FLUORESCENCE PROPERTIES OF SOME ISOQUINOLINE ALKALOIDS

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The fluorescence properties of 28 isoquinoline alkaloids have been investigated. In most of them the chromophore responsible for fluorescence was the benzene ring with electron-donor substituents containing oxygen. The long-wave excitation peak practically coincides with the long-wave absorption peak of these substances, covering the region from 284 to 293 nm, the maximum emission being in the range 320—332 nm. With alkaloids having a number of conjugated rings, both excitation and emission were observed at higher wavelengths. Only protoberberine alkaloids behaved as hydrophobic probes, *i.e.* transfer of these compounds into a less polar medium produced a marked hypsochromic shift and a higher intensity of emission. The effect of polarity on the behaviour of tetrahydroprotoberberines, protopines, pavinanes, aporphines and benzophenanthridines was not so pronounced. Changes of pH manifested themselves most markedly in compounds with dissociable hydroxyl groups; the majority of phenolates did not fluoresce. The phenol group pK values of these compounds in the excited state were lower than in the ground state (which ranged between 8·4 and 10·4). The relations between the apparent pK, determined from fluorescence data, and the pK's of these compounds in the ground and the excited states are discussed.

Some isoquinoline alkaloids are capable of interaction with various biologically active macromolecules. Protoberberine alkaloids were found to bind to nucleic acids[1], biological membranes and their models[2], and to some soluble proteins (*e.g.* alcohol dehydrogenase[3]). Benzophenanthridines can interact with some biopolymers too (*e.g.* aminotransferases[4]). Aporphines bind to alcohol dehydrogenase[5] and glutamate dehydrogenase[6]. Acetylcholinesterase and butyrylcholinesterase pertain to enzymes whose activity is substantially affected by the presence of some alkaloids of the isoquinoline type[7]. ATPase[8], monoamine oxidase[9], adenylate cyclase[8] and phosphodiesterase[10] are also inhibited by some isoquinoline alkaloids. Interaction of certain alkaloids of this group with receptor proteins for catecholamines was described too[8]. The interactions were successfully studied by the fluorescence methods. In the binding of protoberberine alkaloids to biopolymers the fluorescence properties (especially intensity of fluorescence) change markedly[3]. The binding of aporphines to alcohol dehydrogenase[5] and glutamate dehydrogenase[6] was followed by polarization of fluorescence.

The objective of the present paper was to describe the fluorescence properties of compounds representing various subgroups of isoquinoline alkaloids. The effects of polarity and pH of the medium on the fluorescent behaviour of these alkaloids have also been studied. The results of this work might help in choosing a fluorescence technique suited for studying the binding of isoquinoline alkaloids to biological systems.

EXPERIMENTAL

Materials

Cyclanoline iodide, (−)-α-canadine methiodide, (−)-α-stylopine methiodide, escholidine perchlorate, mecambridine, corypalmine, protopine, protopine methiodide, allocryptopine, hunnemanine, sanguinarine chloride, chelerythrine chloride, chelidonine, homochelidonine, bulbocapnine, aporheine, argemonine, californidine perchlorate, platycerine, bisnorargemonine, norargemonine, eschscholtzine and dihydrosanguinarine were isolated at the Department of Medical Chemistry and Biochemistry, Medical Faculty, Purkyně University. Jatrorrhizine chloride, berberrubine chloride and coralyne chloride (prepared by acetylation of papaverine[18]) were supplied by Dr S. Pavelka, Research Institute of Veterinary Medicine, Brno. Compound *I* was a gift from Dr J. Ulrichová, Medical Faculty, Olomouc. Berberine sulphate was a product of the firm E. Gurr, Ltd. England. The structures of all the compounds are given in Table I.

Methods

Optical measurements were carried out at 25°C in a universal Davies buffer (pH 2—12). For the region pH 1—2 we used Clark and Lubs's buffer; for measurements to be performed at even lower pH's solutions of hydrochloric acid were used.

The absorption spectra and absorbances were measured employing a spectrophotometer Cary 118/Varian, solutions of $3 \cdot 10^{-5}$ mol/l and 1·5 ml quartz cells, optical path 1 cm. Uncorrected excitation and emission spectra, as well as fluorescence intensities were obtained with a standard spectrofluorimeter Aminco-Bowman, equipped with a high-pressure Xe discharge lamp and a photomultiplier R446S. To measure excitation or emission we used 1 mm apertures and solutions of $1 \cdot 10^{-5}$ mol/l in 2 ml quartz cells or 0·2 ml microcells. Phosphorescence was measured in the same apparatus equipped with a phosphoriscope and a Dewar vessel with liquid nitrogen. The phosphorescence data were obtained using $1 \cdot 10^{-5}$ mol/l solutions in ethanol and a quartz capillary. Polarization of fluorescence and phosphorescence was measured in an Aminco-Bowman apparatus with an adapter for polarization. The values of polarization (p) were calculated from the equation (refs[5,6]):

$$p = \frac{F_{VV} - (F_{HV}/F_{HH}) \cdot F_{VH}}{F_{VV} + (F_{HV}/F_{HH}) \cdot F_{VH}}, \qquad (1)$$

where F designates the intensity of fluorescence, V and H the vertical or horizontal positions of the excitation and the emission polarizers, respectively.

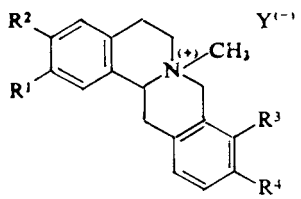
Approximate pH values from absorption and fluorescence data (pK_{abs}, pK_{fl}) were obtained as inflexion points on the plots of absorbance or fluorescence intensity *vs* pH, more accurate

TABLE I
Structures of the alkaloids

Alkaloid	R^1	R^2	R^3	R^4	R^5	R^6	R^7	Type
			Tetrahydroprotoberberines					
Cyclanoline iodide	OH	OCH_3	OH	OCH_3	—	—	—	*a*
Stylopine methiodide	O—CH_2—O		O—CH_2—O		—	—	—	*a*
Canadine methiodide	O—CH_2—O		OCH_3	OCH_3	—	—	—	*a*
Escholidine perchlorate	O—CH_2—O		OCH_3	OH	—	—	—	*a*
Mecambridine	OCH_3	O—CH_2—O		H	OCH_3	OCH_3	CH_2OH	*b*
Corypalmine	H	OCH_3	OH	OCH_3	OCH_3	H	H	*b*
			Protoberberines					
Berberine sulphate	O—CH_2—O		OCH_3	OCH_3	—	—	—	*c*
Berberrubine chloride	O—CH_2—O		OH	OCH_3	—	—	—	*c*
Jatrorrhizine chloride	OCH_3	OH	OCH_3	OCH_3	—	—	—	*c*
			Dibenzoquinolizine					
Coralyne chloride	OCH_3	OCH_3	CH_3	OCH_3	OCH_3	—	—	*d*
			Protopines					
Protopine	O—CH_2—O		O—CH_2—O		—	—	—	*e*
Protopine methiodide	O—CH_2—O		O—CH_2—O		—	—	—	*f*
Allocryptopine	O—CH_2—O		OCH_3	OCH_3	—	—	—	*e*
Hunnemanine	O—CH_2—O		OH	OCH_3	—	—	—	*e*

TABLE I
(*Continued*)

Alkaloid	R^1	R^2	R^3	R^4	R^5	R^6	R^7	Type
				Pavinanes				
Argemonine	OCH_3	OCH_3	H	OCH_3	OCH_3	—	—	*g*
Platycerine	OCH_3	OCH_3	OH	OCH_3	H	—	—	*g*
Bisnorargemonine	OH	OCH_3	H	OCH_3	OH	—	—	*g*
Norargemonine	OH	OCH_3	H	OCH_3	OCH_3	—	—	*g*
Eschscholtzine	$O—CH_2—O$		H	$O—CH_2—O$		—	—	*g*
Californidine perchlorate	$O—CH_2—O$		H	$O—CH_2—O$		—	—	*h*
				Aporphines				
Aporheine	H	H	$O—CH_2—O$		—	—	—	*i*
Bulbocapnine	OCH_3	OH	$O—CH_2—O$		—	—	—	*i*
				Benzophenantridines				
Sanguinarine chloride	$O—CH_2—O$		$O—CH_2—O$		—	—	—	*j*
Chelerythrine chloride	OCH_3	OCH_3	$O—CH_2—O$		—	—	—	*j*
Chelidonine	$O—CH_2—O$		$O—CH_2—O$		OH	—	—	*k*
Homochelidonine	OCH_3	OCH_3	$O—CH_2—O$		OH	—	—	*k*
Dihydrosanguinarine	$O—CH_2—O$		$O—CH_2—O$		—	—	—	*l*
				Indenisoquinoline				
Compound *I*	OCH_3	OCH_3	$O—CH_2—O$		—	—	—	*m*



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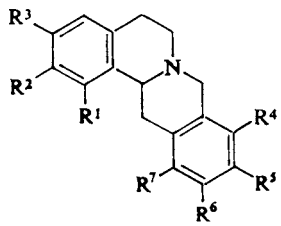

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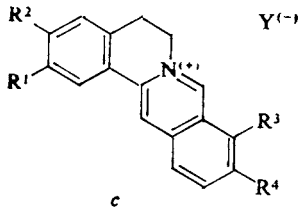

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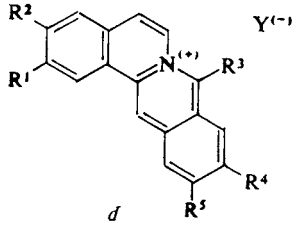

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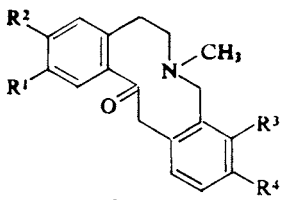

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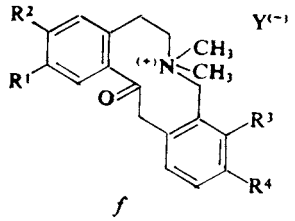

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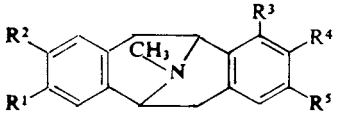

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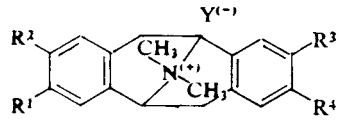

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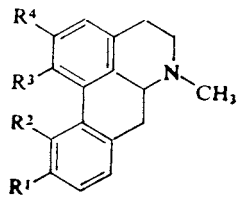

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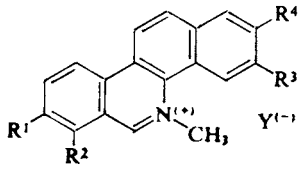

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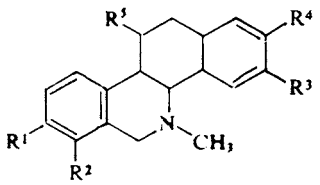

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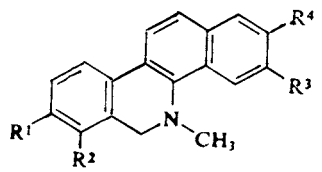

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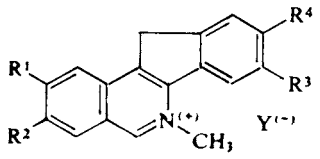


m

values were calculated from the formula:

$$pK = pH - \log [(Y - Y_{HA})/(Y_A - Y)] , \qquad (2)$$

where Y denotes the signal (absorbance or intensity of fluorescence) at a pH, Y_{HA} the signal of the acid, Y_A the signal of the base. Y_{HA} and Y_A were obtained by extrapolation to low and high pH, corrected for the possible quenching by high concentrations of protons or hydroxyl ions in measuring fluorescence. The calculations from equation (*2*) were carried out for at least 5 values of Y at defined pH values for each substance; from these data the average value and the standard deviation were calculated. The value of pK_{abs} is supposed to be identical with pK of the substance in the ground state ($pK_{abs} = pK_g$). The difference in pK between the ground state (pK_g) and the excited state (pK_e), *i.e.* $\Delta pK = pK_g - pK_e$, was calculated from the equation[11]:

$$\Delta pK = 21\,187(\lambda_A - \lambda_{AH})/(\lambda_A \cdot \lambda_{AH}) , \qquad (3)$$

where λ_A and λ_{AH} denote wavelengths of the long-wave absorption peaks of the basic and the acid forms of the compound.

Approximate partition coefficients of alkaloids distributed between octanol and water (K) were obtained as follows: $6 . 10^{-5}$ mol l^{-1} aqueous solutions (3 ml) of the alkaloids, brought to a given pH, were mixed with an equal volume of n-octanol, agitated for 30 min and allowed to stand for about 2 h. The phases were separated by centrifugation (*c.* 2 000*g*) and the absorption spectrum was measured in either phase.

RESULTS AND DISCUSSION

Fluorescence Properties of the Alkaloids in Relation to their Structures

The basic fluorescence properties (*i.e.* excitation and emission peaks, and assessed intensities of fluorescence) in aqueous media are compiled in Table II. Most of the compounds exhibited the last excitation peak which practically coincided with the

TABLE II

Fluorescence properties of some isoquinoline alkaloids. λ_{exc} wavelength of the last excitation band, λ_{em} wavelength of the emission peak; VH, H, M, L, VL very high, high, medium, low and very low intensity of fluorescence (the M intensity of fluorescence is comparable with the fluorescence of tyrosine at excitation by 280 nm and emission of 305 nm, the quantum yield of fluorescence of tyrosine at pH 6 and 23°C is 0·14, ref.[26]). The data in the first three columns refer to measurements of aqueous solutions of the alkaloids, 10^{-5} mol l^{-1}, at 25°C. With compounds differing substantially in optical properties at different pH's, the intensities of fluorescence (and excitation, in some cases) are given for an alkaline medium (10^{-2} mol NaOH/l) and an acid medium (10^{-2} mol HCl/l). The fourth and the fifth column illustrate the effect of the decreasing polarity of the medium (ethanol, dioxan) under the same conditions. (←) a very weak blue shift, ← a rather marked blue shift of the emission peak; increase in fluorescence intensity is assessed, according to magnitude and sense of the effect, by symbols 0, (↑), ↑, ↑↑, ↑↑↑, (↓), ↓, ↓↓

Alkaloid	λ_{exc} nm	λ_{em} nm	Intensity	Shift	Change of intensity
Canadine	290	330	H	0	0
Stylopine	290	326	M	0	(↑)
Escholidine	285	332	M	(←)	↑
Cyclanoline	286	320	L	←	(↓)
Corypalmine	284	325	L	←	↑
Mecambridine	285	365	L	←	↑
Berberine	420	550	L	←	↑↑↑
Berberrubine	426	500	VL	←	↑↑↑
Jatrorrhizine	422	520	VL	←	↑↑↑
Coralyne	422	475	VH	←	(↑)
Protopine	290	332	H	(←)	↓↓
Protopine methiodide	303	325	M	(←)	↓
Allocryptopine	285	329	H	0	↓
Hunnemanine	287	331	M	←	(↓)
Californidine	292	328	H	0	0
Eschscholtzine	293	332	M	0	(↑)
Argemonine	287	320	M	0	↑
Norargemonine	287	321	L	0	↑
Platycerine	283	321	L	0	↑
Bisnorargemonine	287	322	L	0	↑
Aporheine	316	380	H	←	(↑)
Bulbocapnine	340 (alk)	465	H	←	↑
	305 (acid)	390	H	←	↑
Chelidonine	291	330	M	(←)	↑
Homochelidonine	288	325	M	0	↑
Compound *I*	320	460	L	(←)	(↑)

TABLE II
(*Continued*)

Alkaloid	λ_{exc} nm	λ_{em} nm	Intensity	Shift	Change of intensity
Dihydrosanguinarine	324	430 (alk)	M	←	↑↑
		390 (acid)	H	←	↑↑
Sanguinarine	326 (alk)	420	H	(←)	↑↑
	468 (acid)	565	L	0	↓
Chelerythrine	323 (alk)	420	H	(←)	↑↑
	440 (acid)	550	L	0	↓

longest-wave absorption band, in the region 284–293 nm (ref.[12]). In the short-wave UV region these alkaloids had at least one more excitation peak (c. 230–260 nm). The coincidence of this peak (these peaks) with the short-wave absorption peaks of the alkaloids[12] was not so good, because the recorded excitation spectra were not corrected; besides, in the region below 250 nm the luminous intensity of the source rapidly decreased and so did the transmittance of the excitation monochromator. The short-wave excitation peaks are not included in Table II. The emission spectra are single and smooth curves which suggest emission of one form only. The emission peaks of the given groups of compounds cover the range 320–332 nm (uncorrected). These results show that the absorption and the fluorescence properties of the compounds essentially correspond to the optical properties of benzene with OH, OCH_3, and OCH_2O substituents. This chromophore occurs in tyrosine[13] (λ_{exc} = 280, λ_{em} = 304 nm), adrenaline and Dopa[14] (λ_{exc} = 285 nm, λ_{em} = 325 nm). In comparison with phenol[15] (λ_{exc} = 270 nm, λ_{em} = 310 nm) or methoxybenzene[15] (λ_{exc} = = 270 nm, λ_{em} = 300 nm), the excitation and emission peaks of the compounds show a bathochromic shift; this is due to the greater number of substituents, especially OCH_3 and OCH_2O groups. The similarity between the chromophores of the studied alkaloids and the chromophore of tyrosine also follows from the measurement of phosphorescence at 77 K in ethanol (the tested alkaloids were stylopine, protopine, californidine and homochelidonine). The phosphorescence maxima of these alkaloids were in the region 440–450 nm; they were single bands without a characteristic pattern; an exception was stylopine, whose peak was a complex one. The phos-

phorescence excitation peaks were practically identical with the fluorescence excitation peaks. Polarization of the longer-wave band was positive (taking values between 0·1 and 0·25), whereas polarization of the shorter-wave band was negative (approximately −0·1). These values are in accordance with the polarization values of the two excitation peaks of tyrosine and phenol[16]. With all the alkaloids the angle between the absorbing and the emitting oscillators was close to 0° for the longer-wave excitation band (for 0° $p = 0{\cdot}5$) and close to 90° for the shorter-wave excitation band (for 90° $p = -0{\cdot}3$). The transition moments for the short-wave and the long-wave bands are perpendicular to one another[16]. The fluorescence behaviour of the other tested alkaloids was more complex than that of this fundamental chromophore, corresponding to substituted benzene. This is due to the fact that their chromophores contained greater numbers of conjugated aromatic rings. With protoberberines, having a long-wave absorption band above 400 nm and emitting around 500 nm and higher (Table II and refs[3,17]), the chromophore is a substituted isoquinoline system in partial conjugation with substituted benzene; protoberberines are non-planar structures, the angle between the two aromatic moieties of the molecule being *c.* 20−30° (ref.[18]). Coralyne, which has a planar structure with perfectly conjugated aromatic rings, absorbs in the visible spectral region like protoberberines (but about three times as intensely[19]). However, it fluoresces at somewhat shorter wavelengths and very intensely (the fluorescence intensity was the highest of all the alkaloids tested), *cf.* Table II and ref.[17]. According to ref.[19] the quantum yield of the fluorescence of coralyne exceeds 0·6. With the aporphines aporheine and bulbocapnine the wave corresponding to the long-wave absorption and excitation bands are longer than 300 nm, fluorescence can be detected in the region 350−400 nm (ref.[5]). This is consistent with the fact that aporphines have two substituted (but not coplanar) benzene rings. The benzophenanthridine alkaloids show the effect of increasing number of double bonds on the optical properties. Chelidonine and homochelidonine, having the properties of the fundamental chromophore (*i.e.* the substituted benzene ring) absorb around 290 nm and emit at *c.* 320−330 nm. Dihydrosanguinarine, absorbing at *c.* 320−330 nm emitting above 400 nm, can be regarded as a substituted naphthalene in partial conjugation with another substituted benzene ring (the wavelengths of the excitation and the emission peaks of substituted naphthalenes are substantially lower than those of dihydrosanguinarine, but higher than those of benzene[20]). The optical properties of compound *I* resemble dihydrosanguinarine, although the former is a substituted isoquinoline partially conjugated with a substituted benzene ($\lambda_{exc} > 300$ nm, $\lambda_{em} > 400$ nm). The absorptions and emissions of sanguinarine and chelerythrine, which are fully aromatic plane compounds, are associated with much smaller quanta of energy ($\lambda_{exc} > 400$ nm, $\lambda_{em} > 500$ nm). In transition of these cationic forms to pseudobases (ref.[21] and see below) their chromophore assumes a structure and optical properties similar to those of the chromophore of dihydrocompounds (compare sanguinarine in an alkaline medium, *i.e.*

a pseudobase, with dihydrosanguinarine in Table II). Table II also gives some idea on the effect of substituents, though the selection is rather poor. The compounds with OH groups instead of OCH_3 absorb (and are excitable) at higher energies (*cf.* escholidine and cyclanoline *vs* stylopine and canadine; platycerine and bisnorargemonine *vs* eschscholtzine; hunnemanine *vs* protopine). The fluorescence intensity of phenolic alkaloids is also generally lower than that of their methoxyl analogues. The presence of a methylenedioxide group instead of two methoxyls in the molecule raised the values of λ_{exc} and λ_{em} in nearly all the cases (*e.g.* stylopine *vs* canadine, protopine *vs* allocryptopine, sanguinarine *vs* chelerythrine). This phenomenon was especially marked if two OCH_2O groups were present instead of four OCH_3 groups (eschscholtzine *vs* argemonine). The absence of the mentioned electron-donor substituents manifested itself by shifts of the excitation and emission wavelengths to the blue region (aporheine *vs* bulbocapnine). Similar effects of substituents on the optical properties of some of the studied groups of alkaloids have been described before[17].

Effect of Polarity of the Medium on the Fluorescence Properties of the Alkaloids

As is known[3], protoberberine alkaloids pertain to the so-called hydrophobic probes, *i.e.* compounds weakly fluorescing in water and other very polar media, but intensely in less polar media, with a marked hypsochromic shift of the peak. This property can be made use of for following interaction of these compounds with biologically important macromolecules having a hydrophobic binding site.

We have investigated the fluorescence behaviour in ethanol and dioxan (Table II). With the exception of the protoberberine alkaloids, none of the alkaloid groups tested behaved as a typical hydrophobic probe. Apart from the pavinane alkaloids, a weak blue shift of the emission peak was observed in transfer from water into ethanol and dioxan, but the increase in the intensity of fluorescence was not so high as with the protoberberine alkaloids. Alkaloids of the protopine type even exhibited a decrease in fluorescence intensity with increasing hydrophobicity of the medium. A rather marked increase in fluorescence intensity accompanying transfer from water to ethanol was observed with sanguinarine and chelerythrine (in their alkaline, pseudobase forms), but in dioxan their fluorescence was diminished (Table II and ref.[21]). With compound *I* and dihydrosanguinarine, as structurally analogous substances, the fluorescence in dioxan was also weaker than in ethanol. With the other compounds, the intensity of fluorescence in ethanol ranged between those in water and in dioxan (when both increase and decrease were observed with increasing hydrophobicity of the medium).

These results show that interaction of most of the compounds tested with various biopolymers (proteins, nucleic acids, biological membranes, *etc.*) cannot be studied by measuring merely the emission and excitation spectra, or even the intensity

of fluorescence only. These properties cannot be expected to change so profoundly as in the case of protoberberines. Since the studied alkaloids rather intensely fluoresce both in water and in less polar media, and polarization of their fluorescence in solutions at room temperature is close to zero, the method of polarization of fluorescence might prove useful for studying the interactions. Polarization of ligands is known[3,5,6] to be generally rather enhanced by the interaction. However, in following the binding to proteins with the aid of all the fluorescence techniques the key problem will be that of distinguishing the fluorescence of these compounds from the intrinsic fluorescence of proteins which is due to residues of tyrosine and especially tryptophan. These amino acid residues have fluorescence characteristics similar to those of the alkaloids with the basic chromophore; *i.e.* a substituted benzene ring (*cf.* Table II). Apart from protoberberines, promising results in applying the fluorescence methods to studying the interactions with proteins can be expected from aporphines and some benzophenanthridines which absorb and emit at longer wavelengths than tyrosine and tryptophan do (Table II). These alkaloids can even be expected to exhibit transfer of energy from tryptophan or tyrosine by the resonance mechanism[22].

Effect of pH *on the Fluorescence Behaviour of the Investigated Alkaloids*

More than a third of the isoquinoline alkaloids tested were compounds containing one or two dissociable hydroxyl groups. In these compounds, naturally, the effect of a higher pH was the most marked. None of these phenolic compounds (excepting bulbocapnine) was capable of any significant fluorescence at pH $\geqq$ 12. But even the other alkaloids (including those containing a quaternary nitrogen atom and no dissociable group) were appreciably affected in their fluorescence behaviour by extreme pH values. All the compounds exhibited quenching in strongly acid (generally below pH 1 to 2) or in alkaline media (the phenomenon was also observed in moderately alkaline media, with protopines and pavinanes even at pH > 8). This circumstance made it difficult in some cases to evaluate pK of phenolic groups from fluorescence data of the compounds. The considerably strong effect of hydroxyl ions as quenchers was essentially similar to that of iodide ions (ref.[23]). With compounds sensitive to iodide ions the quenching in alkaline media was strong also (this applies especially to protopines and pavinanes). Of the compounds with a substituted benzene as the chromophore system it was only tetrahydroprotoberberines that reacted relatively little both to the presence of iodide ions and hydroxyl ions. In this group potassium iodide failed to exhibit a quenching effect even at concentrations above 0·1 mol l^{-1} and the alkaline medium did not affect the intensity of fluorescence either. Owing to this fact the pK values of the phenolic group of tetrahydroprotoberberines obtained from fluorescence data can well be correlated with values obtained from the absorption data. With the protopines this correlation was not possible, because

the inflexion points on the plots of fluorescence intensity *vs* pH were not the actual pK values.

The effect of pH on the behaviour of some of the alkaloids tested (especially of the phenolic character) is illustrated in Table III. The formation of phenolates was accompanied in all cases by changes in the absorption spectra. In compounds with the basic type of chromophore (*i.e.* a substituted benzene ring) a new absorption band in the region 293–303 nm appeared in alkaline solutions. Phenol, tyrosine and other compounds with the same chromophore were found to behave alike[13–15]. The absorbance-pH plots allow of calculating the value of pK_{abs} (equation (*2*)). From the wave-length shift of the long-wave absorption peak between the neutral and the phenolate forms it was possible to evaluate approximately the pK change between the ground state and the excited state (ΔpK, equation (*3*)). In the alkaloids with OH groups, like in other phenolic compounds, ionization of OH groups in the

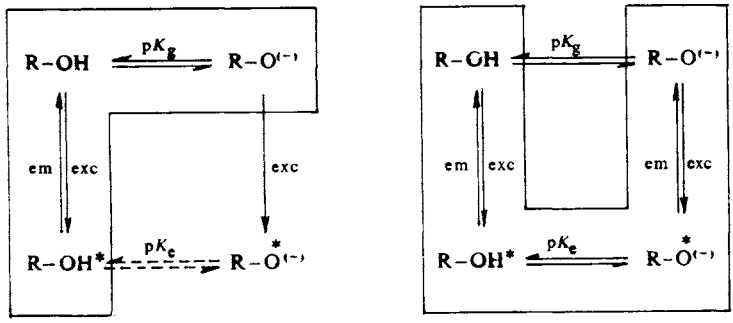
TABLE III

Effect of pH on the absorption and fluorescence properties of some isoquinoline alkaloids. The values of pK_{abs}, pK_{fl} and ΔpK were determined as described in the experimental part. The absorption spectra for determining pK_{abs} and changes of wavelength in the change of pH from 6 to 12 ($\Delta\lambda$) were measured in the visible region and the near UV region (up to 260 nm)

Alkaloid	$\Delta\lambda$, nm	ΔpK	pK_{abs}	pK_{fl}
Escholidine	286 → 294	2·0	8·45 ± 0·1	8·4 ± 0·1
Cyclanoline	284 → 295	2·9	8·4 ± 0·1	8·3 ± 0·1
Corypalmine	284 → 299	3·7	9·55 ± 0·15	9·3 ± 0·2
Jatrorrhizine	422 → 454	3·5	8·2 ± 0·15	<8[a]
Berberrubine	426[b] → 485	[c]	$\approx 5{\cdot}7$[c]	≈ 2[d]
Hunnemanine	285 → 293	2·0	9·0 ± 0·1	$\approx 8{\cdot}5$
Platycerine	282 → 293	2·8	9·85 ± 0·15	≈ 9
Norargemonine	285 → 301	3·9	9·8 ± 0·1	≈ 9
Bisnorargemonine	286 → 303	4·1	9·7 ± 0·15	≈ 9
Bulbocapnine	304 → 342	7·7 8·3[e]	10·1 ± 0·2	1·65[f] 1·75[g]
Dihydrosanguinarine	324[h]	—	2·6 ± 0·3	2·3 ± 0·25
Sanguinarine	468 → 326	—	8·05 ± 0·15	7·95 ± 0·15
Chelerythrine	440 → 323	—	9·0 ± 0·2	8·9 ± 0·2

[a] Difficult to evaluate because of very weak fluorescence; [b] at pH 4; [c] difficult to evaluate because of interference by the non-charged quinoid form; [d] difficult to evaluate because of strong quenching at pH < 1·5; [e] determined from 0–0 transition of the acid and the basic forms; [f] calculated from increase of the acid form; [g] calculated from decrease of the basic form (Fig. 2); [h] at pH > 4 of weak absorption band appeared at 310–340 nm, at pH < 2·5 a high absorption peak at 324 nm.

excited state was energetically easier[24] ($\Delta pK > 0$). With the alkaloids having the basic chromophore with one OH group the values of ΔpK ranged between 2·0 and 4·1; with two OH groups the values of ΔpK were higher (*cf.* cyclanoline *vs* escholidine, and bisnorargemonine *vs* argemonine in Table III). Since pK of the ground state (pK_g), determined by pK from absorption data (pK_{abs}), occurs between 8·4 and 9·9, the pK of the excited state (pK_e) is in a weakly acid region (Scheme 1). With the



SCHEME 1

Mechanism of excitation and emission of alkaloids with an ionizable phenolic group (R—OH); [a] Ionization in the excited state is comparatively slow (- - - - -), this refers to escholidine and cyclanoline; [b] ionization in the excited state is comparatively fast (———), this refers to bulbocapnine. The asterisks denote the excited states, exc stands for excitation, em for emission, pK_g and pK_e are pK values of the OH groups in the ground and the excited states. The processes affecting the measured intensities of fluorescence are framed.

tetrahydroprotoberberine alkaloids escholidine and cyclanoline (only these two compounds with the basic chromophore allow of safe determination of pK from fluorescence data) the values of pK_{abs} and pK_{f1} were practically equal (Table III, Fig. 1). This means that in these two alkaloids even the values of pK_{f1} correspond to pK_g. As is known[25], the rate constant of the association of proton with the basic form is of the order of magnitude 10^{11} s^{-1} l mol^{-1} for the majority of organic compounds capable of releasing H^+; pK of such a compound is governed by the rate constant of the dissociation. The life time of the excited state in a typical $\pi - \pi^*$ transition is generally 10^{-8} to 10^{-9} s^{-1} (ref.[24]) which corresponds to a rate constant of the excited state → ground state transition of the order 10^{+8} to 10^{+9}. If the quantum yield of fluorescence is close to 0·1 (as it was with most of the alkaloids tested), the rate constant for emission of radiation is *c.* 10^7 to 10^8 s^{-1}. With escholidine the value of pK_e equalled approx. 6·45 which corresponds to a rate constant of dissociation of about $3 . 10^4$ s^{-1}; for cyclanoline pK_e was about 5·5 and the rate constant of dissociation *c.* $3 . 10^5$ s^{-1}. Thus it is probable for either compound

that the rate of dissociation in the excited state is much lower than the rate of emission. Hence it follows that pK_{f1} essentially equals pK_{abs} (and thus pK_g). The diminishing fluorescence with increasing pH manifests the decrease of the fluorescing concentration form, with the OH group in the ground state (Fig. 1).

Of interest is the behaviour of the aporphine derivative, bulbocapnine, in relation to pH. The value of pK_g (determined from the dependence of absorbance upon pH) was the highest of all of the phenolic derivatives investigated and equalled *c.* 10·1, whereas pK_e, judging by the change of the absorption spectrum (calculated from equation (*3*)), was about 2·4. Bulbocapnine, being the only one of the alkaloids studied that fluoresces even in the phenolate form (at a greater wavelength than the hydroxyl form, as is the case with other phenolic compounds, *e.g.* naphthol[27]), allowed of determining the change of p*K* more exactly, from the O–O transitions of the OH and $O^{(-)}$ forms[11]. The wavelengths of the O–O transitions are 347 nm and 402 nm, respectively. After inserting these values into equation (*3*), the value of pK_e results as even lower than 2 (Table III). From the dependence of the fluorescence intensity on pH (both OH and $O^{(-)}$) the value of pK_{f1} results as *c.* 1·7

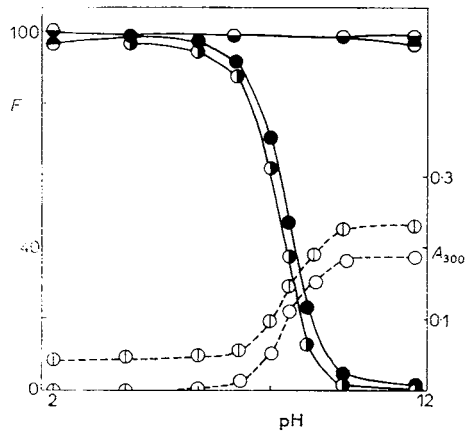


FIG. 1

Absorbance and fluorescence of tetrahydroprotoberberine alkaloids in relation to pH. For conditions of the measurements see Methods. The solid line denotes the relative intensity of fluorescence (F) at λ_{exc} and λ_{em} for the individual alkaloids, as given in Table II; ◒ canadine, ◓ stylopine, ● escholidine, ◑ cyclanoline. The dashed line refers to absorbance at 300 nm (A_{300}); ○ escholidine, ◐ cyclanoline

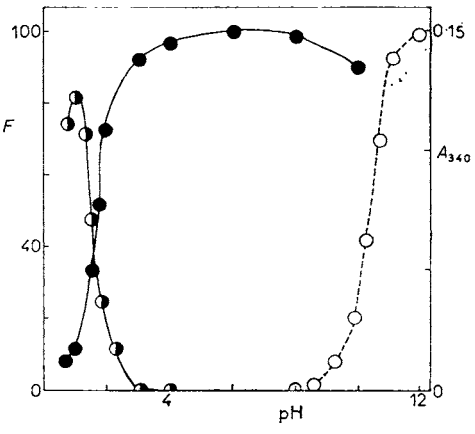
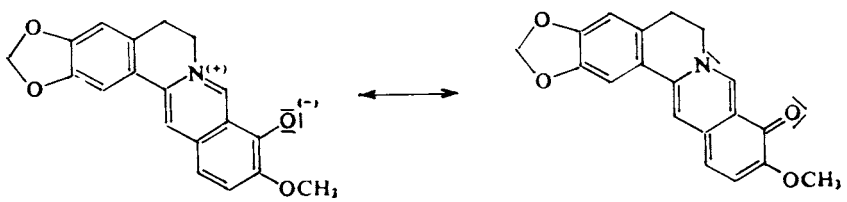


FIG. 2

Absorbance and fluorescence of bulbocapnine in relation to pH. For conditions of the measurements see Methods; ○ absorbance at 340 nm (A_{340}), ● intensity of fluorescence (F) at λ_{exc} = 325 nm and λ_{em} = 465 nm, ◑ intensity of fluorescence at λ_{exc} = 325 nm and λ_{em} = 390 nm

(Fig. 2). In this case, consequently, the measurement of fluorescence essentially gives the value of pK_e. This is due to the fact that at $pK_e = c.\ 1{\cdot}7$ (supposing the rate constant for the association with protons to be $10^{+11}\ s^{-1}\ l\ mol^{-1}$) the dissociation rate constant is of the order 10^9 to $10^{10}\ s^{-1}$. At a pH close to pK_e the effective rate constant of the association (*i.e.* product of the association rate constant and concentration of protons) also takes this high value. From these data it follows that the transfer rate of protons in the excited state of bulbocapnine exceeds the emission rate (*c.* 10^7 to $10^8\ s^{-1}$) by at least one order of magnitude, so that the change of the fluorescence intensity of the alkaline form (at 465 nm) with pH corresponds to the concentration change of the ionized excited molecules, and its course accords with that of fluorescence of the phenolic form at 390 nm (Fig. 2). The mechanisms interpreting the two extreme cases of the alkaloids studied (*i.e.* escholidine, where $pK_{f1} = pK_g$, and bulbocapnine, where $pK_{f1} = pK_e$) are depicted in Scheme 1.

The behaviour of the phenolic alkaloids jatrorrhizine and berberrubine, of the protoberberine class, is interesting too. Although kindred, the two alkaloids very differ in pK_g of their phenolic groups. Whereas the pK_g of the jatrorrhizine hydroxyl group is, like with the other alkaloids, in the alkaline regions (and is comparable, *e.g.* with the pK_g's of escholidine and cyclanoline), the apparent pK_g of the berberrubine hydroxyl group occurs between 5 and 6 (Table III and ref.[28]). This anomalously low pK_g of the berberrubine OH group may be due to a possible tautomeric transition of the berberrubine phenolate form (a zwitterion) into an electroneutral quinoid structure (Scheme 2). The formation of a non-charged form of berberrubine



SCHEME 2
Tautomer forms of berberrubine

in an alkaline medium was demonstrated by measuring the coefficient of distribution of this alkaloid between octanol and water (K). At pH 2, as a result of a positive charge of berberrubine (like with berberine and jatrorhizine), the value of $\log K$ is lower than $-1{\cdot}5$ (ref.[29]). At pH 11 $\log K$ is about $+2$ (with "zwitterion" jatrorhizine and cation berberine the value of $\log K$ is below $-1{\cdot}5$ even at this pH). The increase of the distribution coefficient of berberrubine by more than three orders of magnitude with the change of medium from acid to alkaline testifies to the

presence of a non-charged form. Since the red colouring of berberrubine in a neutral or alkaline medium is strongly deepened by the non-charged quinoid form, the exact values of ΔpK and pK_{abs} cannot be calculated from spectroscopic data (such calculations assume the existence of a phenolic and a phenolate form only). From the fluorescence data (showing concentration of the non-charged form with the OH group, since neither the phenolate nor the quinoid form fluoresces) the value of pK_{f1} results as *c.* 2·0 (here too it probably applies that $pK_{f1} \approx pK_e$).

Another compound whose absorption and fluorescence properties were sensitive to pH was dihydrosanguinarine. The last absorption band of the acid and the neutral forms was around 324 nm (the absorption by the acid form was strong and sharp, whereas the neutral form exhibited a wide but very weak absorption band). From the increase in absorbance at 324 nm it is possible to determine the value of pK_{abs}. Since the ionization is not accompanied by a shift of the long-wave absorption peak, the value of pK_e is likely to be approximately equal to pK_g. This idea is supported by the fluorescence properties of dihydrosanguinarine (pK_{f1} is close to pK_{abs}), even allowing for the rather great inaccuracy of the fluorescence measurements. The fact that protonization of the dihydrosanguinarine nitrogen alters the fluorescence properties of this compound may be due to the circumstance that the nitrogen atom is adjacent to the chromophore (*i.e.* the substituted naphthalene).

Sanguinarine and chelerythrine differ from the other alkaloids by adding an OH^- group in alkaline media, thus becoming pseudobases. This phenomenon has been described before[21]. The absorption and fluorescence properties of the sanguinarine pseudobase resemble dihydrosanguinarine (the two compounds have a common chromophore), the acid (cationic) form of sanguinarine absorbs in the region of longer wavelengths. The pK values of sanguinarine and chelerythrine determined by absorption spectroscopy and fluorometry (Table III) are practically identical with the reported ones[21].

The other alkaloids capable of being protonized on nitrogen (mecambridine, protopine, allocryptopine, eschscholtzine, argemonine, aporheine) exhibited no significant changes of absorption and fluorescence properties in the pK region of the nitrogen atom, so that the ionization of nitrogen cannot manifest itself in these optical properties. This may be due to the circumstance that (unlike dihydrosanguinarine) none of these compounds has a nitrogen atom adjacent to the chromophore system. This accords with the fact that the behaviour of alkaloids having a quaternary nitrogen is much the same as that of their analogues with a protonizable tertiary nitrogen (*cf.* corypalmine and mecambridine *vs* canadine and stylopine; protopine *vs* protopine methiodide; eschscholtzine *vs* californidine), although certain anomalies have been observed (*e.g.* the high λ_{em} of mecambridine or the high λ_{exc} of protopine methiodide). The obtained pK values of the alkaloids (excepting dihydrosanguinarine) cannot be correlated with those in ref.[12], since the latter reflect the pK of nitrogen.

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