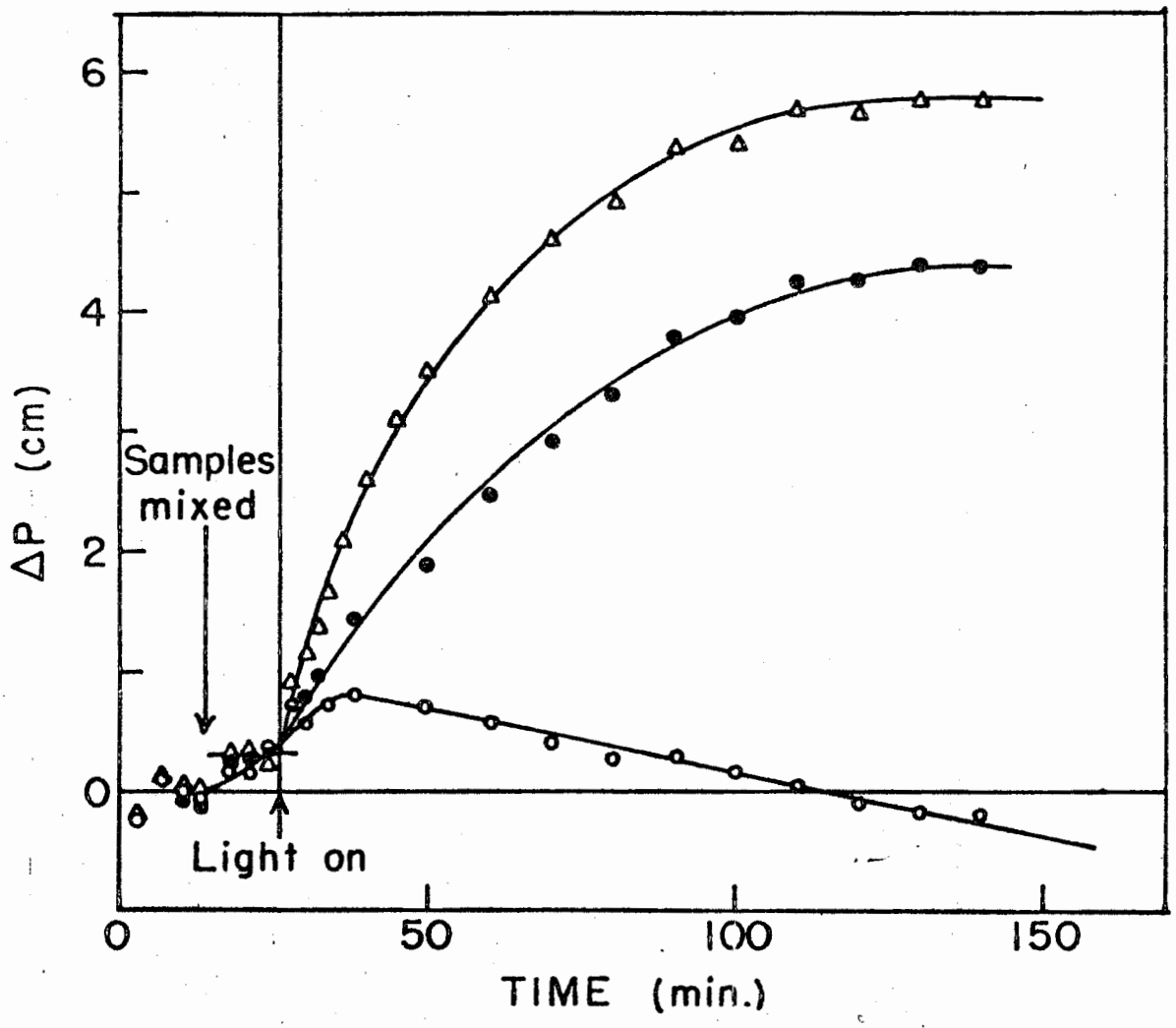
EFFECTS OF VARIOUS CHEMICALS ON PHOTO-INDUCED NITROXIDE DECAY

	DTBN Conc. (M)	DCMU Conc. (M)	DCPIPH Conc. (M)	DCPIP Conc. (M)	$K_3Fe(CN)_6$ Conc. (M)	Oxime* Conc. (M)	Initial Rate** (%/min/mg Chl)
a) Control	5.0×10^{-4}	0	0	0	0	0	-46.0
DCMU	5.0×10^{-4}	4.7×10^{-4}	0	0	0	0	0
b) Control	2.08×10^{-4}	0	0	0	0	0	-18.3
DCPIPH	2.08×10^{-4}	0	7.4×10^{-5}	0	0	0	-6.4
DCMU	2.08×10^{-4}	2.3×10^{-4}	0	0	0	0	-0.5
DCMU + DCPIPH	2.08×10^{-4}	2.3×10^{-4}	7.4×10^{-5}	0	0	0	-0.2
c) Control	2.08×10^{-4}	0	0	0	0	0	-18.3
DCPIP	2.08×10^{-4}	0	0	7.4×10^{-5}	0	0	-16.1
$K_3Fe(CN)_6$	2.08×10^{-4}	0	0	0	5.9×10^{-4}	0	-12.9
d) Control	1.5×10^{-4}	0	0	0	0	0	-32.0
Oxime	1.5×10^{-4}	0	0	0	0	9.6×10^{-3}	-12.0

* Salicyaldoxime-treated chloroplast incubated with oxime 20 minutes prior to experiment.

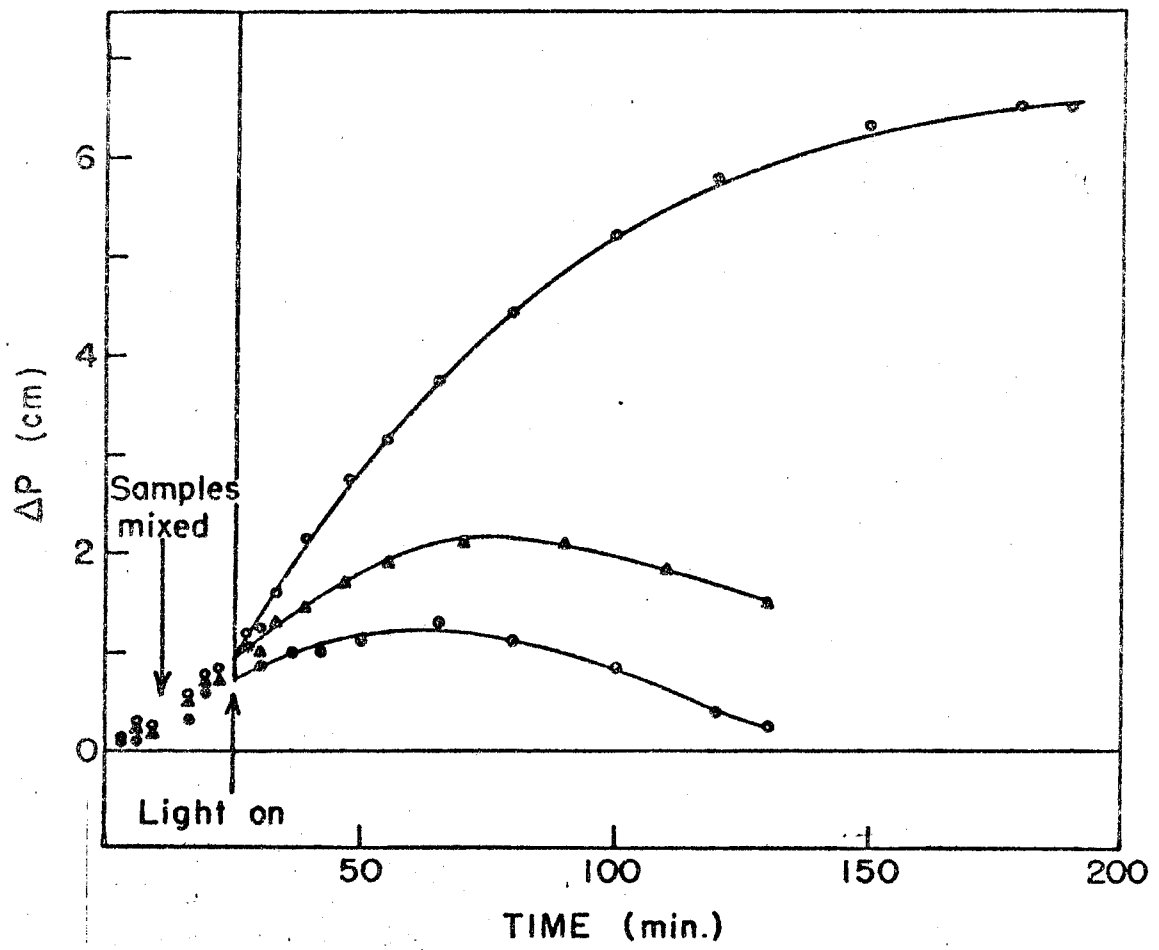
** Total amounts of chlorophyll (a + b) varied between 0.5 mg to 1.4 mg Chl per ml of sample.

Only data enclosed in dashed lines can be compared.



XBL 679-6165

Fig. 1



XBL 679-6157

Fig. 2

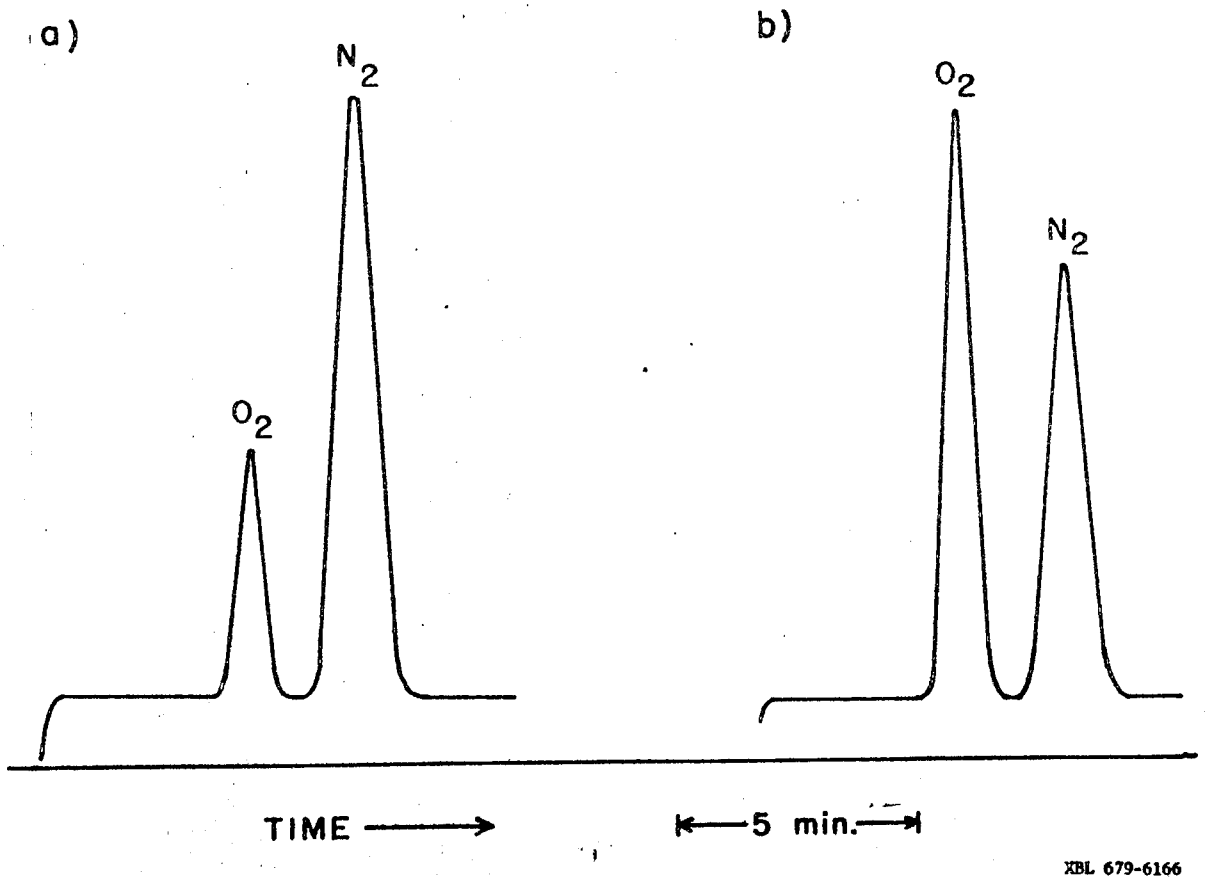
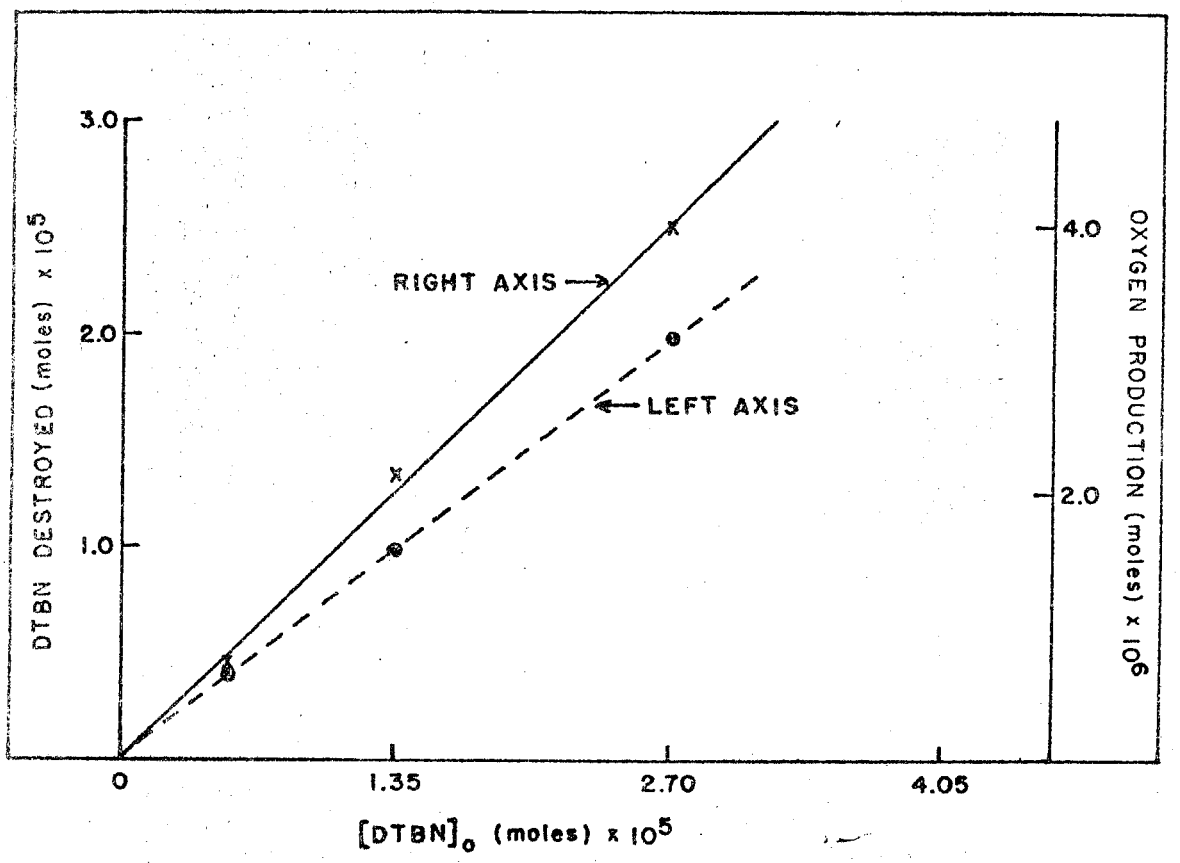
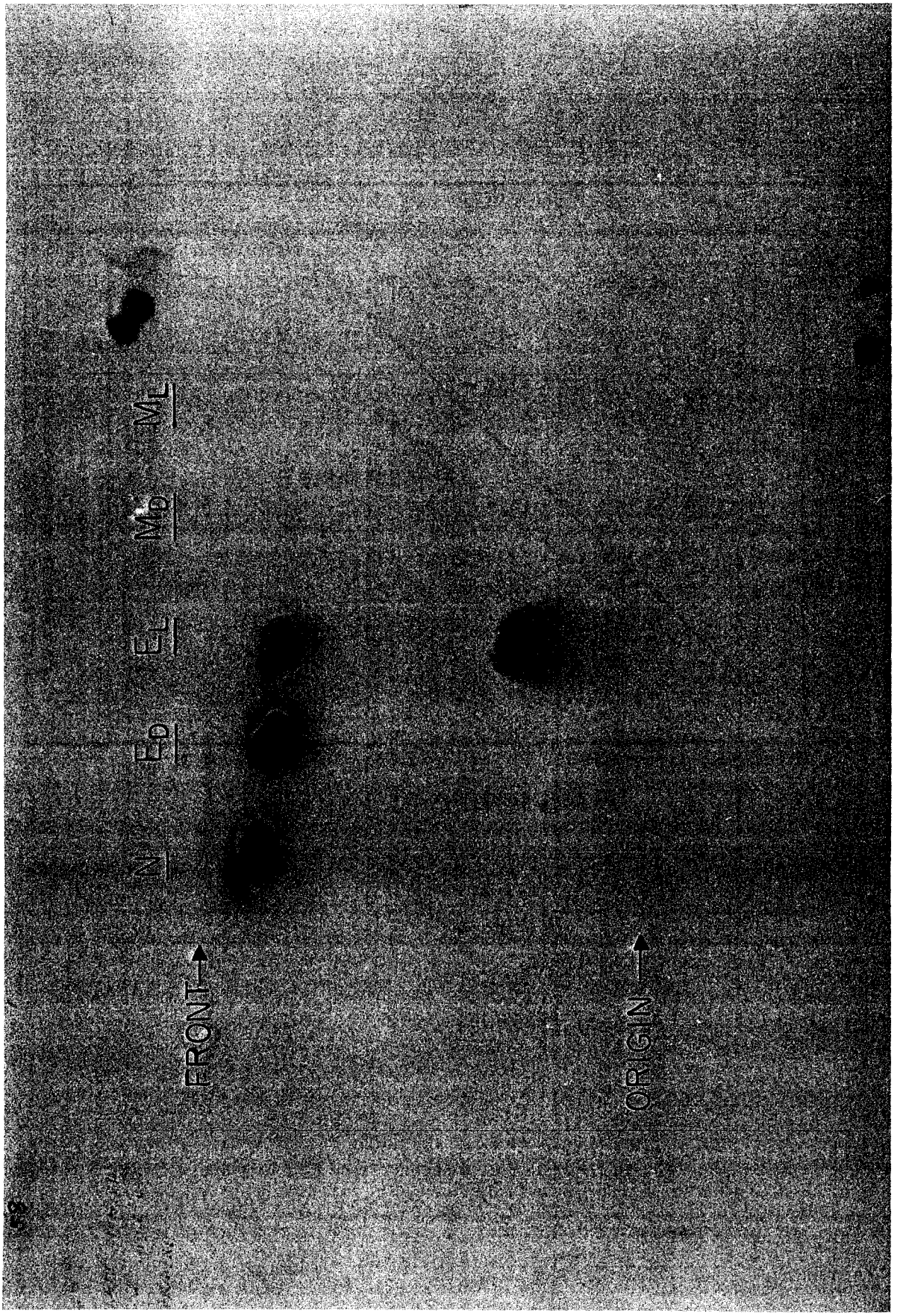


Fig. 3



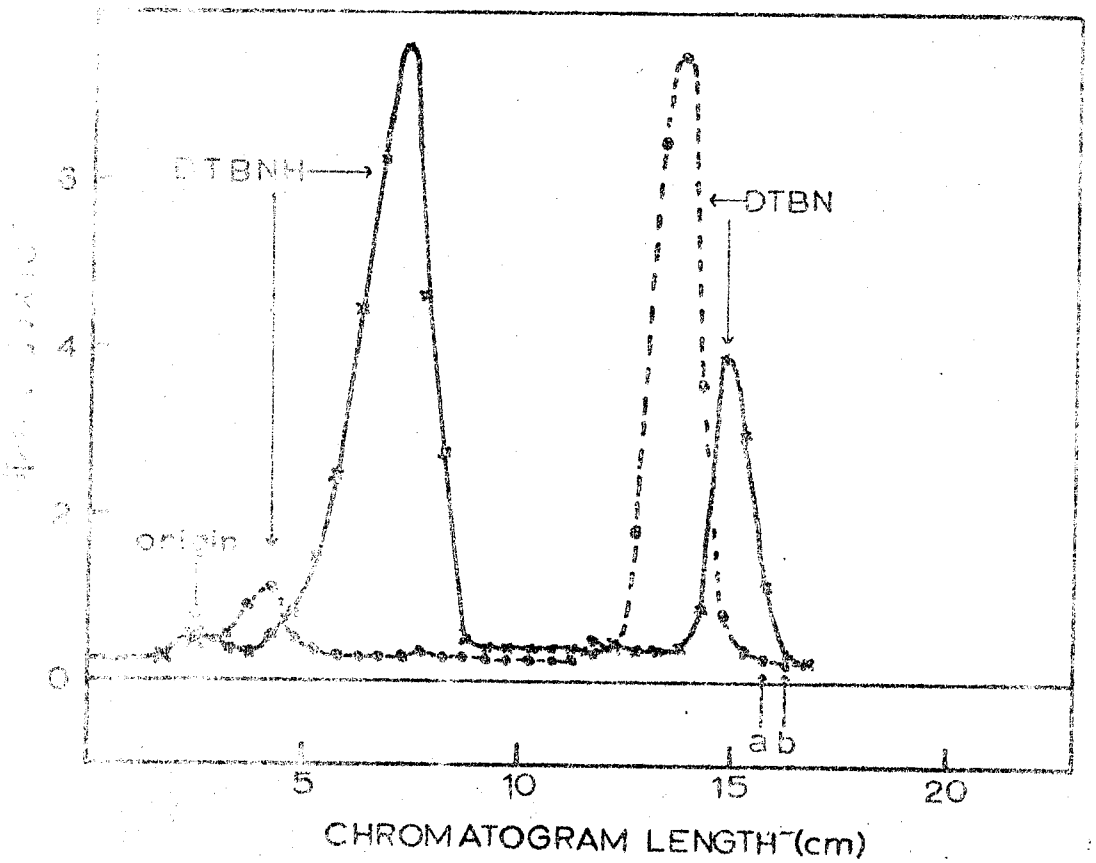
XBL 6710-6202

Fig. 4



XBB 678-4981

Fig. 5



XBL 679-6143

Fig. 6

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.