THE DETERMINATION OF THE CONCENTRATION OF THE DYE T-1824 IN NORMAL AND LIPEMIC PLASMAS

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The customary method of reading the optical density of a dye-stained plasma sample at a standard wave length against dye-free plasma at the same wave length is unsatisfactory in very lipemic plasma for three main reasons: (1) Few spectrophotometers are available which can accurately determine relatively small differences in optical density at high optical densities, such as are encountered in turbid, lipemic plasmas. (2) There appears to be a specific effect of the suspended fat particles in their scattering of light. This scattering results in changes in the T-1824 extinction-concentration curves which are thereby different in lipemic and non-lipemic plasmas. The difference increases with the degree of lipemia though the increase is not linear. (3) At high lipid particle concentrations the fat particles appear to be unstable and tend to coalesce and rise; optical densities of unstained samples of the same lipemic plasma may vary by as much as 10 per cent, so that the blank optical density cannot be measured precisely. Calculations of plasma volumes by the dye method with lipemic plasmas have been reported to give results which are completely erroneous (1), particularly when there is a difference in the degree of lipemia between blank and dye-stained plasmas. Such changes in the degree of lipemia are particularly marked following the intravenous administration of human albumin (2).

Several procedures have been suggested to surmount this difficulty. Luetscher (2) has recommended measuring the optical densities of plasmas at the usual wave length (λ = 620 mμ) and another "indifferent" wave length, (*e.g.* λ = 540 mμ) at which the dye absorbs relatively little light, to compensate for changes in lipemia. This, however, has been found to be impractical with plasmas where the blank optical density is 1.5 or higher in the cuvettes used (cylindrical, about 10 mm. internal diameter) at λ = 620 mμ because the blank optical density increases markedly with decrease in wave length. For photometric measurement Harington, Pochin, and Squire (3) have suggested that the dye be extracted from plasma into butyl alcohol by a procedure which is complicated and is affected by hemolysis. Crooke and Morris (4) have used the filtrate from precipitation of the plasma proteins and lipids with a hydrochloric acid-ethanol-phosphotungstic acid reagent; a correction is required in the presence of hemolysis. In addition the supernatants are not always clear and fading of the dye occasionally occurs, because of an undetermined impurity

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in the phosphotungstic acid (5). Morris (5) has used chromatographic adsorption of the dye from plasma adjusted to pH 10 onto a column of alumina, followed by elution with acetic acid and ethanol. This procedure, too, is affected by hemolysis and requires an elaborate apparatus. Another method based on the reduction of T-1824 by $Na_2S_2O_4$ in alkaline solution has been reported from this laboratory by Phillips (6). The results obtained by this method are not affected by hemolysis and are apparently quite reliable. A type of spectrophotometer more precise than may be generally available is required and it is necessary to wait 12 hours to finish a determination. Further, Phillips' method cannot correct (*a*) for the change in optical density which may occur as a result of the rise of the fat particles to the top of the plasmas after the plasmas have been exposed to the cold for a few hours, (*b*) for the possible coalescence of the fat particles, and (*c*) for the effect of the lipemia in spite of the 1:5 dilution. Other procedures, such as fat extraction with ethylene-dichloride and centrifugation at 18,000 R.P.M., have been reviewed by Gregersen (7) but were found unsatisfactory in the presence of marked lipemia. Gibson, quoted by Thorn (1), has unsuccessfully tried extraction of the fats with triethyl phosphate.

The present paper reports a procedure which permits the determination of T-1824 in lipemic plasmas. It is based on addition of acetone, which dissolves the lipids,[1] extracts[2] the T-1824, and precipitates the plasma proteins. A small fraction of the dye, varying with the plasma protein concentration, remains bound in the precipitated protein. Because of this, it is necessary to prepare a reference solution (8) of the dye in the subject's plasma for each determination. The method is relatively little affected by hemolysis, and, since the lipid particles disappear, is not at all affected by changes in the degree of lipemia. Approximately 1 hour is required for a complete determination.

The method was developed to study plasma and blood volume changes in nephrotic children treated with intravenous human albumin; it is not proposed as a substitute for the usual procedures in the absence of lipemia. Its reliability has been checked in normal individuals and with normal plasmas but not in diseases other than the nephrotic syndrome. The general procedure and precautions for the determination of plasma volume and whole blood volumes with T-1824 have been set forth in detail in the publications of Gregersen and his coworkers and those of Gibson and his associates.

Materials and Technic

Reagents

Acetone, analytical reagent.

T-1824, 0.5 per cent solution in glass ampoules.[3]

NaCl, U. S. P. grade, 0.85 per cent in distilled water.

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[1] Determination of total lipid carbon in plasma and the acetone extract shows that approximately 50 per cent of the total lipid is extracted in the acetone.

[2] "Extracts" is used advisedly. In plasma, the T-1824 is bound to the albumin fraction and is removed from the albumin by the acetone which precipitates the protein. Most of the T-1824 remains in solution in the acetone.

[3] Procurable from the William R. Warner Co., New York.

Procedure[4]

Drawing and Handling of Blood.—Approximately 4 ml. of blood is drawn and transferred to a test tube containing dried heparin. Heparin is preferred as anticoagulant; if oxalate is used the amount should not exceed 1 or at most 2 mg. per ml. of blood. Larger amounts of oxalate may cause hemolysis. The needle is left in place in the vein and a Krogh-Keys syringe pipette,[5] containing the dye, is adapted to the needle; the dye is injected at one stroke. The Krogh-Keys syringe pipette is not washed with blood after the delivery. The syringe and needle are then removed together. After the required time interval (in this laboratory both 10 minute intervals and the falling curve method have been used) another sample of blood[6] is drawn.

The blood samples, after aliquots have been set aside for hematocrit determinations by centrifugation, are centrifuged at 3000 R.P.M. for 10 minutes and the plasmas separated with care to avoid drawing up red blood cells. Very lipemic plasmas may require longer or repeated centrifugation.

One ml. of the dye-stained plasma and two samples of the unstained plasma are pipetted accurately into 12 × 75 mm. Pyrex tubes. To each of the dye-stained samples and to one of the dye-free samples is added, from a 2 ml. burette, exactly 0.2 ml. of 0.85 per cent NaCl.

Preparation of Standard.—The syringe previously used for the intravenous injection of the dye is now refilled with dye solution to the same mark used for the injection and is fitted to a needle filled with saline solution. The dye solution in the syringe is then injected into a volumetric flask suitable to permit exact dilution with 0.85 per cent NaCl to a final volume roughly one-fifth the expected plasma volume. For example, if the expected plasma volume is 2500 ml., a 500 ml. volumetric flask is used. To have the same dead space in the needle it is necessary to use the same needle that was employed for the intravenous injection.

The standard solution is prepared by adding of the diluted dye solution exactly 0.2 ml. to 1 ml. of one of the unstained plasmas in a 12 × 75 mm. Pyrex tube.

Measurement of Dye Concentration in the Plasma

There are now 3 tubes prepared, which are designated as follows: *a*, the plasma blank, *viz.* plasma without dye; D_R, the standard prepared by adding dye to plasma *in vitro*; D_V, the dye-stained plasma drawn from the subject 10 minutes after the intravenous dye injection.

The contents of all 3 tubes are gently swirled to ensure mixing, and to each is added 3.0 ml. of acetone from a 25 ml. burette. The stopcock of the burette is greased with a silicone lubricant. As each tube receives the acetone it is closed with a rubber stopper and shaken vigorously for 10 seconds. The tubes, still stoppered to prevent evaporation of acetone, are

[4] The procedure may be applied when necessary to 0.5 ml. samples of plasma. In that case only 0.1 ml. of 0.85 per cent NaCl, 0.1 ml. of the diluted dye solution, and 1.5 ml. of acetone will be added. The pipetting and the addition of acetone require considerable care under these circumstances to avoid error.

[5] This can be obtained from the Macalaster Bicknell Company, Cambridge, Massachusetts. The screw shaft which is used to adjust the course of the syringe plunger must be filed to a point, and the surface of the syringe plunger must be flattened to a plane surface by grinding. In addition, it is necessary to mark a point on the end of the plunger so that it may be kept in line with a mark on the barrel, and thus used in the same position for delivery and calibration. Ordinary tuberculin type syringes may be used if modified as described.

[6] The requirements of plasma are: Two ml. unstained plasma, 1 ml. of which is used for the blank and 1 ml. for the standardization of plasma plus dye. Subsequent samples require only 1 ml. of plasma. Allowance must be made for the 1 ml. of whole blood required for a hematocrit. Knowledge of the approximate hematocrit in the patients permits economy of the amount of blood drawn.

then centrifuged at 2000 R.P.M. for 15 minutes. The precipitated proteins will be found packed at the bottoms of the tubes. The clear supernatants are transferred to three photometer cuvettes,[7] which are immediately stoppered to prevent evaporation of the acetone. At this point some protein particles may be found suspended in the acetone. If they do not settle in a few minutes they can be brought down by centrifugation for 5 minutes at 1500 R.P.M. Any air bubbles on the walls of the tubes will be removed at the same time.

The optical densities of the solutions are read in the spectrophotometer at $\lambda = 620$ mμ. The supernatants, while usually clear, have been found on occasion to be very faintly turbid. The turbidity has been the same in all tubes of a series and at most increases the optical density by 0.01. The turbidity increases if the tubes are cooled, and disappears if they are slightly warmed. It is desirable, therefore, to avoid any marked change in temperature during the whole procedure.

Calculations

An expression similar to that used by Phillips *et al.* (8) has been found convenient:[8]

$$\text{Plasma volume in ml.} = 5 \times V_R \times \frac{D_R - a}{D_V - a}$$

where D_R = optical density of the plasma to which dye has been added *in vitro*, D_V = optical density of the dye-stained plasma, a = optical density of the plasma blank, and V_R = volume in milliliters to which the dye in the syringe is diluted with saline.

The whole blood volume is calculated from the expression:

$$\text{Whole blood volume} = \frac{100 \times \text{plasma volume}}{100 - \text{true cell volume}}$$

where the true cell volume is found by multiplying by 0.955 the value found by the Wintrobe hematocrit[9] to correct for the 4.5 per cent of plasma trapped by the cells (9).

For calculation of the blood and plasma volumes by the falling curve method, the usual semilog plot of the optical density due to dye against time is used.

EXPERIMENTAL

Agreement with Beer's Law.—A series of experiments was done in which increasing amounts of the dye diluted 1:100 in 0.85 per cent saline were added to 1.0 ml. aliquots of plasma from different patients and normal individuals.

[7] Because centrifugation of the cuvettes may be necessary it is recommended that each investigator calibrate a set of 50 or so 12 × 75 mm. soft glass test tubes. Out of 200 such tubes it was possible to obtaine three sets of approximately 50 tubes, each set having an internal agreement of better than ± 1 per cent.

[8] The derivation (not given in reference 8) of this expression depends on the fact that the product of the concentration of the dye and the volume gives the total amount of dye present. If C_1 is the concentration of the dye in the reference solution, C_2 the concentration in the plasma, V_1 the volume of the reference solution, and V_2 the plasma volume, then $C_1 \times V_1 = C_2 \times V_2$ whence $V_2 = \frac{C_1 \times V_1}{C_2}$. In practice V_2 is made to be 1/5 V_1 so that only 0.2 ml. of the reference dye-NaCl solution need be added to the unstained plasma instead of the 1.0 ml. which would be required if V_1 were to equal V_2. Therefore V_R is multiplied by 5.

[9] The blood is pipetted into Wintrobe hematocrit tubes and centrifuged for 60 minutes at 3000 R.P.M. in a centrifuge with an 18 cm. radius.

The dye-stained plasmas were then carried through the acetone procedure. In all cases the optical density due to the dye was found directly proportional to the dye's concentration in the plasma. An example is given in Fig. 1. It was noted in the plasmas of patients with low protein that the amount of dye extracted was greater than in normal plasmas and tended to increase with decreasing protein concentration. A possible explanation of this effect is given later. With regard to the optical densities of the blank plasmas after treatment with acetone, the variation was so small compared to the optical density due to the dye that this variation could be neglected. For example five serial samples of blood taken from a normal individual after a low fat meal at 15

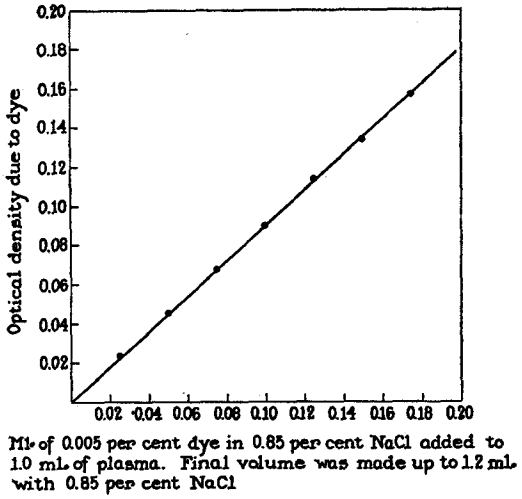

FIG. 1. Extinction-concentration curve of T-1824 in plasma treated with acetone.

minute intervals gave blank plasma optical densities of 0.081, 0.090, 0.071, 0.069, 0.068 by the standard method,[10] and of 0.009, 0.008, 0.008, 0.007, 0.008 after treatment with acetone.

Effect of Lipemia on the Acetone Method and the Standard Method[10].—The degree of lipemia had no effect on the amount of dye measured by the acetone method, as the fat particles disappeared on treatment with acetone. The optical densities of the supernatant acetone solutions from unstained plasmas

[10] The term "standard method" indicates the method in which the concentration of dye in plasma is estimated from the optical density of the dye-plasma mixture, without treatment with acetone. In the present work the standard method is applied as described by Phillips *et al.* (9) with the exception that smaller amounts of plasma are used, and that a separate reference solution of the dye in the unstained plasma of the subject is made as standard for each determination instead of using a single reference solution of the dye in a pooled clear plasma.

varied between 0.002 and 0.018, with the majority below 0.010. The optical densities of the original plasmas calculated to the same dilution varied from approximately 0.03 to 0.70. There was no correlation between the blank optical density and the degree of lipemia in the acetone method.

In an attempt to determine the effect of lipemia on the measurement of the optical density due to the dye in the standard method the following experiment was set up:—

Ten ml. of lipemic plasma from a nephrotic boy was centrifuged for 1 hour at 11,000 R.P.M. The separation into clear and turbid fractions was incomplete and only the upper, more

TABLE I

Effect of Lipemia on the Determination of T-1824 in Plasma by the Standard and Acetone Procedures

Plasma No.	Non-lipemic plasma	Lipemic plasma	0.005 per cent dye solution in 0.85 per cent NaCl	Sodium chloride 0.85 per cent	Standard procedure				Acetone procedure		
					Optical density with H_2O set at zero	Optical density due to dye	Optical density with plasma blank set at zero	Per cent of No. 2 determined	Optical density with H_2O set at zero	Optical density due to dye	Per cent of No. 2 determined
	ml.	*ml.*	*ml.*	*ml.*	*o.d.*	*o.d.*	*o.d.*	*per cent*	*o.d.*	*o.d.*	*per cent*
1	2.0	0.0	0.0	0.4	0.112				0.003		
2	2.0	0.0	0.4	0.0	0.745	0.633	0.640		0.173	0.170	
3	1.7	0.3	0.0	0.4	0.508	0.000			0.005		
4	1.7	0.3	0.4	0.0	1.155	0.647	0.668	104.3	0.175	0.170	100.0
5	1.3	0.7	0.0	0.4	0.915	0.000			0.009		
6	1.3	0.7	0.4	0.0	1.69±	0.775	0.680	106.2	0.180	0.171	100.6
7	1.0	1.0	0.0	0.4	1.120	0.000			0.013		
8	1.0	1.0	0.4	0.0	1.95±	0.830	0.795	124.3	0.185	0.172	101.2

The acetone procedure was carried out on aliquots of the plasmas examined by the standard procedure. The value obtained in No. 2 was taken as the theoretical under these conditions.

lipemic fraction was used (optical density approximately 2.0 at $\lambda = 620$ mμ). Duplicate mixtures were then made in which varying portions of this lipemic fraction were added to 1.0 ml. of non-lipemic plasma and the volume made up to 2.0 ml. with non-lipemic plasma from a normal individual. To each duplicate of one set was added 0.4 ml. of a dye solution containing 50 mg. of dye per liter of 0.85 per cent NaCl solution,[11] and to each of the other sets 0.4 ml. of 0.85 per cent NaCl solution. The optical density due to the dye was then estimated as closely as possible with both water and the appropriate plasmas without dye as blanks. (The densities of the lipemic plasmas were too great (Table I) for very precise measurements.) Then a 1.2 ml. aliquot of solution from each of the tubes was carried through the acetone procedure. From the results in Table I it can be seen that the most dense plasma with the standard method gave an error of + 24.3 per cent while the acetone method has an

[11] As noted by Gregersen and Stewart (10) T-1824 is not stable in 0.85 per cent NaCl. For this reason the 1:100 dilutions were made up fresh for each series of experiments that was carried out within the next few hours.

error of + 1.2 per cent, assuming that the dye concentrations found in mixtures of dye with non-lipemic plasma were exact. Similar results were obtained with plasmas of naturally different degrees of lipemia. Again the deviations from theoretical were greater in the more lipemic plasma, but it was not possible to make a general correction factor relating blank optical density and error. Similar experiments with trypan blue and methylene blue gave similar results. Briefly, then, in a lipemic plasma more dye is estimated by the "standard method" than is actually present. Examples of the extinction-concentration curves in normal and lipemic plasmas are given in Fig. 2. The relationship between percentage of dye present determined by the "standard method" and the optical density of the plasma blank is shown in Fig. 3 for various plasmas.

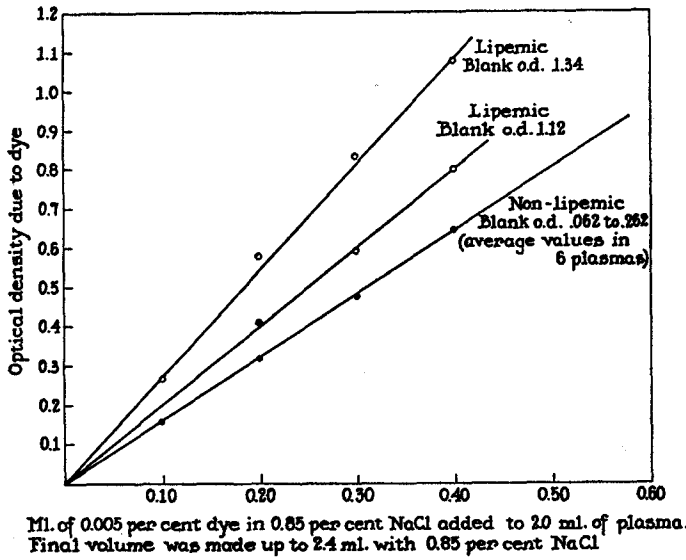

FIG. 2. Extinction-concentration curves of T-1824 in non-lipemic and lipemic plasmas with the standard method without acetone.

That the error encountered in analyses of lipemic plasma by the "standard method" was not attributable to the instrument was shown by the fact that similar results were obtained when duplicates were run with a Beckman spectrophotometer. In a lipemic plasma with an optical density of 1.34 the error with the Beckman, using 1.0 cm. cells, was + 75 per cent, and with the Coleman instrument + 72 per cent. The optical density of the same concentration of dye in a non-lipemic plasma was used as the true value.

A final proof that the lipemia was responsible for the error was furnished by the following experiment:

The same amount of dye was added to equal volumes of non-lipemic and lipemic plasmas (blank plasma optical densities 0.129 and 1.375 respectively). The amount of dye measured in the lipemic plasma was 159 per cent of that found in the normal plasma. The dye-stained

plasmas and their blanks were then centrifuged at 11,000 R.P.M. for 90 minutes (approximately 8,800 × *g*). The separation of the lipid particles was not complete, but the blank optical densities were reduced to 0.058 for the normal plasma and 0.210 for the lipemic plasma. It was therefore possible to apply the correction of Luetscher (2), which holds only for minor degrees of lipemia. With these corrections on both normal and lipemic plasmas (after centrifugation) the amount of dye measured in the lipemic plasma was 100.3 per cent of that found in the normal plasma, well within the limit of experimental error.

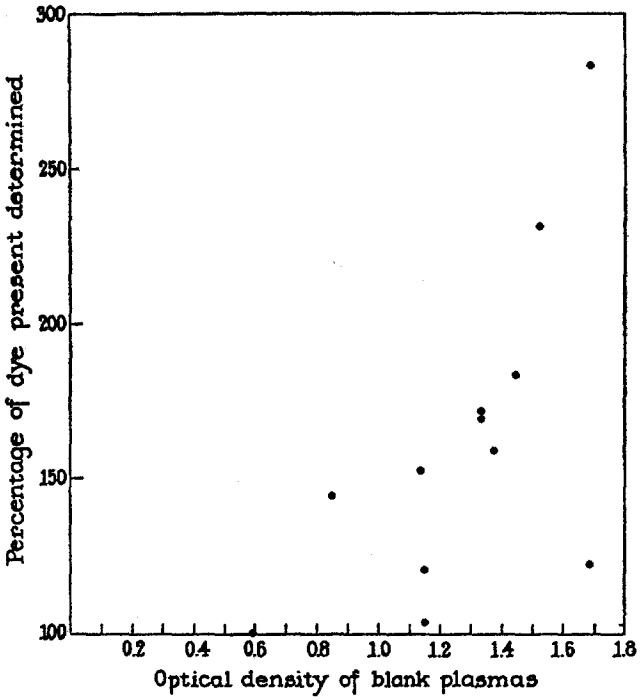

FIG. 3. Effect of lipemia on the percentage of dye present determined by the standard method.

Effect of Hemolysis.—The effect of hemolysis was examined by adding 0.1 ml. of a hemoglobin solution, obtained by laking 3 ml. of red cells with 97 ml. of distilled water and filtering twice, to aliquots of four different plasmas to which dye in some cases and 0.85 per cent NaCl in others had been added (see Table II). A portion of plasma with no added hemoglobin was used as the blank. After the acetone treatment it was found that the variation was approximately ± 3 per cent of the amount of dye that was measured in the same plasmas without adding hemoglobin. The amount of hemoglobin involved was sufficient to cause gross coloration of the plasma to which it was added. Similar experiments with smaller amounts of added hemoglobin gave smaller errors. With

10 times as great a hemoglobin concentration, the error was larger but did not exceed ± 3.5 per cent; in these cases, however, the amount of hemoglobin present in the plasmas not treated with acetone was so great that the very high optical densities of both blank and dye-stained plasmas entirely precluded using Gibson and Evans' (11) correction for hemoglobin with the standard method.

TABLE II

Effect of Added Hemoglobin on the Determination of T-1824 in Plasma with Acetone

Plasma No.		*a* Plasma	*b* 0.005 per cent dye solution in 0.85 per cent NaCl	*c* Sodium chloride 0.85 per cent	*d* Hemoglobin solution (3 ml. red cells laked in 97 ml. distilled H_2O)	*e* Optical density with H_2O set at zero	*f* Optical density due to dye with appropriate blank set at zero $a' - b'$ $c' - d'$
		ml.	*ml.*	*ml.*	*ml.*	*o.d.*	*o.d.*
1	a′	1.0	0.1	0.1	0.0	0.094	0.092
	b′	1.0	0.0	0.2	0.0	0.002	
	c′	1.0	0.1	0.0	0.1	0.096	0.093
	d′	1.0	0.0	0.1	0.1	0.003	
2	a′	1.0	0.1	0.1	0.0	0.092	0.086
	b′	1.0	0.0	0.2	0.0	0.006	
	c′	1.0	0.1	0.0	0.1	0.095	0.086
	d′	1.0	0.0	0.1	0.1	0.009	
3	a′	1.0	0.1	0.1	0.0	0.118	0.100
	b′	1.0	0.0	0.2	0.0	0.018	
	c′	1.0	0.1	0.0	0.1	0.115	0.102
	d′	1.0	0.0	0.1	0.1	0.013	
4	a′	1.0	0.1	0.1	0.0	0.108	0.104
	b′	1.0	0.0	0.2	0.0	0.004	
	c′	1.0	0.1	0.0	0.1	0.110	0.104
	d′	1.0	0.0	0.1	0.1	0.006	

Optical densities of plasmas before treatment with acetone were for No. 1, 0.088, No. 2, 0.134, No. 3, 1.50, No. 4, 1.10. The hemoglobin was added to the plasma *after* the dye had been added.

Comparison of Results Obtained with Standard and Acetone Methods.—In the cases listed in Table III, the standard method was first carried out, then 1.2 ml. aliquots of each sample of plasma were taken through the acetone procedure and a reference solution in dye-free plasma was made in each case. The results have been grouped according to the degree of lipemia; Nos. 1 through 14 are essentially clear, Nos. 15 through 21 are lipemic to a varying degree, indicated by the optical densities of the plasma blanks. For the non-lipemic plasmas,

the mean of the plasma volume measured by the acetone method is 99.46 per cent of the mean by the standard method, with an estimated standard deviation of ± 1.97 per cent. Gross deviations occurred in the lipemic plasmas. In many lipemic cases it was not possible to determine the plasma volume by the

TABLE III

Comparison of Plasma Volume Determinations by the Standard and Acetone Procedures

Plasma No.	Patient	Optical density of blank plasma by standard procedure	Plasma volume determined by		$\frac{100 \times b}{a}$	$b - a$
			Standard procedure *a*	Acetone procedure *b*		
		o.d.	*liters*	*liters*		*liters*
1	A.C.	0.062	2.29	2.33	101.7	+0.04
2	M.B.	0.080	2.56	2.52	98.5	−0.04
3	M.B.	0.080	2.59	2.55	98.4	−0.04
4	M.B.	0.080	2.64	2.57	97.4	−0.07
5	A.H.	0.087	2.27	2.28	100.4	+0.01
6	A.M.	0.091	2.63	2.56	97.3	−0.07
7	P.R.	0.091	1.09	1.06	97.2	−0.03
8	P.R.	0.109	1.39	1.35	97.2	−0.04
9	R.A.	0.117	2.47	2.54	102.8	+0.07
10	W.K.	0.148	3.57	3.61	101.1	+0.04
11	A.M.	0.149	2.53	2.46	97.2	−0.07
12	A.M.	0.159	2.44	2.45	100.4	+0.01
13	A.M.	0.159	2.39	2.45	102.4	+0.06
14	R.A	0.291	2.62	2.63	100.4	+0.01
15	M.B.	0.229	2.25	2.10	93.4	−0.15
16	P.R.	0.282	1.15	1.09	94.4	−0.06
17	A.F.	0.290	1.27	1.20	94.5	−0.07
18	M.M.	0.382	3.25	3.01	92.7	−0.24
19	C.C.	0.387	1.19	1.25	104.4	+0.06
20	M.M.	0.436	3.30	2.94	88.8	−0.36
21	P.R.	0.638	1.30	1.12	86.2	−0.18

Nos. 2, 3, 4, 12, and 13 were calculated from serial samples obtained for a falling curve plot. Nos. 12, 13, and 14 were not lipemic but contained residual dye from previous determinations.

standard method because of the extremely high plasma blanks. With the exception of these omissions, the table gives the results of consecutive determinations.

Effect of Protein Concentration in Plasma on Amount of Dye Extracted by Acetone.—Plasmas from normal individuals were found to yield somewhat smaller recoveries of added dye than did plasmas from patients with hypoproteinemia. Since T-1824 is known to be bound to albumin in plasma (12) it was considered desirable to determine the effect of albumin concentration on the

recovery of the dye. For this purpose a plasma containing 2.20 gm. of albumin per 100 cc. (total protein 4.10 gm. per 100 cc.) was chosen, and to aliquots were added increasing amounts of human plasma albumin. T-1824 was found to be adsorbed by the albumin when the proteins were precipitated by acetone. It was found, as shown in Table IV, that less dye was recovered in the acetone extract as the concentration of albumin in the plasma was increased; the recovery was 95 per cent from plasma that had 2.0 gm. of albumin per 100 cc. and 85 per cent from plasma that had 4.0 gm. (Table IV). The T-1824 adsorbed by the precipitated protein could not be readily recovered by repeated extraction of the precipitate with acetone.

TABLE IV

Effect of Added Albumin on Extraction of T-1824 by Acetone

Solution No.	Concentration of albumin	Optical density due to dye
	gm. per 100 cc.	*o.d.*
1	2.20	0.196
2	2.88	0.190
3	3.50	0.185
4	4.34	0.174
5	5.17	0.163
6	6.00	0.156

No. 1 is the plasma of a nephrotic patient with an optical density of about 1.6 with the standard procedure. Nos. 2 through 6 are samples of this plasma to which human albumin was added in sufficient amounts to bring the albumin concentration to the level indicated. The same amount of dye was present in all the plasmas.

Addition of γ globulin (immune serum globulin) to normal and hypoproteinemic plasmas had a similar effect on the percentage of dye extracted by acetone.

Error from adsorption of dye by the proteins is avoided in the acetone method by preparing the reference solution in each case from a mixture of the dye and the subject's previously drawn plasma, so that adsorption is the same in the reference mixture as in the analysis of the plasma drawn after dye infusion.

Non-Applicability of the Acetone Method to Whole Blood

When the acetone method was applied to whole blood a clear filtrate was obtained, in which the dye concentration was proportional to that in the whole blood, in the case of a given blood. However, about half of the dye was adsorbed by the hemoglobin precipitate. The adsorbed fraction varied with the cell content of the blood, and the dye concentrations of the filtrates were too low (about one-quarter as much as in plasma filtrates) for optimal accuracy of measurement in a Coleman photometer.

DISCUSSION

The principle of using a solvent which will precipitate proteins, dissolve lipids, and extract dye, is not new in its application to the determination of dyes in lipemic plasmas. Among others, Dragstedt and Mills (13) have used acetone in a ratio of 2:1 to plasma for the determination of bromsulfalein. Taran (14) has used acetone in a 1:1 ratio to plasma for the Congo red test for amyloidosis, and Ernst and Förster (15) have employed a similar method for bilirubin. More recently with brilliant vital red Robinow and Hamilton (16) in blood volume studies in infants found 95 per cent ethyl alcohol satisfactory, though approximately one-third of the dye was adsorbed by the protein precipitate formed; with T-1824 these authors found that 75 per cent of the dye was precipitated. Acetone was dismissed because it evaporated too rapidly.

Before acetone was adopted in the present procedure many other substances were tried in an attempt to achieve the results obtained with acetone. Dioxane, methyl cellosolve, collidine, *n*-butanol, *n*-propanol, isopropanol, ethanol, methanol, and anionic and cationic detergents, were all tried unsuccessfully. A series of preliminary experiments with methanol in the same proportions as the acetone seemed at first to be quite promising. They were abandoned because the hemoglobin in plasma from partly hemolyzed blood adsorbed relatively large amounts of dye when the proteins were precipitated with methanol, and caused large errors in the measurement of the dye. In addition it was found necessary to cool the plasmas to which the methanol had been added for 1 hour at 4°C. to complete the flocculation of denatured proteins. Acetone alone was found to be relatively insensitive to the presence of hemolysis and to be adequate for the solution of the lipids and extraction of the T-1824.

The protein concentration in the plasma examined is apparently the determining factor in the percentage of the dye extracted from plasma by acetone. This necessitates establishing a standard calibration curve for each individual, and for each concentration of protein when the protein concentration of the subject's plasma is changed as the result of infusions or otherwise. However, neglect of this precaution would cause relatively small errors only when the protein changes are small.

If one assumes, as have Le Veen and Fishman (17), that the dye protein combination is a reversible one, then the simplest formulation is

$$P + D \rightleftarrows PD \tag{1}$$

with

$$S = [D] + [PD] \tag{2}$$

where P is protein, D is free dye, PD is dye-protein combination, and S is total amount of dye in system. Then

$$\frac{[P][D]}{[PD]} = K \tag{3}$$

whence, combining (2) and (3) we obtain

$$[D] = \frac{KS}{K + [P]}. \tag{4}$$

Here, the concentration of free dye, $[D]$, will depend not only on the total amount of dye present S, but also on $[P]$. However, if $[P]$ is held constant, and it is under these circumstances that the acetone method has been used, then (4) reduces to $[D] = K'S$ and the amount of free dye is determined, with $[P]$ constant, solely by the total amount of dye S. This is adequately demonstrated in Fig. 1. If one were to attempt a semilog plot for a falling curve of dye in plasma in the face of rapidly shifting protein concentrations it would be necessary to determine K in equation (4) for each concentration of protein.

The results reported here do not necessarily contradict those of Gregersen (18), who compared the amounts of dye estimated in non-lipemic plasmas with the amounts of dye estimated in lipemic plasmas from the same individuals after they had taken a fatty meal. He found no difference in the amounts of dye estimated. However, in our experience, the lipemia found after meals in normal individuals has never equalled the extremes of lipemia found in nephrotic individuals. Further, at blank optical densities up to 1.0, the errors in the amount of dye estimated by the standard method are not necessarily very great.

The effect of the lipemia on the determination of the extinction due to dye can best be discussed by referring to the optical properties of sols or suspensions in general. It is generally accepted that Beer's law holds for dilute suspensions. However, in concentrated suspensions deviations from Beer's law are found; the optical density increases less rapidly than the concentration of the suspension. (See for example the work of Lange (19), Wells (20), Dreosti (21), and of Bloch and Renwick (22).) Wells suggested that some of the light scattered in dense suspensions finds its way back into the original beam after multiple reflections in every which way among the particles. This secondary scattering has been well recognized, but it has not been so well recognized that the effect of the suspensions in a dye solution is to permit passage of light through dye molecules which would not be in the normal path of light. Lipemia, then, acts as if it increased the length of the path of light through the cuvette. This results in an erroneously high value for the optical density due to the dye. (Gibson, quoted by Thorn, was apparently the first to draw attention to the error in lipemic plasmas (1).) This error will depend, naturally, on the degree of lipemia, and the greater the lipemia the greater will be the apparent optical density due to the dye, as shown by the extinction-concentration curves of Fig. 2 and the points in Fig. 3. Fig. 3, in addition, shows that the error in the determination of the dye is not necessarily the same in plasmas of the same optical density. That the error is entirely due to the lipemia is demonstrated by the experiment in which the error disappeared after centrifuging out the lipid particles.

Three salient facts must therefore be emphasized. (*a*) At low particle concentrations, the degree of lipemia is directly proportional to the optical density, and corrections for changes in optical density due to lipemia may be made as described by Luetscher (2). In addition, the extinction-concentration curves for dye in plasma will be identical in different plasmas and the specific extinction of the dye in a pooled plasma may be used to determine the concentration of the dye in other plasmas suitable corrected for the plasma blank, provided the lipemia is of a minor degree. (*b*) At high particle concentrations, deviations from Beer's law occur, more of the dye is estimated than is actually present, the extinction-coefficient is not the same in all plasmas even of the same blank optical density, and Luetscher's correction for changes in degree of lipemia cannot be applied. (*c*) If the standard method is used and the extinction of the dye is determined in the lipemic plasma, the results are likely to be inaccurate because the plasma used for the reference standard and the plasma of unknown dye content may vary in degree of lipemia even when the two plasmas are drawn within a few minutes from the same subject.

A word may be said here about the particles responsible for the lipemia. It has been found (*e.g.* Fig. 2) that for a given lipemic plasma Beer's law is roughly obeyed for the dye, but the deviation from a straight line may be as high as ± 5 per cent. This suggests that the lipid suspensions in plasmas are not entirely stable, and that the instability may be one of the reasons for the erratic results obtained with the standard method. If the plasmas are allowed to stand, particularly in the cold, some of the lipid particles will coalesce, thus introducing unpredictable and uncorrectable errors. In addition, in some plasmas, centrifugation at 3,000 R.P.M. for 15 minutes has been sufficient for the formation of a "butter" layer at the top.

While the method has been described for small amounts of plasma, there is no apparent reason why, when larger amounts of plasma are available, all additions cannot be increased proportionately. This would permit the use of larger cuvettes which, giving higher optical densities, might decrease the experimental error.

SUMMARY

A method is presented for estimating the concentration of T-1824 in lipemic plasma. The plasma is mixed with acetone, which precipitates the proteins, dissolves or precipitates the lipids, and yields a clear solution for photometric measurement of the dye. The method is not sensitive to changes in the degree of lipemia and is relatively insensitive to hemolysis.

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