THE ESTIMATION OF AMINES IN BIOLOGICAL MATERIALS WITH CRITICAL DATA FOR COCAINE AND MESCALINE[1, 2]

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During investigations concerning the utility of halogenated sulfonephthalein dyes in studying acid-base equilibria in organic solvents, La Mer and Downes (1933) and Griffiths (1938) observed that minute amounts of organic amines added to bromophenol blue in benzene and chlorobenzene produced an intense yellow color. When more than an equimolar amount of the particular amine was added to the dye solution, the red alkaline form of the dye resulted. Recently Davis *et al.* (1948) demonstrated that dilute solutions of bromophenol blue in benzene when mixed with 0.2 to 0.8 molar equivalents of di-*n*-butylamine gave optical density values which resulted in a straight line relationship following Beer's law when plotted against concentration. It was also observed that if an equimolar ratio of amine to dye was used, the amount of color production (i.e., optical density reading) was identical for each amine when triethylamine, piperidine, mono-, di- and tri-*n*-amylamine were added to 5×10^{-5} *M* bromocresol green in benzene. Such data would suggest that the amount of color formation varies inversely as the molecular weight.

On the basis of the above observations the feasibility of adapting these principles to the estimation of organic amines in biological materials was investigated. It has been possible to establish a general method, the limitations of which will be discussed later, that can be used for a wide variety of aliphatic and alicyclic amines, many of which are of pharmacological interest. Essentially, the procedure involves extraction of the amine from the fluid or tissue in question with a suitable solvent, passage of the extract through filter paper or preferably a sucrose column to remove excess water and perhaps interfering materials, and mixing of the filtrate with bromocresol purple solution in the same solvent. The optical density values of the resulting yellow solutions are determined in a Beckman DU spectrophotometer at 410 mμ. Re-extraction procedures may be used to increase the sensitivity or to reduce the amount of interfering substances.

METHOD. *Reagents.* Chloroform. Dow technical chloroform is passed through a column (made from 18 mm. glass tubing) prepared as follows from below upward: plug of glass wool, about 12–15 cm. of ignited powdered aluminium oxide (Baker and Adamson reagent), plug of glass wool, and 15–20 cm. of 14–20 mesh (Eimer and Amend) silica gel. An automatic siphoning device is useful for supplying the solvent to the column. Such a column will pro-

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vide one-half to one liter of pure chloroform. It is preferable to use the chloroform purified the same day, although if kept at refrigeration temperatures overnight, it may be used a second day.

Benzene. This solvent is shaken with 10 per cent NaOH for ten minutes, then with 1 *N* HCl and finally three times with distilled water.

Bromocresol purple reagent—50 mgm. per cent. Twenty-five mgm. of the dye are dissolved in pure chloroform and diluted to 50 ml. If refrigerated when not in use, this solution is stable for a period of two weeks. Although the dye may be used directly as prepared by the National Aniline Division, Allied Chemical and Dye Corp., New York, it is better to use material purified by the following procedure: One gm. of the dye is dissolved in 15 ml. of boiling glacial acetic acid, the solution filtered and allowed to deposit crystals which are washed with about 5 ml. of glacial acetic acid and dissolved in 8 ml. of boiling glacial acetic acid. The pale yellowish tan crystals (0.5–0.6 gm.) which appear on cooling of the latter solution are placed in a vacuum desiccator (18–20 mm. of Hg) over $CaCl_2$ and KOH pellets for 2–3 days for complete removal of adherent acetic acid.

Phosphate buffer, 1.5 *M*[3]. One hundred sixty-one gm. $Na_2HPO_4 \cdot 7H_2O$ and 20.4 gm. of KH_2PO_4 are dissolved in distilled H_2O and diluted to 500 ml.; the pH of this solution is about 7.0.

Borate buffer.[3] Forty-eight gm. of H_3BO_3 and 15 gm. of NaOH are dissolved in distilled H_2O and diluted to one l.; the pH of this solution is about 9.2.

0.1 *N* NaOH.

0.5 *N* NaOH.

0.1 *N* HCl.

Sucrose. Reagent crystalline sucrose is ground with a mortar and pestle to such a size that about three to seven minutes are required for 5 ml. of $CHCl_3$ to pass through a 5 cm. column. When prepared the sucrose is a mixture of coarse and fine material.

Preparation of the standard curve. Concentrations (calcd. as free amines) are prepared ranging from 1 microgm. to 20 microgm. per ml. (mescaline) or 30 microgm. per ml. (cocaine) of the amine hydrochloride. Five ml. of the aqueous solution, 2 ml. of the phosphate buffer (cocaine) or borate buffer (mescaline), or 0.1 *N* NaOH (mescaline), and 10 ml. of pure chloroform are placed in a 35 ml. g.s. centrifuge tube and shaken twenty minutes in a size 2, International shaker machine, at a frequency of 250–260 oscillations per minute at an angle (see discussion on types of shaking). The aqueous layer is removed using a water aspirator pump and about 5 ml. of the chloroform extract are run through Whatman No. 41 filter paper (cocaine) or preferably a sucrose column[4] (cocaine or mescaline) into a clean, dry test tube. Exactly 3 ml. of the chloroform filtrate are placed in a clean, dry, 10 ml. g.s. volumetric flask. Using an automatic pipette[5] syringe 0.3 ml. of the bromocresol purple reagent is introduced into the volumetric flask. After 25 to 35 minutes[6] the optical density of the solution is determined at 410 mμ. using a Beckman DU spectrophotometer.[7] The op-

[3] Both of these buffer solutions tend to crystallize at room temperature so it is convenient to store them on top of an oven.

[4] The glass columns which are about 40 cm. long are prepared from 7 mm. Pyrex glass tubing. A plug of glass wool is placed in the column followed by about 5 cm. of sucrose. Sucrose columns are much more satisfactory than filter paper as can be seen from the recoveries of cocaine from plasma in table 2. Mescaline in chloroform solution is adsorbed to an extent of 15 to 80 per cent on filter paper depending on the type and previous treatment.

[5] Metal pipette frame for one ml. syringe can be obtained from the Northern Tool and Instrument Co., 50–23 192nd St., Flushing, New York.

[6] The colored solutions give relatively constant optical density readings from ten to sixty minutes after addition of the dye reagent.

[7] The corex cells tend to develop a film of dye unless rinsed with alcohol and acetone, respectively, and dried between each reading. Occasionally it is necessary to clean the cells in dichromate cleaning solution.

tical density readings may be plotted against concentration, or an average constant determined from the constants calculated at each level from the formula, K = opt. dens./concentration (microgm./ml.). Aqueous controls are carried through the procedure. All optical density determinations are read against pure chloroform; control values should range from 0.020 to 0.040, depending upon the humidity, etc. Per cent recovery of the amine in biological fluids and tissues can be determined by use of the optical density-concentration graph or the constant, and suitable correction factors for dilution, etc.

Procedure for plasma. 1. *Direct.* For concentrations of 1 microgm. per ml. and above the estimation is satisfactorily carried out as follows. Ten ml. of pure chloroform, 2 ml. of 0.1

TABLE 1

Percentage recovery of cocaine and mescaline from oxalated dog plasma by the re-extraction and direct methods

RE-EXTRACTION METHOD			DIRECT METHOD			
Amine added	Per cent recovery		Amine Added	Per cent recovery		
	Cocaine	Mescaline		Cocaine		Mescaline
				Filter paper	Sucrose column	Sucrose column
micro-gm./ml.			*micro-gm./ml.*			
0.4	101, 114, 105, 92, 100, 85, 105*		1	125, 65, 130, 108	106, 86, 84	106, 98, 88, 98
			2	78, 93, 108, 103, 97	96, 92, 103, 96	95, 83, 97, 100
0.5	76*	94, 122				
0.8	92, 109, 104, 92, 81, 103, 111*, 75*		4	97, 97, 108, 103	93*	93, 99, 94, 100
			5		93, 93	
1.0	93*	94, 101	6	112, 101, 80, 80		
1.2	83*		8			101, 95, 99, 101
1.5		87, 79	10	88, 98, 99	104, 98, 95, 100, 96*	95, 96, 93, 96
1.6	93, 96, 97, 90, 88, 88, 80*					
1.9	77*		16			104, 105, 105
2.0	94*, 95*	92, 93	20		101, 89, 93*, 96*	114, 103, 104, 101
2.4	88, 90, 91, 90, 80, 88, 83*					
			24	94, 87, 88, 92		
			30		92, 94	
			32	91, 89, 85, 89		

* These results were obtained from the determination of unknowns given to three different individuals familiar with the method.

N NaOH,[8] and 5 ml. of plasma[9] are added in that order to a 35 ml. centrifuge bottle after which the bottle is swirled gently in an upright position to mix the NaOH and plasma but with minimal disturbance of the aqueous-chloroform interface. Then one ml. of distilled water or one ml. of amine solution (for recoveries) is added to the bottle. The glass stopper is wet with distilled water and placed firmly in the bottle and subsequently the bottle is shaken in the horizontal position (see discussion of shaking) for twenty minutes and then centrifuged at 2300–2400 RPM for twenty minutes. As much as possible of the aqueous layer

[8] When 2 ml. of 0.1 *N* NaOH are mixed with 5 ml. of plasma, the pH of the mixture is approximately 8.9 to 9.2.

[9] Must be oxalated; heparin interferes in the method.

is removed and about 5 ml. of the chloroform extract pipetted through a sucrose column and color developed as indicated above. Control values (plasma but no amine) should not be above 0.075 optical density reading. The reliability is about 100 (±12) per cent (table 1).

2. *Re-extraction.* When it is desired to estimate amine concentrations as low as 0.4 or 0.5 microgm. per ml. the following procedure may be employed. To each of two 35 ml. centrifuge bottles are added 15 ml. pure chloroform, 2 ml. 0.1 *N* NaOH and 5 ml. plasma, and the mixture is swirled gently; then one ml. distilled H_2O or one ml. of a suitable amine solution (for recovery studies) is added. The bottles are shaken horizontally for twenty minutes and centrifuged for twenty minutes. After the major portion of the aqueous layer has been removed by the aspirator, 10 ml. of the chloroform extract from each tube are pipetted into a 30 ml. glass stoppered separatory funnel[10] and the 20 ml. of chloroform are shaken vigorously with 3 ml. of 0.1 *N* HCl for 90 seconds. The layers are allowed to separate (three to five minutes) and the chloroform carefully drained until the interface between the two layers just reaches the uppermost part of the stopcock. Then the acid extract is drained into a clean 35 ml. centrifuge bottle, the last drop being removed by compressing a finger over the top of the separatory funnel or dropping in the stopper. Two ml. of 0.1 *N* HCl are introduced into the separatory funnel after the stopcock is closed. The stopper is replaced and the vessel manipulated so that the wash acid comes in contact with all of the interior surface of the funnel. This wash acid is combined with the original 3 ml. acid extract; then 2 ml. borate buffer (cocaine), or 1.2 ml. 0.5 *N* NaOH (mescaline) and 5 ml. pure chloroform are added. After twenty minutes of shaking in the angle position the tubes are centrifuged if necessary and the aqueous layer aspirated, care being employed to avoid removal of chloroform. As much as possible of the chloroform extract is pipetted through a sucrose column. The color is developed in 3 ml. of the filtrate as described previously. Control plasma optical density readings should not be above 0.050. The reliability is approximately 95 (±15) per cent.

Procedure for urine. Five ml. of urine, 2 ml. phosphate buffer (cocaine) or 2 ml. borate buffer (mescaline) and 15 ml. pure chloroform are placed in a 35 ml. centrifuge bottle which is shaken at an angle for twenty minutes and centrifuged if necessary. Most of the aqueous layer is removed by aspiration and 10 ml. of the chloroform extract are pipetted into a 30 ml. separatory funnel and shaken vigorously with 5 ml. of 0.1 *N* HCl. Then 4 ml. of the acid extract are pipetted into a clean 35 ml. centrifuge bottle and two ml. phosphate buffer (cocaine) or 5 ml. 0.1 *N* NaOH (mescaline) are added followed by 5 ml. (cocaine) or 10 ml. (mescaline) of chloroform. The mixture is shaken twenty minutes, centrifuged if necessary and the chloroform treated as usual. The procedure for cocaine gives low control optical density readings, 0.050 to 0.100 for human urines and catheter or cage-collected dog urine. The procedure for mescaline gives about the same, or slightly higher control readings with human urine and some catheterized dog urine. However, many samples of catheterized dog urine, and particularly cage collected dog urine, give high control readings. Since such a large amount of mescaline is excreted in the urine (Cochin *et al.*, 1951), the necessary dilution of samples reduces the difficulty with high control values. The reliability of the urine method is 100 (±12) per cent (table 2).

Procedure for blood. Three ml. of oxalated blood, 10 ml. of borate buffer and 10 ml. pure chloroform are placed in a 35 ml. centrifuge bottle and shaken at an angle for twenty minutes and then centrifuged. The rest of the procedure is identical with that described for the direct method for plasma. This procedure is of no value below concentrations of 2–3 microgm. per ml. of blood. The reliability is about 100 (±15) per cent (table 2).

Procedure for tissues. One gm. of tissue is homogenized in 5 ml. of isotonic salt solution using a Potter-Elvehjem tissue homogenizer (Potter and Elvehjem, 1936) cooled by an ice bath, and transferred as completely as possible to a 35 ml. centrifuge bottle. About 2 ml. of isotonic salt solution are used to wash as much as possible of the residual material from the

[10] J-6025 reaction vessel with stopcock, Scientific Glass Apparatus Co., Bloomfield, New Jersey.

TABLE 2

Percentage recovery of cocaine and mescaline from oxalated whole blood, urine and tissues of the dog

WHOLE BLOOD			URINE			TISSUES (Amine concentration 30 microgm./gm. tissue)		
Amine added	Per cent recovery		Amine added	Per cent Recovery		Type	Per cent recovery	
	Cocaine	Mescaline		Cocaine	Mescaline		Cocaine	Mescaline
micro-gm./ml.			*micro-gm./ml.*					
2.0		88, 100, 78	1.0	91, 102		Liver	90, 90	87, 103, 102, 92
5.0		94, 94, 96	2.0	108, 94		Kidney	92, 95	89, 86, 93, 88
10.0	104, 83, 83, 91	98, 103	5.0		86, 92	Spleen	99, 93	91, 97, 82, 100
			6.0	102, 107		Cerebrum	96, 110	90, 82
20.0	96, 99, 92, 92	88, 90, 95, 90	10.0	97, 95, 96		Fat	108	85, 85
30.0		91, 91	20.0	93, 95, 94, 82	90, 94, 88	Pancreas	112	
			30.0	94, 83, 94	91, 93	Ventricular muscle	104	92, 102
50.0		95, 101, 96	32.0	92, 93				
			40.0		96, 93	Skeletal muscle	108	99, 92
						Tonsil*	104, 114	

* Was obtained from surgical removal in the human.

homogenizing tube. Then 4 ml. of the phosphate buffer (cocaine), or one ml. 0.5 *N* NaOH (mescaline), one ml. distilled water or amine solution (for recovery studies), and 15 ml. of benzene (cocaine) or chloroform (mescaline) are added. For mescaline extraction 6 ml. of the borate buffer may be used instead of the one ml. of 0.5 *N* NaOH; the values are somewhat more consistent and the control values lower. The mixture is shaken at an angle (cocaine) or horizontal (mescaline) position for twenty minutes. Centrifugation is necessary for a satisfactory separation of the layers. Ten ml. of the solvent extract are pipetted into a 30 ml. g.s. separatory funnel (cocaine) or 35 ml. centrifuge bottle (mescaline); 5 ml. of 0.1 *N* HCl are added and the mixture shaken vigorously. Since the chloroform tissue extract containing mescaline forms a rather stable emulsion when shaken with acid, it is necessary to centrifuge for adequate separation. Four ml. of the acid extract are pipetted into a clean 35 ml. centrifuge bottle and 1 ml. of phosphate buffer (cocaine) or 1 ml. 0.5 *N* NaOH (mescaline), and 5 ml. of pure chloroform are added. The rest of the procedure is as described above. Depending upon the solvent used for the extraction, the pH of the extraction mixture, and the tissue, the blank optical density values range from 0.040 to 0.160. Recovery data are included in table 2. The reliability is about 100 (±15) per cent.

Type of shaking. Numerous experiments indicated that tissues, and particularly plasma, when shaken with buffer and solvent at too great an amplitude or frequency form a creamy-appearing emulsion which either cannot be broken or if broken gives very high and erratic readings in the procedure described above. In order to obtain complete extraction without emulsion formation, it is essential to shake the centrifuge bottles, used for the extraction, so that the longitudinal axis is in the horizontal plane. Also the arc of shaking should be perpendicular to the longitudinal axis of the tubes. It is convenient to hold the centrifuge bottles in wooden blocks with the aid of sponge rubber and double thickness one-half inch rubber bands. The ground area of the glass stopper is wet with a drop of distilled water and the stopper pressed firmly in position. The rubber bands can be adjusted to provide enough tension to avoid leakage. When the holder and centrifuge bottles (three per holder) are placed in the head of the International Shaker so that longitudinal axis of the bottles is parallel to radius of shaking, the spring tension clamps of the shaker head serve to keep the bottles plus holder in a very firm position.

For some tissues, urine and other aqueous solutions, satisfactory extraction is obtained by using a 4 inch square wooden block, holding four centrifuge bottles, which is fastened to a one inch by 4 inch board extension that can be held in the shaker by the spring tension clamps so that the longitudinal axis of the tubes makes a 33° angle with the horizontal, the stoppered end of the tubes being higher and more distal from the center of rotation of the shaker than the bottom of the tubes. The tubes are held in this "angle" holder by a sponge rubber lined cap held down by a single bolt and wing nut.

By shaking the tubes in an angle position, and particularly in the horizontal, the interface area between the aqueous and solvent layers is increased four or five fold over that in the vertical position. A comparison is made in table 3 of the recovery of cocaine at two levels in plasma when the centrifuge bottles were shaken in the horizontal and vertical (used wooden block type of holder which fits into the head of shaker) positions at a frequency of 255 oscillations per minute.

Specificity. The appraisal of the specificity is dependent upon the particular type of drug under investigation. In the instance of cocaine the possible degradation products, methyl ecgonine, benzoyl ecgonine and ecgonine, were prepared and demonstrated to show no interference in the above procedures.

The chemical nature of mescaline indicates three possible alterations of the drug in the animal body, demethylation or oxidation to form a phenol, oxidative deamination of the amino group, or methylation of the amino group. Alkaline wash (0.5 *N* NaOH) of the chloroform extracts of both plasma and urine in the dog does not alter the optical density readings of the determinations. How-

TABLE 3

Per cent recovery of 2 microgm. and 10 microgm. per ml. levels of cocaine in plasma when centrifuge bottles were shaken twenty minutes in vertical and horizontal positions

The rate of shaking was 255 oscillations per minute

INITIAL CONC. COCAINE	VERTICAL POSITION		HORIZONTAL POSITION	
	Found	Recovery	Found	Recovery
microgm./ml.	*microgm./ml.*	*Per cent*	*microgm./ml.*	*Per cent*
2	1.37	69	2.06	103
2	1.83	92	1.92	96
10	7.60	76	9.50	95
10	8.25	83	10.0	100

TABLE 4

Extraction of cocaine and mescaline at varying pH values

The figures are per cent extraction of the respective drug at the pH indicated

DRUG	pH*																	
	2.0	2.5	3.0	3.5	4.0	4.4	4.8	5.2	5.6	6.0	6.5	7.0	7.5	8.0	8.3	8.6	9.0	9.2
Cocaine.....	0	4.9	19	24	66	86	94	96	97	99	101	101	100	99	100	98	—	99
Mescaline.....	—	—	—	—	0	0	0	0	4.8	6.4	10	30	63	90	93	97	100	101

* All buffers were adjusted to the correct pH with the aid of a Beckman Line pH Meter.

ever, methoxyphenolic amines related to mescaline are completely removed from chloroform under the same conditions. Loss of the amino group insures non-interference in the method. Methylation of the amino group would give metabolites which would interfere in the method of estimation. However, the fact that mescaline from the urine of the dog (Cochin *et al.*, 1951) and human has been isolated and converted quantitatively to the picrate derivative and identified with known material would militate against there being N-methylation of any significant degree.

Influence of pH on the extraction of cocaine and mescaline from aqueous solutions. Although the general method described previously works equally well with mescaline as with cocaine, it was found that the optimum pH for maximum ex-

traction was somewhat different for the two amines. Aqueous solutions (20 microgm./ml.) of cocaine and mescaline were extracted by chloroform at varying pH values using phosphate and borate buffers. The results are given in table 4.

TABLE 5

Results when 3.0 ml. of chloroform solutions of the various amines in the final concentrations indicated are mixed with 0.3 ml. of 50 mgm. per cent of bromocresol purple reagent

All values are corrected for blank reading. Unless otherwise indicated the amines are made up in chloroform solutions

AMINE	MOL. WT.	FINAL CONC. IN $CHCl_3$ SOLN.	OPT. DENS.	T	ϵ	LOG ϵ
		micro-gm./ml.				
Ethylenediamine*	60.10	1	0.268	0.54	16,056	4.205
Di-*n*-propylamine	101.2	5	0.965	0.11	19,500	4.290
Di-*n*-butylamine	129.2	5	0.805	0.157	20,870	4.319
Diisoamylamine	157.3	5	0.658	0.22	20,300	4.307
Diisoamyl-*tert*-butylethylamine	241.3	5	0.442	0.361	22,700	4.356
Nicotine	162.0	5	0.637	0.231	20,523	4.312
Nicotine (aq.)		5	0.640	0.23	20,680	4.315
Benzylamine	107.0	5	0.945	0.113	20,264	4.306
Meperidine	246.0	5	0.392	0.405	19,313	4.285
Methadon (aq.)	309.0	5	0.292	0.511	18,020	4.255
Physostigmine* salicylate	275.2	5	0.283	0.521	15,584	4.192
Physostigmine salicylate (aq.)		5	0.056	0.88	3,055.7	3.485
Cocaine	303.4	5	0.352	0.445	21,360	4.329
Morphine monohydrate	303.4	5	0.357	0.44	21,650	4.335
Mescaline	211.1	5	0.480	0.331	21,800	4.338
Quinoline*	129.0	5	0.278	0.527	7,177.4	3.855
Isoquinoline*	129.0	5	0.351	0.447	9,022.0	3.955
Atropine (aq.)	289.0	5	0.333	0.465	19,222	4.283
Ephedrine (aq.)	165.0	5	0.577	0.266	18,978	4.278
Procaine (aq.)	236.0	5	0.400	0.40	18,782	4.273
Amphetamine	135.0	6.53	0.927	0.119	19,112	4.281
Strychnine†	334.2	5	0.254	0.558	16,935	4.228
Hexamethylene† tetramine	140.1	5	0.583	0.261	16,349	4.213
Aconitine†	647.4	5	0.109	0.778	14,115	4.149
Codeine (aq.)	317.2	5	0.305	0.497	19,261	4.284
Diethylaminoethanol	117.1	5	0.963	0.11	22,456	4.351
Diethylaminoethanol (aq.)		5	0.333	0.465	7,790.0	3.891
Tripelennamine*	255.0	5	0.225	0.597	11,425	4.057
Priscoline	160.0	5	0.180	0.661	5,753.6	3.759
Lidocaine (aq.)	234.3	5	0.430	0.372	20,100	4.303

* Color faded quite rapidly.

† Available materials of uncertain purity.

Cocaine is completely extracted at pH of 6.0 or above while mescaline is totally extracted only above pH 8.6.

Application to other amines. A wide variety of amines was studied to establish

definitely the relationship of color formation to molecular weight and to determine the feasibility of the estimation of other amines of pharmacological interest. Each amine was prepared in aqueous (as acid salt) and/or chloroform solution to give the final chloroform concentration indicated in table 5. The procedure for the aqueous is that described above under "preparation of the curve" using borate buffer. Except in the case of cocaine and mescaline which are included for purposes of comparison, single determinations were made on each compound. The optical density and transmission were determined at 410 mμ. using the Beckman DU spectrophotometer. The molecular extinction (ϵ) was calculated from the formula,[11]

$$\epsilon = \frac{\text{Mol. wt.} \times (-\log T)}{c \times d},$$

where T = transmission, c = concentration in grams per liter and d = distance of light path (one cm. in this case). The results are given in table 5.

It is quite evident from the calculated values of ϵ and log ϵ that with most amines the color formation is inversely proportional to the molecular weight. Where the compound does not follow this rule there is usually a satisfactory explanation. For example, ethylenediamine and derivatives (i.e., tripelennamine) produce a color which fades, so the method is not practical for such compounds. Likewise, quinoline, isoquinoline, physostigmine and Priscoline give similar results.

For this method to be of any value the particular amine in question must be extractable from aqueous solution. As can be seen, many of the amines of pharmacological interest can be extracted completely. β-Diethylaminoethanol was one compound tested which was incompletely extracted (about 35 per cent). This fact would make the method non-specific for procaine. However, by selection of the proper buffer and re-extraction the method probably could be made specific. Morphine is not appreciably extracted by chloroform from aqueous solution.

A number of compounds were tested to determine the effect on color formation of functional moieties other than the aliphatic and alicyclic amine groups. Aniline and dimethyl-aniline gave a small amount of color at a 5 microgm. per ml. concentration in chloroform; the color faded rapidly. *p*-Aminobenzoic acid gave some color above 100 microgm. per ml. but none at lower levels. Relatively concentrated chloroform solutions of the following gave no color when mixed with the bromocresol purple solution: hippuric acid, phenol, benzoic acid, acetanilide, indole, caffeine and pentylenetetrazol.

Aqueous solutions of the barbiturates, thiobarbiturates and epinephrine do not interfere in this procedure.

Choice of solvents. Numerous other solvents besides chloroform were investigated as to their utility in the color development. Preliminary experiments using mescaline as the test amine indicated that methylene dichloride, methylchloro-

[11] Derived from formulae found on page 4 of Brode: Chemical Spectroscopy, second edition, 1943, John Wiley and Sons, Inc., New York.

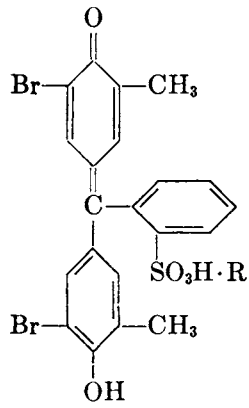

FIG. 1. Suggested colored quininoid form of bromocresol purple.

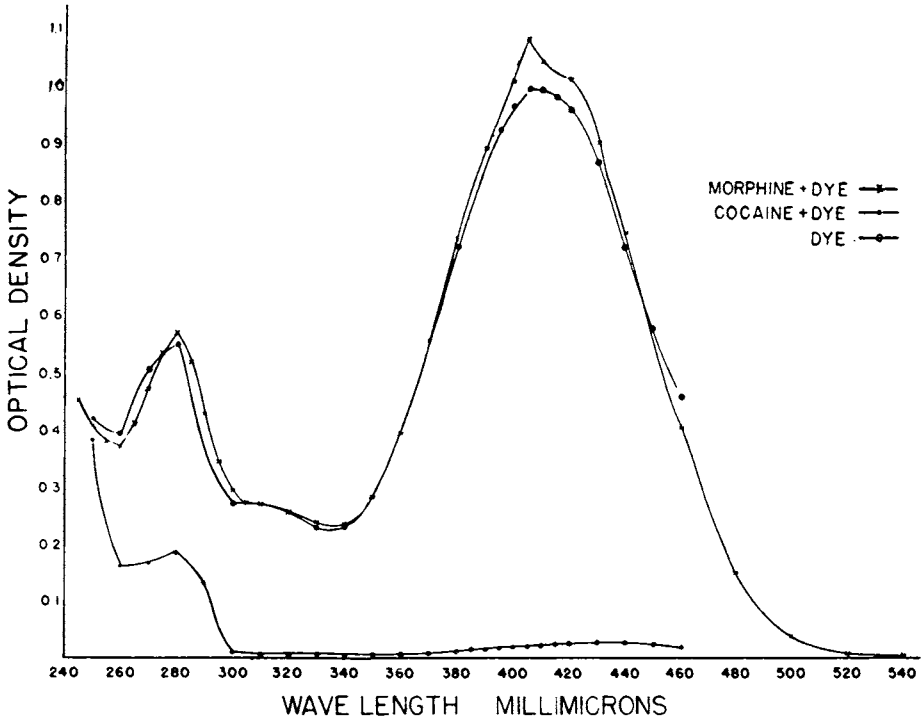

FIG. 2. Absorption (optical density) of 0.1 *mM* solution of bromocresol purple in chloroform as well as mixtures of equal volumes of 0.1 *mM* morphine and cocaine with the dye solution at various wave lengths.

form, anisole, bromobenzene, chlorobenzene and toluene might be used. The petroleum ether fractions, benzene and cyclohexane were not satisfactory. However, benzene can be used for cocaine but with less sensitive results than with chloroform. None of the above solvents demonstrated any particular advantage over chloroform and some are very inconvenient to use.

Nature of the colored material. The pale yellow color of the halogenated sulfonephthaleins when prepared in benzene and chloroform has been attributed (Davis *et al.*, 1948) to the presence of a small amount of the yellow quininoid acid form (fig. 1, less R). Addition of amines shows a gradual intensification of

TABLE 6

Optical density readings after 3 ml. of 10 microgm./ml. cocaine solution in chloroform are mixed with 0.3 ml. of chloroform dye solution in question and allowed to stand thirty-five minutes

DYE		MOLECULAR WEIGHT	ABSORPTION PEAK, mμ	OPT. DENS. AT PEAK ABSORPTION		
Common name	Chemical name*			Blank (B)	Coc. soln. (B)	R − B
Bromocresol purple†	3′,3″-Dibromo-5′,5″-dimethylphenolsulfonephthalein	540.23	410	.021	.742	.721
Bromophenol blue†	3′,3″,5′,5″-Tetrabromophenolsulfonephthalein	669.99	415	.022	.750	.728
Bromothymol blue†	3′,3″-Dibromo-5′,5″-diisopropyl-2′,2″-dimethylphenolsulfonephthalein	624.39	415	.014	.596	.582
Bromocresol green‡	3′,3″,5′,5″-Tetrabromo-2′,2″-dimethylphenolsulfonephthalein	698.05	415	.008	.612	.604
Chlorophenol red‡	3′,3″-Dichlorophenolsulfonephthalein	423.26	405	.013	.635	.622
Iodophenol blue‡	3′,3″,5′,5″-Tetraiodophenolsulfonephthalein	858.01	430	.063	.667	.604
+	3′,3″,5′,5″-Tetrabromophenolphthalein	633.94	Colorless	0.0	0.0	0.0
Thymol blue‡	5′,5″-Diisopropyl-2′,2″-dimethylphenolsulfonephthalein	466.57	Colorless	0.0	0.0	0.0
Metacresol purple‡	2′,2″-Dimethylphenolsulfonephthalein	384.43	Colorless	0.0	0.0	0.0
Cresol red‡	3′,3″-Dimethylphenolsulfonephthalein	384.43	Colorless	0.0	0.0	0.0
Orthocresolphthalein‡	3′,3″-Dimethylphenolphthalein	346.36	Colorless	0.0	0.0	0.0

* According to Chemical Abstracts.
† 50 mgm. per cent solution.
‡ Saturated solution.

absorption in the range 400 to 410 mμ. apparently due to increased conversion of the colorless lactone to the acid salt of the quininoid form (fig. 1, R = primary, secondary, or tertiary amine). That the nature of the amine contributes little to the quantity or location of the absorption peaks is shown in fig. 2. Morphine (free amine) and cocaine (free amine), which have the same molecular weight (303) but different type of chemical structure, were prepared in 0.1 *mM* solu-

tions in chloroform and mixed with equal volumes of a 0.1 *mM* solution of bromocresol purple in chloroform. The optical density readings are plotted against wave length. The two curves are identical within the limits of experimental error, maximum absorption peaks being at 400–410 mμ.

Use of other indicators. A number of indicators were investigated as to the ease of solution, sensitivity and general utility in the method. The general procedure was to mix 3 ml. of cocaine (10 microgm./ml.) in pure chloroform with 0.3 ml. of 50 mgm. per cent, or saturated, solutions of dye in chloroform and to determine the optical density in the Beckman spectrophotometer after 35 minutes. One can conclude from the results in table 6 that the only requirement for use of an indicator in the above method is that it belongs to the halogenated phenolsulfonephthalein type. Non-halogenated phenolsulfonephthaleins, halogenated and non-halogenated phenolphthaleins give absolutely no color when mixed with minute quantities of amines in solvent solution. Bromocresol purple, bromophenol blue and bromothymol blue all have the advantage of satisfactory solubility in chloroform. There is little preference of one over the other of the two most sensitive indicators, bromocresol purple and bromophenol blue.

SUMMARY

1. A general method of estimation of alicyclic and aliphatic amines in plasma, whole blood, urine and tissue with specific reference to cocaine and mescaline has been described.

2. The importance of the type of shaking for extraction of amines from biological material has been discussed.

3. The procedure for mescaline and cocaine has been shown to be specific.

4. Only halogenated sulfonephthalein dyes may be used for color development which is apparently dependent upon quininoid formation from the dye.

5. Investigation of numerous amines indicates that the method has wide application and that color formation is inversely proportional to the molecular weight of the amine.

6. Chloroform appears to be the most convenient solvent to use although preliminary experiments indicate that methylene dichloride, methylchloroform, anisole, bromobenzene, chlorobenzene, toluene and benzene might be employed depending upon the amine, etc.

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