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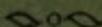
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SOME COLOUR REACTIONS OF ADRENINE AND ALLIED BASES

BY

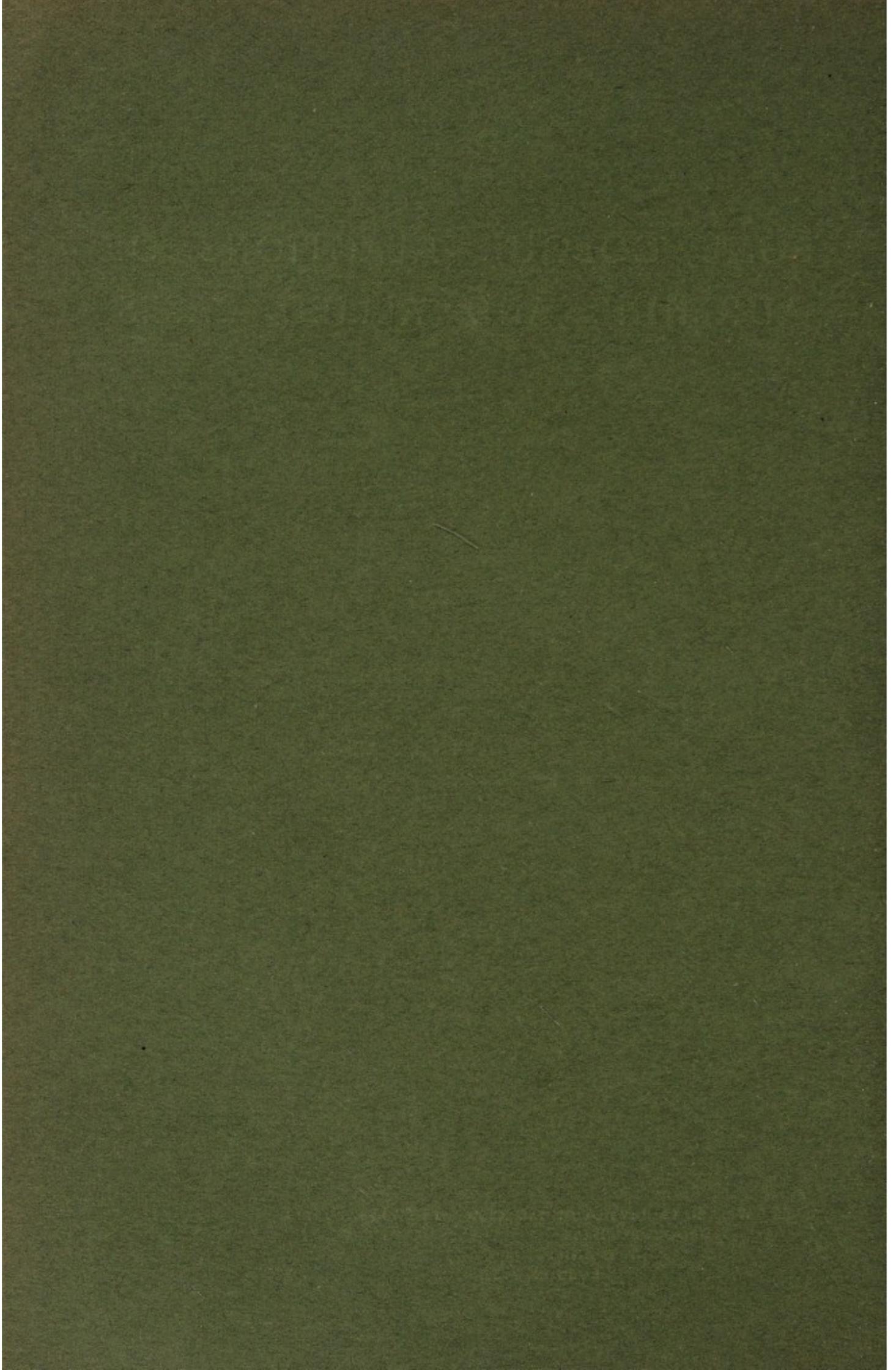
A. J. EWINS, B.Sc.

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SOME COLOUR REACTIONS OF ADRENINE AND ALLIED BASES. BY A. J. EWINS.

(From the Wellcome Physiological Research Laboratories, London.)

THE physiological investigation of a number of bases very closely related to adrenine in chemical constitution has recently been carried out in these laboratories. In view of this relationship it was considered of interest to examine these bases with regard to the more characteristic colour reactions which from time to time have been suggested for the identification or estimation of adrenine in various fluids. Apart from those reactions of the latter base which are due to the fact that it is a catechol derivative, the following characteristic reactions have been described.

(1) The production of a rose red colour on the addition of iodine (Vulpian)¹.

(2) The formation of a red colour on addition of mercuric chloride (Comessatti)².

(3) The production of a reddish violet colour by the action of potassium biiodate and phosphoric acid (Fränkel and Allers)³ or by the action of iodic acid (Krauss)⁴.

The reaction with iodine.

The fact that a substance was present in the supra-renal gland which gave a rose red colour with iodine was first observed by Vulpian (*loc. cit.*).

Later when the isolation of the physiologically active principle of the supra-renal gland had shown that this compound was the chromogenic substance observed by Vulpian this reaction was suggested by

¹ Vulpian. *Compt. Rend.* 1856. XLIII. 663.

² Comessatti. *Münch. med. Wochenschr.* 1908. No. 37.

³ Fränkel and Allers. *Biochem. Ztschr.* XVIII. 40. 1909.

⁴ Krauss. *Apotheker Zeitung*, 1908. 701.

Abelous, Soulié and Toujan¹ as a means of quantitatively estimating adrenine in solution. The method however when applied to aqueous extracts of supra-renal glands does not yield results in conformity with those obtained by physiological standardisation, at present the only satisfactory method of estimating adrenine. With solutions of the pure base the delicacy of this reaction is very great. A distinct reaction is obtainable with solutions of one part of the hydrochloride (or other salt) of adrenine in one million of solution. The rose red colour is apparently due to oxidation under the influence of iodine the same colour being produced by the action of other oxidising agents, or when dilute neutral solutions of adrenine are allowed to stand exposed to light and air.

Comessatti's Reaction.

In a preliminary paper published in 1908 G. Comessatti² recorded a new characteristic colour reaction for adrenine, which he has since employed for the quantitative estimation of the base³. The reaction was stated to be brought about by the addition of a few drops of mercuric chloride (0.2%) to a dilute solution of the base. On standing at the laboratory temperature a distinct rose red colour was produced within a few minutes. The maximum dilution at which the reaction was obtainable was stated to be 1—400,000. K. Boas⁴ was unable to confirm these observations and his experiments were confirmed by Fränkel and Allers (*loc. cit.*) who stated that the reaction as indicated by Comessatti could not be obtained except on very long standing or by carrying out the reaction according to a modification suggested by Boas (heating to boiling and shaking vigorously). With solution of 1—400,000 adrenine they could obtain no reaction in the cold and only a very feeble coloration on heating.

In reply to Boas, Comessatti⁵ asserted that the reaction could be obtained but mentioned the fact that his solution of mercuric chloride was prepared with tap-water.

On repeating these experiments I was able to confirm these statements. If distilled water be used in making up the solution no reaction

¹ Abelous, Soulié and Toujan. *Soc. de Biol.* 1905. I. 301.

² Comessatti. *Münch. med. Wochenschr.* 1908. No. 37.

³ Comessatti. *Archiv für exp. Pathol. u. Pharm.* LXII. 190. 1910.

⁴ Boas. *Centr. für Physiol.* 1909. No. 26.

⁵ Comessatti. *Ibid.* 1909. XXIII. No. 6.

is obtainable while if tap-water is employed the reaction is readily observed. Further investigation showed that the production of the red colour depended on the presence of calcium bicarbonate in the tap-water. For on adding mercuric chloride to solutions of adrenine which had been prepared with distilled water and with a solution of calcium bicarbonate in distilled water respectively, the latter developed a beautiful red colour within a few minutes on standing (15° — 17° C.) while the former remained quite colourless over a long period of time.

It seemed then probable that calcium bicarbonate played the part of a catalyst in the oxidation of adrenine by mercuric chloride. Further experiments showed that mercuric chloride could be replaced by such oxidising salts as silver nitrate, platonic chloride and copper sulphate.

The same reaction occurred in the presence of calcium carbonate, acetate, citrate, or lactate and presumably it occurs in the presence of any salt of calcium with a weak acid. The substitution of such salts as calcium chloride, nitrate and sulphate gave negative results. Salts of barium and strontium acted in the same way as calcium salts. Further investigation showed that the reaction was produced under the influence of sodium acetate and finally it was found that any salt formed by the combination of a metal with a weak acid was capable of acting as catalyst in this reaction. Thus the reaction was obtained with the acetates of sodium, potassium, magnesium, strontium, barium, zinc, cobalt, nickel, manganese and lead; also with lithium formate, potassium tartrate and sodium monochloracetate but not with sodium trichloracetate.

From these facts it would appear that the Comessatti reaction is a particular instance of a general reaction, namely the oxidation of adrenine by relatively weak oxidising agents under the catalytic influence of salts which are of the type strong base + weak acid. Moreover the catalytic action of these salts must be dependent on the presence of free hydroxyl ions in their solution since they are all hydrolytically dissociated by water with consequent setting free of hydroxyl ions. This view is supported by the fact that the reaction is not obtainable with salts of the type strong base + strong acid, *e.g.* calcium chloride, sodium trichloracetate, etc., nor with salts which are formed by the combination of a weak base with a weak acid, *e.g.* ammonium acetate. Such salts are not appreciably hydrolysed under the influence of water and there is no preponderance of free hydroxyl ions in their solutions. The presence of a trace of a mineral acid completely inhibits the reaction.

This result is of interest in view of the recent work of Euler and Bolin¹. These investigators have shown that the so-called "oxydase," medicago-laccase, consists for the most part of calcium glycollate together with calcium salts of such acids as mesoxalic, malic and citric acids. Other laccases also, such for example as that obtained from *Sedum telephium*, have also been shown to consist of mixtures of salts (not of calcium only) of organic oxyacids. From experiments carried out upon the oxidation of hydroquinone under the influence of such naturally obtained "laccases" or "oxydases" and of certain sodium salts of organic oxyacids they arrived at the conclusion that such salts acted in a manner entirely comparable with that of the laccases and might even be more active catalytically than the laccases themselves. There is therefore no justification for considering such laccases as enzymes. In the case of Rhus-laccase, the first of the laccases to be investigated², it appears probable however that the catalytic action is indeed due to a ferment which is destroyed by heat and cannot be dialysed, characteristics which distinguish it from the laccases mentioned above.

It seems probable therefore that the phenomena produced by medicago- and similar laccases in bringing about rapid oxidation of hydroquinone and other polyphenolic derivatives and the oxidation of adrenine (a diphenolic derivative) as brought about by the Comessatti reaction are capable of the same explanation, being due to the catalytic action of salts of strong bases + weak acids hydrolytically dissociated by water, an action therefore which must be dependent on the presence of free hydroxyl ions in solution.

As some difficulty seems to have been encountered hitherto in obtaining the reaction indicated by Comessatti the conditions under which the reaction can be obtained with certainty may be described here in detail. To 1 c.c. of a dilute solution (1—100,000) of adrenine in distilled water is added an equal volume of a solution of sodium acetate (1%) followed by four or five drops (approx. 0.25 c.c.) of a solution of mercuric chloride in distilled water (1—1000). If the solution now be warmed to 40—50° a rose red colour is immediately obtained. At the laboratory temperature (15° C.) a pale rose tint is obtained after some four or five minutes. The use of sodium acetate is to be preferred to tap-water as ensuring uniform results.

¹ Euler and Bolin. *Ztschr. f. physiol. Chem.* LXI. 1. 1909.

² Bertrand. *C. R. de l'Acad. des Sciences* CXXIV. 1355-8.

The Fränkel-Allers Reaction.

This new characteristic reaction for adrenine was recently described by Fränkel and Allers (*loc. cit.*) and is brought about by heating to just below boiling a dilute solution of the base with an equal volume of a N/1000 solution of potassium biiodate and a few drops of dilute phosphoric acid. There is thus produced a rose red colour which with the more concentrated solutions of adrenine (1—50,000) assumes on standing a distinct violet tint. The reaction was stated to be obtainable with a dilution of 1—300,000. The production of the colour is in the opinion of the authors probably due to the formation of an iodo- or iodoso-compound since "at no stage of the reaction can free iodine be detected." This last statement is not strictly accurate as the following experiment shows.

To 5 c.c. of a solution of adrenine (1—1000) were added 4 c.c. of a N/100 solution of potassium biiodate and 1 c.c. of a 1% solution of phosphoric acid and the mixture allowed to stand at the laboratory temperature. After a few seconds a few drops of the solution (which had already become distinctly yellow) gave with a few drops of starch solution a distinct purple colour. At the end of five minutes on repeating the test a distinct blue coloration was obtained. At the end of one hour the solution had assumed the characteristic reddish violet colour normally produced on heating and free iodine could no longer be detected.

This is in agreement with a statement in a note very recently published by L. Krauss¹ who calls attention to the fact that this (Fränkel-Allers) reaction was first described by him². He employed free iodic acid in place of the biiodate phosphoric acid mixture. He states that free iodine is momentarily set free in the reaction, as may be demonstrated by shaking the mixture with chloroform, which on separating shows a pink colour due to iodine in solution, and also by mixing a few drops of a solution of iodic acid (1—20) with a solution of adrenine and adding a little starch solution when the characteristic blue colour is at once obtained.

With the catechol bases of the types of dihydroxyphenylethylamine and of aminoaceto-catechol it is still more easy to show that iodine is set free during the course of the reaction. On standing at the

¹ Krauss. *Biochem. Ztschr.* xxii. 131. 1909.

² Krauss. *Apotheker Zeitung*, 1908. 701.

ordinary temperature the solutions gradually become yellow in colour and the reaction with starch is readily obtained. On heating, the bases of the dihydroxyphenylethylamine series give the normal colour reaction while aminoaceto-catechol and the other bases of this type give yellow coloured solutions.

Further a number of catechol derivatives examined with regard to this reaction gave a similar result. Thus the following catechol derivatives

Catechol	$(\text{OH})_2\text{C}_6\text{H}_4$
Protocatechuic aldehyde	$(\text{OH})_2\text{C}_6\text{H}_3\text{CHO}$
Protocatechuic acid	$(\text{OH})_2\text{C}_6\text{H}_3\text{COOH}$
Nitroprotocatechuic acid	$(\text{OH})_2\text{C}_6\text{H}_2\text{NO}_2 \cdot \text{COOH}$
Dihydroxymandelic ethyl ester	$(\text{OH})_2\text{C}_6\text{H}_3 \cdot \text{CHOH} \cdot \text{COOC}_2\text{H}_5$

all yield free iodine with this reaction while methylene-dioxy compounds, *e.g.*

Piperonylic acid	$\text{CH}_2\text{O}_2\text{C}_6\text{H}_3\text{COOH}$
Methylene dioxymandelic ester	$\text{CH}_2\text{O}_2\text{C}_6\text{H}_3\text{CHOHCOOC}_2\text{H}_5$

as well as

Dimethoxyphenylethylamine	$(\text{CH}_3\text{O})_2\text{C}_6\text{H}_2\text{CH}_2\text{CH}_2\text{NH}_2$
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give no reaction at all.

It would appear therefore that free iodine must play an important part in this reaction, which is however to some extent modified by the conditions obtaining, since the coloration produced is distinctly different from that obtained by the direct action of free iodine on solutions of the bases.

The reaction is again very delicate with pure solutions of the bases. According to my experiments, a distinct reaction may be obtained with a solution of one part of adrenine in one million (1—1,000,000). The same order of sensitiveness is shown by the other reactive bases.

This reaction has been modified and intensified by G. Bayer¹ who finds that by addition of sulphanilic acid to the solution as prepared for the Fränkel-Allers' reaction there is produced on heating a yellow colour instead of the normal rose tint. The reaction was distinctly given with a solution of one part of adrenine in five millions. I have confirmed this result and have found the same reaction to hold good for the remaining reactive bases.

According to Bayer naphthionic acid acts in a similar manner, intensifying the reaction and producing not a yellow but a distinct

¹ *Biochem. Ztschr.* xx. 176. 1909.

violet colour. I have found however that naphthionic acid behaves with adrenine and the other reacting bases in all respects similarly to sulphanilic acid, but owing to the fact that control solutions of naphthionic acid and potassium biiodate-phosphoric acid mixture develop a yellow colour when heated the reaction is only readily observed with comparatively concentrated solutions of the bases.

A new reaction with potassium persulphate.

If to a dilute solution of adrenine a solution of potassium persulphate be added so that the concentration of the latter is approximately 0·1 per cent. and the mixture be then warmed by immersion in a boiling water-bath for a short time there is produced the rose red colour due to the oxidation of adrenine. The reaction thus obtained however appears to be more delicate than that obtained with other oxidising agents such as iodine, etc.; inasmuch as one part of adrenine in five million of solution gives a distinct reaction under these conditions.

The reaction moreover is valuable in that it is readily applicable to such solutions as are obtained by extracting the supra-renal glands or other organs with faintly acidified water. The reagent too possesses the advantage over iodine that it is colourless. Excess, therefore, does not interfere with the colour produced. Moreover the extracts obtained as above described are usually of at least a pale yellow colour. This colour which would, for example, mask the reaction suggested by Bayer, is to a considerable extent discharged under the influence of the persulphate and the resulting red tint is clean and distinct, and, under the conditions indicated, remains practically permanent for some considerable time.

An exact method of quantitatively estimating adrenine by this reaction has not yet been worked out but qualitatively it has been shown that there is a distinct parallel between the depth of colour produced and the physiological activity of the extracts. Further an extract of supra-renal glands, in which, owing to its deep yellow colour, the presence of adrenine could scarcely be detected by the iodine or the Fränkel-Allers' reaction, gave a very deep red colour with persulphate.

The reaction is given in the same way by the other reactive bases already referred to. With catechol and catechol derivatives other than these bases no coloration is obtained.

The reactions of bases closely allied to adrenaline.

As already mentioned in the introduction to this paper a number of bases have been investigated some of which bear a very close relationship both chemically and physiologically¹ to adrenaline. Of these the following catechol bases correspond most closely to it in chemical structure. They may be conveniently arranged in three groups:

I. Methylaminoethanol catechol (adrenaline)	$(\text{OH})_2\text{C}_6\text{H}_3\text{CHOHCH}_2\text{NHCH}_3$
Aminoethanol catechol	$(\text{OH})_2\text{C}_6\text{H}_3\text{CHOHCH}_2\text{NH}_2$
II. Dihydroxyphenylethylamine	$(\text{OH})_2\text{C}_6\text{H}_3\text{CH}_2\text{CH}_2\text{NH}_2$
*Dihydroxyphenylethylmethylamine	$(\text{OH})_2\text{C}_6\text{H}_3\text{CH}_2\text{CH}_2\text{NHCH}_3$
*Dihydroxyphenylethylethylamine	$(\text{OH})_2\text{C}_6\text{H}_3\text{CH}_2\text{CH}_2\text{NHC}_2\text{H}_5$
*Dihydroxyphenylethylpropylamine	$(\text{OH})_2\text{C}_6\text{H}_3\text{CH}_2\text{CH}_2\text{NHC}_3\text{H}_7$
III. Aminoaceto catechol	$(\text{OH})_2\text{C}_6\text{H}_3\text{COCH}_2\text{NH}_2$
Methylaminoaceto catechol	$(\text{OH})_2\text{C}_6\text{H}_3\text{COCH}_2\text{NHCH}_3$
Ethylaminoaceto catechol	$(\text{OH})_2\text{C}_6\text{H}_3\text{COCH}_2\text{NHC}_2\text{H}_5$
Propylaminoaceto catechol	$(\text{OH})_2\text{C}_6\text{H}_3\text{COCH}_2\text{NHC}_3\text{H}_7$
In addition to these one trihydroxy base was examined:	
Aminoaceto pyrogallol	$(\text{OH})_3\text{C}_6\text{H}_2\text{COCH}_2\text{NH}_2$

* For these bases I am indebted to Dr F. L. Pyman. (*Trans. Chem. Soc.* xcvi. 264. 1910.)

Of the foregoing bases those of the first two groups in the catechol series as well as the pyrogallol base gave all the reactions hitherto considered to be characteristic of adrenaline. The catechol bases of the ketone series III, however, gave negative results in all cases.

The following table shows the maximum dilutions at which the reactions are obtainable with the bases in question.

Base	Reaction				
	Iodine	Comessatti	Frankel-Allers	* Bayer	Potass. persulph.
Methylaminoethanol catechol (adrenaline)	1-2,000,000	1-1,000,000	1-1,000,000	1-5,000,000	1-5,000,000
Aminoethanol catechol	1-2,000,000	1-1,000,000	1-200,000	1-1,000,000	1-1,000,000
Dihydroxyphenylethylamine	1-1,000,000	1-1,000,000	1-500,000	1-1,000,000	1-1,000,000
Dihydroxyphenylethylmethylamine	1-1,000,000	1-2,000,000	1-2,000,000	1-5,000,000	1-2,000,000
Dihydroxyphenylethylethylamine	1-1,000,000	1-2,000,000	1-2,000,000	1-5,000,000	1-2,000,000
Dihydroxyphenylethylpropylamine	1-1,000,000	1-2,000,000	1-2,000,000	—	1-2,000,000
Aminoaceto pyrogallol	1-2,000,000	1-1,000,000	1-1,000,000	—	1-5,000,000

¹ The physiological investigation is being carried out by Dr H. H. Dale in conjunction with Dr G. Barger, and the results of their work will be published shortly.

Note on the relative stability of the catechol bases.

It was repeatedly observed that if solutions of the hydrochlorides (or other salts) of the bases of Group II (dihydroxyphenylethylamine, etc.) were allowed to stand under the same conditions exposed to light and air the solution of dihydroxyphenylethylamine remained for a considerable time unchanged while the solutions of the remaining bases soon became oxidised forming pink solutions or depositing black sediments. Further the solutions of the methylamino and propylamino bases appeared to deteriorate more rapidly than the ethylamino base. A solution of aminoacetypyrogallol under similar conditions undergoes very rapid oxidation. As is well known, dilute neutral solutions of adrenaline exposed to light and air soon develop a pink colour: on the other hand solutions of aminoethanol catechol, the corresponding amino base, are comparatively stable under such conditions. Solutions of the ketone bases (Group III) appear to be quite stable and may be kept indefinitely.

From these observations it appeared that marked differences in stability showed themselves in these bases and experiments were made to obtain some idea of the relative reactivity of these compounds in the hope that the results obtained might help to throw some light upon certain aspects of their physiological action.

The series of bases of the type of dihydroxyphenylethylamine were first investigated with regard to their reactivity with potassium persulphate, which gives a comparison of the rate of oxidation, and also with regard to their behaviour towards the reaction with potassium biiodate.

The general result obtained with potassium persulphate from a number of experiments was as follows.

The rate of oxidation was always most rapid with the methylamino base, this rate being about three times that with dihydroxyphenylethylamine, which was always least readily oxidised. The rate at which the propylamino base was oxidised was somewhat slower than that of the methylamino base, but was again more rapid than that of the ethylamino base. Similar results were obtained with the Fränkel-Allers' reaction.

Direct comparison of these bases with those of the adrenaline group showed that adrenaline was the most reactive of the catechol bases but was followed very closely by dihydroxyphenylethylmethylamine, the propyl and ethylamino bases in order. The amino base of both groups reacted very much more slowly than the other members of the series but

of these the amino base corresponding to adrenine was more reactive than dihydroxyphenylethylamine.

Between the bases so far considered and those of Group III (amino-aceto catechol, etc.) there is a wide difference of reactivity. As has been shown these bases give none of the characteristic adrenine reactions. With the Fränkel-Allers' reaction iodine is set free and by a comparison of the amount of iodine set free in a given time by the various bases under similar conditions it was found that here again the methylamino base was most reactive closely followed by the ethyl and propyl bases, between which there seems to be very little difference, while the amino base is by far the least reactive of the series. With potassium persulphate, which on warming leads to the formation of a dark brown solution, similar results were obtained.

SUMMARY.

(1) The colour reactions for adrenine hitherto described—the iodine reaction (Vulpian), the mercuric chloride reaction (Comessatti) and the biiodate reaction of Fränkel and Allers and of Krauss—are due wholly or in part to oxidation.

(2) Potassium persulphate has been found similarly to oxidise adrenine giving a characteristic red colour. This reaction has advantages over those mentioned above both in sensitiveness and in being readily obtained with crude extracts of the glands, etc.

(3) The Comessatti reaction has been shown to be brought about by the oxidation of adrenine by such oxidising agents as mercuric chloride, silver nitrate, or platonic chloride under the catalytic influence of salts of metals with "weak" acids (*i.e.* salts which are hydrolytically dissociated by water). The reaction is compared with the results which have been obtained in the investigation of certain "laccases."

(4) The characteristic colour reactions of adrenine have been shown to be given by certain other closely related bases, namely by (*a*) the amino base corresponding to adrenine, (*b*) dihydroxyphenylethylamine and the corresponding methyl, ethyl and propylamino bases, (*c*) amino-aceto pyrogallol. The bases of the type aminoaceto catechol do not give these reactions.



