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Indocyanine green densitometry in flowing blood compensated for background dye[1]

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EDWARDS, ANTHONY W. T., JAMES ISAACSON, WILLIAM F. SUTTERER, JAMES B. BASSINGTHWAIGHTE, AND EARL H. WOOD. *Indocyanine green densitometry in flowing blood compensated for background dye.* J. Appl. Physiol. 18(6): 1294–1304. 1963.—Blood is nonhomogeneous; hence, the relationship between light transmission and increasing concentration of dye in whole blood is never the perfect exponential curve predicted by Beer's law. Instead, as the concentration of indocyanine green is increased to high levels (40 mg/liter) the light transmission decreases exponentially toward an asymptote at 6–8% transmission for nearly monochromatic densitometers (half-band width: 13–20 mμ), but at 30–40% for densitometers using light of wide-band width. Consequently, following recording of a dilution curve, circulating background dye reduces the change in transmission per unit increase in dye concentration in subsequent curves. This decrease in sensitivity cannot be compensated for by a simple increase in the sensitivity of the densitometer or in the intensity of its light source. Compensation can be attained, however, if increasing densitometer sensitivity is associated with the automatic scale expansion provided when a suppressed zero point is used. At correct zero suppression, the deflection for zero output of the densitometer coincides with the asymptotic transmission value mentioned above.

indicator-dilution in circulation blood flow measurement blood optical density dye dilution technic cardiac output measurement

VARIOUS INSTRUMENTS HAVE BEEN DEVELOPED for observing the optical properties of flowing blood in vitro, usually immediately after withdrawal of the blood from the circulation (11). These have been employed mainly for the estimation of oxygen saturation of the blood and for the detection and estimation of various indicator dyes used for studying the circulation. The Waters cuvette oximeter has been used for both of these purposes in this laboratory during the last 12 years. The present communication describes an instrument (designed by the Section of Engineering, Mayo Clinic; manufactured by the Waters Corp., Rochester, model XC100A) (Fig. 1) which has been designed and used in this laboratory for estimation of the dye, indocyanine green, by densitometry in the near infrared range. In this instrument, an interference filter is used instead of a gelatin filter, and the light detector is a phototube in place of an iron-selenium barrier-layer cell. This instrument presents several advantages, such as more readily reproducible performance characteristics, increased stability, increased output, and better dynamic response.

Several authors have called attention to the effects on calibration of instruments that are produced by varying amounts of background dye in the circulating blood (2, 4, 11). Although indocyanine green is removed rapidly from the circulation by the liver (8), it does accumulate to a significant extent when injected repeatedly at short time intervals (3). Therefore, calibration of the new densitometer for indocyanine green has been carried out against various concentrations of background dye. The effects of varying the oxygen saturation and hematocrit value of the blood on the calibration for indocyanine green also have been studied. A method of setting up the instrument has been developed by which accurate, automatic compensation for background dye can be achieved. This method is applicable to other densitometers for whole blood, the calibrations for all of which are, due to the spectral absorption characteristics of blood, sensitive to some degree to the level of background indicator in the circulatory blood.

MATERIALS AND METHODS

Description of Instrument

The densitometer, seen in cross section in Fig. 2, consists of a light source, lens, interference filter, lumen, phototube, and cathode follower.

The light source is a lens-type, 6.3-v lamp (manufactured by Chicago Miniature Lamp Works, Chicago) with an incandescent tungsten filament. This bulb has an indefinite life span if the filament voltage is kept less than 4 v.

The lens-interference filter assembly consists of two plano-convex lenses cemented one to each side of an interference filter in such a way that light going through the filter is essentially collimated and the emergent light is focused on the lumen. The interference filter (manufactured by Baird-Atomic, Cambridge, Mass.) has a band width of 20 mμ at 50% light transmission (13.5 mμ in two other densitometers tested, D_1 and D_{24}). The proportion of incident light transmitted at the maximal wavelength (795–798 mμ) is 40%.

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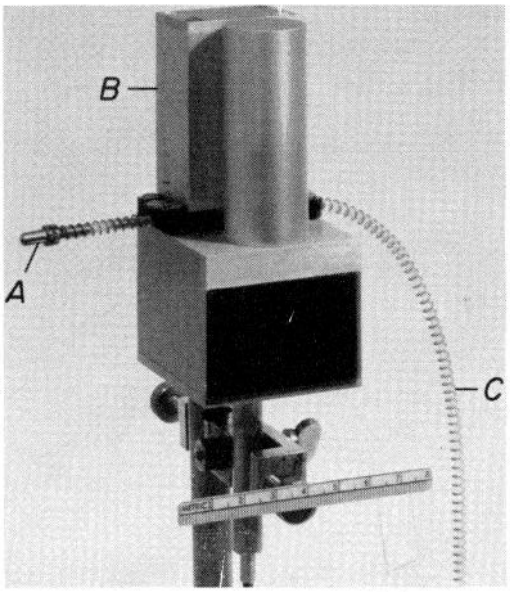

FIG. 1. Densitometer for measurement of dye concentration in flowing blood. A = male hypodermic adapter for connection to catheters or needles; B = housing containing light source and filter assembly; C = nylon tubing protected by coil spring for connection to assembly for withdrawing blood samples at constant rate of flow.

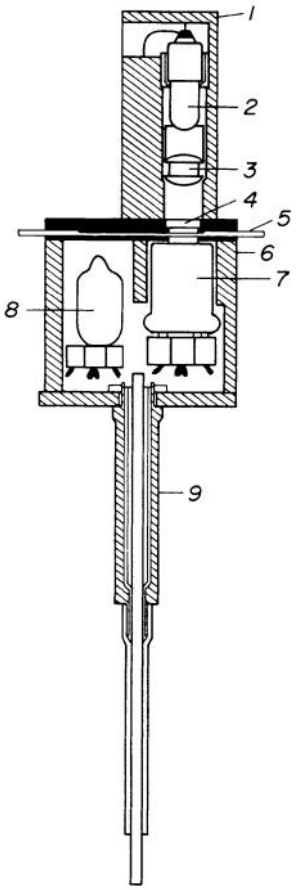

FIG. 2. Cross-sectional diagram of densitometer. 1 = aluminum housing for light-source and interference-filter assembly; 2 = 6.3-v lamp; 3 = interference-filter assembly including lens system for collimating light and then focusing filtered light on lumen (transmission peak of filter = 797 mμ; half-band width = 20 mμ); 4 = clear polymeric methyl methacrylate (Lucite) window covering lumen; 5 = connection to lumen which is milled from black Lucite; 6 = phototube housing; 7 = phototube; 8 = vacuum tube of impedance-matching circuit (cathode follower); 9 = metal mounting rod containing leads to control circuits.

The edges of the filter assembly are masked by painting them with an opaque, rubber-base paint to assure that all light illuminating the lumen passed through the interference filter.

Blood flows through the instrument in a lumen milled from black polymeric methyl methacrylate (Lucite) and is transilluminated through a portion of the lumen having clear Lucite windows. This allows only that light which has passed through the blood to reach the phototube. The transilluminated chamber has a total volume of 5.6 mm^3 (length = 3 mm, width = 2.5 mm, depth = 0.75 mm) and the lumen in the black Lucite leading to this chamber has a volume of 38 mm^3 (diameter = 1.56 mm, length = 19.8 mm). From the lumen, a 9.5-mm length of nylon tubing (i.d. = 2.1 mm) leads to a male hypodermic adapter (i.d. = 1.6 mm, length = 23 mm) for connection to the catheter or intra-arterial needle. The dead space from adapter to transillumination chamber is 0.12 ml.

The light-sensing element (manufactured by Allen B. Du Mont Laboratories, Clifton, N. J.) is a photodiode tube having a semitransparent cathode and a maximal sensitivity in the near infrared. The relationship of incident light intensity to voltage output is essentially linear. With saline in the lumen of the densitometer (incident light on the phototube) and with an exciting voltage of 45 v, the approximate phototube output is 0.1 μa. This low-current output, high-resistance circuit is unsuitable for activating a recording galvanometer. Therefore, an impedance converter (cathode follower) is included in the output circuit.

The cathode follower and control circuitry is shown in Fig. 3. A direct current is produced in the output of this circuit even when the voltage at the grid of the cathode follower is zero (no light on the phototube). Therefore the use of a bias voltage in the output circuit is necessary which can be adjusted so that the recorder indicates zero when there is no voltage output from the phototube. The photographic recording assembly (14) utilizes a D'Arsonval-type galvanometer (manufactured by the Heiland Division of Minneapolis-Honeywell, Denver, Colo.) with a natural frequency of 22 cycles/sec, optimal damping resistance of approximately 350–400 ohms, and a static sensitivity of 0.035 μa/mm deflection with a light arm of 1 m.

Individual Instruments Tested

Five different phototube densitometers were tested. These were assembled in various combinations from three phototube-cathode-follower assemblies, designated as *I*, *II*, and *III*, and four light-source plus filter assemblies designated as *1*, *2*, *3*, and *4*. In *no. 2*, the masking around the filter was absent during the early experiments but was replaced in later experiments. This assembly will be designated *2U* in the unmasked phase and *2* after masking. These individual light-source and detecting units were assembled in various combinations to produce five complete densitometers as follows: D_1 ($I + 1$), D_2 ($II + 2$), D_2U ($II + 2U$), D_3 ($III + 3$) and D_{24} ($II + 4$).

The optical densities of the various solutions of dye studied were determined in a Beckman DU spectrophotometer at a mean wavelength of 800 mμ with a slit width of 0.02 mm (equivalent to a band width at half transmission of 1.3 mμ). The samples were contained in a Waters NAC-22 cuvette with a depth of 1.04 mm, which made it possible to use the same concentrations of the test substance as in the other instruments.

Setting Up Instrument

Three standard scale positions will be referred to throughout the paper:

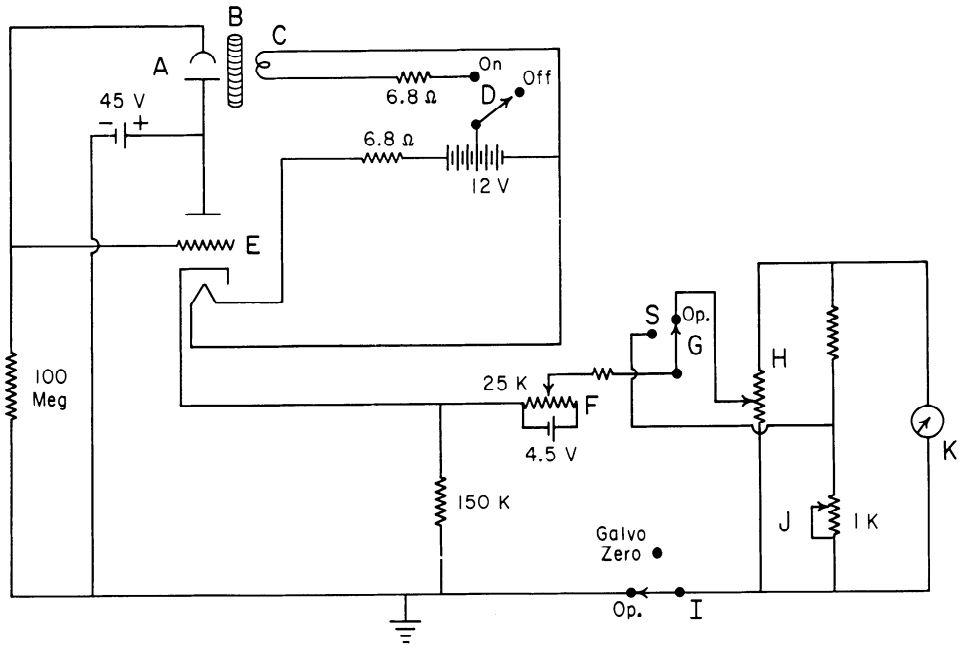

FIG. 3. Control circuit for interference-filter, phototube densitometer. A = phototube (Du Mont K-1573); B = lumen of densitometer through which blood sample is drawn; C = tungsten filament light source (220 cycles/min, 6.3 v, 0.3 amp); D = on-off switch for light source; E = vacuum tube (12AU7) for cathode follower; F = potentiometer control for cathode-follower bias voltage; G = main control switch; H = sensitivity potentiometer for use with blood in the lumen; I = switch for checking mechanical zero of recording galvanometer, K; J = sensitivity potentiometer for use with saline in the lumen; V = battery-voltage source in volts; K = resistances in thousands of ohms.

a) Galvanometer zero position: the scale reading with no current flowing through the galvanometer.

b) Zero-light position: the first resting[5] reading after the phototube light source is switched off. This does not necessarily coincide with position *a*. The position of *a* relative to *b* may be adjusted by altering the bias voltage in the cathode circuit of the cathode-follower tube (potentiometer F, Fig. 3). However except when *a* and *b* coincide, the position of *b* also varies with the setting of the sensitivity potentiometer (H, Fig. 3).

c) Blank or zero-dye reading: the galvanometer reading with the circuit intact, the light source on, and a blank sample of blood flowing through the lumen. For a given densitometer, the magnitude of the deflection between the zero-light position and the blank reading is directly related to its sensitivity to changes in dye concentration. This deflection can be adjusted by means of the sensitivity potentiometer (H, Fig. 3).

In the procedure for setting up the densitometer which follows, the abbreviations and code letters refer to Fig. 3.

The circuit is set up to record dilution curves as follows:

1) The mechanical zero of the recording galvanometer, K, is set to a predetermined point in the lower portion of the scale while switch I is in galvo-zero position.

2) Densitometer light source is turned on with switch D.

3) Blood is allowed to flow through densitometer lumen, B.

4) Switches G and I are changed to their operating (Op) positions.

5) Sensitivity potentiometer, H, is adjusted until the recording galvanometer has deflected to the chosen blank or zero-dye reading on the upper portion of its scale.

6) Light source is turned off with switch D, and with switches G and I in operating positions, cathode-follower bias voltage is adjusted with potentiometer F, until the galvanometer is at a predetermined reading below the mechanical zero of the galvanometer. This is the zero-light reading. The magnitude of this zero suppression determines the degree of increase in sensitivity of the instrument that occurs when the setting of potentiometer H is changed (*step 5*) to compensate for the amount of dye in the blood just prior to the recording of each dilution curve. The amount of zero suppression, that is, the distance between the galvanometer zero and the galvanometer zero-light reading is usually about 10% of the distance between the zero-light and zero-dye readings.

7) Light source is turned on and potentiometer H is readjusted so that the galvanometer beam returns to the zero-dye reading.

8) *Steps 6* and *7* are repeated to obtain proper final settings of potentiometers F and H. Densitometer controls are now properly adjusted for the recording of dilution curves. If *step 5* is repeated just prior to the recording of each successive curve, the sensitivity of the instrument to increments in concentration of the dye in blood will remain constant.

Additional adjustments are required to provide a means of checking the stability of the device over long periods of operation. This can be accomplished by filling the densitometer lumen with saline, and then, with switch G in the "S" (saline) position and switch I in the "Op" position, potentiometer J is adjusted so that the galvanometer beam rests at the zero-dye reading.

At any subsequent time during the procedure, possible drifts in the sensitivity of the instrument can be detected by filling the lumen with saline and recording: *a*) the galvanometer-zero reading with switch I in galvo-zero position, *b*) the cathode bias (zero-light) reading (switches D and G in off and Op positions, respectively), and *c*) the saline reading (switches G, I, and D in S, Op, and On positions, respectively). The over-all sensitivity of the instrument is directly proportional to the difference in the saline and zero-light galvanometer readings.

After completion of the adjustments and readings just described, the initial and all subsequent dilution curves are recorded as follows:

1) Switch G should be in the Op position and, initially, switch I should be in the galvo-zero position.

2) Begin blood flow through densitometer lumen.

3) Change switch I to its Op position.

4) Adjust sensitivity potentiometer H until the galvanometer beam is at its zero-dye reading toward the top of the scale.

[5] The definition has been stated in this manner since the first resting reading is followed by a slow drift.

TABLE 1. *Change in output of three densitometers with time (drift)*

Time,* min	Output as % of Output at Zero Time D_1	D_2	D_3
0	100	100	100
15	100	99.4	99.8
58	100	99.7	100.2
84	100.7	98.8	98.6
101	100.1	98.7	98.5
132	100	100.4	99.0
152	100.1	100.6	99.5
174	99.7	99.1	99.0
185	99.9	98.8	99.0
0	100	100	100
40	99.4	99.0	99.6
65	99.4	99.0	100.1
95	99.2	98.6	100.1
135	98.6	98.8	100.6
175	98.5	98.5	101.0
205	97.3	98.5	101.3
235	96.8	98.5	101.3
255	97.0	98.3	100.6

Saline in the lumens, circuits at constant gain, lights on. *In the first run the instruments were switched on 120 min before, and in the second run, 40 min before zero time.

5) Inject dye and record dilution curve.

In the experiments to be described, the instruments were set up so that zero-light and galvanometer zero positions coincided unless otherwise specified.

Dynamic Response of Densitometer

The response of the complete densitometer assembly, including the hydraulic connections, the lumen, electric circuitry, and recording system, was tested by establishing a fast rate of blood flow (250 ml/min) through the instrument and introducing a stepwise change in optical density in the blood just before it entered the lumen. For this purpose, a solenoid-operated valve, previously described (5), was used, enabling an almost instantaneous change from dye-free blood to blood containing a uniform concentration of indocyanine green. The resultant change in electrical output was recorded photokymographically at a paper speed of 25 mm/sec. The instrument was calibrated for indocyanine green, and the dynamic response for a given blood flow was expressed as the time elapsing between the onset of the deflection of the galvanometer beam and attainment of a deflection representing 90% of the total change due to dye concentration. The dynamic response also was determined at a flow rate of 25 ml/min with a 19-gauge needle on the male hypodermic adapter.

Calibration Procedures

Two substances were used, indocyanine green (supplied as Cardio-green through the courtesy of Hynson, Westcott & Dunning, Inc., Baltimore), a dye having its absorption maximum at about 800 mμ (6), and India ink, a black substance and consequently having a broad, flat absorption spectrum. As the absorption of light by indocyanine green is not completely stable in dilute aqueous solutions (6), it was tested either in plasma or whole blood. Suspensions of India ink (Higgins) are stable in most aqueous dilutions. Optical density readings of these solutions, made 24 hr apart in the Beckman DU spectrophotometer, were found to be identical. Occasionally at certain dilutions, the carbon particles aggregated and came out of suspension, a change obvious to the naked eye. Suspensions of India ink were studied both in distilled water and in whole blood. Optical density readings in the densitometer for suspensions in whole blood were unchanged when repeated 2 hr later.

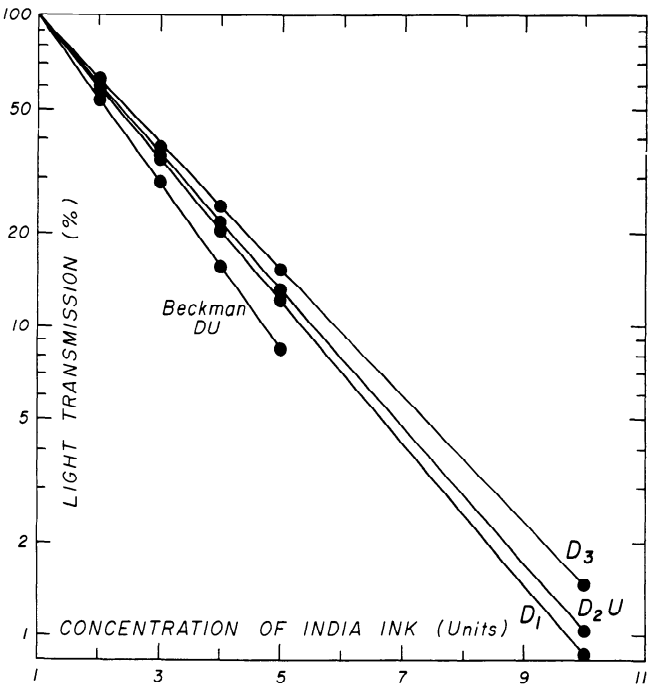

FIG. 4. Comparison of calibration curves for the Beckman DU spectrophotometer and three densitometers using India ink in water. One unit of concentration was 0.2% of the concentration of the stock solution. The origins of all curves were set at 100% (full scale) deflection for 0.2% India ink. The straight lines were drawn through this point and the point in the 10–20% deflection range. The differences in slope may be attributable to differences in cuvette depth. The adherence to Beer's law in each instrument indicates linearity of the detecting system to equal decrements in incident light.

The blood used was either freshly drawn, heparinized dog's blood or heparinized, citrated human blood which had been stored under refrigeration up to 5 days, after use in a mechanical pump-oxygenator during intracardiac surgery. There was no detectable difference between results obtained with blood from either source; the deviation from Beer's law observed with varying concentrations of indocyanine green in blood was present and of the same order with both dog's blood and human blood.

For calibration, serial dilutions of the test substance were prepared and run through the instrument under a constant head of air pressure to produce a steady flow of about 10 ml/min. When testing for the effect of background dye, a calibration run was performed using a blank free of dye followed by a second run using one of the samples containing dye as a blank. Variations in hematocrit values were produced by allowing blood to stand in the refrigerator for several days, and then separating and remixing the plasma and cell layers in various proportions. Hematocrit values as high as 65% were readily obtained by this means, but for higher values, centrifugation was necessary. The hematocrit values of the samples were measured in Wintrobe tubes after spinning at 2,000 rev/min for 55 min.

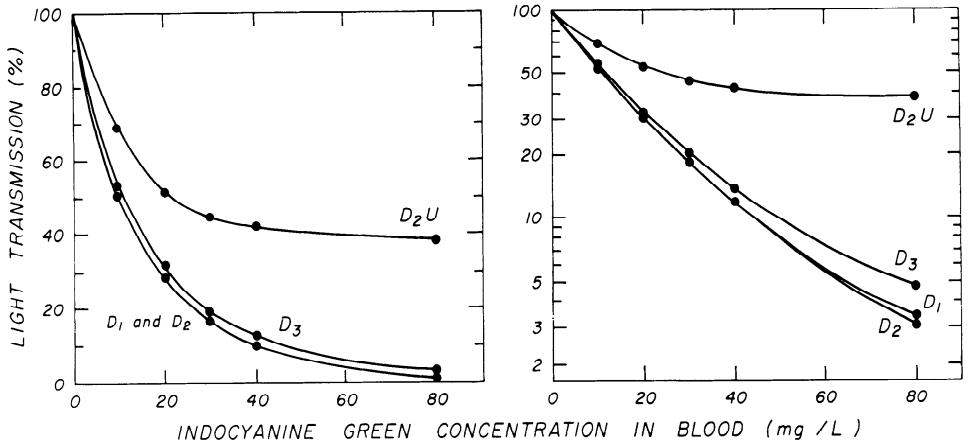

FIG. 5. Calibration curves of four densitometers for indocyanine green in whole blood. The data on densitometers D_1, D_2, and D_3 illustrate that when the waveband of incident light is narrow, that is, nearly monochromatic, the calibration curves are similar and differ little from the relationship expected from Beer's law. In densitometer D_2U, a light leak around the interference filter gave a wide (polychromatic) waveband of incident light and resulted in marked deviation from Beer's law. (Deviations from Beer's law over the range 0–30 mg/liter were: D_1 = 6%; D_2 = 6%; D_3 = 8%; D_2U = 38%.)

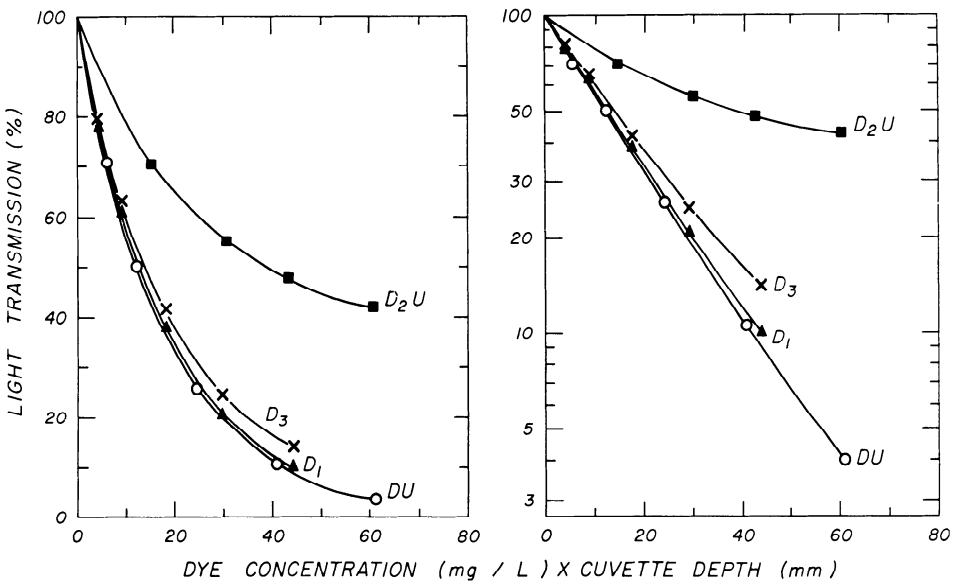

FIG. 6. Calibration curves of four densitometers for indocyanine green in plasma. In comparison with the data given in Fig. 5 for the dye in whole blood, there is less deviation from Beer's law when the dye is dissolved in plasma. Thus, the presence of red cells in itself causes deviation from Beer's law. (Deviations from Beer's law, assessed over the range 0–60 mg/liter were: Beckman DU = 1%; D_1 = 2.6%; D_3 = 5.9%; D_2U = 37%.)

Experiments to Test Effect of Background O_2 Saturation

Two dogs, anesthetized with morphine and pentobarbital, were used. Catheters were placed with their tips in the coronary sinus and main pulmonary artery, and a needle was inserted in the left femoral artery. Blood was withdrawn by suction into burettes from two of these sites simultaneously. The blood was run quickly into a measuring cylinder and pipetted immediately into test tubes containing accurately measured amounts of indocyanine green. The test tubes and cylinder for the venous blood were previously filled with nitrogen to minimize the degree of oxygenation of the blood during manipulation. These known solutions of dye in blood were then immediately run through the densitometers. The degree of oxygen saturation of the freshly drawn blood samples was determined by manometric analysis (Van Slyke). On one occasion these analyses were repeated after the process of adding and mixing known amounts of dye with the blood had been completed. The oxygen saturation values before and after mixing with dye were 78.8 and 81.7%, respectively, indicating that values from fresh blood represented reasonable approximations of the saturation of the blood-dye samples examined in the densitometers.

RESULTS AND COMMENT

Stability of Densitometer

Three instruments, D_1, D_2, and D_3, were observed for periods of 3 and 4 hr. With clear saline in the lumen, the output was recorded at constant gain with switch G at the saline position, S. The results are given in Table 1, expressed as a percentage of the original output. The maximal drift during 4 hr under these circumstances was 3%. The degree of stability was dependent on the state of charge of the batteries supplying power to the instruments, the most troublesome being the 45-v B battery. This battery was always replaced before any experiment that required hour-to-hour stability.

Linearity of Phototube, Cathode Follower, and Galvanometer System

Figure 4 shows a plot of the logarithm of light transmission against concentration when serial dilutions of India ink in distilled water were read in the Beckman DU spectrophotometer at 800 mμ, and in densitometers D_1, D_2, and D_3. The straight-line relationship in the spectrophotometer indicates good agreement with Beer's law. In view of the broad, flat absorption spectrum of India ink, it is likely that India ink will

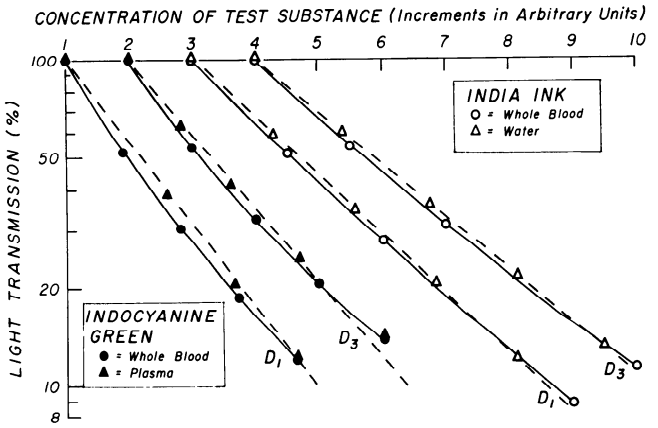

FIG. 7. Calibration curves of dye and India ink in whole blood superimposed on the corresponding curves when in plasma or water, for two densitometers. To superimpose the lower ends of the curves, the concentration scales have been adjusted. The values obtained with whole blood as the diluent are connected by solid lines. The dashed straight lines are visual best fits to the values obtained using water or plasma as the diluent. Note that the presence of red cells caused a systematic deviation whether measuring concentrations of indocyanine green or India ink.

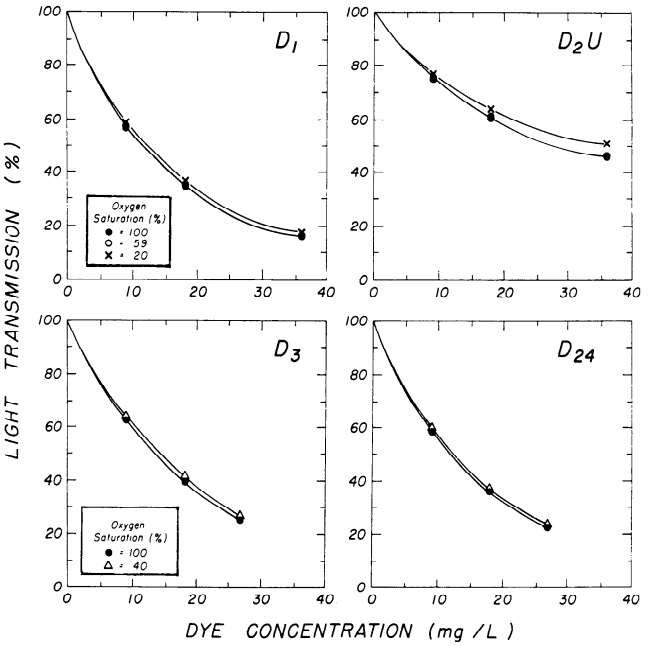

FIG. 8. Effect of the degree of O_2 saturation of the blood on the calibration of four densitometers for indocyanine green in whole blood. In all densitometers, higher O_2 saturations were associated with greater sensitivity to dye. The effect of varying O_2 saturations was less in those instruments in which the curves deviated least from Beer's law. That is, the order of merit of these four instruments with respect to Beer's law and to constancy of calibration in the presence of various O_2 saturations was D_1, D_{24}, D_3, and D_2U. The data from D_2U illustrate the adverse effect of the use of polychromatic incident light on the calibration characteristics of a densitometer.

also obey Beer's law in the presence of the broader bands of incident light in other instruments. Thus the linearity of the semilogarithmic plots from the latter instruments provide evidence of the linearity of the remaining parts of the system, the phototube, cathode follower, and galvanometer of each densitometer. Though linearity appeared to be perfect down to 10% light transmission, the readings at 1–3% transmission were slightly off the line joining the other points. These represented deviations of less than 0.25% transmission and well may have been due to measurement errors.

Dynamic Response Characteristics of Densitometer

With a blood flow of 250 ml/min through the densitometer lumen, a square-wave change in the concentration of dye in the blood was induced (using the solenoid valve), and the time required for the scale reading to change to 90% of the final reading was measured. The values obtained for three instruments were: D_1 = 0.12 sec, D_2 = 0.13 sec, D_3 = 0.12 sec. This represents the 90% response time of each system as a whole under conditions of maximal blood flow. With a 19-gauge needle on the male hypodermic adapter and a flow rate of 25 ml/min, the recorded appearance time of indicator in the densitometer lumen was 0.30 sec and the 90% response time was 0.89 sec.

Calibration Curves for Indocyanine Green in Flowing Blood

Results from four densitometers are given in Fig. 5. The curve for D_2U demonstrates the performance of such an instrument when unfiltered (polychromatic) light is used to transilluminate the blood. The arithmetic plot of the light transmission against concentration of dye in blood for the four instruments showed the expected nonlinear relationship. A similar plot, except that the ordinate represented the common logarithm of the light transmission, measured from the zero-light end of the scale, also is shown. Provided the detecting-recording system responds linearly, the deflection measured from this reference point will be proportional to the intensity of the light reaching the light-sensitive surface. If, in addition, the absorption of the incident light by the test substance obeys Beer's law, the concentration of this substance will be proportional to the logarithm of the transmitted light and hence to the logarithm of the galvanometer deflection measured in this way.[6] Having shown that there is little deviation from linearity in any of the detecting-recording systems, it is clear from Fig. 5 that in densitometers D_1, D_2, and D_3, the dye-blood mixture fits a relationship significantly but only slightly different from Beer's law but that, in densitometer D_2U, there is much greater deviation.

Deviation From Beer's Law: Investigation of Possible Factors

As Nilsson (12) has recently pointed out, deviation from Beer's law may be expected in instruments employing a wide waveband of incident light, the dye itself acting as an additional filter which alters the spectral composition of light falling on the photocell more and more as the dye concentration increases. This is borne out by the far greater deviation from Beer's law observed when incident light of a broad spectrum was used

[6] Beer's law states that $I = I_0 \cdot 10^{-Ecd}$ where I_0 = the incident light falling on the sample, I = the light transmitted by the sample, c = the concentration of the test substance in the sample, d = the lumen depth, and E = a constant for the test substance at that particular wavelength. If the incident light and the lumen depth are constant, as may be assumed here for a given instrument in a given experiment, the equation may be written $I = K_0 \cdot 10^{-K_2 c}$ or $\log I = K_1 - K_2 c$ (where K_0, K_1, K_2 are constants). This indicates the linear relationship between concentration of the test substance and logarithm of transmitted light.

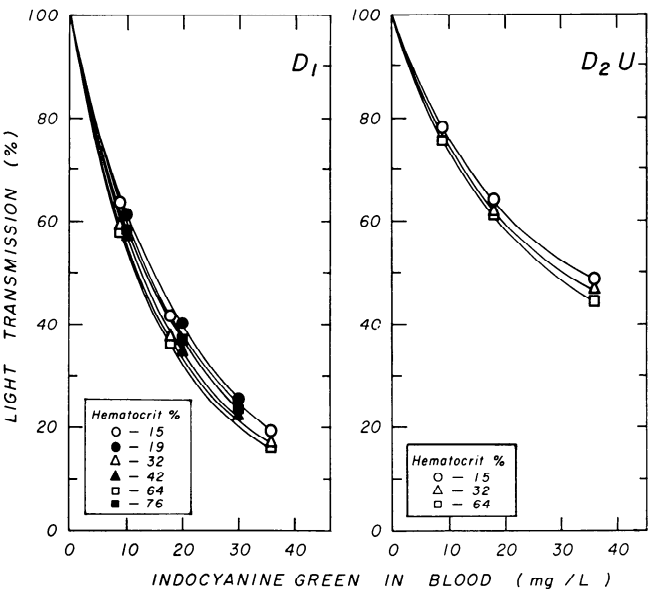

FIG. 9. Effect of hematocrit value on the calibration curves of two densitometers for indocyanine green in whole blood. Variation in hematocrit value from 30 to 70% had little effect on the calibration curve of densitometer D_1, in which the incident light was relatively monochromatic. In D_2U, in which polychromatic incident light was used, the effect of hematocrit value was somewhat more evident. In both, an increase in hematocrit value resulted in an increased sensitivity to increments in concentration of dye.

(D_2U). In an attempt to shed some light on the deviation evident even in the other densitometers, additional calibration studies were carried out with indocyanine green in plasma and with suspensions of India ink in water and blood.

Calibration curves for indocyanine green in plasma. The behavior of four instruments was examined with serial dilutions of dye in plasma: the Beckman DU spectrophotometer at 800 mμ, and densitometers D_1, D_3, and D_2U. The results are shown in Fig. 6, where light transmission relative to the blank has been plotted both arithmetically and logarithmically against the product of dye concentration and lumen depth (the latter differing from one type of instrument to the next). It may be seen that indocyanine green in plasma follows Beer's law closely in a spectrophotometer to an optical density of 1.0 (10% light transmission), that in D_1 there is little deviation, and in D_3 a little more, but that in D_2U there is marked deviation; thus the relative order of the results among the instruments is the same with indocyanine green in plasma as it is in blood, but in the absence of red cells, the degree of deviation from Beer's law is diminished in the instruments utilizing more nearly monochromatic light.

India ink in water. As the light spectrum absorbed by India ink is many times broader than that absorbed by Indocyanine green, it is to be expected that the proportion of incident light transmitted by suspensions of India ink will be much less dependent on the spectral composition of this light. Therefore, the differences between instruments just noted should tend to disappear with suspensions of India ink if those differences were due to differences in the spectral characteristics of the light-source filter assemblies. The results plotted in Fig. 4 demonstrate that this was so, and that Beer's law was obeyed by suspensions of India ink in water in all the instruments tested, including densitometer D_2U.

India ink in whole blood. Figure 7 shows calibration curves of two densitometers for India ink in blood superimposed on the corresponding curves for India ink in water. These results show deviation from Beer's law in the presence of red blood cells when using a substance that otherwise conforms to this relationship in these instruments. Indocyanine green (Fig. 7), whether in whole blood or plasma, shows greater deviation in these instruments than does India ink in whole blood or water. It is concluded that in instrument D_1, and possibly in D_2, the phenomena described were related at least as much to the use of blood as a medium as they were to the failure to achieve an adequate degree of monochromaticity in the detecting system. In the other densitometers, the additional deviations from Beer's law observed were attributable to greater deviation from monochromaticity and possibly to other characteristics of the instruments.

DISCUSSION

In considering the relationship of light transmission of whole blood to its oxygen content, Kramer and associates (9) concluded that such a system obeyed Beer's law in their whole-blood spectrophotometer as long as the hemoglobin and hematocrit values of the blood remained unchanged. However, slight nonlinearity similar to that just noted is apparent on close inspection of their Fig. 4. This was not present when they tested the optical properties of hemoglobin solutions. Implication of the spectral transmission characteristics of whole blood as a factor in the deviation from Beer's law is important in deciding whether to strive for further improvements in the optical design of these devices. If, as it appears from these studies, most of the residual deviation in the better instruments is due to the spectral characteristics of blood, little could be expected from such efforts. However, we will show that the closer the calibration curves of these instruments can be made to approach Beer's law, the simpler will be the measures required to compensate for variations in the characteristics of the background medium.

Effects on Calibration of Variations in Blood O_2 Saturation, Hematocrit Values, and Background Dye Concentration

Effect of changes in O_2 saturation of the blood. In one experiment, blood samples with O_2 saturations of approximately 100%, 60%, and 20%, drawn simultaneously from the femoral artery, pulmonary artery, and coronary sinus of the same dog were used to make up otherwise identical sets of serial dilutions of indocyanine green. These dilutions were run through densitometers D_1 and D_2U with the same instrument sensitivity at all saturation values. The results are given in Fig. 8. Also given are the results of a second similar experiment using D_3 and D_{24} at two O_2 saturation values, 100 and 40%. The resulting calibration curves were slightly different for D_1, D_3, and D_{24}, while those for D_2U were strikingly different, especially at low values of oxygen saturation. All the instruments were most sensitive to dye when the oxygen saturation of the blood was high. The pH and pCO_2 of the blood were not measured. It is possible that the small effects on the sensitivity of the densitometers to indocyanine green in blood of different degrees of oxygenation may have been due in part to nonspecific changes in optical density associated with the increased pCO_2, decreased pH, and increased red-cell volume of venous blood (13).

These results demonstrate that, except in circumstances requiring exceptional accuracy (1), the effect of oxygen saturation on the calibration of the phototube densitometer may be

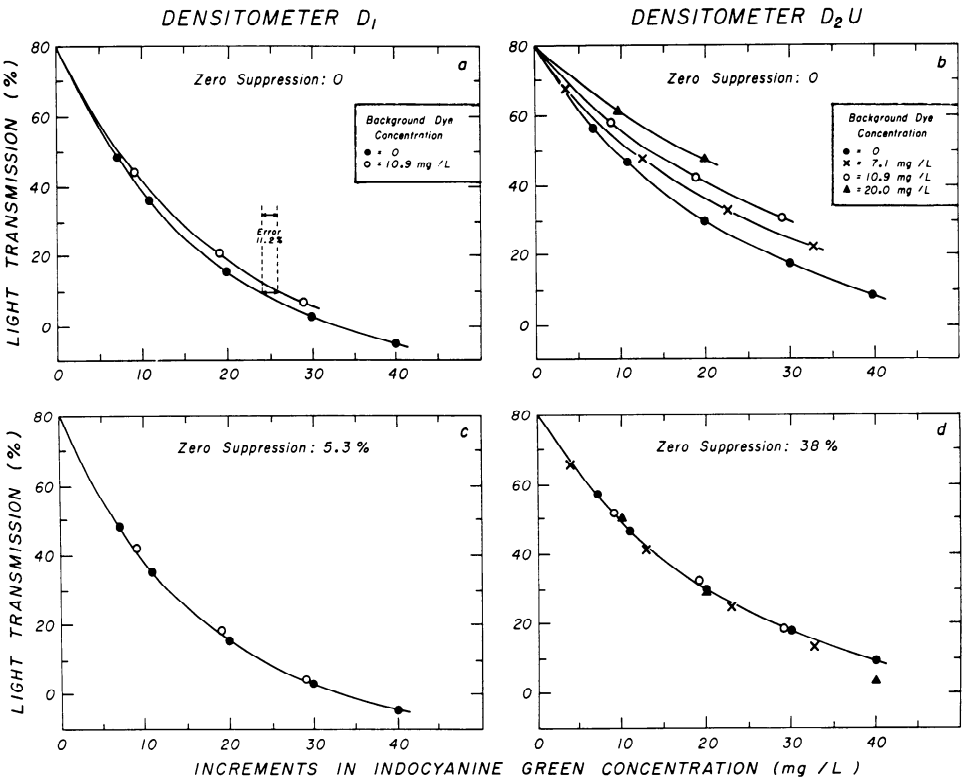

FIG. 10. *Upper panels:* effect of background dye on the calibration curves for indocyanine green in blood in a monochromatic densitometer (D_1) and a polychromatic densitometer (D_2U). *Lower panels:* effect of appropriate zero suppression on compensation for background dye. The degree of zero suppression is defined as the ratio of a to d_0 in which d_0 is the galvanometer deflection produced by changing the phototube illumination from zero to that transmitted through undyed "blank" blood and a is the deflection from zero illumination to the mechanical zero position of the galvanometer. See text and Fig. 13 for additional details.

regarded as negligible but that, with inadequate monochromaticity the effect of variations in oxygen saturation becomes greater.

Effect of changes in hematocrit value. The effect of hematocrit values on the calibration of the densitometers for indocyanine green in whole blood is shown in Fig. 9. Hematocrit values varied from 15 to 76%. The blank reading was set to the same value for each set of samples at the different hematocrit values by adjusting the sensitivity potentiometer (H, Fig. 3). In D_1, there was little difference between the indocyanine green calibration curves at hematocrit values of 32%, 42%, 64%, and 76%. However, at 19 and 15%, the sensitivity to dye was somewhat diminished, so that the use of the calibration curve obtained with blood of the highest red-cell content would result in an error of 6–12% in dye concentration if used for blood with the lowest hematocrit value. In the densitometer with the unmasked filter (D_2U), the corresponding error was about 20%.

Effect of changes in concentration of background dye on calibration for indocyanine green. The major use of indocyanine green has been as the indicator in the dilution method of studying the circulation. Since such studies frequently involve repeated injections of the dye, it is desirable to be able to measure increments in concentration in the presence of varying amounts of background dye in the blood. There are two broad approaches to this goal: *a*) individual calibrations may be carried out for each dye-dilution curve as suggested by McNeely and Gravallese (10), or *b*) special attention may be paid to instrumentation so that a single calibration curve applies throughout a procedure on a given experimental subject. The second approach offers considerable advantage when performing large numbers of dilution-curve studies in each subject. In this instance, the main problem to be overcome is that, as dye accumulates in the blood of the subjects, the light transmitted by the blood diminishes exponentially, with resultant loss in sensitivity to any additional dye that may be added. This could be avoided if a method were used that allowed resetting the instrument before each injection of dye (or each calibration run) so that an appropriate adjustment in sensitivity would be achieved automatically. One method that has been used widely is to alter the sensitivity of the system prior to each determination to bring the base line (blank reading) back to the same position on the galvanometer (11) scale. The basic tests of the effect of background dye described here were made using this method of compensation. The densitometers were set up so that the zero-light position coincided with the galvanometer zero, that is, so that the dark current was bucked out exactly by the cathode bias voltage. Sensitivity was changed with potentiometer H in the phototube densitometer circuit. The results are given in the upper panels of Fig. 10. In spite of using this method of increasing the sensitivity, the presence of background dye caused a decrease in sensitivity from the original calibration curves in both instruments. This was most marked in the polychromatic densitometer, D_2U. In D_1, the effect of as much as 10 mg/liter of background dye appeared to be small, but represented a deviation of about 11% in the dye concentration corresponding to a given galvanometer deflection.

The inadequacy of this simple method of increasing sensitivity is related to the degree of nonconformance with Beer's law exhibited by dye-blood mixtures in the various instruments. If a system such as India ink in water is used, the calibration curve is a simple exponential, and the simple method of increasing sensitivity described above achieves excellent compensation for changes in the background level of India ink (Fig. 11).

Theoretical Considerations in Compensation for Effect of Background Dye on Calibration of Densitometers

The method developed to achieve compensation for the effect of background dye hinges on four basic considerations:

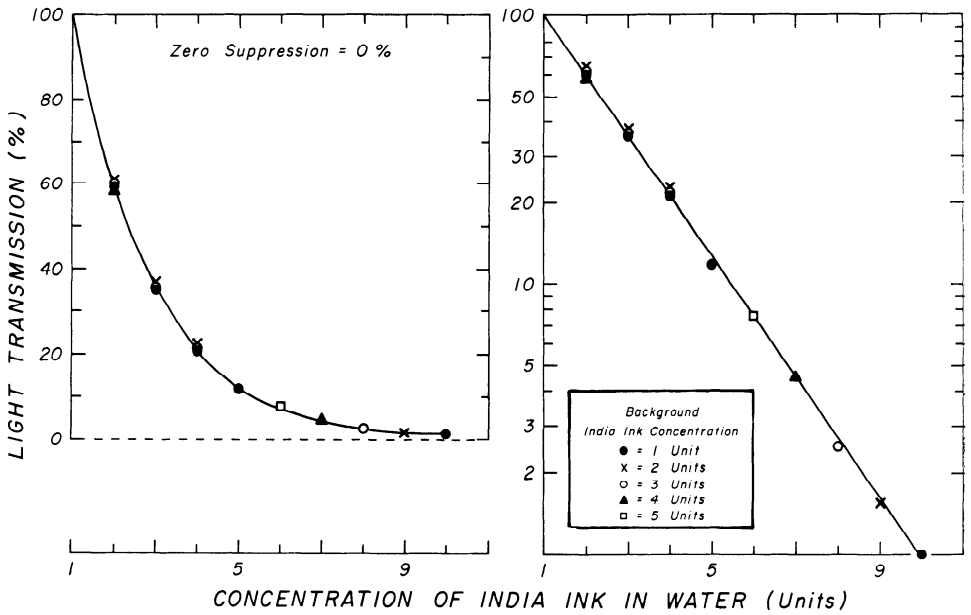

FIG. 11. Arithmetic and semilogarithmic plots of calibration curves of densitometer D_1 for India ink in water in the presence of different amounts of background India ink. One concentration unit of India ink was the concentration of a 1:500 dilution of the stock suspension of Higgins' India ink. In the initial run, a concentration of 1 unit was used as the blank and increments were of 1 unit of concentration. In subsequent runs, concentrations of 2, 3, and more units were used as the blank. No zero-suppression voltage was employed (galvanometer-zero and zero-light positions coincided). Within limits of experimental error, the compensation for background India ink was perfect, there being no change in the sensitivity to corresponding increments in concentration. This excellent compensation was expected since the plot of the logarithm of the transmission versus the concentration was a straight line (*right panel*) indicating conformance to Beer's law.

1) The calibration curve of the densitometer for indocyanine green in blood is nearly, but not quite, exponential.

2) It appears from the experiments with India ink in water that compensation for background substance can be achieved when the calibration curve for the substance is exponential.

3) It is possible to approximate a perfect exponential curve for indocyanine green in blood much more closely if the data are replotted using the mathematical device known as the $X = \log (x + x_0)$ transformation (7). This means, in effect, that the data can be fitted to an exponential curve much better if each of the values is adjusted by some constant x_0.

4) From the point of view of increases in sensitivity, the form of the calibration curve detected by the instrument is that which prevails at the site in the circuit where sensitivity is varied, the sensitivity potentiometer (H, Fig. 3). The recorded current is a function of the potential difference between the central and lower terminals of the potentiometer as drawn in Fig. 3. It is easy to change all values of this current by a constant amount by incorporating a constant voltage source proximal to the potentiometer (for example, at potentiometer F in Fig. 3). The effect produced may be illustrated by a numerical example: The series, 9, 5, 3, 2, 1½, 1¼, when adjusted by subtracting 1 from each value, results in the exponential series, 8, 4, 2, 1, ½, ¼.

Data pertaining to the first consideration listed in this section were presented in preceding sections. *Consideration 2* can be verified graphically as follows: Fig. 12 illustrates the effect of an increase in sensitivity (to S times its original value) on the slope of two calibration curves, the first, an exponential curve (left panel) and the second, a near-exponential curve (right panel). In each case, all points on the curve are shifted by a vertical distance representing the logarithm of S, but the result in the first case is to maintain the slope of the original calibration curve while in the second case it does not. It can be seen from this figure that the explanation of the background dye problem lies in the fact that, when the calibration curve of the instrument being used is not exponential, its slope, on a semilog plot, changes from point to point along its length and hence simple multiplication of deflections obtained at higher dye concentrations cannot reproduce the slope of the original part of the curve. When the curve is exponential, this is not a problem.

In relation to *consideration 3* it is relatively easy for a given instrument to find, by trial and error, the constant ($x_0 = a$) by which to adjust the observed galvanometer deflections ($x = d$) so that the relationship of ($d - a$) to the blood concentration of indocyanine green is exponential over a considerable range (up to 40 mg/liter) (Fig. 13). This finding suggests that, however much dye is added to the blood, there will always be some residual light not absorbed by the dye.

To achieve the desired adjustment of values at the sensitivity potentiometer, the procedure outlined under *consideration 4* was adopted, using the bias voltage regulated by potentiometer F, (Fig. 3). Thus, for a series of equal increases in dye concentration, the sensitivity potentiometer was presented with an exponentially decreasing series of potential differences. The procedure then was to alter the sensitivity as the level of background dye varied, so as always to reset the blank reading to its original mark on the scale, just as in the standard resetting procedure described in the preceding sections. It was anticipated that this would result in maintenance of the same calibration curve regardless of the level of background dye.[7]

[7] Actually, this theory was constructed in reverse, starting with an empirical observation. Trials were being run using a negative (with respect to phototube output) bias voltage to counteract the effect of the dark current in the circuit so that the zero-light galvanometer reading (the reading with no incident light on the phototube) was identical to the reading of the galvanometer when its input circuit was opened (mechanical zero). In this situation, when the zero-light and the mechanical zero position of the galvanometer were identical (no zero suppression), the increase in sensitivity of the instrument without background dye was not sufficient to prevent a diminution in sensitivity to equal increments in dye concentration as the level of background dye increased. It was conceived by one of us (*WFS*) that a greater relative increase in sensitivity of the device would result from this

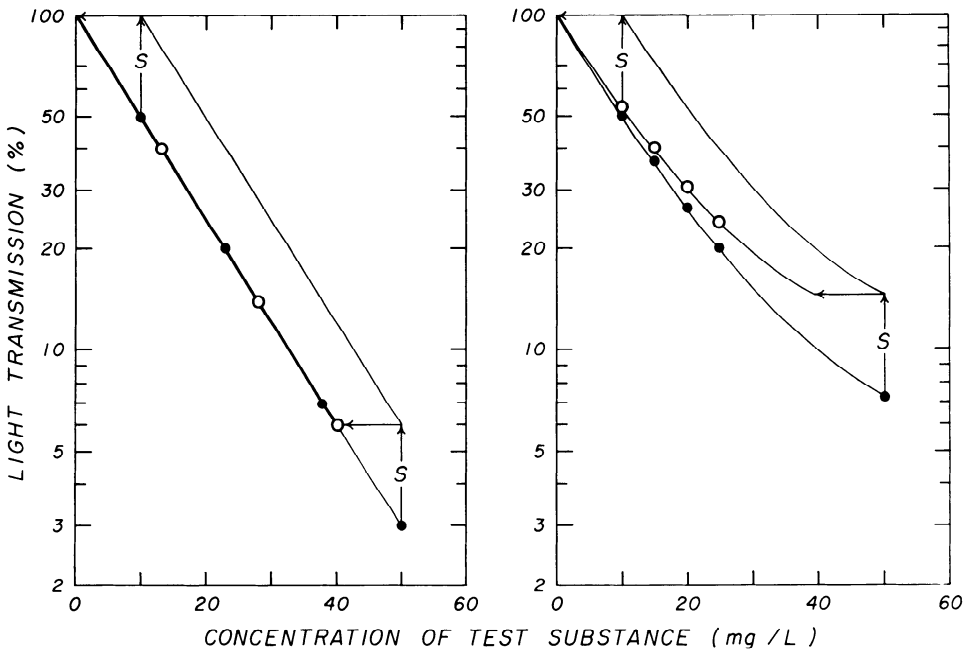

FIG. 12. Theoretical effect on calibration curves when sensitivity of densitometer is adjusted to the same scale reading by resetting on a blank containing some of the test substance. *Left panel* illustrates the situation occurring when calibration of densitometer for the test substance fits Beer's law. With some background concentration of the test substance (10 mg/liter in the illustration) as a blank, the galvanometer deflection was reset by multiplication of the sensitivity by a factor, *S*. This produces a parallel straight line originating at 10 mg/liter = 100% transmission, which, when considered as the origin (by shifting line to the left as indicated by arrows), is identical to the original calibration. Such a situation is illustrated experimentally in Fig. 11 for india ink in water. *Right panel* illustrates the situation expected when the calibration for the test substance in a given instrument does not comply with Beer's law. Multiplication of each reading on the *ordinate* by a factor, *S*, produces a calibration curve originating at 10 mg/liter. However, this curve is no longer parallel, as is seen when the origin is shifted to the left to coincide with the original origin. This is the situation occurring with indocyanine green in whole blood; experimental observations are illustrated by Fig. 10.

Zero Suppression

Definition of percentage zero suppression. Using the cathode-bias voltage as described results in a shift in the zero-light position on the scale, so that it lies negative to the galvanometer zero (phototube output considered positive). This is a form of zero suppression, and the percentage zero suppression will be considered here to mean the separation of the two zero positions as a percentage of the full scale of operation of the phototube between the zero-light reading and the blank reading on blood free of dye.

Practical procedure for determining amount of zero suppression required. A set of serial dilutions of test substance is run through the lumen of the instrument, the deflections d_0, d_1, d_2, $\cdots$ d_n are measured (from the zero-light position), and plotted against dye concentration on semilogarithm paper. This usually results in a curve. Each galvanometer value is then diminished by a constant, a, and replotted against concentration. By trial and error, the value of a is found that yields the best straight-line fit on the semilog plot (as in Fig. 13, right panel); that is,

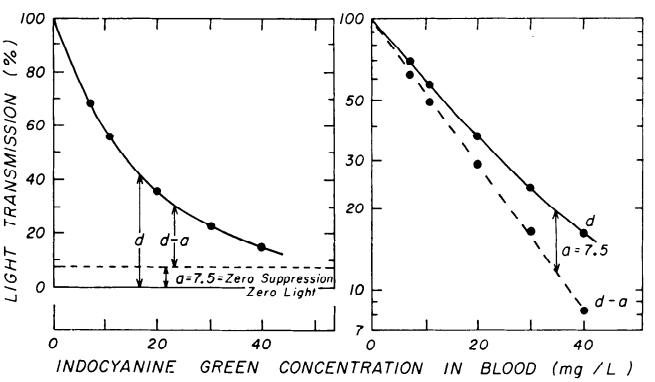

FIG. 13. Arithmetic and semilogarithmic plots of the calibration curve of densitometer D_1 for indocyanine green in whole blood. *Left panel* shows the calibration curve approaching asymptotically a line a divisions (a = 7.5) above the zero-light position. *Right panel* shows that the semilogarithmic plot of the deflection d from zero light versus concentration is curved, thus deviating from Beer's law. The plot of log (d-a) produces the desired straight line. This plot was attained by using various values of a until a straight-line relationship was found. Here, a = 7.5%, which is the amount of zero suppression which should be used so that the calibration curve will remain constant when the sensitivity of the instrument is adjusted on blank blood containing background dye by increasing the sensitivity potentiometer (H, Fig. 3) to the point that the identical deflection is obtained as on the original blank blood which contained no background dye (100% transmission). In this situation, the calibration curve approaches exponentially an asymptote coinciding with the galvanometer-zero position. Therefore, if all measurements of deflections are made from the galvanometer-zero position, the calibration will have an exponential (Beer's law) relationship (the logarithm of the galvanometer deflection will be related linearly to the concentration of indocyanine green).

maneuver if zero suppression were used. That is, if the bias voltage were increased so that the zero-light position fell on the negative side of the mechanical zero position of the galvanometer. Qualitatively this operated in the following manner: as sensitivity was increased, the negative contribution from the bias voltage was also magnified with the result that there was progressive expansion of the scale over which the phototube operated. This automatically produced a greater increase in sensitivity than otherwise would occur when the instrument was reset in the presence of background dye, and a value of zero suppression could be found at which the increase in sensitivity associated with resetting on the blood sample blank compensated perfectly for the concentration of background dye in the blank. The theory outlined was developed as a quantitative description of this phenomenon, and as a means of determining the voltage settings required by referring to the basic optical properties of the individual instruments.

a linear relationship between log $(d - a)$ and dye concentration. A good fit is usually obtained over a limited range of concentrations (0–40 mg indocyanine green/liter blood). If the deflection from zero light to the blank reading on blood free of dye is designated d_0, then the zero suppression required is $a/d_0 \times 100$ and the instrument is set up accordingly (see under MATERIALS AND METHODS, *Setting Up Instrument*).

Unless some factor, such as hematocrit value, is expected to vary widely from one experiment to the next, it has been found that the amount of zero suppression needed for compensation in a given densitometer does not vary from day to day by more than about 2% of full scale; for example, it always may lie between 7 and 9%. It has been found to be satisfactory for most purposes to use 8% suppression for this instrument.

Experimental verification. The arguments presented herein were tested in two phototube densitometers, a nearly monochromatic instrument, D_1, and a polychromatic instrument, D_2U. Both instruments were set up in the manner described, the zero suppression required being 5.3% in D_1 and 38% in D_2U. The effect of varying the concentration of background dye over a wide range was tested and compared with the results obtained without zero suppression (zero light and galvanometer zero coinciding) (Fig. 10). With appropriate zero suppression, compensation was almost perfect even under the rigorous conditions imposed by instrument D_2U, in which the incident light falling on the blood sample was polychromatic.

Advantages of the New Instrument

The interference-filter, phototube densitometer for measuring indocyanine green in flowing blood, described herein, is commercially available, rugged, stable, and has a rapid dynamic response.

Calibration of the densitometer for indocyanine green in blood approximates Beer's law and is affected to only a small degree by changes in degree of O_2 saturation over the range 100–20%, and changes in hematocrit values between 76 and 32%. The small deviation from Beer's law is due in large part to the spectral transmission characteristics of whole blood and is therefore a characteristic of all densitometers for whole blood sensitive to light in the wavelength region of 800 mμ.

None of the statements in the preceding paragraph applied to a densitometer in which white light leaked around the interference filter. This instrument provided an interesting model of the adverse effects of the use of polychromatic light as the incident light beam in these devices.

The effect of background dye concentration on calibration was studied and found to vary inversely with the degree of monochromaticity of the instrument studied, however a practically significant degree of alteration in sensitivity with background dye level was demonstrated for all instruments. A method using a zero-suppression circuit coupled with adjustment to a standard blank sensitivity was developed to compensate automatically for this effect. This method of compensation for background dye is, with minor alterations in the circuitry used, applicable to any densitometer.

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