A Study of Skin Pigmentation by Clofazimine[1, 2]

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Selective staining of lepromatous tissue by a redox dye was described by Wade ([13]), who stated that, during ". . . the period when various dyes were being tried out in leprosy therapy, it is known that after repeated intravenous injection of methylene blue in lepromatous cases the skin lesions become colored . . . because of selective absorption of dye by the lepra cells." Wade cited no authority for this statement, and apparently inferred selective absorption from selective staining.

The preferential staining of lepromatous lesions by another redox dye, clofazimine (B.663), during the treatment of patients with lepromatous leprosy is an important disadvantage of treatment with this effective drug ([2]). Although it has been assumed that the preferential staining results from concentration of the drug in the lepromatous lesions, there is at least one alternative explanation. If the oxidation-reduction (redox) potential of normal skin were lower than that of lepromatous skin, a greater proportion of the drugs would be present as its oxidized (red-orange) species in lepromatous tissue, whereas a greater proportion would be present as its reduced (leuco-, colorless) species in adjacent normal tissue, resulting in preferential staining of the lesions.

A histochemical study has been undertaken to elucidate the mechanism of tissue staining by B.663. A variety of redox dyes has been applied as supravital stains to frozen sections of skin biopsy specimens obtained from lepromatous lesions and from adjacent normal-appearing skin of several patients with active lepromatous leprosy. The dyes selected possessed a wide range of redox potential, and several differed greatly in chemical structure from that of B.663. Frozen sections of skin biopsy specimens from a stained lepromatous lesion and from adjacent normal-appearing skin were also studied.

Finally, studies of the chemistry of B.663 and of some related redox dyes have been undertaken to examine the possibility that differential staining of normal and lepromatous tissue results from differences in pH rather than from differences in redox potential.

METHODS

Patients. Skin biopsy specimens were studied from four patients with lepromatous leprosy. One of the patients (G.R.) had been shown to be harboring dapsone-resistant *M. leprae* ([10]), and had been under treatment with B.663, 200 mg. daily, for approximately nine months. Many of his lesions were nodular, and had by this time acquired a red-purple color; the adjacent, more normal-appearing skin had a slightly reddish tint. One patient (J.J.) had recently been placed on B.663 after the demonstration of dapsone-resistant organisms; no pigmentation of lesions or normal-appearing skin was yet evident. The two remaining patients (H.J. and S.K.) had been recently diagnosed and admitted to original treatment with dapsone.

Direct microscopy. Skin biopsy specimens were obtained with a 5 mm. Orentreich scalp punch after local infiltration of the skin with 1 per cent lidocaine both from a pigmented nodule and from the adjacent, normal-appearing skin of patient G.R. The specimens were immediately mounted in a

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cryostat.[4] After the tissue specimens were frozen, sections approximately 6 μ in thickness were cut and placed on clean glass microscope slides. The sections were covered with pH 7.4 phosphate buffer, and microphotography was performed. Because of the transparency of these sections, and because staining was so subtle, it was necessary to make the photographs under conditions of greatly reduced light intensity.

Supravital staining. Sections of skin biopsy specimens obtained and processed in the manner described were covered by a 0.01 M solution of one of several redox dyes in pH 7.4 phosphate buffer, and then by a coverslip in order to minimize evaporation. After the sections had stood in contact with the dyes for about 30 minutes at room temperature, photomicrographs were obtained. Additional sections cut at the same time were mounted on slides, fixed with formalin, and stained either with hematoxylin and eosin or by the standard acid-fast stain employed routinely in this laboratory (11).

TABLE 1. *Redox dyes employed.*

Redox dye	Derivative of	Redox potential[a] (E_{m7})
Thymolindophenol	Indophenol	+0.174
Thionine	Phenothiazine	+0.062
Cresyl blue	Phenoxazine	+0.047
Methylene blue	Phenothiazine	+0.011
New methylene blue	Phenothiazine	−0.021
Indigtetrasulfonate	Indigo	−0.046
Indigodisulfonate	Indigo	−0.125
B.663	Phenazine	−0.180
Phenosafranine	Phenazine	−0.252

[a] "Redox potential" here means the potential at 50% reduction at pH 7 (the "midpoint" potential—E_{m7}). The values listed here are taken from "Oxidation—Reduction Potentials of Organic Systems," by W. M. Clark, Williams & Wilkins Co., Baltimore, 1960, except for that of B.663, which is given by Barry *et al.* (1).

[4] International Equipment Company Model CTD International-Harris Cryostat, International Equipment Company, Needham Heights, Mass.

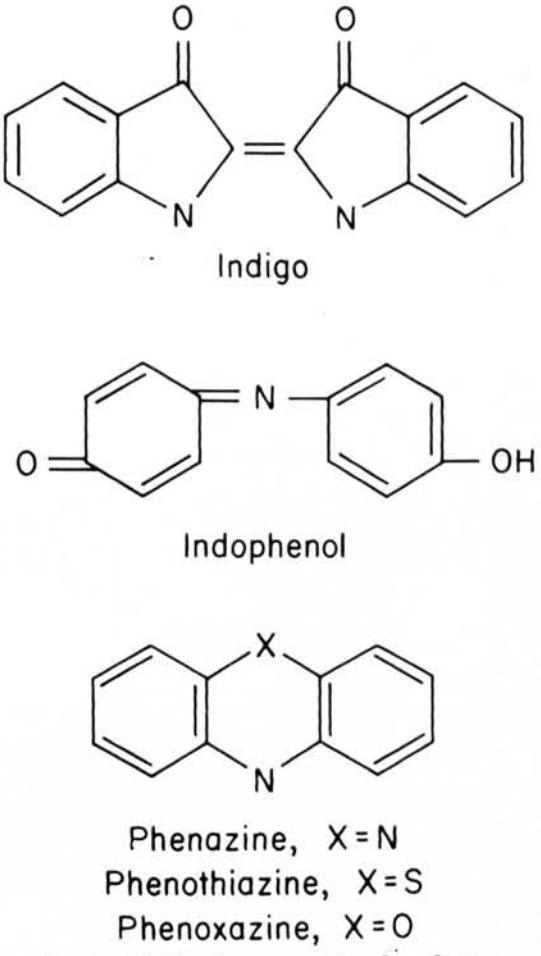


FIG. 1. Basic structures of redox dyes employed.

Redox dyes. The redox dyes employed in this study were selected in such a way that compounds with several completely different chemical structures and a wide range of redox potentials could be compared. The basic chemical structures are illustrated in Figure 1; the compounds are listed in Table 1, along with their structures and redox potentials.

Chemistry of B.663 and related dyes. The apparent acid-base dissociation constants (K_a') of B.663 were estimated from the absorption spectra (employing a Beckman DK2A recording spectrophotometer) of solutions of this compound in H_2SO_4 solutions ranging in concentration from 1.7 per cent to 98 per cent. The hydrogen ion activities of these H_2SO_4 solutions had been previously measured (5). Similar, but

less detailed, studies of methylene blue, phenosafranin, and indigotetrasulfonate were carried out.

RESULTS

Staining by B.663 *in vivo*. Yellowish pigmentation of the section of a skin biopsy specimen obtained from a pigmented lepromatous nodule (patient G. R., after treatment with 200 mg. B.663 daily for 9 months) was quite evident. This pigmentation was difficult to capture and to reproduce in a microphoto (Fig. 2). Pigmentation was most intense in the epidermis, and in a distribution in the dermis suggesting concentration in the lepromatous granuloma. A specimen obtained from adacent normal-appearing skin (Fig. 3) demonstrated, by contrast, only a pale, rather homogenous yellow staining with slightly more intense staining of the epidermis. No selective staining of subcutaneous fat was apparent in either specimen, although the gross specimens were somewhat reddish—both dermis and subcutaneous fat — com-

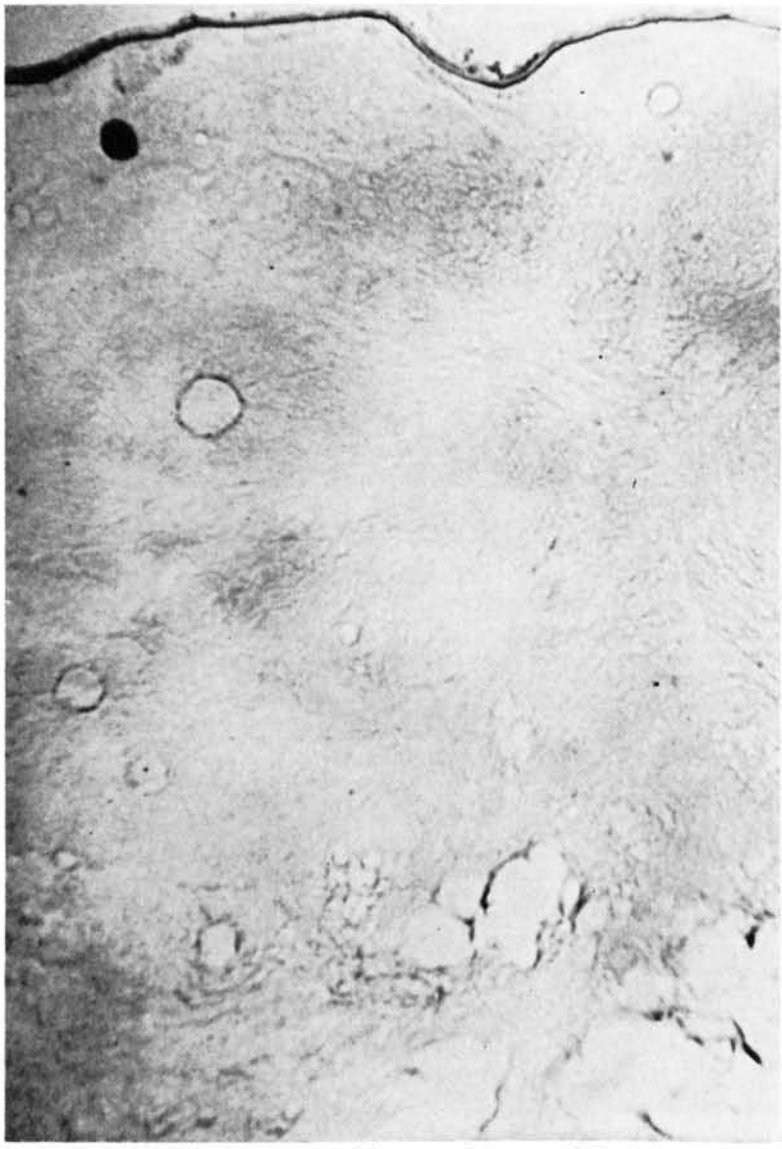


FIG. 2. Frozen section from pigmented lesion of patient G.R. 2.5X. Unstained. Note the staining of the epidermis and the slightly more intense staining of some dermal areas than of others, suggesting localization to areas of granuloma.

TABLE 2. *Absorption maxima of B.663 in various solvents.*

Solvents	Absorption maxima				
	Visible			Ultraviolet	
	λ[a] (mμ)	A_m[b] $\times 10^{-4}$	Color	λ (mμ)	A_m[b] $\times 10^{-4}$
Dichloroethane	449	3.08	orange	287.5	4.52
Ethanol	455	3.63	orange	287	4.82
0.17% H_2SO_4	490	1.26	orange	284	2.60
20% H_2SO_4	530	1.63	red	287.5	2.43

[a] λ is the wave-length of the absorption maximum.
[b] A_m is the molar absorbance—that is, the absorbance of a 1 M solution/cm light path.

pared to specimens obtained from patients being treated with other drugs.

Supravital staining. Supravital staining with several redox dyes of skin biopsy specimens obtained from the lepromatous lesions of three patients resulted in staining of both the epidermis and the lepromatous leproma, but not of surrounding normal dermal tissue (Figs. 4-6). Among these dyes were phenosafranin, new methylene blue, cresyl blue, thionine, and thymolindophenol. Supravital staining with two redox dyes, indigosulfonate and indigotetrasulfonate, on the other hand, resulted in selective staining of the epidermis only.

Chemistry of B.663 and related redox dyes. In a variety of solvents, B. 663 demonstrated the absorption maxima presented in Table 2. These data suggest the existence of four species of B.663: (1) the orange, unionized species which exists in organic solvents; (2) the orange, monoprotonated species which exists in dilute acid (1); (3) the red, doubly-protonated species which exists in maximal concentration in 10-40% H_2SO_4; and (4) a colorless, triply-protonated species, which accounts for the loss of absorbence at 533 mμ in concentrated H_2SO_4.

In Figure 7 are plotted the absorbence at both 533 and 288 mμ of 3.5×10^{-6}M solutions of B.663 in a variety of H_2SO_4 solutions. These data suggest that a species absorbing at both 533 and 288 mμ is present in maximal concentration in H_2SO_4 solution of about 20 per cent. A different species which absorbs strongly at 288 mμ exists in less concentrated H_2SO_4

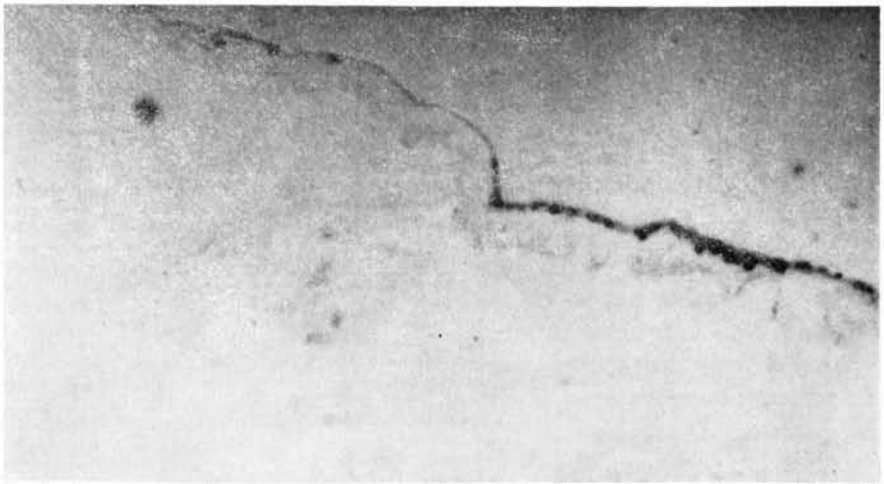


FIG. 3. Frozen section from biopsy specimen of adjacent normal-appearing skin of patient G.R. 2.5X. Unstained. Coloring is limited to the epidermis.

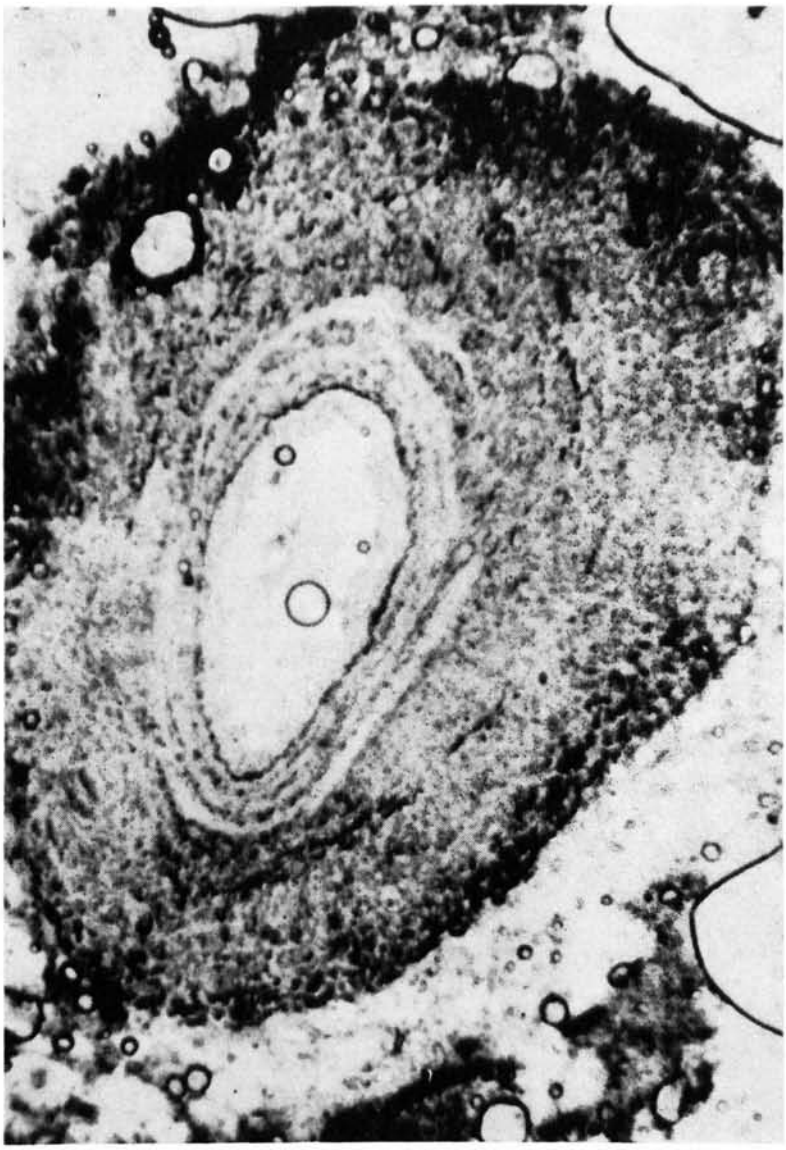


FIG. 4. Frozen section from lesion of patient S.K. 2.5X. Supravital phenosafranin. The perivascular granulomatous process is intensely stained by both of the supravital dyes.

solutions, while a third species which absorbs strongly neither at 533 nor at 288 mμ begins to appear in more concentrated H_2SO_4 solutions. If one may assume that the B.663 is present almost entirely as one (the doubly-protonated) species in 20% H_2SO_4, it is possible to measure the dissociation constants of B.663, according to the equilibrium:

$$\text{B.663}\cdot\text{H}^+ + \text{H}^+ \underset{K^a_2}{\overset{[H_2SO_4] < 20\%}{\rightleftharpoons}} \text{B.663}\cdot 2\text{H}^+ + \text{H}^+ \underset{K^a_1}{\overset{[H_2SO_4] > 20\%}{\rightleftharpoons}} \text{B.663}\cdot 3\text{H}^+$$

1)

orange

red

colorless

A solution of B.663 in 20% H_2SO_4 was diluted so as to yield about the same absorbence at 550 mμ as that produced by an initially equimolar solution of B.663 in 1.7% H_2SO_4, and the difference spectrum between the two solutions was recorded. The difference spectrum between the a 1.69×10^{-5} M solution of B.663 in 1.7% H_2SO_4 and a 1.47×10^{-5} M solution of B.663 in 20% H_2SO_4 (Fig. 8) lies almost entirely above the line of 0 net absorbence. The portion of the difference spectrum above the 0 line represents that substance which is in excess in the 1.69×10^{-5} M solution of B.663 in 1.7% H_2SO_4 ($B.663 \cdot H^+$); the spectrum of the $B.663 \cdot 2H^+$ contained in the 1.7% H_2SO_4 solution of B.663 has been almost completely subtracted out. There is a small excess of a substance absorbing maximally at 550 mμ, which is present in the B.663 solution in 20% H_2SO_4. There was no difference in the absorbence at 550 mμ of a 1.69×10^{-5} solution of B.663 in 1.7% H_2SO_4. and a 1.12×10^{-5} M solution of B.663 in 20% H_2SO_4; if the concentration of $B.663 \cdot 2H^+$ in the 1.69×10^{-5} M solution of B.663 in 1.7% H_2SO_4 is 1.12×10^{-5} M, then the remainder (0.57×10^{-5} M) must be $B.663 \cdot H^+$.

A similar measurement of the concentration of $B.663 \cdot 3H^+$ in a solution of B.663 in concentrated H_2SO_4 was carried out (Fig. 9). There was no difference in the absorbence at 510 mμ between a 1.69×10^{-5} M solution of B.663 in concentrated H_2SO_4 and a 1.32×10^{-5} M solution of B.663 in 20% H_2SO_4, suggesting that the concentration of $B.663 \cdot 3H^+$ in concentrated H_2SO_4 was 0.37×10^{-5} M.

Employing the hydrogen ion activities measured for each of these H_2SO_4 solutions, the first two apparent dissociation constants (K_a') for B.663 may be calculated:

$$2)\quad K^{a}_{1}{}' = \frac{[B.663 \cdot 2H^+]\, aH^+}{[B.663 \cdot 3H^+]} = \frac{(1.32 \times 10^{-5})\,(10^{4})}{(0.37 \times 10^{-5})} = 3.57 \times 10^{4};$$

$$3)\quad K^{a}_{2}{}' = \frac{[B.663 \cdot H^+]\, aH^+}{[B.663 \cdot 2H^+]} = \frac{(0.57 \times 10^{-5})\,(0.60)}{(1.12 \times 10^{-5})} = 0.31$$

Thus, B.663 in solution in 0.31 NH_2SO would be equally divided between singly-protonated $B.663 \cdot H^+$ (orange) and doubly-protonated $B.663 \cdot 2H^+$ (red). The very large $K^{a}_{1}{}'$ indicates that the weakly-colored, triply-protonated species of B.663 accounts for 50 per cent of the B.663 present only at a hydrogen ion activity greater than that found in concentrated H_2SO_4; therefore, a weakly-colored species of fully oxidized B.663 can exist in important concentration only at very large hydrogen ion concentrations. At those hydrogen ion concentrations encountered *in vivo*, only the orange species of B.663 could exist, and differences in pH could not account for differential staining of tissues and cells.

The colors of various ionic species of three other redox dyes chosen from among those employed as supravital stains have been similarly studied. The absorbence of solutions of methylene blue, phenosafranin, and indigotetrasulfonate in a number of concentrations of H_2SO_4 was measured in the recording spectrophotometer, and in a Zeiss PMQ II spectrophotometer; where appropriate, measurements were made at the wave-lengths of the absorption maxima. Methylene blue may be seen (Table 3) to vary from blue to green as the [H+] is increased; there may well be a colorless species which accounts for the generally diminished absorbence in concentrated H_2SO_4. Phenosafranin varies from red to blue to green as the [H+] is increased, whereas indigotetrasulfonate is probably completely ionized at physiologic pH, so that there is no color change as the [H+] is increased. In no case is there a colorless ionic species of the oxidized forms of these redox dyes which could be consistent with differential staining resulting from differences in pH.

DISCUSSION

The therapeutic value of B.663 ([8]) is sharply limited by skin pigmentation which results from its use, and by the fear of toxic effects which may follow the deposition of crystals of the drug in tissues ([9]). Knowledge of the mechanism by which skin pigmentation occurs may assist in the design of analogous compounds which retain the

TABLE 3. *Molar absorbence at indicated wavelengths of several redox dyes in a variety of H_2SO_4 concentrations.*

Molar absorbance $\times 10^{-5}$ / λ (mμ)	% H_2SO_4 5	20	50	98
Methylene blue	.593[b] / 750[a]	.783 / 752	.573 / 764	.047 / 755
	.568 / 672	.415 / 682	.317 / 690	.100 / 680
	.016 / 402	.024 / 402	.042 / 402	.105 / 402
Color	Blue	Green-blue	Blue-green	Green
Phenosafranin	0 / 650	0 / 650	.063 / 650	.090 / 650
	.057 / 575	.198 / 580	.100 / 580	.065 / 580
	.322 / 520	.120 / 530	.045 / 530	.033 / 530
	.020 / 400	.040 / 400	.073 / 400	.112 / 400
Color	Red	Purple	Blue	Green

TABLE 3. *Continued*

	Molar absorbance $\times 10^{-5}$ / λ (mμ)	% H_2SO_4			
		5	20	50	98
Indigo-tetrasulfonate	.142 / 610	.140 / 610	.092 / 615	.092 / 625	
Color		Blue	Blue	Blue	Blue

[a] This number represents the wavelength at which the measurement of molar absorbence has been made. The wavelengths chosen for each substance and each H_2SO_4 concentration are those of the absorption maxima.

[b] This number is the molar absorbence, A_m, defined in a footnote to Table 2, measured at the wavelength noted. This table should be read in the following manner: note that for methylene blue and the absorption maxima in the range 750–764 mμ, A_m decreases as the concentration of H_2SO_4 increases. This is also true for the absorption maxima in the range 672–690 mμ. There is, on the other hand, an increase in A_m with increasing $[H_2SO_4]$ for the absorption maximum at 402 mμ. Solutions absorbing maximally at the higher wavelengths are blue, whereas absorption at the lower wavelengths results in a yellow color. Methylene blue in 98% H_2SO_4, which absorbs equally at 402 and 680 mμ therefore exhibits a green color, which is not intense, because the A_m at each of these wavelengths is low. The data for phenosafranine and indigo-tetrasulfonate may be similarly interpreted.

antimicrobial activity of the drug without causing skin pigmentation to the same degree. This work does not relate to the melanosis, which has been described as an accompaniment of B. 663 therapy ([2]). The structural similarity of B.663 to chlorpromazine, which is known to promote melanosis, suggests a similar mechanism ([7]). These experiments relate to the specific staining of skin lesions by the drug.

It would be desirable to establish that uptake of a dye by a thin section of skin *in vitro* occurs by the same mechanism as does uptake *in vivo*. The fact that the activity of various enzymes can be demonstrated histochemically in frozen sections ([6]) suggests that the tissues studied in these experiments may be expected to remain viable according to at least one criterion of viability. On the other hand, the uptake of certain dyes by cells *in vitro* is taken as evidence of nonviability ([4]). The applicability of these experiments *in vitro* to the study of the staining of tissues by B.663 *in vivo* gains support from the following considerations: (1) specific staining of the cells characteristic of the lepromatous lesion by the several dyes applied supravitally appears to parallel the specific staining of lesions which occurs when methylene blue and B.663 are administered to patients with lepromatous leprosy; (2) selective staining of the lepromatous cells by some dyes but not by others suggests that the uptake of dye by the cells studied here was a process possessing a degree of specificity. Passive diffusion of dyes across the permeable cell membranes of nonviable cells might be expected to permit staining of the lepromatous cells by all rather than by merely some of the dyes studied here; and (3) the distribution within the sections of the dyes applied supravitally appears the same as for B.663 administered *in vivo*.

The observations reported here suggest that differential pigmentation of lesions is neither a function of tissue redox potential nor of tissue pH; rather, differential skin staining appears to result from selective uptake of the compound by tissue elements involved in the pathologic process. This conclusion is supported by the demonstra-

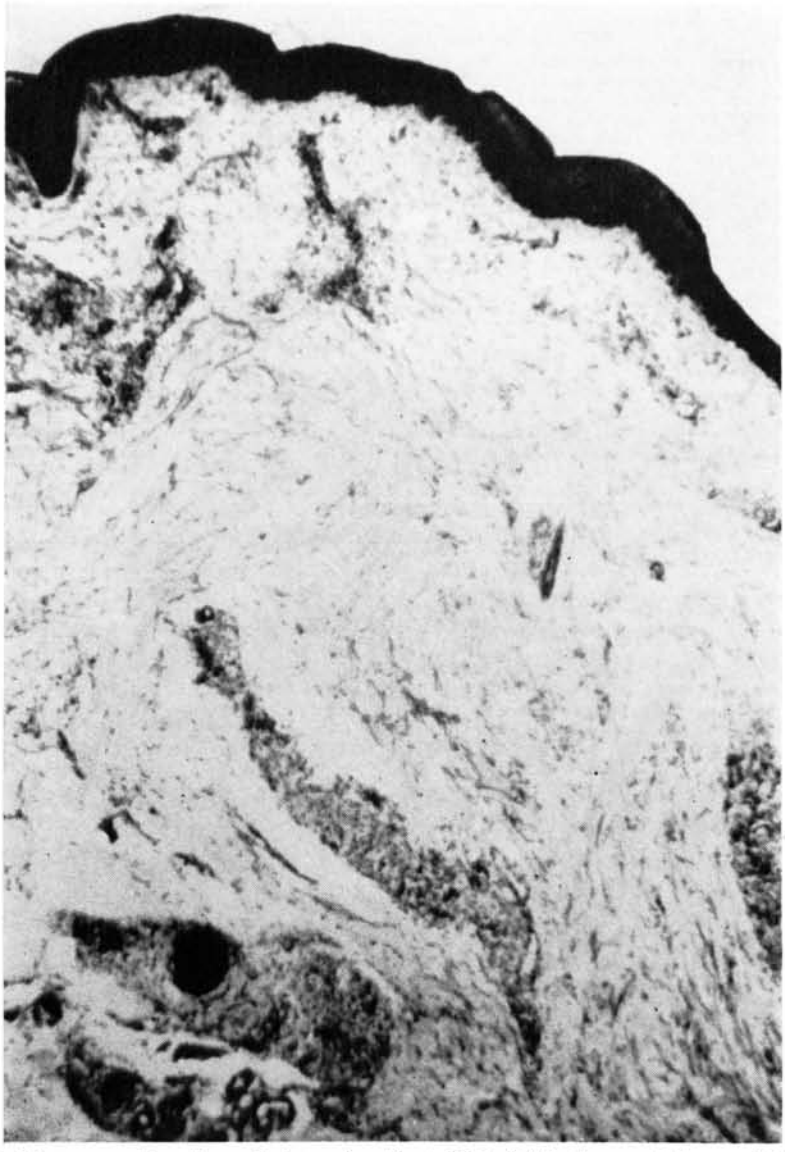


FIG. 5. Frozen section from lesion of patient H.J. 2.5X. Supravital cresyl blue. The granulomatous process is selectively stained by the supravital dye.

tion of B.663 uptake (clearance) by the cells of the reticuloendothelial system ([3, 12]).

If selective uptake is essential to the action of the drug, perhaps by producing a higher concentration of the drug in the environment of the *M. leprae*, then it is also important to learn if less intensely colored analogs of the drug can be prepared which will be selectively taken up and which are potent antimicrobial substances, but which will not lead to so intense skin pigmentation. If selective uptake is not essential to the action of B.663, then a more soluble derivative which retains antimicrobial potency but which is not concentrated in the lesions may be desirable. Experiments designed to study these questions are currently in progress in this laboratory.

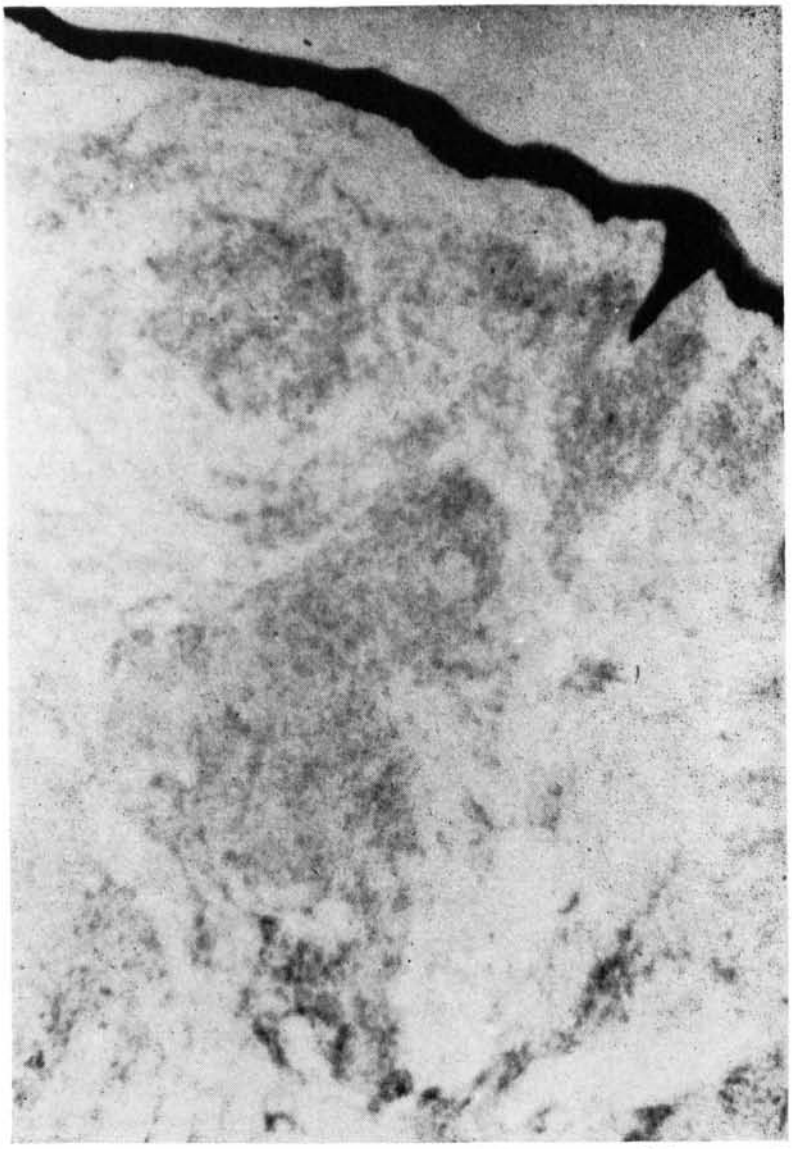


FIG. 6. Frozen section from lesion of patient J.J. 2.5X. Supravital thionine. The granulomatous process is stained by supravital thionine but not by indigo-tetrasulfonate.

SUMMARY

In an attempt to elucidate the mechanism of selective staining of lepromatous lesions by the phenazine dye, B. 663, a variety of studies have been carried out. Direct microscopy of frozen sections of a lepromatous nodule obtained by skin biopsy from a patient under treatment with this compound revealed staining to be more intense in the epidermis and in the lepromatous granuloma than in the adjacent normal dermis. Supravital staining of frozen sections of biopsy specimens of lepromatous lesions with a variety of redox dyes dissolved in neutral phosphate buffer revealed selective staining, also of epidermis and leproma, by the phenazine and phenothiazine dyes studied and also by thymolindophenol, whereas indigo-di- and tetrasulfonate did not stain in a similar pattern; selective staining appeared to de-

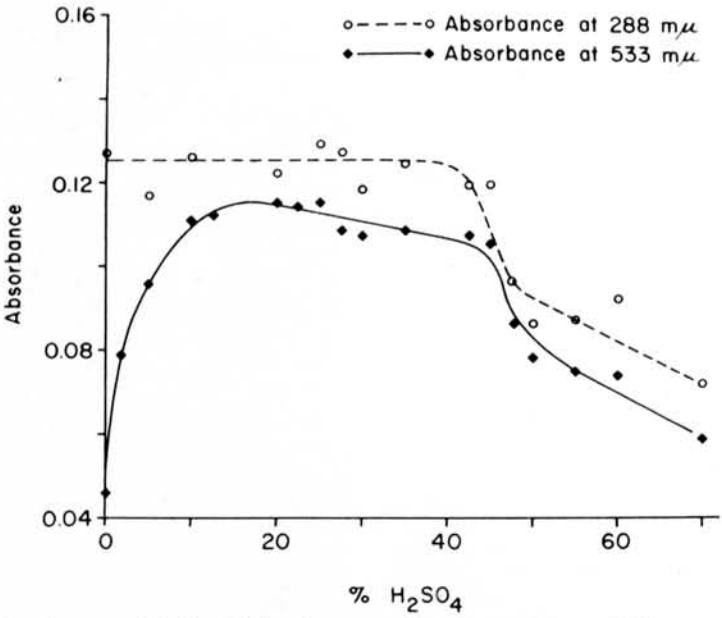
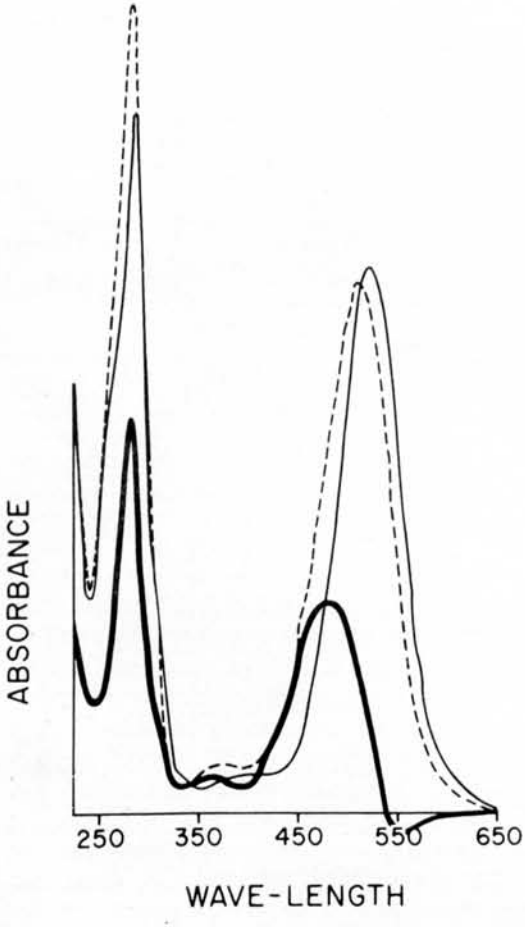


FIG. 7. Absorbence of 3.5 x 10^{-6} solutions of B.663 at 288 and 533 mμ as a function of H_2SO_4 concentration.



pend more on chemical structure than on redox potential. Study of the absorption spectra of the several ionic species of B.663 and of several other redox dyes demonstrated that the selective staining did not depend on differences in pH between lepromatous granuloma and adjacent normal tissue. It is concluded that staining of lepromatous lesions during B.663 therapy results from selective concentrations of the dye by tissue elements present in the lesion. This phenomenon may be essential to the therapeutic effect of the drug.

FIG. 8. Difference spectrum between 1.69 x 10^{-5} M B.663 in 1.7% H_2SO_4 and 1.47 x 10^{-5} M B.663 in 20% H_2SO_4 (heavy line); this represents the spectrum of B.663·H^+ (see text). Spectra shown for contrast are: (a) that of 3.5 x 10^{-6} M in 1.7% H_2SO_4 (broken line), and (b) that of 3.5 x 10^{-6} M B.663 in 20% H_2SO_4 (light line);

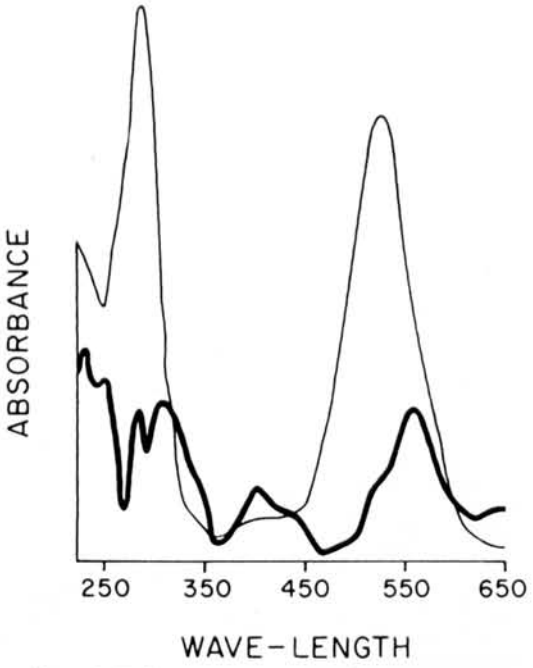


FIG. 9. Difference spectrum between 1.69 x 10^{-5} M B.663 in concentrated H_2SO_4 and 1.32 x 10^{-5} M B.663 in 20% H_2SO_4 (heavy line); this represents the spectrum of B.663·3H$^+$ (see text). The spectrum shown for contrast is that of 3.5 x 10^{-6} M B.663 in concentrated H_2SO_4 (light line).

RESUMEN

Se llevaron a cabo una serie de investigaciones con el propósito de aclarar el mecanismo de tinción selectiva de las lesiones lepromatosas por el colorante fenazínico B.663. La microscopía directa de cortes por congelación de un nódulo lepromatoso obtenido por biopsia de piel de un paciente bajo tratamiento con este compuesto reveló que la coloración era más intensa en la epidermis y en el granuloma lepromatoso que en el dermis normal adyacente. Tinciones supravitales de cortes por congelación de biopsias de lesiones lepromatosas con una variedad de colorantes redox disueltos en solución tampón-fosfato neutra revelaron que los colorantes fenazínicos y fenotiazínicos, como asimismo el timolindofenol, coloreaban también en forma selectiva la epidermis y el granuloma, mientras que el di y tetrasulfonato de índigo no coloreaba en la misma forma; la coloración selectiva parecía depender más de la estructura química que del potencial redox. El estudio del espectro de absorción de las varias especies iónicas de B.663 y de varios otros colorantes redox demostraron que la coloración selectiva no depende de las diferencias de pH entre el granuloma lepromatoso y el tejido normal adyacente. Se concluye que la coloración de las lesiones lepromatosas durante la terapia con B.663 es consecuencia de concentraciones selectivas de colorante en los elementos tisulares que se encuentran en la lesión. Este fenómeno puede ser esencial para el efecto terapéutico de la droga.

RÉSUMÉ

On a procédé à une série d'études, en vue d'essayer d'élucider le mécanisme de la coloration sélective des lésions lépromateuses par le colorant dérivé de la phénazine, le B.663. L'examen microscopique direct de coupes congelées d'un nodule lépromateux obtenu par biopsie cutanée chez un malade en traitement par ce produit, a révélé une coloration plus intense au niveau de l'épiderme et du granulome lépromateux qu'au niveau du derme normal adjacent. La coloration vitale des coupes congelées d'échantillons biopsiques de lésions lépromateuses, par toute une série de colorants oxydo-réducteurs dissous dans un tampon phosphate neutre, a révélé une coloration sélective de l'épiderme et du granulome, à la fois par les colorants dérivés de la phénazine et par ceux de la phénothiazine qui ont été étudiés, et par le thymolindophénol; par contre, le disulphonate indigo et le tétrasulfonate indigo n'entrainaîent pas de coloration similaire. La coloration sélective s'est révélée dépendre davantage de la structure chimique que du potentiel oxydo-réducteur. L'étude des spectres d'absorption de différences analogues ioniques du B.663 et de plusieurs autres colorants oxydo-réducteurs a permis de démontrer que la coloration sélective ne dépendait pas de différent dans le pH entre le granulome lépromateux et le tissu normal adjacent. On en conclut que la coloration des lésions lépromateuses au cours de la thérapeutique par le B.663 résulte de la concentration sélective du colorant par les élements tissulaires présents dans la lésion. Ce phénomène pourrait être essentiel à l'effet thérapeutique du produit.

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