some of the few families containing leuco-anthocyanins which are at present included by him in the Herbaceae.

SUMMARY

1. The presence or absence of leuco-anthocyanins in the leaves of vascular plants is related to their systematic position. They are generally present in ferns, but have not been found in Selaginellaceae, Psilotaceae, or Lycopodiaceae, nor in mosses. They are also generally present in gymnosperms, but are absent from *Gnetum*.* Their occurrence in monocotyledons is common but scattered.

2. In the leaves of dicotyledonous plants, the presence or absence of leuco-anthocyanins appears to be connected with a woody habit in the plant, or in the forms closely related to it. Hutchinson's classification of the families of dicotyledons into a predominantly woody group (Lignosae) and a predominantly herbaceous group (Herbaceae) provides a fairly accurate division into those containing members which have, and those whose members do not have, leuco-anthocyanins in their leaves. There are, however, many members of the Lignosae the leaves of which do not contain leuco-anthocyanins; these are usually either herbaceous in habit, or belong to families containing herbaceous members. A few families of Herbaceae, viz. Saxifragaceae (Hutchinson), Polygonaceae, Oxalidaceae, Limnanthaceae, Balsaminaceae, Aizoaceae and Plumbaginaceae, contain leuco-anthocyanins. In Crassulaceae substances reacting with vanillin occur, but these are not leuco-anthocyanins.

3. The distribution of leuco-anthocyanins in the leaves of Papilionatae has been especially studied. They occur in plants of woody habit in Sophoreae, Dalbergieae, Phaseoleae and Galegeae, and in the herbaceous Hedysareae, but are absent from Podalyrieae, Trifolieae, Loteae and Vicieae. This distribution follows closely the division of Papilionatae by Dormer (1946) on grounds of vegetative morphology.

4. The systematic distribution of leuco-anthocyanins closely follows the recorded incidence of tannins in the botanical literature. They have, in fact, the properties of tannins and are probably the substances most commonly responsible for the reactions in plant tissues attributed to tannins.

* See also footnote p. 127.

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REFERENCES

Bate-Smith, E. C. (1953). *J. exp. Bot.* **4**, 1.

Bate-Smith, E. C. & Swain, T. (1953). *Chem. & Ind.* p. 377.

Bergmann, M. & Gierth, M. (1932). In *Handb. der Pflanzenanalyse*, ed. by G. Klein. Vol. II, ii, p. 807. Berlin: Springer.

Creighton, R. H. J., Gibbs, R. D. & Hibbert, H. (1944). *J. Amer. chem. Soc.* **66**, 32.

Dormer, K. J. (1946). *New Phytol.* **45**, 145.

Hutchinson, J. (1946). *A Botanist in Southern Africa.* London: T. R. Gawthorn.

Hutchinson, J. (1948). *British Flowering Plants.* London: T. R. Gawthorn.

Lawrence, W. J. C., Price, J. R., Robinson, G. M. & Robinson, R. (1939). *Phil. Trans.* B, **230**, 149.

Metcalfe, C. R. & Chalk, L. (1950). *Anatomy of the Dicotyledons*, vol. II. Oxford: The Clarendon Press.

Towers, G. H. N. & Gibbs, R. D. (1953). *Nature, Lond.*, **172**, 25.

Trim, A. R. & Hill, R. (1952). *Biochem. J.* **50**, 310.

Willis, T. C. (1948). *Dictionary of the Flowering Plants*, 6th ed. London: Cambridge University Press.

On the Occurrence of Adrenaline and Noradrenaline in Blood

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Results obtained with a fluorimetric method of estimation (Weil-Malherbe & Bone, 1952, 1953) indicate a concentration of about 2–3 μg. adrenaline and 5–7 μg. noradrenaline/l. human venous plasma under basal conditions. Although these figures are lower than many proposed by previous investigators (see review by Pekkarinen, 1948), even lower levels are postulated by some physiologists. Evidence in support of the specificity of the method has been submitted in our earlier publications (Weil-Malherbe & Bone, 1952, 1953). This paper contains a more detailed report of experiments in which the occurrence and concentration of adrenaline and noradrenaline in blood were studied by paper-chromatographic methods.

A comparison of the R_F values of the fluorogenic substances present in blood with those of adrenaline and noradrenaline provided qualitative evidence

for their identity. Since adrenaline and noradrenaline may be differentiated by the fluorescence ratios obtained with different light filters, a second qualitative criterion was applied by measuring these ratios in the fractions separated by paper chromatography. Particular emphasis was laid on the quantitative aspect of the experiments by drawing up a balance sheet between the amounts determined by the direct fluorimetric method and those recovered from the paper chromatograms and by comparing these recoveries with those of added adrenaline and noradrenaline.

It has been suggested (Lehmann & Kinzius, 1951; Annersten, Grönwall & Köiw, 1949) that plasma contains a biologically inactive, protein-bound form of adrenaline (called 'adrenalinogen' by Lehmann & Kinzius). In view of the fact that precipitation of proteins involves considerable losses of added adrenaline (Gaddum & Schild, 1934; D'Silva, 1937; Lehmann & Michaelis, 1942) an association between adrenaline and plasma proteins is a reasonable assumption. If this association is loose, for instance due to coulombic or van der Waals forces, the complex would be expected to dissociate when the solution containing it is passed through an alumina column or extracted with butanol. Thus it is known that plasma thyroxine is extracted by butanol, although it is non-dialysable and quantitatively adheres to protein precipitates (Taurog & Chaikoff, 1948). However, both procedures failed to liberate the amounts of adrenaline claimed to occur in non-deproteinized plasma by Lehmann & Michaelis (1942, 1949) and by Annersten *et al.* (1949). Since there still remained the possibility of an undissociable link between adrenaline and plasma proteins, such as a peptide bond, it was decided to estimate adrenaline and noradrenaline in partly hydrolysed plasma.

The final problem investigated was the arteriovenous difference of adrenaline and noradrenaline under basal conditions. The rate of adrenaline utilization can be approximately calculated from these figures. As will be shown, there is a good correlation with the known rate of adrenaline discharge from the adrenal gland.

EXPERIMENTAL

Methods

Collection and preparation of blood fractions. Human blood was withdrawn by mixing 3 parts in the syringe with 1 part fluoride–thiosulphate solution (Weil-Malherbe & Bone, 1952). Ox blood was obtained from the slaughterhouse; a few minutes after the death of the animal the blood (3 parts) was run from the severed carotid artery into a bottle containing 1 part of fluoride–thiosulphate solution. Plasma was separated by centrifuging. Red cells were washed 3 times with isotonic NaCl and finally suspended in isotonic NaCl made up to the original blood volume. Fluoride–thiosulphate solution was added (1 vol. to 3 of red cell suspension) and cytolysis was induced, in some instances by the addition of cetyltrimethylammonium bromide (cetrimide) solution as previously described (Weil-Malherbe & Bone, 1953), or, more frequently, by freezing at $-40°$ in an ethanol–dry ice bath (Lovelock, 1953). The cytolysed solution was finally clarified by centrifuging.

The packed-cell volume of the mixture of blood and fluoride–thiosulphate solution was determined by centrifuging in haematocrit tubes for 45 min. at approx. 2000 *g*.

Extraction. Samples of plasma and erythrocyte lysates were extracted with 2 vol. *n*-butanol by vigorous mechanical shaking in stoppered centrifuge tubes for 10 min. The emulsion was broken by centrifuging and the clear supernatant transferred to a separating funnel. The residue, including a creamy intermediate zone, was extracted twice more with butanol (2 vol.). The pooled butanol extracts (6 vol.) were extracted 3 times with 0·05 N-HCl (2 vol./extraction). The combined HCl extracts were concentrated *in vacuo* to about a third of their volume and the pH was adjusted to 3 by the gradual addition, with constant stirring, of Dowex 2 (OH form, 20 to 40-mesh; Microchemical Specialities Co., Berkeley, California). After the resin had been filtered off and repeatedly washed with water, the filtrate and washings were further concentrated *in vacuo* until the volume was reduced to about one-fifth of that of the blood sample used.

To the concentrated extract (50 ml.) were added 0·2 M phosphate buffer pH 6·5 (5 ml.) and enough N-NaOH to raise the pH to 6·5; 3% (w/v) $Na_2S_2O_3$ (5 ml.) was also added. Any precipitate formed (calcium phosphates?) was removed by centrifuging. The solution was adjusted to pH 8·4 by careful addition of 0·2 N-NaOH and passed through a column of acid-washed aluminium oxide. The preparation of this column and the procedure of adsorption and elution were according to Weil-Malherbe & Bone (1952).

Comment on extraction procedure. The use of butanol as solvent was suggested by the method of extracting thyroxine from plasma (Taurog & Chaikoff, 1948). Adrenaline, like thyroxine, is carried down by protein precipitates, a fact which precludes the use of trichloroacetic acid and similar agents.

By partially neutralizing the HCl extract with Dowex 2 the introduction of salt is avoided, but this step is safe only if certain precautions are observed. If the HCl extract is treated with resin while it is still saturated with butanol, adrenaline is lost through adsorption by the resin. After the extract has been concentrated to 0·3–0·5 vol., the butanol has been quantitatively distilled off. If, on the other hand, concentration of the solution is carried too far, adrenaline may again be adsorbed by the resin; this may occur when the HCl concentration exceeds 0·2 N. Rapid mechanical stirring is necessary to ensure a thorough mixing while the resin is slowly added, thus avoiding local zones of alkalinity. The amount of resin required to neutralize a given quantity of acid is fairly constant and should be determined in advance.

Several other anion-exchange resins were found unsuitable since they released fluorescent material.

Paper chromatography. The eluate from the column of alumina was evaporated to dryness *in vacuo*, the process being finished in a small test tube, 75 × 12 mm. The residue was extracted 3 times with 0·5 ml. acid acetone (1 ml. conc. HCl in 100 ml. acetone) and the extract transferred to the starting line of the paper strip in 5 μl. portions under a jet of N_2 to accelerate drying.

Chromatography was carried out by the ascending technique in Pyrex jars, 22 × 9 in., fitted with a ground flange and a tubulated desiccator lid. The jars stood in a thermostatically controlled cabinet at 23°. Each jar held three separate paper strips, usually a reference strip, a blank strip and the strip carrying the blood extract. The strips were suspended from a glass-rod triangle by means of a bent-over flap and were secured by a length of glass rod threaded through slots. A glass rod was also threaded through the paper below the line of application. The paper strips measured 48 × 8 cm.; the line of application was at a distance of 6 cm. from the lower edge.

Whatman no. 1 paper was used for most experiments. The strips, in batches of about 50, were bathed in 0·5 N-HCl (three changes) for 24 hr. and washed in several changes of distilled water. They were then immersed in a solution of 0·1 % ethylenediaminetetraacetate for 24 hr., washed and air-dried. In some experiments with butanol–HCl mixture as solvent the paper used was Grycksbo OB.

A mixture of phenol, freshly distilled over aluminium (Draper & Pollard, 1949), and glass-distilled water (3:1, w/v) was used as solvent in most experiments. A dish containing 5 N-HCl, saturated with phenol, was placed in the bottom of the jar. In some experiments butanol saturated with N-HCl was used as solvent (Hamberg & Euler, 1950). At the end of the run (after 21–24 hr.) the paper strips were twice immersed in benzene (purified by extraction with conc. H_2SO_4 and redistilled) and hung up to dry for 0·5 hr. The reference strip was sprayed with the potassium ferricyanide reagent of James & Kilbey (1950), while the strip containing the blood extract was cut into a series of eight to nine transverse sections: two of these corresponded to the zones occupied by the adrenaline and noradrenaline spots on the reference strip, one corresponded to the clear interval between the spots and the remainder accounted for the paper between the line of application and the noradrenaline spot and that between the adrenaline spot and a line situated about 2–3 cm. below the solvent front. Each section was placed in a test tube containing 10 ml. 0·01 N-HCl and extracted for 12–24 hr. at 3°. Each HCl extract was shaken with three lots of 10 ml. freshly distilled, peroxide-free ether to remove traces of phenol. The extracts were freed from ether by brief evacuation and carried through the condensation with ethylenediamine by the procedure described (Weil-Malherbe & Bone, 1952).

Comment on the chromatographic procedure. The method described is essentially that of Goldenberg, Faber, Alston & Chargaff (1949). Solvent systems consisting of mixtures of butanol with water and various acids (formic, acetic, oxalic and HCl) did not lead to clear-cut separations of the adrenaline and noradrenaline spots in the ascending method. Attempts to use the descending method were abandoned, since the rate of flow was liable to differ in the separate paper strips, a complication not encountered with the ascending technique.

Various modifications of the phenol method were tested. Phenol containing 15 % (v/w) 0·1 N-HCl (Vogt, 1952) slightly decreased the R_F value of both adrenaline and noradrenaline. Filling the jar with CO_2 (Vogt, 1952) improved recoveries a little and this procedure was adopted in later experiments. A slight improvement of recovery was also found when the papers were sprayed with ascorbic acid according to Crawford & Outschoorn (1951) (Expt. 4, Table 5), but this was offset by a wider spread of the spots. The treatment was therefore omitted. None of the modifications reduced the fluorescence of the blank.

A factor of importance for obtaining low blank readings was the purity of the benzene and ether used for the removal of phenol. The use of ether contaminated by peroxides resulted in highly fluorescent blanks, presumably owing to the formation of oxidation products of phenol.

Fluorimetric estimation. This was carried out as described in previous papers, in the earlier experiments by the original method (Weil-Malherbe & Bone, 1952), later by the differential method (Weil-Malherbe & Bone, 1953).

In the differential method, fluorimeter readings are taken first with a yellow secondary filter (Chance OY 4, maximum transmission 550 mμ. and upwards) and next with a blue-green filter (Ilford Bright Spectrum Filter 623, max. transmission approx. 490 mμ.). While the fluorescence of the noradrenaline derivative is of similar intensity at both wavelengths that of the adrenaline derivative is about 4 times stronger in the yellow than in the green region. The results are calculated with the aid of simultaneous equations which, on solution, yield the following formulae:

$$\text{Noradrenaline } (N) = mn(b-y)/(m-n), \quad (1)$$

$$\text{Adrenaline} = y - N/m = b - N/n, \quad (2)$$

where m = ratio of adrenaline/noradrenaline fluorescence with yellow filter; n = ratio of adrenaline/noradrenaline fluorescence with blue-green filter; y = amount of adrenaline corresponding to fluorescence measured with yellow filter; b = amount of adrenaline corresponding to fluorescence measured with blue-green filter. Unfortunately, equation (4) of the paper of Weil-Malherbe & Bone (1953), corresponding to equation (2) above, was erroneously printed with brackets and this opportunity is taken to correct the error.

Other amendments of previous directions are as follows:

(1) Ethylenediamine dihydrochloride is obtained in better yield by dissolving 50 ml. freshly distilled ethylenediamine in 200 ml. ethanol and mixing this solution with 1500 ml. ethanolic HCl containing 150 ml. conc. HCl. After cooling the crystals are filtered off, washed with ethanol and dried.

(2) If the *iso*butanol extract is exposed to atmospheric CO_2 for any length of time, a turbidity is liable to appear owing to formation of ethylenediamine carbonate.

RESULTS

Recovery of adrenaline and noradrenaline. Recoveries were studied separately in the two phases of the analysis, extraction and paper chromatography. The efficiency of the extraction procedure was examined by analysing a sample of the eluate from the alumina column. The results (Table 1) show that adrenaline added to plasma or serum is recovered in 75–85 % yield. The recovery of the unknown fluorogenic material from plasma is of the same order (Table 1, bottom line). Losses, however, tend to become larger when the scale of the process is increased (cf. Table 6). A slightly lower recovery is also found when adrenaline is added to red cell extracts. This may be due to the fact that haemoglobin when treated with butanol turns into a sticky mass which it is impossible to disperse.

Table 1. *Recovery of adrenaline by extraction procedure*

In experiments where adrenaline was added the results were corrected by subtracting 'pre-formed' adrenaline determined in a control sample. In experiments without addition (last line of table) the recovery figures are based on a comparison between an analysis of a plasma sample before extraction and an analysis of the purified extract.

No. of expts.	Blood fraction	Volume extracted (ml.)	Adrenaline added (µg.)	Mean recovery (%)	S.E.M.
3	Horse serum	20	10	74	—
1	Horse serum	20	5	85	—
7	Red cells (human)	20–40	1	66·3	2·3
2	Plasma (human)	20	0·2	82·9	—
10	Plasma (human)	70–115	0	80·2	4·0

Table 2. *Recovery of adrenaline and noradrenaline by paper chromatography*

In Expt. 1 the amines were applied directly to the paper. In Expt. 2 they were contained in 0·2 ml. water and in Expts. 3 and 4 in 15 ml. 0·067 M acetic acid. The solutions were evaporated to dryness and the residue was extracted with acid acetone. The fluorimetric assay of the eluates from the paper sections was performed with the yellow filter only.

	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Adrenaline added (µmg.)	100	200	80	160
Noradrenaline added (µmg.)	500	1000	300	600
Total added in terms of adrenaline fluorescence (µmg.)	214	427	148	296
Recovered: (µmg. in terms of adrenaline fluorescence)				
Residue in tube	—	0	13	15
In noradrenaline section	106	164	43	94
In adrenaline section	98	177	68	103
Total recovered	204	341	124	212
Recovery (%)	96	80	84	71·5

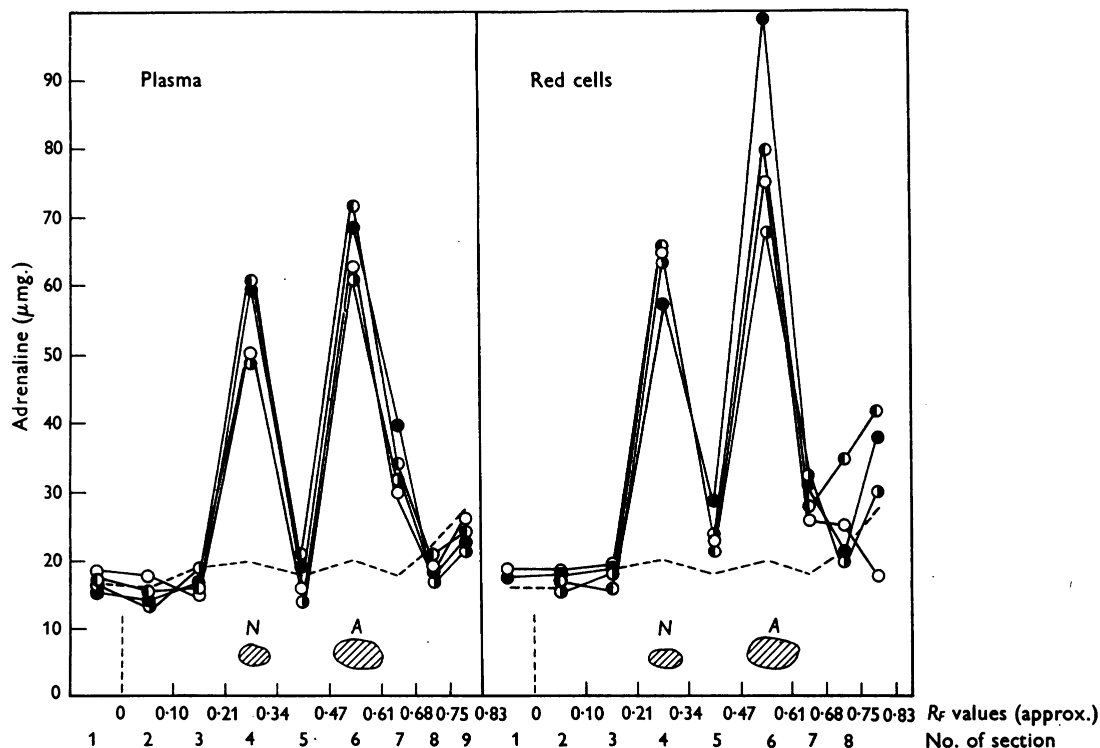

Fig. 1. Paper chromatography of extracts of human plasma and red blood cells. Fluorimetric readings (in terms of µmg. adrenaline) obtained in four experiments are plotted against the approximate R_F values of the cuts. Shaded areas: position of noradrenaline (*N*) and adrenaline (*A*) spots on guide strip.

The recovery of adrenaline and noradrenaline by the paper-chromatographic method was studied in other experiments (Table 2). These experiments were done before the differential method (Weil-Malherbe & Bone, 1953) was available and the estimations are based on fluorimeter readings obtained with a yellow filter. Under these conditions adrenaline gives rise to a fluorescence intensity which is about 4·4 times greater than that given by noradrenaline. This factor was used to calculate the balance of recovery. All readings were corrected by subtracting the appropriate blank. It appears from Table 2 that losses are small during the paper-chromatographic procedure itself, but that some losses are incurred during the preceding stage of evaporation and transfer (see also Table 5).

Analysis of blood extracts. In a first series of experiments, carried out before the development of the differential method, plasma and red cell fractions of ten human blood samples measuring 120–150 ml. were analysed. The qualitative aspect of the results is apparent from Fig. 1, where the fluorescence intensities produced by the eluates (in terms of μmg. adrenaline) are plotted against the approximate R_F value of the cuts (the position of the cuts is not accurately drawn to scale since it varied somewhat from experiment to experiment). The shaded areas indicate the appearance of the noradrenaline and adrenaline spots on the guide strip. Without exception the analysis of both plasma and corpuscle extracts produced two peaks which coincided with the position of these spots.

Some fluorescence also appeared in the eluates of a blank paper strip (Fig. 1, broken line). Its intensity was fairly constant and did not vary appreciably with the size of the paper section, with its position up to an R_F value of about 0·7, or from one experiment to another. Between R_F 0·7 and the solvent front the fluorescence of the blank tended to increase. This effect coincided with the appearance, in these paper sections, of the 'pink front', which formed irregular-shaped streaks near the upper edge of the chromatogram. Despite many precautions, such as the use of acid-washed, ethylenediaminetetraacetate-treated paper and of freshly distilled phenol and the displacement of air by CO_2, the pink front could not be entirely eliminated. The amount of fluorescence produced by the eluates from the upper regions of a blank paper strip showed little uniformity in different experiments and contaminated sections were therefore discarded.

Eluates from sections other than those corresponding to the position of the adrenaline and noradrenaline spots on the guide strip did not yield a fluorescence higher than the general level of the blank. This is particularly significant in the case of the section intermediate between adrenaline and noradrenaline. The only exception was the section following adrenaline (no. 7 in Fig. 1), which often showed a small amount of fluorescence above the blank level. Since the spots tend to spread with increasing R_F and the section was contiguous with the adrenaline section it was assumed that the fluorescence was produced by the same substance as that in the adjoining fraction. Any excess fluorescence found in this section was therefore calculated as adrenaline. It was later found by the differential technique that this procedure was justified.

From a qualitative point of view, it may thus be stated that the fluorogenic matter of the extracts is entirely concentrated in positions corresponding to those of adrenaline and noradrenaline, at any rate up to an R_F value of about 0·8. A quantitative balance sheet is shown in Tables 3 and 4. The amount of fluorescence recovered in the adrenaline and noradrenaline positions of the paper chromatogram and expressed in terms of μmg. adrenaline has been compared with the amount of fluorogenic matter (also in terms of μmg. adrenaline) present in the purified extract before evaporation. Included in the balance is the residue left in the test tube after extraction with acid acetone. It was dissolved in water, treated with ethylenediamine and analysed fluorimetrically. It accounted for about 10 % of the recovered matter.

Extracts of both plasma and red cells gave mean recoveries of over 70 %. The figures compare well with the recovery of authentic adrenaline and noradrenaline shown in Table 2. There is therefore no reason to assume that the substances estimated in the fluorimetric method include components other than those migrating to the adrenaline and noradrenaline positions.

Further experiments were performed after the differential method of estimation had been developed (Weil-Malherbe & Bone, 1953). On applying the formula for the calculation of the results, the material extracted from the noradrenaline position should analyse as noradrenaline and that isolated from the adrenaline position as adrenaline.

The fluorescence of the blank when determined with the blue-green filter was even higher than when determined with the yellow filter; it corresponded with the fluorescence given by 60–80 μmg. adrenaline, increasing near the upper edge of the chromatogram. Similar blank values were obtained with butanol–N-HCl as solvent. Readings were corrected by subtracting the blank value, and the calculations were based on the corrected readings. These corrections were necessarily fraught with some inaccuracy as shown, for instance, by the occasional appearance of a negative result or by the appearance of positive figures at positions separated from the main fraction by a gap. Such results were

Table 3. *Paper chromatography of adrenaline and noradrenaline in human plasma extracts*

Fluorimetric assay with yellow filter. Results expressed in terms of μmg. adrenaline. The plasma extracts of Expts. 6 and 10 were lost.

Expt. no.	Plasma extracted (ml.)	Residue	Zone of application	Noradrenaline zone	Adrenaline zone	Total recovered	Content of extract (before evaporation)	Recovery (%)
1	70	15	0	59	105	179	209	85·6
2	74	8	2	55	76	141	158	89·0
3	95	14	0	50	69	133	239	55·7
4	82	13	4	45	82	144	214	67·3
5	84	6	0	45	73	124	182	68·3
7	76	15	0	45	66	126	161	78·3
8	83	13	0	41	63	117	186	63·0
9	80	20	0	45	60	125	177	70·8
			Mean % recovery (with S.E.)					72·2 ± 4·0

Table 4. *Paper chromatography of adrenaline and noradrenaline in human erythrocyte extracts*

Fluorimetric assay with yellow filter. Results expressed in terms of μmg. adrenaline.

Expt. no.	Volume extracted (ml.)	Residue	Zone of application	Noradrenaline zone	Adrenaline zone	Total recovered	Content of extract (before evaporation)	Recovery (%)
1	120	12	0	73	86	171	218	78·5
2	100	13	6	50	72	141	179	79·0
3	134	19	0	47	51	117	242	48·5
4	90	16	0	45	65	126	238	53·0
5	106	15	0	49	80	144	185	78·0
6	104	23	0	45	66	134	199	67·4
7	104	16	0	52	62	130	180	72·3
8	104	19	0	54	72	145	201	72·0
9	104	25	0	51	63	139	181	77·0
10	115	17	0	49	91	157	187	84·0
			Mean % recovery (with S.E.)					71·0 ± 3·7

Table 5. *Recovery of adrenaline and noradrenaline by paper chromatography*

Fluorimetric assay by differential method. Adrenaline and noradrenaline solutions were directly applied to the paper in Expts. 1–4. In Expts. 5 and 6 they were added to 15 ml. 0·067 M acetic acid and evaporated before application to paper. In Expt. 4 the paper was sprayed with ascorbic acid solution before use. Bracketed values were omitted from calculations.

		Recovered in				
Expt. no.	Added	Residue	Noradrenaline zone	Adrenaline zone	Total	Recovery (%)
			Adrenaline (μmg.)			
1	1000	—	0	830	830	83
2	100	—	(2)	64	64	64
3	100	—	0	83	83	83
4	100	—	(1)	99·5	99·5	99·5
5	600	20	(−15)	301	321	53·5
6	600	5	(2)	330	335	56
			Noradrenaline (μmg.)			
1	1000	—	688	0	688	68·8
2	100	—	66	(7)	66	66
3	100	—	86	(13)	86	86
4	100	—	90	0	90	90
5	900	56	571	0	627	69·7
6	900	17	509	(12)	526	58·5

Table 6. *Balance sheet of extraction and paper chromatography of adrenaline and noradrenaline in plasma*

Fluorimetric assay by differential method (see text). Bracketed values were considered erroneous and were omitted from balance calculation. All quantities in columns 2–8 are in μmg. unless otherwise stated.

		1		2		3		4	
Expt. no.		1		2		3		4	
Species		Bovine		Bovine		Bovine		Human	
Volume extracted (ml.)		1080		1000		800		120	
Corresponding volume of undiluted plasma (ml.)		675		650		491		86	
		Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
Estimated before extraction		2770	5660	2520	3810	2960	4400	263	364
Estimated in extract		2400	3520	1270	1990	1900	2350	201	333
Recovery in extract (%)		86·6	62·2	50·5	52·2	64·3	53·5	76·5	90·6
Paper chromatography with phenol									
Amount used		1170	1710	617	974	1770	2250	181	300
Paper sections: No.	R_F (approx.)								
1	Below 0–0·09	0	0	0	0	0	0	0	0
2	0·09–0·22	0	0	0	0	0	0	0	0
3*	0·22–0·34	(10)	275	(15)	199	(11)	830	(−2)	119
4	0·34–0·48	(−4)	97	(11)	46	(−4)	59	0	0
5†	0·48–0·64	157	(12)	189	0	532	(−52)	81	0
6	0·64–0·74	76	0	10	(7)	9	0	13	0
7	0·74–0·84	0	0	0	0	0	0	0	0
8	0·84–0·93	0	0	0	0	0	0	0	0
Residue after acetone extraction		323	512	76	123	268	326	25	43
Total		556	884	275	368	809	1215	119	162
Recovery (%)		47·6	51·6	45·5	38	45·6	54·0	65·8	54·0
Paper chromatography with butanol–HCl									
Amount used		1170	1710	617	974				
Paper sections: No.	R_F (approx.)								
1	0·11–0·14	0	0	0	0				
2	0·14–0·17	(4)	52	0	0				
3*	0·17–0·20	26	103	(−12)	108				
4*	0·20–0·23	36	89	10	126				
5†	0·23–0·26	55	40	60	38				
6†	0·26–0·29	49	28	98	12				
7	0·29–0·32	42	0	20	(18)				
8	0·32–0·35	12	(10)	8	(2)				
9	0·35–0·39	0	0	0	0				
10	0·39–0·43	0	0	0	0				
Residue after acetone extraction		261	278	146	290				
Total		481	590	352	574				
Recovery (%)		41·2	34·5	57·0	59·0				

* Sections corresponding to position of noradrenaline spot on guide strip.

† Sections corresponding to position of adrenaline spot on guide strip.

considered erroneous and they are shown in Tables 5 and 6 in brackets. It will be noticed, however, that the rejected figures are less than 20 μmg. and that their inclusion would not significantly alter the result.

Recoveries of added adrenaline and noradrenaline were again lower when the solutions were evaporated to dryness before application to the paper (Table 5, Expts. 5 and 6). As was to be expected in recovery experiments, the substance extracted from the noradrenaline position did not contain significant amounts of adrenaline and *vice versa.*

The results of three extractions of bovine plasma and one of human plasma are recorded in Table 6. With the larger quantities of bovine plasma available, more concentrated extracts could be used for paper chromatography, and the relative extent of the corrections imposed by the blank accordingly reduced. Moreover, in two experiments the extract was divided and simultaneously chromatographed with both solvent systems. In the butanol chromatogram the adrenaline and noradrenaline spots on the reference strip were partly overlapping; a length of 10 cm. was cut out of the plasma strip in such a way that the middle zone corresponded to the position of the spots on the reference strip. The paper was then cut into ten equal transverse sections of 1 cm. width. The phenol chromatogram was cut into sections as previously described.

The recoveries in these large-scale experiments were lower than in the analyses of human plasma. There was also a larger proportion of both amines remaining in the undissolved residue after acetone extraction. Nevertheless, the recoveries did not fall far short of, or were equal to, the figures found in model experiments.

The fluorescence ratio obtained with the extracts from the noradrenaline positions was that of the noradrenaline derivative and accordingly the calculation showed adrenaline to be absent from these fractions, within the limits of error. The same is true, *mutatis mutandis*, for the extracts from the adrenaline positions. Separation was incomplete in the butanol chromatogram, but the concentration curves of adrenaline and noradrenaline had different peaks which coincided with the position of the respective spots on the guide strip.

Expt. 4, Table 6, shows that entirely satisfactory results can be obtained when the differential method is applied to extracts from about 80 ml. human plasma.

Adrenaline and noradrenaline in partially hydrolysed plasma. Preliminary experiments showed that 95 % of adrenaline was recovered after refluxing in 2 N-HCl for 6 hr., while an increase in the concentration of HCl led to considerably lower recoveries. When plasma was refluxed in 2 N-HCl the mixture first became flocculent and turbid, but later cleared and was perfectly homogeneous after 6–8 hr. Though hydrolysis was presumably incomplete, at least a partial liberation of bound adrenaline might have been expected.

The experiments (Table 7) were carried out with bovine plasma. A sample was mixed with an equal volume of 4 N-HCl and refluxed for 8 hr. After cooling, the solution was neutralized by the slow addition, with vigorous stirring, of 8 N-NaOH. A second sample of equal size was mixed with an equal volume of 4 N-NaCl and made up to the same volume as the first sample. Both samples were extracted with butanol and the procedure continued as described. The purified extracts were finally chromatographed on paper with phenol as solvent and the eluates from the paper sections analysed by differential fluorimetry. The agreement of the results obtained with the hydrolysed and unhydrolysed samples is as good as can be expected. These experiments therefore do not support the hypothesis of a protein-bound form of adrenaline existing in plasma.

Several unsuccessful attempts were made to achieve a more thorough hydrolysis by digesting plasma with a cation-exchange resin (Dowex 50) in 0·05 N-HCl at 100°, but although adrenaline could be recovered satisfactorily from pure solution, a plasma suspension remained flocculent for up to 6 days.

Arteriovenous differences of adrenaline and noradrenaline in plasma. Arteriovenous differences were determined in six fasting human subjects, all males (Table 8). The adrenaline level was always higher in arterial than in venous plasma by 0·5–1·0 μg./l. The noradrenaline level of the arterial plasma, on the other hand, was found to be higher than in venous plasma in three cases and lower in the

Table 7. *Estimation of adrenaline and noradrenaline in hydrolysed and unhydrolysed bovine plasma*

Fractionation by paper chromatography. Fluorimetric assay by differential method. Amounts given are in μmg.

Expt. no.	Diluted plasma (ml.)	Unhydrolysed		Hydrolysed	
		Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
1	50	35·2	106	38·6	158
2	100	65	194	51	140

Table 8. *Arteriovenous differences in the adrenaline and noradrenaline content of human plasma*

A = arterial blood; V = venous blood; Δ = arteriovenous difference.

Expt. no.	Adrenaline (μg./l. plasma)			Noradrenaline (μg./l. plasma)		
	A	V	Δ	A	V	Δ
1	3·26	2·20	1·06	5·38	6·82	−1·44
2	3·68	2·73	0·95	6·44	5·58	0·86
3	3·76	3·10	0·66	4·40	4·60	−0·20
4	3·80	3·30	0·50	4·40	6·92	−2·52
5	2·49	1·77	0·72	6·32	5·65	0·67
6	2·47	1·87	0·60	6·15	4·98	1·17

other three cases. Although the number of experiments is small, it is felt that the results are probably significant, especially in view of the consistency of the adrenaline results. A negative arteriovenous difference for noradrenaline does not seem improbable if it is remembered that noradrenaline is discharged not only from the adrenal medulla, but also from the sympathetic nerve endings of peripheral tissues.

DISCUSSION

The work described shows that plasma and red blood cells contain two components one of which has the R_F value of adrenaline, the other that of noradrenaline in two solvent systems. Both fractions yield, on treatment with ethylenediamine, fluorescent derivatives whose fluorescence ratios at two different wavelengths agree with those of the adrenaline and noradrenaline derivatives, respectively. Quantitatively these fractions accounted, or almost accounted, for such a proportion of the adrenaline and noradrenaline estimated by the routine procedure as corresponded to the recovery of added amines in model experiments.

Though they do not supply incontrovertible proof, these facts strongly support the supposition that the blood constituents estimated by the fluorimetric method are identical with adrenaline and noradrenaline. Several attempts were made to test the biological activity of the fractions eluted from the paper chromatogram. An experiment in which the rat-uterus method was used has been described in an earlier publication (Weil-Malherbe & Bone, 1952); it was inconclusive owing to the presence of interfering material. The same difficulty arose when a noradrenaline fraction, isolated by paper chromatography from about 80 ml. human plasma, was tested by the rat blood-pressure test; again the extract contained substances which interfered with the action of added noradrenaline. (This experiment was kindly performed by Dr M. Vogt.) Finally, eluates from the adrenaline and noradrenaline zones of a paper chromatogram were tested by Dr R. T. Grant by measuring the constrictor action on the artery of the denervated rabbit's ear (Armin & Grant, 1953). The noradrenaline fraction produced, after a transitory dilator effect, a constriction which was equal to that produced by a standard solution of the expected strength ($1:10^{-11}$). When, however, the adrenaline fraction was tested, a strong and irreversible dilatation of the artery ensued. An extract from the corresponding position of a paper blank had the same effect. The interfering material which was obviously derived from the reagents probably accumulated in the more distal parts of the chromatogram, as does the fluorescent impurity and the 'pink front'. It is intended to continue these experiments with more highly purified fractions.

A method for the estimation of adrenaline and noradrenaline in blood which in sensitivity and specificity is comparable to that used in this laboratory has been described by Lund (1949), but this author was unable to detect measurable amounts of these amines in peripheral venous blood. Lund's method was therefore compared with the ethylenediamine method by analysing samples of the same specimen of plasma. The results were practically identical (Bone, 1953).

The results of Table 8, expressed in μg./l. of whole blood, yield a mean value of 0·50 for the adrenaline arteriovenous difference. Assuming a basal minute volume of 4 l., a mean body weight of 60 kg., and an identical arteriovenous difference in all parts of the vascular system, an adrenaline utilization rate of 0·033 μg./kg./min. is obtained. This figure may be compared with the adrenaline output from a single adrenal gland of the anaesthetized cat, which was found to be 0·015 μg./kg./min. by Dunér (1953) and about 0·02 μg./kg./min. by Kaindl & Euler (1951), or of the anaesthetized dog, which is 0·011–0·027 μg./kg./min. according to Houssay & Rapela (1953). Wada & Kanowoka (1935) reviewed the results of the Japanese school, obtained on dogs without the use of an anaesthetic, and gave as the mean of 377 observations the figure of 0·06 μg./kg./min. for the basal adrenaline discharge from both adrenal glands.

In a first series of blood samples a mean concentration of plasma noradrenaline of about 5·2 μg./l. blood was found (Weil-Malherbe & Bone, 1953). From a large number of analyses since carried out it appears that this figure was probably a little high and that the true mean is approx. 4·5 μg./l. blood.

On the basis of a plasma volume of 55 % of blood and expressed in molar units the concentration of adrenaline in plasma is about 0·015 μM, that of noradrenaline about 0·048 μM. It is of interest to compare these figures with the concentration of other low-molecular hormones in plasma, in so far as they have been determined by reliable methods. The concentration of thyroxine may be calculated if it is assumed as a first approximation that all protein-bound iodine is accounted for by thyroxine. From a value of 4–8 μg./100 ml. of protein-bound iodine in plasma (Kydd, Man & Peters, 1950; de Mowbray & Tickner, 1952) the concentration of thyroxine is calculated as 0·08–0·16 μM. Recent estimates of individual adrenocortical steroids in plasma (Morris & Williams, 1953; Bliss, Sandberg, Nelson & Eik-Nes, 1953; Bush & Sandberg, 1953; Bayliss & Steinbeck, 1953) suggest that they occur in concentrations of 0·06–0·30 μM. In order of magnitude the concentration of adrenaline and noradrenaline in plasma is therefore comparable with that of other hormones.

Note added in proof. Recent experiments have shown that 'plasma adrenaline' and 'plasma noradrenaline' are largely associated with the blood platelets and are liberated under the conditions of blood collection described. It has also been confirmed, with various blood fractions and for both adrenaline and noradrenaline, that the method of Lund (1950) gives, in our hands, results identical with those obtained by the ethylenediamine method within the limits of error.

SUMMARY

1. Methods are described for the extraction and fractionation of adrenaline and noradrenaline from plasma and erythrocytes.

2. It has been shown, using two different solvent systems, that the blood constituents estimated by the fluorimetric method of Weil-Malherbe & Bone (1952, 1953) are concentrated on paper chromatograms in positions corresponding to the R_F values of adrenaline and noradrenaline.

3. The fraction migrating with the R_F value of noradrenaline yields a derivative the fluorescence of which has the same spectral properties as the derivative of noradrenaline. The same correlation exists between adrenaline and the fraction having the R_F value of adrenaline.

4. The quantity of the substances estimated as adrenaline and noradrenaline by the routine procedure was compared with the quantity of the substances recovered in the adrenaline and noradrenaline zones of the paper chromatogram. The proportion accounted for was similar to the recovery of added amines in model experiments. It is concluded that the results are compatible with the identity of the blood constituents estimated with adrenaline and noradrenaline.

5. No increase of adrenaline or noradrenaline was found after the partial hydrolysis of plasma by boiling in 2N-HCl. This result does not support the existence of a protein-bound fraction of adrenaline in plasma.

6. Arteriovenous differences were determined in six human subjects. The arterial concentration of adrenaline was always higher than the venous concentration. The noradrenaline concentration was higher in arterial than in venous plasma in three cases, while the reverse was found in the other three cases.

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REFERENCES

Annersten, S., Grönwall, A. & Köiw, E. (1949). *Scand. J. clin. Lab. Invest.* **1**, 60.
Armin, J. & Grant, R. T. (1953). *J. Physiol.* **121**, 593.
Bayliss, R. I. S. & Steinbeck, A. W. (1953). *The Determination of Adrenocortical Steroids and their Metabolites. Memoirs Soc. Endocrinol.* **2**, 31. London: Dennis Dobson Ltd.
Bliss, E. L., Sandberg, A. A., Nelson, D. H. & Eik-Nes, K. (1953). *J. clin. Invest.* **32**, 818.
Bone, A. D. (1953). *J. Med. Lab. Technol.* **11**, 212.
Bush, I. E. & Sandberg, A. A. (1953). *J. biol. Chem.* **205**, 783.
Crawford, T. B. B. & Outschoorn, A. S. (1951). *Brit. J. Pharmacol.* **6**, 8.
De Mowbray, R. R. & Tickner, A. (1952). *Lancet*, **263**, 511.
Draper, O. J. & Pollard, A. L. (1949). *Science*, **109**, 448.
D'Silva, J. L. (1937). *Biochem. J.* **31**, 2171.
Dunér, H. (1953). *Acta physiol. scand.* **28**, suppl. 102.
Gaddum, J. H. & Schild, H. (1934). *J. Physiol.* **80**, 9 *P*.
Goldenberg, M., Faber, M., Alston, E. J. & Chargaff, E. C. (1949). *Science*, **109**, 534.
Hamberg, U. & Euler, U. S. von. (1950). *Acta chem. scand.* **4**, 1185.
Houssay, A. & Rapela, C. F. (1953). *Arch. exp. Path. Pharmak.* **219**, 156.
James, W. O. & Kilbey, N. (1950). *Nature, Lond.*, **166**, 67.
Kaindl, F. & Euler, U. S. von (1951). *Amer. J. Physiol.* **166**, 284.
Kydd, D. M., Man, E. B. & Peters, J. P. (1950). *J. clin. Invest.* **29**, 1033.
Lehmann, G. & Kinzius, H. (1951). *Pflüg. Arch. ges. Physiol.* **253**, 132.
Lehmann, G. & Michaelis, H. F. (1942). *Arbeitsphysiologie*, **12**, 264.
Lehmann, G. & Michaelis, H. F. (1949). *Arbeitsphysiologie*, **14**, 9.
Lovelock, J. E. (1953). *Biochim. biophys. Acta*, **10**, 414.
Lund, A. (1949). *Acta pharm. tox., Kbh.*, **5**, 231.
Lund, A. (1950). *Acta pharm. tox., Kbh.*, **6**, 137.
Morris, C. J. O. R. & Williams, D. C. (1953). *Biochem. J.* **54**. 470.
Pekkarinen, A. (1948). *Acta physiol. scand.* **16**, suppl. 54.
Taurog, A. & Chaikoff, I. L. (1948). *J. biol. Chem.* **176**, 639.
Vogt, M. (1952). *Brit. J. Pharmacol.* **7**, 325.
Wada, M. & Kanowoka, K. (1935). *Tohoku J. exp. Med.* **27**, 1.
Weil-Malherbe, H. & Bone, A. D. (1952). *Biochem. J.* **51**, 311.
Weil-Malherbe, H. & Bone, A. D. (1953). *Lancet*, **264**, 974.