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OXIDATION OF ADRENALINE IN ALKALINE SOLUTION

BY

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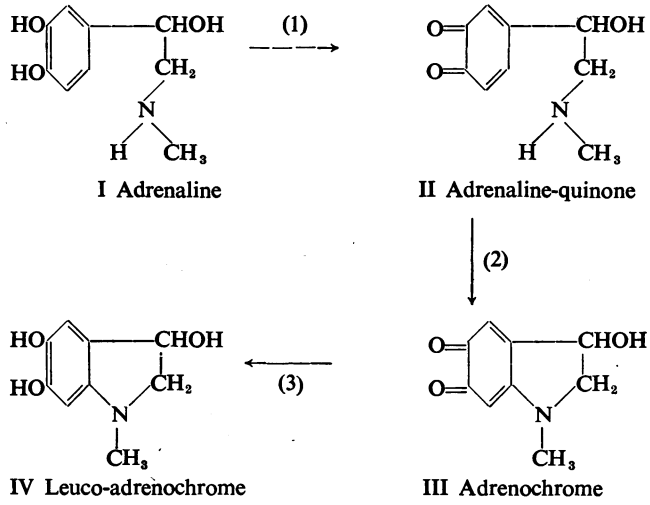
When adrenaline in simple alkaline solutions is exposed to oxygen, the loss of physiological activity is accompanied by various other known phenomena:

1. The solution becomes pink and then red owing to the formation of adrenochrome, which has been isolated in crystalline form and has formula III (Green and Richter, 1937; Richter and Blaschko, 1937). Eventually, this indole quinone is destroyed and the red colour disappears.

2. A green fluorescence develops and then disappears (for references see Jörgensen, 1945). Attention was drawn to the advantages of this fluorescence for the detection of adrenaline by Gaddum and Schild (1934), who found that it provided a sensitive physical test, by which a concentration of 10^{-8} adrenaline could be easily detected. They also found that the reaction was specific at this concentration, many substances chemically related to adrenaline giving no fluorescence, whilst others gave fainter green fluorescences in more concentrated solutions.

3. The solution acquires an increased power of reducing arsenomolybdic acid (Shaw, 1938). This fact has also been made the basis of a specific and sensitive test for adrenaline. If the reducing power of a tissue extract be increased many times by treatment with alkali, the observation can be taken as evidence that the extract contains adrenaline or some other phenol with the same side chain. Shaw stated that, if the treatment with alkali was prolonged, the colour decreased and the more active reducing agent was itself destroyed.

It has been suggested (Utevsky, 1944) that adrenaline undergoes the following chemical changes in these conditions:



I Adrenaline

II Adrenaline-quinone

IV Leuco-adrenochrome

III Adrenochrome

Substance IV eventually undergoes further changes of unknown nature. Substances II and IV have not been isolated, but the theory is supported by a certain amount of indirect evidence.

The object of the experiments described here was to determine the relations which exist between the formation of adrenochrome, the appearance and disappearance of fluorescence, the changes in reducing power and the disappearance of biological activity; in addition, experiments were carred out in order to obtain evidence about the substances responsible for these different effects and to devise an improved method, based on the fluorescence reaction, of detecting adrenaline in low concentrations.

The fluorescence reaction and chemical properties of the oxidation products of adrenaline

Gaddum and Schild showed that oxidation is the cause of the fluorescence of alkali-treated adrenaline, since none was produced if all oxygen was removed from the solution. Utevsky assumed that oxidation is the cause of the weakening as well, since intense oxidation destroys the fluorescent product. By preparing solutions containing large proportions of each of the reaction products, he was able to study some of their properties. He found that the duration of fluorescence when alkali was added to the orthoquinone (II) or to adrenochrome was shorter than when it was added to adrenaline. Formaldehyde inhibited oxidation of adrenaline to adrenaline-quinone before the indole ring had been

formed. On the other hand, he found that strong fluorescence was produced if adrenochrome and formaldehyde together were treated with alkali, suggesting that the fluorescent material was related to adrenochrome. The conclusion reached by Utevsky was that the material giving the fluorescence was leuco-adrenochrome (IV).

In a limited study, the above reactions have been repeated and confirmed, and so far we have found no evidence to disprove Utevsky's conclusions. The fluorescent material is yellow in solution, gives a positive ferric chloride reaction for catechols, a positive *p*-dimethylaminobenzaldehyde test for indoles, and is adsorbed at *p*H 8.5 by aluminium hydroxide. Efforts to isolate the fluorescent material in the dry state have so far failed, and therefore some of these reactions may be due to impurities. The life of the fluorescent material can be lengthened by the addition of ascorbic acid to a solution with maximal intensity, by adjusting the *p*H of the solution to 6 after maximal production, or by adsorption on alumina at *p*H 8.5.

Quantitative estimation of adrenaline

Much work has been done in adapting the fluorescence reaction for quantitative work. The findings described here were obtained before Jörgensen's work was published, but the conclusions are in good agreement with his.

Gaddum and Schild recommended the use of 5*N*.NaOH for obtaining the fluorescence, but the interval of maximal intensity was then very short. The reading was taken after 20 seconds' contact, and after about one minute no fluorescence remained. The use of more

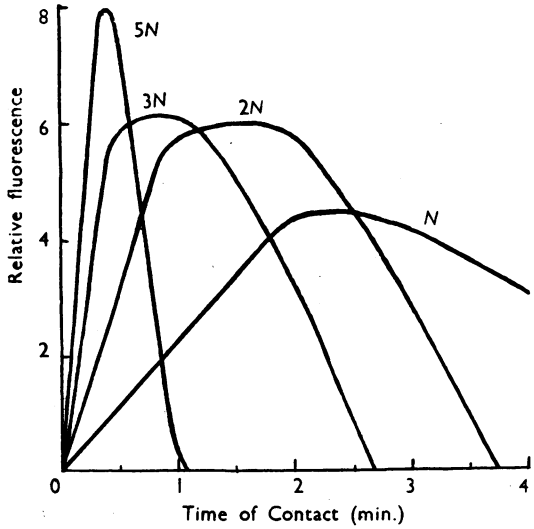


FIG. 1.—The effect of different concentrations (*N* to 5*N*) of NaOH on the intensity and duration of fluorescence of adrenaline solutions. Ordinates: relative fluorescence as measured on the fluorimeter. Abscissae: time of contact between adrenaline (10^{-7}) and alkali (1/10 vol.).

dilute alkali was investigated, therefore, in order to prolong this maximum and enable comparisons to be carried out more accurately. The results of this work, shown in Fig. 1, indicated that a satisfactory strength was 2*N*.NaOH, with which maximal fluorescence was obtained in 1 min. and lasted for 1 min.; only a slight loss of sensitivity resulted. Comparisons were made by two methods: in the first (hereinafter referred to as West's method), the adrenaline fluorescence was compared in Rimington's (1943) fluorimeter against standard eosin solutions, whilst the second was a modification of Jörgensen's method, employing one concentrated standard eosin solution. In the fluorimeter, blank readings for water and alkali only were high, and relative fluorescence readings were obtained by subtracting the scale readings from the blank ones. A linear relationship existed between these readings and the concentration of adrenaline in the solution, for each standard eosin solution used (Fig. 2). Fluorescence was produced by treating 3 ml. of the adrenaline solution with 0.3 ml. of 2*N*.NaOH. The two standard eosin solutions had fluorescent intensities corre-

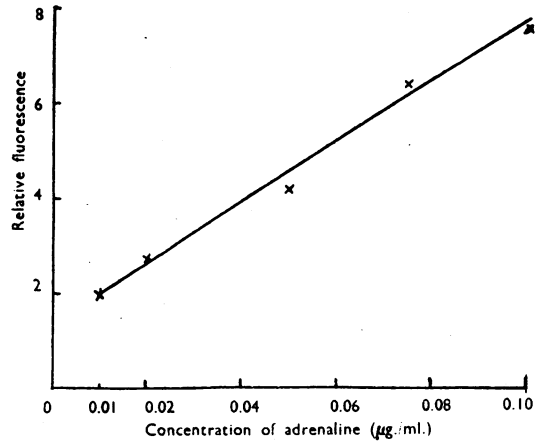


FIG. 2.—Calibration curve for fluorescence of alkali-treated adrenaline, using standard eosin solution "A."

sponding to alkali-treated 10^{-7} and 10^{-8} adrenaline respectively. Monax glass test-tubes have been used throughout this work, as they do not fluoresce in ultra-violet light.

In the second method, the fluorescence was compared with a similar volume of distilled water to which eosin solution (0.1 mg./100 ml.) was added from a burette. Another standard curve was obtained, the volume of eosin solution added being linearly related to the adrenaline content of the solution (Fig. 3). The method was improved by the use of

TABLE I

ESTIMATIONS OF "UNKNOWN" SOLUTIONS OF ADRENALINE BY THE TWO FLUORIMETRIC METHODS

Readings taken after contact with alkali for 1 min. Each experimental figure represents the average of six estimations.

	Adrenaline in μg./ml.									
Solution taken	1.000	0.800	0.500	0.300	0.200	0.100	0.070	0.050	0.030	0.010
Found, West's method	1.010	0.810	0.476	0.303	0.220	0.100	0.074	0.048	0.030	0.014
„ Jörgensen's method	1.020	0.830	0.542	0.300	0.220	0.098	0.075	0.055	0.033	0.012

less alkali (0.2 ml. of 2*N*.NaOH) and less water (2.0 ml. of water), and by carrying out comparisons in the fluorimeter. Solutions of adrenaline prepared by an independent worker were estimated by both methods and good agreement found. The results are recorded in Table I, each figure representing the average of six determinations. The standard error of the test, calculated by the method of Gaddum (1938) using the true values of the " unknown " solutions, was 2.11 per cent with West's method and 2.15 per cent with Jörgensen's method. The limits of error (P=0.99), therefore, are 100±5.44 and 100±5.53 per cent respectively.

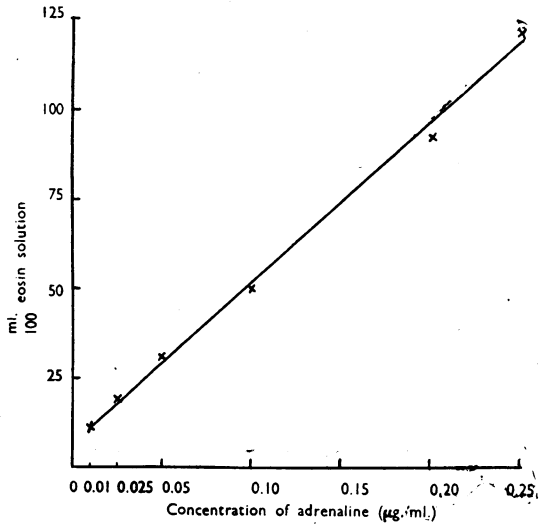


FIG. 3.—Calibration curve for fluorescence of alkali-treated adrenaline, using Jörgensen's eosin solution.

The effect of alkali on strong solutions of adrenaline

In order to measure the biological activity at different stages of the reaction, a more concentrated solution of adrenaline than previously used was treated with a weaker alkali, such as 1.5*N*.Na_2CO_3 (as suggested by Shaw, 1941). The activity was determined using isolated rabbit gut, the perfused frog heart and perfused frog blood vessels, and results are shown in Fig. 4. A loss of 50 per cent was found after 15 min. contact. Chemical estimations by Shaw's (1938) method with and without sodium sulphite and fluorimetric estimations were also carried out.

All the results are reduced to one scale and plotted on one graph (Fig. 5). This graph suggests that the increase in reducing power is not due to the formation of the fluorescent substance (as Utevsky suggested), since maximal fluorescence only appeared after 20 min., and lasted only 5 min. There was a sudden increase in reducing power within 2 min. and this increase (usually five-fold) was maintained for at least 30 min. When the sulphite was omitted from Shaw's test, the values followed the physiological activities very closely, but less than 10^{-6} adrenaline could not be detected by this procedure. There appears to be little or no connexion between loss of physiological activity (measuring rate of

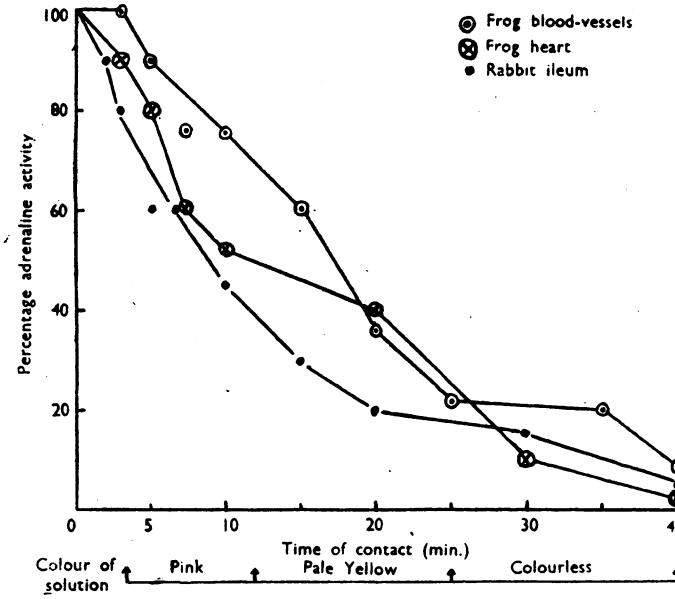


FIG. 4.—The physiological activity of adrenaline (10^{-5}) treated with 1/10 vol. 1.5*N*.Na_2CO_3 for given times. The three methods of assay show fair agreement.

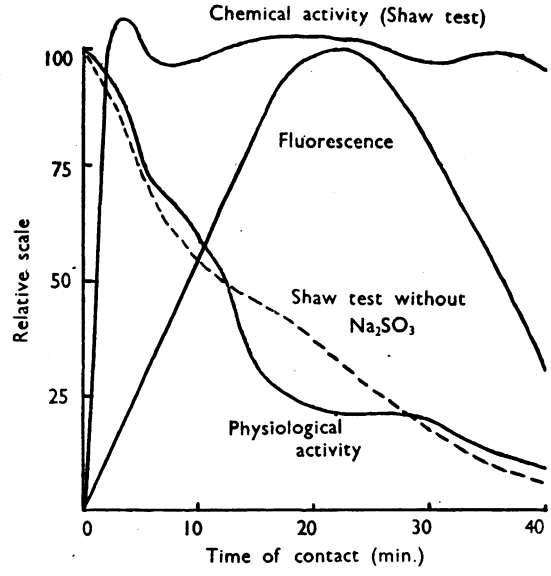


FIG. 5.—The chemical and physiological activities and the fluorescence of adrenaline solution (10^{-5}), when treated with 1.5*N*.Na_2CO_3 for given times.

destruction of adrenaline) and increase in reducing power or production of fluorescence.

Sensitization of tissues to adrenaline by alkali-treated adrenaline

During tests of the physiological activity of alkali-treated adrenaline solutions on the perfused frog blood vessels, an interesting phenomenon was observed. In this preparation, the two anterior venae cavae and the right aorta were ligatured, and Clark's Ringer solution from a Mariotte bottle was perfused via a special arterial cannula into the left aorta and collected from the posterior vena cava into a drop timer (Gaddum and Kwiatkowski, 1938). After about 4 hours' perfusion, during which time the preparation became much more sensitive to doses of adrenaline, consistent responses to 0.01–0.05 μg. were obtained. The presence of oedema was not detrimental to the responses, which were graded according to the dosage (Fig. 6). The perfused blood vessels of female Winter frogs proved the most suitable test objects; moreover, they were relatively insensitive to histamine, acetylcholine and atropine in moderate doses.

A short-lived sensitization of the preparation to a subsequent dose of adrenaline was shown by the same dose of adrenaline previously treated with weak alkali (1.5*N*.Na_2CO_3) for 20 to 25 min. (Fig. 7). No such sensitization was detected in similar experiments when the adrenaline was treated with alkali for 15 min. or less, or for more than 30 min. The time relations suggest that this sensitization is due to the fluorescent substance (Fig. 5).

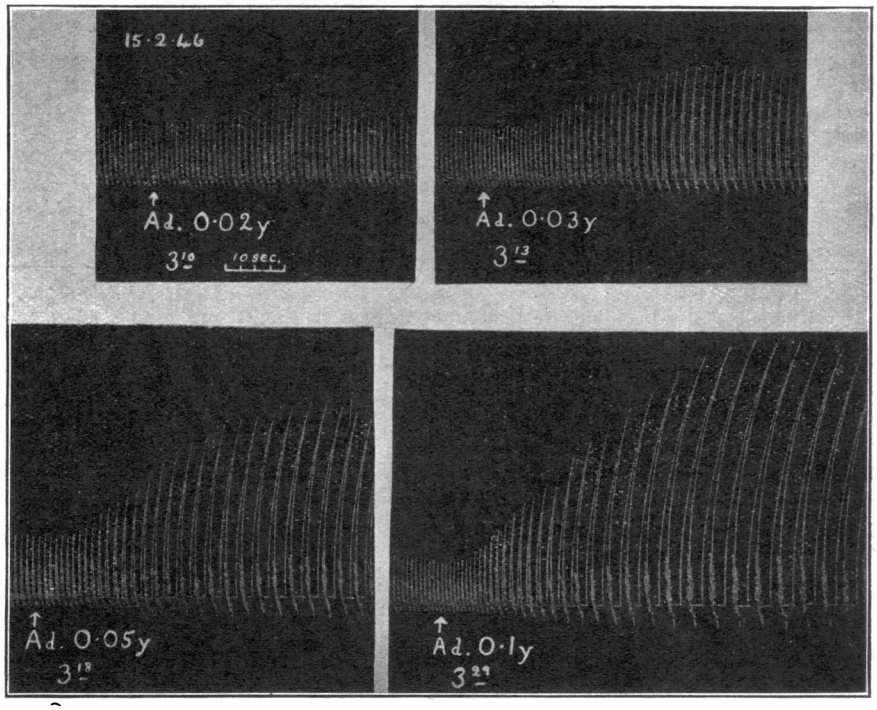


FIG. 6.—Record of outflow of perfused frog blood vessels; the dose-response tracing for adrenaline (Ad.).

because it was only short-lived. That it was not due to the volume of saline injected, the alkali used, or the adrenaline remaining active was shown by injecting these solutions alone just before the adrenaline dose. Jang (1940) has already reported a short-lived sensitization of adrenaline responses by tyramine, sympatol and adrenalone, but a prolonged sensitization

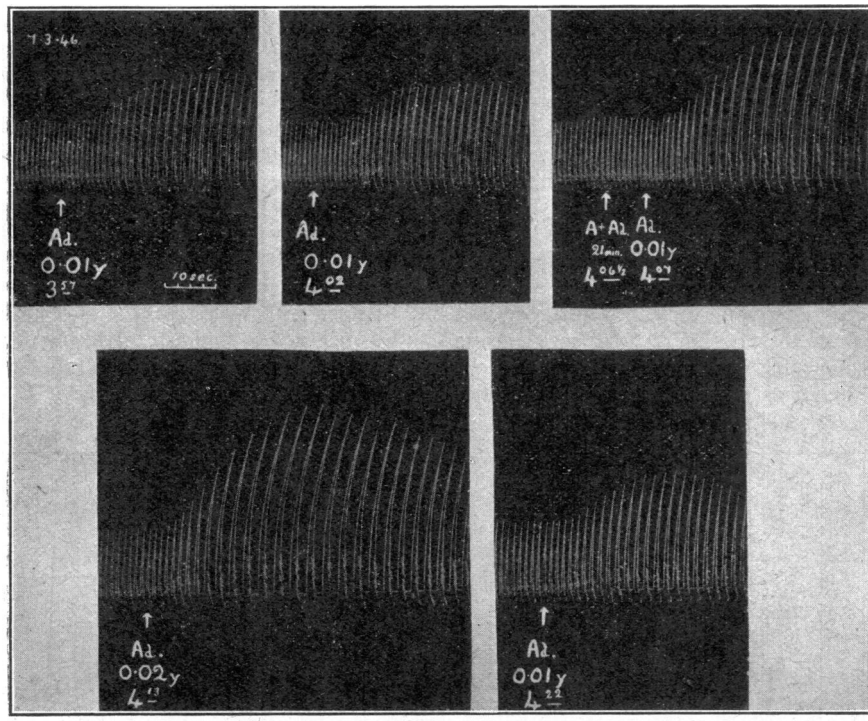


FIG. 7.—The effect of adrenaline, treated with 1.5*N*.Na_2CO_3 for 21 min., and given 1/2 min. before the standard dose of adrenaline (Ad. 0.01 μg.), on the outflow of the perfused frog blood vessels. Enhancement shown was not quite equal to that due to doubling the standard dose.

by phenyl*iso*propylamines such as benzedrine and ephedrine, which are immune to amine oxidase. In the case of the alkali-treated adrenaline, there was some sensitization of the adrenaline response on the spinal cat, rabbit gut, and the Straub frog heart, but none on the perfused frog heart. On the cat's nictitating membrane depression of the adrenaline response sometimes occurred.

DISCUSSION

It is well known that adrenaline is unstable when dilutions are made in Tyrode or Locke solutions. The pink and red colours appear and with them is some fluorescence. Red oxidation products have formed the basis of many chemical methods of estimating adrenaline in solution, the common oxidizing

agents used being ferric chloride, potassium permanganate, persulphate, iodate or dichromate, and iodine. It must not be forgotten, however, that oxidation may occur in the side chain (amine oxidase of Blaschko, Richter, and Schlossmann, 1937).

The fluorescence of adrenaline in alkaline solution has been investigated and most of Utevsky's findings confirmed. That the effect of alkali on adrenaline should be oxidation followed by reduction is an unusual observation, but one explanation might be that a little adrenochrome is first formed and this is reduced at the same time as further adrenaline molecules are oxidized. The process may go on until all the initial adrenaline is oxidized. On the other hand, it is possible that leuco-adrenochrome is formed from adrenaline-quinone and is then oxidized to adrenochrome, in which case the fluorescent material would be another derivative of adrenochrome, giving both catechol and indol reactions.

It has been suggested that the fluorescent reaction is dependent upon a certain grouping such as $\equiv C-CH_2-N=$ (Jörgensen, 1945), or even the phenylethylamine nucleus. Sympathomimetic amines similar to adrenaline, such as *p*-sympatol, epinine, noradrenaline, and "Dopa," produce the same green fluorescence in much stronger solutions (Gaddum and Schild, 1934), but tyramine, ephedrine, and benzedrine do not. Catechol itself produces traces of fluorescence in alkaline solution, so that the basis of the production is not clear. Nevertheless, the test is a specific one for adrenaline in concentrations of 10^{-8} and above, and, when used in conjunction with chemical and biological assays, it can form a good method for the identification of sympathomimetic amines.

From the results shown here, it is certain that the reducing agent in Shaw's test is not entirely composed of the fluorescent material. The brief preliminary treatment of adrenaline with *N*.NaOH for 2 min. produced fluorescence, but the acid nature of the subsequent reagents soon removed any fluorescence before reduction commenced.

Summary

1. Adrenaline in alkaline solution has been shown to produce the fluorescent material, which is probably leuco-adrenochrome, and the reactions leading to its production have been studied. This fluorescent test of Gaddum and Schild has been made of greater use by decreasing the concentration of alkali to 2*N*.NaOH and measuring the fluorescence in a simple fluorimeter.

2. A modified technique for perfusing frogs is described. This preparation is particularly suitable for the assay of adrenaline in tissue extracts since it is relatively insensitive to histamine, acetylcholine, and other substances in these solutions.

3. Fluorescent solutions obtained from adrenaline sensitized the perfused blood vessels of the frog to a subsequent dose of adrenaline, but had little action on cat preparations and rabbit gut.

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