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THE REACTIONS OF AMINOCHROMES WITH THIOLS

A thesis

Submitted to the Faculty of Graduate Studies

in partial fulfilment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in the Department of Chemistry

Dalhousie University

by

W. S. Powell

Halifax, Nova Scotia

January, 1972

Approved by: 

DALHOUSIE UNIVERSITY

Date Jan. 20, 1972

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Title The Reactions of Aminochromes with Thiols

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ABSTRACT

Prior to the commencement of this investigation it had been shown that, in general, aminochromes react with thiols to give three major products: 5,6-dihydroxyindole reduction products (I); 5,6-dihydroxyindole thioethers (II); and aminochrome/thiol addition products (III).

Various derivatives of the two thiosubstituted products (II and III) have been isolated and their structures determined unambiguously. The indolic products II have been shown to be 4-thiosubstituted 5,6-dihydroxyindoles, whilst the addition products III have been shown to be 9-thiosubstituted 4,9-dihydroaminochromes. Epinochrome reacts with thiols to give, besides epinochrome/thiol addition products (cf. III), 5,6-dihydroxy-1-methylindoline and 4-thiosubstituted 5,6-dihydroxy-1-methylindolines. The formation of the 4-thiosubstituted 5,6-dihydroxyindoles (or indolines) in aminochrome/thiol reactions is favoured by low pH's whereas the formation of the addition products III is favoured by somewhat higher pH's. The effects of some substituents upon the courses of several aminochrome/thiol reactions have been investigated.

The preparation of some unhalogenated "nor" aminochromes is described.

PUBLICATIONS RESULTING FROM THIS WORK

W. S. Powell, R. A. Heacock, G. L. Mattok and D. L. Wilson
Chemistry of the Aminochromes. Part X. Some Further Observations on the Reactions of Aminochromes with Thiols.
Can. J. Chem., 1969, 47, 467

W. S. Powell and R. A. Heacock
Chemistry of the Aminochromes. Part XIII. Some Further Observations on the Reactions of Aminochromes with Thioglycollic Acid.
Can. J. Chem., 1969, 47, 2102

W. S. Powell and R. A. Heacock
Chemistry of the Aminochromes. Part XIV. Noradrenochrome.
Can. J. Chem., 1971, 49, 341

R. Marchelli, W. S. Powell and R. A. Heacock
The Adrenochrome-sodium Bisulfite Addition Product.
Chem. Ind. (London), 1971, 1021

W. S. Powell and R. A. Heacock
Adrenochrome-thiol Addition Products.
Experientia, in press

R. A. Heacock and W. S. Powell
Adrenochrome and Related Compounds.
Progr. Med. Chem., in press

GLOSSARY OF ABBREVIATIONS AND SYMBOLS

δ	chemical shift expressed in parts per million
λ_{max}	wavelength of maximum absorbance (nm)
ν	frequency (cm^{-1})
A	absorbance
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
DHMI	5,6-dihydroxy-1-methylindole
DMF	N,N-dimethylformamide
DMSO	dimethylsulphoxide
e.p.r.	electron paramagnetic resonance
GSH	glutathione
Hz	Hertz
i.r.	infrared
J	spin-spin coupling constant (Hz)
m	multiplet
$M^{+\cdot}$	molecular ion
m/e	mass to charge ratio
m.p.	melting point (°C)
n.m.r.	nuclear magnetic resonance
pH	the negative logarithm of the hydrogen ion concentration
PPM	parts per million
R_f	the distance moved by the substance in question divided by the distance moved by the solvent front

s	singlet
sh	shoulder
t	triplet
$T_{\frac{1}{2}}$	half time of reaction (min)
t.l.c.	thin-layer chromatography
TMS	tetramethylsilane
u.v.	ultraviolet
1, 2, 2A, 2B, etc.	these numbers (in italics) refer to either the positions around the indole (or partially reduced indole) nucleus or, in the case of $J_{X,Y}$, to the protons in these positions

ACKNOWLEDGEMENTS

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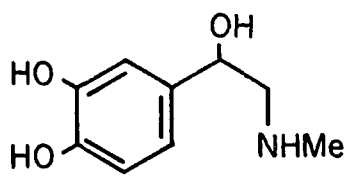
1 INTRODUCTION

1 INTRODUCTION

1.1 Introduction

In the middle of the last century Vulpian reported that aqueous extracts of mammalian suprarenal capsules developed a pink colouration upon standing in air[1,2]. The significance of these observations was not realized at the time and some 40 years elapsed before the potent physiological activity of the original colourless extracts was discovered[3]. The substance responsible for this activity was isolated several years later, around the turn of the century, and shown to be β-hydroxy-β-(3,4-dihydroxyphenyl)-N-methylethylamine (1)[4-6], which is now commonly known as adrenaline or epinephrine.

The colour reactions exhibited by adrenaline and related catecholamines in the presence of oxidizing agents were the basis of early qualitative and quantitative assay procedures for these compounds[7-10]. The first of these oxidation products to be correctly described in the literature was the red product obtained upon oxidation of 3,4-dihydroxyphenylalanine (dopa, 2). In 1927 Raper suggested



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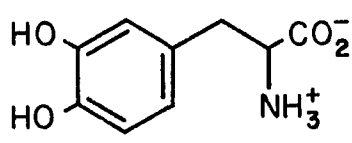

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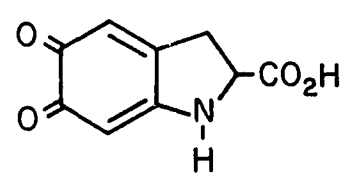
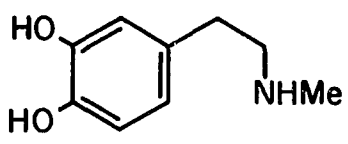


3

that this compound was indoline-5,6-quinone-2-carboxylic acid (3)[11] although he was unable to isolate it in pure form. Seven years later Burton obtained a crystalline monophenylhydrazone of epinochrome, the red oxidation product of epinine (4)[12]. In 1937 Green and Richter isolated the compound obtained upon enzymic oxidation of adrenaline as a red crystalline solid. They suggested that this compound, for which they proposed the name adrenochrome, was 3-hydroxy-1-methylindoline-5,6-quinone (5)[13]. In the same year Richter and Blashko obtained a crystalline iodo-derivative of adrenochrome by the oxidation of adrenaline with potassium iodate[14]. Two years earlier Weinstein and Manning had obtained a red crystalline compound upon oxidation of adrenaline in methanol but they erroneously considered their product to be adrenaline quinone (6)[15].



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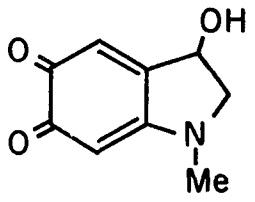

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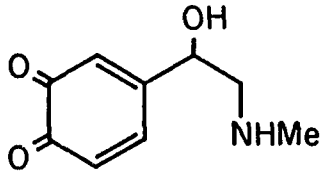

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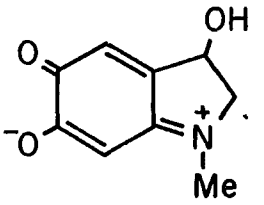

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In order to better explain the physical and chemical properties of adrenochrome Harley-Mason suggested in 1948 that this compound was a resonance hybrid of the structures 5 and 7 with the zwitterion 7 making the major contribution[16,17]. This would explain its formation of mono- rather than di- derivatives with typical ketone reagents and its failure to produce a phenazine derivative readily with o-phenylenediamine. Its colour, which is much more intense than other o-quinones, together with the fact that it is quite soluble in polar solvents but almost insoluble in nonpolar solvents would also be more consistent with the structure 7. These considerations would apply to all compounds of this type and the zwitterionic formulation is now generally accepted for the indoline-5,6-quinones obtained upon oxidation of catecholamines. The generic name "aminochrome" which is now widely used for this series of compounds was proposed in 1951 by Sobotka and Austin[18].

Interest in the aminochromes stems primarily from their occurrence in living organisms. They are intermediates in the formation of melanins, the dark naturally occurring pigments which are responsible for brown or black hair and skin colour. Furthermore, oxidation of catecholamines to aminochromes may constitute a minor, but normal, metabolic pathway for the latter compounds, although this is rather controversial. Secondly, the aminochromes and some of

their derivatives, notably of the substituted hydrazone type, have some interesting physiological activities and, in the case of the latter, may be used therapeutically.

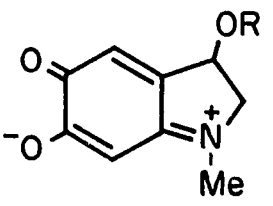
Developments in the chemistry, biochemistry, physiology and pharmacology of the aminochromes have been reviewed by a number of authors over the last 15 years[19-29].

1.2 Formation of aminochromes

1.2.1 Preparative procedures

The usual procedure for the preparation of aminochromes in crystalline form consists of the oxidation of the appropriate catecholamine with silver oxide in methanol. This reaction was first described by Veer in 1942[30] and many extensions and modifications of this general method have been described since this time (cf. Refs. 19-22). The products originally obtained in this manner tended to be somewhat unstable, probably due to the presence of some residual silver, but it was later found that filtration of the reaction mixture through a bed of sodium sulphate or an anion exchange resin directly prior to crystallization resulted in good yields of a pure, stable and relatively silver-free crystalline product[31]. The methyl and ethyl ethers of adrenochrome (cf. 8) were originally synthesized by oxidation of the corresponding catecholamines with silver oxide in acetonitrile[32], but more recently they

have been obtained by carrying out the reaction in methanol and then adding ether after the filtration step[33].



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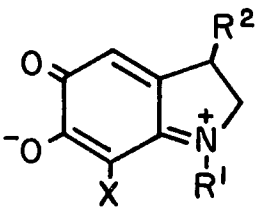

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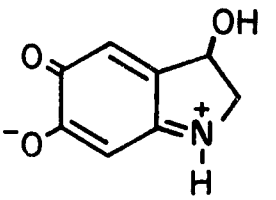

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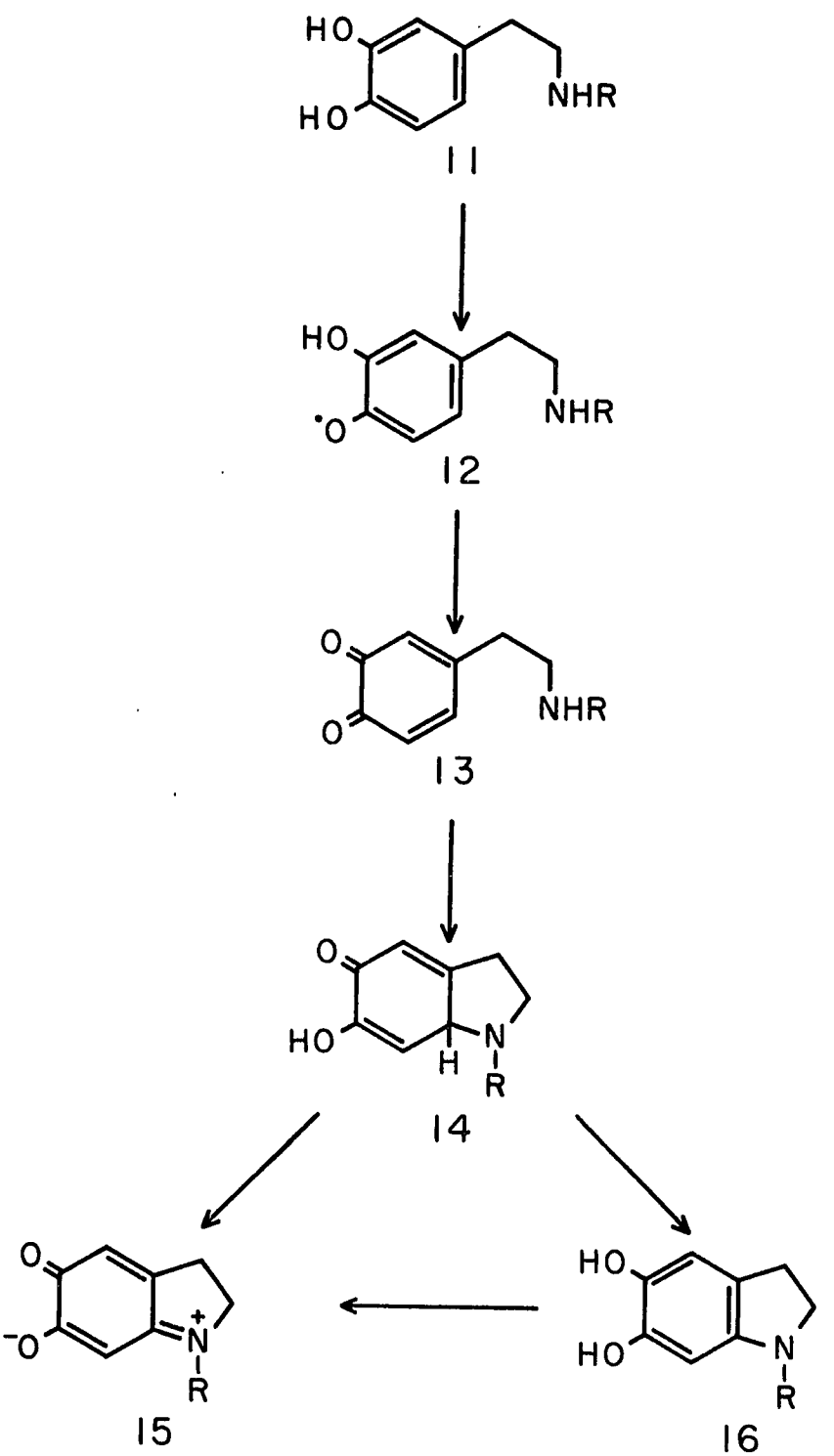
7-Iodoaminochromes (cf. 9) can be obtained by oxidation of catecholamines with iodine, potassium iodate or iodic acid in water and 7-bromoadrenochrome has been synthesized from the reaction of adrenaline with bromine in aqueous solution[19-22]. These reactions involve the initial oxidation of the catecholamine to the aminochrome. The latter then reacts with molecular halogen, which is either present initially or is produced in the preliminary reaction, to give the 7-haloaminochrome[34-36].

Prior to the present work no unhalogenated "nor" aminochromes (i.e. not bearing an alkyl substituent on the nitrogen atom) had been isolated in a pure form and fully characterized. Noradrenochrome (10) had been obtained as "red rings on the side of the flask" but the only physical property of the compound reported was its ultraviolet and visible absorption spectrum[37].

1.2.2 Mechanism of formation

According to the scheme originally proposed by Raper and his colleagues (cf. Scheme 1) in their classical studies on the oxidation of dopa to melanin[11,38], the first stage in the reaction was the oxidation of the catecholamine 11 to the open chain quinone 13. This was followed by cyclization to the leucoaminochrome 16 which was rapidly oxidized to the aminochrome 15.

More recent work has been aimed at establishing the existence of these and other intermediates. Since the formation of aminochromes from catecholamines occurs very rapidly detection of the intermediates is rather difficult and their isolation virtually impossible. The first step in the reaction appears to be a one electron oxidation to give the semiquinone 12[39-42], which is then oxidized to the open chain quinone 13. The reaction stops at this stage in strongly acidic solution[43] (cf. Refs. 21, 22) but in slightly acidic or neutral solution 13 undergoes an intramolecular 1,4-Michael addition reaction, presumably to give the intermediate 14. This may then tautomerize to give the leucoaminochrome 16 which would be oxidized to the aminochrome 15 as was suggested in Raper's original scheme[11,38,43]. Alternatively 14 could be converted directly to the aminochrome. This is, in effect, the type



SCHEME 1

of oxidative cyclization previously mentioned by Harley-Mason[17]. It has been suggested that if 3-hydroxyleuco-aminochromes, which lose water very readily, were intermediates in the reaction the corresponding 5,6-dihydroxyindoles might be expected to occur as by-products[22]. The fact that these products have not been detected in the reaction mixtures would tend to support the oxidative cyclization mechanism. On the other hand, however, if the leuco compound were formed it would be oxidized to the aminochrome very rapidly, either by the open chain quinone 13 or by excess oxidizing agent and consequently would have a very short lifetime. It is therefore rather difficult to state conclusively which mechanism is dominant.

1.3 Physical properties

1.3.1 General comments

The nonhalogenated aminochromes are deep red to red-violet crystalline solids, which are readily soluble in water, methanol, ethanol, dimethylsulphoxide and dimethyl-formamide, slightly soluble in some relatively polar organic solvents, such as acetonitrile or acetone and almost completely insoluble in nonpolar solvents such as benzene or cyclohexane. The halogenated aminochromes are usually deep violet-brown crystalline solids, which are only slightly soluble even in polar solvents. Most aminochromes,

with the exception of the 3-alkoxy derivatives, decompose upon heating without melting.

1.3.2 Ultraviolet and visible spectroscopy

The ultraviolet and visible absorption spectra of the aminochromes have been reported in detail in reviews by Heacock[21,22]. In general, the nonhalogenated aminochromes exhibit three absorption maxima, one in the 200-230 nm region, one at ca. 300 nm and a broad flat maximum in the visible range between 470 and 495 nm. The spectra observed for the 7-halogenated aminochromes are essentially the same except that the maximum observed in the visible range is shifted to longer wavelengths (i.e. ca. 520-535 nm). A similar shift is observed in the case of 7-methyladrenochrome which has a λ_{max} in the visible region at 534 nm[44].

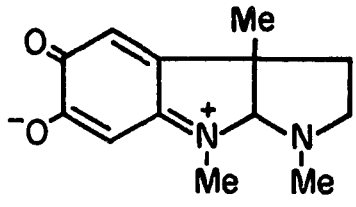
1.3.3 Infrared spectroscopy

The infrared spectra of some aminochromes have also been discussed by Heacock[22]. The infrared spectrum of adrenochrome itself (in Nujol) exhibits an OH stretching peak at 3295 cm^{-1}. Peaks are observed at 1682 cm^{-1} and 1672 cm^{-1} which are probably due to the C^5-carbonyl group and to the C^4-C^9 double bond respectively. There are peaks at 1622 cm^{-1} and 1575 cm^{-1} (the latter being very intense) which may be attributed to the vinylogous amide system

(involving the C^6 polarized carbonyl group and the nitrogen atom). In general, the infrared spectra of other aminochromes show basically similar features in the carbonyl region to that of adrenochrome[22,29].

1.3.4 Nuclear magnetic resonance spectroscopy

Some features of the n.m.r. spectrum of adrenochrome (7) were first reported by Robinson in 1965, who used the compound as a model for studying the structure of the red oxidation product of physostigmine known as rubreserine (17)[45].



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Robinson reported that the n.m.r. spectrum of adrenochrome (7) in DMSO showed singlets at δ, 5.47 and δ, 6.50 which he assigned to the protons at C^4 and C^7 respectively (see Sect. 3.2.1, p. 90).

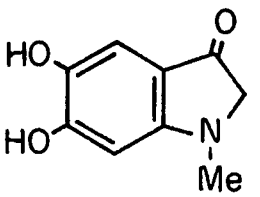
Robinson also reported the n.m.r. spectrum of rubreserine (17) in the same paper and concluded that, as in the case of adrenochrome, the zwitterionic form makes the major contribution to its structure.

1.4 Chemical properties

1.4.1 Rearrangement

One of the most characteristic reactions of the aminochromes is their rapid rearrangement to 5,6-dihydroxyindoles or 5,6-dihydroxyindoxyls. This reaction is catalyzed either by base or by some metal cations, notably zinc ions (cf. Ref. 22). It is the basis of the widely used "trihydroxyindole" or, more appropriately, "lutin" method for the fluorimetric analysis of catecholamines.

The nature of the products formed in this reaction is dependent upon the structure of the aminochrome involved. Aminochromes with 3-hydroxyl substituents rearrange to the highly fluorescent 5,6-dihydroxyindoxyls. In the case of adrenochrome (7) the product obtained is adrenolutin (18). What was essentially the correct structure for this yellow fluorescent compound was first predicted on theoretical grounds by Ehrlén in 1948[46]. One year later it was isolated and named adrenolutin by Lund[47,48] who considered its structure to be 3,5,6-trihydroxy-1-methylindole (19). This



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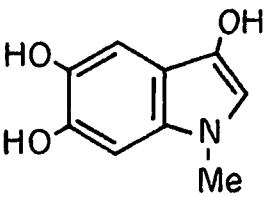

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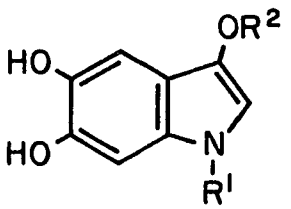
structure is still the one most often encountered in the literature in spite of the fact that Heacock pointed out[49] that the infrared spectrum of adrenolutin indicated that it, like most other indoxyls[50], exists predominantly in the keto form (i.e. 18) in the solid state. When 3-hydroxy-aminochromes are dissolved in mixtures of pyridine and acetic anhydride they first rearrange and are then acetylated to 3,5,6-triacetoxyindoles[22].

3-Alkoxyaminochromes rearrange to form nonfluorescent compounds, probably 3-alkoxy-5,6-dihydroxyindoles[51] (cf. 20). These indole derivatives, however, appear to be readily oxidized in air and have not yet been isolated in pure form.



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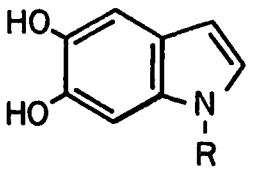

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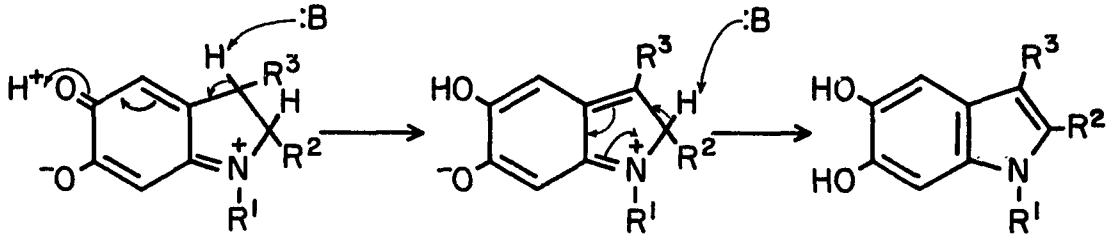
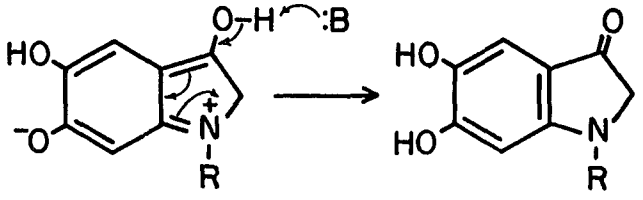
Aminochromes without a substituent in the 3-position rearrange with great facility to 5,6-dihydroxyindoles (cf. 21) which exhibit a characteristic blue fluorescence. If the rearrangement is carried out in pyridine/acetic anhydride the end products for these aminochromes are 5,6-diacetoxy-indoles[29].

As a result of a study of the kinetics of the rearrangement, Heacock and Mattok proposed the mechanism of the reaction to be as shown in Scheme 2[52]. In the case



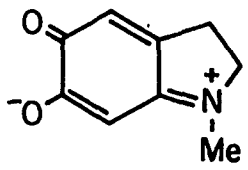
SCHEME 2

of 3-hydroxyaminochromes the last step could possibly be modified as shown in Scheme 3. The presence of a 3-hydroxyl



SCHEME 3

or 3-alkoxyl group slows the reaction down somewhat[52], but in its absence, as in the case of either epinochrome (22) or dopachrome (23) it takes place very readily and even occurs in the solid state[18]. The slower rate in the former case may be due to the negative inductive effect of the hydroxyl or alkoxyl group in the 3-position which would tend to oppose the electron flow required for the first step of the reaction[52].



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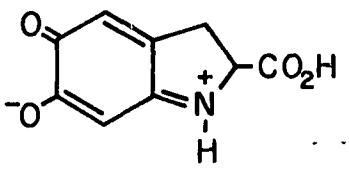

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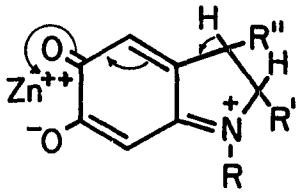

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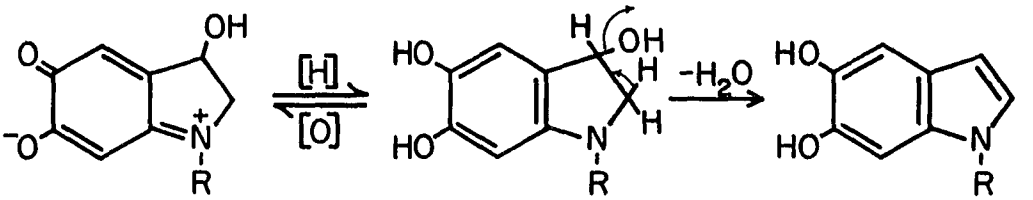
The mechanism of the metal salt catalyzed rearrangement can be explained in essentially the same manner as the alkali catalyzed reaction (cf. 24) (cf. Refs. 19-22 and 52).

1.4.2 Reduction

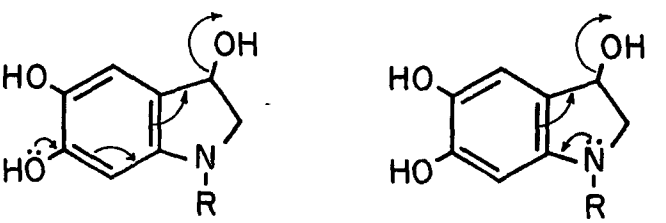
The aminochromes are reduced very readily by a wide variety of reducing agents, the most commonly used being sodium hydrosulphite (i.e. sodium dithionite, $Na_2S_2O_4$), ascorbic acid, sodium borohydride and zinc and acetic acid[19-22].

5,6-Dihydroxyindoles are obtained upon reduction of 3-hydroxyaminochromes. Polarographic[53,54] and electrochemical[43] evidence seems to indicate the initial reversible formation of a 3,5,6-trihydroxyindoline derivative (i.e. the true leucoaminochrome) which readily loses the elements of water to give the fully aromatic 5,6-dihydroxyindole derivative (cf. Scheme 4).



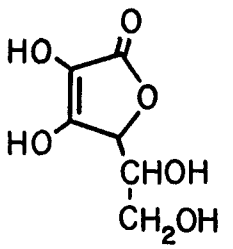
SCHEME 4

Attempts to isolate the 3,5,6-trihydroxyindoline intermediates have, so far, been without success. The ease with which the 3-hydroxyl group is lost may be due to the combined electromeric effects of the indoline nitrogen and the 6-hydroxyl group as shown below. This type of mechanism would also explain the loss of methanol or ethanol which occurs during the formation of 5,6-dihydroxyindole derivatives upon the reduction of aminochrome methyl (or ethyl) ethers[51].



The mechanism of the reduction of adrenochrome by ascorbic acid (25) in aqueous or methanolic solution has been investigated by spectroscopic[55] and polarographic[56] methods. E.p.r. studies indicated that free radicals were

formed as intermediates. The reaction followed second order kinetics, being first order in adrenochrome concentration and first order with respect to the concentration of undissociated ascorbic acid. The mechanism of the reaction was postulated by Mattok to involve the initial formation of the semiquinone 26 which was further reduced by the ascorbic acid to the trihydroxyindoline 27. The latter would then rapidly lose water to give the dihydroxyindole in the manner described above[55].



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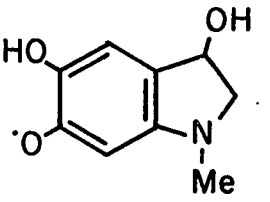

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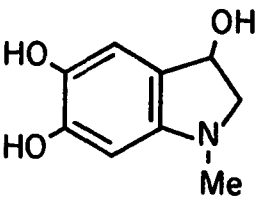

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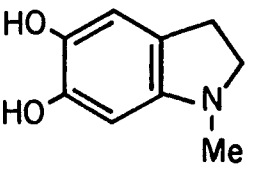
The reduction of aminochromes without a 3-hydroxyl or 3-alkoxyl group is usually complicated by their facile rearrangement to 5,6-dihydroxyindoles and in most cases only the latter type of product could be isolated. With epinochrome (22)[57] and 7-iododopachrome ethyl ester[58], however, the true leuco compounds, 28 and 29, were said to



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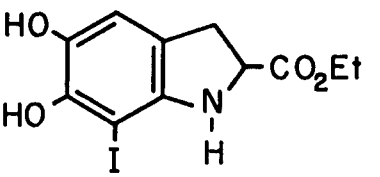

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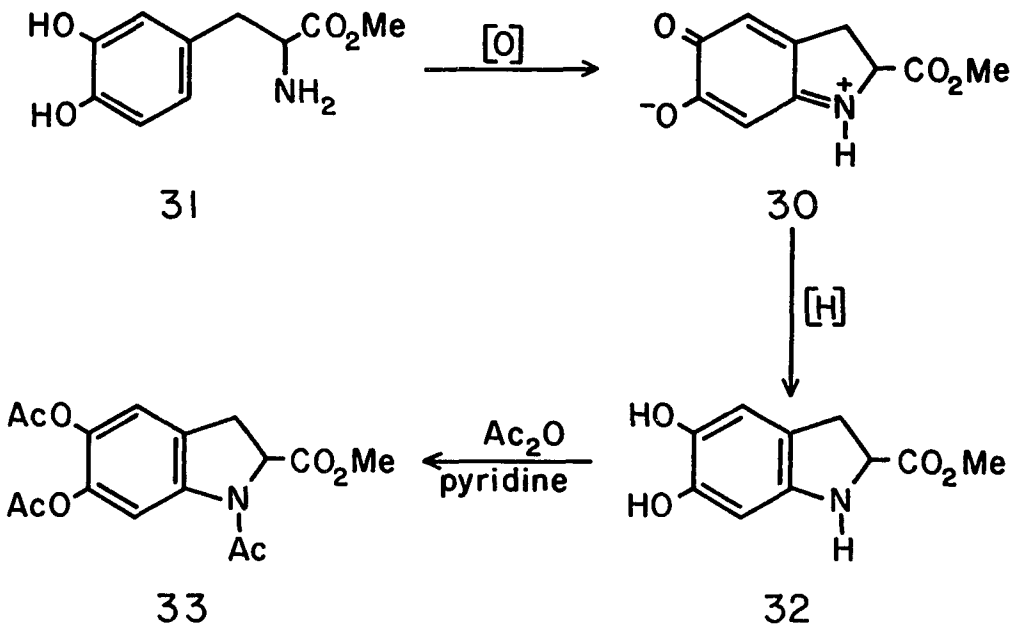
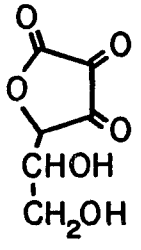
have been obtained in low yields. More recently the reduction of dopachrome methyl ester (30) with sodium hydrosulphite has been studied. Since this aminochrome is very unstable it was formed *in situ* by the oxidation of dopa methyl ester (31) with potassium ferricyanide. It was then immediately reduced to the 5,6-dihydroxyindoline 32 which was acetylated to give the *O*,*O*,*N*-triacetyl derivative 33[59]. A number of related compounds have been synthesized by similar methods[60-62].



The reduction of aminochromes may also be complicated by the occurrence of side reactions between either the aminochrome itself or its reduction products, and the reducing agent or its oxidation products. When sodium hydrosulphite is used as the reducing agent, the sodium bisulphite generated in the reaction can form an

addition product with the unreacted aminochrome. Any bisulphites present are also capable of reacting with the 5,6-dihydroxyindole reduction product to give another type of addition product, the structure of which has not yet been determined[63] (cf. Ref. 22).

In reductions employing ascorbic acid (25), the α-dicarbonyl function of the dehydroascorbic acid (34) produced in the reaction reacts with the o-dihydroxy groups of the reduction product. This reaction has been studied in the case of adrenochrome (7) and was shown to give rise to a compound with the structure 35 in addition to 5,6-dihydroxy-1-methylindole (DHMI, 36) and an unidentified product[64]. The side reactions can be minimized, however, if the reduction is carried out very quickly and the product extracted immediately with ether. In this way DHMI can be obtained in relatively high yields[65].



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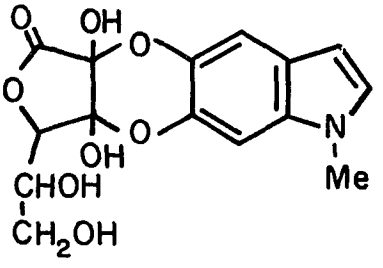

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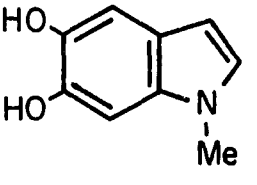

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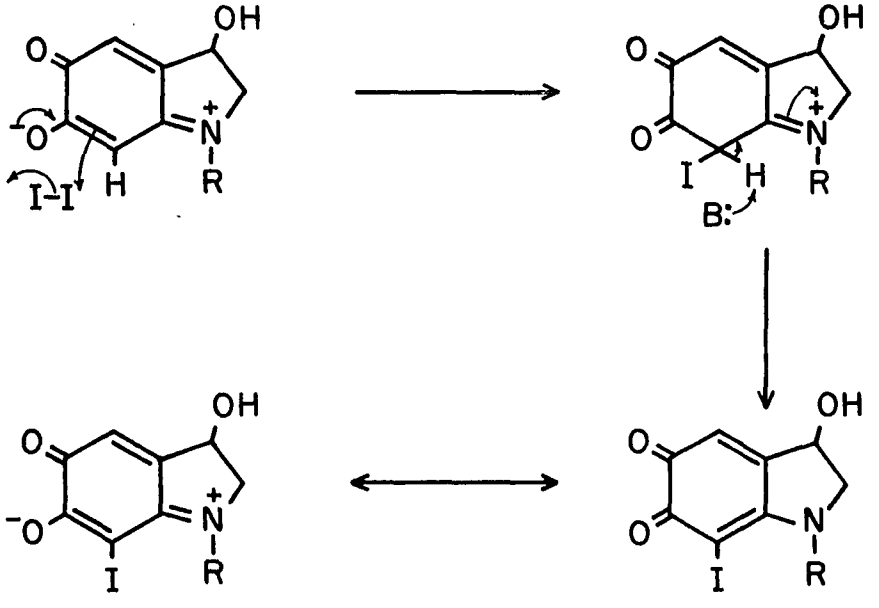
1.4.3 Substitution

The aminochromes readily undergo substitution reactions with molecular iodine or bromine to form 7-iodo- or 7-bromo- derivatives[19-22]. These reactions have been mentioned briefly in Sect. 1.2.1.

The reactions between a number of aminochromes and molecular iodine have been studied in detail by Mattok and Wilson[34-36]. These reactions were shown to obey second order kinetics, being first order with respect to both the aminochrome and the molecular iodine concentrations[35]. The actual reaction rate varies inversely with the iodide concentration due to the equilibrium $I_2 + I^- \rightleftarrows I_3^-$. It is independent of pH, but is subject to general base catalysis, the catalytic activity of the anion increasing in the same order as the pK_a of the corresponding acid[36]. It was suggested by Mattok and Wilson that the overall mechanism for iodination of an aminochrome is as shown in Scheme 5[35].



SCHEME 5

The reaction rate was found to decrease as the electron-donating capacity of the N-substituent increased (i.e. Me > Et > iPr). This fact, together with the general base catalysis that is observed, suggests that the rate-determining step is the loss of the C^7-proton.

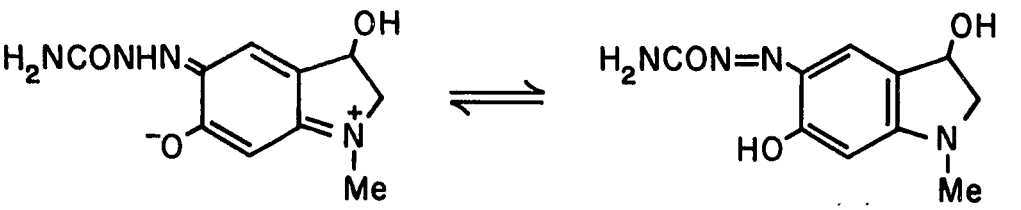
1.4.4 Formation of condensation products

Aminochromes react readily with a wide variety of ketone reagents of the substituted hydrazine type to give monocondensation products involving the C^5-carbonyl group of the aminochrome[66]. Since the C^6-carbonyl is part of a vinylogous amide system it does not react with these reagents. A relatively large number of derivatives of this nature, adrenochrome monosemicarbazone being the most common, have been prepared because of their reported hemostatic activity[21,22,29]. These compounds are usually obtained as well defined crystalline solids varying in colour from yellow to deep red-brown. They are considerably more stable than the parent aminochromes[21].

1.4.4.1 Ultraviolet and visible spectroscopy of aminochrome condensation products

The ultraviolet and visible spectra of these condensation products are dependent upon the pH of their solutions[19-21,67,68]. In neutral solution adrenochrome monosemicarbazone (37) has a λ_{max} at 356 nm with a shoulder

at ca. 447 nm. Under these conditions it is said to exist in a tautomeric equilibrium (37 ⇌ 38) with the quinonoid tautomer 37 predominating[19,21,67].



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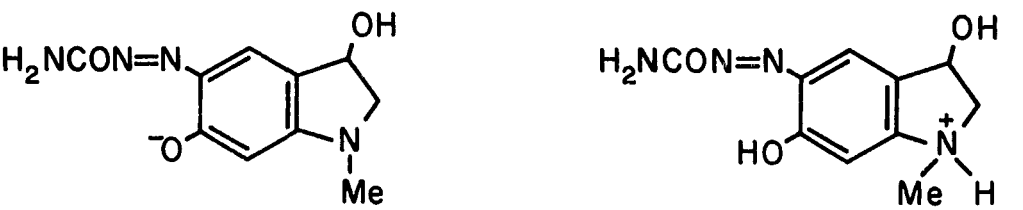
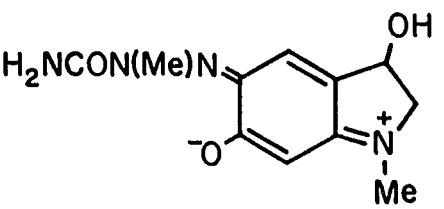


39 40

In alkaline solution the peak at 356 nm disappears and the absorbance at 447 nm becomes much more intense, probably due to the formation of the azophenol anion 39[19-21,67,68]. This was supported by the fact that the spectrum of the 2'-methylsemicarbazone of adrenochrome (41) is the same in both neutral and alkaline solutions[19]. Under acidic



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conditions adrenochrome monosemicarbazone has a λ_{max} at 377 nm and this is attributed to the protonation of the azophenol tautomer 38 to give 40[67].

1.4.4.2 Infrared spectroscopy of aminochrome condensation products

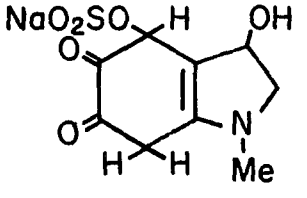
The infrared spectrum of adrenochrome monosemicarbazone (37) has been measured by a number of workers[21,67,69]. Heacock reported that there were two high frequency peaks at 3370 and 3120 cm^{-1} due to OH and NH stretching respectively. In the carbonyl region, peaks were observed at 1703, 1663, 1610 and 1562 cm^{-1}[21]. Since this is a rather complicated molecule it is rather difficult to assign all the peaks with certainty. The bands at 1703 and 1663 cm^{-1} could probably be attributed to the semicarbazone carbonyl and the C=N groups respectively. The peak at 1610 cm^{-1} could be assigned to part of the vinylogous amide system and the one at 1562 cm^{-1} may be due to the other part of this system as well as to NH bending vibrations.

1.4.5 Formation of addition products

The aminochromes are not reduced by sodium bisulphite as was originally thought[70,71], but, as first suggested by Bouvet[72] in 1949, they react readily with this compound to form 1:1 addition products. These compounds are usually pale yellow in colour (the adrenochrome/sodium

bisulphite addition product for example has a λ_{max} in water at 348 nm) and exhibit a deeper yellow fluorescence[73]. They are reasonably stable in neutral solution but upon addition of alkali they decompose very readily, regenerating the aminochrome which subsequently rearranges to the appropriate 5,6-dihydroxyindole or 5,6-dihydroxyindoxyl derivative[21,22]. The adrenochrome/sodium bisulphite addition product has been isolated in the solid state as a pale yellow crystalline solid[73,74].

van Espen found that this substance reacted with either semicarbazide or p-nitrophenylhydrazine to give a monocondensation product[73]. These derivatives could be converted by the action of either heat or alkali to compounds having spectroscopic and chromatographic properties similar to those of the corresponding adrenochrome hydrazone derivatives. This showed that the bisulphite residue in the addition product was not attached to the C^5-carbonyl group. Addition of the bisulphite to the C^6-carbonyl group of



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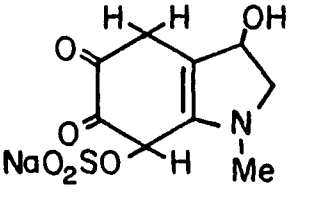

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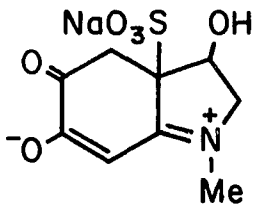
adrenochrome was considered unlikely since it is part of a vinylogous amide system. The addition product reacted only slowly with ferric chloride, indicating that it did not contain any phenolic groups. As a result of these considerations, along with microanalytical data, van Espen proposed that its structure was either 42 or 43[73]. These structures may be criticized, however, since: (a) the addition product forms mono- and not di- condensation products; (b) compounds of this nature would be expected to tautomerize to 3,5,6-trihydroxyindoline derivatives and (c) bisulphite addition products are usually considered to be sulphonates rather than sulphite esters[74].

Tse and Oesterling[74] and Heacock[22] have measured the infrared spectrum of the adrenochrome/sodium bisulphite addition product and found it to be similar to that of adrenochrome. The peak due to the C^5-carbonyl group, however, is observed at a somewhat higher frequency (1725 cm^{-1}) in the spectrum of the addition product suggesting less conjugation with unsaturated systems in the molecule. Peaks are seen at ca. 1630 cm^{-1} and 1575 cm^{-1} which appear to be analogous to those observed for the vinylogous amide system in adrenochrome[74] suggesting that this part of the molecule is essentially the same in both compounds. An OH stretching peak, analogous to that due to the 3-hydroxyl group in adrenochrome is also observed in the spectrum of the

addition product at 3340 cm^{-1}. Bands attributable to the C-SO_3^- grouping were observed at 1248-1220 cm^{-1} and at 1043 cm^{-1}. It was largely on the basis of these observations that Tse and Oesterling proposed that the adrenochrome/ sodium bisulphite addition product has the structure 44 in the solid state[74]. These authors, however, suggested that the enolic structure 45 predominated in aqueous solution[74].



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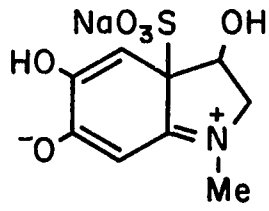

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It can be seen that the structure 44 would explain the formation of mono- rather than di- condensation products since the C^6-carbonyl group is part of a vinylogous amide system[74].

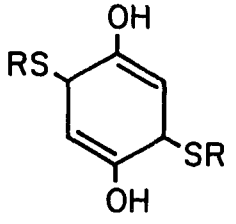
1.4.6 Reactions with thiols

Since reactions of this type form the basis of the research described in this thesis, they will be considered in somewhat more detail than those discussed previously. Firstly, the reactions of thiols with quinones in general will be discussed. Secondly, a consideration of the reactions of thiols with intermediates, including aminochromes, which

occur during the formation of melanin will be presented. Finally, the reactions of thiols with aminochromes themselves will be discussed.

1.4.6.1 Reactions of thiols with quinones

The reactions between quinones and thiols have been known for many years and have been shown to give rise to both reduction and addition products. In 1888 Bongartz reported that p-benzoquinone was reduced by thioglycollic acid to give hydroquinone[75]. Several years later Troeger and Eggert observed that two molecules of thiophenol added to one molecule of p-benzoquinone to give a disubstituted product of the composition $C_6H_4O_2, 2C_6H_5SH$[76]. Shortly after the turn of the century Posner reported that a number of aliphatic and aromatic thiols added to p-benzoquinone to give disubstituted products to which he assigned the general structure 46[77]. These dienols were said to be in equilibrium



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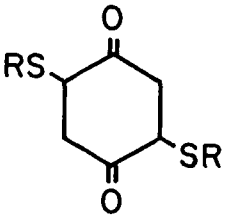
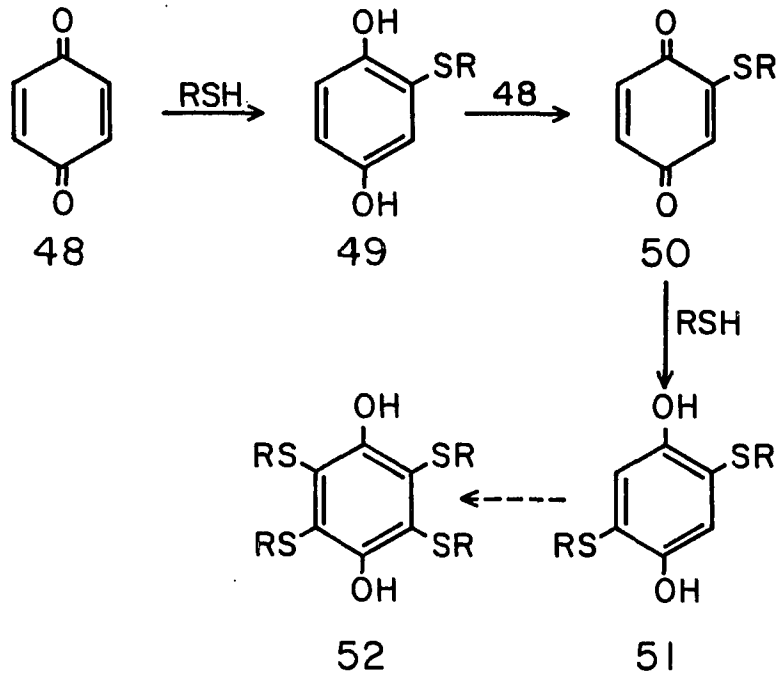


47

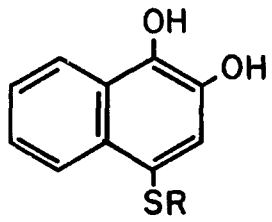
with the corresponding diketo tautomers (cf. 47)[77]. A German patent appeared the following year, however, which described

what is essentially the correct mechanism for the formation of polythiosubstituted products from p-benzoquinone (48). The initial product was said to be the monosubstituted



hydroquinone 49, which was thought to be oxidized to 50 by a second molecule of p-benzoquinone. The quinone 50 then reacted with the thiol to give a disubstituted hydroquinone (cf. 51). This process could then be repeated until the tetrasubstituted product 52 was finally obtained[78].

The first reference to the reaction of o-quinones with thiols appears to be in 1914 when Ghosh and Smiles reported that 1,2-naphthoquinone reacted with either thiolacetic acid or o-mercaptobenzoic acid to give a 4-thiosubstituted naphthohydroquinone (cf. 53)[79].



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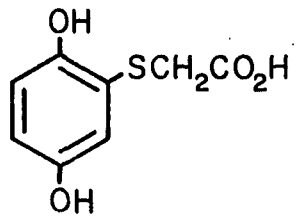
A quarter of a century later Snell and Weissberger studied the reaction of p-benzoquinone with a number of thiols[80]. One equivalent of p-benzoquinone reacted with one equivalent of thioglycollic acid to form the monosubstituted hydroquinone 54 which could be converted to the lactone 55 by heating at 150° for 3 hours. In the presence of 2 moles of quinone, the excess quinone oxidized the initial product to 56. With β-mercaptopropionic acid and ethylmercaptan, even with an excess of the thiol, the initial monosubstituted hydroquinone was oxidized to the quinone to which the thiol again added to give a disubstituted product[80].



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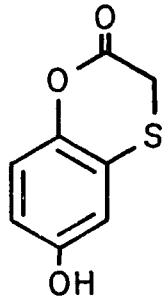

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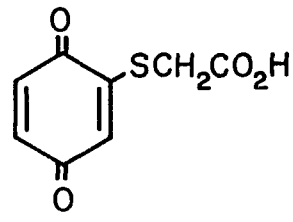

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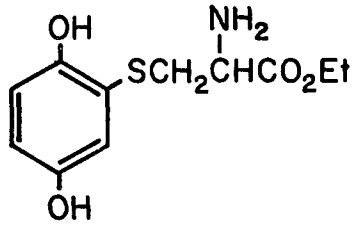
Kuhn and Beinert observed that cysteine ethyl ester reacted with p-benzoquinone to give initially the monosubstituted product 57. The latter was oxidized to the quinone which underwent an intramolecular condensation reaction to yield 58[81]. 2,5-Dimethyl-p-benzoquinone and 1,4-naphthoquinone were found to react similarly with cysteine ethyl ester[82].



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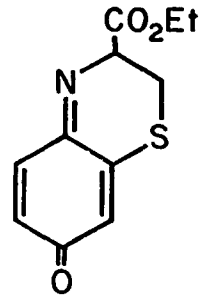

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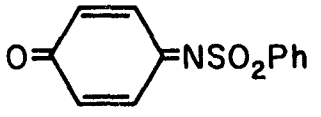
More recently a number of groups have investigated the reactions of p-benzoquinones[82-89] and 1,4-naphthoquinones[82,84,89-95] with several different thiols.

The reactions of quinone mono- and di- imides with thiols have been studied by Adams and his coworkers[96]. p-Benzoquinonemonobenzenesulfonimide (59) reacted with thiophenol to give a mixture of the 2- and 3- monothiosubstituted p-benzenesulfonamidophenols[97]. A number of p-benzoquinone-diimides also reacted with thiols, forming monosubstituted products[96].



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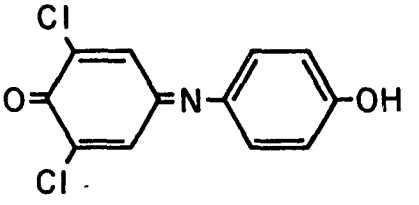

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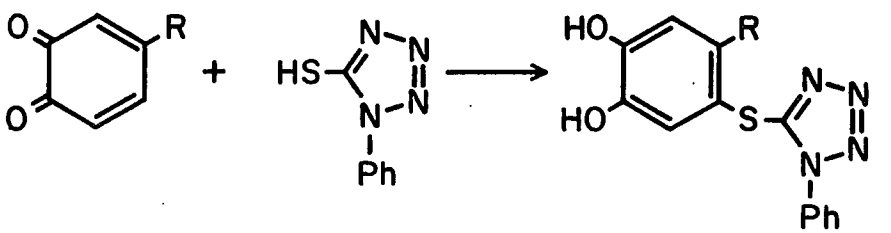
Several workers have investigated the reactions of p-benzoquinonemonoimines with thiols. 2,6-Dichloroindophenol (60) was found to react with cysteine, glutathione and coenzyme A to form thioethers[98,99]. Polysubstitution was said to occur in the case of cysteine if an excess of the dye were present due to the oxidation of the initial reduction product by the latter and subsequent addition of another molecule of the thiol. Polysubstitution was much reduced with glutathione and coenzyme A, however, and this was attributed partially to steric factors and partially to stabilization of the reduced monosubstituted product by hydrogen bonding[98,99]. On the other hand, Coffey and Hellerman concluded that for the reaction of glutathione with 2,6-dichloroindophenol, the formation of monosubstituted products was favoured with a ratio of thiol to dye of one or less, while the disubstituted product predominated when an excess (1.5:1 or 2:1) of glutathione was used[100]. Some years earlier Dimroth and his coworkers found that when

two equivalents of p-benzoquinone were treated with one equivalent of thiophenol the monosubstituted quinone resulted. When the ratio was three equivalents of quinone to two of thiol, however, they obtained a mixture of two disubstituted quinones as well as a trisubstituted product[101].

Several reports have recently appeared concerning the reaction of o-quinones with thiols. 1,2-Naphthoquinone reacted with 1-phenyl-5-mercaptotetrazole (61) to give the 4-thiosubstituted naphthohydroquinone. 4-t-Butyl-o-benzoquinone and 4-phenyl-o-benzoquinone were also said to

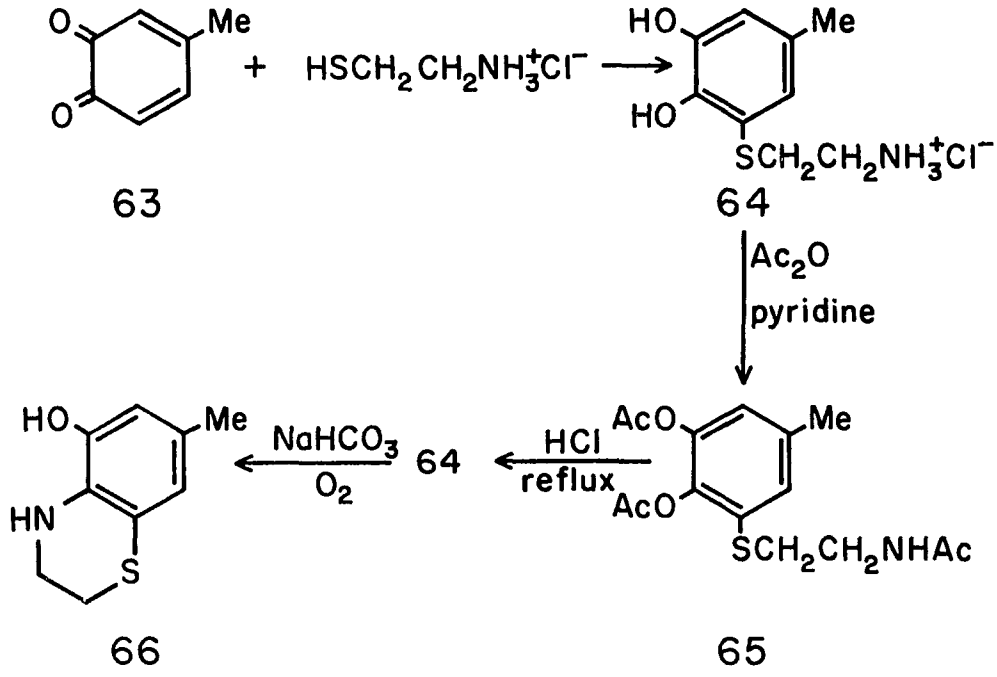


61 62

react with 61 by 1,4-addition to form 5-thiosubstituted products (cf. 62)[87]. Thiourea is also believed to react similarly to 61 with o-benzoquinone and 4-methyl-o-benzoquinone[102]. In these investigations, however, it is not clear on what basis the positions of the thioether linkages were determined and it is possible that, in some cases, the structures were not assigned correctly.

Prota and his coworkers found that 2-amino-ethanethiol hydrochloride added to 4-methyl-o-benzoquinone (63)

by a 1,6-addition mechanism giving 64 which they isolated as

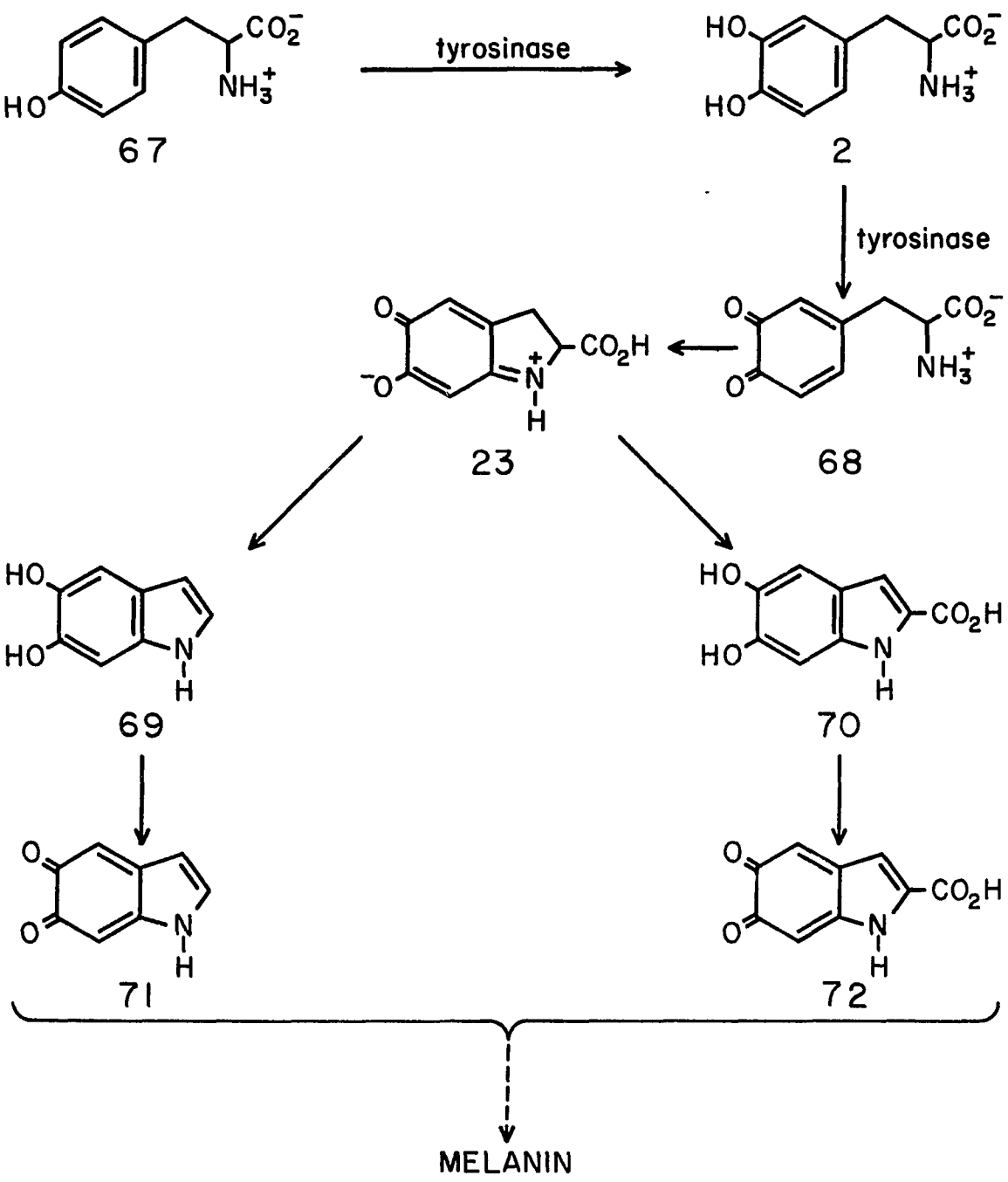


the triacetyl derivative 65. The n.m.r. spectrum of this compound showed the coupling constant between the aromatic protons to be 1.9 Hz, indicative of meta-coupling.[103] Hydrolysis of 65 regenerated the catechol 64 which they were able to isolate. When the latter was dissolved in aqueous sodium bicarbonate and treated with oxygen it was converted to the dihydrobenzothiazine 66[103]. Similar reactions occur in the formation of phaeomelanin and these will be discussed later (see Sect. 1.4.6.2, p. 38).

1.4.6.2 Reactions of thiols with intermediates in melanin formation

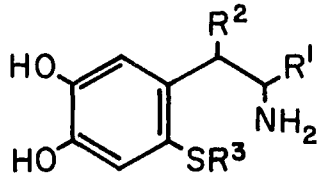
It is well known that thiols inhibit the enzymic formation of melanin from tyrosine (67) or dopa (2) (see Scheme 6, p. 34). There appear to be at least three independent mechanisms involved in this inhibition. Thiols can directly inhibit the enzyme system which is responsible for the hydroxylation of tyrosine and the subsequent oxidation of dopa to dopa quinone (68). It has been suggested that this inhibition is due to chelation of the copper of the enzyme[104,105]. Alternatively, some thiols such as lipoic acid, appear to act by simply reducing the intermediate quinones back to the corresponding leuco compounds[106]. Finally, thiols can form addition products with some of the quinones which are formed as intermediates in the process of melanin formation[107-114].

Bouchilloux and Kodja observed that when either tyrosine, dopa or noradrenaline was oxidized by polyphenol-oxidase in the pH range of 6-7 in the presence of a 2:1 excess of glutathione (GSH) or cysteine, the formation of melanin was inhibited and colourless products resulted[109]. Although these compounds could not be isolated the reaction mixture was studied by paper chromatography and ultraviolet spectroscopy. In each case there was one major product which had the characteristics of an α-amino acid (positive



SCHEME 6

reaction with ninhydrin) and an o-diphenol (positive reaction with Arnow's nitrite/molybdate reagent) but was not indolic (negative reaction with Ehrlich's reagent). It was postulated that this compound was an addition product formed between the open chain quinone (cf. 68) and the thiol and it was suggested that it was a 6-thiosubstituted catecholamine (cf. 73).* When the reaction was carried out with ratios of thiol to dopa of 1.5:1 or 1:1 it was not completely inhibited at the open chain quinone stage and a more complex mixture of products resulted[109]. Traces of 5,6-dihydroxyindole (69) and 5,6-dihydroxyindole-2-carboxylic acid (70) as well as the catecholamine thioether (cf. 73) and a compound which behaved as an α-amino acid (positive reaction with ninhydrin), an o-diphenol (positive reaction with Arnow's reagent) and an indole (positive reaction with Ehrlich's reagent) were observed. It was suggested that the latter compound had the structure 74 and had arisen as a result of the addition of the



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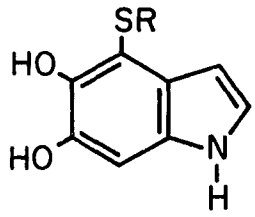

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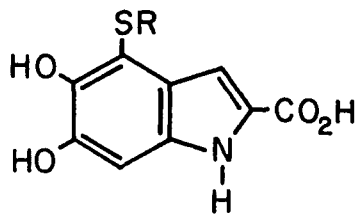

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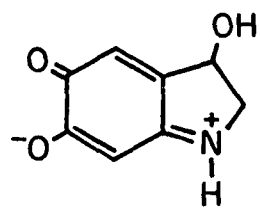
*Dopa will be considered to be a catecholamine in this thesis.

thiol to indole-5,6-quinone (71) which would have been an intermediate in the reaction. When glutathione was added to a solution containing either dopachrome (23) or noradrenochrome (10), compounds having the properties of indole thioethers were again formed. It was postulated that in the former case the dopachrome rearranged to either 69 or 70 (see Scheme 6, p. 34) which was oxidized to the quinone 71 or 72 followed by the addition of glutathione to give either 74 or 75. Noradrenochrome (10) was said to be transformed first to 69 which was then oxidized to the quinone, followed by addition of the thiol to give 74[108,109].



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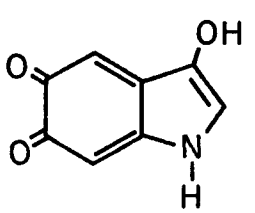
In a more recent study, Mason and Peterson observed that in the tyrosinase catalyzed oxidations of dopa (2) and 5,6-dihydroxyindole (69) in the presence of thiols, products were obtained with spectroscopic properties compatible with those expected for the 5,6-dihydroxyindole thioethers 74 and 75[112]. These authors also postulated that the thiols reacted with the indole-5,6-quinones 71 and 72 formed during the course of the reaction. It was also observed that, whilst aromatic amines appeared to affect

the course of the reaction by combining with some of the intermediates, neither neutral nor basic amino acids had any influence on the nature of the products formed[112].

It has been reported that a thiol, probably glutathione, reacts with an oxidation product of adrenaline within the red blood cell. The mechanism of this interaction and the exact nature of the products were not defined, however[115,116].

Natural melanins usually occur in the form of melanoproteins and thioether linkages such as those mentioned above may be important in the overall structure of these melanoproteins[117,118]. Burton and Stoves suggested that 3-hydroxyindole-5,6-quinone (76), which they considered to be an intermediate in melanin formation, could react with thiols to give an intermediate 4-thiosubstituted 3,5,6-trihydroxyindole which would be oxidized to 77. This thiosubstituted quinone could then be incorporated into the polymerized molecule of natural melanin[85].



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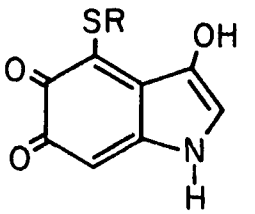

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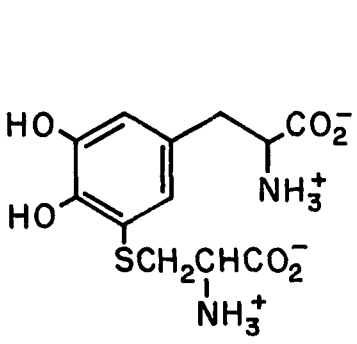
In this regard, several attempts to demonstrate the formation of addition products between the oxidation products of either 5,6-dihydroxyindole (69) or dopa (2) and certain proteins and peptides including ovalbumin[112,119] and bovine serum albumin[119] have been unsuccessful. More recently, however, it has been shown that when tyrosine was oxidized in the presence of bovine lens protein, brown or black melanoproteins were formed[120]. Upon hydrolysis, these pigments gave rise to a compound with ultraviolet spectroscopic and chromatographic properties similar to those of the product (cf. 73) which was formed by the oxidation of dopa in the presence of cysteine. It was suggested that the thiol groups of the protein reacted with some of the intermediates produced by the oxidation of tyrosine[120]. Reactions such as this may be involved in the formation of cataracts in the eye[120].

Quinone/thiol interaction similar to those previously discussed are responsible for the formation of phaeomelanins, the pigments which often cause the brown, red and yellow colours of mammalian hair and bird feathers. It appears that if tyrosine (67) or dopa (2) is oxidized in the presence of an excess of cysteine, the normal process of melanin formation is diverted by the formation of 1,6-addition products between the thiol and dopa quinone (68) (see Scheme 6, p. 34)[121-125]. In reactions carried out

in vitro 5-*S*-cysteinyldopa (78) and 2-*S*-cysteinyldopa (79)



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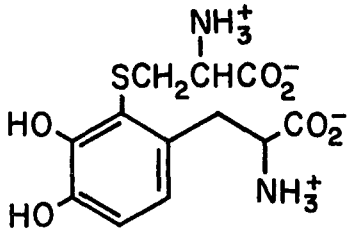

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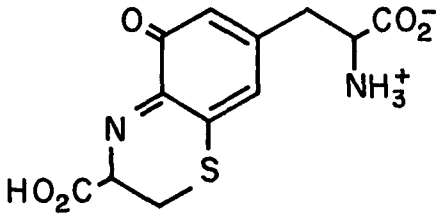

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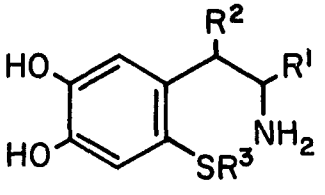

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were isolated from the reaction mixture, the former being the major product. The structures of these compounds were determined unambiguously by chemical and physical methods and it would therefore seem that the structures (*cf.* 73) proposed by Bouchilloux and Kodja for the products formed from the addition of glutathione or cysteine to dopa quinone or noradrenaline quinone should be modified accordingly. The compounds 78 and 79 can then be oxidized to quinones which then form intermediates such as 80 which undergo

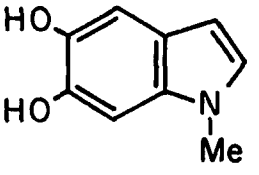
further reactions to give phaeomelanins[121-123].

1.4.6.3. Reactions of thiols with aminochromes

There has been a considerable amount of work carried out on the chemistry of the reactions of aminochromes themselves (other than the tyrosine, dopa and noradrenaline oxidation products mentioned above) with thiols. In their paper describing the original isolation of adrenochrome, Green and Richter reported that its solutions were decolourized by hydrogen sulphide[13]. Thioglycollic acid[126] and 2,3-dimercapto-1-propanol (B.A.L.)[127] were also found to decolourize adrenochrome solutions.

Heacock and Laidlaw studied the reactions between adrenochrome and a number of thiols using paper chromatography with 2% aqueous acetic acid as solvent. They found that cysteine and glutathione reacted with adrenochrome to give 5,6-dihydroxy-1-methylindole (DHMI, 36) and, in each case, a product which exhibited the colour reactions of a phenol, an indole and an α-amino acid and hence must have been some sort of addition compound between adrenochrome and the thiol[128]. These workers also found that thioglycollic acid reacted with adrenochrome to give, besides DHMI, a compound which behaved like a carboxylic acid, a phenol and an indole and was probably analogous to the products obtained from the reactions with cysteine and glutathione[129]. With thioglycollic acid another indolic product which had a fairly low R_f value

was present in the reaction mixture. Penicillamine (β,β-dimethylcysteine), 2,3-dimercaptopropanol (B.A.L.) and hydrogen sulphide all reacted to give mainly DHMI[129]. Ergothioneine (81) was the only thiol studied which did not



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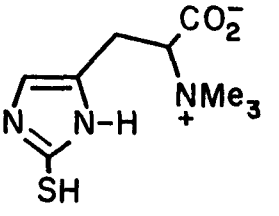


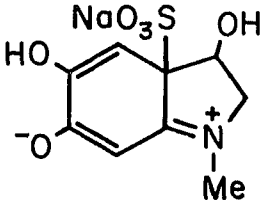
81

give DHMI but another compound showing indole colour reactions was observed. 7-Bromo- and 7-iodo- adrenochrome were found to be rapidly reduced by hydrogen sulphide and B.A.L. to give mixtures of indoles including the corresponding 7-halogeno-5,6-dihydroxy-1-methylindoles[129].

In 1964 Heacock and Mattok reported a more detailed study of the reaction between glutathione and adrenochrome[130,131]. Upon chromatography of the reaction mixture on paper with 2% aqueous acetic acid as solvent, three major products, with R_f values of 0.43, 0.64 and 0.85 were detected.

The product which had an R_f of 0.85 could not be extracted from the reaction mixture with ether. Its concentration varied with the pH and it was found to be formed optimally at a pH of ca. 4.7. The amount formed

dropped off sharply at pH values above and below this and at pH's >6 it was not observed at all. Its properties were very similar to those of the addition product formed between adrenochrome and sodium bisulphite (which was thought to have the structure 45 in aqueous solution[74]), except that the thiol addition product appeared to be less stable. It had a λ_{max} at ca. 350 nm, compared to 348 nm for 45 and, like 45, exhibited a yellow fluorescence. Both of these compounds reacted slowly with Ehrlich's reagent and with ferric chloride, giving similar colours in each case. Both products were readily decomposed by the cautious addition of alkali with the regeneration of adrenochrome. With the thiol addition product this could also be brought about merely by dilution of the reaction mixture with water. By analogy



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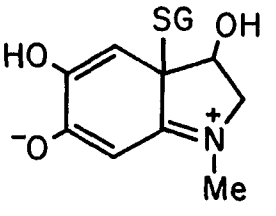

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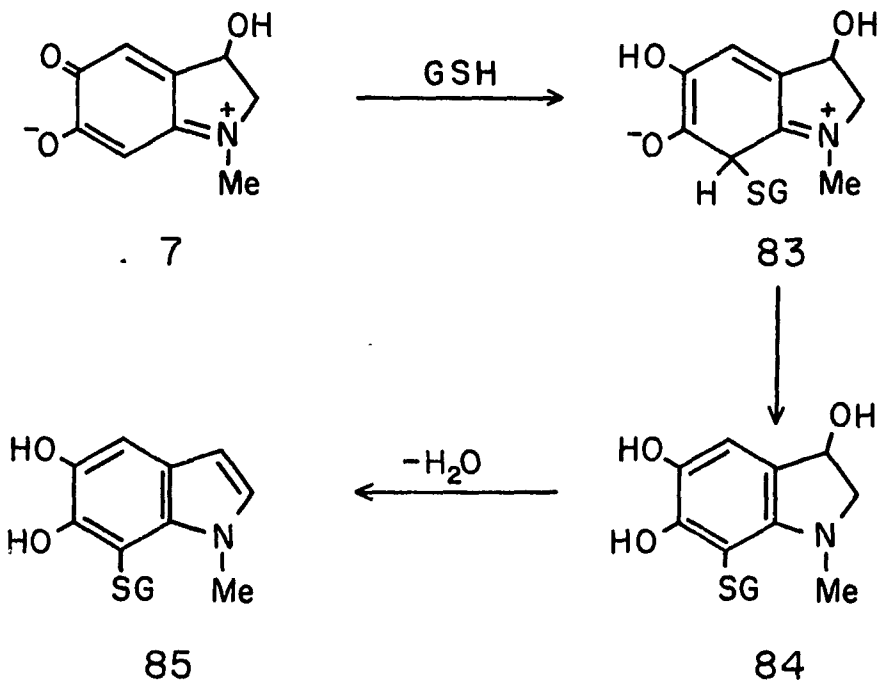
with the structure 45 proposed by Tse and Oesterling[74] for the adrenochrome/sodium bisulphite addition product in aqueous solution it was suggested that the structure of the thiol addition product was 82[131]. This compound could have arisen by the 1,4-addition of glutathione to the α,β-

unsaturated carbonyl system of adrenochrome involving the C^5-carbonyl group and the C^4-C^9 double bond.

The compound with an $\underline{R}_f$ of 0.64 was the one which, as mentioned above, behaved as a phenol, an indole and an α-amino acid with chromogenic reagents. It could not be extracted from the reaction mixture with ether and it had a λ_{max} at *ca.* 300 nm. It was shown that this compound could not have been formed as a result of a reaction between glutathione and DHMI since no reaction was observed between these two compounds under comparable conditions. It was thought to have arisen via a 1,4-addition of glutathione to the α,β-unsaturated carbonyl system of adrenochrome (7) involving the C^5-carbonyl group and the C^6-C^7 double bond. The



7 83 85 84

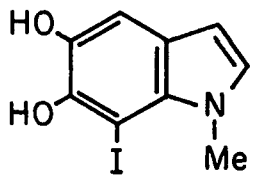
intermediate product 83 would rearrange to the thiosubstituted trihydroxyindoline 84 which would lose the elements of water to give 85[131]. This structure differs in the position of the thiol residue from that (cf. 74) proposed by Bouchilloux and Kodja[109] for the product obtained from the reactions of some catecholamine oxidation products with either glutathione or cysteine (see Sect. 1.4.6.2, p. 35).

The remaining compound (R_f 0.43), which could be extracted from the reaction mixture by ether, was identified as DHMI by comparison of its chromatographic, spectroscopic and chemical properties with those of an authentic sample of this compound. It was suggested that it was produced mainly by the reduction of adrenochrome by the dihydroxyindole thioether 85 or its precursor 84 rather than by the direct action of glutathione, although the latter mechanism was not excluded. In support of this hypothesis it was found that DHMI was capable of reducing 1-isopropylnoradrenochrome to an appreciable extent in a time period comparable to that allowed for the adrenochrome/glutathione reaction[131].

The reactions between glutathione and 1-ethyl-noradrenochrome, 1-isopropylnoradrenochrome, adrenochrome methyl ether and adrenochrome ethyl ether were also investigated with results very similar to those observed for the reaction with adrenochrome[131].

The reactions of glutathione with a number of

halogenated aminochromes were studied using techniques similar to those described above[132]. 7-Iodoadrenochrome reacted with glutathione to give five products. The structures proposed for these compounds as a result of a consideration of their chromatographic, ultraviolet spectroscopic and chemical properties were as follows: (a) 5,6-dihydroxy-1-methylindole (36); (b) 5,6-dihydroxy-7-iodo-1-methylindole (86); (c) 7-S-glutathionyl-5,6-dihydroxy-1-methylindole (85); (d) 4-S-glutathionyl-5,6-dihydroxy-7-iodo-1-methylindole (87) and (e) 9-S-glutathionyl-2,3,6,9-tetrahydro-3,5-dihydroxy-7-iodo-6-oxo-1-methylindole (88).



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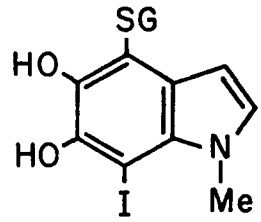

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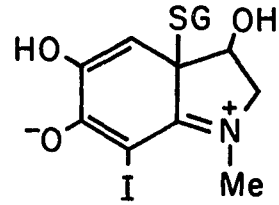

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With glutathione itself (i.e. the diacid) the deiodinated products 36 and 85 predominated in the reaction mixture whereas with the monosodium salt of glutathione the main products were the iodinated indoles 86 and 87. This effect was attributed to a change in the pH of the reaction

mixture. It was suggested that the 4-thiosubstituted 7-iodoindole 87 had arisen by the addition of another molecule of glutathione to the 4-position of the addition product 88, followed by loss of the 9-glutathionyl residue by a β-elimination reaction[132].

In general, the reactions of glutathione with 1-ethyl-7-iodonoradrenochrome, 7-iodo-1-isopropyl-noradrenochrome and 7-iodoadrenochrome methyl ether were similar to those with adrenochrome. In the reaction of 7-bromoadrenochrome with either glutathione itself or its monosodium salt, the brominated products (cf. 86, 87 and 88) predominated in the reaction mixture[132].

Using chromatographic and u.v./visible spectrophotometric techniques Mattok investigated the effect of the structure of the thiol upon the nature and distribution of the products for the reactions of a number of thiols with adrenochrome[133]. Adrenochrome was found to react with primary thiols to form varying mixtures of DHMI, a thiosubstituted 5,6-dihydroxy-1-methylindole (cf. 85) and an adrenochrome/thiol addition product (cf. 82). With cysteine and glutathione (free acid) all three types of products were formed; the same was true with homocysteine and glutathione (monosodium salt) except that in these cases significantly greater amounts of the addition products were formed. When the reaction was carried out

with neutral primary thiols (e.g. 1-propanethiol, 1,2-propanedithiol, 1,3-propanedithiol and B.A.L.) only the addition product (cf. 82) and DHMI could be detected. No reaction appeared to take place between adrenochrome and secondary or tertiary thiols, such as 2-propanethiol and 2-methyl-2-propanethiol. It also appeared that in the case B.A.L. only one of the two thiol groups, presumably the primary one, reacted with the aminochrome. Some reaction did appear to take place with penicillamine (β,β-dimethyl-cysteine) however, but the reaction was slow and much melanin was produced. There was some evidence for DHMI formation but no addition products were detected. The relatively low reactivity of secondary and tertiary thiols towards the aminochromes is presumably due to steric factors[133].

1.5 Biosynthesis and metabolism

The major metabolic pathways for catecholamines in mammals involve two enzyme systems, namely catechol-O-methyltransferase and monoamine oxidase. The former leads to the methylation of the 3-hydroxyl group and the latter to the oxidative deamination of the ethylamine side chain[134,135]. It has been suggested that the oxidation of catecholamines to aminochromes may form a third, although minor, metabolic pathway for these compounds and a number of reports have appeared concerning the detection of aminochromes

or their rearrangement products in body fluids[27,29,136-139]. No incontrovertible evidence has yet been presented for the existence of this pathway, however, since the methods used (cf. Refs. 140 and 141) for the detection and assay of these compounds in body fluids have been questioned[142-145]. Since the aminochromes are very reactive it is unlikely that they would have more than a transient existence in vivo. Consequently it is very difficult to determine with certainty whether or not they are present in the body. A considerable number of workers have reported the presence, in mammals, of enzymes other than tyrosinase which are capable of oxidizing adrenaline and other catecholamines to aminochromes[29,146-154].

In the presence of tyrosinase, catecholamines are oxidized to melanins, which were originally thought to be homopolymers of indole-5,6-quinones[11]. More recent work by Nicolaus[117,155] and Swan[156], however, suggests that their structures are very complex and that their formation involves the copolymerization of a number of the intermediates of catecholamine oxidation (cf. Scheme 6, p. 34). Naturally occurring melanins are usually bound to protein, probably by thioether linkages involving cysteine thiol groups[117,118].

It has been shown chromatographically that when adrenochrome is administered to a number of experimental animals it is metabolized to three different compounds. Two of these are the sulphate esters of adrenolutin and

DHMI and the third is a conjugate, probably a glucuronide of DHMI[157]. Similar results were found previously by a number of Belgian workers[158-161]. Thus it would appear that adrenochrome is metabolized via two pathways, one involving rearrangement to adrenolutin and the other, reduction to DHMI.

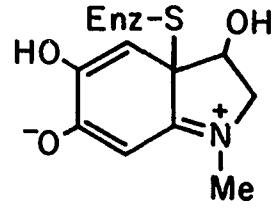
1.6 Physiological activity

1.6.1 Aminochromes

The instability of aminochromes in mammalian fluids and tissues does not mean that they are without any physiological significance. It has been suggested by Heacock and Mattok[130] that aminochromes could be stabilized by the reversible formation of addition products with the thiol groups of naturally occurring amino acids, peptides or proteins. These substances could then act as "aminochrome carriers", regenerating the aminochrome under the appropriate conditions[130].

Alternatively, aminochromes could be synthesized in close proximity to their site of action. In this regard Inchiosa[146-148] has found an adrenaline-oxidizing enzyme in the smooth muscles of a number of mammals. This enzyme was found to oxidize adrenaline to adrenochrome which caused the inhibition of actomyosin adenosine triphosphatase (ATPase). It was suggested[146] that this inhibition was due

to the formation of addition products (cf. 89) of the type



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proposed by Heacock and Mattok[130] for one of the products of the reaction between adrenochrome and glutathione (see Sect. 1.4.6.3, p. 41). Since ATPase is important in the contraction of muscles it was suggested that adrenaline, which relaxes smooth muscles, may act by being first oxidized to adrenochrome[146-148]. Denisov also reported that adrenochrome inhibited myosin ATPase activity and he postulated that this was due both to the addition of the aminochrome to free thiol groups of the enzyme and to the reduction of these groups to disulphides[162].

Adrenochrome has also been found to inhibit a number of other enzymes, including acetylcholinesterase[163], hexokinase[164], phosphokinase[164], glutamic acid decarboxylase[165] and triose phosphate dehydrogenase[166]. Pyruvate oxidation and its associated phosphorylation in brain mitochondria was inhibited by adrenochrome and this was attributed to the binding of free thiol groups of the enzymes[167]. Roston found that oxidation products of adrenaline and noradrenaline,

probably adrenochrome and noradrenochrome, inactivated coenzyme A by reacting with its thiol group[168,169].

The psychotomimetic activity reported to be shown by adrenochrome and adrenolutin and the possible role of these substances in some forms of mental illness, particularly schizophrenia, have been the subject of much controversy since the adrenochrome hypothesis of schizophrenia was first proposed by Hoffer, Osmond and Smythies in 1954[170]. According to this hypothesis, schizophrenia was caused by a defect in the normal metabolism of adrenaline, which resulted in the overproduction of adrenochrome[27,170]. Whether or not this theory is correct, however, adrenochrome does appear to have psychotomimetic properties in man. This was first reported by the above authors in 1954[170] and since that time has been corroborated by a number of authors (see Ref. 27). More recently Grof and his coworkers reached similar conclusions after carrying out extensive studies on the psychological effects of adrenochrome on man[171].

1.6.2 Aminochrome condensation products

The condensation products formed between aminochromes and a number of substituted hydrazine derivatives, especially semicarbazide (see Sect. 1.4.4) are much more stable than the parent aminochromes and have been used therapeutically. A number of these compounds have

been used as hemostatic agents, especially for the control of minor bleeding[29]. Some of these derivatives have also been found to protect the body against the damaging effects of ionizing radiation. Hence they may prove to be useful as adjuncts in the treatment of cancer by radiation therapy[29].

Adrenochrome monosemicarbazone has been shown to be of value in the treatment of erythroblastosis. This condition arises when the erythrocytes of a fetus with Rh positive blood cross through the placenta into the maternal circulation. If the mother has Rh negative blood she then produces antibodies against the fetal Rh antigens and this may be lethal to the fetus. Adrenochrome monosemicarbazone apparently has its effect by reducing the transplacental flow of erythrocytes[29].

2 OBJECTS OF THIS INVESTIGATION

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Prior to the commencement of these investigations it had been proposed that aminochromes reacted with thiols to give three major products. These were thought to be: (1) a 5,6-dihydroxyindole reduction product; (2) a 7-thio-substituted 5,6-dihydroxyindole and (3) an aminochrome/thiol addition product in which the thioether linkage was attached to the 9-position of the aminochrome nucleus. The reduction products were readily identified on the basis of the similarity of their ultraviolet spectroscopic, chromatographic and chemical properties to those of authentic samples of these compounds. The structures of the other two types of products, however, were somewhat more speculative since they had not been isolated and there were no standards, other than the adrenochrome/sodium bisulphite addition product, available for comparison.

It was considered desirable to obtain more conclusive evidence concerning the structures of the two thiosubstituted products because of: (a) a purely chemical interest in the reactions of the aminochromes and (b) the possible biological significance of these reactions. In view of the instability of these compounds it appeared that the best approach would involve the preparation of appropriate stable derivatives of the products of the reactions between various aminochromes and thiols. These

could then be purified and hopefully obtained as crystalline solids, the structures of which could be determined by the usual physical and chemical methods. It was also decided to investigate some of the factors which might influence the rate of the reaction and the distribution of the products.

3 RESULTS AND DISCUSSION

3 RESULTS AND DISCUSSION

3.1 The 5,6-dihydroxyindole thioether

It had been suggested that in the 5,6-dihydroxyindole thioether produced in the reactions of adrenochrome with thiols, the thiol residue was attached to the 7-position of the indole nucleus (see Sect. 1.4.6.3, p. 43). The reaction products, which had not been isolated, had only been studied by paper chromatography and ultraviolet/visible spectroscopy, however, and there was no firm evidence to indicate that the thiol had added to the 7-position of adrenochrome. It was therefore decided to make an attempt to isolate a compound of this type and to characterize it unambiguously.

3.1.1 Reaction of adrenochrome with thioglycollic acid

The reaction first studied was that between adrenochrome (the most common aminochrome) and thioglycollic acid. This thiol was chosen since it should be easy to separate the expected 5,6-dihydroxy-1-methylindole thioether, which would have a free carboxyl group, from the DHMI which would also be formed in the reaction.

3.1.1.1 Preliminary experiments

Thin-layer chromatography of the reaction mixture on cellulose with 2% aqueous acetic acid as developing solvent revealed the presence of two major products with R_f values of 0.45 and 0.48 and one minor product with an R_f value of 0.13.

No product analogous to the adrenochrome/glutathione addition product 82 was detected, however. The compound (R_f 0.45) was identified as DHMI on the basis of its chromatographic behaviour and its reactions with chromogenic reagents (see Table 1, p. 57). The product with an R_f value of 0.48, presumably the indole thioether, was soluble in aqueous sodium bicarbonate while the other two were not. When the bicarbonate solution was acidified, however, this product could be extracted by ether or ethyl acetate. It exhibited the colour reactions expected for an indole (positive reactions with Ehrlich's reagent, cinnamaldehyde and the F_1 reagent) and for a phenol (positive reactions with Folin and Ciocalteu's reagent, ferric chloride and Gibb's reagent) and it gave a blue-green colour with 1 N sodium hydroxide (see Table 1).

3.1.1.2 Structure of the indole thioether

Preliminary attempts to purify this compound were unsuccessful so it was acetylated with acetic anhydride and pyridine. A crystalline acetyl derivative was obtained after column chromatography of the crude product on silica gel. This compound reacted positively with chromogenic reagents for indoles. It did not appear to contain a free carboxyl group since it was insoluble in aqueous sodium bicarbonate and its infrared spectrum showed no absorption in the NH/OH stretching region but had a peak in the

Table 1

Thin-layer chromatography* of the products of the reaction between adrenochrome and thioglycollic acid in aqueous solution

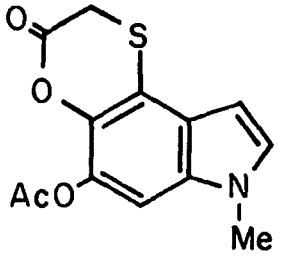
	Colours with chromogenic reagents						
R_f values†	F_1	Ehrlich's reagent	Cin	1 N NaOH	F-C	1% $FeCl_3$	Gibb's reagent
Reaction mixture							
0.13(w)	B→V	B	R→V	BG	Gy	Gy	Br
0.45(s)	V	BV	OBr→VBr	Br	Gy	GyV	VBr
0.48(s)	V	BV	OR→GyV	BG	Gy	GyG	OBr
DHMI							
0.45	V	BV	OBr→VBr	Br	Gy	GyV	VBr

*Ascending development with 2% acetic acid in water on Eastman "Chromagram" cellulose sheets (20 × 20 cm).

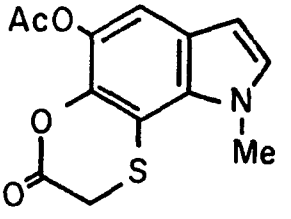
†A spot, R_f 0.85, due to excess thioglycollic acid was also observed on chromatograms of the reaction mixture.

Abbreviations: F_1 = p-N,N-bis(2-chloroethyl)aminobenzaldehyde; Cin = cinnamaldehyde; F-C = Folin and Ciocalteu's reagent.
B = blue; V = violet; R = red; G = green; Gy = grey;
Br = brown; O = orange;
w = weak; s = strong.

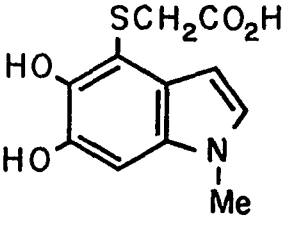
carbonyl region at 1764 cm^{-1}. Microanalytical data and mass spectrometry indicated a molecular formula of $C_{13}H_{11}NO_4S$. It therefore appeared that lactonization had occurred at the same time as acetylation to give a product, probably with either the structure 90 or 91.



90



91



92

Nuclear magnetic resonance spectroscopy appeared to offer the best means of distinguishing between structures 90 and 91 for the acetyl derivative, since the problem was essentially one of determining whether the proton on the benzene ring was in the 4- or the 7- position of the indole nucleus. Long range coupling of ca. 0.7-0.9 Hz between the 3- and 7- protons of the indole nucleus has been observed by several workers in the n.m.r. spectra of a number of indole derivatives[172-175]. There is negligible coupling, however, between the 4- and either the 2- or 3- protons[172-175]. The n.m.r. spectrum (Fig. 1, p. 60) of the acetyl derivative (i.e. 90 or 91) exhibited signals for three protons in the aromatic region (see Table 2, p. 59). The 2-proton of the pyrrole ring was observed as a doublet at δ 7.10

Table 2

Nuclear magnetic resonance spectral* data for some 5,6-dihydroxyindole derivatives

Indole compound	δ-Values				Coupling constants (Hz)	
	H^2	H^3	H^4	H^7	$J_{2,3}$	$J_{3,7}$
5,6-Diacetoxy-1-methylindole	6.99(d)	6.39(dd)	7.37(s)	7.10(d)	3.1	1.0
5,6-Diacetoxy-1,4-dimethylindole	6.98(d)	6.45(dd)	-	6.95(d)	3.0	0.8
5,6-Diacetoxy-1,7-dimethylindole	6.95(d)	6.39(d)	7.22(s)	-	3.0	-
5,6-Diacetoxy-7-iodo-1-methylindole	6.95(d)	6.31(d)	7.33(s)	-	3.1	-
5,6-Diacetoxy-7-iodo-1,4-dimethylindole	6.97(d)	6.35(d)	-	-	3.0	-
3,5,6-Triacetoxy-7-iodo-1-methylindole	7.27(s)	-	7.33(s)	-	-	-
5,6-Diacetoxy-1-isopropylindole	7.21(d)	6.45(dd)	7.37(s)	7.18(d)	3.2	1.0
5,6-Diacetoxy-7-iodo-1-isopropylindole	7.33(d)	6.42(d)	7.37(s)	-	3.3	-
6-Acetoxy-4-carboxymethylthio-5-hydroxy-1-methylindole lactone (90)	7.10(d)	6.45(dd)	-	7.00(d)	3.0	0.8
6-Acetoxy-4-carboxymethylthio-5-hydroxy-1,7-dimethylindole lactone (97)	6.97(d)	6.36(d)	-	-	3.3	-
6-Acetoxy-4-carboxymethylthio-5-hydroxy-1-isopropylindole lactone (98)	7.27(d)	6.47(dd)	-	7.06(d)	3.0	0.9
6-Acetoxy-4-(β-carboxyethyl)thio-5-hydroxy-1-methylindole lactone (99)	7.15(d)	6.68(dd)	-	7.12(d)	3.2	0.9

*The spectra were recorded in $CDCl_3$ with TMS as an internal reference.

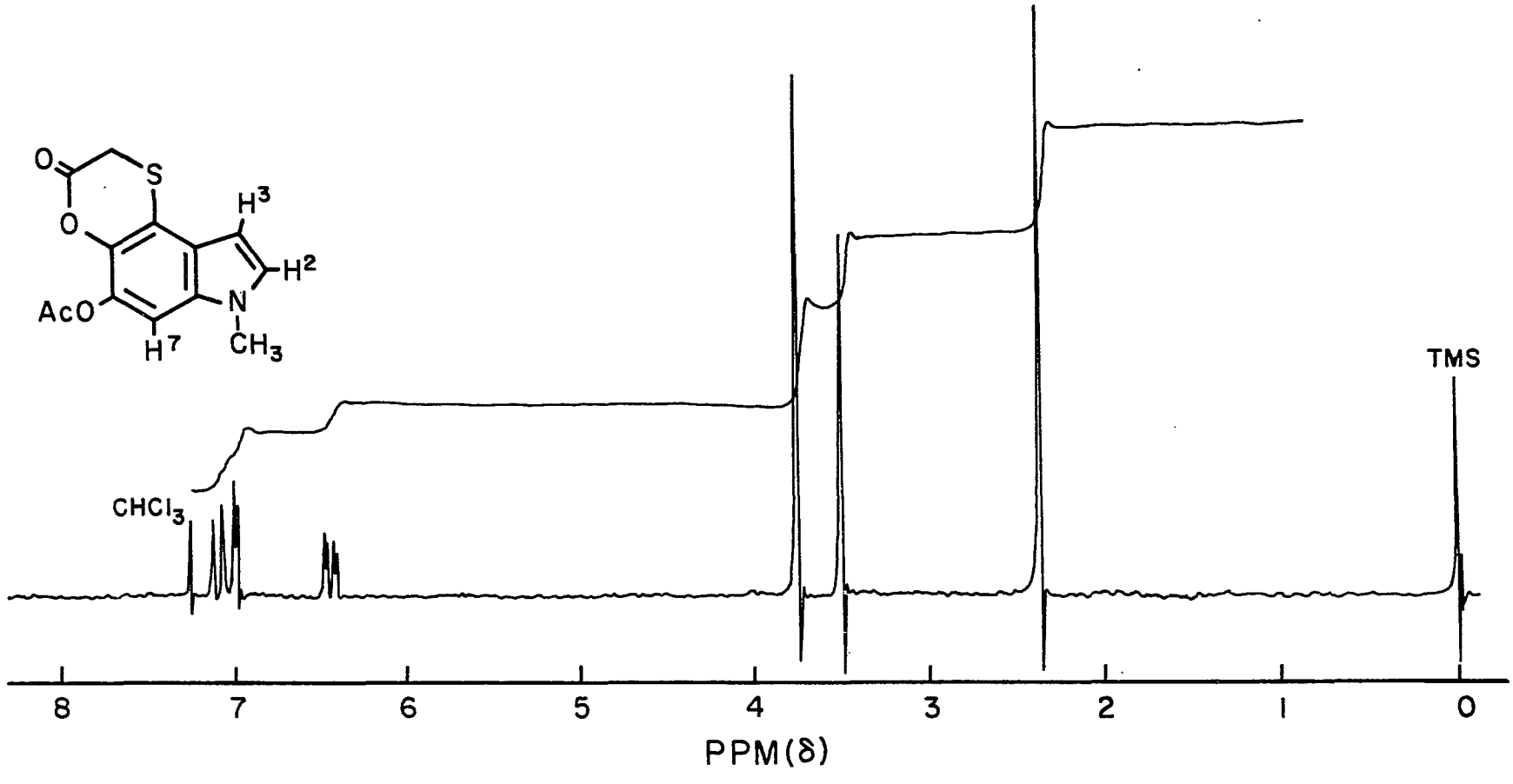


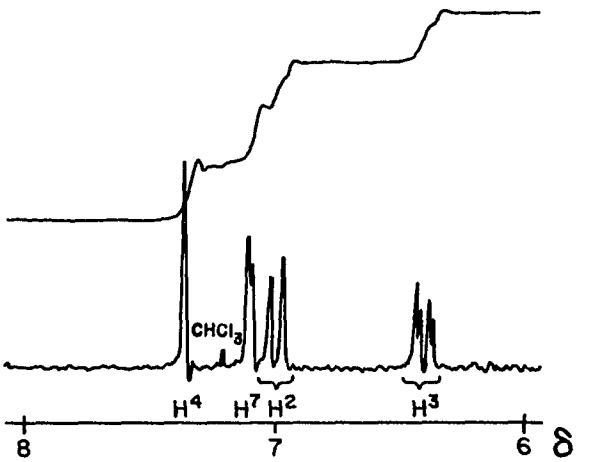
Fig. 1. N.m.r. spectrum of 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindole lactone (90) in $CDCl_3$.

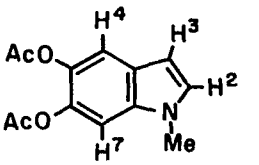
($J_{2,3}$ = 3.0 Hz) while the 3-proton formed a doublet of doublets at δ 6.45. This was due to further coupling (J = 0.8 Hz) with the proton of the benzene ring which was observed as a doublet at δ 7.00. This long range coupling would suggest that the latter proton was in the 7-position of the indole nucleus and that the thiol residue was attached to the 4-position. The thiol must therefore have attacked the 4-carbon of adrenochrome to give the 4-thiosubstituted indole 92 which, upon acetylation, gave 90. The n.m.r. spectrum of the acetyl derivative 90 also revealed peaks at δ 3.75, δ 3.50 and δ 2.35 for the N-methyl, S-methylene and acetyl methyl protons respectively.

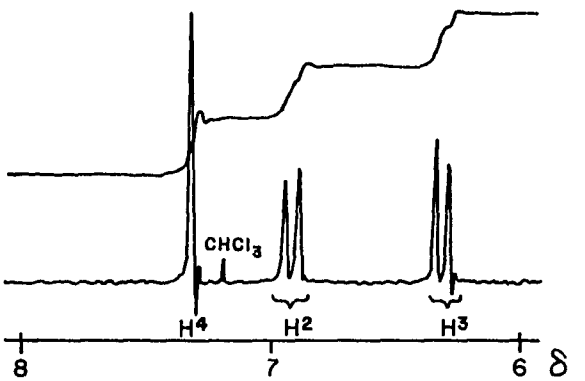
The n.m.r. spectra of a number of related 5,6-diacetoxyindole derivatives were recorded and the relevant chemical shifts and coupling constants are given in Table 2. The aromatic regions of the n.m.r. spectra of four of these compounds are shown in Figure 2, p. 62. In general the signal due to the 4-proton is observed as a well defined singlet downfield from the other aromatic proton signals, whereas the signal due to the 7-proton is seen as a doublet ($J_{3,7} \approx 0.8$ Hz) at slightly higher field. These observations would strengthen the validity of the assignments which have been made for the acetylated indole thioether 90.

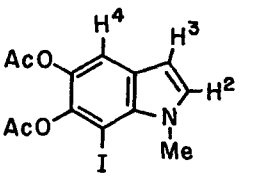
Fig. 2

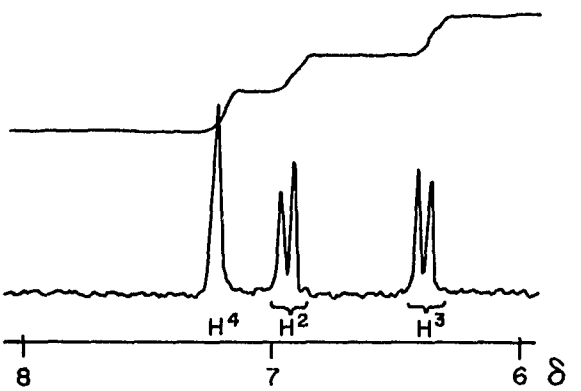
Partial n.m.r. spectra of some 5,6-diacetoxy-1-methylindoles in $CDCl_3$.

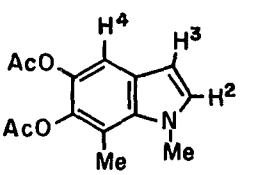

CHCl3
H4
H7 H2
H3
8
7
6
δ

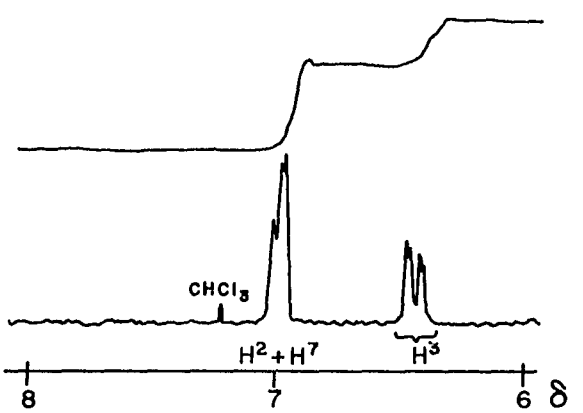

H4
H3
AcO
H2
AcO
N
Me
H7

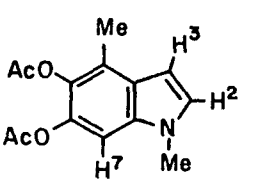

CHCl3
H4
H2
H3
8
7
6
δ


H4
H3
AcO
H2
AcO
N
Me
I


H4
H2
H3
8
7
6
δ


H4
H3
AcO
H2
AcO
N
Me
Me


CHCl3
H2+H7
H3
8
7
6
δ

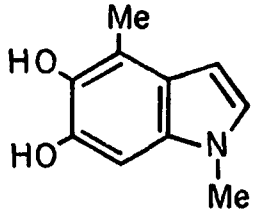

Me
H3
AcO
H2
AcO
N
Me
H7

3.1.1.3 Structure of the minor product (R_f, 0.13)

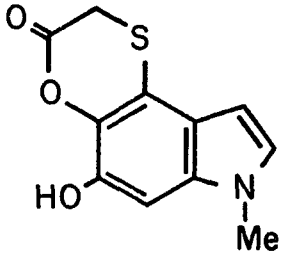
The reaction between adrenochrome and thioglycollic acid was carried out in the absence of water with an excess of the thiol as solvent: (a) at room temperature and (b) at 120°. The reaction mixtures were diluted with water and acidic and phenolic fractions were obtained upon ether extraction at the appropriate pH's. These extracts were compared chromatographically with the corresponding extracts from the aqueous reaction mixture. For the reaction at room temperature in the absence of water there was considerably less DHMI formed while the relative amounts of the indole thioether 92 and the minor product were greater. When the reaction mixture was heated at 120° for 3 hours, however, no DHMI and only a small amount of 92 were detected. The substance with an R_f value of 0.13 was then the only major product observed.

This compound, which was not soluble in aqueous sodium bicarbonate, behaved like an indole and a phenol with chromogenic reagents (see Table 1, p. 57). In a similar manner to the indole thioether 92 it gave a blue-green colour with sodium hydroxide. This colour is probably caused by the blocking, due to ring substitution, of alkali induced melanin formation. 5,6-Dihydroxy-1,4-dimethylindole (93) also gives a blue-green colour with sodium hydroxide whereas DHMI and 5,6-dihydroxy-1,7-dimethylindole, on the

other hand, give brown colours with this reagent, presumably due to melanin formation. These observations would seem to indicate that the compound in question is the lactone 94. A



93



94

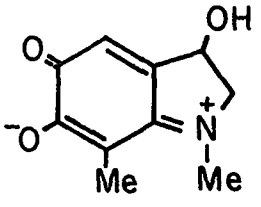
similar lactonization reaction had been observed by Snell and Weissberger[80] when the crude products from the reaction between p-benzoquinone and thioglycollic acid were heated at 150° for 3 hours (see Sect. 1.4.6.1, p. 28).

When the compound thought to be 94 was acetylated with acetic anhydride and pyridine, a crystalline monoacetate was obtained after purification by column chromatography. It was shown to be identical to the acetylated indole thioether 90 by the nondepression of melting points and the identity of its spectral characteristics to those of 90. This would thus confirm that the original compound with an R_f value of 0.13 was the lactone 94.

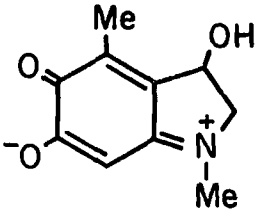
3.1.2 Indole thioethers from other aminochrome/thiol reactions

A chromatographic study of the reactions between thioglycollic acid and 1-ethylnoradrenochrome, 1-isopropylnoradrenochrome and adrenochrome methyl ether was

carried out[176]. In each case two major indolic products and one minor one were observed which were analogous to the compounds (DHMI, 92 and 94) which have been identified in the reaction between adrenochrome and thioglycollic acid. Furthermore, it was demonstrated that 7-methyladrenochrome (95)



95



96

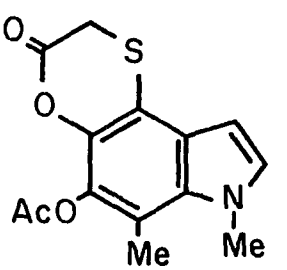
reacted with glutathione to give mainly an indole thioether with only a trace of 5,6-dihydroxy-1,7-dimethylindole. 4-Methyladrenochrome (96), on the other hand, reacted to give the reduction product, 5,6-dihydroxy-1,4-dimethylindole (93) as the major product. These results would therefore confirm that the thioether linkage was in the 4-position of the indole nucleus.*

---

*The results described in this paragraph were obtained in conjunction with G. L. Mattok and D. L. Wilson and have been published jointly with some of the results which have been obtained in the course of the current work (see Ref. 176).

3.1.2.1 Reactions of 4- and 7- methyladrenochrome with thioglycollic acid

Thioglycollic acid reacted in a similar manner to glutathione with 4-methyladrenochrome (96) to give only one major product. This was identified as the reduction product, 5,6-dihydroxy-1,4-dimethylindole (93), on the basis of the similarity of its chromatographic and chemical properties with samples of this compound prepared by the reduction of 4-methyladrenochrome (96) with ascorbic acid (see Table 3, p. 67).



97

7-Methyladrenochrome, however, reacted with thioglycollic acid to give mainly an acidic product with an $\underline{R}_f$ value of 0.40 along with a nonacidic minor product with an $\underline{R}_f$ value of 0.06 which was probably analogous to the lactone 94. A trace of a compound having an $\underline{R}_f$ of 0.33, probably the reduction product, 5,6-dihydroxy-1,7-dimethylindole, was also observed. Acetylation of the product ($\underline{R}_f$ 0.40) gave a crystalline monoacetate. The i.r.,

Table 3

Thin-layer chromatography* of the products obtained in the reactions of thioglycollic acid with 4- and 7- methyladrenochrome in aqueous solution

Aminochrome	R_f values† and colours with the F_1 reagent and 1 N NaOH respectively		
4-Methyladrenochrome			
Total reaction mixture		0.39(s;V,BG)	
Phenolic products		0.39(V,BG)	
Acidic products		-	
5,6-Dihydroxy-1,4-dimethylindole		0.39(V,BG)	
7-Methyladrenochrome			
Total reaction mixture	0.06(m;B,BG)	0.33(w;B,-)	0.40(s;V,BG)
Phenolic products	0.06(B,BG)	0.33(B,-)	
Acidic products			0.40(V,BG)
5,6-Dihydroxy-1,7-dimethylindole		0.33(B,Bn)	

*Ascending development with 2% acetic acid in water on Eastman "Chromagram" cellulose sheets (20 × 20 cm).

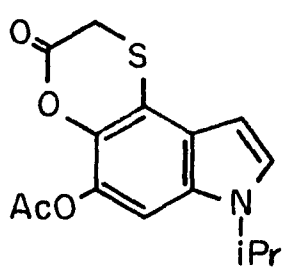
†A spot, R_f 0.85, due to excess thioglycollic acid was also observed in the reaction mixtures and in the acid extracts.

Abbreviations: F_1 = p-N,N-bis(2-chloroethyl)aminobenzaldehyde.
w = weak; m = medium; s = strong.
V = violet; B = blue; G = green; Bn = brown.

n.m.r. (see Table 2, p. 59) and u.v. spectral characteristics of this compound were analogous to those of the acetylated thioether 90 from the adrenochrome/thioglycollic acid reaction, suggesting that it had the structure 97. These results add additional support to the results which were obtained for the reaction between adrenochrome and thioglycollic acid.

3.1.2.2 Reaction of 1-isopropylnoradrenochrome with thioglycollic acid

1-Isopropylnoradrenochrome was found to react with thioglycollic acid in a manner analogous to adrenochrome. The acetylated thioether was obtained in crystalline form and shown by its spectral characteristics (see Table 2, p. 59 for partial n.m.r. spectrum) to be 6-acetoxy-4-carboxymethylthio-5-hydroxy-1-isopropylindole lactone (98).



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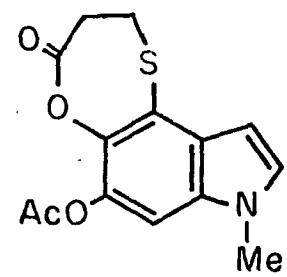

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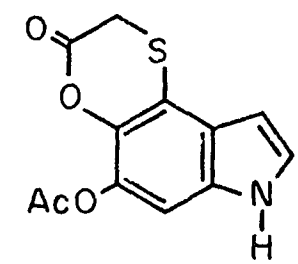


100

3.1.2.3 Reaction of adrenochrome with β-mercaptopropionic acid

Adrenochrome reacted in the same way with β-mercaptopropionic acid as it had with thioglycollic acid. Upon acetylation of the initially formed 5,6-dihydroxyindole thioether, a monoacetyl derivative 99 with a 7-membered lactone ring was obtained (see Table 2, p. 59 for partial n.m.r. spectrum).

3.1.2.4 Reaction of noradrenochrome with thioglycollic acid

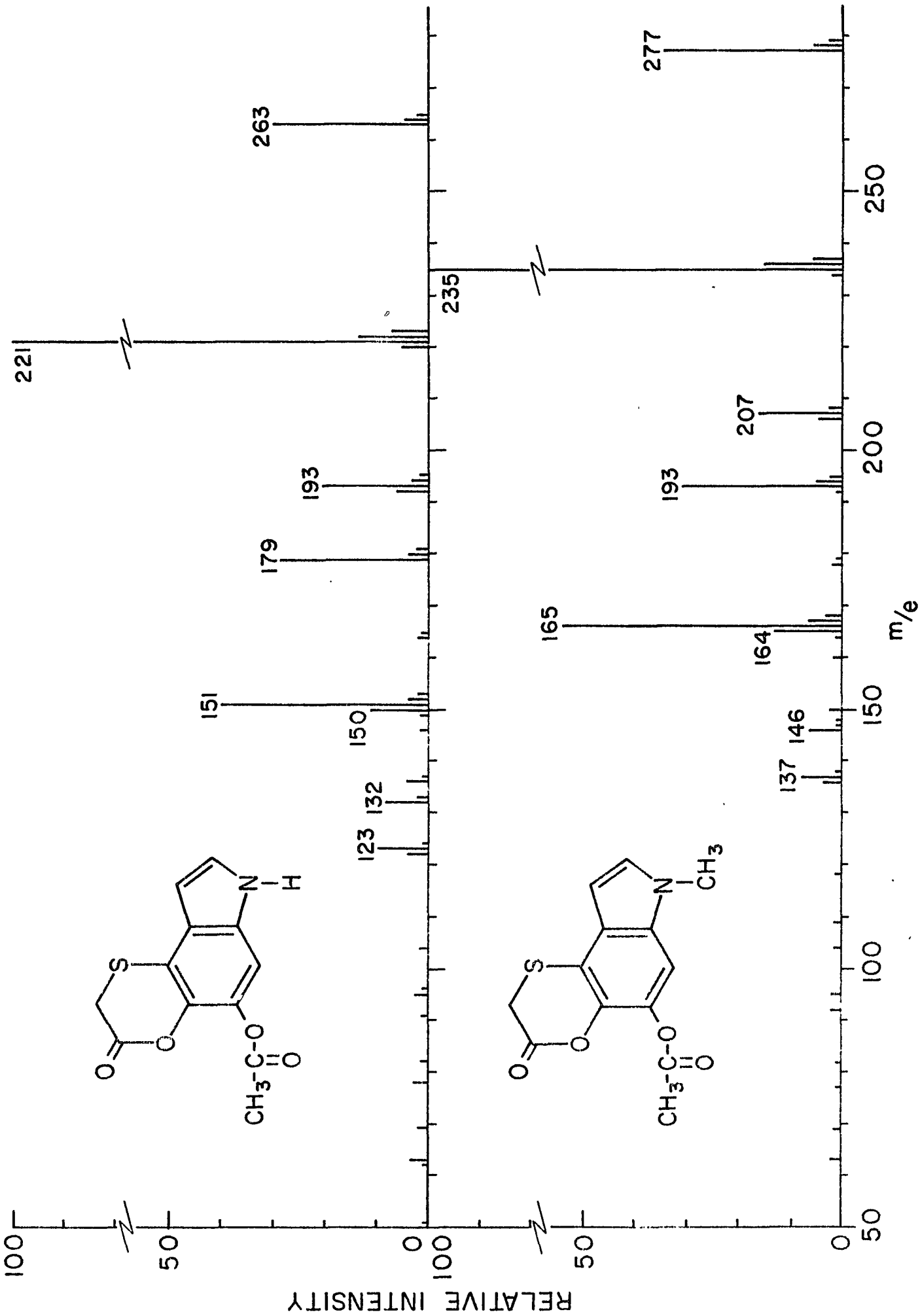
The reaction of noradrenochrome with thioglycollic acid in the absence of water gave mainly 5,6-dihydroxyindole and a compound having the chromatographic and chemical properties expected for a 5,6-dihydroxyindole thioether. The latter compound was acetylated and an acetyl derivative was obtained in rather low yield as a white crystalline solid. The molecular formula of this compound was found to be $C_{12}H_9NO_4S$ by mass spectrometry and its fragmentation pattern was very similar to that of the acetylated thioether from the adrenochrome/thioglycollic acid reaction (see Fig. 3, p. 70). It is quite likely, therefore, that this compound has the structure 100.

3.1.2.5 Reactions of adrenochrome with some thiol amino acids

The reactions of adrenochrome with some thiol amino acids in aqueous solution were investigated, since these reactions would have more relevance to biological systems.

Fig. 3

Mass spectra of the acetylated 5,6-dihydroxyindole thioether from the noradrenochrome/thioglycollic acid reaction (top) and 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindole lactone (90) (bottom).


RELATIVE INTENSITY
100
50
0
100
50
0
m/e
50
100
150
200
250
263
221
235
277
207
193
179
193
165
164
151
150
146
137
132
123
N–H
N–CH3
S
O
O
O
CH3–C–O
C

3.1.2.5.1 Homocysteine

Homocysteine [$HSCH_2CH_2CH(NH_3^+)CO_2^-$] reacted with adrenochrome to give two major indolic products: DHMI and what was probably a 4-thiosubstituted 5,6-dihydroxy-1-methylindole (cf. Ref. 133). After extraction of the DHMI with ethyl acetate the reaction mixture was lyophilized and then acetylated with acetic anhydride and pyridine. This gave rise to a mixture of several compounds, none of which could be isolated in a pure form. Work with this amino acid was therefore abandoned and the reactions of adrenochrome with some N-acetyl thiol amino acids investigated. The blocking of the amino group should: (a) result in the formation of an ethyl acetate extractable indole thioether and (b) eliminate secondary reactions involving the amino group.

3.1.2.5.2 N-Acetylcysteine

N-Acetylcysteine [$HSCH_2CH(NHAc)CO_2H$] reacted with adrenochrome to give two major products as detected by t.l.c. on cellulose with 2% aqueous acetic acid as solvent. One of the products, which had an R_f value of 0.45, was DHMI and the other, which had an R_f value of 0.68, appeared to be a 5,6-dihydroxyindole thioether. The latter substance reacted with chromogenic reagents in a manner similar to the thioether 92 which was formed in the reaction of adrenochrome with thioglycollic acid (see Sect. 3.1.1.1, p. 55). It was soluble

in aqueous sodium bicarbonate but could be extracted from the acidified reaction mixture with ethyl acetate.

Acetylation of the thioether gave a mixture of indolic products and preliminary attempts to separate these compounds by column chromatography on silica gel were not successful. Methylation of the thioether with diazomethane, however, gave only one major indolic product, as shown by t.l.c., with an R_f value of 0.75 in the chromatographic system described above. Attempts to purify this compound by column chromatography on silica gel were again unsuccessful and the only compound isolated was N,N-diacetylcystine dimethyl ester [$(-SCH_2CH(NHAc)CO_2Me)_2$]*. This compound had not been detected with the chromogenic reagents used previously, but it was found to give a positive reaction with the iodine/azide reagent for disulphides and thiols (cf. Ref. 178). Preparative t.l.c. also proved to be unsuccessful in the attempted purification of the methylated indole thioether. The N,N-diacetylcystine dimethyl ester was separated from the main product but there were still some impurities present and the oil which was obtained after elution of the appropriate band of the chromatogram could not be crystallized.

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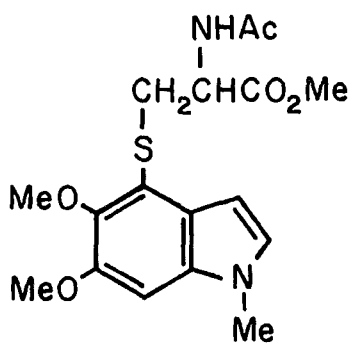
*Microanalytical data indicated that this compound (m.p. 124-126°) had a molecular formula of $C_{12}H_{20}N_2O_6S_2$ (lit. m.p. 125°[177]).

The next method tried was partition chromatography on a Sephadex G-25 column and this proved to be successful. The methylated indole thioether was obtained as a white crystalline solid. Its molecular formula, determined by mass spectrometry, was $C_{17}H_{22}N_2O_5S$. The i.r. spectrum of this compound exhibited peaks at 1737, 1651 and 1545 cm^{-1} for ester carbonyl, amide carbonyl and NH groups respectively. The aromatic region of its n.m.r. spectrum (see Sect. 5.5.7.2 p. 170) was similar to those of the acetylated indole thioethers previously isolated except for the presence of a broad NH peak. There were signals for four N- or O-methyl groups and one acetyl methyl group. An ABX system, with the X proton coupled to the NH was observed, presumably due to the $>CHCH_2-$ of a cysteine residue. One can conclude from these observations that the structure of this compound is 101.



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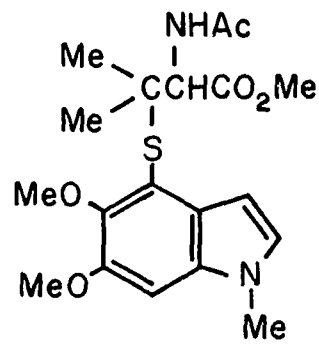


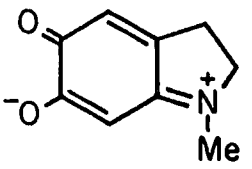
102

3.1.2.5.3 N-Acetylpenicillamine

N-Acetylpenicillamine [$HSC(Me)_2CH(NHAc)CO_2H$] reacted with adrenochrome as did N-acetylcysteine to give a mixture consisting mainly of DHMI and the indole thioether. The amount of the latter compound appeared to be somewhat less in this case, however, probably due to the steric effects of the methyl groups (cf. Ref. 133 and Sect. 1.4.6.3, p. 47) and there was a considerable amount of melanin produced. The methylated indole thioether proved to be more difficult to purify and, after column chromatography, first on Sephadex G-25 and then on silica gel, only a pale yellow oil, which could not be crystallized, was obtained. Mass spectrometry revealed that the molecular formula was $C_{19}H_{26}N_2O_5S$. Its n.m.r. spectrum was analogous to that of the N-acetylcysteine compound 101 so it would appear that the structure of this compound is 102.

3.1.3 Reactions of epinochrome with thiols

Since epinochrome (22) does not have a 3-hydroxyl group the initial products of the reaction could not lose the elements of water from the 2- and 3- positions of the "leuco derivatives" to form indoles, so the corresponding indolines should be obtained. One complication in working with epinochrome, however, is the extreme ease with which this compound rearranges to DHMI (see Sect. 1.4.1, p. 13).



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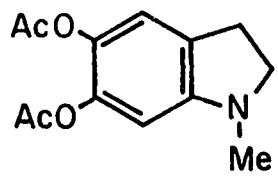
3.1.3.1 Preparation and properties of 5,6-diacetoxy-1-methylindoline (103)

Since 5,6-dihydroxy-1-methylindoline was expected to be one of the products of the reaction being investigated it was decided to synthesize 5,6-diacetoxy-1-methylindoline (103) by an unambiguous route so that it could be used as a standard. This was accomplished using a method analogous to that which had been used by Wyler and Chiovini for the preparation of 1,5,6-triacetoxy-2-carbomethoxyindoline (33) (see Ref. 59 and Sect. 1.4.2, p. 17). Thus epinine was oxidized with potassium ferricyanide to epinochrome, which was rapidly reduced with sodium dithionite to 5,6-dihydroxy-1-methylindoline. Acetylation of this compound gave a crystalline diacetate (i.e. 103). This compound had a λ_{max} at 310 nm in its u.v. spectrum and its i.r. spectrum revealed peaks at 1765 and 1756 cm^{-1} for the two acetyl carbonyl groups. The mass spectrum, together with microanalytical data, indicated the anticipated molecular formula (i.e. $C_{13}H_{15}NO_4$). In the n.m.r. spectrum of this



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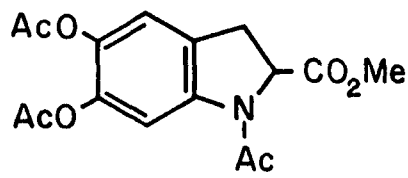

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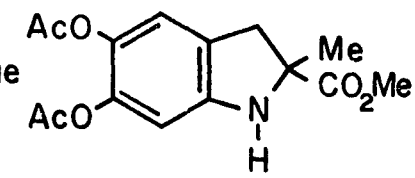


104

compound (see Table 5, p. 86 and Fig. 4, p. 77) benzylic coupling was observed between the proton in the 4-position and the C^3-methylene protons (J = 1.1 Hz). Coupling of this nature had previously been observed in similar compounds[60,61] and Fischer and Dreiding had found the corresponding coupling constant to be ca. 1.0 Hz in the case of 5,6-diacetoxy-2-carbomethoxy-2-methylindoline (104)[60].

3.1.3.2 Preliminary reactions

3.1.3.2.1 Initial products of reaction

Thin-layer chromatography of the products of the reactions between epinochrome and thiols was complicated by the aerial oxidation of the reaction products to red compounds. This occurred when the excess thiol was separated from the initial reaction products as a result of the chromatography. With more acidic thiols such as thioglycollic acid, N-acetylcysteine and glutathione, the major products seemed to be 4-thiosubstituted 5,6-dihydroxy-1-methylindoles, which were identical in their chromatographic and chemical

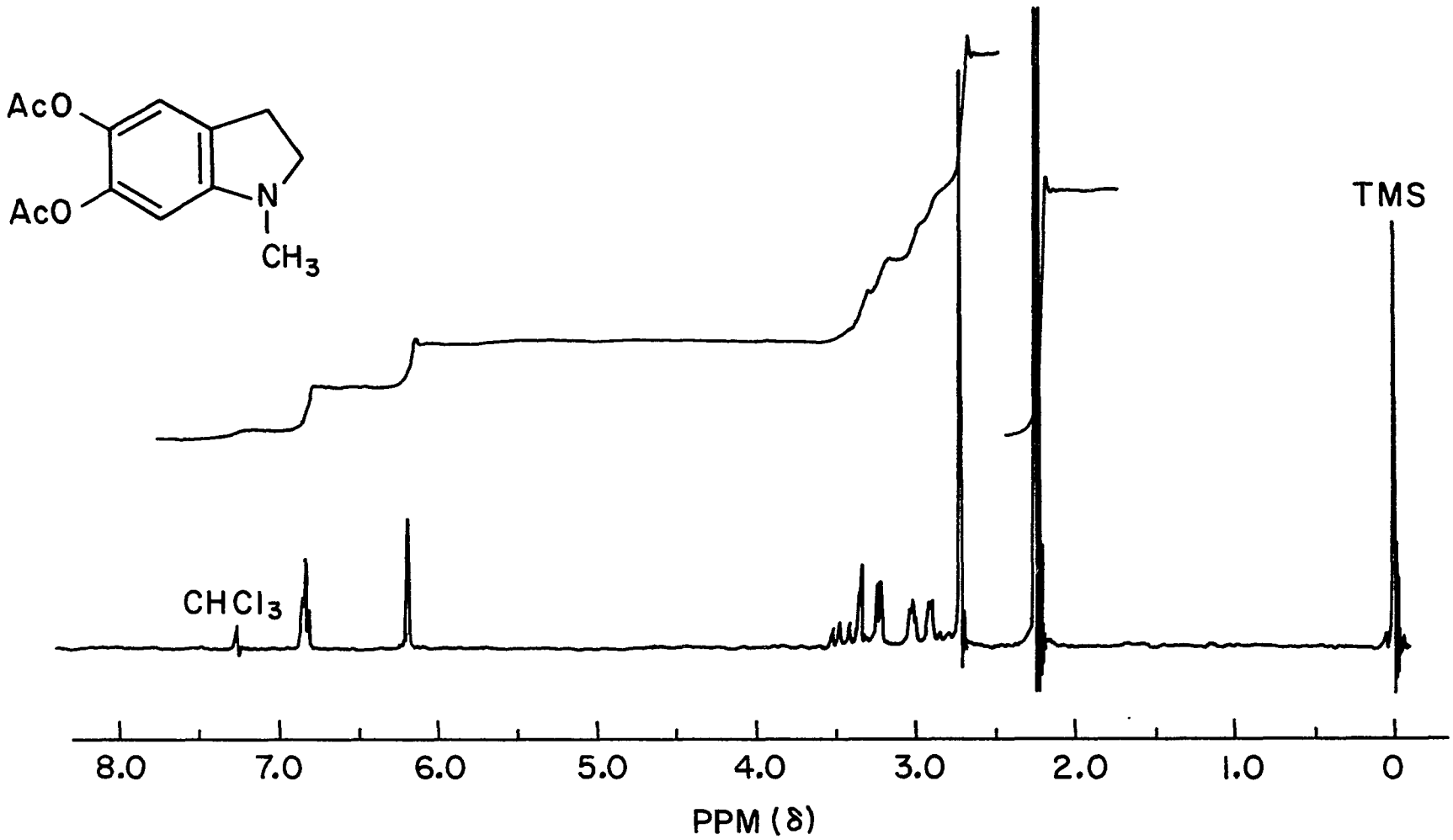
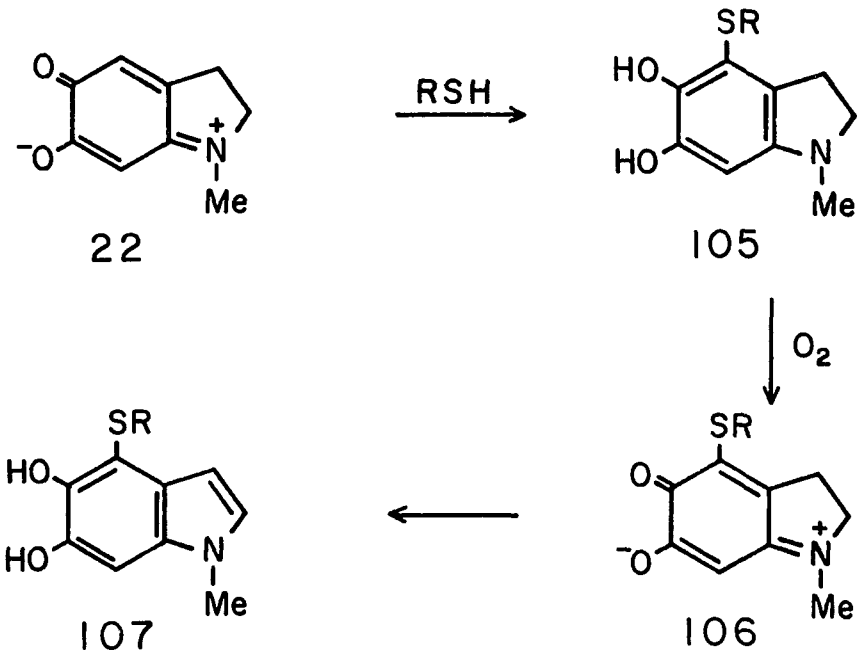


Fig. 4. N.m.r. spectrum of 5,6-diacetoxy-1-methylindoline (103) in $CDCl_3$.

properties to the indole thioethers formed in the reactions between adrenochrome and the corresponding thiols. In addition there was an unidentified nonindolic compound which could, in each case (including the reaction involving GSH), be extracted from the reaction mixture by ethyl acetate indicating that it probably did not contain a thiol residue. With less acidic thiols, such as homocysteine and the monosodium salt of glutathione, the major products were the indole thioether and DHMI. The latter compound was presumably formed by the rearrangement of the epinochrome which would be favoured at higher pH's. The indole thioethers observed in these reactions may have arisen due to the oxidation of an initially formed indoline thioether (i.e. 105) to the indolinequinone 106 followed by rearrangement to the 5,6-dihydroxyindole 107 as shown in Scheme 7. These reactions should be inhibited by the presence of excess thiol, but during the chromatography the thiol would be separated from the indoline thioether and the latter could then be oxidized by air.

The reaction between epinochrome and glutathione at pH 3.3 was followed spectroscopically. The peak at 476 nm due to epinochrome disappeared whilst the other epinochrome peak at 307 nm was replaced by a less intense one at 311 nm. The ether soluble products of the reaction mixture had a λ_{max} at 309 nm in aqueous solution and the



SCHEME 7

mother liquor showed maximum absorption at 312 nm. Mishra and Swan have reported the λ_{max} of 5,6-dimethoxyindoline in ethanol to be 308 nm[179] and the λ_{max} of 5,6-diacetoxy-1-methylindoline is 310 nm in ethanol, whereas DHMI in aqueous solution absorbs maximally at 299 nm with a shoulder at 283 nm. This would suggest that the ether extractable product was 5,6-dihydroxy-1-methylindoline. The indole thioether produced in the reaction between glutathione and adrenochrome has been reported[131] to absorb maximally at ca. 303 nm, somewhat lower than the λ_{max} of the mother liquor in the epinochrome/glutathione reaction. By analogy with the λ_{max} values for the compounds mentioned above, it

would appear that a 5,6-dihydroxy-1-methylindoline thioether (cf. 105), which would be expected to have a λ_{max} at a longer wavelength than the corresponding indole*, may be at least in part responsible for this absorption.

The reaction of epinochrome with N-acetylcysteine at a pH of 6.1, however, gave mainly an ethyl acetate extractable product which exhibited a λ_{max} in water at 299 nm (with a shoulder at 280 nm). This is typical of the spectrum of DHMI and suggests that the rearrangement reaction predominates at higher pH's. In the early stages of this reaction a shoulder was observed at ca. 360 nm, presumably due to the formation of an epinochrome/N-acetylcysteine addition product (see Sect. 3.3.6, p. 135).

3.1.3.2.2 Acetylation of reaction mixture

Since the initial products of the epinochrome/thiol reactions investigated appeared to be susceptible to oxidation, an attempt was made to acetylate the mixture of products obtained from the reaction of epinochrome with thioglycollic acid in the absence of any other solvent. After the reaction was complete a mixture of acetic anhydride and pyridine was added to the reaction mixture whereupon the solution became

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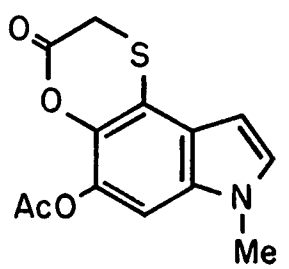
*In aqueous solution indole, for example, has a λ_{max} at 269 nm with peaks of lower intensity at 275 nm and 286 nm. Indoline has a λ_{max} at 288 nm in aqueous solution.

quite warm and changed from yellow to orange in colour and then back again to yellow.

After removal of the solvent, the acetylated reaction mixture was examined by t.l.c. on silica gel with a variety of solvents and chromogenic reagents. For comparison purposes, 5,6-diacetoxy-1-methylindole (108), and 6-acetoxy-4-carboxymethylthio-5-hydroxy-1-methylindole lactone (90), both of which had been prepared from adrenochrome, and 5,6-diacetoxy-1-methylindoline (103) were used as chromatographic standards. The chromatographic results, along with the colours given by the major products with various chromogenic reagents are summarized in Table 4, p. 82.



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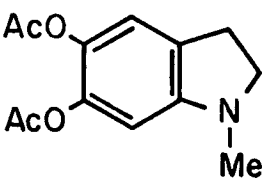

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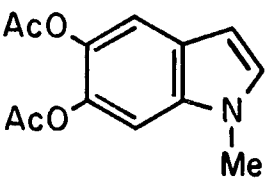

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The reaction gave rise to three major products, two of which appeared to be the acetylated indole thioether 90 and 5,6-diacetoxy-1-methylindoline (103). The latter

Table 4

Thin-layer chromatography* of the acetylated products of the reaction between epinochrome and thioglycollic acid

	R_f values†			Colours with chromogenic reagents				
	S_1	S_2	S_3	Ehr	Ehr (after heating)	Cin	Cin (after heating)	1 N NaOH
Reaction mixture	0.66	0.20	0.63(s)	V	B	R	V	BG
	0.61	0.39	0.37(s)	-	B	-	V	BG
	0.56	0.25	0.51(w)	P	V	O	VBn	Bn
	0.49	0.44	0.08(s)	-	V	-	VBn	V→Bn
6-Acetoxy-4-carboxymethylthio-5-hydroxy-1-methylindole lactone (90)	0.66	0.19	0.62	V	B	R	V	BG
5,6-Diacetoxy-1-methylindole	0.56	0.25	0.51	P	V	O	VBn	Bn
5,6-Diacetoxy-1-methylindoline	0.49	0.43	0.08	-	V	-	VBn	V→Bn

*Ascending development on Merck pre-coated silica gel F-254 t.l.c. plates (layer thickness 0.25 mm; 5 × 20 cm).

†Several other minor Ehrlich positive products were also present, along with products which appeared to be derived solely from the thioglycollic acid (positive iodine/azide reaction).

Solvents: S_1 = benzene/EtOAc (8:2); S_2 = hexane/EtOAc/pyridine (7:2:1); S_3 = benzene/EtOAc/formic acid (33:4:3).

Abbreviations: Ehr = Ehrlich's reagent; Cin = cinnamaldehyde.
s = strong; w = weak
V = violet; B = blue; R = red; G = green; P = pink; O = orange; Bn = brown

gave a violet colour with aqueous sodium hydroxide which changed to brown with time. It reacted with Ehrlich's reagent or cinnamaldehyde, however, only after the chromatogram was heated for several minutes. This was probably due to hydrolysis of the ester linkages followed by oxidation of the resulting 5,6-dihydroxyindoline to epinochrome. This could rearrange very readily to DHMI which would react with the chromogenic reagents for indoles. The third major compound, like 103, reacted with Ehrlich's reagent or cinnamaldehyde only after heating, suggesting that it too was an acetylated dihydroxyindoline derivative. Like the 4-thiosubstituted dihydroxyindoles discussed previously it gave a blue-green colour with sodium hydroxide, suggesting the blocking of melanin formation, possibly due to the presence of a thioether linkage on the aromatic ring. In addition to these three major products there were also several minor ones, including one which behaved like 5,6-diacetoxy-1-methylindole (108).

The indolic compounds in the reaction mixture were probably formed as a result of the removal of the protective effect of the thioglycollic acid due to the acetylation of the thiol group in the residual thioglycollic acid by acetic anhydride and pyridine. The 5,6-dihydroxyindolines present in the reaction mixture at this stage could be oxidized by air to indoline-5,6-quinones, which would be expected to

rearrange to 5,6-dihydroxyindoles in the presence of the pyridine. This would explain the transient orange colour observed in the reaction mixture shortly after the addition of the acetylating agent (see p. 81). When the acetylation reaction was carried out in a nitrogen atmosphere, with air being excluded as much as possible, t.l.c. of the reaction mixture indicated that, although the indoles were still present, they were formed to a lesser extent under these conditions, whereas the indoline compounds appeared to be present in greater amounts.

3.1.3.3 Isolation of products

The next step was to isolate the major products of the reaction and thereby obtain more conclusive evidence for the structures which have been proposed.

Isolation of the compound suspected to be the acetylated indole thioether 90 was fairly straightforward and could be accomplished by column chromatography on silica gel with benzene/ethyl acetate as eluant. After recrystallization this product was identical in all respects to samples of 90 which had been prepared by the methods described previously (cf. Sect. 3.1.1.2, p. 56). It was obtained in a yield of 7.1%.

Purification of the two indoline compounds was accomplished by effecting a preliminary separation from

impurities on a column of silica gel with hexane/pyridine as solvent. This was followed by purification on a second column of silica gel with benzene/ethyl acetate as eluant.

The compound thought to be 5,6-diacetoxy-1-methylindoline (103) was obtained as a white crystalline solid in a yield of 15.1%. It was identical in all respects to samples of 103 which had been prepared by another method (cf. Sect. 3.1.3.1, p. 75).

The remaining product was obtained as a white crystalline solid in a yield of 8.2%. It was shown by mass spectrometry to have a molecular formula of $C_{13}H_{13}NO_4S$ and its infrared spectrum revealed a peak at 1761 cm^{-1} probably due to ester and lactone carbonyl groups. Its n.m.r. spectrum, shown in Table 5, p. 86 and in Fig. 5, p. 87, was similar to that of 103 (see Table 5 and Fig. 4, p. 77) except for the absence of the peak due to the proton in the 4-position and one of the acetyl methyl signals. A peak which can be assigned to an S-methylene group was observed at δ 3.43. This is close to the position of the signal for the S-CH_2- of the corresponding acetylated indole thioether 90 which occurs at δ 3.50. As a result of these considerations it can be concluded that the structure of this compound is 109.

Judging by the yields obtained, the most important product of the reaction after acetylation was 5,6-diacetoxy-1-methylindoline (103) followed by 6-acetoxy-4-

Table 5

Nuclear magnetic resonance spectral* data for some of the acetylated products of the reaction between epinochrome and thioglycollic acid

Compound	δ-Values					
	H^4	H^7	$\underline{S}-CH_2-$	$-CH_2CH_2-$ (AA'BB' system)	$\underline{N}-CH_3$	$\underline{C}-CH_3$
5,6-Diacetoxy-1-methylindoline (103)	6.85 ($J_{3A,4}=J_{3B,4}=1.1$Hz)	6.21	-	3.54-2.72† (centred at 3.13)	2.72	2.25 2.22
6-Acetoxy-4-carboxymethylthio-5-hydroxy-1-methylindoline lactone (109)	-	6.10	3.43	3.63-2.71 (centred at 3.17)	2.73	2.32
6-Acetoxy-4-carboxymethylthio-5-hydroxy-1-methylindole lactone (90)	-	7.00	3.50	**	3.75	2.35

*The spectra were recorded in $CDCl_3$ with TMS as an internal reference.

†The upper half of the AA'BB' system for this compound was coupled to the C^4-proton ($J \approx 1.1$ Hz).

**The chemical shifts and coupling constants for the protons in the 2- and 3- positions of the indole nucleus are given in Table 2, p. 59.

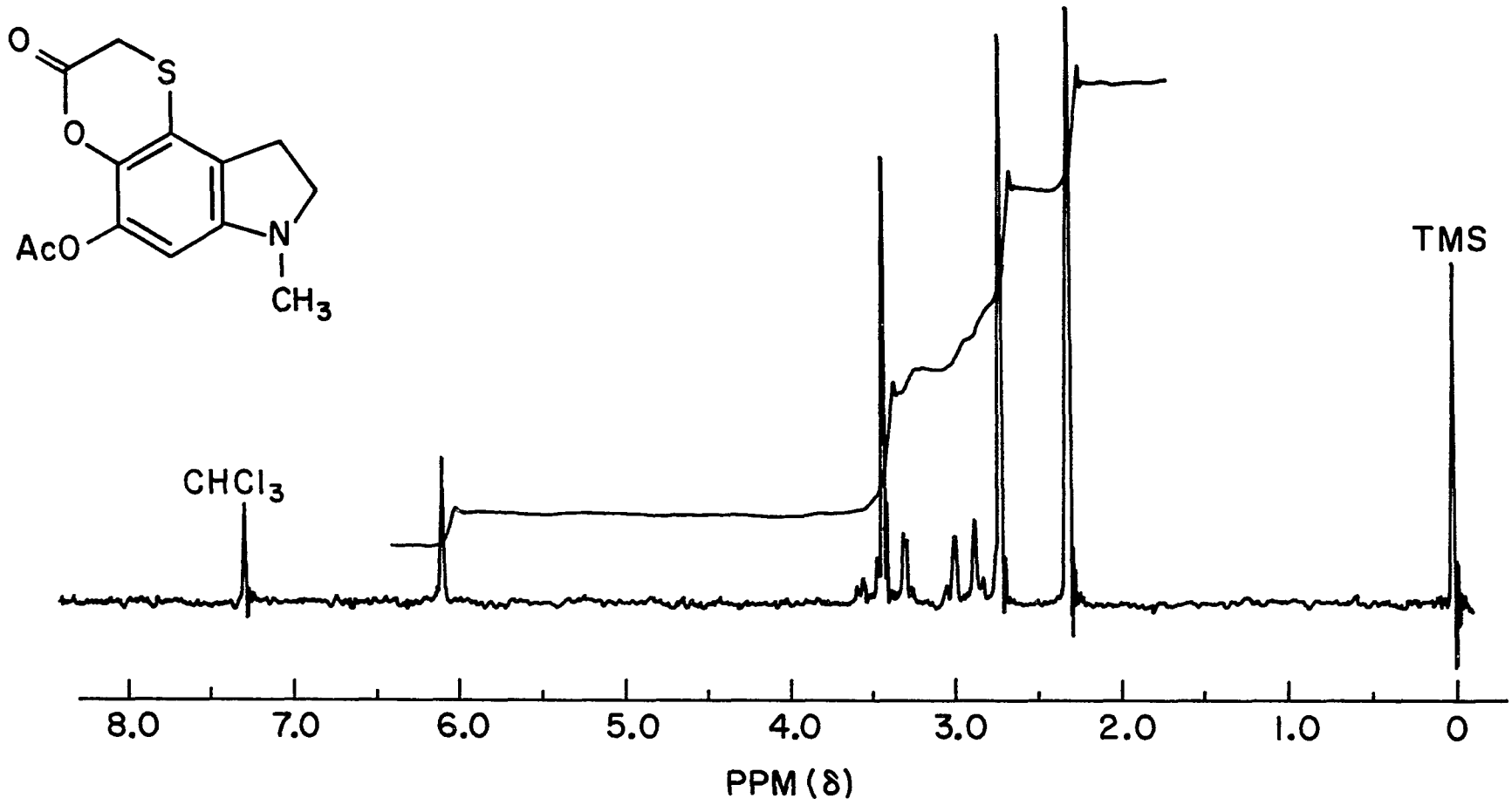
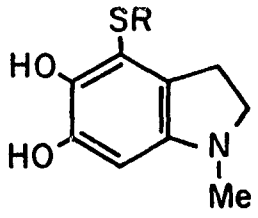


Fig. 5. N.m.r. spectrum of 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindoline lactone (109) in $CDCl_3$.



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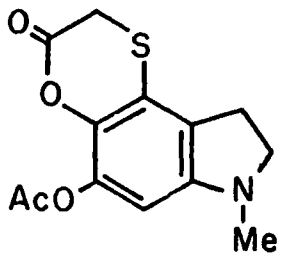

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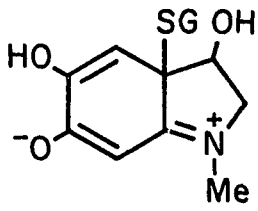
carboxymethylthio-5-hydroxy-1-methylindoline lactone (109) and 6-acetoxy-4-carboxymethylthio-5-hydroxy-1-methylindole lactone (90). The yields of the two indoline compounds with respect to the indole were probably lowered since purification of these compounds involved successive chromatography on two columns. Moreover, the acetylated indoline thioether was rather unstable in the pyridine solvent and this may have resulted in some loss due to decomposition.

The formation of the 5,6-dihydroxyindole thioether 90 may have been due to the oxidation of the initially formed dihydroxyindoline thioether (cf. 105) by oxygen, since complete exclusion of air proved to be rather difficult.

3.2 Aminochrome/thiol addition products

In 1964 Heacock and Mattok[130] proposed that the addition product formed between adrenochrome and glutathione had the structure 82. This product was not

isolated but its structure was proposed on the basis of the similarity of its chromatographic, u.v. absorbance, fluorimetric and some of its chemical properties with those of the adrenochrome/sodium bisulphite addition product (see Sect. 1.4.6.3, p. 42). Tse and Oesterling[74] proposed the structure 44 for the latter compound, largely on the basis



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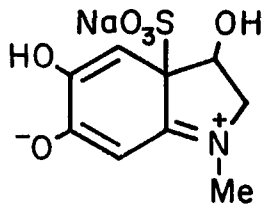

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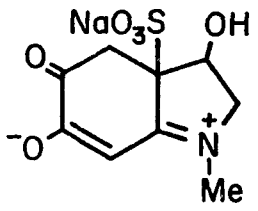

44

of its infrared spectrum, but they speculated that in aqueous solution, however, it would exist as the enol tautomer 45 (see Sect. 1.4.5, p. 25).

Because of the apparent analogy between the adrenochrome/thiol addition product and the adrenochrome/sodium bisulphite addition product it would be desirable to have more concrete evidence, such as n.m.r. spectral data, in support of the structure which has been assigned to the latter compound. It would first be necessary, for comparison purposes, to have an understanding of the n.m.r. spectra of aminochromes.

3.2.1 Nuclear magnetic resonance spectra of aminochromes

In 1965, Robinson[45] described the n.m.r. spectrum of rubreserine (17) as well as a partial spectrum of adrenochrome. For the latter compound the 4- and 7- protons were said to occur as singlets at δ 5.47 and δ 6.50 respectively.*

The n.m.r. spectra of a number of aminochromes and aminochrome semicarbazones have now been measured in solutions in DMSO-$\underline{d}_6$ and the values for the chemical shifts and coupling constants are given in Table 6, pp. 91-93 (see Fig. 6, p. 94 for the spectrum of adrenochrome in DMSO-$\underline{d}_6$).

The proton in the 4-position of the aminochrome nucleus is coupled to the C^3-proton(s) with a coupling constant of between 1.5 and 2.3 Hz. If a substituent is present in the 3-position, the signal due to the C^4-proton appears as a low field doublet. In the case of epinochrome, which does not have a 3-substituent, a triplet is observed for this proton due to coupling to the C^3-methylene protons. Homoallylic coupling between the 4-methyl protons and the proton in the 3-position is observed in the spectrum of 4-methyladrenochrome.

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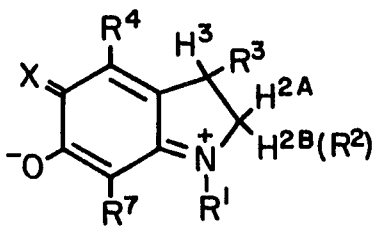
*In a personal communication to Dr. R. A. Heacock in 1966, Dr. Robinson stated that these assignments should have been reversed.

Nuclear magnetic resonance spectral* data for



	X	R^1	R^2	R^3	R^4	R^7
Adrenochrome (I)	O	CH_3	-	OH	H	H
Adrenochrome methyl ether† (II)	O	CH_3	-	OCH_3	H	H
7-Iodoadrenochrome methyl ether (III)	O	CH_3	-	OCH_3	H	I
4-Methyladrenochrome (IV)	O	CH_3	-	OH	CH_3	H
1-Isopropyl-noradrenochrome (V)	O	$\underline{i}$-C_3H_7	-	OH	H	H
Noradrenochrome (VI)	O	H	-	OH	H	H
2-Methyl-noradrenochrome (VII)	O	H	CH_3	OH	H	H
Epinochrome (VIII)	O	CH_3	-	H	H	H
Adrenochrome monosemicarbazone (IX)	$H_2NCONHN$	CH_3	-	OH	H	H
4-Methyladrenochrome monosemicarbazone (X)	$H_2NCONHN$	CH_3	-	OH	CH_3	H
Noradrenochrome monosemicarbazone (XI)	$H_2NCONHN$	H	-	OH	H	H

δ-Values

H^4	OH	H^7	H^3	H^{2B}	H^{2A}	$\underline{N}-CH_3$	Other
6.42(d)	6.02(d)	5.39(s)	4.98(m)	4.02(dd)	3.51(dd)	3.08(s)	-
6.46(d)	-	5.33(s)	4.83(m)	4.13(dd)	3.73(dd)	3.12(s)	3.46(s),O-CH
6.56(d)	-	-	4.63(m)	4.13(dd)	3.79(dd)	3.31(s)	3.60(s),O-CH
-	5.87(d)	5.33(s)	5.03(m)	4.03(dd)	3.52(dd)	3.07(s)	1.95(d),C-CH
6.43(d)	6.03(s)	5.46(s)	5.00(m)	4.01(dd)	3.40(dd)	-	4.07(m),N-CH 1.24(d),C-CH 1.23(d),C-CH
6.45(d)	**	5.48(s)	5.02(m)	4.01(dd)	3.44(dd)	-	
6.39(d)	6.07(s)	5.39(s)	4.50(t)	-	3.76(ddd)	-	9.35(s),NH 1.33(d),C-CH
6.36(t)	-	5.40(s)	-	-	-	3.11(s)	ca. 3.80(m), C^2H_2 ca. 3.00(m), C^3H_2
6.75(d)	5.74(d)	5.38(s)	5.00(m)	3.93(dd)	3.45(dd)	3.00(s)	14.72(s),NH 7.00(s),NH_2
-	5.56(d)	5.29(s)	5.03(m)	3.91(dd)	3.44(dd)	2.98(s)	14.98(s),NH 6.89(s),NH_2 2.17(s),C-C
6.80(d)	5.70(d)	5.39(s)	5.03(m)	3.90(dd)	3.39(dd)	-	14.83(s),N-N 8.55(s),C-N 7.02(s),NH_2

	Coupling constants (Hz)					
	$J_{3,4}$	$J_{3,OH}$	$J_{2B,3}$	$J_{2A,3}$	$J_{2A,2B}$	Other
I	2.0	5.5	6.8	3.4	-12.1	-
II	1.5	-	6.0	2.9	-12.0	-
III	1.6	-	5.6	2.4	-12.1	-
IV	-	7.0	6.2	2.0	-12.5	$J_{3,C-CH_3}$=1.1
V	2.0	#	6.8	3.2	-12.0	J_{CH,CH_3}=6.7
VI	2.0	**	6.7	3.2	-12.5	-
VII	2.2	#	-	3.6	-	J_{2,CH_3}=6.6
VIII	-	-	-	-	-	$J_{3A,4}$=$J_{3B,4}$=2.3
IX	1.7	4.8	6.6	2.8	-11.9	-
X	-	6.7	6.0	2.0	-12.0	-
XI	1.5	5.0	6.8	3.1	-11.8	-

Footnotes to Table 6.

*The spectra were recorded in DMSO-$\underline{d}_6$ (unless otherwise stated) using TMS as an internal reference.

†Spectrum recorded in acetone-$\underline{d}_6$.

#The peaks due to the OH protons in 1-isopropylnor-adrenochrome and 2-methylnoradrenochrome appeared as broad singlets and consequently the coupling constants between these protons and the C^3-methine protons could not be measured. A similar phenomenon was encountered in the case of adrenochrome if its spectrum was not recorded immediately after the solutions were prepared.

**The signals due to the OH and NH protons could not be seen in this case.

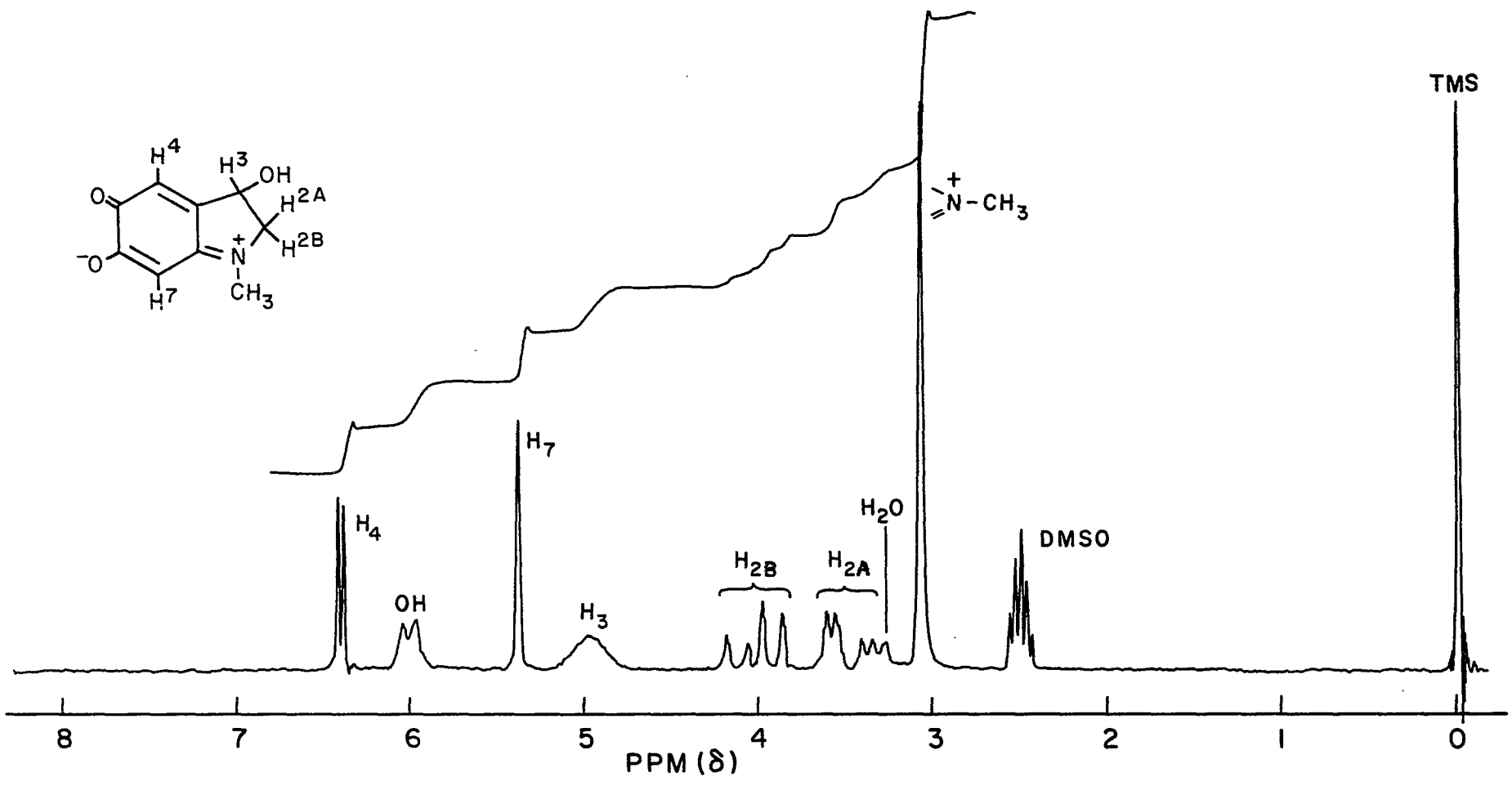


Fig. 6. N.m.r. spectrum of adrenochrome in DMSO-$\underline{d}_6$.

The hydroxyl proton of adrenochrome is coupled to the C^3-methine proton (J = 5.5 Hz), but this peak is not always easy to observe and collapses to a broad singlet with time. As would be expected it disappears in the presence of D_2O. In the case of 1-isopropylnoradrenochrome, there is a broad singlet for the hydroxyl proton. Signals due to either the OH or the NH protons could not be observed in the n.m.r. spectrum of noradrenochrome. The corresponding signals have been observed, however, in the spectra of all the aminochrome semicarbazones which have been investigated.

The signal due to the C^7-proton of aminochromes is observed as a singlet δ *ca.* 5.4. This signal is absent in the spectrum of 7-iodoadrenochrome methyl ether, confirming previous results[181] which suggested that the iodine atom was present in the 7-position. The halogen atom in halosubstituted aminochromes had formerly been considered to be in the 2-position of 3-substituted aminochromes and in the 3-position of aminochromes which did not have a 3-substituent (*cf.* Refs. 19 and 21).

The $-CH_2CH<$ of the 5-membered ring of 3-substituted aminochromes forms an ABX system which is observed as three quartets. The related $-CH_2CH_2-$ grouping of epinochrome forms a more complex system (which has not been analyzed) in which the C^3-methylene protons are coupled to the proton in the

4-position (J = 2.3 Hz). As might have been expected, solutions of epinochrome in DMSO-d_6 were not very stable and rearrangement to DHMI occurred (cf. Sect. 1.4.1, p. 13). Consequently it was impossible to obtain an n.m.r. spectrum of epinochrome free of signals from the rearrangement product.

As would have been anticipated, the n.m.r. spectra of the aminochrome semicarbazones investigated are quite similar to those of the corresponding aminochromes. Peaks due to the NH and NH_2 groups of the semicarbazone function were observed at δ ca. 14.85 and δ ca. 7.0 respectively.

3.2.2 Structure of the aminochrome/sodium bisulphite addition product

The n.m.r. spectrum of the adrenochrome/sodium bisulphite addition product was measured in solutions in DMSO-d_6 and D_2O. The spectrum of this compound in DMSO-d_6 (see Fig. 7, p. 97) is similar to that of adrenochrome (see Table 8, p. 118, and Fig. 6, p. 94), except that the signal for the proton in the 4-position of adrenochrome at δ 6.42 is replaced by a signal at δ 2.91 which integrates for two protons and is presumably due to the C^4-methylene protons of the keto tautomer 44. In solution in D_2O (see Fig. 8, p. 98) these methylene protons (which did not exchange, except in the presence of excess sodium

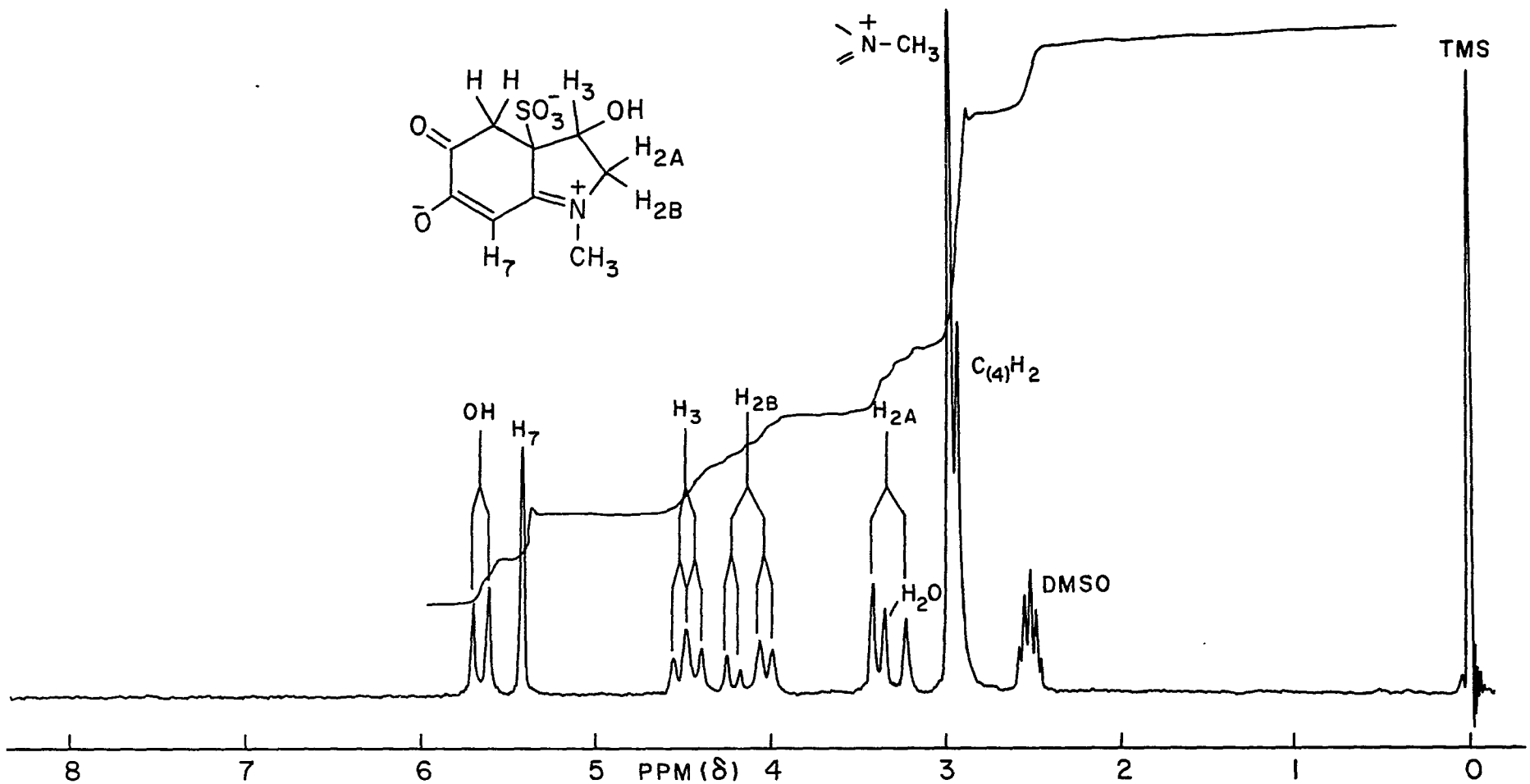


Fig. 7. N.m.r. spectrum of the adrenochrome/sodium bisulphite addition product in DMSO-$\underline{d}_6$.

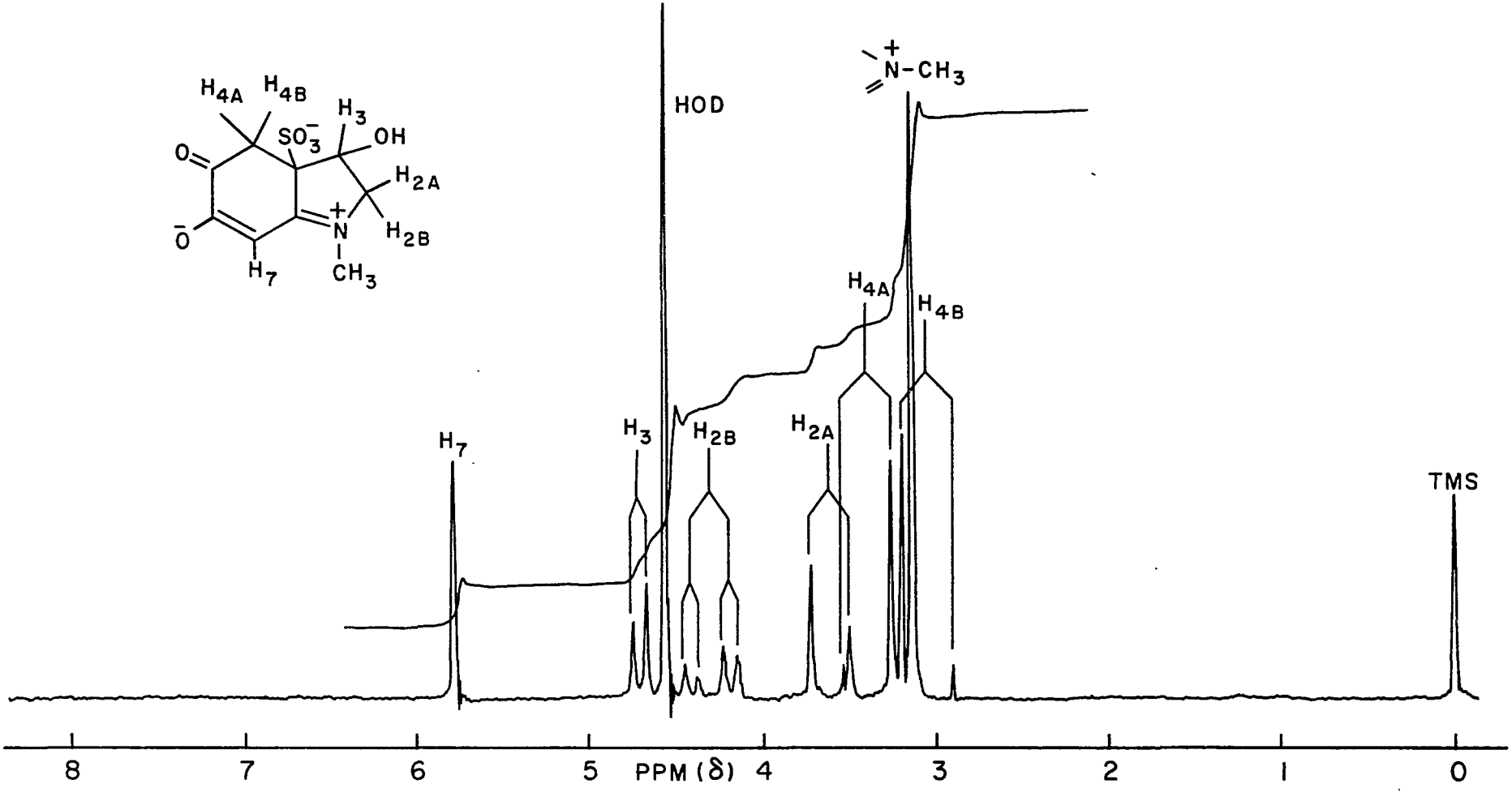
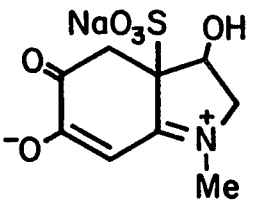
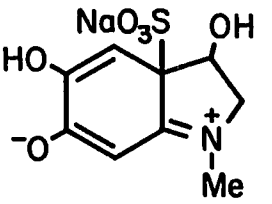


Fig. 8. N.m.r. spectrum of the adrenochrome/sodium bisulphite addition product in D_2O.



44

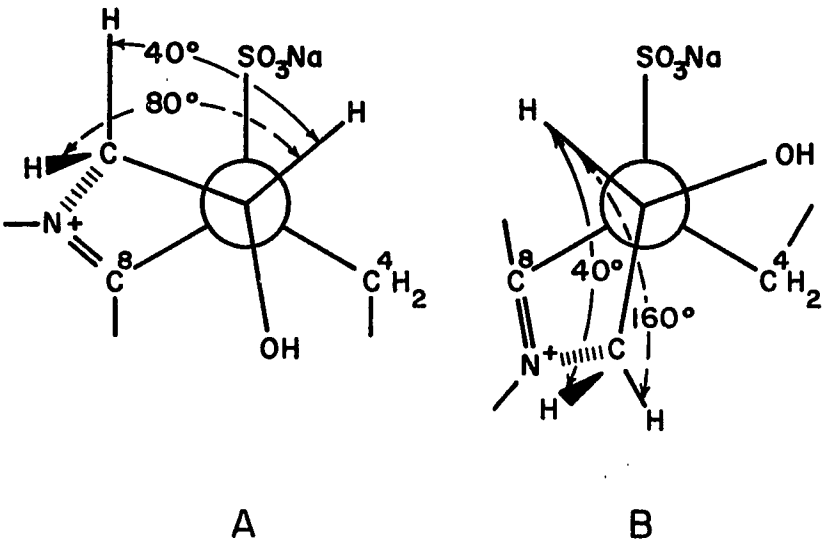


45

bisulphite) form an AB system with a geminal coupling constant of -17.0 Hz. As in the case of adrenochrome, the $-CH_2CH<$ of the 5-membered ring of the adrenochrome/sodium bisulphite addition product is observed as an ABX system but one of the vicinal coupling constants $J_{2A,3}$ is now apparently equal to ca. zero. Consequently, in solution in DMSO-d_6 the proton in the 3-position is observed as a triplet due to further coupling to the hydroxyl proton. One of the C^2-methylene protons, H^{2B}, forms a doublet of doublets and the other, H^{2A}, a doublet. These results would therefore indicate that the structure 44 which was proposed by Tse and Oesterling[74] is, in fact, the correct one for this compound (see Sect. 1.4.5, p. 25). No evidence was found in the course of the current investigations, however, for the existence of the enol form 45 in solution.

The vicinal coupling constants of 4.5 and ca. 0 Hz observed for the ABX system would correspond to dihedral

angles of about 40° and 80°. This could be explained in the case of the <u>trans</u> diastereoisomer, for example, if the most stable conformation resembled A, in which the hydroxyl group is situated between the C^4-methylene group and the sp^2-hybridized C^8-carbon. This conformation would probably be more



A B

favourable than B in which the hydroxyl group has <u>gauche</u> interactions with both the C^4-methylene group and the sulphonate group. Examination of suitable models shows that the dihedral angles between the C^2- and C^3- protons of conformation A would be approximately equal to 40° and 80°, whereas in B the angles would be more like 40° and 160°.

The sodium bisulphite addition products of 1-isopropylnoradrenochrome and adrenochrome methyl ether were synthesized using methods similar to that used by Tse and Oesterling[74] for the synthesis of the adrenochrome/

sodium bisulphite addition product (44). The u.v., i.r. and n.m.r. spectra of these compounds were similar to those of 44 (see Sect. 5.6, p. 177).

3.2.3 Structure of the aminochrome/thiol addition product

The aminochrome/thiol addition products were considerably less stable than the corresponding aminochrome/sodium bisulphite addition products (cf. Ref. 131) and consequently isolation of these compounds in the solid state proved to be impossible. van Espen[73] reported several years earlier that stable semicarbazone and p-nitrophenylhydrazone derivatives of the adrenochrome/sodium bisulphite addition product could be prepared (see Sect. 1.4.5, p. 23). This suggested that similar derivatives might be obtained from the reactions of aminochrome/thiol addition products with ketone reagents of this type.

3.2.3.1 Preliminary experiments

The reaction first studied in the current investigation was the one that occurred between adrenochrome and N-acetylcysteine. Preliminary spectroscopic experiments indicated that, under acidic conditions, this reaction gave rise mainly to products absorbing in the region 300-310 nm, (cf. Fig. 18, p. 129) presumably DHMI (λ_{max} 299 nm) and the

indole thioether*. At neutral or only slightly acidic pH's, however, a product with a λ_{max} at 358 nm, presumably the adrenochrome/N-acetylcysteine addition product, was initially the major product, although it was eventually replaced in the reaction mixture by the indolic products (cf. Sect. 3.3.1.2, p. 131).

The reaction between adrenochrome and N-acetylcysteine was carried out at a pH of 5.2 and the course of the reaction followed spectroscopically. After about an hour, when the concentration of the addition product reached its maximum level as indicated by a λ_{max} at 358 nm (see Fig. 9, p. 103), a solution of semicarbazide at the same pH was added. This gave rise to a much more intense peak at 352 nm, presumably due to the formation of a semicarbazone derivative of the thiol addition product. When the semicarbazide was not added to the reaction mixture, however, the peak at 358 nm diminished in intensity with time and was gradually replaced by another one at about 310 nm due to the formation of the indolic products. Addition of alkali to a solution containing the addition product semicarbazone resulted in its gradual conversion to a compound having spectroscopic properties similar to those of adrenochrome monosemicarbazone (37) (see Fig. 10,

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*The indole thioether formed in the reaction between adrenochrome and glutathione, for example, has been reported to have a λ_{max} at 303 nm[131].

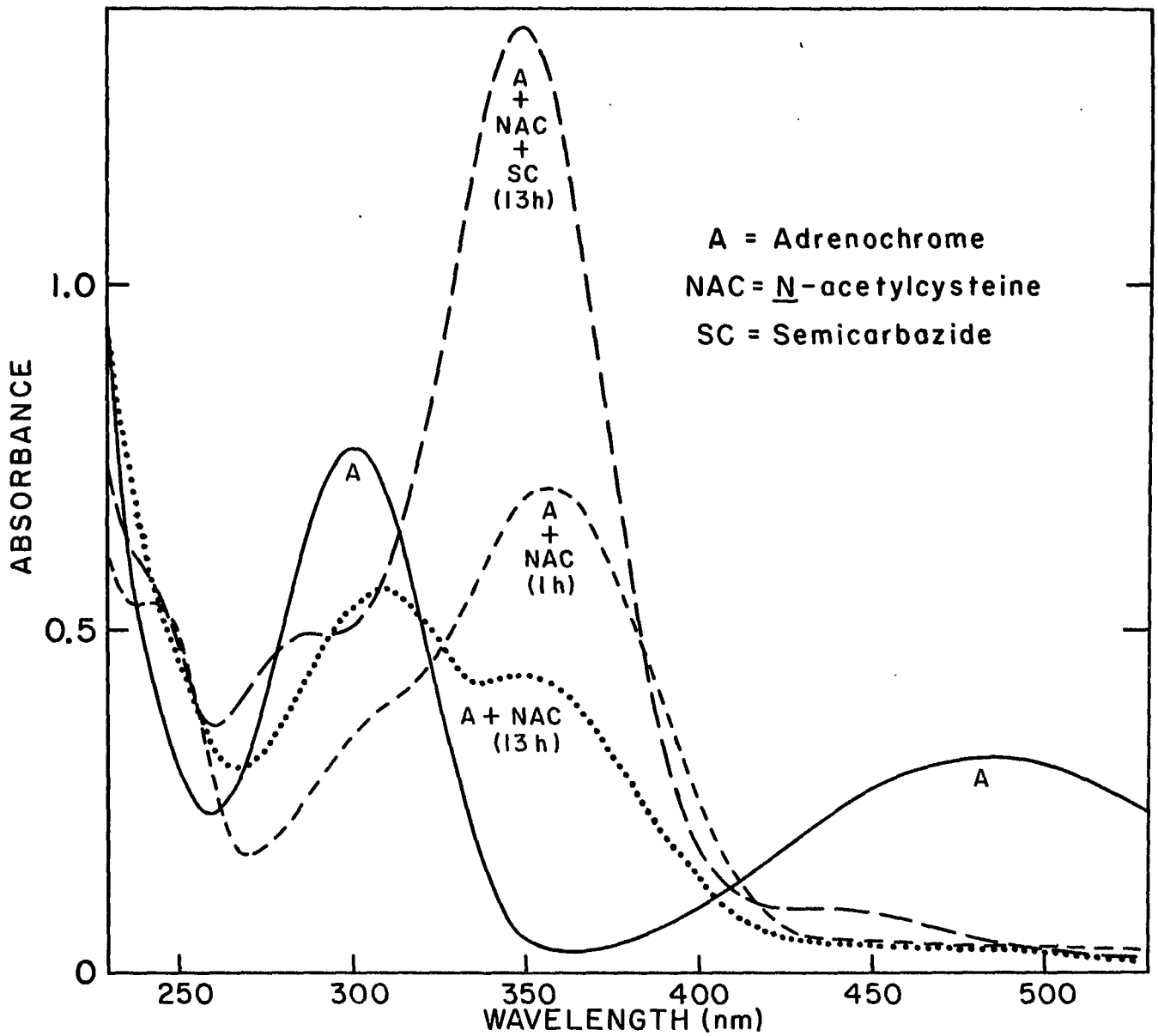


Fig. 9

U.v./visible spectra of adrenochrome, the products of the adrenochrome/N-acetylcysteine reaction (pH 5.2) and the products of the adrenochrome/N-acetylcysteine/ semicarbazide reaction (pH 5.2).

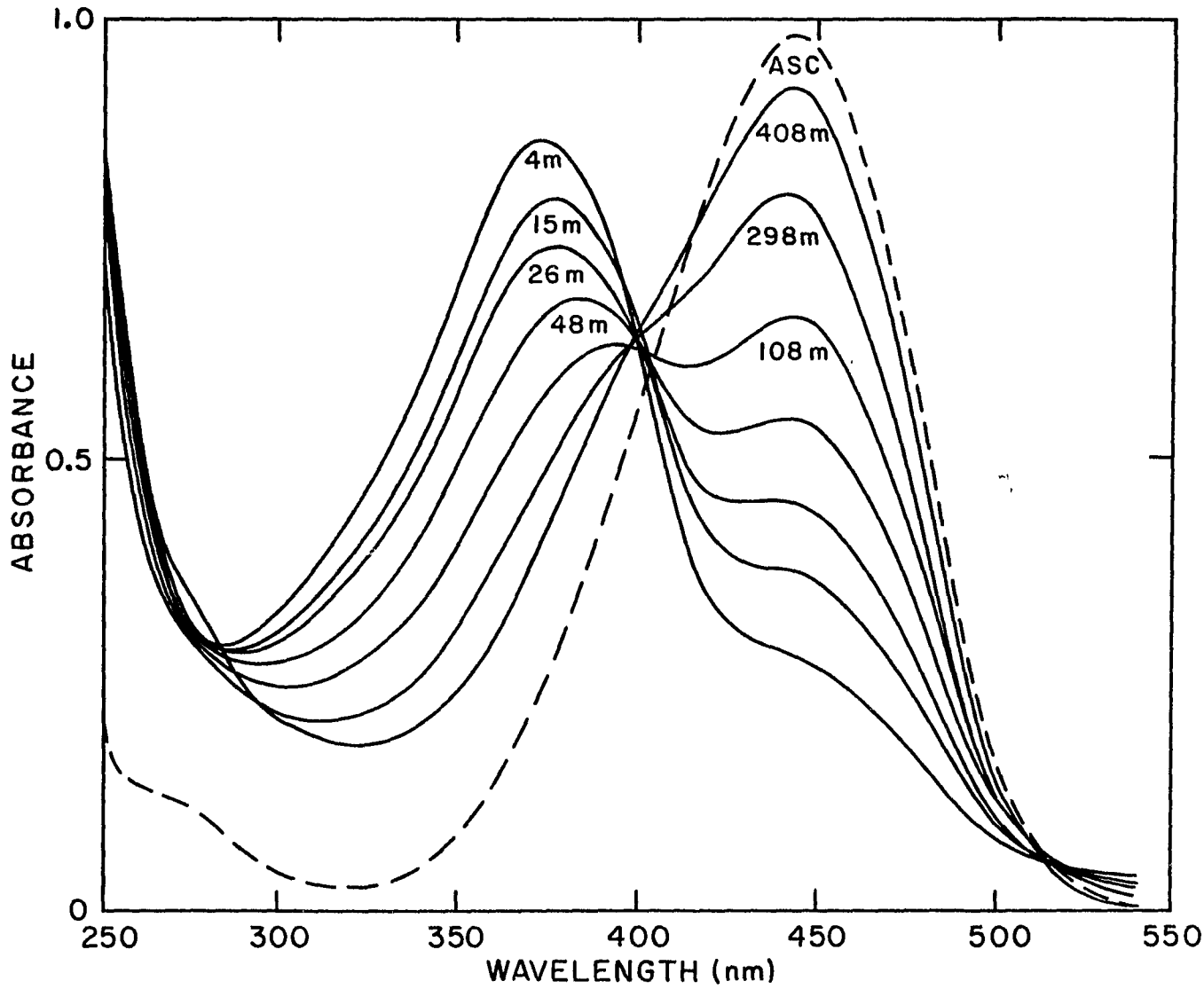
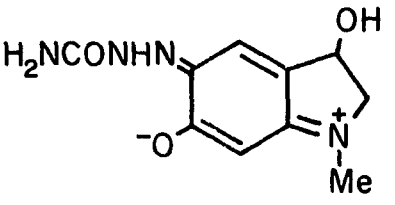


Fig. 10

U.v./visible spectra measured at various times during the course of the reaction between the semicarbazone of the adrenochrome/N-acetylcysteine addition product and sodium hydroxide [the spectrum of adrenochrome monosemicarbazone (ASC) is given for comparison].



37

p. 104). In alkaline solution this degradation product had a λ_{max} at 443 nm which shifted to 356 nm (with a shoulder at 445 nm) in neutral solution and to 374 nm in dilute HCl. The corresponding values for 37 in alkaline, neutral and acidic solutions are 444, 355 (with a shoulder at 445 nm) and 376 nm respectively (see Fig. 11, p. 106).

The reaction mixture containing the addition product semicarbazone was also examined by means of 2-dimensional thin-layer chromatography on cellulose using isopropanol/water/acetic acid (14:5:1) as running solvent (see Fig. 12, p. 107). For comparison purposes the reaction mixture and adrenochrome monosemicarbazone (37) were spotted close to the edges of the chromatogram in the positions indicated on the diagram. After the first run the major component appeared to be a very pale yellow substance (R_f, 0.25) exhibiting an intense blue fluorescence in ultraviolet light. A considerable amount of 37, which was observed as an intense yellow spot (R_f, 0.64), and several minor products were also present. Upon heating the developed chromatogram at 70°

Fig. 11

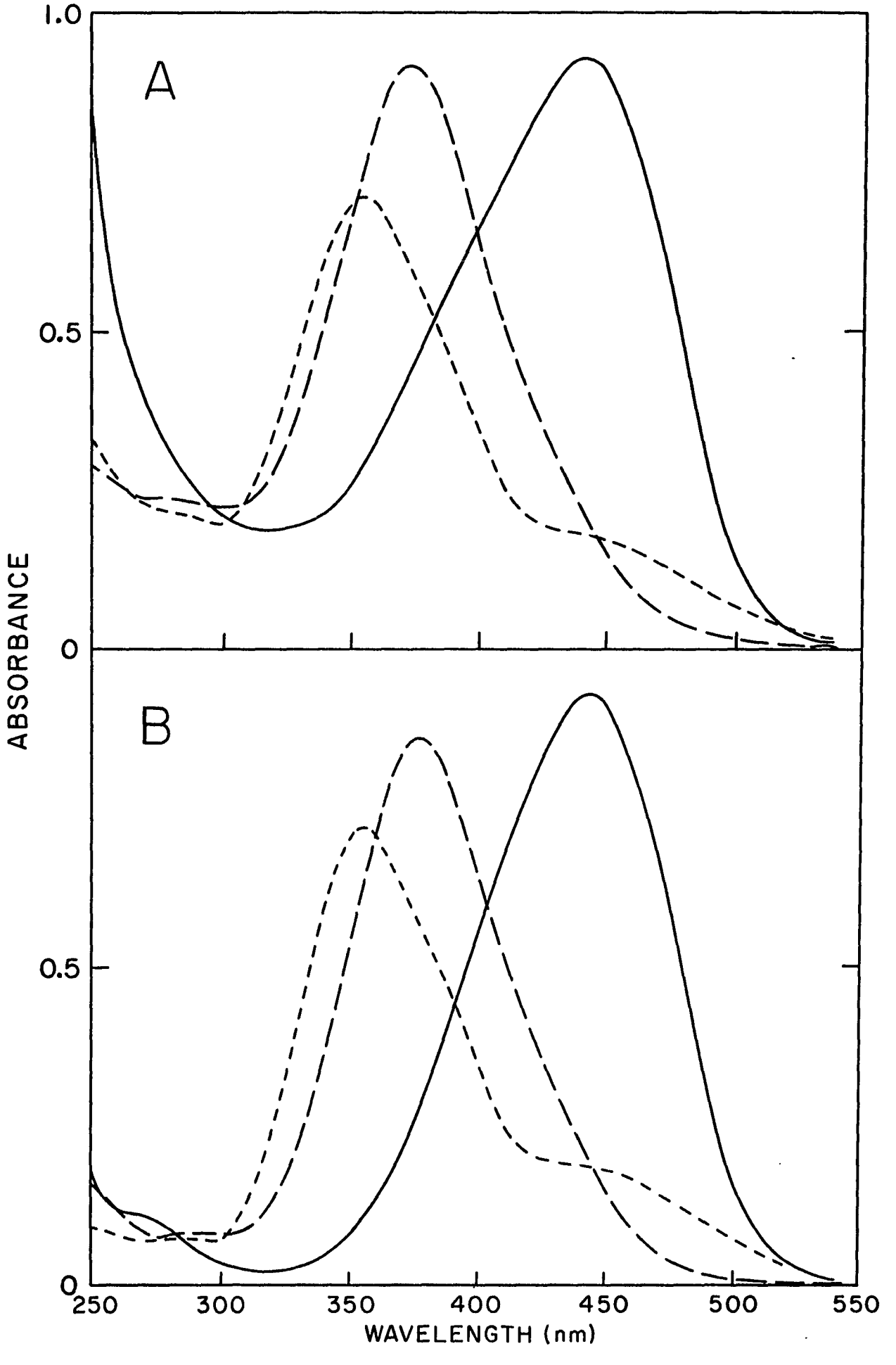
A U.v./visible spectra of the semicarbazone of the adrenochrome/N-acetylcysteine addition product.

B U.v./visible spectra of adrenochrome monosemicarbazone.

——— spectrum in strongly basic solution

- - - - spectrum in weakly acid (pH 5) solution

— — spectrum in strongly acidic solution


A
B
1.0
0.5
0
0.5
0
ABSORBANCE
250
300
350
400
450
500
550
WAVELENGTH (nm)

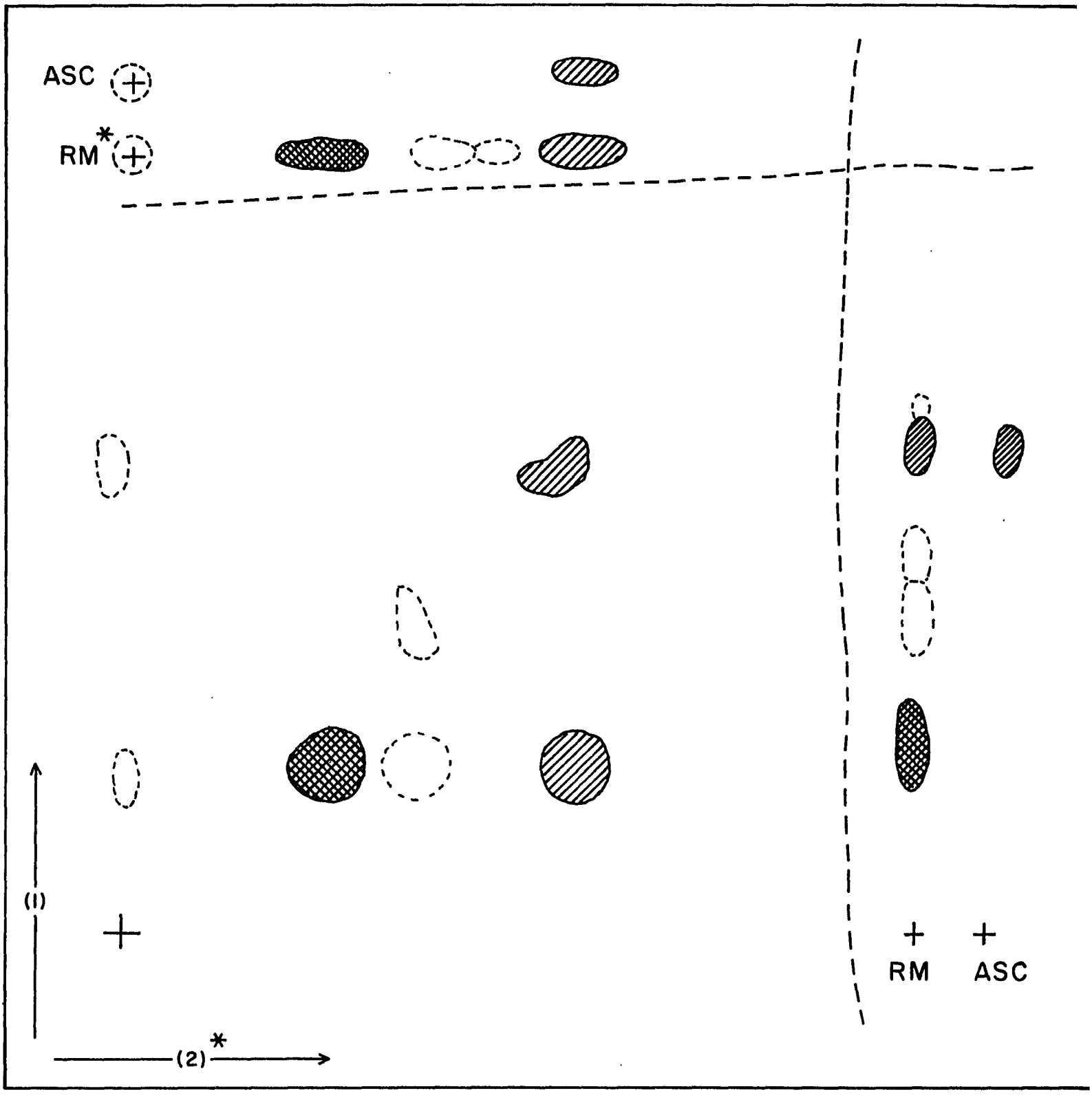
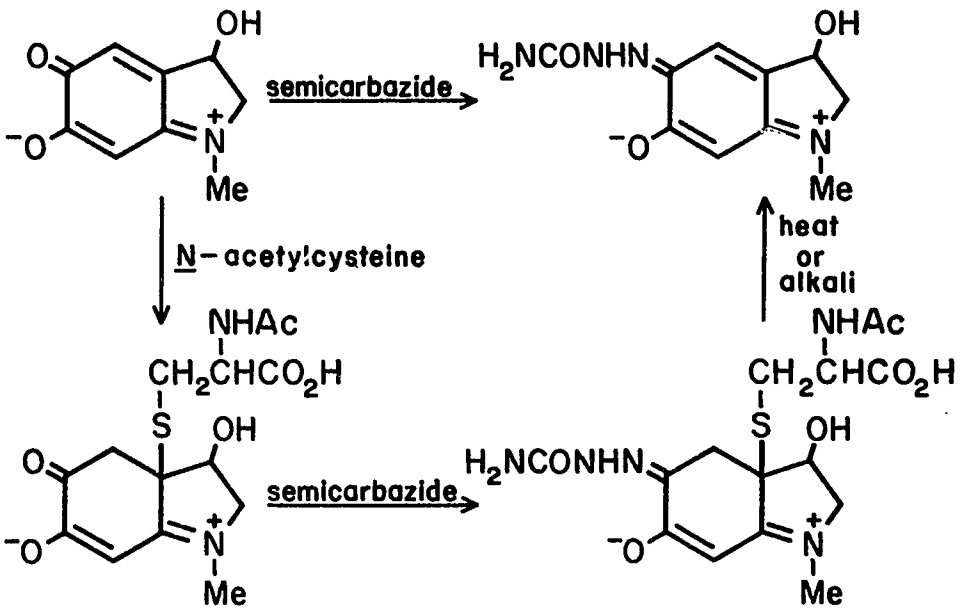


Fig. 12

Two-dimensional t.l.c. of the products of the adrenochrome/ N-acetylcysteine/semicarbazide reaction (see text for experimental details).

overnight the spot with an R_f of 0.25 became darker yellow in colour. After cooling down to room temperature the chromatogram was run again in the same solvent at right angles to the first direction. The yellow substance (R_f, 0.25) gave rise to an additional product which was yellow in colour and had an R_f identical to that of 37. The heating had apparently partially converted the original product (R_f, 0.25), presumably the semicarbazone of the addition product, to adrenochrome monosemicarbazone.

These experiments are comparable to those carried out by van Espen[73] with the adrenochrome/sodium bisulphite addition product. They would seem to indicate that the C^5-carbonyl of the aminochrome/thiol addition product had reacted with the semicarbazide to give a monosemicarbazone, showing that the addition product could not have been a mercaptole or an α-hydroxysulphide. The formation of what would appear to be a mono- rather than a di- semicarbazone could be explained if the C^6-carbonyl of the addition product was part of a vinylogous amide system as it is in adrenochrome. These results would therefore be consistent with the proposed attachment of the thiol residue to the 9-carbon atom of the adrenochrome nucleus and can probably be explained as shown in Scheme 8, p. 109.



SCHEME 8

3.2.3.2 Isolation of derivatives of aminochrome/thiol addition products

Preliminary attempts to isolate the semicarbazone of the adrenochrome/N-acetylcysteine addition product were unsuccessful due to the high solubility of this compound in water, even at acidic pH's. An attempt was made to find a more suitable ketone reagent for derivative formation and the most useful of those investigated proved to be p-nitrophenylhydrazine and p-bromophenylhydrazine. These formed arylhydrazones of the addition products which were insoluble in acidic solution but were quite soluble in

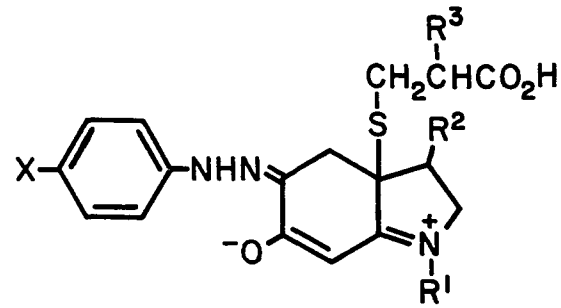
aqueous sodium bicarbonate, allowing them to be separated very readily from the corresponding aminochrome arylhydrazones, which were quite insoluble both in water and aqueous sodium bicarbonate. The p-bromo- and p-nitro- phenylhydrazones of the addition product formed from N-acetylcysteine and adrenochrome were prepared, along with the p-nitrophenylhydrazones of the addition products formed between β-mercaptopropionic acid and adrenochrome, 1-isopropylnoradrenochrome and epinochrome (see Table 7, p. 111). These derivatives appeared to be reasonably stable microcrystalline solids which could be recrystallized in most cases from either methanol or methanol/ethyl acetate. The microanalytical data and equivalent weights obtained for each of these compounds were in agreement with the structures proposed. It was not possible to detect the molecular ions of these compounds by mass spectrometry, which was not surprising since they decomposed without melting over a wide temperature range upon heating. Satisfactory melting or decomposition points could not be obtained for any of the arylhydrazones isolated.

3.2.3.2.1 Infrared spectroscopy

The i.r. spectra of these compounds are, unfortunately, rather difficult to interpret because of their complexity and only tentative assignments have been made. The spectrum (see Fig. 13, p. 112) of the

Table 7

Aminochrome/thiol addition product arylhydrazones which have been isolated



Aminochrome	Thiol	Substituted hydrazine	R^1	R^2	R^3	X
Adrenochrome	N-Acetylcysteine	p-Bromophenylhydrazine	Me	OH	NAc	Br
"	"	p-Nitrophenylhydrazine	Me	OH	NAc	NO_2
"	β-Mercaptopropionic acid	"	Me	OH	H	NO_2
l-isopropylnor-adrenochrome	"	"	iPr	OH	H	NO_2
Epinochrome	"	"	Me	H	H	NO_2

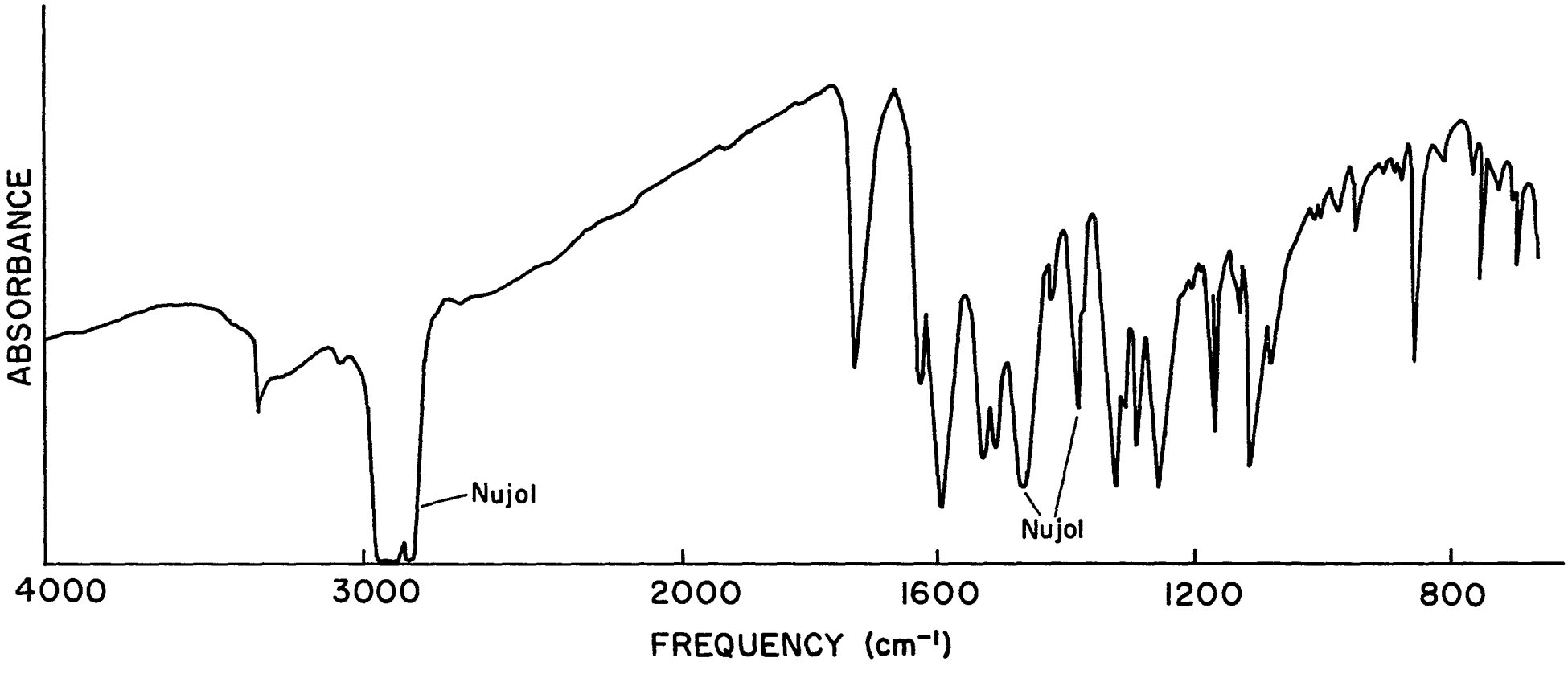


Fig. 13. I.r. spectrum (in Nujol) of the p-nitrophenylhydrazone of the adrenochrome/β-mercaptopropionic acid addition product

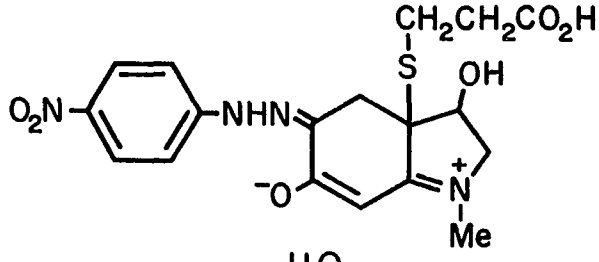
p-nitrophenylhydrazone of the adrenochrome/β-mercaptopropionic acid addition product (in "Nujol") will first be discussed in some detail. The NH/OH stretching region of the spectrum of this compound reveals a peak at 3335 cm^{-1} with a shoulder at 3290 cm^{-1}, presumably due to the NH and C^3-OH groups. The carboxylic acid OH group would be expected to be observed as a fairly broad peak and is probably covered up by the peaks mentioned above as well as the very strong Nujol peak. In the carbonyl region there is a peak at 1723 cm^{-1}, probably due to the carboxylic acid carbonyl group. There are peaks at 1621 and 1590 cm^{-1} which may be due to the vinylogous amide system (cf. Refs. 182 and 183). The corresponding system in adrenochrome gives rise to peaks at 1613 and 1570 cm^{-1} and, in the adrenochrome/sodium bisulphite addition product, to peaks at 1629 and 1571 cm^{-1}. Peaks are observed at 1522 and 1503 cm^{-1} which could be due to NH bending and asymmetric NO_2 stretching vibrations. The other nitro peak due to symmetric NO_2 stretching vibrations occurs at 1317 cm^{-1}. In general the other addition product arylhydrazones have similar i.r. spectra except that the addition products involving N-acetylcysteine are somewhat more complex due to the additional amide absorptions. The spectrum of the p-bromophenylhydrazone derivative is similar except that there are no peaks corresponding to those which are observed for the nitro group in the p-nitrophenylhydrazones.

To summarize, the infrared spectra of these compounds would be consistent with the presence of, firstly, a vinylogous amide system, secondly, a carboxyl group which would be derived from the thiol, and lastly, in the case of the p-nitrophenylhydrazones, a nitro group.

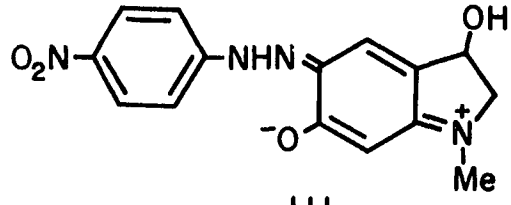
3.2.3.2.2 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy proved to be the most useful tool in determining the structrues of these compounds. It was rather difficult to obtain good spectra and it was found to be essential to use mixed solvents in order to separate the chemical shifts adequately. Changes in the composition of the solvent mixtures used affected the chemical shifts of some protons more than those of others. Evidence obtained in this manner together with relevant spin-spin decoupling experiments enabled some of the assignments to be made.

The n.m.r. spectrum of the p-nitrophenylhydrazone (i.e. 110) of the addition product between adrenochrome and β-mercaptopropionic acid was measured in solution in



110



111

pyridine-$\underline{d}_5/D_2O$ (5:1) and is shown in Fig. 14, p. 116.

The general pattern of the spectrum of this compound is very similar to that of the adrenochrome/sodium bisulphite addition product (see Table 8, p. 118). As in the case of the latter compound an analogy may be drawn with the spectrum of adrenochrome as well as that of adrenochrome mono-p-nitrophenylhydrazone (111) (see Table 8). It should be pointed out, however, that the chemical shifts observed for the protons of 110 cannot be compared directly with those of the other three compounds in Table 8 since the solvent is different. The pyridine solvent used to measure the spectrum of 110 caused a considerable displacement to lower field of the chemical shifts observed for some of the protons.

The signal corresponding to that for the 4-proton of adrenochrome or adrenochrome mono-p-nitrophenylhydrazone (111) is absent in the spectrum of 110. Instead, a pair of doublets (i.e. an AB system), apparently integrating for two protons (see Fig. 15, p. 117), is observed at higher field and is presumably due to the C^4-methylene protons. An AB system was also observed for the corresponding protons in the spectrum of the adrenochrome/sodium bisulphite addition product in D_2O solution (see Fig. 8, p. 99).

The $-CH_2CH<$ of the 5-membered ring gives rise to an ABX system (see Fig. 15) which is quite similar to that of the adrenochrome/sodium bisulphite addition product

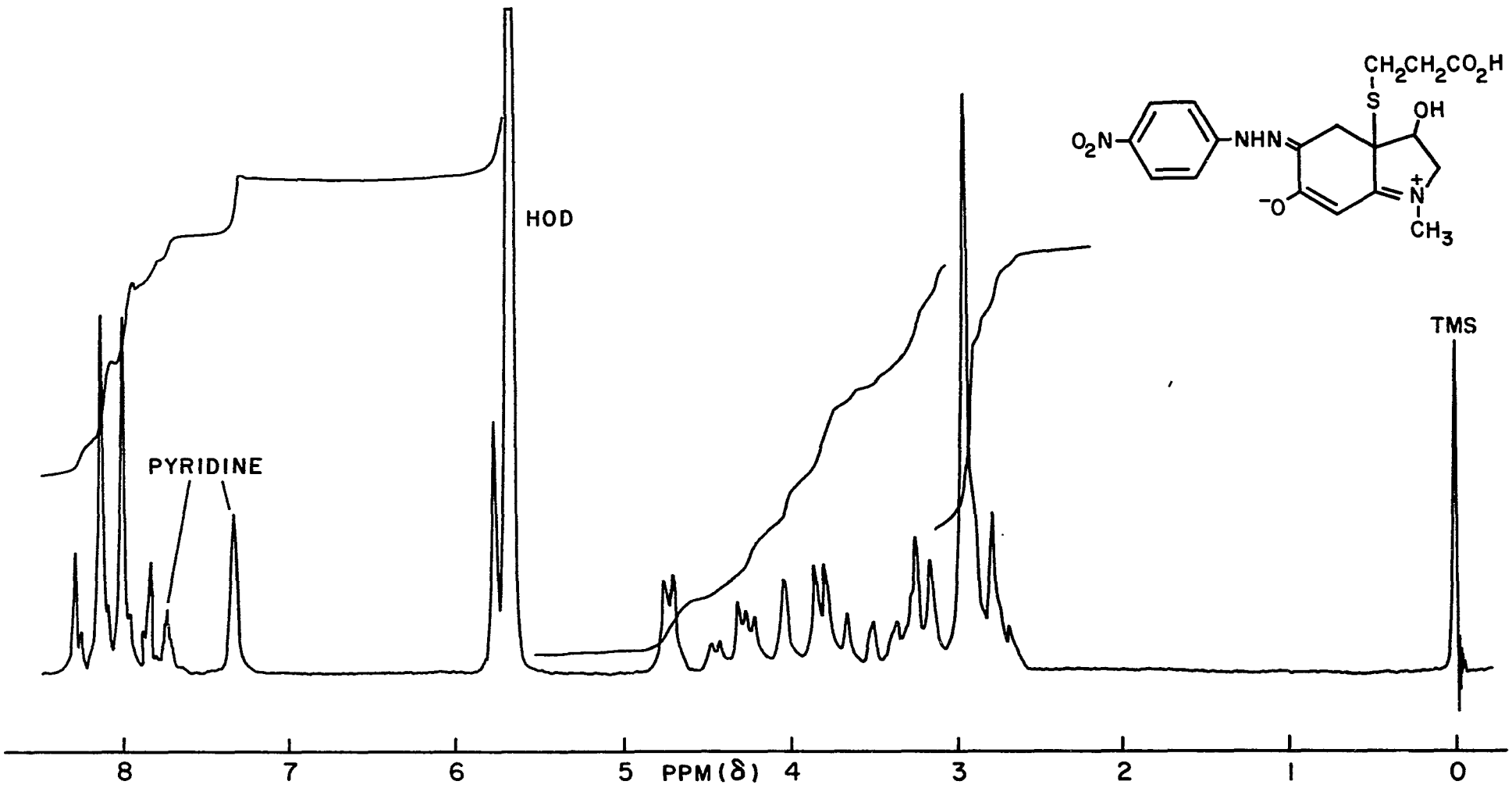


Fig. 14. N.m.r. spectrum of the p-nitrophenylhydrazone of the adrenochrome/β-mercaptopropionic acid addition product in pyridine-d_5/D_2O (5:1).

Fig. 15

Expanded portion of the n.m.r. spectrum of the p-nitrophenylhydrazone of the adrenochrome/β-mercaptoprioponic acid addition product in pyridine-$\underline{d}_5/D_2O$ (5:1).

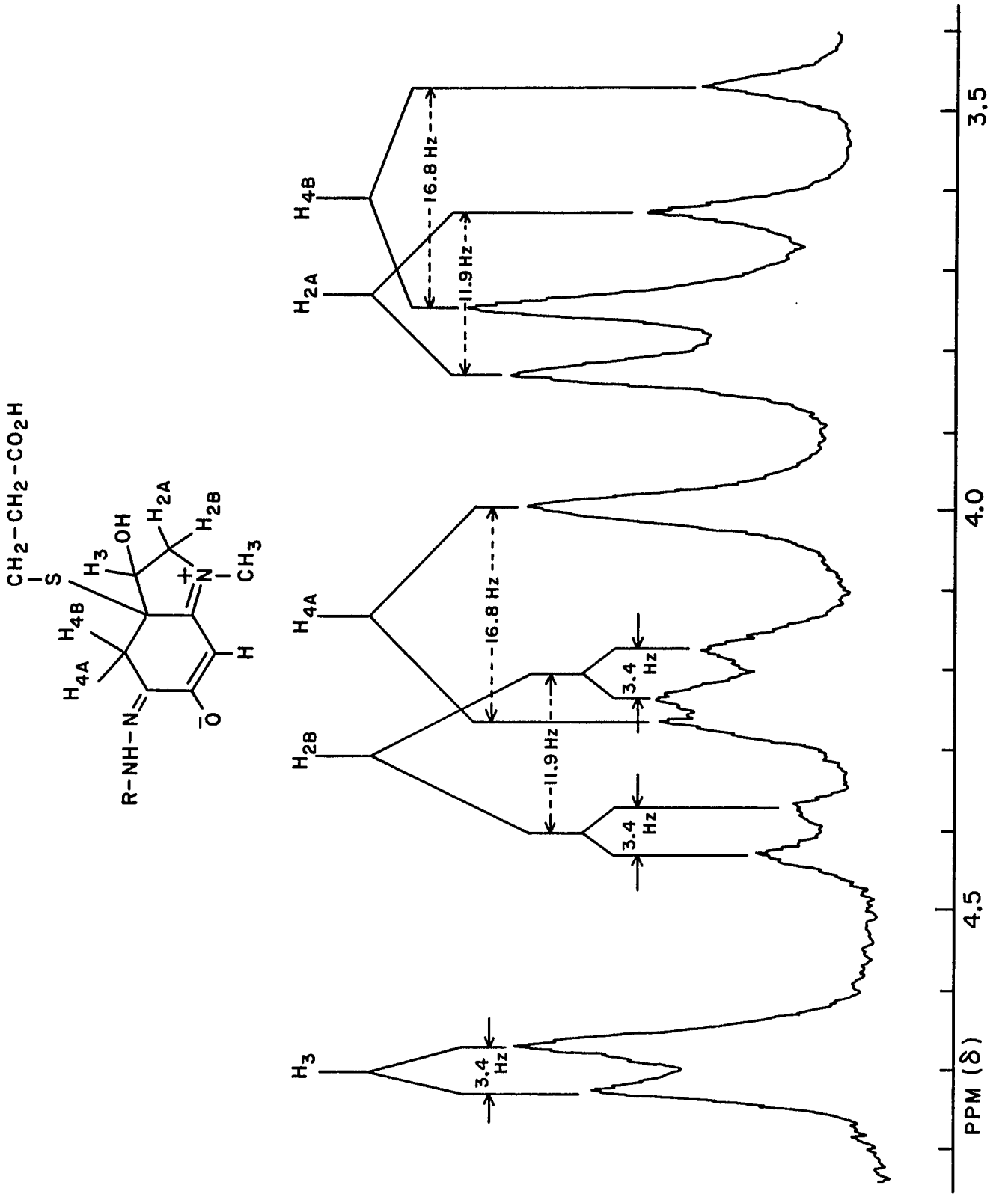

CH2-CH2-CO2H
S
H3
OH
H2A
H2B
H4B
H4A
+N
CH3
H
O
R-NH-N
H3
H4A
H2B
H4B
H2A
16.8 Hz
11.9 Hz
3.4 Hz
PPM (δ)
4.5
4.0
3.5

Table 8

Nuclear magnetic resonance spectral data* for adrenochrome and some of its derivatives and addition products

		Adrenochrome	Adrenochrome-p-nitrophenylhydrazone (111)†	Sodium 4,9-dihydroadrenochrome-9-sulphonate (44)	9-S-(β-carboxyethylthio) 4,9-dihydroadrenochrome mono-p-nitrophenyl-hydrazone (110)#†
δ-Values	H^4	6.42(d)	6.92(d)	-	-
	OH	6.02(d)	5.70(d)	5.65(d)	-
	H^7	5.39(s)	5.52(s)	5.41(s)	5.77(s)
	H^3	4.98(m)	5.02(m)	4.48(t)	4.73(d)
	H^{2B}	4.02(dd)	3.91(dd)	4.12(dd)	4.31(dd)
	H^{2A}	3.51(dd)	3.46(dd)	3.34(d)	3.77(d)
	N-CH_3	3.08(s)	3.02(s)	2.95(s)	2.96(s)
	Other	-	8.36-7.39(aromatic)	2.91(s,C^4H_2)	8.40-7.76(aromatic) 4.13(d,H_{4A});3.67(d,H_{4B}
Coupling constants (Hz)	$J_{3,4}$	2.0	1.2	-	-
	$J_{3,OH}$	5.5	4.3	5.2	-
	$J_{2B,3}$	6.8	6.8	4.5	3.4
	$J_{2A,3}$	3.4	3.2	ca. 0.0	ca. 0.0
	$J_{2A,2B}$	-12.1	-12.0	-11.9	-11.9

Footnotes to Table 8

*The spectra were recorded in DMSO-$\underline{d}_6$ (unless otherwise stated) using TMS as an internal reference.

†Only the signals relevant to the discussion are given for these compounds.

#Spectrum recorded in pyridine-$\underline{d}_5/D_2O$ (5:1).

(see Table 8, p. 118). One of the coupling constants, $J_{2A,3}$ is again equal to zero. Consequently the C^3-methine proton is observed as a doublet, one of the C^2-methylene protons, H^{2B}, as a doublet of doublets and the other, H^{2A}, as another doublet.

A complex multiplet, centred at δ 3.03, which is partially obscured by the N-CH_3 signal, is observed for the four protons of the carboxylic acid side chain in the 9-position. Signals are also observed for the proton in the 7-position and for the aromatic protons and these are similar to those observed in the spectrum of adrenochrome mono-p-nitrophenylhydrazone.

In general the n.m.r. spectra observed for the other compounds listed in Table 7, p. 111, are similar to that which has just been discussed. The spectra of the arylhydrazones of the adrenochrome/N-acetylcysteine addition product (cf. Fig. 16, p. 121) are slightly more complex due to the change in the side chain derived from the thiol but they are still very similar to the spectrum of 110. Peaks were not observed for either the C^3-OH or the carboxylic acid OH of any of the compounds investigated, but there was a broad peak which disappeared in the presence of D_2O, probably due to the N-NH- of the arylhydrazone residue. In solutions in DMF-d_7/pyridine-d_5 (3:2) for example, this peak was observed at δ 10.12 in the case of

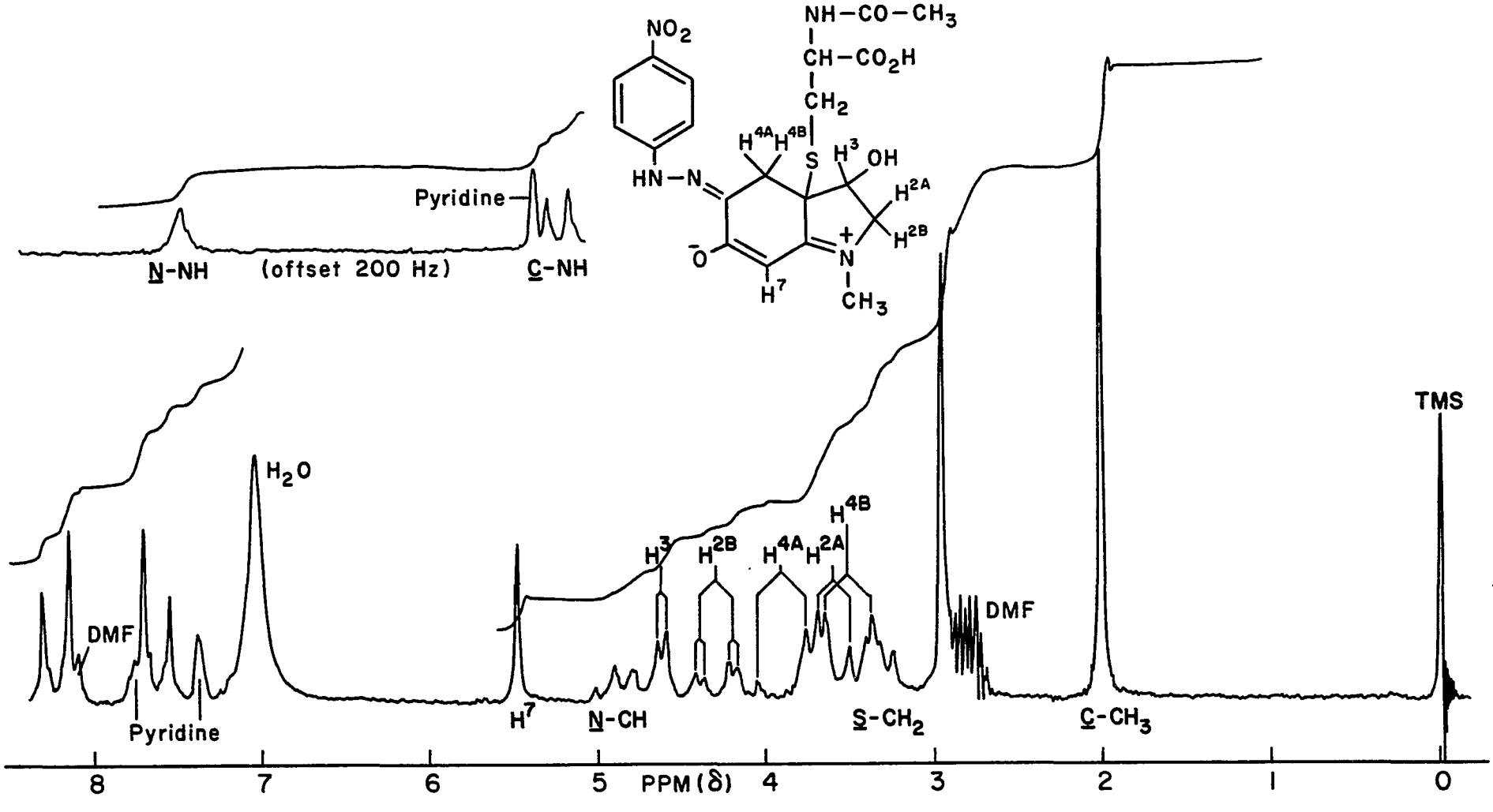
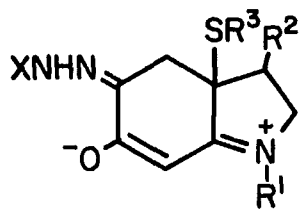


Fig. 16. N.m.r. spectrum of the p-nitrophenylhydrazone of the adrenochrome/N-acetylcysteine addition product in DMF-d_7/pyridine-d_5 (3:2).

the p-bromophenylhydrazone of the adrenochrome/N-acetylcysteine addition product and at δ ca. 10.8 for the p-nitrophenylhydrazones of the addition products between adrenochrome and N-acetylcysteine and between epinochrome and β-mercaptopropionic acid. The spectrum of the latter compound (i.e. the one involving epinochrome) was rather complex and only approximate assignments could be made for some of the peaks (see Sect. 5.7.2.5, p. 189).

3.2.4 Conclusions

On the basis of the u.v., i.r., n.m.r. and microanalytical data as well as the chemical properties of the substituted hydrazone derivatives of the aminochrome/thiol addition products investigated, it would appear that the thiol residue is attached to the 9-position of the aminochrome nucleus as first suggested by Heacock and Mattok[130]. Most of the evidence obtained in the current investigation concerns the structure of condensation products of aminochrome/thiol addition products and it would certainly appear that these compounds exist in the imino form (cf. 112).



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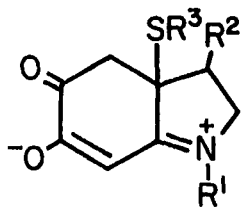

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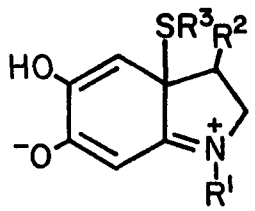

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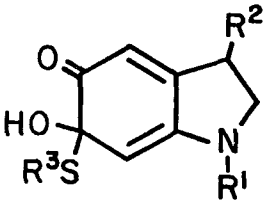
Heacock and Mattok[130,131] have noted the similarity between some of the properties of the adrenochrome/glutathione addition product and the adrenochrome/sodium bisulphite addition product (see Sect. 1.4.6.3, p. 42). Since the latter compound is now known to exist in the keto form in aqueous solution (see Sect. 3.2.2, p. 96) it would appear that the aminochrome/thiol addition products also exist as the keto tautomers (cf. 113) rather than as the enol tautomers (cf. 114) as had been first suggested.[130]

3.3 Effect of pH and substituents upon the courses of the reactions between aminochromes and thiols

The reactions between aminochromes and thiols are rather complex since there are at least three different types of products formed. In addition, side reactions, including the formation of rearrangement products and of melanin, can occur in the buffer solutions used. It would thus be very difficult to conduct a detailed study of the individual rates of the various reactions concerned. An attempt has been made, however, to get a rough idea of the effects of pH and of some of the structural features of the reactants upon the overall reaction rate. This has been done by following the decrease in the absorbance at ca. 490 nm due to the aminochromes in the visible spectrum of the reaction mixture. The absorbances were then plotted against the time and the half times of the

reactions determined (see Table 9, p. 125). The absorbances at ca. 360 nm due to the addition products were also measured as the reactions progressed. Since these compounds have not been isolated and their extinction coefficients are not known, it was impossible to determine their actual concentrations in the reaction mixture.

The λ_{max} values observed for the addition products in these reactions are shifted to higher wavelengths at lower pH's (see Table 9). This shift may be due to the formation of an additional product, possibly an α-hydroxysulphide (cf. 115), involving direct addition of the thiol to the C^6-carbonyl group of the aminochrome. These considerations



115

make it rather difficult to compare the rates of addition product formation at lower pH's with the rates at higher pH's.

The reactions were, in general, carried out in 0.1 M citrate buffer in the pH range 2.7-5.9 with a ratio of thiol to aminochrome of 2:1. At pH's lower than this, melanin formation becomes a problem and at higher pH's the rearrangement reaction becomes significant. The values

Table 9

The half times and reciprocal half times for some aminochrome/thiol reactions together with the λ_{max} values of the addition products formed in these reactions at various pH's*

Reaction	pH	λ_{max} (nm)	$T_{1/2}$ (min)	$1/T_{1/2}$ (min^{-1})
Adrenochrome/	2.80	376	3.3	.303
N-acetylcysteine	3.50	367	5.8	.172
	4.20	363	8.9	.112
	4.95	357	7.1	.141
	5.70	357	5.2	.192
	5.91	357	4.5	.222
Adrenochrome/	2.79	360	7.0	.143
glutathione	3.44	356	10.0	.100
	4.10	356	11.6	.086
	4.70	356	9.4	.106
	5.26	355	7.1	.141
	5.90	354	6.0	.167
1-Isopropylnoradrenochrome/	2.70	382	1.8	.555
N-acetylcysteine	3.37	378	3.0	.333
	4.06	367	5.3	.189
	4.71	363	6.8	.147
	5.29	360	5.0	.200
	5.92	359	3.7	.270
Adrenochrome methyl ether/	2.74	374	1.9	.526
N-acetylcysteine	3.38	374	3.1	.323
	4.08	370	6.2	.161
	4.70	365	8.0	.125
	5.30	362	10.4	.096
	5.93	361	10.4	.096
Epinochrome/	2.71	365(sh)	12.7	.079
N-acetylcysteine†	4.28	360(sh)	11.3	.088
	5.42	360(sh)	7.4	.135

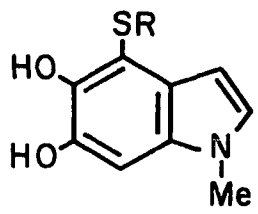
*See Sect. 5.8, p. 190 for experimental details.

†The concentrations of the reactants were increased fourfold in this case.

obtained for the concentrations of the aminochromes and the absorbances due to the addition products with respect to time are given in Table 11, p. 194.

3.3.1 Adrenochrome/N-acetylcysteine reaction

The effect of the pH of the reaction mixture upon the rate (i.e. the reciprocal of the half time) of the reaction between adrenochrome and N-acetylcysteine is shown in Fig. 17, p. 127. It can be seen that the reaction is slowest in the pH range 4-4.5 but is faster at either low or nearly neutral pH's.



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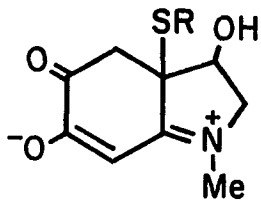

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3.3.1.1 Reaction under acidic conditions (pH 3-4)

At lower pH's the peak due to adrenochrome at 301 nm was replaced by one at 308 nm, probably due mainly to the indole thioether 107* and partially to DHMI

*An extract containing the acidic fraction of the reaction mixture, which consisted mainly of 107 along with N-acetylcysteine and its disulphide (cf. Sect. 3.1.1.1), exhibited a λ_{max} at 308 nm in aqueous solution.

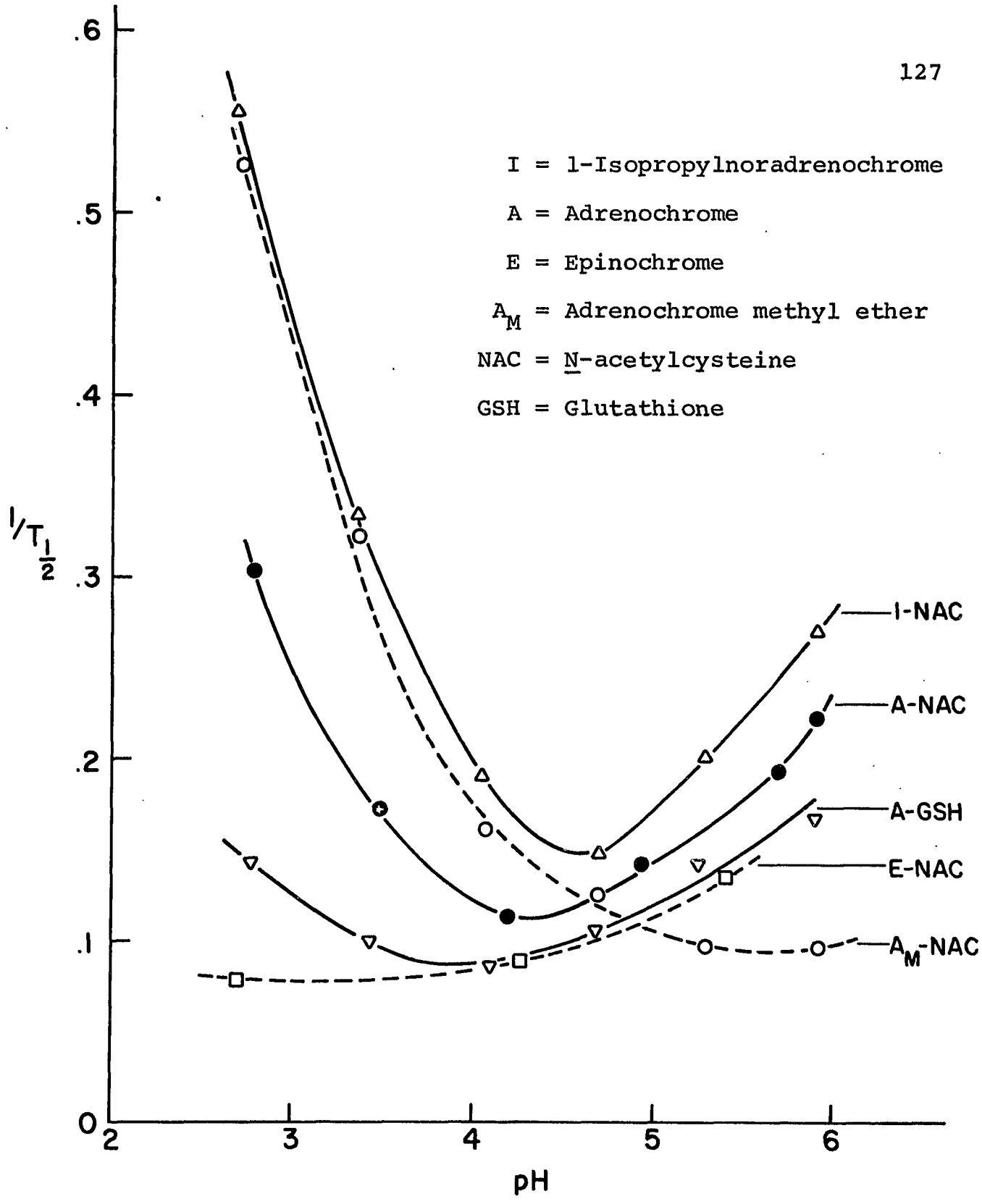
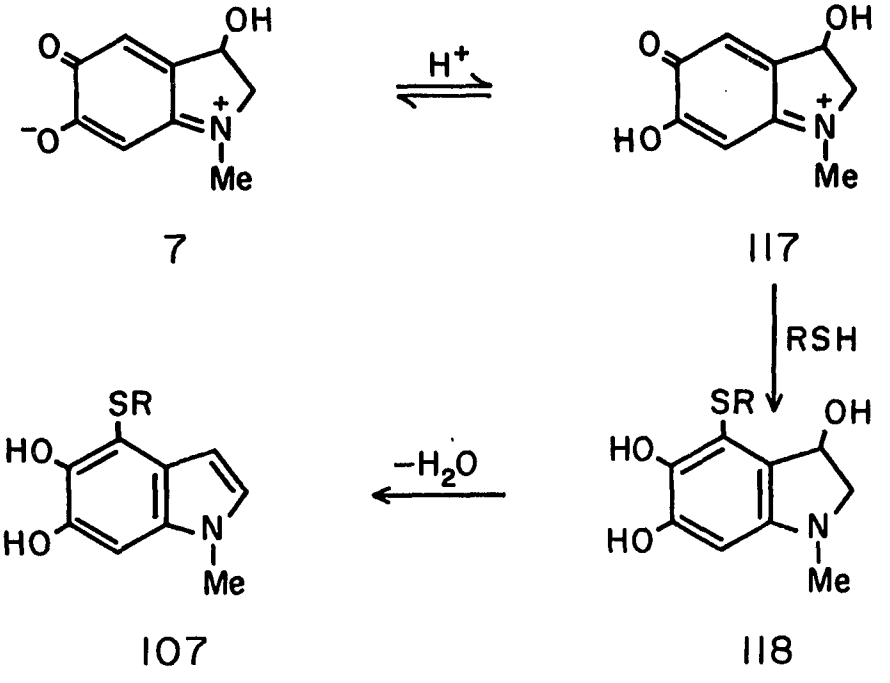


Fig. 17

Effect of pH upon the reciprocals of the half times of some aminochrome/thiol reactions (see Sect. 5.8, p. 190 for experimental details).

(λ_{max} 299 nm) (see Fig. 18, p. 129). An addition product (or products), possibly 115 or (and) 116, appeared to be formed fairly rapidly in the initial stages of the reaction (see Fig. 18 and Fig. 19, p. 130), but after about 4 min its concentration began to decline. This was presumably due to a shift in the equilibrium caused by the removal of adrenochrome by the irreversible formation of the indole thioether.

The acceleration at lower pH's therefore appeared to be due to an increase in the rate of formation of the indole thioether, possibly coupled with an increase in addition product formation. The increased indole thioether formation may be due to a greater degree of protonation of the C^6-carbonyl group to give 117. The partial positive



7 117 107 118

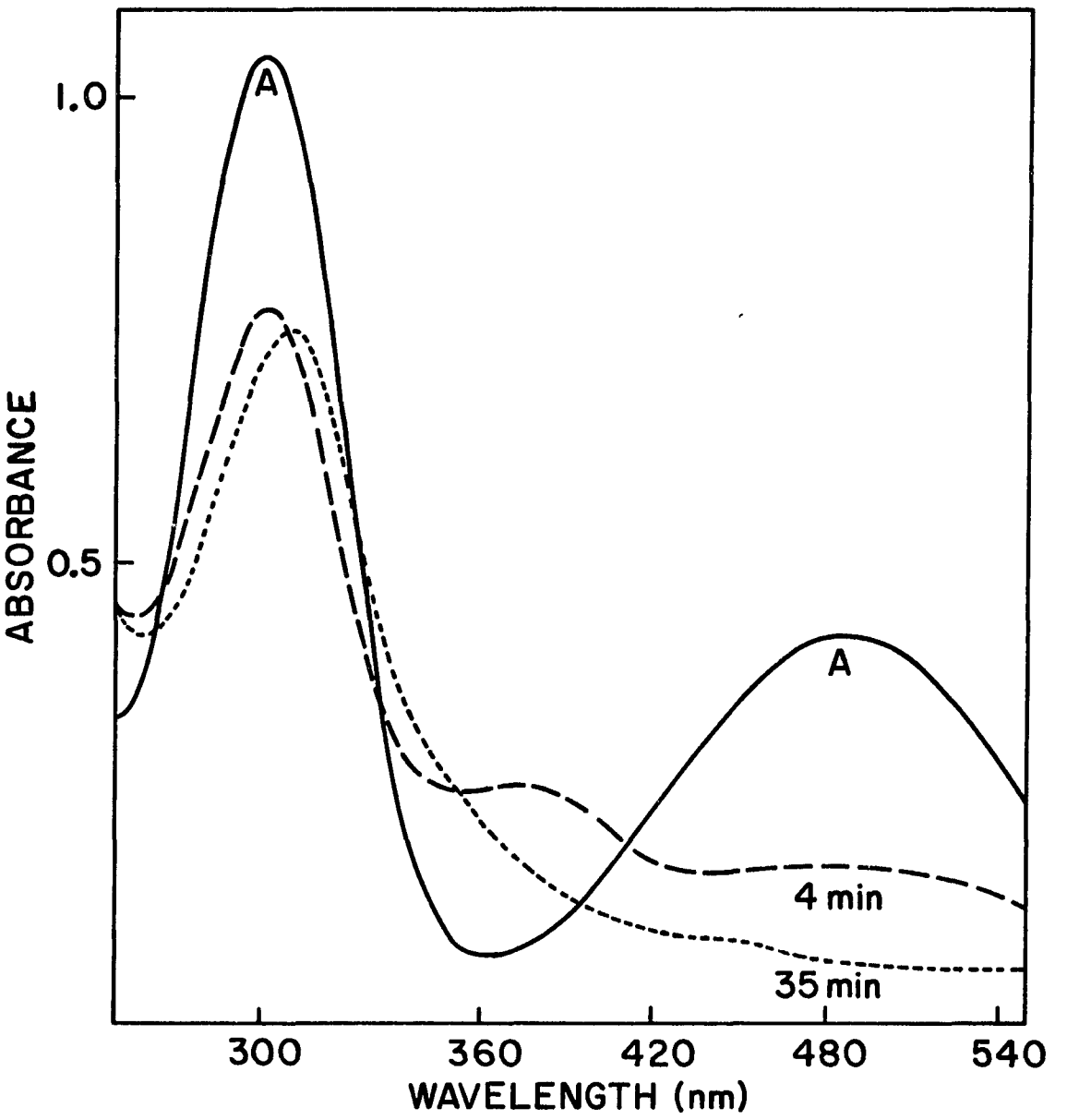
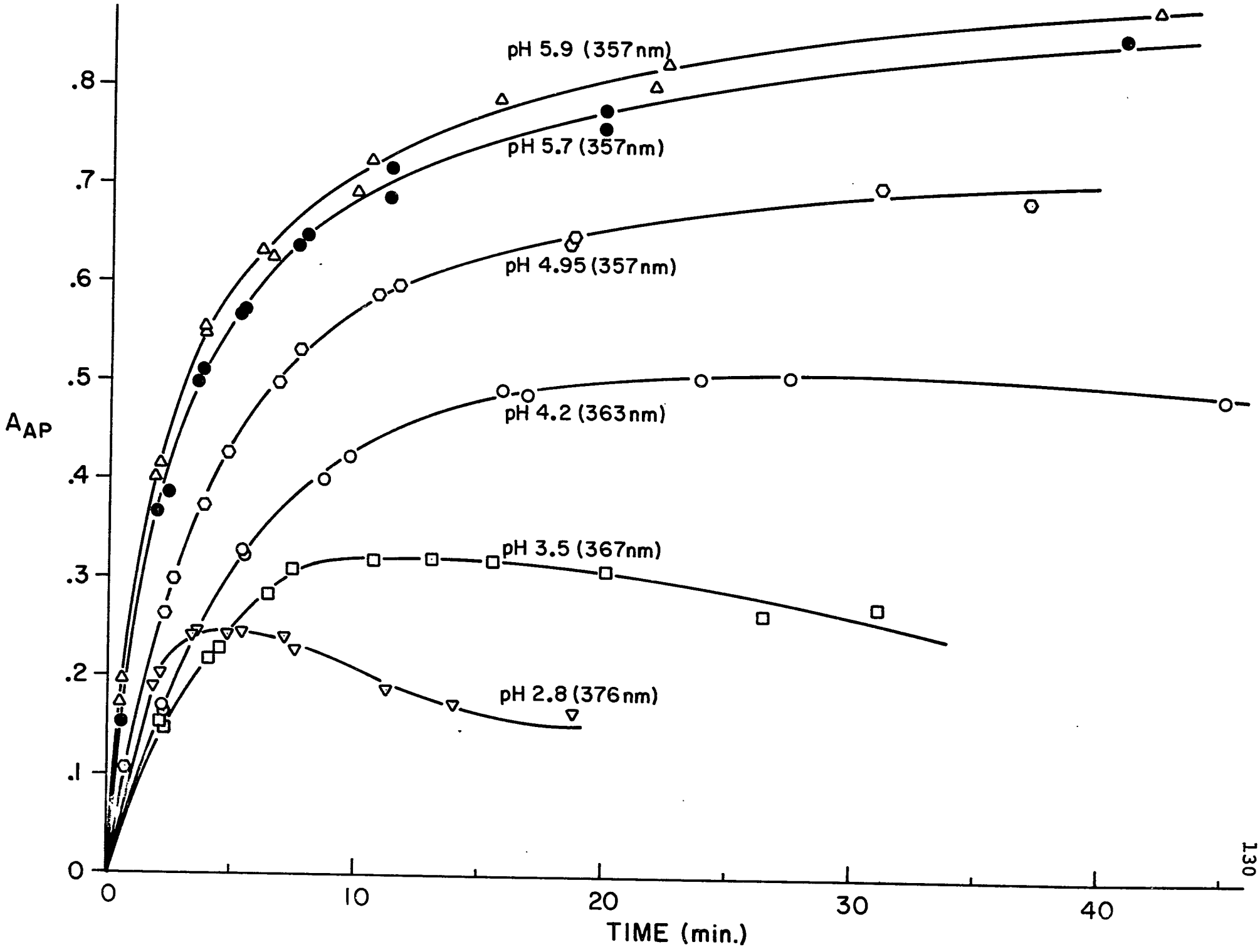


Fig. 18

U.v./visible spectra of adrenochrome (A) and the products of the adrenochrome/N-acetylcysteine reaction (pH 2.8) after 4 min and after 35 min.

Fig. 19

Absorbances (corrected for adrenochrome absorption) of the addition product(s) *vs* reaction time for the reactions between adrenochrome and *N*-acetylcysteine at various pH's (see Sect. 5.8, p. 190 for experimental details) (A_{AP} = corrected absorbance of addition product).


pH 5.9 (357nm)
pH 5.7 (357nm)
pH 4.95 (357nm)
pH 4.2 (363nm)
pH 3.5 (367nm)
pH 2.8 (376nm)
A_{AP}
0
.1
.2
.3
.4
.5
.6
.7
.8
0
10
20
30
40
TIME (min.)
130

charge on the 4-carbon atom of 117 would be considerably greater than that in adrenochrome itself and consequently the rate of formation of the 4-thiosubstituted trihydroxyindoline intermediate 118 would be enhanced. 3,5,6-Trihydroxyindolines of this type are known to dehydrate to the corresponding 5,6-dihydroxyindoles very readily (see Sect. 1.4.2, p. 14) and in the case of 118 this tendency would be increased by the electron donating thiol substituent. The last stage of this sequence (i.e. 118 → 107) would also be acid catalyzed, which would contribute further to the rate of formation of 107 at low pH's.

The thiol could also possibly add to 117 to give an α-hydroxysulphide (cf. 115) under acidic conditions, but the formation of such a compound is highly speculative.

3.3.1.2 Reaction under weakly acidic conditions (pH 5-6)

At weakly acidic pH's the major product appeared to be the addition product 116. Although it was eventually replaced in the reaction mixture by the indolic products (cf. Fig. 9, p. 103) it was much more stable under these conditions than in acidic solution (see Fig. 19, p. 130), presumably because of the much slower rate of formation of the indolic products.

The increased rate which is observed would thus appear to be almost completely due to an enhancement of the

rate of addition product formation. This may be explained by the greater degree of ionization of the thiol at higher pH's. Since protonation of the C^6-carbonyl group of adrenochrome would be negligible under these conditions, the formation of the indole thioether would be much reduced.

3.3.2 Adrenochrome/glutathione reaction

It has been reported[131] that the addition product between adrenochrome and glutathione is formed optimally at a pH of 4.7. Its formation was said to fall off sharply at pH values above and below this and it was said not to be formed at all at pH's above 6 (see Sect. 1.4.6.3, p. 41). Since the decline in the formation of the addition product at pH's above 4.7 was not in agreement with the results obtained for the adrenochrome/N-acetylcysteine reaction, the reaction involving glutathione was reinvestigated. This reaction was also of potential physiological interest since glutathione, unlike N-acetylcysteine, is present in living organisms.

The effect of pH on the reaction between adrenochrome and glutathione was found to be quite similar to that which had been observed for the reaction with N-acetylcysteine. The overall reaction rate was minimal at a pH of ca. 4 and was faster both at lower and higher pH values (see Fig. 17, p. 127). At all the pH's investigated the rates were lower than those for the N-acetylcysteine reaction, probably due

to the greater bulk of the glutathione molecule. As in the case of N-acetylcysteine, the increased rates at higher pH's appeared to be due to an increase in the formation of the addition product (see Fig. 20, p. 134).

3.3.3 Adrenochrome/thioglycollic acid reaction

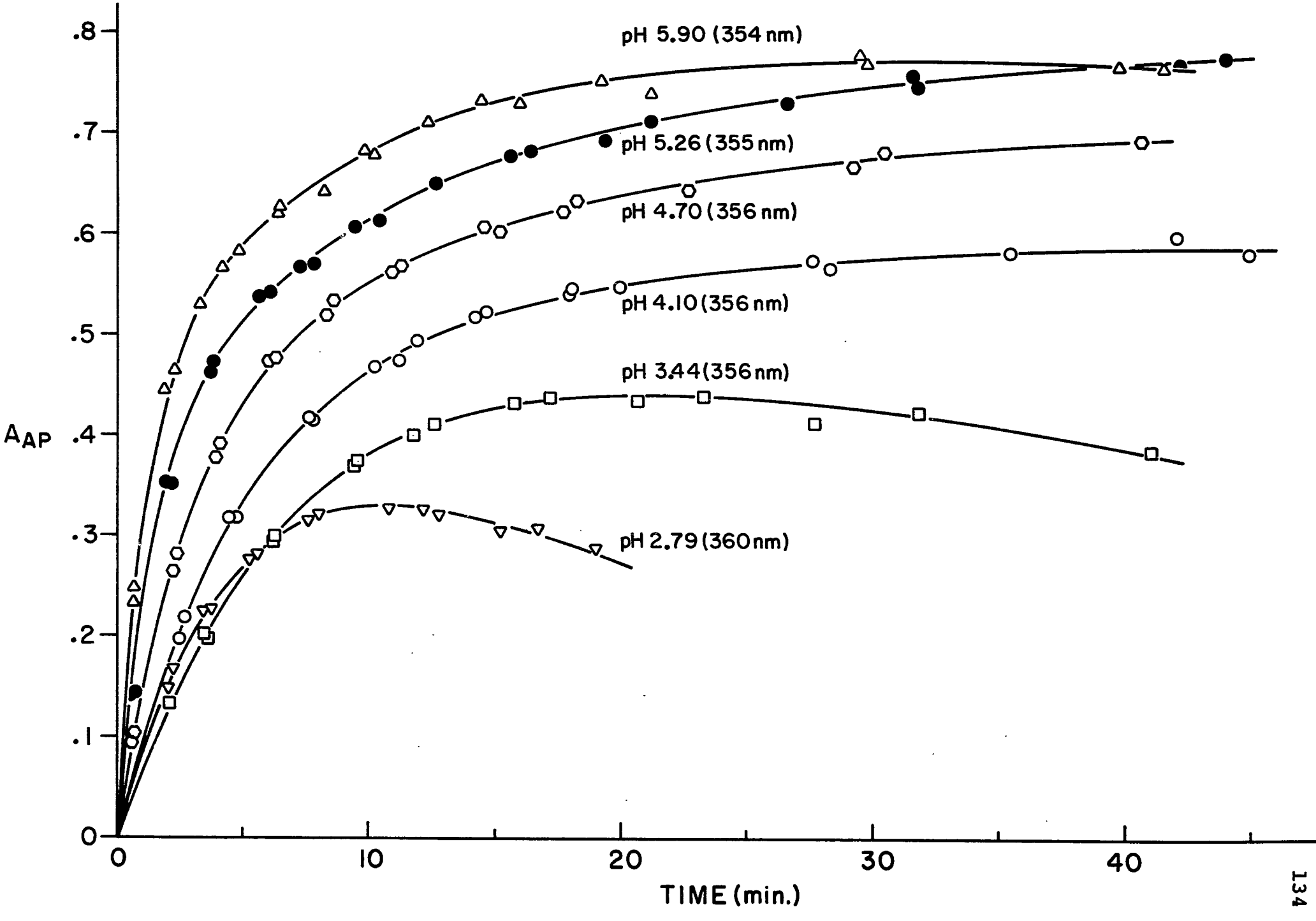
Adrenochrome reacted very slowly with thioglycollic acid in the pH range 2.8-5.9 at the concentrations used (i.e. 1×10^{-2} M aminochrome and 2×10^{-2} M thiol) and the half times could not be readily measured. The slower reaction at lower pH's is probably due to a decrease in the nucleophilicity of the thiol group due to the electronegative effect of the α-carboxylic acid group. At higher pH's, on the other hand, ionization of the thiol group would be repressed by the ionized carboxyl group.

3.3.4 1-Isopropylnoradrenochrome/N-acetylcysteine reaction

The reaction between 1-isopropylnoradrenochrome and N-acetylcysteine was considerably faster than that of adrenochrome with this thiol, especially in the low pH range (see Fig. 17, p. 127). This is probably due to an increase in the rate of formation of the indole thioether. The basicity of the vinylogous amide system would be increased by the more electropositive isopropyl group, resulting in a greater degree of protonation of the 6-carbonyl oxygen. The protonated species would then be readily attacked by the thiol.

Fig. 20

Absorbances (corrected for adrenochrome absorption) of the addition product(s) vs reaction time for the reactions between adrenochrome and glutathione at various pH's (see Sect. 5.8, p. 190 for experimental details) (A_{AP} = corrected absorbance of addition product).


pH 5.90 (354 nm)
pH 5.26 (355 nm)
pH 4.70 (356 nm)
pH 4.10 (356 nm)
pH 3.44 (356 nm)
pH 2.79 (360 nm)
A_{AP}
.8
.7
.6
.5
.4
.3
.2
.1
0
0
10
20
30
40
TIME (min.)

3.3.5 Adrenochrome methyl ether/N-acetylcysteine reaction

Adrenochrome methyl ether reacted considerably faster with N-acetylcysteine at lower pH's than did adrenochrome, but much slower at higher pH's (see Fig. 17, p. 127). Thus it would appear that the rate of formation of the indole thioether is greater and that the addition product is formed less readily. It is difficult to explain the increased rate of formation of the indole thioether in this case. The decrease in the rate of addition product formation may be partially caused by a slight decrease in the partial positive charge on the 9-carbon of adrenochrome methyl ether due to the lesser electronegative effect of the methoxyl substituent. The main reason, however, would probably be the greater steric interaction between the methoxyl group and the attacking thiol molecule.

3.3.6 Epinochrome/N-acetylcysteine reaction

The reaction between N-acetylcysteine and epinochrome was much slower than the one with adrenochrome at all pH's investigated (see Fig. 17, p. 127) and consequently the concentrations of both the aminochrome and the thiol had to be increased fourfold. In the case of the reaction involving the 4-carbon of epinochrome, the partial positive charge on this carbon atom would be reduced due to (a) the replacement of the electronegative hydroxyl group by a proton

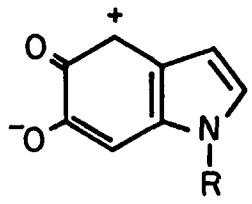
and (b) the greater hyperconjugative effect of the C^3-methylene group. The formation of the epinochrome/*N*-acetylcysteine addition product involving the 9-carbon would be less favourable due to a decrease in the partial positive charge at this position. This can also be explained by the effect of the C^3-methylene group as has been discussed above.

3.3.7 Conclusions

It appears that in general aminochromes react with thiols in the pH range of *ca*. 3-4 to give mainly 4-thio-substituted 5,6-dihydroxyindoles (or indolines) along with reduction products. In the pH range of *ca*. 5-6, the major products are initially the aminochrome/thiol addition products. The increased formation of the indole thioethers at lower pH's is probably mainly due to protonation of the C^6-carbonyl group. The addition product appears to be formed more readily at higher pH's due to the increased ionization of the thiol group.

Bouchilloux and Kodja[108,109] and later Mason and Peterson[112] postulated that it was not the aminochromes themselves which reacted with thiols to form indole thioethers. These authors were of the opinion that the reactive species were the indole-5,6-quinones which were thought to be produced from the aminochromes under the reaction conditions employed

(see Sect. 1.4.6.2, p. 35). This mechanism would appear to be rather unlikely, however, since aminochromes would be expected to react more readily with thiols than indole-5,6-quinones. The partial positive charge on the 4-carbon of the latter compounds (cf. 119) should be reduced by the contribution of 120 to the overall structure. Such a reduction of the partial positive charge on the 4-carbon is not possible in aminochromes. Moreover, the formation of the 4-thiosubstituted 5,6-dihydroxyindolines in the reaction of epinochrome with thioglycollic acid (see Sect. 3.1.3, p. 84) could not be explained by such a mechanism.



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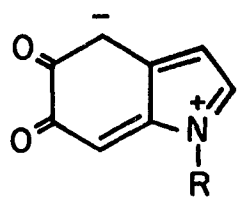
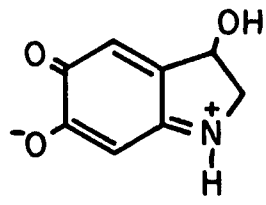


120

3.4 Synthesis of new aminochromes

During the course of these investigations some aminochromes, namely noradrenochrome (10) and 2-methyl-noradrenochrome (121), were prepared for the first time as crystalline solids. These are the first reported syntheses of unhalogenated "nor" aminochromes in crystalline form. In addition, noradrenochrome monosemicarbazone (122) was

also prepared for the first time. The structures of these compounds were determined on the basis of their u.v./visible, i.r. and n.m.r. (see Table 6, p. 91) spectral characteristics along with microanalytical data. These "nor" aminochromes appeared to be somewhat less stable in the solid state than the corresponding aminochromes with N-alkyl groups.



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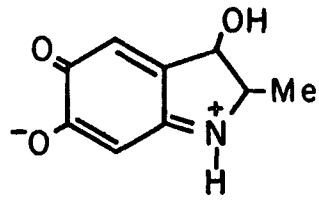
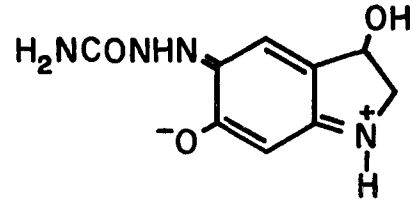


121



122

The difficulties (cf. Ref. 33) which have been encountered in the syntheses of these "nor" aminochromes appear to be due to the decreased basicity of the primary amino groups of the catecholamine precursors[184]. This apparently leads to a lower cyclization rate[39,43,184,185], especially at the acid pH's (cf. Ref. 43) which are normally used for the syntheses of aminochromes (e.g. in solutions in methanol made acidic by the addition of formic acid). Consequently, neutral solutions must be used and the reactions carried out on suspensions of the catecholamine in methanol. The higher yields obtained in the case of 2-methylnoradrenochrome (26%) compared to noradrenochrome (3%) are consistent with the higher cyclization rates observed by Hawley et al. for

α-methylnoradrenaline compared to noradrenaline[43].

Noradrenochrome can be easily reduced by ascorbic acid in aqueous solution to 5,6-dihydroxyindole which can readily be isolated as its diacetyl derivative.

4 SUMMARY AND CONCLUSIONS

4 SUMMARY AND CONCLUSIONS

The 5,6-dihydroxyindole thioethers which were formed in the reactions between a number of aminochromes and several thiols, including some amino acid derivatives, have been isolated as their acetyl or methyl derivatives. An n.m.r. study has shown that the thiol residue in each of these compounds is in the 4-position of the indole nucleus and not in the 7-position as had been suggested previously. This was confirmed by the finding that 7-methyladrenochrome reacted with thioglycollic acid to give the anticipated 5,6-dihydroxyindole thioether, whereas 4-methyladrenochrome gave only the 5,6-dihydroxyindole reduction product.

Epinochrome was found to react with thioglycollic acid to give mainly 5,6-dihydroxy-1-methylindoline and a 4-thiosubstituted 5,6-dihydroxy-1-methylindoline. The acetyl derivatives of these compounds, along with that of the corresponding 4-thiosubstituted 5,6-dihydroxyindole, were isolated after acetylation of the reaction mixture. The latter compound is probably a secondary product of the reaction, having arisen from the 4-thiosubstituted 5,6-dihydroxy-1-methylindoline which is formed initially.

It has been confirmed that the addition products formed between aminochromes and sodium bisulphite are 4,9-dihydroaminochrome-9-sulphonates. These compounds were found to exist as the keto tautomers in aqueous solution

and not as the enol tautomers as had been postulated earlier.

Aminochromes also react with thiols to give addition products analogous to the aminochrome/sodium bisulphite addition products mentioned above. Several of these thiol addition products have been isolated as their arylhydrazone derivatives, the structures of which have been determined by physical and chemical methods. In this way it has been shown that the thiol addition products are 9-thiosubstituted 4,9-dihydroaminochromes.

The pH of the reaction mixture has a marked effect upon the course of the reaction between aminochromes and thiols. At low pH's, the major products appear to be 4-thiosubstituted 5,6-dihydroxyindoles (or indolines) along with the 5,6-dihydroxyindole (or indoline) reduction products. The formation of the former type of compound is probably acid catalyzed due to protonation of the carbonyl group in the 6-position. There is some evidence which suggests that another compound, possibly an α-hydroxysulphide, is also formed during the initial stages of the low pH reactions. Further work would be necessary to test this hypothesis however. At only weakly acidic pH's the major products are 9-thiosubstituted 4,9-dihydroaminochromes. The formation of these compounds would appear to be catalyzed by the increased ionization of the thiol groups at higher pH's.

Some studies have been carried out in order to

determine the effects of some of the substituents upon the half times of several aminochrome/thiol reactions. Aminochromes with a 3-hydroxyl or 3-methoxyl substituent were found to react much more rapidly with N-acetylcysteine than did epinochrome, which is without a substituent in this position. This may have some significance since two (i.e. dopachrome and norepinochrome) of the four (i.e. dopachrome, norepinochrome, noradrenochrome and adrenochrome) aminochromes which are most likely to occur naturally have no substituent in this position. It would be interesting to extend these studies to include "nor" aminochromes and also to work at slightly higher pH's (ca. pH 7) in order to approximate to physiological conditions more closely.

Two "nor" aminochromes along with some of their derivatives have been prepared in crystalline form. These are the first reported syntheses of unhalogenated "nor" aminochromes in crystalline form.

5 EXPERIMENTAL

5 EXPERIMENTAL

5.1 Materials

Solvent mixtures were prepared on a volume to volume basis. Pyridine was purified by distillation from KOH pellets and the fraction distilling in the range 115-116° was used. The light petroleum used throughout this investigation was B.D.H. "Analar" grade (b.p. 60-80°, unless otherwise stated). Peroxide free ether was used throughout this investigation. The formic acid used was B.D.H. "Analar" grade (98-100%).

Silica gel for column chromatography was obtained either from Koch-Light Laboratories Ltd. (100-200 or 200-300 mesh size) or from the Davison Chemical Corporation (100-200 mesh size). The Sephadex G-25 was obtained from Pharmacia Fine Chemicals AB. Dowex-1 × 8 (Cl^-) (200-400 mesh size) and Dowex-1 × 10 (Cl^-) (200-400 mesh size) resins were obtained from the Baker Chemical Co.

The Dowex resin was pretreated by repeated washing by decantation with: (1) 3N HCl, (2) H_2O until neutral to litmus and (3) methanol. The silver oxide was prepared by the dropwise addition of an aqueous solution of NaOH to a rapidly stirred solution of an excess of $AgNO_3$ in H_2O. The resulting spongy brown precipitate was washed extensively with: (1) H_2O until neutral to litmus, (2) methanol and (3) ether. It was then dried in air in the absence of light.

Adrenochrome monosemicarbazone (37) was obtained

from Koch-Light Laboratories Ltd. Epinine hydrochloride was obtained either from Raylo Chemicals Limited or as a gift from Burroughs Wellcome and Co. The following compounds were obtained from Dr. R. A. Heacock: β-methoxyepinine hydrochloride; 5,6-diacetoxy-1,4-dimethylindole; 5,6-diacetoxy-1,7-dimethylindole; 5,6-diacetoxy-7-iodo-1-methylindole; 5,6-diacetoxy-7-iodo-1,4-dimethylindole; 3,5,6-triacetoxy-7-iodo-1-methylindole; 5,6-diacetoxy-1-isopropylindole; 5,6-diacetoxy-7-iodo-1-isopropylindole and 4-methyladrenochrome monosemicarbazone.

The following compounds were prepared by methods which had previously been described in the literature: 5,6-dihydroxy-1-methylindole (DHMI, 36);[65] 5,6-diacetoxy-1-methylindole (109);[180] 5,6-dihydroxy-1,4-dimethylindole (93);[44] 5,6-dihydroxy-1,7-dimethylindole;[44] adrenochrome (7);[31] 1-isopropylnoradrenochrome;[32] adrenochrome methyl ether;[32] 7-iodoadrenochrome methyl ether[32] and sodium 4,9-dihydro-adrenochrome-9-sulphonate (i.e. the adrenochrome/sodium bisulphite addition product, 44).[74] 4-Methyladrenochrome (96) and 7-methyladrenochrome (95) were synthesized by the methods of Heacock and Hutzinger,[44] starting, in both cases, with 3-methylcatechol. In some cases, the 2-methyl-adrenaline required for the synthesis of 4-methyladrenochrome was obtained from Raylo Chemicals Ltd.

The p-bromophenylhydrazine hydrochloride and the

p-nitrophenylhydrazine hydrochloride were purified by dissolving them in H_2O, filtering the resulting solutions and then precipitating out the free bases by the addition of solid K_2CO_3. After washing with H_2O the bases were dissolved in solutions of conc. HCl in methanol, which were concentrated to dryness *in vacuo* to give the hydrochlorides.

5.2 Physical measurements

a) Ultraviolet and visible spectroscopy

Ultraviolet and visible spectra were measured with either a Bausch and Lomb Spectronic 505, a Beckman DK-2 or a Beckman Acta V recording spectrophotometer.

b) Infrared spectroscopy

Infrared spectra were obtained using a Perkin-Elmer 237 grating spectrophotometer. Unless otherwise stated the samples were prepared as mulls in "Nujol". The positions of only the peaks relevant to the discussion are given.

c) Nuclear magnetic resonance spectroscopy

The n.m.r. spectra were measured with a Varian A-60A instrument with a model V-6058A spin decoupler. The chemical shifts were measured relative to tetramethylsilane as an internal reference in organic solvents or as an

external reference (in solution in $CDCl_3$) in D_2O. The chemical shifts are reported in δ-values and the coupling constants in Hz.

d) Mass spectrometry

Mass spectral data were obtained using a Dupont/C.E.C. 21-110B mass spectrometer.

e) Melting points

Melting points were determined on either a Leitz hot stage polarizing microscope or a Fisher-Johns apparatus and are uncorrected.

5.3 Chromatography

a) Stationary phases

All thin-layer chromatography was carried out using ascending development. Either Merck pre-coated silica gel F-254 plates (layer thickness 0.25 mm) or Eastman "Chromagram" cellulose sheets (with or without an added fluorescent indicator) were used.

The paper chromatography was carried out radially in a modified Kawerau apparatus on discs (diam. = 32 cm) of Whatman No. 1 paper. In some cases the paper was pretreated with formamide by dipping into a 30% solution of formamide in acetone. The discs were then blotted free of excess reagent and allowed to dry in air for approximately

20 minutes prior to use.

b) Chromogenic reagents

F_1 reagent: p-N,N-bis(2-Chloroethyl)aminobenzaldehyde (Frinton Laboratories; 1 g) was dissolved in a solution of conc. HCl (25 ml) and methanol (75 ml).

Ehrlich's reagent: p-N,N-Dimethylamino-benzaldehyde (2 g) was dissolved in a solution of conc. HCl (25 ml) and methanol (75 ml).

Cinnamaldehyde reagent: Cinnamaldehyde (5 ml) was dissolved in a solution of conc. HCl (10 ml) and methanol (90 ml).

Folin and Ciocalteu's reagent: This reagent was obtained from B.D.H. and was diluted with two volumes of H_2O before use.

Gibb's reagent: 2,6-Dibromo-N-chlorobenzoquinone-moncimine (1 g) was dissolved in 95% ethanol (100 ml).

Iodine/azide reagent: Iodine (1 g) and sodium azide (6 g) were dissolved in 50% ethanol (100 ml). After spraying with this reagent, the thin-layer plates were oversprayed with a saturated aqueous solution of starch.

5.4 Preparation of some aminochromes and their derivatives

5.4.1 Epinochrome (cf. Sobotka and Austin, Ref. 18)

The free base of epinine was prepared by the addition of solid K_2CO_3 to a stirred solution of epinine hydrochloride in H_2O. After standing in an ice bath for 1.5 hr under a stream of nitrogen, the free base was filtered off and washed with H_2O.

Epinine free base (2 g) was suspended in MeOH (82 ml) and 98% formic acid added dropwise with stirring until a clear solution was obtained. Freshly prepared silver oxide (8.64 g) was added portionwise during a period of 5 min, the reaction temperature being maintained between 16° and 25°. The reaction mixture was then filtered through a Dowex-1 × 8 (Cl^-) (200-400 mesh) resin bed (height = 3.5 cm; diam. = 4.5 cm). Dry ether (260 ml) was added to the filtrate and it was kept in a cold room at -30° for 8 hr. Upon filtration epinochrome (1.17 g, 60%) was obtained as deep red needles, m.p. 80-83° with decomposition (lit. m.p. 78°[18]).

u.v. λ_{max} (H_2O) nm (ε): 217 (31,400), 307 (12,600), 476 (4070).

i.r. ν_{max} (Nujol): 1661, 1652, 1612, 1566 cm^{-1}.

n.m.r. δ(DMSO-d_6): 6.36 (1H, t, $J_{3A,4} = J_{3B,4}$ = 2.3 Hz, H^4); 5.40 (1H, s, H^7); ca. 3.80 (2H, m, C^2H_2); 3.11 (3H, s, N-CH_3); ca. 3.00 (2H, m, coupled to H^4, J = 2.3 Hz, C^3H_2).

5.4.2 5,6-Diacetoxy-1-methylindoline (103) [cf. synthesis of 5,6-diacetoxy-1-acetyl-2-carbomethoxyindoline (33) by Wyler and Chiovini[59]]

A solution of potassium ferricyanide (2 g) in 50 ml of phosphate buffer at pH 8* was added to a stirred solution of epinine hydrochloride (250 mg) in 75 ml of the same buffer. After 4-5 sec, sodium dithionite (1.36 g) in 25 ml buffer was added, resulting in an almost immediate discharge of the red colour due to the epinochrome. Conc. HCl (2.5 ml) was added to the solution 1-2 sec after the addition of the dithionite and it was then concentrated to dryness in vacuo, benzene being added during the latter stages to enable complete removal of the H_2O. A mixture of acetic anhydride and pyridine (1:1, 25 ml) was added to the solid residue which was broken up with a spatula. The acetylation mixture was left at room temperature for 4 hr, filtered and the residue washed with CH_2Cl_2 (3 × 20 ml). The washings and the acetic anhydride/pyridine solution were combined, filtered and concentrated to dryness in vacuo below 50°. The residue was dissolved in a mixture of 0.5 N HCl (25 ml) and CH_2Cl_2 (25 ml) in a separatory funnel. The organic layer was drawn off and washed with saturated aqueous $NaHCO_3$ (4 × 30 ml) and water (2 × 30 ml). The dried (Na_2SO_4) CH_2Cl_2 solution was then concentrated

*0.5 g KH_2PO_4 and 22.6 g $Na_2HPO_4 \cdot 12H_2O$/liter.

to dryness *in vacuo*. The crude product, which also contained a small amount of 5,6-diacetoxy-1-methylindole, was purified on a silica gel (Davison, 100-200 mesh) column (18 × 2.2 cm) with a mixture of hexane/EtOAc/pyridine (15:3:2) as eluant. A series of 25 ml fractions were collected. Fractions 6-10 were combined and the solvent removed *in vacuo* below 50° to give 5,6-diacetoxy-1-methylindoline (200 mg) as a white crystalline solid. Recrystallization from heptane gave colourless rods, m.p. 115-116°.

u.v. λ_{max} (EtOH) nm (ε): 206 (22,200), 257 (9710), 310 (3460).

i.r. ν_{max} (Nujol): 1765, 1756 cm^{-1}.

n.m.r. δ($CDCl_3$): 6.85 (1H, t, $J_{3A,4} = J_{3B,4}$ = 1.1 Hz, H^4); 6.20 (1H, s, H^7); 3.54-2.72 (4H, m, AA'BB' system centred at 3.13, $-CH_2CH_2-$ of 5-membered ring; the C^3 methylene group, which appears as the upper field half of this system is coupled to H^4, J = 1.1 Hz); 2.72 (3H, s, N-CH_3); 2.25 (3H, s, C-CH_3); 2.22 (3H, s, C-CH_3).

mass spectrum: $M^{+\cdot}$ 249; calcd. for $C_{13}H_{15}NO_4$ 249.

Anal. Calcd. for $C_{13}H_{15}NO_4$: C, 62.64; H, 6.07; N, 5.62%.

Found: C, 62.72; H, 6.05; N, 5.83%.

5.4.3 Noradrenochrome (10)

Freshly prepared silver oxide (22.5 g) was added to a suspension of L-noradrenaline base (5 g, Schuchardt) in dry methanol (500 ml) at 32°. The reaction mixture was vigorously shaken for 6 min at this temperature (it was possible to maintain the temperature at 32° without external heating due to the mildly exothermic nature of the reaction), after which time it was filtered firstly through an anhydrous sodium sulphate bed (height = 2 cm; diam. = 11 cm) and secondly through a Dowex-1 × 8 (Cl^-) resin bed (height = 3 cm; diam. = 10 cm). The filtrate was concentrated below 20° in vacuo to small bulk (ca. 30 ml), until crystals began to form in the solution. Noradrenochrome was obtained as dark reddish-violet needles [150 mg (3%); totally decomposed without melting by 105°] on filtration of the solution.

u.v. λ_{max} (H_2O) nm (ε): 217 (22,350), 293 (8580), 488 (3190).

i.r. ν_{max} (Nujol): 3320(sh), 3100, 1673, 1660, 1617, 1579, 1524 cm^{-1}.

n.m.r. δ(DMSO-d_6): 6.45 (1H, d, $J_{3,4}$ = 2.0 Hz, H^4); 5.48 (1H, s, H^7); 5.02 (1H, m, X of ABX, also coupled to H^4, H^3); 4.01 (1H, dd, B of ABX, $J_{2A,2B}$ = -12.5 Hz, $J_{2B,3}$ = 6.7 Hz, C^2-methylene H); 3.44 (1H, dd, A of ABX, $J_{2A,2B}$ = -12.5 Hz, $J_{2A,3}$ = 3.2 Hz, C^2-methylene H).

Anal. Calcd. for $C_8H_7NO_3$: C, 58.18; H, 4.24; N, 8.48%.

Found: C, 58.39; H, 4.08; N, 8.56%.

5.4.4 Noradrenochrome monosemicarbazone

(a) Directly from noradrenochrome

Semicarbazide hydrochloride (27 mg) in acetate buffer (1.5 ml) at pH 5 was added to a stirred solution of noradrenochrome (20 mg) in water (1.5 ml). The crude monosemicarbazone (15 mg) was obtained in brownish-orange needles after the solution had been allowed to stand at room temperature for 1.5 hr. Pure noradrenochrome monosemicarbazone [9 mg (34%); totally decomposed without melting by 221°] was obtained as fine orange needles after recrystallization of the crude product from water.

u.v. λ_{max} (0.1 N HCl) nm (ε): 373 (29,000); λ_{max} (H_2O) nm (ε): 440(sh), 351 (27,000); λ_{max} (0.1 N NaOH) nm (ε): 441 (27,700).

i.r. ν_{max} (KBr): 3410, 3300, 3250, 3170, 1700, 1663, 1594, 1588(sh), 1550 cm^{-1}.

n.m.r. δ (DMSO-d_6): 14.83 (1H, s, semicarbazone NH); 8.55 (1H, s, noradrenochrome NH); 7.02 (2H, s, NH_2); 6.80 (1H, d, $J_{3,4}$ = 1.5 Hz, H^4); 5.70 (1H, d, $J_{3,OH}$ = 5.0 Hz, OH); 5.39 (1H, s, H^7); 5.03 (1H, m, X of ABX, also coupled to H^4 and to the hydroxyl proton, H^3); 3.90 (1H, dd, B of ABX, $J_{2A,2B}$ = -11.8 Hz, $J_{2B,3}$ = 6.8 Hz, C^2 methylene H); 3.39

(1H, dd, A of ABX, $J_{2A,2B}$ = -11.8 Hz, $J_{2A,3}$ = 3.1 Hz, C^2-methylene H). The peaks due to the NH and OH protons disappeared upon addition of D_2O to the DMSO-d_6 solution.

Anal. Calcd. for $C_9H_{10}N_4O_3$: C, 48.65; H, 4.54; N, 25.22%.
Found: C, 48.84; H, 4.46; N, 25.03%.

(b) From noradrenochrome produced in situ from noradrenaline

Noradrenaline (2.5 g) was treated with silver oxide in methanol as described above for the preparation of noradrenochrome. After filtration through the Na_2SO_4 and Dowex resin beds the deep red solution containing noradrenochrome was added to a solution of semicarbazide hydrochloride (1.5 g) and NaOAc (1.5 g) in H_2O (100 ml). This solution was concentrated in vacuo below 25° to 25-30 ml and then kept at 5° for 1.5 hr. The crude monosemicarbazone (177 mg) was obtained as brownish-orange needles upon filtration of this solution. Recrystallization of this product from H_2O gave noradrenochrome monosemicarbazone as orange needles [128 mg (4%), totally decomposed without melting by 222°].

5.4.5 Reduction of noradrenochrome by ascorbic acid; preparation of 5,6-diacetoxyindole

Ascorbic acid (400 mg) was added to a mixture of

noradrenochrome (75 mg) in water (5 ml) and peroxide-free ether (15 ml). The system was vigorously shaken until the red colour was totally discharged (about 1 min). The ether phase was removed and dried (Na_2SO_4) and a mixture of acetic anhydride (2 ml) and dry pyridine (2 ml) added. After removal of the ether *in vacuo* the reaction mixture was allowed to stand at room temperature for 2 hr, and was then concentrated to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2 (15 ml) and washed in turn with the following reagents: 0.1 N HCl (15 ml), saturated aqueous sodium bicarbonate (3 × 15 ml), and water (2 × 15 ml). The CH_2Cl_2 solution was dried (Na_2SO_4) and upon concentration to dryness *in vacuo* gave a white crystalline solid (47 mg), which gave 5,6-diacetoxyindole [38 mg (36%), m.p. 139-142°] on recrystallization from heptane containing a small quantity of benzene (lit. m.p. 139-140°[180], 130-133°[186]).

n.m.r. $\delta(CDCl_3)$: 8.40 (1H, broad s, NH); 7.26 (1H, s, H^4); 7.04 (1H, s, H^7); 6.92 (1H, t, H^2); 6.30 (1H, t, H^3); 2.27 (6H, s, 2 × CH_3).

After shaking with D_2O the peak at δ 8.40 disappeared and the triplets at δ 6.92 and 6.30 collapsed to doublets ($J_{2,3}$ = 2.7 Hz).

mass spectrum: $M^{+\cdot}$ 233.0684 ± .0007, calcd. for $C_{12}H_{11}NO_4$, 233.0688.

5.4.6 2-Methylnoradrenochrome

The free base of 3,4-dihydroxynorephedrine was prepared by the addition of solid K_2CO_3 to a stirred solution of 3,4-dihydroxynorephedrine hydrochloride in H_2O. After standing in an ice bath for 1.5 hr under a stream of nitrogen, the free base was filtered off and washed with H_2O.

Freshly prepared silver oxide (8.3 g) was added to a suspension of 3,4-dihydroxynorephedrine (2 g) in methanol (200 ml) at room temperature. The reaction mixture was vigorously shaken for 8 min after which time it was filtered firstly through an anhydrous sodium sulphate bed (height = 1.5 cm; diam. = 4.5 cm) and secondly through a Dowex-1 × 8 (Cl^-) (200-400 mesh size) resin bed (height = 3.5 cm; diam. = 4.5 cm). Ethyl acetate (250 ml) was added to the filtrate and the resulting solution was concentrated *in vacuo* below 25° to about 30 ml. Ethyl acetate (30 ml) was again added and the solution concentrated *in vacuo* to small bulk (*ca.* 25 ml) until crystals began to form. 2-Methylnoradrenochrome was obtained as a dark red microcrystalline solid [515 mg (26%); totally decomposed without melting by 115°] upon filtration of the solution.

u.v. λ_{max} (H_2O) nm (ε): 216 (26,800), 295 (9600), 488 (3690).

i.r. ν_{max} (Nujol): 3235, 3210, 1667, 1617, 1582, 1543 cm^{-1}.

n.m.r. δ(DMSO-d_6): 9.35 (1H, broad s, disappears upon addition of D_2O, NH); 6.39 (1H, d, $J_{3,4}$ = 2.2 Hz, H^4);

6.07 (1H, broad s, disappears upon addition of D_2O, OH); 5.39 (1H, broad s, sharpens upon addition of D_2O, H^7); 4.50 (1H, broad t, forms a well defined doublet of doublets, $J_{3,4}$ = 2.2 Hz, $J_{2,3}$ = 3.6 Hz, upon addition of D_2O, H^3); 3.76 (1H, ddd, $J_{2,3}$ = 3.6 Hz, J_{2,CH_3} = 6.6 Hz, H^2); 1.33 (3H, d, J_{2,CH_3} = 6.6 Hz, CH_3).

Anal. Calcd. for $C_9H_9NO_3$: C, 60.33; H, 5.06; N, 7.82%.
Found: C, 59.94; H, 4.86; N, 7.79%.

5.4.7 Adrenochrome mono-p-nitrophenylhydrazone (111)

Adrenochrome (200 mg) was added to a rapidly stirred suspension of p-nitrophenylhydrazine hydrochloride (212 mg) in a solution of sodium acetate (115 mg) in water (25 ml). The mixture was stirred for a further 15 min, after which time the resulting red precipitate was removed by filtration. Upon trituration of the precipitate with methanol (2 × 150 ml) adrenochrome mono-p-nitrophenylhydrazone was obtained as a red microcrystalline solid [245 mg (68%); m.p. 255-258°].

u.v. λ_{max} (1 N NaOH) nm (ε): 280 (8100), 298(sh), 421(sh), 585 (43,400).

i.r. ν_{max} (Nujol): 3400(sh), 3185, 1658(sh), 1651, 1594, 1515, 1504, 1338 cm^{-1}.

n.m.r. δ(DMSO-d_6): 16.45 (1H, broad s, NH); 8.36-7.38 (4H, m, aromatic AA'BB' system centred at δ 7.87);

6.92 (1H, d, $J_{3,4}$ = 1.2 Hz, H^4); 5.70 (1H, d, $J_{3,OH}$ = 4.3 Hz, OH); 5.52 (1H, s, H^7); 5.02 (1H, m, X of ABX, also coupled to H^4 and to the hydroxyl proton, H^3); 3.91 (1H, dd, B of ABX, $J_{2A,2B}$ = -12.0 Hz, $J_{2B,3}$ = 6.8 Hz, C^2-methylene H); 3.46 (1H, dd, A of ABX, $J_{2A,2B}$ = -12.0 Hz, $J_{2A,3}$ = 3.2 Hz, C^2-methylene H); 3.02 (3H, s, N-CH_3).

Anal. Calcd. for $C_{15}H_{14}N_4O_4$: C, 57.32; H, 4.49; N, 17.83%.
Found: C, 57.28; H, 4.47; N, 17.37%.

Veer[30] reportedly obtained the p-nitrophenylhydrazone of adrenochrome as a brown crystalline solid [m.p. 200° (dec.)] upon the reaction of adrenochrome with p-nitrophenylhydrazine in boiling 30% acetic acid. The compound which he obtained, however, may well be a degradation product of adrenochrome mono-p-nitrophenylhydrazone (111) since attempts to recrystallize 111 during the course of this investigation led to its decomposition with the formation of an unidentified brown product.

The synthesis of 111 was also reported in a Belgian patent[187] although neither the experimental details nor the physical characteristics of this compound were given. van Espen[73] apparently successfully synthesized 111 and reported that it had a λ_{max} at ca. 580 nm in 1 N NaOH.

5.5 The 5,6-dihydroxyindole thioether

5.5.1 Reaction of adrenochrome with thioglycollic acid

5.5.1.1 Preliminary reactions

5.5.1.1.1 Aqueous reaction

Thioglycollic acid was added dropwise to a solution of adrenochrome (3 mg) in water (1 ml) until the red colour of the solution was totally discharged. The reaction mixture was then treated with an excess of sodium bicarbonate and extracted with ether (4 × 2 ml) to remove phenolic but not acidic products. The mother liquors were acidified with 2 N HCl and then extracted with ether to remove acidic products.

5.5.1.1.2 Nonaqueous reactions

Adrenochrome (3 mg) was dissolved in thioglycollic acid (ca. 0.5 ml) and the resulting dark yellow-brown solution was divided into two approximately equal parts. After being allowed to stand at room temperature for ca. 1 hr, one part of the reaction mixture was poured into water (3 ml) and excess solid sodium bicarbonate added. The reaction mixture was then treated as described above (Sect. 5.5.1.1.1) to give ether extracts containing phenolic and acid products.

The second half of the original reaction mixture was heated in an oil bath at 120° for 3 hr, after which time it was cooled and then treated as described above to

give phenolic and acidic ether extracts.

5.5.1.1.3 Chromatography

The aqueous reaction mixture along with the acidic and phenolic ether extracts mentioned above were examined by t.l.c. on cellulose layers (Eastman "Chromagram" sheets, 20 × 20 cm) using 2% acetic acid in water as running solvent. For comparison, samples of DHMI were chromatographed along with the fractions mentioned above (see Table 1, p. 57).

5.5.1.2 Isolation of 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindole lactone (90) from acidic fraction of aqueous reaction mixture

Freshly prepared silver oxide (30 g) was added portionwise, during a period of about 3-4 min to a stirred solution of adrenaline bitartrate (7.0 g) in water (150 ml). The temperature of the reaction mixture was not allowed to exceed 30° during the addition of the oxidant. The resulting deep red suspension was filtered through a Dowex-1 × 10 (Cl^-) (200-400 mesh size) resin bed (diam. = 4.5 cm; height = 2.5 cm) and thioglycollic acid (4 ml) was added dropwise, with stirring, to the filtrate. The resulting opaque brownish-green solution was acidified with conc. HCl (3 ml) and extracted with ether (6 × 100 ml). The

combined ether extracts, which contained both DHMI and 4-S-carboxymethylthio-5,6-dihydroxy-1-methylindole, were extracted with saturated aqueous $NaHCO_3$ (6 × 100 ml). Conc. HCl (50 ml) was added slowly with stirring to a two-phase system composed of the combined $NaHCO_3$ extracts and ether (100 ml). Small quantities of ether were added from time to time to replace losses due to evaporation. After addition of the acid was complete, the ether layer was separated and the aqueous mother liquors further extracted with ether (5 × 100 ml). The original ether layer and extracts were combined, dried (Na_2SO_4), and filtered. A mixture of acetic anhydride (7.5 ml) and dry pyridine (7.5 ml) was added to the filtrate and the reaction mixture allowed to stand overnight at room temperature. The ether was then removed in vacuo, below 30°, and the resulting solution added dropwise, with stirring, to an ice/water mixture (500 ml). The crude acetyl derivative, which was obtained as a pale yellow gummy solid was dissolved in benzene/light petroleum (3:1), and adsorbed on a silica gel (Koch-Light 200-300 mesh) column (2 × 15 cm). The column was eluted with the same solvent and a series of fractions (50 ml) were collected. Fractions 5-10 and fractions 11-24, which showed positive reactions with Ehrlich's reagent, were combined and concentrated to dryness in vacuo below 30° to give 90 as white crystalline solids

(137 mg, m.p. 159-161° and 119 mg, m.p. 136-157° respectively). The latter product was a somewhat impure version of the former. The presence of 90 in the eluant was determined by radial chromatography on formamide treated paper using benzene/light petroleum mixtures [either (1:1) or (1:2)] as running solvents. The $\underline{R}_f$ values of 90 in these systems are 0.86 and 0.65 respectively. The compound 90 could easily be located on the developed chromatograms by spraying with either the F_1 reagent (violet colour) or 1 N NaOH (blue-green colour).

Pure 6-acetoxy-4-S-carboxymethylthio-5-hydroxyl-1-methylindole lactone (90) (m.p. 161-163°) was obtained as colourless needles upon repeated recrystallization of the crude product from light petroleum (b.p. 80-100°).

u.v. λ_{max} (EtOH) nm (ε): 224 (25,700), 243(sh), 269 (11,800), 299 (7310), 307(sh).

i.r. ν_{max} (Nujol): 1764 cm^{-1}.

n.m.r. δ($CDCl_3$): 7.10 (1H, d, $\underline{J}_{2,3}$ = 3.0 Hz, H^2); 7.00 (1H, d, $\underline{J}_{3,7}$ = 0.8 Hz, H^7); 6.45 (1H, dd, $\underline{J}_{2,3}$ = 3.0 Hz, $\underline{J}_{3,7}$ = 0.8 Hz, H^3); 3.75 (3H, s, N-CH_3); 3.50 (2H, s, S-CH_2-); 2.35 (3H, s, C-CH_3).

Mass spectrum: $M^{+\cdot}$ 277; calcd. for $C_{13}H_{11}NO_4S$, 277.

Anal. Calcd. for $C_{13}H_{11}NO_4S$: C, 56.30; H, 4.00: N, 5.05; S, 11.57%.

Found: C, 56.06; H, 4.02; N, 5.12; S, 11.73%.

5.5.1.3 Isolation of 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindole lactone (90) from phenolic fraction of nonaqueous reaction mixture

A solution of adrenochrome (0.5 g) in thioglycollic acid (15 ml) was heated on an oil bath at 120° for 3 hr. After cooling, the reaction mixture was poured into saturated aqueous sodium bicarbonate solution (200 ml) and the resulting suspension was extracted with ether (3 × 250 ml). A mixture of acetic anhydride (2.5 ml) and dry pyridine (2.5 ml) was added to the combined, dried (Na_2SO_4), ether extracts and the reaction mixture allowed to stand at room temperature overnight. After removal of the ether in vacuo the concentrated acetylation mixture was poured slowly, with stirring, into ice/water (250 ml). The pale yellow gummy solid which was obtained was dissolved in benzene and purified chromatographically on a silica gel (Koch-Light 100-200 mesh) column (1.8 × 10 cm) with adsorption from and elution with benzene. The crude solid obtained on concentration of the benzene eluant gave 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindole lactone (90) as colourless needles (125 mg; m.p. 156-159°). The identity of this product with samples of 90 as described in Sect. 5.5.1.2 was established by the nondepression of m.p. and by the identity of their infrared and nuclear magnetic resonance spectra.

Anal. Calcd. for $C_{13}H_{11}NO_4S$: C, 56.30; H, 4.00; N, 5.05; S, 11.57%.

Found: C, 56.69; H, 3.98; N, 5.07; S, 11.69%.

5.5.2 The reactions of 4- and 7- methyladrenochrome with thioglycollic acid

5.5.2.1 Preliminary reactions

Thioglycollic acid was added dropwise to a solution of the aminochrome (2 mg) in water (1 ml) until no further colour changes occurred. An excess of solid sodium bicarbonate was added to each of the reaction mixtures, which were then treated as described in Sect. 5.5.1.1.1, p. 158 to give phenolic and acidic ether extracts. The reaction mixtures and the ether extracts were examined by t.l.c. on cellulose layers (Eastman "Chromagram" sheets, 20 × 20 cm) using 2% acetic acid in water as running solvent. For comparison purposes, samples of 5,6-dihydroxy-1,4-dimethylindole and 5,6-dihydroxy-1,7-dimethylindole were chromatographed along with the above fractions. The developed chromatograms were dried and sprayed with several different chromogenic reagents (see Table 3, p. 67).

5.5.2.2 Isolation of 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1,7-dimethylindole lactone (97) from acidic fraction of aqueous reaction mixture

Thioglycollic acid (0.5 ml) was added dropwise, with stirring, to a solution of 7-methyladrenochrome (300 mg) The reaction mixture was stirred at room temperature for a further few minutes, after which time excess solid $NaHCO_3$ was added. The neutralized reaction mixture was immediately extracted with peroxide-free ether (5 × 100 ml), cautiously acidified with 2 N HCl, and further extracted with ether (5 × 100 ml). A mixture of acetic anhydride (1.5 ml) and pyridine (1.5 ml) was added to the combined, dried (Na_2SO_4) ether extracts of the acidified reaction mixture. The ethereal acetylation mixture was allowed to stand overnight at room temperature after which time the ether was removed in vacuo and the concentrated reaction mixture poured dropwise, with stirring, into ice/water (150 ml). The crude product, a light green tarry precipitate, was dissolved in benzene and purified chromatographically on a silica gel (Koch-Light 100-200 mesh) column (6 × 1.8 cm). The column was eluted with benzene and a series of 25 ml fractions collected. The fractions were monitored by t.l.c. using the F_1 reagent. Fractions 4-11, which reacted positively with this reagent, were combined and the benzene removed in vacuo. Recrystallization from light petroleum (b.p. 80-100°)

of the crude white crystalline solid so obtained gave 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1,7-dimethylindole lactone (97) as white needles (53 mg, m.p. 202-204°).

u.v. λ_{max} (EtOH) nm: 226, 242(sh), 269, 295, 305(sh).

n.m.r. $\delta(CDCl_3)$: 6.97 (1H, d, $J_{2,3}$ = 3.0 Hz, H^2); 6.36 (1H, d, $J_{2,3}$ = 3.0 Hz, H^3); 4.02 (3H, s, N-CH_3); 3.46 (2H, s, S-CH_2-); 2.55 (3H, s, C-CH_3); 2.38 (3H, s, acetyl CH_3).

mass spectrum: $M^{+\cdot}$ 291; calcd. for $C_{14}H_{13}NO_4S$, 291.

Anal. Calcd. for $C_{14}H_{13}NO_4S$: C, 57.80; H, 4.50; N, 4.81; S, 11.02%.

Found: C, 57.61; H, 4.36; N, 4.85; S, 10.69%.

5.5.3 Reaction of 1-isopropylnoradrenochrome with thioglycollic acid; isolation of 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-isopropylindole lactone (98) from acidic fraction of aqueous reaction mixture

Compound 98 was prepared from N-isopropylnoradrenaline hydrochloride and thioglycollic acid by essentially the same procedure as described for 90 in Sect. 5.5.1.2, p. 159. It was obtained as colourless plates, m.p. 154-157°.

u.v. λ_{max} (EtOH) nm (ε): 224 (24,300), 243(sh), 269 (11,100), 299 (6880), 306(sh).

i.r. ν_{max} (Nujol): 1780(sh), 1771 cm^{-1}.

n.m.r. $\delta(CDCl_3)$: 7.27 (1H, d, $J_{2,3}$ = 3.2 Hz, H^2); 7.06 (1H, d, $J_{3,7}$ = 0.8 Hz, H^7); 6.47 (1H, dd, $J_{2,3}$ = 3.2 Hz, $J_{3,7}$ = 0.8 Hz, H^3); 4.55 (1H, m, J = 6.8 Hz, N-CH<); 3.50 (2H, s, S-CH_2-); 2.36 (3H, s, C-CH_3); 1.52 (6H, d, J = 6.8 Hz, C$(CH_3)_2$).

mass spectrum: $M^{+\cdot}$ 305; calcd. for $C_{15}H_{15}NO_4S$, 305.

Anal. Calcd. for $C_{15}H_{15}NO_4S$: C, 58.98; H, 4.95; N, 4.59; S, 10.51%.

Found: C, 58.85; H, 4.62; N, 4.73, S, 10.17%.

5.5.4 Reaction of adrenochrome with β-mercaptopropionic acid; isolation of 6-acetoxy-4-S-(β-carboxyethyl)thio-5-hydroxy-1-methylindole lactone (99) from acidic fraction of aqueous reaction mixture

This compound was prepared from adrenaline bitartrate by a method analogous to that for the preparation of 90 (see Sect. 5.5.1.2, p. 159). After recrystallization from light petroleum (b.p. 80-100°) it was obtained as colourless plates, m.p. 165-168°.

u.v. λ_{max} (EtOH) nm (ε): 237 (32,800), 304 (8910).

i.r. ν_{max} (Nujol): 1770, 1740 cm^{-1}.

n.m.r. $\delta(CDCl_3)$: 7.15 (1H, d, $J_{2,3}$ = 3.2 Hz, H^2); 7.12 (1H, d, $J_{3,7}$ = 0.9 Hz, H^7); 6.68 (1H, dd, $J_{2,3}$ = 3.2 Hz, $J_{3,7}$ = 0.9 Hz, H^3); 3.78 (3H, s, N-CH_3);

3.55-2.69 (4H, AA'BB' system centred at 3.12, $-CH_2CH_2-$); 2.34 (3H, s, C-CH_3).

mass spectrum: $M^{+\cdot}$ 291; calcd. for $C_{14}H_{13}NO_4S$, 291.

Anal. Calcd. for $C_{14}H_{13}NO_4S$: C, 57.73; H, 4.50; N, 4.82; S, 11.01%.

Found: C, 57.45; H, 4.25; N, 4.81; S, 11.28%.

5.5.5 Reaction of noradrenochrome with thioglycollic acid; isolation of 6-acetoxy-4-S-carboxymethylthio-5-hydroxyindole lactone (100)

This compound was prepared by dissolving noradrenochrome (100 mg) in thioglycollic acid (3 ml) followed by treatment of the reaction mixture as described in Sect. 5.5.1.3 for the preparation of the corresponding compound (i.e. 90) from adrenochrome. After purification by column chromatography and crystallization of the crude product from heptane, 6-acetoxy-4-S-carboxymethylthio-5-hydroxindole lactone was obtained as colourless needles (1 mg, m.p. 176-179°).

u.v. λ_{max} (EtOH) nm: 223, 243(sh), 268, 293, 303(sh).

mass spectrum: $M^{+\cdot}$ 231.0528 ± .0007; calcd. for $C_{12}H_9NO_4S$, 231.0532.

5.5.6 Reaction of adrenochrome with homocysteine

Homocysteine (760 mg, 2 equiv.) was dissolved in

H_2O (150 ml) and conc. HCl was added to bring the pH of the solution to 2.5. Adrenochrome (500 mg, 1 equiv.) was added with stirring to the above solution. Examination by t.l.c. on cellulose of the aqueous reaction mixture at this stage revealed the presence of two major indolic products which exhibited chemical and chromatographic properties anticipated for DHMI and a 4-thiosubstituted 5,6-dihydroxy-1-methylindole. About 10 min after the addition of the adrenochrome the reaction mixture was filtered, extracted with EtOAc (4 × 200 ml) and the mother liquor lyophilized. A 1:1 mixture of Ac_2O and pyridine was added to the residue and the acetylation reaction was allowed to proceed for 3 hr at room temperature. The excess reagent was removed by concentration of the reaction mixture in vacuo below 60°. The residue was dissolved in CH_2Cl_2 (120 ml) and this solution was washed with 0.2 N HCl (2 × 100 ml) and H_2O (5 × 100 ml) and then dried (Na_2SO_4). Examination of the CH_2Cl_2 solution by t.l.c. on silica gel revealed the presence of a mixture of several compounds. Attempts to separate this mixture into its components by column chromatography on silica gel were not successful and work with this amino acid was abandoned.

5.5.7 Reaction of adrenochrome with N-acetylcysteine

5.5.7.1 Preliminary reactions

An excess of N-acetylcysteine was added to a

solution of adrenochrome (2 mg) in H_2O (1 ml). The phenolic and acidic ether extracts were obtained using the procedure previously described (see Sect. 5.5.1.1.1, p. 158). These extracts, along with the reaction mixture were examined chromatographically in the manner described previously (see Sect. 5.5.1.1.3, p. 159).

5.5.7.2 Isolation of 4-S-(N-acetylcysteinyl)-5,6-dimethoxy-1-methylindole methyl ester (101)

Adrenochrome (1 g) was added to a stirred two phase system consisting of EtOAc (250 ml) and a solution of N-acetylcysteine (3.7 g, 4 equiv.) in H_2O (250 ml) (the pH of the reaction mixture was ca. 2.5). Stirring was continued until the red colour of the adrenochrome had been completely discharged (ca. 15 min). After this time the EtOAc layer was removed and the aqueous solution further extracted with EtOAc (2 × 250 ml). The EtOAc extracts were combined and then extracted with a saturated solution of $NaHCO_3$ (1 × 1000 ml). The latter solution was acidified to a pH of ca. 2 by the addition of conc. HCl and then extracted with EtOAc (3 × 1000 ml). Diazomethane (ca. 1.5 g) dissolved in ether (75 ml) prepared by the method of De Boer and Backer[188] was added to the combined dried (Na_2SO_4) EtOAc extracts. This solution was allowed to stand for 3 hr after which time it was concentrated in vacuo to give a dark brown oil. About one half of this oil was then subjected to partition

chromatography on a column (2.5 × 90 cm) of Sephadex G-25 (fine) with the aqueous phase of an H_2O/MeOH/EtOAc/hexane (2:1:2:2) mixture as the stationary phase and the organic phase as the mobile phase.

The Sephadex was prepared by allowing ca. 150 g of this substance to swell overnight in an excess of the aqueous phase. Excess solvent was then removed by filtration and the swollen Sephadex was slurried with the organic phase and poured, a little at a time, into the column. Each new portion of slurry was packed by compressing the Sephadex with a perforated plunger. The column was equilibrated by prolonged washing with the organic phase until the effluent was free of the aqueous phase.

A series of 5 ml fractions were collected, the flow rate being ca. 10 ml/hr. The fractions were monitored by t.l.c. using Ehrlich's reagent. Fractions 26-33 were combined and the solvent removed in vacuo to give a colourless oil. Crystallization from light petroleum (b.p. 60-80°) gave 4-S-(N-acetylcysteinyl)-5,6-dimethoxy-1-methylindole methyl ester as clusters of white crystals (82 mg, m.p. 110-113°).

u.v. λ_{max} (EtOH) nm (ε): 232 (26,900), 295(sh), 307 (9270).

i.r. ν_{max} (KBr): 3295, 1737, 1651, 1614, 1545 cm^{-1}.

n.m.r. δ($CDCl_3$): 6.99 (1H, d, $J_{2,3}$ = 3.2 Hz, H^2); 6.93 (1H, broad d, J ≈ 8 Hz, NH); 6.82 (1H, d, $J_{3,7}$ = 0.9 Hz,

H^7); 6.56 (1H, dd, $J_{2,3}$ = 3.2 Hz, $J_{3,7}$ = 0.9 Hz, H^3); 4.78 (1H, m, X of ABX, $J_{AX} = J_{BX}$ = 4.5 Hz, $J_{CH,NH}$ = 8.2 Hz, cysteine methine H); 3.92 (6H, s, 2 O- or N- CH_3's); 3.72 (3H, s, O- or N- CH_3); 3.56 (3H, s, O- or N-CH_3); 3.48 (1H, dd, B of ABX, J_{AB} = -14.2 Hz, J_{BX} = 4.5 Hz, cysteine methylene H); 3.24 (1H, dd, A of ABX, J_{AB} = -14.2 Hz, J_{AX} = 4.5 Hz, cysteine methylene H); 1.75 (3H, s, C-CH_3).

mass spectrum: $M^{+\cdot}$ 366.1254 ± .0010; calcd. for $C_{17}H_{22}N_2O_5S$, 366.1250.

5.5.8 Reaction of adrenochrome with N-acetylpenicillamine; isolation of 4-S-(N-acetylpenicillaminyl)-5,6-dimethoxy-1-methylindole methyl ester (102)

This compound was obtained from the reaction of adrenochrome with N-acetylpenicillamine using a method similar to that which was employed for the preparation of 101 (see Sect. 5.5.7.2). The solvent system used for partition chromatography with Sephadex G-25 was H_2O/MeOH/EtOAc/light petroleum (4:4:3:5). The yellow oil obtained after chromatography on Sephadex was purified by chromatography on a column (17 × 2.2 cm) of silica gel (Davison, 100-200 mesh) with EtOAc as eluant. Fractions 22-28 (10 ml fractions) were combined and concentrated to dryness in vacuo, giving what was probably 102 as a colourless oil which could not be crystallized.

n.m.r. $\delta(CDCl_3)$: 7.92 (1H, broad d, $\underline{J} \approx 9$ Hz, NH); 6.99 (1H, d, $\underline{J}_{2,3} = 3.2$ Hz, H^2); 6.89 (1H, d, $\underline{J}_{3,7} = 0.8$ Hz, H^7); 6.60 (1H, dd, $\underline{J}_{2,3} = 3.2$ Hz, $\underline{J}_{3,7} = 0.8$ Hz, H^3); 4.53 (1H, d, $\underline{J}_{CH,NH} = 8.5$ Hz, penicillamine CH); 3.94 (3H, s, N- or O- CH_3); 3.92 (3H, s, N- or O- CH_3); 3.75 (6H, s, 2 N- or O- CH_3's); 1.96 (3H, s, acetyl CH_3); 1.58 (3H, s, C-CH_3); 1.07 (3H, s, C-CH_3).

There was also a sharp peak at δ 1.25 and a broad one at δ ca. 1.05, probably due to impurities.

mass spectrum: $M^{+\cdot}$ 394.1557 ± .0012; calcd. for $C_{19}H_{26}N_2O_5S$, 394.1563.

5.5.9 Reactions of epinochrome with thiols

5.5.9.1 Preliminary experiments

a) Chromatography

The reactions between epinochrome and excesses of thioglycollic acid, N-acetylcysteine, glutathione (free acid), glutathione (monosodium salt) and homocysteine were carried out in aqueous solution. The reaction mixtures, their EtOAc extracts and the mother liquors were examined by t.l.c. on cellulose (without fluorescent indicator) with 2% HOAc as developing solvent. Prior to use the cellulose sheets were washed, first with 1 N HCl and then with H_2O and allowed to dry. For comparison the reactions of adrenochrome with the corresponding thiols were carried out and the R_f values of

the products compared with those obtained from the epinochrome/thiol reactions.

b) Ultraviolet and visible spectroscopy

A solution of epinochrome (9.1 mg) in H_2O (1 ml) was added to a solution of N-acetylcysteine (18.2 mg, 2 equiv.) in acetate buffer (1 ml, pH 6.1). A series of samples of the reaction mixture were taken at various times and their u.v./visible spectra recorded after dilution to suitable concentrations. When the reaction was complete, as indicated by the disappearance of the peak due to epinochrome at 476 nm, the reaction mixture was extracted with ether. A small amount of the ether extract was dissolved in water and the spectrum recorded. For comparison the spectrum of DHMI was also measured in aqueous solution. In a similar manner epinochrome was reacted with glutathione at a pH of 3.3 and the reaction followed spectroscopically.

c) Acetylation of reaction mixture and chromatography of products

Epinochrome (50 mg) was added to thioglycollic acid (1 ml) and the mixture shaken until all the epinochrome had dissolved (ca. 15 min). A 1:1 mixture (7 ml) of Ac_2O and pyridine was added and the reaction mixture kept at room temperature for 2 hr. Concentration of this solution in vacuo gave a dark yellow oil which was dissolved in CH_2Cl_2

(25 ml). After washing with a saturated aqueous solution of $NaHCO_3$ (3 × 25 ml) and H_2O (2 × 25 ml) the organic phase was dried (Na_2SO_4) and its composition investigated by t.l.c. on silica gel with a number of solvents and chromogenic reagents (see Table 4, p. 82). Samples of 5,6-diacetoxy-1-methylindoline (103) and 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindole lactone (90) prepared by methods previously described (see Sect. 5.4.2 and Sect. 5.5.1.2) were used as chromatographic standards.

5.5.9.2 Isolation of reaction products

Epinochrome (500 mg) was added to thioglycollic acid (5 ml) and the mixture shaken until all the epinochrome had dissolved (ca. 45 min). The flask containing the thioglycollic acid solution was then evacuated and N_2 was bubbled through the reaction mixture. A 1:1 mixture (40 ml) of Ac_2O and pyridine was added to the thioglycollic acid solution at 0°, the system being maintained under nitrogen at slightly reduced pressure throughout the addition and then for a further 2 hr. Half an hour after the addition of the Ac_2O and pyridine the reaction mixture was allowed to warm up to room temperature and after another 1.5 hr it was concentrated to small bulk in vacuo below 50°. The residue was dissolved in CH_2Cl_2 (50 ml) and this solution was washed with a saturated aqueous solution of $NaHCO_3$ (3 × 50 ml) and then with H_2O (3 × 50 ml). A dark yellow oil was obtained upon concentration of the dried (Na_2SO_4) organic layer in vacuo.

5.5.9.2.1 Isolation of 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindole lactone (90)

The dark yellow oil mentioned above was purified chromatographically on a silica gel (Davison, 100-200 mesh) column (25 × 2 cm) using benzene (500 ml) followed by benzene/EtOAc (97:3; 1000 ml) as eluting solvent. A series of fractions (17 ml) were collected and these were monitored by t.l.c. on silica gel using benzene/EtOAc (8:2) as developing solvent with Ehrlich's reagent and 1 N NaOH as chromogenic reagents. Fractions 65-80 were combined and concentrated to dryness in vacuo to give 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindole lactone (90) as a white crystalline solid (60 mg, 7.1 %), which, after recrystallization from heptane, had a melting point of 160-163°. The identity of this product with samples of 90 which had previously been prepared (see Sect. 5.5.1.2) from the reaction of thioglycollic acid with adrenochrome was established by the nondepression of m.p. and by the identity of their i.r., n.m.r. and mass spectral properties.

5.5.9.2.2 Isolation of 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindoline lactone (109) and 5,6-diacetoxy-1-methylindoline (103)

The dark yellow oil obtained after acetylation of the epinochrome/thioglycollic acid reaction mixture was

chromatographed on a column (20 × 2 cm) of silica gel (Davison, 100-200 mesh) with hexane/pyridine (8:2) as eluant. A yellow oil was obtained after concentration of fractions 8-20 (fraction size 20 ml) to dryness in vacuo below 35°. This oil was rechromatographed on a column (25 × 2 cm) of silica gel (Davison, 100-200 mesh) with the following solvents as eluants: benzene (500 ml); benzene/EtOAc (97:3, 1000 ml) and benzene/EtOAc (95:5, 2000 ml). Fractions 95-120 (fraction size 15 ml) were combined and concentrated to dryness in vacuo to give 6-acetoxy-4-S-carboxymethylthio-5-hydroxy-1-methylindoline lactone as a white crystalline solid (70 mg, 8.2%), which gave colourless needles, m.p. 137-140° after recrystallization from heptane.

u.v. λ_{max} (EtOH) nm (ε): 225(sh), 234 (19,700), 262 (9,020), 319 (4,010).

i.r. ν_{max} (Nujol): 1761 cm^{-1}.

n.m.r. δ($CDCl_3$): 6.10 (1H, s, H^7); 3.43 (2H, s, S-CH_2-); 3.63-2.71 (4H, m, AA'BB' system centred at δ 3.17, -CH_2CH_2- of 5-membered ring); 2.73 (3H, s, N-CH_3); 2.32 (3H, s, C-CH_3).

mass spectrum: $M^{+\cdot}$ 279.0561 ± .0008; calcd. for $C_{13}H_{13}NO_4S$, 279.0566.

Similarly, fractions 150-220 were combined and concentrated to dryness in vacuo to give 5,6-diacetoxy-1-methylindoline (103) as a white crystalline solid (115 mg,

15.1%). Recrystallization from heptane gave colourless rods, m.p. 113-115°. The identity of this product with 103 which had previously been prepared from the reduction of epinochrome with sodium dithionite (see Sect. 5.4.2) was established by the nondepression of m.p. and the identity of their i.r., n.m.r. and mass spectra.

5.6 Preparation of aminochrome/sodium bisulphite addition products

5.6.1 Sodium 4,9-dihydro-3-methoxyepinochrome-9-sulphonate (adrenochrome methyl ether/sodium bisulphite addition product)

Adrenochrome methyl ether (400 mg) was added to a stirred solution of $Na_2S_2O_5$ (186 mg, 0.47 equiv.) in 40 ml H_2O and nitrogen was bubbled through the reaction mixture for 1 hr. After this time MeOH (60 ml) was added and the solution concentrated to dryness in vacuo below 25°. The residue was dissolved in H_2O (2 ml) and EtOH (20 ml), followed by ether (20 ml), was added, resulting in a dark tarry precipitate. The supernatant liquid was decanted off and ether (20 ml) was added to it, giving an oily precipitate which was removed by filtration and then dissolved in H_2O (2 ml) and EtOH (20 ml) and the solution filtered. Addition of ether (30 ml) to the filtrate led to the formation of a light yellow-brown precipitate which was dissolved in H_2O (2 ml) and EtOH (20 ml). A small amount of activated

charcoal was added at this stage and the solution was stirred for a few minutes and then filtered. After the addition of ether (30 ml) to the filtrate a pale yellow precipitate formed which was removed by filtration. Upon repetition of this procedure, the adrenochrome methyl ether/sodium bisulphite addition product was obtained as clusters of pale yellow crystals (224 mg, 34%).

u.v. λ_{max} (H_2O) nm (ε): 245 (7480), 349 (15,400).

i.r. ν_{max} (Nujol): 1712, 1627, 1568, 1242, 1049 cm^{-1}.

n.m.r. δ[Pyridine-$\underline{d}_5$/DMSO-$\underline{d}_6$ (2:1)]: 5.79 (1H, s, H^7); 4.65 (1H, d, X of ABX, $J_{2A,3} \approx 0$, $J_{2B,3}$ = 4.4 Hz, C^3-methine H); 4.40 (1H, dd, B of ABX, $J_{2A,2B}$ = -11.4 Hz, $J_{2B,3}$ = 4.4 Hz, C^2-methylene H); 3.65 (1H, d, A of ABX, $J_{2A,2B}$ = -11.4 Hz, $J_{2A,3} \approx 0$, C^2-methylene H); 3.60 (1H, d, A of AB, $J_{4A,4B}$ = -17.0 Hz, C^4-methylene H); 3.34 (1H, d, A of AB, $J_{4A,4B}$ = -17.0 Hz, C^4-methylene H); 3.33 (3H, s, O-CH_3); 2.95 (3H, s, N-CH_3).

Anal. Calcd. for $C_{10}H_{12}NO_6SNa$: C, 40.40; H, 4.07; N, 4.71; S, 10.79; Na, 7.73%.

Found: C, 40.60; H, 4.00; N, 4.81; S, 10.85; Na, 7.66%.

5.6.2 Sodium 4,9-dihydro-1-isopropylnoradrenochrome-9-sulphonate (1-isopropylnoradrenochrome/sodium bisulphite addition product)

This was prepared from 1-isopropylnoradrenochrome and $Na_2S_2O_5$ by essentially the same procedure as described above (Sect. 5.6.1) and gave a yellow crystalline solid in a yield of 55%.

u.v. λ_{max} (H_2O) nm (ε): 247 (7220), 352 (16,400).

i.r. ν_{max} (Nujol): 3366, 1717, 1602, 1566, 1245, 1052 cm^{-1}.

n.m.r. δ(DMSO-$\underline{d}_6$): 5.61 (1H, d, $\underline{J}_{3,OH}$ = 5.0 Hz, OH); 5.47 (1H, s, H^7); 4.49 (1H, t, X of ABX, $\underline{J}_{2A,3} \approx 0$, $\underline{J}_{3,OH} \approx \underline{J}_{2B,3} \approx$ 4.5 Hz, C^3-methine H); 3.98 (1H, m, $\underline{J}$ = 6.6 Hz, $\underline{N}$-CH<); 3.96 (1H, dd, B of ABX, $\underline{J}_{2A,2B}$ = -11.2 Hz, $\underline{J}_{2B,3}$ = 4.1 Hz, C^2-methylene H); 3.34 (1H, d, A of ABX, $\underline{J}_{2A,2B}$ = -11.2 Hz, $\underline{J}_{2A,3} \approx 0$, C^2-methylene H); 2.91 (2H, s, C^4-methylene H's); 1.17 (3H, d, $\underline{J}$ = 6.6 Hz, $\underline{C}$-CH_3); 1.12 (3H, d, $\underline{J}$ = 6.6 Hz, $\underline{C}$-CH_3).

δ(D_2O): 5.88 (1H, s, H^7); 4.74 (1H, d, X of ABX, $\underline{J}_{2A,3} \approx 0$, $\underline{J}_{2B,3}$ = 4.3 Hz, C^3-methine H); 4.15 (1H, dd, B of ABX, $\underline{J}_{2A,2B}$ = -13.4 Hz, $\underline{J}_{2B,3}$ = 4.3 Hz, C^2-methylene H); 4.14 (1H, m, $\underline{J}$ = 6.7 Hz, $\underline{N}$-CH<); 3.68 (1H, d, A of ABX, $\underline{J}_{2A,2B}$ = -13.4 Hz, $\underline{J}_{2A,3} \approx 0$, C^2-methylene H); 3.31 (1H, d, A of AB, $\underline{J}_{4A,4B}$ =

-17.9 Hz, C^4-methylene H); 3.13 (1H, d, B of AB, $J_{4A,4B}$ = -17.9 Hz, C^4-methylene H); 1.27 (3H, d, J = 6.7 Hz, C-CH_3); 1.22 (3H, d, J = 6.7 Hz, C-CH_3).

Anal. Calcd. for $C_{11}H_{14}NO_6SNa$: C, 42.44; H, 4.53; N, 4.50; S, 10.30; Na, 7.39%.

Found: C, 42.52; H, 4.60; N, 4.42; S, 10.21; Na, 7.31%.

5.7 The aminochrome/thiol addition product

5.7.1 Preliminary experiments

5.7.1.1 Formation of the semicarbazone of the addition product between adrenochrome and N-acetylcysteine

A solution of adrenochrome (40 mg) in H_2O (4 ml) was added to a stirred solution of N-acetylcysteine (72.8 mg, 2 equiv.) in acetate buffer (4 ml) at pH 5.2. Samples were taken from the reaction mixture at various times, diluted appropriately with H_2O and their u.v./visible spectra in the range 230-530 nm were recorded as rapidly as possible. When the concentration of the addition product (λ_{max}, 358 nm) between adrenochrome and N-acetylcysteine reached its maximum level (about 65 min), 4 ml of the reaction mixture were added to a stirred solution of semicarbazide HCl (25 mg, 2 equiv.) in acetate buffer (2 ml) at pH 5.2. The reaction was followed spectroscopically as before, the final spectrum being taken 13 hr after the start of the experiment. The semicarbazone of the adrenochrome/N-acetylcysteine addition product

formed in this way had a λ_{max} at 352 nm. For comparison the spectrum of the original adrenochrome/N-acetylcysteine reaction mixture was also measured at this time (see Fig. 9, p. 103).

5.7.1.2 Degradation of the semicarbazone of the addition product between adrenochrome and N-acetylcysteine

a) By alkali

A solution of the addition product semicarbazone (0.17 ml) prepared in the above manner was diluted to 200 ml with 0.5 N NaOH and the reaction which ensued was followed spectroscopically (see Fig. 10, p. 104). When the reaction was complete (about 7 hr) the reaction mixture was made strongly acidic (pH 1.1) by the addition of conc. HCl and the u.v./visible spectrum measured. The pH of this solution was then adjusted to 5.1 by the addition of conc. aqueous NaOAc and the spectrum was again measured. The λ_{max} values found for the degradation product in strongly acid, weakly acid and alkaline solutions were 374, 356 (with a shoulder at 445) and 443 nm respectively. The values found for adrenochrome monosemicarbazone under identical conditions were 376, 355 (with a shoulder at 445) and 444 nm (see Fig. 11, p. 106).

b) By heating

A sample of the reaction mixture containing the

(582 mg, 1.1 equiv.) and NaOAc (254 mg, 1.1 equiv.) in 30 ml H_2O. After stirring the reaction mixture for 20 min, $NaHCO_3$ (1 g) was added cautiously. The resulting mixture was filtered and the filtrate acidified, with stirring, by the dropwise addition of 2 N HCl to give a light brownish-yellow precipitate which was filtered off and dried *in vacuo*. This material was then triturated with MeOH (4-5 ml) and the remaining solid removed by filtration and dissolved in 150 ml of boiling MeOH. The methanolic solution was filtered, kept overnight at -20°, and the resulting small quantity of yellow product which had separated out was filtered off and discarded. Water (150 ml) was added to the filtrate and the resulting solution was concentrated *in vacuo* to about 150 ml, giving a light brownish-yellow microcrystalline solid (320 mg, 27%). The product so obtained could be recrystallized from a methanol/ethyl acetate mixture to give 9-S-(β-carboxyethylthio)-4,9-dihydroadrenochrome mono-p-nitrophenylhydrazone as a yellow microcrystalline solid.

u.v. λ_{max} (0.1 M $NaHCO_3$ aq.) nm (ε): 231 (13,460), 416 (46,400).

i.r. ν_{max} (Nujol): 3335, 3290(sh), 1723, 1621, 1607, 1590, 1522, 1503, 1317 cm^{-1}.

n.m.r. δ[Pyridine-d_5/D_2O (5:1)]: 8.40-7.76 (4H, aromatic AA'BB' system centred at 8.08); 5.77 (1H, s, H^7); 4.73 (1H, d, X of ABX, $J_{2A,3} \approx 0$, $J_{2B,3}$ = 3.4 Hz,

C^3-methine H); 4.31 (1H, dd, B of ABX, $J_{2A,2B}$ = -11.9 Hz, $J_{2B,3}$ = 3.4 Hz, C^2-methylene H); 4.13 (1H, d, A of AB, $J_{4A,4B}$ = -16.8 Hz, C^4-methylene H); 3.77 (1H, d, A of ABX, $J_{2A,2B}$ = -11.9 Hz, $J_{2A,3} \approx 0$, C^2-methylene H); 3.67 (1H, d, B of AB, $J_{4A,4B}$ = -16.8 Hz, C^4-methylene H); 3.03 (4H, m, $-CH_2CH_2-$ side chain H's); 2.96 (3H, s, N-CH_3). The n.m.r. spectrum of this compound in pyridine-d_5 alone revealed an additional broad peak at δ 11.20 presumably due to the p-nitrophenylhydrazone NH.

Anal. Calcd. for $C_{18}H_{20}N_4O_6S$: C, 51.42; H, 4.79; N, 13.33; S, 7.63%. Equiv. wt. 420.

Found: C, 51.48; H, 4.92; N, 13.08; S, 7.54%. Equiv. wt 416.

5.7.2.2 9-S-(N-acetylcysteinyl)-4,9-dihydroadrenochrome mono-p-nitrophenylhydrazone

Adrenochrome (500 mg) was added to a solution of N-acetylcysteine (1,366 mg, 3 equiv.) and NaOAc (687 mg, 3 equiv.) in 5 ml H_2O. Nitrogen was bubbled through the solution for about 10 min and it was then added to a stirred suspension of p-nitrophenylhydrazine hydrochloride (582 mg, 1.1 equiv.) and NaOAc (254 mg, 1.1 equiv.) in 30 ml H_2O. The reaction mixture was stirred for 20 min and then $NaHCO_3$ (2 g) was added cautiously. The resulting mixture was filtered and the filtrate acidified by the dropwise

addition of 2 N HCl to give a yellow precipitate which was removed by filtration and dried *in vacuo*. This material was triturated with methanol (3-4 ml) and the remaining solid filtered off and recrystallized from methanol, to give 9-S-(N-acetylcysteinyl)-4,9-dihydroadrenochrome mono-p-nitrophenylhydrazone as a yellow microcrystalline solid (265 mg, 20%).

u.v. λ_{max} (0.25 M $NaHCO_3$ aq.) nm (ε): 233 (13,830), 416 (48,300).

i.r. ν_{max} (Nujol): 3615, 3320, 3245, 1690, 1656, 1637, 1625, 1605, 1592, 1573, 1534, 1511, 1338 cm^{-1}.

n.m.r. δ[DMF-d_7/Pyridine-d_5 (3:2)]: 10.80 (1H, broad s, p-nitrophenylhydrazone NH); 8.55 (1H, d, J = 7.7 Hz, cysteine NH); 8.39-7.47 (4H, aromatic AA'BB' centred at 7.93); 5.48 (1H, s, H^7); *ca.* 4.85 (1H, m, cysteine methine H); 4.63 (1H, d, X of ABX, $J_{2A,3} \approx 0$, $J_{2B,3}$ = 3.2 Hz, C^3-methine H); 4.29 (1H, dd, B of ABX, $J_{2A,2B}$ = -11.8 Hz, $J_{2B,3}$ = 3.2 Hz, C^2-methylene H); 3.85 (1H, d, A of AB, $J_{4A,4B}$ = -16.8 Hz, C^4-methylene H); 3.62 (1H, d, A of ABX, $J_{2A,2B}$ = -11.8 Hz, $J_{2A,3} \approx 0$, C^2-methylene H); 3.56 (1H, d, B of AB, $J_{4A,4B}$ = -16.8 Hz, C^4-methylene H); *ca.* 3.33 (2H, m, cysteine methylene H's); 2.95 (3H, s, N-CH_3); 2.01 (3H, s, C-CH_3).

Anal. Calcd. for $C_{20}H_{23}N_5O_7S$: C, 50.31; H, 4.86; N, 14.67; S, 6.72%. Equiv. wt. 477.5.

Found: C, 50.25; H, 4.87; N, 14.77; S, 6.58%. Equiv. wt. 461.

5.7.2.3 9-S-(N-acetylcysteinyl)-4,9-dihydroadrenochrome mono-p-bromophenylhydrazone

This was prepared from adrenochrome by a method analogous to that for the previous compound (Sect. 5.7.2.2) except that p-bromophenylhydrazine HCl was used instead of p-nitrophenylhydrazine HCl. It was obtained as a yellow microcrystalline solid in a yield of 25% after recrystallization from methanol.

u.v. λ_{max} (0.1 M $NaHCO_3$ aq.) nm (ε): 240 (15,170), 263(sh), 387 (33,800).

i.r. ν_{max} (Nujol): 3610, 3325, 3250, 1694, 1661, 1638, 1622, 1601, 1575, 1510 cm^{-1}.

n.m.r. δ[DMF-d_7/Pyridine-d_5 (2:1)]: 10.06 (1H, broad s, p-nitrophenylhydrazone NH); 8.43 (1H, d, J = 8.0 Hz, cysteine NH); 7.44 (4H, s, aromatic H's); 5.37 (1H, s, H^7); ca. 4.77 (1H, m, cysteine methine H); 4.55 (1H, d, X of ABX, $J_{2A,3} \approx 0$, $J_{2B,3}$ = 3.4 Hz, C^3-methine H); 4.26 (1H, dd, B of ABX, $J_{2A,2B}$ = -11.5 Hz, $J_{2B,3}$ = 3.4 Hz, C^2-methylene H); 3.68 (1H, d, A of AB, $J_{4A,4B}$ = -17.0 Hz, C^4-methylene H);

3.56 (1H, d, A of ABX, $J_{2A,2B}$ = -11.5 Hz, $J_{2A,3} \approx 0$, C^2-methylene H); 3.41 (1H, d, B of AB, $J_{4A,4B}$ = -17.0 Hz, C^4-methylene H); ca. 3.25 (2H, m, cysteine methylene H's); 2.92 (3H, s, N-CH_3); 1.97 (3H, s, C-CH_3).

Anal. Calcd. for $C_{20}H_{23}N_4O_5SBr$: C, 46.97; H, 4.53; N, 10.96; S, 6.27; Br, 15.63%. Equiv. wt. 511.

Found: C, 46.89; H, 4.56; N, 10.45; S, 6.33; Br, 15.40%. Equiv. wt. 494.

5.7.2.4 9-S-(β-Carboxyethylthio)-4,9-dihydro-1-isopropylnoradrenochrome mono-p-nitrophenylhydrazone

This compound was obtained in a similar fashion by the addition of a solution containing the addition product formed between 1-isopropylnoradrenochrome and β-mercaptopropionic acid to a suspension of p-nitrophenylhydrazine hydrochloride in sodium acetate solution. After recrystallization from methanol it was isolated as a yellow microcrystalline solid in a yield of 33%.

u.v. λ_{max} (0.1 M $NaHCO_3$ aq.) nm (ε): 231 (12,580), 417 (47,100).

i.r. ν_{max} (Nujol): 3635, 3300(sh), 3265, 1682, 1607(sh), 1595(sh), 1577, 1512, 1501, 1324 cm^{-1}.

n.m.r. δ(DMF-d_7): 10.58 (1H, broad s, p-nitrophenylhydrazone NH); 8.35-7.39 (4H, aromatic AA'BB' system centred

at 7.87); 5.37 (1H, s, H^7); 4.34 (1H, d, X of ABX, $J_{2A,3} \approx 0$, $J_{2B,3}$ = 3.2 Hz, C^3-methine H); 4.01 (1H, dd, B of ABX, $J_{2A,2B}$ = -11.8 Hz, $J_{2B,3}$ = 3.2 Hz, C^2-methylene H); 4.00 (1H, m, J = 6.5 Hz, N-CH<); 3.63 (1H, d, A of AB, $J_{4A,4B}$ = -16.7 Hz, C^4-methylene H); 3.59 (1H, d, A of ABX, $J_{2A,2B}$ = -11.8 Hz, $J_{2A,3} \approx 0$, C^2-methylene H); 3.30 (1H, d, B of AB, $J_{4A,4B}$ = -16.7 Hz, C^4-methylene H); ca. 2.71 (m, $-CH_2CH_2-$ side chain H's); partially obscured by signal from solvent); 1.25 (3H, d, J = 6.5 Hz, C-CH_3); 1.20 (3H, d, J = 6.5 Hz, C-CH_3).

Anal. Calcd. for $C_{20}H_{24}N_4O_6S$: C, 53.56; H, 5.39; N, 12.49; S, 7.15%. Equiv. wt. 448.5.

Found: C, 53.57; H, 5.30; N, 12.41; S, 7.01%. Equiv. wt. 453.

5.7.2.5 9-S-(β-Carboxyethylthio)-4,9-dihydroepinochrome mono-p-nitrophenylhydrazone

This compound was obtained in essentially the same manner by the addition of a solution containing the addition product between epinochrome and β-mercaptopropionic acid to a suspension of p-nitrophenylhydrazine hydrochloride in aqueous NaOAc. The product obtained after addition of 2 N HCl to the reaction mixture was not sufficiently soluble in methanol to enable it to be recrystallized but it was triturated with a large amount of this solvent. This compound

was thus obtained as a yellow microcrystalline solid in a yield of 30%.

u.v. λ_{max} (0.1 M $NaHCO_3$ aq.) nm (ε): 229 (14,420), 416 (46,700).

i.r. ν_{max} (Nujol): 3520, 3215, 1687, 1620, 1610, 1588, 1510(sh), 1502, 1324 cm^{-1}.

n.m.r. δ(1 M $NaDCO_3$ in D_2O): 8.20-7.08 (4H, aromatic AA'BB' centred at 7.64); 5.28 (1H, s, H^7); 3.61 (3H, broad m, C^2-methylene H's and one C^4-methylene H); 2.97 (3H, s, N-CH_3); 2.83-1.70 [7H, m, $-CH_2CH_2-$ side chain H's (δ ca. 2.52), C^3-methylene H's and one C^4-methylene H.]

δ[DMF-d_7/Pyridine-d_5 (2:1)]: 10.75 (1H, broad s, p-nitrophenylhydrazone NH); 8.35-7.45 (4H, aromatic AA'BB' centred at 7.90); 5.34 (1H, s, H^7); 4.11 (1H, d, J = -16.5 Hz, C^4-methylene H); 3.67 (2H, m, C^2-methylene H's); 2.94 (s, N-CH_3, superimposed upon other signals, including those due to the solvent); 2.35 (2H, m, C^3-methylene H's); signals for the other C^4-methylene proton and the protons of the $-CH_2CH_2-$ of the side chain were partially obscured by the signals due to the solvent and consequently their chemical shifts could not be determined.

Anal. Calcd. for $C_{18}H_{20}N_4O_5S$: C, 53.45; H, 4.98; N, 13.85: S, 7.93%. Equiv. wt. 404.5.

Found: C, 53.16; H, 5.07; N, 13.72; S, 8.05%. Equiv. wt. 390.

5.8 Rate studies

All reactions were carried out in duplicate in a water bath maintained at 27°. The aminochrome (10^{-4} moles) was dissolved in 0.1 M citrate buffer* (5 ml) at the required pH. A solution of the thiol (2×10^{-4} moles) in 0.1 M citrate buffer (5 ml) at the same pH was immediately added. Samples (0.5 ml) were taken from the reaction mixture at various time intervals and were diluted to 50 ml with the corresponding buffer.

For the experiments with epinochrome, however, the concentrations of epinochrome and of N-acetylcysteine were greater by a factor of four and 0.25 M citrate buffer was used. Samples (0.125 ml) were taken from the reaction mixture and were diluted to 50 ml with buffer.

The absorbances of the diluted samples at the λ_{max} of the aminochrome (ca. 490 nm) and at the λ_{max} of the addition product (ca. 360 nm) were measured as quickly as possible with a Beckman DK-2 spectrophotometer. The λ_{max} and extinction coefficient in the visible spectrum of each

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*The rates of decomposition of each of the aminochromes in all the buffer solutions used were found to be sufficiently slow so as not to have a significant effect upon the rates of any of the aminochrome/thiol reactions investigated.

of the aminochromes investigated were found not to change noticeably with respect to the pH of the solution within the range studied. Consequently, for each aminochrome, the same value could be used in the calculation of its concentration at different pH's (see Table 10).

Table 10

λ_{max} Values and extinction coefficients in the visible spectra of aminochromes in 0.1 M citrate buffer

Aminochrome	λ_{max} (nm)	ε
Adrenochrome	489	4170
1-Isopropylnoradrenochrome	495	4300
Adrenochrome methyl ether	491	4270
Epinochrome*	476	4070

*Spectrum in 0.25 M citrate buffer.

The λ_{max} values for the addition products were determined by carrying out a preliminary reaction at each pH and scanning the spectra of the samples diluted at varying time intervals. The λ_{max} values found for the addition products formed in each of the reactions at different pH's are given in Table 9, p. 125. The absorbance for each of

the addition products at its λ_{max} was determined by subtracting the absorbance due to the unreacted aminochrome at the corresponding wavelength from the total absorbance at that wavelength. This correction was only significant in the early stages of the reaction.

The half times of the reactions were determined by plotting the concentration of the aminochrome in the reaction mixture against the reaction time and determining the time at which the aminochrome concentration was equal to one half of its original value (see Fig. 21, p. 193). The concentrations of the aminochromes in the reaction mixture and the corrected absorbances of the addition products in the diluted samples at varying times throughout the reactions are given in Table 11, p. 194. The half times and the reciprocals of the half times for each of the reactions are given in Table 9, p. 125.

Fig. 21

Concentration of adrenochrome vs reaction time for the reaction between adrenochrome and N-acetylcysteine at pH 2.80 (see text for experimental details).

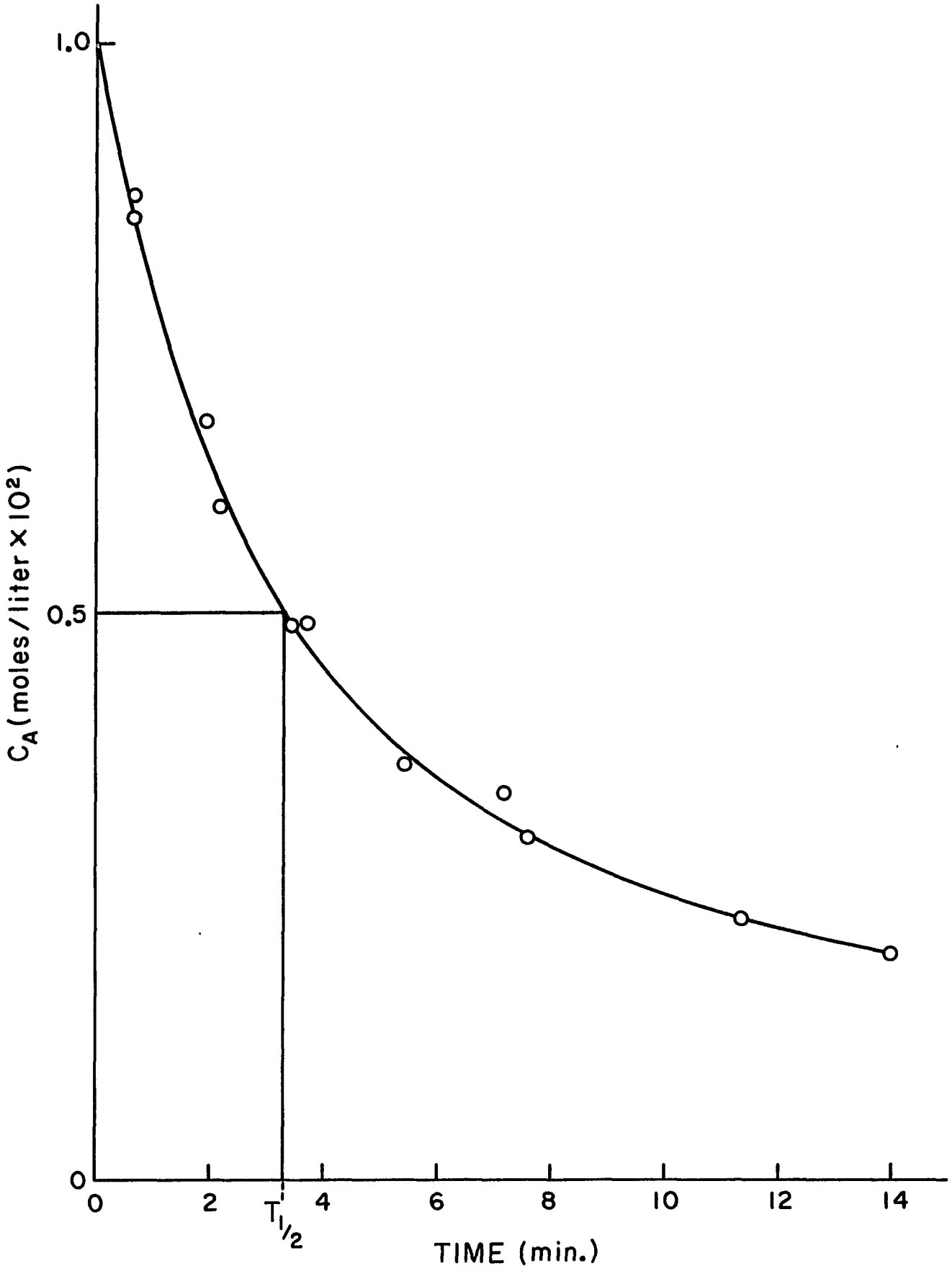
1.0
0.5
0
C_A (moles / liter X 10^2)
0
2
$T_{1/2}$
4
6
8
10
12
14
TIME (min.)

Table 11

Concentrations of aminochromes (in the reaction mixtures) and absorbances of aminochrome/thiol addition products (in the diluted samples) at various time intervals for several aminochrome/thiol reactions*

pH	Reaction time (min)	C_A**	A_{AP}†	Reaction time (min)	C_A	A_{AP}
	a) Adrenochrome/N-acetylcysteine reaction					
2.80	0.67	.867	.082	0.67	.848	.090
	1.92	.669	.189	2.17	.593	.202
	3.42	.490	.241	3.67	.490	.247
	4.92	.407	.242	5.42	.369	.244
	7.17	.343	.240	7.58	.305	.226
	11.33	.231	.188	14.00	.202	.171
	18.83	.205	.165	36.50	.164	.145
3.50	0.67	.910	.059	0.58	.917	.047
	2.17	.762	.151	2.25	.733	.148
	4.08	.598	.218	4.50	.555	.228
	6.50	.469	.282	7.50	.460	.309
	10.75	.352	.317	13.08	.326	.321
	15.58	.279	.317	20.08	.231	.308
	26.50	.181	.264	31.08	.174	.272
4.20	0.58	.948	.059	0.67	.881	.054
	2.25	.819	.168	2.25	.833	.165
	5.42	.650	.327	5.50	.640	.321
	8.75	.495	.400	9.75	.481	.422
	15.92	.371	.492	16.92	.350	.487
	27.50	.274	.506	23.92	.286	.505
	51.08	.210	.476	45.08	.226	.488

Table 11 (Cont'd)

pH	Reaction time (min)	C_A	A_{AP}	Reaction time (min)	C_A	A_{AP}
4.95	0.67	.857	.106	0.75	.905	.107
	2.33	.712	.261	2.67	.707	.299
	3.83	.626	.374	4.83	.583	.427
	6.75	.512	.499	7.75	.483	.531
	10.83	.410	.589	11.67	.410	.599
	18.58	.324	.642	18.75	.355	.647
	37.17	.238	.688	31.17	.286	.702
	71.92	.179	.684	54.00	.248	.729
	148.75	.131	.630	125.50	.152	.666
5.70	0.58	.833	.153	0.58	.786	.153
	2.42	.579	.386	2.00	.683	.365
	3.83	.540	.511	3.58	.571	.498
	5.33	.500	.568	5.58	.483	.571
	7.67	.436	.638	8.00	.443	.648
	11.33	.395	.716	11.33	.376	.687
	20.00	.310	.777	20.00	.331	.761
	47.67	.205	.849	41.08	.286	.854
5.91	0.58	.842	.196	0.58	.840	.172
	1.88	.667	.400	2.07	.650	.415
	3.82	.529	.554	3.83	.529	.549
	6.08	.443	.632	6.58	.443	.625
	10.00	.376	.693	10.53	.390	.725
	22.00	.276	.803	15.72	.333	.788
	42.33	.198	.882	22.42	.286	.825
	78.92	.169	.841	49.58	.200	.874
				100.53	.179	.836

Table 11 (Cont'd)

pH	Reaction time (min)	C_A	A_{AP}	Reaction time (min)	C_A	A_{AP}
	b) Adrenochrome/glutathione reaction					
2.79	0.60	.925	.068	0.82	.893	.069
	2.02	.782	.149	2.27	.772	.167
	3.48	.678	.224	3.75	.651	.226
	5.32	.562	.276	5.60	.545	.281
	8.05	.467	.321	7.63	.482	.315
	12.12	.378	.326	10.83	.397	.326
	15.27	.305	.305	12.83	.349	.320
	19.02	.252	.287	16.75	.303	.307
3.44	0.67	.947	.063	0.55	.966	.050
	2.10	.847	.133	2.08	.862	.140
	3.60	.743	.197	3.57	.748	.200
	6.32	.637	.298	6.32	.617	.296
	9.43	.513	.370	9.57	.516	.372
	12.63	.438	.410	11.80	.465	.398
	17.22	.383	.437	15.83	.397	.431
	23.35	.329	.438	20.68	.339	.432
	31.82	.281	.421	27.80	.271	.411
				41.12	.220	.382
4.10	0.67	.947	.065	0.67	.949	.067
	2.45	.818	.197	2.67	.811	.218
	4.80	.697	.316	4.55	.717	.317
	7.80	.600	.415	7.67	.593	.416
	10.32	.550	.468	11.25	.496	.473
	11.97	.501	.494	14.75	.443	.521
	14.23	.460	.518	18.10	.404	.544
	18.00	.421	.538	27.62	.327	.571
	20.00	.397	.546	45.00	.252	.578
	28.42	.320	.564			
	35.58	.305	.580			
	42.15	.276	.595			

Table 11 (Cont'd)

pH	Reaction time (min)	C_A	A_{AP}	Reaction time (min)	C_A	A_{AP}
4.70	0.67	.903	.104	0.60	.930	.097
	2.25	.751	.265	2.40	.755	.283
	3.97	.654	.379	4.10	.634	.392
	6.10	.581	.473	6.33	.571	.478
	8.32	.518	.519	8.65	.516	.533
	11.00	.475	.561	11.33	.458	.567
	14.65	.441	.606	15.22	.426	.601
	17.75	.404	.621	18.38	.397	.633
	22.70	.375	.643	29.33	.327	.666
	30.58	.329	.681	50.50	.247	.708
	40.67	.278	.694			
5.26	0.63	.886	.143	0.58	.893	.141
	2.18	.685	.350	2.10	.702	.353
	3.75	.605	.462	3.83	.588	.473
	6.17	.523	.542	5.65	.540	.536
	7.88	.475	.569	7.30	.499	.567
	9.50	.462	.606	10.43	.433	.613
	12.77	.421	.650	15.67	.390	.677
	16.45	.375	.681	19.42	.349	.694
	21.25	.334	.712	31.67	.288	.756
	26.65	.312	.730	42.35	.259	.768
	31.87	.276	.745			
	44.08	.249	.774			
5.90	0.67	.826	.233	0.67	.821	.249
	1.87	.673	.444	2.35	.642	.465
	3.33	.596	.530	4.20	.547	.566
	4.87	.533	.584	6.45	.484	.620
	6.53	.501	.627	8.27	.455	.641
	9.97	.400	.682	10.60	.402	.677

Table 11 (Cont'd)

pH	Reaction time (min)	C_A	A_{AP}	Reaction time (min)	C_A	A_{AP}
5.90 (Cont'd)						
	12.40	.392	.711	16.03	.320	.730
	14.47	.373	.734	21.32	.271	.739
	19.30	.305	.751	29.88	.242	.768
	29.60	.257	.776	39.92	.237	.765
				41.62	.220	.763
c) 1-Isopropylnoradrenochrome/N-acetylcysteine reaction						
2.70	0.58	.714	.111	0.62	.721	.127
	1.83	.488	.219	2.00	.488	.249
	3.33	.423	.291	3.58	.419	.279
	4.75	.391	.293	6.00	.358	.282
	6.50	.370	.290	13.25	.307	.253
	12.25	.326	.264	25.25	.251	.194
	21.92	.274	.220			
3.37	0.62	.809	.085	0.65	.833	.092
	2.03	.600	.201	2.12	.591	.205
	3.50	.465	.267	3.58	.463	.264
	5.30	.386	.297	5.53	.384	.301
	8.55	.321	.318	8.83	.333	.319
	13.57	.288	.312	13.88	.267	.301
	21.47	.258	.290	27.23	.230	.266
4.06	0.63	.888	.063	0.67	.856	.067
	1.98	.728	.171	2.08	.733	.178
	3.63	.616	.273	3.48	.593	.246
	5.95	.481	.335	5.43	.486	.324
	8.00	.405	.385	8.38	.379	.382
	12.73	.319	.434	13.60	.295	.428
	20.92	.253	.429	18.70	.274	.438
				23.63	.256	.421

Table 11 (Cont'd)

pH	Reaction time (min)	C_A	A_{AP}	Reaction time (min)	C_A	A_{AP}
4.71	0.60	.898	.087	0.97	.847	.123
	2.03	.765	.235	2.58	.719	.275
	3.58	.670	.345	4.35	.600	.383
	5.50	.556	.449	6.30	.516	.469
	7.50	.477	.506	9.22	.435	.544
	10.75	.405	.563	14.80	.347	.588
	15.67	.330	.588	23.17	.293	.613
	22.25	.295	.606	41.02	.230	.602
	34.97	.244	.615			
5.29	0.67	.851	.153	0.60	.870	.148
	2.27	.677	.344	1.90	.716	.325
	3.90	.551	.477	3.22	.616	.454
	5.55	.477	.545	4.58	.523	.526
	8.30	.423	.616	7.73	.435	.623
	12.60	.353	.677	11.47	.367	.674
	20.92	.295	.723	17.90	.321	.725
	32.62	.244	.759	38.22	.226	.794
5.92	0.60	.828	.219	0.73	.805	.218
	2.17	.614	.470	2.17	.602	.463
	3.83	.502	.600	3.67	.495	.578
	5.57	.453	.665	5.77	.400	.632
	9.47	.365	.730	8.30	.377	.694
	18.23	.270	.812	13.10	.333	.775
	32.35	.219	.862	21.70	.230	.799
	47.12	.153	.880	23.77	.223	.844
				60.43	.149	.903

Table 11 (Cont'd)

pH	Reaction time (min)	C_A	A_{AP}	Reaction time (min)	C_A	A_{AP}
	d) Adrenochrome methyl ether/N-acetylcysteine reaction					
2.74	0.65	.758	.121	0.68	.724	.131
	2.12	.452	.245	2.00	.493	.239
	3.72	.379	.261	3.28	.397	.260
	5.33	.317	.252	4.75	.326	.249
	8.32	.265	.211	9.00	.217	.191
3.38	0.68	.820	.081	0.62	.831	.068
	2.15	.607	.193	2.07	.621	.286
	3.77	.455	.263	3.53	.467	.255
	5.47	.379	.289	5.92	.379	.297
	9.33	.277	.278	8.78	.300	.282
				12.60	.231	.317
4.08	0.68	.939	.066	0.73	.876	.057
	2.07	.763	.157	2.17	.733	.151
	3.72	.628	.232	3.57	.628	.223
	5.55	.529	.290	5.05	.553	.275
	8.07	.438	.335	7.03	.447	.307
	12.28	.356	.372	8.75	.422	.340
	19.00	.281	.355	11.82	.375	.370
				19.05	.283	.351
4.70	0.75	.895	.084	0.67	.885	.077
	2.25	.742	.179	2.33	.749	.198
	3.72	.658	.259	3.97	.658	.281
	5.43	.569	.315	6.00	.574	.337
	7.00	.515	.247	8.85	.478	.368
	10.03	.473	.397	14.33	.386	.397
	13.77	.417	.415	23.70	.326	.410
	19.12	.384	.433			
	24.80	.351	.432			

Table 11 (Cont'd)

pH	Reaction time (min)	C_A	A_{AP}	Reaction time (min)	C_A	A_{AP}
5.30	0.70	.844	.096	0.63	.878	.097
	2.22	.742	.248	2.03	.752	.226
	4.07	.645	.332	3.67	.645	.310
	7.22	.550	.398	6.23	.577	.374
	10.90	.504	.427	9.50	.523	.413
	18.15	.423	.451	13.73	.457	.426
	25.08	.384	.477	15.73	.438	.438
	33.70	.333	.485	20.98	.404	.458
				30.97	.348	.479
5.93	0.67	.833	.127	0.72	.823	.148
	2.17	.688	.285	2.17	.709	.289
	3.70	.649	.354	3.60	.627	.354
	5.18	.608	.403	5.10	.608	.399
	7.88	.542	.420	7.93	.540	.423
	11.75	.494	.452	12.00	.475	.451
	19.62	.404	.492	17.10	.431	.488
	27.83	.356	.529	23.82	.383	.522
	34.50	.329	.553	32.90	.305	.537
				40.27	.298	.561
	e) Epinochrome/N-acetylcysteine reaction					
2.71	1.98	3.47	.094	1.97	3.45	.086
	3.90	3.03	.152	5.83	2.79	.189
	6.53	2.64	.204	11.50	2.10	.265
	10.17	2.24	.264	16.08	1.73	.295
	13.40	1.99	.298	22.50	1.37	.297
	18.32	1.59	.315	27.92	1.14	.285
	20.33	1.51	.322			
	25.65	1.21	.313			
	36.58	0.93	.292			

Table 11 (Cont'd)

pH	Reaction time (min)	C_A	A_{AP}	Reaction time (min)	C_A	A_{AP}
4.28	1.87	3.52	.122	2.17	3.44	.132
	3.32	3.27	.209	4.22	3.16	.248
	4.98	2.86	.267	6.35	2.76	.316
	6.75	2.70	.340	8.55	2.39	.371
	8.77	2.35	.374	11.05	2.00	.399
	12.05	1.92	.412	15.58	1.66	.437
	16.62	1.77	.461	22.80	1.52	.459
	25.85	1.26	.426	30.07	1.20	.427
5.42	2.15	3.20	.301	1.72	3.11	.239
	3.90	2.51	.417	3.65	2.58	.435
	5.48	2.26	.487	5.97	2.14	.503
	7.28	1.97	.526	8.30	1.95	.537
	10.58	1.89	.556	11.92	1.87	.552
	14.02	1.74	.553	19.47	1.63	.529
	19.45	1.49	.509	28.05	1.52	.506

*Initial aminochrome concentration = 10^{-2} M; initial thiol concentration = 2×10^{-2} M for all reactions except the epinochrome/N-acetylcysteine reactions, where initial concentration of epinochrome = 4×10^{-2} M and initial concentration of N-acetylcysteine = 8×10^{-2} M.

**C_A = Concentration of the aminochrome in the reaction mixture (in moles/$\ell \times 10^2$).

†A_{AP} = Corrected absorbance of the diluted sample at the λ_{max} (see Table 9, p. 125) of the addition product.

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