CCLII. THE COLORIMETRIC DETERMINATION OF ADRENALINE IN SUPRARENAL GLAND EXTRACTS.

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SOON after the commencement of the manufacture of extract of suprarenal cortex for the treatment of Addison's disease it became evident that even moderate traces of adrenaline could not be tolerated owing to the large doses sometimes employed, and it became necessary to find a colorimetric method for the rapid estimation of adrenaline at certain stages of the process.

Various tests were tried on these comparatively crude extracts and the ammonium molybdate test, described later, was adopted provisionally since it was found that extracts which showed less than 1 part in 10,000 of adrenaline by this test, when injected, did not give rise to any of the physiological reactions characteristic of adrenaline.

With improved methods of manufacture much purer extracts were obtained which proved to contain no adrenaline when estimated biologically. Nevertheless, such extracts when tested colorimetrically with ammonium molybdate always gave some colour, usually equivalent to about 0.5 part per 10,000 of adrenaline but occasionally as high as 2 parts per 10,000. It was obvious, therefore, that it was useless to depend upon this test and it was decided to investigate further the subject of colorimetric determination of adrenaline in order to find a test which could be relied upon when applied to such gland extracts.

The numerous colour tests for adrenaline described in the literature have been grouped under three headings for the purposes of this investigation:

- (a) those depending primarily on the formation of a red oxidation product;
- (b) those depending on the presence of the catechol grouping;
- (c) miscellaneous.

Included under heading (a) are the following:

- (1) ferric chloride reaction, (2) potassium iodate reaction and its modifications, (3) potassium persulphate reaction, (4) mercuric chloride reactions, (5) other oxidising agents, including hydrogen peroxide, potassium ferricyanide, permanganate and dichromate, sodium bismuthate, sodium hypobromite, manganese dioxide and the halogens.

Under (b) we have included the ammonium molybdate test and Folin's test.

Miscellaneous tests include diazobenzenesulphonic acid, Azzolini's test [1931] and the ninhydrin test [Orru, 1929]. Another reaction (depending upon the formation of a blue ferrous-adrenaline complex) consists in the addition of a trace of ferrous sulphate followed by sufficient sodium phosphate (Na_2HPO_4 , $12\text{H}_2\text{O}$) to bring the mixture to neutrality. A blue colour appears which is fairly stable but which rapidly fades through a purple colour if too much alkali is added. The reaction did not seem sensitive enough for the estimation of traces of adrenaline.

A preliminary examination of any test which appeared suitable for quantitative work was made and it was soon found that some of the tests described were extremely unreliable even when considered as qualitative tests, *e.g.* those described by Azzolini [1931], Paget [1931] and Orru [1929], this last being non-specific.

The reaction with mercuric chloride, originally suggested by Comessatti [1909] and modified by Ewins [1910], was not found to be very sensitive, although its application to gland extracts has been reported by Paget and Lohéac [1928] and Bailly [1924]. The modification of Comessatti's reaction described by Stuber, Russmann and Proebsting [1923] consists in the addition of a sulphanilic acid, potassium iodate and mercuric chloride reagent to adrenaline solution and boiling for 1 minute. They claim an extraordinary sensitivity of 1 in 100 millions for this test. Viale [1930] has applied this test to blood and confirms some of Russmann's statements. This test was, however, found to be unreliable, since the reagents themselves when mixed, on boiling produced an orange-brown colour which rapidly deepened on standing, due to the liberation of iodine. Purification of the sulphanilic acid produced no improvement and attempts to remove the excess of iodine with sodium thiosulphate were unsuccessful owing to the liberation of sulphur.

The reaction between adrenaline and diazotised sulphanilic acid described by Friend [1923] for quantitative application to urine (using a colorimeter) was also found unsatisfactory. No definite red colour was obtained either with pure adrenaline or with urine to which adrenaline had been added. Moreover, the orange-yellow colour which was obtained, while certainly somewhat stronger in the presence of adrenaline, was also given by a blank determination, making the colorimetric estimation of traces of adrenaline unreliable, if not impossible. The sulphanilic acid was recrystallised but still gave a yellow colour when ammonia was added to the diazotisation mixture. Further, the addition of ferric chloride to destroy the adrenaline present (as directed by Friend) did not alter the colour ratios obtained. This test is also described by Sansoni [1930] who states that it is the most sensitive and the easiest to employ, and although we were at a loss to account for its failure further attempts to utilise it were abandoned.

The remaining tests which were satisfactory qualitatively and appeared worthy of further investigation were Folin's test, the ammonium molybdate

test, and three oxidation reactions using ferric chloride, potassium iodate and potassium persulphate respectively as oxidising agents. Two of the latter were subsequently eliminated and will be dealt with first.

The ferric chloride reaction.

This reaction is characterised by the formation in slightly acid or neutral solution of a transient grass-green coloration, which changes to red on standing or by careful addition of dilute alkali. It is said that the more acid the solution the more fugitive and less marked is the green colour produced.

It was found that with pure adrenaline the green colour took about 10 minutes to change to red but that the reaction took place rapidly on boiling. A trial test was made using 2 cc. of a 1:100,000 adrenaline solution and 1 cc. of a 0.05 % FeCl_3 solution. The green colour produced on mixing was hardly discernible, but on boiling the solution and cooling, a colour of 1.8 red, 3.3 yellow Lovibond tintometer units was observed in a 1 cm. cell. The measurement of this orange-red colour seemed promising at the time but it was afterwards found that dilute FeCl_3 solution alone gives a somewhat similar colour on boiling, making the quantitative application of the test impossible. Further, it was found that suprarenal cortex extracts which contained no adrenaline gave definite reactions with this test.

It may be noted that alcohol does not interfere with this test and that if the colour obtained by addition of alkali be examined under ultra-violet light, using a Wood's screen, a very marked yellow fluorescence is observable very similar to that subsequently to be described under the ammonium molybdate test. The colour produced by adrenaline-free suprarenal cortex extracts does not show this property. The test is a very sensitive and reliable qualitative one for adrenaline but does not lend itself to quantitative application.

The potassium iodate reaction.

This test was originally applied quantitatively by Hale and Seidell [1911] who state that the method gives results 30 % below the biological value. Scoville [1920] who investigated the test much more fully found that the orange-red colour produced in neutral solution becomes reddish-violet in the presence of acid (acetic or hydrochloric) and prefers to do the test in acid solution (0.015 % hydrochloric acid) using an optimum temperature of 38° for 15 minutes. The method compares satisfactorily with the biological method provided the acidity of the original solution is known and reducing substances such as bisulphites (sometimes added as preservatives) are absent. By this method it is possible to estimate concentrations of adrenaline greater than 1 in 100,000 and the test is of about the same order of sensitivity as the persulphate test. Moreover, suprarenal cortex extracts free from adrenaline give no colour and the reaction should prove capable of measuring adrenaline in such extracts with a high degree of accuracy.

As, however, the reaction is essentially very similar to the persulphate reaction which is described later and as the latter is easier to use, the test was not further investigated.

The ammonium molybdate reaction.

This test, described by Rae [1930], depends on the presence of a catechol grouping in the adrenaline molecule. On mixing 1 cc. of adrenaline solution with 2 cc. of 10 % ammonium molybdate solution a yellow colour instantly develops and is permanent for several hours.

It was found that this test, while not so sensitive as the persulphate reaction, could be used in pure solutions for the quantitative estimation of adrenaline down to a dilution of about 1 in 20,000. Unfortunately when applied to concentrated gland extracts it gives very misleading results even when allowance is made for the yellow tint due to the extract alone.

The colour-concentration curve obtained with pure adrenaline is a straight line.

Other points noticed were that alcohol does not affect the reaction, except that too strong a concentration will precipitate the ammonium molybdate and also that the test cannot be applied to urine owing to the presence of other phenolic substances.

Paget [1930] uses this test to differentiate adrenaline and adrenalone, since, although both give an orange colour, the addition of sodium hydroxide discharges this colour to a greenish fluorescence with adrenaline but not with adrenalone. When observed under ultra-violet light adrenaline was found to give a very strong greenish-yellow fluorescence with Paget's test and this constitutes a very sensitive qualitative test for adrenaline, less than 1 in 2,000,000 being detectable. The colours given by adrenaline-free suprarenal extracts show no such fluorescence but the test could not be applied quantitatively without special apparatus.

Folin's test.

Folin's reagent [Folin *et al.*, 1912; 1913] was originally devised for the colorimetric determination of uric acid and many modifications of the reagent and technique employed have been suggested for the determination of uric acid in blood. In this connection Folin and Trimble [1924] attribute the inconsistencies met with by various workers to the presence of varying traces of molybdenum in the sodium tungstate used and describe a method for preparing the uric acid reagent free from such impurity. The sodium tungstate used by the present workers gave no reactions for molybdenum when tested by the xanthate test, and the reagent was therefore made without further treatment as directed by Folin and Trimble.

Although not specific for adrenaline this test is extremely sensitive, being about 10 times more sensitive than the persulphate reaction. The colour produced is a deep blue very similar to that given by uric acid, but it is said to be 3 times as sensitive for adrenaline as for uric acid.

The test has been applied to the estimation of adrenaline by Maiweg [1922] who is also of our opinion that the method gives too high results when applied to suprarenal extracts. Baker and Marrian [1927], in a fuller study of the test, estimated adrenaline in protein-free trichloroacetic acid extracts of suprarenal glands and calculated the experimental error as about 7 %.

Our first attempts to utilise this reaction were carried out as follows. 1 cc. of 1 in 100,000 adrenaline solution was added to 0.5 cc. Folin's reagent and 0.5 cc. saturated Na_2CO_3 . The temperature was kept constant at 21° , but even with this precaution results could not be repeated. Elevation of the temperature to 37° resulted in a quicker development of the colour but the same inconsistencies were noticed.

These anomalies were found to be due at least partly to the metal cell used for matching the colours, since, as with the persulphate test, traces of copper dissolved from the metal markedly altered the reaction velocity. This, however, seems to be a peculiarity of the adrenaline test since the colour given by uric acid does not behave in the same way. The matter was not investigated further but the use of an all-glass cell was strictly observed.

Another difficulty encountered was the formation of crystalline precipitates after the mixture had been standing 10 to 15 minutes, the trouble being even worse at 37.5° . Some of the precipitate was removed and analysis showed it to be lithium phosphate which presumably only appears slowly owing to the slow breakdown of the phosphotungstic acid complex. It was found, however, that the use of only half the original quantity of Folin's reagent largely overcame this difficulty, as 45 minutes then elapsed before any precipitate was observed. In spite of these alterations no satisfactory results could be obtained with the test until the importance of the degree of alkalinity of the reaction mixture was discovered. It was found that, owing to the combined buffering action of the carbon dioxide evolved and the phosphates already present, the amount of sodium carbonate added brought the mixture to about p_{H} 8 only, and it was found necessary to make the reaction at least alkaline to thymolphthalein in order to obtain satisfactory results.

The use of sodium hydroxide as the alkali was therefore adopted, and while this causes the colour to fade rapidly if used in too great excess very consistent results are obtained if the following procedure is adopted. The amount of sodium hydroxide necessary is, of course, partly dependent on the Folin's reagent used and it is suggested that each sample should be titrated to thymolphthalein. In calculating the strength of the alkali to be employed an extra 2 % sodium hydroxide should be added to ensure the required alkalinity of the reaction mixture, *i.e.* if titration shows 0.25 cc. Folin's reagent equivalent to 0.75 cc. of 3 % NaOH , use 0.75 cc. of 5 %. This small excess of alkali is quite safe and does not cause any fading.

We have found that the use of sodium hydroxide as the only alkali constitutes a great advantage both in the determination of adrenaline and of uric acid. Not only does it enable remarkably constant figures to be obtained,

but the colour develops very much more rapidly, the maximum being reached within 3 minutes. The colour is just as stable as when sodium carbonate is used and no troubles are caused by the precipitation of phosphates or by the evolution of carbon dioxide. In view of the fact that many workers find the addition of sodium cyanide advantageous we attribute many of the discrepancies obtained with this test to the question of the p_H , not only in the estimation of adrenaline but also in the estimation of uric acid.

The final modification of this test adopted was as follows. Mix 1 cc. of an adrenaline solution of a concentration between 1 in 100,000 and 1 in 1,000,000 with 0.25 cc. Folin's reagent followed by 0.75 cc. of 5 % NaOH. The colour develops in a few minutes at room temperature and the maximum value is observed in a 1 cm. all-glass cell. The authors found that 0.01 mg. of adrenaline under these conditions gave a colour of 10.6 blue, 1.4 yellow Lovibond tintometer units. With pure solutions of adrenaline a straight line is obtained for the colour-concentration curve.

The potassium persulphate reaction.

Ewins [1910] described this test as the most sensitive reaction for adrenaline (giving a lower limit of 1 in 5,000,000). He also examined the effect of persulphate on various bases allied to adrenaline and found that the oxidation products such as aminoacetocatechol and its derivatives did not give the reaction. When applied to suprarenal extracts which were free from adrenaline we found that this test gave no colour and it was decided to investigate it more thoroughly in order to establish it on a quantitative basis.

Preliminary trials soon showed that Ewins had rather overestimated the sensitivity and that for quantitative measurement the adrenaline content should be not less than 1:100,000. The test was carried out by mixing 1 cc. of the solution under test with 1 cc. of 0.2 % $K_2S_2O_8$. The concentration of the adrenaline solution was kept constant at 1:10,000 and was made by diluting Liq. Adrenalin. Hydrochlor. B.P. 1914.

Effect of temperature.

The effect of variation of temperature was first studied. The colour was measured in a 1 cm. cell in the Lovibond tintometer at intervals, and the intensity plotted against the time (see Figs. 1 and 2), at temperatures of 18° and 37.5° respectively. It will be noticed that the maximum colour developed is slightly greater at 37.5°, being about 7.6 red, 3.4 yellow units and that the time required to reach this is much less at this temperature, actually about 25 minutes instead of the 55 minutes required at 18°. Further, the colour is stable over a longer period at 37.5°. The red tint is about double the yellow, but both tints are produced at about the same rate although the yellow starts to fade slightly before the red. When the red does fade, however, it does so much more quickly, especially at 37.5°.

Further experiments showed that at 0° the colour hardly develops at all, whilst at higher temperatures, 60° and 100°, although the colour appears

rapidly, it also fades very rapidly. Attempts to prevent this rapid fading by sudden cooling in a freezing mixture were not very successful and in no case was the colour obtained as high as that at 37.5°.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 1. 1 cc. 1 : 10,000 adrenaline (HCl). 1 cc. 0.2 % potassium persulphate.

Fig. 2. 1 cc. 1 : 10,000 adrenaline (HCl). 1 cc. 0.2 % potassium persulphate.

Fig. 3. 1 cc. 1 : 10,000 adrenaline (H_2SO_4) solution. 1 cc. 0.2 % potassium persulphate.

Fig. 4. 1 cc. 1 : 10,000 adrenaline (H_2SO_4) solution. 1 cc. 0.2 % potassium persulphate, 1.0 % sodium chloride.

Effect of chlorides.

When an attempt was made to confirm the above results, using a freshly made solution of adrenaline sulphate, very much lower figures were recorded. Thus, at 18° the colour development was almost nil, although it was higher at 37.5° (see Fig. 3). The original figures were confirmed, however, by diluting some freshly made Liq. Adrenalin. Hydrochlor. B.P. 1914 and it was obvious therefore that either the chloroform or the sodium chloride present in the B.P. liquor, or perhaps both, was responsible for the increased colour.

Experiment showed that the presence of chloride was the important factor and the effect of the addition of 1.0 % of sodium chloride to the reagent, using a 1 in 10,000 adrenaline sulphate solution, is shown in Fig. 4. This curve showed an improvement on all the previous work since not only was the maximum colour higher but it was established in a shorter time. Further, the colour was stable over a very much longer period. This stabilising effect of chlorides has been utilised in all the subsequent work, 1.0 % of sodium chloride being added to the reagent.

Effect of calcium chloride.

It has been noted by various workers that very dilute solutions of adrenaline in tap water turn pink much more rapidly than similar solutions in distilled water.

Two explanations suggested were:

- (a) the chemical effect of dissolved substances in tap water;
- (b) the buffering effect of tap water.

At first the conclusion was reached that the calcium present in the tap water was responsible for this phenomenon since the addition of 0.2 % CaCl_2 to the persulphate reagent resulted at 18° in a quicker establishment of the maximum colour. The only other observable effects were that slightly less yellow was produced and that the stabilising effect of the sodium chloride largely disappeared at 37.5° although it was retained at room temperature.

A modification of the test using a reagent containing 0.2 % CaCl_2 , 1.0 % NaCl , 0.2 % $\text{K}_2\text{S}_2\text{O}_8$ was tried, but it was discovered that the reduction of the calcium chloride from 0.2 % to 5 parts per million produced no different results. We had to assume, therefore, that the effect of calcium chloride was either catalytic in nature or that it was entirely fortuitous and our attention was turned to a consideration of the p_{H} values during the reaction.

Effect of p_{H} value.

It is well known that the change of colour of adrenaline solutions brought about by oxidising agents is less rapid in acid solution than in neutral solution and more rapid in alkaline solution but we have not been able to find any record of an investigation of the effect of p_{H} upon the maximum colour produced by any given quantity of adrenaline or upon the time necessary for the production of that maximum coloration. This is a factor of great importance if the test is to be employed from the quantitative point of view, for variation of the p_{H} value of the reaction mixture was found to produce widely differing results.

Normally the p_{H} of the mixture was about 4.6, and the maximum colour was obtained in 30 minutes, but after the addition of acid to p_{H} 2 the maximum colour was not reached even after 7 hours. Attempts to determine the optimum p_{H} value for the reaction were at first unsuccessful, but this was traced to an increase in the p_{H} of the mixture as the reaction proceeded. It was therefore

necessary to incorporate a buffer in the reagent and several were tried, but those containing organic acids, especially citric acid, were excluded. The ordinary phosphate buffers were adopted, and the p_H values of the reaction mixtures were determined electrometrically using the quinhydrone electrode. Caution was necessary since the presence of an oxidising agent resulted in the cell becoming more acid on standing, and readings had to be taken immediately after adding the quinhydrone in order to obtain consistent results.

The time required to establish the maximum colour at 22° was plotted against the p_H value, keeping the adrenaline constant (1 in 10,000) and this curve is shown in Fig. 5. It clearly shows the optimum p_H value to be at p_H 5.5 and that if the mixture is either more acid or alkaline than this a very much longer time is required to establish the maximum colour.

Fig. 5.

Fig. 6.

Fig. 5. Relation between time required to establish maximum colour and p_H of mixture (adrenaline 1 : 10,000). All in phosphate buffer.

Fig. 6. Effect of p_H on maximum colour. 1 cc. 1 : 10,000 adrenaline (HCl) solution. 1 cc. 0.2 % $K_2S_2O_8$, 1.0 % NaCl in phosphate buffer.

In Fig. 6 the maximum colour is plotted against the p_H value. It should be noted that only in solutions more alkaline than p_H 5.8 is there any reduction of the maximum colour so that fortunately in all our previous work, where the p_H happened to fall about 4.6, only the time required and not the actual maximum colour was liable to variation.

Also in Fig. 5 is given a similar curve in which 0.2 % $CaCl_2$ was present. This curve, although almost the same as that obtained in the absence of calcium, rises very sharply at p_H 6.0, and 24 hours are required at p_H 6.1 for the development of the maximum colour. This point coincides with the appearance of a precipitate of calcium phosphate. Thus it is obvious that no catalytic effect is associated with the calcium ion and the use of calcium chloride in the reagent was discontinued.

Catalytic effect of copper.

A chance observation emphasised the fact that copper will catalyse reactions of this kind. It was found that the colour developed more quickly in a metal cell than in an all-glass cell and this was found to be due to the dissolution of minute amounts of copper from the imperfectly plated framework of the cell. The curves obtained in the presence of 10 parts per million of copper (as sulphate) are shown in Figs. 5 and 6, using as reagent 0.2 % $\text{K}_2\text{S}_2\text{O}_8$ and 1.0 % NaCl in phosphate buffer solution. The catalytic effect results in the maximum colour being developed in less than half a minute over a wider p_{H} range (5 to 6). Fading is, however, much more marked although it does not occur in solutions more acid than p_{H} 4. This catalytic effect is intimately connected with the stability of the persulphate reagent, which probably functions in a similar manner to hydrogen peroxide. Other heavy metals such as mercury, cobalt, nickel and manganese also show some catalytic effect but not to the same degree as copper.

Iron on the other hand shows a slight inhibitory effect.

Colour-concentration curve.

As a result of the above work the following reagent was adopted and should be made with A.R. materials: 0.2 % $\text{K}_2\text{S}_2\text{O}_8$, 1.0 % NaCl , 0.239 % $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.937 % $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. This solution should have p_{H} 5.5, and is stable if kept away from the light and in a cool place, but caution must be observed since when kept for some weeks at room temperatures the reagent tends to become more acid in spite of the buffer present, presumably owing to the release of sulphuric acid by the decomposition of the persulphate.

The colour-concentration curve for pure adrenaline (using the above reagent) is given in Fig. 7. Both the red and yellow tints give straight lines and it should be observed that both curves meet the horizontal axis at a point

Fig. 7. Colour-concentration curve persulphate test. 1 cc. adrenaline solution (HCl). 1 cc. 0.2 % $\text{K}_2\text{S}_2\text{O}_8$, 1 % NaCl in phosphate buffer at p_{H} 5.5.

corresponding to 0.005 mg. of adrenaline. This point represents the limit of sensitivity of the test.

Application of the test to gland extracts.

Having thus determined the most favourable conditions for carrying out the test in pure solutions the method was applied to gland extracts. It was found that the colour did not develop so rapidly as in pure solutions, requiring about 30 minutes at 22°, probably because of the greater amount of easily oxidisable organic material in such an extract. The addition of a trace of copper sulphate enables the test to be carried out in less than 5 minutes but the results are less accurate and should not be used except as a preliminary indication.

The method recommended for gland extracts is as follows. Adjust the p_H of the extract to 5.4 (methyl red, external indicator). Then to 1 cc. add 1 cc. of the potassium persulphate reagent and immediately measure the colour (red units) in a tintometer using a 1 cm. all-glass cell. Then place the cell in the thermostat at 22° and read the colour again after 30 minutes. The increase of the red units is then compared with that given by a standard solution of adrenaline treated in a similar manner. The method can obviously be modified for comparison in a colorimeter.

The test is seriously interfered with by alcohol of which even 1.0 % affects the time required to produce the maximum colour. It is recommended that alcoholic solutions should be evaporated *in vacuo* making sure that the solution is more acid than p_H 5 and not allowing the temperature to rise above 30°. When free from alcohol, the residue is made up to the original volume with distilled water, and then tested as usual. No loss of adrenaline occurs under these conditions.

Comparison of tests on other substances.

The three tests found suitable for estimating adrenaline in pure solutions, *viz.* molybdate, persulphate and Folin's tests, were applied to a variety of organic substances which might cause interference and the results are given in Table I.

It will be noticed that Folin's test is not specific for adrenaline and, besides uric acid, most easily oxidisable substances give some blue colour, especially the aromatic polyhydroxy-compounds.

The molybdate test is, however, much better, tannic acid, and pyrogallol being the only other substances tried which gave the reaction. This, of course, is to be expected as they contain the catechol grouping. The fact that this test indicates the catechol group also means that substances closely allied to adrenaline, but without its pressor activity, may be the source of error encountered on applying this test to suprarenal extracts.

The persulphate test is the most specific test for adrenaline and (of the substances examined) gives a positive test only with metol and *p*-anisidine

Table I.

Test substance	Ammonium molybdate	Potassium persulphate	Folin's
Adrenaline	Orange-yellow	Red	Deep blue
Histamine	Nil	Nil	Nil
Liquid extract of ergot	Very slight pink	Nil	Prussian blue
Tyramine	Nil	Nil	Nil
Metol. OH NHCH ₃	Nil	Red (on boiling only)	Intense blue
Salicylic acid	Yellow on warming; discharged by NaOH	Slight yellow	Nil
Tannic acid	Reddish orange; no u.v. fluorescence	Slight yellow	Intense blue
Tyrosine	Nil	Nil	Very slight blue
Trypsin	Very slight yellow	Nil	Blue
Vanillin	Nil	Nil	Very slight blue
Pyrogallol	Orange (as tannic acid)	Orange-brown on heating	Intense blue
o-Cresol	Very slight yellow on heating	Yellow on heating	Very slight blue
Salicylic aldehyde	Slight yellow on heating	Nil	Green-yellow precipitate on heating
Uric acid	Nil	Nil	Intense blue
Meat extract	Nil	Nil	Blue
Urine	Nil	Nil	Intense blue
p-Anisidine	Nil	Red on boiling	Nil
Phenacetin	Nil	Nil	Nil
Aniline	Nil	Slight purple	Nil

and then only on boiling. This test seems to depend on the presence of a primary or secondary amino-group in the side-chain and an —OH group in the *para*-position in the benzene ring. If, however, in addition to these groups a ketone group is present in the side-chain no reaction is obtained.

Ewins [1910] found that the following bases allied to adrenaline will give a positive persulphate reaction: arterenol (dihydroxyphenylethanolamine), dihydroxyphenylethylamine and epinine (dihydroxyphenylethylmethylamine), whereas the following will give no colour: homorenol (ethylaminoacetocatechol), methylaminoacetocatechol, ephedrine.

It should be noted that although some of these bases give the persulphate reaction, those which do so possess marked physiological action very similar to, but weaker than that produced by adrenaline. The corresponding ketonic oxidation products on the other hand do not give the colour test and do not generally possess physiological action much greater than that of catechol and its simple derivatives.

Comparison of colour tests with the biological method.

A comparison of the colour tests with biological results was made in order to prove definitely the impressions gained from previous work, *viz.* that Folin's and the molybdate tests were unreliable for the estimation of adrenaline in concentrated suprarenal extracts.

A sample of suprarenal cortex extract was taken and known quantities of standard adrenaline solution were added giving four extracts with upper and

lower limits of adrenaline at about 1 in 5000 and 1 in 100,000 respectively. The adrenaline was then determined colorimetrically in each extract by the ammonium molybdate, potassium persulphate and Folin's tests. Samples were assayed biologically at the same time.

Table II summarises the results obtained, the corresponding curves being shown in Fig. 8.

Table II. *Adrenaline in suprarenal extracts.*

	Results as parts per 10,000.			
	Extract "A"	"B"	"C"	"D"
Adrenaline actually present assuming "A" to contain 0.1 parts per 10,000	—	0.278	1.01	1.91
Persulphate test	<0.1	0.29	1.01	1.98
Folin's test	0.61	—	2.02	3.04
Molybdate test	1.5	3.9	5.4	6.7
Biological test	0.11	0.23	0.78	1.7

Fig. 8. Comparison of tests on gland extracts.

It will be seen that neither Folin's test nor the molybdate test gives reasonably correct results with gland extracts. The error of Folin's test is less than that of the molybdate test and in some cases might be very much less than that shown in Table II, since some samples of suprarenal extract gave much less colour with Folin's reagent, although of the same adrenaline content. The increase from sample "C" to "D" shown by Folin's test is in good agreement with that expected. Since such abnormal samples are always liable to occur, the use of Folin's test for quantitative work must be abandoned in spite of its great sensitivity, but negative results can be safely interpreted as indicating absence of adrenaline.

The results given by the persulphate test are as close as can be expected to the biological figures and are extremely close to the values anticipated, the

increases from samples "B" to "C" and from "C" to "D" being exactly the amounts actually added.

Attempts were made to use the persulphate test at dilutions below 1 in 100,000, and it was found possible by utilising the Klett colorimeter to carry out determinations with a degree of accuracy comparable with that of the biological method.

The estimation of adrenaline in glands.

It must be remembered that the suprarenal gland extracts described in this paper are of high concentration and naturally give the extremes of divergence of the various tests for adrenaline. In the quantitative determination of adrenaline in the suprarenal glands themselves, we obtained satisfactory results by using the protein-free trichloroacetic acid extracts described by Baker and Marrian [1927], who make use of Folin's reagent.

It is important, however, not to neutralise the filtrate before adding the reagents (Folin's or persulphate). Adrenaline is quite stable in trichloroacetic acid but if the solution is neutralised with sodium hydroxide, as the p_H approaches a value of about 3, a colour reaction takes place, a red colour forming which quickly fades to a fluorescent yellow. This results in the destruction of a large part of the adrenaline, but can be avoided by adding first the reagent and then afterwards the requisite quantity of alkali, allowing of course for the effect of the volume added on the dilution of the colour. Ox suprarenal gland by the persulphate test applied in this way showed an average adrenaline content of 3.3 mg./g. fresh gland, with 2 mg./g. in the cortex and 9 mg./g. in the medulla. The filtrates obtained from the trichloroacetic acid precipitation of defibrinated blood showed no adrenaline by the persulphate test. Addition of known amounts of adrenaline to the blood and subsequent precipitation showed that the persulphate test gave correct results (2 % loss) but the Folin's test gave results which were 25 % too high.

SUMMARY.

1. The need for a rapid and accurate quantitative test for adrenaline in highly concentrated suprarenal gland extracts led to the investigation of many of the colour tests described in the literature. Some of these are unsatisfactory even from a qualitative point of view; others are non-specific. The latter criticism applies to the ferric chloride, ammonium molybdate and phosphotungstic acid (Folin's) tests, although the latter is extremely sensitive, and gives good results with comparatively pure adrenaline solutions if applied in accordance with the slightly modified technique described in this communication.

2. The potassium persulphate test has been investigated more thoroughly and although it is not as sensitive as Folin's test it gives results comparable with those obtained by the biological test when the necessary conditions, including control of temperature and p_H , are observed.

3. Preliminary work upon the application of the persulphate test to the determination of adrenaline in the suprarenal glands themselves indicates that it is of sufficient accuracy for this and similar purposes.

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REFERENCES.

- Azzolini (1931). *Boll. Chim. Farm.* **70**, 665.
 Bailly (1924). *J. Pharm. Chim.* (VII) **30**, 404.
 Baker and Marrian (1927). *Biochem. J.* **21**, 1005.
 Comessatti (1909). *Berl. klin. Woch.* **48**, 356.
 Ewins (1910). *J. Physiol.* **40**, 317.
 Folin and Denis (1912). *J. Biol. Chem.* **12**, 239.
 — Cannon and Denis (1913). *J. Biol. Chem.* **13**, 477.
 — and Trimble (1924). *J. Biol. Chem.* **60**, 473.
 Friend (1923). *J. Biol. Chem.* **57**, 497.
 Hale and Seidell (1911). *Amer. J. Pharm.* **83**, 551.
 Maiweg (1922). *Biochem. Z.* **134**, 292.
 Orru (1929). *Ann. Chim. App.* **19**, 239.
 Paget (1930). *Bull. Sci. Pharm.* **37**, 537.
 — (1931). *Bull. Sci. Pharm.* **38**, 77.
 — and Lohéac (1928). *J. Pharm. Chim.* **8**, 159.
 Rae (1930). *Pharm. J.* **125**, 451.
 Sansoni (1930). *Officina*, **3**, 323.
 Scoville (1920). *J. Ind. Eng. Chem.* **12**, 769.
 Stuber, Russmann and Proebsting (1923). *J. Soc. Chem. Ind.* **42**, 739 A.
 Viale (1930). *Bull. Soc. Ital. Biol. Sper.* **5**, 1163.