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Positive Direct Coombs' Test Induced by Various Azo Dyes.* (30352)

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In a previous study, subcutaneous injection of the diazo dye trypan blue, was found to produce an anemia with positive Coombs' tests in rats(1). Since a positive antiglobulin reaction was also produced *in vitro* when normal rat erythrocytes were exposed first to the dye and then to plasma, it was postulated that the protein binding property of trypan blue might be responsible for the *in vivo* coating of the erythrocytes. The capacity of other azo dyes to bind with protein has been related to their chemical structure(2). The present study was undertaken to correlate the molecular configuration of a selected group of these dyes with their ability to bind with serum proteins and to relate the serum concentration of both protein bound and free dye to the development of a Coombs' positive anemia.

Materials and methods. The compounds initially tested are listed in Table I with their commercial sources which are of importance since the actual dye content of different brands has been found to vary from 40 to 80% depending on the manufacturer. Aqueous solutions were injected subcutaneously into male and female Wistar strain rats (Simonsen Laboratories) weighing approximately 350 g.

The concentration of free and protein bound dye in the serum was measured by an adaptation of the method of Judah and Willoughby(3). The protein in 1 ml of serum was precipitated with an equal volume of 10% trichloracetic acid and the residue treated first with 50% ethanol and then with absolute ethanol to remove lipids. Any color in the supernatant was regarded as free dye. The dye bound to protein was extracted by suspending the precipitate in 3 ml of a 25% solution of aqueous pyridine at 70°C for 40 minutes or until extraction was complete. The color of the supernatant was measured in the Coleman Jr. Spectrophotometer at the wave length representing the peak of the absorption spectrum for each dye, and the dye concentration determined by reference to predetermined

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TABLE I. Hematologic Effect of One Injection of H Acid, Orthotolidine Dihydrochloride and Azo Dyes Structurally Related to Trypan Blue.

	Anemia with positive Coombs' test
Orthotolidine dihydrochloride*	No
H acid*	"
H acid/Orthotolidine dihydrochloride*	"
Azidine violet†	"
Azo fuchsin V†	"
Benzoazurine G†	"
Benzopurpurin 4B†	"
Benzo sky blue 2B†	Yes
Chloramine black†	No
Chlorazol sky blue FF†	Yes
Chromotope 2R*	No
Congo red*	"
Dianil blue 2R*	"
Direct brilliant blue C*	"
Evans blue*	Yes
Manchester blue*	No
Niagara blue 2B*	Yes
Niagara sky blue 4B*	"
Niagara sky blue 6B*	"
Orange G*	No
Trypan blue*	Yes

* National Aniline Co., U.S.A.
† Michrome Laboratories, London.

All dyes were given in a 20 mg/100 g dose, except orthotolidine 6 mg/100 g, and H acid 14 mg/100 g of body wt.

calibration curves. Microhematocrits, white blood cell, platelet and reticulocyte counts were done by standard methods. The technique employed in the preparation of the antiglobulin serum and performance of the Coombs' tests has been described(1). A 1:4 dilution of Coombs' serum in saline was used for all tests. In control procedures positive erythrocytes were not agglutinated by absorbed normal rabbit serum and normal cells were not agglutinated by the antiglobulin serum. Histologic studies were carried out on formalin fixed tissues stained with hematoxylin and eosin.

Results. Since trypan blue is prepared by coupling tetrazo tolidine to H acid used in excess, it seemed possible that residuals of these starting substances in the commercial product might be responsible for its biologic activity. However, groups of 5 animals given one injection of H acid or orthotolidine dihydrochloride, either alone or in combination showed neither anemia or other evidence of illness during the 11-day observation periods. Gross and microscopic examination of the tissues failed to reveal significant alterations in the reticuloendothelial system. Each of 17 azo dyes having a structural relationship to trypan blue was injected into a group of 5 animals. Hematocrits and Coombs' tests were performed on the 4th, 7th, 9th and 11th days after injection. As can be seen, 6 of these dyes produced an anemia associated with a positive Coombs' test (Table I).

Of the 8 dyes selected for more extensive study, 3 in addition to trypan blue proved to be active (Fig. 1). With each of these dyes, the hematocrit dropped rapidly, reaching the lowest levels on the 9th to the 11th days, and Coombs' tests were positive with titers rising as high as 1:128 (Table II). The development of the positive Coombs' reactions was always associated with the presence of non-protein bound or (free) dye in the serum (Fig. 2). The highest percentage of free dye was found after injection of trypan blue with a maximum of 32% at one hour.

With Evans blue the percentage of free dye

TABLE II. Direct Coombs' Tests.

		Dilutions of Coombs' serum with saline							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Trypan blue	U44*	3+	3+	2+	1+	±	0	0	0
	U 3	4+	4+	4+	4+	4+	2+	±	±
	U 5	4+	4+	4+	4+	4+	2+	1+	0
Evans blue	U61	2+	2+	1+	±	0	0	0	0
	U18	4+	2+	2+	1+	1+	±	±	0
	U19	4+	4+	4+	3+	1+	±	0	0
Niagara blue 2B	U63	4+	4+	2+	2+	0	0	0	0
	U26	4+	4+	3+	2+	2+	1+	0	0
	U66	4+	4+	3+	2+	2+	±	0	0
Benzo sky blue 2B	U69	3+	2+	1+	±	0	0	0	0

* Designation of individual animals.

was much less, the maximum of only 8.4% at one hour was reduced to 0 in 48 hours. As shown in Fig. 2, the percentage distribution with the other 2 dyes was intermediate. In earlier studies with trypan blue it was shown that the anemia was accompanied by a thrombocytopenia, leukopenia and reticulocytosis (1). In the present study a thrombocytopenia and leukopenia developed concomitantly with the anemia induced by benzo sky blue 2B and Niagara blue 2B, the lowest counts being found on the 11th day following dye administration, and the anemia was followed by a reticulocyte response. Examination of blood smears showed a microcytic hypochromic anemia with the presence of normoblasts and abnormal platelets. With Evans blue these studies were inconclusive because of illness of the animals.

Although, in general, the extent of skin discoloration and severity of illness could be correlated with the concentration of dye in the serum and development of anemia, there was considerable variation in the response of animals given the 4 active dyes. Trypan blue, Niagara blue 2B and benzo sky blue 2B were rapidly absorbed from the injection site and the tissues became deeply dye stained within

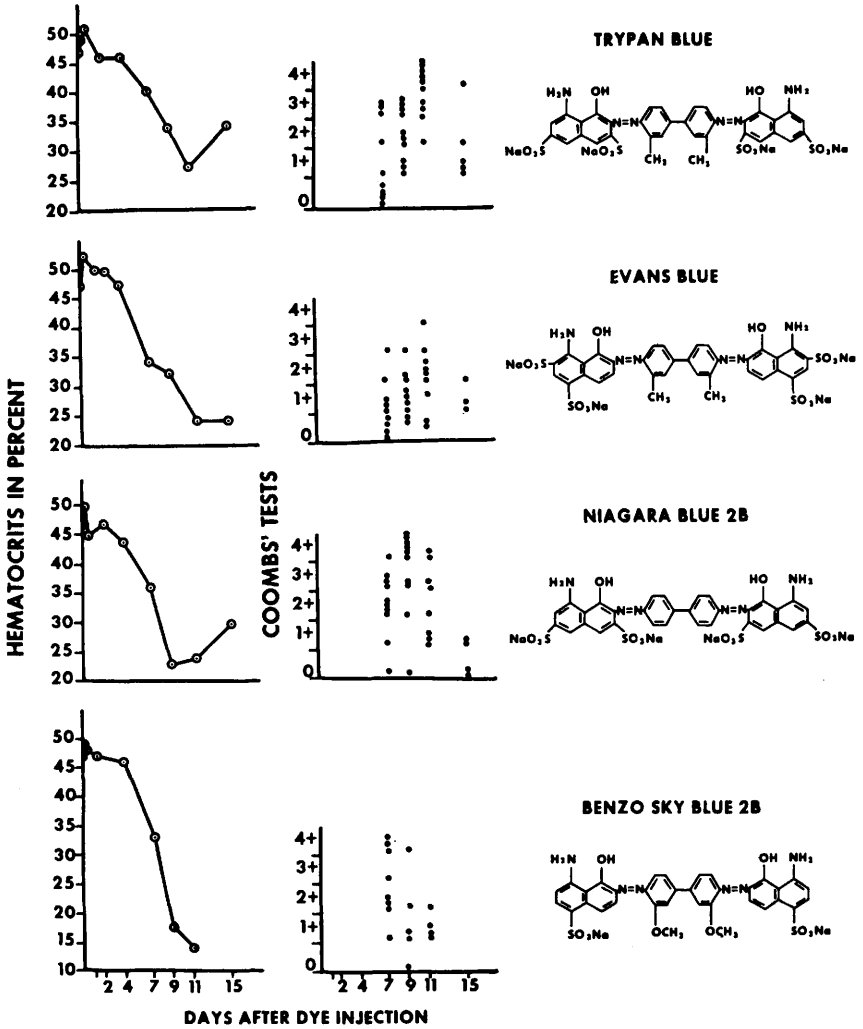


FIG. 1. Mean hematocrit response following a single injection of 20 mg/100 g of 4 active diazo dyes. Individual Coombs' tests are represented by dots. Each dye was tested in a group of 10 animals (normal hematocrit 47.0 ± 2.54).

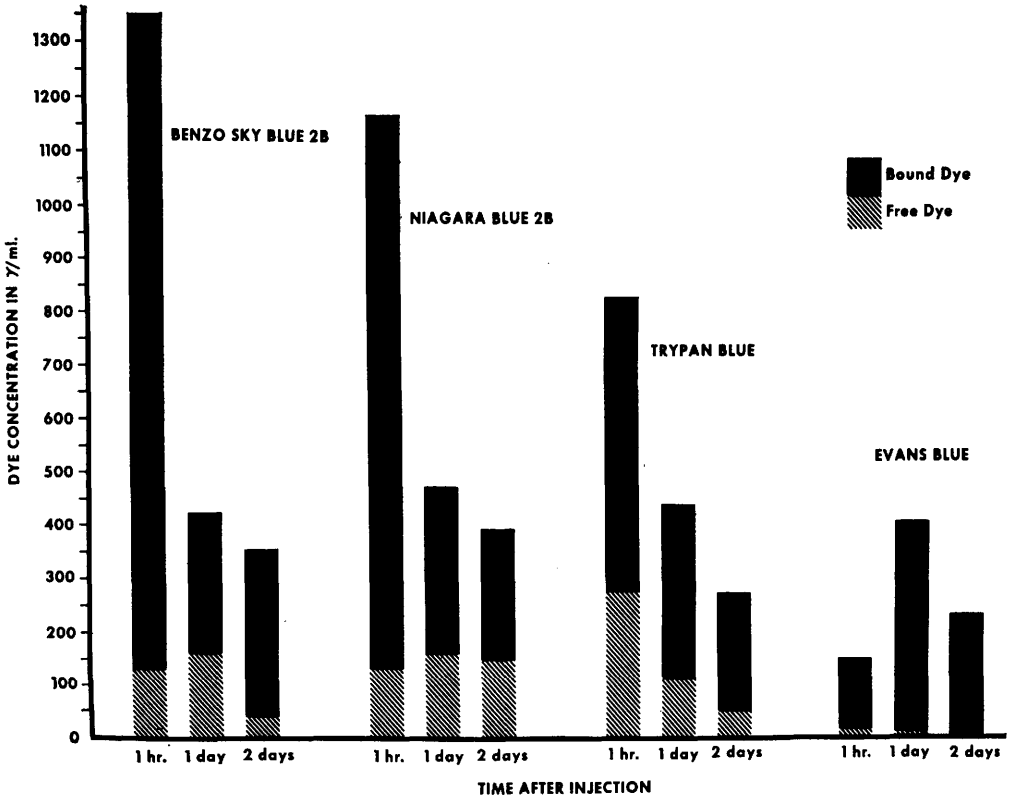
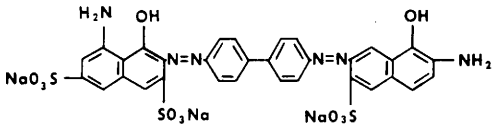


FIG. 2. Mean serum dye levels measured at intervals following injection of 20 mg/100 g of the 4 dyes that caused anemia. Each determination was based on measurements in 3 animals.

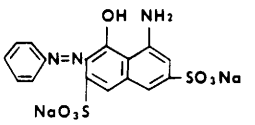
the first hour. The concentration of dye in the serum reached its peak during this period and appeared promptly in the urine (Fig. 2). Evans blue was so slowly absorbed and distributed through the tissues that the maximum concentration in the serum was not attained until the end of the first day. This prolonged dye absorption with retention of dye in the tissues was accompanied by signs of severe illness with loss of muscular control in the extremities and watery diarrhea. Splenomegaly, the most striking gross abnormality in animals injected with trypan blue, was also present to about the same degree in the animals given Niagara blue 2B and benzo sky blue 2B but in the Evans blue group the enlargement was less pronounced. Reticular cell hyperplasia in the portal triads of the liver and lymph nodes was present to about the same extent in the trypan blue and Evans blue treated animals but was most pronounced in those given Niagara blue 2B and benzo sky blue 2B. In the latter group the animals also showed scattered petechial hemorrhages, especially in the lungs and pericardial sac and microscopically there was focal necrosis of liver parenchymal cells without reaction.

The structural formulae of the other 4 dyes tested are shown in Fig. 3. When injected in the same manner they did not produce anemia, and Coombs' tests done on the 7th, 9th, 11th and 15th days were negative. Significant amounts of dye in either protein bound or free form were not demonstrable in the serum. Three of these dyes (chloramine black, benzopurpurin 4B and direct brilliant blue C) were apparently poorly absorbed from the injection site while the fourth (azo fuchsin V) was rapidly taken up but excreted in the urine in large amounts during the first day. With all 4 the animals showed no evidence of illness and at autopsy there were no significant gross or microscopic abnormalities.

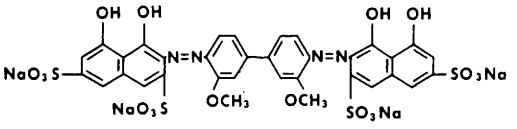
Discussion. Positive Coombs' tests in the



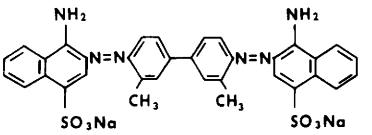
CHLORAMINE BLACK



AZO FUCHSIN V



DIRECT BRILLIANT BLUE C



BENZOPURPURIN 4B

FIG. 3. Structural formulae of 4 diazo dyes which failed to produce anemia following injection of 20 mg/100 g into groups of 10 animals.

anemia induced by trypan blue was shown in our earlier studies to be associated with a relatively high concentration of dye in the serum(1). Trypan blue in low concentration is known to bind preferentially with albumin (4), however it was postulated that the serum dye levels produced by the relatively large doses used exceeded the dye binding capacity of the albumin and free dye was thus available. The strength of the Coombs' reaction was regarded as a function of the amount of dye in this form available for either absorption of plasma proteins onto altered cell surfaces or to serve as a prosthetic group for binding nonimmune proteins to the erythrocytes. The results of the present study appear to support this hypothesis, for dyes which failed to concentrate in significant amounts in the serum were ineffective in producing either anemia or positive Coombs' tests. Evans blue which was associated with low serum dye levels and little or no free dye induced inconstant antiglobulin reactions. On the other hand strongly positive reactions were induced by dyes with high concentration of the free component in the serum and trypan blue which had the highest proportion of circulating free dye proved to be the most effective.

The active dyes, with one exception, had the highest molecular weights and similarities were noted in their structural formulae in that all had 3 or 4 sulphonic acid groups on the napthalene rings with a hydroxyl and an amino group in the 1-8 positions. The amino groups in this location appeared to be essential since dyes in which they were absent or in which their position was shifted were inactive. In general the inactive compounds, including the monazo dyes, had fewer sulphonic acid groups as well as absence or rearrangement of the hydroxyl and amino groups. Even among the active agents, differences in structure were associated with altered effectiveness. In comparing the 2 closely related tolidine dyes, trypan blue and Evans blue, it is known that the shift of the sulphonic acid groups from the 3-6 to the 2-4 position increases both the strength of the protein-dye bond(2), and the binding capacity since one mole of albumin joins with 8-14 moles of Evans blue but with only 2 moles of trypan blue(4). This may well be the explanation for the observation that Evans blue remained bound to tissue proteins with little diffusion into the plasma and little circulating free dye.

The experimental production of positive antiglobulin tests by non-immunologic means with azo dyes is not unique since a variety of other agents have been shown to have a similar action on erythrocytes, both *in vivo* and *vitro*(5,6,7,8,9,10,11). In these instances it has also been suggested that the red cell membrane was either altered or made more reactive to protein. Recently Jandl has demonstrated that the immature red cells of man, rabbit, and the rat are selectively agglutinated by Coombs' sera and has suggested that this may be the explanation for the positive

tests observed in some experimentally induced non-immunologic anemias(12). It is possible that this phenomenon may have contributed to the positive reactions found with the azo dyes since the anemia in each case was followed by a good reticulocyte response. However, our demonstration of strongly positive Coombs' tests *in vitro* with trypan blue suggests that the agglutination of immature red blood cells was not the major factor contributing to the positive *in vivo* tests(1).

Summary. In an earlier investigation the activity of the diazo dye trypan blue in producing an anemia with positive Coombs' test was attributed primarily to its capacity for binding with protein and thus either altering the surface of erythrocytes or serving as a prosthetic group for the attachment of serum proteins. The present study of the activity of a number of structurally related compounds supports this hypothesis, for only those dyes which appeared in the serum in high concentration, with a large proportion of non-protein bound (free) dye, induced protein coating of the erythrocytes. In general, the active dyes had structural formulae similar to trypan blue and the diazo linkage, as well as the presence of the amino group, appeared to be essential.

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Differences in Cytopathic Effects of Adenovirus in Monkey Kidney Tissue Culture. (30353)

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Presence of a soluble group complement fixing antigen(1,2,3), absence of pathogenicity for laboratory animals(1), ether resistance (1), DNA nucleic acid configuration(3), 60-85 mμ size range(3), and production of characteristic cytopathic effect (CPE) in continuous cell cultures of epithelial origin(2) are characteristics which ostensibly place viruses in the adenovirus group. There are at present 28 recognized human types(2), 2 additional serotypes pending recognition(4), and numerous other adenovirus types of simian, bovine and perhaps avian origin(3).

Denny and Ginsberg(5) have suggested 2 subgroups based on neutralization kinetics, virus multiplication cycles and virus yields. Reports from Rosen(6) and Simon(7) indicate the presence of natural subgroups based on hemagglutination tests with monkey and rat erythrocytes.

The classical CPE of adenovirus in monkey kidney tissue culture was first described by Rowe and co-workers(1). This study included only types 1 to 6 and identical changes were described for all types. Rosen *et al*(8) reported differences in CPE in Rhesus monkey kidney cultures with types 1, 2, 3, and 5 as opposed to 9, 10, and 13, but details were not given. Rafajko(9) suggested a possible separation of adenovirus types 1 to 18 into three