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SOME STRUCTURAL FEATURES OF VITAL DYES

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Lymphocytes and macrophages of healthy rats, cells of lymphosarcoma and ovarian tumors (strain OYa) of rats, and cells of sarcoma 37 and lymphosarcoma Nk/Ly of mice were stained intravitally with $1 \cdot 10^{-5}$-$3 \cdot 10^{-2}$ M solutions of 86 dyes. The presence of alkylating amino groups in the molecule of the dye substantially increases its ability to penetrate to living cells and to be deposited in their cytoplasm. Acid radicals considerably reduce the ability of the dye to stain cells intravitally. This has been shown for thiazine, oxazine, triphenylmethane, acridine, diazine, and xanthene compounds. The degree of basicity of the dye molecule and also of its amino group does not play a decisive role in the process of vital staining.

KEY WORDS: *vital dyes; intravital microscopy; cell membranes; permeability.*

The problem of interaction between living cells and dyes has been studied for many years. Nevertheless, many aspects of the chemistry of vital staining still remain unexplained. It has been noted that methylation of the amino groups of some basic dyes can increase their ability to stain cells intravitally [13]. However, the choice of vital dyes is still made empirically.

The object of this investigation was to demonstrate the existence of a definite relationship between the chemical structure of dyes and their ability to stain normal and, in particular, tumor cells intravitally.

EXPERIMENTAL METHOD

Cells of lymphosarcoma and ovarian tumors (strain OYa) and lymphocytes of rats, and also cells of sarcoma 37 and lymphosarcoma Nk/Ly of mice were stained intravitally *in vitro* with $1 \cdot 10^{-5}$-$3 \cdot 10^{-2}$ M solutions of 86 basic and acidic dyes (in Hanks's solution) at 18-20°C at pH 6.8-7.2. The list of dyes tested is given in Table 1.

The results of staining were read 1-3 min after the beginning of contact between the cells and the dye by visual examination under the microscope. The rat macrophages were stained by injecting the dye into the animal in a special chamber for intravital microscopy *in vivo* [9].

To determine the toxicity of the various dyes, the viability of stained lymphocytes and lymphosarcoma cells was tested in tissue culture, and the viability of the tumor cells was tested also by subcutaneous inoculation of animals and in special chambers for intravital microscopy *in vivo* [6, 9].

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TABLE 1. Dyes Tested

Group 1		Group 2
Acid anthraquinine blue	Fast red	Acridine orange
Acid fuchsin	Fluorescein	Auramine OO
Acridine yellow	Gallamine blue	Azure I
Alcian blue	Gallocyanin	Azure II
Alizarine red S	Indophenol	Bismarck brown G
Amido black	Indigocarmine	Bismarck brown
Aniline blue w/s	Janus black	(Basic brown 2K)
Anthracene blue	Janus blue	Bright cresyl blue
Azocarmine G	Light green	Brilliant green
Azafuchsin	Lithium carmine	Chrysoidine
Basic fuchsin	Malachite green sulfate	Crystal violet
Basic parafuchsin	Methyl orange	Dahlia violet
Benzo purpurine 4B	Methyl red (sodium salt)	Gentian violet
Betamine blue	Methylthymol blue	Janus green B
Biryuzov direct	Naphthol green B	Malachite green
Bright Rhine blue 5G	Naphthol yellow	Methylene blue
Brilliant Congo G	Orange G	Methylene green B
Brilliant yellow	Phenol red	Methyl green
Bromphenol blue	Remazol red-violet F	Methyl violet
Bromphenol red	Remazol yellow G	Neutral red
Brompyrogallol red	Potassium eosin	Nile blue sulfate
Bromthymol blue	Potassium indigodisulfonate	Pyronine B
Congo red	Quinoline yellow w/s	Pyronine G
Cresol red (azo dye)	Remazol black B	Rhodamine 6G
Cresol red (triphenylmethane indicator)	Remazol brilliant blue F	Rhodamine S
Diamond fuchsin	Remazol red B	Toluidine blue
Eosin yellowish w/s	Safranin T	Toluylene blue
Evans' blue	Thionine	
Fast acid marine blue	Trypan blue	
	Trypan red	
	Water blue (Patent blue V)	

Note: w/s) water-soluble.

Legend. 1. The dyes used in the investigation were manufactured by: I. G. Farbenindustrie (from the collection of the Department of Dye Technology, Lensovet Leningrad Technological Institute), Reanal (Hungary), E. Merck A. G., Darmstadt (West Germany), Fluka A. G. and Buchs S. G. (Switzerland), Chemapol (Czechoslovakia), Research Institute of Organic Semi-finished Products and Dyes (NIOPIK, USSR), and Voikov Chemical Factory (USSR). The grades of the substances used were: "rein" (pure), "microscopic stain," "ch. d. a." ["analytically pure"], and "ch." ["pure"].

2. Group 1: dyes not staining viable rat lymphosarcoma cells without additional injury; group 2: dyes staining viable rat lymphosarcoma cells without any additional injury. The dyes listed at the end of group 2 (after the line) [as in Russian original, no line shown on Table — Consultants Bureau] are those not giving a constant effect.

EXPERIMENTAL RESULTS

Vital staining of the various objects tested followed in general a very similar course. Some compounds stained fully viable cells (Table 1, dyes of group 2). No additional factor damaging the cell was necessary for the action of these substances. They include dyes whose action is inconstant (mainly as applied to lymphosarcoma cells). Although they do not strain completely intact cells, they evidently can penetrate into them following the slightest injury.

TABLE 2. Structural Differences between Basic Dyes and Their Ability to Stain Cells Intravitally

No.	Class of compound	Number according to Colour Index [11]	Name of compound	Presence of alkylated amino groups	Presence of hydrophilic radicals	Ability to stain living cells †
1		52,000	Thionine	Absent	Absent	–
2	Thiazine	52,005	Azure I	Present	"	++
3		52,015	Methylene blue	"	"	++
4		52,040	Toluidine blue	"	"	++
5		52,020	Methylene green	"	"	+
6		51,010	Fast cresyl blue	Present	Absent	++
7	Oxazine	51,180	Nile blue sulfate	"	"	++
8		51,045	Gallamine blue	"	"	–
9		51,030	Gallocyanin	"	"	–
10	Acridine	46,025	Acridine yellow	Absent	Absent	–
11		46,005	Acridine orange	Present	"	++
12		42,500	Basic parafuchsin	Absent	Absent	–
13		42,510	Basic fuchsin	"	"	–
14	Triphenylmethane	(12) 783	Gentian violet	Present	"	++
15		42,555	Crystal violet	"	"	++
16		42,585	Methyl green	Present*	Present	+
17		42,000	Malachite green	"	Absent	++
18		42,040	Brilliant green (oxalate)	"	"	++
19		42,530	Dahlia violet	"	"	++
20		41,000	Auramine OO	Present	Absent	++
21		45,005	Pyronine G	Present	Absent	++
22	Xanthene	45,010	Pyronine B	"	"	++
23		45,050	Rhodamine S	"	"	++
24		45,160	Rhodamine 6G	"	"	++
25		49,410	Toluylene blue	Present	Absent	++
26		50,040	Neutral red	Present	Absent	+
27	Diazine	50,240	Safranin T	Absent	"	–
28		50,085	Azocarmine T	"	"	–
29		11,050	Janus green B	Present	"	++
30		11,825	Janus black	Absent	Present	–
31		12,210	Janus blue	"	"	–
32		11,270	Chrysoidine	Absent	Absent	+
33	Azo	21,000	Bismarck brown G	"	"	++
34		21,010	Bismarck brown R	"	"	+

Legend. All dyes except No. 7 and No. 18 are hydrochlorides.
*A quaternary nitrogen group is present in the dye molecule.
†The intensity of vital staining is shown first in relation to rat lymphosarcoma cells. Vital staining of other types of cells differed only in unimportant details. ++) Intense staining of all or the overwhelming majority of cells (staining of the nucleolus, granule formation in the cytoplasm, often adsorption of the dye on the nuclear membrane); +) moderate and weak staining of cells (moderate or slight formation of dye granules in the cytoplasm of the overwhelming majority of cells without adsorption on the nuclear membrane); –) no staining of the overwhelming majority of cells.

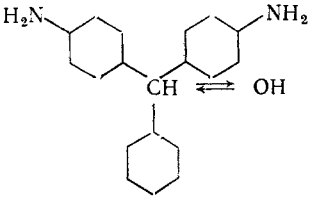
The dyes included in group 1 (Table 1) stained cells only after additional injury, whether thermal, mechanical (centrifugation), or chemical (for example, prolonged exposure to the dye). These compounds are naturally best used to demonstrate injured cells [4]. Differences between the two groups of dyes distinguished in Table 1 are clearly visible if $6 \cdot 10^{-3}$-10^{-3} M solutions are used.

TABLE 3. Ionization Constants of Some Dyes*

Dye	Ionization constants‡		Ability to stain living cells	Temperature, °C	Literature citations
	pKa	pKh			
Debner's violet†	12,7	5,4	—	—	[3]
Malachite green	11,8	6,94	++	18	[1, 2]
Basic para-fuchsin		7,6	—	18	[3]
Basic fuchsin	11,7	7,8	—	18	[3]
Methyl green		6,2	+	—	[2]
Gentian violet (basic violet K)		9,4	++	—	[2]
Crystal violet	12,7	9,69	++	—	[1]
Thionine	11,9		—	—	[8]

*See explanation to Table 2.

†Debner's violet was not tested as a vital dye.



Its hydrolysis constant pKh = 5.4 shows that in a neutral medium it exists almost entirely in the form of the carbinol R≡C—OH, which is very sparingly soluble in water and cannot stain cells. Nevertheless, the basicity constant of the amino group on Debner's violet pKa = 12.7, which is equal to pKa of crystal violet which stains cells well, shows that the properties of this dye relative to living cells are independent of the degree of basicity of the amino group.

‡pKa is the constant of basicity of the amino group; pKh the hydrolysis constant of the dye (the constant of carbinol formation $R \equiv C \rightleftarrows R \equiv C—OH$).

The presence of alkylating amino groups in the dye molecule substantially increases its ability to penetrate into living cells and to be deposited in their cytoplasm. Conversely, acid radicals or groups reducing the basic properties of the dye molecule, and also the presence of a quaternary nitrogen atom, substantially prevents vital staining of intact cells. This was shown for the thiazine, diazine, oxazine, triphenylmethane, acridine, and xanthene compounds. Some azo and diazo dyes, despite the absence of alkylating amino groups, penetrated easily into living cells (Table 2).

On the appearance of methyl radicals in the ortho or para position relative to amino groups (Bismarck brown R) the ability of the substance to stain cells of the lymphoid series (lymphocytes, rat lymphosarcoma cells, mouse Nk/Ly tumor cells) was somewhat reduced, whereas for epithelial tumors (cells of rat ovarian carcinoma) this property of the dye was completely preserved.

Neutral red stained rat macrophages very intensely and lymphocytes and tumor cells much less so. Meanwhile, toluylene blue, which is similar in its structure to neutral red, was

much more easily adsorbed in the cytoplasm of all the cells tested. It is interesting to note that toluylene blue is readily oxidized to neutral red [7]. Experiments showed that the reaction proceeds more rapidly intracellularly than outside the cell. The neutral red formed in the cells remains there for a long time and can easily be detected under the microscope. This shows once again that differences in the behavior of the dyes of groups 1 and 2 were due to their "selection" by the cell and not to the character of their interaction with the cytoplasm [5].

The degree of basicity of the dye molecule and its amino group do not play a decisive role in the process of vital staining (Table 3). This indicates that the dye is transported through the outer membrane in the nonaqueous phase. The basic dye perhaps combines with a lipid of the outer cell membrane, which acts as a carrier. In the course of the reaction a complex may be formed, with transfer of charges [10]. Under these circumstances the tertiary nitrogen atom of the alkylated amino group, on which the excess of the electronic charge is concentrated, may act as electron donor. The primary (unsubstituted) amine in this case will not be active. Electron acceptors are present in the lipids in sufficient amount (for example, as choline).

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