COMBINATION OF EVANS BLUE WITH PLASMA PROTEIN: ITS SIGNIFICANCE IN CAPILLARY PERMEABILITY STUDIES, BLOOD DYE DISAPPEARANCE CURVES, AND ITS USE AS A PROTEIN TAG

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The use of isotopes has proved to be an important tool in the study of the metabolism of body constituents (1). The employment of such tracer substances has developed an awareness for easily identifiable substances which can be combined with a particular body constituent, in order to tag it for study purposes. It is understandable, therefore, that the announcement by Rawson (2) that Evans blue dye (T-1824) combines selectively with the albumin of blood plasma would foster studies employing this dye as a tracer for serum albumin. Thus, for example, Cope and Moore (3) studied the problem of capillary permeability to albumin in burn shock utilizing radioactive dibromo Evans blue as a tracer for albumin.

Evans blue dye (T-1824) has come into wide clinical use as a dyestuff suitable for determining blood volume. Gregersen and Rawson (4), in studies of the disappearance of T-1824 from the blood of dogs, observed that there was an early logarithmic phase of disappearance lasting for approximately one hour. This was followed by a linear phase during which time the dye disappeared from the blood at a constant rate. In view of Rawson's (2) demonstration that the dye combines selectively with albumin, the logarithmic phase of disappearance was considered as a measure of capillary permeability to albumin. Accordingly, its disappearance after the logarithmic phase would possibly mean that a definite quantity of albumin has been removed with the dye from the plasma. Since plasma protein concentration is maintained within narrow limits, that amount of dye-protein removed would have been replaced by protein uncombined with dye. From these considerations, the definite possibility exists that the rate of disappearance of dye from the blood stream would mirror the rate of albumin turnover in the body. The potentialities of this attractive idea for the development of a clinical test for quantitating albumin regeneration rates prompted us to explore this concept experimentally.

It is known that basic dyestuffs accumulate in high concentrations in the juice of actively secreting stomachs (5, 6) and acid dyestuffs, in the alkaline pancreatic juice. In these studies it has usually been considered that it was the permeability to the dye molecules alone which was being investigated. However, in the case of Evans blue, one would be following the behavior of a dye-protein complex, whose chemical and physical properties are necessarily different

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from the dye and protein molecules in the uncombined state. Our present information about the exact nature of the Evans-blue dye-albumin linkage or of its stability in vivo is very inadequate.

Accordingly, a critical study has been made of the significance of the disappearance of T-1824 from the blood stream, its appearance in gastric and pancreatic juices and the chemical nature of the dye-protein linkage.

Twenty-four-hour disappearance of Evans blue from the blood. Fasting blood specimens from patients and normals were drawn over a 24-hour interval beginning 4 hours following the injection of 10 cc. of 0.15% Evans blue and continuing to 28 hours following the injection of the dye. As a rule, 4 blood specimens were taken during this period.

The percentage of disappearance of Evans blue from the plasma was calculated from the 24-hour dye-disappearance curve. From table 1 it is apparent that there are no significant differences in the percentage of disappearance in normals and in hypoproteinemics. Also both groups show wide variations. The average percentage disappearance for the entire group was 46.

From the evidence presented, one is inclined to believe that the percentage of dye disappearance in 24 hours has little or no significance insofar as it may mirror albumin turnover, although the possibility cannot be excluded that perhaps 50% of the circulating albumin may be turned over in a 24-hour period. In the latter case, it would follow that in hypoproteinemia there is no difference from normal in the rate of protein turnover.

Table 1. The disappearance of Evans blue dye from the blood of normal and of hypoproteinemic subjects

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Clinical Diagnosis</th>
<th>Plasma Proteins</th>
<th>Percentage of Disappearance in 24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-1</td>
<td>Normal</td>
<td>7.68</td>
<td>50</td>
</tr>
<tr>
<td>N-2</td>
<td>Normal</td>
<td>7.21</td>
<td>40</td>
</tr>
<tr>
<td>N-3</td>
<td>Normal</td>
<td>7.32</td>
<td>48</td>
</tr>
<tr>
<td>N-4</td>
<td>Normal</td>
<td>7.26</td>
<td>42</td>
</tr>
<tr>
<td>N-5</td>
<td>Normal</td>
<td>7.57</td>
<td>74</td>
</tr>
<tr>
<td>9</td>
<td>Ca. of stomach</td>
<td>6.26</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>Ca. of pancreas</td>
<td>6.64</td>
<td>23</td>
</tr>
<tr>
<td>12</td>
<td>Colostomy</td>
<td>6.58</td>
<td>27</td>
</tr>
<tr>
<td>11</td>
<td>Ca. of pancreas</td>
<td>6.53</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>Ca. of stomach</td>
<td>7.78</td>
<td>35</td>
</tr>
<tr>
<td>16</td>
<td>Ca. of stomach</td>
<td>5.26</td>
<td>38</td>
</tr>
<tr>
<td>13</td>
<td>Cardiospasm</td>
<td>6.70</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>Cirrhosis</td>
<td>6.49</td>
<td>49</td>
</tr>
<tr>
<td>1</td>
<td>Ca. of stomach</td>
<td>5.75</td>
<td>52</td>
</tr>
<tr>
<td>18</td>
<td>Ca. of stomach</td>
<td>6.14</td>
<td>61</td>
</tr>
<tr>
<td>17</td>
<td>Ca. of stomach</td>
<td>4.95</td>
<td>54</td>
</tr>
<tr>
<td>19</td>
<td>Ca. of stomach</td>
<td>6.02</td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>Ca. of esophagus</td>
<td>5.71</td>
<td>33</td>
</tr>
</tbody>
</table>
Appearance of Evans blue in gastric and pancreatic juice. In the course of another study on some dogs with Pavlov pouches and some with pancreatic fistulas, blood volumes were determined with T-1824. On some of these dogs the dye appeared in the secretions. These experiments were repeated to exclude the possibility that the dye was oozing from granulating surfaces. With an intravenous dose of 15 mgm. no dye appeared in the aspirated stomach contents of dogs either before or after the administration of histamine. In three dogs given an intravenous dose of 25 mgm. of Evans blue, the pancreatic ducts were cannulated and juice collected after stimulation with secretin. No dye appeared in the pancreatic juice. In a similar experiment on one dog, 250 mgm. of the dye was given. The dose was sufficient to make the ratio of dye to serum albumin approximately 5. A definite blue tint was seen in the pancreatic juice. The fact that the dye appeared in the secretion of this one dog, even though in minimal concentration, was considered significant. It would seem that if the dye is given in great enough concentration it will appear in the pancreatic juice.

Chemical studies. The purpose of the chemical studies was to measure the extent of the combination of dye with individual plasma proteins. After many preliminary trials conditions were found which enabled this to be done quantitatively. Purified human plasma protein fractions were mixed with varying concentrations of Evans blue. The dye-protein combination was then precipitated with trichloracetic acid and the excess dye remaining in the solution was read in an Evelyn photoelectric colorimeter.

The use of trichloracetic acid has the disadvantage, for purposes of comparison with the work of others (2), that the precipitation is carried out at an acid pH. However, it was found to be free of the more serious drawbacks of other protein precipitants which were tried, such as heavy metals which precipitate Evans blue, and methyl alcohol or (NH₄)₂SO₄ which did not precipitate the dye-protein quantitatively. Although the amount of dye which combines with protein at different pH may vary, the essential nature of the dye-protein linkage will remain unaltered. Accordingly, all precipitations have been done with trichloracetic acid.

A. Combination of Evans blue with purified albumin. Varying concentrations of albumin in normal saline were prepared so that 3 cc. contained from 0 to 2.6 mgm. of albumin. These albumin solutions were added to 6 cc. of 0.03% Evans blue buffered at pH 2.5 in a test tube and mixed by inversion. After a 10-minute interval (even though equilibrium is reached almost instantly), precipitation of dye protein was accomplished by the addition of 5 cc. of 20% trichloracetic acid. The precipitated dye-protein was separated from the solution by centrifugation. (Filtration is unsatisfactory because the dye is absorbed by the filter paper.) The supernatant was transferred to an Evelyn tube, diluted 1:20 with distilled water, and then read in an Evelyn photoelectric colorimeter using a no. 620 filter. The density of the supernatant dye is plotted in figure 1. The determinations were made in triplicate and the data on figure 1 have been reproduced on other occasions.

³ Purified human plasma proteins were obtained through the courtesy of Dr. E. J. Cohn of Harvard University Serum Laboratory.
Since the plot is linear only when a great excess of dye is present and tends to level off and reach a plateau with relatively high protein concentration, a characteristic of dissociation curves of weak acids and bases, one may reasonably assume that the reaction is reversible.

\[\text{dye + protein} \rightleftharpoons \text{dye-protein}\]

In this case, the law of mass action should apply according to the following equation:

\[
\frac{[\text{dye}][\text{protein}]}{[\text{dye-protein}]} = K.
\]

The linear portion of the curve represents the stoichiometric combination of the dye with protein, since the reversible reaction has been displaced to the right by an excess of dye. Under these conditions it can be calculated that 70 moles of dye combine with one of albumin. Let us assume in this experiment that approximately 70 moles of dye are combined with protein on the non-linear portion of the curve in figure 1; then the initial concentrations of dye and protein are known and the concentration of dye-protein can be calculated by the difference from the residual dye. Molar concentrations
are obtained by dividing the gram concentrations by the molecular weight of purified albumin (70,000) and dyestuff (960), respectively. When such calculations are made for several points on the non-linear portion of the curve, the following constant is obtained: $2 \times 10^{-3}$. If one presumes that the combination of the dye with protein is the same in both its dissolved and precipitated phase, this figure ($2 \times 10^{-3}$) represents the dissociation constant.

B. Combination of Evans blue with globulins. Experiments similar to the above were performed with purified human-plasma-globulin fractions. The combination of T-1824 with the various globulin fractions as compared to albumin is pictured in figure 2. The globulins fix less dye than albumin, since the slopes of the linear portions are less steep. Certainly under these conditions Evans blue dye does not combine specifically with albumin. When various known mixtures of albumin and globulin are precipitated with trichloracetic acid in the presence of T-1824, the density of the residual dye is always less than would be expected with albumin alone, indicating that globulin takes up some of the dye.

C. Combination of Evans blue with bovine plasma-protein fractions. When highly purified bovine plasma-proteins are substituted for human plasma-proteins, similar curves as in figures 1 and 2 are obtained. Quantitatively the extent of the combination of dye with bovine or human albumin is identical. There are some individual differences with the bovine globulins as compared to human globulins, but they both combine with dye to approximately the same degree.

D. Combination of Evans blue with cellophane and exchange resins. Cellophane was stained with a concentrated solution of T-1824. The stained cellophane was washed first with repeated changes of distilled water for a period of two weeks and then with repeated changes of normal saline for a similar period. After a short interval the washings were no longer colored by dyestuff. The stained and washed cellophane was then transferred to a 5-gram % albumin solution. Color immediately left the cellophane and was seen in the protein solution. The cellophane had acted in a fashion similar to an ion exchange resin.

A 5½-inch resin column which contained approximately 5 grams of Dualite A-2 (RCl form) was set up. Seven cc. of a 0.05% Evans blue solution containing 5 mgm. per 1 cc. of albumin (ratio of dye to albumin = 7.29) was run through the column at a rate of approximately 1 cc. per minute. Fifty % of the dye was removed by the resin, although the protein was recovered quantitatively. Since the insoluble resin accepts only free dye anions, dissociation of the dye-albumin complex must have occurred. The dye could not be washed from the resin by distilled water but was liberated by sodium hydroxide which changed the resin from the R-dye form to the R-OH form.

DISCUSSION AND CONCLUSIONS. The evidence of Rawson that Evans blue dye attaches itself to albumin is based mainly on electrophoretic data, on ultracentrifugation experiments and on spectrometric studies. It was found that

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4 Purified bovine plasma fractions were obtained through the courtesy of Armour and Company, Chicago, Illinois.
Evans blue in serum migrated exclusively with the albumin fraction except with a high concentration of dye, at which time some dye migrated with a globulin (2). These results have been confirmed by others (7). Such studies do not show the exact nature of the binding, that is, whether it is chemical or physical in nature.

Our chemical studies are explained best on the basis that T-1824 forms a dissociable compound with plasma albumin and globulin. Others (8) like ourselves have found that dye-protein combination follows the law of mass action and that dissociation constants can be derived. The reaction is not specific for albumin but selective at certain concentration. The dissociation constant is an average for the many groups that combine with dye forming a dye-protein complex. On the basis that dyestuffs titrate acid and basic groups in the protein molecule (9), our findings agree with other observations (10).

Also, since Evans blue is an acid dye, the maximum amount of dyestuff will combine with the protein when it is entirely in its basic form, i.e., at a very acid pH. Maximally 70 moles of Evans blue combine with one of albumin. At neutrality, fewer moles of dye would combine with albumin. This explains the discrepancy between our findings and those of others (2).

Careful studies have led to the concept that ions as such are generally not able to permeate cell membranes (11, 12). Since Evans blue occasionally appears in pancreatic juice, one must surmise, at least that the unionized dye molecule is capable of diffusing past the capillary membrane. According to present day concepts, this means that the dye is trapped by ionization (5, 6) in the secreted juice. These authors and many others have observed that neutral red, which is a plasma protein-binding dye, appears in high concentrations in acid gastric juice. Trypan blue, also plasma-binding, appears in pancreatic juice. These observations are most readily explained on the basis of a dissociable dye-protein complex existing in vivo and do not indicate any great or unique stability of this linkage.

That tissue protein also fixes dye is evident from autopsies on dogs which received Evans blue. The lymph nodes are always more deeply stained than other tissues (13). As the dye diffuses from the circulation and is fixed in the tissues, more dye-protein is dissociated to maintain chemical equilibrium.

One may therefore hypothesize the following conditions as occurring in vivo:

\[
\text{Dye-protein} \rightleftharpoons \text{unionized dye} + \text{protein (blood)}
\]

\[\text{(capillary membrane)}\]

\[\text{unionized dye (tissue fluids)}\]

trapping in some secretions by ionization

Fixation of dye by tissue protein.

Because little dye is in the free form in those concentrations obtained in the blood clinically, the diffusion of these free dye molecules into the interstitial
fluid would not completely account for the early rapid phase of disappearance from the blood. It is evident from the scheme that the fixation of the free dye molecules by tissue protein would cause more dye to leave the blood until the tissue proteins become saturated. This may explain the early rapid phase of disappearance. Such an interpretation would be in agreement with the findings of Fine and Seligman (14, 15), who found no rapid initial disappearance of infused radioactive plasma protein. The duration of the ‘logarithmic’ phase of disappearance of protein-binding dyes would probably be determined by the degree of dissociation of the dye-protein complex. The more highly dissociated the complex, the more rapidly would a steady state be established, bringing about a short initial phase. It would follow that the extent of dyestuff disappearance in the ‘logarithmic’ phase would depend upon the amount of dye the tissue proteins are capable of fixing. Apparently, the body can do quite easily what we attempted to do in vitro with the dye-protein ion-exchange resin experiment. Others (16) have approached the problem in a manner different from ours, but, like ourselves, have concluded that T-1824 is fixed to tissue protein during the early phase of blood dye disappearance.

To what extent then does Evans blue measure the capillary permeability to albumin? In those blood dye concentrations obtained clinically, Evans blue exists almost exclusively in the form of dye-protein complex because of the excess molecular concentration of protein which displaces the reversible reaction to the right. Therefore Evans blue appears wherever plasma protein is present. Since the dye-protein combination is reversible, and since it can diffuse in its free form, dye may migrate and slowly become fixed in sites where serum protein fails to appear. In other words, when albumin migrates (as into a burned area), Evans blue must follow. The converse is true only if one can show that there is no fixation by other tissue protein or no ionization of the free dye molecules. The initial disappearance of Evans blue from the bloodstream apparently does not mirror the capillary permeability to albumin.

Should not the dye be a good tag for albumin in metabolic studies even though it may not measure capillary permeability to albumin? The late linear disappearance phase of Evans blue dye from human plasma over a 24-hour period amounts to approximately 50% of the dye concentration. This should mean that 50% of the circulating albumin had been turned over in a 24-hour period. Quantitatively such figures are incompatible with the better isotopic studies of others (14, 15). The rapid disappearance of dye in contrast to the relatively slow rate of disappearance of isotopically tagged plasma protein is readily explained on the basis that dye can be dissociated from the albumin molecule without the latter being metabolized. Once the dye has been removed from the protein molecule, it may return to the circulation and recombine with protein or be lost in metabolism, fixation or excretion. With the isotopic studies the isotopic element is an integral part of the protein molecule, which must be catabolized before the isotope is released. Evans blue or other dyestuffs therefore can not be used as a tag for protein in metabolic studies.
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SUMMARY

Evans blue dye (T-1824) forms a dissociable complex with plasma protein in the present experimental conditions. The dye-protein combination is not specific for albumin, since the dye combines readily with all the globulin fractions. With those blood dye concentrations obtained in the clinical use of Evans blue, most of the dye is probably united to albumin.

The use of Evans blue as a label for protein in studies of capillary permeability and protein metabolism has been critically discussed and its limitations have been pointed out. Because the dye protein compound is dissociable and because the dye is not an integral part of the protein molecule, its clinical use as a tracer for protein becomes limited.

In vitro observations and studies with humans and dogs have provided a basis for interpreting the disappearance and appearance of Evans blue dye in tissue fluids.

Gratitude is expressed to Dr. D. B. Phemister whose active interest made these studies possible. We are indebted to Dr. R. K. Cannan who suggested the ion exchange resin experiment. Thanks are also expressed to Mrs. Evelyn Gordon and Mr. Lamarr Walker for their clinical help.

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