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MEASUREMENT AND NORMAL RANGE OF FREE HISTAMINE IN HUMAN BLOOD PLASMA*

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Abstract—A method is described for measuring histamine in plasma by development of fluorescence with *o*-phthalaldehyde, after separation of the histamine from most other constituents of plasma by: 1) deproteinizing with trichloroacetic acid, 2) adsorption on Decalso, 3) elution with KBr, 4) extraction into butanol at alkaline pH, and 5) extracting back into water after adding acid and heptane. A mean value of 0·6 μg/l. (S.D. 0·3 μg/l.) was obtained for 62 samples of fresh plasma from 46 normal young women. Freezing and thawing the plasma did not significantly affect this value. This level is less than a third of the lowest level previously reported by any chemical method. The specificity of the procedure was tested in several ways. A wide variety of amines were tested with pthalaldehyde in regard to capacity to produce fluorescence and the spectrum of the fluorescence when produced. Large plasma samples were carried through the analytical procedure and chromatographed prior to fluorescence development. Other samples were treated with diamine oxidase. The conclusion is reached that 65–100 per cent of the fluorescence is attributable to histamine. Some samples contained material which accompanied histamine through the entire procedure and reacted with phthalaldehyde to give fluorescence at shorter wavelengths than did histamine. Spermidine seems to be a good candidate for this material.

THE CONCENTRATION of histamine in normal plasma is so low that even in pure solution its measurement would require a sensitive test performed with painstaking technique. When, as in plasma, it is accompanied by substances with somewhat similar properties and in relative abundance, the problem of specificity becomes at least as serious at that of sensitivity. The rigorous demands of measurement of the histamine in plasma are not fulfilled either by known bioassay procedures or current chemical methods. The fluorometric method for measuring tissue histamine, based on condensation of the amine with *o*-phthalaldehyde in alkaline solution,[1] gave promise of being sensitive and specific enough to apply to plasma. By prefacing the development of fluorescence with purification of plasma extract through a Decalso adsorption-elution step, as in the dinitrofluorobenzene method,[2] a method has been developed that gives lower values for normal plasma histamine than any previous chemical method. The levels are so low that even the sensitive bioassay of Adam *et al.*[3] is inadequate. Adam's procedure consists of superfusion of isolated guinea pig ileum with meticulously prepared extracts of human plasma. Out of 5 different plasma samples that he analyzed, the levels of 4 were below the limit of measurement (2·5 ng/5-ml sample or 0·5 μg/l.), while that of the 5th sample was at the limit (0·6 μg/l.).

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The Decalso-fluorometric method described here is more sensitive; it will record the presence of less than 0·001 μg (10^{-11} mole) per 4-ml sample, and yields an estimate of 0·6 μg/l. (5×10^{-9} M) as the average histamine concentration of normal human plasma.

The specificitiy of the proposed method has been tested with a large number of known substances that might interfere. In addition, extracts of pooled plasma samples were tested by treatment with diamine oxidase or fractionated by TLC prior to development of fluorescence. These studies suggest that *bona fide* histamine contributes 65–100 per cent of the total fluorescence, and that spermidine may be responsible for the remainder. It is therefore concluded that the method presented here is more specific than other chemical methods, and that it provides a new and lower baseline above which very small increases in plasma histamine can be detected.

METHOD

Apparatus and reagents. Some of the equipment and techniques of the Decalso-dinitrofluorobenzene method[2] have been modified in a few details for use in the present method. The durability of the glass containers for the Decalso columns was increased by making the narrow segments out of Pyrex capillary tubing (1·8 mm i.d., 6–8 mm o.d.). The layer of sand originally recommended for the bottom of the Decalso columns was omitted, in order to avoid occasional blocking of the constriction or the tip with sand particles. The hot box was lined with asbestos, and two 100-W heating coils, stretched from end to end of the bottom of the box and provided with metal shields, were substituted for the lamp heaters.

Fluorescence intensity was measured routinely in a Farrand fluorometer model A with Corning glass filters No. 7–37 in the primary (activation) light path and Nos. 3–73 and 4–70 in the secondary (emission) path. The primary filter provides maximum transmission at 360 mμ (and therefore isolates the 366 mμ Hg line). The secondary filter transmits maximally at 480 mμ. Fluorometer tubes of 3-ml vol. were selected individually for low and uniform fluorescence and for fit in the fluorometer.* The tubes were rinsed after use and left filled with water until cleaned by boiling in 50–85% concentrated HNO_3, rinsing and boiling in distilled water, rinsing with glass-distilled water and drying in an oven at 105°.

All chemicals were of reagent grade and were used without further purification. The *o*-phthalaldehyde was obtained from Calbiochem., Los Angeles, Calif.

Preparation of plasma extracts. Blood was drawn into a heparinized syringe, and the plasma separated by prompt centrifugation at 4° for 30 min at 2000 *g*. Unless used immediately, the plasma was stored at −18°. Protein was removed by centrifugation after addition of 1/2 vol. of 10% trichloroacetic acid or 1/9 vol. of 50% trichloroacetic acid; the extracts were brought to about pH 4 by addition of 1/10 vol. of 4 M sodium acetate. Plasma samples from blood collected with citrate in a hospital blood bank frequently had higher histamine levels than samples obtained in the laboratory with heparin; this was attributed to less prompt separation of the cells in the blood bank.

Absorption, elution, extraction, development of fluorescence. Aliquots of the plasma extracts, each aliquot representing 2–4 ml plasma, were applied to Decalso columns;

* A volume of nearly 1 ml is required to bring the meniscus above the light beam. Since the volume of sample in the procedure described is only about 0·7 ml, it is suggested that a piece of cork or other material be placed in the bottom of the tube holder to raise each tube 3 or 4 mm.

the columns were washed with 5 ml H_2O and eluted in the hot box (80°) with 0·5 ml of 40% KBr. Each eluate was received in a 6-ml tube containing 0·2 g of solid NaCl, and the mixture was vigorously mixed by "buzzing"* with 0·06 ml of 5 N NaOH and 1·25 ml of *n*-butanol. Most (1·2 ml) of the butanol layer was transferred to a fresh 6-ml tube (tube No. 2) and washed with 1 ml of 0·1 N NaOH saturated with NaCl. An aliquot (1·1 ml) of the washed butanol layer was put into an 8-ml tube with great care not to contaminate the aliquot with the slighest amount of the alkaline aqueous wash. The histamine was extracted from this by buzzing with 0·6 ml of 0·1 N HCl and 2 ml heptane. An aliquot (0·5 ml) of the acid layer was transferred to a fluorometer tube, made alkaline with 0·1 ml of 1N NaOH and treated with 0·025 ml of 0·1% *o*-phthalaldehyde in absolute methanol. After a 4-min wait, timed for each tube from the addition of the phthalaldehyde, 0·05 ml of 2·5 M H_3PO_4[5] was added. Fluorescence was ordinarily read at once, but was stable for 24 hr.

The final aliquot added to the fluorometer tube was found to contain about 40 per cent of the histamine originally added to the Decalso column. The balance was lost chiefly due to transfers of aliquots (rather than the whole sample) plus a rather large loss at the butanol extraction step. There was only a small loss on the Decalso column. The overall losses were quite reproducible. Moreover, known histamine, added to plasma extracts and carried through the recommended procedure, showed complete recovery (average 102 per cent) in comparison to pure standards also carried through the entire procedure. In one series of experiments, the volume of plasma extract applied to the Decalso column was 9·5 ml, i.e. more than double the usual amount. Lower recoveries of added histamine (80–90 per cent) were obtained with this larger volume. For this reason, the maximum volume of plasma in the extract applied to any one column was subsequently restricted to 5 ml.

Proportionality, sensitivity and reproducibility. Fluorometer readings with standards were found to be proportional to histamine over a range of 1–200 ng. With 4-ml plasma samples, most normal values fall in the range from 1 to 5 ng. Since the fluorescence of the blank is equivalent to at least 4 ng histamine, there is only a limited possibility of increasing the accuracy of the readings of amounts smaller than 1 ng by increasing fluorometer sensitivity.

The reproducibility depends largely on the level of histamine. With standards in the range of 5 to 10 ng, carried through the entire procedure, the standard deviation was 3–4 per cent. With the lower amounts of histamine present in normal plasma, the standard deviations were 5–10 per cent, depending on the histamine level.

RESULTS

Contribution of each analytical step to the specificity of the method. The sum of all amines present in normal plasma is at least 250,000 times greater than the amount of histamine present.[2] The necessary specificity is achieved by (1) adsorption and elution from a Decalso column, (2) extraction into butanol from alkaline solution and back into acid, and (3) reaction with a rather specific fluorogenic reagent. The Decalso treatment removes at least 99 per cent of total amines. If the Decalso treatment is omitted, the apparent value for plasma histamine is increased 10-fold. The butanol

* A slightly bent ¼ in. rod is mounted horizontally in the chuck of a rather high-speed (2000 or more rpm) motor or stirrer. When a tube is held at an angle against the rotating rod, the contents are violently agitated.[4] A vortex mixer may be substituted, although it is not quite as satisfactory.

extraction removes amino compounds with acidic groups, such as histidine, as well as other organic-soluble substances which may have native fluorescence. Omission of the butanol step can increase apparent histamine values as much as 100-fold. The importance of this step is emphasized by the results of failure to separate traces of the original aqueous layer from the butanol before reextracting the histamine back

TABLE 1. FLUORESCENCE OF AMINES WITH PHTHALALDEHYDE AND ELIMINATION BY STEPS IN ANALYTICAL PROCEDURE

The compound shown was carried through some or all of the steps of the proposed analytical procedure. Any of the compound not eliminated was allowed to react with phthalaldehyde and the fluorescence measured. Fluorescence at peak wavelength was measured with the Aminco–Bowman spectrofluorometer with activation wavelength of 365 mμ. The other readings were made in the Farrand fluorometer with the excitation and emission filters recommended for histamine.

Compound	Peak emission wavelength (mμ)	Per cent of peak histamine fluorescence (molar basis)			
		No pretreatment		Decalso step omitted*	Complete procedure
		At peak wavelength	Farrand filters	Farrand filters	Farrand filters
Histidine	450	1·9	3·2	0·003	0·002
Histidinol	475	16	25	13·5	8·6
1,4-Methylhistamine	430	0·5	0·5	0·02	
Spermidine†	400	5·2	0·4	0·4	0·4
Acetylspermidine‡	400	0·4	0·05	0·02	
1,3-propanediamine-*N*(3-aminopropyl)	400	3·8	0·15	0·00	0·00
1,4-butanediamine-*N*(2-aminoethyl)	400	0·7	0·1		
1,2-ethanediamine-*N*(2-aminoethyl)	400	0·6	0·4		
Spermine§	400	0·09	0·07	0·04	0·04
1,4-butanediamine-*N*,*N*^{1}-*bis*(4-aminobutyl)	400	0·07	0·02		
Agmatine	435	0·09	0·02	0·02	0·01

* Complete analytical procedure except for Decalso step.

† 1,4-Butanediamine-*N*(3-aminopropyl).

‡ Monoacetylspermidine A ($CH_3CONH(CH_2)_4NH(CH_2)_3NH_2$); monoacetylspermidine B ($CH_3CONH(CH_2)_3NH(CH_2)_4NH_2$) gave even less fluorescence. These derivatives were kindly provided by Dr. Celia Tabor.

§ 1,4-Butanediamine-*N*,*N*^{1}-*bis*(3-aminopropyl).

into acid. Thus, originally, after washing the butanol layer with alkali in tube No. 2, the aqueous layer was removed and the histamine extracted into acid in the same tube. The consequence, as shown by comparison with the recommended procedure, was to increase the apparent histamine content of plasma by an average of 1·2 μg/l. (200 per cent).

The specificity of the phthalaldehyde reaction itself was tested with a wide range of amines. None of 17 monoamines tested gave fluorescence greater than 0·03 per cent of that of histamine. These consisted of alanine, α-aminobutyric acid, ammonia, cysteic acid, glutamic acid, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, taurine, threonine, tyrosine and valine. Of 17 diamines tested, none gave fluorescence greater than 0·25 per cent of that of histamine. These consisted of lysine, ornithine, cysteine, tryptophane, tryptamine, serotonin, urea,

N-methyl-1,4-butane diamine, 2-butene-1,4-diamine and a series of 8 aliphatic amines of formula NH_2-$(CH_2)_x$-NH_2 with values of x from 2 to 8 and 10. This series includes cadaverine (x = 5) and putrescine (x = 4). Certain triamines and tetramines, however, developed more significant fluorescence with phthalaldehyde (Table 1). These included compounds directly related to histamine. The interfering fluorescence of histidine and methyl histamine was eliminated by the butanol extraction step. Histidinol gave $\frac{1}{4}$ as much fluorescence as histamine, with a considerable overlap of fluorescence spectra; its fluorescence was reduced to $\frac{1}{12}$ that of histamine by the butanol and Decalso steps.

Spermidine and related triamines also produce fluorescence with phthalaldehyde. Fortunately their fluorescence peaks are at shorter wavelengths, so that in the histamine peak region the fluorescence of spermidine, for instance, is only about 0·4 per cent that of an equimolar amount of histamine (Table 1). Spermidine must however be considered as a possible interfering substance, since it survives the adsorption and extraction steps in the assay as well as does histamine itself. Spermine, a tetramine analog of spermidine, gives less than $\frac{1}{10}$ as much fluorescence as spermidine; it therefore presents less likelihood of serious interference in the histamine assay.

Fluorescence spectra of plasma samples. A number of plasma samples were examined in the Aminco-Bowman spectrophotofluorometer after reaction with phthalaldehyde. The fluorescence of the reagent blank increases with decreasing wavelengths and is relatively large at wavelengths shorter than that of the histamine peak, 445 mμ. This makes it difficult to measure the spectra with precision. Nevertheless, it was clear that the fluorescence peaks of certain plasma samples fell at wavelengths shorter than that of histamine (Fig. 1(b)), indicating that some substance other than histamine was contributing to the fluorescence. The emission spectra from such samples could be

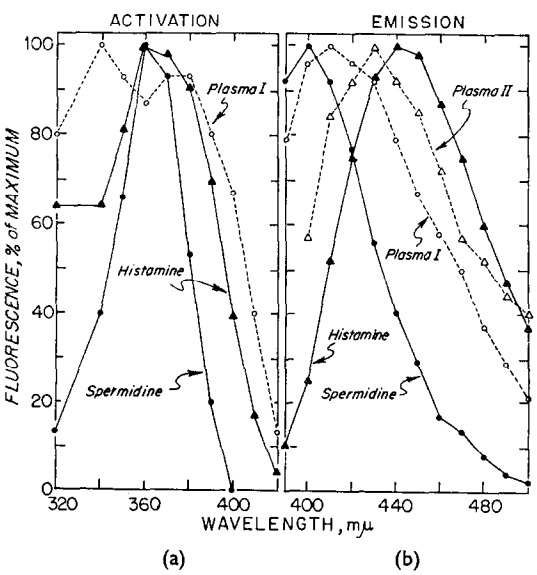

FIG. 1. Fluorescence activation and emission spectra of phthalaldehyde derivatives of histamine, spermidine and plasma samples that had been carried through the standard procedure. The activation spectra were obtained with emission readings at 450 mμ, the emission spectra were obtained with activation at 360 mμ.

duplicated with mixtures of histamine with spermidine, which has its peak fluorescence at 400 mμ (Fig. 1(b)).

The activation spectra of plasma samples were also examined (Fig. 1(a)). The activation peak for histamine was found to be about 365 mμ, in agreement with Michaelson.[6] Plasma samples were likewise maximally activated at this wavelength, but since the phthalaldehyde derivatives of most of the amines examined were also maximally activated in this same region, this observation provides little help in identifying the nonhistamine fluorescent constituent(s) of plasma.

In summary, the evidence from the spectral studies is that a large fraction, but not all, of the fluorescence in the 440–450 mμ region (the region of the histamine peak) could be produced by histamine, and that spermidine might be responsible for some, if not all, of the nonhistamine fluorescence. The use of the recommended secondary fluorometer filter with peak transmission at 480 mμ would appear to be of help in excluding this nonspecific fluorescence.

Effect of histaminase on apparent plasma histamine. Treatment of plasma samples with histaminase strengthened the evidence that some of the samples contained one or more interfering substances with a phthalaldehyde fluorescent peak in the 400 mμ region.

Diamine oxidase (histaminase) was prepared* by the method of Tabor[7] with a modification which gives somewhat higher specific activity. At 25° and pH 6·8 in 0·01 M phosphate buffer, it could deaminate diaminobutane at an initial rate of 1·6 μmole/mg protein per min. Diamine oxidase is known to destroy not only diaminobutane (putrescine) and histamine, but diaminopentane (cadaverine) and agmatine as well; it has not been reported in purified form to destroy spermine or spermidine. The preparation used did not attack spermidine (i.e. it had no effect on the phthalaldehyde fluorescence of spermidine); but fluorescence from spermine was decreased 30 per cent by incubation with the enzyme preparation under the conditions used in the tests on the plasma samples.

Some plasma samples incubated with sufficient enzyme prior to the butanol step lost all ability to fluoresce with phthalaldehyde; the original fluorescence of these samples presumably was due entirely to histamine. Other samples after incubation with the enzyme produced fluorescence with a peak in the 400 mμ region, as illustrated by Fig. 2. Although the fluorescent spectrum of the unincubated plasma in such cases did not conform exactly to the spectrum of authentic histamine, the difference spectrum (decrease on enzyme treatment) conformed very closely to the spectrum from histamine. Conversely, the spectrum of the fluorescence remaining after incubation conformed reasonably closely to that from spermidine.

Readings were made before and after enzyme treatment on a total of 15 plasma samples, some of them selected for previously observed high histamine content. After enzyme treatment, the fluorescence was reduced below the level of detection in the standard procedure. Examination of the spectra of the 15 samples showed, in the case of 9, no fluorescence in the 400–410 mμ region after incubation with diamine oxidase, i.e. in these samples all of the fluorescence was probably attributable to histamine. With the remaining 6 samples, significant fluorescence was observed at 400–410 mμ after enzyme treatment.

* We are grateful to Dr. Charles McEwen for a generous supply of this enzyme.

Chromatographic analysis. To obtain enough material for chromatography, large volumes of plasma extract (500 ml) were adsorbed and eluted from Decalso columns which had 100 times the usual capacity. The eluates were extracted into butanol and out again by the regular procedure, but with reagent volumes increased 100-fold. The final acid solutions were evaporated to dryness in a Virtis centrifuge Bio-dryer.

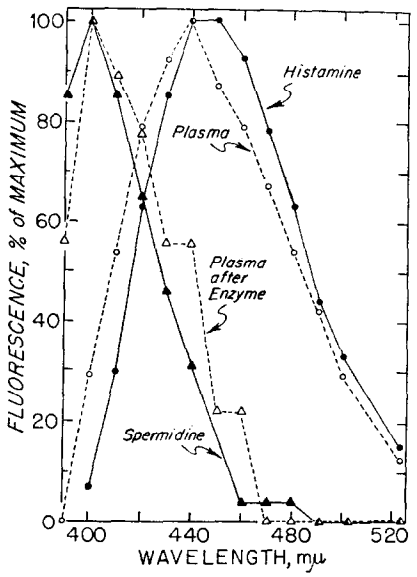

FIG. 2. Effect of diamine oxidase treatment on the fluorescence emission spectrum of a plasma sample. The excitation wavelength was 360 mμ. For comparison, the spectra of histamine and spermidine are shown. The plasma was from a patient with polycythemia and the histamine content was above the normal range (3·4 μg/l.). The plasma spectra before and after enzyme treatment have both been plotted as per cent of the peak fluorescence, but the absolute peak fluorescence from the enzyme-treated sample was reduced to about one-third. There was little change in absolute fluorescence at 400 mμ. This residual plasma fluorescence at 400 mμ would be equivalent to about 90 μg spermidine/l.

The residues were taken up in 2-ml portions of methanol, centrifuged to remove suspended solids and evaporated to about 0·1 ml. Aliquots were chromatographed on Eastman Chromagram sheets in an Eastman Chromagram Developing Apparatus. The chromatograms were developed either with 8% NaCl–1 N NaOH (100:2) (cf. the NaCl–acetic acid developer of Shimizu *et al.*[8]) or with 95% ethanol–30% ammonia (4:1).[9]

Test strips sprayed with ninhydrin–copper nitrate reagent[10] showed detectable color with as little as 2 μg spermidine or spermine, but no color was detected with the methanol extract from 54 ml plasma. This result indicates that there was present in these samples less than 50 μg spermine or spermidine per liter plasma.

Other chromatogram strips were cross-sectioned into 10 parts; the adsorbent was scraped off each section, treated with phthalaldehyde and the fluorescence measured. About 65 per cent of the total fluorescence (as measured under standard conditions)

migrated with each solvent in the same zone as histamine and showed peak fluorescence at the same wavelength (440–450 mμ). The fluorescence near the solvent front usually peaked at 400–410 mμ, the wavelength of the peak fluorescence of such natural amines as spermidine or spermine. The fluorescence in the region of the origin consistently peaked at 400 mμ with the ammonia solvent, whereas no fluorescence peak was observed with the salt solvent. Authentic spermidine and spermine migrated near the solvent front with the salt solvent and remained near the origin with the ammonia solvent. Spermine is ruled out as a major contributor to the fluorescence from plasma because of its low fluorescence yield. An amount sufficient to produce discernible fluorescence would have been detected by the ninhydrin–copper sulfate test on the chromatogram strip. Because of its 10-fold greater fluorescence with phthalaldehyde, spermidine is a much more likely candidate.

Possible presence of spermidine in plasma from stored blood. A number of plasma samples were examined from blood that had been stored in the blood bank. As expected, all had histamine values well above the normal range. In addition, in each case there was found to be much greater than usual fluorescence in the 400 mμ region. This fluorescence could have been accounted for by spermidine present in plasma at levels of 20–90 μg/l. There was a degree of correlation between the histamine and "spermidine" levels, with a mean ratio of about 1:20.

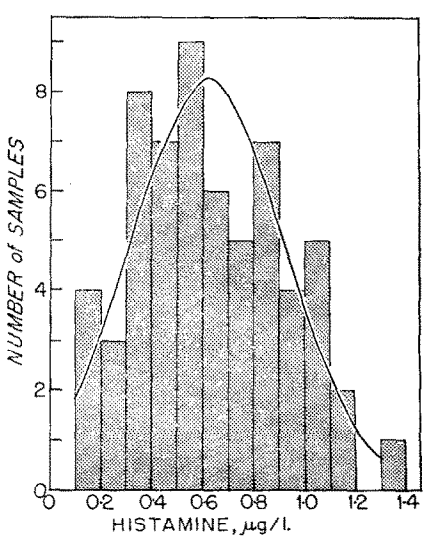

FIG. 3. Histogram of values obtained for histamine in 62 normal plasma samples. A normal distribution curve has been superimposed.

Normal plasma histamine values by proposed method. A total of 62 fresh plasma specimens was obtained from 46 women, 19–45 yr. of age, none of whom had been taking known histamine-releasing drugs. These specimens were assayed by the procedure described. The values had a nearly normal distribution (Fig. 3) about a mean value of 0·62 μg/l., with an S.D. of 0·3 μg/l. The extreme values were 0·10 and

1·3 μg/l. A smaller series of 18 plasma samples, frozen and thawed before analysis, gave a mean value of 0·50 μg/l., with an S.D. of 0·25 μg/l.

DISCUSSION

The proposed method for measuring plasma histamine gives a mean normal value that is much lower than that given by any previous chemical procedure or any bioassay (with the exception of the bioassay of Adam *et al.*[3] which, although not sensitive enough for the purpose, indicated normal levels of 0·6 μg/l. or less). Lowry *et al.*[2] reported an average value of 4·3 μg/l. by a procedure based on color developed with dinitrofluorobenzene, and cited earlier estimates in the literature which ranged as high as 60 μg/l. Dunér and Pernow,[11] using a method involving treatment with acetone, adsorption on and elution from Amberlite IRC-50, and subsequent bioassay on a strip of guinea pig ileum, found a mean of 24 μg histamine/l. of serum (25 healthy subjects). Levels averaging about 50 μg/l. have been reported in the last few years by workers with other methods.[12, 13] Several estimates based on development of fluorescence with phthalaldehyde have appeared. Noah and Brand,[14] using a scaled-down form of the Shore–Burkhalter–Cohn procedure, reported an average histamine value of 13·6 μg/l. for 34 plasma samples. Later, these workers described a micro-method based on the formation of the Schiff base of benzaldehyde and histamine, followed by a cellulose acid succinate adsorption–elution step, and the development of fluorescence with phthalaldehyde.[15] By this method, samples of plasma from 10 normal individuals gave a mean level of 2·1 μg/l. Beall,[16] using the fluorescent method in much its original form, found a mean of 5·6 μg/l. plasma for 15 normal persons. Thompson and Walton[17] and Garden,[18] also using the fluorescent method in slightly modified forms, reported means of 7·7 μg/l. (16 samples) and 5·5 μg/l. (47 subjects) respectively.

The much lower level of histamine reported in the present paper indicates that in the 14 l. of plasma and extracellular fluids of the average person there may be a total of only 5 or 10 μg of this amine. Conceivably, increases above this very low level can have physiological significance. The proposed more specific and sensitive histamine method makes it feasible to study changes in plasma histamine levels in this exceedingly low concentration range.

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