THE PURIFICATION OF HISTAMINE FOR BIOASSAY*

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The purification of histamine for assay purposes has been the subject of reports by several investigators (1–9). The available methods are not readily applicable to large numbers of samples, especially when there is less than 2 γ of histamine per sample. The ultrafiltration method of Emmelin probably is the least objectionable from the standpoint of time consumed and equipment required, but this method has yet to be tested thoroughly, and one should suspect that its selectivity for histamine may be inadequate in some cases.

The purpose of this publication is to present a new method for the purification of histamine which is very suitable for application to large numbers of samples and which promises a high degree of selectivity. The essential features of this method are as follows: An aqueous, histamine-containing extract from tissue is extracted with *n*-butanol under conditions such that nearly all the histamine is removed from the aqueous phase in one extraction. The histamine is recovered from the butanol by means of a new cation exchange medium, cotton acid succinate. The histamine is eluted from the cotton acid succinate with a small volume of dilute hydrochloric acid and the eluate is neutralized with sodium hydroxide to give an isotonic solution suitable for bioassay.

Effect of pH and Salt Concentration on Distribution of Histamine between Water and Butanol—The first step in the development of this histamine purification procedure was to find a simple extraction procedure by which histamine could be separated from most of the other constituents of an aqueous extract of biological origin. We found that the distribution of histamine between water and butanol at a high pH could be shifted in favor of the butanol by the addition of a water-soluble salt to the system. Experiments were then set up to determine quantitatively the effect of both pH and salt concentration. Of several salts which were investigated, sodium sulfate was finally chosen because of its high water solubility, etc.

The data of Fig. 1 show the distribution of histamine between butanol and water (*a*) as related to sodium sulfate concentration in the aqueous phase at an optimum pH, and (*b*) as related to pH at an optimum or near

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optimum salt concentration. These data show the per cent of histamine in the butanol phase at equilibrium when the water and butanol phases are at equal volumes. The data are expressed in this manner rather than in terms of distribution coefficients in order to show more clearly the plateaus in the distribution curves. The per cent of histamine which goes into the butanol phase changes very slightly with salt concentration above about 22 per cent or with pH above 12.5.

In the routine procedure, described later, a mixture of sodium sulfate and trisodium phosphate is employed to yield an aqueous solution of about 22.5 per cent (weight to volume) sodium sulfate and about 3 per cent (0.17 M) trisodium phosphate, with a pH between 12.5 and 13. The salt mixture dissolves readily and produces optimum conditions for the extraction.

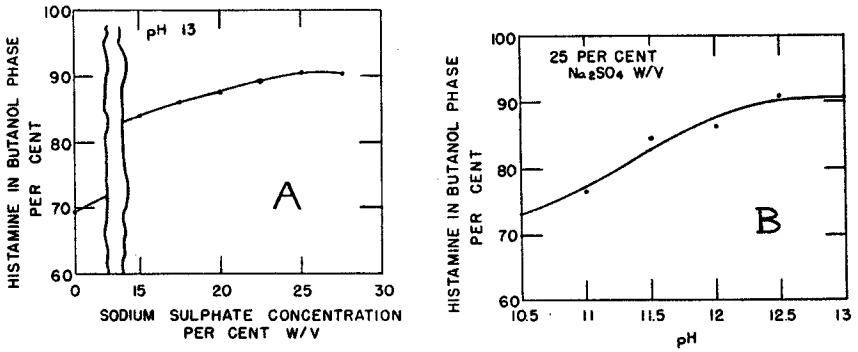

FIG. 1. The effect of salt concentration and pH on the distribution of histamine between water and butanol.

The distribution of histamine between water and *n*-butanol, isobutanol, tertiary butanol, *n*-amyl alcohol, and isoamyl alcohol was determined at pH 13 and 25 per cent sodium sulfate concentration. *n*-Butanol proved to be the alcohol of choice.

Recovery of Histamine from Butanol Solution by Means of Cotton Acid Succinate—Following the development of conditions for the extraction of histamine from aqueous solution by means of butanol, a simple quantitative method of recovering histamine from the butanol was desired. Such a procedure should yield, with a minimum of manipulations, an aqueous solution of physiological salt concentration which is free of organic solvents and in which the histamine is sufficiently concentrated for bioassay. Perhaps the simplest procedure to satisfy these requirements would be the removal of histamine from the butanol by means of a cation exchange medium, or an adsorbent, from which the histamine could be eluted easily with a small volume of an aqueous solution. The available cation exchange

media and histamine adsorbents were investigated. Several materials of both classes would remove histamine quantitatively from butanol, but the recovery of histamine was very unsatisfactory in all cases.

Finally, cotton acid succinate (hereinafter abbreviated as CS) was developed especially for this procedure. This material has the gross mechanical properties of cotton. It is produced by esterifying with succinic anhydride only a fraction of the free hydroxyls of cotton. For every hydroxyl group esterified, there is a free carboxyl which can function in cation exchange reactions. When a butanol solution of histamine is filtered through a small pad of CS, the histamine is quantitatively removed from the butanol. The CS can, without loss of the histamine, be washed with water until free of alcohol. The histamine can then be quantitatively

TABLE I

Recovery of Histamine from Water and Butanol Solutions by Means of Cotton Acid Succinate (CS)

Solvent	Original histamine	
	In CS filtrate	In CS eluate
	per cent	*per cent*
Water*	0.6	102
	0.3	104
Butanol†	1.2	97
	1.4	97

* 5 cc. of aqueous histamine, 80 γ per cc., 0.014 M Na_3PO_4.

† 5 cc. of butanol, 67 γ of histamine per cc.

eluted from the CS with a small volume of dilute hydrochloric acid. The concentration of hydrochloric acid may be chosen so that neutralization with sodium hydroxide will yield an aqueous isotonic solution which may be assayed biologically for histamine.

The data of Table I illustrate the recovery of histamine from water or butanol solutions by means of CS. The solutions were filtered through 100 mg. CS pads. The pads were washed well and then eluted with 1 cc. of 0.4 N hydrochloric acid, followed by 1 cc. of water. In these experiments, a chemical determination of histamine was employed. The data show that the recovery of histamine by cation exchange on CS is quantitative, within the accuracy of the histamine determination.

Detailed Procedure for Purification of Histamine in Blood Plasma—Thus far this procedure for the purification of histamine has been applied only to free histamine in animal blood plasmas. The details of the procedure are as follows:

1. In glass-stoppered test-tubes place 2 cc. of plasma; dilute to 4.5 cc.

with saline and add 1.2 gm. of a salt mixture containing 6.25 gm. of anhydrous sodium sulfate to 1 gm. of trisodium phosphate monohydrate. Stopper the tubes and shake thoroughly.

2. To each test-tube add 5 cc. of butanol; shake vigorously for a few seconds; let stand a few minutes and then shake briefly to break the protein gel. Shake vigorously in a mechanical shaker for ½ hour and then centrifuge at about 2000 R.P.M. for 15 to 20 minutes in a No. 1-SB International centrifuge with a No. 240 head.

3. Transfer 4.6 cc. of butanol solution from each test-tube to a CS tube (Fig. 2, *B*, and Fig. 2, *C*) which contains 100 mg. of CS packed tightly in

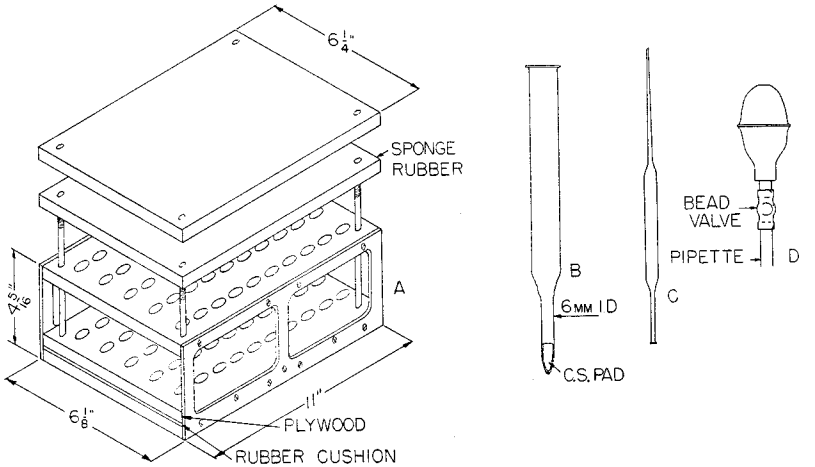

FIG. 2. *A*, special holder for shaking glass-stoppered test-tubes (the bottom, center, and top plates are made of ½ inch aluminum plate; the sides are of 3/16 inch aluminum plate); *B*, CS tube with 100 mg. CS pad; *C*, glass weight which rests on CS pad to keep it firmly packed; *D*, pipetting bulb arrangement.

the 6 mm. tube at the bottom. On account of the protein pad which is packed between the two liquid phases during centrifugation, the complete transfer of the butanol phase to the CS tube would be difficult, and 4.6 cc. of butanol are nearly all that can be taken conveniently and consistently. The total volume of the butanol phase is 5.54 cc.; therefore only 83 per cent of the histamine which goes into the butanol phase is transferred to the CS tube. The transfer of exactly 4.6 cc. of butanol is most conveniently accomplished by means of a serological pipette (calibrated to the tip), to which is fitted a rubber bulb with a bead valve (Fig. 2, *D*). The bulb is attached to the pipette; the bead valve is opened and the bulb is exhausted. The bulb serves as a "vacuum reservoir" which will draw the liquid up into the pipette. The amount of liquid drawn up is easily regulated by the bead valve.

4. After the butanol has passed through the CS pad, wash the CS tube and pad with 3 cc. of 95 per cent ethyl alcohol, followed by 3 cc. of water. By means of a stirring rod, remove the last drop of water from the tip of the CS tube. Place a small test-tube or vial graduated to 2.5 cc. under the tip of the CS tube and elute the histamine with 1 cc. of 0.4 N hydrochloric acid, followed by 1 cc. of water. Neutralize the eluate with 0.5 cc. of 0.8 N sodium hydroxide and adjust the volume to 2.5 cc. if necessary. This solution may be assayed biologically for histamine without further manipulation.

For the purification of large numbers of samples, the following time-saving devices may be employed:

In Step 1, a small metal cup may be used to measure the 1.2 gm. aliquots of salt mixture. Slight deviations from the specified quantity of salt mixture are not of significant consequence.

For Step 2, a 5 cc. Machlett automatic pipette was used in measuring out the butanol. A special rack was built (Fig. 2, *A*) in which 50 glass-stoppered test-tubes could be clamped securely during the mechanical shaking. This rack was clamped firmly in a precision equipoise heavy duty shaker, so that the tubes were lying lengthwise with the stroke of the shaker. The glass-stoppered test-tubes were of the following specifications: Pyrex, 16 to 16.5 mm. outer diameter, 126 mm. over-all length, 104 mm. length below the joint, standard taper 13/14 flat stoppers with the stopper head 18 mm. in diameter. With tubes so designed, No. 356 metal cups with No. 355 three place trunnion carriers may be used in the centrifugation (Step 2). It has been found advisable to coat the test-tube stoppers with a thin film of vaseline before each experiment.

For Steps 3 and 4, 50 CS tubes have been used simultaneously. The CS tubes may be used almost indefinitely without changing the CS pads if suitable precautions are taken. The CS pad must not be treated with an excess of strong bases in aqueous solution; it should be washed thoroughly with water and then with 95 per cent alcohol immediately after each experiment.

With the equipment specified, one technician can purify the histamine from 50 plasma samples in 3 to $3\frac{1}{2}$ hours.

Distribution studies in which histamine was determined chemically indicate that 93.3 per cent (average value) of the total histamine goes into the butanol phase in Step 2. Of this, 83 per cent is transferred to the CS pad in Step 3. With a 100 per cent recovery of histamine from the butanol by means of CS, the over-all yield of this procedure should be 77.4 per cent of the original total histamine. This figure can be increased to 95 per cent by the use of two butanol extractions instead of one (repeating Steps 2 and 3).

Table II shows the recovery, with one butanol extraction, of a known

quantity of pure histamine which had been added to rabbit, guinea pig, and dog blood plasmas. For these data the cat blood pressure assay for histamine was employed. This assay was performed on cats anesthetized with a phenopentobarbital mixture. Samples and histamine standards were administered via the femoral vein with the blood pressure recorded graphically from the carotid artery. Standardization was achieved by the injection of histamine in doses of 0.1 to 0.3 γ until reproducible blood pressure drops were obtained. The fall in blood pressure produced by each sample was compared with histamine standard doses interspersed

TABLE II

Recovery of Histamine from Animal Blood Plasmas

Plasma	Histamine added	Histamine recovered		Per cent recovery
		Calculated	Found	
	γ	γ	γ	
Rabbit	0		0.15	
	0		0.15	
	8	6.2	6.4	101
	8	6.2	6.4	101
	8	6.2	6.7	106
Guinea pig	0		0.32	
	0		0.25	
	8	6.2	6.4	98
	8	6.2	6.55	101
	8	6.2	6.70	103
Dog	0		0.0	
	0		0.0	
	8	6.2	6.4	103
	8	6.2	6.8	110
	8	6.2	6.65	107

throughout the experiment, and intermediate values were interpolated. The accuracy of this method averages within ±10 per cent.

The data of Table II suggest that the above purification procedure is highly selective for histamine. There was no histamine activity extracted from the heparinized dog plasma "blanks." The small amount of histamine extracted from the rabbit plasma "blanks" represents only 5 to 8 per cent of the total histamine found in rabbit blood (2). On several occasions we have found, by means of the isolated guinea pig intestinal strip, that the histamine activity extracted from heparinized rabbit plasma could be nullified by small quantities of antihistamine drugs. The amount of histamine extracted from guinea pig plasma is approximately equal to the total histamine of guinea pig blood (2). Since in this case some hemolysis had occurred before the blood cells were separated from the

plasma, it might be expected that the plasma would contain nearly all of the blood histamine.

This procedure can be made even more selective for histamine by extraction of the plasma first with ether and then with butanol. The ether extract would contain no histamine and *should not be filtered through the CS pad.* Many drugs (amines), which might interfere with the histamine assay, are extracted from water by ether under these conditions and may thus be separated from histamine.

Materials and Methods

The butanol, anhydrous Na_2SO_4, and $Na_3PO_4 \cdot H_2O$ were of reagent grade. The $Na_3PO_4 \cdot H_2O$ was obtained by drying reagent grade $Na_3PO_4 \cdot 12H_2O$ at 100° for 48 hours, then at 300° for 2 to 3 hours. The salts were finely powdered and mixed in a ratio of 6.25 gm. of Na_2SO_4 to 1 gm. of $Na_3PO_4 \cdot H_2O$. In the study of histamine distribution between water and butanol as related to pH, phosphate buffers were used to obtain solutions of pH 10.5, 11, 11.5, and 12 (10). For pH 12.5 unbuffered solutions were made to 0.03 N with sodium hydroxide; for pII 13 0.1 N sodium hydroxide solutions were used.

Cotton acid succinate was prepared as follows: Fused sodium acetate (5 gm.) and succinic anhydride (40 gm.) were dissolved in 300 cc. of glacial acetic acid. Cotton (10 gm.) was immersed in this solution; a drying tube was attached to the flask and the temperature was maintained at 100° for 48 hours. The partially esterified cotton was filtered off, washed well with water, dilute hydrochloric acid, water, and finally with alcohol. The product was dried in a vacuum oven.

The chemical method used for histamine determination was a modification[1] of the method published by Baraud (11).

SUMMARY

A histamine purification procedure has been given in detail. By this procedure free histamine may be purified for bioassay quickly and in good yield. The method is particularly designed for the purification of a large number of samples at one time.

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