THE DETERMINATION OF ADRENALIN IN BLOOD

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The chemical method for adrenalin which best fulfils the requirements for its determination in blood is that of Whitehorn (1) as modified by Shaw (2). For the isolation of adrenalin from the blood filtrate Whitehorn used silica which, however, does not give a clean separation and for which Shaw substituted aluminum hydroxide at about pH 8.5 which does adsorb the adrenalin completely. The determination of adrenalin in this method depends on the production by adrenalin of a blue color by the reduction of an arsenomolybdate reagent in strong acid solution. The amount of blue color is greatly increased by the presence of sulfite which, however, also increases the hazards of the method because of the difficulty of controlling the color produced by the sulfite itself. Increasing the concentration of sulfuric acid makes it possible to reduce the color of the blank to a minimum; but the sensitivity to adrenalin is also much reduced, so that for greatest sensitivity it is better to use a procedure which allows some color in the blank. The blue color is given by a variety of substances in blood which are only partly separated from adrenalin by the adsorption procedures used and the present work indicates that many of the figures given in the literature (3–5) for the adrenalin content of blood represent something else.

In the application of Shaw's procedure to the determination of adrenalin in blood the following difficulties were encountered. (a) In the first aluminum hydroxide adsorption of the blood filtrate (acid adsorption) 1 drop of 1 N sulfuric acid, as directed by Shaw, was not found sufficient to prevent adsorption of adrenalin. It took 16 to 18 drops of acid and thereby much of the aluminum
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hydroxide was dissolved. The hydroxide is a good buffer for either acid or alkali. (b) In the special enhancing treatment described by Shaw in which 0.35 cc. of 4 per cent sodium hydroxide and 2 cc. of water with 2 minutes heating are employed, the aluminum hydroxide did not dissolve completely and the resulting increase of color was small and uncertain. Both these difficulties may possibly be accounted for by differences in the sample of alum used for preparation of the aluminum hydroxide, since Shaw's directions for preparation of the hydrate were carefully followed. In the procedure as finally adopted both these steps were omitted. (c) 5 minutes heating did not give the maximum color, thus adding another factor of uncertainty. It was found by actual measurement of color development (see Fig. 2) that the color increased rapidly for 6 to 8 minutes and after that more slowly, so that a heating time of 10 minutes was used. Tietz, Dornheggen, and Goldman (5), in regard to Shaw's procedure, state, "We have reason to believe that the error in this method as carried out by us is great," and others (personal communications) have had difficulties with the procedure. Both Whitehorn and Shaw found that the control of the blank with sulfite was a serious difficulty in the method and in this we fully agree. The variability of the blank remains one of the major difficulties in the procedure.

Correction for a variable blank color in the ordinary colorimetric matching is not easily made and we decided early that the photoelectric colorimeter, in which the color produced by the substance is automatically corrected by using the blank as the reference solution, offered the best solution of the difficulty. In addition the factors influencing the color in the blank have been studied and changes made, so that difficulty in this direction, while not entirely removed, has been considerably lessened. Changes in procedure have also increased its sensitivity, so that under good conditions amounts of 0.02 γ of adrenalin can be measured with an accuracy of about 5 per cent; that is, the presence of amounts of adrenalin as small as 0.001 γ may be determined. The method, however, remains temperamental—a not unexpected finding considering the minute quantities of material measured—and calls for careful technique with close adherence to a rigid routine throughout the procedure.
The amount of adrenalin in normal blood is not known. Stewart and Rogoff (6), on the basis of many determinations with the biological method of assay, found that the output of the adrenals in cats was about 0.3 to 0.8 \( \gamma \) per kilo per minute, which when distributed in arterial blood on the basis of 80 cc. of blood per kilo of animal would amount to 0.004 to 0.01 \( \gamma \) per cc. Since adrenalin is rapidly removed by the tissues and perhaps also destroyed by the blood, the amount which reaches the venous system would be much smaller. Schlossman (7), also using biological methods, concluded that if present at all adrenalin must be in a concentration of less than 1 part in 1000 million or 0.001 \( \gamma \) per cc. Whitehorn (1) gives a value of 0.025 \( \gamma \) per cc. for cat blood obtained at operation. Shaw (2) gives for rabbit blood 0.05 \( \gamma \) per gm. and for man 0.016 \( \gamma \) per gm. Sarfy (8) using Shaw's procedure found in pigeon blood 0.06 to 0.08 \( \gamma \) per cc. Tietz, Dornheggen, and Goldman (5) found values of about 0.2 \( \gamma \) per cc. in human blood. Giordano and Zeglio (3), using a different method, report values from 1 to 4 \( \gamma \) per cc. in human blood. In the present work, colors corresponding to values of 0.2 to 0.5 \( \gamma \) per cc. of adrenalin were found in venous blood of dogs and humans. This material was, however, found to be stable to alkali under conditions in which adrenalin added to blood or to blood extract was completely destroyed. Blood extracts to which adrenalin has been added, when treated with alkali, give the same or slightly higher values than does the extract containing no adrenalin. This result leaves considerable doubt as to whether there is any adrenalin in venous blood of dogs and humans. This material was, however, found to be stable to alkali under conditions in which adrenalin added to blood or to blood extract was completely destroyed. Blood extracts to which adrenalin has been added, when treated with alkali, give the same or slightly higher values than does the extract containing no adrenalin. This result leaves considerable doubt as to whether there is any adrenalin in venous blood or at any rate amounts greater than about 0.001 \( \gamma \) per cc., which is the limit of measurement of the present method.

Reagents—The reagents used are essentially those employed by Whitehorn and by Shaw.

Arsenomolybdate reagent. Made by dissolving 120 gm. of crystalline sodium molybdate and 20 gm. of crystalline sodium arsenate in 500 cc. of hot water, filtering, cooling, and making up to 1000 cc. 10 cc. of bromine water and 80 cc. of concentrated sulfuric acid are then added. The solution so prepared is unstable, gradually acquires a bluish color, and increases in sensitivity to adrenalin and sulfite until finally it will no longer distinguish between different amounts of material. Protecting it
from light by keeping the solution in a brown bottle greatly slows the change and, if spoiled, the solution may be made useful again by the cautious addition of bromine water. The change in sensitivity is rapid at first, so that if the reagent is used before it is 2 weeks old calibration curves must be adjusted frequently; after that, calibration once a week is necessary.

Sulfuric acid. Four concentrations are used: (a) 1 part of concentrated sulfuric acid to 1 part of water by volume, (b) 1 N sulfuric acid, (c) 0.4 N sulfuric acid, and (d) concentrated sulfuric acid.

Sodium sulfite. A cold, saturated solution made by adding 10 gm. of powdered sodium sulfite (Na₂SO₃) to 25 cc. of boiling water and allowing to cool with occasional shaking. This solution is made fresh each day.

Acid digestion mixture. 5 cc. of the cold, saturated sodium sulfite solution are measured into a 200 cc. Florence flask, 25 cc. of the 1:1 sulfuric acid are added, and the whole well mixed. This solution is made fresh for each set of determinations. It is a disagreeable, fuming mixture and must be handled with care.

Sodium hydrate. Approximately 1 N sodium hydroxide. As ordinarily prepared it contains suspended matter (silica?) which affects the color, especially noticeable in the blank, so that the sample must be allowed to stand for several days or be centrifuged for each day's use and only the clear liquid used. Also, because of its effect on the burette from which it is measured, this instrument must be rinsed out with the solution before use. Impurities in the alkali appear to be an important and perhaps the main factor producing variability in the blanks.

6 N sodium hydroxide (approximately) made by dissolving solid sodium hydroxide in water.

Aluminum hydrate. 100 gm. of potassium aluminum sulfate dissolved in 800 cc. of hot water, filtered, and cooled to 20°. 20 gm. of sodium hydroxide are dissolved in 80 cc. of water, cooled to 20°, and added slowly to the alum solution with continuous stirring. After addition of the alkali, the mixture is well shaken to insure even composition of the hydrate, and then filtered on a Buchner funnel through hardened filter paper. The precipitate is suspended in 800 cc. of distilled water and filtered. The process of washing is repeated twice more, after which the precipitate is
suspended in 400 cc. of water for use, giving a suspension which just does not separate a watery layer and flows freely from a pipette. The suspension has a pH of about 5.3. Care should be taken to avoid getting fibers of filter paper into the suspension. The pipette should be washed free of hydrate after each set of determinations.

Adrenalin standard. Made by dissolving 50 mg. of adrenalin chloride1 and 1.25 mg. of alanine in 0.4 N sulfuric acid to make 50 cc. The first dilution is made by mixing 1 cc. of this standard with 0.4 N sulfuric acid and 2.5 mg. of alanine and making up to 100 cc. (1 cc. = 10 μ of adrenalin). These two solutions kept in the refrigerator are good for several months. A dilution of 10 cc. of the second solution plus 2.5 mg. of alanine made up to 100 cc. with 0.4 N sulfuric acid (1 cc. = 1 μ of adrenalin) and a second 1:10 dilution without alanine (1 cc. = 0.1 μ of adrenalin) are kept in the laboratory. The first of these laboratory solutions must be renewed each month (oftener in hot weather) and the final dilution every week.

Phenolphthalein. 100 mg. in 100 cc. of 0.01 N sodium hydroxide; renewed each week.

Procedure

5 cc. of blood drawn from the vein are run at once into 20 cc. of 10 per cent trichloroacetic acid in a 50 cc. conical centrifuge tube. After thorough mixing, the material is centrifuged for 5 minutes at about 1000 R.P.M. and the clear fluid poured through a filter into a test-tube or other container.

For determination, 1 cc. of the extract (containing the equivalent of about 0.4 μ of adrenalin) is measured into a small centrifuge tube (16 × 105 mm.), 1 cc. of 0.4 N sulfuric acid and 2 drops of the phenolphthalein solution added, and the mixture titrated with 1 N sodium hydroxide to a faint pink color. 2 cc. of the well mixed aluminum hydroxide suspension are added and the mixture again neutralized to a faint pink color, after which the material is well shaken (it should now have only the faintest perceptible pink tint), allowed to stand for 5 minutes, and centrifuged for 5 minutes at about 2000 R.P.M. The supernatant fluid is poured

1 Kindly supplied by Parke, Davis and Company.
off, the tubes drained, and the liquid completely removed from the end of the inverted tube with absorbent paper (the amount of water in the reaction mixture is important). 2 cc. of the ars enomolyb date reagent are added and the precipitate well mixed with it. The mixture is set in boiling water for 5 minutes and then to the clear solution are added 3 cc. of the acid digestion mixture of 1:1 sulfuric acid and sodium sulfite and the digestion in the boiling water continued for 10 minutes, after which the tube is set in ice water (with an excess of ice) for 15 minutes. The solution is then diluted to 40 cc. and the color measured in 10 cc. of this dilution. A blank determination on the reagents alone is run along with the samples. Determinations are carried out in quadruplicate and the average taken as the value. It is emphasized that measurements of time and volume must be exact and the routine strictly adhered to.

Colorimeter—The measurement of the color was made by a photoelectric colorimeter which was an adaptation of that described by Eddy and DeEds (9). The modifications consisted in (1) the use of the Evelyn (10) light system employing a flashlight bulb, focusing device, and a 6 volt storage battery; (2) slots in the inside of the box carrying bakelite plates fitting into the slots, holding in order from below (a) the light system, (b) the filter (Corning, Signal Red), (c) a sliding cup carrier, working between guides and controlled from outside the box by a short rod, with places for two colorimeter cups (or a block suitably perforated to carry a small test-tube), and (d) the barrier layer photoelectric cell connected with a galvanometer.

Readings—The colored solution, after the 15 minutes chilling, was diluted to 40 cc. and 10 cc. were measured into a colorimeter cup. The blank solution was similarly diluted and 10 cc. measured into another colorimeter cup matched to the first one or adjusted to it. The cups were placed in the colorimeter, the cup with the blank solution in the left-hand position. With the blank cup in the path of light, adjustments of the rheostats on the light circuit were made until the indicator on the galvanometer read some convenient figure, ordinarily 20 (200 divisions) or its equivalent to match 20 on the other cup. The cup with the unknown was then moved into position and the reading taken. The difference between the 200 reading of the blank and the reading of the un-
known represents the light absorption of the latter. Its value in terms of adrenalin was then read off the calibration curve (see Fig. 1) and by calculation the value of the original sample was determined. In this way, correction for the blank was made automatically. The amount of colored solution used for the reading contained one-fourth of the total color obtained from 1 cc. of extract, which was one twenty-fifth of the amount from 5 cc. of blood. The values obtained were then multiplied by 100 to obtain the value for 5 cc. of blood. From this it may be seen that if desirable much smaller amounts of blood may be used.

Calibration Curve—A calibration curve was made as follows: Samples of standard adrenalin solution containing from 0.4 to 1.2 γ were measured into the round bottom centrifuge tubes used in the method; 1 cc. of the 10 per cent trichloroacetic acid and 2 drops of the phenolphthalein were added and the neutralization, adsorption on aluminum hydroxide, color development, and readings carried out exactly as described. The galvanometer readings obtained were subtracted from 200 and the values of light absorption plotted against adrenalin values, as in Fig. 1. Since the colored solution was diluted to one-fourth its volume, the values plotted are one-fourth of those of the samples taken.

Development of the Color—In order to determine the optimum time of heating in boiling water for the development of the characteristic color, experiments were made as follows, with the test-tube adapter in the photoelectric colorimeter. A sample of adrenalin solution of known content was carried through the regular procedure to the end of the 5 minute heating with the arsenomolybdate reagent. The 1:1 sulfuric acid was then added, and the whole well mixed and placed in the photoelectric colorimeter which was then adjusted to a convenient value, ordinarily 200 scale divisions on the galvanometer. The tube containing the mixture was then set in the boiling water and at intervals of 2 minutes thereafter removed and readings made. A blank of the reagents was similarly treated throughout. Characteristic results in the form of curves are shown in Fig. 2, from which it is seen that the rate of color development is rapid at first, the maximum color and the maximum difference between blank and test coming at about 6 to 8 minutes. After that the increase in color was slow and was about equal in the test and in the blank.
up to 18 minutes. The time selected for the color development was therefore 10 minutes.

Placing the tubes in ice water for 15 minutes increases the depth of color and the amount of increase of color appears to depend con-}

![Calibration curve](http://www.jbc.org/)

**Fig. 1.** Calibration curve; units of light absorption by micrograms of adrenalin.

siderably on the temperature of the cooling water, hence the need of keeping ice in the water during the cooling period.

Effect of Alkali—Alkali destroys adrenalin, while it has no (or a slight enhancing) effect on the other substances present in blood which give color in this reaction. The test is carried out as follows: The procedure as outlined above is carried to the stage
of draining the supernatant liquid from the aluminum hydroxide after the centrifugation. To the centrifuged precipitate in the tube is added enough 6 N alkali just to dissolve it, generally 5 to 6 drops. Solution is aided by stirring with a glass rod and the tube with the dissolved material is immersed in boiling water for 3 minutes, cooled by a short immersion in ice water, and made just

![Diagram](http://www.jbc.org/)  

**FIG. 2.** Development of color with time by adrenalin on acid arsena-molybdate in boiling water (99°).

acid (to phenolphthalein) with concentrated sulfuric acid. These strong solutions are used to keep down the volume of water, which strongly affects the color production. After neutralization, which results in a white salty mass, the 2 cc. of arsena-molybdate reagent are added, the mixture heated for 5 minutes, and the color production carried out as before. In known adrenalin solutions
the alkali treatment reduces the color production to that of the blank. The color production of blood extracts is increased about 10 per cent by the alkali treatment and is the same whether adrenalin was present or not. The alkali treatment of blood extracts thus serves to distinguish adrenalin from the other substances which give the color under the conditions of the determination.

The demonstration of adrenalin in blood thus requires two determinations, one with and one without the use of alkali. If the result with alkali is higher than that without, adrenalin is absent from the sample. If it is lower, then the amount of adrenalin present is the difference between the two values corrected by the 10 per cent by which the values for the blood reducing substances are elevated by the alkali treatment. Up to the present it has not been possible to demonstrate adrenalin in either dog or human venous blood.

By the procedure described amounts of adrenalin of 0.015 to 0.25 \(\gamma\) can be measured with an accuracy of about 5 per cent; i.e., amounts of adrenalin as small as 0.001 \(\gamma\) or 1 part in 1000 million of solution will be shown.

To obtain this sensitivity and accuracy, it is necessary that quantitative care be taken at every step in the procedure and if departures from the directions given for time and amount are made it should be only after one has made sure that the changes do not affect the values.

Results

Recovery of adrenalin added to trichloroacetic acid extracts of blood was found to be practically complete (95 per cent or better), as reported by Shaw. Shaw also stated that recovery from blood, while good, was not complete. By the present procedure recovery of adrenalin added to freshly drawn dog blood was found to be as complete as from blood extracts. Experiments were made as follows: A known amount of a standard adrenalin solution in 0.4 \(N\) sulfuric acid (from 0.7 to 2.0 \(\gamma\)) was measured into a small test-tube, a drop of the aqueous phenolphthalein solution added, and the mixture titrated just to alkalinity with 1 \(N\) sodium hydroxide. 1 \(N\) sulfuric acid was immediately added to bring the mixture to faint acidity. A blank solution containing the same
amount of acid without adrenalin was similarly treated. About 12 cc. of blood were drawn from the jugular vein of a dog and 5 cc. samples measured at once into the two test-tubes. After mixing, the blood was decanted into 50 cc. conical centrifuge tubes containing 25 cc. of 10 per cent trichloroacetic acid and mixed; the test-tubes were rinsed out, the mixture was well shaken, centrifuged, and the clear filtrate used as in the procedure described above. Recovery is shown in Table I.

The adrenalin-like reducing substances in blood were determined for a number of dogs and a few humans. The values obtained varied from 0.24 to 0.36 γ per cc. in both. This substance is both acid- and alkali-stable. Tests made on human urine showed the presence of a substance which behaved similarly to the reducing substance in blood and was present in about 10 times the concen-

<table>
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<tr>
<th>Dog No.</th>
<th>Adrenalin added</th>
<th>Adrenalin recovered</th>
<th>per cent</th>
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<tbody>
<tr>
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<td>0.75</td>
<td>0.756</td>
<td>101</td>
</tr>
<tr>
<td>40-56</td>
<td>1.6</td>
<td>1.8</td>
<td>112</td>
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</tr>
<tr>
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<td>1.2</td>
<td>1.18</td>
<td>98</td>
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<tr>
<td>40-2</td>
<td>1.8</td>
<td>1.6</td>
<td>90</td>
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<td>1.23</td>
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<td>100</td>
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<tr>
<td>38-101</td>
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<td>1.5</td>
<td>94</td>
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</table>

* The adrenalin values decrease on standing. Thus, in Dog 38-346 above (immediate recovery of 93 per cent), the recovery 3 hours later was 55 per cent and 35 per cent the next day.
Adrenalin in Blood

tration found in blood. It was alkali-stable and was not affected by hydrolysis with sulfuric acid.

The method has been applied to the determination of adrenalin in commercial samples with the following results, expressed in mg. per cc.

<table>
<thead>
<tr>
<th>Manufacturer No.</th>
<th>Sample No.</th>
<th>Found</th>
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<tbody>
<tr>
<td>I</td>
<td>I</td>
<td>1.10</td>
<td>1.0</td>
</tr>
<tr>
<td>I</td>
<td>II</td>
<td>1.00</td>
<td>1.0</td>
</tr>
<tr>
<td>I</td>
<td>III</td>
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<td>II</td>
<td>I</td>
<td>0.87</td>
<td>1.0</td>
</tr>
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</table>

Samples I and II of Manufacturer I kept in cork-stoppered bottles for about a year were found to be brownish in color and to have values of 0.72 mg. per cc. (Sample I) and 0.7 mg. per cc. (Sample II).

Recovery of adrenalin injected directly into the circulation has been tried a few times. After a control blood sample was taken from a dog, a known dose of adrenalin, 0.25 to 0.8 mg., was injected into the jugular vein of one side and samples were taken at intervals from the vein on the other side. Measured quantities of the blood were run at once into 4 volumes of 10 per cent trichloroacetic acid, mixed by shaking, centrifuged, and determinations made on the filtrate. The difference in values between the control and the sample was taken as representing adrenalin, an assumption which was supported by the fact that alkalinization of control and sample gave in all cases the same values.

A typical experiment was as follows: 0.77 mg. of adrenalin (48 γ per kilo of body weight) was given; preliminary control sample 0.208 γ per cc.; 1 minute after injection 0.546 γ per cc.; 10 minutes after injection 0.377 γ per cc.; 20 minutes after injection 0.213 γ per cc.

If 30 cc. of blood per kilo of animal (a total of 1520 cc.) is allowed, the amount of adrenalin present in the blood was 0.514 mg. or 66 per cent of the dose 1 minute after injection, 0.257 mg. or 33 per cent of the dose 10 minutes after injection, and a trace 20 minutes after injection.

SUMMARY

A procedure is described for the determination of adrenalin in blood by which amounts of 0.02 γ can be measured with an accuracy of about 5 per cent.
A means is provided for distinguishing adrenalin from other similarly reacting substances in blood.

With this method, it was found that, if present at all, the adrenalin content of venous blood of man and dog is less than 0.001 \( \gamma \) per cc. or 1 part in 1000 million.

The substances in blood which react similarly to adrenalin are present in amounts of about 0.25 \( \gamma \) per cc.

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