STRUCTURE AND PROPERTIES OF

SPINOCHROME H

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF THE

UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN CHEMISTRY

JUNE 1964

By

Clifford Wah Jun Chang

Thesis Committee:

Paul J. Scheuer, Chairman
Sidney C. Hsiao
Richard G. Inskeep
Edgar F. Kiefer
Harold O. Larson

TABLE OF CONTENTS

STRUCTURE AND PROPERTIES OF SPINOCHROME H
By Clifford Wah Jun Chang

A thesis submitted to the Graduate School of the University
of Hawaii in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

ABSTRACT

Spinochrome H, the major purple pigment in the
spines of Echinometra oblonga *(Blainville) and* Colobocentro-
tus (Podophora) atratus *(Linn.), was isolated in yields of*
0.06 and 0.05% respectively by a previously unreported method
utilizing acid-washed silica gel in column chromatography.
Trace amounts of spinochrome H were also isolated from other
Hawaiian sea urchins, Tripneustes gratilla *(Linn.) and* Echi-
nothrix diadema *(Linn.).*

Spinochrome H crystallizes with one mole of metha-
nol, m.p. 183-184°. The solvent may be removed at 80° under
high vacuum over phosphorus pentoxide yielding solvent-free
pigment, m.p. 192-193°. The molecular formula of $C_{12}H_8O_7$ is
supported by combustion analyses and mass spectrum (M^+, 264).
The compound possesses an acetyl group, one nucleoid proton,
and four hydroxy groups of two types, free and hydrogen-
bonded.

Spinochrome H is a polyhydroxy-1,4-naphthoquinone
and, specifically, a naphthazarin analog as shown by its
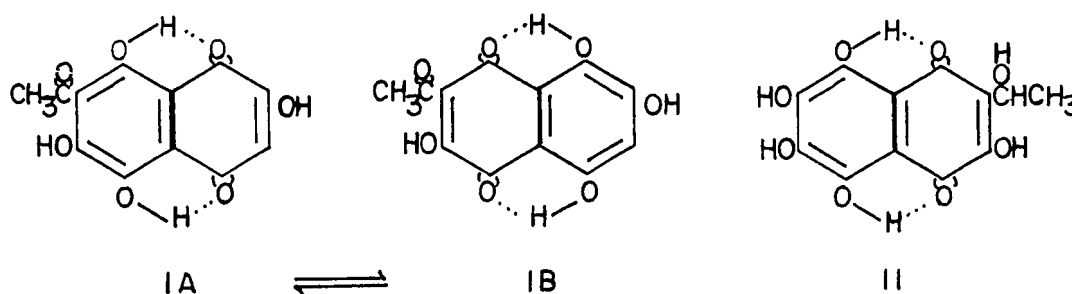*ultraviolet spectrum in methanolic aluminum chloride.*

Spectral and chemical behavior of spinochrome H, of
its monomethoxy derivative, m.p. 239-242°, and of its dimethoxy

derivative, m.p. 224-227°, place one of the unassigned two hydroxy groups ortho to the acetyl group. No decision can be made on the basis of available evidence regarding the fourth hydroxy group. The expression IA⇌IB may therefore be written as a representation of spinochrome H. The remaining structural ambiguity will have to be solved by synthesis or X-ray diffraction.

A similarity of spinochrome H with spinochrome A is suggested on the basis of ultraviolet and visible spectra, melting point, and reported behavior during calcium carbonate column chromatography. Spinochrome A, isolated from the Mediterranean sea urchins, <u>Paracentrotus</u> <u>lividus</u> (Lam.) and <u>Echinus</u> <u>esculentus</u> (Linn.), however, was assigned structure II. No conclusive evidence in support of II has been published.

A biogenetic scheme is presented which favors the 2,7-dihydroxy-6-acetylnaphthazarin structure over its 3,7-isomer on the basis of head-to-tail linkage of activated acetate units.

Several synthetic reaction pathways, designed to lead to spinochrome H or its isomers, have resulted in structural elucidations of some naphthazarin intermediates.

IA ⇌ IB   II

LIST OF FIGURES

Table II.   Summary   of   Spinochrome   Research.

SPINOCHROMES		SOURCE	MELTING POINT, °C	EMPIRICAL FORMULA	Investigator(s)	Year	Ref
European	Japanese						
A		_Echinus esculentus_ (Linn.)	185	$C_{12}H_{10}O_8$	Goodwin & Srisukh	1950	22
		Paracentrotus lividus (Lam.)			Lederer & Glaser	1938	10
					Glaser & Lederer	1939	11
B*		_Echinus esculentus_ (Linn.)	283d	$C_{12}H_{12}O_8$	Goodwin & Srisukh	1950	22
		Paracentrotus lividus (Lam.)	>340		Lederer	1952	19
			350-355	$C_{12}H_8O_7$	Musajo & Minchilli	1940,1942	23,24
		Salmacis sphaeroides	325-330	$C_{10}H_6O_6$	Gough & Sutherland	1964	39
C**		_Arbacia pustulosa_ (Leske)			Lederer & Glaser	1938	10
			229-230s	$C_{12}H_8O_8$	Kuhn & Wallenfels	1941	25
		Paracentrotus lividus (Lam.)	_ca._ 247d		Lederer	1952	19
D	Ak_1	_Pseudocentrotus depressus_ (Ag.)	285-295		Kuroda & Ohshima	1940	26
					Kuroda & Iwakura	1942	27
					Kuroda & Koyasu	1944	28
				$C_{11}H_8O_9$	Kuroda & Okajima	1953	31
E		_Paracentrotus lividus_ (Lam.)	>350		Lederer	1952	19
				$C_{10}H_6O_8$	Smith & Thomson	1960,1961	36,37
		Psammechinus miliaris (Gmelin)			Yoshida	1959	38
F	F_1	_Heterocentrotus mammilatus_ (Linn.)	229		Kuroda & Ohshima	1940	26
			245-247	$C_{12}H_8O_8$	Kuroda & Okajima	1960	33
	B_2	_Hemicentrotus pulcherrimus_ (Ag.)			Kuroda & Koyasu	1944	28
					Kuroda & Okajima	1953,1954	31,32
M	M_1	_Anthocidaris crassispina_ (Ag.)	193		Kuroda & Ohshima	1940	26
					Kuroda & Iwakura	1942	27
	Ak_2	_Pseudocentrotus depressus_ (Ag.)	195-196s		Kuroda & Okajima	1951,1953	30,31
				$C_{14}H_{10}O_9$	Okajima	1959	35
	B_3	_Hemicentrotus pulcherrimus_ (Ag.)			Kuroda & Okajima	1962	34
N***	B_1	_Hemicentrotus pulcherrimus_ (Ag.)	>200	$C_{10}H_6C_6$	Kuroda & Iwakura	1942	27
					Kuroda & Koyasu	1944	28

Group	Subgroup	Species	m.p.	Formula	Author	Year	Ref
				$C_{11}H_8O_9$	Kuroda & Okajima	1953	31
E		Paracentrotus lividus (Lam.)	>350		Lederer	1952	19
				$C_{10}H_6O_8$	Smith & Thomson	1960,1961	36,37
		Psammechinus miliaris (Gmelin)			Yoshida	1959	38
F	F_1	Heterocentrotus mammilatus (Linn.)	229		Kuroda & Ohshima	1940	26
			245-247	$C_{12}H_8O_8$	Kuroda & Okajima	1960	33
	B_2	Hemicentrotus pulcherrimus (Ag.)			Kuroda & Koyasu	1944	28
					Kuroda & Okajima	1953,1954	31,32
M	M_1	Anthocidaris crassispina (Ag.)	193		Kuroda & Ohshima	1940	26
					Kuroda & Iwakura	1942	27
	Ak_2	Pseudocentrotus depressus (Ag.)	195-196.5		Kuroda & Okajima	1951,1953	30,31
				$C_{14}H_{10}O_9$	Okajima	1959	35
	B_3	Hemicentrotus pulcherrimus (Ag.)			Kuroda & Okajima	1962	34
N***	B_1	Hemicentrotus pulcherrimus (Ag.)	>200	$C_{10}H_6O_6$	Kuroda & Iwakura	1942	27
					Kuroda & Koyasu	1944	28
	M_2	Anthocidaris crassispina (Ag.)			Kuroda & Koyasu	1944	28
					Kuroda & Okajima	1950	29
			>260		Smith & Thomson	1961	37
					Lederer	1952	19
P****		Paracentrotus lividus (Lam.)	188	$C_{12}H_{10}O_7$	Musajo & Minchilli	1940	23

* Formerly designated P_1 by Musajo & Minchilli.[23]
** Previously known as isoechinochrome[11] and probably has structure (X) of spinone A.[25]
*** Found to be identical with spinochrome B by Gough & Sutherland[39] who suggested retainment of the name spinochrome B.
**** May be identical with spinochrome A.
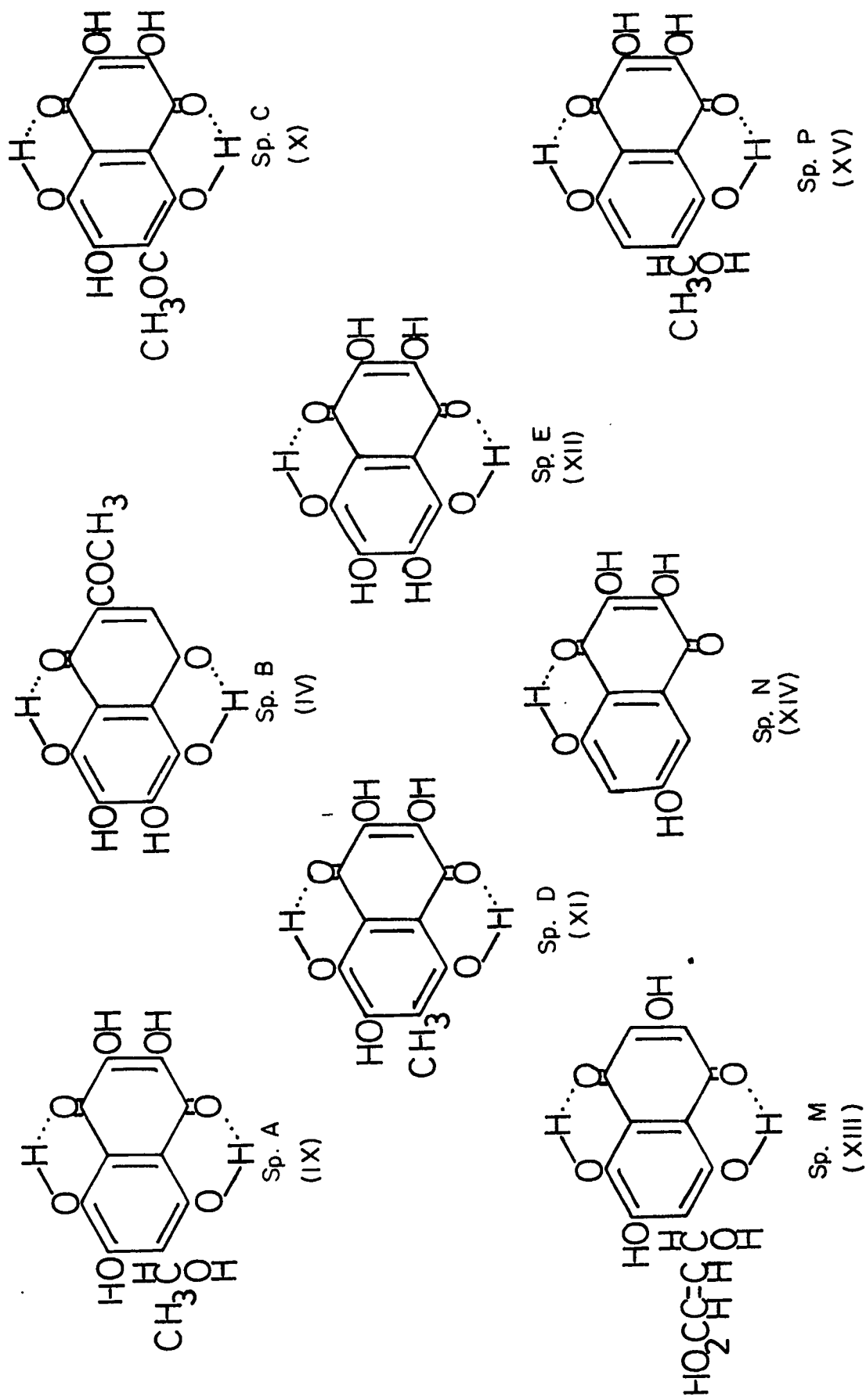
Fig. I. Proposed Structures of Spinochromes.

rigorously proven.

Spinochrome B previously assigned a molecular formula of $C_{12}H_8O_7$ and structure IV by Musajo and Minchilli,[23] was also isolated by two other independent groups, by Goodwin and Srisukh,[22] and by Lederer.[19] Their data on the visible and ultraviolet absorption maxima are in agreement; however, sufficient differences in the combustion analyses and in the reported melting points along with only limited chemical data left reasonable doubt regarding its proposed formula and structure.

This uncertainty has now been removed through the work of Gough and Sutherland.[39] They showed by the NMR spectrum of the methyl ether that spinochrome B is identical with spinochrome N, the structure of which was unequivocally established by Kuroda[29] and was proven through synthesis by Smith and Thomson.[37] The pigment for Gough and Sutherland's work was isolated from the spines of <u>Salmacis sphaeroides.</u> Its properties and those of the trimethyl ether and of the leucoacetate agreed with the data reported for spinochrome N. Identity of spinochromes B and N was conclusively demonstrated by Gough and Sutherland[39] through direct comparison of authentic samples supplied by Thomson of the University of Aberdeen. Retainment of the name spinochrome B is suggested[39] in preference to the numerous designations of P_1,[23] B,[21] B_1,[31] M_2,[31] Ak_1[31] and N.[21]

Spinochrome A, formerly called spinochrome by

Glaser and Lederer[11] and spinochrome P by Musajo et.al.,[23,40]
was the first reported pigment from the violet spines of P.
lividus. It is accompanied in small quantities by echino-
chrome A. Glaser and Lederer found this pigment to possess
five active hydrogens. By methylation with diazomethane,
three derivatives melting at 176, 147, and 265° were
separated by column chromatography on calcium carbonate. No
combustion data were reported.

Structure IX was originally proposed by Kuhn and
Wallenfels[25] for spinochrome A and was supported by Lederer
and Glaser.[10] On chromic acid oxidation one mole of acetic
acid was produced and presumably the position of the alipha-
tic oxygen must therefore be alpha to the aromatic ring.[10]
Goodwin and Srisukh[22] reported analytical data in support
of the formula $C_{12}H_{10}O_8$. The ruby red pigment, m.p. 185°,
appeared violet on calcium carbonate on which it is strongly
adsorbed. Structure IX for spinochrome A is still considered
tentative by the European workers.

Spinochrome C, like spinochrome A, requires
additional confirmatory evidence in support of its proposed
structure X. Little work has been done on this pigment
except for some color reactions. The pigment was isolated
from the spines of A. lixula (Linn.) by Glaser and Lederer[11]
who suggested that it may be similar to the pigment spinone
A (X), which was reported by Kuhn and Wallenfels[25] to occur
in the same sea urchin. Though the melting points varied,

color reactions and the visible absorption spectra were
identical.

Since 1939 four sea urchin species were investi-
gated by Kuroda and coworkers. At least four pigments were
isolated and structures proposed (see Table II and Fig. 1).
The names, spinochrome Ak, Ak_1, Ak_2, M, M_1, M_2, B, B_1,
B_2, B_3, F, and F_1, were advanced at one time or another by
the Japanese investigators. Some of the compounds so desi-
nated were found identical with one another. They were
named for the Japanese sea urchin species, aka-uni, murasaki-
uni, bafun-uni, and Futozao-uni [Pseudocentrotus depressus
(Ag.), Anthocidaris crassispina (Ag.), Hemicentrotus
pulcherrimus (Ag.), and Heterocentrotus mammilatus (Linn.)
respectively].

Spinochrome D was isolated by Kuroda and Ohshima[26]
in the crude state in 0.07% yield. It was characterized
through its trimethyl ether, its pentaacetate, and its
leucoheptaacetate. Structure XI is likely on the basis of
elemental analyses of its derivatives. Although in 1947 an
attempt was made by Weygand et. al.[41] to synthesize XI, the
pigment could not be isolated after the 5,6,8-trimethylether
was demethylated. The compound, though, had the same ultra-
violet absorption spectrum as echinochrome A.

Examination of the benzene-soluble fraction from
Pseudocentrotus depressus produced a second pigment, spino-
chrome M. Structure XIII, which was supported by a yellow

pentaacetate[30,31] and a dimethylether (XVI),[31] was proposed.
The ether upon acetylation, yielded a dimethyl-triacetyl
compound (XVII).[31]

XVI   XVII
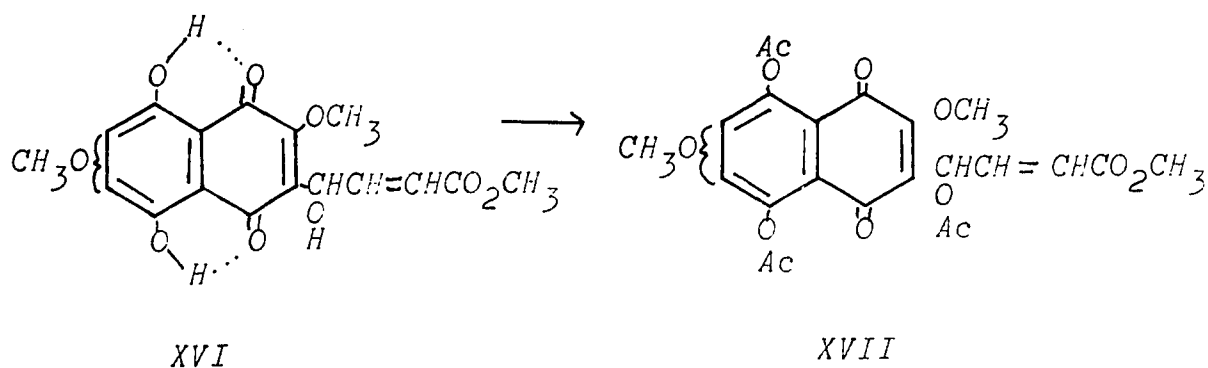
The pentaacetate upon ozonolysis afforded glyoxalic
acid, which was identified by its 2,4-dinitrophenylhydrazine
derivative, while oxidation of the hexamethylether[42] resulted
in what was believed to be the 3,4,6-trimethoxyphthalic
acid.[35,42]

C.   Objective of Research

In part A of this chapter it was shown that
previous work on spinochrome H resulted in two -perhaps
compatible- empirical formulas and in at least seven pro-
posed structural alternatives.   In Part B an attempt was
made to demonstrate that the entire spinochrome literature
suffers from a multiplicity of names and structures and from
a paucity of rigorous chemical and physical data.

This research was undertaken in the hope of estab-
lishing unequivocally the structure of spinochrome H and, by
so doing, of resolving some of the conflicting proposals in
the existing spinochrome literature.

It was also desired to evaluate critically previously reported procedures of isolation and purification in order to arrive at improved methods of obtaining reasonable quantities of pure compounds. Many difficulties of the earlier workers were caused by a lack of pure pigments in adequate quantity for characterization.

Finally, it was a goal of this research to employ physical methods to the greatest possible extent in the area of naphthazarin chemistry. When this study was begun, melting points and ultraviolet-visible spectra were the only physical data which were routinely found in the literature. Even infrared spectral data were almost entirely lacking.

Chapter II

EXPERIMENTAL

General analytical procedures are cited below and will be referred to later.

All melting points reported herein were recorded from a Fisher-Johns hot stage block and are uncorrected.

Unless otherwise noted all combustion analyses reported herein were carried out by Dr. Alfred Bernhardt of the Microanalytisches Laboratorium im Max-Planck-Institut für Kohlenforschung, Mülheim, Germany.

All infrared (IR) spectra unless otherwise stated were measured on samples in chloroform or carbon tetrachloride solutions or potassium bromide disks on a Beckman IR-5 automatic recording spectrophotometer. The solvents were of reagent quality (B.&A. or Merck) and the potassium bromide was of infrared quality (Harshaw Chemical Company).

Relative intensities are defined as follows: b (broad), vs (very strong), s (strong), infl. (inflection), w (weak), and sh (shoulder).

All ultraviolet (UV) spectra were recorded on a Beckman DK-2 ratio recording spectrophotometer. Absolute methanol (B.&A.) was the neutral solvent, anhydrous hydrogen chloride gas in absolute methanol was the acidic solvent, and sodium hydroxide or potassium hydroxide in absolute methanol was the basic solvent.

Nuclear magnetic resonance (NMR) spectra were

observed with a model A-60 Analytical N.M.R. Spectrophoto-
meter (Varian Associates, Palo Alto, Cal.). All values are
expressed in parts per million (ppm) referred to tetra-
methyl-silane (TMS) having δ = 0.

A.   Sources and Isolation

The sea urchin, E. oblonga (Bl.), occurs abundant-
ly in the Blow Hole and Kaena Point regions on the island of
Oahu. Collections were made in these areas and carried out
during the summer months. Taxonomic identification was con-
firmed by Dr. Sidney C. Hsiao of the Department of Zoology.

Another good source of spinochrome H was found in
the purple, scaly spines of Colobocentrotus (Podophora)
atratus (Linn.). Preliminary investigation of the spine
pigments of other Hawaiian sea urchins, Tripneustes gratilla
(Linn.) and Echinothrix diadema (Linn.) has resulted in the
isolation of the same pigment, however in small yields of
ca. 0.0001 and 0.0005% respectively.[43]

The spines were separated from the tests by
shaking the urchins in a five-gallon can. An alternate and
better method consisted of immersion of the urchins in fresh
water, thus allowing the spines to fall off while the urchins
decomposed. The foul-smelling aqueous mixture was decanted;
the spines and tests were washed copiously with water; the
spines were separated by a screen; and finally transferred to
a large beaker.

In order to minimize heavy and continued frothing

during dissolution of the spines in concentrated hydrochloric acid the acid was overlayed with ether. However, this technique became ineffective once the ether was saturated with water and organic material. Furthermore, much ether was lost in the constant stream of carbon dioxide bubbling through the ether layer. This difficulty was overcome by using instead of an open vessel a twenty-gallon pyrex flask equipped with a long reflux condenser. Addition of the acid through the top of the condenser served to collapse the heavy froth of bubbles.

The isolation scheme for the pigment is shown in Fig. 1. In a typical batch, 1 kg of spines was dissolved in 1.8 l of conc. hydrochloric acid. After dissolution was complete (ca. 6 hrs), the acidic and ethereal solutions were filtered with suction and separated. The aqueous layer was continously extracted with ether for 72 hrs. The resulting ethereal extract (O-2) was combined with the original ether phase (O-1) and w shed with 10% sodium bicarbonate solution in order to extract the pigments from the ethereal solution. The resulting prussian-blue bicarbonate solution was then washed with ether, acidified with hydrochloric acid, and reextracted with ether yielding the crude spinochrome fraction (O-4). After drying over anhydrous sodium sulfate the solvent was stripped in vacuo and the residue triturated with benzene. Column chromatography of the benzene solution followed. The use of benzene, however, was not entirely

SPINES

12N   HCl

ether

A-1

ether

aqueous
discard

O-2   **O-1**

NaHCO$_3$

A-2

1. ether wash
2. H$^+$
3. ether

ether
discard

aqueous
discard

O-4

COLUMN
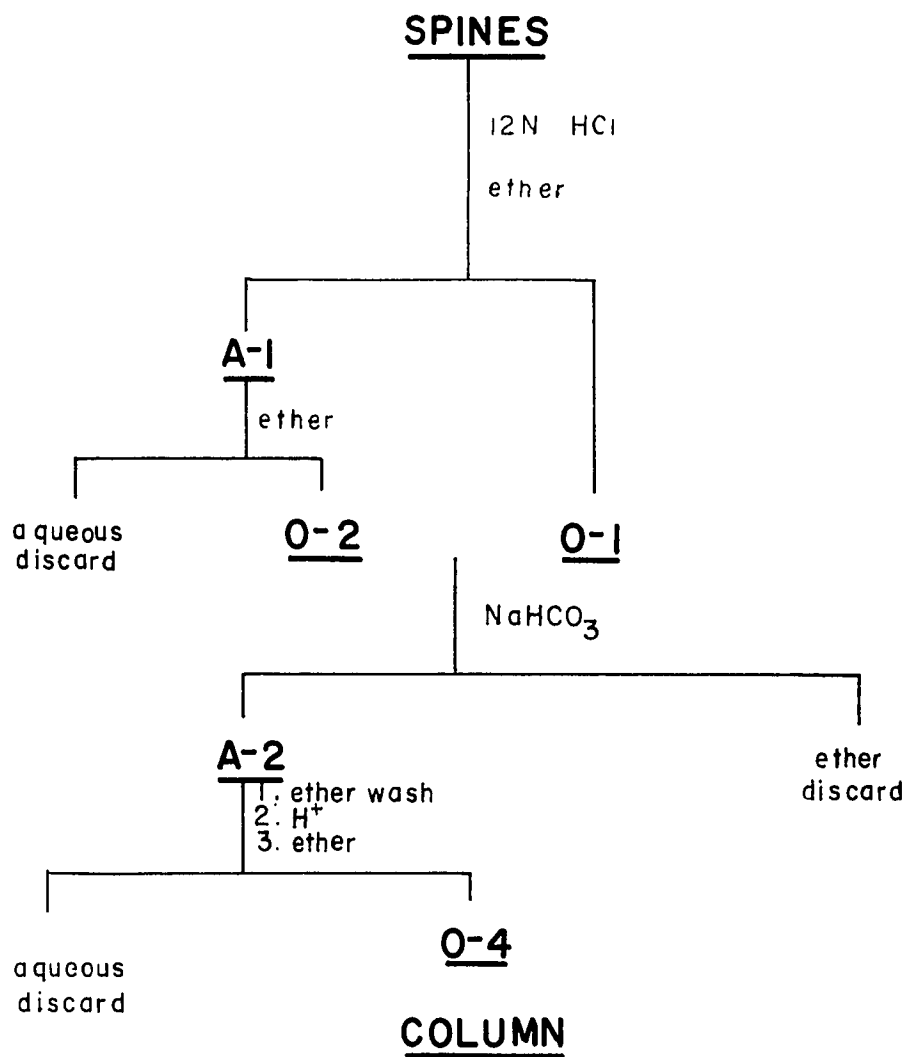
Fig. 2.   Isolation   Scheme   for   Spinochrome   H.

satisfactory because of the limited solubility of the pigment

and because of troublesome emulsions.

Rapid manual extraction rather than continuous

extraction was found preferable once the pigments came in

contact with base in order to avoid oxidative degradation.

Procedures of isolating the pigments from the two

species of urchins were as follows.

1.   From Echinometra oblonga (Bl.)

Eight hundred milliliter of conc. hydrochloric

acid was required to effect solution of 430 g of spines.

The dark red acidic solution, after filtration through ce-

lite, was exhaustively extracted with ether in a liquid-

liquid extractor until it was light orange in color (ca. 72

hrs). The pigments were then extracted into a 10% solution

of sodium bicarbonate leaving only neutral oily material in

the ether. After acidification (HCl) of the basic solution

the pigments were again extracted into ether and the ethe-

real solution was concentrated in vacuo to aproximately 300

ml and dried over sodium sulfate. After drying of the ethe-

real concentrate the remaining solvent was removed in vacuo

leaving ca. 400 mg of crude residue which was triturated

with benzene, followed by development of the benzene filtrate

on 900 g of acid washed silica gel (see page 29). Elution

with benzene and benzene-chloroform (1/1,v/v) afforded 250

mg (0.058%) of the major pigment, spinochrome H, as long

purple needles (from benzene-methanol), and 49 mg (0.011%) of

a minor orange pigment as orange-red needles.

Crystalline purple pigment was obtained from the respective combined eluents by concentration *in vacuo* to a minimun volume after drying over sodium sulfate. The volumes were further reduced under a rapid stream of argon; methanol was added to the residue; the resulting solution was warmed and seeded, leading to a compound of m.p. 191-192°.

Further purification of the minor orange pigment was achieved in the following way. The combined eluates of the orange band from several columns were concentrated and the residue taken up in benzene, followed by washing with dilute phosphoric acid solution to insure that the compound was present in the acid form. The benzene solution containing the orange pigment was then introduced into a column of acid-washed silica gel using a ratio of adsorbent to pigment of 1000-1. The chromatogram was achieved by a gradual increase of the concentration of chloroform in benzene. Elution of the orange pigment occurred at a chloroform:benzene ratio of 15:75 (v:v). Isolation of the minor pigment in a similar manner as above led to the compound of m.p. 240-244°, clouding at 238°.

2. From Colobocentrotus (Podophora) atratus (Linn.)

Six hundred and ninety gram of the flat purple spines was dissolved under an ethereal layer in a total of 1.6 l of 12N hydrochloric acid. After a work-up as described

above the yield of major pigment, after chromatography, was
354 mg (0.05%), m.p. 189-190°, after crystallization from
methanol.

No orange pigment was isolated in this case since
it was present in only trace amounts.

The identity of the purple pigment from this
source with that of *Echinometra* *oblonga* (Bl.) was proven by
thin layer chromatography and by an admixture of both samples
which caused no depression in the melting point. Solution
of the admixture yielded upon recrystallization the same
long purple needles of unchanged melting point.

B.   Purification

1.   The Neutral Fraction

Amai[1] and Temple[2] had observed that column chroma-
tography on calcium carbonate resulted in yellow and pink
bands which could be eluted with ether and in a broad violet
band at the top of the column which remained on the column
even when methanol was the eluant.

This observation could be confirmed. It seemed
likely that the relatively non-polar yellow and pink bands
were not phenolic in nature. This hypothesis was proven for
the yellow band in the following manner.

It seemed probable that the yellow band originated
from the fatty tissue which joined the spines to the test of
the sea-urchin. To test this point, the spines were clipped
from their bases and the bases were combined and pulverized

in a Waring Blendor. A methanol extract (Soxhlet, 20 hrs) of this material was concentrated and the residue taken up in ether and developed on a calcium carbonate column with continued ether elution. The resulting diffuse yellow band was thus shown to be derived from the fatty tissues in the stumps of the spines. This was further confirmed by observing the absence of any yellow band when the base-less spines were dissolved in hydrochloric acid, extracted with ether, dried, and finally developed on a calcium carbonate column.

The yellowish brown gum, obtained after stripping the solvent from the yellow band eluate, exhibited a negative ferric chloride test and was insoluble in 10% sodium hydroxide. No further attempt was made to identify this non-phenolic fraction.

No attempt was made to confirm the nature of the pink band which was present only in trace amounts.

2. Selective Precipitation

Okajima[35] reported the use of magnesium bicarbonate as a preliminary step in separating two pigments, spinochrome M_1 and M_2. These were finally purified by differential solubilities in organic solvents. We checked this procedure in our work and found that a saturated solution of magnesium carbonate and dry ice served as a convenient means of precipitating the spinochromes from an organic extract. It was, however, no more effective than our ether washes of the sodium bicarbonate solution of the pigments

and was therefore not adopted for our work.

3.   Column Chromatography

a.   On calcium carbonate

A 2.5 x 45 cm column was prepared with 50 g of powdered calcium carbonate (Baker & Adamson) slurried in benzene. A portion of the crude extract (O-4, Fig. 2) dried over magnesium sulfate, was concentrated and applied to the top of the column. Elution with benzene containing increasing percentages of ether failed to move the main purple band. Mixtures of methanol and ether did move the purple band, but caused it to become diffuse and also caused channelling of the column. The resulting eluates failed to yield pure samples of spinochrome H.

b.   On acid-washed alumina

Neutral alumina (Woelm) washed with 0.5N hydrochloric acid and air dried was not satisfactory as an adsorbent since the pigment could not be eluted with ether or methanol. The characteristic deep violet band remained on the top of the column.

c.   On Florisil

A column of Florisil (60-100 mesh, Floridin Company, Tallahassee, Fla.) behaved in a similar fashion as the alumina column. Attempted elution with ether or methanol was unsuccessful.

d.   On powdered nylon

The column material was obtained from Badische

Anilin & Soda-Fabrik, Germany. Elution with methanol
resulted in a purple band at the top portion of the column
and a bright red diffuse band immediately below. These
bands remained stationary upon further elution.

> *e. On acid-washed cellulose*

Cellulose powder (Whatman, W.&R. Balston, Ltd.,
England) treated with 1.5N hydrochloric acid was filtered
and washed with acetone. This adsorbent, after drying at
room temperature, was used in a small column and a portion
of the crude ethereal extract was introduced. At first
channelling occurred, but the appearance of bands was evi-
dent. Some impure crystals resulted from several fractions.
However, no single major band ever developed.

> *f. On silica gel*

One hundred and fifty gram of 80-200 mesh silica
gel (Baker & Adamson, chromatography grade) was prepared for
column chromatography by the slurry method. Acidic methanol
solutions consisting of 0.2 to 0.4 ml of glacial acetic acid
in 250 ml of methanol were used as the eluating solvent.
Crude pigment (390 mg), when first introduced, appeared as
a broad, nearly black band which turned reddish when the
acidic methanol was added. No resolution was achieved.

> *g. On acid-washed silica gel*

Separation of the crude spinochromes by the fol-
lowing procedure was employed for all subsequent purifica-
tion runs. After several experiments designed to test

preparation of columns, choice of solvents, and load factor, the following standard procedure was adopted for the separation of spinochrome H and of the minor pigment.

Silica gel (B.&A., 80-200 mesh, chromatography grade) was washed with 0.5 N hydrochloric acid and filtered and dried with suction. The resulting deactivated silica gel (DSG) was spread on porcelain trays and air-dried in a fume hood. Preliminary drying with a heat lamp may be used with caution, but the adsorbent must not be exhaustively dried to its original activity. A resulting free flowing powder was bottled tightly and stored for later use.

Solvents which were used include carbon tetrachloride, benzene, methylene chloride, chloroform, and ethyl acetate. A choice of elution solvents was made after preliminary trials with thin-layer chromatography.

C.   Characterization

1.   Thin-Layer Chromatography

A chromatographic procedure as a rapid means of determining purity was developed by thin-layer chromatography (TLC). Deactivated silica gel and non-polar solvents as used for the column work served as phases for the plates, yielding excellent results without tailing. Because of the pigmentary nature of these compounds no spray reagent was required. Occasionally a 10% sodium hydroxide spray solution was used, since this reagent led to bathochromic shifts whereby intensifying spots on the plate. Upon standing,

exposure to air caused the well-defined spots to fade.

The plates were prepared according to the method
of Stahl[44] using a Desaga/Brinkmann standard applicator
(Brinkmann Instruments, Inc.) which produces a standard
layer, approximately 250 μ thick. A mixture consisting of
a slurry of 30 g of silica gel G (E. Merck, Germany) and
60 ml of 0.5N hydrochloric acid was stirred in a mortar and
applied to a series or combination of plates (20 x 20 and/
or 20 x 5 cm), which were then allowed to dry at room tem-
perature for at least six hours and finally stored in a
wooden cabinet.

Since observed R_F values depend not only upon the
adsorbent and the developing systems, but also upon the
thickness and acidity of the layer, the concentration of the
solute, and the temperature and humidity of the room, these
values are only valid when direct comparisons are made on
the same plate.

The results of thin-layer chromatography of spino-
chrome H and the minor orange pigment are shown below for
several solvent systems:

Solvent	Spinochrome H	Minor Pigment
benzene	0.02	0.00
methylene chloride	0.28	0.04
chloroform	0.24	0.08
chloroform- ethyl acetate (9-1)	0.32	0.06

In all cases the runs were made by the ascending method over a distance of 10 cm.

The observed colors of the pigments were deep reddish-purple and orange for spinochrome H and the minor pigment, respectively. When sprayed with base the respective colors changed to violet and pink.

2. Combustion Analyses

An analytical sample was prepared by recrystallization from methanol, m.p. 183-184°, after softening at 170°. Drying was carried out for 12 hr at 80° under high vacuum over calcium chloride.

	C	H	Active H
Found:	52.31	3.57	1.17
	52.75	3.13	
Calcd.for $C_{13}H_{12}O_8$:	52.71	4.08	

A second sample was prepared, m.p. 192-193, after clouding at ca. 178°. Drying was for 15 hr at 80° under high vacuum over phosphorous pentoxide.

	C	H	O	Active H
Found[*]:	54.18	3.45	41.60	0.13
	54.37	3.19		
Calcd. for $C_{12}H_8O_7$:	54.55	3.05	42.39	

---

[*] *Analysis by Dr. W. Zimmermann of the Australian Micro-analytical Service, University of Melbourne, Australia.*

3. *Spectra*

 a. *Ultraviolet*

The visible and ultraviolet spectra of spinochrome H are reproduced in Figs. 3 and 5. The following maxima and minima were observed:

In methanol (Figs. 3 & 5); λ_{max}: *514 mμ (log ε, 3.67), 316.5 (4.10), 270sh (4.14), 251 (4.16);* λ_{min}: *385.5 (3.35), 290.6 (4.08), 234.7 (4.14).*

In methanol/satd. hydrogen chloride gas (Figs. 3 & 5); λ_{max}: *514 mμ (log ε, 3.71), 317 (4.09), 271sh (4.10), 249.6 (4.16);* λ_{min}: *403 (3.34), 290.6 (4.06), 233.1 (4.14).*

In 0.02 N sodium hydroxide in methanol (Figs. 3 & 5); λ_{max}: *569 mμ (log ε, 3.80), 469 (3.49), 328 (4.28), 287sh (4.21), 236 (4.24);* λ_{min}: *490 (3.48), 456 (3.53), 4.20 (3.48), 261 (4.08), 217 (4.17).*

A mixture was prepared of 10 ml of a 5.17 x 10^{-5} :. solution of spinochrome H in methanol and 1 ml of 2% aluminum chloride in methanol and allowed to stand for 1 hr. The following spectrum was measured (Figs. 4 & 6); λ_{max}: *560 mμ (log ε, 3.87), 494 (3.93), 462 (3.69), 381 (3.74),* ca. *337 (3.99), 317 (4.10), 254 (4.45);* λ_{min}: *512 (3.75), 472 (3.64), 440 (3.36), 353.5 (3.63), 291.5 (3.95).*

The following visible and ultraviolet spectra were observed in a variety of other solvents.

In ether; λ_{max}: *522, 315, 251 mμ.* λ_{min}: *432-365b, 290 mμ.*
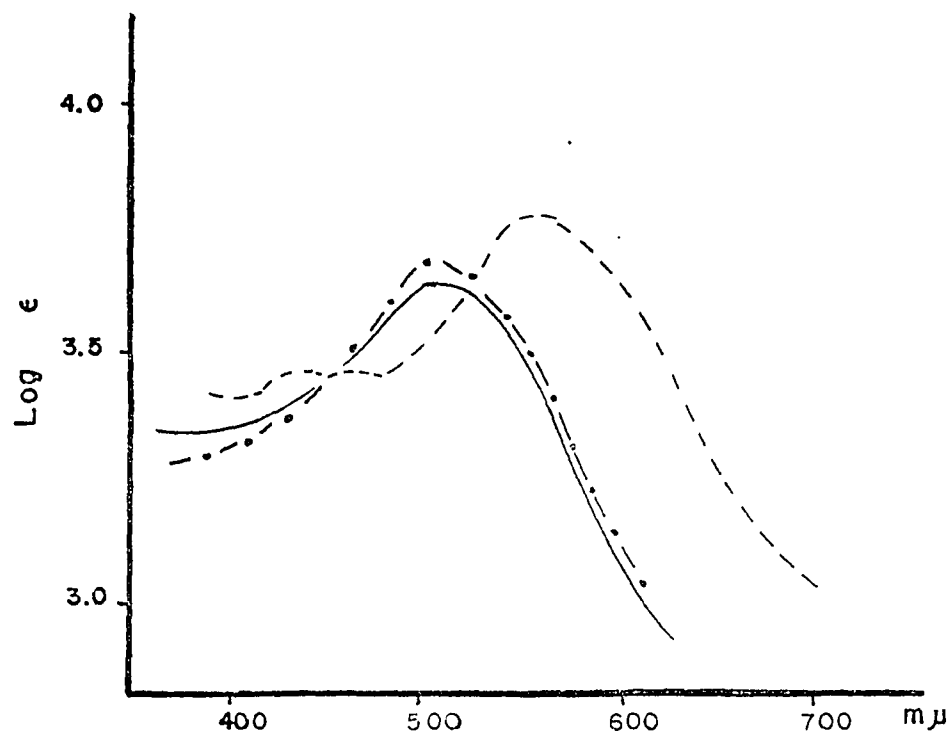
Fig. 3. Visible Absorption Spectra of Spinochrome H
in MeOH(——), MeOH/HCl(g)(—·—·—), and 0.02 N
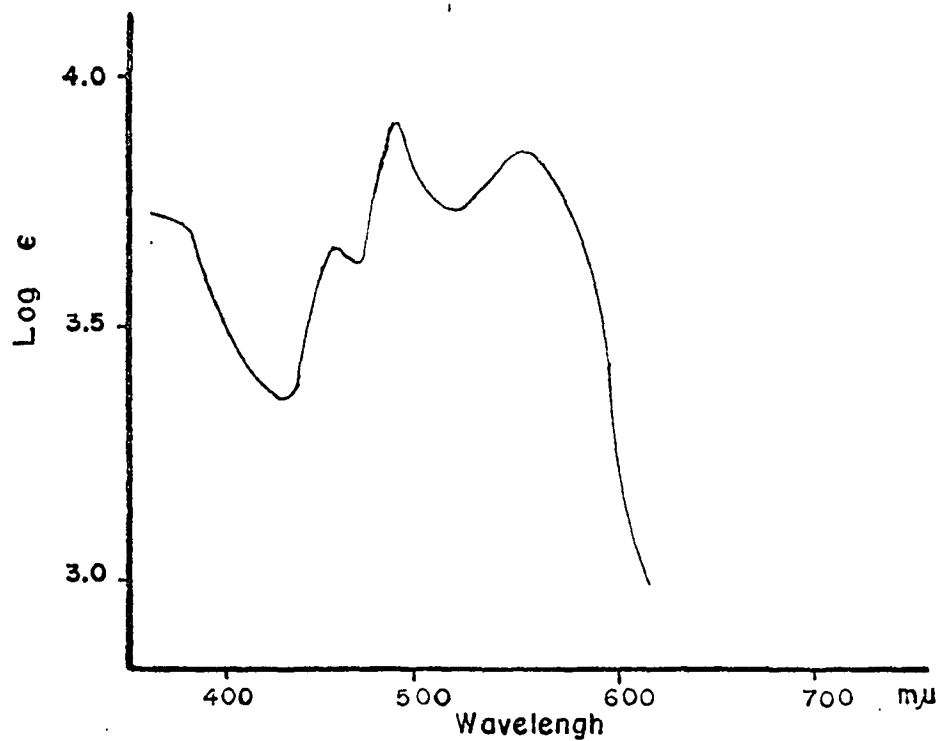NaOH/MeOH (———).

Wavelengh

Fig. 4. Visible Absorption Spectrum of Spinochrome H
in Methanolic AlCl$_3$.
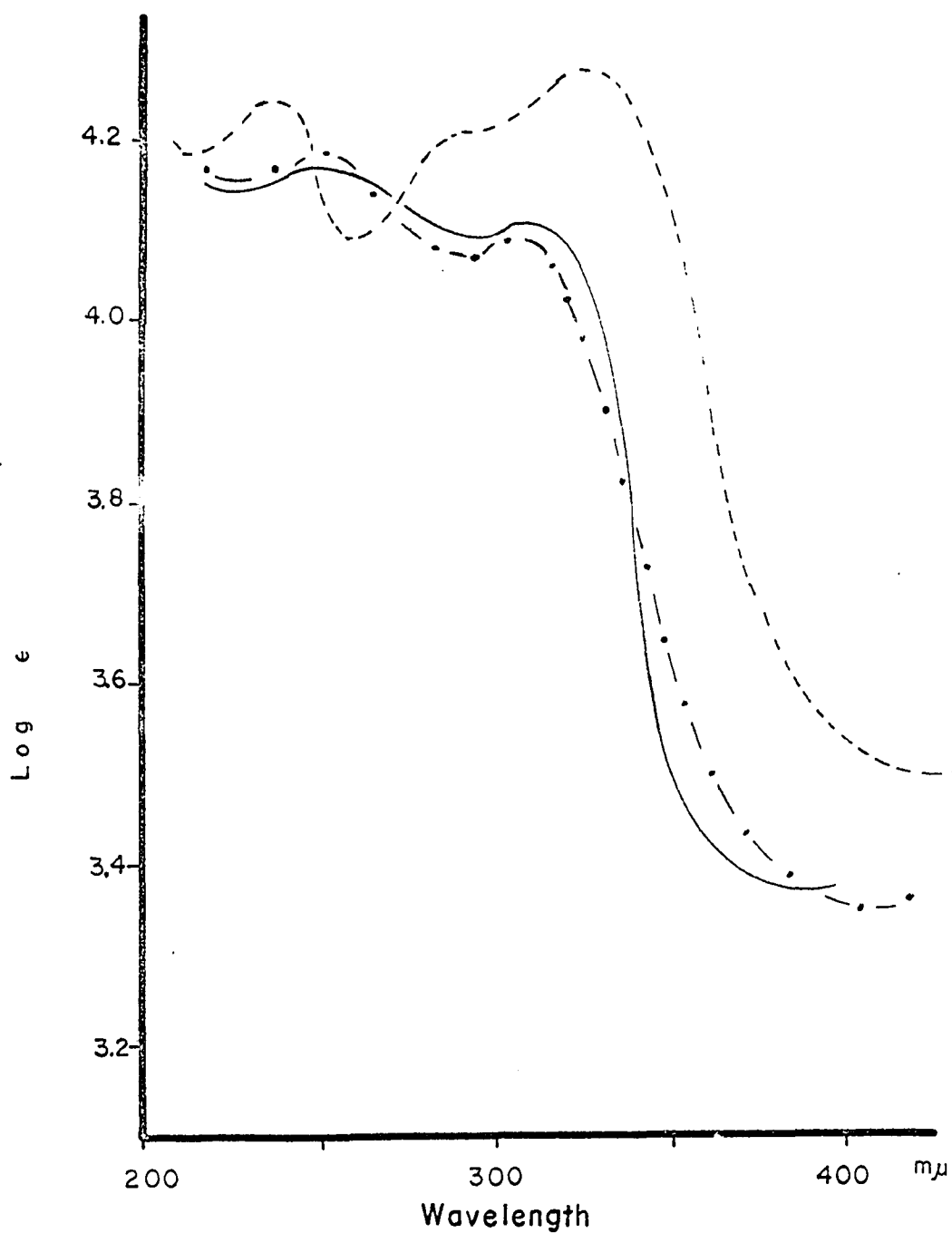
Fig. 5. Ultraviolet Spectra of Spinochrome H
in MeOH(—) , MeOH/ HCl(g) (·—·—·), and 0.02 N
NaOH/MeOH (----).
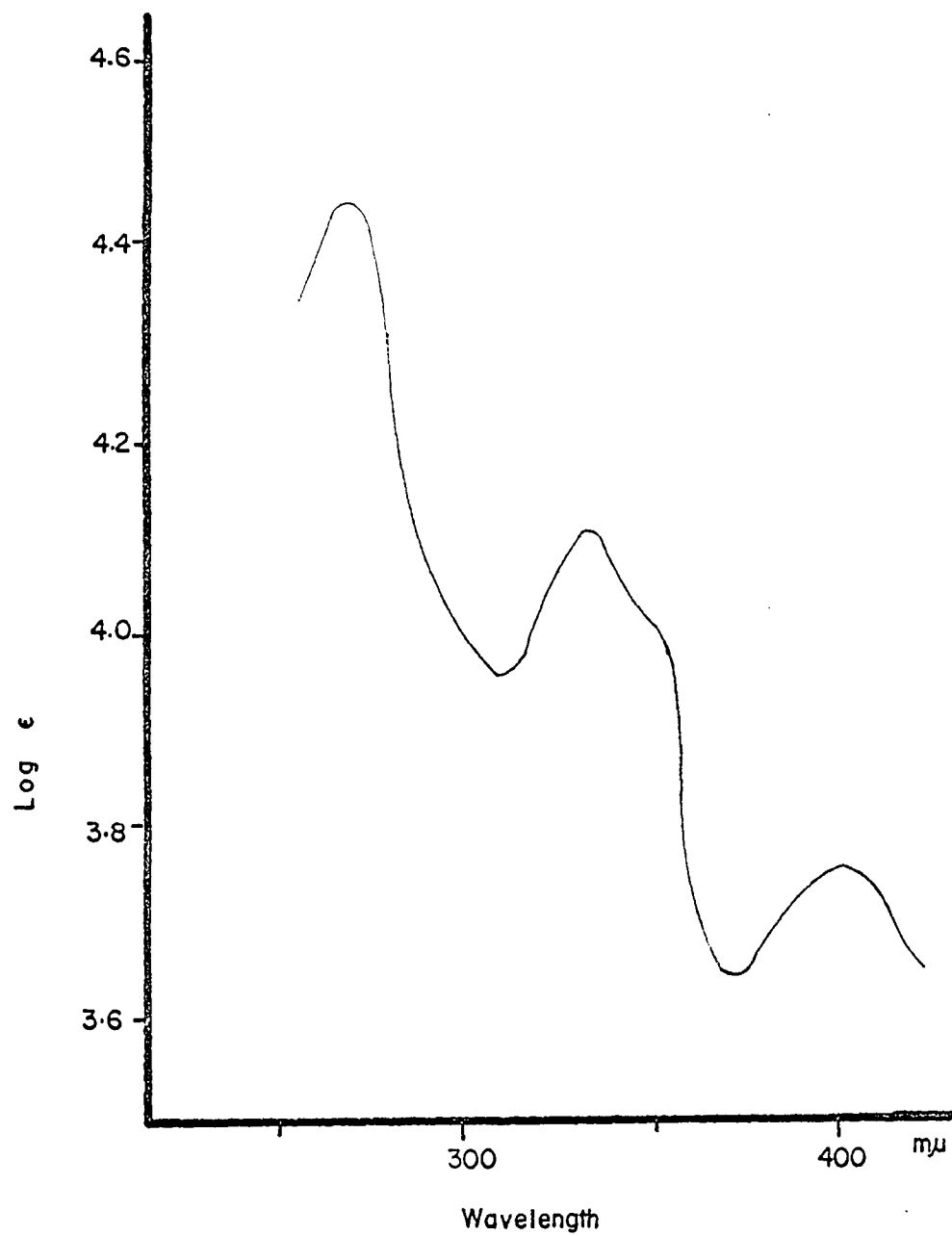
Fig. 6.   Ultraviolet   Spectrum   of   Spinochrome H
in   Methanolic   AlCl₃.

In conc. sulfuric acid; λ_{max}*: 572, 530,* <u>ca.</u> *496 359sh, 320sh, 271 mμ.* λ_{min}*: 401 mμ.*

In carbon tetrachloride; λ_{max}*: 585, 543, 532, 512, 315 mμ.* λ_{min}*: 450-370b, 292.4 mμ.*

In benzene; λ_{max}*: 588, 541, 508, 316.5 mμ.* λ_{min}*: 442, 388b, 289 mμ.*

In 95% ethanol; λ_{max}*: 519, 311, 268 mμ.* λ_{min}*: 375, 289 mμ.*

The minor orange pigment in absolute methanol (Fig. 7) showed maxima at 540sh, 458, 340sh, and 294 mμ and minima at 390 and 260 mμ.

In other solvents, the maxima and minima were recorded as indicated below:

In 10% methanol/water; λ_{max}*: 462, 340, 294 mμ.* λ_{min}*: 395, 277 mμ.*

In 10% 0.1N sodium bicarbonate in methanol after 5 min standing; λ_{max}*: 470, 365, 307, 253 mμ.* λ_{min}*: 414, 350, 273 mμ.*

In hexane; λ_{max}*: 540, 502, 457,* <u>ca.</u> *334,* <u>ca.</u> *299,* <u>ca.</u> *287 mμ.* λ_{min}*: 526, 483, 377, 316.5 mμ.*

In conc. sulfuric acid; λ_{max}*: 490, 308, 250 mμ.* λ_{min}*: 411, 266.5, 229 mμ.*

In benzene; λ_{max}*: 548, 509, 461, 333.5, 286 mμ.* λ_{min}*: 537, 492, 389, 280 mμ.*

b. Infrared

The infrared absorption bands of spinochrome H
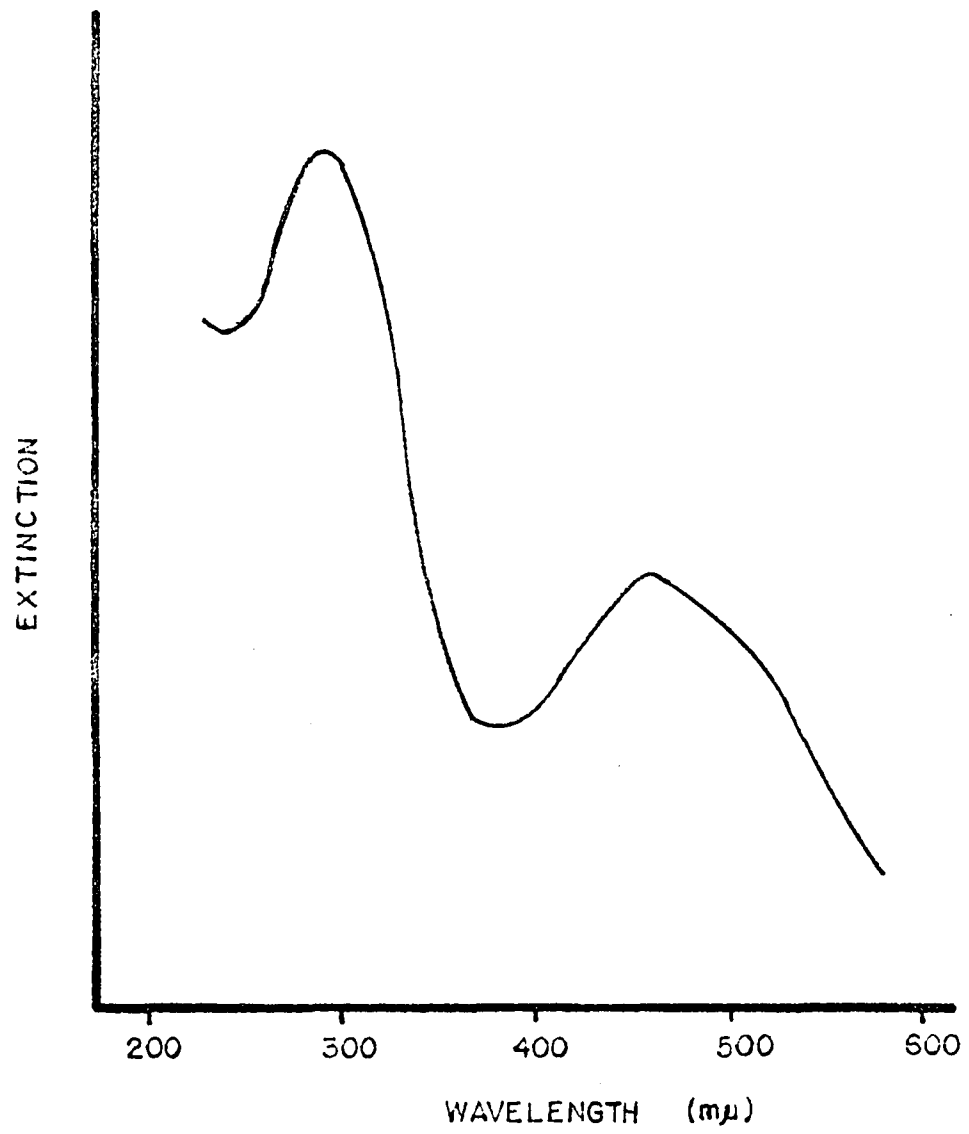
Fig. 7.   Ultraviolet and Visible Absorption Spectrum
of the   Minor Pigment in Methanol.

measured in potassium bromide* (Fig. 8) are reported in
wavelengths (μ) as follows: 2.80s, 2.92s, 3.12-4.00b, 6.21b,
6.56, 6.85infl., 8.44s, 8.61w, 9.09s, 9.80, 10.10vs, 10.36w,
11.08s, 16.81b.

The IR spectrum of the minor orange pigment (Fig.
9) was measured in potassium bromide and exhibited bands at
the following wavelengths (μ): 2.88, 2.95, 6.05infl., 6.17
infl., 6.26s, 6.42infl., 6.85, 7.30, 7.75b, 8.25w, 9.13w,
9.35, 9.97s, 10.9b, 12.05w, 13.04.

Good resolution of the absorption bands in the
2.8-3.0 μ region of the IR spectrum was achieved on the
Beckman IR-5 spectrophotometer by preparing the potassium
bromide pellet under a pressure of 18,000 lb/sq in. The
pellet was held in a circular hole cut in the center of a
4.9 x 7.6 cm file card and sandwiched between two polished
steel plates. This technique produced a translucent disk
while previous trials with opaque disks prepared under lower
pressure in a conventional pellet press resulted in a single
broad band in the 2.9 μ region.

c. Nuclear magnetic resonance

The NMR spectrum** of spinochrome H in deuteriated

---

* Kindly determined by Dr. I.L. Barnes of this Department
on a Beckman IR-9 spectrophotometer.

** Originally determined by Dr. Eugene A. Pier of Varian
Associates, and now reproduced in our Laboratory.

WAVELENGTH IN MICRONS

WAVENUMBER IN CM⁻¹
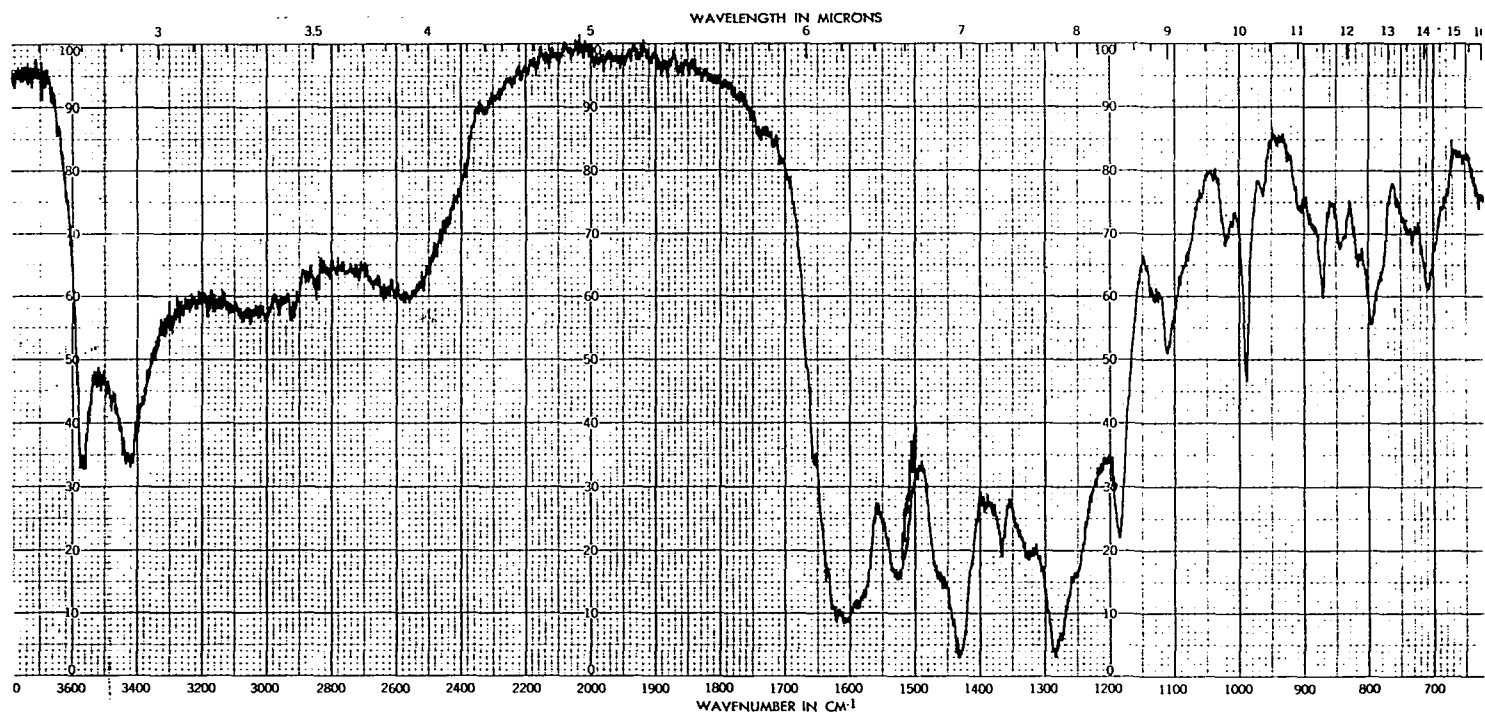
Fig. 8.   Infrared Spectrum of Spinochrome H in KBr.

41
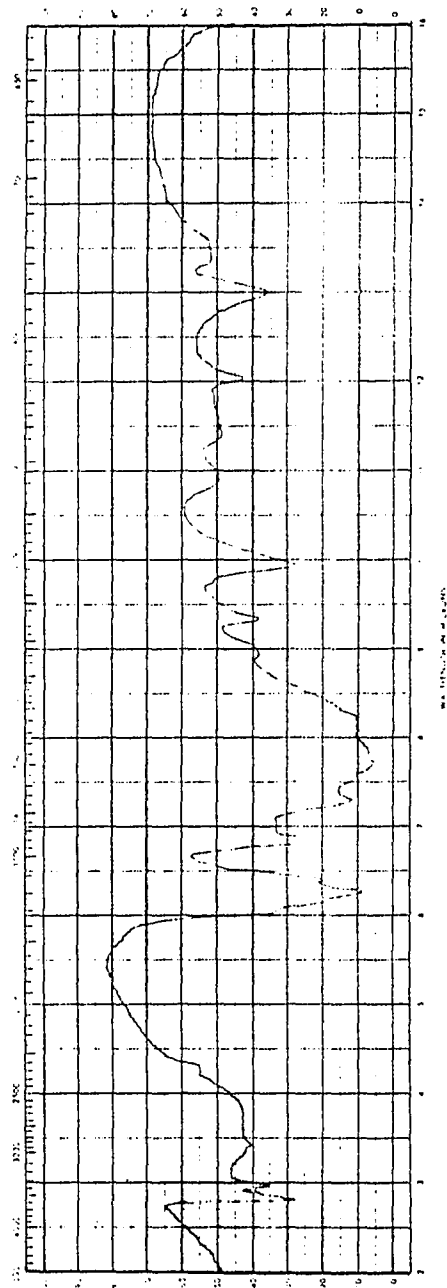
Fig. 9.   Infrared Spectrum of the Minor Pigment in KBr.
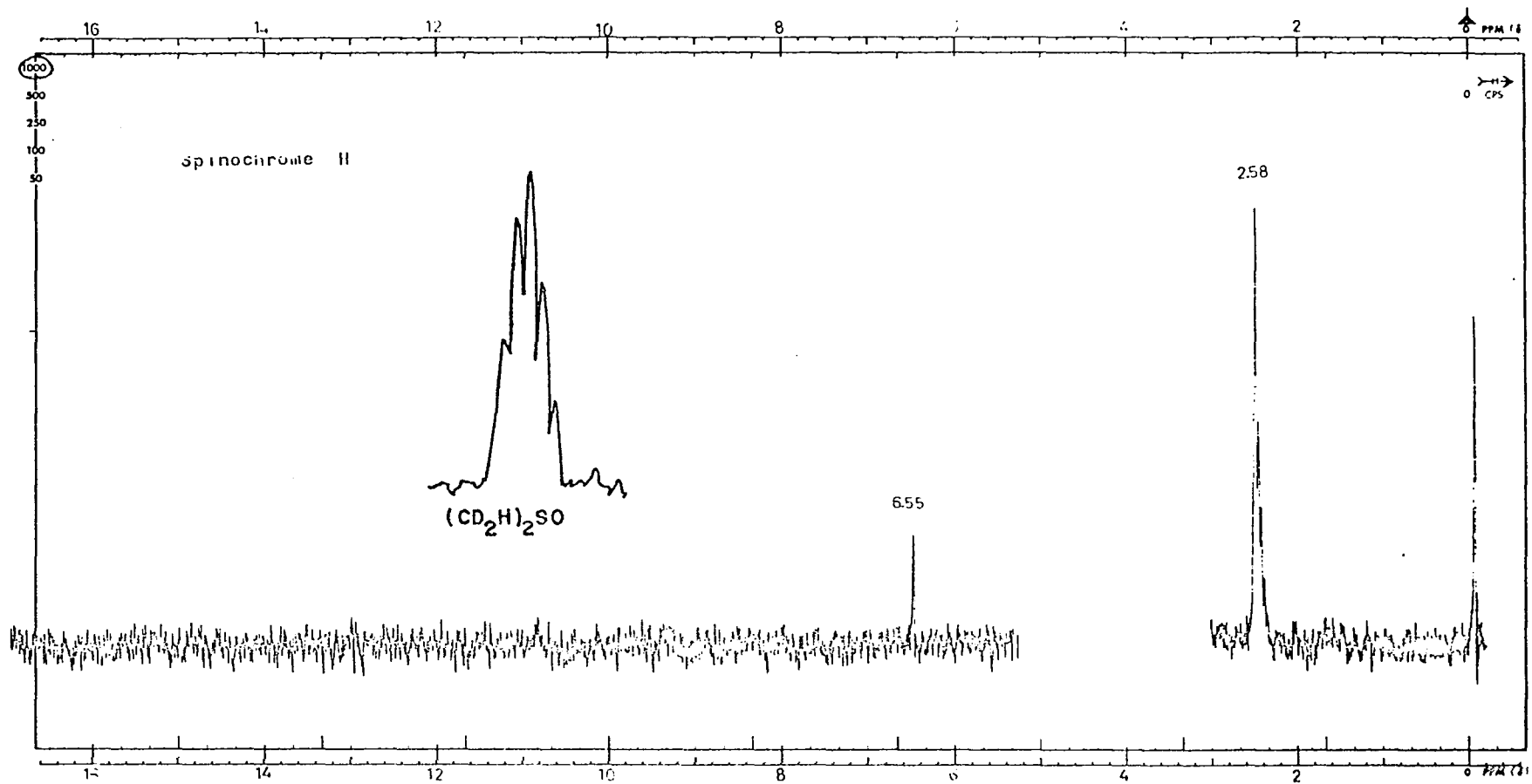
Fig. 10.   Nuclear Magnetic   Resonance Spectrum of Spinochrome H in DMSO-d$_6$.

dimethyl sulfoxide $(DMSO-d_6)$ (Fig. 10) was determined at a sweep width of 1000 c.p.s. and at a sweep time of 500 sec. Integration of the spectrum showed a ratio of 5 (high field multiple peaks centered at δ 2.5) to 1 (low field peak at δ 6.55).

d. Mass

A molecular weight of 264 was established by the low intensity M+1 peak in the high m/e region of the mass spectrum[*] (Fig. 11). The relative intensities of the fragments in the mass spectrum are given in Table III. The relative intensities are based on the largest peak of the spectrum (m/e 69, 100%).

4. Stability of Spinochromes

Spinochrome H is stable in the crystalline state. It is a sufficiently strong acid to be soluble in dilute sodium bicarbonate solution, from which it may be regenerated by lowering the pH of the solution. Upon long contact with this base the pigment eventually decomposed and imparted a yellowish color to the solution.

In stronger bases the pigment decomposed rapidly. This was illustrated by measuring the electronic spectrum of spinochrome H in methanolic potassium hydroxide) 15 min and 1 hr after solution. The results are shown in Fig. 12.

---

[*] Kindly determined by Dr. C. Djerassi of Stanford University.

Fig. II.   Mass   Spectrum   of   Spinochrome   H.

Table III. Mass Spectrum of Sp. H

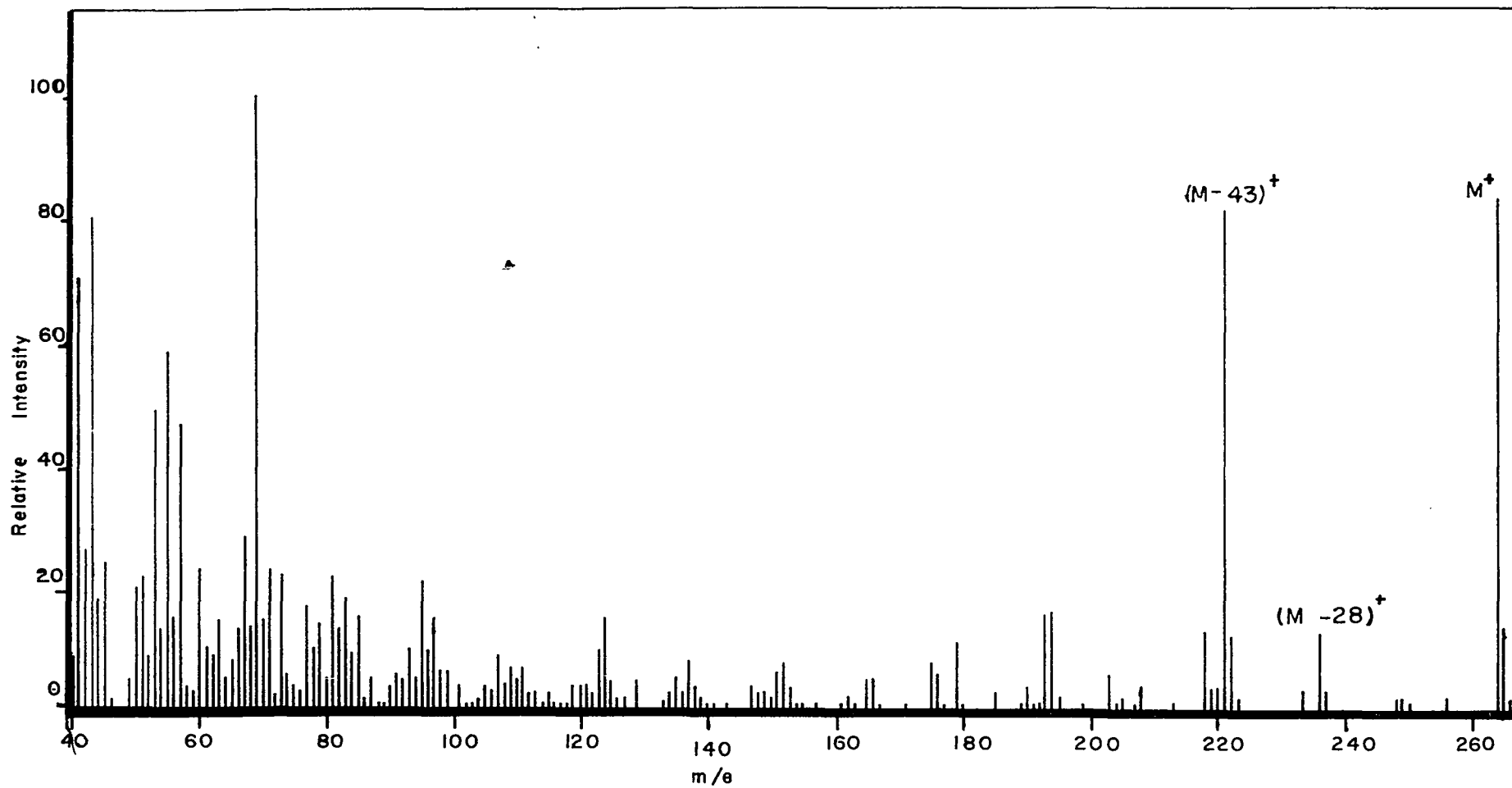| m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI
40	9	86	2	132		178		224	
41	70	87	5	133	1	179	11	225	
42	26	88	1	134	5	180	1	226	
43	80	89	1	135	5	181		227	
44	18	90	4	136	3	182		228	
45	24	91	6	137		183		229	
46	2	92	5	138	4	184		230	
47		93	10	139	2	185	3	231	
48		94	5	140	1	186		232	
49		95	21	141	1	187		233	3
50	20	96	10	142		188		234	
51	22	97	15	143	1	189	1	235	
52	9	98	6	144		190	4	236	12
53	48	99	7	145		191	1	237	3
54	13	100		146		192	1	238	
55	58	101	4	147	4	193	15	239	
56	15	102	1	148	3	194	16	240	
57	46	103	1	149	3	195	2	241	
58	4	104	2	150	2	196		242	
59	3	105	3	151	6	197		243	
60	23	106	3	152	8	198		244	
61	10	107	9	153	4	199	1	245	
62	9	108	4	154	1	200		246	
63	15	109	7	155	1	201		247	
64	5	110	5	156		202		248	2
65	8	111	7	157	1	203	6	249	2
66	13	112	3	158		204	1	250	1
67	28	113	3	159		205	2	251	
68	13	114	1	160		206		252	
69	100	115	3	161	1	207	1	253	
70	14	116	1	162	2	208	4	254	
71	23	117	1	163	1	209		255	
72	3	118	1	164		210		256	
73	22	119	4	165	6	211		257	2
74	7	120	4	166	5	212		258	
75	4	121	4	167	1	213	1	259	
76	3	122	3	168		214		260	
77	17	123	10	169		215		261	
78	10	124	15	170		216		262	
79	14	125	5	171	1	217		263	
80	5	126	2	172		218	15	264	93
81	22	127	2	173		219	3	265	13
82	13	128		174		220	4	266	2
83	18	129	5	175	8	221	91	267	
84	9	130		176	6	222	12		
85	15	131		177	1	223	2		
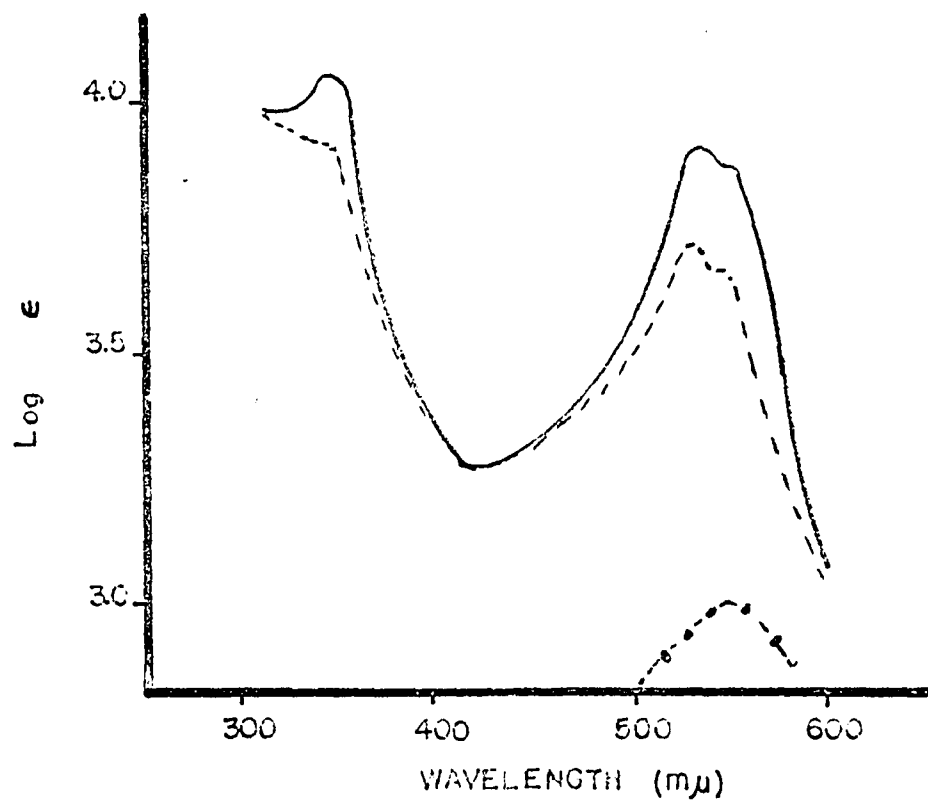
Fig 12. Visible Spectra of Spinochrome H in 0.25 N
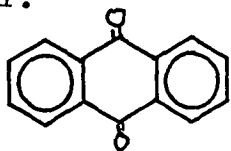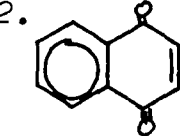Methanolic KOH after 0.5 min (—), 15 min (---),
and I hr (—-.).
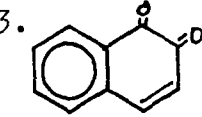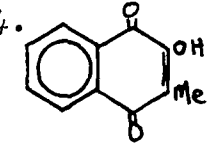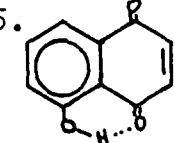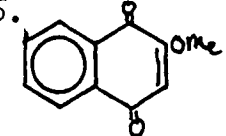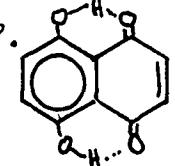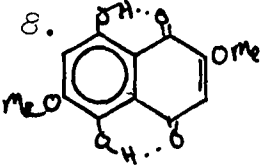
The minor orange pigment proved to be even less stable to base than was spinochrome H. The ultraviolet spectrum of a sample dissolved in ammonium carbonate solution and left to stand for one day with exposure to air was reduced to a single broad maximum at 301 $m\mu$. Upon acidification with dilute hydrochloric acid a hypsochromic shift to 286 $m\mu$ was observed, which could be reversed upon rebasification.

5. Craven Test

Hydrogen or halogen atoms in a quinone nucleus may be detected by the Craven test.[45] A positive test is indicated by a color change from bluish violet to bluish green and finally to reddish brown, when 2 to 3 drops of ethyl cyanoacetate are added to 3 ml of an alchoholic ammonia solution of the test compound. The test is sensitive for concentrations of 0.1 mg/ml and is recognizable with concentrations 1/10 as strong.

A series of 8 quinones was subjected to the Craven test and the results compared with spinochrome H. All concentrations were at least 0.1 mg/ml and a blank of each compound was run in the absence of ethyl cyanoacetate. Compounds 1 to 5 (9,10-anthraquinone, 1,4-naphthoquinone, 1,2-naphthoquinone, pthiocol, and juglone) were commercial samples and 6 to 8 (2-methoxy-1,4-naphthoquinone, naphthazarin, and 2,6-dimethoxynaphthazarin) were prepared by published procedures. The results are summarized in Table IV.

Table IV. Craven Test for Quinones.

		0	½"	10"	30"	1'	16'	60'
1.	test	C	C	C	C	C	1y	Y
	blank	C	C	C	C	C	C	1Y
2.	test	1Y	dBV	dBV	dBV	dB	Br	RBr
	blank	1Y	1Br	Br	Br	Br	Br	Br
3.	test	YBr	YBr	YBr	YBr	1R	1R	Y
	blank	1Y	1Y	1Y	C	C	C	Y
4.	test	O	O	O	O	O	1Y	1Y
	blank	O	O	O	O	1Y	1Y	1Y
5.	test	Y	B	B	B	1O	1OY	
	blank	Y	1O	1O	1O	1O	OR	OR
6.	test	C	dB	dB	dB	dB	1G	1G
	blank	C	C	C	C	C	C	C
7.	test	RO	BV	dB	G	G	G	BrY
	blank	RO	V	V	V	V	V	P
8.	test	P	1R	1R	1R	1R	1R	1R
	blank	P	P	P	OR	OR	OR	OR
9. Spinochrome H	test	R	dV	dV	dV	dV	1Bl	1R B
	blank	R	V	V	V	1Bl	1Bl	

Symbols used for the above qualitative test: d(dark), l (light), C(colorless), Y(yellow), B(Blue), Br(brown, Bl (black), V(violet), R(red), O(orange), G(green), & P(purple).

D. Chemical Transformations

   1. Haloform Reaction

      Forty-three milligram of non-crystalline but homo-geneous (single spot on TLC) spinochrome H was dissolved in a mixture of 0.5 ml of 1,4-dioxan and 0.5 ml of 10% sodium hydroxide in a small test tube. The solution immediately turned brown-black. Iodine solution, prepared by adding 1 g potassium iodide to 0.5 g iodine in 4 ml water, was added until the color of the solution appeared light brown. The solution was then heated at 60° on a water bath for 2-3 min with stirring. Sodium hydroxide solution (10%) was added (pH 14) and the aqueous basic solution was extracted with chloroform. The yellow chloroform solution was stripped to dryness after preliminary drying over magnesium sulfate. A few crystals (ca. 3 mg) of iodoform resulted which were recognized by their characteristic odor and their melting point of 118-119° (literature m.p.,[46] 119°).

   2. Methylation

      a. With dimethyl sulfate

      To a magnetically stirred mixture of 150 mg spino-chrome H in 15 ml dry acetone and 7.5 g anhydrous powdered potassium carbonate was added 4.5 ml freshly distilled dimethyl sulfate. The reaction mixture was kept in the dark and was run in an inert (argon) atmosphere for 26 hr. An additional 7.5 g potassium carbonate and 4.5 ml of dimethyl sulfate were added and the mixture was refluxed for 3 hr and

then allowed to cool. Water (75 ml) was added and the resulting orange solution was extracted with chloroform which became tinged orange-yellow. The aqueous solution was acidified to pH 4 with phosphoric acid with occassional additions of water to dissolve the precipitated inorganic salts. Some orange-red crystals of a product precipitated and these were collected and washed with water.

Extraction of the acidified solution with ether gave a mixture of two major and two minor components as seen by TLC. The spots were designated MS-1, MS-2, MS-2.5, and MS-3. The R_F values and colors on DSG are recorded in Table V.

Table V. TLC of Products from the Dimethyl Sulfate
Reaction with Spinochrome H

Spot	R_F	Color before base spray	after
MS-1	0.26	pink	violet
MS-2	0.17	yellow	brown
MS-2.5	0.06	pink	violet
MS-3	0.04	yellow	yellow

Compounds corresponding to these spots could be separated and eluted with benzene as sharp bands on a DSG column. MS-1, later shown to be monomethoxyspinochrome H, appeared as a purple band which was immediately followed by a yellow band (MS-2) and two trace components as pink-orange (MS-2.5) and yellow (MS-3) bands.

Monomethoxyspinochrome H (MS-1) was crystallized

from chloroform as purple needles, m.p. 239-242°. *Anal.*

Calcd for $C_{13}H_{10}O_7$: C, 56.12; H, 3.62. Found: C, 56.29,

56.16; H, 3.86, 3.84.

Dimethoxyspinochrome H (MS-2) was crystallized

from chloroform-petroleum ether (30-60°) as dark-orange

needles, m.p. 224-227°. *Anal.* Calcd for $C_{14}H_{12}O_7$: C, 57.54;

H, 4.14. Found: C, 57.60, 57.58; H, 4.54, 4.55.

The visible and ultraviolet spectra of monomethoxy-

spinochrome H are reproduced in Figs. 13, 14, and 15. The

following maxima and minima were observed.

In methanol, λ_{max}: 508 mμ (log e, 3.69), 312.5

(4.08), 271 (4.17), ca. 224 (4.20); λ_{min}: 371 (3.24), 288

(4.02), 252 (4.12), 216.3 (4.18).

In 0.05N potassium hydroxide in methanol after one

minute from the preparation of the solution; λ_{max}: 570 mμ

(log e, 3.83), 532 (3.98), 503 (3.92), ca. 334 (3.82),

287.5 (4.22), 235 (4.26). λ_{min}: 558 (3.80), 511 (3.91),

389 (3.28), 258.5 (3.96), 220 (4.20).

In methanolic aluminum chloride; λ_{max}: 614sh, 560,

529sh, 355, ca. 337, 289, 228.5 mμ. λ_{min}: 440, 317, 263-

250b, 220 mμ.

The visible and ultraviolet spectra of dimethoxy-

spinochrome H are shown in Figs. 16 and 17. Pertinent data

follow.

In methanol; λ_{max}: 450 mμ (log e, 3.43), 302 (4.18),

Fig. 13. Visible Absorption Spectra of
Monomethoxyspinochrome H in Methanol (—)
and Methanolic KOH(----).
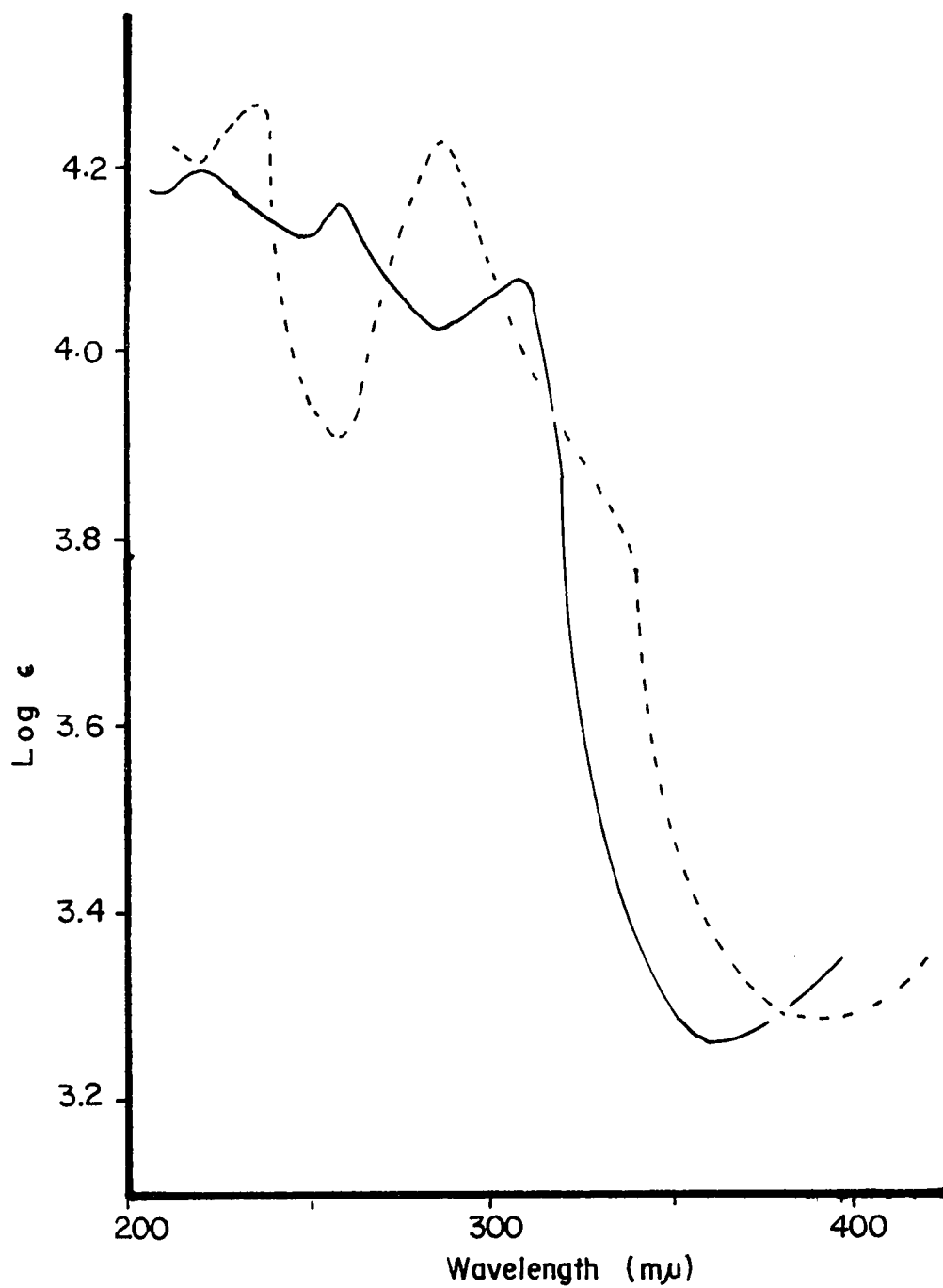
Fig. 14.   Ultraviolet   Spectra   of   Monomethoxyspinochrome   H
in   Methanol (—) and Methanolic KOH (---).

Fig. 15.   Visible and Ultraviolet Spectrum of
           Monomethoxyspinochrome H in Methanolic
           $AlCl_3$.
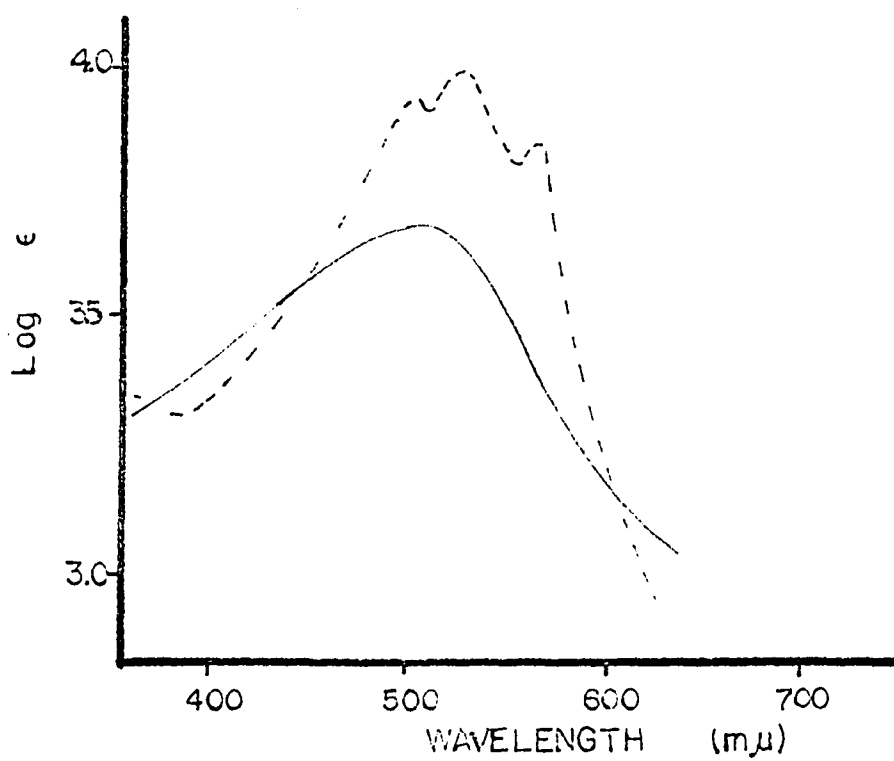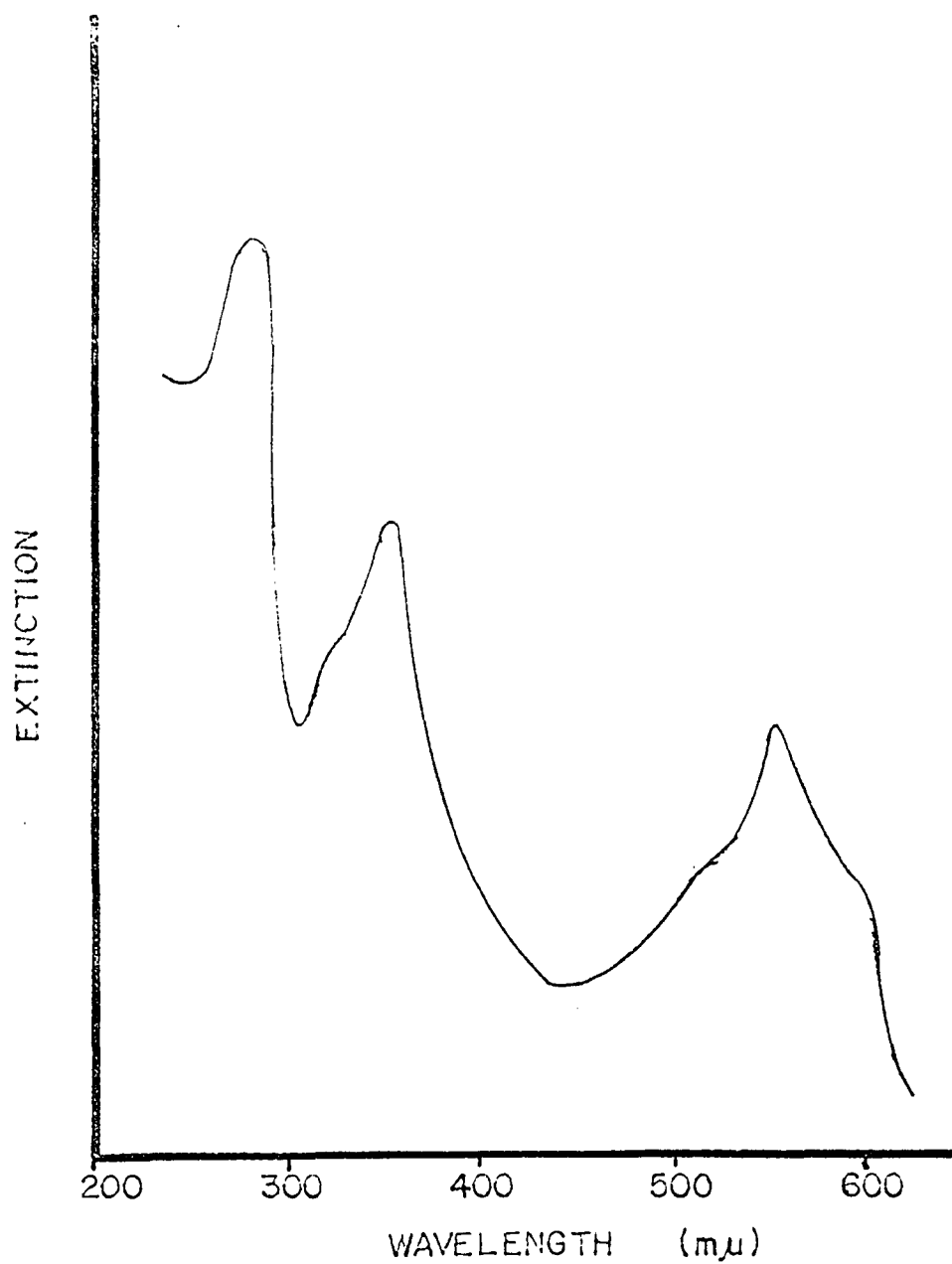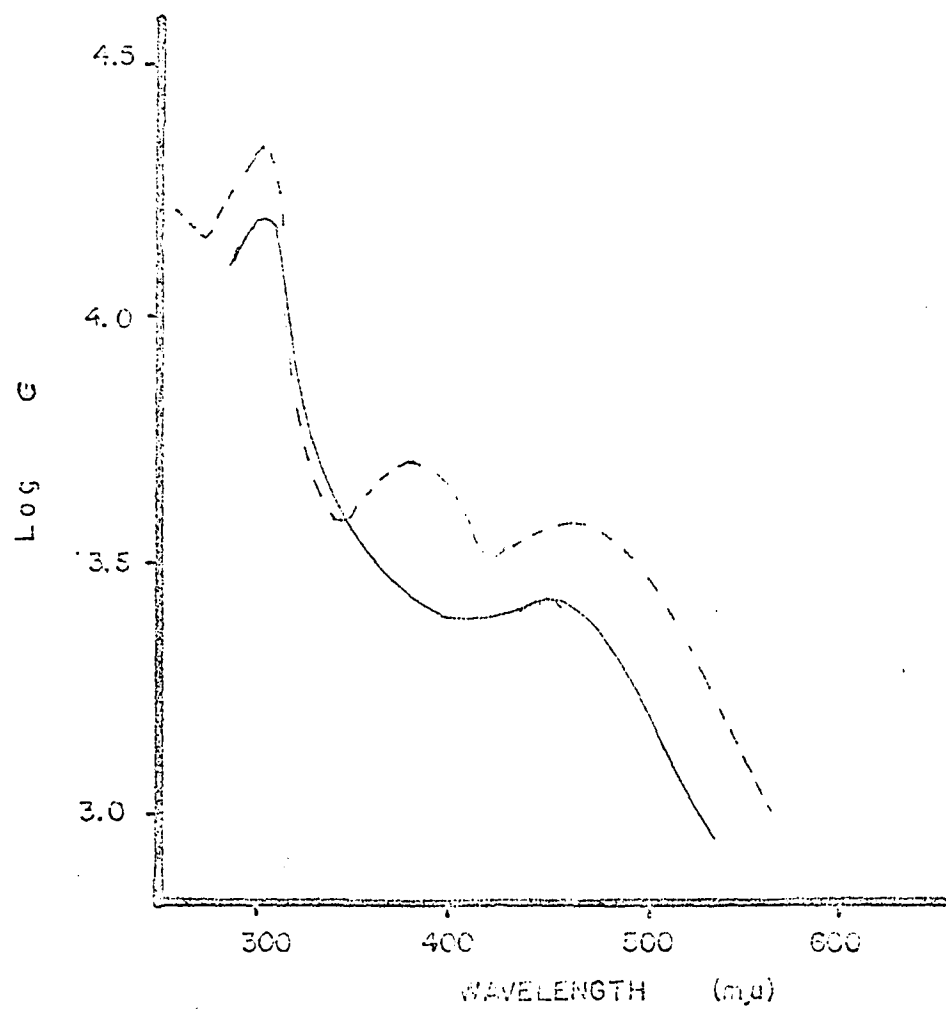
Fig. 16. Visible Absorption Spectra of Dimethoxy-
spinochrome H in Methanol (—) and
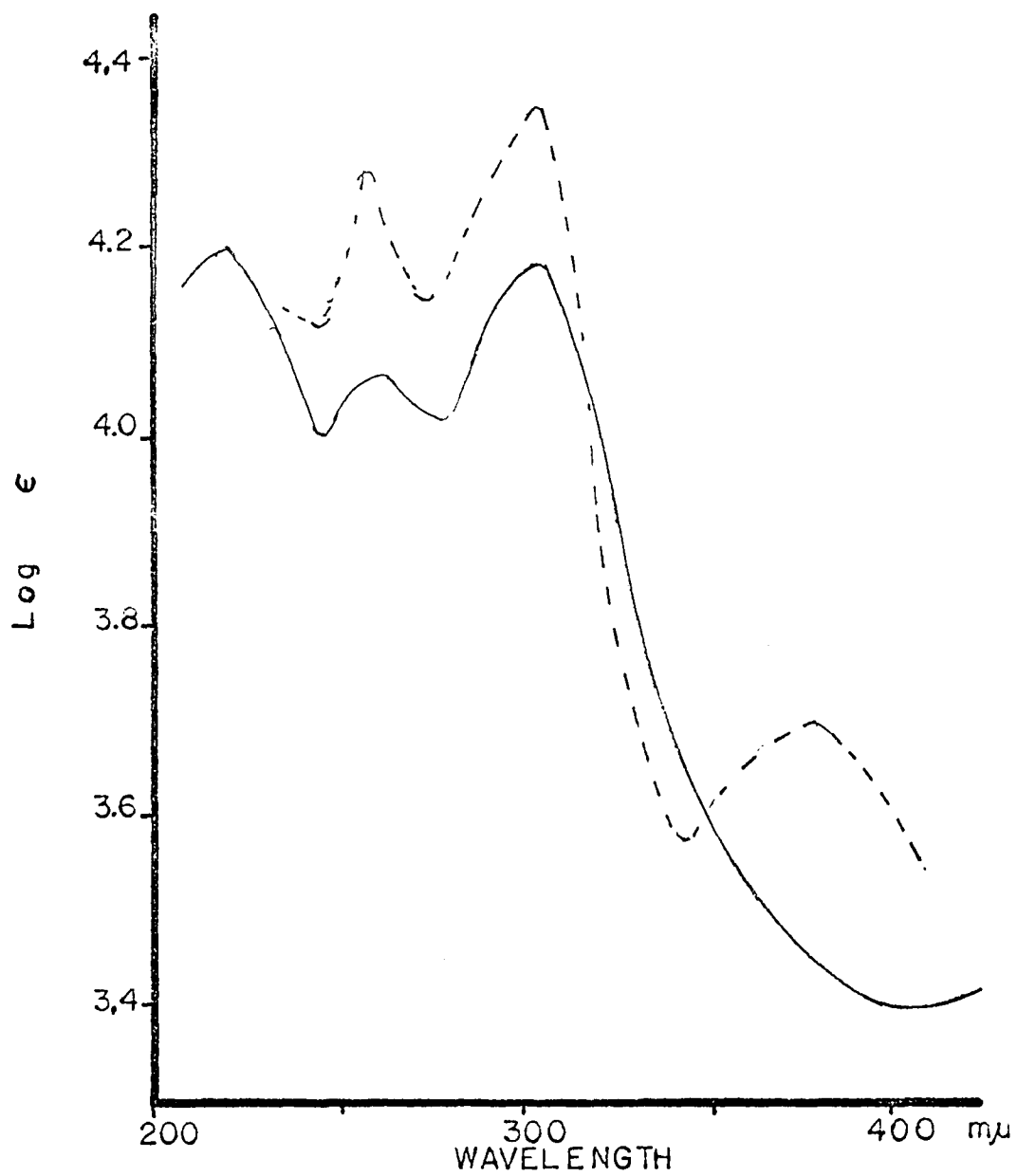Methanolic KOH(---).

Fig. 17.   Ultraviolet Spectra of Dimethoxyspinochrome H
in   Methanol (—) and Methanolic KOH (-----).

263 (4.07), ca. 235 (4.12), 222.5 (4.20). λ_{min}*: 408 (3.38),*
279.3 (4.02), 248.5 (4.00).

In 0.05N *methanolic potassium hydroxide after one*
minute from time of sample preparation; λ_{max}*: 460 mμ (log e,*
3.58), 377 (3.70), 301.5 (4.34), 259.5 (4.28). λ_{min}*: 422*
(3.52), 344 (3.58), 275 (4.14), 245.5 (4.12).

Infrared spectra of mono- and dimethoxyspinochrome
H are reproduced in Figs. 18 and 19.

Major bands of the monomethoxy compound occurred
at 6.2s, 6.35infl., 6.8w, 6.85w, 6.98, 7.1, 7.5w, 7.7w, 7.8w,
8.2s, 8.8w, 8.95w, 10.1, 10.35, 11.6b μ.

Principal peaks of the dimethoxycompound were
observed at 3.4, 5.95s, 6.15, 6.45w, 6.75w, 6.99infl., 7.05w,
7.15infl., 7.85b, 8.1, 8.2w, 8.45, 8.75s, 9.75s, 9.85w,
10.1s, 10.15b, 11.45s, 12.0, 12.2 μ.

The NMR spectrum of dimethoxyspinochrome H (Fig.
20) was measured in deuteriochloroform at a sweep width of
1000 c.p.s. and a sweep time of 500 sec. In order to repro-
duce the spectrum in Fig. 20 the sweep width was offset 40
c.p.s. upfield from TMS. Signals (intensity) were observed
at δ 2.83 (3), δ 3.88 (3), δ 3.96 (3), δ 6.75 (1), δ 13.50
(1) and δ 17.20 (1). The resonance signal due to trace
amounts of chloroform present in the deuteriated solvent
occurred at the normal position of -436 c.p.s. relative to
TMS.

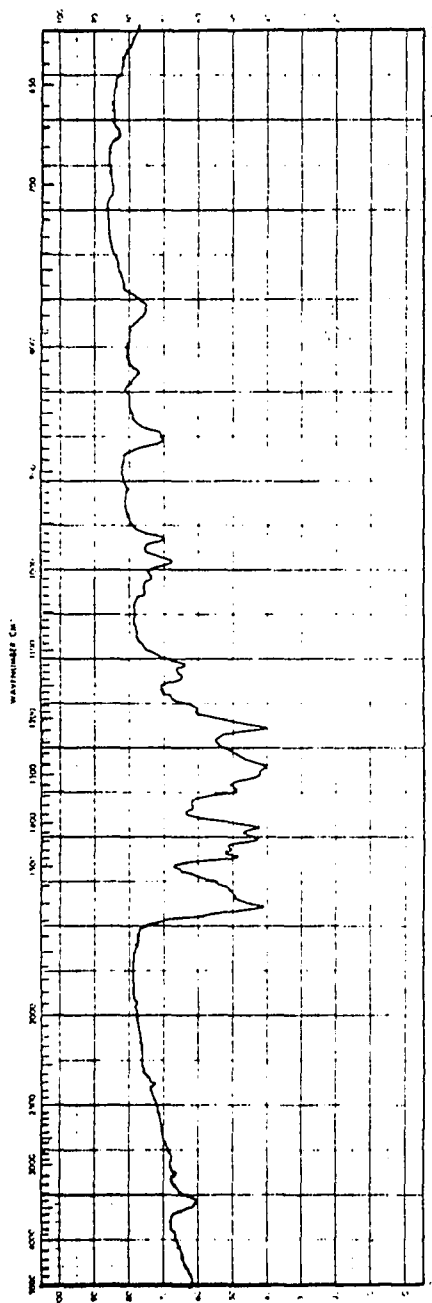*A NMR spectrum of the crystalline monomethoxy-*

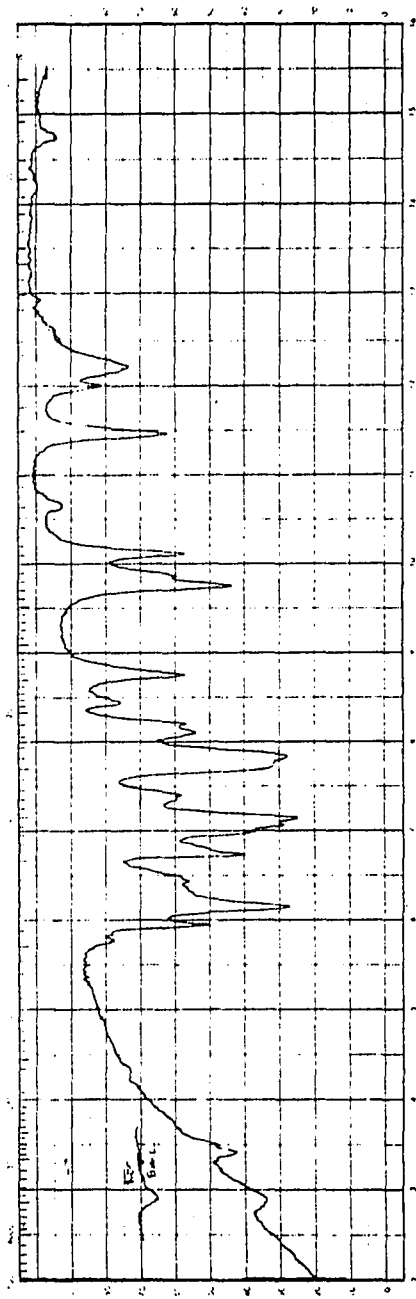Fig. 18. Infrared Spectrum of Monomethoxyspinochrome H in KBr.

Fig 19. Infrared Spectrum of Dimethoxyspinochrome H in KBr.
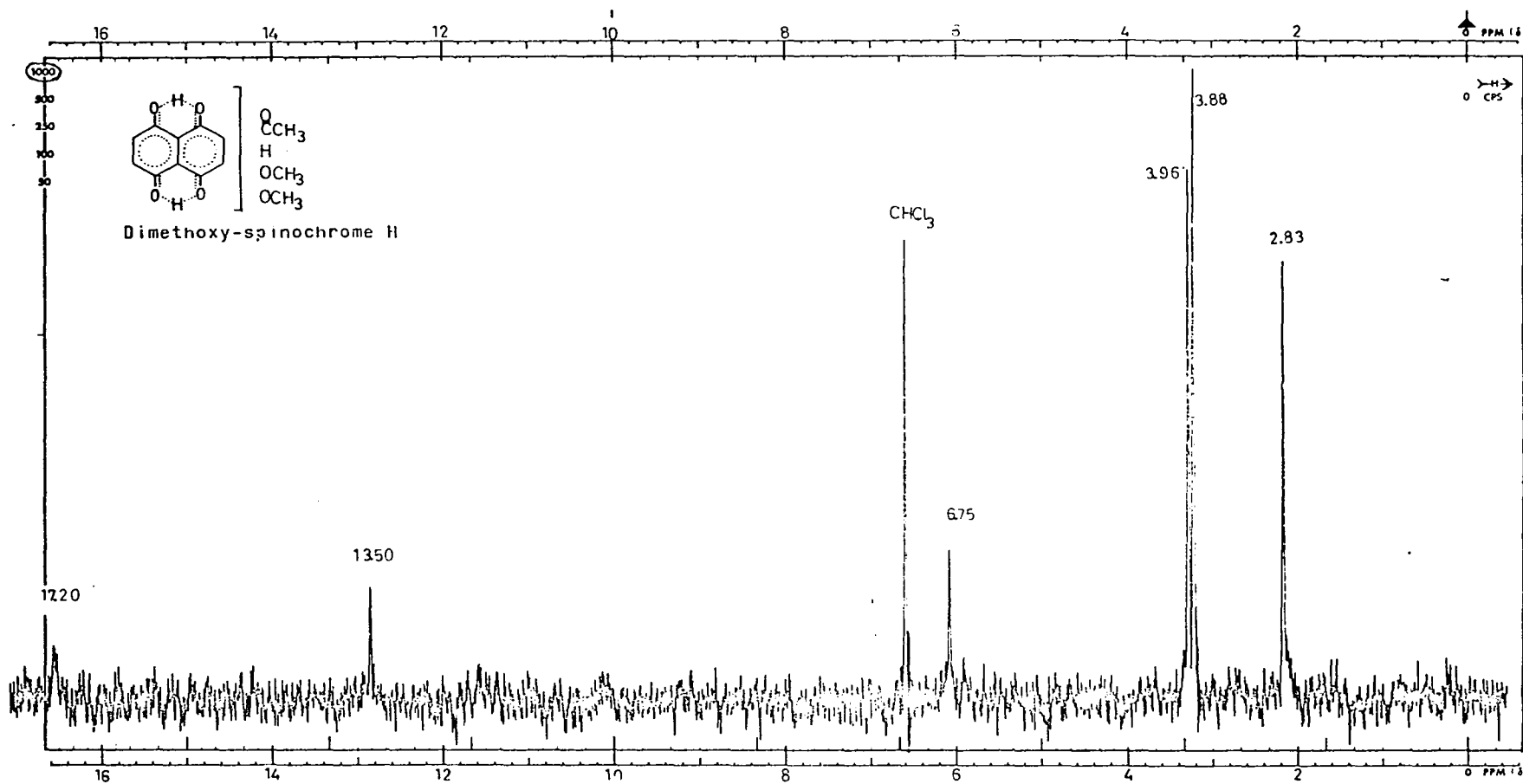
Fig. 20. Nuclear Magnetic Resonance Spectrum of Dimethoxyspinochrome H in CDCl₃.

derivative was difficult to obtain because of its low solu-
bility in chloroform. In this respect the compound behaved
like its parent compound. A crude sample, as eluted off the
column, could be measured in deuteriochloroform with a trace
of methanol. The spectrum exhibited only one signal in the
aromatic methoxy region at <u>ca.</u> δ 4.0.

Since only trace amounts were obtained of the
other two products, MS-2.5 and MS-3, no combustion data
could be determined. Spectra (Fig. 21) of the two trace
products showed the following maxima and minima. MS-2.5 in
chloroform; λ_{max}: 489, 363, 316sh, 272 mμ. λ_{min}: 427, 336,
310, 262.5 mμ. MS-3 in chloroform; λ_{max}: 428, 361, 303 mμ.
λ_{min}: 408, 347, 279.5 mμ.

An infrared spectrum of MS-3, measured in chloro-
form solution is reproduced in Fig. 22.

b. With diazomethane

<u>Preparation of diazomethane</u>: A solution of 4.3 g
(0.02) mole Diazald (Aldrich Chemical Co.) in 25 ml ether
was added through a dropping funnel over a period of 25 min
to a solution of 5 ml 95% ethanol and 1 g potassium hydrox-
ide in 2 ml water. The temperature of the water bath was
maintained between 60 and 70°. The resulting ethereal solu-
tion of diazomethane was distilled in a modified 100 ml
Claisen flask and was collected in an erlenmyer flask
partially immersed in a dry ice bath. The yield was <u>ca.</u>
14.3 mmole diazomethane in 40 ml ether or 0.35 mmole/ml.
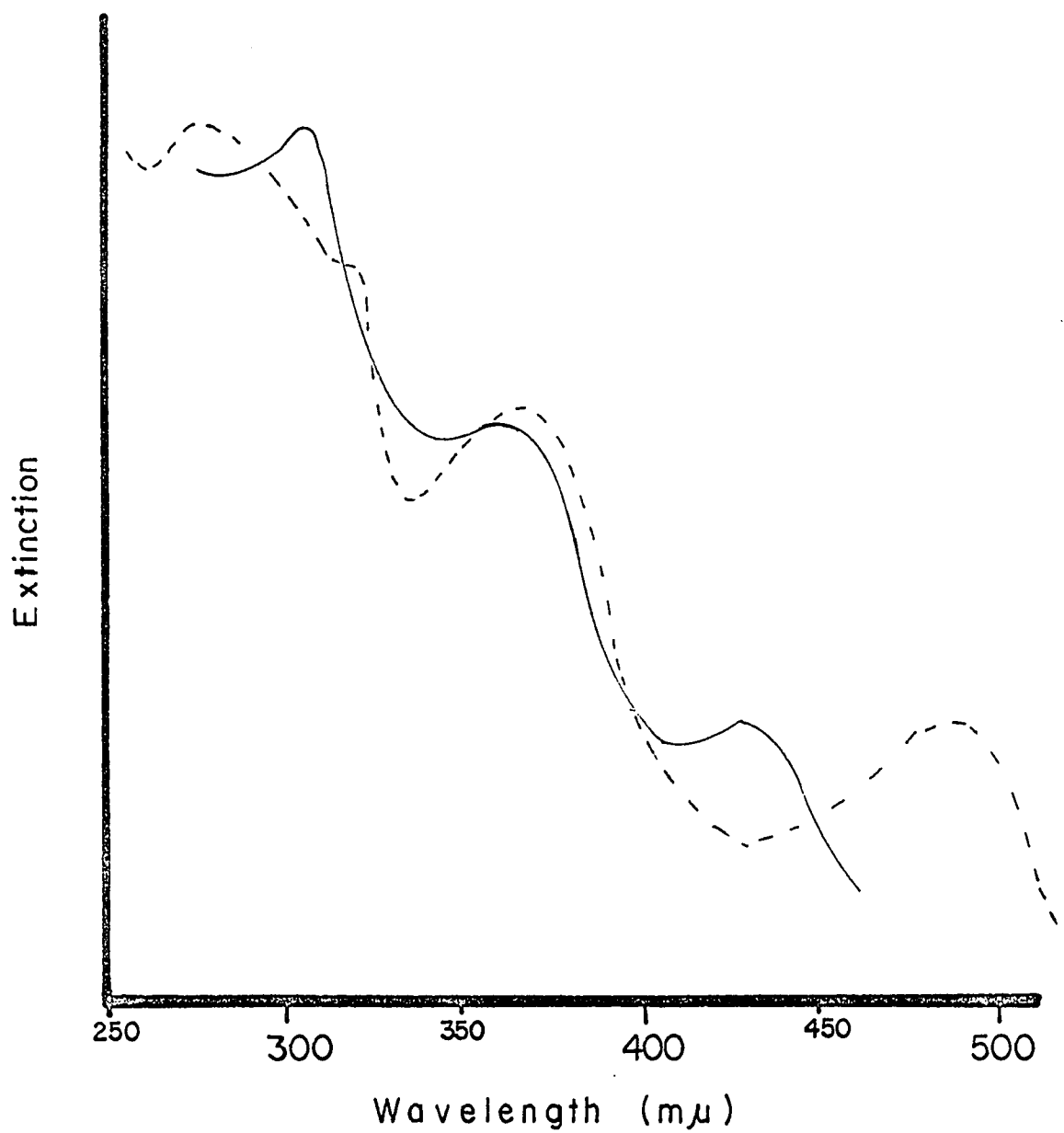
Fig. 21.   Visible and Ultraviolet Spectra of MS-2.5(---) and
MS-3(—) in CHCl$_3$.

WAVENUMBER CM⁻¹

WAVELENGTH IN MICRONS
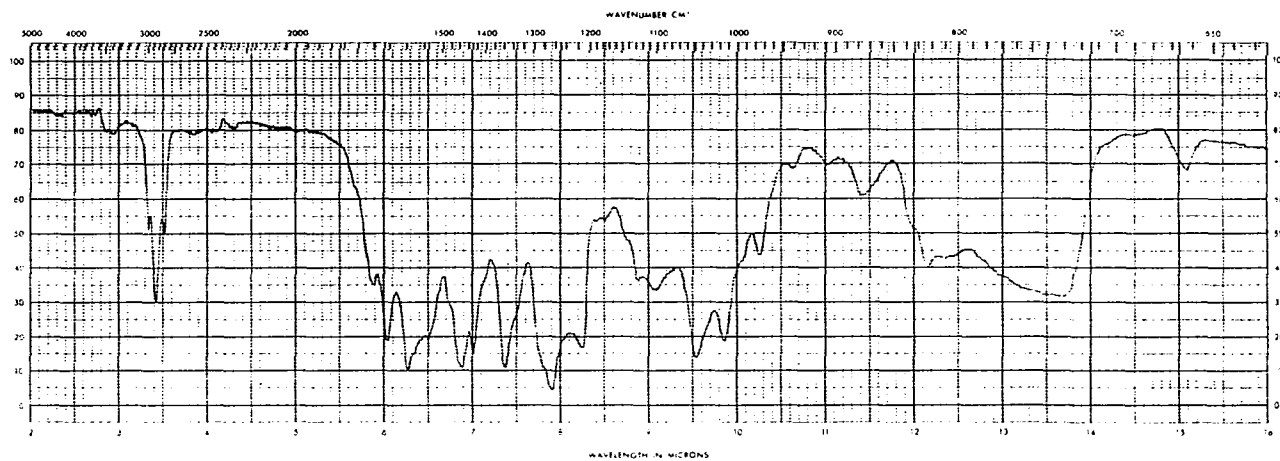
Fig. 22.   Infrared Spectrum of Fraction MS-3 from the Dimethyl Sulfate
            Reaction of Spinochrome H in $CHCl_3$.

Reaction with diazomethane: To a magnetically stirred solution of spinochrome H (100 mg, 0.38 mmole) in 100 ml ether was added 4 ml of the above solution. The purple ethereal solution of the pigment instantaneously turned orange with rapid evolution of nitrogen gas. The solution was allowed to stand for 3 hr and then washed with a 5% solution of sodium bicarbonate. The basic solution was deep purple. It was acidified with hydrochloric acid and exhaustively extracted with ether to give a yellow ethereal solution, which was dried over sodium sulfate and stripped *in vacuo.* The residue was taken up in benzene and chromatographed over acid-washed silica gel.

TLC results of the reaction are shown in Table VI. For comparison the samples were all chromatogrammed simultaneously with the products of the dimethyl sulfate reaction.

Table VI. TLC of Products from the Diazomethane Reaction with Spinochrome H.

Spot	R_F	Color before base spray	after
CHN-1	0.27	purple	l. violet
CHN-2	0.44	l. yellow	yellow-orange
CHN-3	0.14	l. red	dk. violet
CHN-4	0.11	dk. yellow	purple
CHN-5	0.41	orange	pink
CHN-6	0.00	yellow-orange	blue

The deep red spot of unreacted spinochrome H showed a R_F value of 0.06.

Fraction CHN-1 proved to be identical with MS-1 by TLC, melting point, UV and IR spectra and was therefore the monomethoxy derivative. It was the only major product and was isolated in a quantity of ca. 30 mg.

Small amounts of orange hexagonal crystals were obtained from the fraction designated CHN-3, m.p. 174-175°, after recrystallization from chloroform-methanol. Fraction CHN-5 furnished orange-yellow needles from chloroform-methanol, m.p. 220-221°. The visible and ultraviolet spectra of these compounds, which were homogeneous by TLC, were measured in chloroform. No crystalline material was obtained from fractions CHN-2, CHN-4, and CHN-6. Table VII records the absorption maxima and minima.

Table VII. Visible and Ultraviolet Spectra in Chloroform of Diazomethane Reaction Products.

Fraction	Wavelength (mμ)	
	Maxima	Minima
CHN-1	507, 312.5, 268, ca. 234	366, 287, 252
CHN-2	472infl., 443, 340sh	373, 281.5, 320
CHN-3	546infl., 513, 485, 313	364, 290.5
CHN-4	443, 296	345, 276.5
CHN-5	535, 496, 468	437
CHN-6	485, 287	423, 276

3. Acetylation

    a. With acetic anhydride

No pure crystalline product was obtained from acetylation attempts using acetic anhydride with traces of the following catalysts: sodium acetate, conc. sulfuric acid (70%), and pyridine. In each case, after decomposing the excess acetic anhydride in water, the product hydrolyzed during crystallization attempts to at least three compounds recognizable by well defined spots on TLC. One of the trials was carried out as follows.

Spinochrome H (35 mg) was reacted with 0.4 ml of acetic anhydride and a drop of conc. sulfuric acid. The reaction flask was then heated in a water bath (60°) for 10 min. The yellow solution was treated with water, then washed with sodium bicarbonate solution, followed by carbon tetrachloride extraction. The organic solution, washed again with water, was dried with sodium sulfate and stripped in vacuo. The residue was sublimed at 130-150°/0.05 mm. to yield microcrystals, m.p. 160-164°. A portion of the NMR spectrum is reproduced in Fig. 23.
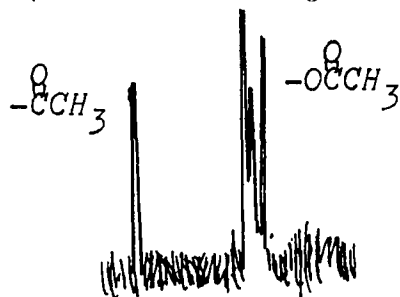

Fig. 23. NMR Spectrum of Acetylation Product of Spinochrome H in CDCl$_3$ in the δ 2-3 region.
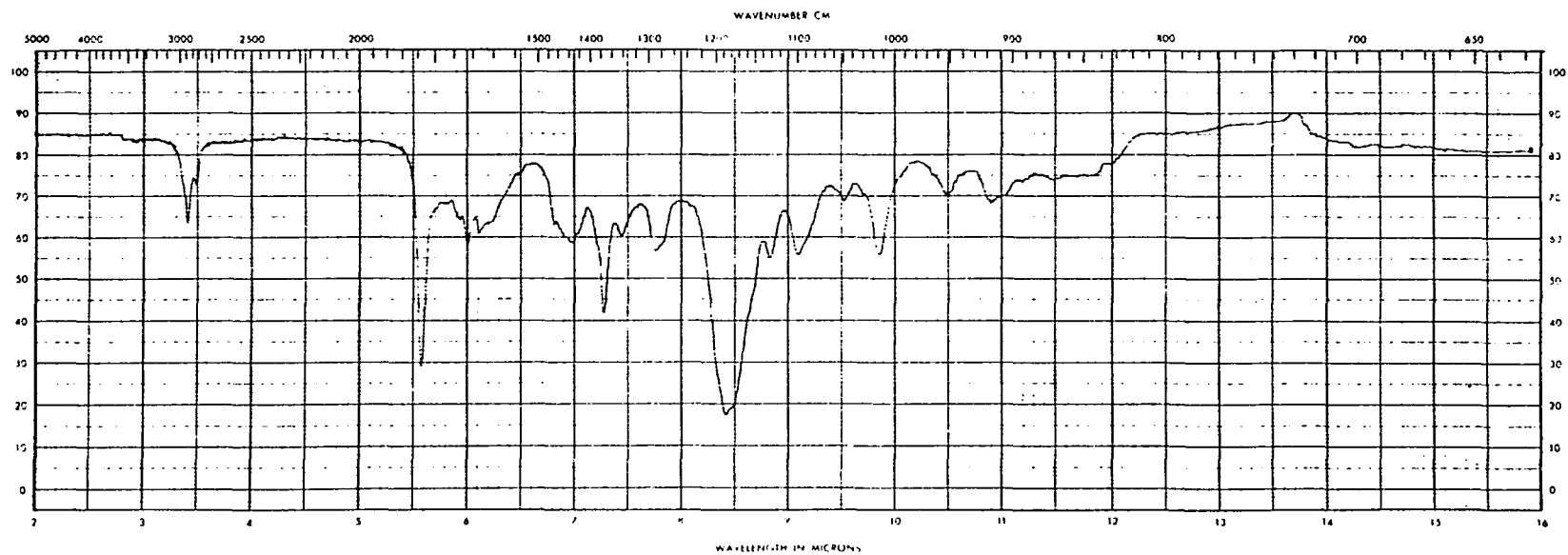
Fig. 24.   Infrared   Spectrum   of   the   Acetate   Derivative   of   Spinochrome   H   in   CCl$_4$.

An infrared spectrum (Fig. 24) of this sample had prominant bands at 5.6 and 8.4 μ.

b. With ketene

Ketene was generated by pyrolysis of acetone and was bubbled for ca. 5 min into a stirred solution of 45 mg spinochrome H in dry benzene. After the benzene solution was allowed to stand for 30 min a sample was spotted on TLC resulting in at least 3 spots. Three hours later a single yellow spot resulted when the benzene solution was spotted. The results are shown schematically in Fig. 25.

○ orange-pink   ● *yellow*

● yellow

◑ orange

⊗   *⊗ purple*

————————————ᴍ ⊦———————————— *Base line*

Sp.H   *Rxn. mixt.*   *Rxn. mixt.*
after 0.5 hr   *after 3 hr*

Fig. 25. TLC of Reaction Solution of Spinochrome H and Ketene

After 3 hr the solution was stripped in vacuo, fresh portions of benzene were added, and the solution stripped again. The yellow oily residue could not be crystallized.

Chapter III

RESULTS AND DISCUSSION

A.   Isolation Scheme

Improvement of the isolation and purification was developed in the course of this work. The new scheme should be generally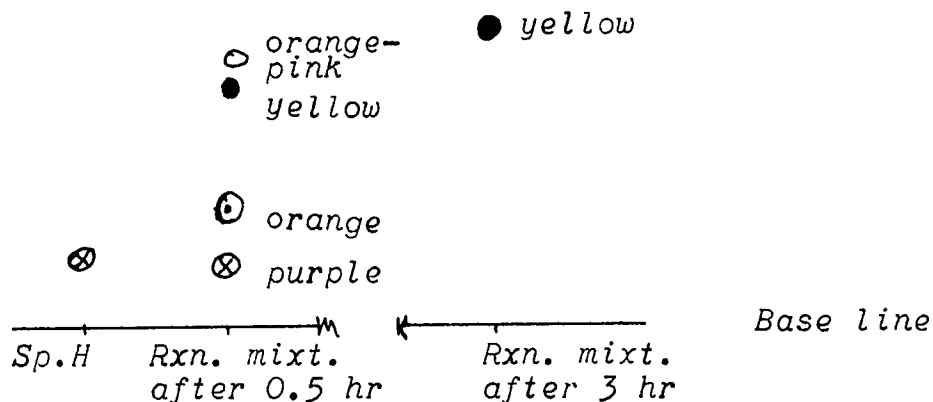 applicable to spinochrome research. Calcium carbonate, which was universally used as the adsorbent by previous workers, seemed unsatisfactory since its basicity did not permit elution of the acidic pigments. Instead, following considerable experimentation, partition-related chromatography with acid-washed silica gel, was successfully utilized employing benzene, benzene-chloroform, chloroform, and chloroform-ethyl acetate as solvents, singly or in pairs. Chloroform-ethyl acetate pair was used but solvents more polar than ethyl acetate were unnecessary except to wash the column.

The yellow band that was observed by Amai[1] and by Temple[2] in the forerun of the carbonate column is possibly a lipid originating from the fleshy portions of the spine stumps. Recent investigations by Bergmann and Domsky[47] and Fursch et.al.[48] on the neutral components from the internal organs of sea urchins revealed the presence of steroidal compounds. Separation of this neutral fraction was efficiently achieved by sodium bicarbonate washes. Long contact of the pigment with basic solutions was avoided as much as possible. It was absolutely essential that peroxide-free ether was used in view of the extreme instability of the orange pigment in basic medium.

The resulting crude mixture of spinochromes was chromatographed on DSG. It was found that the column required a high adsorbent to compound ratio (ca. 1000:1) for an effective separation of the spinochromes. Flooding of the column was observed even at a normally high ratio of 500:1. The purple and orange bands were readily distinguishable and the dark red eluate was homogeneous with respect to spinochrome H.

The pure pigment, m.p. 189-190°, crystallized nicely from methanol as long needles and had a composition of $C_{12}H_8O_7 \cdot CH_3OH$. It was reddish-purple, but when dried under high vacuum over phosphorus pentoxide, it lost methanol of crystallization and became black. Melting points of these darkly colored compounds were difficult to observe, but no decomposition seemed to occur at the melting point as was believed earlier. Instead, it appeared to sublime at 170-180°, but this may be rationalized as a physical disruption of the solvated crystals, which were strongly hydrogen-bonded.

To test the purity of the sample, a thin-layer chromatographic procedure was developed which would effectively resolve mixtures of spinochromes. Since these compounds are colored, information on the homogeneity of the test sample could be achieved qualitatively in ten to twenty minutes with non-polar developing systems. The value of TLC is enhanced by the information which it provides

for subsequent preparative chromatography. By observing the chromatogram of TLC conditions could be selected for use on a preparative scale, provided that the activity of the adsorbent on the plate and column was approximately the same.

Examination of the literature revealed that prior to 1961 only one publication on chromatographic analysis of spinochromes had been published. n-Butanol-water (150/25), with samples spotted on a chromatogram of filter paper pretreated with 6N hydrochloric acid, constituted the system developed by Kuroda and Harada.[49] The process, however, required nine hours for development of the chromatogr m and, besides, a delicate touch in handling the paper which tended to crumble because of its acid impregnation.

An improved paper chromatographic method by Yamaguchi[50] was reported in 1961. The solvent systems generally employed were mixtures of formic acid in methanol (80-20), in methanol-water (60-30-10), in 2N hydrochloric acid (70-30), etc. The pigments were dissolved in 90% formic acid and developed for about eight hours by a one dimensional ascending process.

After our TLC system was developed there appeared an article on the application of TLC to benzoquinone derivatives.[51] Development of the chromatogram for hydroxybenzoquinones with ordinary organic solvents on neutral silica gel plates resulted in tailing of the spots. Pettersson,[51] however, found that ethanol-ammonia (5-1) successfully

resolved the hydroxyquinones without extensive tailing on a
silica gel plate. Although the use of ammonia did not seem
propitious for the strongly acidic spinochrome H, Pettersson's
system was tried. As was forseen, the familiar bluish-violet
spot did not move from the base line. It should be pointed
out that the R_F values obtained by our system of TLC and
reported here would not be valid for comparative studies by
another investigator. Because of the variability of the DSG
it is necessary to conduct comparative studies on the same
thin-layer plate. R_F values on different plates but pre-
pared from the same batch were found to be reproducible pro-
*vided the runs were made within one month of each other.*

B.   The $C_{12}H_8O_7$ Formula

Amai (see p. 4), on the basis of combustion data,
had proposed a molecular formula of $C_{12}H_8O_7$ for spinochrome
H. Temple (see p. 5), on the other hand, had arrived at a
$C_{13}H_{12}O_8$ formulation for the same pigment. The present work
demonstrated conclusively that Temple's analytical sample
must have contained one mole of methanol of crystallization.
Our data leading to a $C_{12}H_8O_7$ formula could be obtained when
the sample was dried at 80° over phosphorus pentoxide in
high vacuum. Less rigorously dried samples retained the
solvent.

The mass spectrum of spinochrome H (Fig. 11), mea-
sured on a solvent-free sample, fully confirmed a molecular
weight of 264 and therefore a formula of $C_{12}H_8O_7$.

C.   Structural Considerations.

A total of seven structures had been proposed for spinochrome H by previous workers, four by Amai (see p. 5) and three by Temple (see p. 6). Temple's three structures V, VI, and VII may be eliminated from further consideration since they were based on an incorrect molecular formula. A Zeisel determination carried out on Temple's sample proved the absence of methoxy groups in spinochrome H, thereby eliminating Amai's structures I, II, and III. Only Amai's structure IV needs to be examined in the light of the new evidence available through this research.
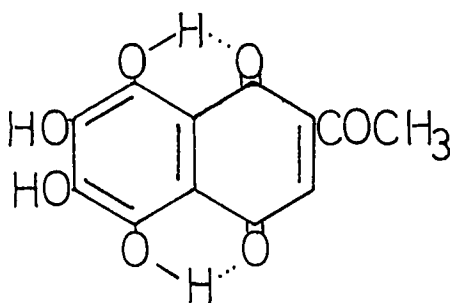

IV

A 1,4-naphthoquinone nucleus for spinochrome H is well substantiated by its UV spectrum in methanol (Figs. 3 & 5), which is a typical 1,4-naphthoquinone spectrum.[52] Closer examination of the UV spectra in various media, however, forces one to conclude that spinochrome H is a 2-hydroxy-1,4-naphthoquinone and that the last of the remaining previous structures, Amai's structure IV, should be dropped from further consideration.

2-hydroxy-1,4-naphthoquinones are strongly acidic

because its enolic hydroxyl permits resonance contributors
such as XIX. In support of this structural assignment for
spinochrome H may be cited its ready solubility in sodium
bicarbonate solutions and the bathochromic shift of the 514

XVIII ↔ XIX
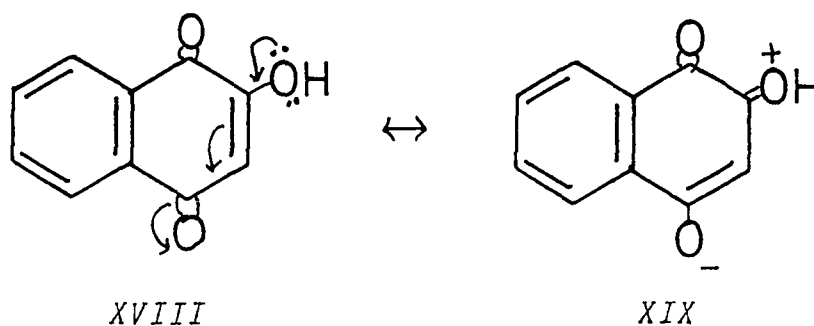
mμ band by 55 mμ concomitant with an increased extinction
coefficient when base was added to a methanolic solution
(Fig. 3). This behaviour is in accord with Spruit's[53]
observations in his study of the spectra of hydroxynaphtho-
quinones.

It should be noted here that UV spectra of spino-
chrome H in base were reproducible only at low concentra-
tion of base within short times after the solutions were
prepared. This aspect is documented by a comparison of Fig.
12 with Figs. 3 and 5.

Spinochrome H possesses two carbons in addition to
the ten-carbon naphthoquinone nucleus. Conclusive evidence
that these two carbons are present as an acetyl group was
derived from a positive iodoform test and from strong mass
peaks at m/e 43 and at 221 (molecular ion minus acetyl) in
the mass spectrum (Fig. 11 and Table III). Integration of

the NMR spectrum of spinochrome H (Fig. 10) showed that the principal absorption centered at δ 2.55 was caused by five protons. This must be interpreted as an overlay of a three-proton peak of partially deuteriated solvent, $(CHD_2)_2SO$. The normal quintet of this solvent impurity is shown in Fig. 10. Additional proof of the acetyl group (δ 2.83, singlet) may be found in the NMR spectrum of dimethoxyspinochrome H (see Fig. 20).

The evidence cited up to this point showed that spinochrome H possesses a 2-hydroxy-x-acetyl-1,4-naphthoquinone structure. Only three oxygen atoms remain to be placed with respect to four vacant positions on the naphthoquinone nucleus. The NMR spectrum of spinochrome H (Fig. 10) showed that the single remaining carbon bearing no oxygen must be a ring proton, probably quinonoid in nature. This is supported by the sharp singlet at δ 6.55 for spinochrome H (Fig. 10) and also by an equally sharp signal at δ 6.75 for dimethoxy-spinochrome H (Fig. 20). Evidence for a quinonoid type proton is supported by the Craven test. Three oxygen atoms, present as hydroxy groups, remain to be assigned.

The IR spectrum of spinochrome H (Fig. 8) indicated that free hydroxy (at 3570 cm^{-1}) as well as strongly intramolecularly hydrogen-bonded hydroxy groups in a system of -OH···X (where X represents an electron-attracting heteroatom) are present. The hydrogen-bonded hydroxy groups exhibited absorption maxima at 3425 cm^{-1} along with a charac-

teristic broad band at 3200-2500 cm^{-1}. These observations supporting two types of hydroxy groups are in accord with the extensive studies on hydrogen bonding by Bellamy,[54] Nyquist,[55] Hilbert,[56] and others.

We have assigned two of the remaining three hydroxy groups to the peri positions of the 1,4-naphthoqui-

$$\left[\; C_{10}H_4O_5 \;\right] \quad \begin{array}{l} \text{-COCH}_3 \\[1em] \text{-OH} \\[1em] C_2H_4O_2 \end{array}$$
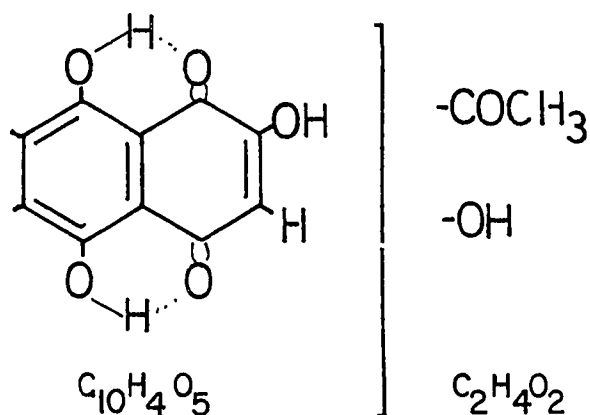
XX

none system, leading to part structure XX. That spinochrome H is a naphthazarin derivative is strongly suggested by previous studies on sea urchins, whose pigments have been reported to be almost exclusively naphthazarin derivatives. A naphthazarin-type structure was substantiated by UV studies in methanolic aluminum chloride (Fig. 26).

Compounds containing a hydroxy group ortho or peri to a carbonyl group show a large bathochromic shift ranging from 40-75 mμ when a trace of aluminum chloride is added to methanolic or ethanolic solution,[57,58] For example, the 330 mμ band of 1,4-naphthoquinone (compound A, Fig. 26) is
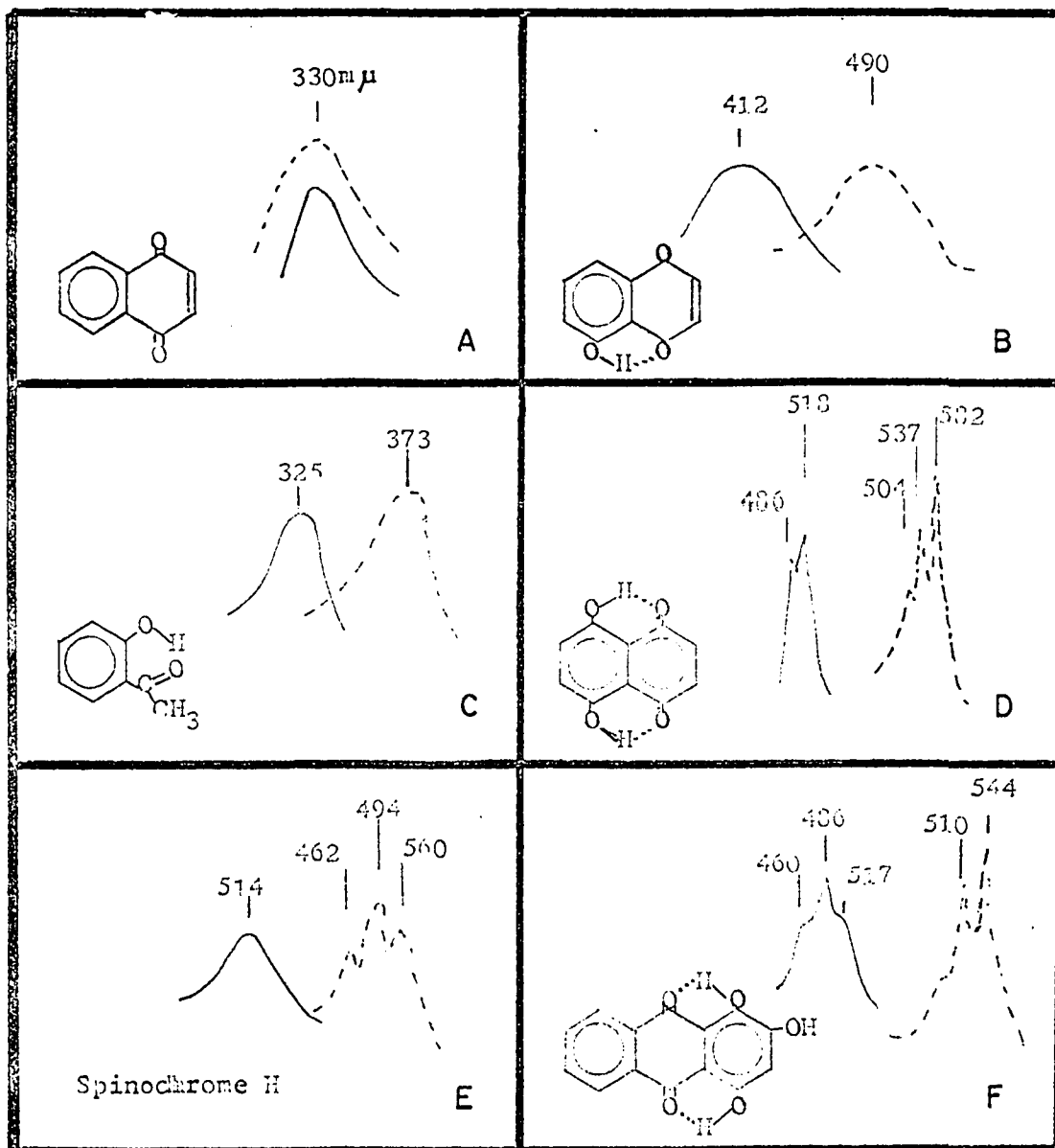
Fig. 26. Visible Spectra of Some Chelated Compounds in Methanol (—) and Methanolic Aluminum Chloride (---).

not influenced by this reagent, but the λ_{max} of juglone (compound B, Fig. 26) in methanolic aluminum chloride is shifted by 78 mu farther unto the visible region from its normal position at 412 mμ in methanol.

Another interesting feature of this technique may also be seen in Fig. 26. Only three of the compounds, naphthazarin (D), spinochrome H (E), and purpurin (F) exhibited a characteristic triplet in the visible spectrum, thereby placing spinochrome H clearly into this class of compounds.

The structural possiblities of spinochrome H are now reduced to two: spinochrome H is either 2,5,6,8-tetrahydroxy-7-acetyl-1,4-naphthoquinone, compound XXIA, or 2,5,7, 8-tetrahydroxy-6-acetyl-1,4-naphthoquinone, compound XXIIA.

XXIA   XXIIA
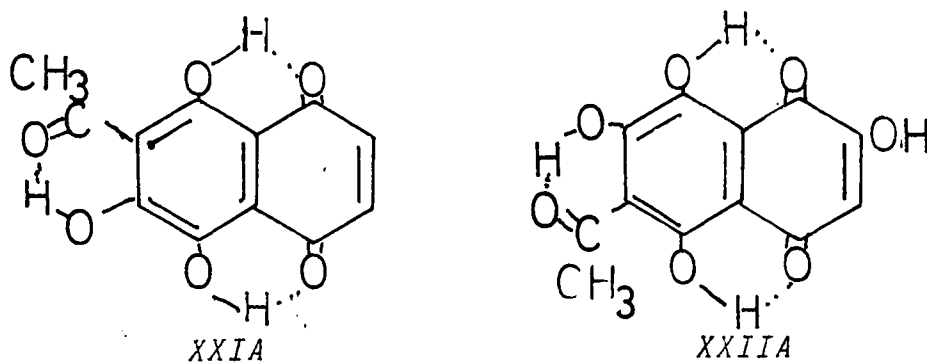
Additional support for either structure may be adduced by the following observations. IR spectral analysis of monomethoxyspinochrome H (Fig. 18) revealed an almost identical spectrum with that of the parent compound(Fig. 8). The IR spectrum of dimethoxyspinochrome H (Fig. 19), however,

showed a band at 5.95 μ (-COCH$_3$ conjugated with an aromatic ring), which was absent in the spectra of spinochrome H and its monomethyl derivative. This finding may be explained as follows.

The unbonded 2-hydroxy group of spinochrome H is not hindered and is sufficiently acidic to react first with either diazomethane or dimethyl sulfate. The color of the resulting monomethyl derivative in the crystalline state and on the DSG column resembled closely that of the parent compound. Once the second hydroxy group is methylated, presumably the one ortho to the acetyl group, the methyl ketone is no longer hydrogen-bonded and therefore has its absorption band in the normal position in the IR spectrum. The color of this compound, incidentally , also approaches the dark red color of naphthazarin.

A comment should be made on the unusual behavior of the dimethoxy derivative, which was transformed to monomethoxyspinochrome H during column chromatography. This facile demethylation is not without precedent. Cram[59,60] had found that the only successful method to acetylate 3-hydroxy-2-acetyl-1,4-naphthoquinone was achieved by silver oxide and acetyl chloride.. The resulting acetate, however, hydrolyzed very easily and in this respect resembled an anhydride rather than an ester. In a similar case, Spruit found that facile cleavage of a methyl ether to yield a hydroxyquinone was analogous to the saponification of an

ester.[61] *Spinochrome H behaved in the same way and must*

therefore be structurally related to these compounds. A

ready explanation may be that loss of acetate or methyl

ether adjacent to the carbonyl relieves steric crowding

through hydrolysis or demethylation to form a spatially

favored, hydrogen-bonded, 6-membered ring.

Spinochrome H, therefore, represents a strongly

hydrogen-bonded system. With three of the four hydroxy

groups bonded, the polar nature of the polyhydroxy molecule

is reduced. This is reflected in its relatively low melting

point in contrast with the high melting points of spinochrome

E (m.p. 350°)[37] and spinochrome B (m.p. 325-330°).[39]

The highly hydrogen bonded nature of spinochrome

H receives further and independent support from the mass

spectral data, in analogy with the extensive studies by

Beynon and coworkers[62,63] on hydroxyanthraquinones. It may

be seen, for example, from Table VIII that the position of

hydroxy groups in dihydroxyanthraquinones may be predicted

from their mass spectra. The intensity of $(M-OH)^+$ peaks,

characteristic of hydroxyanthraquinones is highly dependent

on the relative positions of the hydroxy groups with respect

to the quinone carbonyls in the molecule. The hydroxy

groups which are substituted in the 2,3,6, or 7 positions

around the ring are lost in fragmentation but those substi-

tuted in the 1,4,5, or 8 positions which are intramolecular-

ly hydrogen-bonded tend to lose oxygen atoms only.[63]

Table VIII. * *Relative Intensities of the* $(M-OH)^+$ *Peaks from Some Dihydroxyanthraquinones*

Compound	RI (%)
1,2-dihydroxyanthraquinone	0.49
1,4-dihydroxyanthraquinone	0.31
1,5-dihydroxyanthraquinone	1.46
1,7-dihydroxyanthraquinone	7.71
1,8-dihydroxyanthraquinone	4.46
2,6-dihydroxyanthraquinone	13.0

* *Compiled from Beynon and Williams (Ref. 62).*

In accordance with these findings, the $(M-OH)^+$ peak of spinochrome H is predicted to occur at m/e 247. This peak, as seen in Fig. 11 and Table III, is absent in the spectrum thereby suggesting again that structure IV can not represent spinochrome H.

The corollary requirement that carbon monoxide is lost with difficulty (three hydrogen-bonded carbonyls) is also borne out by the mass spectrum. The m/e 236 $(M-28)^+$ peak is 12% of the base peak and the fragmentation pattern of spinochrome H does not suggest that another CO-fragment was formed readily (m/e 208, 4%). This behavior is in sharp contrast

$M^+_.\ 158$ 100% $\xrightarrow{-CO}$ m/e 130 27.85% $\xrightarrow{-CO}$ m/e 102 31.17% $\longrightarrow$ m/e 76 34.49%
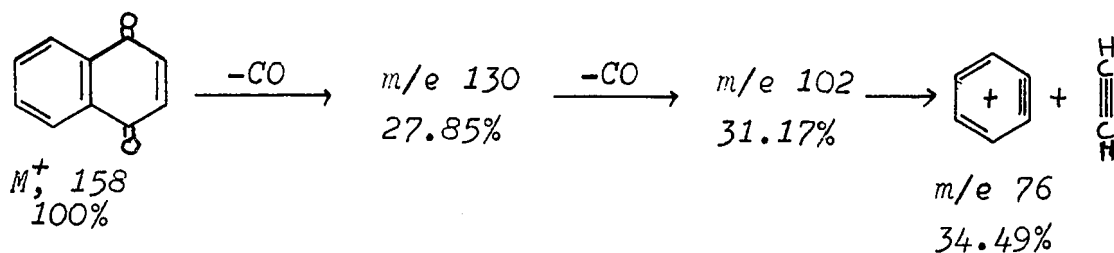
with the facile formation of carbon monoxide fragments from 1,4-naphthoquinone.[62]

A considerable amount of evidence has now been cited to support XXI and XXII (see p. 77) as the only two possible structures of spinochrome H. The question now arises, whether the structures represented by XXI and XXII adequately represent the chemical behavior of spinochrome H.

From the NMR spectrum of naphthazarin (Fig. 27) it appears that the benzenoid and quinonoid protons are indistinguishable and therefore equivalent. The spectrum measured in deuteriochloroform at room temperature showed only two singlets at δ 7.13 and δ 12.43 in a ratio of 2 to 1. The high field signal was assigned to the four ring protons and the low field signal to the two strongly bonded hydroxy protons. It would therefore seem appropriate to draw the structure of naphthazarin as a fully electron-delocalized molecule (XXIII). A structure of this type would agree with the observations of Bruce and Thomson[64] that the quinonoid protons of naphthazarin-like compounds are not as reactive toward condensing agents as the protons of 1,4-naphthoquinone
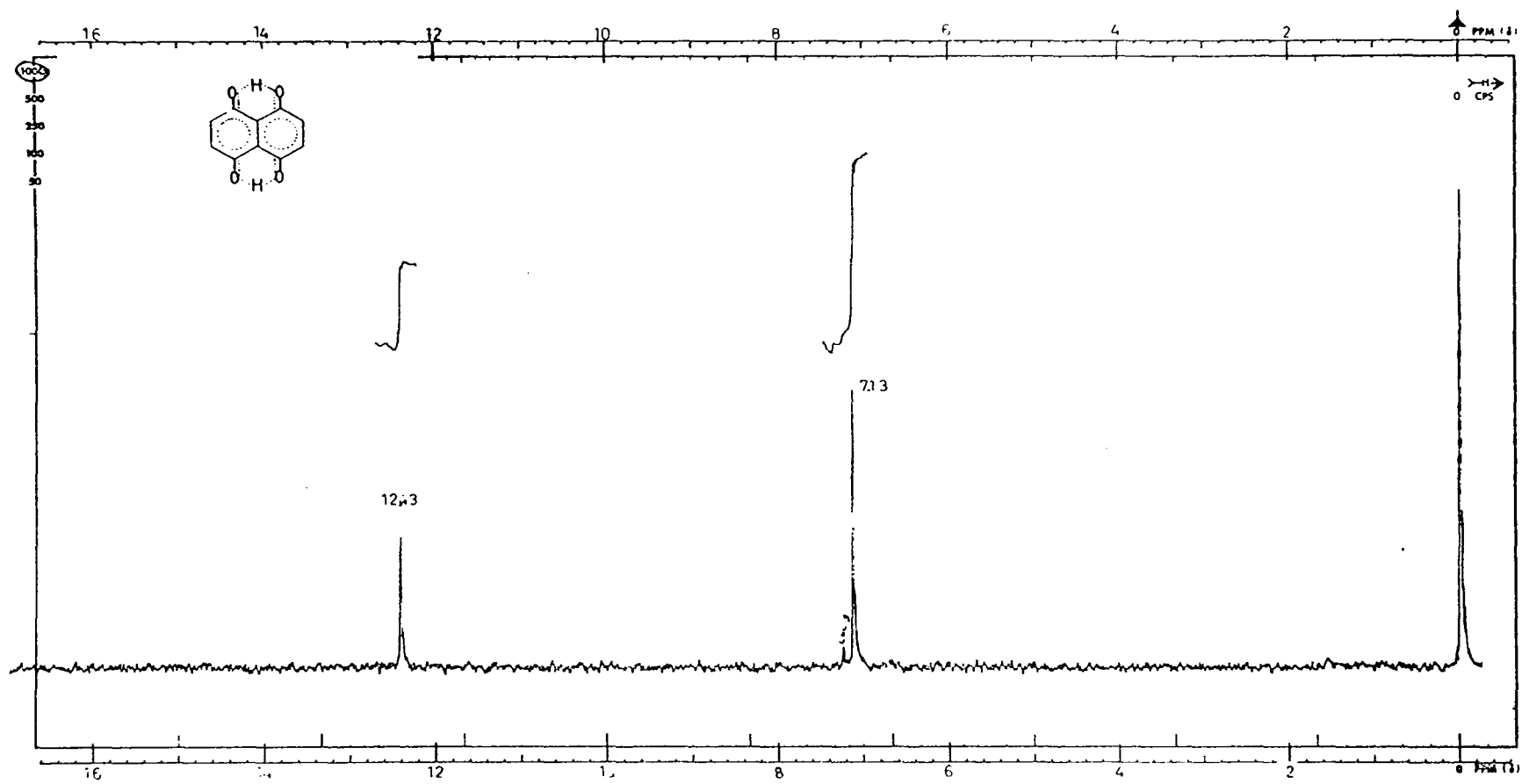

XXIII   XXIV
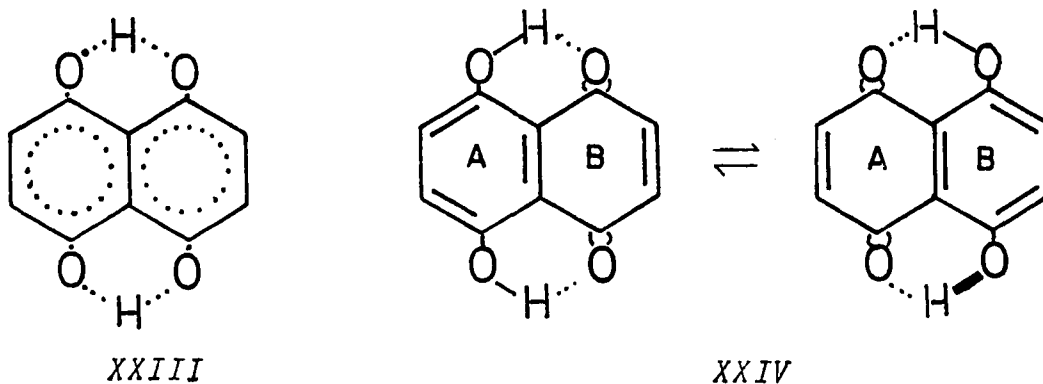
Fig 27. Nuclear Magnetic Resonance Spectrum of Naphthazarin in CDCl$_3$.

because of the lowered activity due to the tautomerism depicted in XXIV. However, the structure of naphthazarin is not fully represented by XXIII itself, since Blinc, Hadži, and Pirkmajer[65] reported on the basis of electronic and infrared spectra, and dipole moment studies that the hydrogen bonds are not symmetrical, i.e. the hydrogen atom is not equidistant from the two oxygen atoms as is suggested by XXIII.

We should like to suggest that rapidly equilibrating tautomers such as XXIV are a valid representation of spinochrome H, although one should bear in mind that XXIII also describes its chemistry. Rapid equilibration of the protons in the fused rings of naphthazarin allows no discrimination between the benzenoid and quinonoid hydrogens. However, since a positive and unambiguous Craven test was recorded for naphthazarin (see Table IV), it may be concluded that the species as represented by XXIV are present in solution.

Spinochrome H, which also exhibited a positive Craven test, though its color changes were not as strong as those recorded for naphthazarin, has only one quinonoid proton in one of its two tautomers as may be seen, for example, in XXIIA ⇌ XXIIB. In solution only XXIIA would react with ethyl cyanoacetate while XXIIB would not.

Related to this question is the work of Batterham and Weiss[66] who reported that elsinochrome A from the

XXIIA   *XXIIB*

XXVA   *XXVB*
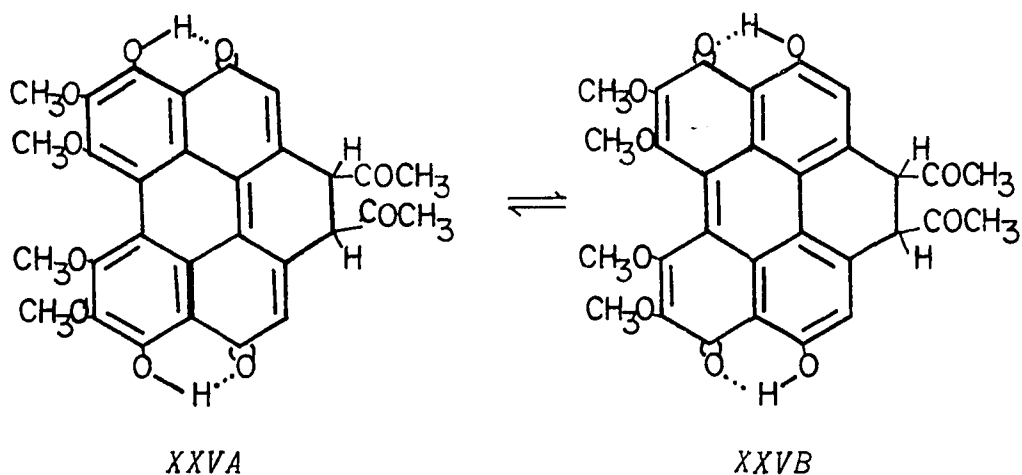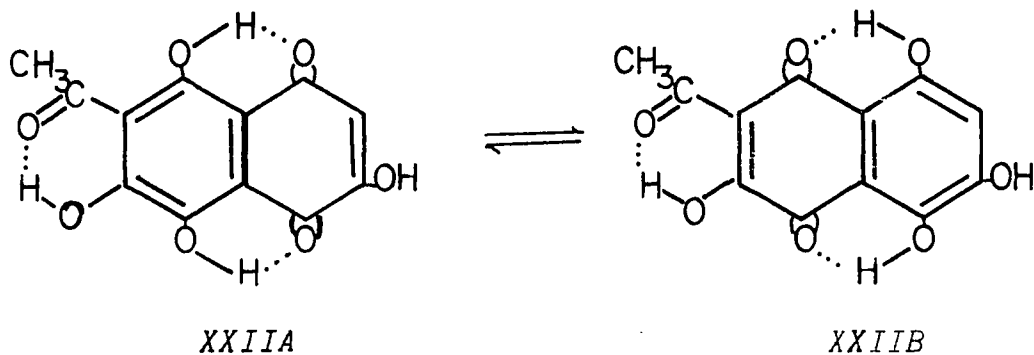
extracts of the fungus, <u>Elsinoe</u> <u>annonae</u>, has the structure
as shown in the tautomers *XXV A* and *B*. The structure was
supported by NMR studies of the natural pigment and its
hexamethyl-ether.

Preparation of derivatives of spinochrome H was
difficult because of the strongly bonded hydroxyls. The
peri hydroxy groups could not be methylated easily and are
therefore in analogy with Spruit's[61] failure to dimethylate
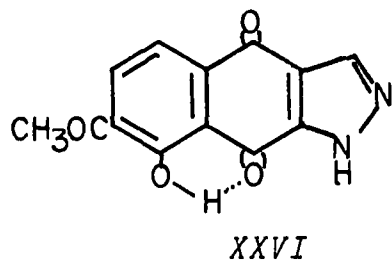
XXVI

the naphthindazolquinone structure XXVI. Only a N-methyl derivative was obtained, even on prolonged treatment with alkali and dimethyl sulfate.

The trace fractions which resulted from the dimethyl sulfate reaction of spinochrome H, MS-2.5 and MS-3, are believed to be the tri- and tetramethyl ethers respectively. Methylation of the peri hydroxy groups would prevent electron delocalization throughout the naphthoquinone system, and correspondingly, the ultraviolet absorption maxima would shift hypsochromically as each hydroxy group is methylated (see Fig. 21). Visible color and ultraviolet features would then approximate those of a substituted 1,4-naphthoquinone. Absence of any color change upon spraying with 10% sodium hydroxide solution on the chromatogram of MS-3 also indicated it to be a fully methylated compound.

It is unfortunate that these and other derivatives were not identified because of the limited amounts of starting material and the numerous products resulting from any given reaction.

A final choice between the two remaining structures by physical means could be made by X-ray crystallography. Without resorting to this method, it therefore appears that the solution of the structural problem of spinochrome H will have to be found in an unambiguous synthesis.

D.   Identity of Spinochrome H

Structure IV, proposed for spinochrome H by Amai

and also erroneously assigned to the pigment from Paracentrotus lividus by Musajo and Minchilli[23] on the basis of their combustion analysis, is in fact unknown in the chemical literature. Prior to the publication of Gough and Sutherland[39] we considered the possible identity of spinochrome H with spinochrome B. Because of the difficulty in observing the melting points of these deeply colored compounds and also because of incorrect assignments of structures based on combustion data without regard to possible solvent of crystallizations, one is compelled to place small reliance on melting points and empirical formulas and to examine other possible means of identification available in the chemical literature. As was mentioned in Chapter I, the only physical data other than melting points which were determined routinely by the different investigators was the measurement of electronic spectra. It logically follows, then, that a summary of all reported ultraviolet and visible spectra data should be compiled; this has now been done and is shown in Table IX. The summary is incomplete since some workers reported no data at all for their pigments, and others published no exact values but only small graphs from which it is almost impossible to extract meaningful quantitative data.

Examination of the Table clearly indicates the difference in the visible and ultraviolet absorption features between spinochrome B and spinochrome H. If spino-

Table IX. Summary of UV Data on Spinochromes

Spinochrome	Solvent	Wavelength (mμ)					Ref.
A	n-hexane	252,	316,	510,	543,	585	22
	$CHCl_3$			515,	530,	572	22
		255,	320,	520,	533,	580	19
	benzene			515,	540,	582	22
	ether	254,	318,	525			22
		255,	315,	510			38
	EtOH	271,	310,	518			22
	conc. H_2SO_4	262,	316,	495,	530,	565	22
B	$CHCl_3$			397,	474		22
	ether	272,	320,	388,	480		22
	EtOH	272,	320,	388,	480		22
	conc. H_2SO_4	276,	360,	445			22
C	$CHCl_3$	290,	330,	455,	510,	542	19
	benzene			460,	515,	551	25
	ether			460,	508,	545	25[*]
	conc. H_2SO_4			462,	513,	553	25[*]
D							
E	MeOH	267,	360,	475			19
F							
G	$CHCl_3$	305,	390,	490			19
M	MeOH	271,	312,	510			35[**]
N	MeOH	325,	385,	476,	516,	537	35[**]
P	$CHCl_3$	255,	300,	450,	510		19

[*] Values reported for spinone A (X) which may be similar to spinochrome C.

[**] Approximate values extracted from small graph. The values for spinochrome N are from Gough and Sutherland (Ref. 39).
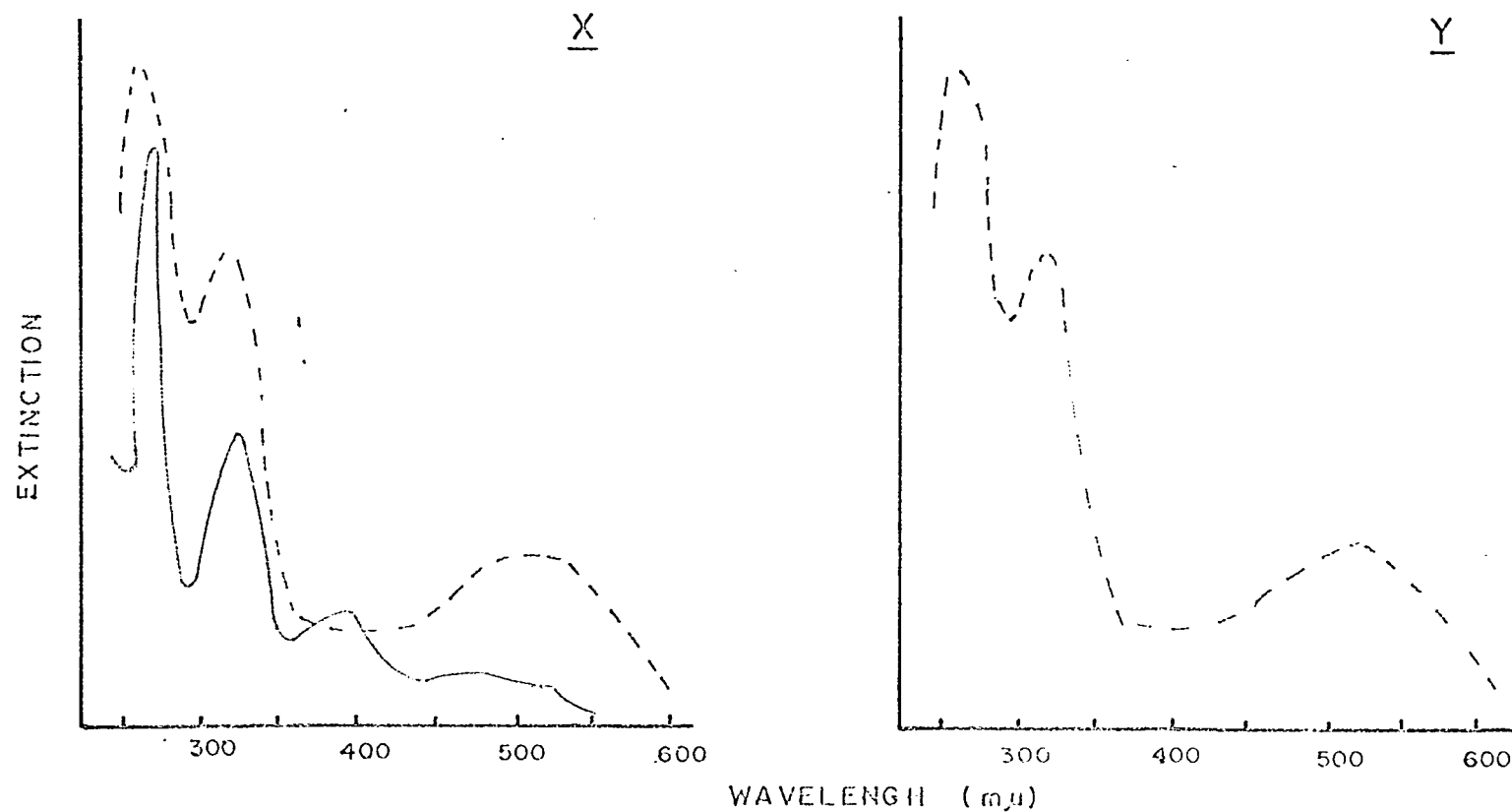
Fig. 28.   Visible and Ultraviolet Spectra of Spinochrome A (---) and Spinochrome B (——) in Ether according to Yoshida (Ref. 38), X. Visible and Ultraviolet Spectrum of Spinochrome H in Ether, Y.

chrome H were to be identical with any of the previously reported pigments, it is spinochrome A which should be seriously considered. The general appearance of the spectra of spinochrome A, spinochrome B, and spinochrome H in ether is depicted graphically in Figs. 28 X and Y.

In addition, the similarities of the ultraviolet spectra of spinochrome A and H in organic solvents of varying polarities are striking. The visible and UV absorption maxima of spinochrome H, which are summarized in Table X, compare very well within experimental error with those reported by Goodwin and Srisukh[22] and again shown in Table X.

Table X. Summary of UV-Visible Maxima ($m\mu$) of Spinochrome H and A

n-hexane	H	249.5	261	315	508	543	585
	A	252		316	510	543	586
benzene	H			316	508	541	588
	A				515	540	582
ether	H	251		318	528		
	A	254		318	525		
ethanol	H		268	311	519		
	A		271	310	518		
conc. H_2SO_4	H		271	320sh	496	530	572
	A		262	316	495	530	565

The comparison would suggest that spinochrome H is identical with spinochrome A. The reported melting point of 185° [10,22] and reported behavior on a calcium carbonate

$column^{22}$ support this supposition. On the other hand, it
is conceivable that the two pigments are closely related and
that one is represented by structure XXI and the other by
XXII. However, it is difficult to reconcile the reported
molecular formula $C_{12}H_{10}O_8$ of spinochrome A which Goodwin
and Srisukh supported by combustion analyses.[22] Although
Lederer and Glaser[10] reported five active hydrogens for
spinochrome A, our own experience with the active hydrogen
determinations carried out in two independent analytical
laboratories showed that no satisfactory result may be ex-
pected. None of the three products obtained by Glaser and
Lederer[11] from the diazomethane reaction and believed to be
the mono-, di-, and trimethyl derivatives of spinochrome H
had melting points of either our mono- or dimethoxyspino-
chrome H. It was established in our Laboratory that more
than three products may be obtained from the diazomethane
reaction on spinochrome H and that the major product was
monomethoxyspinochrome H.

Though the ultraviolet data strongly suggest that
spinochrome H is identical with spinochrome A, the designa-
tion spinochrome H should be retained until an authentic
sample of spinochrome A can be compared with our isolated
compound.

E.   The Minor Pigment

Only one other spinochrome was isolated as a minor
component from the acidic extracts of Echinometra oblonga(Bl.)

and *Colobocentrotus* (*Podophora*) *atratus* (*Linn.*). Spectral data and melting point suggest that this pigment has not been reported previously.

This minor pigment may be a juglone derivative. This relationship is suggested by its sensitivity towards even mild base (see p. 47). This behavior in sodium bicarbonate solution is also reported for juglone and its related compounds.[64] The minor pigment also exhibited two kinds of hydroxy groups as are indicated by its IR spectrum (see Fig. 9).

We have refrained from naming this compound until it can be fully characterized.

Chapter IV

POSSIBLE BIOGENESIS

A biogenetic scheme of many natural products, *e.g.*
of terpene constituents of plants, may be suggested to de-
rive from head to tail linkage of isoprene units. This hy-
pothesis has been proven in some cases by isotope labelling.
In 1953 Birch and Donovan[67] in their extensive studies of
plant natural products reinterpreted the "acetate rule" by
which the origin of some natural products may be explained.
A hypothesis of head to tail linkages of activated acetic
acid units, with the positions of oxygenated substituents
marking the position of the units, was suggested as one
possible pathway for some phenolic natural products. To
support this hypothesis Birch and Donovan in 1955 compiled
a frequency diagram of the then known anthraquinone com-
pounds isolated mainly from moulds and plants.[68]

The biosynthetic pathways leading to these aromatic
phenolic compounds are now firmly established. One is based
on shikimic acid which is recognized in the use of mutants of
microorganisms, and the other is based on the acetate hypothe-
sis mentioned above.[69] The natural pigments of naphthoquinone
nature, however, are too few in number to permit a thorough
examination of the frequency distribution of their substi-
tuents.[68]

Structural considerations, nevertheless, may be
formulated in lieu of the acetate hypothesis as is shown for

flaviolin (XXVIII). Its structure was in question as to the hydroxy group in the quinonoid ring.[70] The structure


XXVII   XXVIII
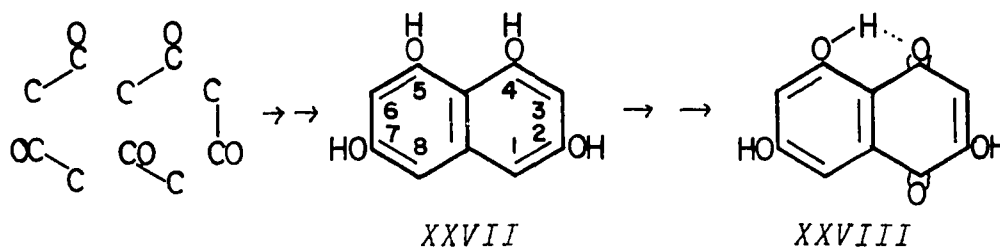
was recently proven by synthesis,[71] whereas biogenetically it may be predicted from the above scheme. The remaining quinone oxygen is commonly introduced by oxygen in the biogenetic pathways of aromatic natural products. Since three of the oxygens (4,5, & 7) are in positions consistent with the formation from acetate units, and the fourth oxygen conceivably introduced by oxidation (on the 1 position of XXVII), the fifth oxygen atom should be in the 2-position if the biogenetic scheme is correct.

The biosynthetic pathway for spinochrome H may
a priori be postulated by a combination of the acetate rule and in vivo oxidation of the parent unit (XXIX) to form the

XXIX   XXX
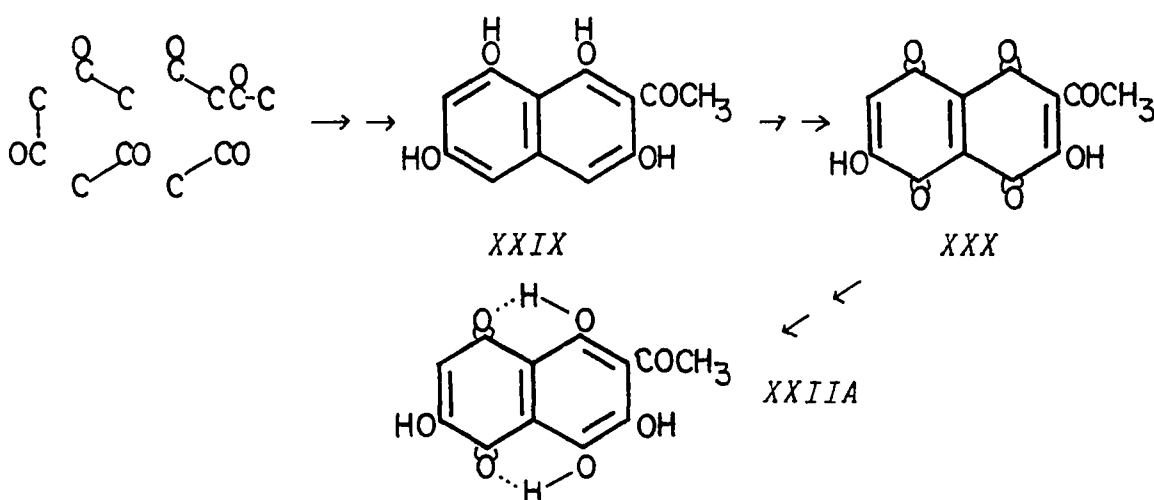
XXIIA

final product. This requires a head to tail pattern as illustrated on the preceding page, followed by the introduction of two perihydroxy groups which may oxidatively be converted to the stable hydrogen bonded form XXIIA. It therefore follows that a 2- or 7-acetyl-naphthazarin structure is favored biogenetically. Its possible biogenesis pattern is analogous to that of kermisic acid, suggested by Birch and Donovan[68] to possess structure XXXII. This possible biosynthetic pattern of spinochrome H remains to be proven as does the general biosynthesis of sea urchin pigments from acetate units. Further detailed investigations are required to explain, for example, in vivo introduction of oxygenated groups as in spinochrome E, whose ten nucleoid carbons are fully substituted by oxygen.

XXXII
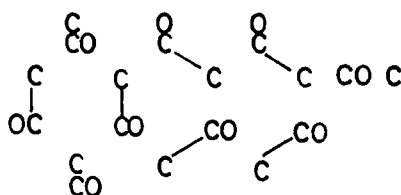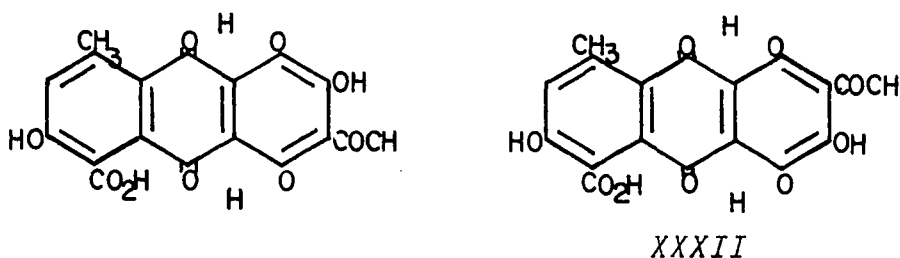
PART II

SYNTHETIC APPROACHES

Chapter I

INTRODUCTION

A.   Previous Synthetic Routes

In addition to the synthesis of echinochrome A by Wallenfels and Gauhe[16] in 1943, the only other synthesis of a chemically related compound, that of 2,3,5,7-tetra-hydroxy-1,4-naphthoquinone (XXXVII) was reported by Smith and Thomson[37] in 1961. Starting with 5,7-dimethoxy-1,4-naphthoquinone, the dihydrodiacetate (XXXIV) was formed, which on air oxidation in basic solution yielded the quinone. XXXV.

XXXIII   XXXIV   XXXV

XXXVII   XXXVI

Treatment of this compound in an aluminum chloride-sodium chloride melt resulted in the desired product, XXXVII. The overall yield was poor because of the competing reaction of XXXIV in base to form 2-hydroxy-5,7-dimethoxy-1,4-naphthoquinone. Depending on the reaction time and concentration of base, varied amounts of this by-product were obtained.[37]

Following the procedure by Zahn and Ochwat,[72]
Kuroda[73,74] synthesized spinochrome-like compounds by the
direct Friedel-Crafts method utilizing substituted benzene
and maleic anhydride compounds in aluminum chloride-sodium
chloride melts.

Recently Farina and coworkers[75] reported the
application of a diene synthesis to the preparation of
naphthazarins and indicated that this method may be useful
in the synthesis of spinochrome D (see XI in Fig. I). For
example, the synthesis of 3-hydroxy-2-methyl-naphthazarin
(XXXVIII) was achieved by the following pathway.

XXXVIII
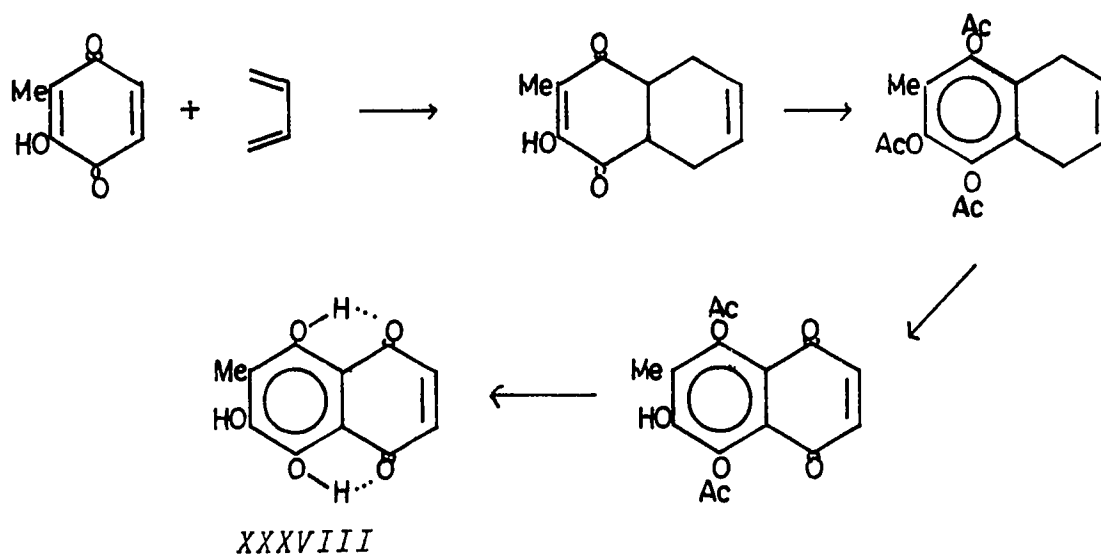
The general chemistry of naphthazarin was reviewed
by Bruce and Thomson[64] in 1955. Several methyl and chloro
derivatives were prepared from 1,5-dinitronaphthalenes by
the reductive-oxidative method of Roussin[76] in a medium of
powdered sulfur and fuming sulfuric acid. From 2-methyl-
1,5-dinitronaphthalene, for example, 2-methyl-5,8-dihydroxy-

1,4-naphthoquinone (XXXIX) was obtained which on acetylation produced the diacetate (XL).[64] The diacetate (XLI) which represents the derivative of the complementary tautomer of XL could not be isolated.[64] Structural proof of compound XL was established by ozonolysis and by its dichloro derivative, which readily lost hydrogen chloride to form the 3-chloro derivative of XL.

XXXIX   XL   XLI
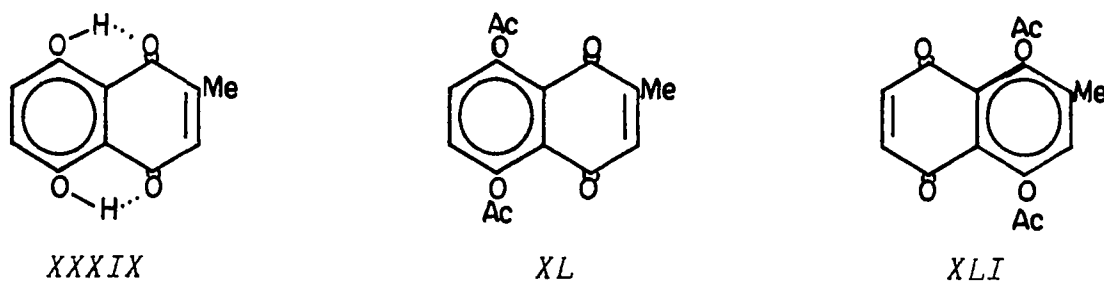
The above evidence would suggest that the species XXXIX predominates in the acetylation reaction as its tautomer would lead to XLI. However, Fieser and Dunn[77] obtained exclusively 1,2,4-triacetoxy-1,4-naphthoquinone (XLIII) when naphthopurpurin, whose structure is generally considered

A

B

XLII   XLIII   XLIV
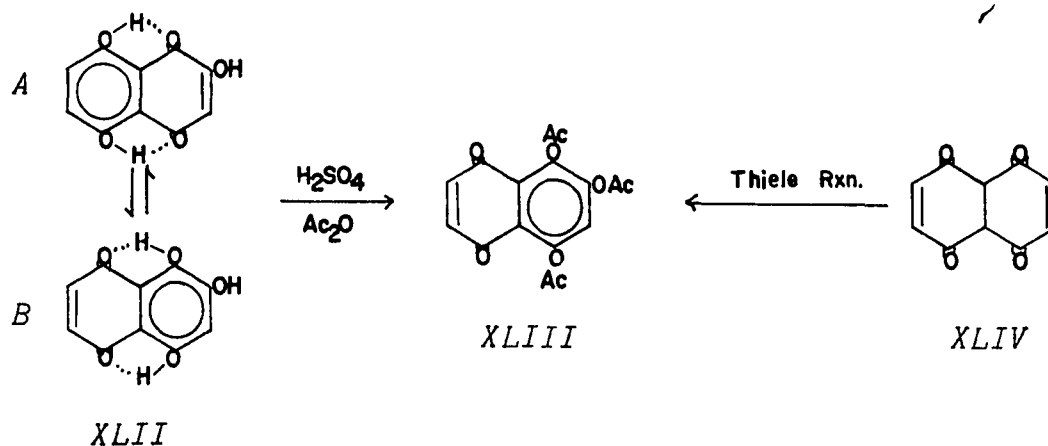
to be XLIIA (see, for example, Fieser[78]), was reacted with

acetic anhydride in the presence of a sulfuric acid catalyst. That the triacetate possessed the structure XLIII was proven by its synthesis from the 1,4,5,8-diquinone, XLIV. It is therefore conceivable that the tautomerism as exhibited by XLIIA ⇌ XLIIB does, in fact, occur in other naphthazarin analogs as was illustrated in the last example, whereby the triacetate XLIII is implied to have arisen from XLIIB. The hydrogen-bonded systems of these naphthazarin compounds permit electron delocalization throughout the entire molecule. A simple representation of this phenomenon is shown in XXIV (see p. 81) for the case of naphthazarin and XLIIA ⇌ XLIIB for naphthopurpurin. This property is ocassionally represented by the fully delocalized structure XXIII (see p. 81). Whether a reaction involving this type of compound would yield preferentially a product of one tautomer would depend on the type of reaction and the conditions by which the reaction would be carried out.

The reactivity of naphthazarin in addition reactions with chlorine, aniline and other reagents is slower compared with its dimethyl ether or diacetate derivatives or with 1,4-naphthoquinone. This behavior of the relatively unreactive quinone is commonly attributed to the tautomeric nature of naphthazarin compounds.[64]

B.   Possible Approaches

In the previous section it was shown that for a given naphthazarin compound no one tautomer would best

describe its overall chemical properties. The substituents on the nucleus and the reaction conditions with regard to polarity of solvents, temperature, etc., may favor the predominance of one tautomer over the other. Examination of the chemical literature reveals few chemical studies regarding this matter. With this in mind one must recognize, therefore, that reaction sequences designed to lead to spinochrome H do not necessarily yield the desired compound as represented by one tautomer as drawn for any one proposed pathway. The following exploratory proposals are based on analogous reactions in the chemical literature.

The example by Wallenfels and Gauhe[16] in the synthesis of echinochrome A may be modified to utilize 2-acetyl-1,3,5-trimethoxynaphthalene (XLV) and benzoyl- or methoxymaleic anhydride (XLVI) in an aluminum chloride melt.

XLV

XLVI

XLVII

XLVIII
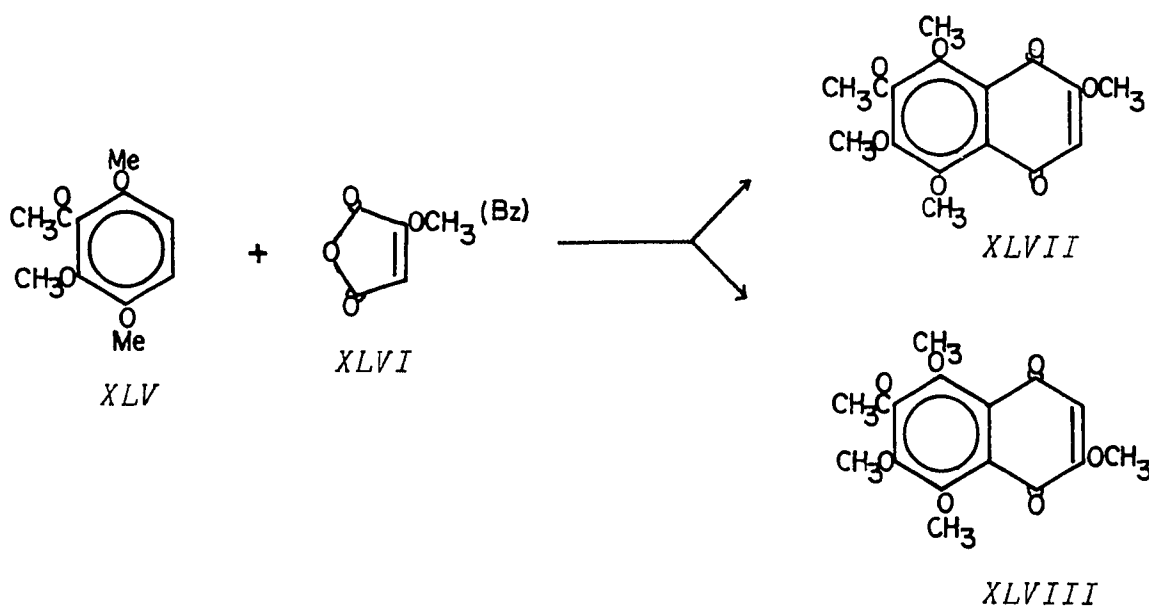
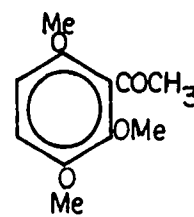*Compound XLV may possibly be obtained by a Thiele reaction on*

2-acetyl-1,4-naphthoquinone with boron trifluoride catalyst
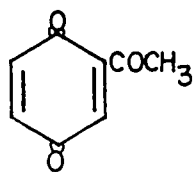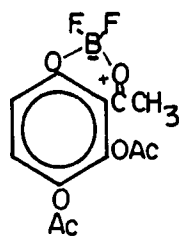
| XLIX | L | XLV |

*to give the boron complex, L, which may be hydrolyzed in
ethanol to form the free phenol. Reaction with dimethyl
sulfate will afford the trimethylether, XLV. Preparation
of this compound is analogous to Cram's effort in preparing
3-hydroxy-2-acetyl-1,4-naphthoquinone from 2-acetyl-1,4-
naphthoquinone.*[60]

*The reaction mixture after boiling in hydrochloric
acid to hydrolyze the aluminum chloride complexes, will un-
doubtedly consist of a mixture of compounds, XLVII, XLVIII,
and their partially demethylated ethers. Separation of
these fractions, however, may be achieved by column chroma-
tography with deactivated acid-washed silica gel.*

*Reasoning by analogy with the method of Farina and
coworkers*[75] *one may predict the following reaction steps
leading to spinochrome H (Fig. 29). Silver oxide oxidation
of the hydrolyzed product of L will presumably afford the
3-hydroxy-2-acetyl-1,4-benzoquinone; though it is possible
that the o-quinone (see page 104) may also form. One
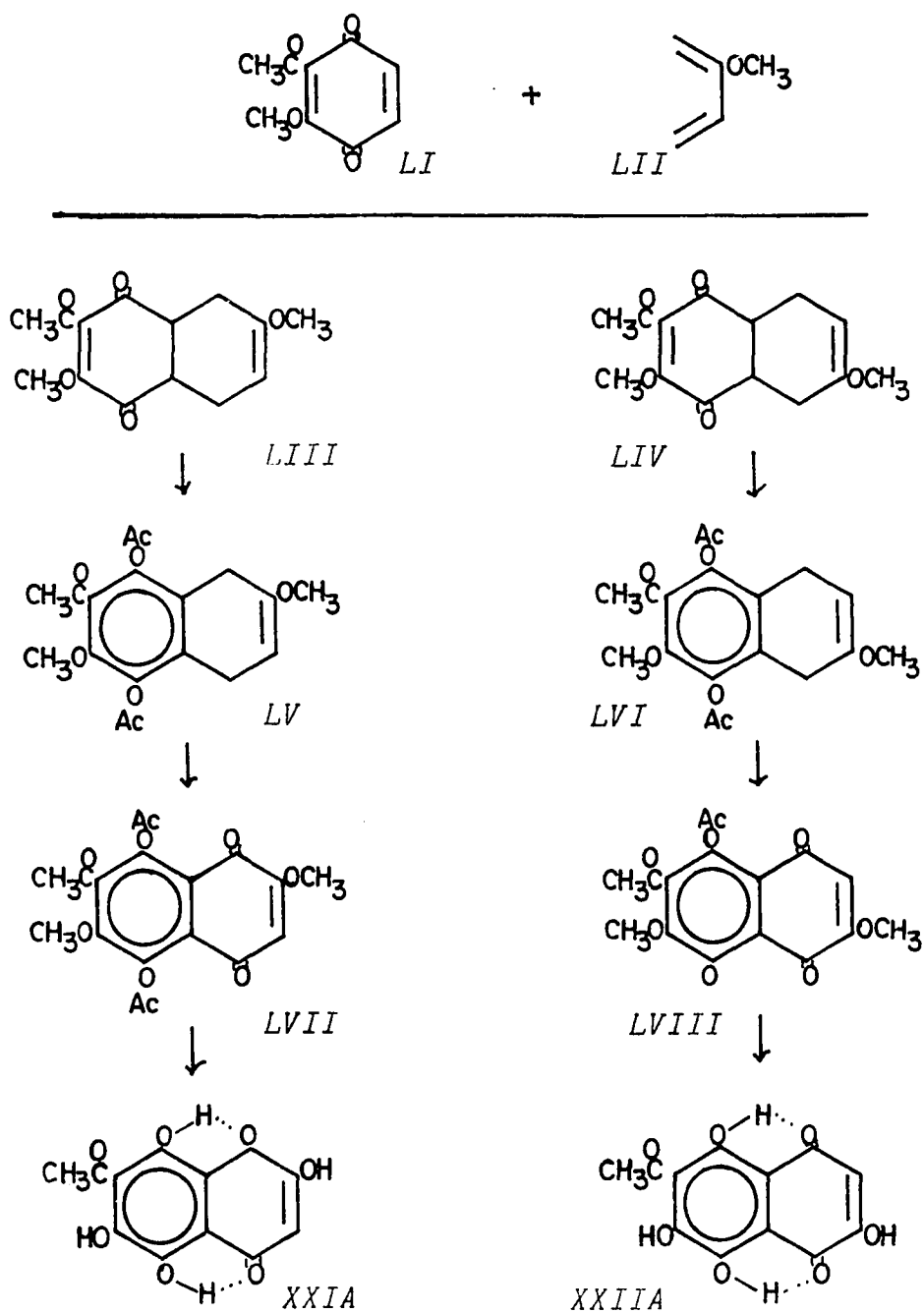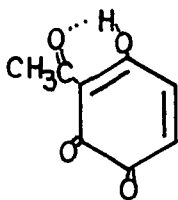should bear in mind that the intermediates in this proposed*

Fig. 29. Proposed Synthetic Scheme for Spinochrome
*H according* to the method of Farina et.
al. (Ref. 75).

scheme possess certain structural features which require
precautions in treatment of the compounds. It has already
been shown (see Part I) that a methyl or acetate group ortho
to the acetyl suffers facile cleavage to form the phenolic
hydroxy which is then free to bond with the acetyl group.
The quinone-diene adducts also have a vinyl ether structure
which is susceptible to hydrolysis under mild conditions.

In 1963 Shibata[79,80] synthesized rubrofusarin
dimethyl ether, LIX, a derivative of the natural product
from *Fusarium* *culmorum* (W.G. Smith) Sacc. Examination of
one of the reaction intermediates, LX, reveals another
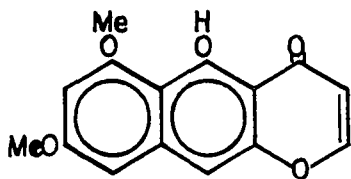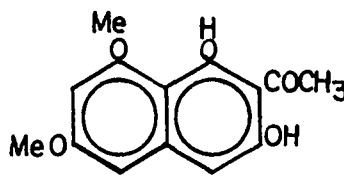
LIX   LX

possible method leading to spinochrome H or an isomer.
Nitration of LX will most likely introduce the first nitro
group in the peri position which is ortho and para to the
two methoxy groups thereby enhancing the electrophilic
character of that position. A second nitro group may then
be introduced into the remaining peri position; such a
reaction is rendered possible by the two ortho and para
electron releasing hydroxy groups. Reduction of the

demethylated dinitro compound, LXI, and oxidation of the reduced compound or its N,N'-diacetylamino compound with nitric acid may afford spinochrome H or its isomer.
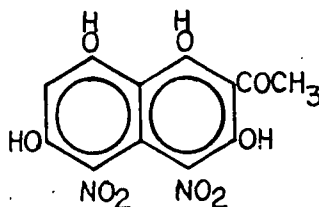
LXI

None of the proposed synthetic pathways have been investigated since they are based on publications during the past few months. However, it seemed possible that a synthesis of spinochrome H via the nitro intermediates by the method of Roussin[76] might be feasible. A plausible reaction sequence is outlined in Fig. 30. Before embarking on this synthesis it was necessary to carry out a preliminary study of the nitration products of several dimethoxy- and dimethoxy-(or dihydroxy-)-acetyl compounds.
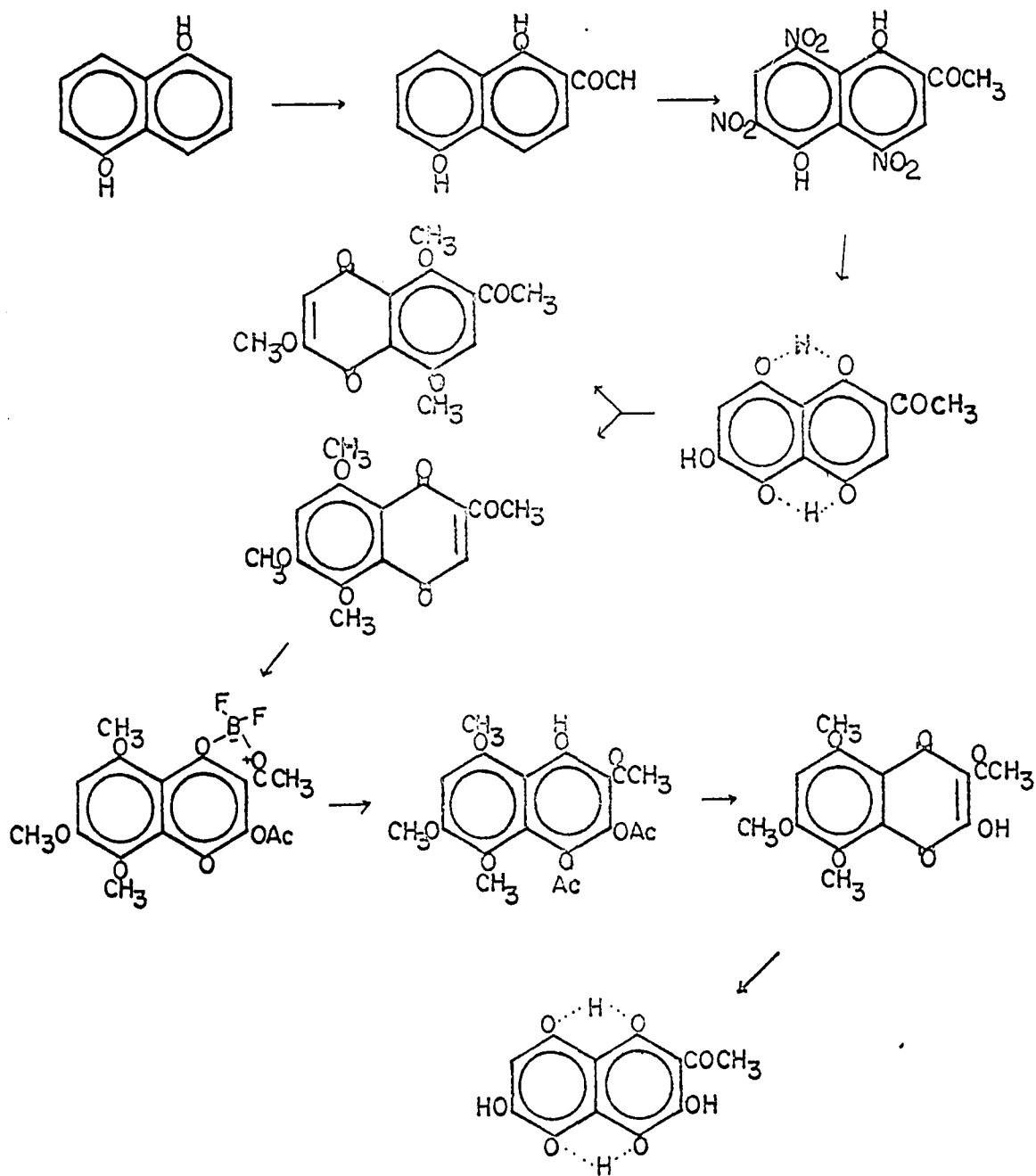
Fig. 30.   Proposed Synthetic Pathway to Spinochrome
H from 1,5-dihydroxynaphthalene.

EXPERIMENTAL

In addition to the general experimental procedures mentioned in Part I (see pp. 20-21) the following conventions are used in describing nuclear magnetic resonance data.

Resonance signals in tabulations of NMR spectra are abbreviated by the symbols s (singlet), d (doublet), q (quartet), and m (multiplet). A question mark (?) indicates a probable but not definite assignment.

In dimethyl sulfoxide-d_6 solvent the resonance signals of some samples were reported as chemical shifts (δ) using the DMSO-d_5 quintet as the internal standard when TMS was not introduced into the sample. The DMSO-d_5 quintet has its signal centered at δ 2.62 with reference to TMS having δ = 0.

A. Synthesis of 2-Acetyl-3-hydroxy-1,4-naphthoquinone (LXII)

Synthesis of the titled compound was according to the method of Cram.[60]

1. 2-Acetyl-1-naphthol: The compound was prepared by a modified procedure of Witt and Braun.[81] A stirred mixture of 60 g 1-naphthol (0.42 mole), 45 ml acetic anhydride, and 30 g of freshly fused zinc chloride was heated under reflux for one hour. Long, green needles (crystallized from methanol, 52g, 72%) of 2-acetyl-2-naphthol, m.p. 99-100° (reported,[60] 102°), were obtained after spearation of the crude gum from the decomposed (ice water) reaction mixture and

after purification with Norite in boiling chloroform.   NMR

spectrum in carbon tetrachloride: C_2-COCH$_3$, δ 2.84 (s);

C_1-OH, 13.95 (s); C_8-H, 8.31 (m); C_3-H, 7.31 (d): C_4-H,

7.02 (d, J_{34} = 8.5); C_5-H, C_6-H, C_7-H, 7.4-7.7 (complex m).

2. *2-Acetyl-4-nitro-1-naphthol:* *Nitration of 2-acetyl-*

1-naphthol (10 g, 0.54 mole) in acetic acid with 3.4 ml

nitric acid (d. 1.42) afforded 6.2 g (60%) of 2-acetyl-4-

nitro-1-naphthol, m.p. 160-161° (from ethanol) reported,[60]

157-158° . Approximately 2 g of starting material was re-

covered by sublimation of the gummy residue from the crys-

tallization mother liquor at 60-70°/0.5mm.   NMR spectrum in

deuteriochloroform: C_2-COCH$_3$, δ 2.72 (s); C_1-OH, 14.55 (s);

C_3-H, 8.66 (s).   In DMSO-d_6 as the solvent the C_3-proton

appeared at δ 8.86 (s) with the DMSO-d_5 quintet as the

internal standard.

When excess nitric acid (ca. 3 equiv.) was added

to 2-acetyl-1-naphthol (1 equiv.), a mixture of products was

obtained.   After separation by acid-washed silica gel column

chromatography with carbon tetrachloride as the eluant, 2-

acetyl-4-nitro-1-naphthol and 2,4-dinitro-1-naphthol in a

ratio of ca. 3:5 were obtained.   The latter melted at 139-

140° (reported,[82] 138°) and showed no resonance signal in

the δ 2 to 3 region in the NMR spectrum.   NMR spectrum in

deuteriochloroform: C_1-OH, δ 12.5 (s); C_3-H, 8.94 (s).   In

DMSO-d_6 as the solvent the C_3-proton appeared at δ 9.06 (s)

with the DMSO-d_5 quintet as the internal standard.

Anal. Calcd. for $C_{10}H_6N_2O_5$: C, 51.28; H, 2.56;
N, 11.95. Found: C, 51.67, 51.79; H, 2.84, 2.87; N, 11.70,
11.84.

3. 2-*Acetyl-4-N-acetylamino-1-naphthol:* Reduction
of 2-acetyl-4-nitro-1-naphthol (10 g, 0.043 mole) by cata-
lytic hydrogenation with platinum oxide catalyst was carried
out at 25 lb pressure until no further uptake of hydrogen
was noted (*ca.* 3.25 hr). 2-Acetyl-4-amino-1-naphthol was
characterized as the free amine (1.5 g of yellow orange
crystals from ethanol), m.p. 125° (reported,[60] 126-127°).
NMR spectrum of 2-acetyl-4-amino-1-naphthol in deuterio-
chloroform: C_1-OH, δ 13.50 (s); C_2-COCH$_3$, 2.55 (s); C_3-H,
6.75 (s); C_4-NH$_2$, 3.71 (broad s); C_5-H, C_6-H, C_7-H, 7.1-
7.7 (complex m); C_8-H, 8.42 (m).

To the crude 2-acetyl-4-amino-1-naphthol obtained
after neutralization of the amine hydrochloride was added
acetic anhydride. The light greenish-yellow N-acetate
immediately precipitated. It was washed with water and
dried (3 g) which after two recrystallizations from ethanol
had a m.p. of 216-217° (reported,[83] 212°).

4. 2-*Acetyl-3,4-diacetoxy-1-naphthol:* Oxidation of
2-acetyl-4-N-acetylamino-1-naphthol (3.7 g, 0.015 mole) in
glacial acetic acid with nitric acid (1.5 g, d. 1.42) yielded
1.2 g (40%) of 2-acetyl-1,4-naphthoquinone, m.p. 79-80°
(reported,[60] 80-81°). NMR spectrum of 2-acetyl-1,4-naphtho-
quinone: C_2-COCH$_3$, δ 2.58 (s); C_3-H, 7.09 (s); C_5-H, C_6-H,

C_7-H, C_8-H, 7.6 - 8.2 (complex m); in carbon tetrachloride.

A Thiele reaction mixture of the quinone (1 g, 5 mmole) and acetic anhydride (3 g) catalyzed by boron trifluo-ride in ether (45%, 0.5 g) resulted in 1.4 g (77%) yellowish green needles of 2-acetyl-3,4-diacetoxy-1-naphthol-boron complex. The crude needles, m.p. 223-224° with decomposi-tion (reported,[60] 234-236° with decomposition).

The boron complex (1.24 g) was hydrolyzed in boiling ethanol to yield 0.98 g of 2-acetyl-3,4-diacetoxy-1-naphthol, m.p. 182-183° (reported,[60] 184-185°). NMR spectrum of 2-acetyl-3,4-diacetoxy-1-naphthol in deuterio-chloroform: C_1-OH, δ 14.65 (s); C_2-COCH$_3$, 2.65 (s); C_3- or C_4-OAc, 2.42 (s); C_3- or C_4-OAc, 2.65 (s).

5.   <u>2-Acetyl-3-hydroxy-1,4-naphthoquinone</u>: Saponifi-cation of the diacetate (500 mg) and oxidation with ferric chloride solution resulted in a brownish amorphous solid which in recrystallization attempts using glacial acetic acid failed to crystallize. Removal of the acid <u>in vacuo</u> and sublimation of the residue afforded <u>ca.</u> 150 mg of yellowish crystals, m.p. 134-135.5° (reported,[60] 134-135°). NMR spectrum of 2-acetyl-3-hydroxy-1,4-naphthoquinone in deuteriochloroform: C_2-COCH$_3$, δ 2.80 (s); C_5-H, C_6-H, C_7-H, C_8-H, <u>ca.</u> 8.05 (complex m).

<u>Anal</u>. Calcd. for $C_{12}H_8O_4$: C, 60.66; H, 3.70. Found: 66.46, 66.61; H, 3.82, 3.91.

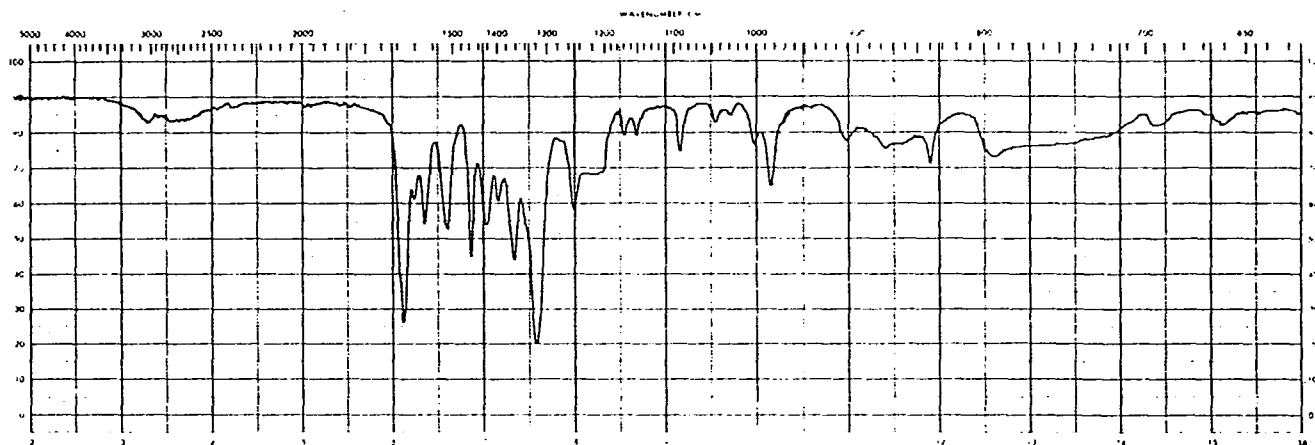The IR spectra of the reaction intermediates are

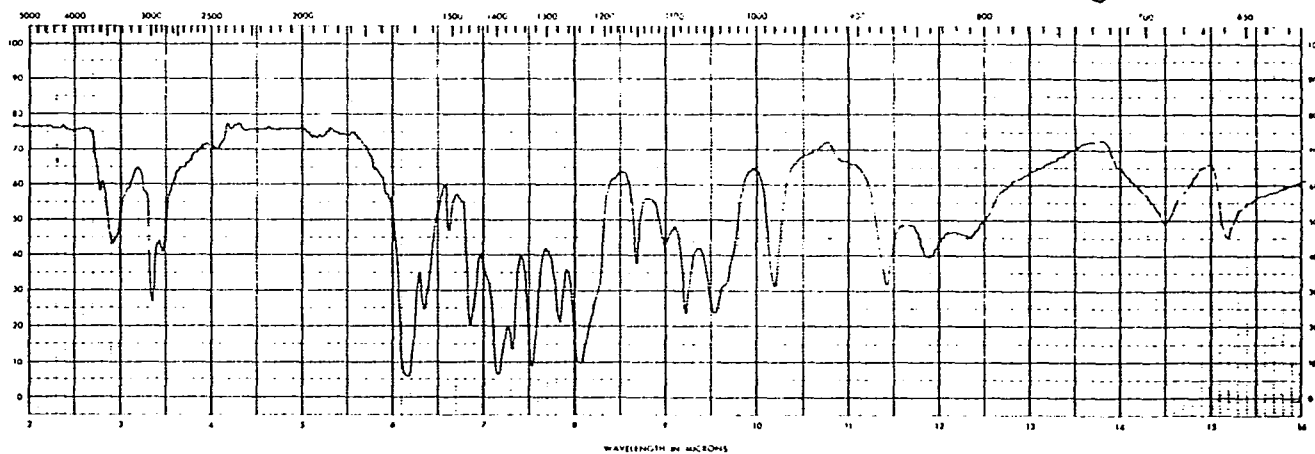Fig. 31   Infrared   Spectrum   of   2-Acetyl-4-nitro-I-naphthol   in   CHCl₃.

Fig. 32.   Infrared   Spectrum   of   2-Acetyl-4-amino-I-naphthol   in   CHCl₃.
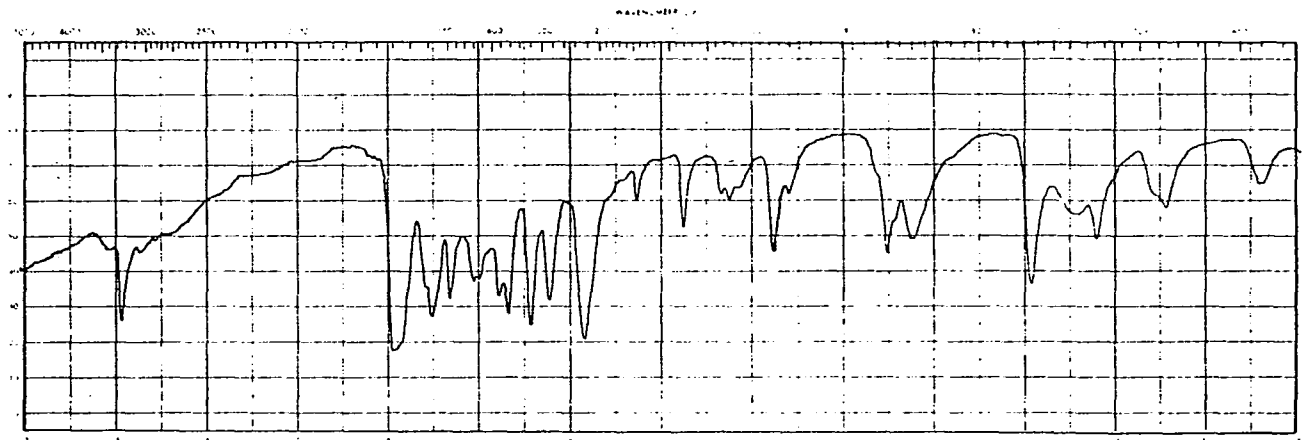
Fig. 33.   Infrared   Spectrum   of   2-Acetyl-4-N-acetylamino-1-naphthol   in   KBr.
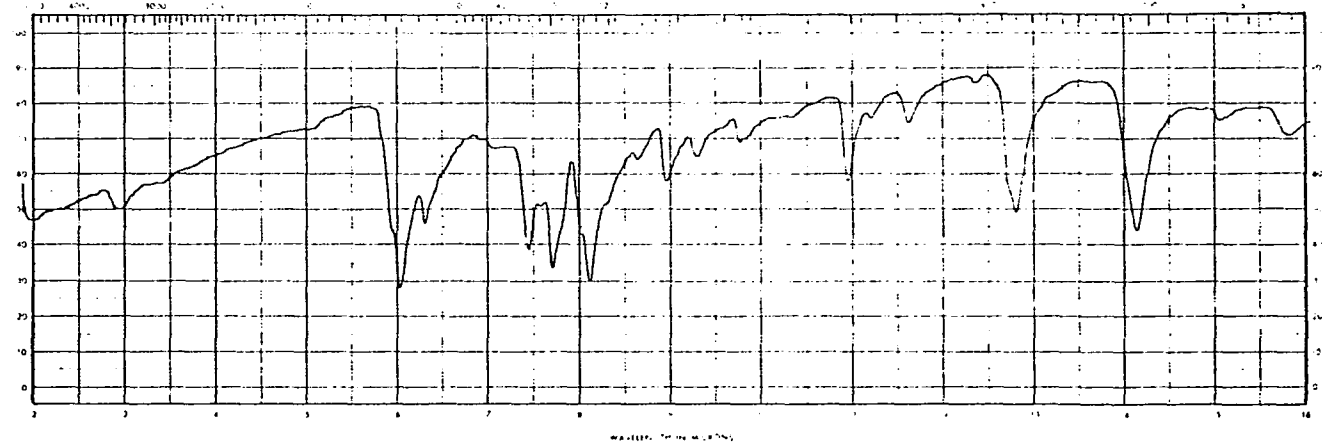

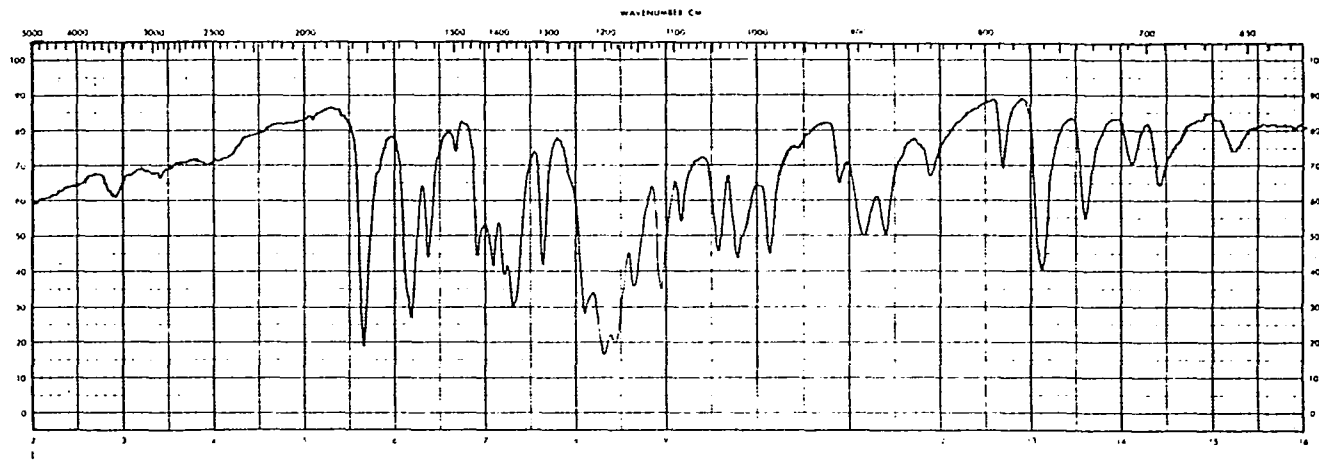Fig. 34   Infrared   Spectrum   of   2-Acetyl-1,4-naphthoquinone   in   KBr.

Fig. 35. Infrared Spectrum of 2-Acetyl-3,4-diacetoxy-I-naphthol in KBr.
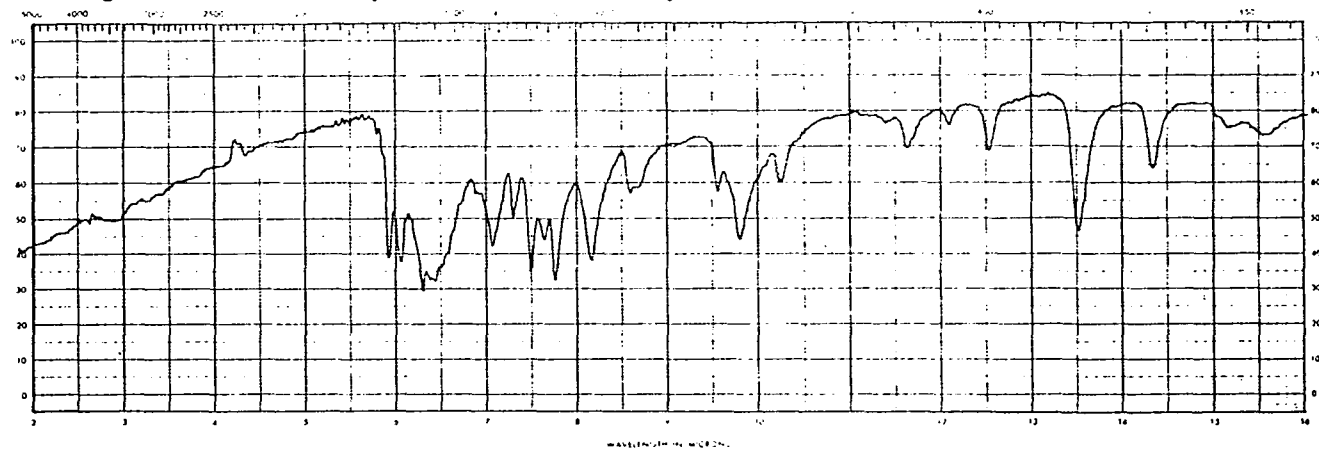

Fig. 36. Infrared Spectrum of 2-Acetyl-3-hydroxy-I,4-naphthoquinone in KBr.

shown in Figs. 31-36.

B.   *Nitration of 2,3-Dimethoxynaphthalene.*

Preparation of 2,3-dimethoxynaphthalene was carried out by the method of Fischer and Kern,[84] yield 68%, m.p. 115-117.5° (reported,[86] 118°).

1.   *With Nitric Acid in Glacial Acetic Acid.*

A solution of 2 ml of nitric acid (d 1.52) and 5 ml of glacial acetic acid was added dropwise at room temperature to a stirred mixture of 1.88 g (0.01 mole) of 2,3-dimethoxynaphthalene in 10 ml of glacial acetic acid. The resulting precipitate was separated by filtration, washed with water, and recrystallized from ethanol to give 0.46 g of 1,6-dinitro-3-methoxy-2-naphthol (LXIII) as light tan needles, m.p. 260-260.5°.

Anal. Calcd. for $C_{11}H_8N_2O_6$: C, 50.01; H, 3.05; N, 10.60. Found: C, 50.27, 50.23; H, 3.07, 3.12; N, 10.46, 10.62.

The filtrate was poured onto ice and the reddish brown precipitate was removed by filtration, washed with water, dried, and chromatographed on deactivated acid-washed silica gel. The chromatogram was developed and eluted with carbon tetrachloride and the following fractions were collected: a pale yellow band (fraction 1), a yellow band (fraction 2), an orange-yellow band (fraction 3), and two reddish orange bands which moved very slowly (fractions 4 and 5) and were more easily removed with benzene-carbon tetrachloride.

Fraction 3 provided an additional 55 mg of 1,6-dinitro-3-methoxy-2-naphthol after recrystallization from ethanol. Fraction 1 crystallized readily from ethanol to give 0.59 g of 1-nitro-2,3-dimethoxynaphthalene (LXVI) as pale yellow plates, m.p. 88.5-89°.

Anal. Calcd. for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75. Found: C, 62.06, 62.03; H, 4.80, 4.85.

Fraction 2 was a mixture of 5-nitro- and 6-nitro-2,3-dimethoxynaphthalene and was separated into its components only after several laborious chromatographies. 5-Nitro-2,3-dimethoxynaphthalene (LXIV) was eluted first and crystallized as bright yellow needles, 270 mg, by slow evaporation of a carbon tetrachloride solution, m.p. 157-158° after recrystallization from ethanol.

Anal. Calcd. for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 61.83, 62.01; H, 4.82, 4.90; N, 6.01, 6.23.

6-Nitro-2,3-dimethoxynaphthalene (LXV) also crystallized as bright yellow needles, 220 mg, by slow evaporation of a carbon tetrachloride solution, m.p. 162-163°, after recrystallization from ethanol.

Anal. Calcd. for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 61.88, 61.72; H, 4.71, 4.85; N, 5.95, 6.20.

Fraction 4 crystallized from acetone to give 25 mg of 3-methoxy-1,2-naphthoquinone (LXVII) as dark red needles, m.p. 189-190° (reported,[85] 186-187°). NMR spectrum of 3-methoxy-1,2-naphthoquinone in acetone: C_4-H, δ 6.78 (s);

C_3-OCH_3, *3.83 (s);* C_8-*H, 7.9 (m);* C_5-*H, 7.6 (m);* C_6-*H and*
C_7-*H, 7.2 - 7.5 (m).*

Anal. Calcd. for $C_{11}H_8O_3$: C, 70.21; H, 4.29.
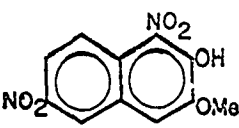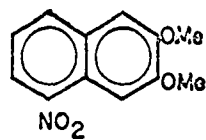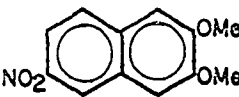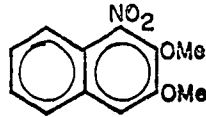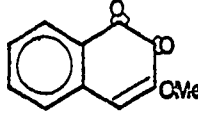*Found: C, 70.00, 70.23; H, 4.28, 4.15.*

Ten milligram of 3-methoxy-1,2-naphthoquinone and
6 mg of o-phenylenediamine in 1 ml glacial acetic acid was
allowed to stand for 8 hr at room temperature. The acetic
acid was removed in vacuo, columned over acid-washed silica
gel with benzene, and the yellow, slightly fluorescent eluent
was stripped to form a residue which was sublimed at 110-
120° under high vacuum. The yellow sublimate melted at 159-
161° (reported,[85] 162-163°) and exhibited UV absorption
identical to that reported by Teuber and Gotz.[85] NMR spec-
trum of 6-methoxybenzo(a)phenazine in deuteriochloroform:
C_6-OCH_3, *δ 4.08 (s);* C_5-*H, 6.96 (s);* C_1-*H, ca. 9.2 (m);* C_8-*H,*
C_{11}-*H, 8.1 - 8.4 (m);* C_9-*H,* C_{10}-*H, ca. 7.6 - 7.8 (m);* C_2-*H,*
C_3-*H,* C_4-*H, 7.3 - 7.6 (m). On integration all hydrogens of*
the $C_{17}H_{12}N_2O$ molecular formula were accounted for.

The infrared spectrum is reproduced in Fig. 42.

The fifth fraction was obtained in trace amounts
after crystallization from carbon tetrachloride as lustrous
black needles, m.p. 266-268°. A sample in methanol showed
absorption maxima at 438, ca. 290sh, 252 mμ and minima at
384 mμ.

The infrared spectrum (Fig. 43) of the quinone
showed the following maxima at 6.0, 6.05sh, 6.2, 6.24, 6.55s,

Table XI.   Infrared Absorption Maxima (Wavelength, μ)
of the Nitration Products of 2,3-Dimethoxy-
naphthalene in KBr.

| Compound	Absorption Maxima
LXIII	2.99s, 3.25w, 3.41w, 6.12w, 6.21s, 6.30, 6.40sh, 6.55b, 6.75b, 7.05s, 7.30sh, 7.49, 7.55sh, 7.80, 8.22-8.40b, 8.48w, 8.75sh, 9.17s, 9.65sh, 10.15s, 10.55s, 10.97s, 11.10, 11.68s, 12.00s, 12.24s, 12.85sh, 12.92s 13.05sh, 13.65s, 14.10s, 15.45s
LXIV	6.18w, 6.25, 6.36w, 6.60, 6.75, 6.90sh, 7.00, 7.20, 7.28sh, 7.45, 7.50, 7.62s, 7.90s, 8.15, 8.32, 8.45, 8.55w, 8.62w, 8.80, 8.90, 9.25w, 9.55s, 9.70w, 10.48, 10.25w, 11.05w, 11.18w, 11.30, 11.55s, 11.70w, 11.83w, 11.96s, 12.35, 12.80, 13.00s, 13.35s
LXV	6.25s, 6.60, 6.75s, 6.99, 7.05sh, 7.20sh, 7.50s, 7.65, 7.90s, 7.98, 8.10sh, 8.35s, 8.45sh, 8.55s, 8.80w, 8.87, 9.24s, 9.60w, 9.70w, 9.85w, 9.96s, 10.40w, 11.05s, 11.30w, 11.55, 11.70, 11.95sh, 12.05, 12.35, 12.45, 13.00, 13.30s
LXVI	6.13w, 6.24, 6.36w, 6.57s, 6.65sh, 6.75, 6.90, 7.00, 7.19w, 7.28sh, 7.34, 7.99, 7.83, 7.94s, 8.15, 8.32, 8.42, 8.50sh, 8.65, 8.90s, 9.52s, 9.70, 9.86s, 10.48s, 11.19, 11.31, 11.50s, 11.95s, 12.80, 13.00s, 13.35s, 13.50, 13.94, 14.70
LXVII	5.93sh, 6.00s, 6.07sh, 6.20, 6.30, 6.75, 6.91, 7.30s, 7.60w, 7.70, 7.87s, 8.18w, 8.32, 8.65, 8.95s, 9.05sh, 10.10w, 10.30s, 10.75s, 11.15, 11.25w, 11.45s, 12.10w, 13.29s, 15.50
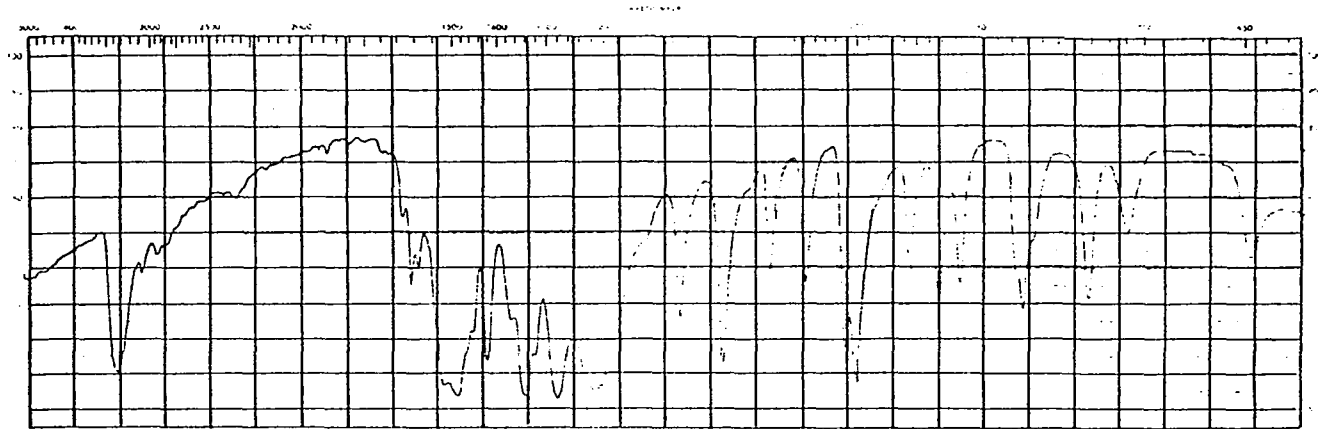
Fig. 37. Infrared Spectrum of 1,6-dinitro-3-methoxy-2-naphthol in KBr.
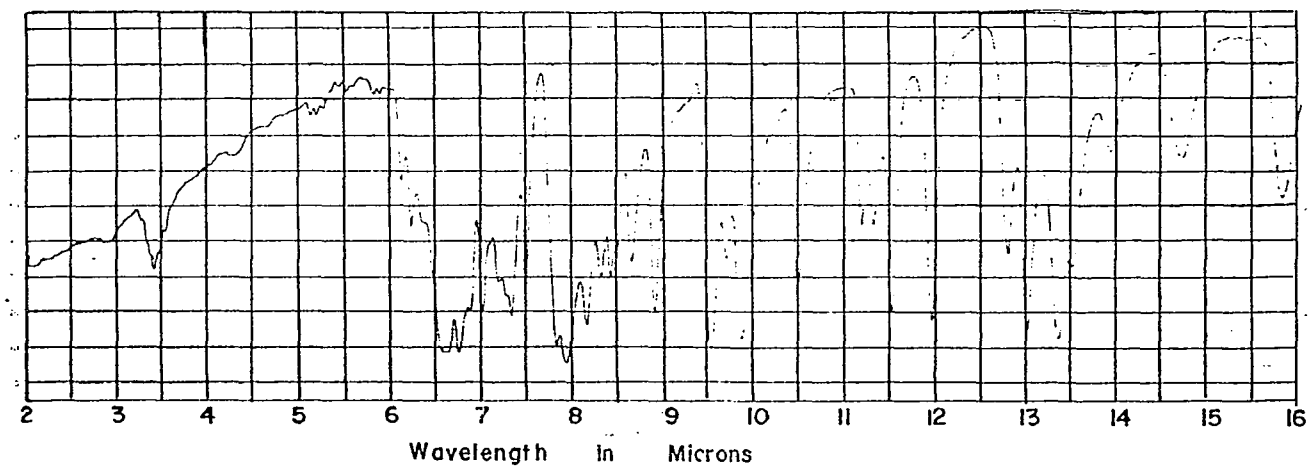

Wavelength in Microns

Fig. 38. Infrared Spectrum of 1-Nitro-2,3-dimethoxynaphthalene in KBr.
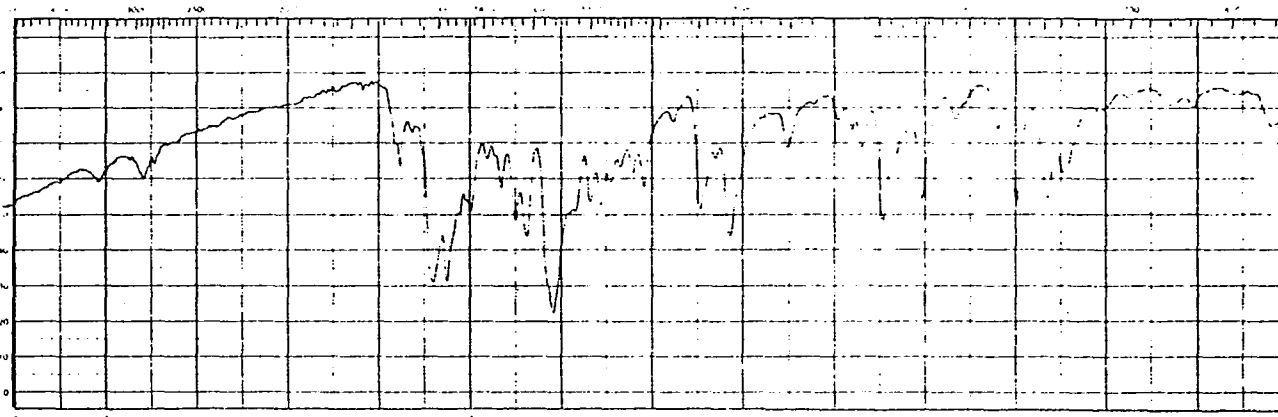
Fig. 39. Infrared Spectrum of 5-Nitro-2,3-dimethoxynaphthalene in KBr.
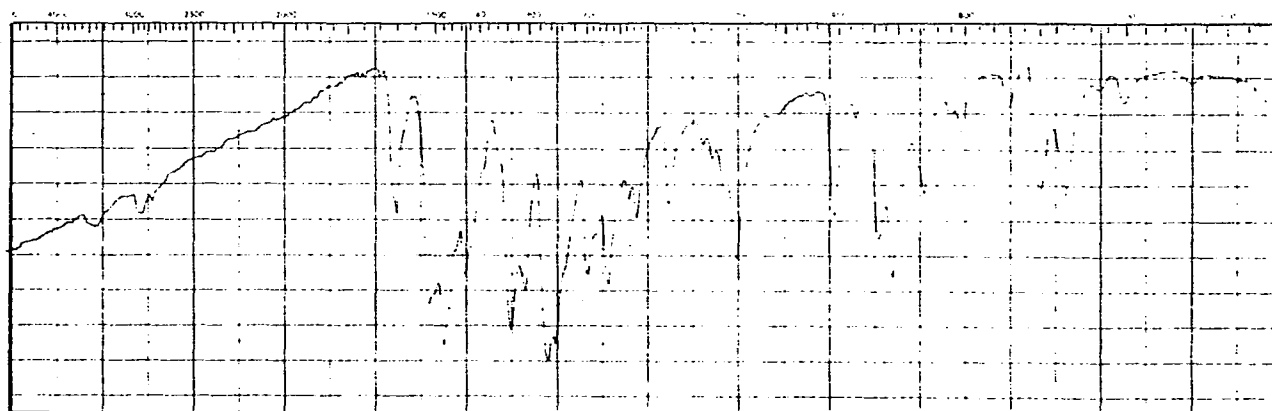

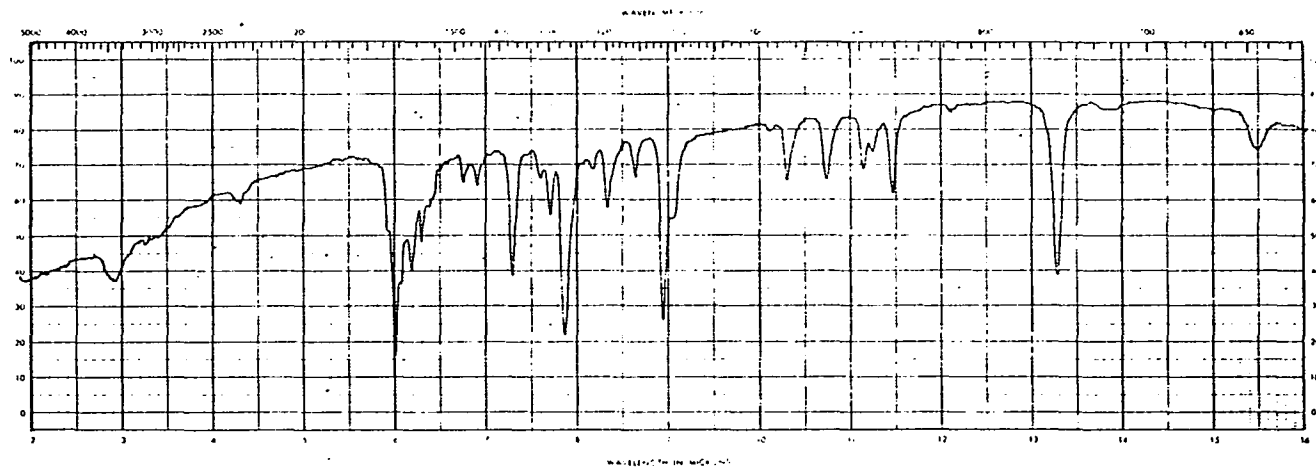Fig. 40. Infrared Spectrum of 6-Nitro-2,3-dimethoxynaphthalene in KBr.

Fig. 41.   Infrared Spectrum of   3-methoxy-1,2-Naphthoquinone in KBr.
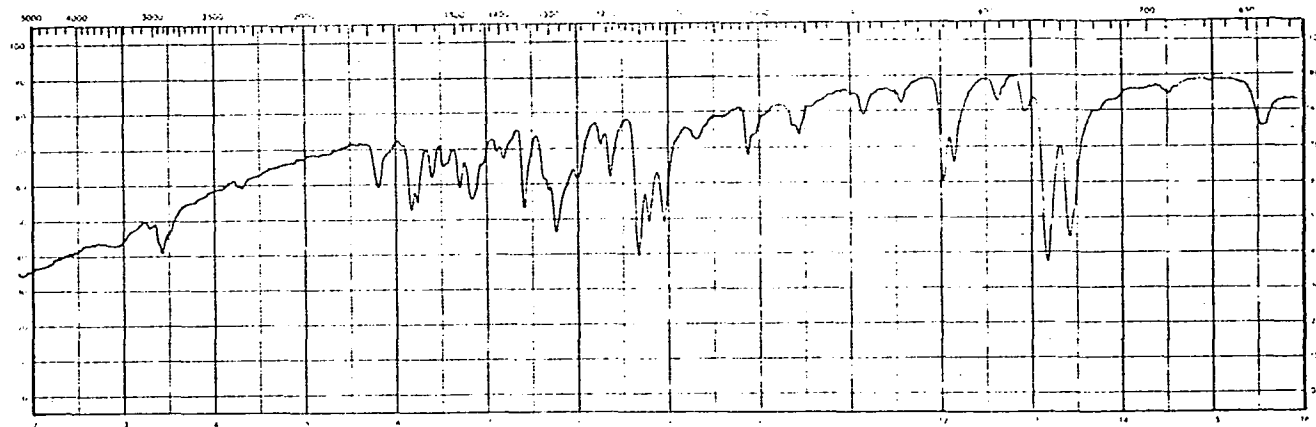

Fig. 42.   Infrared   Spectrum of Phenylenediamine Adduct of 3-methoxy-1,2-Naphthoquinone
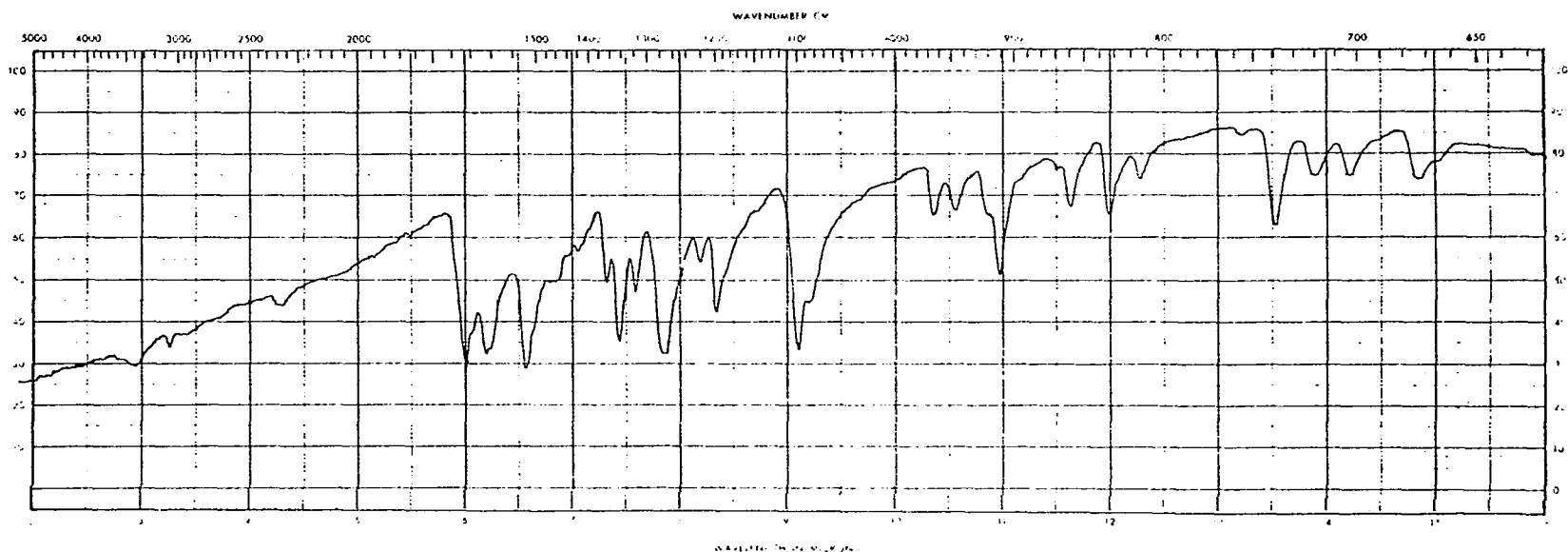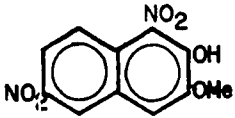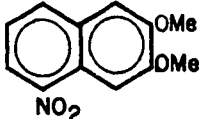in   KBr.
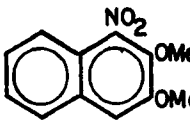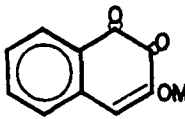
Fig. 43. Infrared Spectrum of the Quinone from Fraction 5 of the Nitration Reaction of 2,3-Dimethoxynaphthalene in KBr.

Table X11. *Ultraviolet Spectra of the Nitration Products of 2,3-dimethoxynaphthalene (in Methanol) in mμ (log ε).*

LXIII		
	λ_{max}	322.7(4.07), 261.8(4.29)
	λ_{min}	288.7 (3.88)
	$\lambda_{max}^{MeOH/KOH}$	401(4.19), 297sh(3.77), 263sh(4.05)
	$\lambda_{min}^{MeOH/KOH}$	329(3.77), 284.5(3.73)

LXIV		
	λ_{max}	355(3.77)
	λ_{min}	291(3.02)

LXV		
	λ_{max}	339.5(3.98), 260.7(4.21)
	λ_{min}	291.4(3.46), 248.4 (4.14)

LXVI		
	λ_{max}	327(3.45), 313.5sh(3.31)
	λ_{min}	302(3.18)

LXVII		
	λ_{max}	456(3.18), 3.43.5(3.29)
	λ_{min}	389(2.95), 302(2.85)

Fig. 44. Ultraviolet Spectra of 1,6-dinitro-3-methoxy-2-naphthol in Methanol (—) and Methanolic KOH (---).

Fig. 45. Ultraviolet Spectrum of I-Nitro-2,3-dimethoxy-naphthalene in Methanol.

Wavelength
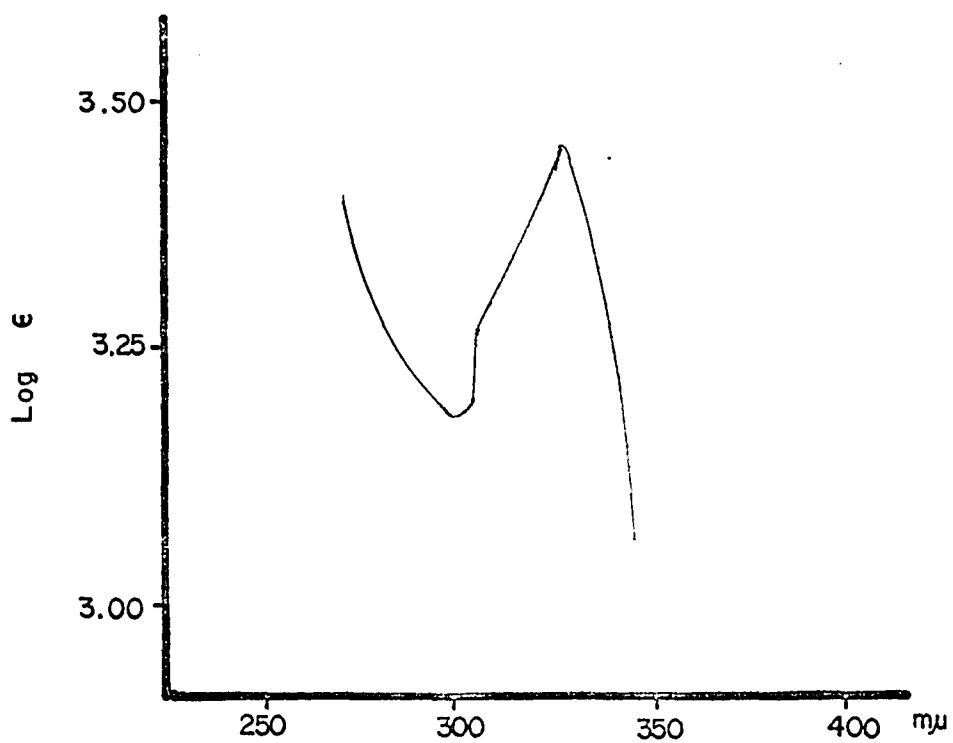
Fig. 46. Ultraviolet Spectrum of 5-Nitro-2,3-dimethoxy-naphthalene in Methanol.
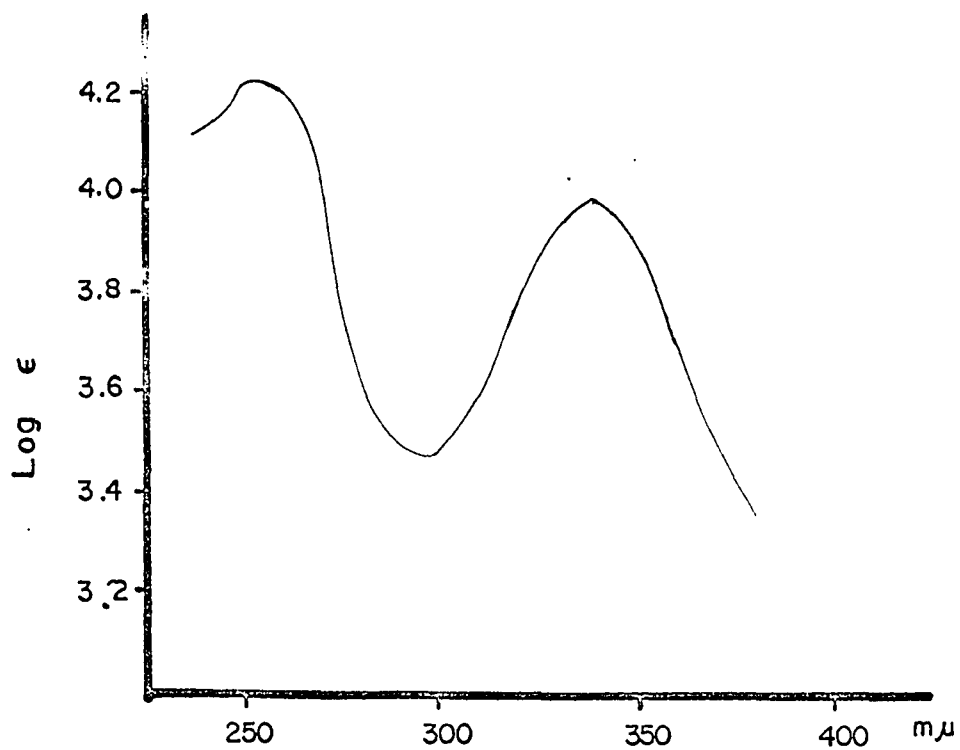
Fig. 47. Ultraviolet Spectrum of 6-Nitro-2,3-dimethoxy-
naphthalene in Methanol.
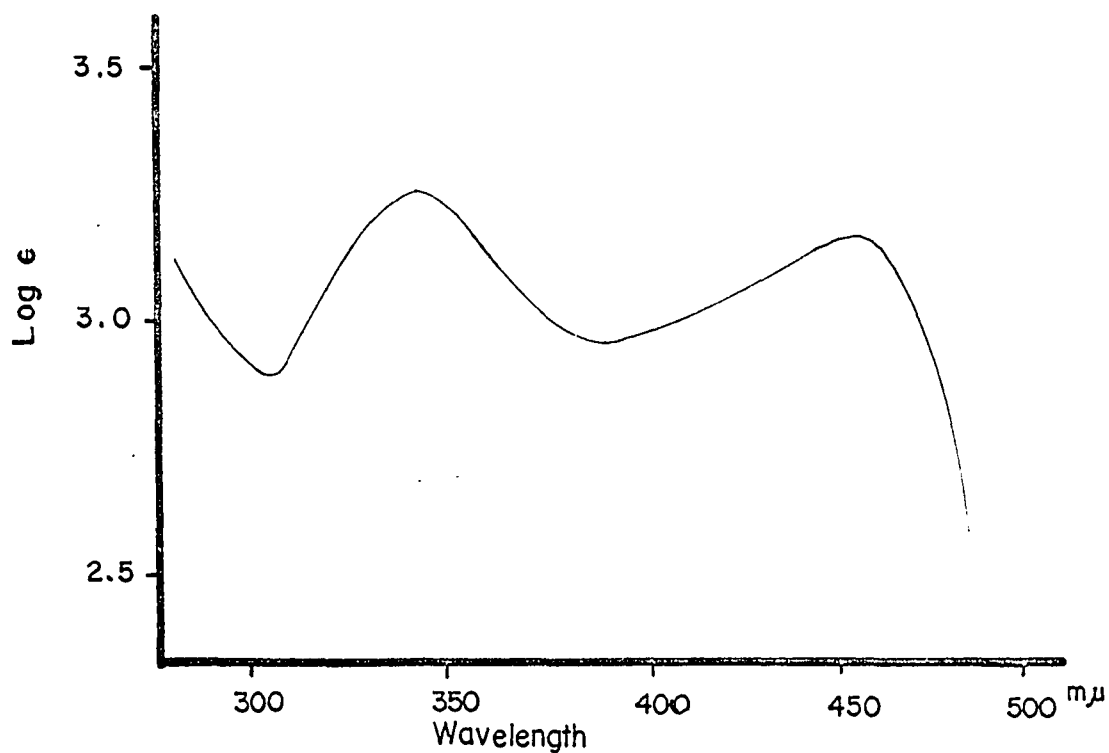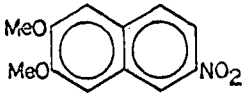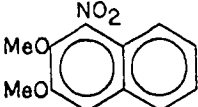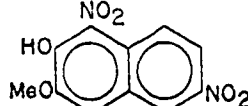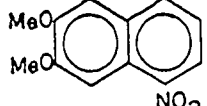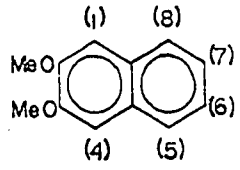
Fig. 48. Ultraviolet and Visible Absorption Spectrum of
3-Methoxy-1,2-naphthoquinone in Methanol.

Table XIII. Nuclear Magnetic Resonance Data of the Nitration Products of 2,3-Dimethoxy-naphthalene.

Compound	Aromatic Protons						2-OCH$_3$	3-OCH$_3$
	H_1	H_4	H_5	H_6	H_7	H_8		
LXV	7.46 ? (s)	7.58 ? (s)	8.72 (d) $J_{57}=2.4$	–	8.02 (q)	7.91 (d) $J_{78}=9$	4.01 (s)	4.01 (s)
LXVI	–	7.58 (s)	7.92 (m)	7.4 – 7.7* complex multiplet			4.06 ? (s)	4.00 ? (s)
LXIII	–	7.89 (s)	8.81 (d)	–	8.26 (q)	7.82* (d) $J_{78}=9$	–	4.13
LXIV	7.50 (s)	7.94* (s)	–	8.15	7.48	8.15 AB_2-type spectrum. $J_{67}=J_{78}$ 8	4.00 (s)	4.00 (s)
	7.26 (s)	7.26 (s)	7.70	7.32	7.32 A_2B_2-type spectrum	7.70	3.91 (s)	3.9- (s)

* *See Wells and Alcorn (Ref.94). The solvent for the determinations is reagent acetone which is transparent in the NMR spectrum below δ 3.5 (with reference to TMS).*

6.85wb, 7.32, 7.44s, 7.59, 7.85s, 8.2, 8.35, 9.10, 9.20w, 10.35, 10.55, 10.86sh, 10.98s, 11.63, 12.00, 12.30, 13.52, 13.90, 14.20, 14.85 μ.

The nitration products of 2,3-dimethoxynaphthalene exhibited infrared maxima as reported in Table XI (Figs. 37-41), visible and ultraviolet absorption maxima and minima as reported in Table XIII (Figs. 44-48) and nuclear magnetic resonance signals as reported in Table XIII.

2.   With Aqueous Nitric Acid.

A suspension of 1.86 g (0.01 mole) of powdered 2,3-dimethoxynaphthalene in 10 ml of 35% nitric acid cooled to -10° was allowed to warm to room temperature with stirring and after 15 min the dark reddish brown gum was separated and triturated with absolute ethanol. Crystallization of the residue from ethanol gave 200 mg of 1,6-dinitro-3-methoxy-2-naphthol as tan needles, m.p. 250°. The ethanol-soluble material was chromatographed on deactivated acid-washed silica gel and an orange-yellow band (fraction 1) and a dark orange-red band (fraction 2) were removed by elution with benzene. The material from fraction 2 readily crystallized from acetone to give 120 mg of 3-methoxy-1,2-naphthoquinone as dark red needles, m.p. 189-190°. Extraction of the material from fraction 1 with hot petroleum ether (b.p. 60-100°) and recrystallization of the insoluble portion from ethanol afforded an additional 125 mg of 1,6-dinitro-3-methoxy-2-naphthol. The petroleum ether-soluble material

consisted mostly of unreacted 2,3-dimethoxynaphthalene.

C.   *Nitration of 2-Acetyl-1,5-dihydroxynaphthalene in*

       Acetic Acid

2-Acetyl-1,5-dihydroxynaphthalene was prepared from 1,5-dihydroxynaphthalene by the method of Spruit[61] in 58% yield, m.p. 262-265° (reported,[61] 265°).

A solution of 5 ml of nitric acid (d. 1.42) and 45 ml of glacial acetic acid was added dropwise over a period of 1.5 hr to a well-stirred suspension of 5.05 g (0.025 mole) of 2-acetyl-1,5-dihydroxynaphthalene in 60 ml of glacial acetic acid, never allowing the temperature of the reaction to exceed 30°. The dihydroxynaphthalene dissolved; the initial green color of the mixture changed to a deep orange-red; and a yellow crystalline precipitate gradually formed. The mixture was cooled in ice and the main nitration product (2.78 g) was removed by filtration, washed with water and cold acetone, and recrystallized from ethanol to give small yellow needles of 2-acetyl-4,6,8-trinitro-1,5-dihydroxynaphthalene, m.p. 266-268°. NMR spectrum in DMSO-d$_6$ with the DMSO-d$_5$ quintet as internal standard: C_2-COCH$_3$, δ 2.86 (s); C_3-H, 8.27 (s); C_7-H, 8.56 (s).

Anal. Calcd. for $C_{12}H_7N_3O_9$: C, 42.74; H, 2.09; N, 12.46. Found: C, 42.95, 43.00; H, 1.97, 1.92; N, 12.45, 12.46.

The filtrate of the nitration mixture was poured into ice water and the reddish brown precipitate was

separated, dried, and extracted with benzene. The benzene
extract was chromatographed on deactivated acid-washed
silica gel and the following fractions were collected using
benzene as the eluant: an orange-yellow band (fraction 1)
and a yellow band (fraction 2). Fraction 1 crystallized
from chloroform-carbon tetrachloride to give 130 mg of 2-
acetyl-6-nitro-1,5-dihydroxynaphthalene *as orange needles,*
m.p. 180-181°. NMR spectrum in deuteriochloroform: C_2-
$COCH_3$, δ *2.75 (s);* C_3-H, *7.96 (?,d,* J_{34}=9 *cps);* C_4-H, *7.73*
(?,d, J_{34}=9 *cps);* C_7-H, *8.07 (?,d,* J_{78}=9 *cps);* C_8-H, *7.87*
(?,d, J_{78}=9 *cps);* C_1-OH, *13.68 (s);* C_5-OH, *11.93 (s).*

Anal. Calcd. for $C_{12}H_9N_2O_5$: *C, 58.30; H, 3.67.*
Found: C, 58.32, 58.28; H, 3.79, 3.90.
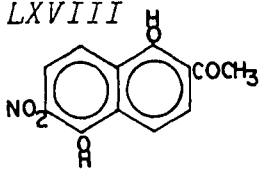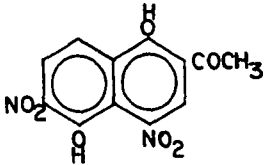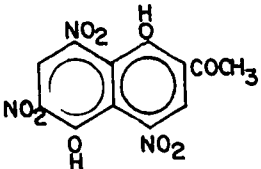
After repeated chromatography of fraction 2 an
addtional 28 mg of the trinitro compound and 450 mg of 2-
acetyl-4(?), 6-dinitro-1,5-dihydroxynaphthalene *was obtained.*
The latter compound, recrystallized from carbon tetrachloride
as orange needles, melted at 239-240°. NMR spectrum of 2-
acetyl-4(?),6-dinitro-1,5-dihydroxynaphthalene *in DMSO-d$_6$*
with the quintet of DMSO-d$_5$ as internal standard: C_2-$COCH_3$,
δ *2.85 (s);* C_3-H, *7.98 or 8.34 (d,* J_{34}=ca. *8 cps);* C_4-H,
7.98 or 8.34 (d, J_{34}=ca. *8 cps);* C_7-H, *8.49 (s).*

Anal. Calcd. for $C_{12}H_8N_2O_7$: *C, 49.32; H, 2.74; N,*
9.59. Found: C, 49.58, 49.60; H, 2.75, 2.89; N, 9.33, 9.33.

Homogeneity of the samples was determined by TLC
on deactivated silica gel plates which were developed with

Table XIV. *Infrared Absorption Maxima (Wavelength, μ) of the Nitration Products of 2-Acetyl-1,5-dihydroxynaphthalene in KBr.*

Compound	Absorption Maxima
LXVIII	6.10, 6.30s, 6.55s, 6.65sh, 6.78, 7.04s, 7.18w, 7.36, 7.65sh, 7.75, 7.95, 8.04w, 8.35w, 8.45, 8.75w, 8.78w, 8.90w, 9.85s, 10.42, 10.55w, 11.00, 11.42s, 12.25sh, 12.39, 12.72, 13.55, 13.18, 14.10b, 14.45b, 15.10wb
LXIX	6.10, 6.29s, 6.51s, 6.95sh, 7.05, 7.30s, 7.56w, 7.68w, 7.89sh, 8.00s, 8.30s, 8.39s, 8.62w, 8.85, 9.72, 10.30, 11.20, 11.35, 11.50, 12.05, 12.32, 12.45sh, 12.70, 13.00w, 13.17, 13.57w, 13.90b, 14.35, 15.5b
LXX	6.11, 6.28s, 6.50s, 6.70sh, 7.10sh, 7.20sh, 7.38s, 7.75, 7.85w, 8.05s, 8.25, 8.55, 8.80, 9.37, 9.70, 10.29, 11.08, 11.25, 11.57, 11.90b, 12.62w, 13.05, 13.30, 13.30, 13.60, 13.80sh, 14,60s, 14.80sh, 15.55b
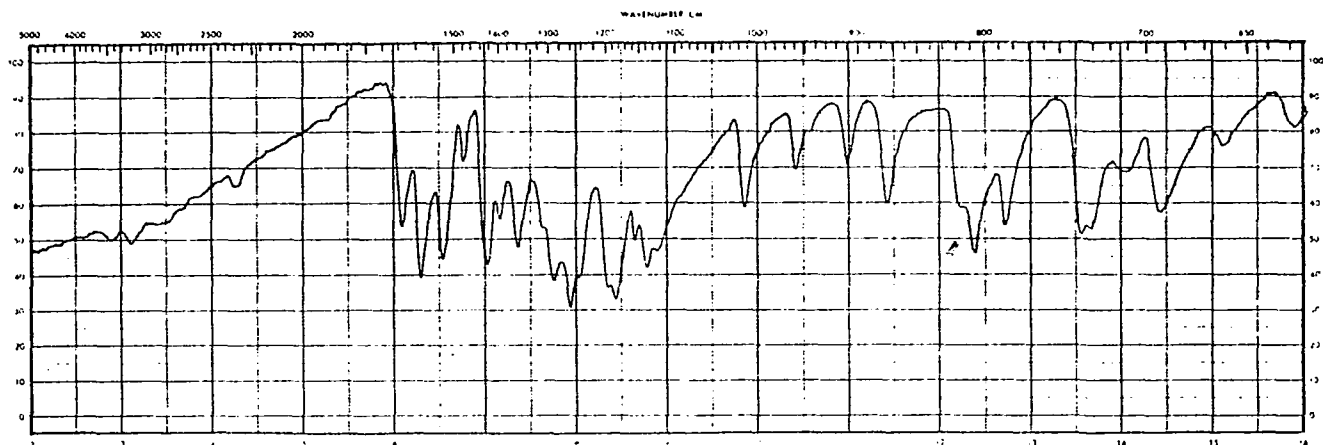
Fig. 49. Infrared Spectrum of 2-Acetyl-6-nitro-I,5-dihydroxynaphthalene in KBr.
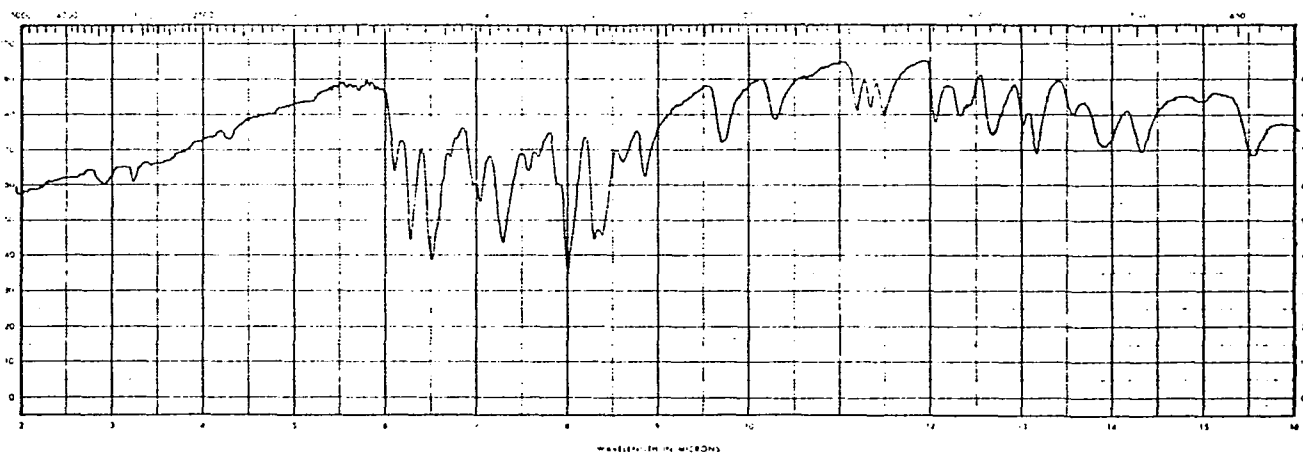
Fig. 50. Infrared Spectrum of 2-Acetyl-4(?),6-dinitro-I,5-dihydroxynaphthalene in KBr.
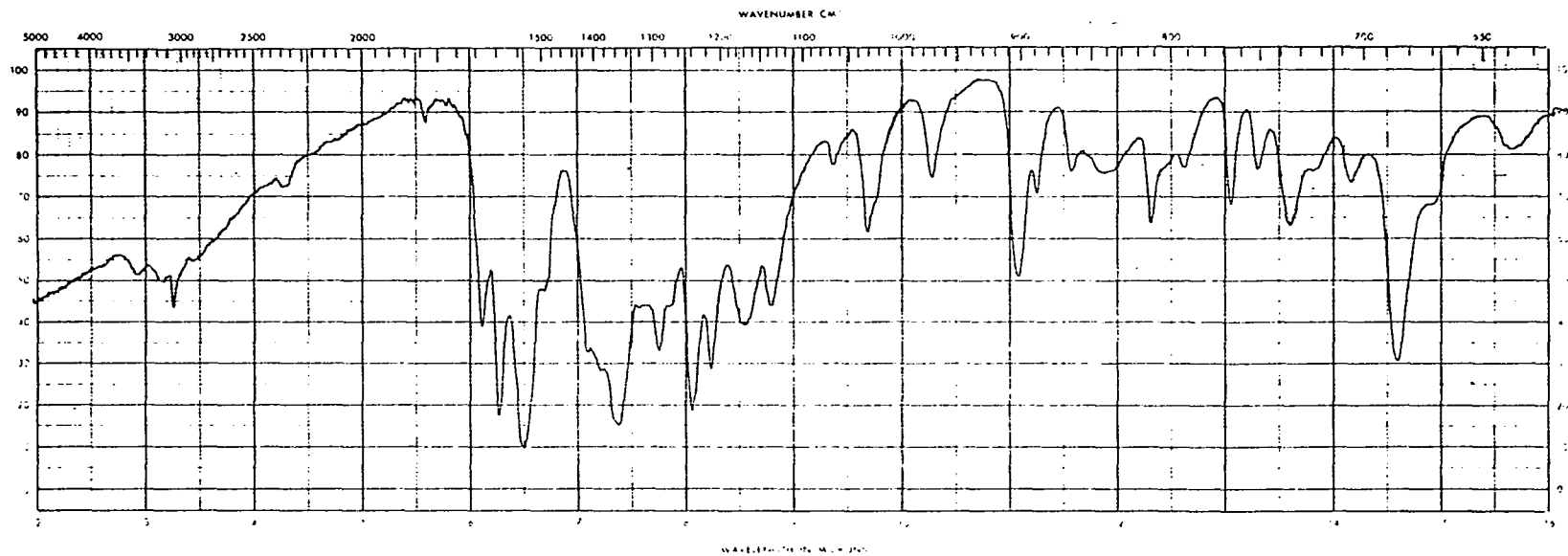
Fig. 51.   Infrared   Spectrum   of   2-Acetyl-4,6,8-trinitro-1,5-dihydroxynaphthalene   in   KBr.

Table XV.   *Ultraviolet Spectra of Nitration Products of 2-Acetyl-1,5-dihydroxynaphthalene.*

2-Acetyl-6-nitro-1,5-dihydroxynaphthalene

> *In methanol,* λ_{max}: *438 mμ (log e, 3.88), 303sh (4.11), 270.6 (4.57).*
>
> λ_{min}: *354 (3.27), 249.5 (4.17).*
>
> *In KOH/MeOH,* λ_{max}: *509 mμ (log e, 4.06), 318sh (4.23).*
>
> λ_{min}: *401 (2.95), 291.6 (4.53), 260 (4.17).*

2-Acetyl-4(?),6-dinitro-1,5-dihydroxynaphthalene

> *In methanol,* λ_{max}: *430 (4.03), 268 (4.49).*
>
> λ_{min}: *340.5 (3.50).*
>
> *In KOH/MeOH,* λ_{max}: *492 (4.17), 283.5 (4.43).*
>
> λ_{min}: *374 (3.55), 249 (4.11).*

2-Acetyl-4,6,8-trinitro-1,5-dihydroxynaphthalene

> *In methanol,* λ_{max}: *445 (3.93), 401sh (3.86), 264 (4.33).*
>
> λ_{min}: *350.5 (3.46)*
>
> *In KOH/MeOH,* λ_{max}: *484 (4.15), 422sh (4.04), 334sh (3.78), 278.2 (4.37).*
>
> λ_{min}: *353 (364), 250.7 (4.08).*
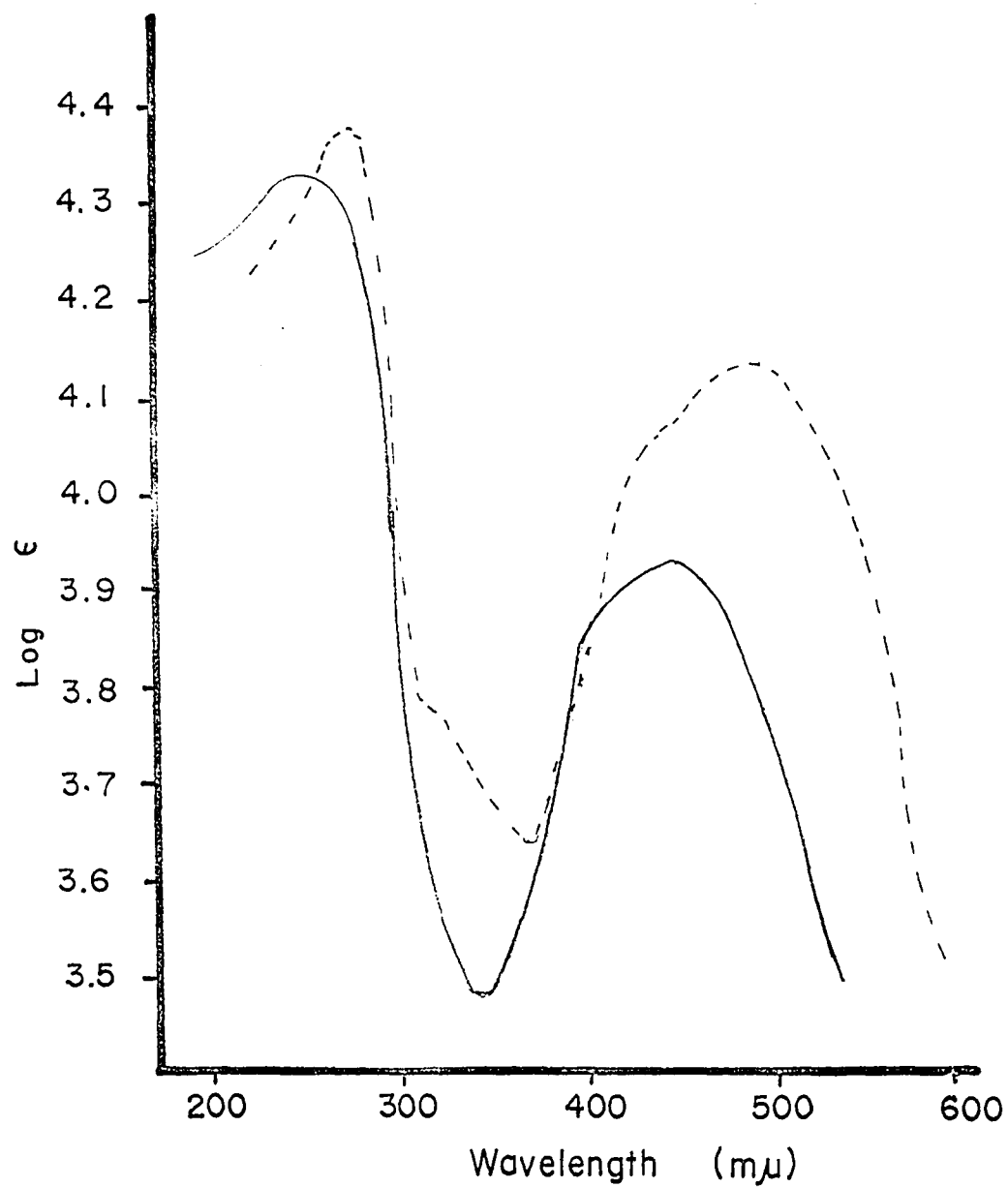
Fig. 52. Ultraviolet and Visible Absorption Spectra of
2-Acetyl-4,6,8-trinitro-1,5-dihydroxynaphthalene in
Methanol(—) and Methanolic KOH.
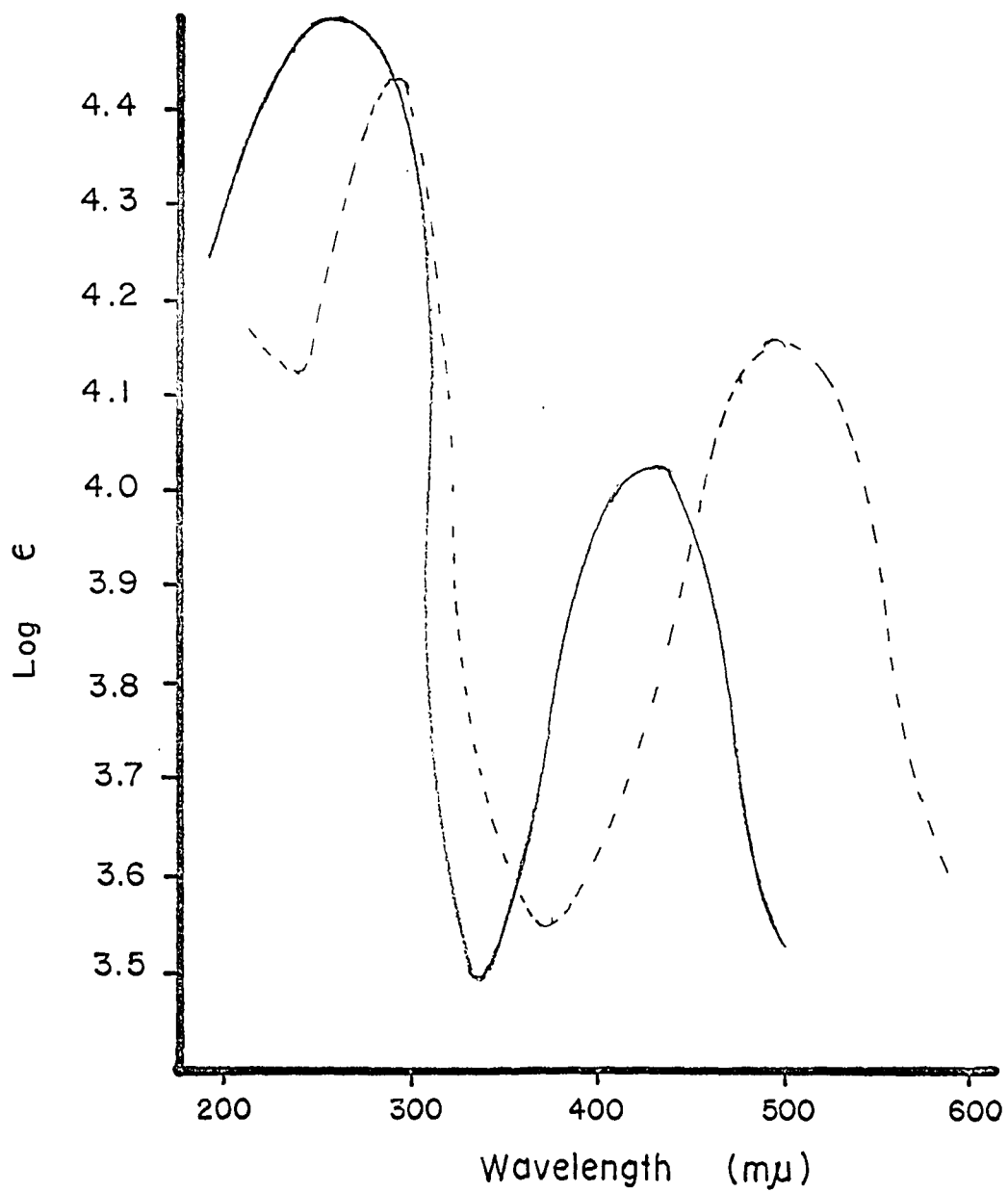
Fig. 53.   Ultraviolet and Visible Absorption Spectra of
2-Acetyl-4(?),6-dinitro-1,5-dihydroxynaphthalene
in Methanol (—) and Methanolic KOH(---).
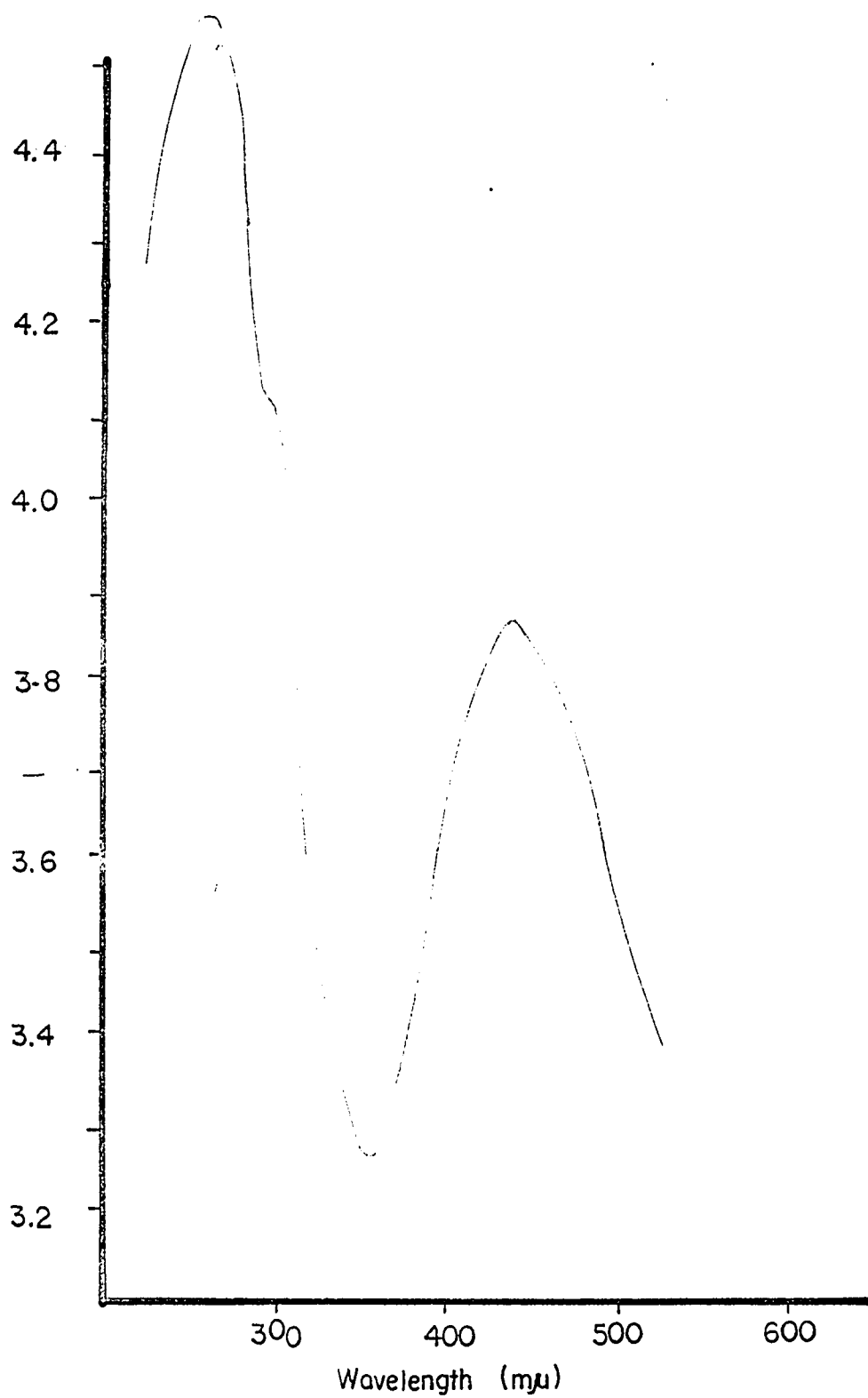
Fig. 54.   Ultraviolet and Visible Absorption Spectrum of
2-Acetyl-6-nitro-1,5-dihydroxynaphthalene in Methanol.

benzene: mononitro (R_F, 0.63), dinitro (0.47), and tri-nitro (0.46).

Other minor products indicated by their reddish spots on the thin layer plates were not isolated.

The ultraviolet and infrared spectra of the reaction products are recorded in Tables XIV and XV and illustrated in Figs. 49-51 and 52-54 respectively.

D. Nitration of 6-Acetyl-2,3-dimethoxynaphthalene in Acetic Anhydride.

Preparation of 6-acetyl-2,3-dimethoxynaphthalene by the method of Buu-Hoi and Lavit[86] resulted in 33% yield after sublimation at $160°/HV$, m.p. $106-107°$ (reported,[86] $109°$). NMR spectrum in carbon tetrachloride: C_6-COCH_3, δ 2.52 (s); C_2-OCH_3, 3.84 or 3.87 (s); C_3-OCH_3, 3.84 or 3.87 (s); C_1-H, 6.93 or 7.00 (s); C_4-H, 6.93 or 7.00 (s); C_5-H, 8.19 (d, J_{57}=ca. 2); C_7-H, 7.80 (d, J_{78}=ca. 8); C_8-H, 7.39 (d, J_{78}=ca. 8).

A mixture of 6-acetyl-2,3-dimethoxynaphthalene (1.15 g, 5 mmole) in 5 ml acetic anhydride was nitrated at room temperature by adding dropwise 1 ml of fuming nitric acid (d. 1.50) dissolved in 5 ml acetic anhydride. From the orange-red solution, 0.36 g of yellow crystals (m.p. ca. 160-162°) precipitated which were then washed, dried, and columned on deactivated acid-washed silica gel with benzene. The first yellow band was collected, stripped in vacuo and

*after crystallizing from ethanol and recrystallizing from
acetone afforded 280 mg of yellow needles, m.p. 181-183°
which was shown by NMR to be* 6-acetyl-4-nitro-2,3-dimethoxy-
naphthalene *. NMR spectrum in deuteriochloroform:* C_6-$COCH_3$,
δ *2.64 (s);* C_2-OCH_3 *and* C_3-OCH_3, *4.03 (s);* C_1-H, *7.26 (s);*
C_7-H, C_8-H, ca. *7.80 (m).*

Anal. *Calcd. for* $C_{14}H_{13}NO_5$: *C, 61.09; H, 4.76; N,
5.09. Found: C, 61.22, 61.22; H, 4.68, 4.69; N, 5.12, 5.18.*

By the method of Furst and Moore[87] *reduction of
the above compound was carried out by suspending 235 mg of
6-acetyl-4-nitro-2,3-dimethoxynaphthalene in 10 ml of boiling
ethanol with Raney nickel and 0.3 ml of 100% hydrazine hy-
drate in 1 ml of ethanol. The product was isolated after
filtering to remove the Raney nickel and stripping off the
filtrate* in vacuo *followed by purification by column chroma-
tography (deactivated neutral-silica gel/benzene-ethyl ace-
tate, 1:1/v:v). Sublimation of the residue at 120°/HV after
removing the combined eluates* in vacuo *afforded* 6-acetyl-4-
amino-2,3-dimethoxynaphthalene *as yellow needles, m.p. 142-
143°. NMR spectrum in deuteriochloroform:* C_6-*acetyl,* δ *2.64
(s);* C_2-OCH_3, *3.94 (s);* C_3-OCH_3, *3.87 (s);* C_1-H, *6.66 (s);*
C_4-NH_2, *4.50 (broad sharp s);* C_5-H, *8.43 (d,* J_{57}=ca. *2 cps);*
C_7-H, *7.91 and* C_8-H, *7.66 (AB q,* J_{78}=9 *cps). Integration
accounted for all fifteen protons of the molecule. UV spec-
trum in methanol,* λ_{max}: *380, 318 mμ.* λ_{min}: *345.5, 305, 273
mμ. In methanol with a trace of conc. hydrochloric acid,*

λ_{max} at 307.7 mμ, which could be reversed with base.
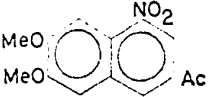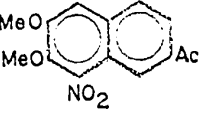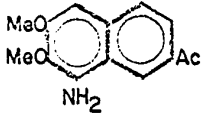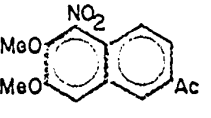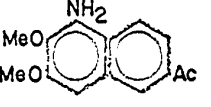
Anal. Calcd. for $C_{14}H_{15}NO_3$: C, 68.55; H, 6.16; N, 5.71. Found: C, 68.45, 68.37; H, 6.06, 6.21; N, 6.70, 6.80.

The second yellow band was collected and after purification by the usual manner, 46 mg of 6-acetyl-8-nitro-2,3-dimethoxynaphthalene as yellow needles were crystallized from acetone, m.p. 261-262°. The compound, sparingly soluble in chloroform, exhibited in the NMR spectrum (in acetone) the C_6-acetyl protons as a doublet (J=ca. 2 cps) at δ 2.70, two methoxy singlets at δ 4.05 and 4.13, and three broad singlets appearing at δ 7.75, 7.90, and 8.23 with the relative intensities of 1:2:1. The singlets appear to be doublets but are collapsed to form broad singlets.

Anal. Calcd. for $C_{14}H_{13}NO_5$: C, 61.09; H, 4.76. Found: C, 61.66, 61.34; H, 4.92, 4.76.

The crude mixture (0.70 g) isolated from the filtrate of the reaction solution by precipitation with ice water was separated by several column chromatographies using deactivated acid-washed silica gel and deactivated neutral silica gel respectively. Traces of both of the 4- and 8-nitro isomers were isolated but the main fraction, 6-acetyl-1-nitro-2,3-dimethoxynaphthalene was obtained as crystalline yellow clusters of needles (352 mg from ethyl acetate) having a melting point of 156-157°. NMR spectrum in deuteriochloroform: C_6-COCH$_3$, δ 2.55 (d, J=2.5 cps); C_2-OCH$_3$, 4.02 (s); C_3-OCH$_3$, 4.01 (s); C_4-H, 7.28 (s); C_5-H, C_7-H, C_8-H, complex

Table XVI. *Infrared Absorption Maxima of the Nitration Products of 6-Acetyl-2,3-dimethoxynaphthalene. Infrared Absorption Maxima of 6-Acetyl-4-amino-2,3-dimethoxynaphthalene and 6-Acetyl-1-amino-2,3-dimethoxynaphthalene.*

Compound[*]	Absorption Maxima (μ)
LXXI 	3.45, 3.52sh, 5.98s, 6.40, 6.63, 6.78sh, 6.81, 7.02, 7.28w, 7.38sh, 7.51, 7.69w, 7.85, 8.05sh, 8.15, 8.30w, 8.42, 8.49sh, 8.75w, 9.20w, 9.65, 9.81s, 10.10, 10.30, 10.99, 11.11, 11.76s, 11.90sh, 12.90s, 13.15w, 15.38
LXXII 	3.25w, 3.41, 3.52w, 6.00s, 6.15, 6.54, 6.80, 6.85sh, 7.04, 7.30sh, 7.37, 7.65s, 7.79, 7.90w, 8.09, 8.15, 8.39, 8.62, 8.88, 9.28s, 9.60s, 9.82, 10.39, 10.45, 11.00s, 11.32sh, 11.49s, 12.25s, 12.96s, 13.55, 14.05, 14.92
LXXIII 	2.95, 3.35w, 3.41, 3.52w, 6.00s, 6.18s, 6.40sh, 6.80s, 6.90w, 7.00w, 7.14w, 7.22w, 7.35s, 7.70, 8.00, 8.25sh, 8.35, 8.50w, 8.85s, 8.95sh, 9.30, 9.82, 10.15, 10.45w, 10.95, 11.02, 11.60w, 11.85, 11.96, 14.16
LXXIV 	3.25w, 3.41, 3.52sh, 6.00s, 6.15, 6.24w, 6.38sh, 6.55, 6.80, 6.86sh, 7.00, 7.15, 7.38, 7.62s, 8.05s, 8.37, 8.60, 8.86s, 9.28s, 9.58s, 9.80, 9.92, 10.37, 10.42sh, 10.80, 11.00, 11.12, 11.25sh, 11.48s, 11.70w, 12.25s, 12.95s, 13.55, 13.85, 14.00, 14.90, 15.40, 15.75
LXXV 	292sh, 2.97s, 3.42, 3.52sh, 6.01s, 6.17s, 6.68w, 6.80s, 6.88w, 7.01s, 7.16, 7.32, 7.70, 8.37, 8.48w, 8.85w, 8.95, 9.90, 10.25, 10.34sh, 10.65w, 11.01s, 11.92, 12.25, 12.45, 13.00w, 13.65w, 13.92, 14.50, 15.30w, 15.65

[*] *Except for compound LXXIII, which was measured in chloroform, all other compounds were prepared in KBr disks.*
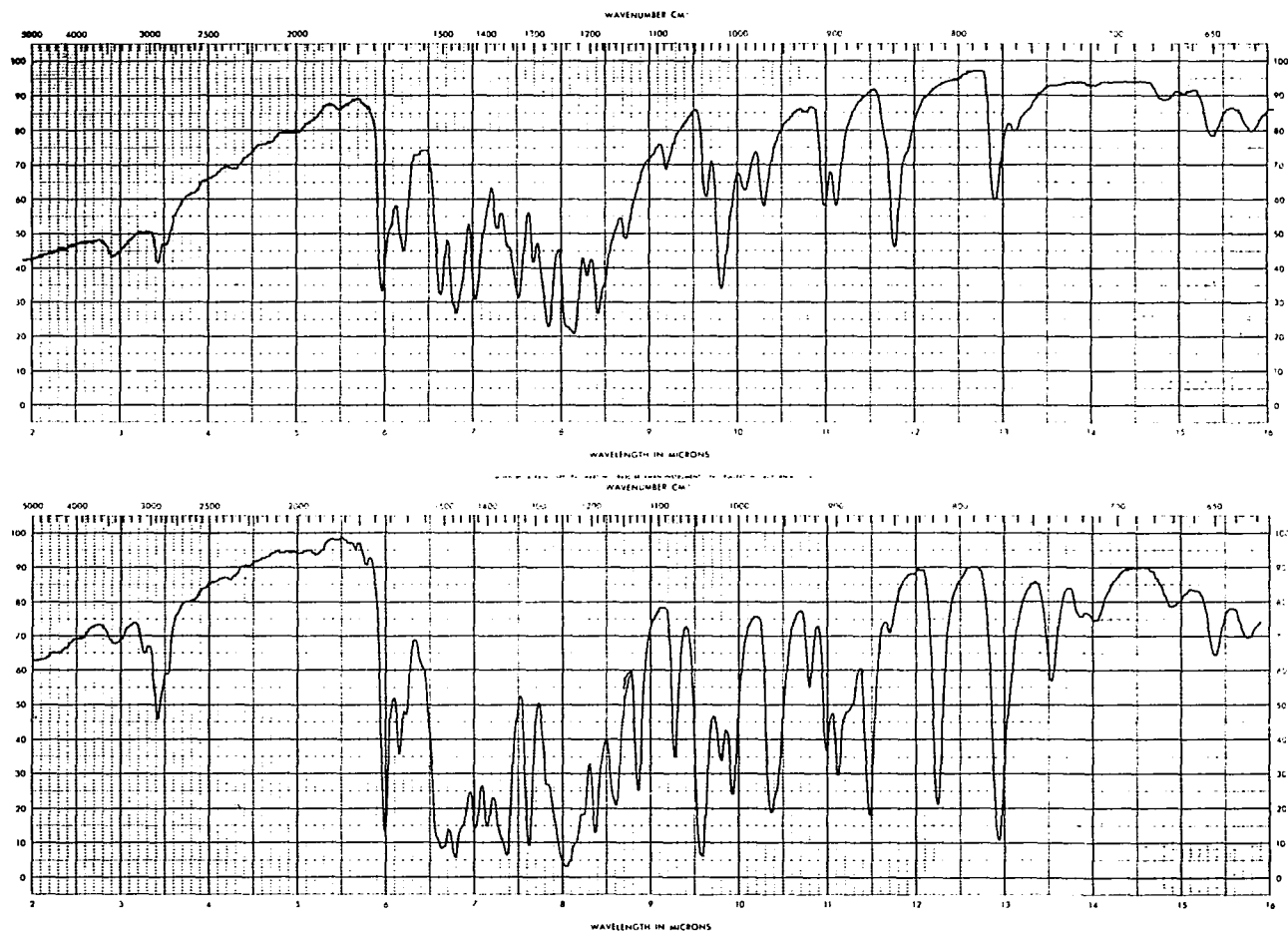
Fig. 55. Infrared Spectrum of 6-Acetyl-8-nitro-2,3-dimethoxynaphthalene in KBr (A).
Infrared Spectrum of 6-Acetyl-1-nitro-2,3-dimethoxynaphthalene in KBr (B).
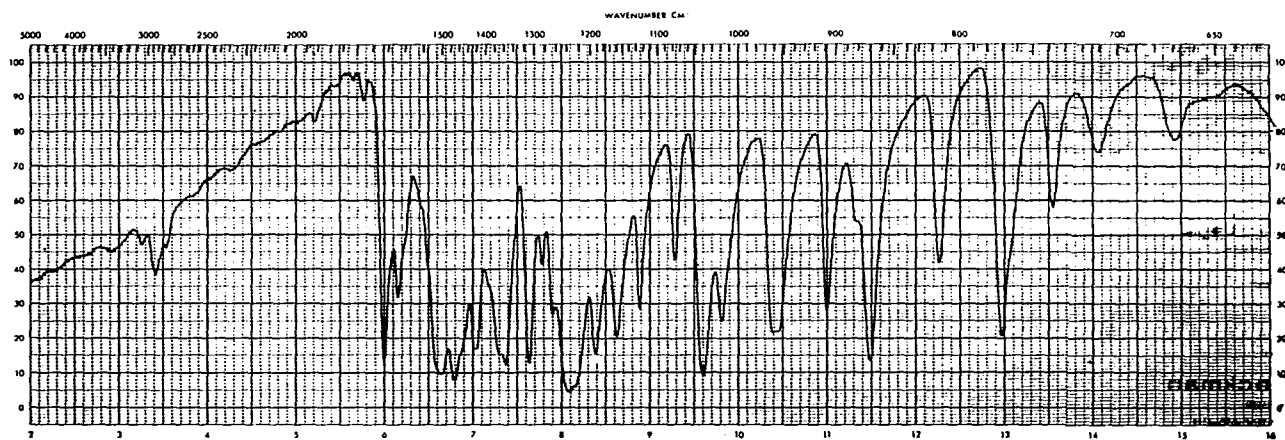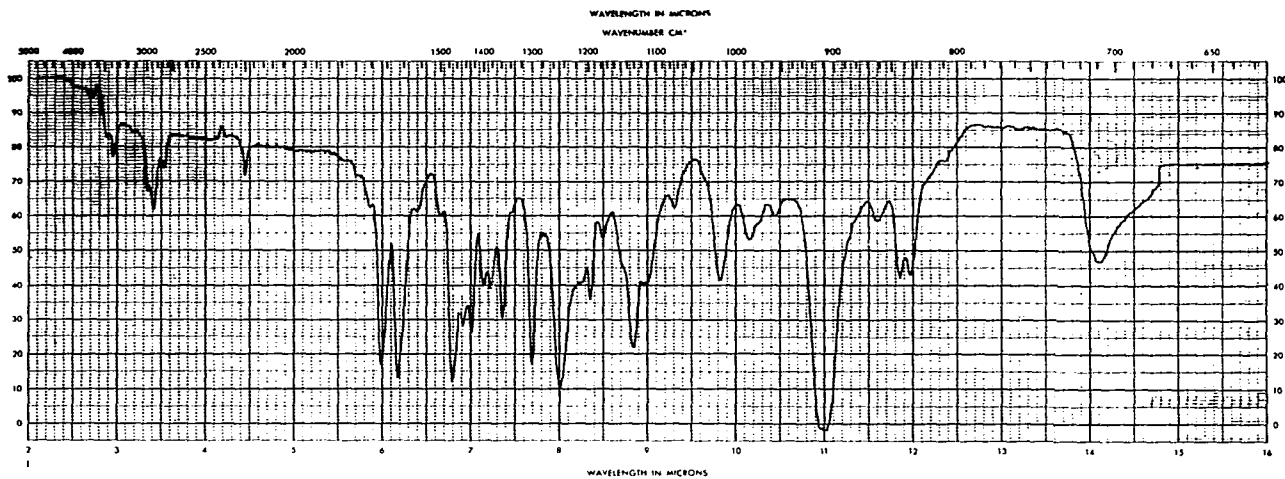
Fig. 56. Infrared Spectrum of 6-Acetyl-4-amino-2,3-dimethoxynaphthalene (A) in KBr. Infrared Spectrum of 6-Acetyl-4-amino-2,3-dimethoxy-naphthalene (B) in KBr.

Table XVII. Ultraviolet Spectra of Nitration Products of 6-Acetyl-2,3-dimethoxynaphthalene

6-Acetyl-4-nitro-2,3-dimethoxynaphthalene

In methanol, λ_{max}: 299.8 mu (log c, 3.72), 255.4

(4.39, 249.4 (4.39)

λ_{min}: 275.7 (3.54

6-Acetyl-8-nitro-2,3-dimethoxynaphthalene

In methanol, λ_{max}: 361 (3.63), 288 (4.14), 251.5 (4.44)

λ_{min}: 328.5 (3.56), 271.3 (4.05)

6-Acetyl-1-nitro-2,3-dimethoxynaphthalene

In methanol, λ_{max}: 292 (3.97), 243.6 (4.67)

λ_{min}: 276 (3.93)
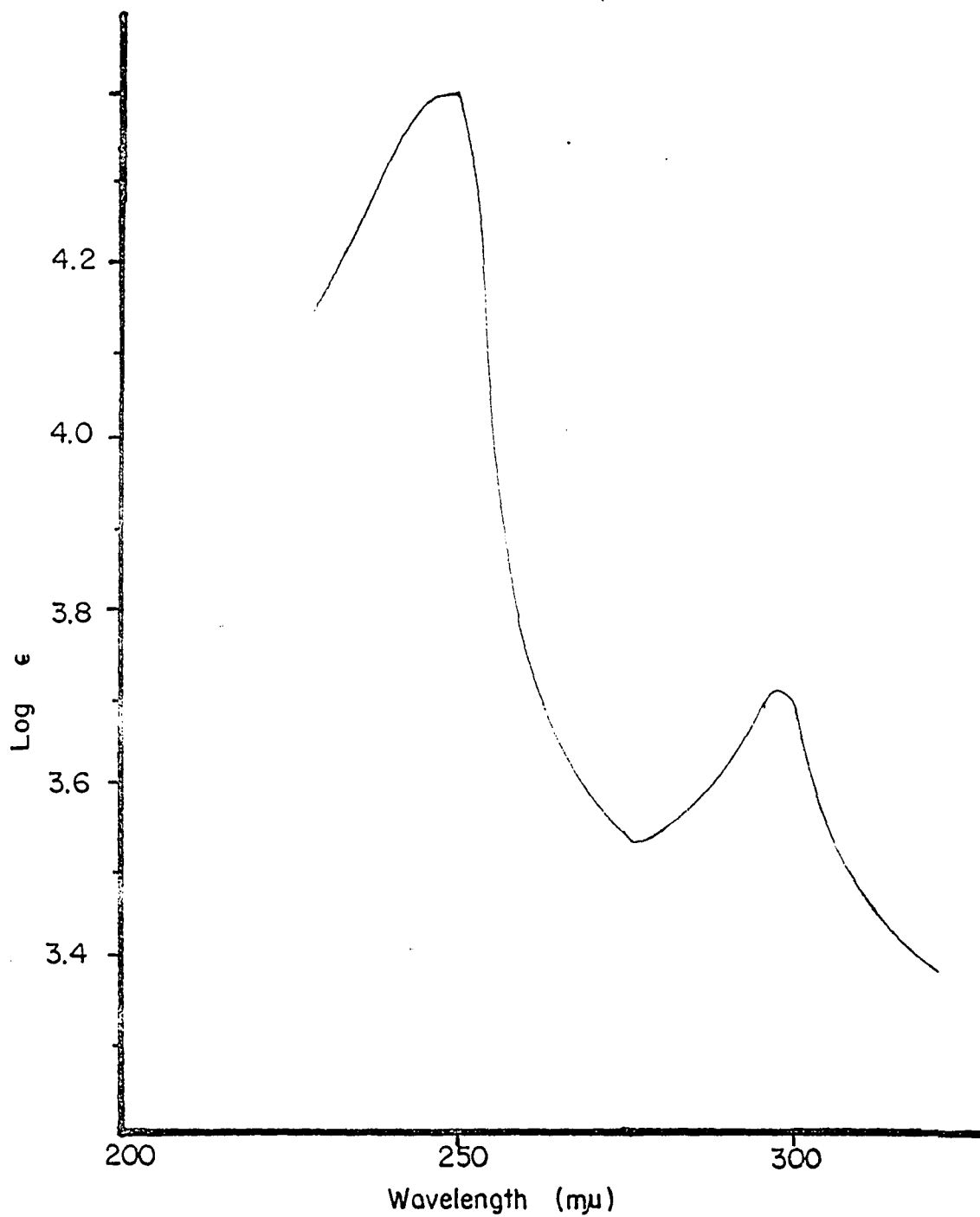
Fig. 57.   Ultraviolet Spectrum of 6-Acetyl-4-nitro-2,3-dimethoxy-
naphthalene in Methanol.
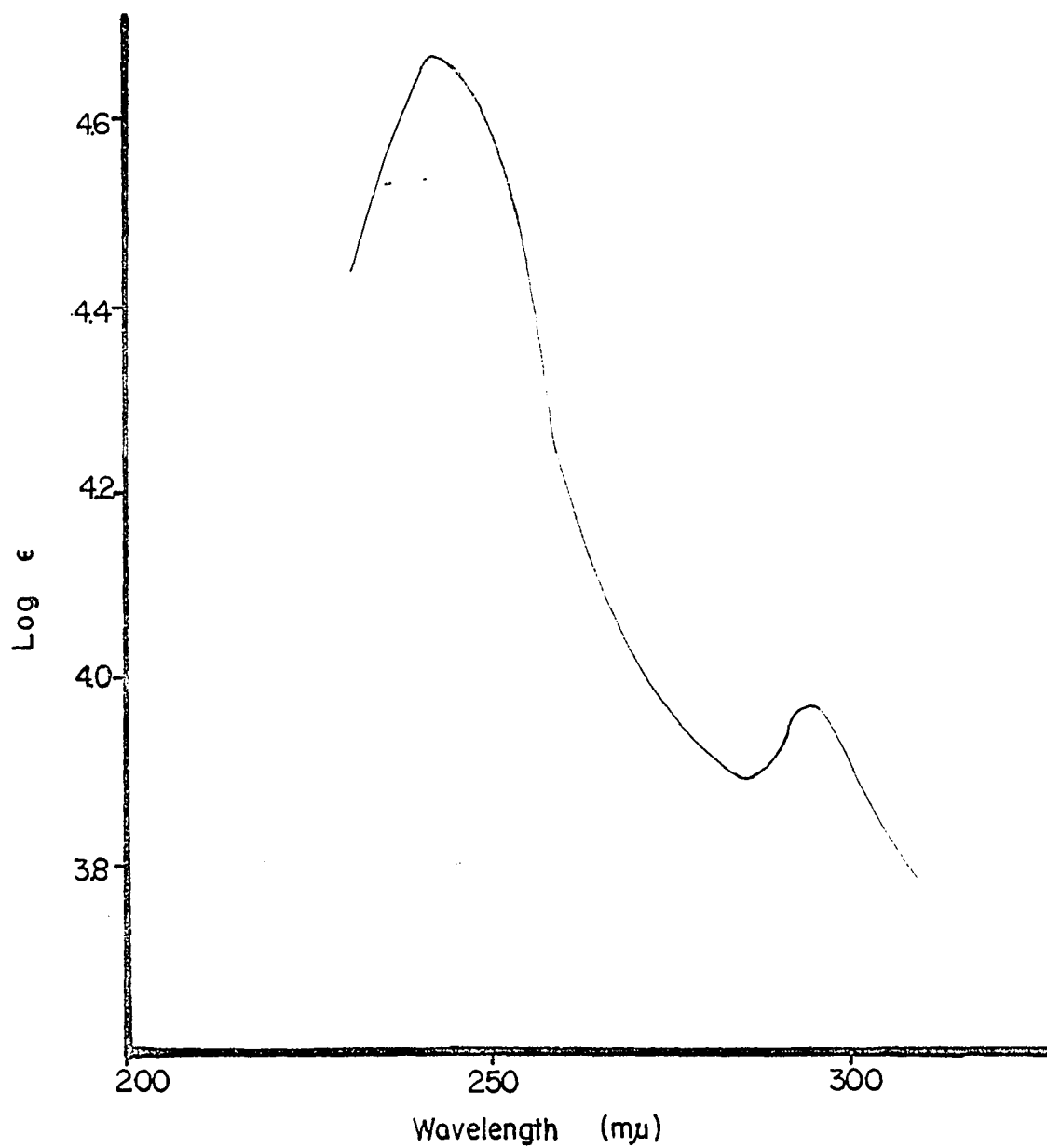
Fig. 58.   Ultraviolet   Spectrum of 6-Acetyl-l-nitro-2,3-dimethoxy-
            naphthalene in Methanol.

Fig. 59. Ultraviolet Spectrum of 6-Acetyl-8-nitro-2,3-dimethoxy-
naphthalene in Methanol.

multiplets.

<u>Anal</u>. Calcd. for $C_{14}H_{13}NO_5$: C, 61.09; H, 4.76; N, 5.09. Found: C, 61.23, 61.29; H, 4.75, 4.72; N, 5.21, 5.10.

Reduction of 500 mg of 6-acetyl-1-nitro-2,3-dimethoxynaphthalene by the method of Furst and Moore[87] afforded <u>6-acetyl-1-amino-2,3-dimethoxynaphthalene</u> whose NMR spectrum in deuteriochloroform was as follows: C_6-COCH$_3$, δ 2.65 (s); C_2-OCH$_3$, 3.90 (s); C_3-OCH$_3$, 3.94 (s); C_1-NH$_2$, 4.10 (broad s); C_4-H, 6.78 (s); C_5-H, 8.26 (d collapsing to broad doublet); C_7-H, and C_8-H, centered <u>ca</u>. 7.65 (m). Sublimation at 12-130°/HV afforded yellow micro crystals which upon recrystallization from ethanol yielded yellow plates, m.p. 146-147°. UV spectrum in methanol: λ_{max}: 383, 325sh, 296 mμ. λ_{min}: 354, 274mμ.

The IR spectra of the nitration products and two of their reduced products are recorded in Table XVI (Figs. 55-56). The IR spectrum of the 6-acetyl-1-amino compound (LXXV) is not reproduced here. A summary of the UV data on the nitration products are found in Table XVII and illustrated in Figs. 57-59.

E.   Synthesis of 2,6-Dimethoxynaphthazarin from 2,6-Dihydroxynaphthalene.

2,6-Dimethoxynaphthalene was prepared from 2,6-dihydroxynaphthalene and diazomethane. Isolation and purification in the usual manner yielded a compound of m.p. 149° (reported,[88] 150°) after sublimation at 60°/3mm. Upon

nitration of 2,6-dimethoxynaphthalene in glacial acetic acid by the method of Chakravarti and Pasupati,[88] 1,5-dinitro-2, 6-dimethoxynaphthalene was obtained in 65% yield, m.p. 268-270° (reported,[88] 265°). Its IR spectrum is shown in Fig. 60.

To a well-stirred mixture of 1 g of 1,5-dinitro-2,6-dimethoxynaphthalene in 10 g concentrated sulfuric acid, 0.35 g of powdered sulfur in 12 g oleum (30-33%) was added dropwise at such a rate that the temperature was not allowed to exceed 40°. The dark purple reaction solution, after an additional hour's stirring, was poured into ice water. It was filtered through a sintered glass funnel; the filtrate was collected and boiled for approximately 0.5 hr and finally extracted with benzene. The extract, after drying over sodium sulfate and reduced in volume, was columned over acid-washed silica gel with benzene. Six bands were observed. In order of elution they were yellow (fraction 1), orange (fraction 2) and dark orange (fraction 3) travelling closely together, dark red (fraction 4), yellow (fraction 5) and purple (fraction 6) bands.

Fraction 2, the major product, was isolated in only 20 mg yield. It was recrystallized from acetone as dark red needles, m.p. ca. 295-296°. NMR spectrum in deuteriochloroform: C_2-OCH$_3$, and C_6-OCH$_3$, δ 3.93 (s); C_5-OH, and C_8-OH, 13.07 (s); C_3-H, and C_7-H, 6.36 (s). The NMR spectrum of 2,6-dimethoxynaphthazarin is reproduced in Fig.

62.

     Anal.   *Calcd. for* $C_{12}H_{10}O_6$: *C, 57.60; H, 4.03.* *Found: C, 57.80, 57.70; H, 4.01, 4.13.*

     Absorption maxima in the IR spectrum (Fig. 61): *3.26b, 6.10sh, 6.25s, 6.35sh, 6.50sh, 6.75, 6.96, 7.10, 7.20sh, 7.60, 7.80, 7.95w, 8.25, 8.55, 8.76, 9.40wb, 10.00s, 10.30, 10.38sh, 11.05w, 11.50s, 12.00bs, 12.35sh, 13.15, 13.35sh, 13.85, 14.50b* μ .

     Visible-ultraviolet spectrum in methanol (Fig. 63), λ_{max}: *522 mμ (log ϵ, 3.44), 487 (3.66), 458 (3.62), 382sh (3.35), 304 (4.09), 267.6 (3.97);* λ_{min}: *513 (3.40), 470 (3.61), 411 (3.41), 350.5 (3.19), 279.8 (3.90), 251.8 (3.82).*

     The ultraviolet-visible data on other solvents are as follows:

     In methanolic potassium hydroxide (Fig. 64), λ_{max}: *578, 537, 501sh, 413, 316, 274.3 mμ.* λ_{min}: *556, 511, 459, 356.5, 287, 265.8 mμ.*

     In methanolic aluminum chloride (Fig. 65), λ_{max}: *538, 499, 468, 362.5, 348, 328, 314sh mμ.* λ_{min}: *565, 477, 419, 355, 335, 301 mμ.*

     Fraction 4 also crystallized from acetone as black micro crystals, m.p. 264-268° with decomposition. No combustion analyses was obtained because of its limited quantity. Ultraviolet data are as follows, λ_{max}^{MeOH} : *529sh, 492, 467sh, 308sh, 287.7bsh mμ.* λ_{min}^{MeOH}: *263, 278.5 mμ.*

     Fractions 1,3,5, and 6 failed to crystallize and

were not furthur characterized.
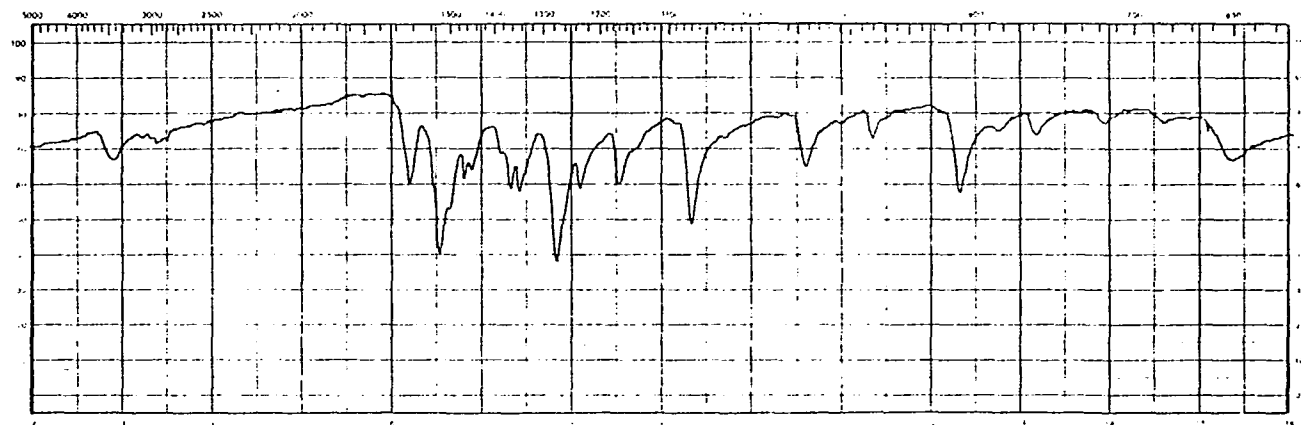
Fig. 60. Infrared Spectrum of 1,5-dinitro-2,6-dimethoxynaphthalene in KBr.
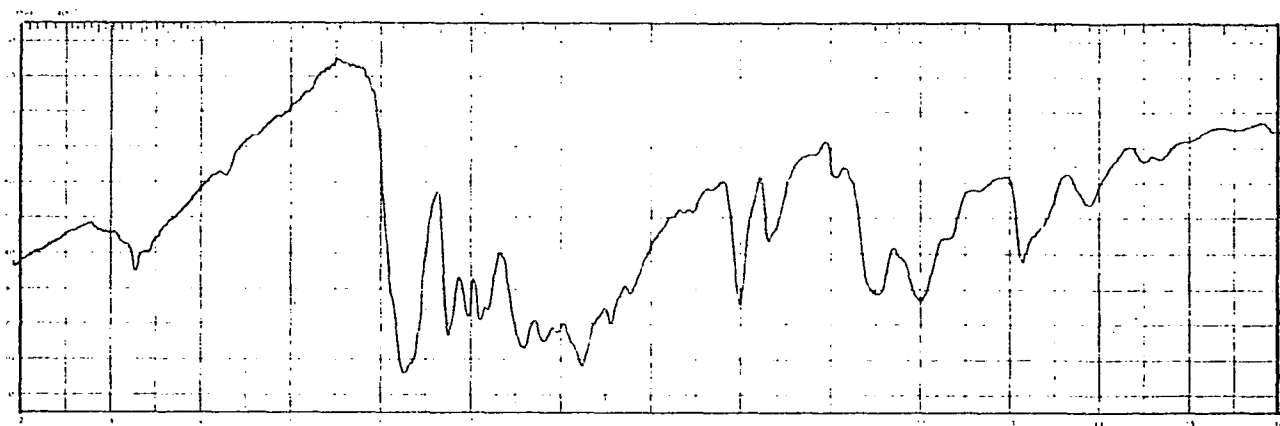
Fig. 61. Infrared Spectrum of 2,6-dimethoxynaphthazarin in KBr.
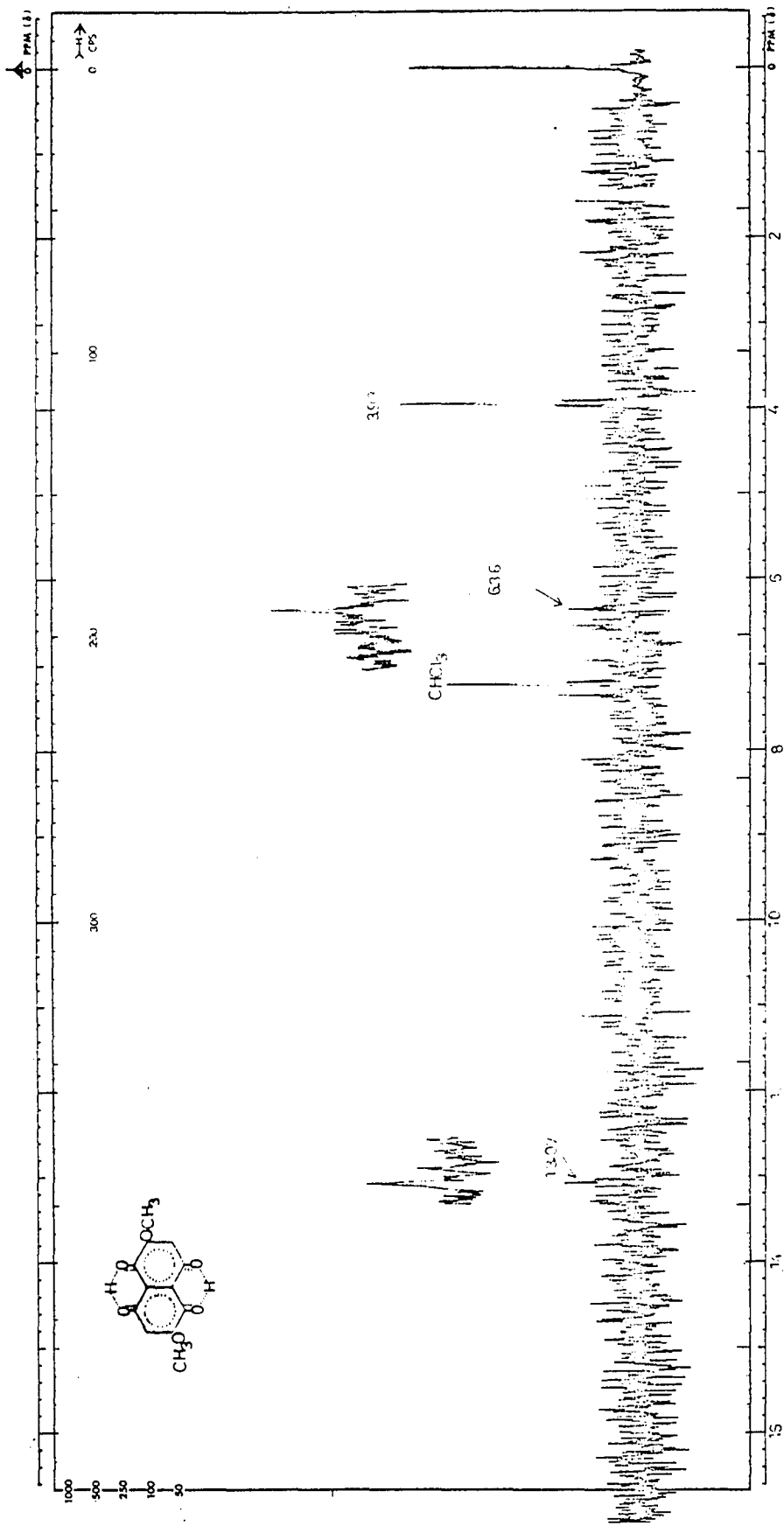
Fig. 62.   Nuclear Magnetic Resonance Spectrum of 2,6-dimethoxynaphthazarin in CDCl₃.
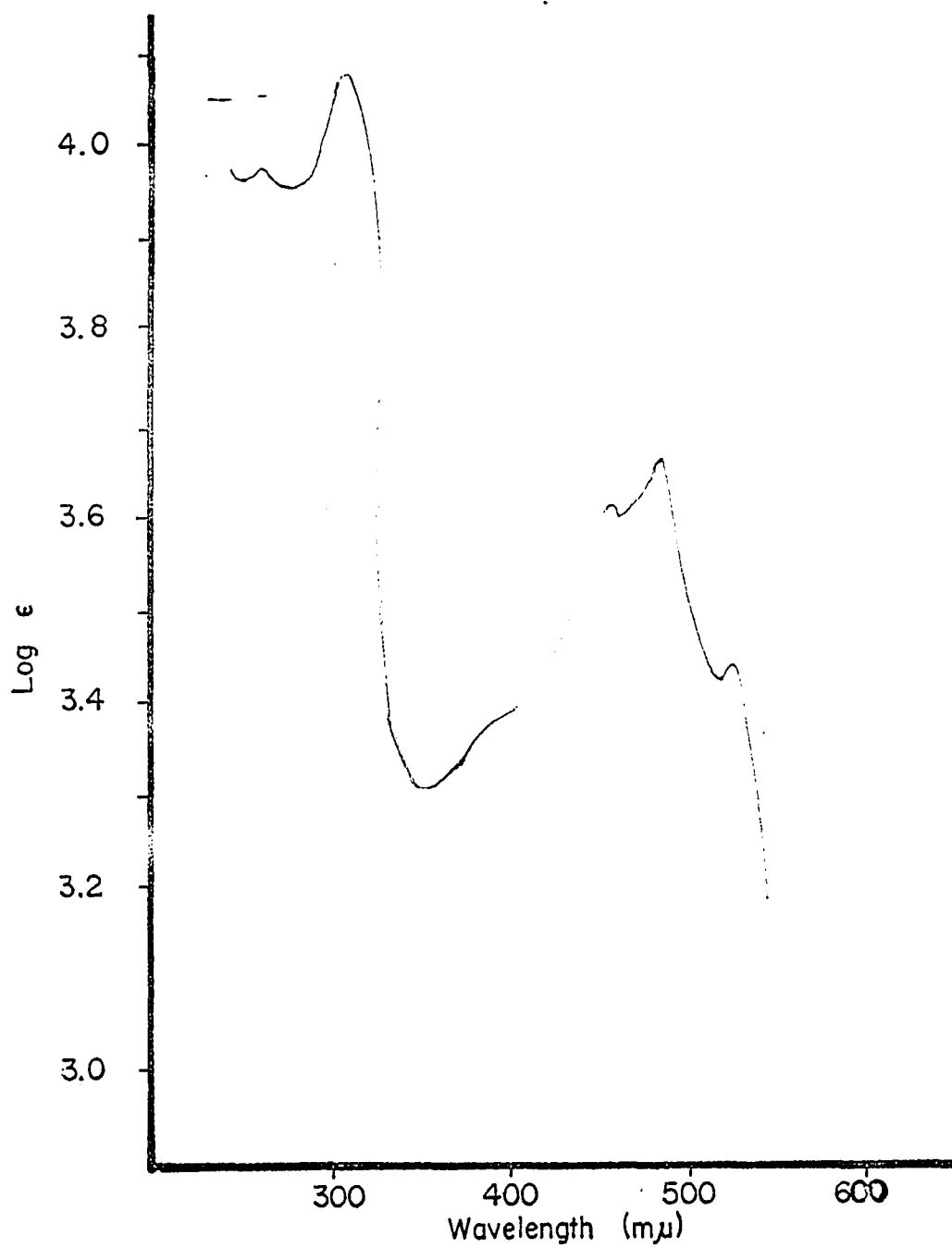
Fig. 63. Ultraviolet and Visible Absorption Spectrum of 2,6-dimethoxynaphthazarin in Methanol.
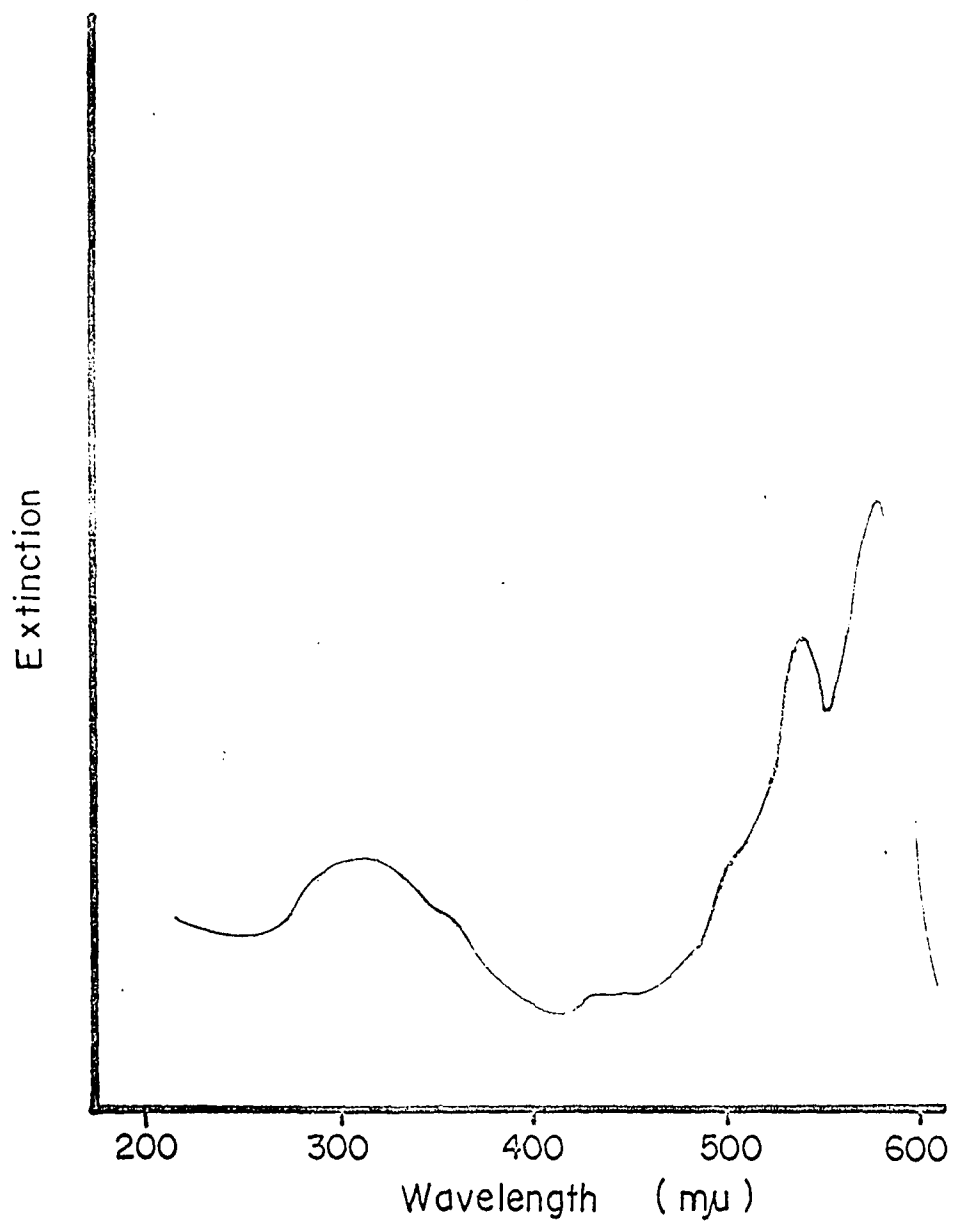
Fig. 64.   Ultraviolet and Visible Spectra of 2,6-Dimethoxy-
naphthazarin in Methanolic Potassium Hydroxide.
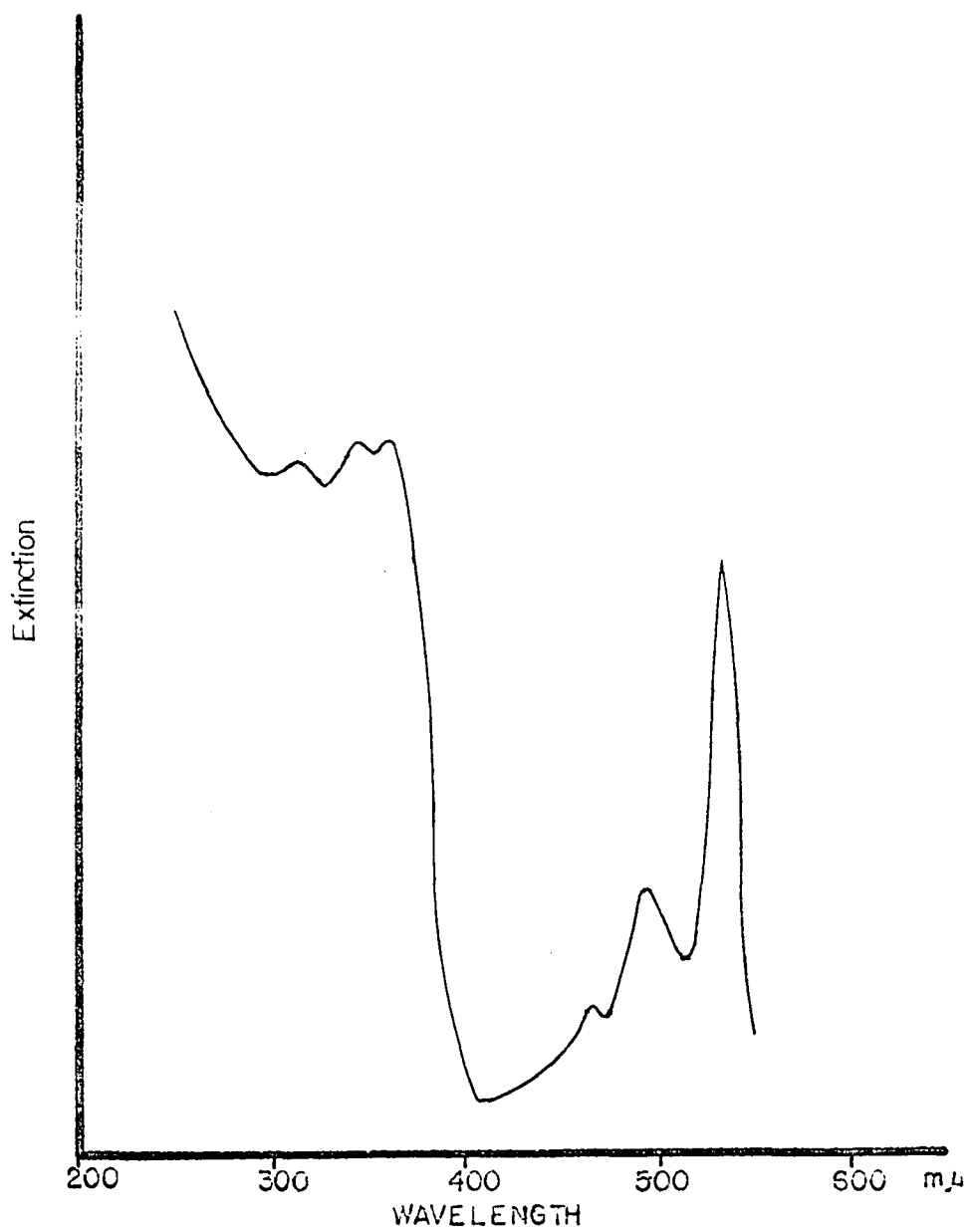
Fig. 65.   Ultraviolet and Visible Spectra of 2,6-Dimethoxy-
naphthazarin in Methanolic Aluminum Chloride.

Chapter III

RESULTS AND DISCUSSION

This part of the present investigation was concerned with reaction intermediates which might lead to spinochrome H and other naphthazarin analogs. Specifically, these compounds were dihydroxy- or dimethoxy-naphthalenes with nitro groups favorably placed in the peri positions so that the reductive-oxidative method of Roussin[76] might lead to naphthazarin compounds. With the requirement that the nitro groups are substituted in the desired peri positios of the naphthalene nucleus, two approaches seem plausible: firstly, nitration of the naphthalene compound bearing an acetyl group in the _beta_ positions, and secondly, introduction of the acetyl group after formation of the naphthazarin compound. In either case a hydroxy group would have to be introduced ortho to this acetyl group. This step is a known reaction which is commonly referred to as the Thiele or Thiele-Winter reaction. The problem of introducing an acetyl group into a naphthazarin intermediate, however, is more complicated. However, Fieser and coworkers were successful in introducing aliphatic gr ups into the quinonoid nucleus utilizing either red lead and an ester[89] or a diacetyl peroxide.[90] These analogies seemed encouraging and might be applicable in our own attempts to synthesize spinochrome H.

The preparation of 2-acetyl-3-hydroxy-1,4-naphtho-

quinone from 1-naphthol following the procedure of Cram[60]

led to the compound believed to be the desired one. The

product possessed a melting point identical with that

reported by Cram and combustion analyses agreed with the

molecular formula of the compound in question. The NMR

spectrum proved to be interesting. Integration of the spec-

trum was in agreement with the assignments of the acetyl and

aromatic protons (ratio of 3:4), but no proton hydrogen-

bonded to an acetyl group could be located. No IR compari-

son of this compound could be made since neither Cram[60] nor

Spruit[61] reported any IR data.

We had attempted to obtain 2-acetyl-4,8-dinitro-

1- naphthol (LXXVI) by using an excess of nitric acid on

2-acetyl-1-naphthol, a reaction which at first seemed possi-

ble since nitration of naphthalene results in a good yield
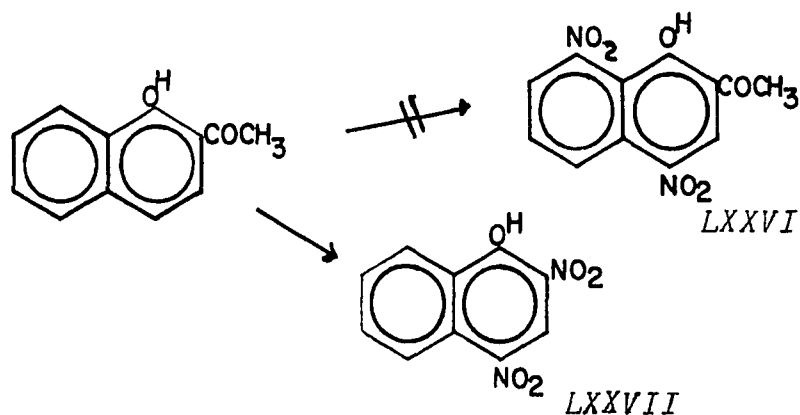
of 1,5-dinitro-naphthalene. Instead product LXXVII was

isolated along with some 2-acetyl-4-nitro-1-naphthol. This

represents an example of an anomolous nitration reaction, a

subject which was reviewed by Nightingale.[91]

Nitration of 2,7- and 2,6-dimethoxynaphthalene by established procedures afforded unambigous products in good yields (ca. 80% for both compounds). When 2,3-dimethoxynaphthalene was nitrated only a gummy precipitate was obtained which at first appeared intractable. The reaction products, however, were cleanly separated by column chromatography, characterized, and identified by NMR.

While our work was in progress, Bell and Buck[92] published experimental data on the nitration products of 2,3-dimethoxynaphthalene. Nitration in acetic acid yielded an oil from which only one product having melting point 152-154° was isolated and assigned the structure of 1-nitro-2,3-dimethoxynaphthalene.[92] With a two-fold excess of fuming nitric acid 1,4-dinitro-3-methoxy-2-naphthol was assigned via IR the structure for the compound isolated having melting point 214-216°.[92] When the nitration was carried out in aqueous nitric acid another compound, also possessing the melting point of 214-216°, was obtained. However, this compound did not show a positive ferric chloride test and an admixture with the reported 1,4-dinitro-3-methoxy-2-naphthol caused a depression. The structure 1,4-dinitro-2,3-dimethoxynaphthalene was also assigned by infrared spectroscopic data.

From the products which were isolated in our work it was shown that 1-nitro-2,3-dimethoxynaphthalene has the melting point of 88.5-89° and not 152-154° as reported.[92] The NMR spectrum confirmed our structure (C_4-H, δ 7.58, s).
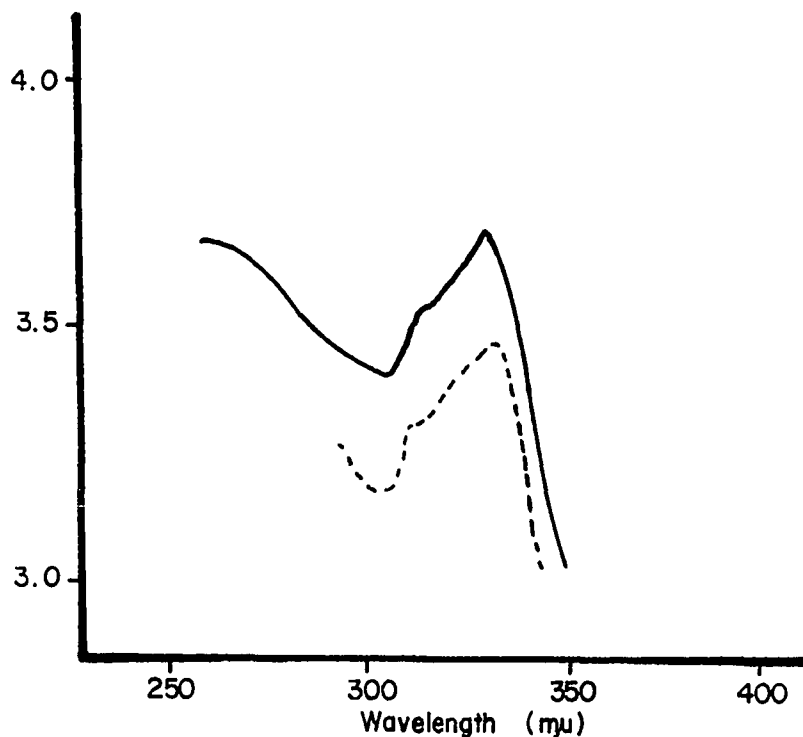
Fig. 66. Comparison of Ultraviolet Spectra of I-Nitro-
2,3-dimethoxynaphthalene (---) and I-Nitro-
2,7-dimethoxynaphthalene (—) in Methanol

In addition, 1-nitro-2,3-dimethoxynaphthalene had the same

ultraviolet spectrum as 1-nitro-2,7-dimethoxynaphthalene

(see Fig. 66).

2,3-Dimethoxynaphthalene exhibits the typical

A_2B_2-*type spectrum for the four protons of the unsubstituted*

ring of the naphthalene system. The two alpha *protons are*

at lower field than the two beta *protons due to the ring*

current effect.[93] *In both the 1-nitro- and 1,6-dinitro-*

compounds, the chemical shift of the C_8-*H was shifted up-*

field due to the steric effect of the ortho substituted

methoxy group, an effect already noted in 1-nitro-2-methyl-

naphthalene.[94] *Normally a -0.47 p.p.m. shift is observed*

for the C_8-*H in 1-nitronaphthalenes having no substituent in*

the 2-position. This is compared with the shift of the C_4-H

in the 5-nitro-2,3-dimethoxynaphthalene compound, whose

structure is also confirmed by the AB_2-type spectrum of the

C_6-, C_7-, and C_8-protons.

Nitration of 1,5-dihydroxy-2-acetylnaphthalene

resulted in two products whose structures were unambigously

assigned to be 2-acetyl-6-nitro-1,5-dihydroxynaphthalene and

2-acetyl-4,6,8-trinitro-1,5-dihydroxynaphthalene. In the

latter case only two aromatic singlets confirmed that the

two para and one ortho positions with respect to the 1,5-

dihydroxy groups in the starting material have been substi-

tuted by three nitro groups into those positions. In the

former case, two low field protons assigned to the hydrogen-

bonded proton of the remaining hydroxy group b a nitro sub-

stituent were confirmed by NMR data. Two AB quartets cor-

responding to a pair of ortho protons (J = ca. 8.5 cps)

further confirmed the assigned structure.

Though we were able to ascribe the low field

signal (б 13.68) to the C_1-OH bonded to C_2-$COCH_3$ on the

basis of model compounds, no definite assignments of chemi-

cal shifts of the aromatic protons could be made. All four

aromatic protons are either influenced inductively by the

acetyl and nitro substituents or by the anisotropic effect

of the hydroxy groups on the peri protons or by a combina-

tions of these effects.

The model compounds used for the assignments of

the hydrogen-bonded hydroxyls were 4-nitro-2-acetyl-1-

naphthol and 2,4-dinitro-1-naphthol whose hydroxy protons

occurred at δ 14.55 and δ 12.50 respectively. Since both of

these compounds have a nitro substituent in the 4-positions

the only influence of the chemical shift of the hydroxyl

group is the ortho substituent in their respective mole-

cules. Thus the hydroxyl group bonded to the oxygen atom

of the acetyl group occurs at lower field relative to the

hydroxy group bonded to a nitro substituent.

While 1,5-dinitro-2,6-dimethoxynaphthalene afforded

2,6-dimethoxynaphthazarin in only three per cent yield with

a mixture of other products which were not fully character-

ized, the reaction of 1,8-dinitro-2,7-dimethoxynaphthalene

with sulfur in fuming sulfuric acid resulted in at least 15

distinct bands on a column of acid-washed silica gel. The

experimental details were not reported in the Experimental

because many of the fractions were trace components and

therefore could not be fully characterized. Fierz-David and

Stocker[95] also reported poor yields of naphthazarin using

oleum and reducing agents such as sulfur or tin.

Attempts to isolate the di- or trinitroderivatives

with structures such as LXXVIII & LXXIX were unsuccessful,

LXXVIII   *LXXIX*
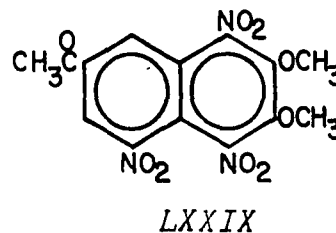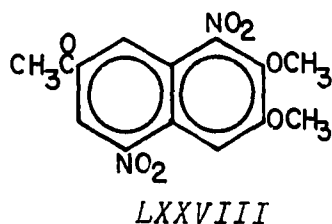
even when the reaction was carried out at 60° in acetic anhydride.

These compounds would be valuable in that they are precursors of the napthazarin compound, IV

SUMMARY AND CONCLUSIONS

Structural studies on spinochrome H have resulted in additional information and some clarification in the area of spinochrome research. Spinochrome H not only occurs in _Echinometra oblonga_ (Bl.), the sea urchin studied by Amai and by Temple, but also in major amounts in _Colobocentrotus_ _(Podophora) atratus_ (Linn.) and in smaller amounts in _Trip-neustis gratilla_ (Linn.) and _Echinothrix diadema_ (Linn.). It is also found in the Japanese sea urchins, _Anthocidaris crassispina_ (Ag.), and _Psuedocentrotus depressus_ (Ag.), a finding which is based on direct comparison of authentic samples generously supplied by the Japanese workers (see Appendix). Reported UV-visible data, behaviour of the pigments on the column, and almost identical melting points also suggest strongly that spinochrome A is identical with spinochrome H. These observations, whose confirmation is pending until an authentic sample can be obtained or pre-pared, therefore suggest that spinochrome H occurs also in the Mediterranean sea urchins, _Echinus esculentus_ (Linn.) and _Paracentrotus lividus_ (Lam.). Its presence in the spines of the Californian sea urchin, _Paracentrotus (Strongylocen-trotus) purpuratus_, whose crystals (m.p. 187-188) were ob-tained by Tyler[20] but were never analyzed may also prove to be identical with our isolated product. All these observat-ions gathered from the work in our Laboratory and from a reexamination of the chemical literature therefore lead one

to suspect that the purple pigment isolated from Hawaiian sources is, in fact, found extensively in the temperate regions (Hawaii, California, Japan, and the Mediterranean area). Gough and Sutherland[39] proved that spinochrome N from the Japanese sea urchins[27-29] was identical with spinochrome B which was isolated from Atlantic and Mediterranean sources. Their sample was isolated from Salmacis sphaeroides, probably common to the Australian shores, although the authors did not mention where their animals had been collected. The identity of spinochrome H with the pigment from Japanese sources (spinochrome M) was proven (see Appendix) by direct comparison with an authentic sample. The melting point of spinochrome H (192-193°) is identical with the melting point of spinochrome M, which was reported by the workers for the sample supplied to us as melting at 193°. The melting point for spinochrome M has been revised to occur at 195-196.5° in one of their later publications.[34]

Though the signal to noise ratio was high, the NMR spectrum of the pigment in deuteriochloroform showed no recognizable signals characteristic of the olefinic protons of the double bond in their proposed structure for spinochrome M. Comparison of spinochrome H and spinochrome M by TLC reveal similar R_F ratios and likeness in color on the plate (see Appendix). UV-visible data also proved that spinochrome M and spinochrome H are identical. The spectra were measured in methanol, methanolic aluminum chloride, and

methanolic potassium hydroxide and are shown in Table XVIII.

In view of these findings one is forced to conclude that the proposed structure for spinochrome M is incorrect. It should be pointed out here that the uncertainty of the UV data for spinochrome M obtained by us from a small graph (see Table IX) in Okajima's paper[35] and the number of papers which seemingly supported the proposed structure had made it implausible to relate our pigment with spinochrome M. Most of the work published in support of its structure had been based solely on combustion data, a criterion which in spinochrome research must be sup.lemented by other physical data.

No definite statement can be made regarding the possible identity of spinochromes H and A. Although UV-visible spectral data suggest identity, it has not been possible to secure an authentic sample.

Two alternate structures have been proposed for spinochrome H, differing only in the placement of a hydroxy group in the beta positions of the naphthazarin nucleus. The naphthazarin structure was demostrated by UV-visible studies with methanolic aluminum chloride. Assignment of a hydroxy group ortho to the acetyl group was supported by chemical and physical studies.

A 2,7-dihydroxy-3-acetyl-naphthazarin structure is favored biogenetically for spinochrome H by the acetate rule. Though biosynthetic pathways of many naturally occurring

phenolic compounds have been worked out, these have been
demonstrated exclusively for compounds isolated from the
plant kingdom, and practically no biosynthetic work invol-
ving invertebrates or other animal sources have been re-
ported. A function of the chemically related compound,
echinochrome A as an oxygen carrier in the internal organs
of sea urchins was postulated by Hoffmann-Ostenhof,[96] *who*
also suggests that echinochrome may be linked to a protein
molecule. No hypothesis of the function of spinochromes in
the tests and spines of sea urchins has been presented.

Several plausible pathways involving the synthesis
of spinochrome H were proposed. Preliminary work was carried
out on the reductive-oxidative method utilizing powdered
sulfur in oleum on nitronaphthalenes. Structural assign-
ments of some of the nitration products were based on NMR
studies and other physical methods and in some instances, on
chemical behavior.

Spinochrome H and its congeners have structural
features, such as ortho dihydroxyls or hydroxyl ortho to a
carbonyl group, which make them attractive for chelation
studies. For example, rhodizonic acid was used to preci-
pitate strontium ions preferentially in the presence of an
excess of calcium ions.[97] *Recently West and coworkers*[98,99]
postulated a class of new aromatic anions whose precursors
were given the name <u>*oxocarbons*</u>. *A study of complex forma-*
tion of spinochrome H and its relatives would add to our

knowledge of this type of compounds.

chrome D is stable to base).

C.   *NMR Spectrum of Spinochrome M in Deuteriochloroform.*

Signals were observed at δ 6.68 (s, aromatic proton), 2.85 (s, acetyl protons), and 13.03 (s, chelated hydroxy proton). Because of the high signal to noise ratio the reported chemical shifts were confirmed by integration. The relative intensities accounted for the nucleoid proton (1) and acetyl protons (3). However, only one of the chelated hydroxy protons was located.

Table XVIII. Visible-Ultraviolet Spectra of Spinochromes from Japanese Sea Urchins.*

Compound in solution	Wavelength ($m\mu$)					Color of solution
Spinochrome D						
In MeOH,	λ_{max}: 495, 369, 266.5					pink
	λ_{min}: 420, 247.5					
In KOH/MeOH,	λ_{max}: 560, 483, 398.5, 292.5					violet
	λ_{min}: 492, 445, 344, 257.3					
In $AlCl_3$/MeOH,	λ_{max}: 501, 472, 365					violet
	λ_{min}: 481, 437, 342.5					
Spinochrome F						
In MeOH,	λ_{max}: 459, 339.5sh, 293.2					orange
	λ_{min}: 389.5, 260					
In KOH/MeOH,**	λ_{max}: 400sh, 330sh, 298					colorless
In $AlCl_3$/MeOH,	λ_{max}: 509, 480sh, 359, 316					pink
	λ_{min}: 337, 340.5, 300					
Spinochrome M						
In MeOH,	λ_{max}: 509, 315					pink
	λ_{min}: 389, 291					
In KOH/MeOH,	λ_{max}: 556, 467, 445, 325, 284.6sh					violet
	λ_{min}: 485, 452, 420, 296					
In $AlCl_3$/MeOH,	λ_{max}: 556, 489, 450, 364, 336.7sh, 316.5					violet
	λ_{min}: 510, 468, 438, 353, 298.2					

Table XVIII, continued.

<u>*Spinochrome*</u> <u>*N*</u>

In *MeOH*, $\{$ λ_{max}: 479, 387.5, 320   orange
             λ_{min}: 444, 354, 287

In *KOH/MeOH*,[**] λ_{max}: 393sh, 345sh   *l. green*
                                                                   colorless

In *AlCl₃/MeOH*, $\{$ λ_{max}: 472, 440, 367, 281, 275   *green*
                      λ_{min}: 429, 332, 278.3

---

[*]   *Cf. Tables II and IX. Samples of the above compounds were obtained by Professor Scheuer from Dr. Kuroda and Dr. Okajima Laboratories while attending the Fourth IUPAC symposium on the Chemistry of Natural Products held at Kyoto, Japan, on April 12-18, 1964.*

[**] *Samples are unstable in methanolic potassium hydroxide.*

Table XIX. Thin-Layer Chromatography Results of the Spinochromes from Japanese Sea Urchins.*

R_F, using solvent:**

Compound	I	II	III	IV
Spinochrome N	0.02	0.10	0.66	ca. 0.90
Spinochrome D	0.03	0.10	0.64	ca. 0.90
Spinochrome F	0.09	0.22	0.75	0.91
Spinochrome M	0.25	0.45	0.87	0.93
Spinochrome H	0.29	0.44	0.85	0.95

---

* Samples supplied by the Japanese workers.

** Thin layer plates prepared according to the method of Stahl (see p. 31) using deactivated silica gel. The solvent systems were equilibrated in the developing chambers at room temperature (28°) and the runs were made over a distance of 10 cm. Solvent systems: (I) chloroform; (II) chloroform-ethyl acetate, 9:1/v:v; (III) chloroform-ethyl acetate, 1:1/v:v; (IV) ethyl acetate.

REFERENCE LIST

1. Robert L.S. Amai, "A Study of the Purple Pigment of *Echinometra* *oblonga* (Blainville)," M.S. Thesis, University of Hawaii, 1956.

2. Ralph E. Temple, "Structural Studies on Spinochrome H," M.S. Thesis, University of Hawaii, 1960.

3. On the chromatography of the blood of some invertebrates. C.A. MacMunn. Quart. J. Micropscop. Sci., 25, 469-490 (1885).

4. R.H. Thomson, "Naturally Occurring Quinones," Butterworths Scientific Publications, London, 1957.

5. Studies on pigments of marine animals-III. Echinochrome A from the spines of sand-dollar, *Echinarachnius* *mirabilis.* Kokichi Nishibori. Bull. Japan Soc. Sci. Fisheries, 22, 708-712 (1957).

6. A new pigment from the sea cucumber, *Polycheria* *rufescens* (Brandt). Toshihiko Mukai, and Tokuichi Tsumaki. Mem. Fac. Sci., Kyushu Univ., Ser. C, 4, 193-195 (1961).

7. Conversion of namakochrome into spinochrome E. Masaru Yamaguchi, Toshikiko Mukai, and Tokuichi Tsumaki. Mem. Fac. Sci., Kyushu Univ., Ser. C, 4, 193-195 (1961).

8. Echinochrome, a red substance in sea urchins. J.F. McClendon. J. Biol. Chem., 11, 435-441 (1912).

9. Echinochrome, its isolation and composition. E.G. Ball. J. Biol. Chem., 114, Sci. Proc. XXV, vi (1936).

10. Sur l'échinochrome et le spinochrome. Edgar Lederer and Rachel Glaser. Compt. Rend., 207, 454-456 (1938).

11. *Echinochrome et spinochrome; dérivés méthylés; distribution; pigments associés. Rachel Glaser and Edgar Lederer. Compt. Rend.,* <u>208</u>, *1939-1942 (1939).*

12. *Über die chemische natur des stoffs, den die eier des seeigels (<u>Arbacia pustulosa</u>) absondern, um die spermatozoen anzulocken. Richard Kuhn and Kurt Wallenfels. Chem. Ber.,* <u>72</u>, *1407-1413 (1939).*

13. *Echinochrome als prosthetische gruppen hochmolekularer symplexe in den eiern von <u>Arbacia pustulosa</u>. Richard Kuhn and Kurt Wallenfels. Chem. Ber.,* <u>73</u>, *458-464(1940)*

14. *Isolation of echinochrome A from the spines of the sea-urchin, <u>Diadema setosun</u> (Leske). K. Nishibori. Nature,* <u>184</u> *1234 (1959).*

15. *Isolation of echinochrome A from the spines of the sea-urchin, <u>Stomopneustes variolaris</u> (Lamarck). K. Nishibori. Nature,* <u>192</u>, *1293 (1961).*

16. *Synthese von echinochrom A. Kurt Wallenfels and Adeline Gauhe. Chem. Ber.,* <u>76</u>, *325-327 (1943).*

17. *Echinochrome. Robert K. Cannan. Biochem. J.,* <u>21</u>, *184-189 (1927).*

18. *Pigment of Arbacia eggs as an indicator of intracellular pH. Fred Vlès and Edmond Vellinger. Bull. Inst. Oceanogr.,* <u>513</u>, *1-16 (1928).*

19. *Sur les pigments naphtoquinoniques des épines et du test des oursins <u>Paracentrotus lividus</u> et <u>Arbacia pustulosa</u>. Edgar Lederer. Biochim. Biophys. Acta.,* <u>9</u>,

92-101 (1952).

20. *Crystalline echinochrome and spinochrome. Their failure to stimulate the respiration of eggs and of sperm of Strongylocentrotus. Albert Tyler. Proc. Nat. Acad. Sci. U.S., 25, 523-528 (1939).*

21. *The nomenclature of the spinochromes of sea urchins. T.W. Goodwin, E. Lederer, and L. Musajo. Experientia, 7, 375 (1951).*

22. *A study of the pigments of the sea-urchins, Echinus esculentus L. and Paracentrotus lividus Lamarck. T.W. Goodwin and S. Srisukh. Biochem. J., 47, 69-76 (1950).*

23. *Lo spinochromo P. Luigi Musajo and Marcherita Minchilli. Gazz. Chim. Ital., 70, 287-292 (1940).*

24. *Su di un secondo pigmento degli aculei di Paracentrotus. Luigi Musajo and Margherita Minchilli. Boll. sci. fac. chim. ind. Bologna, 31, 113-116 (1942).*

25. *Über den stachelfarbstoff von Arbacia. Richard Kuhn and Kurt Wallenfels. Chem. Ber., 74, 1594-1598 (1941).*

26. *The pigments from the sea urchins and the syntheses of related compounds. Chika Kuroda and Hana Ohshima. Proc. Imp. Acad. (Tokyo), 16, 214-217 (1940).*

27. *Spinochrome B from Strongylocentrotus pulcherrimus. Chika Kuroda and H. Iwakura. Proc. Imp. Acad. (Tokyo), 18, 74 (1942).*

28. *The studies on the derivatives of naphthoquinones,VIII. The pigments from the sea-urchins, III. Chika Kuroda*

and Kisako Koyasu. Proc. Imp. Acad. (Tokyo), 20, 23-25 (1944).

29. The studies on the derivatives of naphthoquinone, IX. The pigments from sea-urchins, IV. Chika Kuroda and Masaye Okajima. Proc. Japan Acad., 26, 33-36 (1950).

30. The studies on the derivatives of naphthoquinone, X. The pigments from the sea urchins, V. Chika Kuroda and Masae Okajima. Proc. Japan Acad., 27, 343-345 (1951).

31. The studies on the derivatives of naphthoquinone, XI. The pigments from sea urchins, VI. Chika Kuroda and Masae Okajima. Proc. Japan Acad., 29, 27-29 (1953).

32. Studies on the derivatives of naphthoquinones, XII. The pigments from sea-urchins, VII. Chika Kuroda and Masae Okajima. Proc. Japan Acad., 30, 982-986 (1954).

33. Studies on the derivatives of naphthoquinones, XV. The pigments from sea-urchins, X. Chika Kuroda and Masae Okajima. Proc. Japan Acad., 36, 424-427 (1960).

34. Studies on the derivatives of naphthoquinones, XVI. The pigments from sea-urchins, XI. Chika Kuroda and Masae Okajima. Proc. Japan Acad., 38, 363-355 (1962).

35. A study on pigments of Japanese sea-urchins. Masae Okajima. J. Sci. Research Inst. (Tokyo), 53, 356-376 (1959).

36. Spinochrome E. J. Smith and R.H. Thomson. Tetrahedron Letters, 1963(1), 10-12.

37. Naturally occurring quinones. Part V. Spinochrome E

and N.   J. Smith and R.H. Thomson.   J. Chem. Soc.,
   1961, 1008-1012.

38. Naphthaquinone pigments in *Psammechinus miliaris*
   (Gmelin).   M. Yoshida.   J. Marine Biol. Assoc., U.K.,
   38, 455-460 (1959).

39. The structure of spinochrome B.   J. Gough and M.D.
   Sutherland.   Tetrahedron Letters, *1964*(6), 269-275.

40. Spinochrome P and echinochrome P.   Luigi Musajo and
   Maria DiFonzo.   Boll. sci. fac. chim. ind. Bologna, *29,*
   231-234 (1940).

41. Synthese von 2,3-dioxy-naphthochinonen aus substi-
   tuierten o-phthalaldehyden und glyoxal.   Friedrich
   Weygand, Kurt Vogelbach, and Konrad Zimmermann.   Chem.
   Ber., *80,* 391-401 (1947).

42. Studies on the derivatives of naphthoquinones, XIV.
   The Pigments from sea-urchins, IX.   Chika Kuroda and
   Masae Okajima.   Proc. Japan Acad., *34,* 616-618 (1958).

43. Paul J. Scheuer, Richard E. Moore, and Clifford W.J.
   Chang, unpublished results.

44. Technical Bulletin # 22.   Thin Layer Chromatography.
   Brinkmann Instruments Inc., N.Y., 1962.

45. A sensitive color reaction for certain quinones.
   Reginald Craven.   J. Chem. Soc., *1931,* 1605.

46. "Handbook of Chemistry and Physics," 42nd. Ed., The
   Chemical Rubber Publishing Co., Cleveland, 1961, p.
   1051.

47. *Sterols of some invertebrates. A new method for separation of sterols, new sterol from the slate pencil sea urchin.* Werner Bergmann and Irving I. Domsky. Ann. N.Y. Acad. Sci., 90, 906-909 (1960).

48. *Occurrence of cholesterol in Renilla reniformis and Echinometra lucunter.* Bernard Fursch, Helio Barreto, and Nikolai Sharpin. Bull. Soc. Chim. Belges., 72, 807-808 (1963).

49. *Studies on the derivatives of naphthoquinones, XIII. The pigments from the sea urchins, VIII.* Chika Kuroda and Miye Harada. Proc. Japan Acad., 31, 305-308 (1955).

50. *Paperchromatography of polyhydroxynaphthoquinones.* Masaru Yamaguchi. Mem. Fac. Sci., Kyushu U., Ser. C, 3, 189-192 (1961).

51. *Chromatographic analysis of benzoquinones.* Gösta Pettersson. J. Chromatog., 12, 352-357 (1963).

52. A.E. Gillam and E.A. Stern, *"An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry,"* Edward Arnold Ltd., London, 1954, p. 204.

53. *Absorption spectra of quinones. I. Naphthoquinones and naphthohydroquinones.* C.J.P. Spruit. Rec. trav. chim., 68, 309-324 (1949).

54. L.J. Bellamy, *"The Infrared Spectra of Complex Molecules,"* 2nd. Ed., John Wiley and Sons, Inc., New York, 1958, pp. 95-106.

55. *The OH out of plane deformation in intramolecularly*

hydrogen bonded phenols. R.A. Nyquist. Spectrochim. Acta, 19, 1655-1664 (1963).

56. *The hydrogen bond between oxygen atoms in some organic compounds. G.E. Hilbert, O.R. Wulf, S.B. Hendricks and U. Liddel. J. Am. Chem. Soc., 58, 548-555 (1936).*

57. *Spectral studies on phenolic compounds. T. Swain. Chem. & Ind. (London), 1954, 1480-1481.*

58. *T.A. Geissman, "The Chemistry of Flavonoid Compounds," Pergamon Press, Oxford, 1962.*

59. *Donald J. Cram, "Synthesis and Reactions of 2-(Keto-alkyl)-3-hydroxy-1,4-naphthoquinones," Ph.D. Thesis, Harvard University, 1947, pp. 8-9.*

60. *Preparation and Reactions of 2-Acyl-3-hydroxy-1,4-naphthoquinones. Donald J. Cram. J. Am. Chem. Soc., 71, 3953-3962 (1949).*

61. *Carbonyl-substituted naphthoquinones. Part I. Methyl ketones unsubstituted in the side chain. C.J.P. Spruit. Rec. trav. chim., 66, 655-672 (1947).*

62. *Mass spectra of various quinones and polycyclic ketones. J.H. Beynon and A.E. Williams. Appl. Spectr., 14, 156-160 (1960).*

63. *J.H. Beynon, "Mass Spectrometry and Its Applications to Organic Chemistry," Elsevier, Amsterdan, 1960, p.330.*

64. *Quinones. V. The chemistry of naphthazarin. D.B. Bruce and R.H. Thomson. J. Chem. Soc., 1955, 1089-1096.*

65. *The hydrogen bonds and the structure of naphthazarin*

(1,4-dihydroxy-5,8-naphthoquinone) by R. Blinc, D. Hadži and E. Pirkmajor in "Hydrogen Bonding," D. Hadži, Ed., Permagon Press, New York, 1959, pp. 333-338.

66. *The structure of elsinochrome A. T.J. Batterham and U. Weiss. Proc. Chem. Soc. (London), 1963, 89-90.*

67. *Studies in relation to biosynthesis. I. Some possible routes to derivatives of orcinol and phloroglucinol. A.J. Birch and F.W. Donovan. Australian J. Chem., 6, 360-368 (1953).*

68. *Studies in relation to biosynthesis. V. The structure of some natural quinones. A.J. Birch and F.W. Donovan. Australian J. Chem., 8, 529-533 (1955).*

69. *The biosynthesis of phenolic compounds from activated acid units by R.K. Rickards in "Chemistry of Natural Phenolic Compounds," W.D. Ollis, Ed., Permagon Press, New York, 1961, pp. 1-19.*

70. *Studies in mycological chemistry. Part I. Flaviolin, 2(or 3):5:7-trihydroxy-1,4-naphthoquinone, a metabolic product of Aspergillus citricus (Wehmer) Mosseray. B.D. Astill and J.C. Roberts. J. Chem. Soc., 1953, 3302-3307.*

71. *Studies in mycological chemistry. Part X. Synthesis of flaviolin (2,5,7-trihydroxy-1,4-naphthoquinone). B.W. Bycroft and John C. Roberts. J. Chem. Soc., 1962, 2063-2064.*

72. *Zur Konstitution und reacktionsweise mehrkerniger*

poly-chinone. Über naphtazarin und chinizarin.

Karl Zahn and Paul Ochwat. Ann., 462, 72-97 (1928).

73. *The new synthesis of hydroxydroserone. I. The pig-*
   ments of Drosera Whittakeri, pthiocol. II. The pig-
   ment of human tubercle bacillus and naphthopurpurin
   and the studies of the related compounds. Chika
   Kuroda. Proc. Imp. Acad. (Tokyo), 15, 226-229 (1939).

74. *The studies on the derivatives of naphthoquinone. The*
   syntheses of spinazarin, methyl-spinazarin and related
   compounds. Chika Kuroda. J. Sci. Res. Inst. (Tokyo),
   46, 188 (1952).

75. *C.A., 59, 9920 (1963). [Application of the diene*
   synthesis to the preparation of naphthazarins. F.
   Farina, M. Lora-Famayo and C. Suarez. Anales Real.
   Soc. Espan. Fis. Quim. (Madrid) Ser. B, 59 (3), 167-
   178 (1963).]

76. *See Ref. 64. Roussin, Compt. Rend., 52, 1033 (1861).*

77. *The addition of dienes to halogenated and hydroxylated*
   naphthoquinones. Louis F. Fieser and J.T. Dunn. J.
   Am. Chem. Soc., 59, 1016-1021 (1937).

78. *The tautomerism of hydroxy quinones. Louis F. Fieser.*
   J. Am. Chem. Soc., 50, 439-465 (1928).

79. *Synthesis of rubrofusarin dimethyl ether. Shoji*
   Shibata, Eisaku Morishita, and Yasuo Arima, Chem. &
   Pharm. Bull. (Tokyo), 11, 822-823 (1963).

80. *Shoji Shibata. Private communication through Professor*

Scheuer.

81. *Über umlagerungen in der gruppe der aceto-naphthole.*
    Otto N. Witt and Otto Braun. Chem. Ber., 47, 3216-
    3232 (1914).

82. *"Beilstein Handbuch Der Organischen Chemie," Band VI,*
    Berlin, Verlag von Julius Springer, 1923, p. 136.

83. *Some derivatives of 2-acetyl-naphthol-1. H.A. Torry*
    and E.J. Cardarelli. J. Am. Chem. Soc., 32, 1477-
    1488 (1910).

84. *C.A., 11, 1644 (1917). [Dihydroxynaphthalenes. IV.*
    2,7-Dihydroxynaphthalene dimethyl ether. O. Fischer
    and Wilh. Kern. J. prakt. Chem., 94, 34-48 (1916).]

85. *Reaktionen mit nitrosodisulfonat, V. Mitteil. Über*
    die bildung von naphthochinonen. Hans-Joachim Teuber
    and Norbert Götz. Chem. Ber., 87, 1236-1251 (1954).

86. *Compounds with potential activity against lethal*
    radiations. VI. Homologs of 2,3-dihydroxynaphthalene.
    Ng. Ph. Buu-Hoi and Denise Lavit. J. Org. Chem., 21,
    21-23 (1956).

87. *Reductions with hydrazine hydrate by raney nickel. II.*
    Aromatic nitro compounds to intermediate products.
    Arthur Furst and Richard E. Moore. J. Am. Chem. Soc.,
    79, 5492-5493 (1957).

88. *Naphthalene series. I. Preparation of polyhydroxy*
    derivatives of naphthalene. S.N. Chakravarti and
    V. Pasupati. J. Chem. Soc., 1961, 1859-1862.

89. *Alkylation of α-naphthoquinones with esters of tetravalent lead.* Louis F. Fieser and Frederic C. Chang. J. Am. Chem. Soc., 64, 2043-2052 (1942).

90. *Alkylation of para quinones with acyl peroxides.* Louis F. Fieser and Albert E. Oxford. J. Am. Chem. Soc., 64, 2060-2065 (1942).

91. *Anomolous nitration reactions.* Dorothy V. Nightingale. Chem. Revs., 40, 117-140 (1947).

92. *Some reactions of dimethoxynaphthalenes.* F. Bell and K.R. Buck. J. Chem. Soc., 1963, 1108-1118.

93. J.A. Pople, W.G. Schneider, and H.J. Bernstein. "High-resolution Nuclear Magnetic Resonance," McGraw-Hill, New York, 1959, pp. 250-251.

94. *Proton magnetic resonance spectra of nitronaphthalenes in dimethylacetamide solution.* P.R. Wells and P.G.E. Alcorn. Australian J. Chem., 16, 1108-1118 (1963).

95. *Zur Kenntnis des naphthazarins.* Hans Eduard Fierz-David and Walter Stockar. Helv. Chim. Acta., 26, 92-98 (1943).

96. *Die Biochemie der Chinone. Part II.* Otto Hoffmann-Ostenhof. Experientia, 3, 176-186 (1947).

97. *Herstellung reiner kristallisierten rhodizonsäure.* B. Eistert and G. Bock. Angew. Chemie., 70, 575 (1958).

98. *New aromatic anions. III. Molecular orbital calculations on oxygenated anions.* Robert West and David L. Powell. J. Am. Chem. Soc., 85, 2577-2584 (1963).

99. *New aromatic anions. VII. Complexes of squarate ion with some divalent and trivalent metals.* Robert West and Hsien Ying Hiu. *J. Am. Chem. Soc.,* <u>85</u>, 2589-2590 *(1963).*