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NEW METHODS FOR THE QUANTITATIVE ESTIMATION OF FREE AND CONJUGATED HISTAMINE IN BODY FLUIDS

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Chromatographic purification, combined with very sensitive biological methods of assay, can greatly facilitate the quantitative estimation of physiologically active compounds occurring at extremely low concentrations in body fluids. New techniques of this kind appear to be essential before further progress can be made in the investigation of any role histamine might play in normal human physiology. Free, pharmacologically active histamine has not yet been reliably estimated in human plasma. Human urine has been shown to contain fair quantities of histamine, but almost entirely in a conjugated form (Anrep, Ayadi, Barsoum, Smith, and Talaat, 1944). Urbach's work (1949) suggests that urinary conjugated histamine is acetylhistamine [4(β-acetylaminoethyl)imidazole], and Tabor and Mosettig (1949) were able to isolate this compound from the urine of dogs fed with histamine and to identify it chemically. Evidence for the normal appearance of free histamine in human urine is not so clear-cut. Ackermann and Fuchs (1939) prepared from a single sample of 10 litres of urine, by adsorption on Lloyd's Reagent and purification through the phosphotungstate, a quantity of histamine the bioassay of which implied a concentration of 11.7 μg./litre of free base in the original urine. But Anrep *et al.* (1944), using a charcoal-adsorption method, were able to detect no more than traces of free histamine in normal human urine, a result confirmed later by Adam (1950).

In attempting to work out simpler and more sensitive methods, we have examined a number of chromatographic materials for their capacity to adsorb histamine and acetylhistamine. Adsorbents able to take up these substances directly from body fluids were specially looked for. Such materials would make easier the concentration of these compounds (free histamine in particular) from relatively large volumes of fluid. An adsorbent which could separate histamine and acetylhistamine quantitatively would be extremely valuable, and would make possible the independent determination of these substances in fluids where they occur together. As the quantities expected are minute, sensitive and relatively specific biological methods of assay have been used in preference to chemical methods.

The adsorption of histamine and its separation from acetylhistamine

Permutit-like materials have been tried out for histamine by Whitehorn (1923), Schwartz and Riegert (1936), and Code and Ing (1937). Such adsorbents act by cation-exchange, and are capable of taking up the ions of organic bases from very

dilute solutions. After investigating a number of commercially available cation-exchangers, we decided to use columns of a synthetic zeolite known as Decalso* for the adsorption of free histamine from body fluids, and its subsequent elution in a state pure enough for pharmacological assay. Acetylhistamine and urinary conjugated histamine are not taken up by Decalso from solutions on the alkaline side of neutrality, and their separation from free histamine is quantitative over the *p*H range 8 to 10.

The most difficult problem encountered in using Decalso for the determination of free histamine was to find a reasonably efficient way of eluting the adsorbed base. Strong salt solutions (e.g., saturated sodium or potassium chlorides) can be used, but are not suitable in pharmacological work. A better device is to treat the Decalso with a high concentration of a displacing base, and to elute the liberated histamine in an organic solvent. Strong ammonia is convenient, because it can be removed readily by evaporation. Various organic solvents were tried; the best were those immiscible or only partially miscible with water, because they provided an eluate free of inorganic ions; *n*-butanol and chloroform are probably equally efficient, but chloroform is the more convenient owing to its lower boiling-point, and was chosen for the final method (see p. 529).

The fundamental steps of the Decalso method, therefore, are adsorption of the histamine, and elution of the base with strong ammonium hydroxide followed by chloroform. Certain small variations have occurred in the method during the course of the work, but there has been no evidence that these have made any important difference in the efficiency of the elution. The final technique described later (p. 529) was adopted largely for reasons of maximum simplicity.

The Decalso method for free histamine can be applied to urine, and, with slight modification, to blood plasma and gastric juice. The technique has been tested by recovering known amounts of histamine added to these fluids, over the range of concentration 10 to 250 μg./litre. Recoveries were not, on the average, better than 67 per cent, but this disadvantage is outweighed by the applicability of the method to fluids having a free histamine concentration well below that measurable by existing techniques; this is due to the concentrating effect of passing large volumes of fluid through the Decalso column, and taking up the extract in much smaller volumes for assay.

The adsorption of histamine conjugates

The method of Anrep *et al.* (1944) depends on the adsorption of total histamine, free and conjugated, by shaking the urine sample with activated charcoal. Elution is carried out with ethyl alcohol containing hydrochloric acid. The difference in the activity of the hydrolysed and unhydrolysed portions of the eluate represents the amount of conjugate present in the sample. Adam (1950) used a simplified form of the method to follow the excretion of administered histamine in human urine.

The method to be described here is still based on the use of activated charcoal, but has undergone two important modifications. First, the conjugate is separated from the free histamine on columns of Decalso and determined independently. Secondly, the conjugate is adsorbed on columns of activated charcoal mixed with fine washed sand to aid filtration. The method has been tested by carrying out

* Decalso, Grade F, was obtained from the Permutit Co., Ltd., London, W.4.

recovery experiments with acetylhistamine added to urine. Recoveries were good, and comparable results were obtained by adding to the urine a sample of urinary conjugated histamine which had been extracted in a partly purified state from a urine containing large amounts of this substance. Two experiments were done in which histamine and acetylhistamine were added to the same urine, and recoveries of each evaluated by a combination of the Decalso and the charcoal-sand techniques.

Application of the new methods

These methods have been applied to the independent estimation of the 24-hour excretions of free and conjugated histamine in the urines of five healthy men over a period of six days. As free histamine has appeared in measurable amounts in every urine so far examined, and seems to be continuously excreted, attempts were made to confirm its authenticity. Evidence was obtained by the use of the specific antagonist mepyramine maleate (Reuse, 1948), and by parallel assays on the guinea-pig ileum and the cat blood pressure. The stability of the active substance to acid, alkali, and diamine oxidase was also investigated.

EXPERIMENTAL

Adsorption-pH curves for free and conjugated histamine on Decalso

The data on which the methods are based are best expressed by curves showing the degree of adsorption of the substances over a range of *p*H. Fig. 1 relates the percentage adsorption of 100 μg. histamine, and of 200 μg. synthetic acetylhistamine (m.p. 147° C.), to *p*H over the range 5 to 13 on columns of Decalso 8 cm. high and 1 cm. in diameter (for preparation, see p. 529). For *p*H 5 to 10, buffer solutions

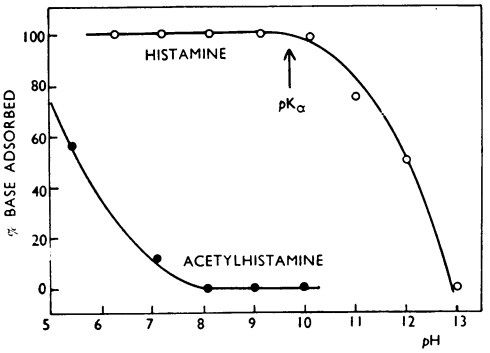

FIG. 1.—Adsorption-*p*H curves for 100 μg. histamine (open circles), and 200 μg. acetylhistamine (blackened circles), on columns of Decalso. Buffer solutions used: NaOH+potassium hydrogen phthalate (*p*H 5); NaOH+NaH_2PO_4 (*p*H 6–7); NaOH+H_3BO_3+NaCl (*p*H 8–10). NaOH solutions, containing about 1% NaCl, were used for *p*H 11–13.

were made up and standardized electrometrically. For *p*H 11 to 13, sodium hydroxide solutions were used (0.001 to 0.1 N), containing sodium chloride to bring the ionic strength very approximately up to that of the buffer solutions. The Decalso columns were first washed with 15 ml. of buffer or sodium hydroxide; 10 ml. of buffer containing the histamine or acetylhistamine were then added to the column, and collection of the eluate started immediately. Further washing with solution of the same *p*H was continued till 25 ml. of eluate had come through. The *p*H of the eluate was again checked, but very little shift had occurred, except with acid buffers. Histamine in the eluate was assayed biologically; acetylhistamine colorimetrically by McPherson's modification (1946) of the Pauly Reaction.

Bioassays showed that histamine below the *p*H corresponding roughly to its pK_a value is more than 99.8 per cent adsorbed on Decalso columns (pK_b of the NH_2-group $= \sim 4.3$. $pK_a = 14 - pK_b = \sim 9.7$. Levy, 1935). Above a *p*H of 9.7, where the ionization of histamine is suppressed, the adsorption falls off, and does not occur at all at *p*H 13. There is evidence of slight adsorption of the weaker base acetylhistamine ($pK_a = \sim 6$) in the acid range of *p*H, but the degree of adsorption is very sensitive to slight differences of salt concentration. Over the *p*H range 8 to 10, acetylhistamine is not adsorbed, however low the salt concentration. A quantitative separation of histamine and acetylhistamine can be carried out on Decalso columns between these *p*H limits. Similar results are obtained with urinary conjugated histamine, and complete separation of the histamine and conjugated histamine in urine can be effected by passing the urine at *p*H 8 through Decalso.

Adsorption-pH curves for acetylhistamine on charcoal

Fig. 2 shows a curve relating percentage adsorption of acetylhistamine to *p*H over the range 2 to 10 on charcoal. Charcoal-sand columns 8 cm. by 1.2 cm. (for preparation, see p. 534) were washed with 10 ml. buffer solution, and 400 μg. acetylhistamine in 10 ml. buffer added. Collection of the eluate was begun at once, and the columns washed with more buffer till 25 ml. eluate had percolated through.

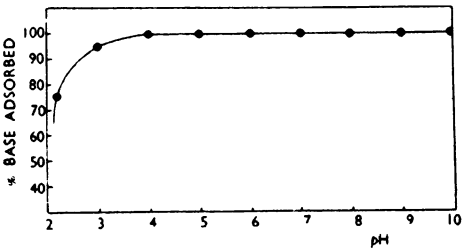

FIG. 2.—Adsorption-*p*H curve for 400 μg. acetylhistamine on charcoal-sand columns. Buffers used: HCl+potassium hydrogen phthalate (*p*H 2.2–3); NaOH+potassium hydrogen phthalate (*p*H 4–5); NaOH+NaH_2PO_4 (*p*H 6–8); NaOH+H_3BO_3+NaCl (*p*H 9–10).

Any acetylhistamine in the eluate was determined colorimetrically by McPherson's modification of the Pauly Reaction. Adsorption is quantitative at *p*H 4 to 10, but falls off in more strongly acid media. The complete adsorption at *p*H 8 was confirmed by hydrolysing part of the eluate (p. 533), and testing for free histamine activity on the guinea-pig ileum.

The Decalso method for free histamine

Details of the final method

(*a*) *Columns:* We generally used a glass tube 20 cm. long and 1 cm. internal diameter, with a narrower tube (1–2 cm. long; 4 mm. internal diameter) fused at one end. For larger volumes of fluid, a column with a 50 ml. bulb, of the type illustrated in Fig. 3, is more suitable (but with internal diameter 1 cm. as before). A pad of cotton-wool is placed in the constricted end of the tube, and Decalso Grade F (mesh 60 to 80; no preliminary treatment) is introduced in small quantities at a time and packed down firmly with a glass tamper (cf. Fig. 3, part *b*). A column 6 cm. high is prepared (containing about 3 g. Decalso), and a small pad of cotton-wool placed on top.

(*b*) *Preparation of the fluids for adsorption:* Urine is adjusted with dilute hydrochloric acid or sodium hydroxide to a *p*H of about 8 (BDH Universal Indicator: green) and then filtered. Blood plasma requires no adjustment of *p*H, but is better diluted with 1 volume of distilled water to increase the rate of percolation. No removal of protein is necessary.

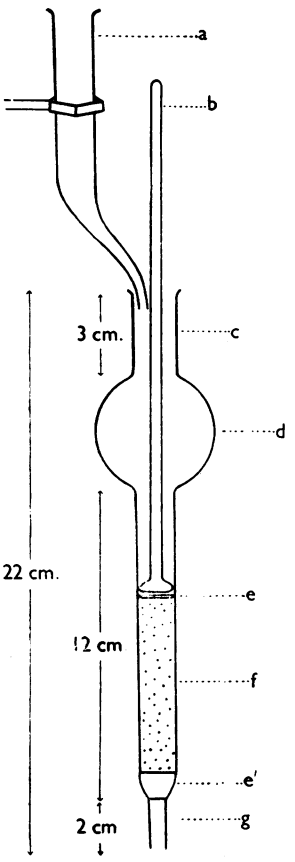

FIG. 3.—Parts required for the charcoal-sand adsorption column: (*a*) glass feed with tube tapering to 2 mm. internal diameter; (*b*) tamper with head of 1.1 cm. diameter; (*c*) neck of spherical bulb 1.5 cm. internal diameter; (*d*) bulb of 50 ml. capacity; (*e*, *e'*) cotton-wool pads; (*f*) charcoal-sand column, 1.2 cm. internal diameter, height 8 cm.; (*g*) tip, 3.5 mm. internal diameter.

Gastric juice is first filtered through glass wool, and mucins, which would otherwise seriously impede percolation, are removed by adding one volume of acetone (AR) to the juice at *p*H<4 and warming the mixture to 60° C. After storing the juice at 0° C. for a few hours the precipitate is removed by centrifugation at 3,000 r.p.m. for 20 minutes. The *p*H of the decanted fluid is adjusted carefully to 8 (BDH Universal Indicator), and centrifuged again at 3,000 r.p.m. for 20 minutes just before adsorption. The clear liquid passes easily through Decalso; the presence of acetone does not interfere with the adsorption of histamine.

(*c*) *Adsorption and elution procedures:* The Decalso column is washed with 10 ml. distilled water before the fluid to be adsorbed is added. Percolation of urine is usually about 50 ml. per hour. Plasma and gastric juice percolate rather more slowly, and the rate may be increased if desired by application of a small positive pressure (about 1 cm. of Hg). But rates of percolation greater than about 60 to 80 ml./hr. are not recommended, or adsorption may not be complete. After the fluid has passed through, the column is washed five times with 5 ml. of 0.9 per cent (w/v) sodium chloride solution, followed by 15 ml. absolute alcohol to remove most of the water.

Elution is carried out by adding 3 ml. AnalaR ammonium hydroxide (sp. gr. ~ 0.880), followed, just as soon as the top of the column dries, with 50 ml. AR chloroform saturated with ammonia gas (prepared by bubbling dry ammonia gas through the solvent until gas ceases to dissolve). Collection of the eluate is begun just after addition of the strong ammonium hydroxide.

(*d*) *Evaporation:* The chloroform-ammonia eluate is collected and evaporated in the apparatus already described by Adam (1950). The solvents (chloroform, alcohol, and traces of water) and ammonia are removed on a water-bath under reduced pressure at a temperature below 40° C. The slight residue is dried off at 60° C., first with 10 ml. absolute alcohol containing 3 per cent (v/v) concentrated HCl to neutralize traces of alkali, and finally with 10 ml. absolute alcohol. The acidified extract may be stored in a desiccator for several days without loss of activity.

Plasma extracts sometimes, gastric juice extracts always, contain acid-labile substances causing a slow contraction of the guinea-pig ileum not inhibited by mepyramine maleate. These extracts, therefore, are boiled on a sand-bath for 60 minutes with 20 per cent HCl (2 vol. concentrated AR HCl + 1 vol. water). The acid is evaporated off under reduced pressure, and the residue dried off with two 10 ml. portions of absolute alcohol.

(*e*) *Assay:* As the chloroform-ammonia elution eliminates inorganic ions (except traces of NH_4^+, insufficient to affect the assay), the extract is taken up in a small volume (5 to 10 ml.) of normal physiological saline. After addition of neutral red indicator (1 to 2 drops 0.01 per cent solution) and careful neutralization with sodium hydroxide, the

extract is ready for assay on the isolated guinea-pig ileum suspended in 2 ml. Tyrode's solution containing atropine (0.1 μg./ml.). All histamine values quoted in this paper are expressed in terms of the base, on the assumption that the histamine acid phosphate (BDH) used as standard contains 36.16 per cent histamine.

Recoveries of free histamine by the Decalso method

From human urine.—Table I records a series of experiments to estimate the recovery of histamine added to human urine over the concentration range 10 to 100 μg./litre (0.5–5.0 μg. added to 50 ml.). The experiments were carried out on urines from healthy men, and were usually done at the same time as the 24-hour excretion determinations (see Table VII). Recoveries were estimated by subtracting the histamine activity in a control sample of urine from that measured in the urine with added histamine. At the lowest concentration (10 μg./litre—i.e., 0.5 μg. to 50 ml.),

TABLE I

RECOVERIES OF HISTAMINE ADDED TO HUMAN URINE

Histamine (μg.) added to 50 ml. filtered urine at *p*H 8	0.5	1.0	2.5	5.0
Number of estimations	10	10	10	10
Mean percentage recovery	63.1	65.7	77.9	68.7
Range	45–100	49–85	68–92	64–77
S.D.	15.7	11.5	8.1	5.0
S.E. of the mean	5.0	3.6.	2.6	1.6

NOTE.—In two estimations, acetylhistamine was also added to the urine and subsequently determined by charcoal adsorption. (See Table VI.)

the amount of histamine normally present in the urine is liable to be greater than the amount added, and estimates of the recovery become very sensitive to errors in assay. Seven of the ten recoveries at this level, therefore, were carried out on urine from which most of the free histamine had been removed by previous passage through a short Decalso column. (The urine used in five of these experiments, about 300 ml., had been passed through a column too small to remove all the histamine, but the concentration was reduced to 8 μg./litre.) The technique was consistent throughout the series of determinations, and differed from the "final method" already described only in this respect: that the elution was performed with chloroform-ammonia heated to about 50° C. Later it appeared that elution with chloroform-ammonia was no less efficient in the cold.

Recoveries appeared to be maximal at an added concentration of 50 μg./litre (2.5 μg. to 50 ml. urine), all the other concentration levels (10, 20, and 100 μg./litre) giving significantly lower values. This anomaly is difficult to explain, but it should be emphasized again that the recoveries quoted are all determined by difference between a control sample and a sample with added histamine. This calculation assumes the *actual* recovery of histamine to be constant over the whole range of concentration. If in fact the actual recovery increases with the concentration to reach a steady level at (say) about 50 μg./litre, the somewhat less efficient recovery of the control histamine could lead to quite a big exaggeration of the estimated recovery, so long as the added amount of histamine was not arge compared with the control. As the added quantity increased, the exaggeration effect would become

TABLE II

RECOVERIES OF HISTAMINE FROM OTHER BODY FLUIDS

	10 ml. stored human plasma + 10 ml. water		25 ml. human gastric juice precipitated with 25 ml. acetone		50 ml. dog urine, *p*H 8		
Histamine added (μg.)	1.0	5.0	1.0	5.0	1.0	2.0	5.0
Number of estimations	1	4	3	3	7	1	1
Mean % recovery	60	63	76	75	70	59	54
Range	—	58–67	68–80	63–82	59–90	—	—

TABLE III

RECOVERIES OF HISTAMINE FROM SALINE

Quantity of histamine adsorbed (μg.)	1.0	5.0
Number of estimations	9	16
Mean percentage recovery	57.8	65.4
Range	50–71	50–83
S.D.	8.2	12.0
S.E. of the mean	2.7	3.0

relatively less serious. This hypothesis could explain the appearance of a maximum in the measured recovery-concentration curve, and so avoid the unlikely conclusion that the *actual* recovery rises to a maximum and then falls off again.

From other body fluids.—A few recovery experiments were done with stored human plasma (containing no detectable free histamine), human gastric juice, and dog urine. Mean recoveries and range of values are recorded in Table II, and are comparable with those obtained with human urine. Values, as before, were calculated from the difference between a control and fluid plus added histamine.

From pure solution.—Table III shows some recoveries of 5 μg. and 1 μg. histamine added to 0.9 per cent NaCl solution. Most of these were done during the early stages of working out the Decalso method, when the technique was liable to undergo slight modifications. There was no significant difference between recoveries at the two levels ($P>0.05$).

The hydrolysis of histamine conjugates

Some anomalous results obtained with urinary conjugated histamine led us to reopen the question of the conditions necessary to ensure complete liberation of free histamine by acid-boiling. The problem was made easier by having for investigation a pure sample of acetylhistamine (m.p. 147° C.). The hydrolysis was carried out with 10 ml. 20 per cent hydrochloric acid in a 150 ml. spherical flask fitted with an air-condenser. Table IV shows the results of treating in this way various quantities of acetylhistamine, first on a boiling water-bath for one and a half to six hours, secondly on a sand-bath heated sufficiently to produce steady boiling of the acid inside the flask. Similar experiments were performed with a urine extract, practically devoid of free histamine, prepared by charcoal-adsorption and elution from a urine containing large amounts of conjugated histamine.

TABLE IV

HYDROLYSIS OF CONJUGATED HISTAMINE

Conjugate	Amount treated with strong HCl (μg.)	Conditions	Amount hydrolysed (μg.) after 1	1.5	3	6 hours	
N-acetylhistamine ..	10	Water-bath at 100° C.	—	3.1	3.8	8.3	(as acetyl-
	10		—	3.0	4.1	7.6	histamine)
Urine extract A containing conjugated histamine	1 ml.	Water-bath at 100° C.	—	4.8	10	12	(as histamine)
N-acetylhistamine ..	10	Boiling on a sand-bath	10				(as acetyl-histamine)
	10		10				
	5		5				
	2.5		2.3				
	2.5		2.4				
Urine extract A containing conjugated histamine	1 ml.	Boiling on a sand-bath	15.4				(as histamine)
	1 ml.		16.4				
L-histidine (pure) ..	100	Boiling on a sand-bath	<0.1 (as histamine)				

The results show that on a water-bath hydrolysis of acetylhistamine is not complete even after six hours. But one hour on a sand-bath, with steady boiling of the acid, is sufficient to liberate the theoretical amount of histamine activity. Comparable results were obtained with the urinary conjugate, which yielded its maximum activity only after boiling on a sand-bath. Decomposition of L-histidine to histamine was not detected under these conditions, either in the pure state or when added to a urine charcoal extract.

In all our experiments, therefore, urinary conjugated histamine was hydrolysed by boiling for one hour on a sand-bath with 10 ml. 20 per cent HCl (2 vol. AnalaR concentrated HCl + 1 vol. water). Care must be taken, however, to avoid desiccation on the sides of the flask, by adjusting the boiling so that very little evaporation of the acid occurs. If a tendency to loss of volume is noticed, the amount of acid should be maintained at 10 ml. by adding more. If there is serious desiccation, exaggerated histamine activity may be found in the hydrolysed extract, possibly owing to decarboxylation of histidine. With the precautions outlined above, there is no danger of this happening.

The charcoal-sand method for conjugated histamine

Apparatus and reagents.—The apparatus used for the evaporations, and the source and method of preparation of the activated charcoal, have already been described (Adam, 1950). The charcoal is of small particle size, but can be used in a column provided it is mixed with an inert filter-aid. Fine sand (BDH, mesh 60–80, washed with acid) is suitable but it must first be freed from substances which interfere with the bioassay. These can be removed by washing the sand with the prospective eluant in the following way. About 300 g. sand are made into a column 24 cm. high, and washed first with tap-water. A total of 300 ml. of 3 per cent acid-alcohol (95 ml. absolute alcohol + 2 ml. water + 3 ml. AR

conc. HCl) is then allowed to percolate through the column. The sand is washed with tap-water until all the acid is removed, and then with distilled water. Finally, it is treated with absolute alcohol, transferred to a porcelain dish, and heated to dryness.

Preparation of the charcoal-sand columns.—The parts required are shown in Fig. 3. The adsorbent mixture consists of 140 mg. of activated charcoal mixed with 14 g. sand. If the proportion of charcoal to sand is greater than 1 per cent, the percolation time becomes unduly prolonged. A disadvantage of this mixture is the tendency for the charcoal to separate from the sand, and for this reason packing of the column requires care. The following method was adopted. Previously weighed lots of charcoal and sand are mixed in a stoppered 50 ml. conical flask, and the mixture transferred to the glass feeding device (Fig. 3, *a*), from which it flows into the chromatography tube. Firm strokes of the tamper *b* are applied at a rate of about one in two seconds. The final column is about 8 cm. high.

Adsorption procedure.—Wash the column with 10 ml. distilled water to remove traces of loose charcoal, and then add the solution for adsorption. This consists usually of 5 ml. of urine that has passed through a Decalso column (" Urine Decalso Percolate," or UDP), made up to 10 ml. with distilled water. After the urine adsorption, wash the column again twice with 10 ml. distilled water. Up to this stage, the percolations are allowed to proceed by gravity.

Elution, hydrolysis, and assay.—A few experiments indicated that elution of adsorbed acetylhistamine was maximal with alcohol containing at least 3 per cent (v/v) of concentrated HCl. Not less than 30 ml. of the mixture were necessary for satisfactory elution. Add to the column, therefore, 30 ml. 3 per cent acid-alcohol (95 ml. absolute alcohol + 2 ml. water + 3 ml. AR conc. HCl). The percolation can be safely accelerated by applying a pressure of 2 cm. Hg to the top of the column; this about doubles the flow-rate. The total time required for adsorption and elution is about one hour.

After the eluate has been taken to dryness, hydrolyse the extract in the way already described (p. 533). The residue left after evaporating off the 20 per cent acid under reduced pressure is small, and easily taken up in 9.5 ml. 0.9 per cent saline. Add 2 drops of neutral red indicator (0.01 per cent solution) to the suspension and neutralize with dilute sodium hydroxide. Make the volume up to 10 ml. and centrifuge at 2,000 r.p.m. The supernatant is then ready for bioassay.

Recoveries of conjugated histamine by the charcoal-sand method

The charcoal-sand column method of estimating conjugated histamine was tested by the recovery of acetylhistamine added to Urine Decalso Percolate (UDP). In two experiments partially purified urinary conjugate of known activity was added to UDP. Mean percentage recoveries and other data are tabulated in Table V. Results were corrected, as before, for the amount of conjugate present in control

TABLE V

RECOVERIES OF CONJUGATED HISTAMINE ADDED TO HUMAN URINE

	N-acetylhistamine				Urine extract A
Conjugate (μg.) added to 5 ml. urine Decalso percolate	2.5	5.0	10	100	3.3 (as histamine)
Number of estimations	10	10	10	1	2
Mean percentage recovery	81.8	82.7	90.3	87	83
Range	68–91	70–93	79–103	—	—
S.D.	7.5	7.0	7.6	—	—
S.E. of the mean	2.4	2.2	2.4	—	—

samples of UDP. Recoveries were consistently good over the range of concentrations tested (0.5 to 20 μg. acetylhistamine/ml.).

It was found that the optimum volume of UDP for adsorption was 5 ml. When the volume was 10 ml. traces of conjugated histamine appeared in the percolate, and when 20 ml. the loss was usually greater than 10 per cent. Unless the urine was very dilute, 5 ml. UDP from various subjects contained enough conjugate for the assay. Loss in the percolate can easily be measured by passing it through a second column. This was done in many of the recovery experiments, and in none was there evidence of incomplete adsorption, even when 100 μg. of acetylhistamine were added to 5 ml. UDP.

Recoveries of free and conjugated histamine

In two experiments, free histamine and acetylhistamine were added simultaneously to human urine, and recoveries of each determined. Results of the expected order were obtained, and are shown in Table VI. Further details are given here to illustrate the method of evaluating the recoveries summarized in Tables I, II, and V.

TABLE VI

SEPARATION AND INDEPENDENT DETERMINATION OF HISTAMINE AND ACETYLHISTAMINE ADDED TO HUMAN URINE

Sample	Total free histamine by Decalso (μg.)	Total conjugate by charcoal (μg. as histamine)	% recovery of added free histamine	% recovery of added acetyl-histamine
47 ml. urine A (control) ..	0.7	6.5	—	—
47 ml. urine A+5 μg. of histamine and 20 μg. (≡ 14.5 μg. histamine) of acetylhistamine	4.0	22.0	66	107
48 ml. urine B (control) ..	1.3	4.4	—	—
48 ml. urine B+1 μg. of histamine and 10 μg. (≡ 7.25 μg. histamine) of acetylhistamine	2.1	10.0	80	77

Application of the new methods

The new methods were applied to the determination of the 24-hour excretions of free histamine and conjugated histamine in 5 healthy men. The estimations for each subject were carried out for six, not necessarily consecutive, days. The sixth-day output was collected in three eight-hourly periods, and separate determinations done on each fraction.

Method of collecting and treating the urines

The day urines were collected in chemically clean, stoppered flasks containing sufficient 2 N-hydrochloric acid (about 2–5 ml.) to keep the *p*H below 5. No other preservative was used. The day urines were kept overnight at 0° C. The night samples were also collected in the presence of 2–5 ml. 2 N-HCl, and mixed the following morning with the day specimen; 50 ml. of the total 24-hour sample were adjusted to *p*H 8, filtered, and adsorbed on Decalso; 5 ml. of the UDP were taken for extraction of the conjugated histamine on charcoal-sand columns.

Results

These are given in Table VII. Means, range of urine volumes, and range of histamine concentrations are also included. The mean free histamine excretion is considerably lower than the mean excretion of conjugated histamine, and is remarkably constant for a given subject, and even between subjects. The conjugated histamine excretion is usually extremely variable, and may alter widely from day to day in the same subject. The ratio of conjugated to free histamine in any given 24-hour sample varies 44-fold from 0.63 to 27.7. The implications of these findings are discussed later.

Any variations of the free histamine excretion within the 24-hour period still remain for further investigation. The figures for the 8-hour excretions all show a greater average excretion during waking hours than during sleep, but differences are hardly big enough for definite conclusions to be drawn.

TABLE VII

24-HOUR EXCRETIONS OF FREE HISTAMINE (μG.) AND OF CONJUGATED HISTAMINE (μG. CALCULATED AS HISTAMINE) IN FIVE MEN

	Subject:	A		B		C		D		E	
	Histamine:	Free	Conj.	Free	Conj.	Free	Conj.	Free	Conj.	Free	Conj.
Day 1		16.8	95	26.2	727	26.2	84	30.0	72	26.0	167
Day 2		16.8	134	18.3	475	28.2	32	16.7	55	36.0	206
Day 3		18.9	119	15.5	253	18.1	60	12.1	111	24.6	39
Day 4		18.6	105	25.0	82	24.2	65	13.9	89	41.4	63
Day 5		18.2	109	20.5	31	19.9	33	13.8	35	25.2	81
Day 6*		18.2	—	16.8	54	25.3	16	13.3	88	23.0	159
Mean		17.9	112	20.4	270	23.7	48	16.6	75	29.4	119
*Day 6	1st 8-hrs.	6.2	—	7.3	21	9.4	4.6	6.0	40	6.7	84
	2nd 8-hrs.	7.2	—	5.3	12	10.6	8.0	3.2	21	9.4	51
	3rd 8-hrs. (sleep)	4.8	—	4.2	21	5.3	3.6	4.1	27	6.9	24

		Number of estimations	Grand mean (μg.)	Range	Range of urine volumes	Range of free histamine concentrations
24-hr. histamine excretion	Free	30	21.6	12.1–41.4	630–2100 ml.	8–33 μg./litre
	Conjugated	29	125	16–727		

The identification of urinary free histamine

The substance extracted on Decalso from human urine produced contractions of the guinea-pig ileum indistinguishable from those due to authentic histamine. Further tests were carried out in order to make as certain as possible that this urinary activity was in fact due to free histamine. Most of the tests were, of necessity, pharmacological. They are summarized in Table VIII and illustrated in Figs. 4 and 5.

Stability to various agents.—The histamine-like activity of the urinary extracts was stable to boiling for one hour in 20 per cent HCl, but about 60 per cent was

destroyed by treatment with boiling 1/7 N-sodium hydroxide. These are well-known chemical properties of histamine, not shared by other common gut-contracting substances. The fact that acid boiling does not significantly increase the activity emphasizes the efficiency with which the histamine is separated from conjugated histamine.

The histamine activity could also be more than 99 per cent destroyed by incubation with a diamine oxidase preparation. This was made by shaking 1 g. of a pig's kidney acetone-powder with 1/15 M-phosphate buffer of *p*H 7.4. The supernatant after centrifugation contained diamine oxidase activity. Under our conditions, about 0.5 ml. of this solution could decompose 2 μg. authentic histamine in 60 minutes at 37.5° C. About three to four times as much of the enzyme was required to break down 2 μg. of the histamine-like substance extracted from normal human urine. This is not surprising, since impurities having an inhibiting effect on the enzyme, or competing for it, are likely to occur in the final extract. Authentic histamine added to a blank extract, prepared by putting sodium chloride solution through the Decalso procedure, was also decomposed less rapidly than histamine in pure solution.

Parallel assays.—Larger volumes of urine were extracted on columns of Decalso (10 cm. × 1 cm.). Complete adsorption probably did not occur, but sufficient urinary histamine (5–6 μg.) could be obtained for parallel assays. The Decalso eluate was taken up in 5 ml. saline and assayed on the blood pressure of an anaesthetized and atropinized cat (Fig. 5). A suitable dilution was tested on the isolated guinea-pig ileum (Fig. 4). Agreement between the two assays was satisfactory (Table VIII).

TABLE VIII

IDENTIFICATION OF URINARY FREE HISTAMINE

Decalso eluate from	Direct assay of extract on guinea-pig ileum (μg.)	Assay after boiling extract in strong HCl for 60 min. (μg.)	Assay after boiling with 1/7 N-NaOH for 60 min. (μg.)	Direct assay of extract on cat B.P. (μg.)
50 ml. urine C ..	1.1	1.2	0.5	—
450 ml. urine D ..	5.0	—	—	6.0
1,200 ml. urine E ..	6.0	—	—	5.7

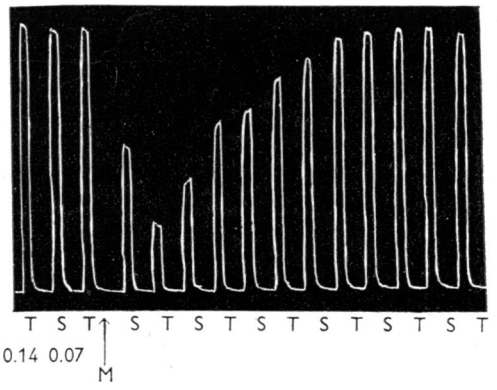

FIG. 4.—Responses of guinea-pig ileum to equiactive doses of urine D extract (T) and histamine standard (S, 0.2 μg./ml.). Test solution was 450 ml. human urine extracted on Decalso columns and concentrated ninefold (cf. Table VIII). Arrow represents addition of 0.1 ml. mepyramine maleate (concentration: 2×10^{-8}).

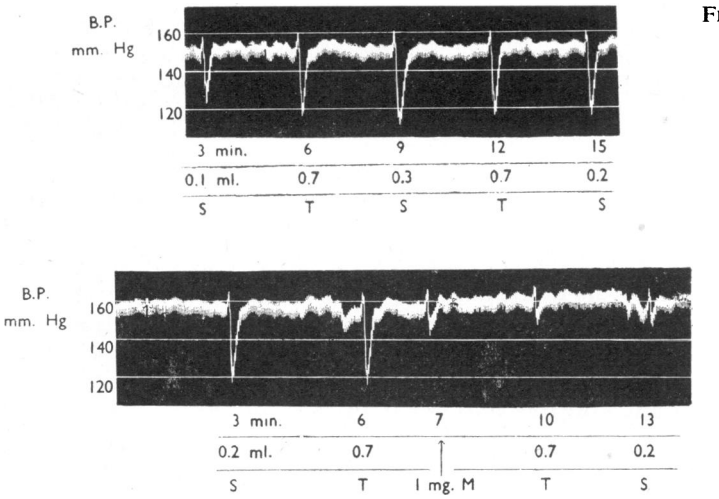

FIG. 5.—Top part of tracing shows assay of urinary free histamine on the blood pressure of a 3.1 kg. atropinized cat. Test was 1,200 ml. human urine E (Table VIII), extracted on Decalso columns and taken up in 10 ml. 0.9% NaCl. Concentration of the histamine standard (S) was 2 μg./ml. Result: 0.7 ml. T was equivalent to 0.2 ml. S. Arrow in second part of tracing represents injection of 1 mg. mepyramine maleate. Subsequent doses of T and S were inhibited to an approximately equal degree.

Inhibition by mepyramine maleate.—Fig. 4 shows that the response of the guinea-pig ileum to a urinary Decalso extract can be inhibited by 0.1 ml. of a solution of mepyramine maleate (May and Baker, Ltd.) in a concentration of 2×10^{-8}. Inhibition of the same order occurs with equiactive doses of the standard histamine solution, and recovery of the sensitivity of the gut to both test and standard occurs at a similar rate. Inhibition of the depressor effect on the cat's blood pressure of both histamine and urinary extract was comparable after intravenous injection of 1 mg. mepyramine maleate (Fig. 5).

DISCUSSION

The Decalso method for free histamine

The main disadvantage of the method is that recoveries of adsorbed histamine are not quantitative, and no way of making them so has been found. The grand mean of 116 recovery experiments, carried out with different fluids and by differing techniques, is 67 per cent, with a standard error of 1.1. This corresponds to the experience of Coulson, Ellinger, and Holden (1944), who could recover only two-thirds of nicotinamide adsorbed on to Decalso. None the less, a 67 per cent recovery is fairly consistently obtained, and makes possible the application of a correction factor, if desired.

The question of possible variations in the adsorptive properties of different batches of Decalso is an important one. No detectable differences, even of percentage recovery, have been observed in the several batches used in the course of our work. It is wise, however, to test every fresh batch of Decalso for quantitative separation of histamine and acetylhistamine at *p*H 8, and to carry out a few recovery experiments as a further check.

As each column contains only 3 g. of Decalso, it is probably not worth while to try to reactivate the adsorbent for further use; we did not do so.

The disadvantage of incomplete recovery is outweighed, in our opinion, by the many advantages of the method. Free histamine can be adsorbed directly from

urine or plasma, and from gastric juice after very mild precipitation of mucins with acetone. This has not merely the advantage of simplicity, but greatly increases the probability that any free histamine extracted by so mild a method was really in the original fluid. It also makes feasible the concentration of free histamine from fluids containing very small quantities of the substance. This is essential in studies on normal human free histamine, since concentrations in the urine are of the order of 10 to 30 μg./litre, and in the plasma undoubtedly much less. There has been no evidence in urine extractions of the simultaneous concentration of other active substances liable to interfere with the bioassay. The interfering substances occurring in plasma and gastric juice can be easily destroyed by acid-boiling. In particular, the Decalso method eliminates potassium.

The charcoal-sand method for conjugated histamine

The method has so far been applied to urines only. Recoveries of acetylhistamine, and also of urinary conjugated histamine, are very good over the usual range of concentrations found. The method is rapid and simple, but suffers from the disadvantage that charcoal is a non-specific adsorbent. The other substances adsorbed and eluted from urine, however, did not interfere with assay of the hydrolysed samples.

Identity of the urinary free histamine

It is not possible, short of complete chemical identification, to prove conclusively that the gut-contracting substance present in normal urine is histamine. We can say that it is indistinguishable from histamine, both quantitatively and qualitatively, over a range of tests. The inhibiting effect of *small* doses of mepyramine maleate distinguishes histamine from many other substances, but not from N-methylhistamine (Schild, 1947). The latter compound would, however, give discordant results when tested against histamine by parallel assays on guinea-pig gut and cat blood pressure (Vartiainen, 1935). Present methods of parallel assay would probably not differentiate histamine from N-dimethylhistamine (Gaddum, 1948).

The destruction of the urinary substance by diamine oxidase is further useful confirmatory evidence, but it should be emphasized that little is known about the action of this enzyme on pharmacologically active compounds closely related to histamine, such as the N-alkyl and N-dialkyl histamines.

Twenty-four-hour excretions of free and conjugated histamine

It is likely that the conjugated histamine found in human urine is formed in the alimentary tract, and that this explains the wide variations in the amount excreted daily. Histamine is present in the food (Anrep *et al.*, 1944), and more is probably formed by the bacterial decarboxylation of histidine. When histamine is given in large doses by mouth, about 1 per cent is excreted in the urine as the conjugate, and only a minute fraction as free histamine (Urbach, 1949; Adam, 1950). The small but definite increase in the urinary free histamine shows, however, that it is slightly absorbed under these conditions. The fate of the remainder of the dose is unknown; presumably it is inactivated in the mucosa of the intestine, or excreted in the faeces as free or conjugated histamine.

The regular, almost constant, daily excretion of free histamine stands in sharp contrast to the unpredictable daily excretion of the conjugate, and suggests a source of origin other than the gut. If free histamine is absorbed from the gut, its excretion would be expected to vary like that of the conjugate. There is, however, no obvious correlation between the values for the conjugate and those for free histamine; but this does not altogether exclude the gut as a possible source of urinary free histamine.

It is also possible that the free histamine is formed or liberated locally in the renal tract. A local source cannot be easily excluded, unless a quantitative relation can be established between the concentration of histamine in the plasma and its excretion in the urine. There is as yet no reliable method of estimating physiological quantities of free histamine in the plasma. It is known, however, that about 1 per cent of histamine introduced directly into the blood stream appears in the urine as free histamine (Adam, 1950). If, therefore, histamine passes continuously into the plasma, either as a result of absorption, or of liberation or formation in tissues elsewhere, a proportion of the amount circulating in the plasma would be expected to appear in the urine.

By infusing histamine intravenously it is possible to simulate its passage into the plasma and in this way to obtain indirect evidence for the assumption that the plasma is the immediate source of free histamine in the urine. In earlier experiments Adam (1950) employed doses that produced generalized effects. More recently the same problem has been studied by infusing histamine in the dose range required to produce graded effects on the acid gastric secretion (Adam, Card, Riddell, Roberts, and Strong; in the press). When histamine was infused at a rate of 8 mμg./kg./min. for three hours, the effect on the stomach was slight, and the excretion of free histamine in the urine was about doubled. These results imply that the histamine normally in the urine could be derived from histamine circulating in the plasma in concentrations too low to have a significant action even on the acid gastric secretion.

Summary

1. New methods of the chromatographic type have been worked out for the separation and purification of free and conjugated histamine in body fluids.

2. Histamine and acetylhistamine (or urinary conjugated histamine) can be separated over the *p*H range 8 to 10 on columns of the cationic exchanger Decalso. The free histamine is quantitatively adsorbed, and can be eluted from the adsorbent by treatment with concentrated ammonium hydroxide followed by chloroform saturated with ammonia gas. After evaporation of the solvent, the extract is assayed biologically on the guinea-pig ileum.

3. The Decalso method for free histamine has been applied to human and canine urine, human plasma, and gastric juice. Recovery experiments from these fluids and from 0.9 per cent saline gave a mean recovery of 67 per cent $\pm$ 1.1, over the concentration range 10 to 250 μg./litre.

4. Conjugated histamine and acetylhistamine are not adsorbed on Decalso at *p*H 8–10, and these substances can be estimated in urine after passage through Decalso. The conjugate is adsorbed on columns of charcoal mixed with fine sand, and eluted with acid-ethanol. After evaporation of the solvent, the extract is

hydrolysed on a sand-bath for one hour with boiling 20 per cent hydrochloric acid, and the liberated histamine assayed biologically.

5. The charcoal-sand method for conjugated histamine (tested mainly with synthetic acetylhistamine) gave recoveries of 80 to 90 per cent over the concentration range 0.5 to 20 μg./ml.

6. The new methods have been applied to the determination of free and conjugated histamine in the 24-hour urinary excretions of five healthy men for six days. Free histamine was found in every urine examined; the mean was 21.6 μg./day (or 32.4 μg./day, if corrected for two-thirds recovery on Decalso), with a range of 12.1 to 41.4 μg. (18.2 to 62.1 μg. if corrected). The mean excretion of conjugated histamine was 125 μg./day (as histamine), with a range of 16 to 727 μg.

7. Evidence, mainly pharmacological, is presented to support the assumption that the active substance extracted on Decalso from normal human urine is histamine.

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