ADDITION OF SODIUM HYDROXIDE TO SERUM FOR THE COLORIMETRIC DETERMINATION OF ITS EVANS BLUE CONCENTRATION

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Evans blue is nowadays among the most favored of the dyes which are employed for the estimation of plasma volume. However, exact determination of its concentration in plasma (serum) is, on occasion, hardly accomplished, because of its turbidity which makes photoelectric colorimetry unavailable. Simple dilution with water, both distilled and saline, fails to remove it, but addition of alkali alone clears its cloudiness. The procedure to be presented below is based on this phenomenon, and enables the photoelectric colorimetry of Evans blue in a completely limpid medium.

METHOD

Reagent

1) 0.5 per cent Evans blue solution for intravenous injection (Daiichiseiyaku)

2) Standard Evans blue solution: Transfer 5.0 ml. of 0.5 per cent Evans blue solution into a 100 ml. volumetric flask with the same syringe which is used for the injection of this dye, make to volume with distilled water, stopper, mix by inversion. Preserve in a refrigerator as mother solution. Dilute it tenfold with 10 g./dl. sodium hydroxide solution on use.

3) 10 g./dl. aqueous solution of sodium hydroxide

Procedure

(1) Withdraw and transfer into centrifuge tube (1°) about 5 ml. of blood from the person to be examined, who has been lying on a bed for at least fifteen minutes.

(2) Inject 5.0 ml. of 0.5 per cent Evans blue solution (exact amount as measured with the syringe) into antecubital vein for thirty to sixty seconds, withdraw about 3 ml. of blood from the opposite antecubital vein 10 minutes later, and transfer it into centrifuge tube (2°).

(3) Allow the centrifuge tubes (1°) and (2°) to stand at room temperature until serum commences to separate from blood clot. Detach gently the clot from the inner surface of tubes with a slender glass rod lest the serum should be stained by hemolysis, and centrifuge. The supernantants thus obtained in centrifuge tubes (1°) and (2°) are denoted serum (1°) and (2°), respectively.

(4) Into separate test tubes A, B and C introduce serum and reagents as listed below in the order left to right, and mix thoroughly.

	Standard solution	Serum (1°)	Serum (2°)	10 g./dl. NaOH
A			1.00	4.00
B	0.5	1.00		3.50
C	0.25	1.00		3.75 (ml.)

(5) Within ten minutes thereafter measure the absorbances a, b and c for the mixtures A, B and C, respectively, at 610 mμ (Filter S 61) in a photoelectric colorimeter with cuvettes of 1.0 cm. optical path. Construct a calibration curve, plotting absorbance against concentration of Evans blue in which mixtures B and C are denoted as 0.5 and 0.25, because they have a half and a fourth amounts of Evans blue contained in 1 ml. of the standard solution, respectively. Read therefrom the concentration x for the absorbance a. Then the plasma volume PV is given by the equation

$$PV = \frac{1}{x} 1.$$

Discussion

Aqueous solution of Evans blue is, as the name implies, blue in color, and turns to red when alkali is added. However, little change in hue takes place if addition of alkali is preceded by mixing with serum protein.

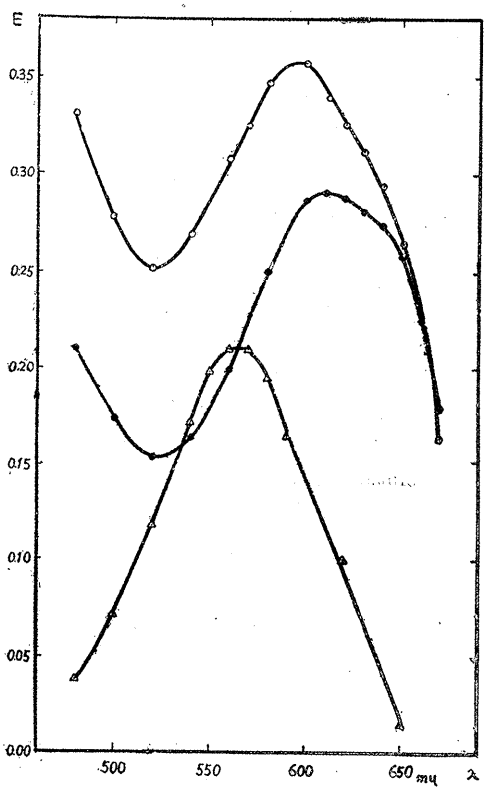

Fig. I. Light absorption curve of Evans blue.
△—△ : Standard Evans blue solution 0.10 ml. + physiologic salt solution 1.90 ml. + 10 g./dl. NaOH 2.0 ml.
○—○ : Standard Evans blue solution 0.10 ml. + physiologic salt solution 1.90 ml. + serum 2.0 ml.
●—● : Standard Evans blue solution 0.10 ml. + 10 g./dl NaOH 1.90 ml. + serum 2.0 ml.
E indicates absorbace, and λ the wave length of light

Figure 1 shows the light absorption curve of theses solutions, which was traced by Erma's photoelectric spectrophotometer over the whole range of visual wave lenghths. The maximum absorbance of Evans blue solution which was alkalinized after serum had been added did not accord exactly with that of its simple aqueous solution, because the former lay at 610 mμ whereas the latter at 600 mμ.

Nevertheless, accurate photoelectric colorimetry was possible with the serum-added alkalinized solution, since this followed Beer's law around its maximum range of light absorption as depicted in Figure 2.

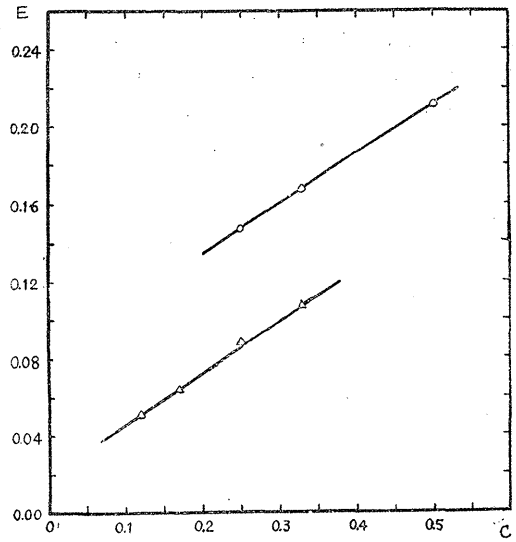

Fig. 2. Serum-added alkalinzed solution of Evans blue obeys Beer's law at 610 mμ. (E: absorbance, C: Concentration)
△—△ : The mixture of 0.12–0.33 ml. of standard solution and 1.0 ml. of serum was diluted to 5.0 ml. with 10 g./dl. NaOH
○—○ : 0.25–0.50 ml. of standard solution was added to 1.0 ml. of serum of the person who received injection of Evans blue 48 hours previously, and was made to 5.0 ml. with 10.0 g./dl. NaOH

The alkali specified by us was sufficient in amount to clear up any turbidity which might entail error in determination, but colorimetry was to be accomplished within several minutes after mixing, inasmuch as it could no longer assure complete clarity after ten minutes had passed.

The standard solution represents one liter of plasma volume when 5.0 ml. of Evans blue solution is injected to the person to be examined, since it is prepared by diluting 5.0 ml. of the dye solution to one liter. Therefore, if the serum (2°) obtained in step (2) were the same in concentration as was the standard solution, the plasma volume would be one liter in amount, and if it had x fold

of dye concentration as compared with standard solution, the volume would be $\frac{1}{x}$ 1, for it is computed that the dye has been diluted to $\frac{1}{x}$ times of one liter with plasma. The equation in step (5) was derived in this way. In step (4) tubes B and C were arranged to denote 2 and 4 liters of plasma volume, employing $\frac{1}{2}$ and $\frac{1}{4}$ amounts of dye as compared with that of the standard solution.

Normal value for plasma volume was 43.7 ml./kg. on the average (S.D.2.27 ml.), being in good accordance with that reported by American and European workers[1]. The fact that duplicate determination about the same persons with interval of forty-eight hours exhibited little discrepancy, as listed below, gives assurance for the reliability of this procedure.

Duplicate determination			
First time	45.4 ml./kg.	Second time	47.6 ml./kg.
	43.8	(48 hours later)	43.3
	42.9		41.2

Summary and Conclusion

A new method for the determination of Evans blue in serum was presented. Sodium hydroxide was added to serum to secure its limpidity, which was mandatory for the accurate photoelectric colorimetry of this dye.

References

1) Sunderman, F.W. and Boerner, F. : *Normal values in Clinical Medicine,* Philadelphia and London (Saunders), 1950