LUMINESCENCE STUDIES ON PHYSOSTIGMINE, RUBRESERINE AND ADRENOCHROME

B. SAVORY and J. H. TURNBULL

Department of Chemistry, Royal Military College of Science, Shrivenham, Wilts. (Gt. Britain)

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Summary

Fluorescence and phosphorescence spectra of physostigmine (eserine) are determined and compared with those reported for the phenyl carbamate pesticides Matacil and Zectran. The conversion of physostigmine to rubreserine is studied in the presence of nucleophilic reagents and inhibitors. Spectral characteristics of rubreserine and adrenochrome are compared with particular reference to the adrenolutin reaction. The fluorometric estimation of physostigmine is described.

1. Introduction

The alkaloid physostigmine (eserine) which occurs in the Calabar bean (*Physostigma venenosum*) is well known as an inhibitor of acetylcholinesterase [1, 2]. The drug has found application as a miotic agent in *myasthenia gravis*, and in anticholinesterase therapy. Physostigmine also appears to facilitate memory recall, and as such shows promise in the treatment of Alzheimer's disease [3].

The molecular structure of physostigmine (I) (Fig. 1) is derived from the *p*-amino-*N*-methylcarbamate grouping which is present in the pesticides Matacil (II, R ≡ H) and Zectran (II, R ≡ Me) [4]. This grouping determines the electronic excited state parameters of physostigmine as well as the intrinsic biological activity of the molecule.

Physostigmine is unique among the carbamates in its susceptibility to oxidative hydrolysis to give the scarlet derivative rubreserine (III) [5]. This reaction is a potential source of instability in pharmaceutical solutions of physostigmine. Rubreserine itself possesses a striking resemblance to the red compound adrenochrome (V) which is a primary oxidation product of adrenaline [6].

The estimation of physostigmine in biological fluids is currently a problem [7]. It is feasible to perform spectrophotometric determination utilizing the rubreserine conversion but this method lacks sensitivity.

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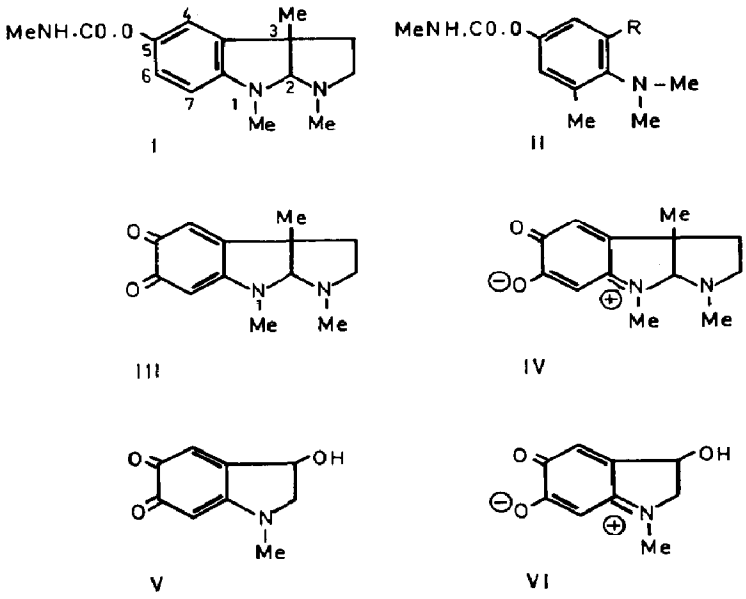


Fig. 1. Chemical structures of physostigmine (I), Matacil and Zectran (II), rubreserine (III and IV) and adrenochrome (V and VI).

With these points in mind we have undertaken a study of the luminescence spectra of physostigmine, rubreserine and adrenochrome. The data obtained provide a basis for the fluorometric estimation of physostigmine, and allow a comparison of the spectral properties of rubreserine and adrenochrome in solution to be made.

2. Experimental details and materials

The spectroscopic techniques and corrections were as described in our earlier papers [8]. Physostigmine was supplied as a crystalline free base by BDH. Adrenochrome (free base) was obtained from Sigma, London. Rubreserine was prepared by the method of Robinson [9].

3. Results

Spectral measurements were made in neutral ethanol and HCl–ethanol (pH 2). Certain additional spectra were recorded in dimethyl sulphoxide (DMSO).

3.1. Absorption spectra

3.1.1. Physostigmine

The absorption spectrum of physostigmine (I) in neutral ethanol (Fig. 2 and Table 1) shows well-defined peaks at λ_{max} values of 253 and 313 nm.

TABLE 1

Absorption			*Fluorescence (298 K)*		*Fluorescence (77 K)*		*Phosphorescence (77 K)*		
λ_{max} (nm)	ϵ (dm^3 mol^{-1} cm^{-1})	λ_{ex} (nm)	λ_{max} (nm)	ϕ_f	λ_{max} (nm)	ϕ_f	λ_{max} (nm)	τ_p (s)	ϕ_p
Physostigmine									
253	11902	253	364	0.17	348	7.3×10^{-2}	425	3.7	0.10
313	2537	313	363		348		425		
Physostigmine (acidic)									
246									
305		313	342						
Physostigmine (alkaline)									
298	7194	298	364	6.0×10^{-3}	349		427		
476	3353	476	—		—		—		
Matacil									
		248	357		341		459	1.12	
		254	366						

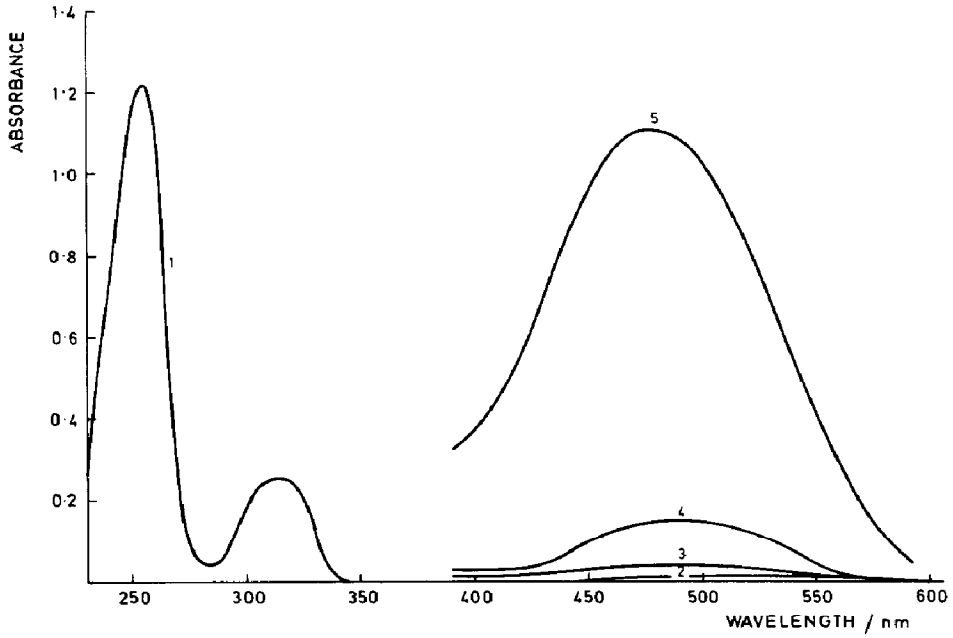


Fig. 2. Absorption spectrum of physostigmine. Curve 1, in neutral ethanolic solution and curves 2 - 5, at increased concentrations after the addition of M/50 KOH: curve 2, 5 min; curve 3, 10 min; curve 4, 15 min; curve 5, 70 min.

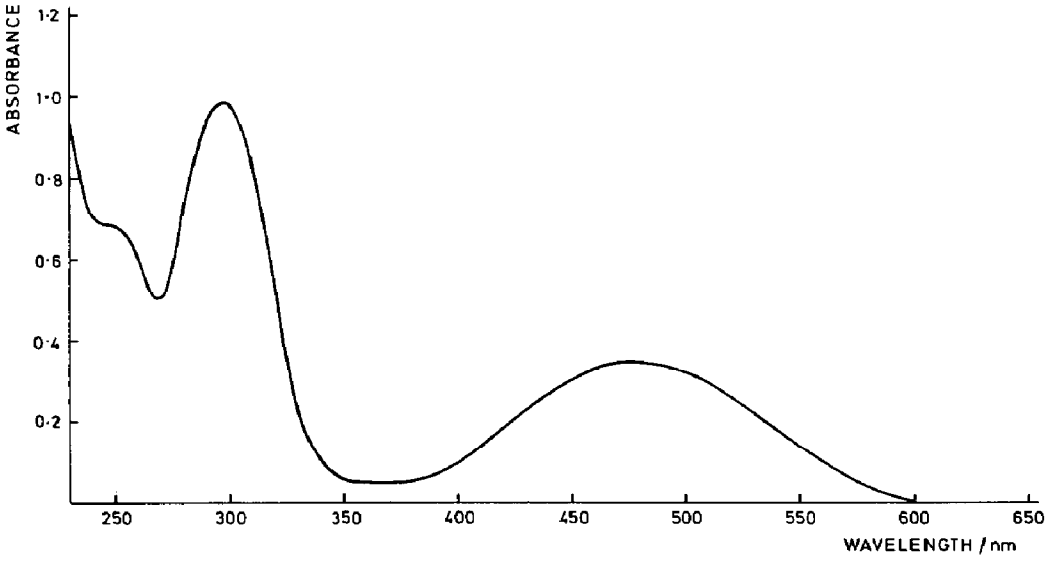


Fig. 3. Absorption spectrum of rubreserine in ethanolic solution.

This spectrum is shifted somewhat to shorter wavelengths in acidic (HCl) ethanol [10].

In aqueous alkali (pH 12) solutions of physostigmine a cherry-red colour rapidly develops as a result of the formation of rubreserine (III and

IV) (Fig. 1). The spectra of these solutions (Figs. 2 and 3) display absorption peaks at λ_{max} values of 253, 312 and 476 nm [5]. The emergence of the 476 nm peak, from which the red colour originates, provides a convenient means of following the rubreserine conversion (Fig. 2). The reaction takes place in the dark; it is suppressed in the absence of oxygen or in the presence of ascorbic acid.

3.1.2. Adrenochrome

The absorption spectrum of adrenochrome (V and VI, Fig. 1) (*cf.* ref. 5) in neutral ethanol (Fig. 4 and Table 2) shows peaks at λ_{max} values of 220, 302 and 485 nm. This spectrum is virtually unchanged on acidification, but is markedly altered in basic solution. When ethanolic or aqueous solutions of adrenochrome were made alkaline (pH 12) by adding sodium hydroxide, the red colour rapidly changed to yellow and a transient green fluorescence developed. These solutions were unstable and quickly became brown in colour. The reactions involved are due to the well-known conversion of adrenochrome to adrenolutin (VII) (Fig. 5) and its subsequent oxidation products [11, 12]. In order to record the absorption spectrum of adrenolutin itself, it is necessary to prevent destructive oxidation of the adrenolutin molecule. This can be achieved by excluding oxygen or by adding ascorbic acid [11]. The spectrum measured at pH 12 in the presence of ascorbic acid revealed weak absorption in the blue region (λ_{max} = 396 nm) and this is attributable to the presence of the yellow adrenolutin [11].

Adrenochrome solutions in DMSO showed the characteristic absorption bands of adrenochrome at λ_{max} values of 305 and 490 nm, the latter band being shifted slightly to the red. This spectrum was changed on the addition of the base potassium *tert*-butoxide, with some weakening of the band at 490 nm and the development of a shoulder at 365 nm (Fig. 6).

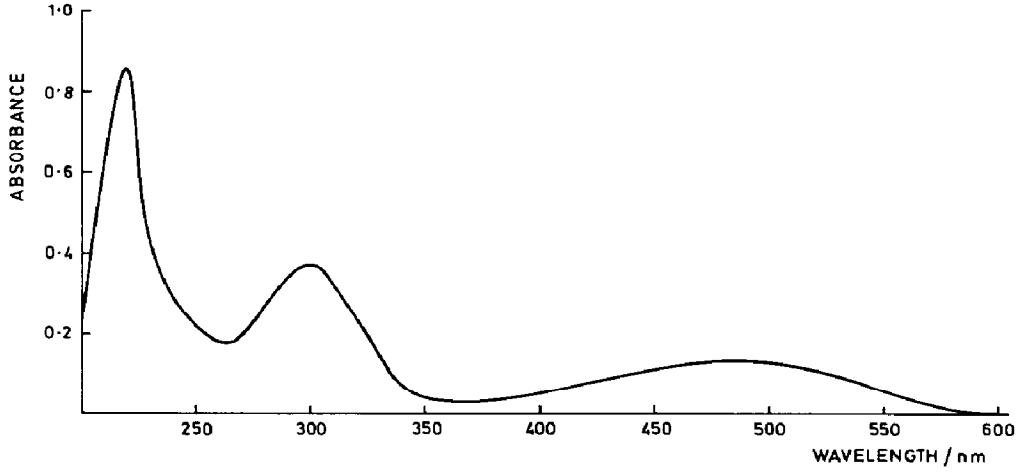


Fig. 4. Absorption spectrum of adrenochrome in neutral ethanolic solution.

TABLE 2

Absorption			*Fluorescence (298 K)*		*Fluorescence (77 K)*		*Phosphorescence (77 K)*		
λ_{max} (nm)	ϵ (dm^3 mol^{-1} cm^{-1})	λ_{ex} (nm)	λ_{max} (nm)	ϕ_f	λ_{max} (nm)	ϕ_f	λ_{max} (nm)	τ_p (s)	ϕ_p
Adrenochrome (neutral)									
220	22861								
302	13815	302	339	2.5×10^{-3}	341, 476	—	—	—	—
485	5487								
Adrenochrome (acidic)									
219	18048								
300	14479	302	340	1.3×10^{-2}	330	4.3×10^{-2}	—	—	—
462	3919								
Adrenochrome (alkaline)									
224 (shoulder)	16578								
268	8957								
318	16578	302	—		395, 489	Very weak	442		Very weak
380	7487								
Adrenochrome in DMSO–t-BuOH									
304		302	341, 485						
365		302	341, 555, 611						
490		365	555, 611						

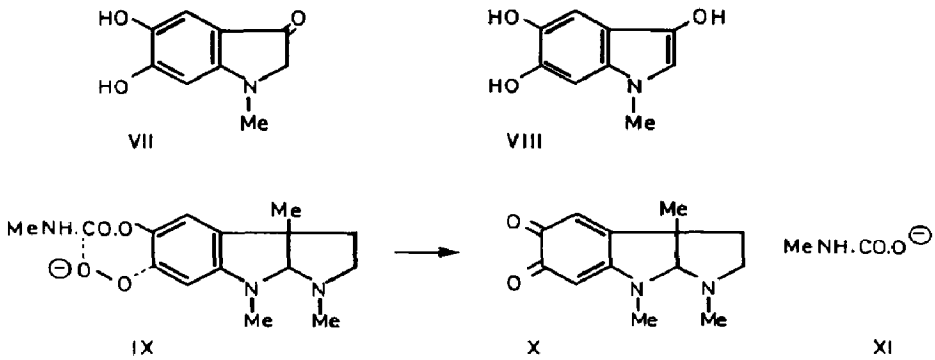


Fig. 5. Chemical structures of adrenolutin (**VII**), *N*-methyl-trihydroxyindole (**VIII**), the peroxy transition state (**IX**), rubreserine (**X**) and *N*-methyl carbamate anion (**XI**).

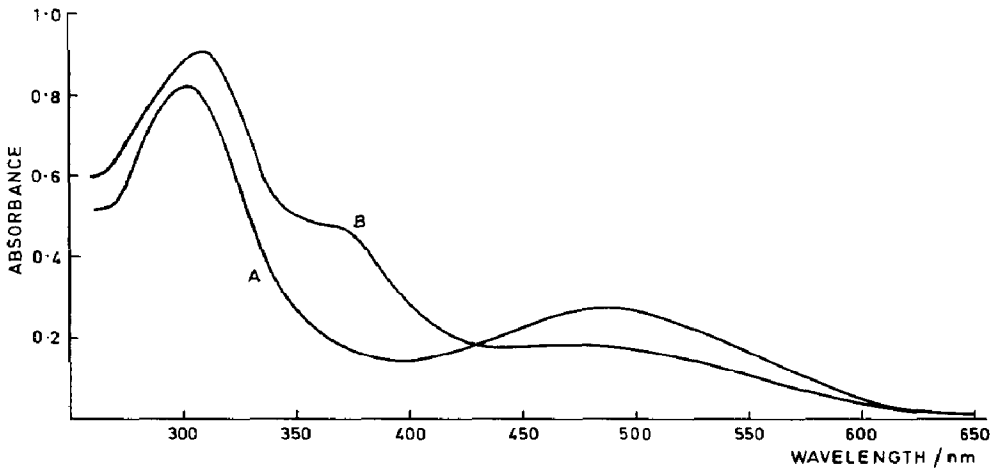


Fig. 6. Absorption spectrum of adrenochrome in DMSO (curve A) and in DMSO after the addition of potassium *tert*-butoxide (curve B).

3.2. Emission spectra

3.2.1. Physostigmine

The fluorescence spectrum of physostigmine in neutral ethanol reveals strong emission at λ_{max} = 364 nm (Fig. 7, Table 1) with a quantum yield ϕ_f = 0.17. The spectrum of physostigmine in acidic (HCl) ethanol is essentially the same. At 77 K the fluorescence peak is weaker and is shifted (normally) towards the blue.

The phosphorescence spectrum of physostigmine (Fig. 7) has a strong emission band (λ_{max} = 425 nm) with a triplet lifetime of 3.7 s (Table 1).

These emission parameters are characteristic of the π–π^* transitions of the *p*-aminophenyl esters related to the carbamates Matacil (Table 1) and Zectran [4]. The fluorometric estimation of physostigmine in solution is easily achieved by measuring the singlet emission intensity at 364 nm (Fig. 7). This method is effective down to physostigmine levels of 0.1 μg ml^{-1} (Fig. 8).

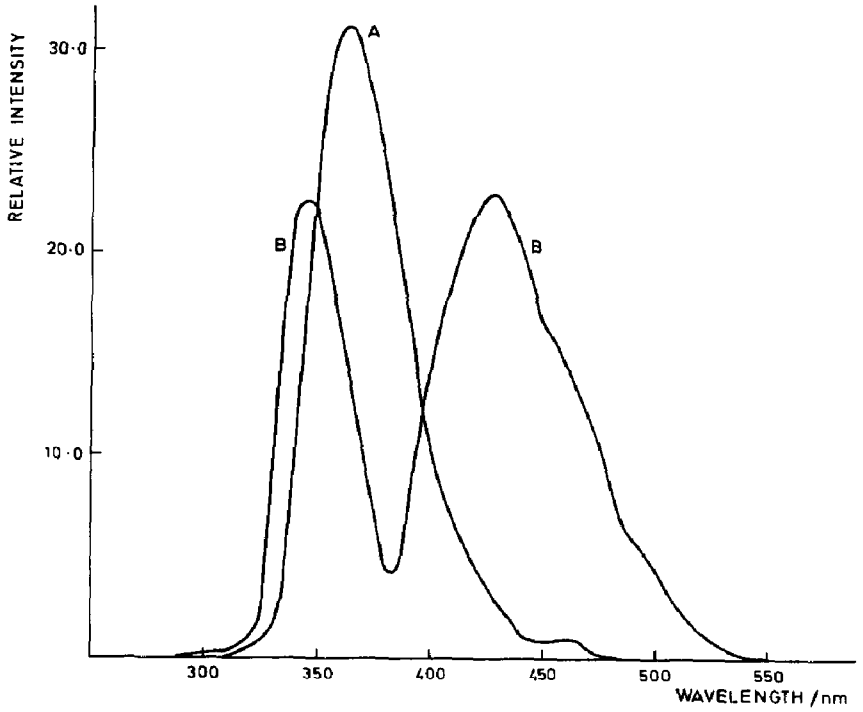


Fig. 7. Emission spectra of physostigmine in neutral ethanolic solution at 298 K (curve A) and at 77 K (curve B).

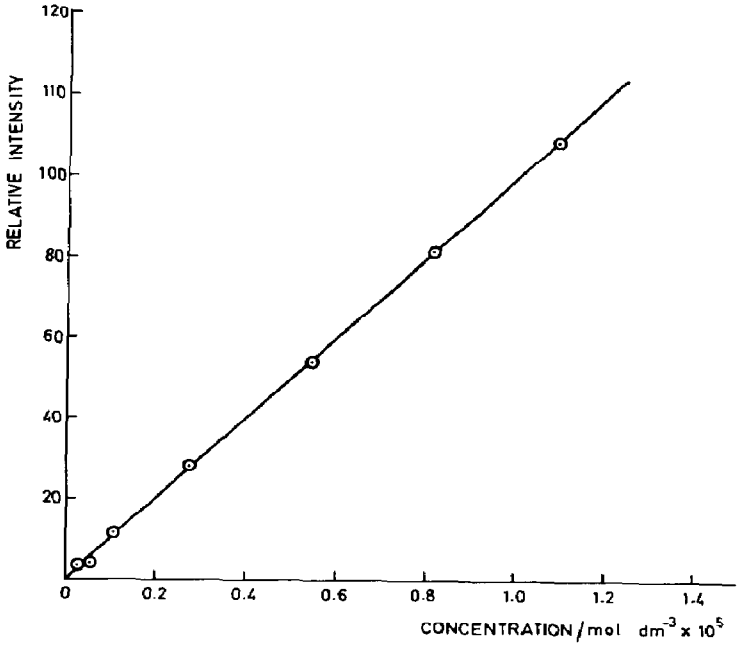


Fig. 8. Fluorescence intensity of physostigmine concentration in ethanol at 298 K.

3.2.2. Adrenochrome

The fluorescence spectrum of adrenochrome in neutral ethanol reveals a weak emission at $\lambda_{max} = 340$ nm ($\phi_f = 2.5 \times 10^{-3}$) with a broad band at about 490 nm (Fig. 9, Table 2). The latter corresponds to the faint blue–green fluorescence observable from the solution under a BTL 3560 UV lamp. In HCl-acidified ethanol, the peak at 340 nm increases in intensity ($\phi_f = 4.3 \times 10^{-2}$) and the visible emission disappears (Fig. 10). Similar results were found in aqueous solution.

Low temperature (77 K) emission spectra (Table 2) revealed similar fluorescence bands at very low quantum yields. No definitive phosphorescence was detectable.

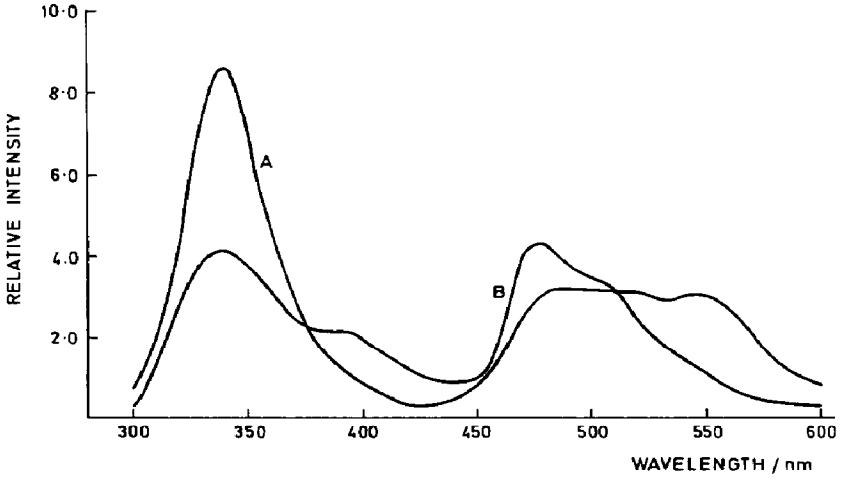


Fig. 9. Emission spectra of adrenochrome in neutral ethanolic solution at 298 K (curve A) and 77 K (curve B).

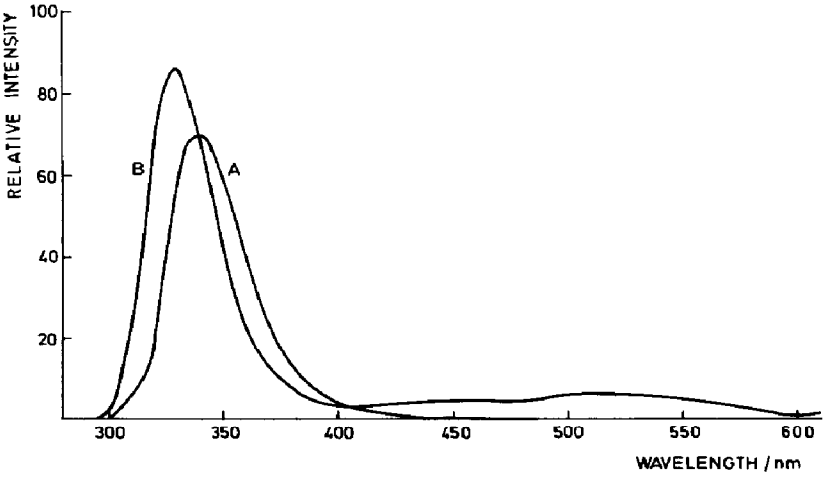
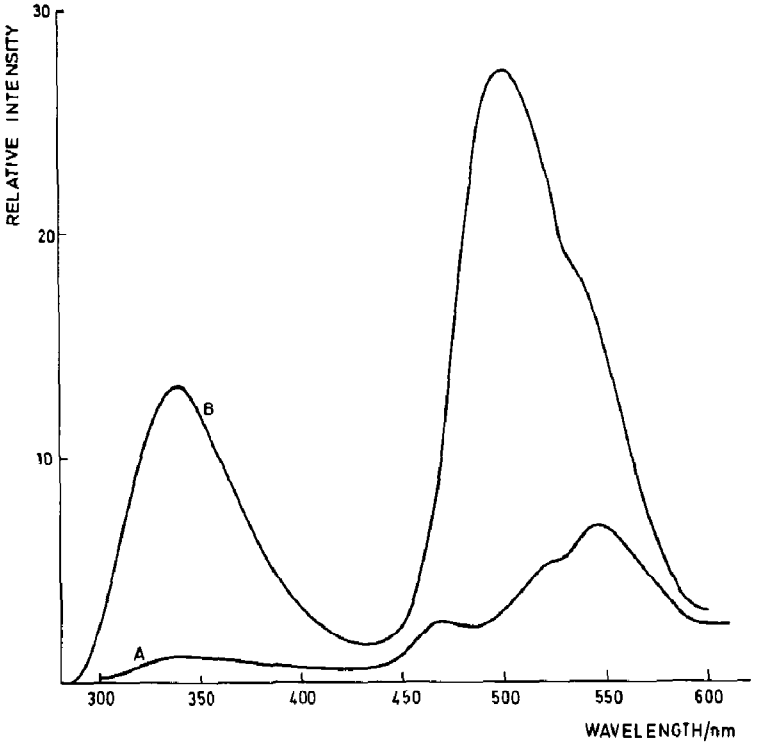


Fig. 10. Emission spectra of adrenochrome in acidic ethanolic solution at 298 K (curve A) and at 77 K (curve B).



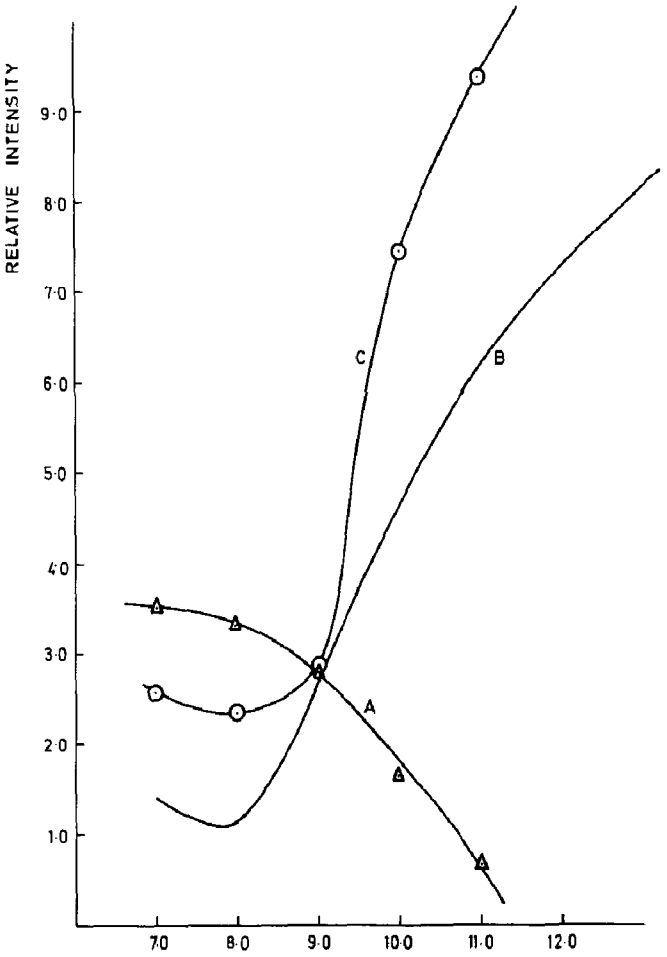


Fig. 11. Emission spectra of aqueous adrenochrome at pH 11, stabilized with ascorbic acid (curve A) and after the addition of glacial acetic acid (curve B).

Fig. 12. Fluorescence intensity *vs.* initial pH of buffered aqueous solutions of adrenochrome stabilized with ascorbic acid for bands at 340 nm (curve A), 550 nm (curve B) and after the addition of glacial acetic acid at 500 nm (curve C).

In alkaline ethanol (NaOH, pH 12) no fluorescence was evident, as a result of the rapid oxidative degradation of the adrenochrome molecule. With ascorbic acid present to inhibit oxidation [13], alkaline solutions of adrenochrome developed the yellow colour of adrenolutin and displayed a characteristic green fluorescence (λ_{max} = 500 nm) (Fig. 11) after acidification with acetic acid. The intensity of the fluorescence peak increased with the initial pH of the buffered alkaline solutions over the pH range 7 - 12. The pH dependence of the fluorescence spectra of these solutions is shown in Fig. 12. It is evident that the adrenochrome fluorescence band (λ_{max} = 340 nm) disappears as the initial pH of the solution is increased to pH 11.

Solutions of adrenochrome in DMSO showed characteristic adrenochrome emission bands in the λ_{max} regions of 340 and 490 nm (Fig. 13). The spectrum was markedly changed on the addition of potassium *tert*-butoxide. The fluorescence bands at 340 and 490 nm disappeared and a prominent emission peak appeared at λ_{max} = 555 nm with a distinct shoulder at 611 nm. Under the 3560 UV lamp the solution displayed a weak orange fluorescence consistent with this longer wavelength emission.

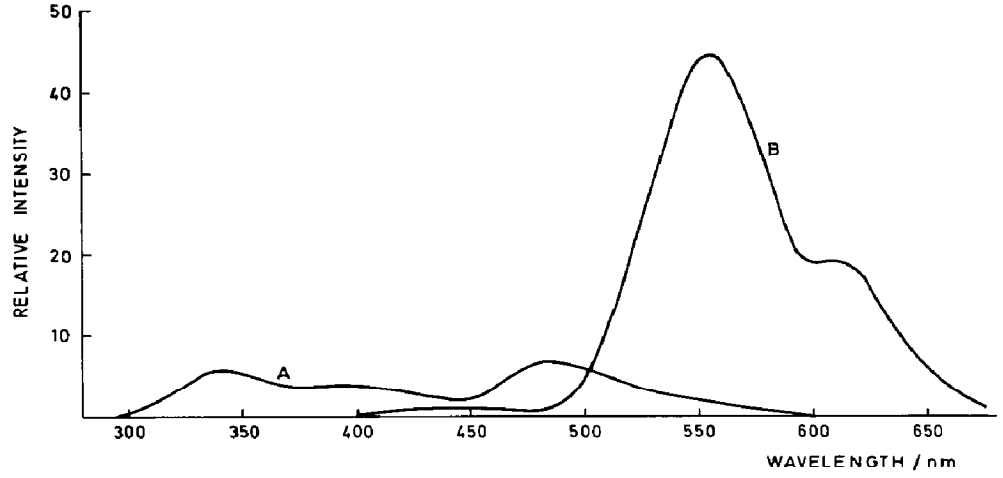


Fig. 13. Emission spectra at 298 K of adrenochrome in DMSO (curve A) and after the addition of potassium *tert*-butoxide (curve B).

4. Discussion

The conversion of physostigmine to rubreserine [7] proceeds very rapidly in solutions of potassium *tert*-butoxide in DMSO. This is significant in view of the remarkable ability of DMSO to act as an oxygen carrier in the perhydroxylation of indoles [14] (*cf.* refs. 15 - 18) and other unsaturated systems [19 - 22]. The reactive intermediate appears to be the oxygen radical anion ($O_2^{\cdot-}$), which arises from electron transfer to molecular oxygen from the butoxide anion with spin relaxation. In the present case we suggest

that the oxidative hydrolysis of the physostigmine molecule is the result of the production of the peroxy transition state (IX) and finally rubreserine (X) and carbamate (XI) (Fig. 5) in the attack by the nucleophilic oxygen radical anion at the ester carbonyl. The primary role of the reaction medium in this process is to generate oxygen radical anions from the molecular oxygen present in the solution.

It has been clearly shown in the comprehensive studies of Russell and coworkers that the effectiveness of oxygen in promoting oxidation reactions in basic media increases with the nucleophilic strength of the base anion. This principle seems to apply to the rubreserine reaction, as has been illustrated in our experimental data (Fig. 14). The rates of rubreserine formation in basic ethanolic solution measured spectrophotometrically at λ_{max} = 490 nm are plotted for the three basic anions OH^-, $^-OC(Me)_3$ and imidazolyl$^-$. The rate of reaction increases in the order hydroxyl < *tert*-butoxyl < imidazolyl. The high reactivity of the imidazolyl anion is consistent with the strongly nucleophilic character of the imidazole nucleus.

The close similarity between the rubreserine and adrenochrome molecules is evident from a comparison of their structural formulae (III and IV) (Fig. 1). This is reflected in the absorption spectra of the two compounds (Figs. 3 and 4) which reveal identical bands arising from the common red *ortho*-quinonoid chromophore. The structures of these aminochromes may be depicted by the dipolar forms IV and VI (Fig. 1) which probably contribute significantly in the excited state. In the ground state, the quinonoid

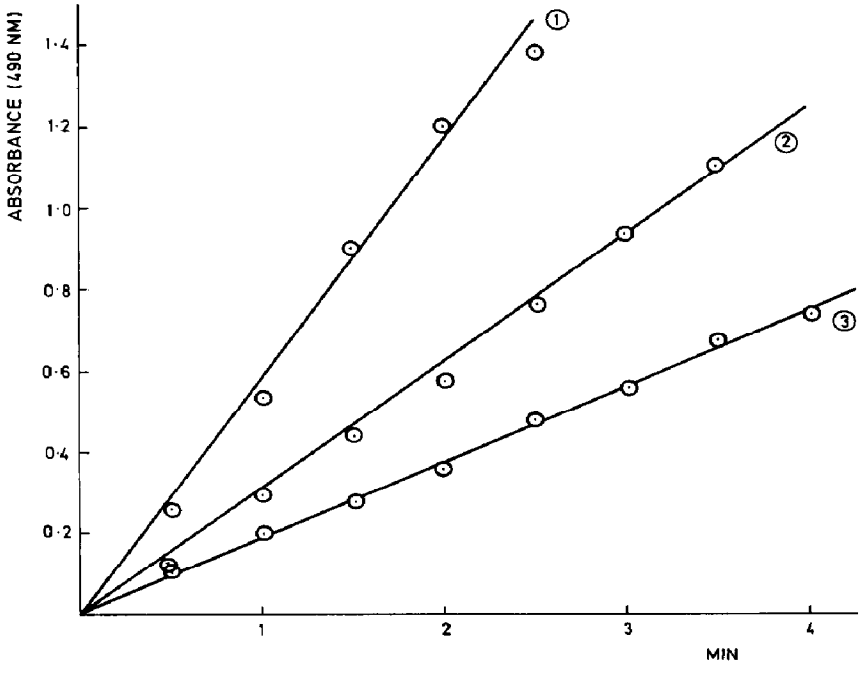


Fig. 14. Rates of rubreserine formation in aqueous ethanol in the presence of M/50 Na–imidazole (curve 1), potassium-*tert*-butoxide (curve 2) and KOH (curve 3); concentration of physostigmine, M/50.

character of these molecules is seen in their susceptibility to reversible reduction by reagents such as hydrogen sulphide, sulphur dioxide and ascorbic acid [14, 23 - 26] to give colourless leuco compounds.

The significant structural difference between rubreserine and adrenochrome is the presence in the former of a tetrahydropyrrolidindole ring system with an angular methyl group at position 3. As a result there is no available hydrogen at position 3 for internal rearrangement. Adrenochrome, however, possesses the requisite 3-hydrogen atom. Consequently adrenochrome can rearrange by internal hydrogen transfer to give the trihydroxy indole (VIII, Fig. 5) [27, 28]. This isomerization is promoted by making the solution alkaline, when subsequent acidification with acetic acid yields the highly fluorescent adrenolutin (VII). Rubreserine cannot rearrange in this way, and remains unchanged under such conditions.

The spectral characteristics of adrenolutin, manifest in the yellow colour (λ_{max} = 396 nm) and intense green fluorescence (λ_{max} = 500 nm) of its solutions, are not easily reconciled with the classical structure VII. We are inclined to attribute the green emission to a contributing dipolar excited state of the molecule represented by the indolinium cation (Fig. 15) (XII). A similar green fluorescence (λ_{max} = 520 nm) is associated with protonated 5-oxyindoles in their cationic form [29, 30].

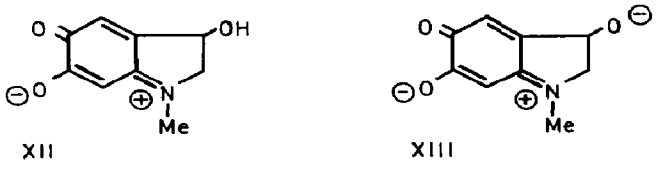


Fig. 15. Chemical structures of adrenolutin zwitterion (XII) and the anionic form (XIII).

Owing to the presence of identical absorption bands (λ_{max} = 476 nm) in the visible region of the spectra of rubreserine and adrenochrome, which is associated with the red colour of these compounds, we decided to search for complementary fluorescence bands in the orange region. In the event no emission was detectable. It is evident that the high dipolar character of the excited states involved favours efficient radiationless deactivation of the singlet levels in solution.

The yellow fluorescence (λ_{max} = 555 nm) which is observable in the basic red solutions of adrenochrome in DMSO–potassium *tert*-butoxide appears to arise from the dissociated anionic form of the molecule (XIII). Rubreserine, which lacks the ionizable hydroxyl group at position 3, shows no fluorescence in this medium.

5. Conclusions

The absorption and emission spectra of physostigmine possess characteristics similar to the spectra of the phenyl carbamates Matacil and Zectran. It is inferred that similar excited states are involved.

Physostigmine differs from Matacil and Zectran in its susceptibility to oxidative hydrolysis. In basic media this process converts physostigmine into the red quinonoid product rubreserine through the absorption of oxygen. Rubreserine resembles adrenochrome in its essential spectroscopic characteristics, but unlike adrenochrome it does not rearrange in alkaline solution. Rubreserine shows only feeble UV fluorescence and affords no visible fluorescent emission in solutions of DMSO–*tert*-butoxide. These differences are ascribed to the absence of the hydrogen atom at position 3.

In view of the non-fluorescent character of rubreserine, fluorometric analysis of physostigmine cannot be performed by using its conversion to the aminochrome. However, the fluorescence of physostigmine itself in the UV region can be utilized to give a direct estimation. Our results show that this is achievable down to levels of about 0.1 $\mu g\ ml^{-1}$.

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