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A METHOD OF ESTIMATING HISTAMINE IN PLASMA

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The method is intended for the estimation of minute quantities of histamine in blood plasma. A neutralized trichloracetic acid extract of plasma is adsorbed at *p*H 8 on a column prepared by mixing a quantity of the cationic exchange resin Amberlite XE-64 with powdered cellulose as a supporting medium. Histamine is adsorbed but *N*-acetylhistamine is not: the amines can therefore be estimated independently. Elution of the histamine is by displacement with HCl. The eluate is converted into the solution for bioassay on the superfused guinea-pig ileum.

In recovery experiments histamine was added in the range 25 to 100 ng. to solutions of known composition and to plasma (5 ml.) obtained from man and the cat. The mean recovery in this range for histamine added to plasma was 82.5% ±2 S.E. (17 estimations).

The histamine equivalent of human plasma obtained from the antecubital vein was found to be less than 1 ng./ml.

It was possible by this method to follow the concentration of histamine in the arterial plasma of the cat when histamine was infused intravenously at a rate of 330 ng./kg./min. There was no perceptible change in the arterial blood pressure during the infusion, but the plasma histamine rose from <0.3 ng./ml. to 3.4 ng./ml. (mean of 2 estimations).

When the quantity of histamine extractable from plasma is less than can be estimated by conventional methods of bioassay, the sensitivity of the test can be increased by applying the undiluted extract directly to the strip of guinea-pig ileum; this may be suspended in a minute bath (Mongar and Schild, 1950) or in air, as in the technique of superfusion (Gaddum, 1953). The method is applicable if the histamine has been sufficiently purified and if the test solution is similar in composition to the solution bathing the intestine between the doses.

If plasma is extracted by the method of Barsoum and Gaddum (1935) as modified by Code (1937a) and tested in this way, the activity of the extract is only partially abolished by the histamine antagonist mepyramine maleate (Mongar and Whelan, 1953). Moreover, the method does not distinguish between free histamine and histamine which may be released from pharmacologically inactive forms at the stage of refluxing the extract in strong acid (unpublished results).

Various chromatographic methods of purifying histamine have been proposed and are reviewed in the article by Code and McIntire (1956). Some employ paper chromatography, others weak cationic exchangers which are used to remove histamine from solutions containing organic solvents. These methods are designed mainly for the chemical determination of histamine, but some have been combined with bioassay.

In the method to be described the purification is conducted entirely in aqueous solution with the aid of a carboxylic ion exchange resin, at room temperature and at *p*H values not far from neutral. Advantage is taken of the fact that carboxylic resins have a high exchange capacity and can be buffered over a wide range of *p*H. They can therefore be used to separate weak organic bases in a mixture depending on whether or not the bases are ionized at the *p*H of the adsorption. Unionized bases and non-electrolytes remain in the aqueous phase. Since the resins have a high affinity for hydrogen ions, adsorbed cations can be readily eluted with acidic solutions (Kunin and Meyers, 1950; Samuelson, 1952; Bregman, 1953; Partridge, 1954).

The essential steps in the method are: (1) precipitation of the plasma proteins with trichloracetic acid followed by centrifugation, (2) preparation of an aliquot of the supernatant for adsorption on buffered columns of the resin (usually *p*H 8), (3) elution of histamine and other bases by displacement with HCl, and (4) conversion of the eluate proper into the solution for bioassay by

methods that have already been described (Adam, Hardwick, and Spencer, 1954).

The method at first depended on adsorbing the histamine on Amberlite IRC-50 at *p*H 5.9; later it was made more sensitive by performing the adsorption on Amberlite XE-64 at *p*H 8. Some of the results reported in this paper were obtained with IRC-50 at *p*H 5.9; details of this earlier procedure are therefore included in the sections that follow.

Materials and Methods

Resins.—Amberlite IRC-50 (Analytical Grade: B.D.H.) is supplied in the hydrogen form (R–COOH) and consists of moist, adherent beads. It was therefore dried at 50° until the beads ran freely. Amberlite XE-64 (Rohm and Haas Company, Philadelphia, Pa.) is chemically identical with IRC-50 but consists of a fine powder of unspecified particle size. A fraction of the powder containing the larger particles was obtained in the following way. A quantity of resin was suspended in water (30 g./l. in a beaker) and the suspension allowed to stand for 10 min. The particles remaining in suspension were poured off and the sediment was resuspended. This procedure was repeated until the supernatant was more or less clear (6 times). The coarse fraction obtained finally was dried at 50° for 24 hr. (There is now available "Amberlite chromatographic grade resin" CG-50 Type I and II (B.D.H.). Type I (100 to 200 mesh) corresponds fairly closely to the fraction of large particles obtained from XE-64.)

The exchange capacity of the resin for a metallic ion such as sodium is estimated to be 10 m.equiv./g. dry resin (Kunin and Meyers, 1950). This represents a maximum value obtained under conditions of high *p*H and ionic strength, and after prolonged contact with the ambient solution; in practice, it is nearer 9 m.equiv./g. dry resin. According to Bergström and Hansson (1951), 1 ml. IRC-50 (0.7 g. dry resin) adsorbs 190 mg. histamine at *p*H 7.2; the salt used is not mentioned.

Powdered cellulose (Whatman Standard Grade for Chromatography) was used as a supporting medium for columns containing XE-64. These are referred to as composite columns.

Solutions.—"AnalaR" materials were used. Stock buffers used in the method had the following composition: *p*H 7.92, 53 ml. 0.2 M NaH_2PO_4+947 ml. 0.2 M $NaHPO_4$ (calculated to be approximately 0.39 N in terms of Na^+). *p*H 5.9, 840 ml. 0.2 M NaH_2PO_4+160 ml. 0.2 M Na_2HPO_4 (calculated to be approximately 0.23 N in terms of Na^+). The solutions were standardized electrometrically and stored in the cold. Trichloracetic acid (TCA), 6% w/v. Hydrochloric acid, 0.1 N and 0.25 N; prepared from concentrated volumetric solution (B.D.H.). Sodium hydroxide, 0.1 N and 1 N. Neutral Red, 0.01% w/v aqueous solution.

Drugs.—Histamine acid phosphate (B.D.H.); the values quoted in this paper refer to the base on the assumption that this corresponds to 36.16% by weight of the salt.

N-acetylhistamine (4-(β-acetyl-amino-ethyl)-imidazole) m.p. 147° was prepared synthetically. (Tabor and Mosettig, 1949; Dr. C. R. Ricketts, personal communication).

Adrenaline (B.D.H.) and 5-hydroxytryptamine creatinine sulphate [5-HT] (May and Baker); the quantities are expressed as the base.

Heparin *B.P.* (Boots) 103 to 115 international units/mg.

Siliconing of Glass.—Glass pipettes for measuring exact volumes of plasma were freshly coated with silicone DC 1107 obtained from Midland Silicones.

Plastic and Other Materials.—Vessels and centrifuge tubes made of polythene were used throughout in handling the blood and plasma. Open tubes were covered with the non-wettable material "Parafilm" (Marathon Corporation, Menasha, Wis.) to keep out dust and to make mixing possible. Teat pipettes with upturned tips were drawn out from polythene tubing (o.d. 8 mm.) as described by Condon (1954) and were used to separate the plasma.

Preparation of Columns

The resin was first treated with NaOH so as to convert about the right proportion into the sodium salt for the *p*H required; the final adjustment of *p*H was obtained by equilibrating the column with buffer solution. The column was made in a glass tube (6 mm. i.d. × 170 mm.) surmounted by a bulb of 30 ml. capacity and closed by a tap. A small plug of glass wool was carefully packed into the bottom of the tube.

Composite Columns (*pH 8*).—50 mg. of the prepared XE-64 was intimately mixed with 300 mg. powdered cellulose in a 25 ml. stoppered, conical flask. 4 ml. 0.1 N NaOH was added from a burette and the suspension allowed to stand overnight at room temperature (if for longer, it was stored at 5° to lessen the danger of bacterial growth).

Stock buffer *p*H 7.92 (0.39 N Na^+) was diluted to 0.1 N or 0.15 N depending on whether the volume of plasma extracted was 2.5 ml. or 5 ml. The tube was filled with 4 ml. of the diluted buffer. 10 ml. of this buffer was added to the resin in the conical flask and the suspension poured into the bulb. When the column had begun to form, the tap was opened; the flow was finally adjusted to 0.3 ml./min. The column was 40 mm. high and had a volume of just over 1 ml. The total volume from the top of the column to the point of outflow was 1.5 ml. The column did not run dry and solutions were allowed to pass through until the tube above the column was empty. Buffer of the appropriate strength was applied to the column until the *p*H of the effluent reached that of the buffer (to within 0.1 unit); 15 ml. usually sufficed.

IRC-50 Columns (*pH 5.9*).—10 ml. 0.1 N NaOH was added to 325 mg. IRC-50 and the mixture allowed to

stand at room temperature for 24 hr. The resin was transferred to the tube with the aid of a wide-mouthed pipette and allowed to settle in the buffer. The height of the column was 38 mm. The solution above the column was drained off and the flow rate adjusted to 0.3 ml./min. Fresh buffer was then applied to the column until the *p*H of the effluent reached that of the buffer (to within 0.1 unit); 15 ml. usually sufficed.

Preliminary Experiments on Adsorption and Elution

The results on which the method is based are best expressed by curves showing the degrees of adsorption of sodium, histamine, and acetylhistamine over a range of *p*H. Experiments were also carried out to test the effect of sodium ion concentration on the adsorption of histamine and to follow the elution of histamine with HCl.

Sodium.—Columns made with 325 mg. IRC-50 were brought to a definite *p*H by treatment with buffer solution (Fig. 1). They were then washed with water and eluted with enough 0.1 N HCl to displace all the adsorbed Na^+. The difference between the acid concentration and the chloride concentration of the eluate was taken to represent the amount of sodium retained on the column. Similar results were obtained with composite columns containing XE-64 (30 mg.) mixed with cellulose (300 mg.). The curves (Fig. 1) show approximately the quantity of sodium ion which the resin will take up when it is equilibrated with buffer of known strength and *p*H, and consequently the quantity of sodium ion which the resin will release into the eluate when the column is neutralized with an excess of HCl.

Histamine and Acetylhistamine.—The curves shown in Fig. 2 relate % adsorption of 100 μg. histamine and

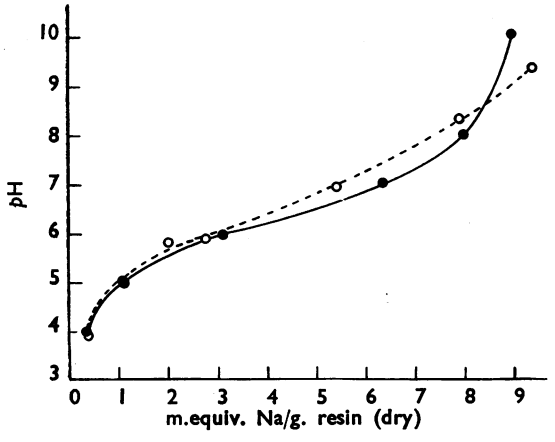

FIG. 1.—Adsorption/*p*H curves for sodium. Adsorption on composite columns containing Amberlite XE-64 (solid circles) and on columns made with Amberlite IRC-50 (open circles). The columns were treated with buffer solutions (0.2 N [Na^+]) until the *p*H of the effluent rose to that of the buffer. The sodium was eluted with HCl and determined as sodium chloride. Buffer solutions used: $CH_3COOH+CH_3COONA$ (*p*H 4 to 5), $NaH_2PO_4+Na_2HPO_4$ (*p*H 5.8 to 8), $H_3BO_3+NaCl+NaOH$ (*p*H 8 to 10), Na_2HPO_4+NaOH (*p*H 10 to 12).

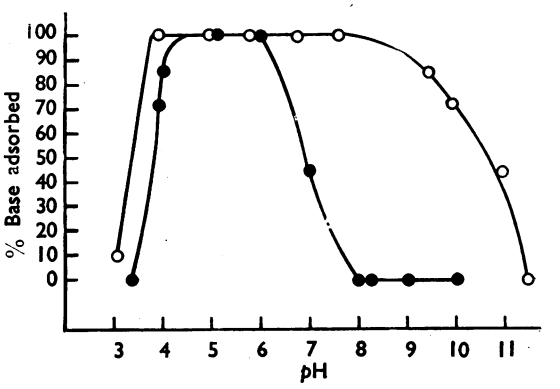

FIG. 2.—Adsorption/*p*H curves for 100 μg. histamine (open circles) and 100 μg. acetylhistamine (solid circles) on columns of Amberlite IRC-50. Buffer solutions used: HCl+potassium hydrogen phthalate (*p*H 3.2), $CH_3COOH+CHCOONa$ (*p*H 4 and 5), $NaH_2PO_4+Na_2HPO_4$ (*p*H 5.8 to 8), $H_3BO_3+NaOH+NaCl$ (*p*H 8 to 10), $NaOH+Na_2HPO_4$ (*p*H 10 to 12). All the solutions containing sodium were 0.2 N in terms Na^+.

of 100 μg. acetylhistamine on buffered columns of IRC-50 (1 g. dry resin: 6 mm. × 60 to 120 mm., depending on *p*H). The results were obtained by measuring the unadsorbed amines colorimetrically (Roberts and Adam, 1950). At *p*H 3.2 only a small fraction of the column is ionized and adsorption of the amines is slight. Histamine is completely adsorbed in the *p*H range 4 to 8; thereafter the adsorption declines as the ionization of the amine is suppressed. At the *p*H corresponding roughly to its $pK\alpha$ (9.7, Levy, 1935) about 80% is adsorbed. Acetylhistamine is completely adsorbed in the *p*H range 4.3 to 6 and not at all at *p*H 8. At this *p*H the two bases are completely separable. This result was confirmed with smaller quantities of the amines which were estimated biologically. The results are presented in Table I. Acetylhistamine was estimated as histamine after hydrolysis with HCl (Roberts and Adam, 1950). The residue obtained after removal of the HCl by evaporation was dissolved in water (10 ml.), buffered (5 ml. stock buffer *p*H 5.9) and applied to a column of IRC-50 (*p*H 5.9); this made it possible to separate hist-

TABLE I

ESTIMATES OF HISTAMINE AND ACETYLHISTAMINE AFTER SEPARATION FROM MIXTURES

Column	Buffer		Histamine			Acetylhistamine		
	*p*H	Normality of Na^+	Added	Eluted	%	Added	Unadsorbed	%
			μg.	μg.		μg.	μg.	
IRC-50 (500 mg.)	7	0·23	5	4·5	90	10	7·6	76
	8	0·23	5	5·2	108	10	8·6	86
	9	0·23	5	4·0	80	10	9·0	90
			ng.	ng.		ng.	ng.	
XE-64 (30 mg.) + Cellulose (300 mg.)	8	0·12	50	40	80	200	144	72
	8	0·12	50	50	100	75	56	75

amine from phosphate ions (for details of method see below). (The variable recoveries for acetylhistamine were partly explained by losses incurred during these procedures. Control experiments showed that a loss of 20 to 50 ng. was likely to occur when 100 ng. histamine was subjected to the hydrolysis only.)

Effect of Sodium Ion Concentration.—10 ml. buffer containing 50 ng. histamine was applied to composite columns made with only 30 mg. XE-64. The columns were subsequently washed with water and eluted with HCl. The sodium ion concentration of the buffer varied from 0.04 to 0.4 N. In the range 0.04 N to 0.2 N, the mean recovery was 97% (range (5) 87 to 110); at 0.3 N the recovery was 60% and at 0.4 N only 40%. It was evident that the composite columns were critically sensitive to salt concentration and that when the volume applied to the column was 10 ml. the sodium ion concentration should not exceed 0.2 N.

Effect of Washing Buffered Columns with Water.—The *p*H of the effluent rose by 0.5 to 1.0 unit presumably owing to the diffusion of Na^+ into the aqueous phase: there was, however, no detectable loss of histamine at this stage.

Elution of Histamine with Acid.—100 ng. histamine was applied to a column of IRC-50 (325 mg.; *p*H 5.9). The column was washed with 5 ml. water and treated with 15 ml. 0.1 N HCl. The eluate was collected in three 5 ml. fractions. The sodium appeared mainly in the first and second fractions and the histamine in the second and third fractions after the *p*H had fallen to below 4. Since the third fraction (*p*H 1.1) contained mostly free acid, it was concluded that elution should be performed with a quantity of acid slightly exceeding the theoretical equivalent of base on the column.

Preparation of Plasma

Collection of Blood.—Human blood was obtained from the antecubital vein of healthy donors and allowed to run freely into a chilled polythene bottle containing 0.9% NaCl (10% by volume) and enough heparin to give a final concentration of about 10 units/ml. of the mixture. Cats were anaesthetized with sodium pentobarbitone (Veterinary Nembutal, Abbott) and given heparin (5 mg./kg. i.v.). Polythene tubing (i.d. 0.30 in. × o.d. 0.48 in.; Intramedic, Clay-Adams, Inc.) was introduced centrally into the femoral or carotid artery, and the blood flowed into graduated centrifuge tubes in ice containing 0.1 volumes of saline and enough heparin to give 5 units/ml. of the mixture.

Centrifugation.—The blood was centrifuged at 900 *g* for 10 min. (4°). The plasma was then separated and centrifuged at 2,200 *g* for 60 min. (4°).

5 ml. human plasma or 2.5 ml. cat plasma was placed in 10 ml. centrifuge tubes for extraction. In recovery experiments histamine or other drugs were added at this stage from a micrometer syringe. The drugs were dissolved in 0.9% NaCl and delivered in volumes of 0.1 to 0.2 ml. Mixing was obtained by inverting the tube several times.

Separation of Histamine

Precipitation of Plasma Proteins.—5 ml. TCA was added to 5 ml. centrifuged plasma. The mixture was well stirred and left at room temperature for 60 min. The precipitate was removed by centrifugation at 2,200 *g* for 30 min. (4°). The volume of supernatant (SN) obtained from different samples of plasma was 7.2 ± 0.2 (25).

Preparation of the Solution for Adsorption.—A 5 ml. aliquot of the SN was transferred to a 10 ml. stoppered, graduated tube. One drop of neutral red solution (0.01% w/v) was added as internal indicator. The free trichloracetic acid was neutralized with N NaOH (0.70 ml. $\pm$ 0.08 (25)), a very pale pink being taken as the end point. A final adjustment was usually made with 0.1 N HCl and 0.1 N NaOH. These solutions were run from microburettes (1 to 5 ml. capacity; Grade A) fitted with polythene adaptors which provided drops of 0.01 to 0.02 ml. The solution for adsorption was brought to *p*H 8 ± 0.05 by the addition of 1 ml. of buffer solution (*p*H 7.92) and the volume was made up to 10 ml. with water. The sodium ion concentration of this solution was calculated to be roughly 0.15 N on the assumption that each of the following contributed in m.equiv. sodium trichloracetate (NaTCA) 0.7, NaCl from the plasma 0.35 and 1 ml. buffer 0.39.

When the volume of plasma extracted was 2.5 ml., the volume of SN was 3.6 ml., of which 3 ml. was taken for the adsorption. The titratable TCA (0.42 m.equiv.) and the estimate of NaCl (0.21 m.equiv.) from the plasma were then proportionately less. The sodium ion concentration of the final solution (10 ml.) was calculated to be roughly 0.1 N.

A control solution containing NaCl and the reagents was prepared for adsorption in parallel with the plasma extract.

Adsorption and Elution.—The solution was applied to a composite column buffered at *p*H 8 which was then washed with 5 ml. water. The total effluent (15 ml.) was collected when it was desired to estimate acetylhistamine. The flow rate did not exceed 0.3 ml./min.

The neutral red indicator was adsorbed at the top of the column, where it appeared as an orange-coloured zone.

2 ml. 0.25 N HCl (0.5 m.equiv.) was carefully pipetted into the tube. The tap was opened and the flow adjusted to 0.2 ml./min. The acid was followed by 1.5 ml. water. The eluate was collected in a 10 ml. stoppered, graduated tube; the total volume was 3.6 ml. and contained 24 mg. Na^+ as NaCl (0.41 m.equiv.). As the *p*H fell, the column became shorter and contributed 0.1 ml. to the eluate.

Note on Stability.—The procedure could be interrupted without loss of histamine at the step preceding the adsorption or after the elution. The solutions were then stored at −17°. Control solutions containing known amounts of histamine (usually 50 ng.), and the solutions from which the histamine standards

were prepared, were also kept in the frozen state. Under these conditions the solutions retained their activity for months.

Reconstitution of the Eluate for Bioassay.—The final volume of the solution was 5 ml. and so provided 8 doses for the assay (using 0.6 ml./dose). The method has already been discussed and will only be summarized here.

1 drop of the neutral red indicator was added to the eluate and the free HCl neutralized with N NaOH (0.09 ml. ± 0.01 (25)), until the pink colour disappeared. The colour was brought back with 0.1 N HCl (0.02 to 0.05 ml.) and a further adjustment made, if necessary, with a trace of 0.1 N NaOH. The additional NaCl formed in back-titrating the NaOH could usually be neglected; if it exceeded 5% of the stoichmetric value (29.2 mg. NaCl), allowance was made in calculating the balance required to make the final solution isotonic. It was normally assumed that the neutralized eluate (3.69 ml.) contained 30 mg. NaCl. The following solutions were added: NaCl (80 g./l.), 0.125 ml. Solution A (which consists of a mixture of equal volumes of the following solutions in g./l.: KCl (8) + $CaCl_2$ (5.5) + $MgCl_2$ (4) + NaH_2PO_4 (0.05), 0.5 ml. $NaHCO_3$ (36.6 g./l.), 0.05 ml., and water to 5.00 ml. The sodium bicarbonate was added at the time when the histamine standards were prepared. The *p*H of the reconstituted solutions was 7.7 ± 0.2 (35) and differed only slightly from that of the Tyrode used (*p*H 7.7). The *p*H values were obtained from freshly made solutions.

Separation of Histamine by Adsorption on IRC-50 Columns

A 5 ml. aliquot of SN obtained after precipitating the proteins in 5 ml. plasma was neutralized with N NaOH (0.7 ml.), and diluted to 12 ml. with buffer *p*H 5.9. The final sodium ion concentration of the solution was calculated to be 0.21 N.

The solution for adsorption was applied to the column (prepared as directed above), which was afterwards washed with 5 ml. water. Elution was performed with 5 ml. 0.25 N HCl (1.25 m.equiv.), followed by 1.5 ml. water. The eluate was collected in a 10 ml. stoppered, graduated tube; the total volume was 6.7 ml. and contained 54 mg. NaCl. Shrinkage of the column contributed 0.2 ml. 1 drop of neutral red indicator was added and the remaining acid was neutralized with N NaOH (0.32 ml. ± 0.07 (25)). The total amount of NaCl was thus 73 mg. in 7.0 ml.; this solution was reconstituted to 10 ml. and assayed.

Assay and Identification of Histamine.—The assay was performed on the superfused guinea-pig ileum in comparison with histamine standards prepared in Tyrode solution containing atropine (100 ng./ml.).

At the end of the assays, Tyrode solution containing mepyramine maleate (2 to 4 ng./ml.) was allowed to flow over the intestine. The assays were repeated to test for active substances other than histamine. The concentration of histamine in the plasma was obtained by multiplying the result of the assay in ng./ml. by a factor depending on the volume of supernatant taken for the adsorption.

Six to eight plasma samples could be conveniently treated at one time. The method, including the assays, required two days.

RESULTS

Recovery Experiments

The method was tested in a series of experiments in which histamine was added to solutions of known composition and to plasma.

Histamine Added to Solutions of Known Composition

Reconstituted Eluates Obtained from Blank Columns.—The effect of solutions from composite columns which had received only buffer or the reagents occasionally exceeded the zero effect of Tyrode solution; but it was always equivalent to less than 0.5 ng./ml. and often to less than 0.25 ng./ml. of the histamine standard. When histamine was added to give a concentration of 1 to 2.5 ng./ml. in the solution, the mean recovery was 101% ± 6 (10). In similar experiments with IRC-50 columns, the effect of the solution was usually greater than the zero effect of Tyrode solution. When histamine was added to give a concentration of 2 ng./ml., the mean recovery was 102% ± 15 (12).

Solutions Applied to Composite Columns (pH 8).—50 ng. histamine was added to solutions of the following composition: 2 ml. stock buffer (*p*H 7.92) + 0.77 ml. N NaCl + N NaTCA (0.5 to 3.0 ml.); the volume was made up to 14 ml. The solutions were applied to columns containing 50 mg. XE-64 which had been previously equilibrated with buffers (*p*H 8) of similar strength. In the range 0.2 N to 0.25 N, the recovery was 87% (2 estimations), at 0.35 N, 80%, and at 0.45 N, 72%. These results confirmed those previously obtained with buffer solutions and showed that the sensitivity of the columns to sodium ion concentration was slightly reduced by increasing the quantity of resin from 30 to 50 mg.

Solutions Applied to IRC-50 Columns (pH 5.9).—When 50 ng. histamine was added to 10 ml. of the stock buffer (*p*H 5.9; 0.23 N [Na^+]), the mean recovery was 89% ± 9 (10). Similarly, when 100 ng. was added to 25 ml. of the buffer the recovery was 95% (range (5) 85 to 100). Recoveries in this range were also obtained when 50 ng. was added to 25 ml. of a solution containing NaTCA (12 ml. 0.23 N NaTCA + 5 ml. stock buffer *p*H 5.9), and to Tyrode solution (5 ml.) diluted with stock buffer (5 ml.). It was apparent from these experiments

that IRC-50 could be used to adsorb histamine from volumes of solution greater than 20 ml., when the sodium ion concentration was approximately 0.2 N.

Potassium ions and the divalent cations present in Tyrode solution would be expected to exchange with sodium ion and to appear in the eluate. The extent to which this occurred was not investigated, since there was no evidence that the recoveries from Tyrode solution differed in any way from those obtained with buffer solution.

Histamine Added to Plasma

The results obtained with composite columns (*p*H 8) are contained in Table II. They are uncorrected for the effect of the plasma control, since

TABLE II

RECOVERY OF HISTAMINE ADDED TO PLASMA

Adsorption on composite column (XE-64) buffered at *p*H 8. Expt. Nos. 1 to 5, 5 ml. human plasma; expt. Nos. 6 and 7, 2.5 ml. cat plasma. Volume of reconstituted eluate 5 ml. The amounts of histamine are given in ng.

Expt. No.	Amount of Histamine Added			
	0	25	50	100
	Amount of Histamine Recovered			
		%	%	
1	<2·5	20 (80)	44 (88)	80
2	<2·5	15 (60)	40 (80)	84
3	<2·5	18 (72)	45 (90)	88
4	<2·5	20 (80)	45 (90)	90
5	3·0	21 (84)	45 (90)	90
6	<3·5	— —	44 (88)	—
7	<1·5	— —	— —	76
Mean		18·8 (75·2)	43·8 (87·6)	84·6

Mean % recovery in the range 25 to 100 ng. 82·5±2·0 S.E.

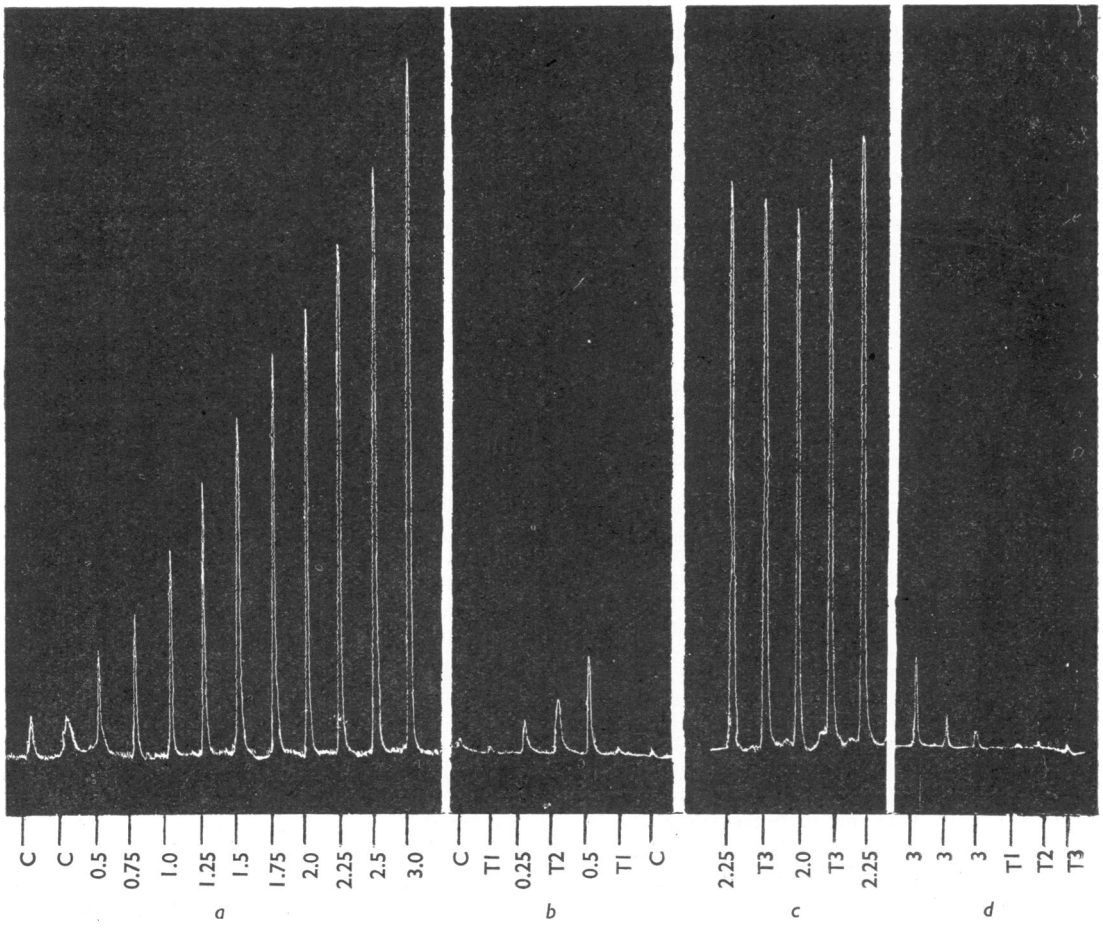

FIG. 3.—Record of assays (expt. 5, Table II). *a*, Zero effect of Tyrode solution (C). The numerals give the doses of histamine standard solutions in ng./ml. Volume of each dose 0.6 ml. *b*, Later in the assay zero effect of Tyrode solution (C) is smaller. T1, reagent control solution (5 ml.). T2, plasma control (5 ml.). *c*, T3, Recovery of histamine (25 ng.) added to plasma (5 ml.). Between *c* and *d* Tyrode solution containing mepyramine maleate (3 ng./ml.) was allowed to flow over the ileum until the effect of doses of the standard solution (3 ng./ml.) was abolished. *d*, Doses of T1, T2, and T3 were reapplied.

in six out of the seven experiments this had less activity than could be measured or was equal to the zero effect of the Tyrode solution or of the reagent control. Tracings obtained in experiment 5 are shown in Fig. 3. In this experiment the histamine equivalent of the control plasma was estimated to be 0.6 ng./ml. (uncorrected for the 10% dilution of the blood sample with saline). The mean recovery of histamine added in the range 25 to 100 ng. was 82.5% ± 2 S.E. Results obtained in earlier experiments by adsorption on IRC-50 columns (*p*H 5.9) are shown in Table III. The mean recovery (81.4%) did not differ significantly

TABLE III

RECOVERY OF HISTAMINE ADDED TO PLASMA

Adsorption on IRC-50 columns buffered at *p*H 5·9. Expt. Nos. 1 to 5, 5 ml. human plasma; expt. Nos. 6 and 7, 5 ml. cat plasma. Volume of reconstituted eluate 10 ml. The values for histamine are in ng.

Expt.	Amount of Histamine Added		
	0	50	
	Amount of Histamine Recovered		%
1	< 5	39	(78)
2	< 5	40	(80)
3	< 5	40	(80)
4	< 5	44	(80)
5	< 5	45	(90)
6	< 5	40	(80)
7	<10	41	(82)
	Mean	40·7	81·4±1·7 S.E.

($P>0.05$) from the mean (87.6%) obtained with the same quantity of histamine applied to composite columns.

Histamine was also added in a concentration of 1 to 3 ng./ml. to reconstituted eluates of control plasma samples. The mean recovery was 98% ± 8 (15). The result shows that the final solution appeared to be free from substances in plasma which might interfere with the assay. This conclusion was strengthened by the results of experiments in which either adrenaline (50 ng.) or 5-HT (50 ng.) was added to plasma which was extracted by the final method. The reconstituted eluates (5 ml.) were devoid of activity on the guinea-pig ileum. When histamine was added to them in a concentration of 2 ng./ml., the recoveries fell within the same range. If the eluate had contained half of the adrenaline added to the plasma, the concentration (5 ng./ml.) would have reduced the response to histamine (2 ng./ml.) by about 10%. Similarly, if it had contained half of the 5-HT (5 ng./ml.), the response would have been increased by about 10%, and the effect of the 5-HT been detectable in the presence of mepyramine (4 ng./ml.).

Application of the Method.—The results of experiments in which histamine was administered by slow intravenous infusion to anaesthetized cats are shown in Table IV. In one experiment IRC-50 columns (*p*H 5.9) were used for the adsorption; in the other, composite columns buffered at *p*H 8 which made it possible to employ smaller volumes (2.5 ml.) of plasma without loss of sensitivity. Infusion at the rate of 330 ng./kg./min. produced no obvious fall in the arterial blood pressure, yet the plasma histamine rose to a measurable concentration in samples of arterial blood. The sample taken 10 min. after the infusion of histamine had stopped showed that the concentration of extractable histamine had fallen to the control value. The total dose infused in each experiment was 20 μg. in 20 min.

TABLE IV

ESTIMATION OF PLASMA HISTAMINE AFTER INTRAVENOUS INFUSION OF HISTAMINE IN THE CAT

Pentobarbitone sodium anaesthesia. Heparin 5 mg./kg.

Time (min.)	Infusion	Blood Sample (Femoral Artery) (ml.)	Haematocrit	Vol. Plasma Extracted (ml.)	Plasma Histamine (ng./ml.)
Experiment 1. Male Wt. 3.0 kg. Adsorption on composite column (*p*H 8)					
−5 to 0	0·9% NaCl	5	44	2·5	<0·3
0 to +15	Histamine 330 ng./kg./min. in 0·9% NaCl	5	43	2·5	3·1
+15 to +20	,,	5	42	2·5	3·7
+20 to +30	0·9% NaCl	5	42	2·5	<0·3
Experiment 2. Male Wt. 3·2 kg. Adsorption on IRC-50 column (*p*H 5·9)					
		(Carotid Artery)			
−5 to 0	0·9% NaCl	10	—	4·0	1·6
0 to +15	Histamine 330 ng./kg./min. in 0·9% NaCl	10	—	4·5	5·6
+15 to +20	,,	10	—	4·0	5·5
+20 to +30	0·9% NaCl	10	—	4·0	<1·3

DISCUSSION

The method makes it possible to purify TCA extracts of plasma so that the bioassay can be performed by placing the final solution in direct contact with the suspended strip of guinea-pig ileum. It involves the assumption that the histamine extracted by the TCA is evenly distributed between the supernatant and the precipitate. The results of recovery experiments support this assumption. The purification achieved at the adsorption step can only be partial by chemical standards; nevertheless it appears to be sufficient for the method of bioassay used. The reconstitution

procedure makes use of the sodium ion displaced from the resin, and losses which would occur by evaporating the eluate and extracting the histamine with ethanol or in other ways are thereby avoided.

The purification depends as much on the initial treatment of the blood and plasma as on the subsequent procedures. Early in this work an attempt was made to adsorb histamine and acetylhistamine cations directly from plasma which had been diluted with buffer (*p*H 5.9). Evidence was obtained that the amines could be removed quantitatively in this way, but the results were complicated by the formation of a slow contracting substance which appeared in the acid eluate (Gaddum, 1956). A method was devised for the separation of histamine from this unidentified substance (X), but it proved to be too laborious for the routine estimation of histamine. It was therefore decided to estimate instead the extractable histamine after precipitation of the proteins with TCA. Substance X, which shares some of the properties of bradykinin (Rocha e Silva, Beraldo, and Rosenfeld, 1949), may have appeared because of contact between plasma and glass (Armstrong, Jepson, Keele, and Stewart, 1954) or resin, or because the plasma was diluted with buffer (Schachter, 1956). Careful treatment of the blood and plasma by methods similar to those employed by Armstrong *et al.* (1954) probably delayed the formation of substance X, so that when the plasma proteins were precipitated two to three hours after obtaining the blood samples, none was detectable in the solution for assay.

The blood was centrifuged at a low value of *g* to separate histamine contained in the cells. The plasma was centrifuged to remove 5-HT and histamine contained in the platelets. The concentration of platelets remaining in the centrifuged plasma was apparently not high enough to influence the result through the release of 5-HT or histamine or an unidentified plain-muscle stimulating substance (Humphrey and Jaques, 1954).

The quantity of resin chosen for the adsorption was limited, on the one hand, by the minimum needed for the complete removal of histamine from the solution, and, on the other, by the exchange capacity of the resin at the *p*H of the adsorption. The final volume of the test solution depended on the quantity of sodium released from the resin when this was treated with acid. For example, the exchange capacity of 325 mg. IRC-50 at *p*H 5.9 is approximately 0.9 m.equiv. Na^+ (or 54 mg. Na^+ as NaCl); the eluate must then be reconstituted to 10 ml. At *p*H 8, the capacity of the resin increases by nearly 3-fold and the final solution could not be less than 20 ml. It was possible to reduce the sodium, and hence the volume of the solution, by using the finely divided form of the resin. At *p*H 8.0, 50 mg. of the resin (XE-64) has a capacity of 0.4 m.equiv. Na^+ (or 24 mg. Na^+ as NaCl) which leaves a margin of 16 mg. NaCl for the final neutralization of the eluate and its reconstitution to 5 ml. The powdered cellulose mixed with the resin adds only slightly to the exchange capacity. However, the adsorption of histamine on composite columns may be incomplete if the solution for adsorption contains too much sodium ion; it is important that the quantity of Na^+ as NaTCA in the solution (10 ml.) should not exceed 1 m.equiv. (185 mg.). This limits the volume of plasma that can be extracted by the method as described to 5 ml. or less.

Adsorption at *p*H 8 makes it possible to estimate acetylhistamine independently; it may also provide better purification of the histamine than could be obtained at a lower *p*H, since pharmacologically active bases that are weaker than histamine would be less strongly adsorbed. Adrenaline ($pK\alpha$ 10.7 [cation]; Albert, 1954) and 5-HT ($pK\alpha$ 9.1; Rapport, Green, and Page, 1948) are probably adsorbed at *p*H 8, but they are unstable in an alkaline medium and so lose pharmacological activity. Thus at *p*H 8, 50 ng. of adrenaline in pure solution may be expected to become inactive in less than 30 min. (Dr. T. B. B. Crawford, personal communication) and 50 ng. 5-HT to lose more than 20% of its activity in the same time (Rapport *et al.*, 1948).

IRC-50 columns buffered at *p*H 5.9 have the disadvantage that the volume of the reconstituted eluate is large (10 ml.). When small volumes of plasma are extracted (2.5 ml.), the histamine contained in the aliquot of the SN (3 ml.) is diluted 3.3-fold and may not be detectable at the assay. Further, adrenaline and 5-HT are stable at *p*H 5.9 and will therefore be adsorbed and eluted. Nevertheless, the recoveries at this *p*H indicate that these bases were not present in the plasma in sufficient quantity to interfere with the recovery of histamine when the assay was performed on the superfused guinea-pig ileum.

The quantity of histamine extractable from plasma obtained from man and the cat was in all instances less than 1 ng./ml. This value is lower than most of the published results (Code, 1952). Thus Emmelin (1945) estimated histamine in ultrafiltrates and in TCA extracts (Code, 1937a) of plasma obtained from the cat and found values in the range 25 to 50 ng./ml. Born and Vane (1952) estimated the freely diffusible histamine in cat blood and obtained similar results (9 to 55 ng./ml.).

The low values for human plasma agree with those previously obtained by a different method (Adam, Card, Riddell, Roberts, and Strong, 1954) and with those reported by Lowry, Graham, Harris, Priebat, Marks, and Bregman (1954) and by Code (1937b). Lowry *et al.* (1954) determined the concentration of histamine in human plasma by a sensitive chemical method and found it to be less than 3 ng./ml. Code (1937b) found that when he protected the blood against trauma and did not use anticoagulants, the plasma histamine in man was less than could be detected by his method of assay (probably 20 ng./ml. plasma). High values for the plasma histamine may be due to damage of the blood or to the liberation of histamine from pharmacologically inactive forms. It is uncertain whether or not human plasma contains detectable quantities of acetylhistamine. This would in any case not be hydrolysed by the TCA used in the extraction (Dr. V. R. Pickles, personal communication). However, when heparin is used as an anticoagulant and the TCA extract is boiled in strong HCl, there is evidence to suggest that some of the histamine activity derives from the heparin (unpublished results). Impurities in the TCA extract (Code, 1937a) may also contribute to the activity of the test solution (Mongar and Whelan, 1953).

The results of the infusion experiments suggest that the present method might be as sensitive as that of the gastric secretion for detecting histamine in the plasma of anaesthetized cats. According to Emmelin, Kahlson, and Wicksell (1941) and Wood (1948), the rate of infusion for a submaximal effect on the gastric secretion varied from 700 to 1,500 ng./kg./min., which is still 10 to 20 times higher than the rate required for a submaximal effect on the gastric secretion in the conscious dog (Hanson, Grossman, and Ivy, 1948) or man (Adam, Card, Riddell, Roberts, Strong, and Woolf, 1954).

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