Effect of Protein and Electrolyte on the Spectral Stabilization of Concentrated Solutions of Indocyanine Green[1] (37433)

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In the measurement of flow by dye-dilution techniques, the optical density of the dye–blood mixture must stabilize in the interval between dilution and detection if an accurate measurement of flow is to be obtained. For many years indocyanine green (ICG), the most widely used dye in hemodynamic studies, was thought to stabilize within 1.5 to 2 sec after dilution (1). However, a recent study (2) failed to reproduce these results. Another study (3) concluded that slow optical stabilization of ICG results in marked overestimation of flow if appearance times of the dilution curves are under 6 sec, a common occurrence in many applications. Others (4) have also found errors suggestive of slow stabilization. Since multiple potential sources of error complicate these studies, and since the good agreement reported between direct flow measurements and values determined by ICG (5–7) involved long stabilization times, we studied the optical stabilization of ICG directly in order to define the circumstances in which this could cause errors in flow measurement.

Materials and Methods. Concentrated solutions of ICG[2] at 1 mg/ml were prepared in media varying in protein and ionic composition, shown in detail in Fig. 2, by adding various solutions to aliquots of a 2.5 mg/ml solution of ICG in the aqueous diluent supplied. Plasma from fasted dogs, anticoagulated with ACD or heparin, was used with or without sodium phosphate buffers.

Changes in optical density (OD) of ICG after dilution in plasma were continuously measured on a split-beam, dual-wavelength recording spectrophotometer.[3] Mixing-dilution was accomplished with a plunger assembly,[4] mounted on top of a 1 cm cuvette, consisting of a cup on a spring-loaded shaft which on rapid depression into the cuvette, created turbulence, mixing the ICG in the cup with the plasma in the cuvette before re-emerging. Nonspecific OD changes, for example due to bubble formation during mixing-dilution, necessitated use of the dual-wavelength mode (8). In this mode light beams at two wavelengths are alternately passed through the cuvette, and the difference in OD of the cuvette contents at these wavelengths is recorded. Wavelengths used were 805 nm, which is in the region used for detection of ICG in hemodynamic applications, and 890 nm, where ICG has no significant absorbance (9–11). The degree of compensation for nonspecific OD changes thus achieved was assessed by repeating the plunger depressions after the OD had stabilized following dilution. With this method, the duration of the mixing artifact was always less than 1.6 sec, so that OD changes due only to ICG could be measured 2 sec after dilution.

Results are reported in percentage of stabilized OD because it was found that a variable amount of dye was emptied from the cup during the mixing, resulting in variations of up to 15% in the stabilized OD

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[2] Cardiogreen dye, lots 222 and 658, Hynson, Westcott and Dunning, Inc., Baltimore, MD.

[3] Aminco Model DW-2 Dual-Beam, Dual-Wavelength Spectrophotometer, adapted for RCA 7102 photomultiplier tube, American Instrument Co., Bethesda, MD.

[4] Aminco Models B2-65085 and B3-65085, American Instrument Co., Bethesda, MD.

attained. Intentional variations in the amount of dye diluted did not affect the time course of stabilization. For each dilution, 9, 10, or 12 μl of the 1 mg/ml, or 4 μl of the 2.5 mg/ml dye solution were diluted in 3 ml of plasma to give a final concentration of 3–4 mg/ml, which was within the linear region of the ICG concentration vs OD plot determined in each experiment. After the plasma had been equilibrated to 25 or 37° for 5 min, the mixing-dilution was accomplished by 2 rapid plunger depressions.

Our study was directed primarily at comparing the stabilization rate of concentrated ICG in distilled water with that in mixtures similar to those used by others (2, 3). Dilutions of these three solutions were performed throughout each experiment to rule out changes in the dye solutions or plasma, both used within 8 hr, and to study the effect of varying anticoagulant, dye lot, and plasma pH.

Results. A total of 131 dilutions was made and of these 22 are not included in the results because of irregular fluctuations in OD. Of these 22 curves, 19 were typical for the particular dye solution during the initial 16 sec, prior to the occurrence of the irregularities. Spillage and subsequent drying of plasma on the optical surfaces of the cuvette appeared to account for most of the irregularities. Abnormal recordings were scattered among the different dye solutions. There was no significant difference in the stabilized OD's attained by the different dye solutions ($p > 0.05$). In approximately 90% of the curves the maximum deviation from the mean OD, after stabilization, was less than 1% of the mean, while for the remaining 10% it averaged 1.5%.

Original recordings of three successive dilutions are shown in Fig. 1. Compare the rapid stabilization when concentrated ICG in distilled water was diluted in plasma at 37° with the slowed stabilization when protein and electrolytes were present in the concentrated solution. Results of all experiments are summarized in Fig. 2 in percentage of stabilized OD. For A1 and A1†, prepared in water, the values at 2 sec were 99 and 100%, respectively. Lowering the temperature to 25°, A1*, reduced the value at 2 sec to 96% ($p < 0.005$), but did not significantly affect the value at 3 sec ($p > 0.05$). Variation of

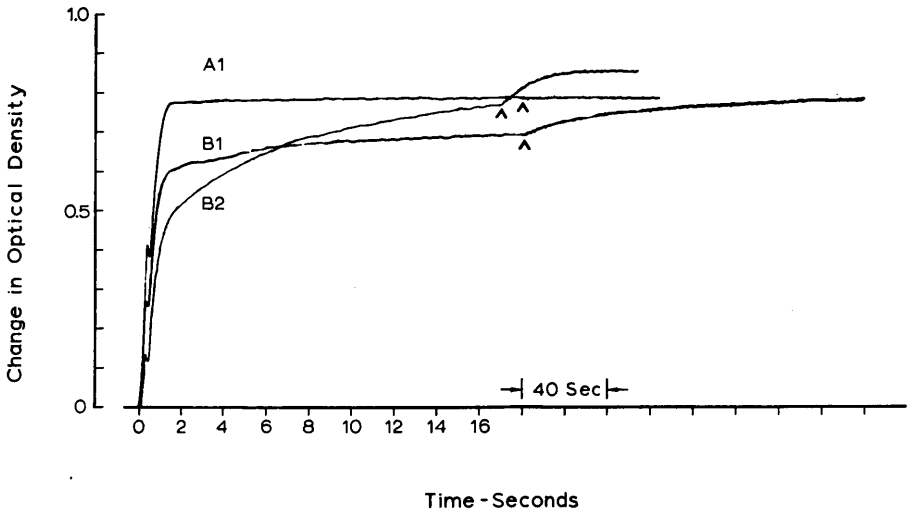

FIG. 1. Original successive recordings of changes with time in OD (805–890 nm) at 37° following mixing-dilution with plasma of ICG (1 mg/ml), prepared in 3 different solutions. Note the rapid (<2 sec) spectral stabilization of ICG in distilled water (A1) compared to the slowed stabilization of dye in a solution containing a moderate concentration of protein (B1) or of dye in a low protein–high ionic strength medium (B2). Time base was changed at the arrows from 2 to 20 sec/in.

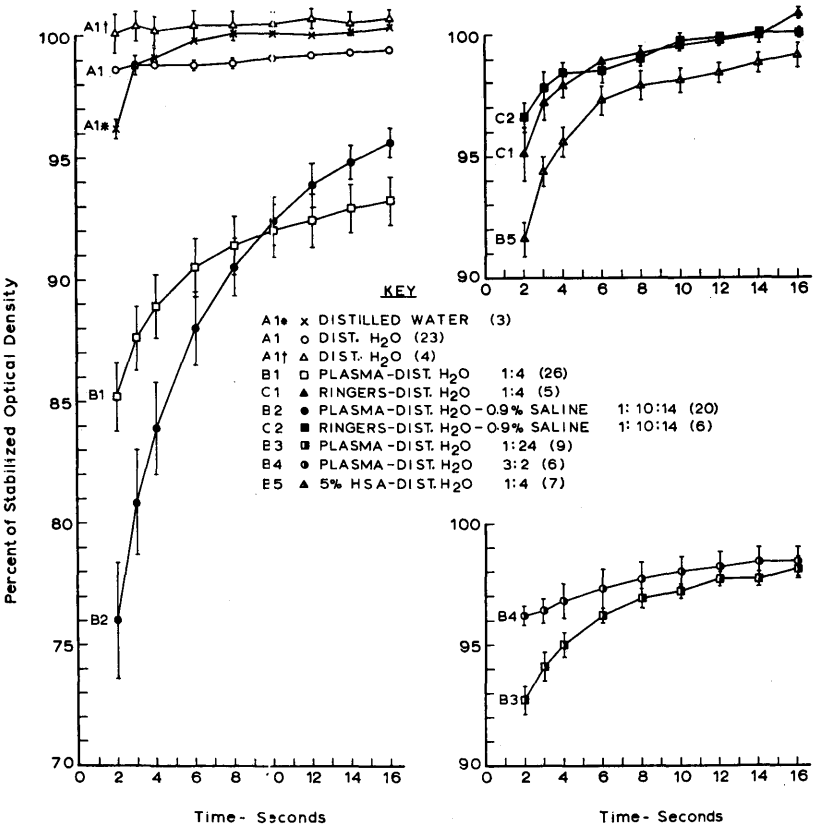

FIG. 2. Comparison of mean changes with time in OD (805–890 nm) as percentage of stabilized OD, following mixing-dilution with plasma of all of the different concentrated ICG solutions listed in the key. Vertical lines are ±SEM. In the key: numbers in parentheses denote number of dilutions performed with the particular solution; A1* refers to dilutions performed at 25°, all others performed at 37°; A1† denotes ICG solution at 2.5 mg/ml, all others were at 1 mg/ml. Left panel: Mean changes in OD with time of ICG solutions original recordings of which are shown in Fig. 1. Also shown are effect on optical stabilization rate of temperature and concentration of the dye in distilled water (see text). Right panel, upper: Note the small delay in stabilization in an ionic medium, C1, C2. Also note the more rapid stabilization of ICG in HSA (B5) compared to ICG in plasma (B1). Right panel, lower: Note the more rapid stabilization as the protein concentration of the medium was increased, B3 vs B4 (see text).

plasma pH between 6.70 and 7.98, of dye lot, or of anticoagulant did not affect the value at 2 sec.

In contrast to these results, for concentrated dye in water, all concentrated solutions containing protein and/or electrolytes had slowed rates of stabilization. Solution B1 (Fig. 2, plasma: distilled water in 1:4 proportions) had a markedly slowed rate of stabilization, with values of 85 and 93% at 2 and 16 sec, respectively. Stabilization of B2 (plasma:water:isotonic saline, 1:10:14) was similarly slowed with values of 76 and 96% at 2 and 16 sec, respectively. For solutions B1 and B2 variations of plasma pH, of dye lot, or of the plasma donor resulted in small but statistically insignificant ($p > 0.05$) changes in the transient OD's. This is reflected in the large variability shown for B1 and B2. Solution B3, with the same low protein concentration as in B2 but with a low ionic strength, stabilized more rapidly than B2, having a value at 2 sec of 93% ($p < 0.05$). Solution B4, with the highest

protein concentration, surprisingly had only a small decrease in stabilization rate, the value at 2 sec of 96% being higher than the values for B1, B2, B3, ($p < 0.05$) but lower than the values for A1, dye in water ($p < 0.05$). Solution B5, prepared with human serum albumin,[5] had an increased stabilization rate compared to B1, which was similar in protein concentration and ionic composition, the value at 2 sec being 92% ($p < 0.05$). When the dye solution had an ionic composition similar to that of B1 or B2 but contained no protein, a small effect on stabilization rate was evident, with the values at 2 sec being 95 and 97% for C1 and C2, respectively. The values for C1 and C2 were significantly lower than the values for dye in water until 6 and 4 sec, respectively ($p < 0.05$).

Discussion. The results show that if ICG is prepared in distilled water, delayed optical stabilization will not be a significant source of error in the determination of flow by dye dilution curves with appearance times of at least 2 sec, which is less than the time between dilution and detection in most hemodynamic applications. Representative methods of anticoagulation, plasma pH values and concentration changes upon dilution were shown to have no effect 2 sec after dilution. The use of plasma instead of whole blood for the dilution should not alter this conclusion (3, 12). Changes in OD after the initial stabilization were not investigated since earlier studies had established the stability of the dye over long periods following dilution in plasma and blood (13, 14). Our results indicate that the marked temperature effect for dye prepared in protein solutions (3) is much reduced when dye is prepared in water.

Most calibration procedures involve the preparation of standards several minutes before their OD's are determined, allowing time for stabilization. When dilution curves are measured on the basis of such a calibration, the true concentration is underestimated whenever the curves are recorded before the dye has stabilized. In such cases flow is overestimated since dye concentration enters the denominator of the equations for flow.

Our results indicate that the erroneous flows reported by Saunders *et al.* (3) were a direct result of the method of dye preparation. Since a dye curve is not describable by a simple function (15), only an approximate comparison of results is possible. They report a 14% error when flow in a circulation model was measured using a dye solution similar to B1 at 37°. Our data would predict an error of between 16 and 12% for such a curve, using the reciprocals of the values for B1 at 2 and 4 sec, respectively. Our results also appear to explain the results of Spangler *et al.* (2). They found that areas of left atrial (LA) dye curves were 8% larger than the mean of the areas of curves recorded simultaneously from the LA and pulmonary artery (PA). The difference in the values for B2 at 4.6 and 9.1 sec, the mean transit times for their PA and LA curves, respectively, is 7.1% of the mean of the two values. This approximate comparison suggests that delayed stabilization was the primary cause of their result, contrary to their conclusion that the 8% difference was "more likely due to a combination of poor mixing, extrapolation errors, time-averaged sampling, and perhaps some small effect of delayed color development." Area differences in their densitometers-in-series experiments are similarly explicable by our data. The other study (4) suggestive of slow stabilization did not report the dye preparation method.

The mechanism by which proteins and electrolytes in the concentrated dye solution cause slow optical stabilization following dilution in plasma may be considered a "stabilization" of dye aggregates existing in the concentrated solution. The colloidal behavior of ICG, which has the structure of a detergent, and the associated spectral changes have been reported for aqueous solutions (11). Similar spectral changes have been reported for concentrated dye solutions containing protein (3). The spectral changes that are associated with dilution of the order seen in this study and in the hemodynamic applications of the dye have been used as evidence of dissociation into the monomer form for acridine orange and

[5] Albumisol, Merck, Sharp & Dohme, West Point, PA.

thiocyanine dyes (16, 17). It is also known that ICG's absorption peak shifts from 775 to 800 nm with protein binding at these low concentrations (13). Therefore, our results for concentrated dye prepared in water are evidence that for this case, disaggregation and binding to protein are essentially completed in less than 2 sec, as evidenced by the OD at 805 nm. After dilution in plasma, dye which was prepared in protein solutions has an absorption curve closely similar to that for dye prepared in water (3). Therefore the final binding step and state are probably the same in both cases. This leaves disaggregation as the rate limiting step causing the slow increase in OD at 805 nm following dilution in plasma for all of our solutions except dye in water. Moreover, as shown in the present study, protein type, protein concentration, and ionic composition of the concentrated dye solutions significantly influenced the rate of disaggregation. These results suggest that, compared to low dye concentrations, different, slowly reversible dye–protein associations occur at high concentrations when the amount of protein is limited, relative to the amount of dye available for binding in the concentrated dye solutions, thus "stabilizing" the aggregates, and that electrolytes alone exert a lesser "stabilizing" effect on dye aggregates.

Summary. The presence of protein and electrolyte in the concentrated indocyanine green solution was shown to slow the rate of optical stabilization following dilution of the concentrated solution in plasma. This finding explains some of the reported discrepancies in flow measurement with the dye. When the concentrated dye was prepared in distilled water the spectral stabilization after dilution was rapid (<2 sec) and should not be a source of error in flow measurement.

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