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INHIBITION OF HEMOLYSIS BY TRICYCLIC DYESTUFFS—FLUORESCEIN–PHENOTHIAZINE ANTAGONISM

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Abstract—Several dyestuffs with a condensed three-ring molecular structure inhibit hemolysis induced by phenothiazines or other tricyclic amines *in vitro*. The fluorescein derivative eosin B appears to be one of the most potent phenothiazine antagonists. Eosin B inhibited *in vitro* the premature breakdown of the abnormally fragile erythrocytes of a person with a hereditary nonspherocytic hemolytic anemia associated with a membrane defect.

SEVERAL GROUPS of heterocyclic dyestuffs have in common a condensed three-ring nucleus with a side chain attached to the center ring. These chemicals resemble, therefore, structurally the pharmacologically important group of phenothiazines, several members of which are extensively used as major tranquilizers and antihistaminics. The structural relationship of phenothiazines and tricyclic dyestuffs raises the possibility of their synergism or antagonism with respect to such pharmacologic actions as tranquilization, hemolysis[1] or local anesthesia.[2] Many heterocyclic dyestuffs are used as biologic stains.[3] Some, e.g. the fluoresceins rose bengal, merbromin and fluorescein sodium (uranine), have also found topic or systemic medical application.[4]

The pharmacology of the fluoresceins, especially eosin Y, rose bengal, erythrosin and fluorescein sodium, has been explored by Kudo and Jodlbauer,[5] Rost,[6] Jodlbauer and Haffner,[7,8] Blum[9] and others who found that these fluorescent dyes act as hemolysins when irradiated with ultra-violet light or in darkness when present at a high concentration or in combination with hydrogen peroxide. Concentrations greater than those required for hemolysis precipitated the organic material of red cell suspensions.[7–9]

The present report shows that several fluoresceins and other related dyestuffs are capable of another interesting pharmacologic action hitherto not described, namely the prevention of both the spontaneous hemolysis of pathologically fragile erythrocytes and the inhibition of chemically induced hemolysis of normal erythrocytes.

METHODS

Under strict sterile precautions, aliquots of 44 μl of fresh, washed human red cells were incubated at 37° in $6{\cdot}7 \times 10^{-2}$ M Sörensen buffer, pH 7·60, for 24 hr in darkness. The incubating suspensions, total volume 2·12 ml each, contained $9{\cdot}4 \times 10^{-3}$ M glucose, 3×10^{-3} M magnesium chloride, dye (usually 5×10^{-4} M)

and various concentrations of either chlorpromazine hydrochloride (10^{-5} M to 5×10^{-3} M) or another hemolysin. The dye was omitted in the control suspensions. Hemolysin, red cells and buffered solution were mixed simultaneously. The extent of hemolysis in each incubated tube was measured spectrophotometrically at 415 nm in a Coleman model 124 double-beam spectrophotometer. Details of the method have already been described.[10]

To evaluate the effect of eosin B on stored blood, aliquots of 0·1 ml of whole blood were suspended in $6{\cdot}7 \times 10^{-2}$ M Sörensen buffer, pH 7·60, containing 10^{-3} M glucose and eosin B concentrations ranging from 5×10^{-7} M to 10^{-3} M.

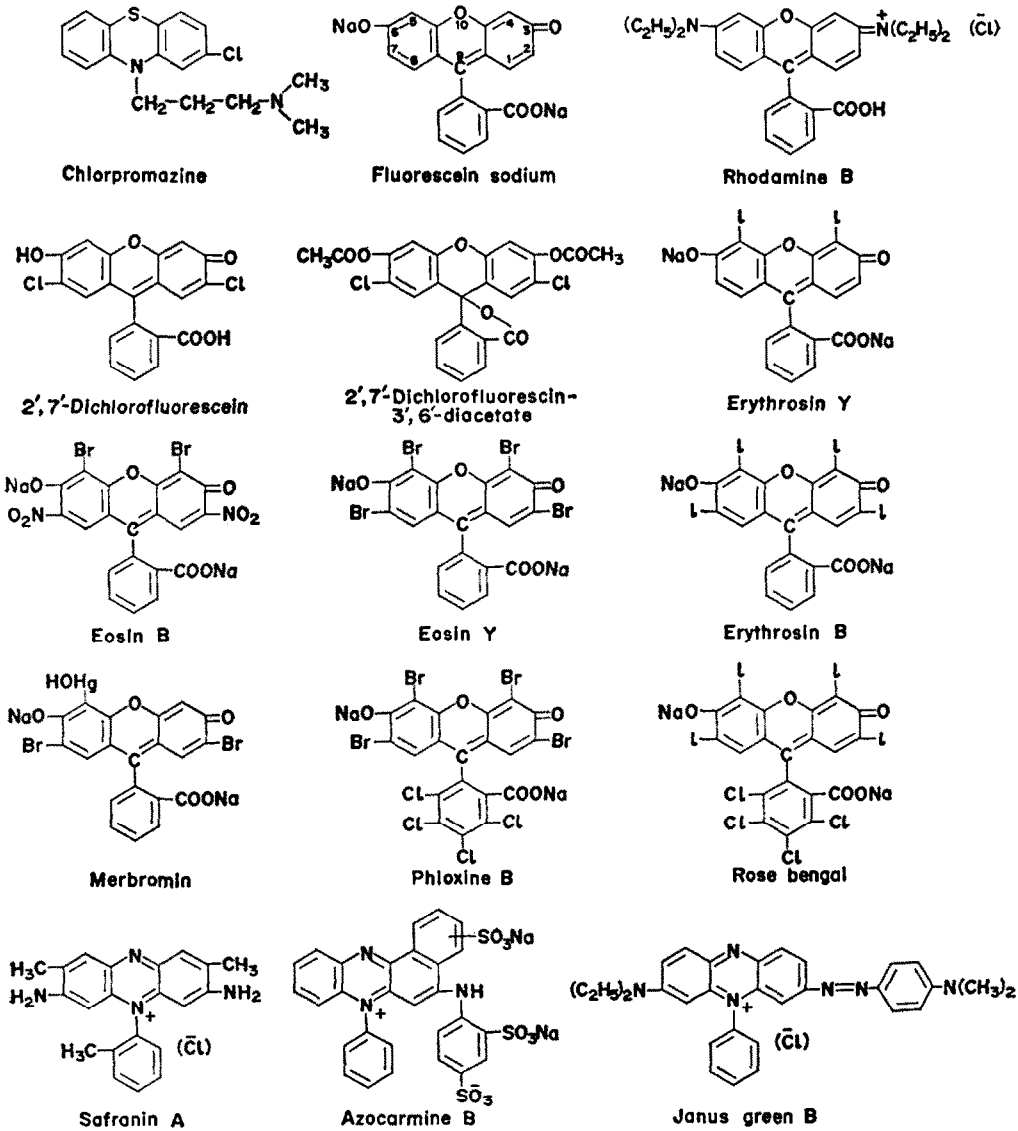

FIG. 1

Figure continued on the following page.

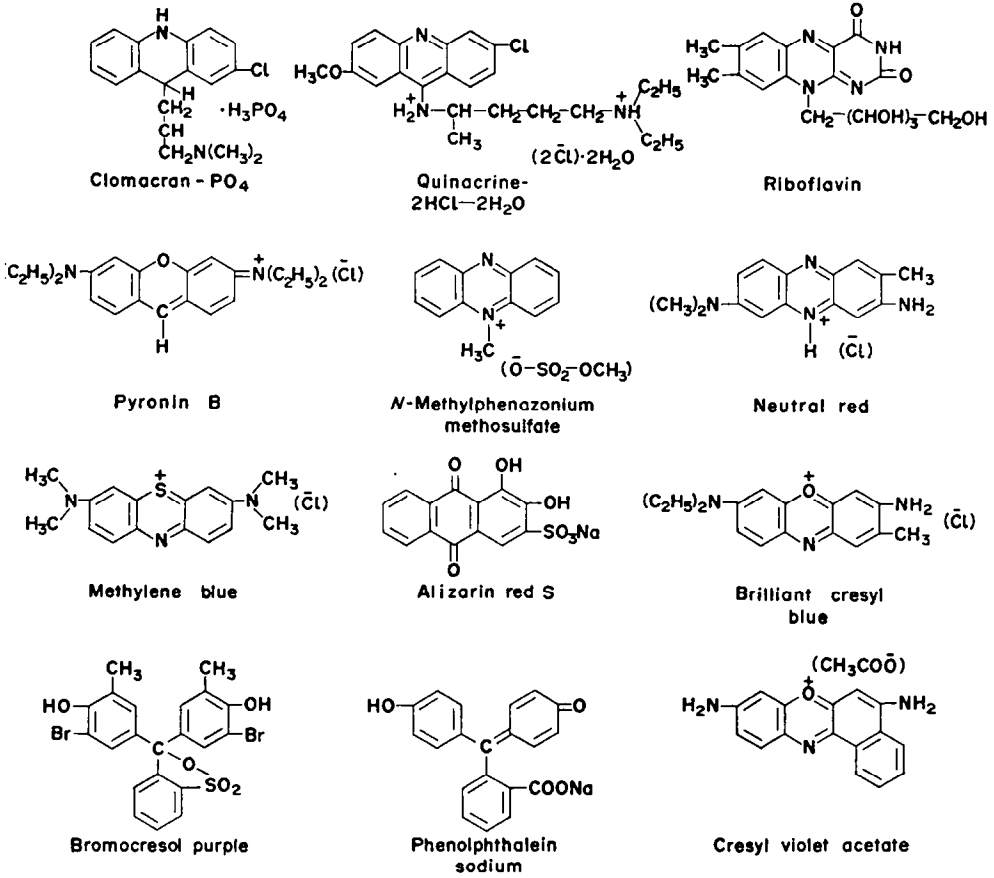

FIG. 1 (CONT.)

FIG. 1. Molecular structures of chlorpromazine and dyestuffs. The structures of all fluoresceins with the exception of 2′,7′-dichlorofluorescin-3′,6′-diacetate are presented in the quinoid form regardless of whether the quinoid or the lactoid form occurs predominantly in solution.

The percentage of hemolysis was determined after storage for 5 days at 37° or for 6 weeks at 4°. Thin-layer chromatography was performed on Gelman ITLC type SG sheets in *n*-propanol–formic acid (v/v 80:20).

The investigated dyestuffs included alizarin red S, fluorescein sodium (uranine), neutral red, rhodamine B (Allied Chemical Corp.); 4′,5′-dichlorofluorescein, pyronin B (J. T. Baker Chemical Company); safranine A (The Coleman & Bell Company); 4′,5′-dibromofluorescein, 2′,7′-dichlorofluorescein, 2′,7′-dichlorofluorescin-3′,6′-diacetate, 4′,5′-diiodofluorescein (erythrosin Y), Janus green B, phloxine B (Eastman Organic Chemicals); riboflavin (General Chemicals Inc.); merbromin (Hynson, Westcott, Dunning Inc.); 2′,4′,5′,7′-tetrachlorofluorescein, *N*-methylphenazonium methosulfate (phenazine methosulfate) (K & K Laboratories); azocarmine G, eosin bluish (4′,5′-dibromo-2′,7′-dinitrofluorescein, eosin B), phenothiazine, rose bengal (Matheson, Coleman & Bell); azocarmine B, eosin bluish, eosin yellowish (2′,4′,5′,7′-tetrabromofluorescein, eosin Y), erythrosin bluish (2′,4′,5′,7′-tetraiodofluorescein, erythrosin B) (National Aniline & Chemical Company Inc.).

The following drugs were supplied through the courtesy of the manufacturers: clomacran phosphate (SK & F 14336) (Bristol Laboratories); imipramine (Geigy Pharmaceuticals); amitriptyline (Merck, Sharp & Dohme); thioridazine (Sandoz Pharmaceuticals); acetophenazine (Schering Corp.); chlorpromazine, prochlorperazine (Smith, Kline & French Laboratories); quinacrine dihydrochloride dihydrate (atabrine, atebrin) (Sterling-Winthrop Research Institute); triflupromazine (Squibb Institute for Medical Research); pyrathiazine (The Upjohn Company); fluphenazine (White Laboratories); propiomazine (Wyeth Laboratories).

RESULTS

The molecular structures of the dyestuffs are presented in Fig. 1. The effect of dyes on the log–dose response (LDR) of red blood cells to the hemolysin chlorpromazine (CPZ) is demonstrated in Fig. 2. The midpoints of the LDR curves (ED_{50}, average for CPZ $6{\cdot}3 \times 10^{-5}$ M) were used for the calculation of the lysis (LI), inhibition (II) or synergism indices (SI). The LI (ratio $ED_{50_{CPZ}}$ to ED_{50_X}) expresses

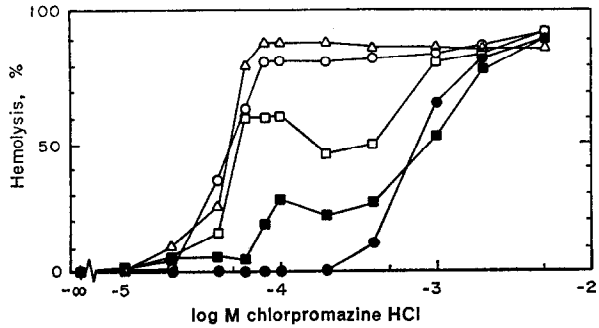

FIG. 2. Inhibition of chlorpromazine-induced hemolysis by fluorescein derivatives (5×10^{-4} M). ○, no inhibitor; △, 2′,4′,5′,7′-tetrachlorofluorescein; □, 2′,4′,5′,7′-tetrabromofluorescein (eosin Y); ■, 2′,4′,5′,7′-tetraiodofluorescein (erythrosin B); and ●, 4′,5′-dibromo-2′,7′-dinitrofluorescein (eosin B).

the potency of a hemolysin (X) in relation to the potency of the arbitrarily chosen standard, CPZ.[10] The II (ratio $ED_{50_{CPZ}}$ to $ED_{50_{CPZ+X}}$) indicates the potency of a dye in preventing CPZ-induced hemolysis. If no inhibition of CPZ and no synergism occurred, the II of a dye is equal to one. If a dye enhanced the hemolytic activity of CPZ, the resulting II is less than one. In the latter case, the SI which represents the reciprocal value of the II is listed in Table 1. The data in Table 1 represent averages of multiple determinations obtained with different human blood samples. The inhibitory activity of dyes was tested at 5×10^{-4} M with the exception of compounds that were either insoluble or induced hemolysis by themselves at the mentioned concentration. If an LDR curve was shaped other than sigmoid, as for instance was the case with eosin Y or erythrosin as inhibitor (Fig. 2), the ED_{50} was determined by extrapolation of the upper part of the curve to the 50 per cent hemolysis line. The percentage level at which the break occurred in the CPZ–LDR curve with eosin Y as inhibitor varied with the blood sample but showed a persistent tendency to either a high, medium or low percentage for any one person.

TABLE 1. LYSIS INDICES AND INHIBITION OR SYNERGISM INDICES OF DYESTUFFS*

	Lysis index	Inhibition index	Synergism index	Molar concn
Xanthenes				
Fluorones				
Fluorescein sodium (uranine)	0·008	1·0		5×10^{-4}
2′,7′-Dichlorofluorescin-3′,6′-diacetate	0·013	3·91		5×10^{-4}
2′,7′-Dichlorofluorescein	0·003	1·16		5×10^{-4}
4′,5′-Dichlorofluorescein	0·003	2·53		5×10^{-4}
4′,5′-Dibromofluorescein	0·003	1·58		5×10^{-4}
4′,5′-Diiodofluorescein (erythrosin Y)	0·003	12·8		5×10^{-4}
2′,4′,5′,7′-Tetrachlorofluorescein	†	1·20		5×10^{-4}
2′,4′,5′,7′-Tetrabromofluorescein (eosin Y)	0·014	12·47‡		5×10^{-4}
2′,4′,5′,7′-Tetraiodofluorescein (erythrosin B)	0·09	13·3		5×10^{-4}
4′,5′-Dibromo-2′,7′-dinitrofluorescein (eosin B)	0·008	19·4		5×10^{-4}
Phloxine B	0·45		2·04	10^{-5}‡
Rose bengal	5·11		1·94	5×10^{-6}‡
Merbromin	3·43		1·39	10^{-6}‡
Rhodamines				
Rhodamine B	0·07	1·38		10^{-4}
Pyronins				
Pyronin B	0·14		1·24	5×10^{-4}
Phenazines				
Azins				
N-methylphenazonium methosulfate (phenazine methosulfate)	0·036	1·0		5×10^{-4}
Amino-azins (eurhodins)				
Neutral red	< 0·008	1·0		10^{-4}‡
Safranins				
Safranin A	0·05	1·12		10^{-4}‡
Azocarmine G	< 0·003	2·22		5×10^{-4}
Azocarmine B	< 0·003	2·32		5×10^{-4}
Azosafranins				
Janus Green B	0·80		1·22	10^{-5}‡
Thiazins				
Methylene azure C	0·09	1·07		5×10^{-4}
Alloxazins				
Riboflavin	< 0·004	1·0		5×10^{-4}
Anthraquinones				
Alizarin red S	0	3·22		5×10^{-4}

* The antagonistic or synergistic activity of the dyes was measured against chlorpromazine-induced red cell lysis.

† Omitted because dye was partially insoluble at 10^{-2} M.

‡ Dye was either partially insoluble or induced hemolysis at 5×10^{-4} M concentration.

Values of II or SI greater than 1·20 are significant.

Several groups of tricyclic compounds inhibited CPZ-induced hemolysis (Table 1). Rhodamine B, which caused methemoglobin formation, acted mildly hemolytic by itself at 5×10^{-4} M, but inhibited CPZ-induced lysis slightly at the lower concentration of 10^{-4} M. Also, safranins but not azosafranins inhibited lysis. In fact, the

azosafranin Janus green B enhanced CPZ-induced hemolysis (*vide* SI > 1 and relatively high LI). CPZ–LDR curves indicated some inhibitory activity of the anthraquinone alizarin red S, but the LDR curve in the presence of the dye was not shifted parallel and its slope was reduced. A strongly antagonistic effect was obtained with several fluoresceins. Although the unsubstituted fluorescein proved ineffective, the 4′,5′-dihalogenated and more so, the 2′,4′,5′,7′-tetrahalogenated or partially nitrated derivatives antagonized CPZ. The 3′,6′-acetylated congener of the 2′,7′-dichlorofluorescein inhibited stronger than the non-acetylated congener. Phloxine B, rose bengal and merbromin, however, enhanced CPZ-induced hemolysis. Triphenylmethane dyes, e.g. phenolphthalein sodium and bromocresol purple, and dyes with a condensed three-ring molecular structure but without a center ring side chain, e.g. *N*-methylphenazonium methosulfate, methylene azure C, pyronin B, brilliant cresyl blue, cresyl violet acetate and neutral red, did not inhibit hemolysis significantly.

Eosin B, the dyestuff with the strongest inhibition of CPZ, antagonized also the hemolytic action of all other tested phenothiazines. Phenothiazine, pyrathiazine, propiomazine, acetophenazine, prochlorperazine, triflupromazine, fluphenazine, and thioridazine as well as the tricyclic amines imipramine and amitriptyline were inhibited at a variable degree. For instance, the II for the eosin B-thioridazine antagonism was found very high (770); the II for prochlorperazine was medium (59) and for imipramine relatively low (2·6). A scattergram (not shown), however, revealed no correlation between the LI of phenothiazines[10] and their II with eosin B. Hemolysins other than phenothiazines were also inhibited by eosin B, e.g. rose bengal (LI 5·11, II 20·7) and the acridines quinacrine dihydrochloride dihydrate (LI 0·20, II 3·9), an antimalarial drug, and clomacran phosphate (LI 0·53, II 20·5), an experimental tranquilizer. Although eosin B inhibited phenothiazines strongly, it was little effective against digitonin-induced hemolysis (LI 5·94, II 1·3).

The sequence in which hemolysin and antagonist were added to the erythrocytes influenced the ED_{50} of CPZ and thus, the II. For instance, the strongest antagonism occurred when CPZ and either eosin Y or eosin B were added simultaneously (II 19·4 for eosin B); less inhibition (II 8·8) was obtained when either dye was added 15 min prior to CPZ and the weakest antagonistic action (II 2·2) occurred when the sequence was reversed. Spectrophotometric scanning (from 210 to 700 nm) of buffered solutions containing eosin B, CPZ or a mixture of both compounds revealed no evidence

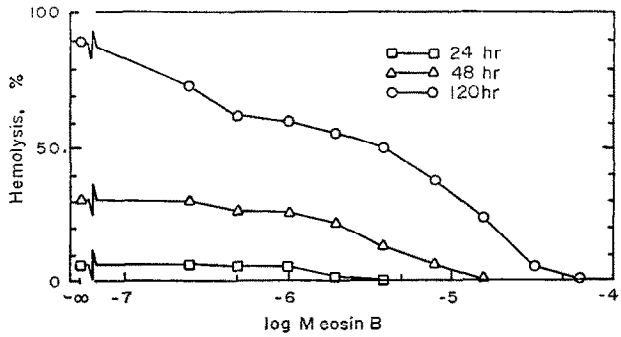

FIG. 3. Inhibition of autohemolysis by eosin B. The blood sample was taken from a person with a dominantly inherited, nonspherocytic hemolytic anemia.

of a chemical reaction between the two compounds. Thin-layer chromatography of mixtures of eosin Y and various concentrations of CPZ yielded only single zones of fluorescent dye with a similar mobility for each concentration.

Eosin B did not prevent autohemolysis of stored normal erythrocytes (approximately 3 per cent hemolysis after 5 days at 37°) but diminished lysis of hemoglobin SS erythrocytes and of red cells from a patient with an idiopathic acquired hemolytic anemia with cold antibodies at 10^{-4} M. The preservative effect of eosin B on the erythrocytes of a 38-yr-old female with a dominantly inherited, nonspherocytic hemolytic anemia became manifest already at a relatively low dye concentration (Fig. 3). The latter condition was not associated with an unstable hemoglobin and no evidence of an enzymopathy* of the anaerobic glycolytic or the pentose phosphate shunt pathway or of an abnormal erythrocyte oxidoreductase was obtained.

DISCUSSION

Many fluorescein derivatives and several other dyes act as antagonists of phenothiazine-induced hemolysis. In the absence of phenothiazines, but at concentrations approximately 20–100 times higher than effective in the inhibition of phenothiazines, many of the same fluoresceins cause hemolysis. Fixation or precipitation as described by Jodlbauer and Haffner[7] and Blum[9] occurs when a dye concentration exceeds that required for hemolysis. Inhibition of hemolysis and "fixation" are, therefore, separate events.

Because of the *o*-carboxyl group, fluoresceins may exist in either a lactoid or a *p*-quinoid form.[11] This raises the question of whether either or both of the isomeric forms are effective antihemolytics. The available evidence thus far is too equivocal to answer the question clearly. The enhanced activity of 2′,7′-dichlorofluorescin-3′,6′-diacetate over 2′,7′-dichlorofluorescein would favor the lactoid form as the more active structure. This view seems to be supported by an increase in efficacy from 4′,5′-dibromofluorescein, which gives a stable quinoid structure, to 2′,4′,5′,7′-tetrabromofluorescein, which favors the lactone form.[11] However, no similar increase in activity was noticed with the corresponding chloro and iodo congeners.

Halogenation and more so, nitration of the ring nucleus clearly increase the antihemolytic efficacy of fluoresceins; dihalogenation of the center ring at positions 4 and 5 enhances the potency more than dihalogenation at positions 2 and 7. On the other hand, halogenation of the side chain ring abolishes inhibition and converts fluorescein congeners into strong hemolysins (cf. eosin Y and phloxine B or erythrosin B and rose bengal). Mercuration of the ring nucleus causes a similar reversal of activity (cf. 4′,5′-dibromofluorescein and merbromin). The molecular mechanism of the inhibition of lysis is not clear. The abrupt break in the LDR curves of CPZ in the presence of eosin Y or erythrosin B may suggest a direct drug–inhibitor interaction and the fact that the simultaneous addition of drug and inhibitor resulted in the strongest inhibitory activity may support this assumption. However, no evidence of a drug–dye interaction is available spectrophotometrically and chromatographically. However, the structural similarity of the hemolysins and their antagonists and the fact that these compounds are adsorbed onto cell and protein surfaces[7,12,13] may

* The enzyme analyses were carried out in the laboratory of Professor W. N. Valentine, University of California Medical Center.

suggest a competitive mechanism of action. The inhibitory action mechanism of the anthraquinones may differ from that of the fluoresceins and safranins. In view of the reported complex formation of CPZ with the isoalloxazine part of flavins,[14] it is surprising that riboflavin does not inhibit CPZ-induced hemolysis.

Eosin B seems to convey membrane stability not only to normal erythrocytes exposed to chemical stress but also to erythrocytes with an innate membrane defect as demonstrated by the case of the non-spherocytic hemolytic anemia. It remains to be seen whether eosin B can also inhibit other phenothiazine drug effects such as local anesthesia and central tranquilization. The antihemolytic activity of eosin B can perhaps be enhanced further by an appropriate modification of the molecule. Eosin Y was found too toxic for parenteral application and pharmacologic data on eosin B are not available.[6,15] The possible usefulness of eosin B as a diagnostic agent for the differentiation of hemolytic anemias associated with a membrane defect and those associated with an intracellular enzymatic defect requires further study.

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