THE BINDING OF T-1824 AND STRUCTURALLY RELATED DIAZO 
DYES BY THE PLASMA PROTEINS

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The purpose of the present study was to find out if the differences in disappear-
ance rates of T-1824, trypan blue, niagara sky blue and niagara sky blue 6B 
depend upon the binding of these dyes by the plasma proteins (Gregersen and 
Rawson, 1943). The affinity of the various dyes for the plasma proteins was 
investigated: 1, with the electrophoresis method of Tiselius (1937); 2, by the 
ultracentrifuge; 3, by the effects of the plasma proteins upon the spectral absorp-
tion of the dyes, and 4, by a cellophane-staining test.

ELECTROPHORESIS EXPERIMENTS. The protein solutions were prepared for 
electrophoresis as follows: 4 cc. of serum or plasma were diluted with 12 cc. of a 
0.2 M phosphate buffer, varying in pH from 7.40 to 7.60, and containing 0.15 
M sodium chloride. The mixture was dialyzed at 5°C. in a cellophane bag for 
two or more days against two liters of the buffer. The buffer solution was 
changed at least once during the dialysis period. The dye was added from stock 
solutions before dialysis.

Figure 1a shows the electrophoretic pattern of normal titrated human plasma. 
The pattern in figure 1b is produced by the same plasma containing T-1824 at a 
concentration of 0.004 per cent. The light absorption of the dye causes a well-
defined shaded area. The fact that the shading begins with the ascending al-
bumin boundary and ends with the descending albumin boundary demonstrates 
that the dye migrates entirely with the albumin. The same results were obtained 
with trypan blue, niagara sky blue, niagara sky blue 6B and also with brilliant 
vital red. In dog serum, as in human plasma, all of the dyes also migrated en-
tirely with the albumin fraction. At the close of each dye-plasma experiment 
the protein boundaries were pushed by clockwork and plunger in order to separ-
ate the alpha, beta and gamma globulins on the descending side from the rest of 
the solution. Chemical and spectrophotometric examination of the globulin 
solution showed the presence of protein and the absence of dye.

The electrophoretic pattern of human plasma containing 0.098 per cent T-1824 
(after dialysis) showed the dye boundary to begin with the ascending albumin 
boundary and to end with the descending beta globulin boundary. Hence if 
sufficient dye is present it will be bound by the alpha and beta globulins as well 
as by the serum albumin. The descending gamma globulin was free of dye. At 
the end of four hours of electrophoresis, the fractions listed in table 1 were

1 Submitted in partial fulfillment of the requirements for the degree of Doctor of Phi-
losophy, in the Faculty of Pure Science, Columbia University.

2 Since trypan blue and niagara sky blue stained the cellophane bags during dialysis it 
was necessary to add these dyes to the plasma samples after dialysis.
separated. The concentrations of dye and protein in the different fractions were obtained by the König-Martens spectrophotometer and micro-Kjeldahl determinations. It should be noted that the ratio of T-1824 to albumin in the albumin fraction was approximately eight moles of dye per mole of albumin.

Experiments with T-1824 and electrophoretically separated human serum albumin. T-1824 was added to a sample of electrophoretically separated human serum albumin in high concentration. The final concentration of dye (after dialysis) was 0.019 per cent in 0.098 per cent albumin, the ratio of dye to albumin being fourteen moles of dye per mole of albumin. After four hours the T-1824 migrated ahead of the albumin on the ascending side. Table 2 gives the mobility of the albumin before and after the adding of T-1824. The mobility of albumin is not affected by T-1824 at a concentration of 0.004 per cent (ratio of

* See page 710 for method of calculating dye concentration.
† Molecular weights used in calculating ratio: Albumin 70,000, Globulin 150,000, Dye 960.

According to Blix, Tiselius, and Svensson (1941) the shading beginning and ending with the beta globulin boundaries is caused by the presence in the plasma of the lipids which migrate with the beta globulin.

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Table 1: The distribution of T-1824 (0.098 per cent) in human plasma after electrophoresis

<table>
<thead>
<tr>
<th>ELECTROPHORETIC SAMPLE</th>
<th>PROTEINS PRESENT</th>
<th>PERCENT DYE</th>
<th>MOL. N PER CC</th>
<th>MOLES OF DYE†</th>
<th>MOLES OF PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Before electrophoresis</td>
<td>Albumin and globulins</td>
<td>0.098</td>
<td>1.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Albumin</td>
<td>0.063</td>
<td>0.76</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Globulins</td>
<td>0.006</td>
<td>0.41</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Gamma globulins</td>
<td>0.00</td>
<td>0.086</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 1. Electrophoretic pattern of normal human plasma. a, without, and b with T-1824 added.
dye to albumin, 1 to 28) but if the dye concentration is increased to 0.019 per cent (ratio of dye to albumin, 14 to 1) the mobility is increased from $5.0 \times 10^{-5}$ to $7.0 \times 10^{-5}$. The significance of this change in mobility will be discussed later.

**Electrophoresis of T-1824 in globulin solution.** A 2.3 per cent globulin solution prepared from normal human plasma by the method of Howe (1921) showed the presence of two protein fractions corresponding in mobilities to alpha and gamma globulin. When T-1824 was added to this globulin solution to a concentration of 0.002 per cent, the dye migrated wholly with the alpha globulin.

**Ultracentrifuge and diffusion experiments.** The observation that the dyes migrate preferentially with the albumin fraction has been confirmed by the sedimentation of T-1824 with serum albumin in the ultracentrifuge. The molecular weights of the albumin-dye complexes in solutions of various dye-albumin ratios were calculated from the sedimentation rates and diffusion constants according to the formula of Svedberg (Svedberg and Pedersen, 1940).

$$M = \frac{RTS}{D(1 - VP)}$$

where $R = 8.212 \times 10^{-7}$

$T = 293^\circ$ absolute

$D = $ Diffusion constant at 20°C.

$S = $ Sedimentation rate at 20°C.

The sedimentation rates were determined in an air driven ultracentrifuge at 800 revolutions per second (158,000 X gravity) and at 24° to 31°C. The diffusion constants were determined at 2° or 8°C. in an electrophoresis cell and diffusion curves were obtained at intervals up to 75 hours on photographic plates using the Longsworth schlieren scanning method (1939). The sedimentation rate and diffusion constant of electrophoretically separated human serum albumin in phosphate buffer (pH 7.49) were determined under the following conditions: 1, without dye; 2, containing 0.00049 per cent T-1824, and 3, containing 0.038 per cent T-1824 (see table 3).
In each of the three sedimentation experiments only one boundary was present. When dye was present it sedimented with the albumin leaving the supernatant buffer colorless. Table 3 lists the data obtained. It should be noted that in a preliminary experiment in which T-1824 in 0.004 per cent concentration in phosphate buffer was centrifuged at 900 revolutions per second, no sedimentation was observed after 1/2 hours.

Discussion of electrophoresis and ultracentrifuge data. The binding of the dyes by the plasma proteins is clearly demonstrated in the electrophoresis experiments (fig. 1). This is further borne out in the case of T-1824 by the ultracentrifuge studies. The dyes, T-1824, niagara sky blue 6B, trypan blue, niagara sky blue and brilliant vital red in 0.004 per cent concentration in plasma are wholly and preferentially bound by the albumin fraction.

The experiments show that if the dye concentration is increased sufficiently, the acid dyes may also be bound by the globulin fraction. Furthermore when

<table>
<thead>
<tr>
<th>CONCENTRATION OF Dye per cent</th>
<th>Albumin (mgm/cc) (N X 7.0)</th>
<th>MOLES OF DYE</th>
<th>MOLES OF ALBUMIN</th>
<th>DIFFUSION CONSTANT AT 20°C X 10^7</th>
<th>SEDIMENTATION RATE AT 20°C X 10^13</th>
<th>MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum albumin</td>
<td>0.0</td>
<td>5.60</td>
<td>0.0</td>
<td>6.3</td>
<td>4.56</td>
<td>70,300</td>
</tr>
<tr>
<td>Serum albumin plus dilute T-1824</td>
<td>0.00049</td>
<td>5.60</td>
<td>0.067</td>
<td>5.9</td>
<td>4.32</td>
<td>71,000</td>
</tr>
<tr>
<td>Serum albumin plus concentrated T-1824</td>
<td>0.038</td>
<td>4.97</td>
<td>4.8</td>
<td>6.0</td>
<td>5.13</td>
<td>83,000</td>
</tr>
</tbody>
</table>

* The serum albumin used in these experiments was electrophoretically separated from human plasma.

T-1824 is added to a solution of alpha and gamma globulins it migrates preferentially with the alpha globulin.

The data make it possible to estimate the approximate number of molecules of T-1824 which can be bound by a molecule of albumin at physiological pH and salt concentration. This dye in plasma migrates only with the albumin fraction unless the ratio of dye to albumin is increased beyond eight moles of dye per mole of albumin. When T-1824 is added to an albumin solution so that the ratio of dye to albumin is 14, some of the dye slowly leaves the albumin during electrophoresis. This would indicate that albumin can bind somewhat less than 14 molecules of dye per molecule of albumin. The ultracentrifuge data show that T-1824 is bound by the albumin in increasing proportions as the concentration of dye is increased, and this is also indicated in the electrophoresis experiments by the effect of T-1824 in low and high concentrations on the mobility of human albumin (table 2). The increase in mobility of human albumin containing relatively high concentrations of T-1824 at pH 7.4 may be explained by the assumption that as T-1824 is attached to the albumin it prevents the dissociation.
of some of the basic groups. This shows that when a sufficient amount of dye is bound to albumin the isoelectric point of the albumin-dye complex is significantly lower than that of albumin.

**Absorption Spectra of the Dyes in Protein Solutions.** The absorption spectra for the dyes (T-1824, Niagara sky blue 6B, trypan blue and Niagara sky blue) were determined by Gregersen and Gibson (1937) in water, 0.9 per cent sodium chloride and plasma. The curves of T-1824 and Niagara sky blue 6B in plasma show an asymmetry about the point of maximum absorption which is not present in the water solutions. This suggests that the effect of albumin and globulin on the absorption spectra of these dyes might be related to the binding of these dyes by the proteins.

The observations were made with a König-Martens visual spectrophotometer. The albumin solutions prepared by ammonium sulfate precipitations were found by electrophoretic analysis to be free of globulin. Howe's method (1921) for preparing the globulin solution was used, and according to electrophoretic analysis, this solution contained only alpha and gamma globulins. The protein solutions were dialyzed (5°C.) for three days against running water, and for two days with several changes of distilled water or phosphate buffer (pH 7.40).

Figure 2 shows the absorption curves of the four blue dyes in human plasma, human albumin and human globulin solutions. In each instance the shape of the curve obtained in albumin solutions (1.0 per cent) was the same as that in plasma. Globulin solutions in concentrations as great as those occurring in plasma depressed the optical density and shifted the maxima of the dyes from that observed with buffer (Gregersen and Gibson, 1937), but did not produce the asymmetry as seen in the plasma curves of T-1824 and Niagara sky blue 6B.

That the changes in the absorption spectra of the dyes produced by plasma are caused mainly by the albumin fraction is demonstrated by the results presented in figure 2. The effect of various concentrations of electrophoretically pure albumin were therefore studied in greater detail and the results are given in figure 3. Figure 4 shows the change of optical density at the maximum for the four dyes as plotted against the ratio, moles of albumin per mole of dye.

Comparison of the optical density at the point of maximum absorption of solutions of 0.001 per cent and 0.002 per cent T-1824 in various concentrations of albumin showed that the Lambert-Beer Law holds in this range of concentration when protein-dye solutions of the same ratio of protein to dye are compared. This was the range of concentration in which the optical density measurements were made.

From the above and figure 3 it is evident that within certain ranges of dye and albumin concentration the concentration of dye cannot be determined unless the ratio of dye to albumin is known. However, by plotting a full curve for the unknown solution (520 mμ to 610 mμ) and by matching this curve in respect to both shape and point of maximum absorption with those obtained on solutions of known dye albumin concentration, and relating the optical density at the point of maximum absorption as in the equation below, the true concentration of dye in the unknown can be determined.
Per cent concentration of dye in unknown = \[ \frac{\text{O.D. of unknown}}{\text{O.D. of the 0.002 per cent curve}} \times 0.002 \text{ per cent} \]

A check on the ratio of dye to protein obtained by this method was made by a micro-Kjeldahl determination of the protein concentration and in all experiments the two values checked quantitatively.

**Fig. 2** Absorption spectra of the dyes in 1, plasma; 2, plasma albumin, and 3, plasma globulin.

**Fig. 3** The effect of varying the albumin concentration on the absorption spectra of the four dyes (0.002 per cent). The curves for T-1824 were obtained in phosphate buffer at pH 7.3; the curves for the other three dyes were obtained in water. The moles of albumin per mole of dye were as follows: T-1824 (1) 0.000 (2) 0.057 (3) 0.096 (4) 0.11 (5) 0.48 (6) 4.8; niagara sky blue 6B (1) 0.00 (2) 0.099 (3) 0.14 (4) 0.20 (5) 0.99; trypan blue (1) 0.00 (2) 0.053 (3) 0.096 (4) 0.24 (5) 0.48 (6) 7.7; niagara sky blue (1) 0.00 (2) 0.10 (3) 0.14 (4) 0.20 (5) 0.50 (6) 7.9.

**Discussion of spectral absorption experiments.** The observations that the changes in the absorption curves of the four blue dyes produced by plasma are caused mainly by the albumin fraction (fig. 2) is in agreement with the preferential binding of these dyes by the albumin as observed in the electrophoresis studies. It should be noted that Robinson and Hogden (1941) have recently found that the albumin fraction of serum is also mainly responsible for the
changes produced by a serum-buffer system on the absorption spectrum of phenol red.

Definite differences were observed among the four dyes when the effect of various albumin concentrations on the absorption spectra were compared (figs. 3 and 4). A shift of about 20 m$\mu$ to the red end of the spectrum was observed with all the dyes in albumin solutions. This would indicate a dampening of the bond energies of the dye as binding with protein takes place. As the albumin concentration was increased in the presence of a constant amount of dye (0.002 per cent) the optical density at the point of maximum absorption of all four dyes fell (fig. 4). The optical density of T-1824 fell to a minimum value when the ratio moles of dye per moles of albumin was 11. The optical density rose when the concentration of albumin was increased and at high albumin concentrations reached that observed in water. A similar variation in optical density was obtained with niagara sky blue 6B. The optical density fell until the ratio, moles of dye per moles of albumin was 8.3 and then increased in value. The rise in optical density from the point of maximum depression was small in trypan blue (fig. 4). Efskind (1940) has observed a similar result for trypan blue using dog plasma. No rise was observed with niagara sky blue.

The experiments suggest that the depression of the optical density by albumin represents binding of the dye, and the continuous fall in optical density with increasing protein concentration represents the conversion of free dye to dye-protein. According to this concept the minimum value of the optical density

\[ \text{Fig. 4. Variation in optical density at maximum absorption with increases in albumin—dye ratio.} \]

4 The depression in optical density produced by albumin cannot be explained as a salt effect since Gregersen and Gibson (1937) found that sodium chloride produced no depression of the optical density in plasma solutions.
should represent the point at which the maximum number of dyes are bound per molecule of albumin. This interpretation is supported by the electrophoretic studies with T-1824, in which it was observed that the maximum number of moles of dye that could be bound by a mole of albumin lay between 8 and 11. When the concentration of albumin is further increased, the optical density rises as the composition of the dye-protein complex changes from a high ratio of dye to protein to lower ratios. It should be noted that in plasma volume determinations the effect of changes in albumin concentration on the optical density of T-1824 does not have to be considered since the ratio of moles of albumin per moles of dye is greater than 10. This fact is also evident from figure 10 of Gregersen and Gibson (1937).

Cellophane staining test. Water solutions of the dyes do not stain cellophane, but if a trace of salt is added to the system, the dyes are immediately and completely deposited on the cellophane. The dye so deposited cannot be removed by washing with water. When 0.002 per cent solutions of these dyes in plasma were placed in cellophane bags and left in the icebox dialyzing against buffer, it was found at the end of a month that the bags containing niagara sky blue and trypan blue were heavily stained and the respective plasma solutions colorless, while the T-1824 and niagara sky blue 6B bags remained unstained and the dyes were still in solution. These observations are striking in view of the fact that niagara sky blue and trypan blue escape from the circulation much more rapidly than T-1824 or niagara sky blue 6B (Gregersen and Rawson, 1943). Since the relative concentration of dye to protein was the same in all bags, and since the electrophoresis experiments have shown that the dyes in this concentration are wholly bound by the albumin, the above observation indicates a difference in the degree of dissociation of the various dye-protein complexes in the presence of a cellophane surface.

The staining of cellophane by the four blue dyes in various concentrations of human albumin was therefore studied more carefully. For each dye a series of dye-albumin solutions were prepared by adding 0.2 cc. of 0.04 per cent dye to 3.8 cc. of albumin giving final concentrations of albumin ranging from 0.17 to 4.20 grams per cent. A strip of cellophane (approximately 6 x 12 mm.) was immersed in each tube and observations were made at intervals up to twenty-four hours.

The albumin solutions used in these experiments had been dialyzed against 0.02 M phosphate buffer, pH 7.4, and contained 0.15 M sodium chloride. The pH of the dye protein solutions lay between 7.3 and 7.4 as determined by the glass electrode. An abbreviated protocol of the experiments with the four blue dyes in albumin solutions is given in table 4.

From the protocol, table 4, it is apparent that there is a difference in the affinities of the dyes for albumin in the presence of cellophane. Niagara sky blue-albumin solutions stained the cellophane strips within 1 to 2 hours even in tubes where the ratio of albumin to dye was 3 to 1. T-1824, in solutions of albumin having a ratio of one or more molecules of albumin to one of dye, did not stain cellophane strips in a period of twenty-four hours. The niagara sky
blue 6B albumin solutions stained the cellophane to only a slightly greater extent than the T-1824 albumin solutions. Deeper staining was observed with trypan blue-albumin solutions and the deepest staining occurred with niagara sky blue.

**Discussion.** The progressive difference in the affinities of the four blue dyes for albumin in the presence of cellophane is apparent from these tests. Since the electrophoresis experiments have shown that these dyes, in the concentrations employed in the disappearance studies, are wholly bound by the albumin fraction of plasma these differences in their affinities for albumin are significant. It will be noted that the relative affinities of the dyes for albumin bear an inverse relation to their disappearance rates from the circulation. T-1824 has the highest affinity and the lowest disappearance rate (8 to 10 per cent per hour), while niagra sky blue has the lowest affinity and the highest disappearance rate (54 per cent per hour) as shown by Gregersen and Rawson (1943).

**Table 4**

*Protocol of cellophane staining test*

<table>
<thead>
<tr>
<th>Albumin Concentration (N X 7.0)</th>
<th>Mole of Albumin per Mole of Dye</th>
<th>T-1824 (0.002 per cent)</th>
<th>N.S.B.6B (0.002 per cent)</th>
<th>Trypan blue (0.002 per cent)</th>
<th>N.S.B. (0.002 per cent)</th>
<th>Time in hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>mgm./cc.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>4.20</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1.40</td>
<td>0.96</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.70</td>
<td>0.48</td>
<td>-</td>
<td>±</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>0.28</td>
<td>0.19</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>0.20</td>
<td>0.13</td>
<td>-</td>
<td>++</td>
<td>±</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>0.17</td>
<td>0.12</td>
<td>-</td>
<td>+++</td>
<td>±</td>
<td>+++</td>
<td>++++</td>
</tr>
</tbody>
</table>

- , No staining; ±, cellophane same shade as solution; +, cellophane darker than solution.

N.SB6B = niagra sky blue 6B; N.SB = niagra sky blue.

**Summary and Conclusions**

The electrophoresis experiments show that T-1824, niagra sky blue 6B, trypan blue, and niagra sky blue in serum or plasma are wholly bound by the albumin fraction when they are present in low concentrations, i.e., about 0.004 per cent or less. This no longer holds, however, when the dye concentration exceeds certain limits representing the binding capacity of the albumin. The evidence indicates that each mole of albumin can bind a maximum of 8 to 14 moles of T-1824.

Ultracentrifugation of serum albumin solutions containing 5 moles of T-1824 to 1 mole of albumin also demonstrates that the dye is bound by the protein. The dye comes down with the albumin forming a single boundary leaving the supernatant buffer solution colorless.

Addition of plasma to aqueous solutions of the dyes shifts the point of maximum absorption toward the red end of the spectrum and changes the contour of the spectral absorption curve (Gregersen and Gibson, 1937). These effects are
caused mainly by the albumin fraction (see fig. 2). There is however some
difference in the effect of albumin on the absorption of the four dyes. With the
two dyes, niagara sky blue and trypan blue, the optical density falls as the al-
bumin concentration is increased to the point where its molecular concentration
is \( \frac{1}{3} \) that of the dye. Further increase in the albumin concentration is without
much effect. With T-1824 and niagara sky blue 6B the maximal effect of the
addition of albumin is reached at much lower concentrations, approximately
\( \frac{1}{3} \) moles of albumin per mole of dye, and the optical density then increases as the
albumin concentration is raised still further (figs. 3 and 4). This ratio for the
maximum number of T-1824 molecules bound by a molecule of albumin is in
agreement with the value obtained from the electrophoretic studies.

The cellophane staining tests reveal differences in the affinities of the four
dyes for albumin. There is a direct relationship between the rates at which
these four dyes leave the circulation (Gregersen and Rawson, 1943) and their
tendency to stain cellophane (see table 4). Thus although all four dyes are
preferentially bound in the plasma by albumin an explanation for their different
disappearance rates is found in their different affinities for albumin.

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