CCLXV. THE CHEMICAL DETERMINATION OF ADRENALINE IN SOLUTIONS CONTAINING PROTEIN

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Many attempts have been made in the past to place qualitative tests for adrenaline on a quantitative basis. The literature on this subject is well covered in a paper by Barker *et al.* [1932] who point out the shortcomings and merits of the various known tests for the drug. In spite of the comparatively large literature on the subject, only three methods have been subjected to detailed study, namely, Ewins's persulphate method, the iodine method originally described by Vulpian, and Folin's method which depends on the reduction of phosphotungstic acid by adrenaline to produce a blue colour.

The persulphate method was improved by Barker *et al.* [1932] but Schild [1933] has shown that the development of the colour is influenced by unknown factors.

The relative merits of the iodine oxidation method and Folin's method are discussed by Barker *et al.* [1932], Schild [1933], and Devine [1937]. The use of the one or the other depends to some extent on the conditions of the experiment. Thus, when solutions of adrenaline are oxidized with iodine (Lugol's solution) at a suitable *p*H, a red compound is produced, but it must be established that chemical analogues which may produce the same compound under the same conditions are absent. Folin's method must be modified in the presence of uric acid and ascorbic acid.

Hitherto experiments have been carried out on protein-free fluids; where necessary, proteins have been removed by precipitation with trichloroacetic acid.

It seemed desirable to attempt to work out a method with a view to determining adrenaline in solutions containing protein without diluting the solution further (as the addition of trichloroacetic acid usually does), and also because the quantitative recovery of adrenaline added to the plasma of defibrinated blood has not been achieved after the precipitation of proteins.

Methods

The plasma from defibrinated ox blood was used as the protein-containing fluid to which was added 1/1000 adrenaline chloride or tartrate solution. Experiments were made with a view to recovering the adrenaline quantitatively (*a*) by precipitating the proteins and determining the drug in the protein-free filtrate and (*b*) from the protein-free ultrafiltrate.

The iodine oxidation method was used to determine adrenaline.

Determination of adrenaline by the iodine (oxidation) method in protein-free solutions

Schild [1933] has described the conditions necessary for a satisfactory determination of adrenaline by oxidation to a red compound with Lugol's solution in fluids free from protein. At *p*H values below 6 a yellow component of the oxidation product is prominent in the colour of the red oxidation product; at *p*H about 6·2, little yellow component exists, whereas at a definitely alkaline reaction, a blue component is introduced. These facts have been confirmed in the present work and some additional information obtained which shows the effect of change of *p*H on the determination of adrenaline.

Two separate 1 ml. portions (V_1 and V_2) of a standard acidified solution of adrenaline hydrochloride containing 0·1 mg. of adrenaline per 1 ml. were added to equal volumes (0·2–0·5 ml.) of 10% disodium hydrogen phosphate and the colours of the oxidation products obtained with 1 ml. of diluted (1 : 14 with water) Lugol's solution followed by 1 ml. of 0·02*N* sodium thiosulphate after 40 sec. were compared in a Duboscq colorimeter.

The results are shown in Table I. When 0·2 ml. of alkali was used, the *p*H of the solution was about 6·2 as determined by the indicator method. Larger

Table I. *Effect of alkali on the determination of adrenaline by the iodine method*

Exp.	Alkali (ml.) added to V_1	Alkali (ml.) added to V_2	Ratio of the concentrations of adrenaline in V_1 and V_2
1	0·2	0·2	1·03
2	0·2	0·2	1·02
3	0·3	0·3	0·97
4	0·4	0·4	0·98
5	0·5	0·5	0·99

volumes of alkali resulted in a tendency towards the introduction of the blue component into the oxidation product; nevertheless, accurate matching of the colours was possible. The error was less than 3%.

In another series of experiments the effect of a different *p*H in each sample was investigated. Thus to each of two 1 ml. aliquots (V_1 and V_2) of an acidified adrenaline solution were added different amounts of 10 or 5% alkaline sodium phosphate. The volumes of the two solutions were equalized and the colours developed as above. The results are in Table II.

Table II. *Effect of alkali on the determination of adrenaline by the iodine method*

Exp.	Alkali (ml.) added to V_1	Alkali (ml.) added to V_2	Ratio of the concentrations of adrenaline in V_1 and V_2	Remarks
	10% Na_2HPO_4, $12H_2O$			
1	0·5	0·2	1·09	Incomplete development of colour in V_2 (?)
2	0·5	0·3	1·06	
3	0·5	0·4	1·02	Easy colour match
4	1·0	0·2	1·15	Accurate colour match impossible
	5% Na_2HPO_4, $12H_2O$			
5	1·0	1·0	1·02	Easy colour match
6	1·0	0·85	1·12	
7	1·0	0·75	1·09	Accurate colour match impossible
8	1·0	0·5	—	

In Exps. 1–3, the addition of 0·5 ml. of alkali to 1 ml. of the adrenaline solution produced a *p*H of 6·2–6·4. In Exps. 1 and 2 the red components of the colours could be matched only approximately, probably because of incomplete colour development in V_2. In Exps. 5–8, the addition of 1 ml. of 5% Na_2HPO_4, $12H_2O$ to the acidified adrenaline solution gave a *p*H of about 7. It will be seen that when V_2 was adjusted to the same *p*H, a good analysis was obtained. Discrepancies between the *p*H values of V_1 and V_2 in the region of *p*H 7 are revealed in very erratic results due to considerable inaccuracies in the matching of the red colours which are much modified by blue components.

Effect of the removal of proteins with trichloroacetic acid on the recovery of adrenaline added to plasma

A weighed amount of adrenaline hydrochloride dissolved in a drop of 2*N* HCl was added to the plasma from defibrinated ox blood so that the resulting solution contained 0·1 mg. of adrenaline per ml. The proteins were precipitated in different experiments by shaking with different volumes of 20% trichloroacetic acid. The protein-free fluid obtained by centrifuging was brought to *p*H 6·4 with 10% disodium hydrogen phosphate and the colour developed after oxidation with iodine was compared with that from a control which also contained trichloroacetic acid. The results are given in Table III.

Table III. *Recovery of adrenaline, added to plasma, from protein-free (trichloroacetic acid) filtrates*

Exp.	Vol. of plasma in ml. containing 0·1 mg. adrenaline per ml.	Vol. of 20% $CCl_3.COOH$ added in ml.	Vol. of protein-free fluid (I) in ml.	Adrenaline content of (I) in mg. per ml.	% recovery of adrenaline
1	10	10	13·7	Very low	—
2	10	3·5	7·5	0·047	35
3	10	2·5	6·4	0·08	51
4	10	2·0	6·8	0·085	58
5	10	2·0*	7·2	0·085	61
6	10	2·0	7·0	0·079	55

* Followed by 1 ml. of 10% Na_2HPO_4, $12H_2O$.

The results seem to depend on the volume of trichloroacetic acid used to precipitate the proteins. A high concentration (Exps. 1 and 2) is associated with a poor recovery of adrenaline. The method was most unpromising because of the low recovery (maximum of 61%) of the drug, owing possibly to the tendency of the precipitated proteins to carry down part of the adrenaline.

Ultrafiltration of plasma containing adrenaline

Preparation of membranes. Collodion (B.D.H.; "special for preparing permeable membranes") in ether was diluted with a little less than its own volume of pure ether dried over anhydrous sodium sulphate. The resulting fairly viscous fluid was poured in a thin stream down the side of a clean test-tube until it was nearly full. The test-tube was then placed vertically in a rack and covered for some time so that the bubbles might rise to the surface. (A few drops of dry ether on the surface of the liquid caused the bubbles to burst as soon as they gained the surface.) When the contents were clear, the tube, held between the palms of the hands, was inverted at an angle of about 45° and gently rotated for 2 min. The tube was refilled with collodion and when the bubbles had risen to the surface it was inverted again and rotated as before for 75 sec. A gentle stream of air was directed from a distance of a few inches into the mouth of the inverted tube which was continuously rotated for 2 min. The odour of ether was no longer observed in the tube

which was then filled with distilled water and set aside for 0·25–0·5 hr. The upper edge of the sac was eased away from the glass tube with a knife after which the sac was usually free to be removed and stored in water.

Successful sacs were also prepared by slight modifications of the above procedure. The conditions for success are that the collodion should be spread evenly on the glass and that the membrane should be sufficiently dry before it is immersed in water.

Ultrafiltration procedure. A membrane was selected and suitably trimmed to have a capacity of about 12 ml.; the uppermost 1 in. of its inside surface was dried with filter-paper. The structure which carried the membrane was a glass tube (as wide as practicable) with a thin piece of rubber tubing slipped over one end. The rubber was smeared with collodion and the sac carefully worked on. The joint was sealed with more collodion and silk tied round as added support. An internal pressure, measured by a manometer, was supplied by a sphygmomanometer bulb. The sac was tested for the absence of air leaks.

Ox plasma derived from defibrinated blood was added to 1/1000 adrenaline solution to make up a suitable concentration of adrenaline. The inside of the membrane was drained free from water and washed three times with successive small quantities of the plasma containing adrenaline, the fluid being drained from the sac after each washing. The remaining fluid was introduced into the sac and a pressure of 160–180 mm. of mercury applied. The ultrafiltrate obtained during the first 15 min. was rejected and the outside of the sac dried with filter-paper. During the succeeding 2–2·5 hr. the ultrafiltrate was collected in a weighed tube containing a known weight of 2*N* HCl (less than 0·1 g.) which served to stabilize the adrenaline filtered out. After filtration, the period of which was maintained constant to obviate the effect of increasing protein concentration in the sac, the tube and contents were again weighed. The rate of filtration was usually 1·0–1·3 g. per hr.

0·5 ml. of the acidified filtrate was brought to a *p*H of about 6·4 with 5% alkaline phosphate, as was a like sample of a suitable adrenaline standard made up by diluting 1/1000 adrenaline with water and 2*N* HCl (0·3 ml. per 10 ml. standard). Adrenaline was then determined in 1 ml. of filtrate according to the procedure described previously. Knowing the amount of acid originally present in the collecting tube, the concentration of adrenaline in the "true filtrate" was calculated. The colours developed in the experimental and control samples could only be accurately matched if the *p*H values were the same. Only those experiments in which protein was absent from the filtrate are included. In fact, in the presence of proteins, no colour match was possible.

Table IV. *Recovery of adrenaline, added to plasma, in protein-free ultrafiltrates*

Exp.	Concentration of adrenaline in mg.per ml.		% recovery of adrenaline
	Plasma	Ultrafiltrate	
1	0·05	0·042	84
2	0·05	0·043	86
3	0·05	0·044	88
4	0·1	0·079	79
5	0·1	0·089	89
6	0·1	0·077	77
7	0·1	0·077	77
8	0·1	0·083	83
9	0·2	0·172	86
10	0·2	0·162	81
			Mean 83

An ultrafiltrate of the plasma alone gave no colour when oxidized with iodine at *p*H 6·4.

The results obtained are tabulated in Table IV.

Table IV shows that 83 % (± 6 %) of the adrenaline is ultrafiltrable within the concentration range 0·05–0·2 mg. per ml. The loss (17 %) might be accounted for by oxidation or combination of adrenaline with the plasma proteins.

Effect of glycine and guanidine

Wiltshire [1931] has shown that glycine, amongst other amino-acids, protects adrenaline from oxidation and that the protein-free ultrafiltrates of plasma and serum act similarly. Burns & Secker [1936] have demonstrated a similar property for guanidine. The effect of incorporating these two substances in concentrations of 2γ per ml. in the plasma is shown in Table V.

Table V. *Effects of glycine and guanidine on the recovery of adrenaline, added to plasma, in protein-free ultrafiltrates*

Exp.	Concentration of adrenaline in mg. per ml.		Protective agent (2γ/ml.)	% recovery of adrenaline
	Plasma	Ultrafiltrate		
1	0·1	0·076	Guanidine	76
2	0·2	0·168	„	84
3	0·1	0·087	Glycine	87
4	0·2	0·162	„	81
				Mean 82

The results show that the substances tried exerted no protective action in addition to that already exercised by the plasma.

Other experiments were carried out in which solutions of adrenaline in plasma were allowed to stand in open test-tubes for periods up to 4 hr. before ultrafiltration, the duration of which was kept constant at 2·5 hr. The recovery of adrenaline was unchanged as shown in Table VI.

Table VI. *Effect of atmospheric oxygen on the recovery of adrenaline, added to plasma, in protein-free ultrafiltrates*

Exp.	Concentration of adrenaline in mg. per ml.		Duration of standing in hr.	% recovery of adrenaline
	Plasma	Ultrafiltrate		
1	0·1	0·08	1	80
2	0·05	0·039	2	78
3	0·1	0·085	3	85
4	0·1	0·078	4	78
				Mean 80

Quantitative recovery of adrenaline added to plasma

2*N* hydrochloric acid (1 ml.) was thoroughly mixed with plasma (14 ml.): 1/1000 adrenaline solution was added to the acidified plasma to give concentrations of adrenaline varying from 0·05 to 0·2 mg. per ml. and the ultrafiltration carried out as before except that the ultrafiltrate (which had a *p*H below 6) was not collected in acid. The determination of adrenaline in the filtrate was carried out as before. Some experiments were performed in which 0·6 ml. of 2*N* HCl was used to acidify 14·4 ml. of plasma but the results indicate (Table VII) that the larger amount of acid gives the more consistent recoveries of adrenaline.

Table VII. *Recovery of adrenaline from solutions of adrenaline in plasma acidified with HCl*

Exp.	Concentration of adrenaline in mg. per ml.		Ratio 2*N* HCl to plasma	% recovery of adrenaline
	Plasma	Ultrafiltrate		
1	0·05	0·053	0·6 : 14·4	106
2	0·05	0·052	0·6 : 14·4	104
3	0·1	0·101	0·6 : 14·4	101
4	0·1	0·098	0·6 : 14·4	98
5	0·2	0·184	0·6 : 14·4	92
6	0·2	0·212	0·6 : 14·4	106
7	0·2	0·194	0·6 : 14·4	97
8	0·1	0·113	1·0 : 14·0	113
9	0·2	0·214	1·0 : 14·0	107
10	0·05	0·055	1·0 : 14·0	110
11	0·1	0·107	1·0 : 14·0	107
12	0·2	0·218	1·0 : 14·0	109
13	0·05	0·056	1·0 : 14·0	112
			Mean of 8–13	110

Control

2*N* HCl (1 ml.) was thoroughly mixed with plasma (14 ml.). To this solution (14·25 ml.) was added water (0·75 ml.) and the solution was ultrafiltered. (*a*) The ultrafiltrate gave no colour with iodine followed by sodium thiosulphate at *p*H 6·4. (*b*) 1 ml. of a standard solution of adrenaline, containing 0·1 mg. of the drug per ml., was introduced into each of two colorimeter cups. To one was added 1 ml. of the filtrate; to the other, 1 ml. of water. The adrenaline present was oxidized as usual after adjusting the *p*H of each solution to 6·4. The adrenaline contents of both solutions were identical which indicated that the filtrate alone of acidified plasma contributed nothing to the recovery of adrenaline in the experiments described in Table VII.

Any destruction of adrenaline which occurred in the course of manipulation might have led to the presence of nearly related oxidation products, which might conceivably have given rise to the same pink colour with iodine as did adrenaline. This source of error was negligible for (*a*) it has been shown by the rabbit intestine method [cf. Wiltshire, 1931] that plasma and serum protect adrenaline from oxidation, and (*b*) solutions of adrenaline at distinctly acid *p*H values are stable. The period during which the drug was not stabilized under one or other of the above conditions was less than 30 sec. and no appreciable amount of oxidation would be expected to occur in that time.

Greenberg & Gunther [1930] have measured the ratio of the concentration of the chloride ion in the ultrafiltrate of serum to its concentration in serum and find a value very near to that (1·11 : 1) obtained by Loeb & Nichols [1927] in membrane distribution experiments. They regard sodium chloride as a completely ultrafiltrable substance; the higher concentrations of chloride in the ultrafiltrate than in the serum can be accounted for on the basis of the Donnan equilibrium theory as applied to heterogeneous equilibria. The ratio found for the concentration of adrenaline in the ultrafiltrate to that in the acidified plasma was 1·10 : 1 (Table VII), which suggested that under the conditions of the experiment the adrenaline was completely ultrafiltrable. When solutions of adrenaline in plasma were ultrafiltered, the concentration of the drug in the ultrafiltrate was 0·83 times that in the plasma (Table IV). The results may be interpreted as evidence of a loose combination between adrenaline and plasma proteins which is broken up completely when sufficient acid is added.

For the purpose of the present experiments it may be taken as an experimental observation that a constant recovery of 110% (average) of adrenaline is achieved by ultrafiltering plasma containing adrenaline under the conditions described above. That is, in any given ultrafiltrate of sufficiently acidified plasma, the concentration of adrenaline multiplied by 0·91 gives the concentration of adrenaline in the original plasma within about 4%.

SUMMARY

1. The influence of *p*H on the determination of adrenaline by the iodine method has been studied.

2. Recoveries of no more than 61% of adrenaline added to plasma (0·1 mg. per ml.) were achieved when the solutions were deproteinized with trichloroacetic acid.

3. About 83% (77–89%) of adrenaline added to plasma (0·05–0·2 mg. per ml.) was recovered in the ultrafiltrate. The loss was not due to oxidation because (*a*) glycine and guanidine, and (*b*) allowing the solutions of adrenaline in plasma to stand in air up to 4 hr., did not alter the yield in the ultrafiltrate.

4. The ultrafiltrates of solutions of adrenaline in plasma to which acid had been added contained 1·10 times (±4%) the concentration of the drug in the plasma.

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REFERENCES

Barker, Eastland & Evers (1932). *Biochem. J.* **26**, 2129.
Burns & Secker (1936). *J. Physiol.* **88**, 2 *P.*
Devine (1937). *Biochem. J.* **31**, 545.
Greenberg & Gunther (1930). *J. biol. Chem.* **85**, 491.
Loeb & Nichols (1927). *J. biol. Chem.* **72**, 687.
Schild (1933). *J. Physiol.* **79**, 455.
Wiltshire (1931). *J. Physiol.* **72**, 88.