

STUDIES IN THE APPLICATION OF JANOVSKY'S AND MOHLER'S  
REACTIONS TO TOXICOLOGICAL ANALYSIS

by

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Thesis

Submitted by

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INTRODUCTION

Forensic chemistry is a branch of applied chemistry and toxicological analysis is fundamentally a specialised application of analytical technology. Toxicology did not really make any great advance until the development of modern chemistry and the application of accurate analytical methods.

Orfila's "Toxicologie Generale" published in 1814 laid the foundations of modern toxicology. His treatise is mainly concerned with the account of systematic experiments on the effects of poisons on animals and with the descriptions of the symptoms observed in cases of human poisoning and hardly touches on the chemistry of the subject. "Medical Jurisprudence" published by Paris and Fonblanque in 1823 includes sections on poisons and describes chemical tests for the detection of some of them. Christison (1829) produced the first systematic treatise in which the chemical tests for poisons were described as fully as their physiological effects. The rapid advances that were then being made were reflected in the succeeding works on Toxicology: Guy's "Forensic Medicine" (1844) and Taylor's "Principles and Practice of Medical Jurisprudence" (1844).

One of the earlier German toxicologists, Plenck, laid down the dictum that the only conclusive proof of poisoning was the identification of the poison in the body, but this view was not accepted by/

by British toxicologists. Christison (1829) held that the medical probability, in conjunction with the general evidence, might be so strong that no rational being could entertain a doubt that poisoning had been perpetrated. Guy (1844) considered that chemical analysis, though not absolutely necessary, when the symptoms, post-mortem appearances and moral evidence confirmed each other, was yet of the very first importance. Taylor repeatedly expressed a similar opinion.

A host of new synthetic drugs, generally of complex chemical character have been introduced into modern therapeutic practice and as tests for their identity in the small amounts usually recovered from biological material do not exist in many cases, the toxicologist is faced with the difficult problem of devising them. When poisons of which little is known are used, there must still from time to time be cases in which there can be no direct chemical evidence of poisoning and in which the toxicologist thus remains in much the same position as his predecessor at the beginning of the last century in cases of morphine poisoning. These and other problems met with in everyday toxicological work are but little appreciated except by those actively engaged in the field.

The different stages in any chemical toxicological analysis are (1) Isolation in a crude form, (2) Purification, (3) Identification and (4) Determination. The separation of the organic poisons from biological material is the most difficult of the toxicologist's analytical problems, partly because of their complex chemical

character/

character and partly because of the small amounts of them to be recovered from relatively large quantities of other organic matter. The principles underlying the methods of isolation are simple but in actual practice great skill and experience are required to obtain the organic poisons in a state of purity necessary for identification. The basic procedure of Stas<sup>1</sup> as modified by Otto<sup>2</sup> and Dragendorff<sup>3</sup> has remained the classical method for the isolation of organic poisons. The large number and tedious nature of the operations involved in this method, render it unreliable for the quantitative isolation of the less stable alkaloids such as aconitine and cocaine. The need for a rapid and satisfactory method for the isolation of alkaloids is keenly felt. With this object in view Stewart, Chatterji and Smith<sup>4</sup> tried trichloroacetic acid as protein precipitant followed by adsorption of the alkaloids upon kaolin; Daubney and Nickolls<sup>5,6</sup> used ammonium sulphate to precipitate proteins followed by chloroform extraction of the alkaloids; Valor<sup>7</sup> concerned mainly with the isolation of barbiturates used tungstic acid followed by ether extraction. These methods have yielded excellent results but as yet insufficient work has been done to demonstrate their general validity. The Stas-Otto process, in view of its general use and its general utility, must remain the safest general procedure.

Purification of crude residues isolated from biological material is a difficult but essential process. Since identification is for the most part dependent on colour reactions the necessity to work with/

with pure extracts cannot be over-emphasised. Stolman and Stewart<sup>8,9</sup> have successfully used adsorption methods for the quantitative separation of mixtures of morphine, codeine, heroin and barbiturates. Their work demonstrates the general utility of chromatographic methods in this field.

Whenever the quantity of material permits, the sharpness and constancy of the melting-point, the preparation and subsequent identification of a definite derivative are undoubtedly of immense value in the identification of an organic compound, but there are unfortunately numerous instances, particularly in the alkaloid group, where it is seldom possible to obtain a pure residue in sufficient quantity. Identification in these circumstances is dependent on colour reactions. Particular mention must be made of Bamford's<sup>10</sup> excellent scheme for the identification of alkaloids, which has lightened considerably the work of the toxicologist in this field.

In quantitative work, standard gravimetric and volumetric procedures are of little or no help to the toxicologist. The problem has been met by the quantitative development of colour reactions. When visual comparison is replaced by the photo-electric measurements the accuracy of colorimetric methods compares favourably with that of other analytical processes.

The object of this investigation is to develop simple but sensitive colour tests based on Janovsky's<sup>11</sup> and Mohler's<sup>12</sup> reactions, for the detection and estimation of alkaloids and other substances met/

met with in toxicological work. Group VII in Bamford's<sup>10</sup> systematic scheme contains the alkaloids for which no satisfactory colour reactions are known. This group includes cocaine, tropa-cocaine, cinnamyl-cocaine, psicaine, amydracaine, benzamine and aconitine. Various colour tests have been described for aconitine but are of doubtful value; for the identification of cocaine in extracts from biological source, by the micro-chemical test with potassium permanganate it is essential to isolate the alkaloid in a state of absolute purity, otherwise the permanganate is reduced and the test is rendered useless.

Several investigators have studied the scope of the Vitali-Morin<sup>13,14</sup> reaction which is an instance of the reaction between aromatic polynitro compounds and acetone in the presence of alkali (Janovsky's reaction). This reaction, in which fuming nitric acid alone is used as nitrating agent, has been applied to a variety of compounds for their detection and estimation. These compounds have one thing in common, they all contain a readily nitratable benzene nucleus. In many cases fuming nitric acid fails to bring about intensive nitration to polynitro derivatives and in these cases a more energetic nitrating agent is required. Using a mixture of potassium nitrate and sulphuric acid the author<sup>15,16,17,18</sup> has in his preliminary work achieved a certain measure of success in the case of cocaine, amphetamine, the phenyl barbiturates and benzoic acid.

Janovsky's/

Janovsky's and Mohler's reactions have been used only in a few isolated cases in toxicological work and there is no reference in the literature to the systematic study of the application of these reactions to toxicological analysis. The reactions are simple enough to carry out, the fundamental reaction involved in both being nitration to polynitro derivatives.

This investigation is mainly concerned with the basic organic poisons which in the Stas-Otto method are extracted with immiscible solvents from alkaline medium. It was hoped that the investigation would yield results which would be of value to those engaged in toxicological analysis and the work has been done with this aim in view, rather than with the object of elucidating precisely the chemical reactions involved in the two tests.

REVIEW OF LITERATURE

A characteristic property of aromatic compounds is the readiness with which they may be converted into nitro-derivatives by the substitution of nitro-groups for hydrogen of the nucleus. Generally speaking the number of hydrogen atoms displaced by the nitro-groups is the larger the higher the temperature and the more concentrated the acid or mixture of acids employed but depends to an even greater extent on the availability of a dehydrating agent and on the nature of the substance undergoing nitration; as a rule the introduction of nitro-groups is facilitated when other groups especially alkyl groups have already been substituted for hydrogen of the nucleus. The nature of these atoms or groups moreover determines the position taken up by the entering nitro-groups.

The method which is almost always used for the preparation of aromatic nitro-derivatives is the action of nitric acid on an aromatic compound. The direct nitration of an aromatic compound was first carried out by Mitscherlich in 1834 when he obtained nitro-benzene by the action of fuming nitric acid on benzene. The most commonly used nitrating agent is a mixture of nitric acid and sulphuric acid. According to Graebe<sup>19</sup> a mixture of nitric and sulphuric acids was first used about one hundred years ago when Muspratt and Hofmann<sup>20</sup> converted nitro-benzene into dinitro-benzene with <sup>the</sup> mixed acids. This mixture has since come into universal use, particularly as a very powerful agent for relatively difficult nitrations. A modification of the usual nitric-sulphuric acid mixture/

mixture is to use sulphuric acid and solid potassium nitrate. This mixture may also be used to introduce a number of nitro-groups. Nitric acid in acetic acid or acetic anhydride is also sometimes used as nitrating agent. Acetyl nitrate is a powerful nitrating agent.

Although the nitro-paraffins are neutral compounds those of the general formula  $RR'CHNO_2$  (primary and secondary nitro-paraffins) can yield by reaction with aqueous alkali sodium salts represented by the formula  $RR'C = N \begin{smallmatrix} O \\ \parallel \end{smallmatrix} ONa$ . In aromatic nitro compounds the nitro-group is of necessity attached to a tertiary carbon atom and hence they cannot react with bases to form salts. The aromatic mono-nitro derivatives of benzene and its homologues are indifferent to cold aqueous alkali and thus resemble the tertiary nitro-paraffins. The presence of two or more nitro-groups in the nucleus appears to make them more active and these show a remarkably interesting behaviour towards alkali; the polynitro derivatives form addition compounds with sodium hydroxide and sodium ethoxide which are often highly coloured.

#### 1. JANOVSKY'S REACTION

In 1886 Janovsky and Erb<sup>21</sup> observed that nitro-derivatives of azo-benzene gave characteristic colourations with acetone and potassium hydroxide. In 1891 Janovsky<sup>11</sup> investigated this colour reaction. He found that the reaction with acetone and alkali was a general one for dinitro substitution products of benzene, toluene and naphthalene and that the reaction was not given by mono-nitro derivatives. He made the following observations. The reaction is carried out by dissolving a few milligrams of the dinitro-compound in acetone and adding aqueous potash drop by drop. A characteristic colour/

colour is formed the intensity of which is increased by further addition of alkali. Mono-nitro compounds produce similar colourations only if they are contaminated with traces of dinitro-compounds. Dinitro-benzene gives a reddish-violet colour which after a time becomes as dark as potassium permanganate and is changed by acetic acid to dark red and by hydrochloric acid to yellow. 1:2:4 dinitrotoluene gives a blue colour which is changed by acetic acid to violet-red and  $\alpha$ -dinitronaphthalene a blood-red colour which becomes eosin-red on adding acetic acid.

A year later in 1892 Bitto<sup>22</sup> extended Janovsky's reaction to apply as a test for aldehydes and ketones. He observed that many aldehydes and ketones gave with certain aromatic nitro-compounds in the presence of potassium hydroxide a more or less intense colour reaction. With m-dinitrobenzene, for example, a blue and in the case of  $\alpha$ -dinitronaphthalene an intense red colour is produced. He investigated the action of aldehydes and ketones on m-dinitrobenzene, dinitro-toluene and  $\alpha$ -dinitronaphthalene in the presence of potassium hydroxide and came to the conclusion that many other aldehydes and ketones served the same purpose as acetone in Janovsky's reaction. He has recorded the behaviour of a large number of aldehydes, ketones and ketonic acids with m-dinitrobenzene in the presence of alkali. He concludes from his observations that a colour reaction is given by all fatty aldehydes and ketones which contain a non-substituted  $-\text{CH}_n\text{CHO}$  or  $-\text{CH}_n\text{CO}$  group respectively; by all mixed ketones and by those aromatic aldehydes which contain a fatty radical/

radical. He has also recorded at the same time some exceptions so far as the nitro-compounds were concerned. He found that tri-nitro-resorcinol did not give any colouration with acetone and alkali whilst trinitro-xylene gave a green colour with alkali alone.

Reitzenstein and Stamm<sup>23</sup> observed that an acetone solution of 1-chloro-2;4-dinitrobenzene gave an intense bluish-violet colour with sodium hydroxide or potassium hydroxide — an instance of Janovsky's reaction for dinitro compounds. They have recorded the colour reactions given by numerous nitro-compounds with acetone and potassium hydroxide and also those exhibited by m-dinitrobenzene, potassium hydroxide and aliphatic and cyclic substances containing the group  $-\text{CH}_2\text{CO}-$ .

Rudolph<sup>24</sup> in the course of the examination of explosives during the first world war observed that the addition of sodium hydroxide or ammonium hydroxide to the alcohol or acetone solutions caused colourations which were more or less characteristic of the nitro-compounds present. He has reported the colourations obtained on adding sodium hydroxide and ammonium hydroxide to acetone and alcoholic solutions of some di- and tri-nitro compounds.

Taylor and Rinkenbach<sup>25</sup> have also reported the colours produced by a few mono-, di- and tri-nitro compounds and point out that the colour produced by tri-nitro compounds will obscure those produced by di-nitro compounds when the two types are present in mixtures.

Desvergnès<sup>26</sup> has reported the colour reactions of one hundred and/

and thirty two nitro-compounds. He prepared solutions containing 0.05% of the nitro-compound in alcohol and acetone and 5 ml. of each of these were treated with 2 drops of 5% potassium hydroxide and 5% ammonium hydroxide. The colours that developed at room temperature and at 40°C were recorded.

Bose<sup>27</sup> in his researches on nitro-compounds observed instances where Janovsky's reaction failed and concluded that the reaction was hardly suitable for the general detection of aromatic poly-nitro compounds.

Bost and Nicholson<sup>28</sup> studied the colour reactions obtained from different aromatic nitro-compounds when treated with acetone and alkali. They summarize their observations as follows:-

"Phenomenal as it may seem mono-nitro compounds give no colour with the reagent, dinitro compounds give a purplish-blue colour while tri-nitro compounds produce a blood-red colour, except when an amino, substituted amino or hydroxyl group is present. Acetylation of the amino or hydroxyl group does not alter the inhibiting effect of such groups on the colour test. The substitution of an alkyl group for an amino-hydrogen does not alter the inhibition of the amino group whereas the substitution of a methyl group for an enolic hydrogen permits the compound to respond to the general colour test. Noteworthy examples are 2:4 dinitro aniline, 2:4 dinitro phenol, 2:4 dinitro acetanilide, 2:4 dinitro phenyl acetate, 2:4 dinitro diethyl aniline and 2:4 dinitro anisole. In cases where the benzene nucleus/

nucleus is richly substituted as in 2:4 dinitromesitylene, 2:4:6 trinitromesitylene and 2:4:6 trinitro-1:3 dimethyl-5 ter-butyl benzene no colour is produced. The steric effect of the nitro group in the isomeric dinitrobenzene is noteworthy. m-Dinitrobenzene readily responds to the test, p-dinitrobenzene gives a reddish-yellow colour which soon fades to a greenish yellow while o-dinitrobenzene gives no colour at all. The test is extremely delicate in most cases".

Nisida<sup>29</sup> studied the reaction with many varieties of nitro-compounds and concluded that there exists some relation between the colouration and chemical structure. He summarizes his observations as follows:- "The colouration of di- and tri-nitro derivatives may be classified into the following three groups (1) red, (2) blue and (3) colourless or slightly coloured. Observing these results from the standpoint of chemical structure it was noted that those belonging to the blue group are 2:4 dinitrotolual and such type of compounds as to contain  $\text{CH}_3:\text{NO}_2:\text{NO}_2$  in 1:2:4 position. Those belonging to the red group also have two nitro-groups in meta position containing, however, other radicals than  $-\text{CH}_3$  or if any in other position as above mentioned. Those belonging to the colourless or slightly coloured group have two two nitro-groups in ortho or para position. Further those tri-nitro compounds which have the above said relation belong to the blue group and same is the case with dimethyl or trimethyl dinitro compounds".

Parkes/

Parkes and Farthing<sup>30</sup> have reported the colour reactions of some derivatives of tri-nitro-toluene with sodium hydroxide in both acetone and alcoholic solutions.

English<sup>31</sup> who studied the colour reactions of certain dinitro aromatic compounds states that in general, the hue, intensity and stability of the colour formed are largely influenced by the concentration of sodium hydroxide and to a lesser extent by the amount of water present. The different dinitro compounds studied required somewhat different concentrations of sodium hydroxide to produce maximum colour.

James and Roberts<sup>32</sup> have established the fact that the Vitali-Morin<sup>13,14</sup> reaction for the solanaceous alkaloids is yet another instance of Janovsky's reaction. The colour reaction for atropine, whereby evaporation of the alkaloid with fuming nitric acid and treatment of the residue with alcoholic potash gives an intense purple colour was proposed by Vitali<sup>13</sup> in 1880. He examined over sixty alkaloids and found that only three, daturine, hyoscyamine and duboisine gave the purple colour. Withaus<sup>33</sup> reported that in addition hyoscyne gave the purple colour. Morin<sup>14</sup> introduced an important modification to Vitali's test. He added the alcoholic potash to an acetone solution of the nitrated residue thereby increasing manifold the intensity of the colour produced and consequently the sensitivity of the test.

Several investigators have examined the scope of the Vitali reaction/

reaction.

Hardy<sup>34</sup> reports that the test is given by atropine, hyoseyamine, scopolamine and isoatropyl-cocaine but not by homatropine. He states that the reaction is a general one for esters of certain acids the constitution of which is analogous to that of tropic acid and that the grouping  $C_6H_5CH-$  seems essential since the ethyl ester of phenyl ethyl acetic acid  $C_6H_5CH(C_2H_5)COOH$ , likewise gives the reaction. He also points out that cocaine gives no colour in Vitali's test and that any violet colour obtained with cocaine reveals with certainty the presence of isoatropyl-cocaine.

Van Urk<sup>35</sup> applied the Vitali reaction to a large number of aromatic compounds and found that a great many of them gave colour reactions with the development of orange, red, green and even violet colour. He makes the following observations:- (1) The reaction is probably dependent on the nitration of a phenyl group, (2) the reaction is readily obtained when the phenyl group is linked to nitrogen and the colour intensified by the presence of hydroxyl groups, (3) where the phenyl group is linked to carbon as in benzoic acid, cinnamic acid, saccharin and luminal no colour develops except a yellow in the case of phenols, ketones and aldehydes.

Celsi<sup>36</sup> discusses the relation between the reaction and chemical constitution of the reacting substances. He states the reaction is negative with homatropine and positive with the ethyl ester of phenyl glycollic acid. The reaction is positive with the esters/

esters of aromatic acids but negative with the free acids.

Poe and Clemens<sup>37</sup> tested a large number of organic compounds by the Vitali test. The object of their investigation was to determine whether or not the characteristic purple colour was given by any number of the organic compounds and also to determine whether or not the colour was due to any organic grouping. They have reported that twenty seven of the compounds which they tested gave various shades of violet, lavender or purple which might to some degree be confused with Vitali's test for atropine. They conclude that in general the compounds giving an atropine-like reaction do not belong to any definite group of organic compounds nor does any special organic radical seem to be responsible for the characteristic test. Nearly all of them contained nitrogen in some form.

More recently James and Roberts<sup>32</sup> have investigated the nature and specificity of the Vitali-Morin reaction for the solanaceous alkaloids. They examined the effect of the reaction on benzene and a series of simple derivatives. Colours were obtained which varied from red through shades of purple to a pure blue. They consider nitration of the benzene ring to be the fundamental reaction of the Vitali test for the following reasons:-

1. Benzene itself responds with a purple colouration and simple derivatives give a series of related tints which can be duplicated by corresponding alkali treatment of the appropriate nitro-compound of known constitution.

2./

2. All the compounds known to give such colours belong to the aromatic series with the sole exception of thiophene which closely resembles benzene in its properties.
3. Further in the solanaceous alkaloids themselves while hydrolysis destroys the power of reaction any esterification of the tropic acid fraction restores the capacity.
4. Finally ketones and some other carbonyl compounds such as malonic ester are capable of intensifying the colours given by simple aromatic nitro compounds and this effect is also reproduced in their results for atropine.

They also observed that in general intense red colours are given by all 2:4:6 trinitro derivatives whereas 2:4 dinitro derivatives give blue colours. *m*-Dinitrobenzene gives a blue colour which fades rapidly to a purple indistinguishable visually from the colour given by the solanaceous alkaloids after Vitali treatment. The stability of the colours varies a good deal from one compound to another but as a rule the blues are the least stable going off to a purple tinge and then more slowly to a red. The change is accelerated by the addition of water. The red colours obtained from symmetrical trinitrobenzene and sym-trinitro toluene fade quickly with precipitation in anhydrous acetone but a few drops of water render them stable for many days.

They also found that the reaction failed with many aromatic compounds that might have been expected to give it. Among the aromatic/

aromatic substances which failed to give a reaction the occurrence of hydroxyl or carbonyl groups in or near the ring is almost invariable. The deactivating effect of these groups is apparent not only when they are directly attached to the aromatic ring but also when they are in its neighbourhood. Mandelic acid,  $C_6H_5CH(OH)COOH$ , gives virtually no reaction but when its (OH) group is acetylated a recognisable Vitali reaction is obtained.

They have established the fact that the Vitali-Morin reaction is a special case of the colour reaction of aromatic polynitro compounds with alkalis. They conclude from the results of their investigation that intense formation of Vitali-purple colours appears to be associated with the grouping  $C_6H_5 CHXCOOR$  providing that X is not hydroxyl and R implies that the carboxyl group is esterified. In general hydrocarbon side chains increase the production of colour in the reaction and the presence of readily replaceable hydrogen particularly in -OH and -COOH reduces it.

Canback<sup>38</sup> has studied the influence of acidic and basic groups on or in the neighbourhood of the ring, on Janovsky's reaction and from the results of his investigation draws the following conclusions:-

1. Compounds with only one nitro in the ring do not react, those with two nitro groups in meta position give a blue or purple colour and maximum absorption at 5500 to 6000Å, while tri-nitro compounds give a red colour with maximum absorption at 5000Å.
2. Presence of a hydroxyl group inhibits the reaction but if the hydroxyl/

hydroxyl group is alkylated the reaction will proceed normally. Acetylation of the hydroxyl group will not prevent its inhibiting action. 3. The presence of a carboxyl group on the ring disturbs the nitration process so that only one nitro group enters the ring. 4. An amino group on or in the neighbourhood of the ring has a remarkable effect and neither alkylation nor acetylation will change this.

The formation of strikingly coloured substances by the action of alkali on certain aromatic nitro-compounds is mentioned by Salkowski<sup>39</sup> in his paper on "Chrysanisic acid" where he describes the nearly black compound formed from chrysanisic acid and the rose coloured derivative of ethoxy dinitrobenzoic ethyl ester.

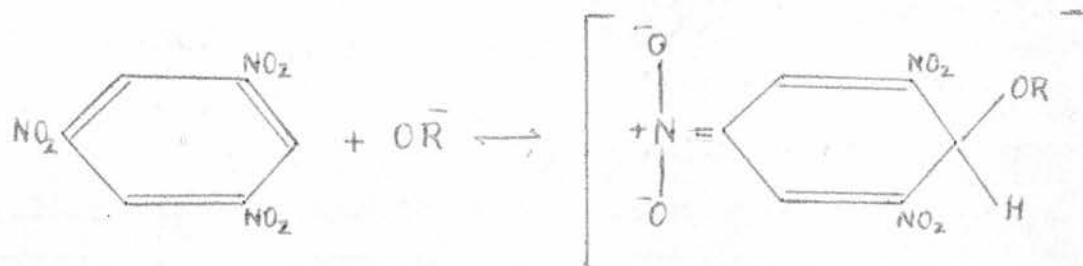
Hepp<sup>40</sup> also observed the appearance of a blood-red colour when sym-trinitro benzene was treated with potassium hydroxide.

Victor Meyer<sup>41</sup> called attention to the curious phenomena attending the action of sodium hydroxide on sym-dinitrobenzoic acid which with one equivalent or less of alkali gave a colourless solution, with more dilute alkali a yellowish-red colour and with excess of strong alkali a dark violet colour.

A large volume of literature exists on the coloured compounds obtained from certain aromatic nitro-compounds with sodium-alcoholate. Of the many publications dealing with this subject may be mentioned the work of Jackson<sup>42,43,44,45</sup> and his school, Meisenheimer<sup>46</sup>, and Hantzsch and Kissel<sup>47</sup>.

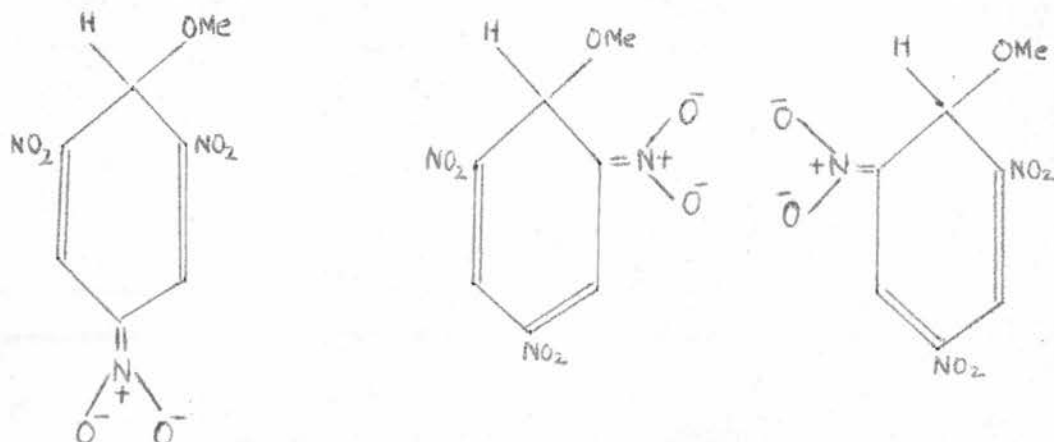
Meisenheimer<sup>46</sup>/

Meisenheimer<sup>46</sup> represents the mechanism of the reaction between aromatic polynitro compounds and sodium alcoholate as follows;



Sidgwick, Taylor and Baker<sup>48</sup> give a survey of the reaction between aromatic nitro-compounds and alkali in which they in the main sustain Meisenheimer's opinion of the mechanism of the reaction. They conclude with the following statement "Meisenheimer's formula however cannot be considered as completely satisfactory, it does not take into account the fact that more than one nitro-group must be attached to the nucleus for this type of salt formation to take place nor does it offer any explanation of the deep colour of the salts. It is evident that the stability of the compounds and their colour has something to do with all the nitro groups and not only with one. The compounds in which the salt formation occurs are those in which two or more nitro-groups are in the meta-position to another. The anion from such a compound can be written in as many separate formulae as there are nitro groups e.g. the anion of the addition product of potassium methyllate and sym-trinitrobenzene can be written in three ways and these formulae do not differ in the positions of the constituent atoms but only in the kind of valencies uniting the atoms/

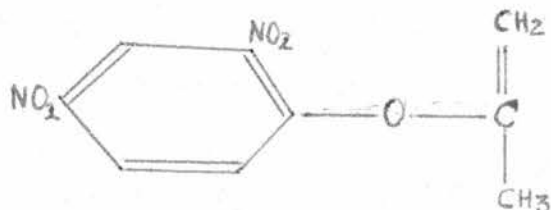
atoms. Hence the anion is almost certainly a resonance-hybrid of



all three formulae, a view which offers the best explanation of the necessity for the presence of more than one nitro group; the extra stability arising from the resonance will account for the formation of the compounds and their colour may well be due to the resonance between quinonoid systems in much the same way as in the triphenyl methane dyes".

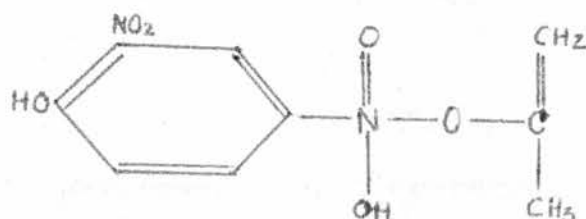
The theoretical interpretation of the reaction between aromatic nitro compounds and active methylene groups in the presence of alkali has been attempted only by a few investigators.

Reitzenstein and Stamm<sup>23</sup> proposed the following structure for the coloured compound obtained by the reaction between 2:4 dinitrobenzene and acetone in the presence of alkali.

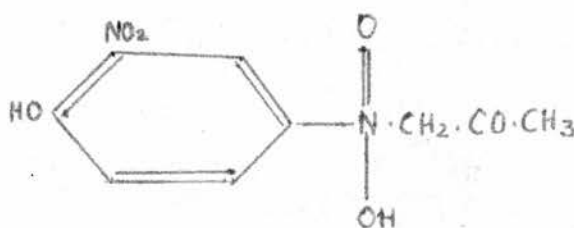


Anslo-

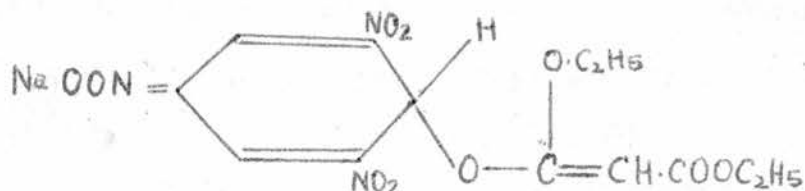
Anslow and King<sup>49</sup> represent the reaction product of 2:4 dinitrophenol and acetone in alkaline medium thus:-



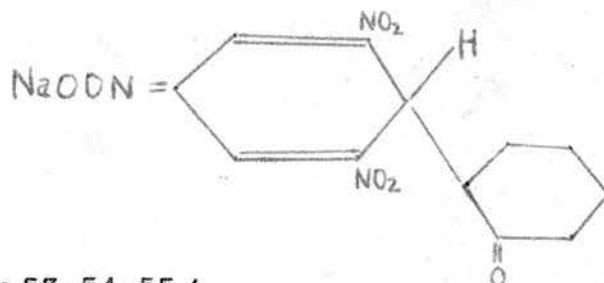
Reissert<sup>50</sup> represents the same reaction product as follows:-



Jackson<sup>51</sup> proposed the following structure for the compound obtained from 1:3:5 trinitrobenzene and diethylmalonate in alkaline solution.



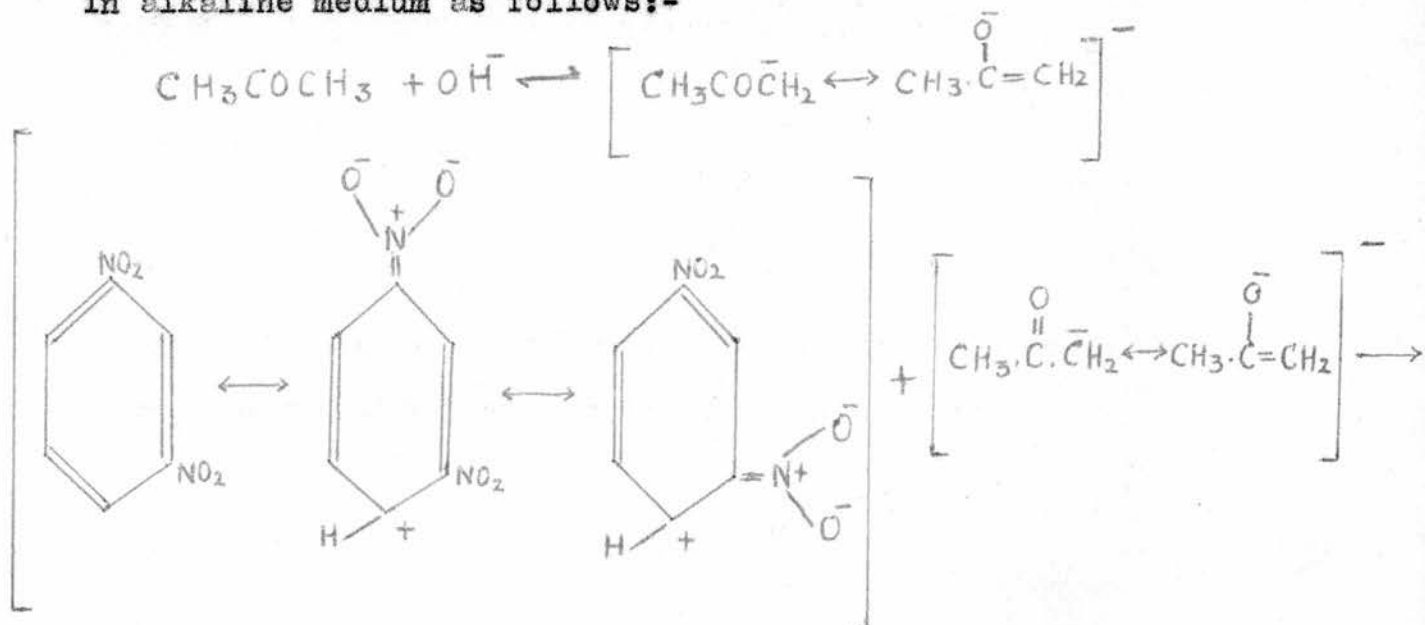
Zimmerman<sup>52</sup> represents the condensation product of 1:3:5 trinitrobenzene and cyclo-hexanone in alkaline medium as follows:-

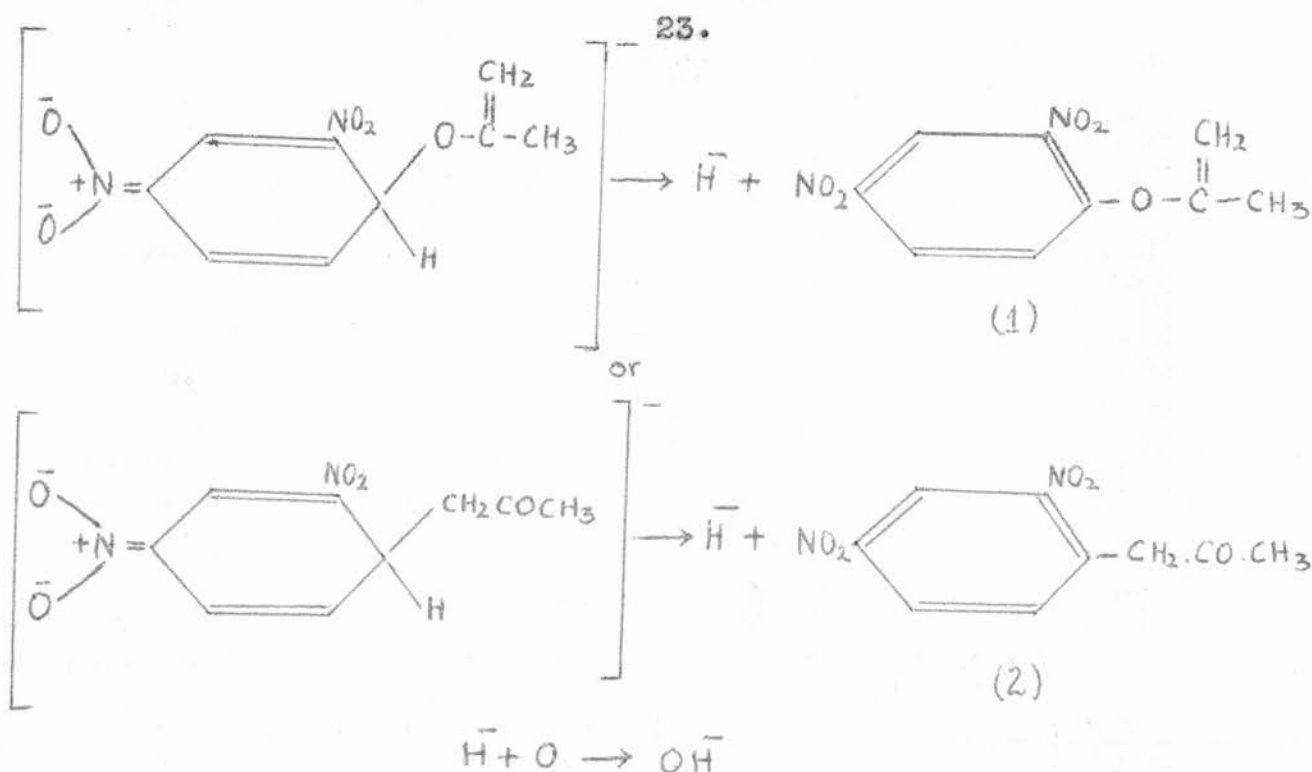


Canback<sup>53, 54, 55/</sup>

Canback<sup>53,54,55</sup> who has made a special study of the reaction between aromatic nitro compounds and active methylene groups in the presence of alkali by means of spectrophotometric methods suggests a mechanism of the reaction based on the modern concept of the relation between light absorption and constitution of organic compounds on the basis of the influence of resonance phenomena in the production of colour in organic molecules. He investigated the difference in behaviour of twenty-nine derivatives of m-dinitrobenzene in their reaction with acetone in alkaline solution and discusses the influence of substituents in the benzene ring on the reaction and light absorption of the addition products. He states that to obtain a positive reaction it is necessary that two nitro groups are present on the benzene ring, that they are in meta-position to each other and that at least one of the ortho-positions of the nitro group must be occupied by a hydrogen atom. He considers that the benzene ring acts as a pseudo-acid when it contains several nitro-groups in meta-positions and that the essential part of the reaction is an addition of the anion to the nucleus.

He represents the reaction between m-dinitrobenzene and acetone in alkaline medium as follows:-





He also states that attempts to isolate 2:4 dinitro phenyl isopropenyl ether (1) or 2:4 dinitro phenyl acetone (2) from the reaction products have been unsuccessful and the course of the reaction therefore not proved.

From a study of the absorption data of the various addition products of m-dinitrobenzene and active methylene compounds Canback concludes that the power to activate the methylene group so much that a positive reaction is obtained with m-dinitrobenzene in alkaline solution is not restricted to the grouping  $-\text{CH}_2\text{CO}-$ . Other groups such as  $-\text{CH}_2\text{CN}$ ,  $-\text{CH}_2\text{NO}_2$  and  $-\text{CH}_2\text{CH}=\text{CH}_2$  are on principle equally effective.

Janowsky's reaction has been used for the detection and estimation of substances capable of giving rise to poly-nitro derivatives on nitration in the following cases.

Illing/

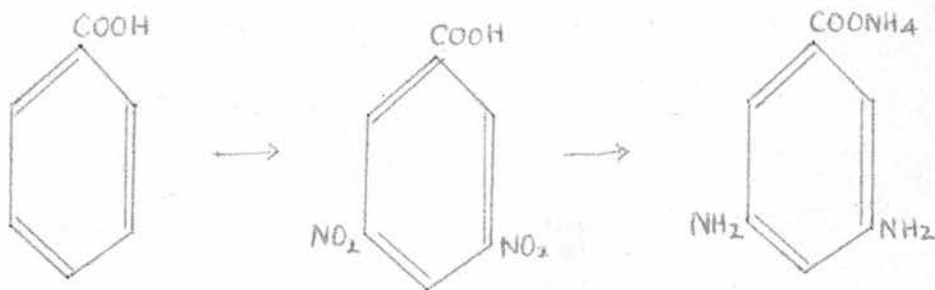
Illing and Stephenson<sup>56</sup> and Schechter et al<sup>57</sup> have applied the reaction for the determination of dicophane (D.D.T.) and related compounds. The author<sup>15,16,17,18</sup> has used the reaction for the detection of cocaine, amphetamine, phenylbarbiturates and benzoic acid. The Vitali-Morin reaction which as has already been mentioned is an instance of the reaction between aromatic nitro compounds and acetone in alkaline medium, has been used for the detection and estimation of a variety of compounds. Roberts and James<sup>58</sup> and Allport and Wilson<sup>59</sup> used the reaction for the assay of belladonna and stramonium, Canback<sup>60,61</sup> for the determination of syntropan (tropic acid ester of 3-diethyl amino - 2:2 dimethyl - 1 - propanol), trasentine (diethylamino cyclo hexenyl phenylacetate hydrochloride) and 2-arylmethyl-imidazolines and Seydlitz<sup>62</sup> for the estimation of amethocaine (tetracaine).

The detection and estimation of cardiac glycosides by Raymond<sup>63</sup> and of sex hormones by Zimmerman<sup>64</sup> are also based on Janovsky's reaction. Weise and Tropp<sup>65</sup> point out that the reaction between picric acid and creatinine in alkaline solution (Jaffe's reaction) is a special case of the reaction between aromatic nitro compounds and compounds containing active methylene groups.

## 2. MOHLER'S REACTION.

In 1890 Mohler<sup>12</sup> described a test for benzoic acid depending on the production of m-diaminobenzoic acid, the ammonium salt of which has an intense red colour in solution. This test is probably the most delicate of all tests for benzoic acid.

Mohler's original directions were to treat the benzoic acid with 2 ml. of concentrated sulphuric acid, to heat to about 240°C when white fumes are evolved from the acid and to add at this temperature several decigrams of sodium nitrate in small portions at a time. The reaction mixture is then cooled and poured into excess of dilute ammonia solution. The liquid which is light yellow in colour owing to the presence of m-dinitrobenzoic acid becomes deep red on the addition of a drop of ammonium sulphide solution owing to the reduction of the nitro-acid to m-diaminobenzoic acid, the ammonium salt of which is red in solution.



This test was used with minor modifications by Reed<sup>66</sup>, Halphen<sup>67</sup>, Robin<sup>68</sup>, Filandeau and Bonis<sup>69</sup> and Liverseige and Evers<sup>70</sup>.

Von der Heide and Jakob<sup>71</sup> showed that nitration at a high temperature/

temperature gave erratic results and that the test was much more sensitive if the acid-mixture be heated not higher than 130°C and kept at this temperature for 10 minutes. Equally good results were obtained by heating the acid-mixture in a steam-bath for 20 minutes.

Grossfeld<sup>72</sup> introduced an important modification. He substituted hydroxylamine for ammonium sulphide as the reducing agent and showed that hydroxylamine gave a purer and more permanent colour. He recommended the following method: The residue of benzoic acid is heated for 20 minutes on a steam-bath with 1 ml. of sulphuric acid and 0.1 g. of potassium nitrate. The mixture is cooled, 2 ml. of water added, again cooled and treated with 10 ml. of 15% ammonia solution and 2 ml. of 2% hydroxylamine hydrochloride solution.

The colour develops slowly and may be hastened by warming on the steam-bath, but attains its maximum colour on subsequent cooling. He also pointed out that the accuracy of the method depended on the correct duration of nitration; too short or too long a time led to low results.

Illing<sup>73</sup> investigated the application of Mohler's reaction to the colorimetric estimation of benzoic acid and found that Grossfeld's modification of the test gave inconsistent results. Illing studied each stage in the reaction and summarises his observations as follows:-

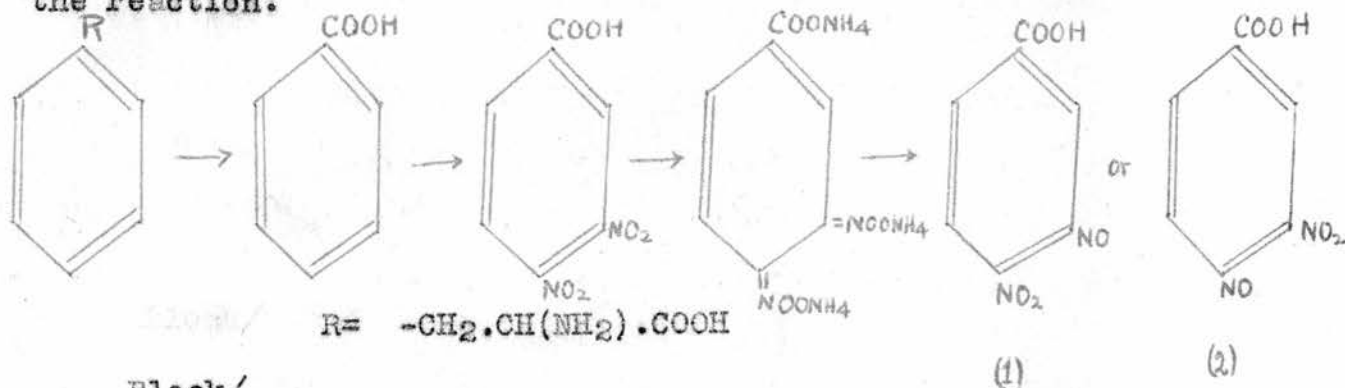
Nitration: This must be carried out under specified conditions.

Temperature and time are important factors as is also the quantity of sulphuric acid. The best results were obtained by the use of a boiling/

boiling tube immersed in boiling water for 20 minutes, but continuation of the nitration for a further 5 minutes has no effect on the final result. It is better to add first the potassium nitrate and then the sulphuric acid.

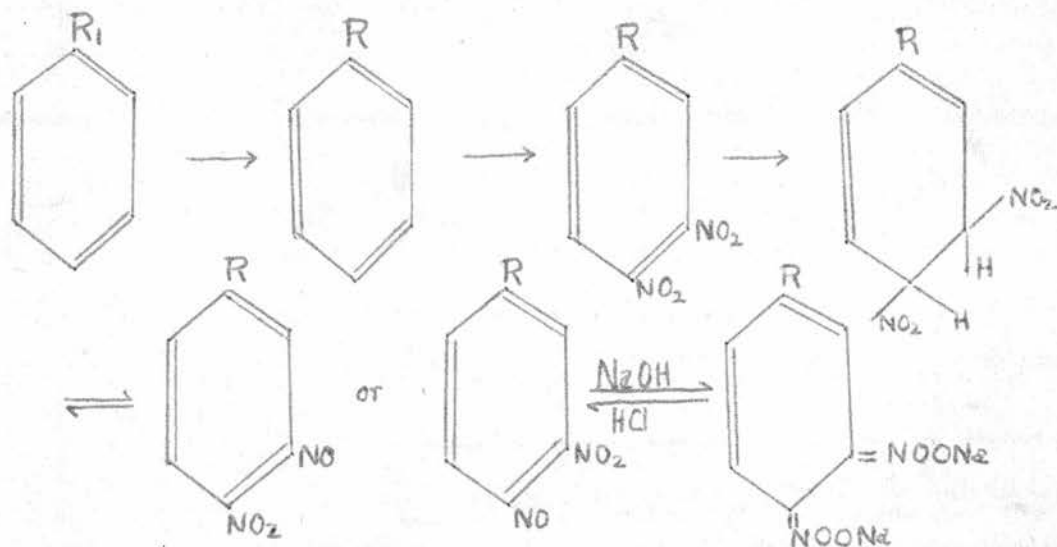
**Reduction:** The reduction must also be carried out under strictly standard conditions. The two essential factors are again time and temperature. The temperature must be carefully regulated as the amino-compound formed is decomposed if it is heated at too high a temperature. It was found that the amino-compound showed signs of decomposition when heated for 20 minutes at 70°C but decomposition could not be detected when the duration of the heating was for 3 to 10 minutes. At a temperature appreciably below 60°C the reduction was not complete in 20 minutes but at 65°C only 5 minutes were required for the maximum development of colour. A variation of 3 to 4°C either way during the heating has no appreciable effect.

Kapeller-Adler<sup>74</sup> applied Mohler's reaction for the determination of phenylalanine in protein hydrolysates. She expected the reaction to proceed as in the case of benzoic acid but found that instead of the expected cherry-red colour a violet colour was obtained. To explain this she proposed the following mechanism of the reaction.



Block/

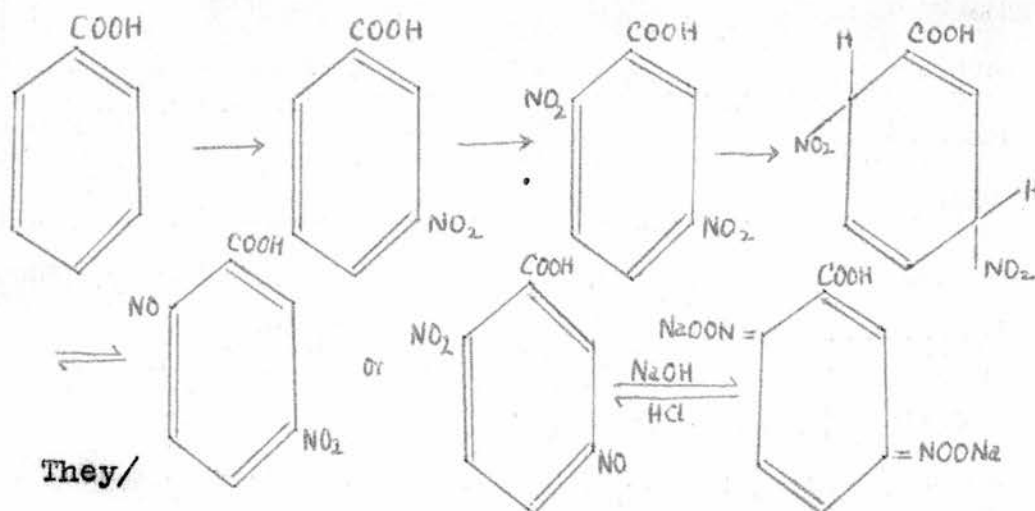
Block and Bolling<sup>75</sup> failed to isolate either compound (1) or (2). They carried out experiments to elucidate the reactions involved in the formation of the violet colour from phenylalanine. They proposed the following mechanism of the reaction.



R<sub>1</sub> may be  $-\text{CH}_2\text{CH}(\text{NH}_2)\cdot\text{COOH}$ ;  $-\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$  or  $-\text{CH}_2\text{COOH}$ .

R may be  $-\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ ;  $-\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$ ;  $-\text{CH}_2\text{COOH}$  or  $-\text{COOH}$ .

They are of opinion that the cherry-red colour obtained with benzoic acid in Mohler's reaction is not due to the reduction of 3:5 dinitrobenzoic acid but is probably formed from 2:5 dinitrobenzoic acid.



They summarize the results of their investigation as follows:-

1. Nitration of phenylalanine, phenylacetic acid and  $\beta$ -phenylpropionic acid yields the p-nitro derivatives, these are further nitrated to 3:4 dinitro compounds.
2. Derivatives of o-dinitrobenzene are relatively easily reduced by hydroxylamine or hydrogen sulphide to the corresponding derivatives of diaci-o-dinitrohydrobenzene which in alkaline solution form the violet o-quinone-like compounds of o-isodinitrohydrobenzene. On the other hand reduction of derivatives of p-dinitrobenzene yields under the same conditions cherry-red p-quinone-like compounds of p-isodinitrohydrobenzene.

They also observed that of the four dinitrotoluenes 2:3, 3:4, 2:4 and 2:5 the two o-dinitrotoluenes (2:3 and 3:4) gave violet colours on reduction with hydroxylamine while 2:5 dinitrotoluene gave a cherry-red colour.

Mohler's reaction has been applied only in a few cases. Illing<sup>25,73,76</sup> used the reaction for the detection and estimation of benzoic acid and amphetamine; Illing and Stephenson<sup>56</sup> for dicophane (D.D.T.) and Kapeller-Adler<sup>74</sup> for the estimation of phenylalanine.

## EXPERIMENTAL

### Methods used in the study of the application of Janovsky's and Mohler's reactions.

#### NITRATION.

Nitration to polynitro derivatives is a feature common to both reactions. Fuming nitric acid has been successfully used for this purpose in a few cases but in a majority of cases it fails to introduce more than one nitro-group (e.g. amphetamine, cocaine, phenylbarbiturates).

A mixture of acetic anhydride and fuming nitric acid is a very energetic nitrating agent; such a mixture was therefore tried, hoping that it would succeed, where fuming nitric acid had failed, in producing intensive nitration. An advantage with such a nitrating mixture is that the by-product of nitration is acetic acid which is easily removed thereby obviating the necessity for isolating the products of nitration by solvent extraction as is done when a mixture of nitric and sulphuric acids is used for nitration in Janovsky's test. Preliminary experiments with amphetamine and cocaine, however, proved unsuccessful. Nitration was carried out at room temperature and at 55°C. At the latter temperature the mixture reacted with explosive violence. Further experiments with this nitrating mixture were abandoned.

A mixture of potassium nitrate and sulphuric acid, as recommended by Grossfeld<sup>72</sup> was finally chosen as the nitrating agent best suited/

suited for the purpose of this investigation. Nitration was carried out in test-tubes (provided with glass stoppers) by heating with 0.1 g. of potassium nitrate and 0.5 ml. of concentrated sulphuric acid, in a boiling water bath. A nitration time of 15 minutes was found to be sufficient; heating for a further 5 minutes made no appreciable difference.

#### REDUCTION (Mohler's test)

Hydroxylamine hydrochloride was used as reducing agent. The rate of development of colour in Mohler's test depends on three factors:- (1) Temperature, (2) Concentration of ammonia and (3) Concentration of hydroxylamine hydrochloride.

#### Temperature:

Grossfeld who studied the application of Mohler's test to the determination of benzoic acid, observed that heat accelerated the development of colour. This was later confirmed by Illing who found, in the case of benzoic acid, that while reduction was not complete in 20 minutes at a temperature appreciably below 60°C only 5 minutes were required for maximum development of colour at 65°C. He also observed that the amino-compound formed on reduction was, however, liable to decomposition if heated at too high a temperature. The use of heat to accelerate the development of colour, therefore offers no advantage as it can also cause decomposition of the chromogen. It was therefore preferred to allow the development of colour to proceed at room-temperature (15 to 17°C).

#### Concentration of ammonia and hydroxylamine hydrochloride.

Neither/

Neither Grossfeld nor Illing appear to have studied the influence of the concentration of ammonia and hydroxylamine hydrochloride on the rate of development of colour. They used 15% ammonia and 2% hydroxylamine hydrochloride. It was found, however, that higher concentrations of ammonia and hydroxylamine hydrochloride hastened the development of colour quite appreciably. The time required, in the case of amphetamine, for maximum development of colour with different concentrations of ammonia and hydroxylamine hydrochloride is as follows:-

|                    | 2 ml. of 2% W/V hydroxyl-<br>amine hydrochloride + 5 ml.<br>of AMMONIA |     | 5 ml. of 30% W/V ammonia<br>solution + 2 ml. of<br>HYDROXYLAMINE HYDROCHLORIDE |    |
|--------------------|------------------------------------------------------------------------|-----|--------------------------------------------------------------------------------|----|
|                    | 20%                                                                    | 30% | 2%                                                                             | 5% |
| Time in<br>minutes | 150                                                                    | 100 | 100                                                                            | 50 |

With 30% ammonia and 5% hydroxylamine hydrochloride maximum intensity of colour is attained in a much shorter time. It was observed, however, that when 5% hydroxylamine hydrochloride solution was used, a light yellow colour developed in the 'blank' whereas with 2% hydroxylamine hydrochloride the 'blank' remained practically colourless. It was therefore preferred to use a 2% solution of hydroxylamine hydrochloride as reducing agent. Using 30% w/v solution of ammonia and 2% w/v solution of hydroxylamine hydrochloride, maximum intensity, in the case of benzoic acid, was reached in about 15 minutes.

The/

# MOHLER'S REACTION

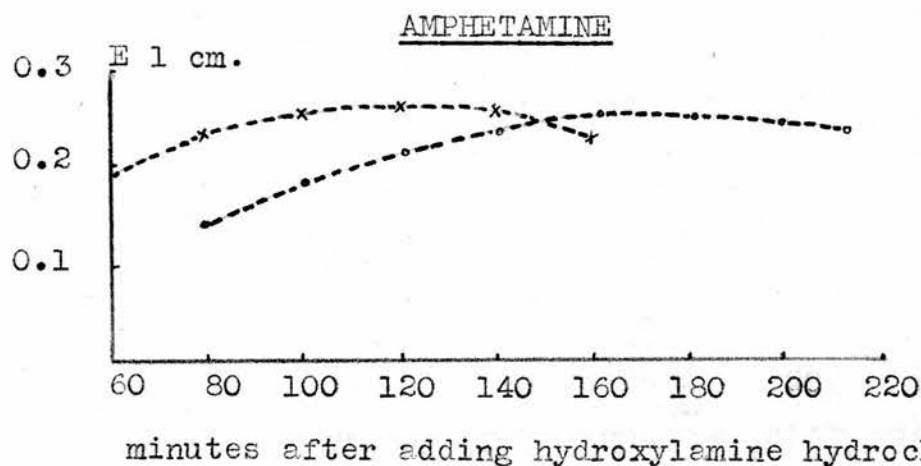


Fig. 1. Effect of concentration of ammonia on the rate of development of colour at 550 mμ.

x-----x = 5 ml. of 30% ammonia solution.  
 o-----o = 5 ml. of 20% ammonia solution.

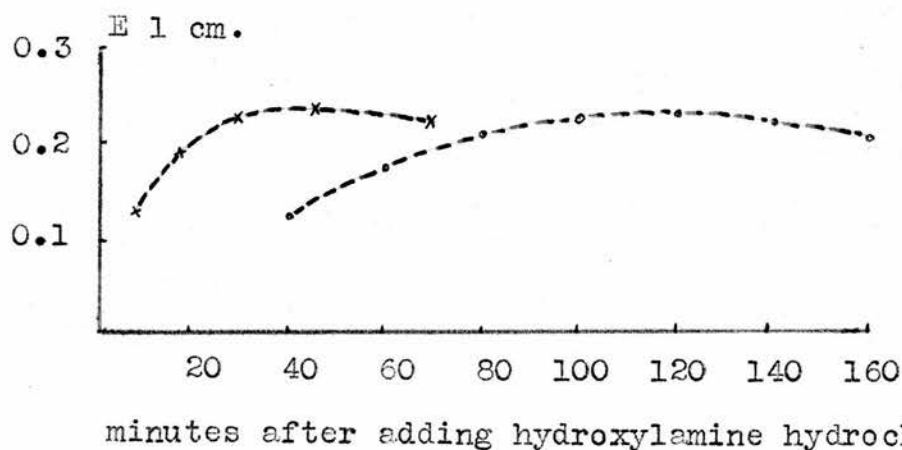


Fig. 2. Effect of concentration of hydroxylamine hydrochloride on the rate of development of colour at 550 mμ.

x-----x = 2 ml. of 5% hydroxylamine hydrochloride soln.  
 o-----o = 2 ml. of 2% " " "

The effect of the concentration of ammonia and hydroxylamine hydrochloride on the rate of development of colour is shown in Figs. 1 and 2.

REAGENTS:

|                             |                            |                                    |
|-----------------------------|----------------------------|------------------------------------|
| Sulphuric acid              | Analar Grade               | Sp.Gr. 1.84.                       |
| Potassium nitrate           | " "                        |                                    |
| Ammonia solution            | " "                        | 30% w/v                            |
| Hydroxylamine hydrochloride | " "                        | 2% w/v aqueous solution            |
| Ether                       | Analar Grade - redistilled |                                    |
| Chloroform                  | " "                        | "                                  |
| Acetone                     | " "                        |                                    |
| Sodium hydroxide            | " "                        | 10% w/v (2.5N) aqueous soln.       |
| Potassium hydroxide         | " "                        | 3% w/v solution in methyl alcohol. |

Acetone-Alcohol mixture : 2 vols. of acetone + 1 vol. of absolute alcohol.

Procedure: Using alcohol transfer the alkaloidal residue or an aliquot thereof to a test-tube. Remove the alcohol by heating in a boiling water bath, care being taken not to lose any of the sample mechanically during the evaporation of the solvent. Remove the last traces of alcohol either by evacuating the test-tube or by means of a gentle stream of air from a hand blower, care being taken not to blow out any of the residue. Unless the alcohol is completely removed it may react violently with the nitrating mixture. To the residue in the test-tube add 0.1 g. of potassium nitrate and 0.5/

0.5 ml. of concentrated sulphuric acid and heat in a boiling water bath for 15 minutes. Remove the test-tube occasionally from the bath, tilt slightly and rotate it gently so that the contents wet the sides completely about half-way up the test-tube.

1. MOHLER'S REACTION.

Cool the test-tube and contents in iced water, add 2.5 ml. of distilled water and mix well. While still in the cold bath add slowly 5 ml. of ammonia solution along the sides of the test-tube and mix gently so as to prevent any undue evolution of heat. Stopper the test-tube, mix the contents thoroughly by shaking and allow the test-tube to stand in the cold bath for 2 minutes longer. At this stage potassium sulphate is precipitated but redissolves later. Transfer the test-tube and contents to a beaker of water at room temperature ( $15^{\circ}$  to  $17^{\circ}\text{C}$ ), add 2 ml. of hydroxylamine hydrochloride solution and mix well. When maximum intensity of colour is attained, remove the test-tube from the bath, mix the contents gently by inverting the test-tube a few times, transfer to a 1 cm. cell and read extinction.

All readings were taken against a blank prepared in the same way.

2. JANOVSKY'S REACTION.

(a) Qualitative test:-

(i) Acid-ether extract:- After nitration, dilute the contents of the test-tube to about 20 ml. and transfer to a separator. Extract once/

once with 15 ml. of ether. Wash the ether extract with a little water and filter through a plug of cotton wool layered with anhydrous sodium sulphate. Remove the ether and reserve the residue for testing as described below.

(ii) Alkaline-Chloroform extract:- Make the aqueous liquid remaining after the ether extraction, alkaline to litmus with dilute ammonia solution and extract once with 15 ml. of chloroform. Wash the chloroform extract with a little water and filter through a plug of cotton wool layered with anhydrous sodium sulphate. Remove the chloroform and reserve the residue for testing as described below.

#### Tests.

(i) Acid-ether extract:- Dissolve the residue in 2 ml. of acetone-alcohol mixture, transfer to a test-tube, add 2 to 3 drops of methyl alcoholic potash and mix well. Observe any colour that develops; allow to stand for some time and note any change in colour.

In the case of the acid-ether extracts, acetone-alcohol mixture was found to be the best solvent for obtaining a more or less stable colour; when acetone alone is used the colour that develops fades almost immediately. Ether was found to be the best solvent for the extraction of nitro-derivatives that come out from acid-medium. Chloroform is a poor solvent for them.

(ii) Alkaline-chloroform extract:- Dissolve the residue in 2 ml. of acetone, transfer to a test-tube, add 2 drops of sodium hydroxide solution/

solution and shake vigorously for a few seconds. Observe any colour that develops. Note any change in colour on standing.  
or Dissolve the residue in 2 ml. of acetone, transfer to a test-tube, add 2 drops of methyl alcoholic potash and mix well. Note any colour that develops.

With aqueous sodium hydroxide, the colour develops slowly and is stable whereas with methyl alcoholic potash the colour develops immediately and is more intense but fades very rapidly.

(b) Quantitative method.

(i) Acid-ether extract:- After nitration, transfer the contents of the test-tube quantitatively to a separator, with small portions of distilled water at a time until the total volume is about 20 ml. Extract twice with ether using 15 ml. of ether for each extraction. Wash each ether extract in turn with the same 4 ml. of water, filter through a plug of cotton wool layered with anhydrous sodium sulphate and finally wash the filter with a little ether. Transfer the ether extract to a test-tube in small portions at a time and remove the ether. Dissolve the residue in 10 ml. of acetone-alcohol mixture, add 0.2 ml. of methyl alcoholic potash, mix well, and transfer to a 1 cm. cell. Read maximum extinction.

All readings were taken against a blank consisting of 10 ml. of acetone-alcohol mixture and 0.2 ml. of methyl alcoholic potash.

(ii) Alkaline-chloroform extract:- Add the aqueous wash of the ether extract, to the main bulk of aqueous liquid, make alkaline to litmus with dilute solution of ammonia and extract twice with chloroform/

chloroform using 15 ml. of chloroform for each extraction. Wash each chloroform extract with the same 5 ml. of water, filter through a plug of cotton wool layered with anhydrous sodium sulphate and finally wash the filter with a little chloroform. Transfer the chloroform extract to a test-tube, in small portions at a time and remove the chloroform. Dissolve the residue in 10 ml. of 80% v/v aqueous acetone, add 0.2 ml. of sodium hydroxide solution, stopper the tube and shake vigorously for a few seconds. Transfer to a 1 cm. cell and read maximum extinction.

All readings were taken against a blank consisting of 10 ml. of 80% acetone and 0.2 ml. of aqueous alkali.

Spectrophotometric measurements were made with the S.P.350 Diffraction Grating Spectrophotometer.

The colour reactions indicated in the succeeding pages were those obtained using about 0.2 mg. of each substance for the test.

Studies in the application of Janovsky's and Mohler's reactions.

The alkaloids and other substances studied in this investigation were arranged into the following groups.

Group I. The Sympathomimetic Amines.

- (i) Noradrenaline, adrenaline, isoprenaline, epinine.
- (ii) Neosynephrin, pholedrine, paredrine.
- (iii) Propadrine, ephedrine; amphetamine, dexamphetamine and methylamphetamine.

Group II. The alkaloids of the atropine group.

- (i) Atropine, hyoscyamine and hyoscine.
- (ii) Homatropine.

Group III. The local anaesthetics of the benzoic ester group, consisting of cocaine and the cocaine substitutes.

- (i) Benzocaine, butyl-p-amino-benzoate, orthocaine, procaine, butacaine and amethocaine.
- (ii) Cocaine, metycaine, benzamine, amydracaine and amylocaine.

Group IV. The aconite alkaloids.

- (i) Aconitine.

Group V. (i) The opium alkaloids consisting of morphine, codeine, narcotine and the artificial morphine derivative, diacetyl morphine - diacetylmorphine or heroin.

- (ii) The synthetic morphine substitutes.

Pethidine and amidone.

Group VI. The antipyretic drugs

- (i)/

(i) phenazone, amidopyrine.

(ii) Phenacetin, acetanilide.

Group VII. Miscellaneous alkaloids containing no benzene or naphthalene nucleus.

Caffeine, strychnine, brucine, veratrine, quinine and yohimbine.

Group VIII. The barbiturates.

(i) Barbitone, allobarbitone.

(ii) The phenyl-barbiturates: pheno-barbitone, phemitone, and rutonal.

(iii) The cyclohexenyl derivatives: cyclobarbitone and hexobarbitone.

Group I.            The Sympathomimetic Amines.

Numerous ethylamine derivatives possessing powerful physiological properties have been isolated from various plant and animal sources. Many of these are derivatives of p-hydroxyl-phenyl-ethylamine and have the property of producing effects very similar to those produced by stimulation of the sympathetic nervous system. The most important compound of this class is adrenaline the active principle of the supra-renal glands.

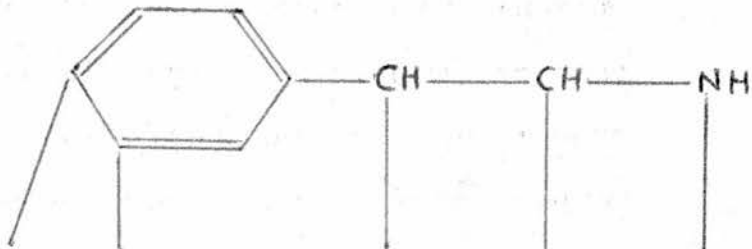
The first extensive study of compounds chemically related to adrenaline was the classical investigation of Barger and Dale<sup>77</sup>. They examined a large series of related synthetic amines, defined the structural requirements for pharmacological activity and introduced the term "Sympathomimetic" to describe the type of action manifested by the group as a whole.

Ephedrine was first isolated by Nagai<sup>78</sup> from the chinese herb Ma Huang a drug which has been used by the Chinese from early times. Chen and Schmidt<sup>79</sup> made a systematic pharmacological investigation of ephedrine and demonstrated its adrenaline-like action. It differs chemically from adrenaline in that it lacks the two hydroxyl groups in the benzene nucleus and contains an extra carbon atom in the aliphatic side chain. These differences account for the many properties of ephedrine which are distinct from those of adrenaline, particularly greater stability and action on the central nervous system.

The discovery of ephedrine stimulated the search for other sympathomimetic/

sympathomimetic amines which would possess advantages over adrenaline.

Adrenaline and ephedrine are the two older members of the group, the others which have subsequently been introduced are noradrenaline, synephrin, neosynephrin, paredrine, propadrine, pholedrine, isoprenaline, amphetamine, methylamphetamine, etc.



|     |                         |    |    |    |                 |                                   |
|-----|-------------------------|----|----|----|-----------------|-----------------------------------|
| (a) | Noradrenaline           | OH | OH | OH | H               | H                                 |
|     | Adrenaline              | OH | OH | OH | H               | CH <sub>3</sub>                   |
|     | Isoprenaline            | OH | OH | OH | H               | CH(CH <sub>3</sub> ) <sub>2</sub> |
|     | Epinine                 | OH | OH | H  | H               | CH <sub>3</sub>                   |
| (b) | Paredrine               | OH | H  | H  | CH <sub>3</sub> | H                                 |
|     | Pholedrine              | OH | H  | H  | CH <sub>3</sub> | CH <sub>3</sub>                   |
|     | Neosynephrin            | H  | OH | OH | H               | CH <sub>3</sub>                   |
| (c) | Propadrine              | H  | H  | OH | CH <sub>3</sub> | H                                 |
|     | Ephedrine               | H  | H  | OH | CH <sub>3</sub> | CH <sub>3</sub>                   |
|     | Amphetamine             | H  | H  | H  | CH <sub>3</sub> | H                                 |
|     | Methyl-amphet-<br>amine | H  | H  | H  | CH <sub>3</sub> | CH <sub>3</sub>                   |

From a chemical standpoint the sympathomimetic amines may be/

be divided into three groups:-

Group (a) Consisting of the amines which have two hydroxyl groups in the benzene nucleus:- nor-adrenaline, adrenaline, isoprenaline and epinine.

Group (b) Consisting of those that contain only one hydroxyl group in the benzene nucleus:- paredrine, pholedrine and neosynephrin.

and Group (c) Consisting of the amines which have no substituents in the benzene ring:- propadrine, ephedrine, amphetamine and methylamphetamine.

#### 1. Mohler's reaction.

The members of groups (a) and (b) give only yellow colours. Of the members of group (c) amphetamine, dexamphetamine and methylamphetamine give beautiful purple colours whereas ephedrine and propadrine give brownish violet colours. The observation that ephedrine gives a brownish-violet colour is contrary to that made by Illing<sup>76</sup> who states that ephedrine gives a deep yellow colour in Mohler's test.

| <u>Substance</u> | <u>Colour obtained in Mohler's test</u> |
|------------------|-----------------------------------------|
| Noradrenaline    | yellow                                  |
| Adrenaline       |                                         |
| Isoprenaline     |                                         |
| Epinine          |                                         |
| Paredrine        | yellow                                  |
| Pholedrine       |                                         |
| Neosynephrin     |                                         |
| Amphetamine/     |                                         |

| <u>Substance</u>  | <u>Colour obtained in Mohler's test.</u> |
|-------------------|------------------------------------------|
| Amphetamine       | purple                                   |
| Dexamphetamine    |                                          |
| Methylamphetamine |                                          |
| Propadrine        | brownish-violet.                         |
| Ephedrine         |                                          |

The absorption curves (Figs. 5 and 6) of the colours obtained with amphetamine and methylamphetamine are similar; both curves exhibit a broad peak. Maximum absorption is in the region of 550 m $\mu$ . The rate of development of colour is shown in Figs. 3 and 4. In the case of amphetamine and methylamphetamine maximum intensity of colour is obtained in about 100 minutes and 85 minutes respectively and thereafter remains practically unchanged for a further 30 minutes.

The absorption curves of the colours (Figs. 11 and 12) obtained with propadrine and ephedrine are similar but differ from those of amphetamine and methylamphetamine. The curves do not exhibit any peak. The rate of development of colour is shown in Figs. 9 and 10. With propadrine and ephedrine maximum intensity of colour is reached in about 75 minutes and remains practically unchanged for a further 25 minutes.

Calibration data:- Extinctions measured in a 1 cm. cell are given below. The readings were taken after 110 minutes from the time of addition of hydroxylamine hydrochloride in the case of amphetamine, /

# MOHLER'S REACTION

## METHYLAMPHETAMINE

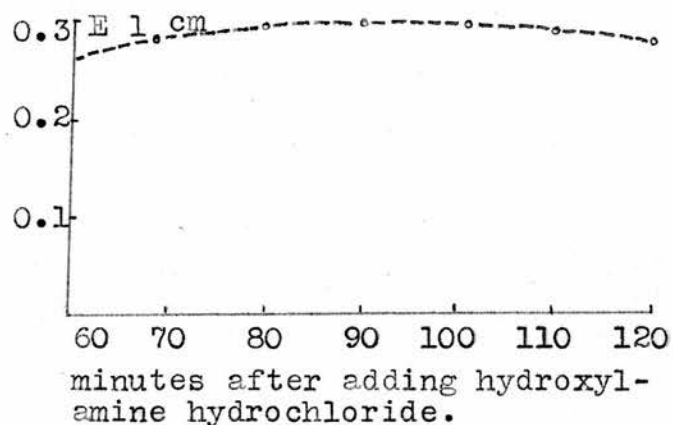


Fig. 4. Rate of development of colour at 550 mμ.

## AMPHETAMINE

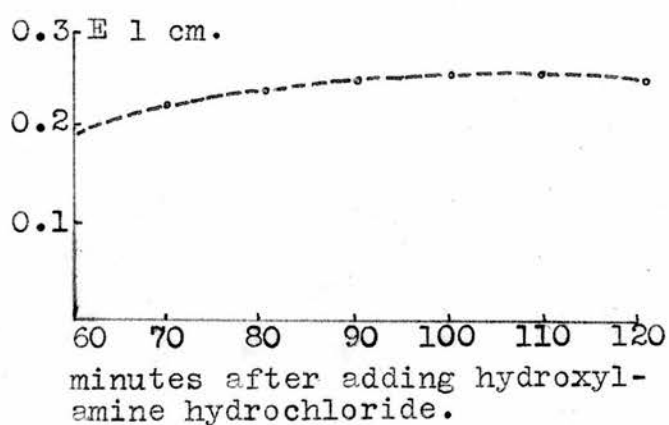


Fig. 3. Rate of development of colour at 550 mμ.

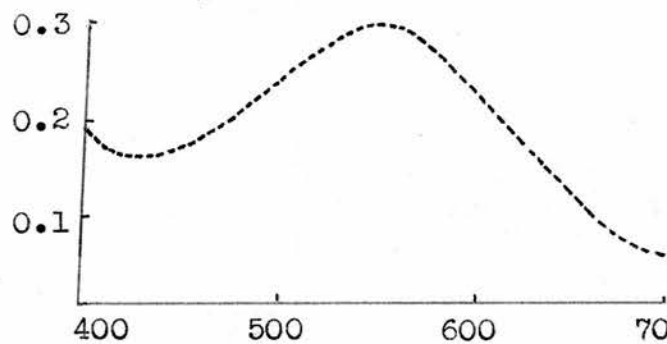


Fig. 6. Absorption curve of colour.

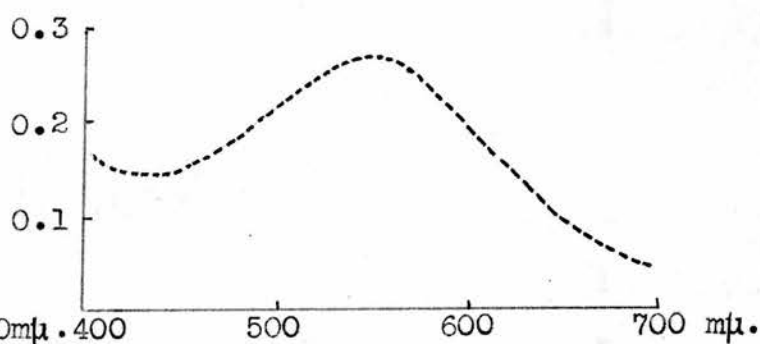


Fig. 5. Absorption curve of colour.

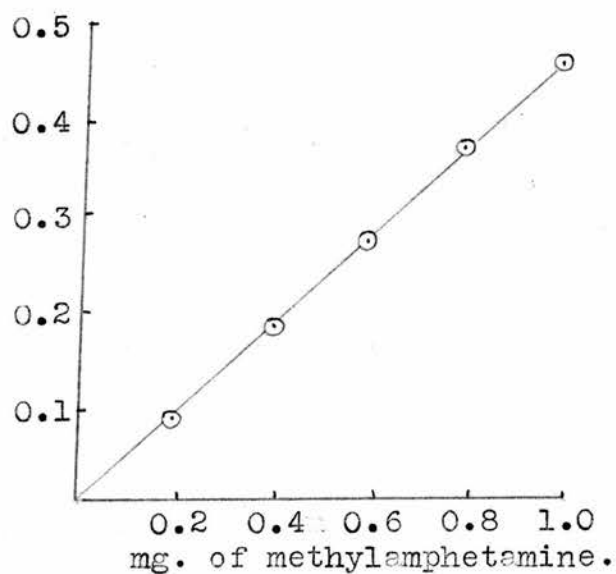


Fig. 8. Calibration curve at 550 mμ.

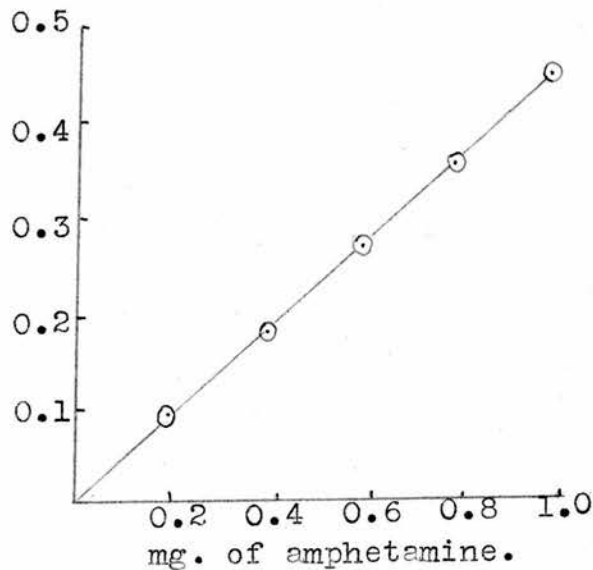


Fig. 7. Calibration curve at 550 mμ.

# MOHLER'S REACTION.

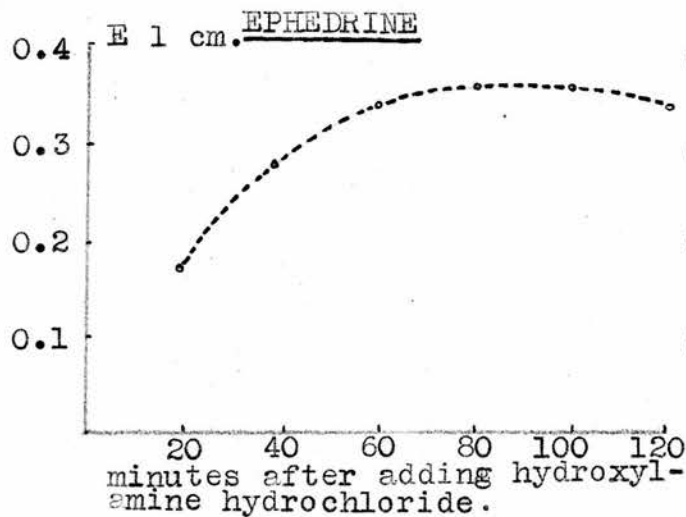


Fig.10. Rate of development of colour at 500 mμ.

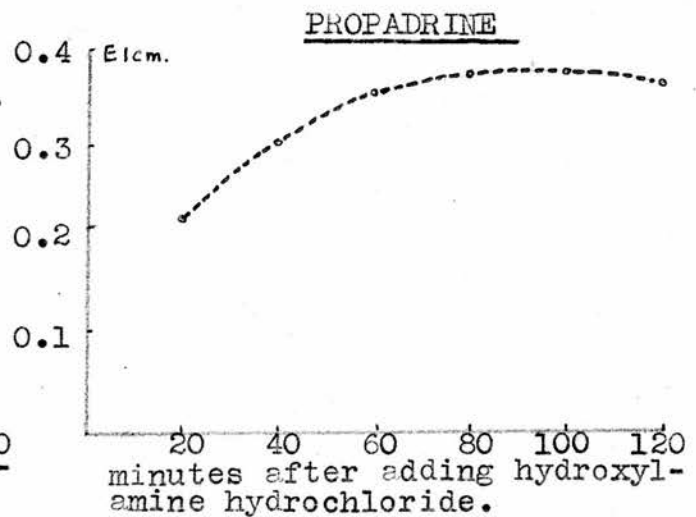


Fig.9. Rate of development of colour at 500 mμ.

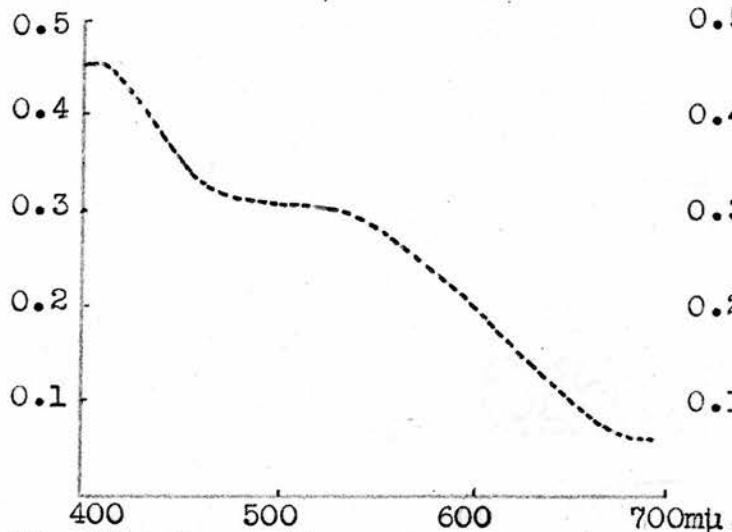


Fig.12. Absorption curve of colour.

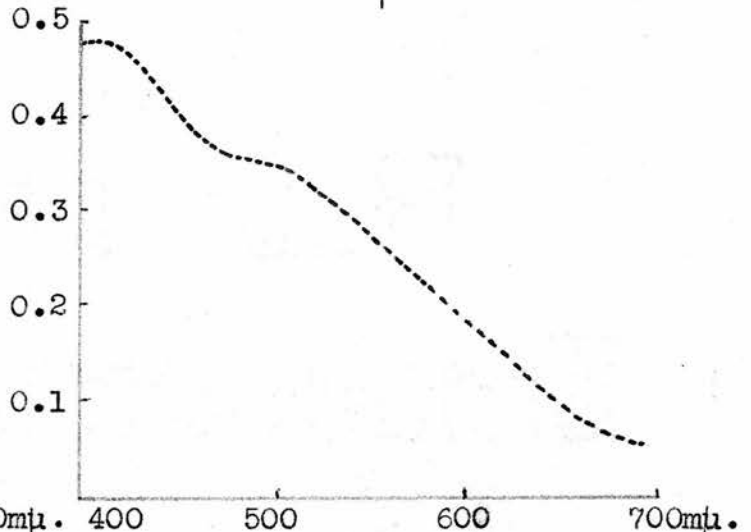


Fig.11. Absorption curve of colour.

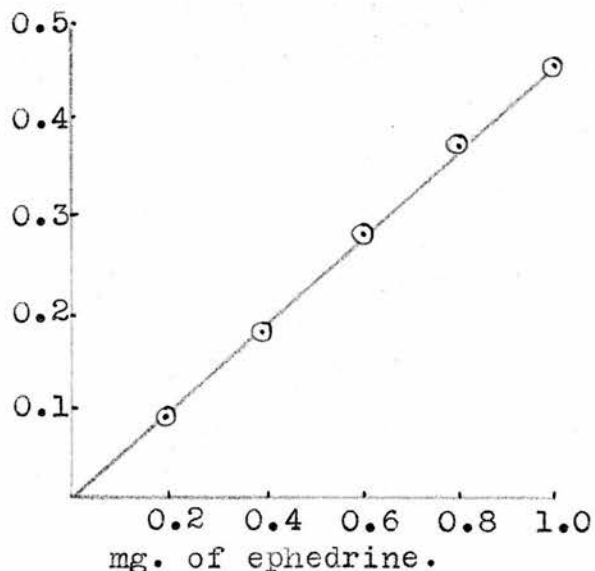


Fig.14. Calibration curve at 500mμ.

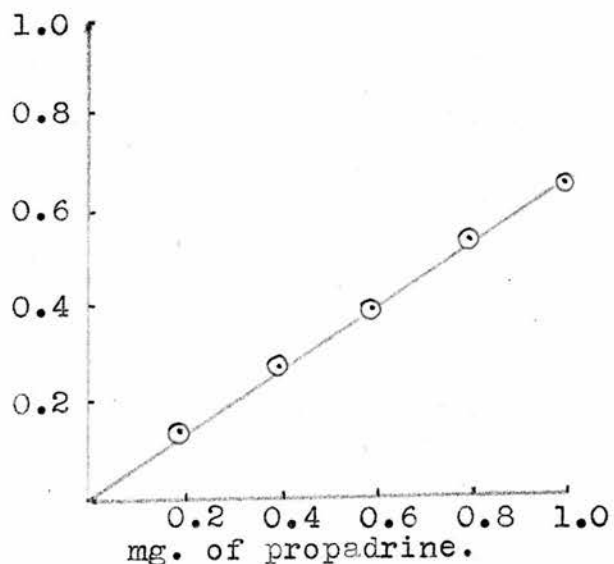


Fig.13. Calibration curve at 500mμ.

amphetamine, after 90 minutes in the case of methylamphetamine and after 80 minutes in the case of ephedrine and propadrine.

| <u>mg base</u> | <u>E 1 cm at 550 m<math>\mu</math></u>     |                                                  | <u>E 1 cm at 500 m<math>\mu</math></u> |                                           |
|----------------|--------------------------------------------|--------------------------------------------------|----------------------------------------|-------------------------------------------|
|                | <u>amphetamine</u><br><u>hydrochloride</u> | <u>methylamphetamine</u><br><u>hydrochloride</u> | <u>ephedrine</u><br><u>sulphate</u>    | <u>propadrine</u><br><u>hydrochloride</u> |
| 0.2            | .090                                       | .088                                             | .085                                   | .132                                      |
| 0.4            | .181                                       | .180                                             | .175                                   | .280                                      |
| 0.6            | .267                                       | .272                                             | .280                                   | .399                                      |
| 0.8            | .355                                       | .370                                             | .372                                   | .558                                      |
| 1.0            | .450                                       | .460                                             | .459                                   | .668                                      |

The calibration curves are shown in Figs. 7, 8, 13 and 14.

## 2. Janovsky's reaction.

The nitrating mixture is also an oxidising agent. Any non-basic nitro-derivative formed is, of course, extracted with ether from acid medium. The intensity of the colour obtained with the acid-ether/<sup>extract</sup> depends on the extent of the oxidation which appears to be complete in the case of pholedrine and paredrine and only slight in the case of ephedrine and propadrine.

The members of group (a) give no colour or only slight brownish-yellow colours. Of the members of group (b) those containing the hydroxyl group in para-position (pholedrine and paredrine) appear to be converted to tri-nitro derivatives, probably picric acid. With these two substances the predominant colour/

colour reaction is obtained with the acid-ether extract. The initial colour obtained is brownish-yellow which changes slowly to an orange colour. Picric acid behaves similarly. The alkaline-chloroform extracts give no colour reaction. In the case of neosynephrin which contains the hydroxyl group in meta-position no colour reactions are obtained either with the acid-ether extract or the alkaline-chloroform extract.

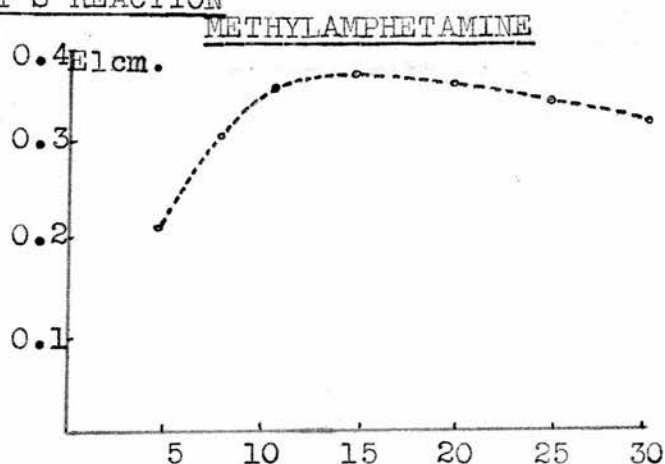
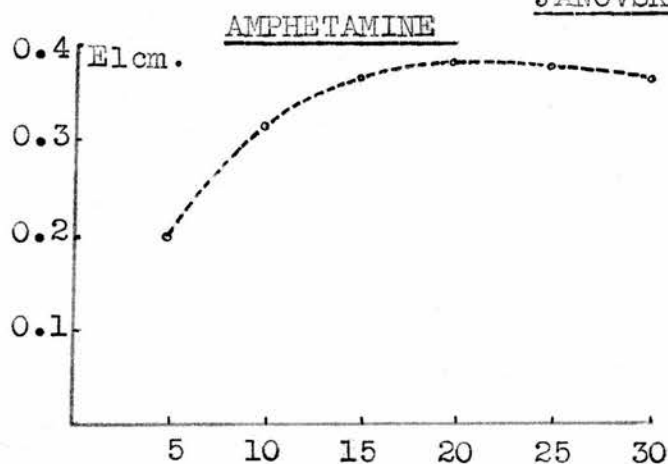
The predominant colour reaction in the case of the members of group (c) is obtained with the alkaline-chloroform extract. The acid-ether extracts in the case of amphetamine, dexamphetamine and methyldamphetamine give no colour reactions at all whereas with propadrine and ephedrine light reddish colours are obtained. The alkaline chloroform extracts from amphetamine, dexamphetamine and methyldamphetamine give blue colours with alcoholic potash and bluish-violet colours with aqueous sodium hydroxide solution. Ephedrine and propadrine give turbid-violet and bluish-violet colours with alcoholic potash and aqueous sodium hydroxide respectively. The colours obtained with alcoholic potash fade rapidly whereas those obtained with aqueous sodium hydroxide are stable.

Acid-ether extract/

|               | <u>Acid-ether extract</u> | <u>Alkaline-chloroform extract</u> |                                                |
|---------------|---------------------------|------------------------------------|------------------------------------------------|
|               |                           | <u>Alcoholic potash</u>            | <u>Aqueous sodium hydroxide.</u>               |
| Noradrenaline | no colour                 | no colour                          | no colour                                      |
| Adrenaline    |                           |                                    |                                                |
| Isoprenaline  |                           |                                    |                                                |
| Epinephrine   |                           |                                    |                                                |
| Pholedrine    | brownish-yellow           | no colour                          | no colour                                      |
| Propadrine    | changing to orange        |                                    |                                                |
| Neo-          |                           |                                    |                                                |
| synephrin     | no colour                 | no colour                          | no colour                                      |
| Amphetamine   | no colour                 | blue colour                        | bluish-violet                                  |
| Dex-          |                           |                                    |                                                |
| amphetamine   |                           |                                    |                                                |
| Methyl-       |                           |                                    |                                                |
| amphetamine   |                           | fades rapidly                      | develops slowly and changes to violet. Stable. |
| Ephedrine     | light reddish             | turbid violet                      | bluish-violet                                  |
| Propadrine    | colour                    | colour fades rapidly.              | develops slowly and changes to violet. Stable. |

The absorption curves (Figs. 15 and 16) of the colours obtained with amphetamine and methyleamphetamine are similar; both curves/

# JANOVSKY'S REACTION



mins. after adding sodium hydroxide. mins. after adding sodium hydroxide  
 Fig.15. Rate of development of colour at 560 mμ. Fig.16. Rate of development of colour at 560 mμ.

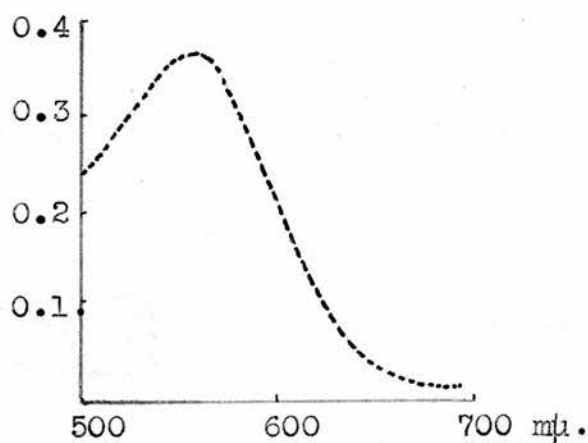
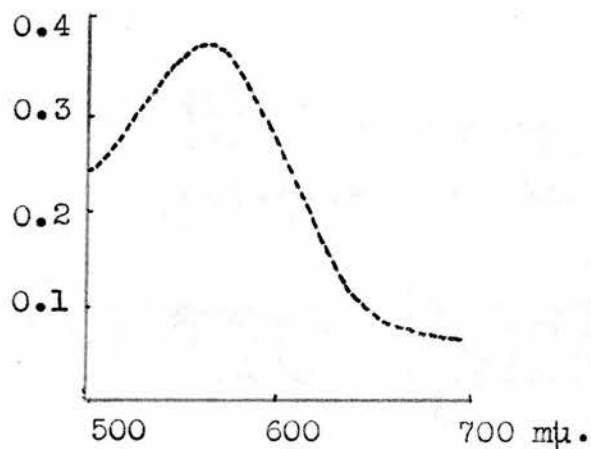


Fig.17. Absorption curve of colour. Fig.18. Absorption curve of colour.

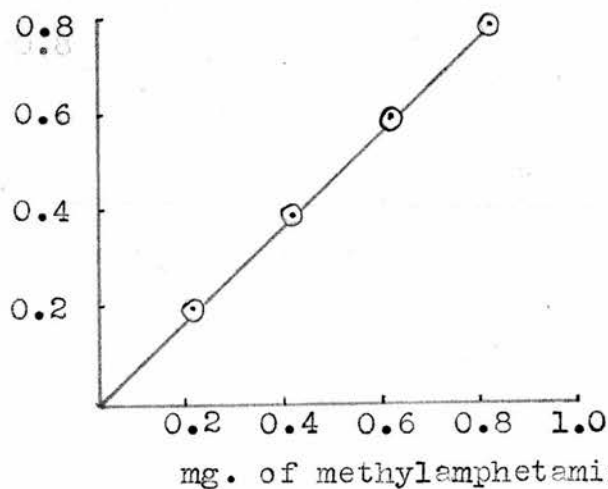
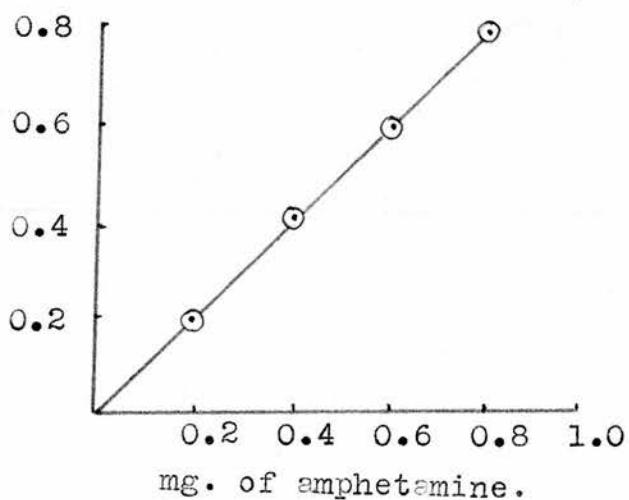


Fig.19. Calibration curve at 560mμ. Fig.20. Calibration curve at 560 mμ.

curves exhibit a fairly narrow peak. Maximum absorption is in the region of 560 m $\mu$ . The rate of development of colour is shown in Figs. 17 and 18. Maximum intensity is attained in about 15 minutes and thereafter the colour fades slowly.

Calibration data. The maximum extinctions measured at 560 m $\mu$  in a 1 cm. cell are given below.

Solvent = 10 ml. of 80% aq. acetone.

Alkali = 0.2 ml. of aq. Sodium hydroxide (10%)

E 1 cm. at 560 m $\mu$

| <u>mg. base</u> | <u>amphetamine hydrochloride</u> | <u>methyl amphetamine hydrochloride</u> |
|-----------------|----------------------------------|-----------------------------------------|
| 0.2             | .198                             | .190                                    |
| 0.4             | .404                             | .395                                    |
| 0.6             | .585                             | .587                                    |
| 0.8             | .776                             | .785                                    |

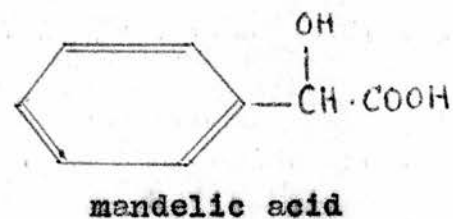
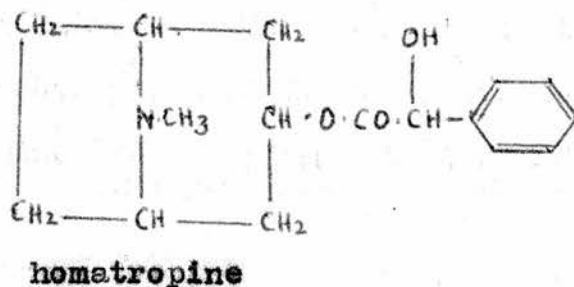
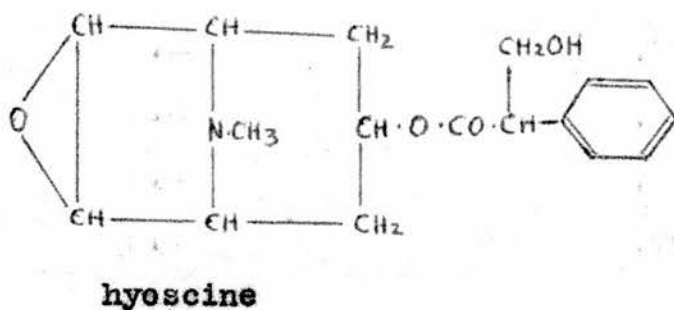
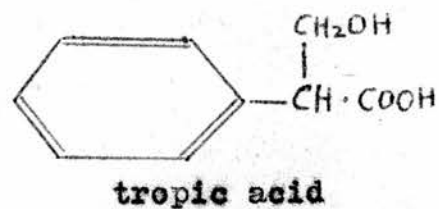
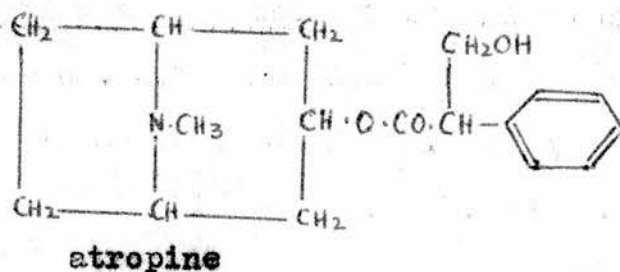
The calibration curves are shown in Figs. 19 and 20.

Janovsky's test is more sensitive than Mohler's for these substances. With Mohler's test 0.1 mg. is just detectable whereas Janovsky's test is sensitive to 0.02 mg. of amphetamine, dexamphetamine and methyamphetamine and 0.05 mg. of propadrine and ephedrine.

### Group II

This group consists of the naturally occurring tropic alkaloids/

alkaloids atropine, hyoscyamine and hyoscine and the synthetic alkaloid homatropine. The three naturally occurring alkaloids are esters of tropic acid while homatropine is the ester of mandelic acid.



The/

The well known Vitali<sup>13</sup> test for the solanaceous alkaloids atropine, hyoscyamine and hyoscine was introduced by Vitali in 1880. The test as described by Vitali is not very sensitive but the modification introduced by Morin<sup>14</sup> renders it a very sensitive test for these alkaloids. Methods based on the Vitali-Morin reaction have been developed by Allport and Wilson<sup>59</sup>, Roberts and James<sup>58</sup> and Canback<sup>80</sup> et al for the determination of very small quantities of these alkaloids. Several investigators, who studied the scope of the Vitali-Morin reaction, have found that it fails with many substances that might have been expected to give it. The test is more or less specific for the solanaceous alkaloids and the rich purple colour obtained with these alkaloids and usually referred to as "Vitali-purple" is indeed quite characteristic of these alkaloids. Homatropine does not respond to the test. Although the free acid does not respond to the test it has been shown by James and Roberts<sup>32</sup> that the methyl and ethyl esters of tropic acid give purple colours comparable to that of the tropic alkaloids.

#### 1. Mohler's reaction.

All the four alkaloids give violet colours, the colours obtained with hyoscine and homatropine, however, being more brownish than those obtained with atropine and hyoscyamine.

The portion of the molecule responsible for the production of colour is the tropic acid fraction in atropine, hyoscyamine and hyoscine and the mandelic acid fraction in homatropine. Both tropic/

# MOHLER'S REACTION

## ATROPINE

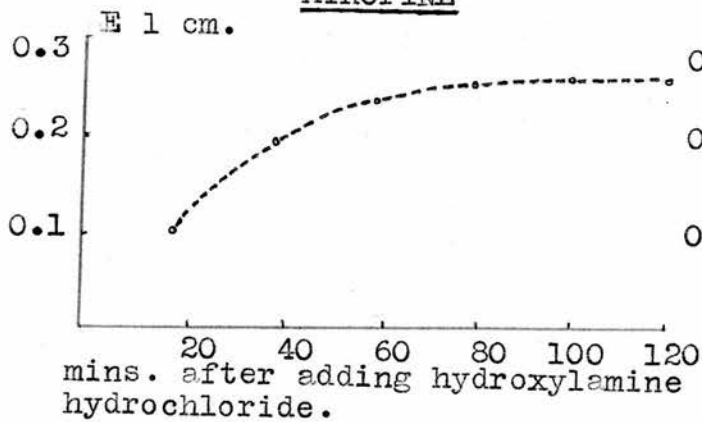


Fig. 22. Rate of development of colour at 530 mμ.

## HYOSCINE

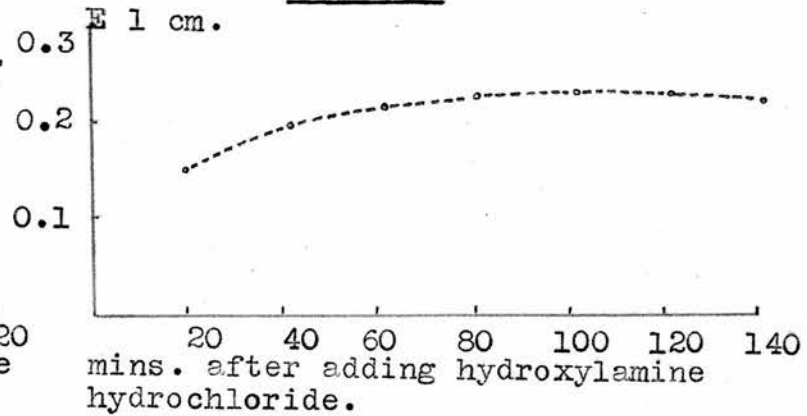


Fig. 21. Rate of development of colour at 530 mμ.

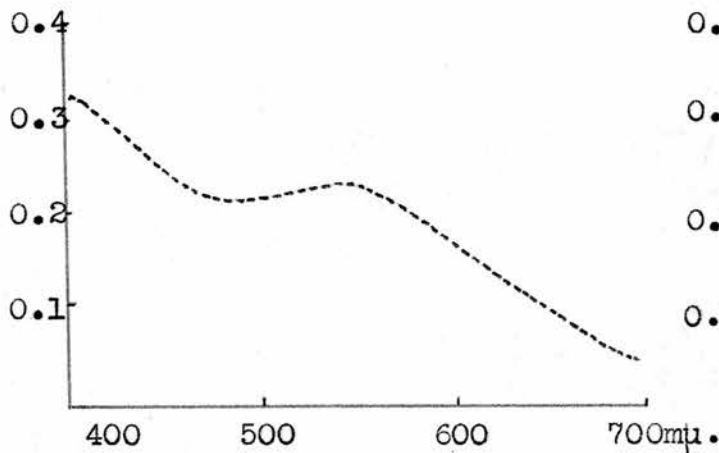


Fig. 24. Absorption curve of colour.

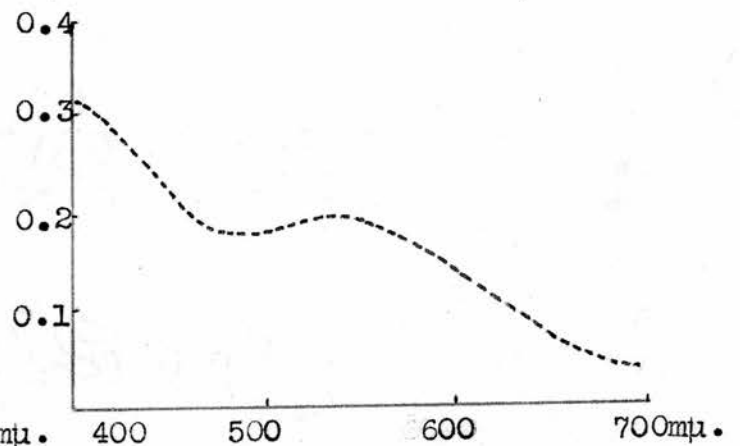


Fig. 23. Absorption curve of colour.

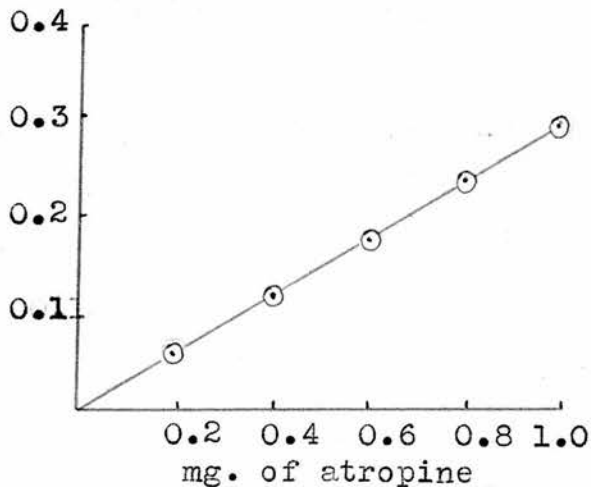


Fig. 26. Calibration curve at 530mμ.

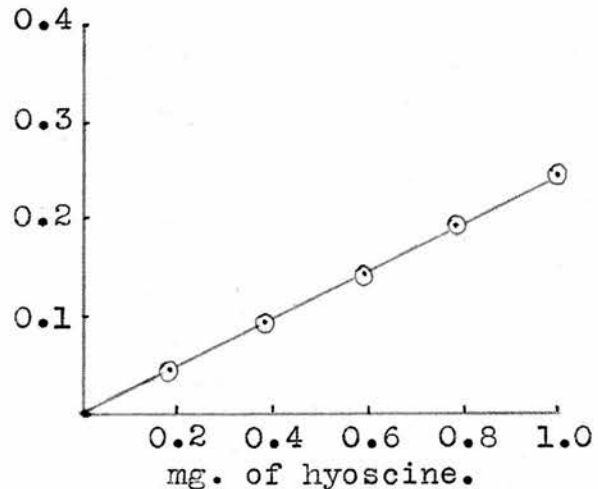


Fig. 25. Calibration curve at 530mμ.

# MOHLER'S REACTION

## HYOSCYAMINE

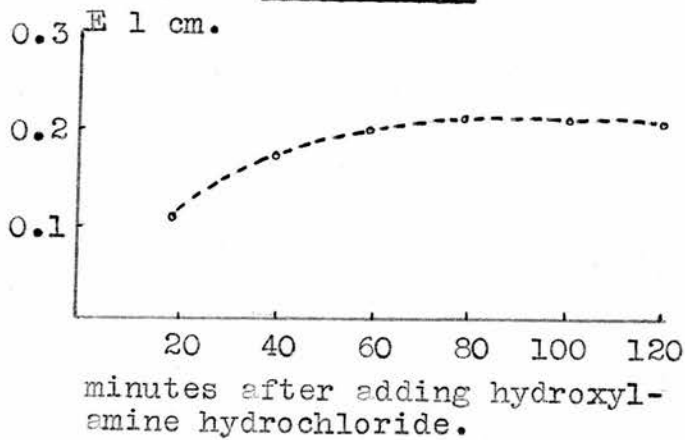


Fig. 28. Rate of development of colour at 530 mμ.

## TROPIC ACID

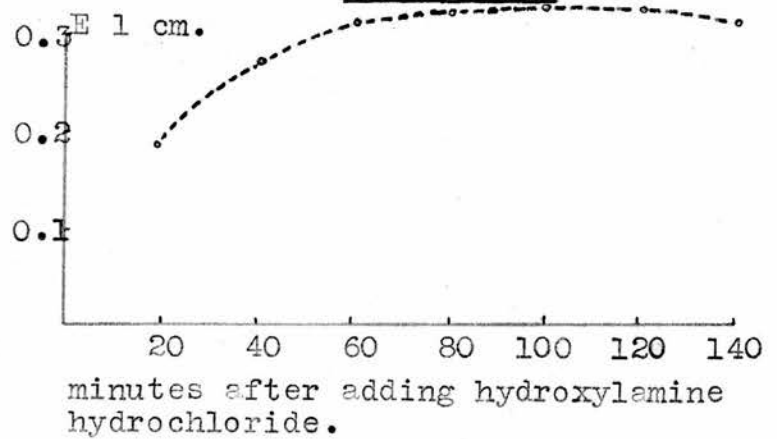


Fig. 27. Rate of development of colour at 530 mμ.

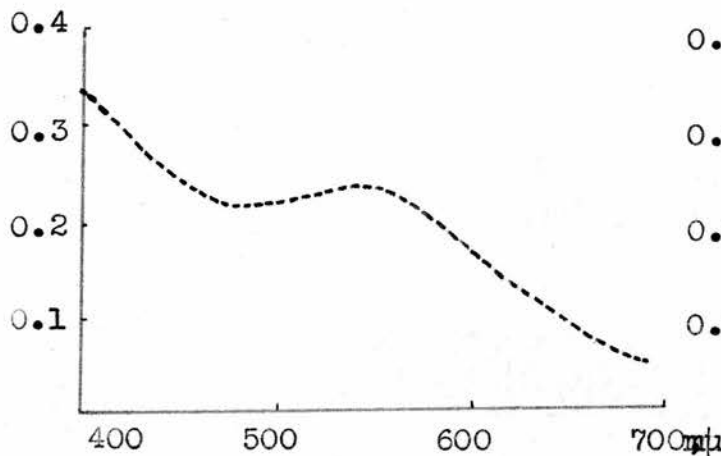


Fig.30. Absorption curve of colour.

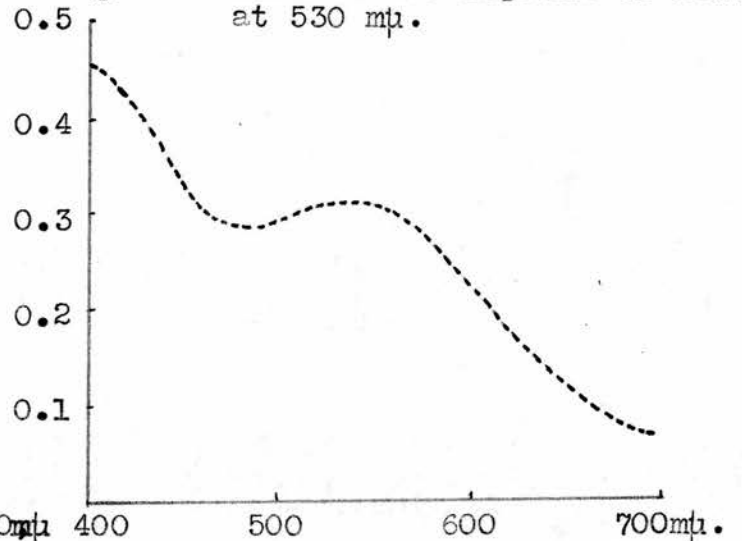


Fig.29. Absorption curve of colour.

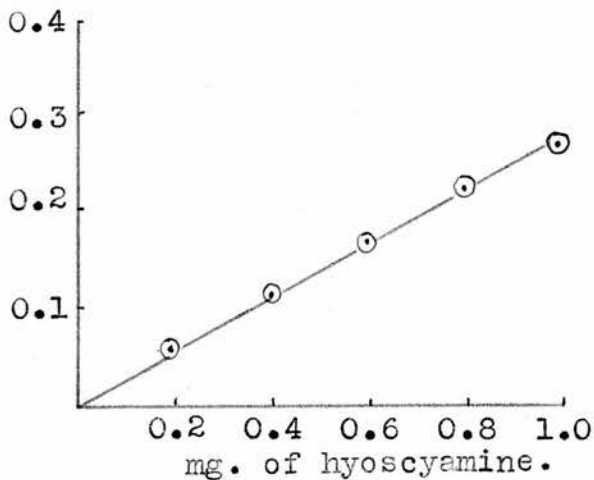


Fig.32. Calibration curve at 530mμ.

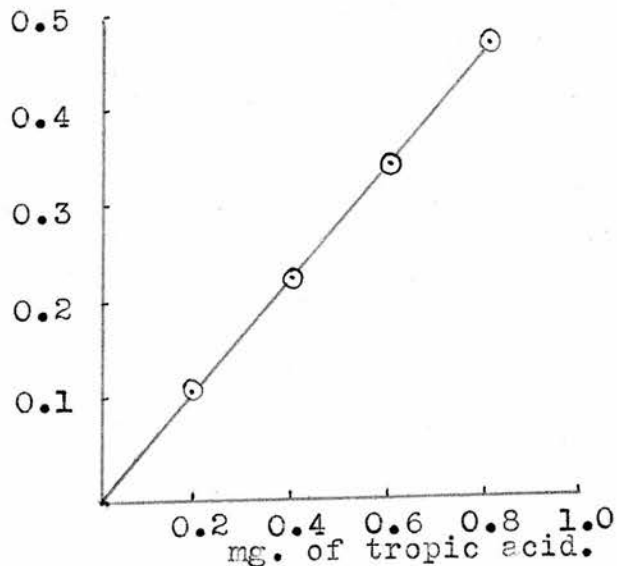
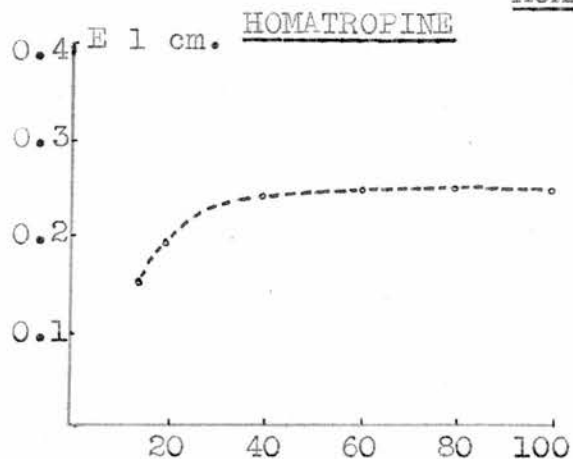


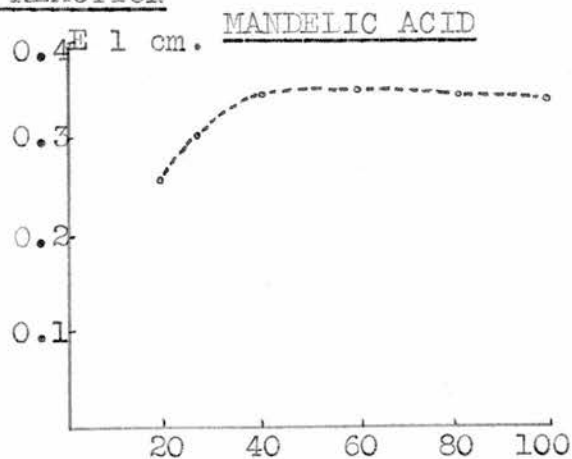
Fig.31. Calibration curve at 530mμ.

# MOHLER'S REACTION



minutes after adding hydroxylamine hydrochloride.

Fig.34. Rate of development of colour at 530 mμ.



minutes after adding hydroxylamine hydrochloride.

Fig.33. Rate of development of colour at 530 mμ.

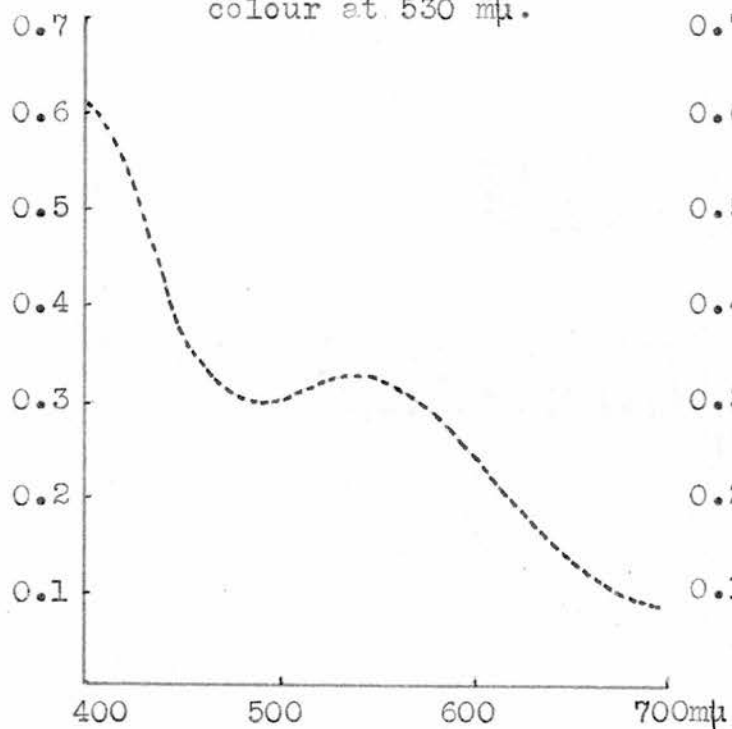


Fig.36. Absorption curve of colour.

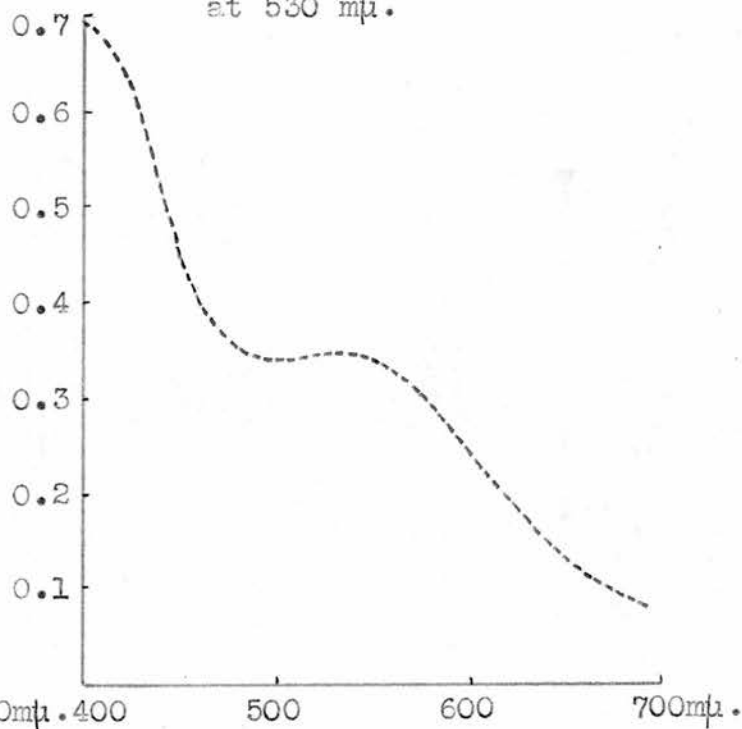


Fig.35. Absorption curve of colour.

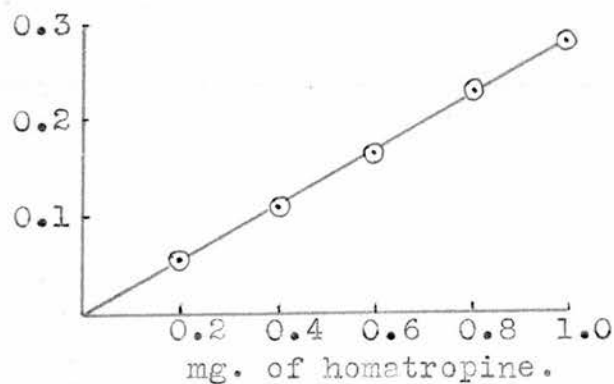


Fig.37. Calibration curve at 530 mμ.

tropic and mandelic acids give violet colours in Mohler's test and the absorption curves (Figs. 23, 24, 29, 30, 35, and 36) of the colours obtained with the four alkaloids and the two acids are similar. They all show rather broad peaks. Maximum absorption is in the region of 530 m $\mu$ . The rate of development of colour is shown in Figs. 21, 22, 27, 28, 33 and 34. In the case of atropine, hyoscyamine, hyoscine and tropic acid maximum intensity is attained in about 80 minutes and remains practically unchanged for a further 60 minutes. With homatropine and mandelic acid maximum intensity of colour is attained in about 45 minutes and remains practically unchanged for a further 40 minutes.

Atropine, hyoscyamine and hyoscine are readily nitrated to poly-nitro derivatives with fuming nitric acid. Since intensive nitration is the first stage in Mohler's reaction, it was thought that it would be much simpler to use fuming nitric acid instead of a mixture of potassium nitrate and sulphuric acid as nitrating agent for these alkaloids. Surprisingly enough it was found that the alkaloids did not respond to Mohler's test when fuming nitric acid alone was used for nitration; if, however, a mixture of fuming nitric acid and sulphuric acid is used a positive reaction is obtained. Sulphuric acid is known to have a directing influence on nitro-groups entering an aromatic nucleus. On the basis of the mechanism of Mohler's reaction put forward by Block and Bolling<sup>75</sup>, it is suggested as an explanation of the difference in/

in behaviour under the influence of the two nitrating agents, that the nitro groups enter positions meta- to one another with fuming nitric acid while in the presence of sulphuric acid the positions occupied are para- to one another. According to Block and Bolling colour reactions are obtained only when the nitro-groups are in ortho- or para- positions to each other. This difference in behaviour under the influence of the two nitrating agents was also observed in Janovsky's reaction; the colour reactions obtained with alcoholic potash though somewhat similar were not identical, whereas with aqueous sodium hydroxide the reactions were quite different.

Mohler's test is a fairly sensitive reaction for these alkaloids, 0.1 mg. being just detectable.

Calibration data: The extinctions measured at 530 m $\mu$  in a 1 cm. cell are given below. Readings were taken after 90 minutes from the time of additions of hydroxylamine hydrochloride in the case of atropine, hyoscyamine and hyosine and after 45 minutes in the case of homatropine.

| E 1 cm. at 530 m $\mu$ |                 |                    |                |                    |
|------------------------|-----------------|--------------------|----------------|--------------------|
| <u>mg. alkaloid</u>    | <u>Atropine</u> | <u>Hyoscyamine</u> | <u>Hyosine</u> | <u>Homatropine</u> |
| 0.2                    | .060            | .058               | .053           | .056               |
| 0.4                    | .118            | .117               | .100           | .107               |
| 0.6                    | .174            | .169               | .145           | .164               |
| 0.8                    | .243            | .226               | .200           | .231               |
| 1.0                    | .299            | .274               | .251           | .284               |

The/



The calibration curves are shown in Figs. 25, 26, 32 and 37.

## 2. Janovsky's reaction:

The alkaloids of this group being esters a certain degree of hydrolysis is to be expected under the conditions of nitration used in the test. Since in Janovsky's test the products of nitration are isolated by solvent extraction first from acid-medium and then from alkaline medium, whether the acid-ether extract or the alkaline-chloroform extract or both give colour reactions depends no doubt on the degree of hydrolysis that has taken place during nitration. If no hydrolysis has taken place then a colour reaction is obtained only with the alkaline-chloroform extract; if hydrolysis is complete only the acid-ether extract will give a colour reaction. When only partial hydrolysis has occurred both acid-ether and alkaline-chloroform extracts give colour reactions.

In the case of atropine and hyoscyamine the acid-ether extracts give only slight colour reactions, the predominant reaction being obtained with the alkaline-chloroform extracts. Hyoscine undergoes hydrolysis to a greater extent than atropine or hyoscyamine and colour reactions are obtained with both the acid-ether and alkaline-chloroform extracts. The sensitivity of the test is thereby appreciably reduced. In the case of homatropine the predominant reaction is obtained with the acid-ether extract. Atropine and hyoscyamine give purple colours; hyoscine/

hyoscine gives a reddish-violet colour quite distinct from that given by atropine and hyoscyamine. Homatropine gives a violet colour and tropic and mandelic acids give bluish-violet colours.

| <u>Acid-ether extract</u> |                                            | <u>Alkaline-chloroform extract</u>            |                                      |
|---------------------------|--------------------------------------------|-----------------------------------------------|--------------------------------------|
|                           |                                            | <u>Alcoholic potash</u>                       | <u>Aqueous sodium hydroxide</u>      |
| Atropine                  | very light violet                          | intense purple                                | intense purple                       |
| Hyoscy-<br>amine          | colour develops<br>slowly.                 | colour fades<br>quickly                       | colour develops<br>slowly. Stable.   |
| Hyoscine                  | light violet<br>colour develops<br>slowly. | reddish-violet<br>colour fades<br>quickly.    | reddish-violet<br>colour. Stable.    |
| Homatropine               | violet colour                              | very light<br>violet colour<br>fades rapidly. | very light violet<br>colour. Stable. |
| Tropic acid               | bluish-violet                              | —                                             | —                                    |
| Mandelic<br>acid          | colour.                                    |                                               |                                      |

Janovsky's reaction is sensitive to 0.05 mg. of atropine and hyoscyamine, 0.2 mg. of hyoscine and 0.1 mg. of homatropine. The reaction has not been studied quantitatively since methods based/

based on the more sensitive Vitali-Morin reaction have been successfully developed for the determination of atropine, hyoscyamine and hyoscine by Allport and Wilson,<sup>59</sup> Roberts and James<sup>58</sup> and Canback<sup>80</sup>.

#### Tropic acid:

The application, both qualitative and quantitative, of Mohler's and Janovsky's reactions to tropic acid has been studied with a definite purpose in view. The separation of the tropic alkaloids in admixture with non-phenolic alkaloids such as ephedrine and codeine is difficult. Brunzell<sup>81</sup> et al have devised an elegant method to get over this difficulty. The tropic alkaloids are hydrolysed in alkaline medium and after acidification the resulting tropic acid is extracted exhaustively with ether. The tropic acid thus obtained is re-esterified either with diazomethane or via the acid chloride with ethyl alcohol and estimated by the Vitali-Morin reaction. The method is based on the observation of James and Roberts<sup>32</sup> that the methyl and ethyl esters of tropic acid respond to the Vitali-Morin reaction. They state that the method is of special value for the estimation of the amount of decomposition in highly dilute solutions of the tropic alkaloids, when sterilized.

When the quantity permits it, it would be much simpler, however, to nitrate the tropic acid to polynitro derivatives and carry out the estimation either by Janovsky's or Mohler's tests.

The

The absorption curve (Fig. 39) of the colour obtained in Janovsky's reaction exhibits a fairly narrow peak. Maximum absorption is in the region of 555  $m\mu$ . The rate of development of colour is shown in Fig. 38. Maximum intensity of colour is attained in about 15 minutes and thereafter remains practically unchanged for a further 10 minutes.

Calibration data:

1. Mohler's reaction: The extinctions measured at 530  $m\mu$  in a 1 cm. cell are given below. The readings were taken after 90 minutes from the time of addition of hydroxylamine hydrochloride.

| <u>mg. tropic acid</u> | <u>E 1 cm. at 530 <math>m\mu</math>.</u> |
|------------------------|------------------------------------------|
| 0.2                    | .117                                     |
| 0.4                    | .230                                     |
| 0.6                    | .358                                     |
| 0.8                    | .486                                     |
| 1.0                    | .620                                     |

2. Janovsky's reaction: Maximum extinctions measured at 555  $m\mu$  in a 1 cm. cell are given below.

| <u>mg. tropic acid</u> | <u>E 1 cm. at 555 <math>m\mu</math>.</u> |
|------------------------|------------------------------------------|
| 0.1                    | .075                                     |
| 0.2                    | .154                                     |
| 0.3                    | .231                                     |
| 0.4                    | .314                                     |
| 0.5                    | .407                                     |
| 0.6                    | .484                                     |

JANOVSKY'S REACTION

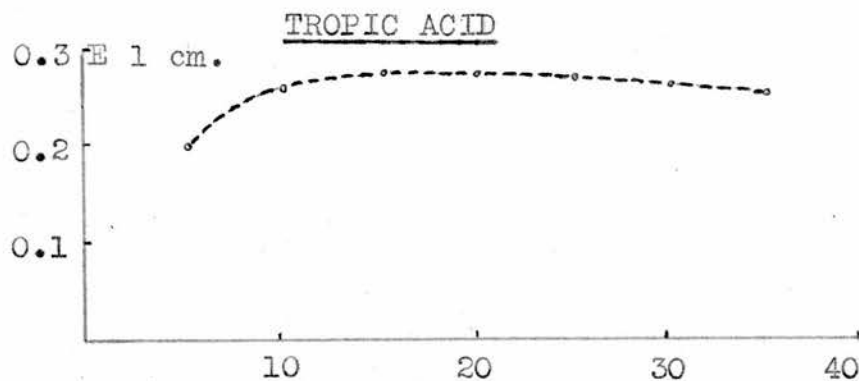


Fig.38. Rate of development of colour at 555m $\mu$ .

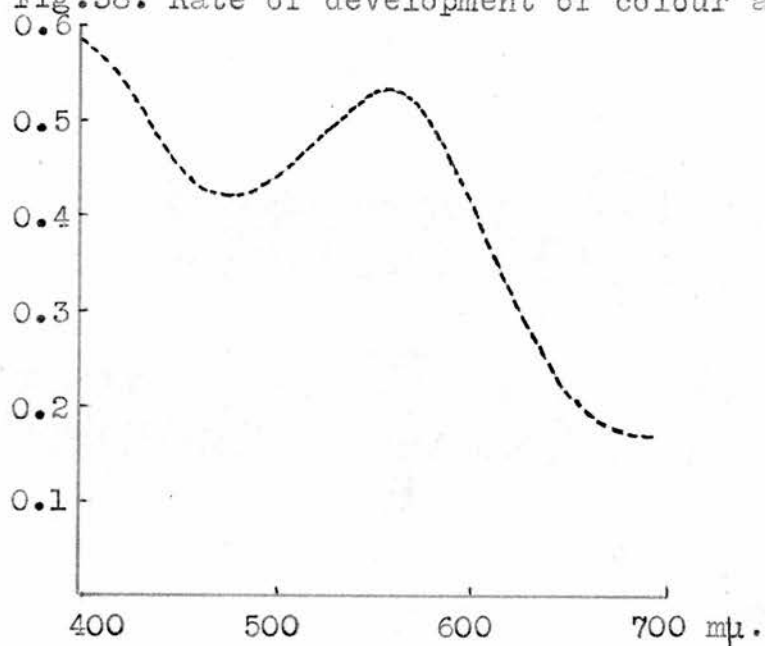


Fig.39. Absorption curve of colour.

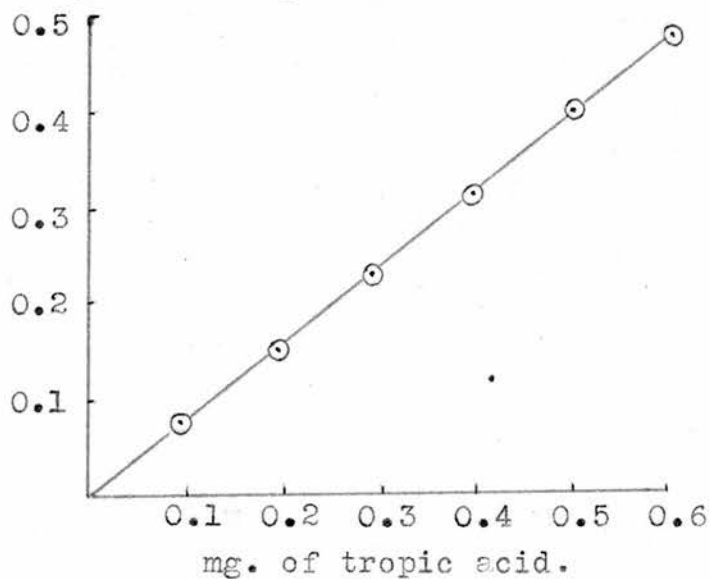


Fig.40. Calibration curve at 555 m $\mu$ .

The calibration curves are shown in Figs. 31 and 40.

1.0 mg. of atropine was hydrolysed in alkaline medium, acidified with dilute sulphuric acid and the tropic acid was extracted with ether eight times using 15 ml. of redistilled ether for each extraction. The ether extracts were filtered, the ether removed and the tropic acid was estimated by Mohler's reaction. The tropic acid found was equivalent to 0.7 mg. of atropine. This low result is probably due to incomplete extraction of tropic acid. Tropic acid is water-soluble and difficult to extract completely from aqueous solutions.

Satisfactory results were obtained by keeping the aqueous volume as low as possible and extracting with ether after saturating with salt:-

An alcoholic solution of atropine containing 1.0 mg. of atropine was transferred to a test-tube (provided with a glass stopper) and the alcohol was removed by heating in a boiling water bath. The residue was dissolved in 2.5 ml. of water containing 2 drops of N  $\text{H}_2\text{SO}_4$  and hydrolysed by heating with 1 ml. of 2.5N sodium hydroxide solution, for 20 minutes in a boiling water bath. The basic-products of hydrolysis were removed by extracting twice with 10 ml. of ether. It was then acidified with N.  $\text{H}_2\text{SO}_4$ , saturated with salt and the tropic acid extracted with ether seven times using 15 ml. of ether for each extraction. The ethereal extracts were filtered through a plug of cotton wool layered with anhydrous/

anhydrous sodium sulphate. The ether was removed and the tropic acid estimated by Mohler's test. Tropic acid found was equivalent to 0.91 mg. of atropine.

The ether-extractions were carried out in the test-tube itself, the ether layers being removed by a 'wash-bottle' arrangement.

Group III. The local anaesthetics of the benzoic ester group.

The group composed of the benzoic esters is the largest and most important of the local anaesthetics. The discovery that cocaine is an effective local anaesthetic stimulated the search for other local anaesthetics which would possess advantages over cocaine as regards toxicity and addiction. A large number of benzoic esters has been synthesised and studied as 'Cocaine Substitutes'.

The members of this group are basic in character and in the Stas-Otto process are obtained in the non-phenolic alkaloid group. Notable exceptions are the structurally similar benzocaine and butyl-p-aminebenzoate which are extracted by ether from acid solutions. Orthocaine, which is phenolic in character, undergoes decomposition with alkali and is best extracted with ether from aqueous solutions acidified with tartaric acid.

These drugs, particularly the cocaine substitutes, are rapidly broken down in the body. In cases of fatal poisoning, the/

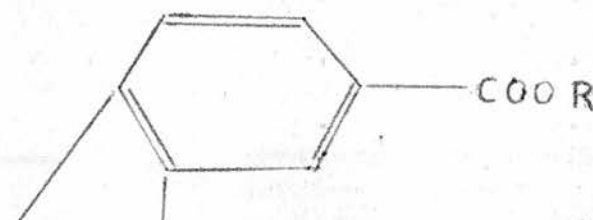
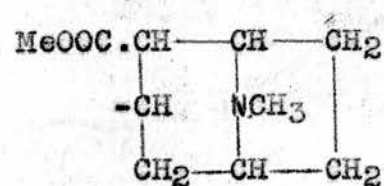
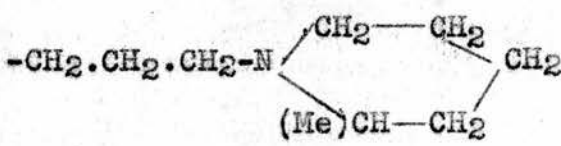
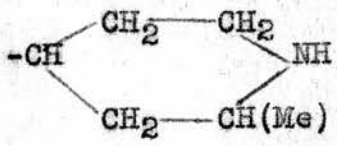
the detection of unchanged drugs other than cocaine is unusual, except where considerable doses have been administered.

In Bamford's systematic scheme these drugs are included in Group V, which consists of those alkaloids that give colour reactions with p-dimethyl aminobenzaldehyde (procaine, benzocaine, amethocaine and amylocaine) and in Group VII which consists of those alkaloids for which no satisfactory colour reactions are known (cocaine, benzamine and amydriscaine).

The members of this group may be arranged into two groups. Group (a) consisting of those which contain one or more substituents in the benzene ring and Group (b) consisting of those which have no substituents. Of the members of group (a) benzocaine, butyl-p-aminobenzoate, procaine and butacaine contain only one substituent, a primary amino-group in para-position. Amethocaine also contains a single substituent, a substituted amino group in para-position. Orthocaine contains a phenolic hydroxyl group in para-position and a primary amino-group in meta-position. Procaine, butacaine and amethocaine, contain, in addition, a tertiary nitrogen in the aliphatic side chain.

Of the members of group (b), cocaine metycaine and benzamine contain/

contain a ring system in the aliphatic side chain.

|                            |                 |                 |                                                                                    |                                                                                         |
|----------------------------|-----------------|-----------------|------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|
|                            |                 |                 |  |                                                                                         |
| (a) Benzocaine             | NH <sub>2</sub> | H               |                                                                                    | -CH <sub>2</sub> .CH <sub>3</sub>                                                       |
| Butyl-p-amino<br>-benzoate | NH <sub>2</sub> | H               |                                                                                    | -CH <sub>2</sub> .CH <sub>2</sub> .CH <sub>2</sub> .CH <sub>3</sub>                     |
| Orthocaine                 | OH              | NH <sub>2</sub> |                                                                                    | -CH <sub>3</sub>                                                                        |
| Procaine                   | NH <sub>2</sub> | H               |                                                                                    | -CH <sub>2</sub> .CH <sub>2</sub> .N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>       |
| Butacaine                  | NH <sub>2</sub> | H               |                                                                                    | -CH <sub>2</sub> .CH <sub>2</sub> .CH <sub>2</sub> .N(Bu) <sub>2</sub>                  |
| Amethocaine                | (Bu).NH         | H               |                                                                                    | -CH <sub>2</sub> .CH <sub>2</sub> .N(CH <sub>3</sub> ) <sub>2</sub>                     |
| (b) Cocaine                | H               | H               |                                                                                    |    |
| Metycaine                  | H               | H               |                                                                                    |     |
| Benzamine                  | H               | H               |                                                                                    |     |
| Amydricaine                | H               | H               |                                                                                    | -C(C <sub>2</sub> H <sub>5</sub> )(CH <sub>2</sub> NMe <sub>2</sub> ) <sub>2</sub>      |
| Amylocaine                 | H               | H               |                                                                                    | -C(CH <sub>3</sub> )(C <sub>2</sub> H <sub>5</sub> )(CH <sub>2</sub> NMe <sub>2</sub> ) |

Bu = butyl = -CH<sub>2</sub>.CH<sub>2</sub>.CH<sub>2</sub>.CH<sub>3</sub>

Me = methyl = -CH<sub>3</sub>

1. Mohler's test./

1. Mohler's test.

All the members of group (a) give yellow colours. In group (b) with the exception of amydriscaine all the others give orange or reddish-orange colours. Amydriscaine gives a yellow colour.

| <u>Substance</u>       | <u>Colour obtained in Mohler's test.</u> |
|------------------------|------------------------------------------|
| Group (a) Benzocaine   | yellow                                   |
| Butyl-p-amino benzoate |                                          |
| Orthocaine             |                                          |
| Procaine               |                                          |
| Butacaine              |                                          |
| Amethocaine            |                                          |
| Group (b) Cocaine      | orange                                   |
| Metycaine              |                                          |
| Benzamine              |                                          |
| Amylocaine             | reddish-orange                           |
| Amydriscaine           | yellow                                   |

Benzoic acid gives a reddish-orange colour.

Mohler's test, however, is not a very sensitive test for cocaine, metycaine and benzamine; this is to be expected since the benzoic-acid portion, responsible for the production of colour, forms only a small fraction of the molecule (1 mg. of cocaine is equivalent to about 0.4 mg. of benzoic acid). With amounts of these alkaloids less than 0.5 mg. yellowish or brownish-yellow colours/

# MOHLER'S REACTION

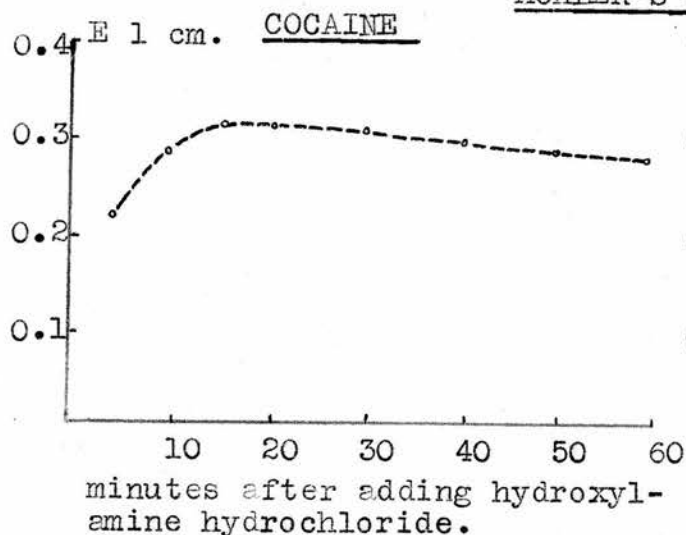


Fig.42. Rate of development of colour at 500 mμ.

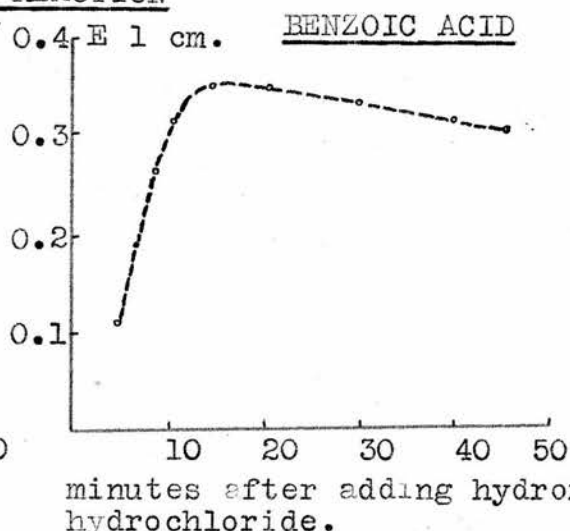


Fig.41. Rate of development of colour at 500 mμ.

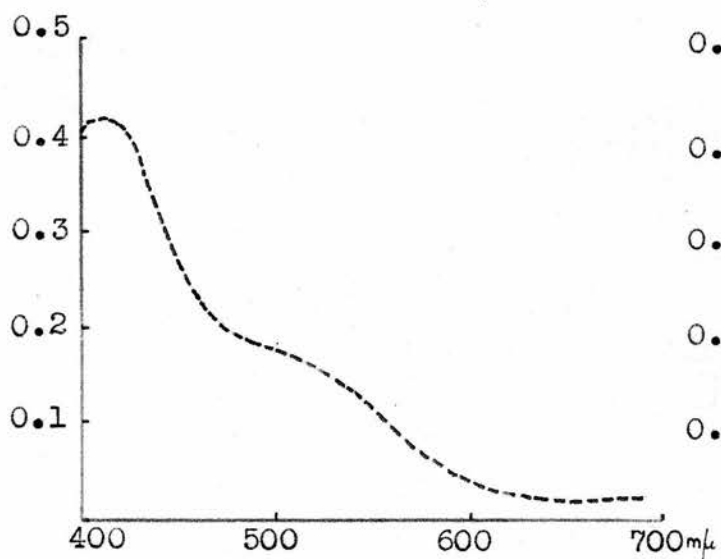


Fig.44. Absorption curve of colour.

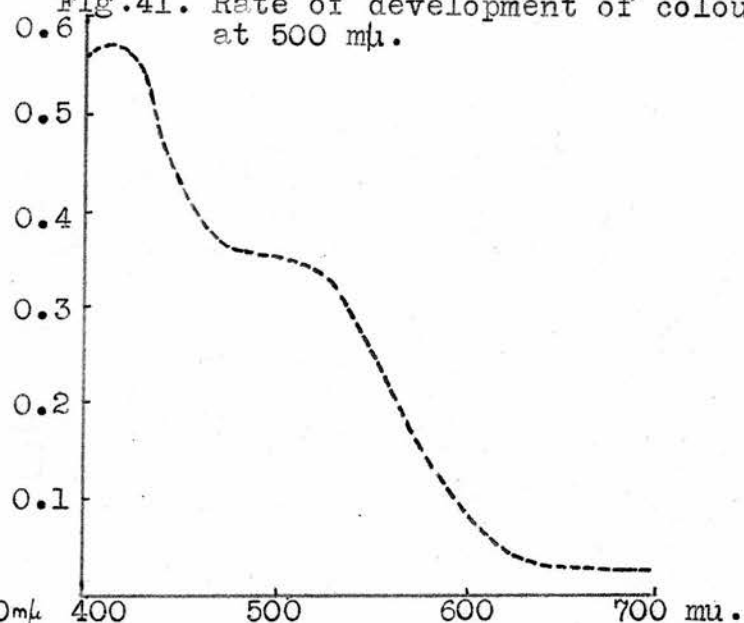


Fig.43. Absorption curve of colour.

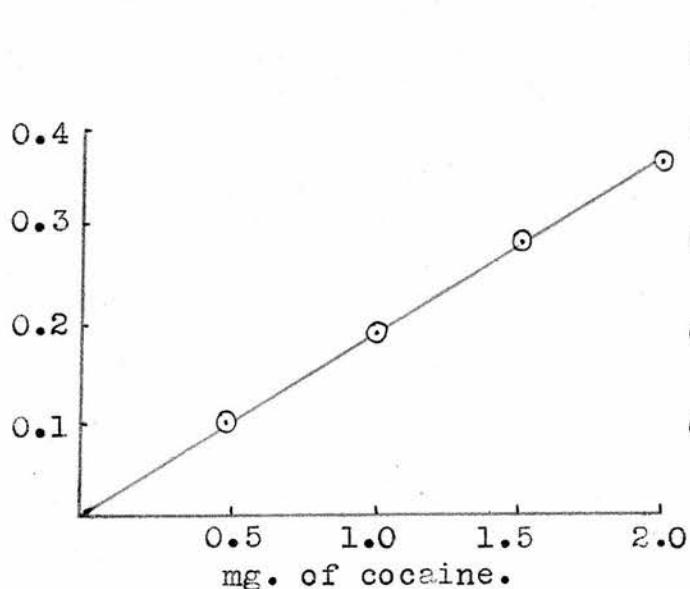


Fig.46. Calibration curve at 500mμ.

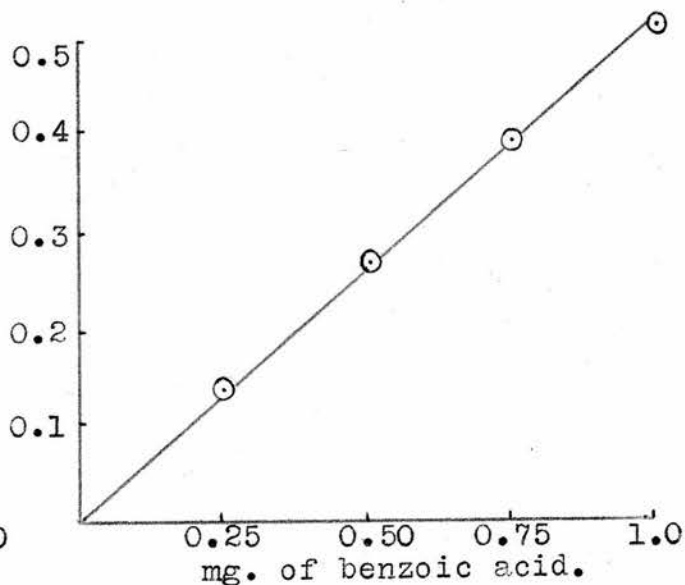


Fig.45. Calibration curve at 500mμ.

colours predominate but with larger amounts the orange colour is quite distinct. Amounts of cocaine of the order of 0.5 mg. or more are readily determined by Mohler's test.

The quantitative application of the reaction has been studied in the case of cocaine and benzoic acid only. Preliminary experiments have shown, however, that Mohler's test could be used for the determination of metycaïne, benzamine and amylocaine.

The absorption curves (Figs. 43 and 44) of the colours obtained with cocaine and benzoic acid are similar. They do not exhibit any peak. The rate of development of colour is shown in Figs. 41 and 42. Maximum intensity of colour is obtained in about 15 minutes, remains practically unchanged for 5 minutes and then fades slowly.

Calibration data. Maximum extinctions measured at 500 mμ in a 1 cm. cell are given below.

| <u>mg. cocaine</u> | <u>E 1 cm. at 500 mμ.</u> | <u>mg. benzoic acid</u> | <u>E 1 cm. at 500 mμ.</u> |
|--------------------|---------------------------|-------------------------|---------------------------|
| 0.5                | .105                      | 0.25                    | .140                      |
| 1.0                | .190                      | 0.50                    | .277                      |
| 1.5                | .285                      | 0.75                    | .400                      |
| 2.0                | .370                      | 1.00                    | .524                      |

The calibration curves are shown in Figs. 45 and 46.

## 2. Janovsky's reaction.

All the drugs in this group are esters and may undergo hydrolysis under the conditions of nitration used in this test.

The/

The same considerations as with the alkaloids of the atropine group hold here as well.

The acid-ether extracts in the case of benzocaine and butyl-p-amino-benzoate give reddish-brown colours. No colour reaction is obtained with the alkaline-chloroform extracts. With butacaine the acid-ether extract gives a reddish-brown colour and the alkaline-chloroform extract a light violet colour. Orthocaine gives no colour reaction.

Procaine and amethocaine behave similarly. The acid-ether extracts give no colour reactions but the alkaline-chloroform extracts give violet colours which change rapidly to greenish-yellow or deep yellow depending on the nature of the alkali used. The colour obtained with amethocaine is, however, much weaker than that obtained with procaine.

Amethocaine gives a beautiful red colour in the Vitali-Morin test whereas the other members give no recognisable colour in the test. The Vitali-Morin reaction is a very sensitive one for amethocaine and a method based on this reaction, for the determination of amethocaine, has been developed by Seydlitz<sup>62</sup>.

Janovsky's reaction is not a very sensitive test for the detection of members of group (a) except procaine and amethocaine. The test is sensitive to 0.1 mg. of procaine and 0.2 mg. of amethocaine.

Group (b). Janovsky's reaction is a very sensitive test for this group./

group. With cocaine, metycaine and benzamine the predominant colour reaction is obtained with the alkaline-chloroform extracts. These give bluish-violet colours with both alcoholic potash and aqueous sodium hydroxide. The acid-ether extracts give only slight colour reactions, light bluish-violet or violet colours. In the case of amydricaine the alkaline-chloroform extract gives a blue colour which changes slowly to a bluish-violet colour; the acid-ether extract gives practically no colour. Amylocaine undergoes complete hydrolysis during nitration and the predominant colour reaction is, therefore, obtained with the acid-ether extract — it gives a bluish violet colour which slowly changes to a beautiful violet colour. No colour reaction is obtained with the alkaline-chloroform extract.

The test is sensitive to 0.05 mg. of cocaine, metycaine, benzamine, amydricaine and amylocaine.

|                | <u>Acid-ether extract</u> | <u>Alkaline-chloroform extract.</u> |                                     |
|----------------|---------------------------|-------------------------------------|-------------------------------------|
|                |                           | <u>Alcoholic potash</u>             | <u>Aqueous sodium hydroxide</u>     |
| Benzocaine     | } reddish-brown           | no colour                           | no colour                           |
| Butyl-p-       |                           |                                     |                                     |
| amine-benzoate |                           |                                     |                                     |
| Orthocaine     | no colour                 | no colour                           | no colour                           |
| Butacaine      | reddish-brown             | very light violet<br>fades rapidly  | very light violet<br>fades rapidly. |
| Procaine/      |                           |                                     |                                     |

| <u>Acid-ether extract</u> |                                                | <u>Alkaline-chloroform extract</u> |                                        |
|---------------------------|------------------------------------------------|------------------------------------|----------------------------------------|
|                           |                                                | <u>Alcoholic potash</u>            | <u>Aqueous sodium hydroxide</u>        |
| Procaine                  | } no colour                                    | violet changing                    | violet changing                        |
| Amethocaine               |                                                | rapidly to deep yellow.            | rapidly to greenish-yellow.            |
| Cocaine                   | } light bluish-violet changes slowly to violet | bluish-violet                      | stable bluish-violet.                  |
| Metycaine                 |                                                | fades rapidly                      |                                        |
| Benzamine                 |                                                |                                    |                                        |
| Amydri-<br>caine          | no colour                                      | violet fades rapidly               | blue changes to a stable bluish-violet |
| Amylocaine                | bluish-violet changes slowly to violet.        | no colour                          | no colour                              |

The colour reactions of amydrine and amylocaine in Mohler's and Janovsky's tests serve to distinguish them from the other members of the group. Cocaine, metycaine and benzamine give identical colour reactions in both Mohler's and Janovsky's tests.

Janovsky's reaction : Calibration data

Volume of solvent used = 5 ml. of 80% aq. acetone

Volume of alkali used = 0.1 ml. of 2.5N aq. sodium hydroxide solution.

The colour develops almost immediately on adding the alkali and maximum intensity is attained within a minute. As the colour fades/

JANOVSKY'S REACTION

COCAINE

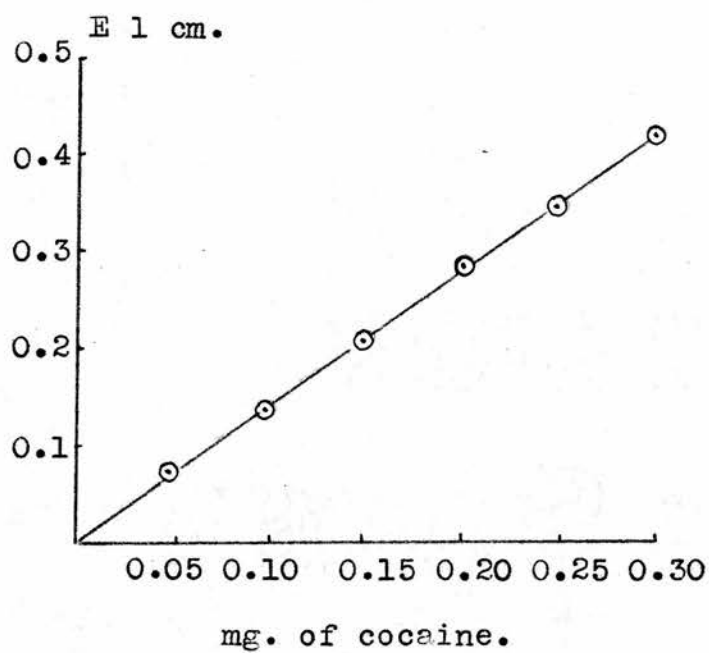


Fig.47. Calibration curve at 560 mμ.

fades rapidly extinction measurements should be made within a minute of adding the alkali. Maximum absorption is in the region of 560 mμ. Extinctions measured at 560 mμ. in a 1 cm. cell are given below.

| <u>mg. cocaine</u> | <u>E 1 cm. at 560 mμ.</u> |
|--------------------|---------------------------|
| 0.05               | .075                      |
| 0.10               | .140                      |
| 0.15               | .220                      |
| 0.20               | .295                      |
| 0.25               | .355                      |
| 0.30               | .428                      |

The calibration curve is shown in Fig. 47.

Preliminary experiments have shown that Janovsky's reaction could also be used for the determination of small amounts of mety-caine, benzamine, amylocaine and amydricine. In the case of amylocaine, since the predominant colour reaction is given by the acid-ether extract, the solvent used should be acetone-alcohol mixture and the alkali, alcoholic potash.

Cocaine and other members of group (b) when present with other non-phenolic alkaloids may be estimated by hydrolysing them in alkaline medium and determining the benzoic acid so formed. The hydrolysis and isolation of the benzoic acid are carried out using the method described under atropine for the isolation of tropic acid. Since benzoic acid is volatile 1 to 2 drops of alcoholic potash/

potash are added to the ethereal extracts before removing the ether. The residue is nitrated and estimated by Mohler's or Janovsky's tests.

Using Mohler's test the following results were obtained:-

| <u>Amount of cocaine in mg.</u> | <u>cocaine found in mg.</u> |
|---------------------------------|-----------------------------|
| 0.70                            | 0.75                        |
| 1.00                            | 1.15                        |

#### Group IV

#### The aconite alkaloids

Numerous and complex alkaloids occur in the various species of Aconitum. The alkaloids are mainly of two kinds (1) the highly toxic aconitines which are diacyl esters of a series of polyhydric amino-alcohols (aconines) and (2) the less toxic atisines which are amino-alcohols. The aconitines are very unstable alkaloids and readily undergo hydrolysis under the influence of acids and alkalis. This property renders it very difficult to isolate them from biological material.

Aconitine. is a diacyl ester and on hydrolysis gives acetic and benzoic acids.

1. Mohler's test. Aconitine gives a brownish-orange colour. The test, however, is not a very sensitive one for aconitine; this is to be expected since the benzoic acid portion, responsible for the colour, forms only a small fraction of the large aconitine molecule (1 mg. of aconitine is equivalent to about 0.2 mg. of benzoic acid). With amounts of aconitine less than 0.8 mg. yellowish or brownish-yellow/

yellow colours predominate but with larger amounts the orange colour is quite distinct. The colour is similar to that given by cocaine. Maximum intensity of colour is reached in about 15 minutes. On account of its low sensitivity Mohler's test has not been studied quantitatively but preliminary experiments have shown that the test could be used for the determination of amounts of aconitine of the order of 1 mg. or more.

## 2, Janovsky's reaction:

This is a more sensitive test for aconitine. During the course of the nitration complete hydrolysis takes place and colour reactions are obtained with the acid-ether extract only. A bluish-violet colour develops slowly, deepens on standing and then changes slowly to a beautiful violet colour. The test is sensitive to 0.05 mg. of aconitine.

The rate of development of colour is shown in Fig. 48. Maximum intensity is reached in about 20 minutes and remains more or less unchanged for a further 10 minutes. The absorption curve (Fig. 49) exhibits a fairly narrow peak. Maximum absorption is in the region of 550 m $\mu$ .

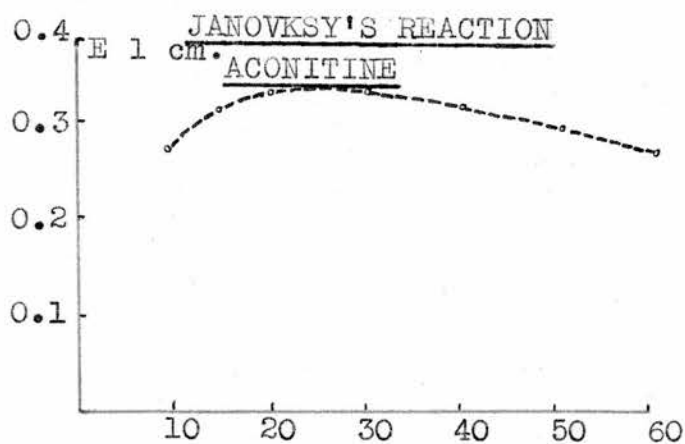
## Calibration data.

Volume of solvent used = 5 ml. of acetone-alcohol mixture

Volume of alkali used = 0.1 ml. of methyl alcoholic potash.

The maximum extinctions measured at 550 m $\mu$  in a 1 cm. cell are given below.

mg. aconitine/



minutes after adding alcoholic potash.  
Fig. 48. Rate of development of colour at 550 mμ.

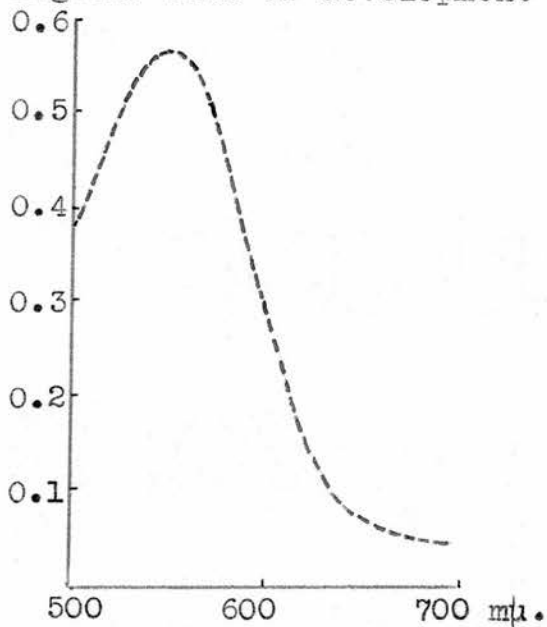


Fig. 49. Absorption curve of colour.

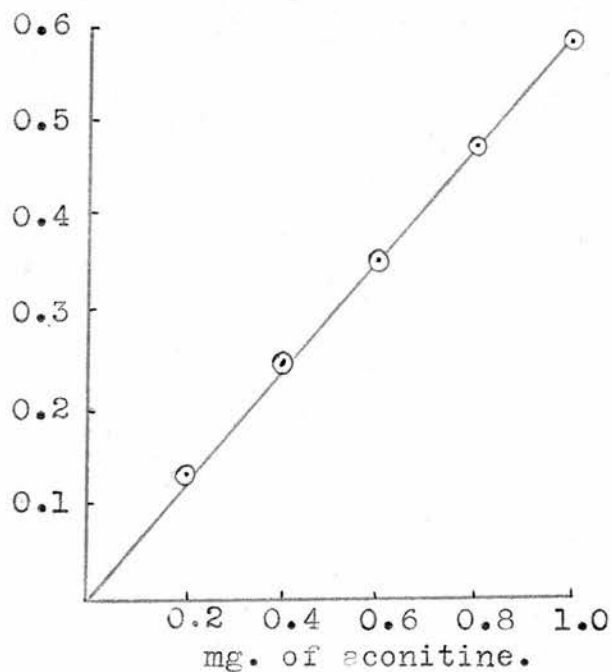


Fig. 50. Calibration curve at 550 mμ.

| <u>mg. aconitine</u> | <u>E 1 cm. at 550 mμ.</u> |
|----------------------|---------------------------|
| 0.2                  | .130                      |
| 0.4                  | .250                      |
| 0.6                  | .361                      |
| 0.8                  | .483                      |
| 1.0                  | .595                      |

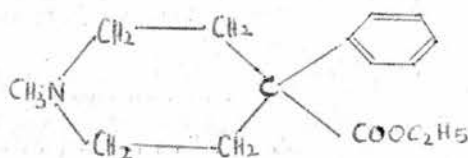
The Calibration curve is shown in Fig. 50.

### Group V

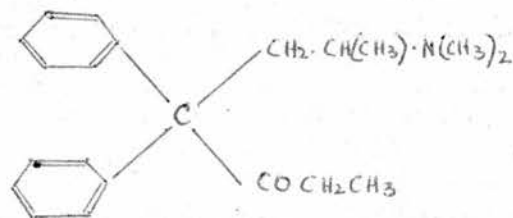
This group consists of the opium alkaloids, morphine, codeine and narcotine, the artificial diacetyl derivative of morphine - heroin and the synthetic morphine substitutes pethidine and amidone.

(a) The opium alkaloids and heroin give yellow colours in Mohler's test and no colour or only light brownish-yellow colours in Janovsky's reaction.

(b) The synthetic morphine substitutes.



Pethidine



amidone

1. Mohler's test:

### 1. Mohler's test:

Pethidine gives a violet colour whereas amidone gives a dirty brownish-red colour. With amounts of amidone above 0.2 mg. precipitation takes place and this renders the test unsuitable for quantitative work. Amidone gives a beautiful rose-pink colour on heating with potassium nitrate and sulphuric acid and this may serve as a test for its detection. It gives no colour reaction on heating with sulphuric acid alone.

The rate of development of the violet colour obtained with pethidine is shown in Fig. 51. Maximum intensity is attained in about 65 minutes and thereafter remains practically unchanged for a further 60 minutes. The absorption curve (Fig. 52) exhibits a broad peak. Maximum absorption is in the region of 555 mμ.

### Calibration data.

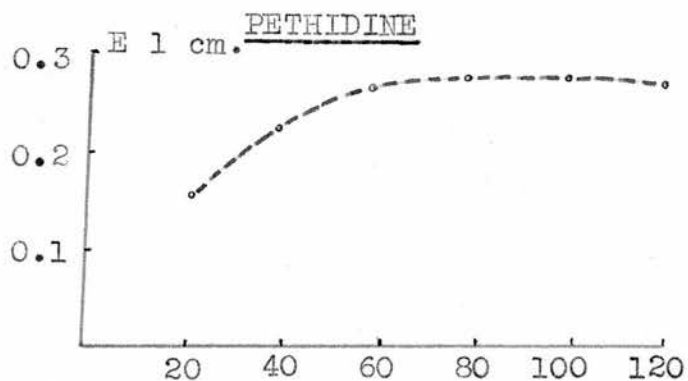
The extinctions measured at 555 mμ in a 1 cm. cell are given below. The readings were taken after 70 minutes from the time of addition of hydroxylamine hydrochloride.

| <u>mg. pethidine hydrochloride</u> | <u>E 1 cm. at 555 mμ.</u> |
|------------------------------------|---------------------------|
| 0.2                                | .072                      |
| 0.4                                | .147                      |
| 0.6                                | .217                      |
| 0.8                                | .288                      |
| 1.0                                | .365                      |

The calibration curve is shown in Fig. 53

The reaction is sensitive to 0.1 mg. of pethidine.

# MOHLER'S REACTION



minutes after adding hydroxylamine hydrochlor.

Fig.51. Rate of development of colour at 555 mμ.

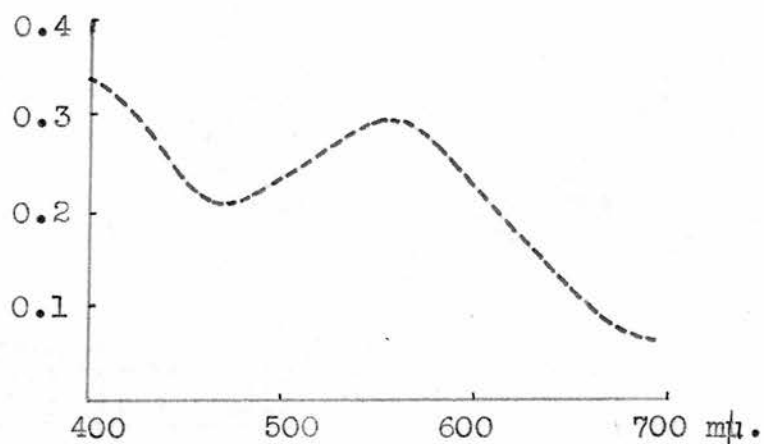


Fig.52. Absorption curve of colour.

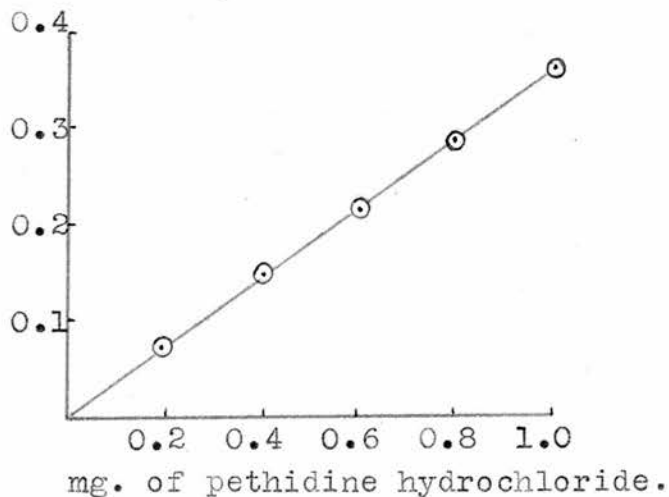


Fig.53. Calibration curve at 555 mμ.

## 2. Janovsky's reaction:

With both pethidine and amidone predominant colour reactions are obtained with the alkaline-chloroform extracts. Pethidine gives a bluish-violet colour and amidone a violet colour, with both alcoholic potash and aqueous sodium hydroxide. The colour obtained with alcoholic potash, however, fades rapidly.

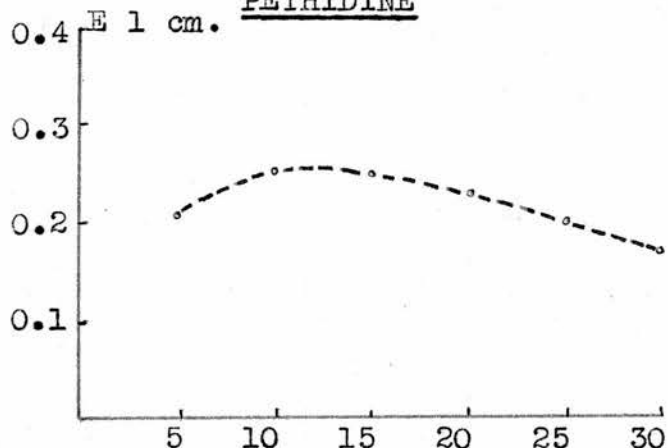
The acid-ether extract in the case of amidone, gives a light yellowish-brown colour which changes slowly to a light reddish colour. Pethidine gives no colour reaction.

| <u>Acid-ether extract</u> |               | <u>Alkaline-chloroform extract</u> |                                                       |
|---------------------------|---------------|------------------------------------|-------------------------------------------------------|
|                           |               | <u>Alcoholic potash</u>            | <u>Aqueous sodium hydroxide</u>                       |
| Pethidine                 | no colour     | bluish-violet<br>fades rapidly     | bluish-violet<br>changes slowly<br>to violet. Stable. |
| Amidone                   | light reddish | violet colour<br>fades quickly     | violet colour.<br>Stable.                             |

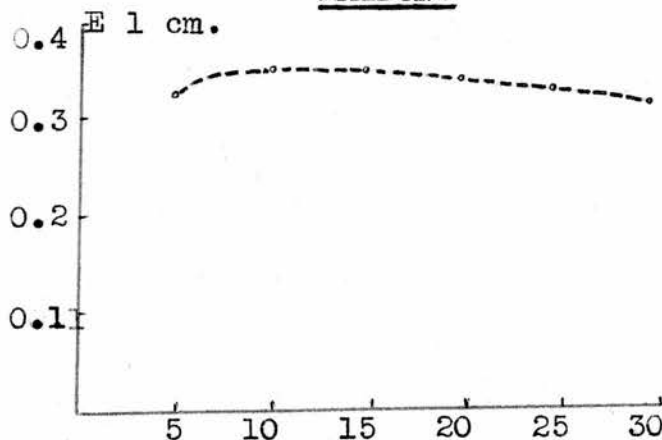
The reaction is sensitive to 0.05 mg. of pethidine and amidone. The rate of development of the colour obtained with pethidine and amidone is shown in Figs. 54 and 55. Maximum intensity is reached in about 8 minutes and 13 minutes with pethidine and amidone respectively. The absorption curves are shown in Figs. 56 and 57. Maximum absorption is in the region of/

# JANOVSKY'S REACTION

PETHIDINE



AMIDONE



mins. after adding sodium hydroxide. mins. after adding sodium hydroxide.  
Fig. 54. Rate of development of colour at 540 mμ. Fig. 55. Rate of development of colour at 520 mμ.

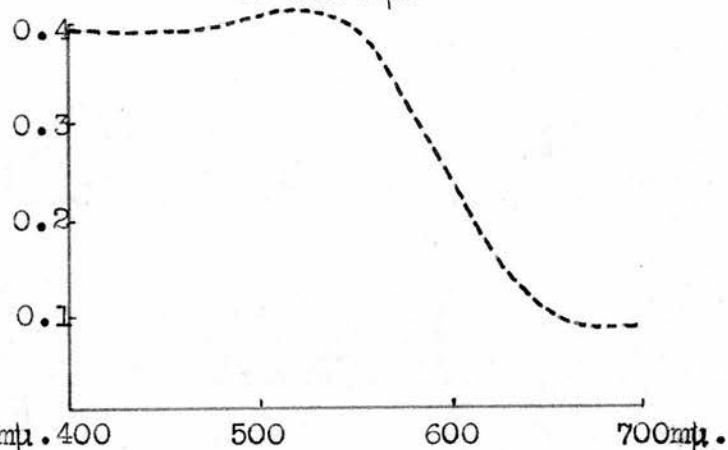
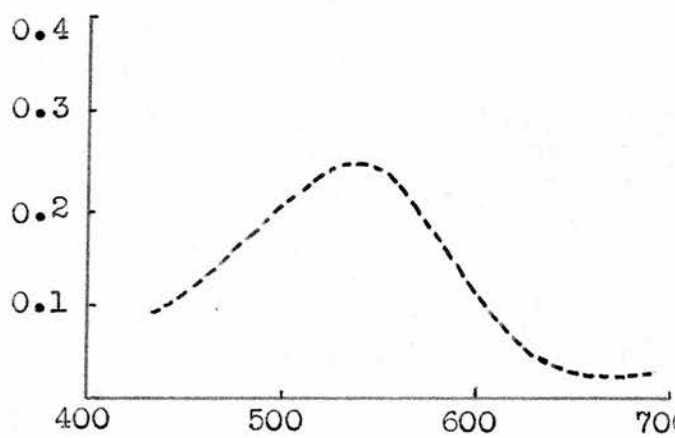
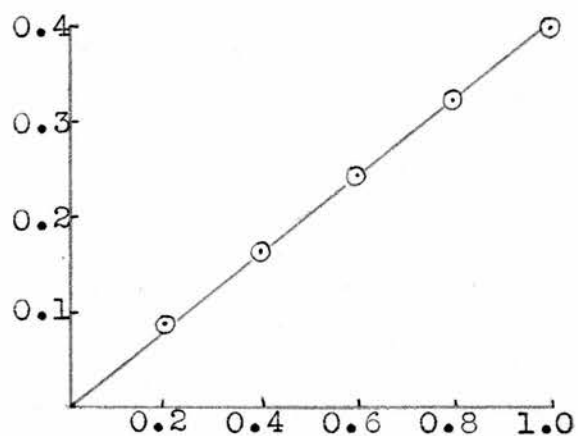
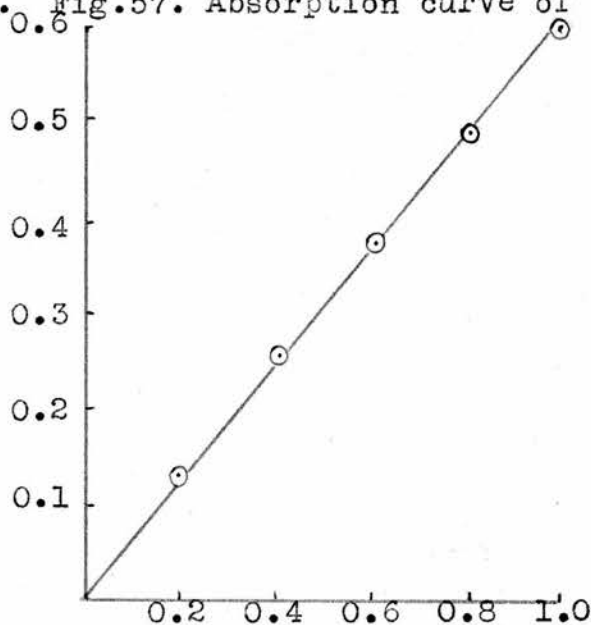


Fig. 56. Absorption curve of colour. Fig. 57. Absorption curve of colour.



mg. of pethidine hydrochloride.



mg. of amidone hydrochloride.

Fig. 58. Calibration curve at 540 mμ. Fig. 59. Calibration curve at 520 mμ.

of 540 mμ and 520 mμ with pethidine and amidone respectively.

Calibration data

Volume of solvent = 10 ml. of 80% aqueous acetone.

Volume of alkali = 0.2 ml. of 2.5N Sodium hydroxide solution.

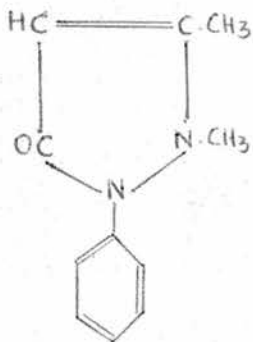
The maximum extinctions measured in a 1 cm. cell are given below.

| <u>mg. hydrochloride</u> | <u>Pethidine</u>  | <u>Amidone</u>    |
|--------------------------|-------------------|-------------------|
|                          | E 1 cm. at 540 mμ | E 1 cm. at 520 mμ |
| 0.2                      | .089              | .134              |
| 0.4                      | .166              | .255              |
| 0.6                      | .242              | .370              |
| 0.8                      | .322              | .485              |
| 1.0                      | .400              | .598              |

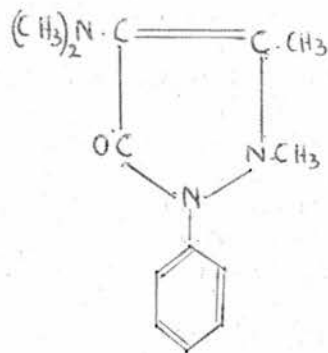
The calibration curves are shown in Figs. 58 and 59.

Group VI      The anti-pyretic drugs.

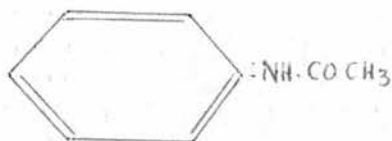
This group consists of phenazone, amidopyrine, phenacetin and acetanilide.



phenazone



amidopyrine



acetanilide



phenacetin

Acetanilide and phenacetin are extracted with ether from acid solutions in the Stas-Otto process whereas phenazone and amidopyrine are obtained in the non-phenolic alkaloid group.

1. Mohler's test:

All four drugs in this group give yellowish colours.

2. Janovsky's reaction:

The predominant colour reaction in the case of phenazone and amidopyrine are obtained with the alkaline-chloroform extracts. These give eosin-red colours with both alcoholic potash and aqueous sodium hydroxide. The colour with alcoholic potash, however, fades rapidly. The test is sensitive to 0.05 mg. of phenazone and amidopyrine. The acid-ether extracts give light brownish-red colours.

Acetanilide behaves like phenazone and amidopyrine but gives a much weaker colour. The test is sensitive to about 0.2 mg. of acetanilide. Phenacetin gives no colour reaction.

| <u>Acid-ether extract</u> |                              | <u>Alkaline-chloroform extract</u>   |                                 |
|---------------------------|------------------------------|--------------------------------------|---------------------------------|
|                           |                              | <u>Alcoholic potash</u>              | <u>Aqueous sodium hydroxide</u> |
| Phenazone                 | } light brownish-red colour. | eosin-red fades                      | eosin-red                       |
| Amidopyrine               |                              | rapidly                              | stable                          |
| Acetanilide               | light brownish-red colour    | light eosin-red colour fades rapidly | light eosin-red colour stable   |
| Phenacetin                | no colour                    | no colour                            | no colour                       |

Group VII    Miscellaneous alkaloids containing no benzene or naphthalene nucleus.

The drugs studied in the previous groups, except the opium alkaloids contained a benzene nucleus which could be nitrated. In this group a few of the alkaloids which contain neither a benzene nor a naphthalene ring were examined.

A mixture of potassium nitrate and sulphuric acid is not only a nitrating<sup>agent</sup>/but is also a drastic oxidising agent and under its influence the more complex alkaloids undergo degradation. If these degradation products are benzene or naphthalene derivatives nitration may also take place.

The alkaloids examined in this group consist of caffeine, strychnine, brucine, veratrine, quinine and yohimbine.

1. Mohler's test.

All/

All the alkaloids in this group gave yellow or brownish-yellow colours except caffeine which remained colourless.

## 2. Janovsky's reaction.

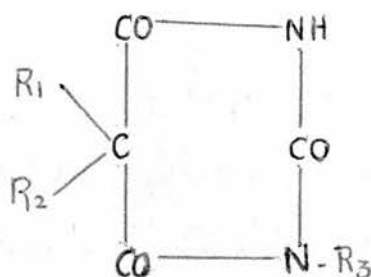
The alkaloids except strychnine gave either no colour reaction or only light brownish-yellow colours. In the case of strychnine the acid-ether extract gave a blood-red colour and the alkaline-chloroform extract gave no colour reaction.

The colour reaction obtained with strychnine is probably due to naphthalene derivatives formed on degradation.

## Group VIII      The barbiturates

The barbiturates are derivatives of barbituric acid. They are mainly 5:5 di-substituted derivatives. They are extracted with ether from acid solution in the Stas-Otto process. There are about 18 clinically important barbiturates and their individual identification is a very difficult problem. Turfitt<sup>82</sup> has described a micro-chemical procedure for the routine identification of barbiturate drugs based on colour reactions and crystal tests. Final absolute identification is however accomplished by mixed melting-point determinations.

Of the barbiturates examined only the phenyl and cyclohexenyl derivatives, as expected, responded to Mohler's and Janovsky's tests.



|                                                            | <u>Substituents</u>           |                               |                      |
|------------------------------------------------------------|-------------------------------|-------------------------------|----------------------|
|                                                            | <u>R<sub>1</sub></u>          | <u>R<sub>2</sub></u>          | <u>R<sub>3</sub></u> |
| Barbituric acid                                            | H                             | H                             | H                    |
| Barbitone (5:5-di-ethyl barbituric acid)                   | C <sub>2</sub> H <sub>5</sub> | C <sub>2</sub> H <sub>5</sub> | H                    |
| Allobarbitone (5:5-di-allyl barbituric acid).              | C <sub>3</sub> H <sub>5</sub> | C <sub>3</sub> H <sub>5</sub> | H                    |
| Phenobarbitone (5-phenyl-5-ethyl barbituric acid).         | C <sub>2</sub> H <sub>5</sub> | C <sub>6</sub> H <sub>5</sub> | H                    |
| Phemitone (N-methyl-5-phenyl-5-ethyl barbituric acid).     | C <sub>2</sub> H <sub>5</sub> | C <sub>6</sub> H <sub>5</sub> | CH <sub>3</sub>      |
| Rutonal (5-phenyl-5-methyl barbituric acid).               | CH <sub>3</sub>               | C <sub>6</sub> H <sub>5</sub> | H                    |
| Cyclobarbitone (5-ethyl-5-Δ-cyclohexenyl barbituric acid). | C <sub>2</sub> H <sub>5</sub> | C <sub>6</sub> H <sub>9</sub> | H                    |
| Hexobarbitone (5-methyl-5-Δ-cyclohexenyl barbituric acid). | CH <sub>3</sub>               | C <sub>6</sub> H <sub>9</sub> | H                    |

#### 1. Mohler's test.

The phenyl-barbiturates, phenobarbitone, phemitone and rutonal gave beautiful purple colours. The cyclohexenyl derivatives cyclobarbitone and hexobarbitone gave brownish-violet colours quite/

quite distinct from those given by the phenyl-barbiturates. Allobarbitone gave a yellow colour and barbitone no colour. The test is sensitive to 0.1 mg. of the phenyl and cyclohexenyl derivatives.

The quantitative application of Mohler's test was studied only in the case of the three phenyl-barbiturates. Preliminary experiments however showed that it is applicable to the cyclohexenyl derivatives as well.

The absorption curves (Figs. 62, 63 and 67) of the colours obtained with the phenyl barbiturates are similar. They exhibit a broad peak. Maximum absorption is in the region of 550 m $\mu$ . The rate of development of the colour is shown in Figs. 60, 61 and 66. Maximum intensity is attained in about 60 minutes and thereafter remains practically unchanged for a further 25 minutes.

Calibration data.

Extinctions measured at 550 m $\mu$  in a 1 cm. cell are given below. The readings were taken after 65 minutes from the time of addition of hydroxylamine hydrochloride.

| <u>mg. barbiturate</u> | <u>E 1 cm. at 550 m<math>\mu</math></u> |                  |                |
|------------------------|-----------------------------------------|------------------|----------------|
|                        | <u>phenobarbitone</u>                   | <u>phemitone</u> | <u>rutonal</u> |
| 0.25                   | .110                                    | .122             | .104           |
| 0.50                   | .214                                    | .240             | .206           |
| 0.75                   | .333                                    | .367             | .317           |
| 1.00                   | .458                                    | .492             | .432           |

The/

# MOHLER'S REACTION

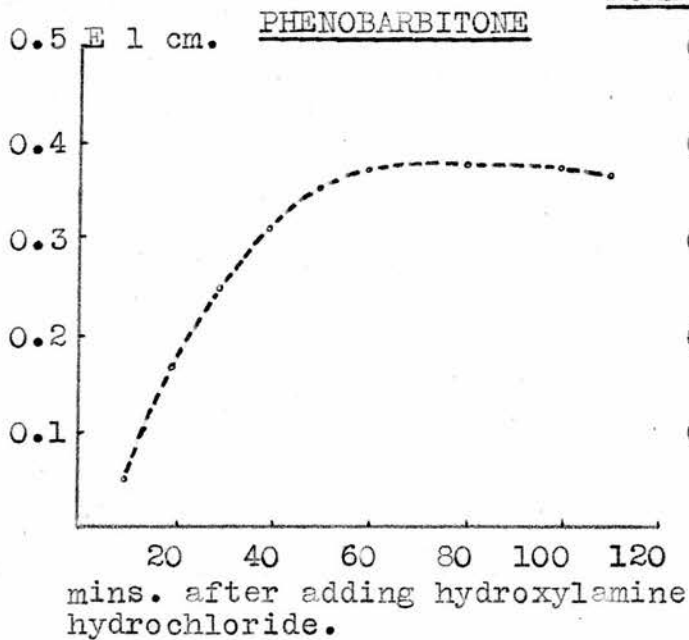


Fig.60 . Rate of development of colour at 550 mμ.

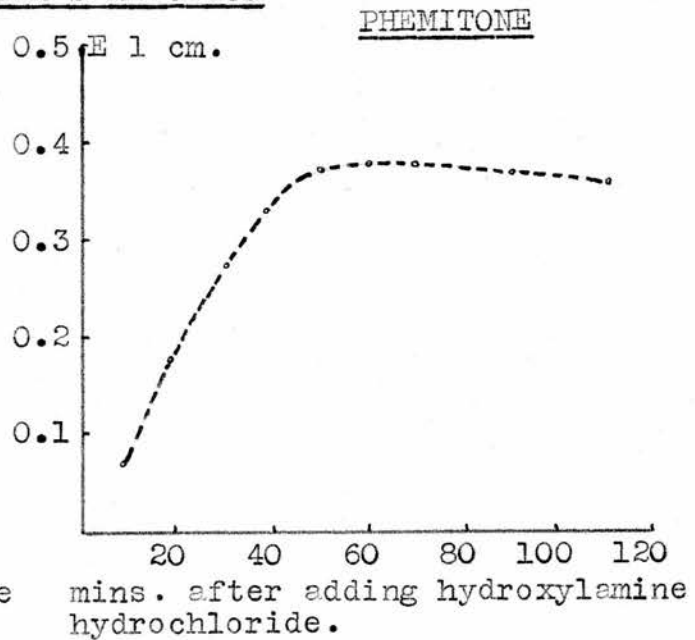


Fig.61. Rate of development of colour at 550 mμ.

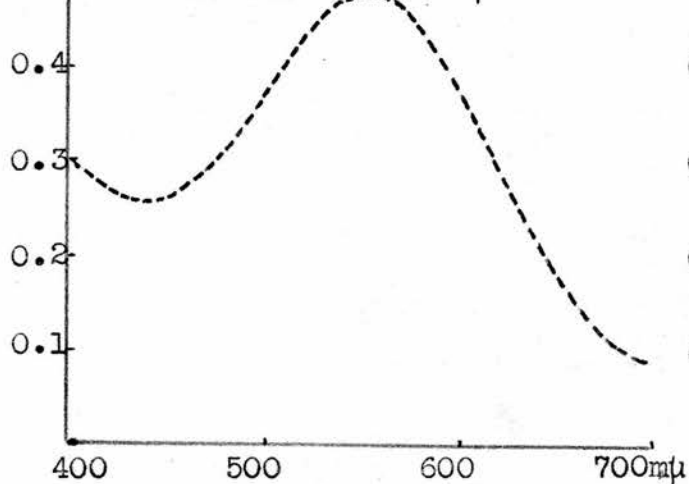


Fig.62. Absorption curve of colour.

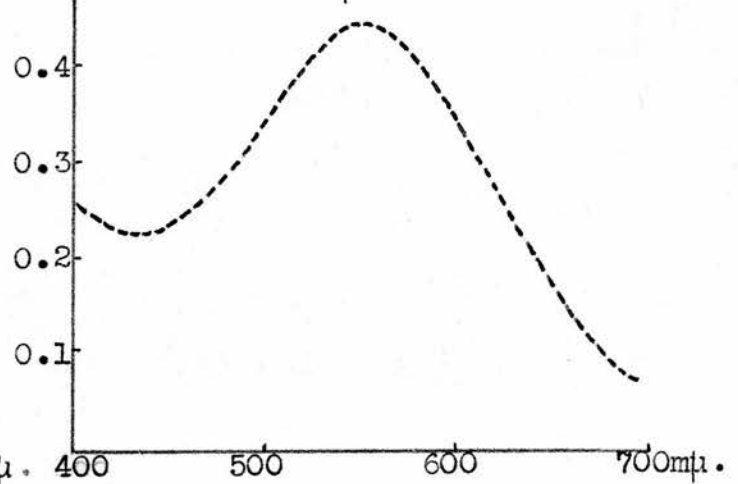


Fig.63. Absorption curve of colour.

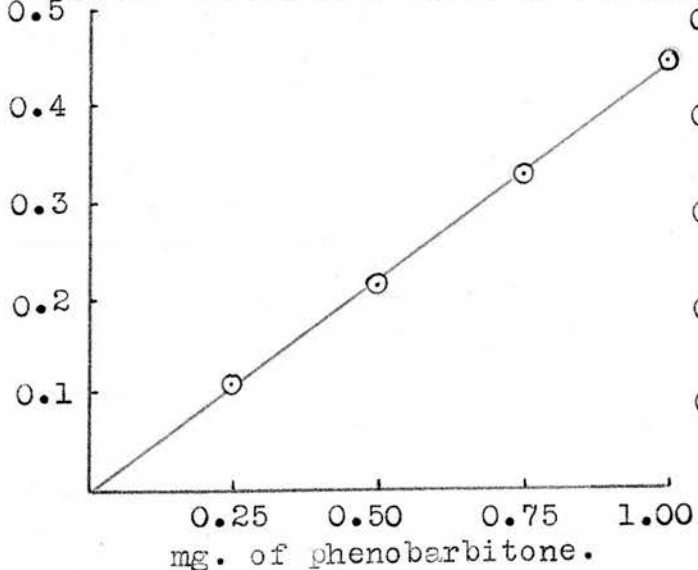


Fig.64. Calibration curve at 550 mμ.

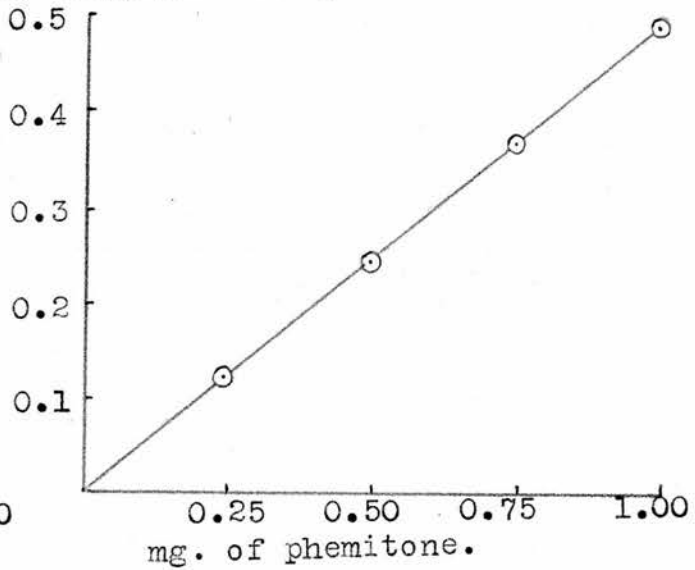


Fig.65. Calibration curve at 550 mμ.

# MOHLER'S REACTION

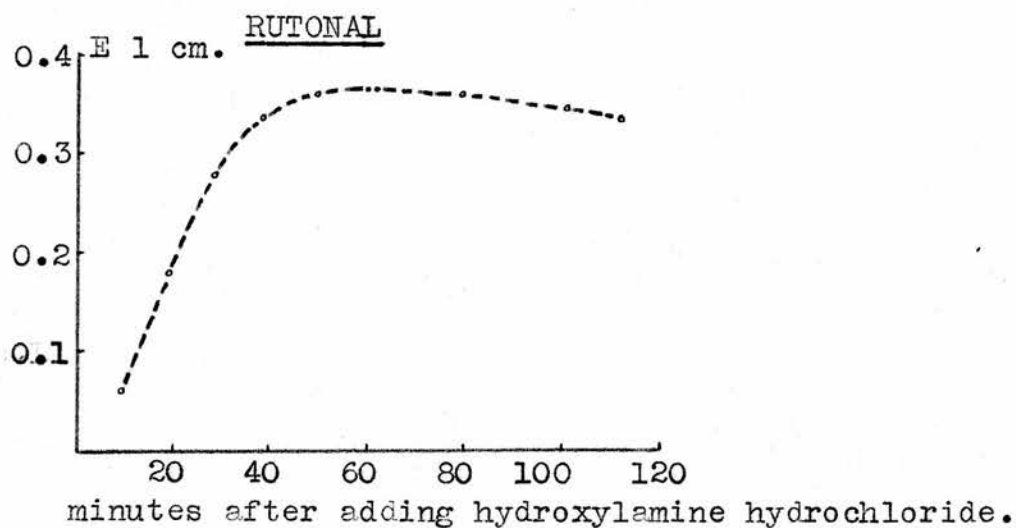


Fig.66. Rate of development of colour at 550 mμ.

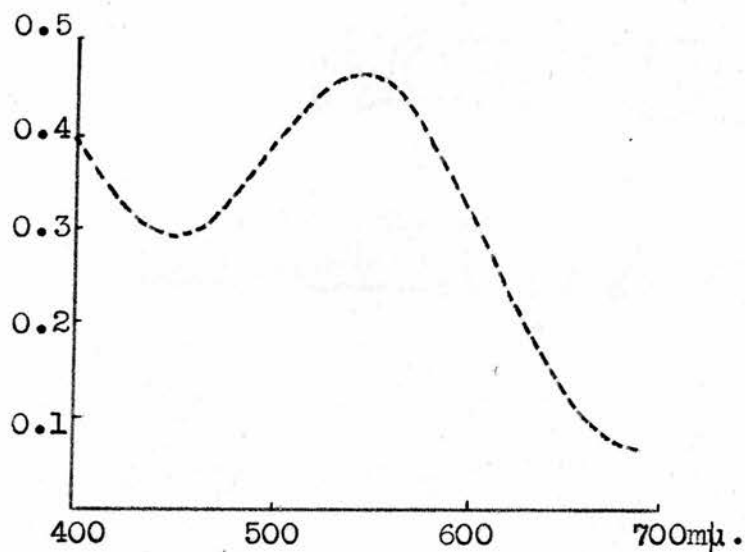


Fig.67. Absorption curve of colour.

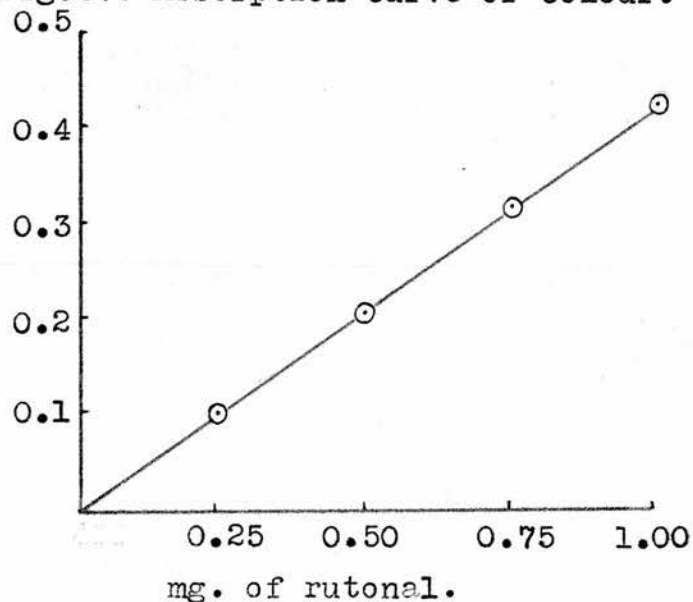


Fig.68. Calibration curve at 550 mμ.

The Calibration curves are shown in Figs. 64, 65 and 68.

Mohler's test serves to distinguish the phenyl and cyclohexenyl derivatives from each other and from the other barbiturates. It is a more sensitive test for these barbiturates than Janovsky's test.

## 2. Janovsky's reaction.

As in Mohler's test only the phenyl and cyclohexenyl derivatives gave colour reactions. The predominant colour reaction is, of course, obtained with the acid-ether extract. The alkaline-chloroform extracts give no colour reactions. Barbitone and allobarbitone gave no colour reaction and a brownish colour respectively.

Phenobarbitone gives a violet colour whereas phemitone and rutonal give bluish-violet colours which change slowly to violet. Hexobarbitone gives a violet colour and cyclobarbitone a reddish-violet colour. The reaction is sensitive to 0.2 mg. of these barbiturates.

Effect of the chloride ion in Janovsky's and Mohler's reactions.

The initial experiments on hyoscine were carried out using hyoscine hydrobromide. Instead of the expected violet colour as in the case of atropine and hyoscyamine, a greyish colour was obtained in Mohler's test. However, when the free alkaloid was used a violet colour was obtained. This behaviour in the presence of the bromide ion led to the investigation of the influence of the chloride ion on the colour obtained in Janovsky's and Mohler's tests.

On account of the volatile nature of some basic substances it is sometimes necessary in toxicological work to convert the free bases into their non-volatile salts. The usual procedure is to 'strip' an ether or chloroform solution of the base with dilute acetic acid and remove the excess of acid by evaporation. Where the acetate too is volatile (e.g. amphetamine) the base is converted into its hydrochloride. The influence of the chloride ion on the two reactions is therefore of particular interest in toxicological work.

The effect of the chloride ion was studied both qualitatively and quantitatively by carrying out the reactions on the free base (or the sulphate) as well as the hydrochloride. The results show that in Mohler's test, the chloride ion has no effect on the hue of the colour but has an appreciable effect on the intensity in some cases. In Janovsky's test neither the hue nor the intensity is affected.

1. Mohler's test.

| <u>mg. cocaine</u> | <u>cocaine alkaloid</u> | <u>cocaine hydrochlor.</u> |         |
|--------------------|-------------------------|----------------------------|---------|
| 0.2                | .071                    | .074                       |         |
| 0.4                | .142                    | .144                       | E 1 cm. |
| 0.6                | .211                    | .215                       | at      |
| 0.8                | .270                    | .269                       | 440 mμ. |
| 1.0                | .328                    | .330                       |         |

| <u>mg. ephedrine</u> | <u>ephed. sulphate</u> | <u>ephed. hydrochlor.</u> |         |
|----------------------|------------------------|---------------------------|---------|
| 0.2                  | .085                   | .085                      |         |
| 0.4                  | .175                   | .180                      | E 1 cm. |
| 0.6                  | .280                   | .280                      | at      |
| 0.8                  | .372                   | .366                      | 500 mμ. |
| 1.0                  | .459                   | .450                      |         |

| <u>mg. amphetamine</u> | <u>amphet. sulphate</u> | <u>amphet. hydrochlor.</u> |         |
|------------------------|-------------------------|----------------------------|---------|
| 0.2                    | .070                    | .090                       |         |
| 0.4                    | .150                    | .181                       | E 1 cm. |
| 0.6                    | .235                    | .267                       | at      |
| 0.8                    | .317                    | .355                       | 550 mμ. |
| 1.0                    | .398                    | .450                       |         |

The chloride ion has an appreciable intensifying effect on the colour in the case of amphetamine and hardly any effect in the case of cocaine and ephedrine.

2./

2. Jenovsky's test.

| <u>mg. cocaine</u>     | <u>cocaine alkaloid</u> | <u>cocaine hydrochlor.</u> |         |
|------------------------|-------------------------|----------------------------|---------|
| 0.10                   | .140                    | .140                       | E 1 cm. |
| 0.20                   | .295                    | .290                       | at      |
| 0.30                   | .428                    | .438                       | 560 mμ. |
| <u>mg. amphetamine</u> | <u>amphet. sulphate</u> | <u>amphet. hydrochlor.</u> |         |
| 0.2                    | .200.                   | .198                       |         |
| 0.4                    | .410                    | .404                       | E 1 cm. |
| 0.6                    | .590                    | .585                       | at      |
| 0.8                    | .780                    | .776                       | 560 mμ. |

In quantitative work, whenever the hydrochloride is used for the test, reference should be made to Calibration curves constructed using the hydrochloride.

Excess of bromide (a few mg.) added as potassium bromide prior to nitration either inhibited the reactions or gave only weak colour reactions. This may be due to liberation of bromine by the nitrating mixture and subsequent bromination of the benzene nucleus. No such inhibiting effect was observed with potassium chloride.

Some practical applications.

Known amounts of alkaloids were added to 50 ml. of urine and the alkaloids were extracted and estimated by Mohler's or Janovsky's reactions.

The usual 'double-extraction' process was used to recover the alkaloids from the urine (i.e. extract alkaloid with an immiscible solvent, 'strip' the solvent extract with dilute sulphuric acid and re-extract the alkaloid from the acid-liquid after alkalising). To prevent precipitation of phosphates, saturated sodium citrate solution was added to the urine before alkalising.

To obtain clean alkaloidal residues filtration of solvent extracts at every stage is essential. Filtration, entailing minimum loss of material, is best carried out by using a plug of cotton wool layered with anhydrous sodium sulphate. The use of filter paper is not to be recommended as it involves appreciable loss.

The results obtained are given below. A blank on the urine was done at the same time. The extinction of the light brownish-yellow colour obtained in the blank is expressed in terms of its alkaloidal equivalent.

Alkaloid/

| <u>Alkaloid</u> | <u>mg. added</u> | <u>mg. found</u> | <u>method used</u> | <u>Blank equivalent in mg.</u> |
|-----------------|------------------|------------------|--------------------|--------------------------------|
| Atropine        | 1.0              | 0.98             | Mohler             | .03                            |
|                 | 0.7              | 0.68             |                    |                                |
| Aconitine       | 0.8              | 0.71             | Janovsky           | .02                            |
|                 | 0.5              | 0.44             |                    |                                |
| Methyl-         | 1.0              | 1.0              | Mohler             | .05                            |
| amphetamine     | 1.0              | 1.0              | Janovsky           | negligible                     |
| Amphetamine     | 0.7              | 0.78             | Mohler             | .05                            |
|                 | 0.5              | 0.54             | Janovsky           | negligible                     |
| Pethidine       | 0.5              | 0.4              | Janovsky           | nil.                           |
| Ephedrine       | 0.6              | 0.65             | Mohler             | .04                            |
| Cocaine         | 1.0              | 0.95             | Janovsky           | negligible                     |
|                 | 1.0              | 1.17             | Mohler             | 0.1                            |

The nitrating mixture destroys to a large extent impurities associated with alkaloidal residues isolated from biological sources, by oxidation and conversion to alkali-soluble products. However such interfering substances are not destroyed completely.

The error introduced by the extraneous colour though not serious is greater in Mohler's test than in Janovsky's. This no doubt is due to the fact that in Janovsky's test extraction of the products of nitration with solvents eliminates to a large extent interfering substances. Moreover, the size of the blank depends on the steepness of the Calibration curve. Mohler's test is/

is less sensitive and consequently the Calibration curves are less steep than in Janovsky's test.

The extinctions measured at different wavelengths in Janovsky's and Mohler's tests on a few specimens of normal urine are given below. The results show that (1) the intensity of the brownish-yellow colour obtained varies with the colour of the urine (2) the error introduced by this extraneous colour is not important when extinction measurements are made in the range 500 to 700 mμ. (3) in Janovsky's test the error introduced is negligible in the case of the alkaline-chloroform extracts.

1. Mohler's test.

| E 1 cm.           |          |       |      |       |      |       |
|-------------------|----------|-------|------|-------|------|-------|
| Wavelength<br>mμ. | Spec. 1. | 2.    | * 3. | 4.    | * 5. | 6.    |
| 440               | .022     | .019  | .049 | .025  | .036 | .023  |
| 480               | .011     | .009  | .025 | .012  | .018 | .011  |
| 500               | .008     | .007  | .020 | .010  | .014 | .009  |
| 540               | .005     | .006  | .015 | .007  | .010 | .005  |
| 580               | <.005    | <.005 | .012 | <.005 | .008 | <.005 |
| 620               | "        | "     | .013 | "     | .009 | "     |
| 660               | "        | "     | .011 | "     | .008 | "     |

\* dark specimens of urine

2. Janovsky's test: 5 ml. of solvent + 0.1 ml. alkali.

E 1 cm.

| Wavelength<br>mμ. | Acid-ether extract |      |      |      | Alkaline-chloroform extract |       |      |      |
|-------------------|--------------------|------|------|------|-----------------------------|-------|------|------|
|                   | Spec. 1.           | 2.   | * 3. | * 4. | Spec. 1.                    | 2.    | * 3. | * 4. |
| 440               | .047               | .047 | .074 | .060 | .007                        | .006  | .009 | .008 |
| 480               | .032               | .034 | .052 | .041 | .006                        | .006  | .008 | .007 |
| 500               | .025               | .028 | .044 | .034 | .005                        | .005  | .006 | .005 |
| 540               | .015               | .020 | .032 | .024 | <.005                       | <.005 | .006 | .005 |
| 580               | .010               | .015 | .022 | .016 | "                           | "     | .007 | .006 |
| 620               | <.005              | .010 | .015 | .010 | "                           | "     | .006 | .005 |
| 660               | "                  | .006 | .010 | .008 | "                           | "     | .006 | .005 |

\* dark specimens of urine

Pure redistilled solvents should be used for the extractions. With ordinary ether and chloroform light brownish-yellow colours develop in Mohler's and Janovsky's tests and is particularly noticeable with ether.

Mohler's/

| m $\mu$ | Mohler's test |            | Janovsky's test.   |            |                        |            |
|---------|---------------|------------|--------------------|------------|------------------------|------------|
|         |               |            | Acid-ether extract |            | Alk-chloroform extract |            |
|         | Ether         | Chloroform | Ether              | Chloroform | Ether                  | Chloroform |
| 440     | .035          | .013       | .065               | .027       | .005                   | <.005      |
| 480     | .020          | .010       | .053               | .021       | <.005                  | nil        |
| 500     | .016          | .007       | .046               | .017       | "                      | "          |
| 540     | .011          | .005       | .026               | .010       | "                      | "          |
| 580     | .011          | <.005      | .019               | .007       | "                      | "          |
| 620     | .012          | "          | .014               | .005       | "                      | "          |
| 660     | .013          | "          | .010               | .005       | "                      | "          |

DISCUSSION

The fundamental reaction involved in Janovsky's and Mohler's tests is nitration to polynitro-derivatives. The Vitali-Morin test for the solanaceous alkaloids is in fact an instance of Janovsky's reaction. The scope of the Vitali-Morin test using fuming nitric acid as nitrating agent has been extensively studied.

A large number of drugs both basic and non-basic met with in toxicological work contain a benzene nucleus and thus provide the necessary basis for the development of colour tests based on Mohler's and Janovsky's reactions, for their detection and estimation. Fuming nitric acid fails to produce intensive nitration in many cases and it is indeed surprising to find that a mixture of potassium nitrate and sulphuric acid, a more energetic nitrating agent capable of introducing more than one nitro group into an aromatic nucleus, has been used only in a few cases.

Nitration of an organic compound rarely gives a 100 per cent yield of a single product but is usually accompanied by smaller amounts of isomeric derivatives. The nitrating mixture destroys to a large extent impurities associated with alkaloidal residues isolated from biological sources, by oxidation and conversion to alkali soluble products. This is an advantage when dealing with slightly impure residues.

In the case of the alkaloids recovered from urine it has been demonstrated that interference due to associated impurities is/

is negligible or almost nil with the alkaline-chloroform extracts in Janovsky's test. In Mohler's test and with the acid-ether extracts in Janovsky's test this interference is not serious when spectro-photometric measurements are made in the range 500 to 700 mμ. It should however be pointed out that the alkaloids were recovered from urine by direct extraction and have not been submitted to any process of purification.

The nitrating mixture is also a drastic oxidising agent and under its influence the alkaloids, the more complex ones in particular, undergo degradation as well. These degradation products give rise to light brownish-yellow colours on alkalisng and tend to modify to some extent the colour obtained in Mohler's test. In Janovsky's test where solvent extraction is used to isolate the products of nitration such interference is largely eliminated and purer colours are obtained.

Hydrolysis of esters which also occurs during nitration is only slight in the majority of cases but complete in a few. In the former case the predominant colour reaction in Janovsky's test is obtained with the alkaline-chloroform extract and in the latter with the acid-ether extract. This difference in behaviour of the two extracts sometimes serves to differentiate between members of the same group (e.g. amylocaine from cocaine, benzamine and metycaine). The two tests since they depend on the presence of a nitratable benzene nucleus, serve to differentiate the phenyl/

phenyl derivatives from the others (e.g. phenylbarbiturates from the others).

Both Mohler's and Janovsky's tests are sensitive reactions for the substances studied. The former gives a measurable colour with 0.1 mg. and the latter with 0.05 mg. or less in most cases. In general drugs containing a benzene nucleus respond to the two tests. The colour reactions obtained are, however, not specific; substances closely allied to each other chemically give identical colour reactions (e.g. amphetamine and methylamphetamine; cocaine, metycaine and benzamine).

In toxicological work proving the absence of a particular drug or group of drugs is as difficult as proving its presence. The two tests, Janovsky's test in particular, may be used either to obtain provisional evidence of the presence of a drug or as a negative test to eliminate its presence with reasonable certainty. Their great value however lies in the fact that once identity is established the two reactions could be used for the determination of small amounts of the drugs.

SUMMARY

1. Janovsky's and Mohler's reactions depend on the presence of a nitratable benzene or naphthalene nucleus. The fundamental reaction involved in both being nitration to polynitro-derivatives. Many drugs met with in toxicological work contain a benzene nucleus, thus providing the necessary basis for the development of colour tests for their detection and estimation. Using a mixture of potassium nitrate and sulphuric acid as nitrating agent, it has been shown that the two reactions are capable of wide application.
2. In Mohler's test hydroxylamine hydrochloride is used as reducing agent and the reduction is allowed to proceed at room temperature. Grossfeld has shown that the rate of development of colour is accelerated by heat. Illing however has pointed out that at higher temperatures decomposition of the chromogen may occur. In the method finally adopted the disadvantage in carrying out the reduction at room temperature is offset by the use of stronger ammonia solution. Higher concentrations of ammonia or hydroxylamine hydrochloride accelerate the rate of development of colour.
3. In the test based on Janovsky's reaction, the products of nitration are isolated by solvent extraction first from acid medium with ether and then from alkaline medium with chloroform, prior/

prior to applying the test. A more or less stable colour is obtained when the 'acid-ether residue' is dissolved in acetone-alcohol mixture and treated with alcoholic potash. For the 'alkaline-chloroform residue' the solvent used is acetone and the alkali, alcoholic potash or aqueous sodium hydroxide.

4. Alkaloids which are esters of benzoic acid or its homologues also undergo hydrolysis during nitration and colour reactions are therefore obtained with both the acid-ether and alkaline-chloroform extracts. Hydrolysis is however slight in the majority of cases.
5. Both reactions are sensitive tests for many of the drugs examined. The tests however are not specific but provide presumptive evidence only. Drugs which are closely allied to each other chemically give identical colour reactions. Once identity is established the two reactions are of value in the determination of small amounts of the drugs.
6. The drugs examined fall into the following groups (1) the sympathomimetic amines, (2) the alkaloids of the atropine group, (3) the local anaesthetics of the benzoic ester group, (4) the aconite alkaloids, (5) the opium alkaloids and the synthetic morphine substitutes pethidine and amidone, (6) the antipyretic drugs and (7) the barbiturates.

The colour reactions observed are recorded and quantitative methods for their determination have been worked out in many cases.

7. In some cases, chloride ions appear to have an effect on the intensity of the colour produced in Mohler's test. Whenever the hydrochloride is used for the test reference should be made to Calibration curves constructed using the hydrochloride. The hydrobromide may not be used as bromide ions inhibit the reaction.
8. Colour intensities were measured spectrophotometrically using the S.P. 350 Diffraction Grating Spectrophotometer.
9. The colour reactions obtained in Mohler's test remain stable for an appreciable length of time and are therefore suitable for visual comparison also.

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