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Use of an optical density ruler in measurements of dye-dilution curves¹

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Abstract

The inapplicability of Beer's law to densitometry of dyes in whole blood or other nonhomogeneous media has been long known. When using a densitometer whose output is directly proportional to the light transmitted, a simple transformation— $\log X = \log (x - x_0)$ —can be employed to allow rapid, accurate estimation of dye concentration. This is facilitated by use of an optical density ruler. The transformation, $X = x - x_0$, can be made mechanically, by shifting the infinity position of the ruler with reference to the zero light transmission position, or electrically, by appropriate use of zero suppression in the densitometer circuit. The transformation results in an exponential (logarithmic) relationship between light transmitted and concentration of the dye (indocyanine green). Readings of relative optical density obtained by use of the ruler are multiplied by a calibration constant to calculate dye concentration. The principles of the transformation have been applied to the determination of the optimal zero suppression required for maintenance of constant sensitivity of the densitometer in the presence of various levels of background dye.

Keywords

densitometer calibration; indocyanine green densitometer; calibration of dye-dilution curves; correction of dye-dilution curves

Continuous direct densitometry of transiently exteriorized blood has greatly facilitated the measurement of cardiac output by dye-dilution technics. The light in the densitometers used currently in such investigations is not monochromatic, and blood, of course, is not a homogeneous solution. For these reasons, the calibration curves of these instruments for dye in blood do not obey Beer's law.⁵

This paper presents a method of estimating a calibration constant from recorded serial dilutions of dye and a method of estimating the areas under dilution curves; both methods utilize a standard optical density (logarithmic) ruler, and the need for any other ruler or scale

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⁵Beer's law, relating light transmission at a single wavelength to the concentration of a substance in a homogeneous solution, may be stated $I_c = I_0 \cdot 10^{-Ecd}$ where I_0 is intensity of incident light, I_c is intensity light transmitted through a depth (d) of a solution of concentration (c) of the test substance having an extinction coefficient (E) at the chosen wavelength of light.

is obviated. Exponential extrapolation of the downslope to exclude recirculation from the primary curve is necessary but may be simplified to avoid replotting.

The method is based on the observation (3) that as the concentration of indocyanine green increases (over the range 0–30 mg/liter), the per cent light transmission decreases exponentially, not toward zero light transmission, as it would if Beer's law were obeyed, but toward a value greater than zero. The difference between this asymptotic transmission value and zero light transmission is directly related to the magnitude of the deviation from Beer's law of the concentration-optical density relationship and is least in densitometers utilizing nearly monochromatic light.

METHODS AND EXPERIMENTAL DATA

Description of ruler

The rulers are made of transparent plastic with the scale printed on the undersurface to avoid parallax (made in Engineering Section, Mayo Clinic; similar rulers are available from Baird Atomic, Inc., Cambridge, Mass.) The optical density scale ranges from zero to infinity and may be of any desired length. The relationship

$$\text{optical density} = \log_{10} \left(\frac{100}{\% \text{ light transmission}} \right)$$

is fundamental to this scale. Just as "100% transmission" may be selected arbitrarily (for example, as the light transmission through undyed whole blood), zero relative optical density also may be selected. Figure 1 illustrates the per cent transmission and optical density scales.

Dye dilution curves

After single sudden injections of indocyanine green into the central circulation of dogs, dilution curves were recorded from arterial sites downstream (1) by continuous sampling at a constant rate through densitometers (Waters XC100A, Waters Corp., Rochester, Minn.) (3). The densitometers were calibrated with serial dilutions of the dye in the dogs' blood traversing the densitometer lumen at the same rate as that used during the experiments. The densitometer output was recorded photokymographically (7) without electrical amplification.

Determination of calibration factor and scale position

To determine the calibration constant (K) of the dye dilutions illustrated in Fig. 2, the vertical position of the ruler on the recording was adjusted so that the calibration factor, $K = C/(r_c - r_0)$, was constant for all values of C . The factor r_c is the reading on the optical density ruler corresponding to a given concentration (C) of dye in whole blood. The factor r_0 is the corresponding reading obtained when blank blood ($C = 0$) is flowing through the densitometer. This is a mechanical means of performing the $\log X = \log (x - x_0)$ type of transformation (4) for the fitting of data. The position of the infinity point of the ruler obtained in this way was the position of the asymptote to the exponential of the concentration-optical density curve.

Example: the readings (r) on the optical density ruler of the calibration dilutions illustrated in Fig. 2 were recorded (Table 1) with different settings of the infinity position of the ruler on the per cent transmission scale. The differences from the blank reading ($r_c - r_0$) were

plotted on a linear scale against dye concentration (Fig. 3). It is evident that if the infinity point of the ruler is placed at the zero (0% T) position of the per cent transmission scale, the line is convex upwards, which indicates that Beer's law does not apply to the relationship between the concentration of indocyanine green in blood and the light transmission as determined by this densitometer. As readings are repeated with the infinity position set at higher values on the transmission scale, the line first becomes less curved (7.7% T) and then becomes straight when the 10% transmission position was used. At still higher positions, the line becomes concave upwards (14.8 and 16.7% T).

When the relationship between relative optical density ($r_c - r_0$) and concentration was linear (infinity position at 10% T), the calibration factor (K) was constant for all values of concentration, C . The expression, $K = C/(r_c - r_0)$, is the reciprocal of the slope of the straight line obtained when the infinity point of the ruler was set at 10% transmission (Figs. 2 and 3). The units of K are (mg/liter)/OD unit and, in this instance, $K = 5/.155 = 10/.309 = 20/.619 = 32.4$ (last column, Table 1). When a constant value for K is obtained, there is no necessity to plot the calibration curve, as in Fig. 3, since constancy of this value proves that the relationship between C and $r_c - r_0$ is linear.

Determination of areas under dilution curves

The area under the time-concentration curve may be estimated by measuring the relative optical density ($r_c - r_0$) values at chosen time intervals. This is done with the infinity point of the OD ruler set at the position determined in the manner just described, as illustrated in Fig. 4. The dye concentration, C , at each point $= K(r_c - r_0)$ and the area under the curve

$$(\text{mg sec/liter}) = K \left(\sum_{i=1}^{i=n} r_{c_i} - nr_0 \right) \div \text{no. of readings per second}$$

For dilution curves which are complicated by recirculation, the n th observation must be made somewhere on the down-slope and the remainder of the area calculated on the assumption that the dye washes out exponentially (5). In such cases, let the area under the curve up to the n th observation be called A_1 and the area under the subsequent exponential curve, A_2 . Then A_2 may be calculated in one of two ways: *a*) replot the downslope of the curve on semilogarithm paper and extrapolate by means of a straight line, making observations until the readings decrease to a chosen small value (5); or *b*) obtain an estimate of the time constant of the slope of the exponential and use a simple integration to sum the area beneath the curve to infinity.

By method *a*

$$A_2 (\text{mg sec/liter}) = K \frac{\left(\sum_{i=n+1}^{i=n+n'} r_{c_i} - n' r_0 \right)}{\text{no. of readings per second}}$$

and by method *b*

$$A_2 = \frac{K(r_{c_n} - r_0)(t_b - t_a)}{\log_e [(r_{c_a} - r_0)/(r_{c_b} - r_0)]} \text{mg sec/liter}$$

where $K(r_{c_n} - r_0)$ = dye concentration when $t = n$ (the n th reading), r_{c_a} and r_{c_b} are the optical density ruler readings which are directly proportional to the concentrations of dye at times t_a and t_b on the downslope of the curve.

The second method has the disadvantage that the determination of the time constant (τ) of the exponential, $\tau = (t_b - t_a)/\log_e[(r_{c_a} - r_0)/(r_{c_b} - r_0)]$ depends on two points only, τ can be estimated more accurately, and very nearly as quickly, by visually fitting the best straight line to a semi-logarithmic replot of $r_c - r_0$. Then τ is the time for the readings to decay from any value, $r_{c_i} - r_0$, to $0.37 (r_{c_i} - r_0)$.

Example: figure 4 shows a dilution curve recorded from the aortic root of a dog after injection of dye into the pulmonary artery. The infinity point of the OD scale was set at a position equivalent to 7.7% light transmission and readings were made at 1-sec intervals (Table 2). Estimation of area in the conventional manner (Hamilton) using the replot of the down-slope gave a total area = 98.7 mg sec/liter. The area (A_2) beneath the exponential extrapolation by method $a = K(.173) = 5.6$ mg sec/liter. Using the abbreviated method b without replotting and choosing points on the downslope at $t = 16$ and $t = 18$ sec,

$$A_2 = \frac{K(r_{c_{18.5}} - r_0)(18 - 16)}{\log_e[(r_{c_{16}} - r_0)/(r_{c_{18}} - r_0)]} = \frac{32.4(.039)(2)}{\log_e(.068/.044)} = 5.8 \text{ mg sec/liter}$$

For r_{c_n} , $r_{c_{18.5}}$ is used since A_1 includes the area up to half of the time interval beyond its last reading, and its value is estimated approximately or can be read directly from the record. The difference in estimates by the two methods amounts to less than 0.3% of the total area and is in the usual direction. Extrapolation to infinity (method b) results in calculated areas slightly larger than those obtained by extrapolation to about 1 % of the peak concentration (6).

Calibration in presence of background dye

Adjustment of the densitometer circuit so that there is no change in sensitivity to increments in dye concentration as the background level of dye in the blood increases has been described previously (2,3). For each densitometer, there is an empirically determined optimal zero suppression (Z) which brings this about. The zero suppression, Z , is given by $100 (G - O)/(B - O)$, where, for any densitometer whose output (galvanometer deflection) is linear with respect to light transmission O = zero per cent transmission (the deflection occurring when no light falls on the photocell); G = galvanometer zero (the deflection occurring when there is no output from the densitometer circuit—a direct current bias is used to adjust this value relative to O); and B = blood, or control blank, setting = galvanometer position equivalent to r_0 = the 100% transmission value.

Figure 2 illustrates these settings and the deflections obtained from a series of dye dilutions when the zero suppression was 7.7%. The determination of optimal zero suppression for this densitometer is illustrated by Table I and Fig. 3: for complete compensation for increases in background dye, the galvanometer zero (G) should have been at 10% transmission, or the same position at which the infinity point of the optical density ruler was placed to obtain a linear relationship between concentration and optical density. When optimal zero suppression is used, this relationship is linear when the infinity point of the optical density ruler is set at the galvanometer zero setting and, under these conditions, the calibration constant (K) remains constant in the presence of varying levels of background dye.

When optimal zero suppression is not used, the methods described above can be simplified and utilized for the estimation of the calibration constant and the dilution curve areas. This involves some minor approximations only.

Approximation of calibration curves by straight lines

In Fig. 3, the calibration curve obtained by placing the infinity point of the ruler at the galvanometer zero setting (see Fig. 2) is indicated by 7.7% *T*. Although this line is slightly curved, it can be approximated reasonably well by a straight line (Fig. 5, background dye concentration, *B*, = 0.0). The other calibration curves shown in Fig. 5, obtained with the same zero suppression but with increasing levels of background dye, similarly can be approximated by straight lines. The closer the zero suppression is to optimal, the straighter are the calibration curves obtained in this manner and the smaller are the differences in slope. The slopes of the lines decrease (*K* values increase) as the level of background dye increases.

This relationship between background dye concentration (*B*) and the calibration constant (*K*) is more or less linear, as illustrated in Fig. 6. The data from Fig. 5, for which the densitometer circuit zero suppression was set at 7.7%, is represented in Fig. 6 by the line $K = 0.19 B + 33.7$. Calibration curves at different levels of zero suppression, similarly approximated by a straight line (all optical density ruler measurements having been made with the infinity point set at the galvanometer zero setting), are also represented in this graph. In all cases, the relationship between *K* and *B* is apparently linear. The general equation for this relationship is: $K = mB + K_0$, where *m* is the slope of the line and *K*₀ is the value of *K* when the background dye level is zero. The slope (*m*) is an expression of the change of sensitivity to increments in dye concentration with changes in the level of background dye. (Sensitivity loss = $(K - K_0)/K_0 = mB/K_0 = m/K_0$ for each milligram of background dye per liter.) For practical purposes, this equation may be determined empirically from two sets of calibration dilutions if the background dye concentrations of each set are known and are sufficiently different. If zero suppression is optimal, then *m* = 0 and $K = K_0$ —there will be no change in the sensitivity of the densitometer to increments in dye concentration with changes in the level of background dye in the blood.

Individualization of *K* for each dilution curve

There are various simple methods for the estimation of background dye at the time of inscription of each dilution curve. These have no importance if zero suppression is optimal but, if it is not, then the use of a specific *K* for each level of background dye can contribute significantly to the accuracy of estimation of the area under the dilution curve. When the experimental procedure necessary to permit evaluation of *B* takes no extra time (0–3 sec), the method is practical: the appropriate value of *K* is simply read from the graph, or calculated by means of the equation, $K = mB + K_0$.

In estimating the areas under a series of dilution curves, such as the one shown in Fig. 4, which are recorded with zero suppression of 7.7% and the base lines of the dilution curves set at the blank setting obtained for undyed blood, all measurements are made with the infinity point of the optical density scale coincident with the galvanometer zero setting. The sum of the readings and the extrapolation for each curve (as in Table 2) results in estimates of area in optical density units. Multiplication of these by the appropriate *K* for each curve gives the estimated area in mg sec/liter.

Consideration of equation, $K = mB + K_0$, as a member of a family of lines

The data of Fig. 6 have been represented by straight lines fitted visually (solid lines). On the basis that these lines appeared to have a possible common point and that the slopes of the

lines were related to the degree of zero suppression used, an equation representing a family of lines intersecting at one point was obtained empirically:

$$K=m(B+9.0)+32.2$$

or

$$K=mB+K_0, \text{ where } K_0=32.2+9.0\ m$$

The point of intersection has no readily apparent significance except that, at optimal zero suppression ($m = 0$), K equals 32.2. The appropriate members of this family are also plotted in Fig. 6 (broken lines). Support for this approach can be seen from Fig. 7 in which the slopes of the lines fitted visually (open circles) and the slopes of lines of the family (closed circles) are plotted against the degree of zero suppression. The line drawn through these points represents the equation, $m = 0.67 - 0.066 \times \text{percentage zero suppression}$. The value of Z at $m = 0$ represents the optimal value for zero suppression (10.2%) since, at this point, the sensitivity of the densitometer to increments in dye concentration is independent of the level of background dye in the blank blood. This estimate of optimal zero suppression is completely independent of that previously obtained (Table 1 and Fig. 3 indicate 10%). The small difference necessitates the difference between the value of K obtained from Fig. 3, 32.4, and that obtained from the family of lines, 32.2.

DISCUSSION

The methods described above have general application to densitometry when Beer's law does not apply. Most of such relationships can be approximated, over a limited range of concentration, by an exponential relationship whose asymptote differs from 0% transmission. A shift of the functional axis to this asymptote permits the use of the exponential relationship instead of a more complex relationship. Such a transformation has been proposed by Gaddum to simplify the relationships in certain populations. This $\log X = \log (x - x_0)$ transformation will, in appropriate cases, reduce the data to linear relationships which are much easier to analyze.

The transformation used here (Figs. 2–4) is identical: x represents percentage light transmission, x_0 represents the asymptote of percentage light transmission approached by the concentration-light transmission curve of indocyanine green in the range of concentrations below 30 mg/liter, and X represents the per cent transmission difference from this asymptote ($X = x - x_0$). The optical density ruler converts per cent transmission to a logarithmic scale so that a linear relationship can be obtained between optical density and the concentration of indocyanine green dye.

The principles illustrated are germane to the construction and use of linear densitometers, that is, those whose deflection from the blank reading is linear with respect to dye concentration (Waters XC250, Waters XC300). These instruments cannot be expected to maintain constant calibration under conditions of increasing amounts of background dye unless the increase in sensitivity is made with respect to the asymptote of the concentration-optical density curve. Increasing the light intensity cannot produce complete compensation for background dye because the reference point assumed is the 0% transmission position. Since this is an optical phenomenon, it is unaffected by the electrical circuitry of linear densitometers, which makes the 0% transmission reading unobtainable. The corollary is that compensation can be achieved only electrically and not optically. The sensitivity loss in the

Waters densitometer XC250A (a linear densitometer) is about 1 %/mg of background indocyanine green per liter. When no zero suppression is used in the Waters densitometer XC100A or in their cuvette oximeter-densitometer, the sensitivity losses are 1.6 and 2–3%, respectively, per milligram of background dye per liter. This degree of loss is important when making repeated dye injections frequently and is even more important when areas under curves recorded by different densitometers are being compared (1).

The error introduced by measuring the relative optical density with the galvanometer zero setting as the assumed asymptote varies with the difference between galvanometer zero setting and the real position of the asymptote as estimated by the method illustrated in Table 1 and Fig. 3. In the densitometer used in the work described here (XC100A), the narrowness of the wave band of light transmitted by the interference filter results in the asymptote being at or below the position of 10% light transmission. In Waters cuvette oximeter-densitometers, the wide wavelength band of transmitted light resulted in an asymptote at about 30% transmission; the $\log X = \log (x - x_0)$ transformation was applied equally well to this instrument. However, if the zero suppression is far from optimal, then greater errors occur in the fitting of the calibration curve to a straight line. The dye curves recorded by the cuvette can be analyzed in precisely the fashion described (as in Fig. 4 and Table 2) after obtaining the estimate of the calibration factor (as in Table 1 and Fig. 3), and if the cuvette is calibrated at two different levels of background dye, the same linear relationship, $K = mB + K_0$, may be applied.

The use of the relationship, $K = mB + K_0$, is of great convenience for two reasons: 1) Perfect setting of the zero suppression is technically difficult. Changes in calibration with background dye can be compensated for, even if the zero suppression of the densitometer circuit is not optimal. 2) The use of straight lines approximating a curved calibration line allows the use of a fixed setting for all measurements of optical density and the errors induced are very small. Reference to Fig. 5 (data with zero suppression less than optimal) shows that at 5 mg/liter, the use of the calibration constant slightly overestimates the dye concentration and at 20 mg/liter, tends to underestimate it. With zero suppression greater than optimal (the two upper lines of Fig. 3), the errors are in the reverse directions.

The observation that the relationship between the calibration constant (K) and the background dye level (B) forms a family of lines related by the degree of zero suppression used (Figs. 6 and 7) provides an independent method of estimating the optimal degree of zero suppression for a given densitometer. These data also indicate the linearity of the densitometer recording system used for this study.

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FIG. 1.

Per cent transmission and optical density scales. Optical density = $\log_{10} (100/\%$ light transmission). Thus, at 50% light transmission the OD = $\log_{10} (100/50) = \log_{10} 2 = .30$; at 10% transmission, the OD = $\log_{10} (100/10) = 1$ and at 1% transmission, the OD = $\log_{10} (100/1) = 2$.

FIG. 2.

This photokymographic record of calibration dilutions of indocyanine green in whole blood is a segment of the recording providing the data of Figs. 3, 5, 6, and 7 and Table 1. Vertical lines show 10-sec intervals. With the infinity point of the optical density scale set at 10% of the distance from the zero light position to the blank setting, the “relative optical densities” ($r_c - r_0$) are linearly proportional to the dye concentration, C ; that is, the calibration constant, K , equals $C/(r_c - r_0) = 5/.155 = 10/.309 = 20/.619 = 32.4$ (mg/liter)/OD unit.

FIG. 3.

Relative optical density readings ($r_c - r_0$) on calibration curve of indocyanine green at various positions of the infinity point of the OD ruler. Data are from Fig. 2. With the infinity reading appropriately positioned (at 10% transmission in this case), the calibration factor is constant (the line is straight) and equals the reciprocal of the slope. Vertical displacement of the infinity point of the ruler upward or downward from the 10% transmission reading resulted in nonlinear calibration curves. Small displacements (for example, moving the infinity point to 7.7% transmission) result in curves that can be approximated quite well by a straight line.

FIG. 4.

Indicator-dilution curve recorded by sampling from the aortic root of a dog after the injection of indocyanine green dye into the pulmonary artery. Vertical lines show 1-sec intervals (equivalent to 2.5 cm on the recording). With the infinity position of the OD ruler at 7.7% light transmission, relative optical densities were read at 1-sec intervals and recorded in Table 2. This recording was made using a different densitometer from the one providing the recording shown in Fig. 2.

FIG. 5.

Optical density-concentration curves at each background dye level (B) were approximated by straight lines. These data were recorded with the densitometer zero suppression at 7.7% (optimal = 10%). The line labeled $B = 0.0$ is the line labeled 7.7% T in Fig. 3. The presence of increasing levels of background dye (2.5, 5.0, 7.5, and 10.0 mg/liter) resulted in a loss in sensitivity to increments in dye concentration—the slopes decreased and K increased as B was increased.

FIG. 6.

Relationship between the calibration constant (K) and the background dye concentration (B) with different levels of zero suppression. The values of K have been estimated by approximating with straight lines the calibration curves obtained from optical density readings made with the infinity point of the scale at the galvanometer zero setting. The solid lines have been fitted visually to the observations; the broken lines are members of the family $K = m(B + 9.0) + 32.2$, or $K = mB + K_0$, where $K_0 = 32.2 + 9.0m$ (these latter equations are marked with asterisks). They have a common point of intersection at $B = -9.0$, $K = 32.2$.

Fig. 7.

Influence of the degree of zero suppression (Z) on the slope (m) of the lines relating the calibration constant (K) to the level of background dye (B). The open circles represent the slopes (m) of the lines fitted visually to the data in Fig. 6; the closed circles represent the slopes of the lines of the theoretical family, $K = m(B + 9.0) + 32.2$. The change in sensitivity of the densitometer to increments in dye concentration at each degree of zero suppression is m/K_0 per milligram of background dye per liter. When no zero suppression is used, the sensitivity loss for this instrument is $0.67/(32.2 + 9.0 \times 0.67) = 1.7\%/mg$ liter of background dye. At $Z = 8\%$, the loss is $0.141/(32.2 + 9.0 \times 0.141) = 0.4\%/mg$ liter. Thus the sensitivity change is insignificant if the zero suppression is close to optimal.

Optical density readings of densitometer calibration dilutions obtained with different settings of infinity point of optical density ruler

TABLE 1

C, mg/liter	Position of Infinity Point of OD Ruler					
	%T = 0		%T = 7.7		%T = 10	
	r_c	$r_c - r_0$	r_c	$r_c - r_0$	r_c	$r_c - r_0$
0	.090		.125	.136		
5	.228	.138	.227	.152	.291	.155
10	.356	.266	.422	.297	.445	.309
20	.590	.500	.711	.586	.755	.619

Data taken from Fig. 1. The symbols are defined as: C = concentration of indocyanine green in the blood sample; r_c = optical density ruler reading of galvanometer deflection obtained when blood of dye concentration, C , was flowing through the densitometer lumen; r_0 = optical density ruler reading obtained with blank blood ($C = 0$) flowing through the lumen; % T = setting of the infinity point of the optical density ruler expressed as its position on the per cent transmission scale. The value for % T equals 100 times the deflection in centimeters from the zero light position of the densitometer to the selected setting for the ruler's infinity position, divided by the deflection in centimeters from the zero light position to the deflection obtained when the blank blood sample ($C = 0$) was flowing through the densitometer (see Fig. 2).

TABLE 2

Readings from optical density ruler for estimation of area under dye-dilution curve

Time, sec	Ruler Readings	
	r_c	$r_c - r_0$
0	.248	
1	.252	
2	.296	
3	.324	
4	.475	
5	.540	
6	.567	
7	.561	
8	.540	
9	.505	
10	.469	
11	.433	.185
12	.400	.152
13	.377	.129
14	.356	.108
15	.333	.085
16	.316	.068
17	.302	.054
18	.292	.044
19		.035
	$\sum_{i=1}^{i=18} r_{ci} = 7.338$	
20		.028
21		.022
22		.018
23		.015
24		.012
25		.010
26		.008
27		.006
28		.005
29		.004
30		.003
31		.003
32		.002
33		.002

Time, sec	Ruler Readings	
	r_c	$r_c - r_0$
		$\sum_{i=19}^{i=33} r_{c_i} - r_{c_0} = 0.173$

These data are from the recording shown in Fig. 4. The total area was calculated by the use of the Hamilton extrapolation to determine values for the 19th to the 33rd sec (down to 0.5% of the peak concentration).

$$\text{The total area} = A_1 + A_2 = \left[K \left(\sum_{i=1}^{i=18} r_{c_i} - 18r_0 \right) + K \sum_{i=19}^{i=33} (r_{c_i} - r_{c_0}) \right] \div \text{no. of readings per second}$$

$$= [32.4(7.388 - 18 \times 0.248) + 32.4 \times 0.173] \div 1.0 = 98.7 \text{ mg sec/liter}$$

. Amount of dye injected =

$$2.5 \text{ mg. Cardiac output} = 2.5 \times 60 / 98.7 = 1.52 \text{ liters/min.}$$