A T H E S I S

ON

PHOTOMETRIC METHOD FOR THE DETERMINATION OF ALKALOIDS IN TOXICOLOGICAL ANALYSES WITH SPECIAL REFERENCE TO STRYCHNINE AND BRUCINE.

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A C K N O W L E D G E M E N T

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PART I : INTRODUCTION

INTRODUCTION.

Alkaloids are now generally defined as basic compounds of vegetable origin. In describing all the vegetable bases as alkaloids we are therefore collecting in one class a number of substances of widely differing constitution with a corresponding variety of chemical properties. All members of this group[2I] contain one or more nitrogen atoms. A few contain nitrogen in an open chain, but the nitrogen in general is present in a cyclic structure such as in pyridine, quinoline, isoquinoline or pyrrole. Still other alkaloids are derived from purine or from complex dicylic systems as in the second half of the chinchona alkaloids and in the tropine group. The nitrogen atom possesses basic characteristics which show in almost all members of this group of compounds. A few are soluble in water forming solutions which are alkaline to litmus, but the majority are only slightly soluble in water and only feebly basic. Morphine is the most conspicuous example of a small group of alkaloids, containing phenolic groups in the molecule which mask the basic nature of the nitrogen atom.

Nearly all the alkaloids are powerful drugs, many of them being very poisonous. The toxicity varies over a wide range from aconitine, one of the most deadly poisons known, to quinine which is poisonous only in very large dose. Many of these compounds possess curative properties and are of great value in medicine. Some of them are illegally used for

homicidal and suicidal purposes, in which case methods for their estimation are of importance in toxicological analysis.

From the analytical point of view, alkaloids together with the analogous basic synthetic drugs, which have come into use as substitutes for natural alkaloids, are grouped by the toxicologist under the name of 'non-volatile basic poisons.'

The general methods for the estimation of alkaloids may be roughly classified thus:-

1. Gravimetric methods. Of the well known alkaloidal precipitants gold chloride, picric acid, phosphomolybdic acid and silico tungstic acid are used for quantitative methods. Hazard (1939) used silicotungstic acid for the determination of solanaceous alkaloids in aqueous solutions while Wochsmith (1951) recommended precipitation in ethereal solution in which case the sensitivity is high and the precipitate has a constant composition. Antimony triiodide has also been used as an alkaloidal precipitant, the concentration of the alkaloid being estimated iodometrically by the amount of the iodine content of the precipitate. These methods are unsatisfactory in toxicological analysis because of the great difficulty of extraction and purification of the alkaloids from biological material and the great losses which are involved.

2. Volumetric methods. These depend on the titration of the alkaloidal salt base with a standard acid. The end point of the titration is determined either visually, using indicators,[I4] or potentiometrically,[I2] the latter being more

accurate especially when an alcoholic solution of the alkaloid is used. Recently the use of ion exchange chromatography was introduced to this method by percolating the solution of the alkaloidal salt through a column of alumina or anion exchangers, (5,18,19) the resultant liquid containing the free alkaloidal bases which could be titrated directly. These methods demand the presence of several milligrams of alkaloids which means a very large quantity in toxicological work.

3. Colorimetric methods. One well known method used in this group is the Reineckate method (2) in which case the results are reliable in the range of from 2 - 10 mgm, a quantity not always or even often obtainable in toxicological practice. However, methods of this group have great potentialities, especially with the modern sensitive photometers.

As there is, at present, a tendency towards an increasing reliance upon colorimetric methods, we adopted as a foundation Prudhomme's original technique with Brodie's later work, extending this method to a large number of different alkaloids. We hoped to get ultimately an accurate and sensitive general method for the estimation of alkaloids in toxicological and pharmaceutical analyses.

Prudhomme (1940), was the first to draw attention to the reaction of alkaloids as organic bases with acid dyes, resulting in the production of stable additional compounds. He treated quinine in chloroform with eosin in water in the presence of phosphate buffer. The quinine-eosin compound formed is readily

soluble in chloroform, giving rise to pink solution, the intensity of which being measured spectrophotometrically. Prudhomme stated that the same reaction applied to other alkaloids; for example, physostigmine, pilocarpine, atropine and ephedrine.

Allen (1945), modified Prudhomme's technique by (a) washing the eosin reagent with chloroform several times to free the eosin from the chloroform soluble fraction of the dye. (b) recommending the shaking of the eosin-quinine compound in chloroform with diluted phosphate buffer. Introducing these two modifications, Allen claimed that the alkaloids mentioned in Prudhomme's results have no longer any significant interference if they are present in quantities of the same order of the useful range of quinine (5 - 30 ug/10 ml. plasma). Strychnine, brucine, hyoscine, morphine, diamorphine and codein, when used in similar concentration to that of quinine, were found not to interfere. He also stated that other common chinchona alkaloids respond to the same extent as quinine, while emetine gives a response of approximately one-tenth of that of quinine.

Barlow & Climenko (1943), were the first to apply this method to synthetic basic drugs. Taking into consideration Prudhomme's original technique, they proposed a method for the determination of demerol in urine. They extracted demerol with benzene and treated it with bromo-thymol blue at pH 7.4. The compound, demerol-bromothymol blue, imparted a yellow tint

to the benzene layer, the intensity of which was measured spectrophotometrically.

In their preliminary study of demerol excretion in man, Lehmann & Aitken (1943), modified the Barlow & Climenko method by extending the procedure a step further. After the formation of the demerol-bromothymol blue compound, an aliquot of the benzene extract was shaken with a small volume of sodium hydroxide solution, when the compound decomposed and the dye passed to the aqueous layer giving a blue colour. The intensity of the colour is indirectly proportional to the amount of demerol present.

Oberst (1943), followed the procedure of Barlow & Climenko but used a smaller volume of benzene and bromothymol blue solution. The intensity of the yellow colour of the benzene layer was measured at 420 mu.

Marshall & Rogers (1945), also used benzene and bromothymol blue for the determination of cinchona alkaloids. They added a solution of bromothymol blue to the aqueous solution of the alkaloid adjusted to pH 7.0; after standing for an hour they extracted the compound formed by shaking with benzene for half an hour, and reading the intensity of the yellow colour of the benzene layer.

Brodie & Udenfriend (1945), found that most of the basic organic compounds combine with sulphonic acids to form molecular complexes which are highly soluble in immiscible organic solvents; ethylene chloride was the solvent of their choice. Taking

cinchona alkaloids (cinchonidine) as an illustrative example of basic organic compounds, they recommended methyl orange as the most suitable member of the coloured sulphonic acids. In 1947, they found that ethylene chloride give higher tissue and reagent blanks and replaced it with benzene, which gave lower blanks and more accurate results.

Cronheim & Ware (1948), studying the different members of the sulphone phthalein dyes group for the determination of amidone in urine found that:

(a) nonhalogenated sulphone phthalein dyes gave no reaction with amidone;

(b) halogenated sulphone phthalein dyes were suitable for this purpose. From the latter group bromocresol purple was chosen as the most suitable member. Cronheim et al. (1950) used bromcresol purple for the determination of tropine alkaloids in pharmateutical preparations.

Aim of the Work:

This method, which is based on the reaction between acidic dyes and basic drugs proved to be a sensitive and accurate method for the determination of minute amounts of cinchona alkaloids (Brodie et al., 1945). As it was not extensively studied, it was thought that it would be of analytical value to extend this method to a number of acidic dyes and alkaloids using different solvents. It was hoped ultimately to get an accurate and sensitive general method for the estimation of

alkaloids in toxicological analysis for routine purposes. This thesis deals with the critical examination of the method, and its application to toxicological analysis.

A number of acidic dyes of different chemical nature were tested for their reactivity with a variety of alkaloids. Quinine was chosen as a representative example of alkaloids for its pronounced basic character and its great sensitivity to this reaction. Preliminary experiments showed that 5 ug. gave an easily detected colour. The dyes found suitable for use were tested for their reactivity with some of the important alkaloids usually met with in toxicological analysis.

The critical examination of the method was further studied for quantitative purposes. Strychnine was chosen as an example of an alkaloid. Strychnine and brucine, which are commonly present together and so closely related to be difficult to separate completely, especially in small quantities, are often required to be determined separately in naturally occurring mixture. The recovery of strychnine from aqueous solution was studied and applied to biological materials. The method was extended for the estimation of strychnine in very low concentrations by concentrating the dilute samples followed by estimating the alkaloid by the above method.

Toxicological samples due to poisoning with naturally occurring mixtures of strychnine and brucine, for example tincture Nux Vomica, the powdered nux vomica seeds, or ignatus beans, are expected to contain both alkaloids. As strychnine and brucine both respond to the alkaloid dye formation method,

they should be separated before their individual estimation. For this purpose a chromatographic study of the separation of these two alkaloids was discussed in a part of this work.

A technique was finally evolved for the estimation of strychnine and brucine in mixture, present in very low concentrations in aqueous and biological material.

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PART II :

THE ALKALOID-DYE ADDITION COMPOUND METHOD

DIVISION I: QUALITATIVE STUDY.

I. (i) CRITICAL STUDY OF THE METHOD INCLUDING LISTS OF DYES, SOLVENTS AND THE ALKALOIDS SUGGESTED FOR USE.

This part of the work is the study of the reaction between acidic dyes and basic drugs; they react with one another forming addition compounds to which we shall refer as the "alkaloid-dye compound" (this in the case of alkaloids which are of interest). These addition compounds are freely soluble in most of the immiscible organic solvents and are decomposed by strong alkali or acid to the alkaloid and the free dye. The dye will give a coloured aqueous layer and thus the concentration of the alkaloid is indirectly estimated colorimetrically. It has been suggested* that the mechanism of reaction for the formation of the alkaloid-dye compound is the combination of the acid radical of the dye with the nitrogen of the basic part of the alkaloid or drug molecule.

Several dyes and alkaloids were subjected to this qualitative study using different solvents.

DYES. The dyestuff must fulfil the following conditions:

(i) it must be of acidic nature.

(ii) it must give colourless blanks with the different solvents.

(iii) it must react with quinine.**

(iv) the quinine dye compound must be easily decomposed with dilute solution of strong alkali or acid.

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* Barlow & Climenko, personal communication, 1943.

** Quinine was chosen as a typical example of an alkaloid for its pronounced basic character and its great sensitivity for this reaction (5 ug. give an easily detected colour).

TABLE 1.

List of the Dyes used in the study.

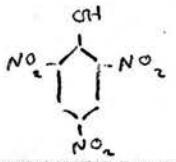
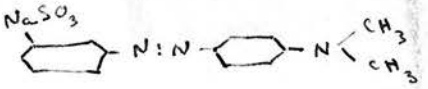
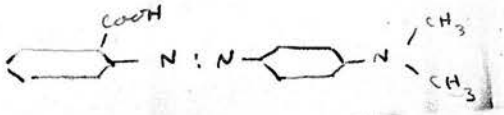
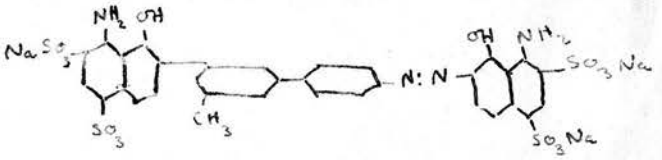
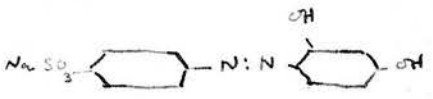
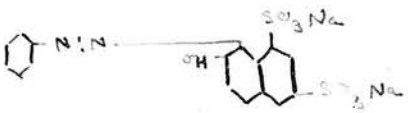
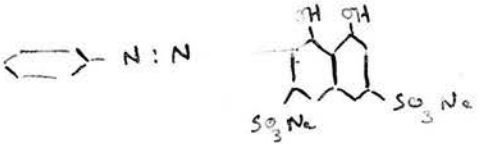
Name	Structural Formula	pH range
NITRO DYES Picric Acid		no pH range
AZO DYES Metanil Yellow		1.2 - 2.3
Methyl Orange		3.1 - 4.4
Methyl Red		4.4 - 6.2
Evans Blue		9.0 - 11.0
Tropoaelin O.		11.1 - 12.2
Orange G.		no pH range
Chromotrope 2R		no pH range

TABLE 1. (Contd).

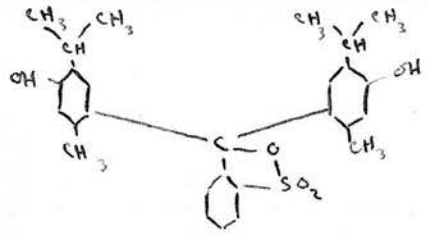
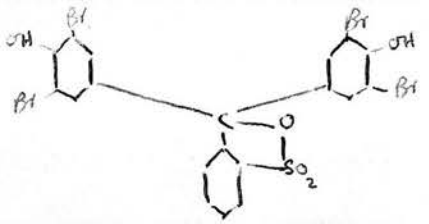
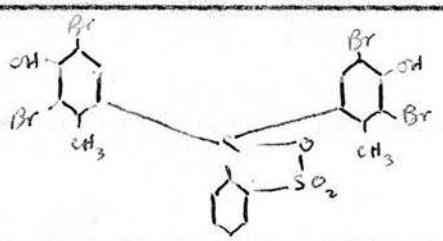
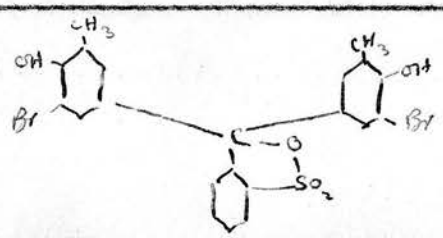
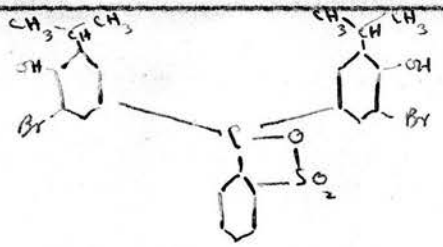
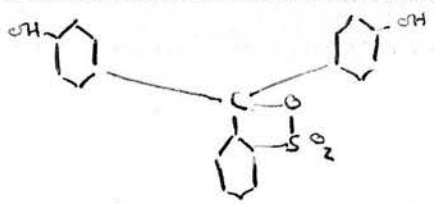
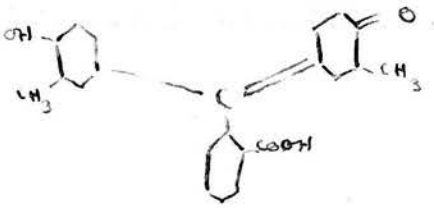
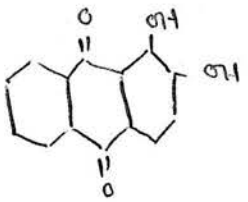
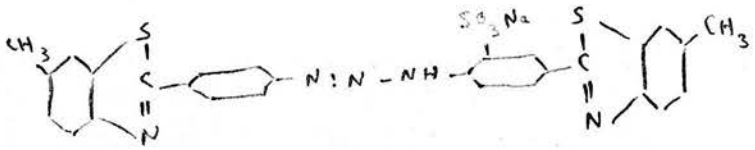
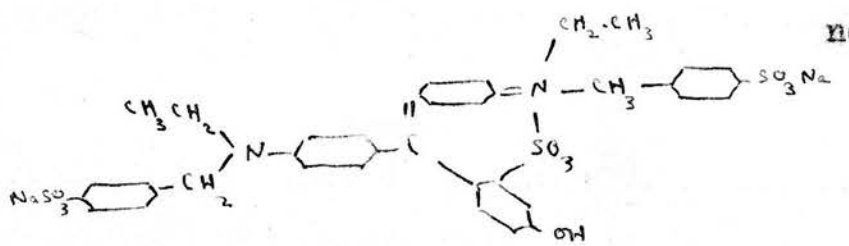
Name	Structural Formula	pH range
XANTHENE DYES		
Thymol Blue		1.2 - 2.8
Bromphenol Blue		3.0 - 4.6
Bromcresol Green		3.8 - 5.4
Bromcresol Purple		5.2 - 6.8
Bromthymol Blue		6.0 - 7.6
Phenol Red		6.8 - 8.4
o-Cresol Phthalein		8.2 - 9.8

TABLE I (Contd).

Name	Structural Form	pH range
ANTHRAQUINOLINE DYES		
Alizarin		about 5.0 - 5.5
TRIAZOLE DYES		
Titan Yellow		no pH range
PHENYL METHANE DYES		
Fast Green		no pH range

A number of dyes of different chemical structure and acidic nature were chosen for the study. (Table 1).

A. As regards the chemical nature of the dyes particular attention was given to the azo and xanthene dyes, coloured sulphonic acids some of which were used by previous investigators.

The Azo dyes group is classified thus:-

1. Dyes having colour change at different pH levels, i.e. acid-base indicators, which are subdivided into:

(a) indicators with pH range on the acid side of neutrality, e.g. metanil yellow 1.2 - 2.3, methyl orange 3.1 - 4.4 and methyl red 4.4 - 6.2.

(b) indicators with pH range on the alkaline side of neutrality, e.g. Evan's blue 9.0 - 11.0 and tropaeolin 0 11.1 - 12.7.

2. Azo dyes without colour change, as orange G and Chromotrope 2R.

Indicator dyes of the Xanthene group, covering a wide range of pH, were picked. These are grouped thus:

(a) nonhalogenated xanthene dyes, thymol blue 1.2 - 2.8, phenol red 6.8 - 8.4 and o-cresolphthalein 8.2 - 9.8.

(b) halogenated xanthene dyes, bromphenol blue 3.0 - 5.4, bromcresol green 3.8 - 5.4, bromcresol purple 5.2 - 6.8 and bromthymol blue 6.0 - 7.6.

Some other dyes were also tried:

Nitro dyes : picric acid, m-dinitrophenol.

Anthraquinoline dyes : alizarin,

Thiazole dyes : titan yellow,

Phenyl methane dyes : fast green.

B. As regards the acidic nature, the dyes and indicators may be classified as follows:-

1. Dyes with phenolic groups, picric acid, alizarin.
2. Dyes with sulphonic acid group(s) as methyl orange, metanil yellow.
3. Dyes with both phenolic and sulphonic acid groups, e.g. Evan's blue and all the sulphone phthalein dyes.
4. In a few cases the acidic group was a carboxylic group as in the case of methyl red.

In the case of o-cresol phthalein the carboxylic group was accompanied with a phenolic group.

All the dyestuff tried in this study are given in Table I.

SOLVENTS. The solvent must fulfil the following conditions to be suitable for use:-

(i) immiscible with water,

(ii) good solvent for the alkaloids,

(iii) the dyestuff to be insoluble in it,

(iv) the alkaloid dye compound is to be freely soluble in it.

The solvents suggested for this work are:-

(a) Ethers : diethyl ether.

(b) Alcohols : as alcohols are known to be good solvents for alkaloids some of the higher members of the aliphatic alcohols which are immiscible with water were tested; those were butanol, pentanol and octanol.

TABLE 2.

Alkaloids used in this Study.
Their Solubilities in Certain Organic Solvents.

Alkaloid	Gm. of alkaloid dissolves in 100 ml. Solvent				
	Water	Alcohol	Benzene	Ether	Chlorofor
Strychnine	0.0156	.666	.556	slightly soluble	20
Brucine	.312	77	1	0.535	20
Atropine	.22	50	soluble	40	100
Hyoscine	Slightly soluble			soluble	soluble
Hyoscine	.357	pH9 freely soluble	.666	1.45	100
Morphine	.02	.476	insoluble	.0160	1220
Diamorphine	.0588	.322	soluble	1.0	.666
Cocaine	.167	15.4		28.6	142.9
Procaine	.5	soluble	soluble	soluble	soluble
Benzocaine	.040	20		25	50
Quinine	.0642	12.5	.125	soluble	90.9
Lobeline	soluble in hot	soluble	soluble	soluble	soluble
Yohimbine	sparingly soluble	soluble	soluble in hot	soluble	soluble
Aconitine	.032	3.57	14.3	1.54	50

(c) Halogenated aliphatic hydrocarbons : Members of this group are excellent solvents of alkaloids. Ethylene chloride was chosen as representative because it is readily recoverable and it keeps well with no production of acidic products as a result of hydrolysis on standing, as is the case with chloroform.

(d) Aromatic hydrocarbons : benzene.

THE ALKALOIDS. Table 2 includes a list of the important alkaloids usually met with in toxicological analysis and covering the various types. The cinchona alkaloids are known by their pronounced basic characters; from this group quinine was chosen as an illustrative example of the alkaloids for the qualitative work as preliminary experiments showed that it responds in minute quantity (5 ug.) to the alkaloid-dye formation method. From the strychnos alkaloids strychnine and brucine were chosen. Atropine, hyosine, hyoscyamine represent the alkaloids of the atropine group. Morphine, an exceptional member of the alkaloids, possesses amphoteric characteristics due to a free phenolic group contained in its molecule; it was of interest for it to be tested for its response to this method, comparing it with one of its artificial derivatives "heroin", in which the phenolic group is acetylated. Cocaine as the chief member of the alkaloids of coca, together with two of its substitutes, procaine and benzocaine, were also tested. Other alkaloids, lobeline, yohimbine and aconitine were included in the list of alkaloids

used in this qualitative study.

The above mentioned alkaloids were tried for their reactivity with acidic dyes in order to test the possibility of applying this method as a general one for all the alkaloids.

(ii) EXPERIMENTAL

A. Testing the Dyes for their Suitability.

(a) Each of the dyes and indicators in Table I was tested for its solubility in the different solvents.

A concentrated solution (0.1 gm.%) of the dyestuff was prepared*; when a buffered solution was used the aqueous solution was diluted with an equal volume of buffer solution

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* Xanthene dyes are provided as their free acid form. To render them water soluble, the monosodium salt was prepared by adding a certain amount of sodium hydroxide as given in the table below.
The volume of the 0.01 N. sodium hydroxide is per 0.1 gm. of the dye.

Table 3.

Dye	ml. NaOH
bromphenol blue	14.9
bromcresol purple	18.5
bromthymol blue	16.0
thymol blue	21.5
phenol red	28.2
bromcresol green	14.3

Koch & Hanke , ' Practical Biochemstry'.

of the required pH**.

1.0 ml. of the dye solution was shaken with 20 ml. of the different solvents in turn for 10 minutes. The mixture was centrifuged and 15 ml. of the solvent layer transferred to a glass stoppered tube containing 5 ml. of N/10 hydrochloric acid,

** (1) The Phosphate Buffer — Rappaport,F. , Rapid Microchemical Methods for Blood and C.S.F. Examinations' 1949 p.387.

Solution A. $NaH_2PO_4.2H_2O$ 2.34 gm/100 ml.
Solution B. Na_2HPO_4 anhyd. 1.906 gm./100 ml.

Table 4.

Solution A. ml.	Solution B. ml.	pH
10.0	...	4.6
9.8	0.2	5.0
9.5	0.5	5.5
7.5	2.5	6.0
7.0	3.0	6.3
6.0	4.0	6.5
3.3	6.7	7.0
2.5	7.5	7.2
1.5	8.5	7.4
1.0	9.0	7.6
0.5	9.5	8.0
0.0	10.0	8.6

(2) The Citrate Buffer.

Solution A. 21.01 gm. citric acid dissolved in 200 ml. of N. NaOH and made up to 1 litre with water.
Solution B. 0.1 N HCl.

Table 5.

Solution A. ml.	Solution B. ml.	pH
6.0	4.0	4.15
5.5	4.5	3.94
5.0	5.0	3.69
4.75	5.25	3.2
4.50	5.5	3.30
4.00	6.00	2.97
3.33	6.67	2.27
3.0	7.0	1.92
2.0	8.0	1.41
1.00	9.0	1.17

TABLE 6.

A Study of the Solubility of Various Dyes and Indicators in Organic Solvents.

Dye	pH of solution	Blank		
		Benzene	Ether	Ethylene Chloride
NITRO DYES:				
Picric acid	aq. soln.	x	x	x
m dinitrophenol	" "	ø	ø	ø
AZO DYES:				
Orange G.	aq. soln.	ø	ø	ø
Chromotrope 2R	" "	ø	ø	ø
Metanil Yellow	" "	ø	ø	ø
	4.6	ø	ø	ø
Methyl Orange	" "	ø	ø	ø
	5.0	ø	ø	ø
Methyl Red	aq. soln.	x	x	x
	6.5	x	x	x
Evans Blue	aq. soln.	ø	ø	ø
	9.0	ø	ø	ø
	11.0	ø	ø	ø
Tropoaelin O	11.0	ø	ø	ø
	12.0	ø	ø	ø

ø - colourless blank

x - coloured blank

TABLE 6 (Contd.)

Dye	pH of solution	Blank		
		Benzene	Ether	Ethylene Chloride
XANTHENE DYES:				
Thymol Blue	1.25	x	x	x
	1.40	x	x	x
	1.64	x	x	x
	2.6	x	x	x
	2.9	x	x	x
	4.6	ø	ø	ø
	8.6	ø	ø	ø
	10.0	ø	ø	ø
Bromphenol Blue	2.6	x	x	x
	2.9	x	x	x
	3.6	x	x	x
	4.4	x	x	x
	4.6	x	x	x
	5.0	ø	ø	x

ø - colourless blank

x - coloured blank.

TABLE 6. (Contd).

Dye	pH of solution	Blank		
		Benzene	Ether	Ethylene Chloride
Bromcresol Green	3.67	x	x	x
	3.99	x	x	x
	4.41	x	x	x
	5.0	ø	x	ø
	5.5	ø	ø	ø
Bromthymol Blue	5.5	x	x	x
	6.0	x	x	x
	6.5	x	x	x
	7.4	ø	x	x
	7.6	ø	x	x
	8.0	ø	x	x
Bromcresol Purple	5.5	x	x	x
	6.0	x	x	x
	6.3	ø	x	ø
	6.5	ø	ø	ø
	7.0	ø	ø	ø
Phenol Red	4.0	ø	ø	ø
	6.5	ø	ø	ø
	7.0	ø	ø	ø
	7.6	ø	ø	ø
	8.0	ø	ø	ø
o-Cresol Phthalein	8.0	x	x	x
	8.6	x	x	x
	above 12	x	x	x
Alizarin	aq. soln.	x	x	x
	5.0	x	x	x
	5.5	x	x	x
	6.0	x	x	x
Titan Yellow	aq. soln.	ø	ø	ø
Fast Green	" "	ø	ø	ø

ø = colourless blank
x = coloured blank.

or sodium hydroxide*. If, after shaking, any colour appeared in the aqueous layer, i.e. if a colour blank was given with a solvent, the dye was excluded for unsuitability with that particular solvent.

(b) Those dyes that passed the previous test were further tested for their reactivity with quinine.

Solutions of quinine in the different solvents were prepared (3 ug./ml).

20 ml. of the quinine solution were shaken with 1 ml. dye solution for 10 minutes, then centrifuged. About 15 ml. of the organic solvent were transferred to a stoppered tube containing 5 ml. of N/10 hydrochloric acid or sodium hydroxide, followed by shaking for 5 minutes. A coloured aqueous layer indicated a positive reaction between the dye and quinine.

B. Reactivity of the Alkaloids with the Dyes.

Solutions of the various alkaloids chosen for this study, Table 2, in the various solvents were tested for reactivity with dyes and indicators selected as suitable for the purpose, following the above procedure A(b).

2. DISCUSSION.

(i) The Blanks of the Dyes Tested.

The study of the blanks of the different dyes (Table 6) showed that:

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* Depending on the colour of the dyestuff in acid and alkaline medium.

(A) All those dyes containing carboxylic and/or phenolic groups gave coloured blanks both in their free acid form and monosodium salt, with benzene, ether and ethylene chloride. The dyes were those of the Nitro dyes; picric acid and m. nitro phenol,

Azo dyes : methyl red,

Xanthene dyes : o-cresol phthalein, and

Anthra quinoline dyes : alizarin.

The sodium salt of the sulphonic acid dyes gave colourless blanks with benzene, ether and ethylene chloride.

(B) The sulphonated azo dyes tested gave colourless blanks with the different solvents at all the pH tested, e.g. methyl orange gave colourless blanks with benzene, ether, ethylene chloride in aqueous solution and at pH 4.0 and 5.0.

(C) The non-halogenated sulphone phthalein dyes, phenol red and thymol blue gave colourless blanks at all the pH lying in their pH ranges. Phenol red (pH range 6.8 - 8.4) gave colourless blanks with the three solvents used at pH 4.0, 6.5, 7.0, 7.6 and 8.0.

(D) It was found that there was a relation between the depth of the colour of the blank and the pH of the dye solution of the halogenated sulphone phthalein dyes. The dyes tested gave deeply coloured blanks at the acid pH of their colour range which decreased on shifting the pH of the buffered dye solution towards the alkaline pH of the indicator range. As

TABLE 7.

Reactivity of the dyes that gave colourless blanks with quinine using the different solvents.

Dye	pH of solution	Reactivity		
		Benzene	Ether	Ethylene Chloride
Orange G.	aq. soln.	-	-	-
Chromotrope 2R	" "	-	-	-
Metanil yellow	" "	+	-	+
	pH 4.6	+	-	+
Methyl orange	aq. soln.	+	-	+
	pH 5.0	+	-	+
Evans blue	aq. soln.	-	-	-
	pH 9.0	-	-	-
	11.0	-	-	-
Tropaeolin O	aq. soln.	-	-	-
	pH 11.0	-	-	-
	12.0	-	-	-
Thymol Blue	pH 4.6	-	-	-
	8.6	-	-	-
	10.0	-	-	-
Bromphenol blue	5.0	+	+	coloured blank
Bromthymol blue	7.4	+	coloured blank	coloured blank
Bromcresol green	5.5	+	+	+

\+ = Reaction with quinine

\- = No reaction

TABLE 7. (Contd).

Dye	pH of dye solution	Reactivity		
		Benzene	Ether	Ethylene Chloride
Phenol red	pH 4.0	-	-	-
	6.5	-	-	-
	7.6	-	-	-
	8.0	-	-	-
Alizarin	aq. soln.	-	-	-
	pH 5.0	-	-	-
	5.5	-	-	-
	6.5	-	-	-
Titan yellow	aq. soln.	-	-	-
Fast Green	aq. soln.	-	-	-

\+ = Reaction with quinine

\- = No reaction

an example bromcresol purple (pH range 5.2 - 6.8) gave highly coloured blank at pH 5.5, at pH 6.0 the blank became faintly coloured and at pH 6.5 the blank became colourless with the solvents used. Bromthymol blue (pH range 6.0 - 7.6), gave coloured blanks at pH 5.5 - 6.5, but the blank was still coloured with both ethylene chloride and ether even at pH 8.0. This might be due to physical solution of bromthymol blue in these two solvents.

(ii) The Reactivity of the Dyes with Quinine. (Table 7).

The reactivity of the acidic dye with the alkaloids depends mainly on the following conditions:

(a) The molecular structure of the dye; as shown above, the nonsulphonated acidic dyes gave coloured blanks, yet some were tested for their reactivity with quinine. With alizarin (acidity due to phenolic groups in the ortho position) and methyl red (carboxylic acid group)*, it was found that there was no reaction.

It could not be concluded from this that nonsulphonated acid dyes do not react with alkaloids because eosin reacts with some alkaloids though it is not sensitive, since strychnine, brucine, hyoscine, morphine diamorphine and codein, when present in the same order as that of the useful range of quinine in plasma were found not to react with eosin.

(b) A colour change at a certain pH range; in order that the

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* A blank was done as well for the comparison of the produced colour.

dye may react with the alkaloids it must have a colour change at a certain pH range. All those dyes with no colour change, e.g. orange G, chromotrope 2R, titan yellow and fast green did not react with quinine.

Concerning those dyes that reacted with quinine:-

(A) The Azo Dyes. Only those dyes with pH range on the acid side of neutrality, that reacted with quinine as methyl orange and metanil yellow, while Evan's blue 9.0 - 11.0 and tropoaeolin O 11.1 - 12.2, did not react because their pH ranges are on the alkaline side of neutrality.

The pH of the dye solution might have an effect on the reaction between the dye and the alkaloid. To investigate this point solutions of methyl orange at pH 8.6 and tropaeolin O at pH 4.6 were prepared and shaken with quinine in benzene and ethylene chloride. It was found that methyl orange did not react with quinine at pH 8.6 while it readily reacts with it at pH 4.5; tropaeolin O was found to react with quinine at pH 4.6 but the colour produced was very weak compared to that of methyl orange. This proves that the reaction between the acid dyes and alkaloids proceeds readily at an acid pH. (Azo dyes).

(B) The Xanthene Dyes. Only those halogenated dyes of this group reacted with quinine as bromphenol blue, bromcresol green, bromcresol purple, bromthymol blue. The nonhalogenated xanthene dyes, phenol red and thymol blue, did not react with quinine. This is in agreement with the results of Cronheim

TABLE 8.

The dyes found to be suitable for use in this study, the pH of their buffered solutions, their blanks with different solvents and the wave lengths at which maximum absorption of the different dye is obtained.

Dye	pH buffer soln.	Blank			max. absorption mu
		Benzene	Ether	Ethylene Chloride	
Metanil yellow[a]	4.6	Colorless	Colorless	Colorless	550
Methyl orange[a]	5.0	"	"	"	515
Bromphenol blue[x]	5.0	"	"	Colored	580
Bromcresol green[x]	5.5	"	"	Colorless	620
Bromcresol purple[x]	6.5	"	"	"	580
Bromthymol blue[x]	7.4	"	Colored	Colored	610

[a] - colour produced with alcoholic N/2 HCl.

x - " " " " 0.1N NaOH.

& Ware (1948).

The dyes found to be suitable for use in this study, the pH of their buffered solutions, their blanks with different solvents and the wave lengths at which maximum absorption of the different dyes are given in Table 8.

The Solvents.

A. Ethers: although ether gave colourless blanks with some of the dyes, yet it is an unsatisfactory solvent for both the alkaloids and their compounds with the dyes.

(a) the alkaloid dye compounds of methyl orange and metanil yellow are insoluble in it.

(b) the high volatility of ether may cause inaccuracy of the quantitative technique.

(c) although ether is a good solvent for some alkaloids, yet it is not for strychnine which is of special interest in this study.

B. Alcohols: the group of immiscible alcohols was excluded as well, because they gave very highly coloured blanks. This was mainly due to the great affinity of these alcohols for water, so that on shaking the alcohol with the aqueous solution of the dye a part of the water passes to the alcohol dissolved in it and resulting in a high blank.

Brodie and Udenfriend in their technique, using benzene and methyl orange found that the blank was coloured. This coloured blank was due to the presence of iso amyl alcohol

(which they added to the benzene layer after the extraction of the alkaloid from the sample to minimise the adsorption of the alkaloid to the glass); repeating their technique after excluding the addition of iso amyl alcohol the blank was colourless.

C. Halogenated Hydrocarbons: Ethylene chloride proved to be satisfactory as it is an excellent solvent for both the alkaloids and their dye compounds.

D. Aromatic Hydrocarbons: Benzene also proved an efficient solvent for these alkaloids which are freely soluble in it, and whenever possible, should replace ethylene chloride since:

(a) ethylene chloride gives a coloured blank with brom thymol blue;

(b) it gives high urine and tissue blanks which is not the case with benzene (as will be shown later);

(c) ethylene chloride readily forms emulsion on shaking with the samples.

(d) in the case of benzene 4/5 of the original volume used could be used for the colour production, i.e. to be shaken with the alkali or acid, while in the case of ethylene chloride only 2/5 of the original volume could be used because of the difficulty of separating the two layers and removing the aqueous layer;

(e) ethylene chloride is more toxic than benzene.

TABLE 9. (a)

BENZENE

Alkaloid	Methyl Orange	Metanil Yellow	Brom-thymol Blue	Brom-cresol Purple	Brom-phenol Blue	Brom-cresol Green
Strychnine	+	+	+	+	+	+
Brucine	+	+	+	+	+	+
Atropine	+	+	+	+	+	+
Hyoscine	+	+	+	+	+	+
Hyoscyamine	+	+	+	+	+	+
Cocaine	+	+	+	+	+	+
Procaine	+	+	+	+	+	+
Benzocaine	-	-	-	-	-	-
Morphine	Morphine is insoluble in benzene					
Heroin	+	+	+	+	+	+
Quinine	+	+	+	+	+	+
Lobeline	+	+	+	+	+	+
Aconitine	+	+	+	+	+	+
Yohimbine	Only soluble in hot benzene					

\+ = positive reaction

\- = negative reaction

TABLE 9(b).

ETHYLENE CHLORIDE

Alkaloid	Methyl Orange	Metanil Yellow	Bromcresol Purple
Strychnine	+	+	+
Brucine	+	+	+
Atropine	+	+	+
Hyoscine	+	+	+
Hyoscyamine	+	+	+
Cocaine	+	+	+
Procaine	+	+	+
Benzocaine	+	+	+
Morphine	-	-	-
Heroin	+	+	+
Quinine	+	+	+
Lobeline	+	+	+
Aconitine	+	+	+
Yohimbine	+	+	+

\+ = positive reaction

\- = negative reaction

TABLE 9. (c)

ETHER

Alkaloid	Methyl Orange	Metanil Yellow	Bromcresol Green	Bromcresol Purple	Brompheno Blue
Strychnine	-	-	+	+	+
Brucine	-	-	+	+	+
Atropine	-	-	-	-	-
Hyoscine	-	-	-	-	+
Hyoscyamine	-	-	-	-	-
Cocaine	-	-	-	-	-
Procaine	-	-	-	-	-
Benzocaine	-	-	-	-	-
Morphine	the alkaloid is insoluble in ether				
Heroin	-	-	-	-	-
Quinine	-	-	+	+	+
Aconitine	-	-	-	-	-
Lobeline	-	-	+	+	+
Yohimbine	-	-	+	+	+

\+ = positive reaction

\- = negative reaction

(IV). THE ALKALOIDS.

The results of tests with various alkaloid dye solvent combinations are given in Table 9a, b, c. The solubility of the alkaloid and its dye compound in the different solvents is the governing factor for choosing the dye-solvent system for the particular member or group of alkaloids.

A. The alkaloid-dye compounds of the azo dye group, e.g. methyl orange and metanil yellow, of all the alkaloids tested, were found to be insoluble in ether. With the sulphone phthalein dyes, bromcresol purple, bromphenol blue and bromphenol green the dye compounds of the atropine group, cocaine and its substitutes and aconitine were also found to be insoluble in ether. The dye compounds of strychnine, brucine, heroin, quinine, lobeline and yohimbine with the dyes of sulphone phthalein group were partially soluble in ether as the colour obtained was very weak compared with that produced with benzene and ethylene chloride and on re-extracting with a fresh amount of ether a colour was produced in the sodium hydroxide layer.

B. Benzene and ethylene chloride proved to be satisfactory solvents for the different alkaloid-dye compounds with the exception of morphine which is insoluble in benzene and slightly soluble in ethylene chloride.

C. Morphine did not react with any of the dyes because there is a free phenolic group in its molecule which masks the basic characters of the nitrogen atom. Heroin which has no free phenolic group reacted readily with all the suitable dyes.

PART II :

THE ALKALOID DYE ADDITION COMPOUND METHOD

DIVISION II : QUANTITATIVE STUDY.

TABLE 10.

A List of Dyes and Indicators which appear to be suitable since a colourless blank could be obtained, and which were active with alkaloids.

(A) Used with Benzene	(b) Used with Ethylene Chloride
Bromcresol purple	Bromcresol purple
Bromcresol green	Bromcresol green
Bromthymol blue	
Bromphenol blue	
Methyl orange	Methyl orange
Metanil yellow	Metanil yellow.

TABLE 11.

A Comparison of the Reactivity of Certain Dyes and Indicators (See Table 10A).

1. Solvent used - benzene
2. Alkaloid - quinine (20 ug.)

Dye	Anti Log Transmittance
Bromcresol purple	0.500
Bromcresol green	.300
Bromthymol blue	.530
Bromphenol blue	.430
Methyl orange	.410
Metanil yellow	.420

TABLE 12.

A Comparison of the Reactivity of Certain Dyes and Indicators (See Table 10B)

1. Solvent used - Ethylene chloride
2. Alkaloid - quinine (20 ug.)

Dye	Anti Log Transmittance
Bromcresol purple	0.490
Bromcresol green	.410
Methyl orange	.420
Metanil yellow	.600

Instrument : Unicam Spectrophotometer G.P. 350.
Wave-lengths: (see Table 9). At the maximum absorption.

Chapter 3. Conditions for the Quantitative Technique.

(i) Choice of the Dye.

For the quantitative work the most sensitive dye of those found to be suitable should be chosen. From the previous qualitative work, a list of the suitable dyes that reacted with all the alkaloids (except morphine) is given in Table 10.

A solution of quinine in benzene containing 2.0 ug/ml. was prepared by shaking 0.2 ml. quinine sulphate solution 1 mgm./ml.), 1.0 ml. sodium hydroxide 10% and 100 ml. Benzene. 10 ml. of the benzene layer and 0.5 ml. of the different dyes (Table 10) were shaken for 5 minutes.

Shake 6 ml. of the benzene solution of the different quinine dye compounds with 4 ml. sodium hydroxide or hydrochloric acid. Read the colour intensity at the suitable wave length. (Table 8).

Repeat the same with ethylene chloride instead of benzene.

A fixed amount of quinine was allowed to react with the different dyes in turn, using benzene and ethylene chloride. (Table 11) With benzene, bromthymol blue gave the highest colour intensity, bromcresol purple was the next, then promphenol blue, metanil yellow and methyl orange, and the least bromcresol green. (Table 12) When ethylene chloride, was used the highest extinction was given by metanil yellow followed by bromcresol purple,

methyl orange and bromcresol green.

Bromcresol green gave a higher reading with ethylene chloride than with benzene although the amount of quinine used with both was the same. This might be due to the incomplete solubility of the quinine-bromcresol green compound in benzene and its free solubility in ethylene chloride. It was found that benzene was not a good solvent for the quinine-metanil yellow compound and that ethylene chloride was superior to it because it gave a higher reading with the same quantity of quinine.

From the above it is noticed that certain solvent-dye combinations should be used. As bromthymol blue gave the highest colour extinction with benzene, this combination was therefore recommended whenever the use of benzene as a solvent is possible. The most suitable dye for use with ethylene chloride is metanil yellow.

Further work was done to confirm the above mentioned solvent-dye combinations. Brodie & Udenfriend (1945), used methyl orange-ethylene chloride for the determination of the cinchona alkaloids in biological material, while in a later communication (1947), they replaced ethylene chloride with benzene. When this work was in progress an article published by Gettler & Sunshine (1951) was noticed. They used methyl orange - chloroform in their studies for extending Brodie's method to the estimation of a variety of alkaloids in tissues.

From the preliminary work mentioned above, the bromthymol blue-benzene and metanil yellow-ethylene chloride dye-solvent combinations gave the best results. It was of interest to construct comparison curves between:

(a) methyl orange and bromthymol blue with benzene

(b) methyl orange and metanil yellow with ethylene chloride.

Procedure:

In a glass stoppered 60 ml. capacity bottle, place 25 ml. of the solvent, 1 ml. 2N sodium hydroxide and the volume of the alkaloidal solution containing the required amount of the alkaloid. Shake for 10 minutes. Stand till the two layers separate, decant the solvent layer in a tube and centrifuge for 10 minutes (to break any emulsion formed and to remove any droplets of the aqueous solution that might be in the organic solvent).

In a clean bottle containing 1.0 ml. of the dye solution, transfer 20 ml. of the organic solvent containing the alkaloid, shake for 10 minutes and centrifuge for quarter of an hour.

In a stoppered 25 ml. measuring cylinder 15 ml. of the alkaloid dye compound solution were shaken, with 4 ml. of sodium hydroxide or hydrochloric acid (according to the dye used). Centrifuge for 5 minutes. The organic solvent layer removed by aspiration and the aqueous layer transferred to a colorimetric tube. Read the colour intensity at the

TABLE 13.

Comparison of two Indicators for the Determination of Quinine in Aqueous Solution

A. Benzene/Methyl Orange
B. Benzene/Bromthymol blue.

Reading (A)*		Quinine(μg)	Reading (B)*	
0.065	0.072	5 μg.	0.135	0.135
.140	.135	8	.190	.200
.172	.180	10	.250	.255
.210	.210	12	.305	.310
.240	.230	14	.345	.375
.270	.280	16	.440	---
.340	.335	18	.475	.475
.370	.370	20	.510	.515

TABLE 14.

C. Ethylene chloride/Methyl orange
D. Ethylene chloride/Metanil Yellow

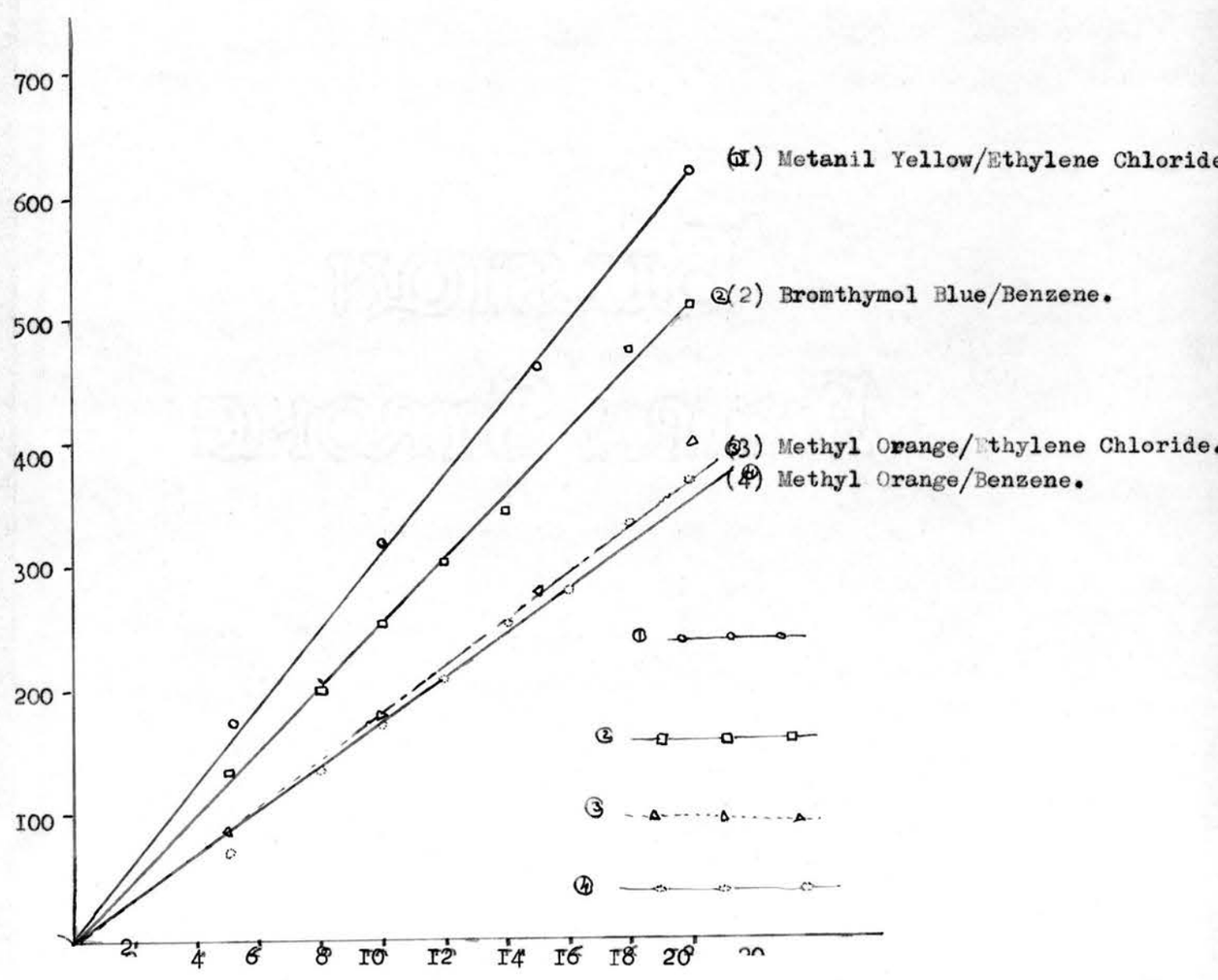
Reading (C)		Quinine (μg)	Reading (D)*	
0.085	0.080	5 μg.	0.175	0.175
.160	.180	10	.340	.320
.280	.290	15	.460	.460
.400	.420	20	.620	.630

Wavelength:

A. 515 mu.
B. 610 mu.
C. 515 mu.
D. 550 mu.

FIGURE I.

COMPARISON OF INDICATORS FOR THE DETERMINATION OF QUININE IN AQUEOUS SOLUTIONS.



suitable wave length.

Perform a blank using distilled water to adjust the zero of the instrument.

For quantitative work the bromthymol blue dye proved to be superior to methyl orange with benzene as the slope of quinine bromthymol blue curve (Fig. 1) was higher than that of quinine methyl orange curve. Metanil yellow ethylene chloride combination gave better results than those of methyl orange ethylene chloride, as shown by the curve (Fig. 1).

As strychnine was the alkaloid of interest in this study, comparison curves between methyl orange and bromthymol blue with strychnine, using benzene as solvent, were again constructed to show strychnine's response to the two dyes.

Fig. 2 shows that bromthymol blue gave higher reading with strychnine than methyl orange. The bromthymol blue curve followed Beer's law in concentrations of 0-50 ug. of strychnine, while methyl orange gave a typical curve only at concentrations higher than 15 ug. (15 - 50 ug of strychnine). This might be explained by the weaker acidic character of methyl orange compared to that of bromthymol blue.

The reaction between the alkaloid and the dye depends on the basic character of the alkaloid and the acidic character of the dye. Quinine being a strong base reacted with methyl orange quantitatively at all concentrations, while strychnine did not react with methyl orange quantitatively at very low concentration from 0 - 10 ug. Strychnine was one

TABLE 15.

The Determination of Strychnine

A Comparison of Readings

A. using methyl orange and benzene

B. using bromthymol blue and benzene.

Strychnine (ug.)	Reading of methyl orange A.		Reading of bromthymol blue B.	
5	0.003	0.000	0.050	0.050
10	.008	.010	.150	.140
15	.030	.036	.220	.230
20	.065	.45	.295	.310
25	.105	.105	.370	.370
30	.135	.160	.450	.470
35	.170	.180	.518	.520
40	.245	.240	.620	.615
45	.280	.290	.645	.675
50	.350	---	.790	.770

Instrument used : Unicam Spectrophotometer G.P. 350

Tubes : small diameter. Wave-length: A 515 mu.
B 610 mu.

Solvent: Benzene Water blank : Nil.

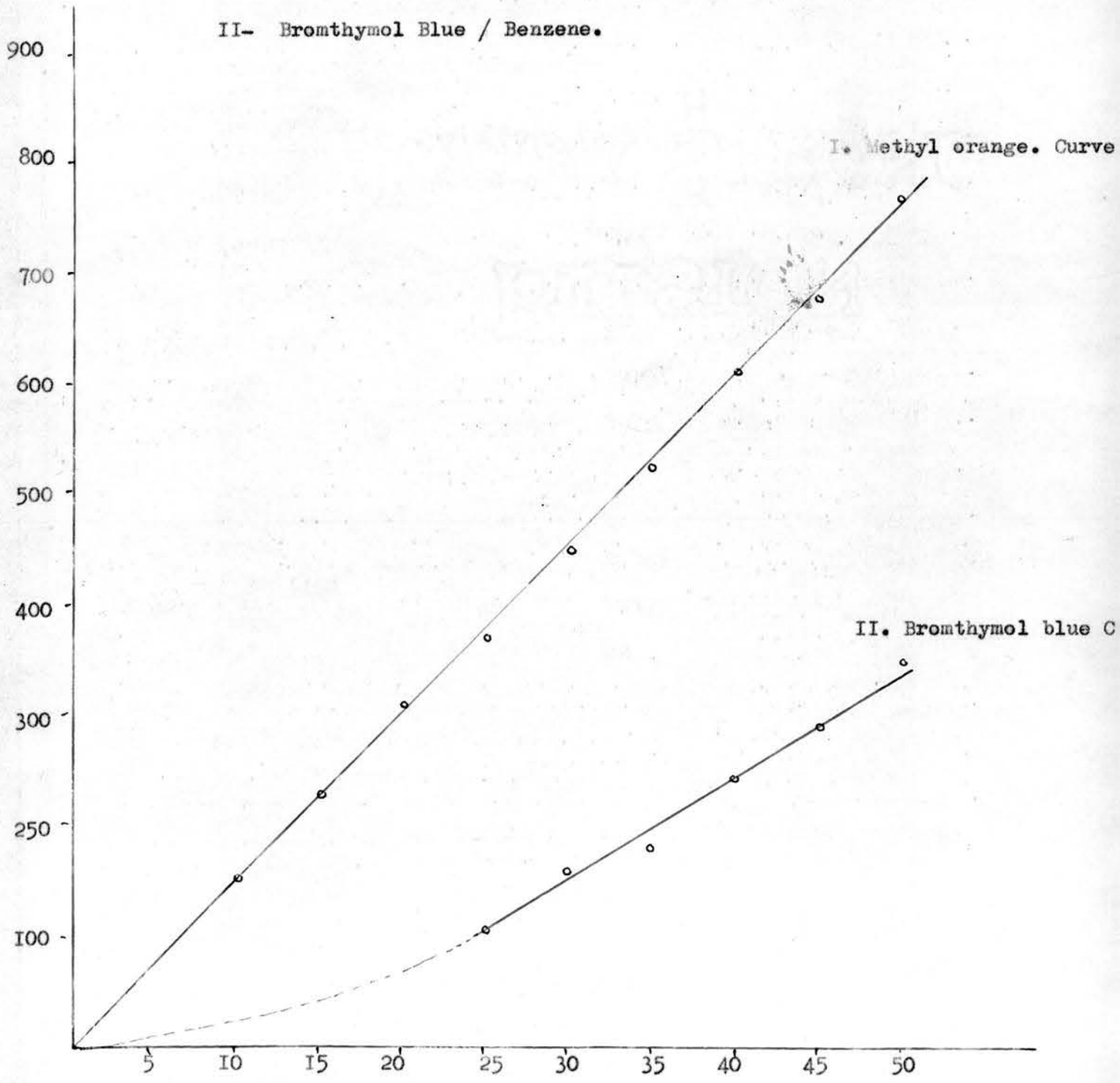
FIGURE 2.

The Determination of Strychnine.

A Comparison of Curves

I- Methyl Orange / Benzene

II- Bromthymol Blue / Benzene.



of the alkaloids (brucine, hyosine, morphine, and codein) mentioned by Allen which do not react with eosin when present in the same concentration as that of the useful range of quinine in plasma (5 - 30 ug./10 ml. plasma).

The use of a strongly acidic sensitive dye, e.g. bromthymol blue, is recommended for the accurate estimation of small amounts (0 - 50 ug.) of alkaloids.

(ii) Extraction Method.

Extraction of the alkaloid from aqueous solutions, after being rendered alkaline, by shaking with organic solvents was satisfactory. The ratio between the volumes of the organic solvent and the aqueous solution was usually between 2.5 : 1 and was kept the same in each series of experiments. This was to avoid any variation in the extraction conditions which might result in inaccuracy due to change of the partition of the alkaloid between the organic solvent and aqueous phases resulting from changes of the volumes used.

Extraction of the alkaloids from biological material will be discussed in Chapter 3, page 32.

Another method of extraction was lately thought of, using ion exchange resins. The aqueous solution of the alkaloid is percolated through a column of anion exchanger, the alkaloidal base is then eluted with benzene or chloroform followed by shaking with the suitable dye, bromthymol blue in

TABLE 17.

A Study of the Effect of Time of Shaking upon the Formation of the Alkaloid-Indicator Addition Compounds.

Volume of dye solution	Reading
0.5	0.510
0.7	.520
1.0	.500
2.0	.530

TABLE 16.

A Study of the Effect of Volume of Dye Solution upon the Formation of the Alkaloid-Indicator Addition Compounds.

Time of Shaking	Reading
2 minutes	0.530
5 "	.510
7 "	.520
10 "	.515
15 "	.510

Reaction Employed:-

Alkaloid - quinine
Indicator - bromthymol blue
Solvent - benzene
Colorimetric details - as in Table No. 15.

the former, and metanil yellow in the latter. This method will be studied in detail to find its accuracy with minute amounts of alkaloids in the order of 10-50 ug. in another communication.

(iii) Time Required for the Reaction Between the Dye and The Alkaloid.

Marshall and Rogers added bromthymol to the aqueous solution of the alkaloid adjusted to pH 7.0. After standing for an hour they extracted the compound formed with benzene by shaking for half an hour. Brodie et al. extracted the cinchona with the organic solvent followed by shaking with the dye for 5 minutes. The method used here was that of Brodie et al.

A preliminary study of the time required for the reaction between the dye and the alkaloid was performed by shaking volumes of 20 ml. benzene solution of quinine (1 ug./ml.) with 2.0 ml. bromthymol blue for different periods. 12 ml. of the benzene solution of the compound formed were shaken with 4 ml. sodium hydroxide.

It was found that the reaction proceeded instantaneously, as the results obtained on shaking for a range of time 2 - 15 minutes were the same. Shaking for 5 minutes was satisfactory. (Table 16).

(iv) Volume of the Dye Solution.

Different volumes of 1% bromthymol blue, 0.5 ml. 0.7, 1.0 and 2.0 ml., were shaken with 50 ml. volumes of benzene solution of quinine (1 ug./ml.) for 5 minutes. 10.0 ml. of N/10 sodium hydroxide were used for colour development with 30 ml. of the benzene layer because the blue colour produced with 4 ml. was very strong.

The readings given with the different volumes of bromthymol blue were the same.

As the quinine (alkaloid) bromthymol blue (dye) compound is soluble to some extent (sparingly soluble) in water, it was advisable to use the minimum volume of the dye solution, 0.5 ml. was recommended to be used.

3. Toxicity of Strychnine.

Strychnine was chosen as an example of alkaloids for the quantitative study of this method, since it is frequently met with in toxicological analyses.

Strychnine is a powerful drug that comes from the seeds of Nux Vomica and Ignatus beans. It is marketed individually in the form of colourless crystals of the alkaloidal base or as its hydrochloric or sulphate. The most important source of this poison for the public is medicinal preparations, e.g. Easton syrup (syr. ferri phos. c. quin. et strych.) contains 1/60 gr. of strychnine in each drach and the tablets of the same strength. Its use in rat and vermin killers is prohibited except for moles and seals.

Posioning with strychnine may be due to accidental, suicidal or homicidal causes. The intensely bitter taste of the poison and the rapidity of its action should presumably prevent its use for homicidal purposes, but in spite of these characteristics it is used for this purpose from time to time. It may be administered frequently in the form of the free alkaloid (among professional people handling this alkaloid), or the powdered nux vomica seeds may be used. Suicides occasionally use these preparations.

Death from accidental causes is perhaps more common and this may arise from taking an overdose of medicinal preparations containing the drug, or from the poison being administered by

mistake. A child about 3 years of age found a small bottle of sugar coated tabloids of Easton's syrup and swallowed two of them in the belief that they were sweets. A convulsion occurred in a few minutes and the child died within an hour. In another case a prescription for powder containing quinine, phenacetin, and one grain of exalgin was dispensed with strychnine instead of exalgin, owing to the chemist having his poisons on the same shelves as the other drugs. Strychnine is precipitated in alkaline medium and may settle to the bottom of a bottle of medicine; in this event, failure to observe this fact or to shake the bottle sometimes results in an accidental overdose. For example, a woman was given a prescription for liquor arsenicalis and liquor strychninae, half ounce each, in a one ounce bottle, with directions to take 6 drops as a dose. The medicine was dispensed as written and the patient took the greater part of the bottle without mishap; a short time afterwards, however, she wished to take another dose but found the bottle empty. A sediment at the bottom of the bottle caught her eyes, and she poured in a little water to dissolve it and drank the resulting fluid. The classical symptoms of strychnine poisoning appeared in two hours and twenty minutes. The alkali in the liquor arsenicalis had precipitated some of the strychnine and this was all taken in the last dose.

TABLE 18.

Calibration Curve - Strychnine

ug. of Strychnine	Readings 1.	2.	3.
10	155	175	160
15	238	230	230
20	310	315	315
25	360	370	375
30	415	470	480
35	540	550	555
40	620	620	630
45	710	733	730
50	800	810	815

Alkaloid : Strychnine

Instrument: Unicam Spectrophotometer G.P. 350.

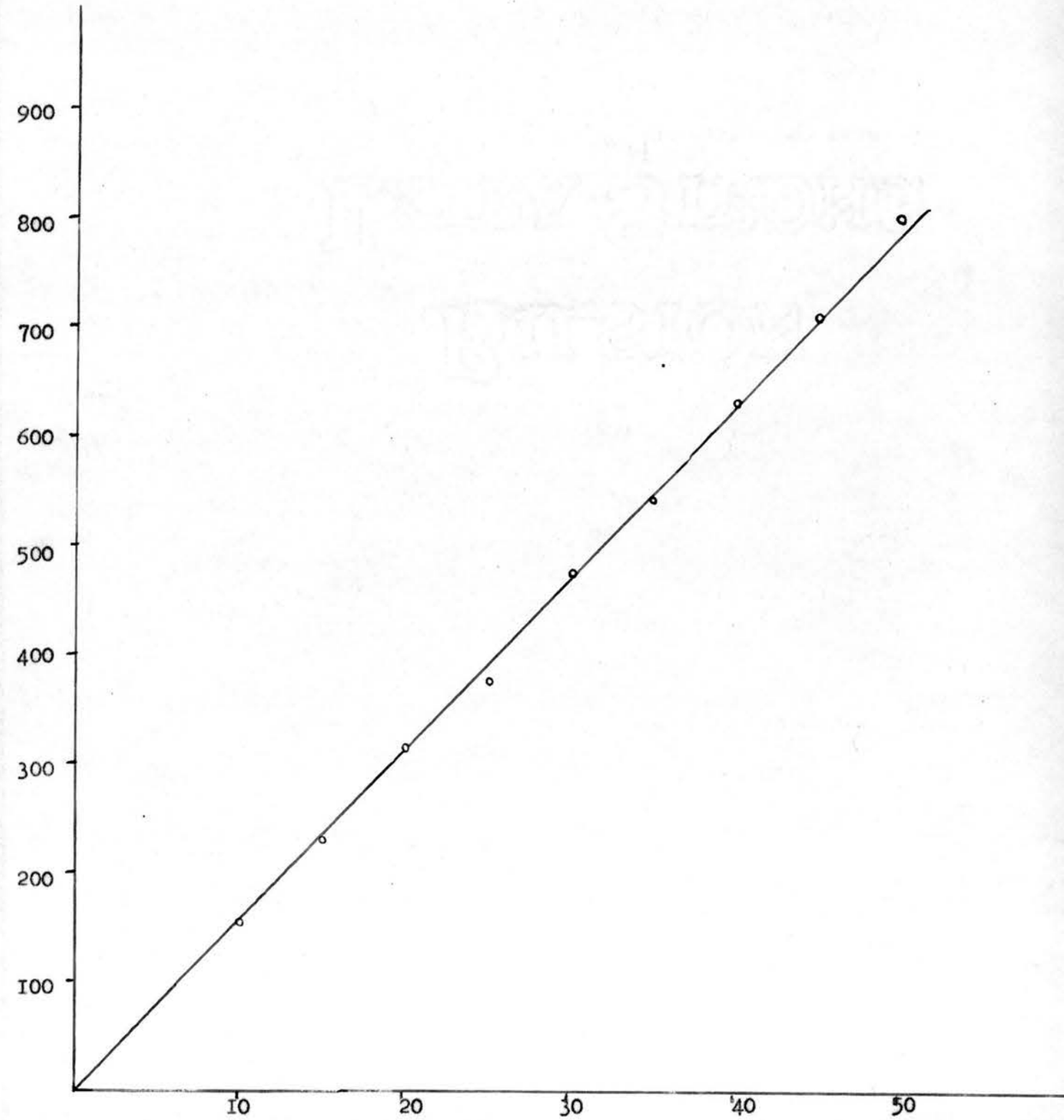
Solvent : Benzene

Wavelength: 610 mu.

Water blank : Nil.

FIGURE 3.

Caliberation Curve Of Strychnine.



3. Recovery of Strychnine from Aqueous Solution.

To justify the accuracy of this method for the estimation of alkaloids, the recovery of a known amount of strychnine in aqueous solution was studied.

Calibration Curve. A standard curve of strychnine was constructed. Slight modifications were introduced to the original procedure of Brodie et al., 1945.

To 1.5, 2.0 ..... 5.0 ml. of strychnine sulphate solution 10 ug. strychnine/ml. were added N sodium hydroxide solution, enough to bring the pH of the solution to 8.5 - 8.6; then the volume was brought to 10 ml. with Na_2HPO_4 solution (1.906 gm/ml.) of pH 8.6, followed by the addition of 25 ml. benzene. This was shaken for 10 minutes and centrifuged.

Shake 20 ml. of the benzene layer with 0.5 ml. bromthymol blue solution for 10 mimutes and centrifuge*.

Shake 15 ml. of the benzene extract with 4 ml. of 0.1N sodium hydroxide and centrifuge.

Remove as much as possible of the benzene by aspiration, then transfer the sodium hydroxide layer by a dropper to a colorimetric tube. Read at 610 mu. The colour remains stable for at least 3 hours.

The zero of the instrument was adjusted with a blank.

Recovery Results.

A series of estimations was performed on different concentrations of strychnine, standard strychnine solution being used. The results are given in Table 19.

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* In this step both the bottles and the centrifuge tubes must be dry as the complex is soluble to some extent in water.

TABLE 19.

The Recovery of Strychnine from Aqueous Solution.

µg. Strychnine added	µg. Strychnine Recovered	% of Recovery
10 µg.	10	100
	10.25	102.5
	11.00	110
	9.75	79.5
	9.25	92.5
	Average Recovery 96.9%	
15 µg.	15.00	100
	14.5	99.3
	14.5	99.3
	13.25	88.3
	14.0	93.3
	Average Recovery 96.0%	
20 µg.	19.75	98.7
	20.00	100
	20.00	100
	19.00	95
	20.00	100
	Average Recovery 98.7%	
25 µg.	23.00	92
	23.5	94
	23.5	94
	25.5	102
	24.0	96
	Average Recovery 95.6%	
30 µg.	26.5	88.3
	30.0	100.0
	30.0	100.0
	30.0	100.0
	30.8	102.6
	Average Recovery 98.2%	
35 µg.	34.0	97.1
	35.0	100
	35.5	101.4
	35.75	102.0
	Average Recovery 100.1%	

TABLE 19 (Contd.)

µg. Strychnine added	µg. Strychnine Recovered	% of Recovery
40 µg.	39.25	98.1
	39.25	98.1
	40.00	100.0
	41.00	102.5
	40.50	101.2
	Average Recovery 100%	
45 µg.	45.00	100
	46.25	102.3
	46.25	102.3
	45.7	101.5
	44.7	99.3
	Average Recovery 101.1%	
50 µg.	50.00	100.0
	50.75	101.5
	50.75	101.5
	49.50	99.0
	Average Recovery 100.5%	

AVERAGE RECOVERY = 98%.

The estimation of strychnine by the alkaloid-dye method gave satisfactory results. Application of the method to biological material is of importance, as the specimens dealt with in toxicological practice are not simple aqueous solutions.

TABLE 20.

Application to Normal Urine.

Solvent	Dye	Results
Benzene	Bromphenol blue	colourless blank with sodi hydroxide
	Bromcresol purple	colourless blank with sodi hydroxide extract
	Methyl orange	colourless blank with hydr chloric acid extract.
	Bromcresol green	colourless blank with sodi hydroxide extract.
	Bromthymol blue	coloured blank with sodium hydroxide extract.
Ether	Bromcresol purple	colourless blank with sodi hydroxide extract.
	Bromphenol blue	colourless blank with sodi hydroxide extract.
Ethylene chloride	Bromcresol purple	Coloured blank with sodium hydroxide extract.
	Bromcresol green	coloured blank with sodium hydroxide extract.
	Methyl orange	coloured blank with hydroc ic acid extract.
	Metanil yellow	coloured blank with hydroc acid extract.

4. Estimation of Strychnine in Biological Material.

In toxicological analysis the samples subjected to the detection and estimation of the suspected alkaloid are mostly biological material such as vomit, urine, blood or tissues. To test the efficiency of the method for its application to biological material the recovery of added amounts of strychnine to samples of urine, blood and tissues was studied.

(i) Recovery of Strychnine from Urine.

Urine blanks were performed with the different dyes and solvents.

The urine was shaken with benzene after being rendered alkaline with sodium hydroxide, the benzene extract then treated with the different dyes in turn. The work was repeated with ether and ethylene chloride. The results are given in Table 20.

From Table 20 it should be noticed that with benzene only bromthymol blue gave coloured blank. This might be due to the high sensitivity of the dye to basic substances (other than alkaloids) present in urine and extracted with benzene.

In the case of ether the blanks were colourless; this might be attributed to the insolubility of the basic compounds in ether.

Ethylene chloride extract of the urine gave highly coloured blanks with all the dyes, due perhaps to the free solubility of the interfering substances in ethylene chloride, being

TABLE 21.

Application to Urine not containing Organic Baric Drugs.

Technique: Benzene/bromthymol blue/sodium hydroxide.

* Unicam Spectrophotometer G.P. 350 with small tube at 610 mμ.

Urine	Origin	Reading *
1	Diabetic, glycosuria	14%
2		23
3		76
4	Patients receiving no medicine 24 hours before sampling	28%
5		17
6		57
7		30
8	Normal individuals	45%
9		50
10		88
11		81
12		48
13		62
14		81
15		83
16		90
17		64
18		79

an excellent solvent for organic basic compounds.

Urine blank with bromthymol blue was further studied in detail. Different samples of urine were tested for their blanks. As shown in Table 21 all samples gave coloured blanks and were found to vary from one person to another in both pathological and normal urines.

Oberst (1943), studying demerol excretion in man, used a fixed value for interfering substances in urine and found that the average value of 28 persons (24 hours sample) was equivalent to 2.1 mgm. demerol. This value was subtracted from the total amount of demerol found in 24 hours specimen. This is not an accurate method, especially when dealing with minute amounts. He stated that "when a small dose of demerol is administered a correspondingly small amount is excreted in the urine. This amount may increase the colour intensity of the benzene layer*. Only a little more of the blank which is not determined fairly accurately would introduce a large error. Occasionally, a demerol free urine gives a high value for a blank, which when subtracted from the corresponding total value would yield a false result. When large amounts of demerol are administered the blank becomes comparatively insignificant."

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* the demerol estimated by the yellow colour of the benzene solution of demerol-bromthymol blue compound.

B. Removal of Interfering Substances from Urine.

The removal of the interfering substances from urine was tried in different ways.

(a) Treatment of the urine (prior to alkaline extraction) with immiscible organic solvent at an acid pH.

The interfering substances might be freely soluble in organic solvents and could be removed by shaking the urine with one of these solvents. As alkaloids are extracted by organic solvents from alkaline solutions, the treatment of the urine should be at an acid medium. The following method was tried:-

Two 7 ml. samples of urine were adjusted to pH 5 with 0.1N hydrochloric acid, one was shaken for 15 minutes with 20 ml. benzene, the other with 20 ml. ethylene chloride. Both were centrifuged and the organic solvents completely removed. Urine was placed in three clean 60 ml. bottles. 5 ml. of untreated urine was placed in the first, 5 ml. of the urine sample treated with benzene in the second, and 5 ml. of urine previously treated with ethylene chloride in the third. The three samples were made alkaline with sodium hydroxide and each extracted with 25 ml. benzene. The bensene extracts were shaken with bromthymol blue and 15 ml. of the benzene layer were used for colour production with 4 ml. volumes of sodium hydroxide.

The untreated urine gave a reading of	0.190
Urine treated with benzene " "	0.180
Urine treated with ethylene chloride "	0.095

Shaking the urine with ethylene chloride at an acid pH removed a part of the interfering substances, but the method was still unsatisfactory.

(b) Adsorption of the Interfering Substances.

Cronheim and Ware (1940) recommended the estimation of true amidone in urine by the difference between the apparent amidone content in the untreated urine and the value of the interfering substances in the sample after the adsorption of amidone on superfilterol.

Removal of the interfering substances from urine was tried by treating the urine with some adsorbents. As superfilterol was not available other adsorbents were used, such as charcoal, keisulguhr, talc and fuller's earth. Both whole urine (alkaline medium) and its benzene extract were treated with the above-mentioned adsorbents. The urine was made alkaline to phenolphthalein (pH > 10). 20 ml. volumes were shaken with about 2 g. of each adsorbent for 10 minutes. The mixture was filtered. 5 ml. of each specimen were shaken with 25 ml. benzene and the technique for the urine blank continued as usual. The benzene extract of urine was prepared by shaking 50 ml. alkaline urine (pH >10) with 250 ml. benzene. 25 ml. volumes of the benzene extract were shaken with different adsorbents and filtered. The benzene extracts were then shaken with bromthymol blue.

TABLE 22.

Application to Urine not containing Organic Basic Drugs- The Effect of pH of Extraction upon the Separation of Non-specific Material.

pH of Extraction	Reading
8.0	.005
8.6	.012
9.0	.005
9.5	.045
11.0	.120
above 12.0	.222

Technique:

Solvent : Benzene

Dye : Bromthymol blue

Reading : at 610 mu wave length.

TABLE 23.

Experiments to illustrate that Minimal Amounts of Non-Specific Substances are separated by extraction at pH 8.6 in contrast to extraction at pH 12.0.

Solvent: Benzene

Indicator: Bromthymol blue

Values obtained for the Urinary 'blank'.

	A. after extraction at pH 12.0	B. After extraction at pH 8.6
1.	0.240	0.012
2.	.195	.005
3.	.270	.022
4.	.060	.000
5.	.222	.012
6.	.102	.010

Instrument:

Unicam Spectrophotometer G.P. 350 - small tubes.

Wavelength: 610 mu.

1. Urine made alkaline and treated with the different adsorbents, gave coloured blanks in all cases, indicating that no adsorption of the interfering substances from alkaline aqueous solutions took place.
2. Treatment of the benzene extract of the urine with the different adsorbents removed the interfering substances.
3. All the adsorbents removed both the interfering substances and strychnine from the benzene extracts of urine to which strychnine was added.

This method was inefficient as there was no individual removal of either the interfering substances or the strychnine from the benzene extract of the urine.

(c) Extraction of the Urine at different pH.

In the original method the urine was treated with one ml. 2N sodium hydroxide which brought the pH of the urine over 12, followed by extracting with benzene. This method resulted in highly coloured blanks due to the extraction of interfering substances present in the urine and which reacted with bromthymol blue. The extraction of these interfering substances might be affected by the pH of the solution. To justify this assumption, urine blanks over a range of pH 8 - 12 were performed.

It was found that there was a relation between the pH of

of extraction and the colour of the urine blank. Adjusting the pH of the urine to pH 8 - 9 before extraction with benzene, the foreign substances present in the urine no longer interfered with the estimation of strychnine (or any other alkaloid). (Table 22) Table 23 shows results of normal urine blanks at pH 8.6 and pH 12.

C. Recovery of Strychnine.

Urine containing 10 ug./ml. was prepared by diluting 1 ml. of strychnine standard 1 mgm./ml. to 100 ml. with urine of normal individuals receiving no drugs whatsoever.

Procedure:

Add enough sodium hydroxide 0.1 N to 5 ml. urine containing strychnine, to bring the pH to 8 - 8.5, followed by the addition of 25 ml. benzene.

Shake for 10 minutes, then centrifuge the benzene layer.

Treat 20 ml. of the benzene layer with 0.5 ml. bromthymol blue for 5 minutes and centrifuge.

Shake 15 ml. of the benzene extract with 4 ml. sodium hydroxide and centrifuge.

Transfer the sodium hydroxide layer to a colorimetric tube. Measure the colour intensity at 610 mu.

TABLE 24.

Recovery of Strychnine Added to Urine.

Indicator : Bromthymol blue Wave-length : 610 mu.

Solvent : Benzene Instrument : Unicam G.P. 350. (small tubes).

Technique : Benzene/Bromthymol Blue/Sodium Hydroxide.

No.	Strychnine added ug.	Strychnine recovered ug.	% Recovery
1. Specimen 1.	50	44.4	88.8
2.	50	50.0	100.0
3.	50	49.5	99
4. Specimen 2	50	51.1	102.0
5.	50	51	102
6.	50	50.0	100
7. Specimen 3	50	51.1	102.2
8.	50	45.0	90
9.	50		

TABLE 25.

The Recovery of Strychnine from Stored Urine.

Strychnine was added to urine which was then placed in a refrigerator (3-5°C).

The strychnine content was determined at 0, 1 day and 7 days later. The analyses were carried out in triplicate.

Time of analysis	Strychnine recovered ug.	% Recovery
1. 0 days	51.1	102.2
	51	102
	50	100
2. 1 day later	50.0	100
	50	100
	51.5	103
3. 7 days later	49.5	99
	50	100
	50	100

Technique : Benzene/Bromthymol Blue/Sodium Hydroxide.

Instrument : Unicam Spectrophotometer G.P. 350 - small tub

Wave-length: 610 mu.

Three urine specimens from different individuals to which known amounts of strychnine sulphate solution (1 mgm. strychnine/ml.) had been added, so that 1 ml. urine contained 10 ug. strychnine, were used for the study of the recovery of added strychnine from urine.

The recovery of the added strychnine from three specimens was 96% - 101.3% and 96.1%, which is considered to be satisfactory for toxicological purposes. (Table 24)

The effect of storage on the strychnine content of the urine was studied by placing one of the previous specimens (specimen 2, the recovery of which was 101.3%) in the refrigerator for 24 hours and for 7 days. The percentage of recovery in the former case was 101.0%, and in the latter 99.6%. This shows that (Table 25)

(a) the strychnine content was not changed due to its stability, and

(b) the extraction of the urine at pH 8.0 - 8.6 was still practically colourless even after standing for seven days in the refrigerator.

(ii) Estimation of Strychnine in Plasma.

The alkaloid-dye compound formation method was applied to plasma to which known amounts of strychnine were added, to find the extent of strychnine recovery. Benzene was used as the solvent on the basis of experience with urine (p. 32) and because Brodie (1945) used benzene for the extraction of cinchonidine from biological material on account of its low blank compared to that of ethylene chloride.

A. Plasma Blank.

The plasma blank was first studied, both whole plasma and the protein-free filtrate being tested.

In the former 10 ml. of plasma were made alkaline with sodium hydroxide and then shaken with 25 ml. benzene. 20 ml. of the benzene layer were treated with 0.5 ml. bromthymol blue, the mixture was centrifuged and 15 ml. of it were shaken with 4 ml. sodium hydroxide N/10. Three different samples of plasma gave blanks corresponding to 1.0 - 2.0 ug. strychnine.

Trichloracetic acid (26%) tungstic acid (equal volumes of 10% sodium tungstate and 0.67N sulphuric acid and meta-phosphoric acid (3.5%) were used for precipitating the plasma proteins. To 10 ml. plasma diluted with 5 ml. distilled H_2O, 5 ml. of the protein precipitant were added. After standing for 15 minutes the tubes were centrifuged, and 15 ml. of the clear supernatant fluid were made alkaline and extracted

with 25 ml. benzene. The benzene layer was treated with bromthymol blue and then shaken with N/10 sodium hydroxide. The blanks using the three precipitants were of the same value as that given by the whole plasma (equivalent to 1.0 - 3.0 ug. strychnine).

From the above, it was concluded that the whole plasma and the protein free filtrate gave negligible readings and could safely be ignored when quantities of alkaloid above 50 ug. were in question. For smaller amounts a blank value was required, and was incorporated in the procedure. In actual toxicological analyses the amount of alkaloid present was not likely to be known in advance, but only an arbitrary blank would be possible.

B. Recovery of Strychnine

Plasma containing 5 ug. strychnine per ml. was prepared by diluting 2.5 ml. strychnine standard (0.2 mgm/ml) to 100 ml. with plasma.

Procedure:-

-Shake 10 ml. plasma (made alkaline to pH 8.0 - 9.0) with 25 ml. benzene for 10 minutes. Stand till the two layers separate, decant the benzene layer and centrifuge.

-Transfer 20 ml. of the benzene layer to a tube (30 ml. capacity) containing 0.5 ml. bromthymol blue and shake for 5 minutes. Centrifuge.

TABLE 26.

The Recovery of Strychnine added to Plasma.

µg. Strychnine added	µg. Recovered	% Recovery
50 µg.	49.5 µg.	99.0%
	49.5	99.0
	48.0	96.0
	50.0	100.0
	50.5	101.0
30 µg.	28.0 µg.	93.3%
	29.0	96.6
	24.5	81.6
	28.0	93.3
15 µg.	12.0 µg.	80.0%
	14.0	93.0
	13.5	80.0

Indicator : Bromthymol blue Wave-length : 610 mu.

Solvent : Benzene Instrument : Unicam G.P.

-Use 15 ml. of the benzene layer with 4 ml. N/10 sodium hydroxide for colour production.

-Read the colour intensity at 610 mu.

-Perform a blank of plasma (without strychnine) to set the zero of the instrument.

The method was repeated using 6 and 3 ml. plasma equivalent to 30 and 15 ug. of strychnine.

Results:-

These recoveries of strychnine from plasma were regarded as satisfactory. Although the average percentage of the recovery of 15 ug. was only 87.6% and that of 30 ug. was 91.2%, yet the recovery of 50 ug. was 99.0%.

(iii) ESTIMATION OF STRYCHNINE IN TISSUES

In toxicological analyses one frequently gets tissue specimens (muscle, liver and stomach) for the identification and estimation of the suspected poison. Although the well known Stas-Otto method is a classical method for the extraction of the suspected alkaloid for qualitative work, it is nevertheless unsatisfactory for quantitative estimations because it is tedious and considerable loss of the alkaloid is usually involved. Stewart, Chatterji, and Smith (1937) used trichloracetic acid, followed by the adsorption of the alkaloid on kaolin from which it was eluted with hot chloroform, Daubney and Nickolls (1937-1938) used ammonium sulphate in the presence of acetic acid for the precipitation of proteins and extracting the alkaloid from the protein free filtrate with chloroform.* In the present work the alkaloid was extracted by treating the homogenised tissue with acidulated water (0.5 N hydrochloric acid) and precipitating any dissolved proteins with trichloracetic acid in N. hydrochloric acid. The alkaline protein filtrate was shaken with benzene. The recovery of strychnine from both liver and muscles was studied to find the extent of recovery of known amounts of strychnine added to the tissue by applying this extraction technique and the alkaloid dye compound method.

* Gettler & Sunshine (1951) precipitated the tissue proteins by passing steam through the homogenized liver acidified with tartaric acid.

A. LIVER

Liver brei was prepared by cutting 75 gm. of liver to small pieces, transferred with 75 ml. saline to the container of a high speed macerator. It was macerated for 10 minutes to a paste so fine that it could be sucked with a pipette.

(a) Liver Blank.

10 ml. of liver brei, 10 ml. distilled water were heated in a boiling water bath for 10 minutes. 1 ml. 5N hydrochloric acid* was added and the mixture heated again for another 10 minutes and centrifuged.

Enough** 20% trichloracetic acid in N hydrochloric acid was added to precipitate any proteins dissolved in the extract.

The clear supernatant fluid after centrifuging was used for the blank. It was rendered alkaline and extracted with benzene; the benzene extract, as usual, was treated with bromthymol blue and finally shaken with sodium hydroxide.

The liver blank gave a very faint colour with negligible reading which was equivalent to about 2 ug. strychnine for each 5 gm. of the liver.

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* To bring the concentration of the acid in the mixture to 0.5 N.

** In a control experiment the hydrochloric acid extract was treated with 20% trichloracetic acid drop by drop till there was no apparent precipitation. After centrifuging a drop of the trichloracetic acid was added. This process was repeated till there was no turbidity in the supernatant fluid on the addition of trichloracetic acid.

(b) Recovery of Strychnine

In two sets of tubes 10 ml. of liver brei and 10 ml. distilled water were placed. To one set 0.5 ml. of strychnine standard (0.2 mg./ml.) and 0.25 ml. to the other set were added. To avoid any possibility of the destruction of strychnine with the liver tissue each tube was placed in the boiling water bath immediately after the addition of the strychnine solution.

The tubes were placed in the water bath for 10 minutes, 1 ml. 5N hydrochloric acid was then added and the tubes placed in the water bath for another 10 minutes.

After cooling, the volume of each was made up to 25 ml. with distilled water.

20 ml. of the clear supernatant fluid, after centrifuging, were treated with enough* trichloracetic acid (20%) in N hydrochloric acid (1 ml.) and 4 ml. distilled water. Centrifuged.

10 ml. portions (a duplicate for each) were used for the estimation of the strychnine content. After rendering alkaling, the strychnine was extracted with benzene, the treatment with bromthymol blue was followed by colour production with sodium hydroxide. The colour intensity was measured and the quantity of the recovered strychnine was calculated.

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* See foot-note ***, p. 43.

TABLE 27.

The Recovery of Strychnine added to Liver.

ug. Strychnine added	% Recovery
50 ug.	94.8%
	106.8
	102.5
100 ug.	96.4
	105.2
	100.0
	91.2

Indicator : Bromthymol blue Wave-length : 610 mu.

Solvent : Benzene Instrument : Unicam G.P.

TABLE 27.

The Recovery of Strychnine Added to Muscle Tissue.

ug strychnine added	% Recovery
100 ug	101.5 %
	101.5%
	100 %
50 ug	100 %
	102 %

Results:-

The recovery of strychnine from liver by extracting the alkaloid with 0.5 N hydrochloric acid and precipitating the dissolved proteins with T.C.A. 20% in N hydrochloric acid followed by estimating the strychnine with the alkaloid-dye method was quantitative. The average recovery from 5 g. liver of 50 ug. strychnine was 101.0% and the recovery of 100 ug. was 98.2%.

B. MUSCLE.

The same technique used in the case of liver was followed.

Results:-

The recovery of strychnine added to muscle tissue was quantitative. For 50 ug. strychnine added to 5 gm. of muscle the average recovery was 101.0% and for 100 ug. strychnine added to the same amount of muscle the recovery was 101.0%.

5. Estimation of Strychnine in Very Low Concentrations.

In toxicological practice, minute quantities of alkaloids may sometimes be present in a large volume of urine or plasma, in which case the direct estimation method is not accurate due to the high dilution of the alkaloid in the plasma or urine and the limitation of the volume used in this method (10 ml.).

In this part of the work, concentration methods were studied for application to dilute specimens containing minute amounts of strychnine followed by estimating the alkaloid in the concentrate.

(i) Concentration Methods.

The following concentration methods were studied with a view to selecting the most efficient for the purposes of this work.

A diluted aqueous solution of strychnine sulphate was used to test the efficiency of each method and where efficient was applied to urine, plasma and liver.

A. The Freezing Method.

Principle: If an aqueous solution of a salt be gradually cooled, comparatively pure ice first separates from the solution till the concentration of the salt reaches the saturation. At this stage the saturated solution starts to

TABLE 29.

The Recovery of Strychnine Using the Freezing Method for Concentration

Initial Strychnine Content - 1200 µg.

	Volume of fraction	Strychnine Content	% of Fractional Recovery of Total Strychnine
1.	50 ml.	520 µg.	42.1%
2.	25	212.5	17.3
3.	25	242.5	19.6
4.	25	91	7.3
5.	50	122.5	9.9
6.	25	20	1.6
7.	25	10	0.8
8.	50	12.5	1.0
9.	25	4.5	0.4

Indicator : Bromthymol blue Wave-length : 610 mu.

Solvent : Benzene Instrument : Unicam G.P. 350.

freeze, and the so-called eutetic mixture is obtained. On allowing the solid mixture (pure ice and the eutetic mixture) to melt, a saturated solution of the salt is obtained first, followed by comparatively pure water. In this case, when freezing a solution of strychnine sulphate, and then allowing it to melt, it is supposed that most of the strychnine sulphate would be contained in the first few mls. of the solution collected. Preliminary trials were done to find the minimum volume of solution that would contain practically most of the alkaloidal salt.

(a) Procedure:-

-Place 300 ml. strychnine solution (4 ug/ml) in a centrifuge bottle.

-Centrifuge at a low temperature, -20°C, until the solution solidifies.

-Wrap the bottle with cotton wool*, and allow to melt.

-Collect fractions with the aid of suction.

-Estimate the strychnine content of each fraction.

Conclusion: The extent of concentration by this methid is of no appreciable value as about 96% strychnine was recovered in the first 175 ml. of the 300 ml. of the solution used. This inefficiency was due to mechanical entangling of the strychnine in the ice separated at the beginning of the freezing process.

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* To minimise the effect of atmospheric temperature which causes the rapid melting of the layer adjacent to the wall of the bottle and results in uneven melting.

Another way of applying this technique was:- 100 ml. of the dilute strychnine sulphate solution in a beaker was placed in the ice box of a refrigerator. When there was partial freezing, the unfrozen solution was thoroughly drained into another beaker and the method was repeated once more. The ice was allowed to melt and its strychnine content was estimated. This step was repeated with the other beaker, the strychnine content in the second solid mass and the unfrozen solution was determined.

Results:-

The first	21 ml.	contained	1% of total strychnine
	25 ml.		12.5% " "
	54 ml.		86.5% " "

From this it could be noticed that 86.5% are obtained in 54 ml. out of 100 ml. of the original volume, in which case the concentration is only about twice, which is not satisfactory for the purpose of this work.

B. Dehydration Method.

Principle: Treating an aqueous solution with excess anhydrous sodium sulphate will lead to the concentration of the solution due to the loss of some water which takes part in the formation of decahydrate sodium sulphate.

Procedure: To 100 ml. strychnine solution 4 mg./ml. add 0.5 sulphuric acid 5N, dissolve anhydrous sodium

TABLE 30.

The Recovery of Strychnine Using the Dehydration Method* of Concentration.

Initial Strychnine Content = 400 µg.

	Volume of solution recovered	Strychnine recovered	% recovery	Extent of Concentration
1	33 ml.	385	96.2%	3.0 times
2	30	350	78.5	3.3
3	31	364	91	3.2
4	40	372	93	2.5
5	30	330	82.5	3.3

* Anhydrous Sodium Sulphate was used for dehydration.

Indicator : Bromthymol blue Wave-length : 610 mu.

Solvent : Benzene Instrument : Unicam G.P. 350.

sulphate A.R. by heating at 80°C until a saturated solution is obtained.

-Stand overnight, filter under suction.

-Measure the volume of the solution obtained and estimate its strychnine content.

Conclusion: Although the recovery (82.5 - 96%) was considered good, yet the degree of concentration is still not high enough (about three times) to be satisfactory. This method may be applied to the direct estimation method in plasma to concentrate the plasma protein filtrate and to compensate for the dilution due to the precipitation of proteins.

C. Extraction Method.

Principle: Extraction of the alkaloid by shaking its alkaline solution with an immiscible solvent, collecting the organic solvent and evaporating it was tested. The residue was dissolved in benzene and the alkaloid recovered was estimated.

Procedure:-

-To 100 ml. of strychnine solution 1 ug./ml., add 75 gm. ammonia sulphate*, to adjust the pH to 8.6** .

-Add 50 ml. of the extracting mixture (ether 3 parts, ethanol 1 part). Shake for 10 minutes.

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* To precipitate the protein in case of urine.

** The pH at which the urine blank was colourless.

-Stand until the layers separate, collect the ethereal layer and evaporate it to dryness over a boiling water bath.

-Dissolve the residue in about 15 ml. benzene with the aid of heating, cool and complete the volume to 25 ml. Use 10 ml. for the estimation.

Results:-

Preliminary experiments showed that the recovery of strychnine was very low (about 30%). This might be due to the presence of alcohol in the extracting mixture, in which case part of it passed to the aqueous layer, resulting in binding part of the alkaloid to the aqueous layer.

Another explanation for the low recovery of strychnine using this particular solvent was that the alkaloid is sparingly soluble in ether.

Other extracting mixtures might be used (amyl alcohol 1: chloroform 3) but it was not recommended because:-

(a) The interfering substances in the biological material are freely soluble in such solvents, so they will be extracted together with the alkaloid.

(b) The presence of alcohol resulted in low recovery due to its solution in the aqueous layer. Alcohols were chosen as a main constituent of such extracting solvents because they are excellent solvents for alkaloids and there is no point in using benzene, chloroform or ethylene chloride alone as large volumes are required to extract minute quantities of the alkaloid

contained in a large volume of solution.

(c) As will be seen later another aim of these concentration methods was for application in chromatographic separation of strychnine and brucine on paper. The case of urine, preliminary experiments showed that applying this method to urine resulted in the extraction of the urine pigments, which spread on the paper chromatogram on development, hindering the revelation of the alkaloidal spots.

Chromatographic Adsorption Method.

As chromatography proved to be a useful method for the purification and the separation of the alkaloids, it was tried as a concentration method and it proved to be efficient. The dilute solution of the alkaloid was to be percolated through a column packed with a suitable adsorbent and the alkaloid was recovered from the column by elution with an alkaline strong eluent.

An aqueous solution of strychnine sulphate was percolated through a column of florisil, the strychnine was recovered by alkaline ethanol ammonia mixture. The eluent was evaporated and the strychnine extracted with benzene.

Florisil was chosen because

(a) It is granular 60/100 mesh, so the liquids are easily filtered through it.

(b) It is a strong adsorbent.

(c) It is alkaline in reaction and this helps in the

recovery of the adsorbed alkaloid from the column.

The eluant used was ethanol (a good solvent for the alkaloids) made alkaline with ammonia. Ammonia was chosen because it leaves no residue on evaporation, avoiding a bulky residue using sodium carbonate which might cause some loss of strychnine on extracting the residue with benzene.

Procedure:-

- Pack a column with florisil.
- Wash with 100 ml. ammonia solution 10%.
- Wash with 100 ml. distilled water.
- Percolate 100 ml. strychnine solution.
- Wash with distilled water, followed by 50 ml. of the eluant (10% ammonia in ethanol).
- Evaporate to dryness, dissolve the residue in 25 ml. benzene. Estimate the strychnine recovered.

Results:-

Strychnine used 100 ug.

Strychnine recovered 137 ug, 145 ug., 120 ug.

The recovery of strychnine from the florisil column was high (120% - 145%). Performing a blank, it was found that untreated florisil gave a coloured blank.

Treatment of Florisil to Remove the Interfering Substances.

A blank of the column was performed, using water instead of the strychnine solution; it was found that the blank gave a high reading. As this method was a promising one, it was worthwhile to study it in detail.

A. It was thought that this coloured blank might be caused by interfering substances contained in the florisil.

1. The florisil was refluxed for 3 hours, using a mixture of ethanol 70 parts, acetone 15 parts, ammonia 15 parts. The florisil was then washed freely with water.

A blank was performed but still was coloured.

2. The florisil was treated with concentrated hydrochloric acid followed by washing freely with distilled water to different pH, pH 6.5 - 7.0, 7.6 - 8.0. The blank was still coloured and the adsorption of the strychnine on the column was not quantitative.

B. The eluant was then examined in case it was the cause of the coloured blank.

1. 100 ml. of the eluant (10% ammonia in 90% ethanol), were evaporated, the residue dissolved in benzene, and shaken with bromthymol blue. A coloured blank was obtained. This proved that the eluant was responsible for this coloured blank of the florisil column.

2. In another experiment 100 ml. of ethanol and 10 ml.

of strong ammonia were evaporated separately. Each was tested for the blank and each gave a very faint blank which did not account for the high reading given on evaporating both together.

It was assumed that on evaporating the eluent an amine (ethyl amine) was formed which reacts with bromthymol blue, to which the coloured blank was attributed.

$$C_2H_5OH + NH_3 = C_2H_5NH_2 + H_2O$$

To justify this assumption 100 ml. of the eluent was evaporated to dryness, dissolved in 5 ml. hydrochloric acid N and tested for the amines by diazotization and coupling*.

A positive reaction was obtained supporting the assumption of the amine formation.

3. To prevent amine formation, neutralisation of ammonia before the evaporation process was suggested.

5N hydrochloric acid - enough to neutralise the ammonia present in the eluent -- was added. The residue was dissolved in 10 ml. water, transferred to a glass stoppered bottle, sodium hydroxide added till alkaline, and then shaken with benzene. The blank was colourless, but when the benzene extract was shaken with bromthymol blue, the latter acquired a strongly alkaline colour. This may be attributed to the

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* The residue was dissolved in o.5 ml N hydrochloric acid , 0.5 ml of .5% aqueous solution of sodium nitrite was added and the mixture was cooled in ice. Few drops of 2% urea was added to destroy the excess nitrite present. After standing for two minutes .5 ml of dimetyl-a-naphthylamine (.4 % in 96 % alcohol) was added. A red violet colour was developed.

solution of ammonia evolved on the addition of sodium hydroxide to the solution of the residue. To eliminate this source of doubt, the residue obtained on evaporation of the eluant was treated with strong alkali and heated on a boiling water bath, until no ammonia was evolved. This solution was then shaken with benzene and tested for the blank.

The blank was colourless.

4. Another alkaline eluant was tried, when the ammonia was replaced with sodium carbonate, (ethanol 175 ml. - distilled water 65 ml., 10% sodium carbonate 10 ml.). The florisil blank, using this eluant, was colourless.

Using this eluant was only satisfactory to determine the extent of the strychnine recovery from the florisil column using chromatographic concentration method. For the chromatographic separation on paper of the alkaloids (strychnine and brucine) this eluant was not satisfactory because, on evaporating the eluant, a residue of sodium carbonate was left which will interfere with the chromatographic separation.

(a) Following the concentration of the alkaloidal solution on florisil volume, the residue was to be dissolved in a small volume of a suitable solvent (1 ml. ethanol), in which case the sodium carbonate residue will affect the volume of the alcohol recovered for application on the paper strip.
(b) On applying the alcoholic solution of the residue the spot would be loaded with sodium carbonate, which might affect the chromatographic separation.

The sodium carbonate ethanol mixture was found to be suitable for the purpose as an eluant because it gave a colourless florisil blank and was alkaline enough to suit the favourable conditions of elution.

The other point to be investigated was the pH at which the strychnine was quantitatively adsorbed on florisil. Solutions of strychnine (50 ml. 2 ug./ml.) adjusted to different pH values were percolated through florisil columns. The percolated solutions were made alkaline and extracted with 30 ml. benzene. The benzene extract was treated with bromthymol blue and 25 ml. of the benzene layer were shaken with 2 ml. N/10 NaOH. Any blue colour in the aqueous layer indicated incomplete adsorption.

Preliminary experiments showed that adsorption of strychnine from strongly acidic solutions pH 1 - 2, and alkaline solution pH 7.0-7.5 was incomplete. Quantitative adsorption of strychnine on florisil was from solutions of weakly acidic pH 5 - 6 or alkaline 6.5 - 7 reaction.

Concentration of Dilute Solutions of Strychnine by the Adsorption Technique.

Procedure:-

1. Pack a column with florisil, wash with 100 ml. ammonia 10%, followed by 200 ml. distilled water.

2. Percolate the strychnine solution (100 ml. - 1 ug./ml.) Adjust to pH 5.-6.

3. Wash with 50 ml. distilled water; when the water is only 0.5 cm. at the top of the column, add 6 ml. sodium carbonate solution 5% to furnish a strong alkaline medium for the elution of the alkaloid.

4. Elute with 75 ml. of the alkaline eluent (ethanol 75: distilled water 20 : sodium carbonate solution 5% 5).

5. In the flask used for collecting the eluant was placed 2 ml. 5N hydrochloric acid to remove the carbonate and render the medium acid to avoid any possibility of destruction of strychnine on evaporating the eluant, which might take place in an alkaline medium.

6. Dissolve the residue in 10 ml. H_2O.

7. Estimate the strychnine recovered in a 5 ml. portion.

Results:

In three experiments using 100 ml. strychnine solution containing 1 ug. alkaloid/ml. solution, the recovery was 95.6% - 97.0% and 101.5%, which was considered quantitative.

By this method a high degree of concentration of very dilute solutions could be achieved, where the quantity of the alkaloid present was 50-100 ug., irrespective of the volume, since the sensitivity of the method does not depend on the volume of the solution used.

Concentration of Urine by Adsorption Technique.

The concentration method was applied to urine to find the extent of recovery of known amounts of strychnine added to the urine. Urine containing 0.5 ug./ml. was used.

Procedure:

- Pack a column of florisil; place a cotton wad at the top of the florisil as a filter to any solid particles present in the urine. Wash it with 100 ml. 10% ammonia, followed with 200 ml. distilled water.

- Percolate 250 ml. of the urine through the column after adjusting the pH of the urine to pH 5.0 - 6.0.

- To remove the adsorbed urine pigments, wash the column with 100 ml. 0.5% ammonia, 100 ml. distilled water, followed by 50 ml. of 30% acetone. All the urine pigments were washed out without disturbing the alkaloid on the column.

- Add 6 ml. of 5% sodium carbonate to the column and elute with 50 ml. of the eluant.

- Add 2 ml. 5N hydrochloric acid to the collected eluant to render it acidic, evaporate to dryness over a water bath.

- Dissolve the residue in 10 ml. distilled water, use 4 ml. for the estimation of the strychnine recovered. Adjust the pH to 8.0 and extract with 25 ml. benzene.

Results:

A blank was performed and found to be colourless. In three experiments the recovery of strychnine was 95.0% - 90.0% - 92.0%, with a mean value of 92.3%.

Concentration of Plasma on Florisil.

In the original method of the estimation of strychnine in plasma 10 ml. plasma were used and the strychnine was extracted with 25 ml. benzene. The use of more plasma requires larger volumes of benzene for extraction, which renders the technique tedious and inaccurate, especially when strychnine is found in a low concentration (1 - 2 ug./ml.).

The concentration of plasma on florisil was suggested. As the protein content of plasma is high, the plasma was diluted five times with distilled water. The pH was adjusted to pH 5 - 6* and percolated through a florisil column. The column was washed with 100 ml. water. 6 ml. sodium carbonate 5% were added to the top of the column and elution with 75 ml. sodium carbonate-ethanol proceeded.

After evaporation the residue** was dissolved in 10 ml. N/10 hydrochloric acid and the strychnine recovered was estimated in 4 ml. portions.

Results:-

It was found that the recovery of 100 ug. strychnine in 50 ml. plasma was not quantitative (80%) at an acid pH 5 - 6. With plasma at pH 8.0 - 9.0, it was found that the recovery in two experiments was 94.0%, 92.5%.

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* the pH found suitable for aqueous solutions and urine.

** a flaculent residue was left after evaporation due to adsorption of a part of plasma proteins on the column which was eluted with the alkaline eluant.

Concentration of Liver Extract Containing Strychnine on Florisil.

The adsorption of strychnine from plasma on florisil was complete from diluted plasma without the precipitation of the proteins.

In this method the strychnine was extracted from liver brei with diluted hydrochloric acid and the strychnine adsorbed on florisil. Preliminary experiments showed that strychnine was quantitatively* adsorbed on florisil at pH 7.0 - 8.5.

Technique:-

50 gm. liver were homogenised with 100 ml. saline to a fine paste and transferred to a round bottom flask and 1 ml. strychnine solution (0.1 mgm./ml.) was added. The flask was placed in a boiling water bath for 5 minutes. 50 ml. of 2N hydrochloric acid were added and the mixture heated for another 10 minutes and allowed to cool. It was transferred to 250 volumetric flask and completed to the mark with distilled water. Filter, use suction if necessary.

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* The liver extract after percolating through florisil was collected, made alkaline and shaken with 30 ml. benzene. The benzene layer after centrifuging was treated with 1 ml. brom-thymol blue solution. 25 ml. of the benzene extract was treated with 2 ml. sodium hydroxide solution N/10. The aqueous layer was colourless indicating that there was no strychnine present in the liver extract after percolation through the florisil column.

The adsorbed strychnine was eluted from the column with 6 ml. sodium carbonate solution 5%, followed by 75 ml. ethanol-carbonate mixture*

The eulted solution was made acidic with hydrochloric acid and evaporated to dryness on a water bath. The residue was dissolved in 20 ml. N/10 hydrochloric acid and the strychnine recovered was estimated in 10 ml. portions.

Results:-

The recovery of 100 ug. strychnine present in 50 gm. liver in triplicate was 92.5%, 94.7% and 96%.

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* See page 57.

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PART III :

CHROMATOGRAPHIC SEPARATION OF CLOSELY RELATED ALKALOIDS.

Chromatographic Separation of Strychnine and Brucine.

Introduction.

A. Strychnine and Brucine are commonly present together in nature. They occur most abundantly in nux vomica seeds (strychnos nux vomica L.) and ignatus beans (S. ignatii Berg.). Both drugs contain from 2 to 3 per cent of alkaloids of which about one half is strychnine in the former, and two thirds in the latter, the rest being mainly brucine. Other species of strychnos are S. lucida, the seeds containing .84% strychnine and 1.5% brucine; S. lignstina B. contains 0.6% to 1.5%, nearly all brucine, while S. Tieute Lesch seeds contain mainly strychnine with traces of brucine.

Naturally occurring mixtures of these alkaloids, mainly tincture nux vomica are used in medicine. The powdered seeds of nux vomica and ignatus beans are used for homicidal purposes. In toxicological analyses these two alkaloids might be found together.

Although strychnine and brucine behave differently with qualitative tests, yet they both respond to the alkaloid dye compound formation method.

El Ridy and Khalifa (1952) overcame the difficulty of the undesired company of brucine by its removal from solution through its destruction with potassium persulphate at 60 - 70°C. and strychnine then directly estimated by ultraviolet spectro-

photometry. Biggs (1952) and Demoen & Jenssen (1952) estimated the presence of both strychnine and brucine spectrophotometrically.

In this part of the work, chromatographic separation of strychnine from brucine was studied to enable the estimation of each separately, using the alkaloid dye compound method. Strychnine-brucine chromatographic separation was studied in both forms of

(a) the alkaloid dye compounds,

(b) the free alkaloidal bases.

B. Chromatographic Separation of Alkaloids.

A vast amount of work has been done in the field of chromatographic separation of alkaloids.

Adsorption chromatography was the first branch that received particular attention. Alumina was the adsorbent generally used and the solvents were chloroform, benzene, and ethanol.

Alumina and chloroform were chosen for the separation of the senecio alkaloids (Adams & Govindachari, 1949), erythrina alkaloids (Folkers & Shavel, 1942) and for the purification of colchicine (Ashley & Harris, 1944). The recovery of the alkaloids from the column was by elution with chloroform-ethanol mixtures. Jacobs and Craig (1941) used

benzene instead of chloroform for the separation of the alkaloids of aconite on alumina. Stewart and Stolman (1949) have separated morphine alkaloids on florisil by changing the pH conditions of elution.

Chromatographic separation of alkaloids by partition chromatography has also been investigated. Evans and Partridge (1948-1949-1950) used Keisulguhr wetted with 0.2 ml. phosphate buffer and ether as the eluant for a series of studies in the chromatographic separation of the solanaceous group, the alkaloids of Datura Stramonium (1948) and Atropa Belladonna and the Datura Ferox (1949). Chilton and Partridge (1950) used the same adsorbent-eluant combination for the separation of Punica Granatum alkaloids. Jensen and Svendsen (1950) separated strychnine and brucine on keisulguhr wetted with phosphate buffer pH 7.0, chloroform-ethanol mixture as stationary phase, and the mobile phase was ether saturated with the buffer.

Silica Gel moistened with N/15 phosphate buffer pH 6.8 was used by Tappi (1950) for the chromatographic separation of strychnine and genostrychnine, developing the chromatogram with chloroform.

All the above methods are useful when appreciable amounts of the alkaloids, 25 mgm. upwards, are dealt with.

A comprehensive investigation of paper chromatography of alkaloids was published by Munier & Macheboeuf in a series of studies (1949-1951), and reviewed in one article (1952). They

found a relationship between the form of the spot on the paper chromatogram and the pK solubility of the alkaloid and the pH of the solvent. They experienced good separation of the alkaloids in acid medium. Hydrochloric and acetic acids were recommended. R_F values of different alkaloids using these acids were given by Munier et al. They also got good separation of alkaloids on paper impregnated with KH_2PO_4. Careless and Woodhead (1951) used buffered filter paper (M/15 phosphate or citrate), using the ascending technique for the development of the chromatograms. Drey and Foster (1953) used phosphate buffer pH 7.2 for impregnating the paper in the course of separating the solanaceous alkaloids. Schute (1951) separated atropine alkaloids in an alkaline medium, using butanol saturated with 5% ammonia.

Butanol is a classical solvent used in paper chromatography. Butanol saturated with water was used for the development of impregnated paper while untreated paper was developed with butanol acidified with hydrochloric acid or acetic acid.

Lussman et al. (1951) used cyclohexanol: hydrochloric acid mixture for the paper chromatography of the cinchona alkaloids.

Paper chromatography is superior to column (adsorption or partition) chromatography because:

A). the alkaloid is very easily located on the paper chromatogram and never escapes detection, (if present in a concentration above 20 ug.), because there is no chance for it to leave the chromatogram as it might in the case with columns during development, especially when such minute amounts (20 ug) are used.

B). Column chromatography requires large amounts of eluants for the recovery of the alkaloid from the column, while in paper chromatography elution of the alkaloidal spot can easily be accomplished by using 3-5 ml. N/10 Hydrochloric acid.

C). it permits the use of minute quantities of alkaloids ranging between 15 - 100 ug.

Strychnine and brucine chromatographic separation was studied in both forms, e.g.:

(a) separation of the alkaloid dye compounds.

(b) separation of the free alkaloidal bases.

An Attempt of Chromatographic Separation of Strychnine and Brucine as their Alkaloid Bromthymol Compounds

Experimental

(a) Adsorption Chromatography.

Of the well-known adsorbents used in adsorption chromatography, florisil, alumina, floridin-X were tried. The first two have alkaline reaction that decomposed the complexes when the solvent contained any trace of water. (The pH at which the complexes are stable is pH 6.5 - 7.0)*. This alkalinity was removed by treating the adsorbents with concentrated hydrochloric acid in the first, and diluted in the latter, followed by washing with ample amount of distilled water till the washings were neutral and then drying in an air oven at 110°C.

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* The pH of decomposition of the compounds were found by shaking an aliquot of benzene solution of the compounds with a few mls. of phosphate buffer at different pH range 5.5 - 8.6. Centrifuge. Note the colour of the aqueous layers; any blue tint indicated decomposition of the compound.
It was found that the compounds are stable at pH 6.5-7.0.

A. The adsorbents were tested for suitability in the following way:

(a) solutions of strychnine-bromthymol blue compound in the different solvents (mentioned below), were prepared as follows.

A solution of the compound 50 ug./ml. in benzene was prepared. In each of a series of beakers place 2 ml. of the solution, evaporated to dryness. The residue was dissolved in 50 ml. of each of the following.

acetone, acetone 3 : benzene 1, benzene
benzene 3 : ethylene chloride 1, ethylene chloride,
ethylene chloride 3 : butanol 1, butanol,
ethylene chloride 3 : ethanol 1, ethanol,
ethanol 3 : water 1.

(b) The columns were packed by the wet method, using the different solvents.

(c) The effluent solvent in each case was shaken with 10 ml. 0.1 N. sodium hydroxide. Any blue coloration in the aqueous layer indicated incomplete adsorption and the adsorbent or solvent to be excluded.

Adsorption from benzene and ethylene chloride and their mixture was quantitative with all the adsorbents used. From the other solvents no adsorption of the compounds took place.

B. <u>The development of the chromatograms</u>.

From the previous observations acetone was suggested as

a developing agent. Florisil was chosen as a good representative of the above mentioned adsorbents, and benzene was chosen as a solvent. A group of three columns were prepared on which strychnine/bromthymol blue compound, brucine compound and a mixture of both, were adsorbed.

Using up to 50% acetone in benzene no movement of the bands was noticed, but on using 60% acetone in benzene the bands moved with the same rate without any separation in the column containing the mixture of strychnine and brucine compounds.

Other developing mixtures were tried with no success. These were:

Butanol - Benzene,	Butanol,
Ethanol - Benzene,	Ethanol.

Celite, as will be seen later, was found to adsorb the addition compounds quantitatively from benzene, and as celite is a weak adsorbent it was worthwhile to repeat the work done on florisil, hoping to get separation.

Using 2% acetone in benzene the bands moved at the same rate without any separation.

C. Conclusion.

There was no separation of the alkaloid dye addition compound of strychnine and brucine, using the adsorption chromatography. This, most probably, is due to the difficulty already experienced in separating strychnine and brucine by adsorption chromatography because of their close resemblance

in molecular structure, and which is rendered more difficult on adding a bromothymol blue molecule to each, thus making the molecular structure similarity critical.

The separation of the alkaloids as their dye compounds may be possible with alkaloids of different groups and not so closely related as strychnine and brucine.

TABLE 31.

The Partition Coefficient of Strychnine Complex.

% Ethanol	Strychnine		Partition Coefficient	
	Benzene	Ethanol	Benzene	Ethanol
70%	...	102		
60	...	102		
50	1.2	100.8	1 :	84
45	3.5	98.5	1 :	28
40	15	87	1 :	6.4
20	52	50	1 :	1
5	81	21	4 :	1
40	17	115	1 :	6.6
42.5	8.4	123.6	1 :	14
45	4.8	127	1 :	26.5

TABLE 32.

The Partition Coefficient of Brucine Complex

% Ethanol	Strychnine		Partition Coeff	
	Benzene	Ethanol	Benzene	Eth
70%	...	95		
60	...	95		
50	...	95		
45	1.2	93.8	1 :	
40	6.3	88.7	1 :	
20	51	44	1.2 :	
5	68	27	2.5 :	
40	7.2	120.8	1 :	
42.5	3.6	124.4	1 :	
45	1.7	126.3	1 :	

(ii) PARTITION CHROMATOGRAPHY.

A. Column Partition Chromatography.

As adsorption chromatography failed to give any separation we had to try partition chromatography, hoping to get satisfactory separation.

A good starting point which is of value in partition chromatography is the partition coefficients of the substances between the solvents intended to be used; the mobile phase, benzene, the stationary phase, ethanol-water mixture, was suggested.

(a) Determination of the partition coefficients of strychnine and brucine compounds with bromthymol blue between benzene and ethanol of different concentrations.

- Shake equal volumes of benzene and ethanol of different concentrations (from 5% - absolute) in a separating funnel. Leave to separate. This is to attain equilibrium between the two solvents.

- In two sets of tubes (A and B) place 9 ml. of the benzene and 10 ml. of the alcohol.

- To each tube of group A add 1 ml. of strychnine bromthymol blue compound in benzene (of known concentration); to the tubes of group B add 1 ml. of brucine bromthymol blue complex.

- Shake the tubes for 10 minutes. Centrifuge.

- Estimate the concentration of each alkaloid in the benzene.

Results:-

1. Ethanol concentrations higher than 70% were found to be completely miscible with benzene.

2. 40% ethanol was a suitable concentration with partition coefficients favourable for partition chromatography.

Packing the Columns.

Celite* was used as the supporting substance, 40% ethanol as the stationary phase and benzene as the mobile phase. A certain weight of celite was mixed thoroughly with 40% ethanol 0.85 ml./gm. celite, and enough benzene to form a slurry was added. The column was packed with the aid of a Martin Packer. The efficiency of the column was tested with Sudan III.

0.2 ml. benzene solution of the alkaloid-dye compound (1 mgm./ml.) was added to the column from a micro pipette and the column was developed with 100 ml. benzene previously equilibrated with 40% ethanol.

B. Recovery.

The recovery of strychnine-bromthymol and brucine-bromthymol blue compounds from the celite column was investigated. In preliminary experiments the recovery of brucine compound was found to be 40.5% and in the case of strychnine compound

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* Celite was first treated with concentrated hydrochloric acid (to remove the alkaline impurities) for 24 hours, then washed with distilled water till the washings were neutral, dried in an air oven at 110°C. and kept in a dessicator.

the recovery was 42.5%. This poor recovery was due to partial adsorption of the compound on celite. In the latter column (strychnine bromthymol blue the recovery of which was 42.5%) 0.2 ml. of benzene solution of brucine compound was added to the top of the column, followed by development of the chromatogram with benzene saturated with 40% ethanol; the recovery was 82.0%. This comparatively high recovery of brucine compound might be mainly due to the previous saturation of celite with the strychnine compound.

Loading the celite with an alkaloid dye compound (quinine-methyl orange) before packing the column might result in good recovery. Methyl orange was chosen because its alkaline colour, (sodium hydroxide is used for the colour production in the original method for breaking down the strychnine and brucine bromthymol blue compounds) yellow, which would have little interference with the blue colour of bromthymol should the quinine-methyl orange compound escape from the column during the development of the chromatogram. Celite after being treated with the proper amount of 40% ethanol was covered with a solution of quinine-methyl orange in benzene. The excess of benzene was then filtered, the celite was washed with benzene, and packed as usual. The column was washed with 50 ml. benzene (saturated with 40% ethanol) and the washings tested for quinine-methyl orange by shaking with N/10 hydrochloric acid. No red colour was produced, indicating that the compound was not eluted.

0.2 ml. of strychnine bromthymol blue was added to the top of the column, followed by development of chromatogram. The recovery was still poor and was about 35%.

Another supporting substance, starch, was tested for its adsorptive power of the compounds; it was found that starch adsorbed the compounds from benzene, so it was not suitable for partition chromatography of the alkaloid-dye compounds.

(ii) Qualitative Paper Chromatography.

The conditions of paper chromatography of alkaloids were studied to achieve the suitable separation of strychnine and brucine.

A. Technique.

Of the many techniques devised for paper chromatography the following were chosen:

(a) Descending chromatography, Consdon, Gordon & Martin, 1944.

(b) Horizontal chromatography, Meredith and Sammons, 1952.

(c) Ascending chromatography, Williams and Kirby, 1948.

(d) Bridge method. Kawerau, 1951.

Preliminary work using the above mentioned techniques, for the separation of strychnine and brucine on paper with butanol 100, hydrochloric acid 15, as a developing solvent, showed that:-

1. The rate of flow of the solvent was the fastest in the descending technique because the liquid travelled downwards with the gravity, the slowest rate of flow was that of the ascending method as it was against gravity. With the horizontal technique the rate of flow is in between the ascending and descending techniques.

2. The spots were compact and well defined with the ascending technique, while the descending and horizontal techniques gave diffused spots. The Bridge method gave spots as good as those obtained with the ascending technique.

The ascending technique was the one used in this study because it had advantages over the others, such as consistency of results, simplicity of apparatus and the ease with which a large number of analyses could be done at one time. Moreover the spots obtained with this technique were compact with well defined margin. The bridge unit was also satisfactory, but the only disadvantage of it was that only one strip 2 cm. could be run at one time.

B. Paper and Solvents.

The paper used was Whatmann No. 1 and No. 4; the latter was more suitable for the ascending method due to the quicker flow of the solvent along the strip.

Munier experienced good separation of alkaloids on paper in acid medium. Butanol acidified with hydrochloric acid or acetic acid was used for the development of the chromatograms.

Butanol 15, hydrochloric acid and Butanol 14, acetic acid mixtures saturated with water were used for preliminary study of the separation of strychnine and brucine on paper. Saturation of the solvent with water was done either by adding the distilled water to the acidified butanol drop by drop with constant shaking till permanent turbidity was obtained, or by shaking the butanol mixture with an equal volume of distilled water. In both cases the mixtures were allowed to stand overnight for complete separation of the layers, as the butanol

layer must be completely free from any water droplets, the presence of which upsets the equilibrium on the paper during the development of the chromatogram. With both solvents good separation of strychnine and brucine was obtained, the only drawback was that the spots were close to each other - 0.5 - 0.7 cm. between the two spots.

Butanol : HCl	R_F strychnine = 0.86	R_F brucine = 0.70
Butanol : Acetic Acid	R_F strychnine = 0.68	R_F brucine = 0.52

Isobutanol was used instead of butanol with practically the same results being obtained.

The acidic medium required for the separation of alkaloids and paper was achieved by impregnating the paper strips with organic acids and acidic inorganic salt, the development of the chromatograms with butanol saturated with water. Saturations of M/4 strength of tartaric acid, oxalic acid, citric and boric acid - potassium and sodium dihydrogen phosphate were used for the impregnation of the paper.

All the organic acids used, except boric acid gave unsatisfactory separations and due to tailing and diffusion of the spots. With boric acid the separation was fairly good except that the spots were slightly diffused. Replacing neutral butanol with butanol 100 : water 100 : Hydrochloric acid 100, good separation was obtained.

The best separation was given with paper impregnated with both potassium dihydrogen phosphate and sodium dihydrogen

phosphate.

R_F strychnine $=0.515$	R_F strychnine $=0.434$
M/4 KH_2PO_4	NaH_2PO_4
R_F brucine $=0.346$	R_F brucine $=0.260$

C. Spraying Reagents.

In revealing the positions of the spots due to alkaloids on the paper chromatograms after development and drying, several agents were used.

1. The most important is a group of reagents containing mainly iodine.

(i) Iodine vapour: Exposing the sheet of paper to iodine vapours the alkaloidal spots turn brown, and on removing the paper from the atmosphere of the iodine, the alkaloidal spots vanish again. This method is tedious and is now replaced by other agents containing iodine either in the simple or complex forms.

(ii) Iodine solution in potassium iodine: Different concentrations of iodine in Kl solution were used by a number of investigators. Munier (1949) used .06% I in .6% KI solution and found it useful for the detection of most of the alkaloids, especially mescaline, hordennine and ephiderine (spots containing less than 10 ug.). Detecting the atropine alkaloids on paper

chromatograms Brindle et al. (1951) used a higher concentration of iodine (.5%). It detected solanaceous alkaloids in concentrations greater than 20 ug. One of the merits of this reagent was that it gave colours with the various alkaloids, the hyoscine was coloured brown, while the atropine was blue grey.

Gore and Adshead (1952) used a concentration of iodine (.2% w/v) and was found to be just sufficiently strong to give a good reaction with the alkaloids without producing a more than faint transient staining of the rest of the paper. They stated that the character and the application of the spray is critical and recommended the use of an atomising device which could be operated by a sustained force of compressed air. They also found that relative rates at which the stains from the various alkaloids faded could be reproduced. The stains from the solanaceous alkaloids fade rapidly, particularly the hyoscine stain, whilst the quinine, strychnine and brucine stains are very persistent.

3. Pot. iodoplatinate: this reagent was recommended by Munier (1949) for revealing spots due to yohimbine, epidenine, hydrastine, trigonelline, hordenine and nicotinamide in quantities from 10 to 20 ug. It was also used by Zaffaroni (1949) and Burton and Kentman for spraying chromatograms of cinchona alkaloids to show the spots due to cinchonine and cinchonidine (which are not fluorescent).

4. Modified Braggendorff reagents: this is the most sensitive member of this group, two solutions were prepared. Solution A

contained .85 gram of bismuth subnitrate, 40 ml. distilled water and 10 ml. glacial acetic acid. Solution B contained 8 gram pot. iodide and 20 ml. distilled water. To prepare the spraying reagent, 5 ml. of A and 5 ml. of B are mixed with 20 ml. acetic acid and a 100 ml. water. The alkaloids form red spots on a pale orange background.

5. Modified Nessler Reagent: gave satisfactory results and as high sensitivity as that of Draggendorff reagent.

Solution A: in a heavy walled flask are mixed 5 ml. of distilled water, 7.5 gm. pot iodide and 5.5 gm. iodine. To this solution add 7.5 gm. of mercury, drop by drop, with shaking over a period of 15 minutes. It is well shaken till the red colour of the solution begins to fade. Cool under running water with shaking until the colour changes green. The supernatant liquid is decanted and the remaining mercury is washed several times with distilled water, the washings being added to the supernatant liquid. Make up the volume to 100 ml.

Solution B: 25% KI solution. The spraying solution was prepared by mixing 15 ml. of solution A to 10 ml. solution B and 20 ml. acetic acid, making up the volume to 100 ml. Spots due to 5 ug. of strychnine, brucine, morphine, heroin, cocaine, atropine and hyoscine gave orange red spots on a pale yellow background.

6. Phosphomolybdic acid Reagent: Phosphomolybdic acid 2.5 gm;
acetic acid 3 drops
Dist. water to 100 ml.

The paper was dipped in the solution for one minute followed by

thorough washing with distilled water and then dipped in a solution of stannous chloride 1 in 10% acetic acid. The alkaloidal spots appeared blue. This reagent was tried for use in the case of the rough determination of ratio between strychnine and brucine in mixture. (see page 80).

(b) 1% pot. permanganate in N H_2SO_4 was of value as a spraying reagent in the case of strychnine, brucine, morphine, heroin, cocaine, procaine, atropine and hyoscine. The alkaloids appeared as pale yellow spots on a pink background.

(iii) Quantitative Paper Chromatography.

After obtaining a satisfactory separation of brucine and strychnine on paper strips, using Bc 15, we had to find a technique for the estimation of the separated alkaloids.

The methods usually applied for quantitative purposes in the field of paper chromatography are:

1. Measurement of spot area. This is an inaccurate method for there are some sources of error, mainly the determination of the periphery of the spots. Standard spots of the same diameter gave spots (after development of the chromatogram) not of the same area.

2. Estimation by measurement of colour density. This method was tried for the determination of the ratio between the strychnine and brucine in the mixture. The colour intensity of the spots was measured by scanning. This method gave only a very rough estimate of the proportional amount of the two alkaloids.

3. Microchemical estimation of eluted substances. In this method the chromatographed alkaloids are eluted from the paper and determined by bromthymol blue.

(a) Known quantities of strychnine were applied to the paper strips, the spots were rendered visible by spraying the strips with the spraying reagent and left to dry. The areas containing the spots (2 x 2 cm.) were cut out separately, each

placed in a tube containing 2 ml. N. sodium hydroxide (sodium hydroxide breaks down the alkaloid-iodine complex, setting the alkaloid free). Add 10 ml. benzene, shake for 10 minutes, centrifuge. Decant the benzene layer into another clean tube containing 0.5 ml. of bromthymol blue, shake for 5 minutes, centrifuge. Use 6 ml. of the benzene layer for colour production.

Applying 50 ug. strychnine to paper strips the recovery in a number of experiments was 27.5 ug., 30 ug., 40 ug., 40 ug., 33.5 ug., and 42.5 ug. The recovery of strychnine was incomplete, which might be due to partial adsorption of the alkaloid on the paper.

(b) To minimise the adsorption of the alkaloid on the paper the addition of a strong eluant to benzene was tried. Ethyl acetate was recommended but unfortunately it gave a very high blank value with bromthymol blue.

(c) Another attempt was made using ethylene chloride instead of benzene and metanil yellow as the dye.

The recovery was still not quantitative although the readings are reproducible. Addition of ethyl acetate did not improve the recovery.

In such cases it is recommended to construct a special curve for the recovery of the alkaloid from paper by using paper strips loaded with known amounts of the alkaloid.

TABLE ___.

The Recovery of Strychnine and Brucine after Separation by Paper Chromatography.

Strychnine		Brucine	
µg. added	µg. recovery	µg. added	µg. recovery
(i) Applied SEPARATELY			
30 µg.	29 µg.	30 µg.	30 µg.
	27.5		30
40 µg.	40	50 µg.	49.5
	39.5		49.5
(ii) Applied in MIXTURE			
30 µg.	29 µg.	30 µg.	29 µg.
	25		30
	31		31.5
40 µg.	40	40 µg.	37.5
	41.5		37.5
	40.5		39

The Recovery of Strychnine and Brucine from paper.

Strychnine		Brucine	
Standard Readings	Recovered	Standard Readings	Recovered
0.680	0.680	0.580	0.570
0.700	0.680	0.580	0.570
0.700	0.650	0.590	0.585
	0.690		0.585
	0.690		0.590

Ug. of Strychnine : 50 ug.

ug. of Brucine : 50 ug.

Eluent : N/10 Hydrochloric acid

Solvent : Benzene

Dye : Bromthymol Blue

Instrument: Unicam Spectrophotometer G.P. 350.

Wavelength: 610 mu.

B. Another method of elution was tried which proved to be satisfactory and reliable.

0.050 ml. portions of strychnine in benzene (1 mgm./ml.) accurately measured from a micropipette, were applied on paper strips. They were sprayed and left to dry. The areas of paper strips containing the spots were placed in tubes each containing 4 ml. N hydrochloric acid and heated in a boiling water bath for 10 minutes. Stand for 6 hours. Squeeze any fluid off the piece of paper, wash with distilled water and squeeze again. Add enough 5N sodium hydroxide to make the reaction strongly alkaline, and follow with 10 ml. benzene, shake and centrifuge.

The standards were prepared as follows:- Exactly 0.05 ml. of the strychnine solution in benzene was placed in tubes containing 4 ml. hydrochloric acid. Heated in a water bath to evaporate the benzene. To have uniform conditions pieces of paper 2 x 2 cm. were dropped in each tube. Proceed as given above.

Results:-

The recovery of strychnine and brucine by this method is quantitative and reliable and this is the method to be chosen as the most suitable for our purpose.

Recovery:-

The recovery of strychnine and brucine from paper in the above steps performed after applying the benzene solution of the alkaloid on paper and then estimating the alkaloid without any development.

In this step known amounts of strychnine and brucine separately and in mixture were applied to paper strips and

developed with butanol 85 parts, hydrochloric acid 15 parts, saturated with water.

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PART IV :

THE TECHNIQUE FINALLY EVOLVED FOR THE ESTIMATION OF STHYCHNINE AND BRUCINE IN MIXTURE.

The Technique Finally Evolved for the Estimation of Mixtures of Strychnine and Brucine found in Very Low Concentrations.

In the previous part the determination of strychnine was applicable only when the alkaloid (strychnine) was present alone. In this part, the method was extended to the estimation of mixtures of closely related alkaloids found in very low concentration. Strychnine and brucine were used in this method because they are closely related to each other. The method comprised the following three steps:-

A. The concentration of the alkaloids on florisil. This step was modified to meet the new conditions of this technique.
B. The chromatographic separation of the alkaloids on paper.
C. Quantitative estimation of the separated alkaloids.

Estimation of Strychnine and Brucine in Mixtures Aqueous Solution.

The chromatographic concentration of the dilute solutions of the mixture was the same as that used for the estimation of strychnine (Part II, Chapter 6, page 57). For this particular technique the ethanol-sodium carbonate mixture used for elution of strychnine from the florisil column was unsuitable because on evaporation a bulky residue of sodium carbonate was left which would interfere with the next step. After the evaporation of the eluent the residue was to be dissolved in 1 ml. ethanol 96% in which case the sodium carbonate residue would affect the volume of the alcohol recovered for application to the paper.

In this respect the ammoniacal ethanol eluent was superior to the sodium carbonate one, as the former left hardly any residue on evaporation. The only disadvantage of the ammoniacal ethanol was the formation of ethylamine (page 54), which would not interfere in this technique as it has a different R_F value from that of strychnine and brucine.

R_F ethylamine = about .43
R_F strychnine = .69
R_F brucine = .52

Solvent: butanol 86
acetic acid I4
Paper : Whatman No 4

Procedure:-

A. The Concentration of the dilute solution:-

1. Pack a column with florisil (about 10 cm. long), wash with 100 ml. concentrated ammonia followed by 200 ml. distilled water. Percolate 200 ml. strychnine-brucine solution (containing 100 micrograms of each and wash the column with 100 ml. distilled water.

2. When the water is only 0.5 cm. from the top of the column add 5 ml. of concentrated ammonia to furnish a strong alkaline medium for the elution of the adsorbed alkaloids. Elute with 75 ml. of the alkaline eluent (ethanol 80 parts: ammonia 15: water 5). Evaporate the eluent on a boiling water bath to dryness.

3. Dissolve the residue in 1 ml. 96% ethanol.

B. The Chromatographic Separation of the Concentrated Alkaloids.

4. Paper strips 8 cm. x 60 cm. impregnated with M/4 sodium dihydrogen phosphate were used. 0.4 ml. of the alcoholic solution of the residue was applied along a line 4 cm. from one edge of the

paper strip. The alcohol was allowed to dry before each addition, drying of the alcohol was helped by holding the strip over a hot plate. Duplicate strips were prepared.

5. Expose the paper strips to steam for 5 minutes and hang them in the chromatographic tank (containing the solvent) for 2-6 hours to attain equilibrium of water vapour and butanol vapours between the paper and the atmosphere of the tank.

6. Dip the strips in the solvent (butanol saturated with water). Allow the liquid front to travel along the sheet of the paper to a distance of 35-40 cm; this took place in about 24 hours.

7. Remove the paper strips and allow to dry at room temperature. When dry, spray with modified Dragenddorff reagent to reveal the alkaloid spots.

C. Estimation of the Chromatographed Alkaloids

8. The elution of the alkaloids from paper is done as follows:-

Cut the areas containing the alkaloidal spots off the paper strips and place them in separate tubes, each containing 3 ml. N. hydrochloric acid. Heat the tubes in a boiling water bath for 10 minutes and leave for two hours, at room temperature. Squeeze the pieces of paper with a glass rod and wash with 1 ml. distilled water; repeat this step three times and discard the paper. Add 5 N sodium hydroxide till the reaction is alkaline to litmus.

9. The extracted alkaloids are then estimated by shaking the alkaline solution with 25 ml. benzene. Treat 20 ml. of the benzene

layer with 0.5 ml. bromthymol blue solution, shake for 5 minutes and centrifuge. Use 15 ml. of the benzene layer with 4 ml. of sodium hydroxide for colour production, centrifuge. Remove as much benzene as possible and transfer the sodium hydroxide layer to a colorimetric tube. Read the colour intensity at 610 mu. The amount of the recovered alkaloid is then calculated.

Results:-

Recovery of Strychnine.

	ug. recovered on paper	mean value recovered on paper	Total recovery	% Recovery
1.	38 36.5	37.25	92.1	92.13
2.	36 37	36.5	91.7	91.75
3.	37 34.5	35.75	89.4	89.4

Recovery of Brucine

1.	30 35	32.5	81.25	81.25
2.	40.0 36.2	38.1	95.25	95.25
3.	37.5	37.3	93.75	93.75

Estimation of Strychnine and Brucine in Mixtures found in Biological Material.

The method of estimating brucine and strychnine present together in the same solution gave satisfactory results. Application of the method to biological material is of importance as the specimens dealt with in toxicological practice are of this type and are not simple aqueous solutions.

Sometimes in toxicological practice, where naturally occurring mixtures of alkaloids are used, and in pharmaceutical preparations, more than one alkaloid is bound to be found in the same specimen. The alkaloid-dye compound method is not specific and all basic organic poisons (alkaloids and synthetic basic drugs) respond to different extents to this method. By the direct method it is impossible to estimate more than one alkaloid in the sample, but by this technique it is quite possible to estimate a mixture of closely related alkaloids found in one sample.

The biological materials tried in this part are urine, plasma and liver.

Estimation of a Mixture of Strychnine and Brucine in Urine.

The technique for urine needed no special modification of that previously used for the estimation of strychnine above, in low concentrations found in urine (p. 59).

Urine pH 6.0 containing strychnine and brucine (250 ml. urine contained 100 ug. of each) were percolated through a florisil column. The interfering colouring substances were removed by 1% ammonia and 40% acetone. The adsorbed alkaloids were recovered by elution with 75 ml. ammoniacal ethanol. After evaporation, the residue was dissolved in 1 ml. ethanol and 0.4 ml. portions were applied to paper strip and developed. The chromatographed alkaloids were eluted and estimated as given in the general technique (p. 86).

Results:-

Strychnine.

	ug. recovered on paper	mean value recovered on paper	Total recovery	% recovery
1.	42 40	41	107.5	102.5
2.	37.7 39.5	38.6	96.5	96.5

Brucine

1.	39.1 37.6	38.4	95.8	95.8
2.	38.0 36.5	93.13	93.13	93.13

The recovery of strychnine and brucine found together in urine was good, for the former 96.5 - 102.5% and for the latter 95.8 - 93.13%, using 100 ug. of each. Greater volumes of urine could be used easily as the concentration method on florisil is not affected by the volume of urine percolated through it.

Estimation of a Mixture of Strychnine and Brucine in Plasma.

In the method used for the estimation of strychnine in plasma, the concentration was obtained by percolating plasma diluted four times with distilled water through a florisil column. A portion of the plasma proteins was found to be adsorbed on the florisil and this was washed out with the alkaloid on elution leaving a flocculent residue after the evaporation of the eluant. As this residue interfered with the method, concentration of the protein free filtrate was suggested to be used instead of the whole plasma.

The blanks of both the protein precipitants and the protein free filtrate together with the recovery of strychnine from the filtrate were studied.

A. Blanks of plasma protein free filtrate.

The plasma protein precipitant gave colourless blanks. The plasma protein filtrate gave a faintly coloured blank which was equivalent to 1-3 ug./10 ml. plasma, with trichloracetic acid and tungstic acid (p. 39).

B. The Recovery of Strychnine from Plasma Protein Filtrate.

The proteins of plasma to which known amounts of strychnine had been added were precipitated and the strychnine was estimated in the protein free filtrate. 5 ml. plasma (10 ug. strychnine/ml. plasma) were treated with 5 ml. 10% sodium tungstate and 5 ml. 2/3 N. sulphuric acid. For trichloracetic

5 ml. volumes of plasma were diluted with equal volumes of distilled water, to each 5 ml. 20% trichloracetic acid were added.

After standing for an hour the tubes were centrifuged. 10 ml. of the water clear supernatant liquid, made alkaline with sodium hydroxide, were shaken for 15 minutes with 25 ml. benzene. The benzene layer was treated with 0.5 ml. of bromthymol blue. The colour was produced by shaking 15 ml. of the benzene layer with 4 ml. sodium hydroxide and the colour intensity read at 610 mu. The amount of strychnine recovered was calculated.

ug. strychnine added to 5 ml. plasma	Recovery	
	Tungstic acid	Trichloracetic acid
50 ug.	7.5	37.5
	20	36.0
	10.5	40.2
		30.8

The recovery of strychnine from the gungstic acid filtrate was very low; only 7.5 - 20 ug. were recovered from 50 ug. of strychnine added to 5 ml. plasma. This low recovery might be attributed to the adsorption of the alkaloid on the precipitated protein. It was found that tungstic acid causes slight variation in the pH of the filtrate which was not strongly acidic enough to hold the strychnine in the aqueous solution. Another explanation for the low recovery was the possibility of partial precipitation of strychnine by tungstic acid. With trichloracetic acid the recovery of 50 ug. strychnine in 5 ml. plasma was 30-40 ug which was not satisfactory. The use of stronger acidic precipitant

Technique for the estimation of a mixture of Strychnine and Brucine in Plasma.

A. Concentration of the alkaloids.

1. Place 75 ml. of plasma (containing 100 ug. each of strychnine and brucine) and 45 ml. 26% trichloracetic acid in N hydrochloric acid in a 500 ml. volumetric flask. Add distilled water to the mark. Stand for an hour, shaking at intervals, and filter. Treat 400 ml. of the filtrate with 40% sodium hydroxide solution till the pH of the solution is 5-6.

2. Percolate through a florisil column and wash with 100 ml. distilled water. Add 5 ml. strong ammonia followed by 75 ml. of the ammoniacal ethanol eluent.

3. Evaporate to dryness on a boiling water bath, and dissolve the residue in 1 ml. ethanol.

B. Chromatographic Separation.

C. Estimation of the separated alkaloids.

Results:-

ug. strychnine recovered	mean value	% recovery
37.6 35.5	36.55	91.4
38.0 39.2	38.6	96.5
ug. brucine recovered	**mean value**	**% recovery**
38.0 36.5	37.25	93.1

26% trichloracetic acid in N hydrochloric acid gave results of 95-100% for 50 ug. strychnine present in 5 ml. plasma.

Trichloracetic acid 26% in N hydrochloric acid was used for the preparation of plasma protein filtrate used for the estimation of strychnine in plasma present in low concentrations.

C. Concentration of plasma protein filtrate on florisil.

It was found (page 56) that complete adsorption of strychnine from aqueous solution on florisil was at pH 5-6, the pH of the trichloracetic acid filtrated was adjusted to pH 6.0 before percolation through florisil.

50 ml. plasma (2 ug. strychnine/ml.) were treated with 30 ml. T.C.A. acid 26% in N hydrochloric acid. The volume was made up to 250 ml. in a volumetric flask. After standing for an hour the plasma was filtered. 200 ml. of the clear filtrate were adjusted to pH 6.0 and percolated through a florisil column treated with ammonia and washed with distilled water. The percolation of the trichloracetic acid filtrated was followed by 100 ml. water and the adsorbed alkaloid was recovered with 6 ml. sodium carbonate 5%, followed by 75 ml. ethanol eluent. The eluent was collected in a flask containing 2 ml. N hydrochloric acid, and was then evaporated to dryness on a water bath. The residue was dissolved in 10 ml. H_2O and the strychnine recovered was estimated in 5 ml. portions.

In three experiments the recovery was 91.5%, 89.0 , 96.0%, with a mean value of 92.1%.

Estimation of a Mixture of Strychnine and Brucine in Liver.

Method:-

A. Concentration of the alkaloidal content of the liver.

50 gm. liver were homogenised with 100 ml. saline to a fine paste and transferred to a round-bottomed flask and 1 ml. strychnine solution (0.1 mgm./ml.) was added. The flask was placed in a boiling water bath for 5 minutes. 50 ml. of 2N hydrochloric acid were added and the mixture heated for another 10 minutes, and allowed to cool. It was transferred to a 250 ml. volumetric flask and completed to the mark with distilled water. Filter, use suction if necessary.

Treat 200 ml. of the filtrate with 10 ml. trichloracetic acid 26%, N. Hydrochloric acid. Make up the volume to 250 ml. with distilled water and filter.

200 ml. of the filtrate was adjusted to pH 8.0 with sodium hydroxide solution. Percolate through a florisil column, followed by 100 ml. distilled water. The adsorbed pigments were washed off the column with 50 ml. volumes of 0.5% ammonia, distilled water, 30% acetone in water and distilled water in the given order.

The adsorbed alkaloids were eluted with 5 ml. strong ammonia, followed by 75 ml. of ethanol ammonia* mixture.

The eulted alkaloidal solution was evaporated to dryness and the residue dissolved in 1 ml. ethanol.

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* See page .

B-Chromatographic Separation of the Concentrated Alkaloids. (page 85)

C- Estimation of the Chromatograhed Alkaloids (page 86)

RESULTS:

	ug. recovered on paper	mean value recovered on paper	Total Recovery	% Recovery
		Strychnine		
1.	30.0 37.5	33.75		84.4
2.	38.0 36.5	37.25		93.1
		Brucine		
1.	32.0 33.5	32.75		81.9
2.	39.5 37.6	38.5		96.3

ADDENDUM I :

ELECTROCHROMATOGRAPHY OF ALKALOIDS.

ADDENDUM II :

OBSERVATIONS ON PAPER CHROMATOGRAPHY IN TOXICOLOGICAL ANALYSIS FOR THE IDENTIFICATION AND ESTIMATION OF ORGANIC BASIS POISONS.

Electrochromatography of Alkaloids.

A. Electrophoretic Separation of Alkaloids from Plasma.

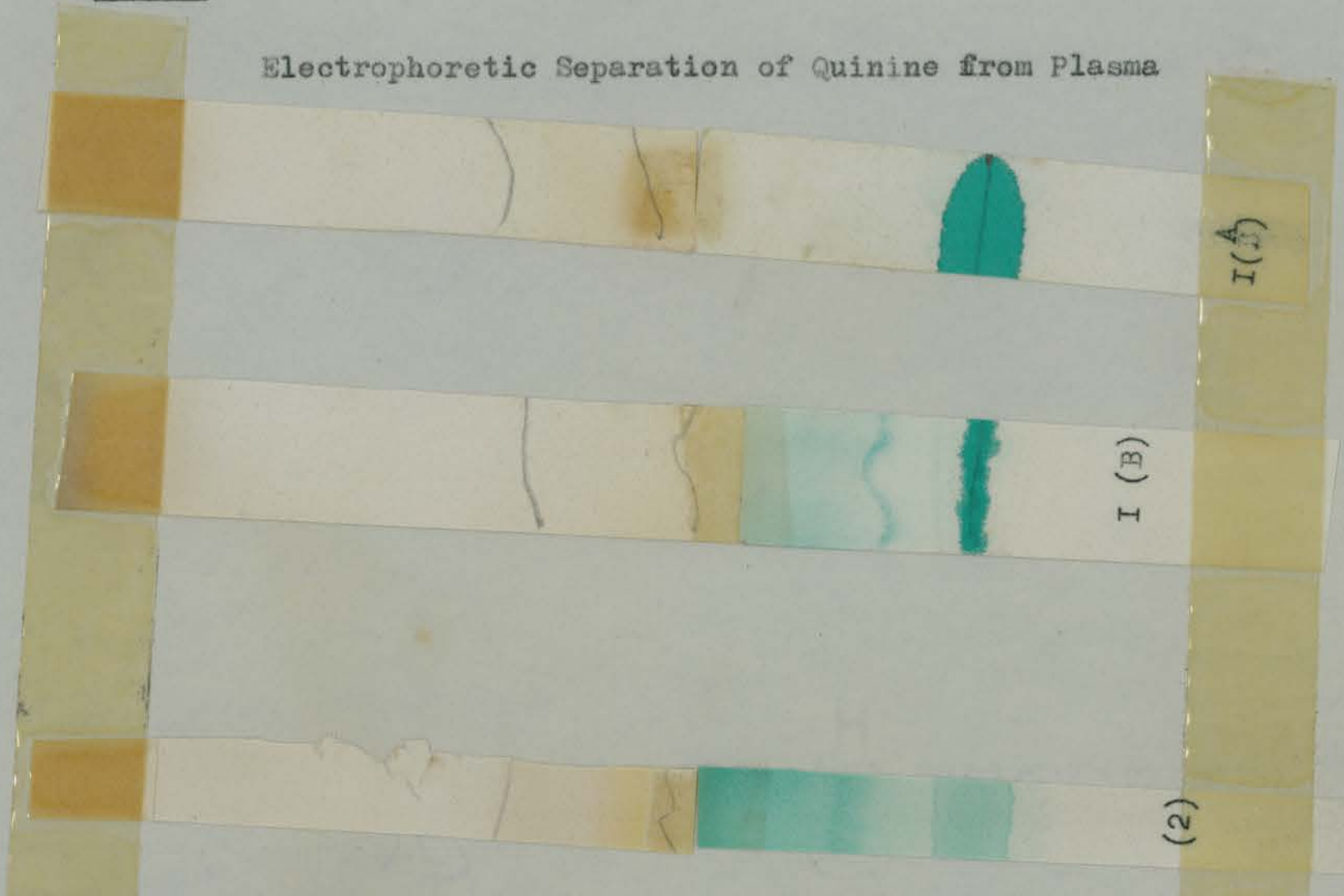
The aim of this part of the work was to separate the alkaloid(s) from plasma for identification and estimation purposes. After the separation of the alkaloids from plasma, the portion of the paper strip containing the proteins was to be cut off and discarded. The paper strip was to be developed with a suitable solvent. The alkaloid was identified by its R_F value and its quantity estimated if required.

Theory: Alkaloids have basic characters due to the presence of a basic nitrogen atom in the molecule. All the alkaloids used in this study are monoacidic bases, i.e. one molecule of the alkaloid combines with one molecule of a monobasic acid, e.g. HCl with a general formula B.HCl, with a dibasic acid, e.g. H_2SO_4 two molecules of the alkaloid combine with one molecule of the acid $B_2.H_2SO_4$. In aqueous solutions alkaloids give rise to positively charged ions which move to the cathode when subject to an electric field.

As acid phosphate impregnated paper gave satisfactory results for the separation of strychnine and brucine, it was suggested that the buffer solution used in this study should be phosphate solution of the same concentration as that used

CHART I.

Electrophoretic Separation of Quinine from Plasma



I (A). Plasma containing quinine apllied to paper followed with Trichloroacetic acid.

I (B) Trichloroacetic acid applied to paper before the application of the plasma.

II Plasma applied to paper without any protein precipitate.

Buffer used M/4 sod. dihydrogen phosphate.

Current : 250 Volts for 4 hours.

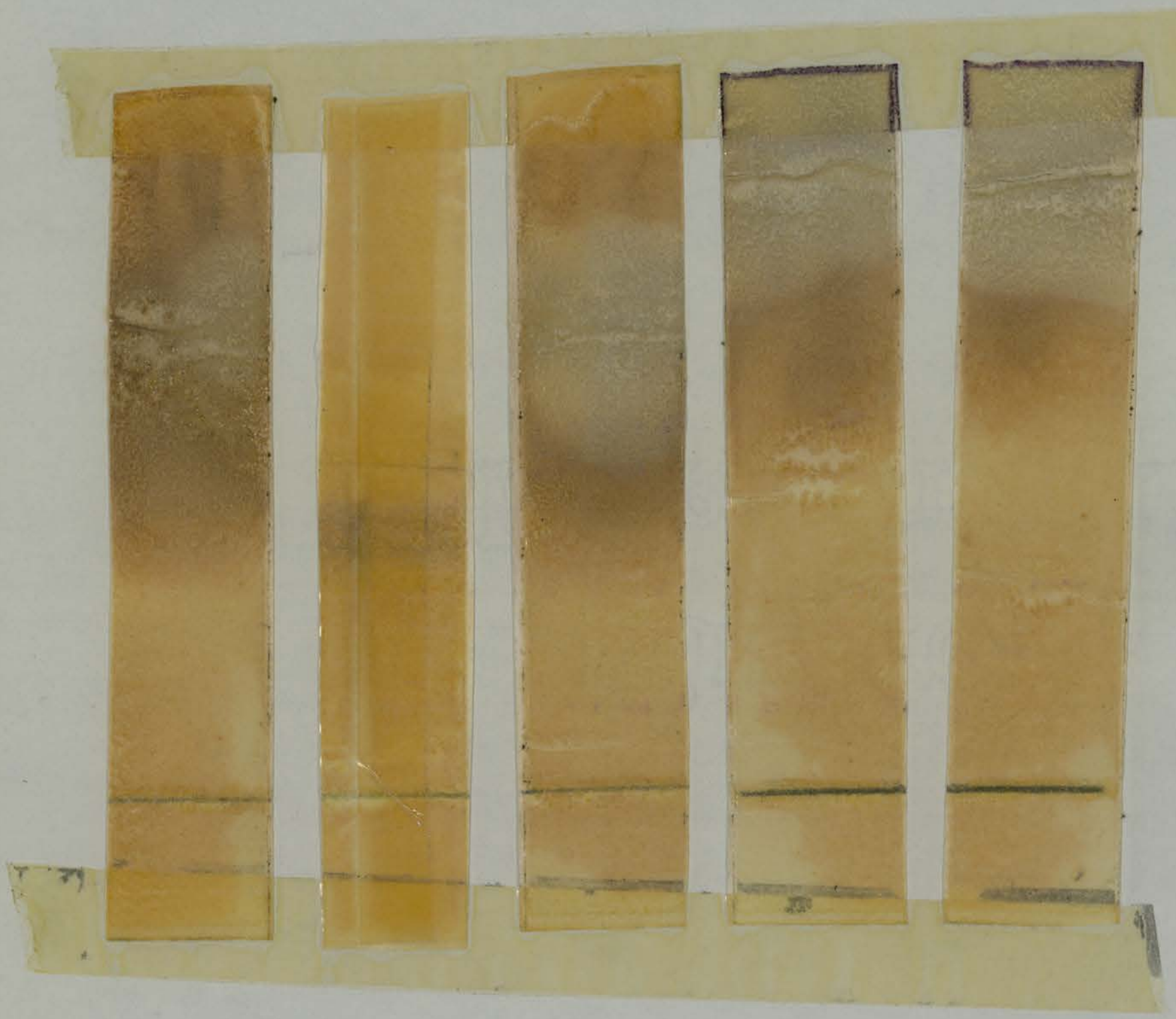
for impregnating the paper. After the electrophoretic separation of the alkaloid from plasma, the strip of paper containing the alkaloid was dried and developed with butanol saturated with water.

Experimental: Plasma to which quinine had been added (1 mgm quinine/ml. plasma) was used. 0.025 ml. plasma was applied to the paper strip in each experiment. Plasma was allowed to dry over a hot plate. It was then soaked in M/4 NaH_2PO_4 solution and the excess was removed by pressing the paper strip between blotting paper. The strip was placed in the electrophoretic chamber containing N/4 NaH_2PO_4 and a current of 250 V was applied for 2 hours. The strip was dried at 100°C and exposed to ultraviolet rays to mark the position of quinine on the paper strip. The portion of the strip below the quinine was cut off and discarded, the rest was developed with butanol H_2O. After development, the strip was sprayed with Dragendorff reagent to reveal the quinine spot.

Preliminary experiments (Chart Ia) showed that the proteins moved along the paper strip in the same direction as the quinine. To eliminate any possible interference of of the proteins, it was suggested that it should be trapped and fixed in their place of application on the paper strip by precipitation. The protein precipitants used were 20% trichloracetic acid, 20% sulphosalysilic acid and tungstic acid. The precipitate was applied either after or before

CHART II.

Electrophoretic Separation of Alkaloids From Plasma.



(i) Quinine

(ii) Brucine

(iii) Strychnine

(iv) Atropine

(v) Cocaine.

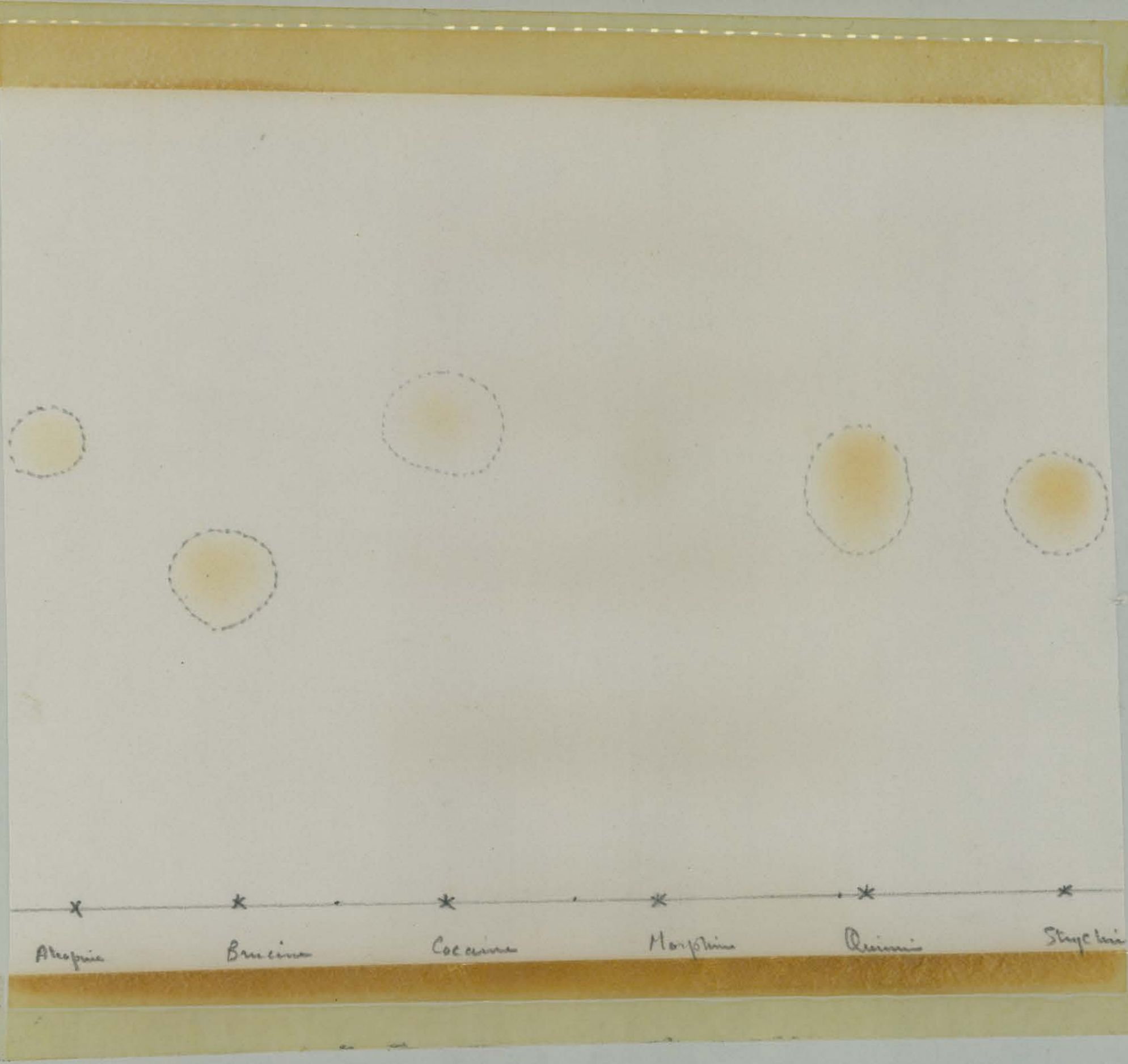
the application of plasma. By this method the plasma proteins were held in their place of application and the alkaloid (quinine) travelled alone along the paper strip.

Results: Tungstic acid was unsuitable for this particular purpose because it precipitated the quinine together with the proteins. In strips with tungstic acid the quinine did not move.

Both trichloracetic acid and sulphosalysilic acid gave satisfactory results. Although the latter precipitated the proteins more completely than the former, yet the trichloracetic acid was preferred. Sulfosalysilic acid was fluorescent in ultraviolet rays and a deep yellow coloured zone around the band of the quinine which interfered with the colour of the quinine spot, especially when present in lower concentrations than 25 ug.

Trichloracetic acid was the protein precipitant chosen for use in the electrophoretic separation of alkaloids from plasma. As shown in Chart 7, the application of T.C.A. acid before the plasma gave the best results, as the plasma did not spread and was restricted to a narrow band, and more plasma could be applied by this method.

The above method was applied to plasma containing various alkaloids (atropine ,strychnine, brucine, cocaine & quinine). Good separation of the above alkaloids from plasma was obtained with M/4 sodium dihydrogen phosphate as buffer.


Atropina
Brucina
Coccinina
Morphina
Quinina
Strychnia

By this method it would be possible to detect and identify any alkaloid suspected to present in plasma especially when a small volume is only available.

The disadvatage of this method that it is only possible when the alkaloid is present in high concentration (.2-.5 mgm/ml) which is not often met with in toxicological practice.

B- Electrophoretic Separation of Alkaloids into groups.

The above work was extended to the electrophoretic separation of a mixture of alkaloids.

Preliminary work showed that there was no satisfactory separation by this method. From chart III it could be noticed that a mixture of quinine, strychnine , brucine, cocaine and atropine could be possibly separated into three groups:

(a) atropine, cocaine

(b) quinine strychnine

(c) brucine.

This method of electrophoretic separation of alkaloids will be studied in detail in a later communication.

Addendum II.

Observations on Paper Chromatography in Toxicological Analyses for the Identification and Estimation of Organic Basis Poisons

Paper chromatography in the field of toxicological analyses could be applied for the following purposes.

1. Paper chromatography of alkaloids could be applied as a qualitative test for the identification of the different organic basic drugs by their R_F values.

2. Separation of mixtures of basic organic poisons to enable their individual estimation as they all respond to the alkaloid-dye compound method.

3. In toxicilogical specimens where the specimen is putrified, paper chromatography may serve as a tool for the purification of the drugs present due to the putrifactive products which may interfere with the qualitative tests and the estimation methods.

Proposed Technique.

1. The basic drug may be extracted and concentrated using the suitable method, depending on the nature of the specimen under examination (urine, plasma, tissues, methods - Part II, Division II, Chapter 6).

2. The concentrated drug is to be applied to paper and chromatographed with suitable solvent. A duplicate

chromatogram should be prepared, one for identification purposes by calculating the R_F value of the spot, which is to be eluted and confirmatory tests to be applied. The other sheet is to be used for quantitative estimations by eluting the spot and estimating its content with the alkaloid dye addition compound method (Part II, Division II). A control chromatogram of drugs suspected to be present should be run at the same time as the unknown.

In this way minute amounts of basic drugs could be identified and estimated.

This work will be studied in detail in another communication.