SUPRAVITAL STAINING OF MITOCHONDRIA WITH PHENOSAFRANIN DYES

by

SYDNEY BRENNER*

Department of Physiology, University of the Witwatersrand, Johannesburg (South Africa) and Physical Chemistry Laboratory, Oxford (England)

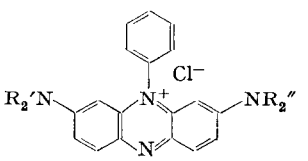
Although Janus green B is widely used as a supravital stain for mitochondria there is no adequate theory to explain the mechanism of its action. Janus green B is diethylphenosafraninazodimethylaniline and COWDRY[1] demonstrated that diethylsafranin itself, as well as other dyes containing it, stained mitochondria supravitally. Because dimethylphenosafranin and its derivatives failed to give the selective supravital staining reaction, COWDRY claimed that the specificity of Janus green B depends on the diethylphenosafranin part of the molecule. This hypothesis was confirmed by the demonstration that amethyst violet (tetraethylphenosafranin) is a selective supravital stain for mitochondria[2].

Alkyl substitution on an amino group attached to an aromatic ring increases the positivity of the nitrogen atom and ethyl groups exert a stronger effect than methyl groups. Thus the diethylphenosafranins would be expected to be more strongly basic than the dimethylphenosafranins and this difference between the two types of dyes might be related to the staining specificity. To test this hypothesis, a series of symmetrical and unsymmetrical phenosafranin dyes were investigated for their supravital staining activity. The absorption spectra of the dyes were measured as well, since the position of the absorption maximum can be used to assess the basicity of the terminal cationic amino groups.

EXPERIMENTAL

Dyes

All the dyes contain the same ring system but have different groups substituted on the amino groups.



	R_2'	R_2''
I. Phenosafranin	H_2	H_2
II. Dimethylphenosafranin	Me_2	H_2
III. Tetramethylphenosafranin	Me_2	Me_2
IV. Diethylphenosafranin	Et_2	H_2
V. Diethyldimethylphenosafranin	Et_2	Me_2
VI. Tetraethylphenosafranin	Et_2	Et_2
VII. Janus green B	Et_2	$NC_6H_4NMe_2$

* Overseas Scholar of the Royal Commission for the Exhibition of 1851.

References p. 486.

Phenosafranin was a commercial preparation obtained from the Coleman and Bell Company and used without further purification.

Dimethylphenosafranin was synthesized by a modification of the method of BINDSCHEDLER[3]. 15 g (0.08 moles) of *p*-nitrosodimethylaniline hydrochloride was reduced in 160 g of 50% aqueous acetic acid with 16 g of zinc dust. The solution was filtered and to the filtrate were added 15 g (0.16 moles) of aniline dissolved in 20 ml of glacial acetic acid and 31.5 g (0.107 moles) of potassium dichromate in hot water. The solution was gently boiled for 1 hour, filtered hot, and the residue in the flask repeatedly extracted with boiling water and the extracts filtered. Solid NaCl was added to the cooled combined filtrates to salt out the dye and the red precipitate was collected by filtration on a Buchner funnel. The dye was dried and crystallized from boiling water with the addition of a few drops of concentrated HCl.

Tetramethylphenosafranin was synthesized by the method of BALLS *et al.*[4] 15 g (0.08 moles) of *p*-nitrosodimethylaniline hydrochloride was dissolved in 160 g of 50% aqueous acetic acid and reduced with 16 g of zinc dust. The solution was filtered and to the filtrate were added 10 g (0.08 moles) of dimethylaniline dissolved in 10 ml of glacial acetic acid and 15.75 g (0.053 moles) of potassium dichromate in hot water. The green indamine formed immediately and after ½ hour 7.5 g (0.08 moles) of aniline was added and the solution allowed to stand for a further ½ hour. 15.75 g (0.053 moles) of potassium dichromate in hot water was then added and the solution gently boiled for 1 hour. The solution was filtered hot and the residue in the flask repeatedly extracted with boiling water and the extracts filtered. The filtrates were combined and cooled and solid NaCl was added to salt out the dye. The precipitate was collected by filtration on a Buchner funnel, dried, and crystallized from boiling water with the addition of a few drops of concentrated HCl.

Diethylphenosafranin was prepared according to the method of COWDRY[1]. 10 g of Janus green B (G. T. Gurr) was reduced in 10% HCl with 10 g of zinc dust. The leucobase formed first but after aeration the red diethylphenosafranin appeared. The solution was filtered and the dye salted out by the addition of solid NaCl. The red precipitate was collected by filtration on a Buchner funnel, dried, and crystallized from boiling water with the addition of a few drops of concentrated HCl.

Diethyldimethylphenosafranin was synthesized in an analogous way to tetramethylphenosafranin; diethylaniline was used instead of dimethylaniline for the preparation of the indamine. The violet dye was crystallized from boiling water with the addition of a few drops of concentrated HCl.

Tetraethylphenosafranin (amethyst violet; C.I. No. 847) was a commercial product obtained from the National Aniline Company and used without further purification.

Janus green B (diethylphenosafraninazodimethylaniline) was obtained from the National Aniline Company. The sample had a certified dye content of 72% and was used without further purification.

Absorption spectra

Absorption of light was measured with a Beckman quartz spectrophotometer, model DU, using 1 cm Corex cells. The molar extinction coefficient ε was calculated according to the formula

$$\varepsilon = \frac{1}{cd} \log_{10} \frac{I_0}{I}$$

References p. 486.

where c is the molar concentration of the dye, d the thickness of the cell in centimetres, I the intensity of light emerging from the dye solution and I_0 the intensity of light emerging from the solvent.

Staining procedures

Dilutions of the dyes were made in Locke's solution. Human lymphocytes were used to test the supravital staining action of the dyes; in these cells the mitochondria can be readily observed. The preparations were made according to the method of COWDRY[1]. A drop of the dye solution was placed on a clean glass slide and a drop of blood from a finger prick added and the preparation covered immediately with a glass coverslip. The preparations were examined on a warm stage at 37° C with an oil-immersed objective N.A. = 1.3. The source of illumination was a 200 watt tungsten filament lamp cooled by a water filter.

RESULTS

Absorption spectra of the dyes

Table I shows the molar extinction coefficients obtained for the dyes at the corresponding absorption maxima.

TABLE I

MOLAR EXTINCTION COEFFICIENTS AND ABSORPTION MAXIMA OF PHENOSAFRANIN DYES
SOLVENT: DISTILLED WATER

Dye	λ_{max} $m\mu$	εmax
I. Phenosafranin	525	24,550
II. Dimethylphenosafranin	541	14,120
III. Tetramethylphenosafranin	557	13,330
IV. Diethylphenosafranin	556	33,250
V. Diethyldimethylphenosafranin	575	16,300
VI. Tetraethylphenosafranin	590	41,820
VII. Janus green B	606	27,700

Supravital staining of mitochondria with phenosafranin dyes

Progressive dilutions of the dyes were made in Locke's solution and the supravital staining reaction of the mitochondria recorded. The results are shown in Table II.

TABLE II

SUPRAVITAL STAINING OF MITOCHONDRIA WITH VARYING CONCENTRATIONS OF PHENOSAFRANIN DYES

Dye	*Molarity of dye solution added to blood*					
	$1 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	$2.5 \cdot 10^{-4}$	$1 \cdot 10^{-4}$	$5 \cdot 10^{-5}$	$2.5 \cdot 10^{-5}$
I. Phenosafranin	—	—	—	—	—	—
II. Dimethylphenosafranin	—	—	—	—	—	—
III. Tetramethylphenosafranin	+	—	—	—	—	—
IV. Diethylphenosafranin	+	+	—	—	—	—
V. Diethyldimethylphenosafranin	+	+	+	—	—	—
VI. Tetraethylphenosafranin	+	+	+	+	+	—
VII. Janus green B	+	+	+	+	+	—

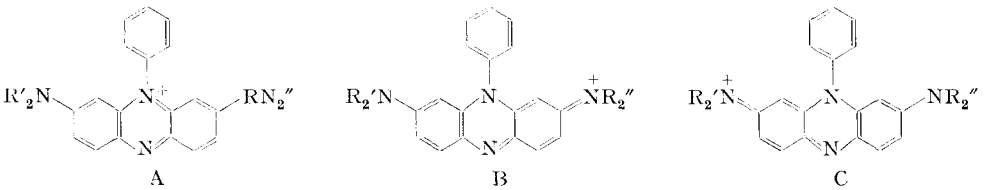
References p. 486.

DISCUSSION

The absorption spectra of the dyes

Spectra of some of these dyes have been recorded by previous authors. LEWIS[5] gives the absorption maxima of phenosafranin, dimethylphenosafranin and tetraethylphenosafranin as 519 mμ, 557 mμ and 590 mμ respectively. The present results confirm only the last value. The maxima found for phenosafranin and tetraethylphenosafranin in the present study agree with the values of 525 mμ and 589 mμ recorded by DEWAR[6]. CONN[7] gives the absorption maximum of Janus green B as 592.7 mμ, but this is incorrect as the dye would then be violet in colour. The value of 606 mμ found in the present study accords more closely with the blue-green colour of the dye.

The dyes are of the general cyanine type, existing as mesomeric cations. According to DEWAR[6,8] the colour in such dyes is due to the oscillation of the positive charge between the terminal nitrogen atoms with resonance of the intervening conjugated chain. Thus, although the usual formula for phenosafranin dyes shows the positive charge placed on the phenyl-substituted azine nitrogen (A), the dyes are more correctly represented by the two extreme resonance forms (B and C) in which the 2- or the 7-amino nitrogens are quaternarized. In symmetrical dyes these two forms are equivalent and, as shown below, the same applies to the unsymmetrical compounds.



A B C

The spectra obtained with the present series of dyes confirm the theory that the more basic the terminal atoms the longer the wavelength of the absorption maximum. Marked bathychromic shifts of the absorption maximum are associated with alkyl substitution of the terminal nitrogen atoms in the phenosafranin dyes. In the symmetrical dyes, there is a shift in the absorption maximum of 32 mμ to the red end of the spectrum when methyl groups are substituted for hydrogen on the terminal amino groups (compare I and III), and a further shift of 33 mμ when the methyl groups are replaced by ethyl groups (compare III and V). An interesting feature is that the unsymmetrical dyes have absorption maxima about midway between those of the two symmetrical compounds to which they are related. BROOKER *et al.*[9] have shown that the absorption band of an unsymmetrical cyanine dye is intermediate in position between the bands of the parent symmetrical compounds only if the terminal nuclei have the same or nearly the same basicity. If the basicity differs widely, then absorption in the unsymmetrical dye occurs at a shorter wavelength than the calculated position, the difference being termed the deviation. Table III shows that the deviation in the unsymmetrical phenosafranins is negligible; when the intermediate band is calculated harmonically, the observed maximum occurs at slightly longer wavelengths.

Because the deviation is absent in the unsymmetrical phenosafranins, it may be concluded that their terminal groups have the same basicity in the molecule and that the extreme resonance forms, though structurally different, are degenerate, making the same contribution to the energy of the compound. They therefore resemble the symmetrical dyes in this respect.

References p. 486.

TABLE III

RELATION BETWEEN OBSERVED AND CALCULATED ABSORPTION MAXIMA IN UNSYMMETRICAL PHENOSAFRANIN DYES

Unsymmetrical dyes	*Parent dyes*	*Observed* λ_{max} $m\mu$	*Calculated* λ_{max}	
			Arithmetic Mean $m\mu$	*Harmonic Mean* $m\mu$
II. Dimethylphenosafranin	I and III	541	541	540.5
IV. Diethylphenosafranin	I and VI	556	557.5	554
V. Diethyldimethylphenosafranin	III and VI	575	573.5	573

From the spectral properties of the dyes, a series can be constructed differing in the basicity of the terminal atoms; this is shown in Table IV. It should be noted that Janus green B, although it has the same terminal groups as diethyl-dimethylphenosafranin, is deeper in colour and this is probably due to increase in the length of the conjugated chain.

The relation of supravital staining action to the basicity of the dye

The results (Table II) demonstrate that there is a definite relation between the basicity of the dye and the concentration at which it will stain mitochondria supravitally. If allowance is made for differences in purity of the dyes (as judged from the molar extinction coefficients) the results remain significant.

TABLE IV

RELATION BETWEEN THE BASICITY OF PHENOSAFRANIN DYES AND THE EXTINCTION COEFFICIENTS OF THE LOWEST SUPRAVITAL STAINING CONCENTRATIONS

Dyes in increasing order of basicity as assessed from λ_{max}	*Extinction coefficient of the lowest staining concentration*
I. Phenosafranin	No staining
II. Dimethylphenosafranin	No staining
IV. Diethylphenosafranin	16.6
III. Tetramethylphenosafranin	13.3
V. Diethyldimethylphenosafranin	4.1
VI. Tetraethylphenosafranin	2.1
VII. Janus green B	1.4

Table IV shows clearly that the more basic the dye, the lower the concentration required to stain lymphocyte mitochondria supravitally. The specificity of the diethylphenosafranins as compared to the dimethylphenosafranins is therefore due to a difference of basicity and not to the structural difference. This is demonstrated by the fact that tetramethylphenosafranin stains mitochondria supravitally at about the same level as diethylphenosafranin which it resembles in basicity. The selectivity of tetraethylphenosafranin and Janus green B which stain mitochondria in very low concentrations is due to their pronounced basicity.

The implications of this result may be examined in relation to the various theories that have been put forward to explain the mechanism of supravital staining of mitochondria. LAZAROW and his co-workers[10,11] have concluded that the supravital staining of mitochondria depends on a non-specific adsorption of Janus green B onto cell protein

References p. 486.

and its rapid reduction in the cell except on the mitochondria where the presence of the cytochrome system prevents reduction and maintains the colour of the dye. It is difficult to explain the differences in the phenosafranin dyes on the basis of this theory. The redox potentials of phenosafranin ($E_o' = -0.252$ V) and dimethylphenosafranin ($E_o' = -0.260$ V) do not essentially differ from that of Janus green B ($E_o' = -0.256$ V). If supravital staining depended only on the difference in oxidation-reduction potential between the mitochondria and the rest of the cell, then the former two dyes should stain like Janus green B, which is not the case. The LAZAROW theory can also be controverted on other grounds. In a previous investigation[12] it was shown that when cells are supravitally stained with Janus green B or with methylene blue and then exposed to 10^{-3} M KCN, the dyes become reduced on the mitochondria. The staining reappears when the cells are washed in Locke's fluid which removes the substrates for the dehydrogenases; the dyes can be decolourised again by placing the cells in lactic acid. If the LAZAROW theory were correct, then the cyanide poisoned cells should show diffuse staining when washed with Locke's solution. Since this was never observed, the theory cannot hold. Although it is true that the high redox potential does maintain the colour of dyes that stain mitochondria supravitally, some specific interaction between the dyes and the mitochondria must occur.

ZACKS AND WELSH[13] have suggested that specific combination of the dyes with cholinesterase may be responsible for the supravital staining process. Supravital mitochondrial stains like Janus green B and methylene blue are potent cholinesterase inhibitors, competing with acetylcholine for the negatively charged site on the enzyme. In this system, the greater the amount of positive charge on the quaternary nitrogen of the dye, the more effectively would it compete for the enzyme. MASSART AND DUFAIT[14] have shown that $M/500{,}000$ Janus green B inhibits 92% of cholinesterase activity whereas the same molarity of phenosafranin gives only 35% inhibition. The effect of basicity on the supravital staining activity of the phenosafranin series might thus be explained on the basis of combination with cholinesterase, the more basic dyes combining more avidly. This theory would also explain the supravital staining of the mitochondria obtained with pinacyanol[15], since cyanine dyes are potent cholinesterase inhibitors[16]. However, there are dyes like methyl violet which strongly inhibit cholinesterase[14] but which do not stain mitochondria supravitally. Moreover, ZACKS AND WELSH[13] found that cholinesterase is not exclusively localised on the mitochondria.

It appears that the combination of Janus green B with the mitochondria is a specific and intimate one even if cholinesterase is not involved. Isolated mitochondrial preparations will stain with Janus green B but not with neutral red[17]. In such preparations, Janus green B potently inhibits oxidative phosphorylation, but this property is shared by phenosafranin and other dyes which are not supravital mitochondrial stains[18]. The possibility must be considered that while many basic dyes may adsorb onto isolated mitochondria all do not permeate with equal facility into the living cell. Weakly basic dyes like neutral red penetrate the cell membrane as undissociated bases and stain the vacuoles forming coacervates with ribonucleoprotein[19]. The phenosafranin dyes, however, are completely ionised at neutral pH and would have to penetrate as dye cations. These dyes never enter the vacuolar system of the cell. It is therefore possible that the basicity of phenosafranin dyes is primarily related to their permeation into the cell, the more basic dyes penetrating as cations more easily, combining with the mitochondria and staining them supravitally.

References p. 486.

SUMMARY

Supravital staining of lymphocyte mitochondria was studied for a group of symmetrical and unsymmetrical phenosafranin dyes. The basicity of the dyes was assessed from their absorption spectra. It was found that the more basic the dye, the lower the concentration necessary to stain mitochondria supravitally. The implications of this finding are discussed in relation to the theories of supravital staining.

RÉSUMÉ

La coloration vitale des mitochondries des lymphocytes a été étudiée pour un groupe de colorants symétriques et asymétriques de la phénosafranine. La basicité des colorants a été déterminée d'après leur spectre d'absorption. Il a été constaté que plus le colorant est basique, moins sa concentration doit être élevée pour qu'il colore vitalement les mitochondries. Les conséquences de cette constatation sont discutées en rapport avec les théories de la coloration vitale.

ZUSAMMENFASSUNG

Es wurde die Supravitalfärbung von Lymphocytenmitochondrien mit einer Reihe symmetrischer und unsymmetrischer Phenosafraninfarbstoffe untersucht. Die Basizität der Farbstoffe wurde aus ihren Absorptionsspektren abgeschätzt. Es wurde gefunden, dass je basischer der Farbstoff ist, desto geringer ist die zur Supravitalfärbung der Mitochondrien nötige Konzentration. Die Folgerungen dieser Entdeckung werden in Bezug auf die Theorien der Supravitalfärbung besprochen.

REFERENCES

1 E. V. COWDRY, *Contributions to Embryology*, Carnegie Inst., Washington, 8 (1918) 39.
2 S. BRENNER, *Stain Technol.*, 25 (1950) 163.
3 R. BINDSCHEDLER, *Ber. deutsch. chem. Ges.*, 16 (1883) 864.
4 K. BALLS, J. T. HEWITT AND H. N. NEWMAN, *J. Chem. Soc.*, 101 (1912) 1840.
5 G. N. LEWIS, *J. Am. Chem. Soc.*, 67 (1945) 770.
6 M. J. S. DEWAR, *Electronic Theories of Organic Chemistry*, Oxford University Press, (1949).
7 H. J. CONN, *Biological Stains*, Biotech Publications, Geneva, N.Y., (1946).
8 M. J. S. DEWAR, *J. Chem. Soc.*, (1950) 2329.
9 L. G. S. BROOKER, G. H. KEYES AND W. W. WILLIAMS, *J. Am. Chem. Soc.*, 64 (1942) 199.
10 A. LAZAROW AND S. J. COOPERSTEIN, *Biol. Bull.*, 99 (1950) 322.
11 A. LAZAROW, S. J. COOPERSTEIN AND J. W. PATTERSON, *Anat. Rec.*, 103 (1949) 482.
12 S. BRENNER, *S. Afr. J. Med. Sci.*, 14 (1949) 13.
13 S. I. ZACKS AND J. H. WELSH, *Am. J. Physiol.*, 165 (1951) 620.
14 L. MASSART AND R. P. DUFAIT, *Enzymologia*, 9 (1941) 364.
15 D. C. HETHERINGTON, *Stain Technol.*, 11 (1936) 153.
16 E. HASE, Y. MIZUNO AND M. KATAYANAGI, *Bull. Chem. Soc. Japan*, 22 (1949) 250. Abstract in *British Abstracts*, A III (1951) 1439.
17 G. A. HOGEBOOM, W. C. SCHNEIDER AND G. E. PALLADE, *J. Biol. Chem.*, 172 (1948) 619.
18 J. D. JUDAH AND H. G. WILLIAMS-ASHMAN, *Biochem. J.*, 48 (1951) 33.
19 P. DUSTIN, *Symposia Soc. Exptl. Biol.*, 1 (1947) 114.

Received February 9th, 1953