CHEMICAL DETERMINATION OF HISTAMINE IN HUMAN BLOOD*

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The normal histamine content of human blood as determined by bio-assay ranges from 1 to 8 \( \gamma \) per 100 ml., with an average value of about 4 \( \gamma \) (1,2). These low levels in human blood have limited the development of a procedure for chemical analysis, although a chemical method applicable to blood would facilitate the accumulation of data from large groups of subjects.

The two chemical methods available for the analysis of histamine in biological fluids are not directly applicable to human blood. The simpler technique of Rosenthal and Tabor (3), which utilizes the non-specific coupling reaction of imidazoles with 4-nitroaniline, requires a specimen of at least 20 ml. In McIntire’s method, the histamine is isolated from plasma under specified conditions (4) and the 2,4-dinitrofluorobenzene derivative is used for the determination (5). This procedure has the required degree of sensitivity but involves two counter-current extraction steps and a scaling down to micro levels for analysis of human blood.

By modifying the purification procedure of McIntire to make it applicable to whole blood filtrates without appreciable loss of histamine, and by scaling down the chemical method of Rosenthal to increase its sensitivity 5-fold, a procedure was developed for the colorimetric analysis of 0.1 to 1.0 \( \gamma \) of histamine in a 5 to 10 ml. specimen of whole blood.

EXPERIMENTAL

It has been reported that the histamine of the blood is concentrated in the leucocytes (6). Analysis of white cells or whole blood, therefore, seems preferable to plasma, and, of these, analysis of whole blood is the simpler procedure.

Prior to application of McIntire’s method (4) for the isolation of histamine from plasma, it is necessary to precipitate the proteins in whole blood. Several protein precipitants were tested. Trichloroacetic acid gives the clearest filtrates but interferes with subsequent adsorption on the ion exchange medium. Histamine is precipitated by alkaline zinc, barium hy-

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dioxide, and ethyl alcohol. Metaphosphoric acid filtrates contain a small amount of protein but they are satisfactory since the filtered protein is later precipitated during butanol extraction.

In McIntire's method for the purification and concentration of histamine from biological fluids advantage is taken of the fact that histamine is extracted from an aqueous solution by an equal volume of n-butanol containing at least 20 per cent by weight of salt at pH 12 or more. Under these conditions, and particularly in plasma, extraction is highly selective for histamine. When whole blood filtrates are treated similarly, other substances, particularly ergothioneine, are also extracted to some extent by butanol and these substances must be treated as interferences in the subsequent analysis.

Histamine is adsorbed from the butanol extract by an ion exchange medium which contains free carboxyl groups. McIntire proposed the use of cotton acid succinate. In our hands the cotton ester was found to vary in efficiency and, for quantitative recoveries, it was necessary to reduce the flow of the butanol solution through the column to an impractical rate. The substitution of Amberlite IRC-50, a resin of the carboxylic acid type which has similar adsorption properties, for the cotton ester was found to facilitate the isolation of histamine. The resin was found to be inefficient in column operation but quantitative adsorption was obtained in batch operation by shaking the resin beads with the butanol solution for a specified time. Neither cotton acid succinate nor Amberlite adsorbs histamine when more than trace amounts of inorganic salts are present. Contamination of the butanol extract by the aqueous phase must, therefore, be avoided. Prevention of losses during the elution step due to the solubility of histamine in water and the presence of traces of inorganic salts on the resin beads is achieved by washing the resin free of butanol in 95 per cent ethyl alcohol alone.

It was found that the quantitative elution of histamine from the Amberlite required the eluting acid to be in contact with the resin for 15 minutes. Subsequent rinsing of the acid with sodium nitrite, necessary for the chemical analysis, provided conditions for the complete elution of histamine. The separate elution step is necessary, since the resin itself otherwise releases a large amount of material which produces color during the subsequent chemical analysis.

Rosenthal's method (3) is sufficiently sensitive for detection of 0.5 \( \gamma \) of histamine in pure solution as the red azo compound which results from the coupling of 4-imidazole ethanol with the diazotate of 4-nitroaniline. For our purposes it was necessary to reduce the volumes and change the proportions of the reagents in order to obtain reproducible readings in the range from 0.1 to 0.5 \( \gamma \).
Quantitative determination of these small amounts of histamine in whole blood is complicated by several sources of interference. The copper present in whole blood, presumably because it is bound to protein, does not interfere with the determination, although copper ion inhibits the coupling reaction. The fact that histamine added to intact whole blood is not recovered has been attributed to the buffer action of intact blood proteins on a wide variety of active substances, of which histamine may be one. It was found that the prior treatment of whole blood with a small amount of concentrated sulfuric acid permitted quantitative recoveries of added histamine. This acid was, therefore, added routinely to all blood specimens to prevent protein binding and to inhibit bacterial growth.

Other interfering substances which are extracted from whole blood filtrates by butanol are not removed by an initial extraction with ether and are also adsorbed by the ion exchange medium and eluted under the same conditions as histamine. These include large amounts of a number of blood extractives. Analysis of whole blood from the pig failed to reveal histamine, although at least one other compound, presumably ergothioneine, was extracted and gave appreciable color on analysis. Such interference is minimized by prolonged heating of the eluate with a higher concentration of nitrous acid than was originally suggested by Rosenthal.

Although butanol extraction removes some substances which interfere with the coupling reaction, and heating with nitrous acid minimizes the effect of other interfering substances present in the butanol extract, extraneous color still develops during the coupling reaction. Rosenthal found on analysis that of the substances in the blood filtrates which couple with 4-nitroaniline only the azo compound of histamine retains its red color at pH 7.7, while the other azo compounds range in color from yellow to orange at this pH. Blood which had been stored in the blood bank for 1 month and found to be free of histamine yielded a yellow azo compound at pH 7.7 on analysis. This yellow color showed no change over a pH range from 7.7 to 3.0. In contrast, solutions containing only the azo compound of pure histamine showed a color range from red to orange to yellow over a pH range of 7.7 to 4.0.

These color changes were utilized to correct for the extraneous color developed during the blood analysis. The tubes were read at pH 7.7, i.e. after equilibration with veronal buffer, at which only histamine produced a red color, and at pH 3.0, obtained by the addition of a measured drop of glacial acetic acid, at which all the reaction products were yellow. The difference between the optical densities of the two readings was assumed to be proportional to the amount of histamine present. Differences in optical density plotted against concentration of histamine standards gave good agreement with Beer's law. Both readings were made at a wave-
length of 525 μm, at which a maximum difference between red and yellow absorption occurs.

Reagents—All reagents must be made up in copper-free water.

1. Metaphosphoric acid, approximately 6 per cent. Prepare fresh each week and store in the refrigerator.1

2. Sodium nitrite, 4 and 1.6 per cent. Prepare fresh every week and store in the refrigerator.

3. 4-Nitroaniline, 2 per cent. Dissolve 2.0 gm. of 4-nitroaniline in 100 ml. of 4 per cent (by volume) HCl. Store in the refrigerator.

4. Diazonium working solution. Add 0.5 ml. of 4 per cent sodium nitrite to 5 ml. of cold nitroaniline solution in an ice bath immediately before use.

5. Sodium carbonate. 21.4 gm. of anhydrous salt per 100 ml. of solution.

6. Veronal buffer. 9.2 gm. of diethylbarbituric acid and 2.0 gm. of NaOH per liter, adjusted to pH 7.7 with molar acetic acid.

7. Dry salt mixture. Mix anhydrous sodium sulfate and tertiary sodium phosphate monohydrate (4) in a ratio of 6.25 to 1 gm.

8. Standard solutions. Solutions of histamine dihydrochloride in 3.8 per cent (by volume) HCl equivalent to concentrations of 0.2, 0.5, and 0.8 γ of free base per ml. These should be prepared fresh for each determination from a stock solution containing 1 mg. of free base per ml in 3.8 per cent HCl. The stock solution may be stored for 1 month in the refrigerator.

9. HCl. 3.8 per cent by volume of concentrated (37.5 per cent) HCl.

10. Amberlite IRC-50.3

11. NaOH, 20 per cent.

12. n-Butanol, reagent grade. Redistil if a blank gives color with the diazonium reagent.

13. Methyl isobutyl ketone, commercial purified grade.

Procedure

It is necessary to use calibrated constriction pipettes for all measurements of volumes of 1 ml. or less. These may be made or may be purchased from the Microchemical Specialties Company, Berkeley, California.

1 The commercial c.p. acid in pellet or stick form contains a minimum of 25 per cent sodium phosphate and is not as effective a protein precipitant as acid prepared by dehydrating c.p. orthophosphoric acid. For the preparation of metaphosphoric acid 300 ml. of 85 per cent orthophosphoric acid are heated in a 1 liter graphite crucible until the water is drawn off and fumes of phosphorus pentoxide appear. The crucible is heated for 2 additional hours. The contents are poured into a metal container with a tight fitting cover and allowed to cool. This preparation does not solidify.

2 Obtained through the courtesy of the Resinous Products Division, Rohm and Haas Company, Philadelphia.
Concentration of Histamine from Whole Blood—A 5 to 10 ml. sample of oxalated blood, collected under sterile precautions, is measured and delivered into a sterile tube. 1 drop (0.01 ml.) of concentrated sulfuric acid is added for each 2 ml. of blood and the contents are mixed. In this condition the blood may be analyzed immediately or stored at -15°C. The blood is transferred to a 50 ml. round bottomed centrifuge tube and the storage tube is rinsed with an equal volume of water and 2 volumes of metaphosphoric acid. The rinsings are added to the blood, giving approximately a 1:4 dilution. After thorough mixing the tube is centrifuged at 2500 r.p.m. (radius 30 cm.) for 15 minutes, and the clear supernatant solution is poured into a 100 ml. round bottomed centrifuge tube equipped with a ground glass stopper. The solution is made alkaline to nitrazine paper with 2 to 5 ml. of 20 per cent NaOH, and 1 gm. of dry salt mixture is added for each 3.75 ml. of the supernatant solution. A volume of n-butanol equal to the total alkaline solution is added. The tube is stoppered and shaken mechanically for 30 minutes. After centrifugation at 1500 r.p.m. to separate the layers, most of the butanol layer is transferred to another glass-stoppered tube containing 400 mg. of dry Amberlite IRC-50. The aqueous layer is extracted a second time with butanol and the extracts are combined. Removal of the last traces of the second butanol layer is facilitated by the addition of several 5 ml. portions of fresh butanol. Contamination of the butanol extract by the aqueous phase should be avoided. The combined butanol extracts are shaken mechanically for 1 hour with the Amberlite. All of the Amberlite is then transferred to a 140 X 21 mm. tube with a narrowed section 25 X 9 mm. above a stop-cock and burette tip. The stop-cock opening is covered by a small plug of glass wool. The transfer is best accomplished by pouring the butanol through the tube, the separated butanol being used to rinse the Amberlite into the tube. The tube and Amberlite are rinsed once with about 40 ml. of 95 per cent ethyl alcohol which is drained by applying gentle suction to the tip of the tube. The stop-cock is closed and 1 ml. of 3.8 per cent HCl is delivered to the Amberlite and allowed to stand in contact with it for 15 minutes. The acid is drained dropwise into a 12 X 75 mm. Pyrex tube. The Amberlite is then rinsed with 1.0 ml. of 1.6 per cent sodium nitrite, which is also delivered into the Pyrex tube. The last drops of rinse solution are blown into the Pyrex tube.

Analysis of Histamine—The tube containing the Amberlite eluate is heated in an oven at 100°C for 30 minutes. At this stage the tubes may be stoppered and stored overnight in the refrigerator, or chilled in an ice bath and analyzed immediately. To the cold Amberlite eluate in an ice bath, 0.2 ml. of the diazonium reagent is added with mixing, followed by 0.4 ml. of sodium carbonate. The contents are mixed and allowed to stand for
3 minutes. 0.25 ml. of methyl isobutyl ketone is pipetted into the tube and the contents are shaken vigorously thirty times, allowed to separate in the ice bath, and the tube is finally centrifuged. By means of a capillary pipette, the largest possible portion of uncontaminated ketone layer is transferred to another 12 X 75 mm. tube containing 1 ml. of veronal buffer measured by burette. The ketone is shaken with the buffer twenty times and allowed to stand in contact with it for 30 minutes in the refrigerator with occasional shaking. The ketone layer is then transferred to a 6 X

75 mm. colorimeter tube and centrifuged if not perfectly clear. The tube is read in the Coleman junior spectrophotometer at a wave-length of 525 m\(\mu\) against a blank prepared similarly with 1.0 ml. of 3.8 per cent HCl. For the standard curve, 1 ml. volumes of standard histamine solutions containing 0.2, 0.5, and 0.8 y per ml. are carried through the analysis in duplicate at the same time. Following these readings, 1 measured drop

3 These tubes are made from Kimble hydrometer glass tubing stock, 6 mm. outside diameter, wall thickness less than 1 mm. to fit the Coleman adapter No. 6-110. The slit in this adapter is partially filled with Plicene cement so that a volume of 0.2 ml. of ketone may be read.
(3 to 5 c.mm.) of glacial acetic acid is added to each tube and the contents are mixed. The tubes are again read at 525 μ. The concentration of histamine is proportional to the difference between the optical densities of the two readings.

**Table I**

Recovery of Added Histamine from 10 Ml. of Histamine-Free Blood

<table>
<thead>
<tr>
<th>Added histamine</th>
<th>Found on analysis</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ</td>
<td>γ</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.20</td>
<td>100</td>
</tr>
<tr>
<td>0.2</td>
<td>0.19</td>
<td>95</td>
</tr>
<tr>
<td>0.5</td>
<td>0.46</td>
<td>92</td>
</tr>
<tr>
<td>0.5</td>
<td>0.51</td>
<td>102</td>
</tr>
<tr>
<td>0.8</td>
<td>0.74</td>
<td>93</td>
</tr>
<tr>
<td>0.8</td>
<td>0.81</td>
<td>101</td>
</tr>
<tr>
<td>1.0</td>
<td>0.95</td>
<td>95</td>
</tr>
<tr>
<td>1.0</td>
<td>1.01</td>
<td>101</td>
</tr>
</tbody>
</table>

**Table II**

Chemical Determination of Histamine Levels in Human Blood

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Volume of specimen</th>
<th>Total histamine content</th>
<th>Hista-</th>
<th>per ml.</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ml.</td>
<td>γ</td>
<td></td>
<td>100 ml.</td>
<td></td>
</tr>
<tr>
<td>HN</td>
<td>Adult</td>
<td>10</td>
<td>0.16</td>
<td>1.6</td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.14</td>
<td>1.4</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td>NE</td>
<td>“</td>
<td>9</td>
<td>0.40</td>
<td>4.4</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td></td>
<td>“</td>
<td>9</td>
<td>0.42</td>
<td>4.6</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td>RL</td>
<td>“</td>
<td>7</td>
<td>0.30</td>
<td>4.3</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td></td>
<td>“</td>
<td>7</td>
<td>0.26</td>
<td>3.7</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td>PG</td>
<td>“</td>
<td>10</td>
<td>0.06</td>
<td>0.6</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.08</td>
<td>0.8</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td>760</td>
<td>“</td>
<td>10</td>
<td>0.27</td>
<td>2.7</td>
<td></td>
<td>Asthma</td>
</tr>
<tr>
<td>750</td>
<td>1½</td>
<td>7</td>
<td>0.49</td>
<td>7.0</td>
<td></td>
<td>Infantile eczema</td>
</tr>
<tr>
<td>751</td>
<td>2½</td>
<td>9</td>
<td>0.82</td>
<td>9.1</td>
<td></td>
<td>“</td>
</tr>
<tr>
<td>752</td>
<td>11</td>
<td>9.5</td>
<td>0.07</td>
<td>0.7</td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>823</td>
<td>9½</td>
<td>12</td>
<td>0.42</td>
<td>3.5</td>
<td></td>
<td>“</td>
</tr>
</tbody>
</table>

**Results**

In Fig. 1, the curve obtained on analysis of standard solutions of histamine is presented together with the logarithms of the colorimetric readings at pH 7.7 and 3.0.

In Table I the percentage recovery of standard solutions of histamine
from 10 ml. of acidified blood is given. The blood used had been stored for at least 1 month and had been found to be free of histamine on analysis. Recoveries ranged from 92 to 102 per cent.

In Table II, blood histamine levels in micrograms per 100 ml. of blood are given for nine subjects. The four duplicate determinations show a reproducibility within 10 per cent. Of the nine determinations, the two values higher than 5 γ per 100 ml. were obtained in children with infantile eczema whose white cell counts were at the upper limit of normal. The significance of the wide range in levels below 5 γ per 100 ml. is unknown.

DISCUSSION

In a limited series of subjects, a wide range in blood histamine was found (0.1 to 9.1 γ per 100 ml.). The values obtained for normal subjects fell within the lower limits of normal reported by bioassay (1); two patients with active allergy had blood levels at the upper limit of the normal. These findings are consistent both with the reported results of bioassay and with the observation that patients with allergic manifestations have high blood histamine levels (7). A study of the blood histamine levels in a large series of children with various conditions is now in progress.

Probably whole blood histamine alone is measured by the proposed method. It is less sensitive but more easily reproducible than methods based on bioassay. With careful technique, its reproducibility is within 10 per cent. The lack of analytical precision is compensated for in part by the fact that the chemical analysis permits storage of specimens over a long period of time and the procedure is less time-consuming.

SUMMARY

The histamine purification method of McIntire and the chemical method of Rosenthal and Tabor have been modified to permit the analysis of 0.1 to 1.0 γ of histamine in human blood samples of 5 to 10 ml., with an error of 10 per cent. The levels obtained by this method in a small group of subjects are reported.

BIBLIOGRAPHY

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