THE DETERMINATION OF HISTAMINE IN BACTERIAL CULTURES

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The production of histamine in bacterial cultures has been the subject of a number of investigations. According to these reports, the power to produce this amine is confined to certain strains of only a few species of bacteria: *Escherichia coli* and related organisms (Mellanby and Twort, 1912–13; Koessler and Hanke, 1919c; Hirai, 1933); *Salmonella enteriditis*, *S. schottmuelleri*, and *S. morgani* (Koessler, Hanke, and Sheppard, 1928); a variety of *Aerobacter aerogenes* designated as *B. aminophilus-intestinalis* (Bertrand and Berthelot, 1913; Jones, 1918); and *Clostridium welchii* (Kendall and Schmitt, 1926; Kendall and Gebauer, 1930).

Several methods have been used to identify histamine in cultures. Ackermann (1910), Bertrand and Berthelot (1913), Mellanby and Twort (1912–13), Hirai (1933), and Kendall and Gebauer (1930), prepared the crystalline di-picrate of the base. This method, while reliable when positive, is qualitative only, because of the large losses in purification. The process is laborious, and rather large quantities of culture must be worked up.

Mellanby and Twort (1912–13) and Jones (1918) also determined histamine in cultures by the Dale and Laidlaw (1910–11) technic, employing the isolated guinea pig uterus. Kendall and Schmitt (1926) used guinea pig intestine in a similar technic. These, as well as other physiological methods, are open to the objection that substances other than histamine may provoke the same response. As Best and McHenry (1931) point out, no one single physiological method for histamine assay is reliable; if the

physiological method is to be used, only a complete biological analysis can establish the presence of histamine.

Koessler and Hanke (1919a) determined histamine colorimetrically by the Pauly diazo reaction. As many other substances react with the diazo reagent, it was first necessary for them to isolate the histamine in a fairly pure form (Koessler and Hanke, 1919b). This was done by extracting the cultures with amyl alcohol after making them strongly alkaline. The histamine was recovered from the amyl alcohol by shaking with dilute acid. It was then determined quantitatively by means of the diazo reaction, for which they described an improved technic.

In our investigation of histamine production by bacteria, we first made use of the extraction method of Koessler and Hanke (1919b). It was soon found that this method presented certain difficulties. It was very time-consuming. It required large amounts of reagents, some of which are expensive. The colors obtained with the extracts often do not match well with the standard, especially if glucose is present in the culture medium. We therefore changed the extraction method to overcome these drawbacks.

As extracting solvent, we used a mixture of three parts of chloroform to one part of amyl alcohol. "Reagent" grades of these solvents were used; after being mixed, they were shaken with about one-fifth of their volume of 1 per cent sulfuric acid; then with several changes of distilled water. This removes impurities that would be extracted later with the histamine.

This mixture of chloroform and amyl alcohol is a more selective solvent for histamine than is amyl alcohol alone. Its use avoids the extraction of the interfering substance produced from glucose, as well as some other interfering substances.

For the extraction, we used the excellent method devised by Widmark (1926), by which quantitative extractions of small amounts of material can be made with a minimum of labor and attention. Widmark's apparatus consists of two small connected chambers; in one of these is the solution to be extracted; in the other is placed a solution in which the extracted substance is

changed to a form insoluble in the extracting solvent. A suitable immiscible organic solvent connects the two chambers; when filled, the apparatus is tilted continually, so that the extracting solvent flows back and forth between the two solutions. The apparatus described by Widmark was intended for solvents lighter than water; as the chloroform-amyl alcohol mixture is heavier than water, we devised a different form of apparatus. This consists of two glass bulbs connected by a vertical U-tube

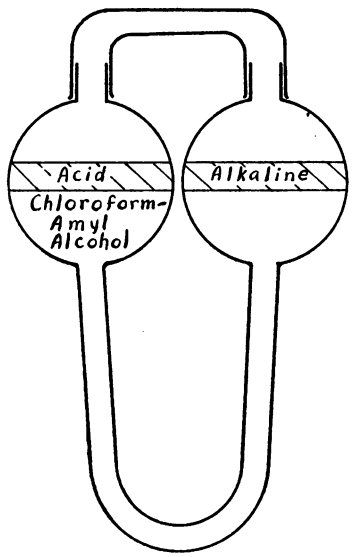

FIG. 1. DOUBLE BULB EXTRACTION APPARATUS

(fig. 1). The bulbs have a diameter of 4.5 to 5.0 cm., a size that is suitable for extracting 10 cc. of fluid. The removable tube connecting the two orifices was added to prevent loss of chloroform by evaporation.

The tilting apparatus consists of a rack with slots to hold 12 such "double bulbs." The rack is pivoted in the middle; the tilting is done by a motor with a reducing gear, which tilts the rack 8 times per minute through an angle of 8 degrees on each side of the horizontal.

By our method, four main steps are involved in the determination of histamine in bacterial cultures:

I. The preparation of the acid extract.
II. The removal of volatile bases and amyl alcohol.
III. The quantitative determination of histamine by means of the diazo reaction.
IV. The preparation of histamine di-picrate from the acid extract (not done in every case.)

I. THE PREPARATION OF THE ACID EXTRACT

About 30 cc. of the washed chloroform-amyl alcohol mixture were introduced into a double bulb. Then, 10 cc. of 0.5 per cent sulfuric acid were pipetted into one bulb. Ten cubic centimeters of the test fluid (bacterial culture) were neutralized, then made alkaline by adding 1.5 cc. of 15 per cent Na_2CO_3 solution, and introduced into the other bulb. (Stronger alkali must not be used, otherwise interfering substances are extracted and the histamine readings become too low.)

The connecting bridge over the two orifices was then put into place, and the double bulb set in a slot in the tilting rack. The apparatus was then run for 24 hours at room temperature.

As the solution to be extracted is strongly alkaline, the histamine is present as the free base. This is slightly soluble in the extracting fluid; and a little of it dissolves in the chloroform-amyl alcohol at the interface. The tilting moves the extracted histamine to the other bulb, where it comes in contact with the dilute sulfuric acid, forming the sulfate. This is no longer soluble in the extracting fluid; it enters the aqueous layer, from which it cannot return. This process continues until all of the histamine is in the acid extract. Other bases, such as ammonia and the volatile amines, will also be transported, but the great bulk of impurities will be left behind. Not the slightest trace of such substances as histidine, peptone, etc., ever appears in the acid extract. The presence of proteins in no wise interferes. The chloroform amyl-alcohol layer often becomes very turbid during the extraction; this turbidity has no significance and can be disregarded.

Sulfuric acid is the acid of choice in this extraction, as none of it will be transported to the alkaline side. Organic acids that are soluble in the choloroform amyl-alcohol will be rapidly carried over. Hydrochloric acid is transported slowly; it can be used in this extraction if allowance is made for the fact that some of it will pass over to the alkaline bulb.

Factors that influence the speed of extraction:

The first factor that influences the speed with which histamine will be extracted is the composition of the extracting fluid. Pure chloroform, in this apparatus, will extract histamine, but the rate is too slow to be of use. The addition of as little as 5 per cent of amyl alcohol greatly increases the speed of extraction. Our experience indicates that 25 per cent of amyl alcohol is most practical. With still more amyl alcohol, the speed of extraction is increased, but the specific gravity of the mixture becomes dangerously low; more interfering impurities will also be extracted.

Either the normal or the iso-amyl alcohol can be used; we found no difference. Butyl alcohol may be substituted for the amyl alcohol. Even ethyl or methyl alcohol can be utilized; with these lower alcohols, much of the alcohol will be in the aqueous layers. Instead of chloroform, one may use tetra-chlor-ethane or carbon tetrachloride. In our hands, however, chloroform and amyl alcohol gave the most satisfactory results.

Another important factor that determines the speed of extraction is the ratio of the area of the extracting surface to the volume of fluid to be extracted. This ratio should be high; in other words, the fluid to be extracted must form a *shallow* layer. In our apparatus, this layer was less than 1 cm. deep.

Other factors that influence the rate of extraction are the speed of tilting, the angle of tilt, and the internal diameter of the connecting U-tube. A large quantity of the extracting solvent must stream from one bulb to the other at each tilt of the rack. If the rate of tilting is too rapid, or if the angle of tilt is insufficient, or if the connecting U-tube is too narrow, then only small quantities of solvent will surge back and forth and the

rate of extraction is diminished. With our apparatus, 8 to 15 tilts per minute were found optimal.

We determined the rate of extraction of histamine in two different ways. By the first method, pure solutions of histamine di-hydrochloride were extracted for different periods and both the acid extract and the alkaline residue were assayed. By

TABLE 1

Rate of extraction of histamine from a solution containing 0.730 mgm. of histamine di-hydrochloride in 10 cc.

	FOUND IN ACID EXTRACT		FOUND IN ALKALINE RESIDUE	IN CHLOROFORM-AMYL ALCOHOL (BY DIFFERENCE)
	mgm.	*per cent*	*mgm.*	*mgm.*
2-hour extraction	0.370	50.7	0.240	0.120
4-hour extraction	0.490	67.1	0.150	0.090
8-hour extraction	0.690	94.5	0.020	0.020
18-hour extraction	0.730	100.0	0	0

TABLE 2

Rate of extraction of histamine from a culture of Salmonella schottmuelleri on meat extract-peptone-histidine-glucose medium

	FOUND IN ACID EXTRACT (10 cc.)		REMAINING IN ALKALINE RESIDUE* (10 cc.)
	mgm.	*per cent*	*mgm.*
2-hour extraction	0.640	28.8	1.580
4-hour extraction	1.050	47.7	1.150
8-hour extraction	1.610	73.9	0.570
12-hour extraction	1.870	83.9	0.360
24-hour extraction	2.13	98.1	0.040

* Determined by re-extracting the alkaline residue for 36 hours.

the second method, a culture of a histamine-producing bacterium was extracted for different time intervals; the acid extracts were removed and the alkaline residues were extracted again for 36 hours. The results of such experiments are shown in tables 1 and 2.

Table 1 shows that histamine is very rapidly extracted from solutions of pure histamine; in 8 hours, 94.5 per cent was removed,

and within 18 hours, all of it. Extraction of cultures, however, takes place more slowly. In 8 hours, only 73.9 per cent of what was probably the total histamine was extracted; even after 24 hours, a slight trace was left. According to table 2, 0.040 mgm. of histamine was recovered by a 36-hour extraction following a 24-hour extraction (this figure may be too high, as the color match with the standard was only fair, and some part of this color value was undoubtedly due to some substance not histamine).

When a known amount of histamine is added to a bacterial culture, the added histamine is removed quantitatively by a 24-hour extraction.

On the basis of these and other similar experiments, we have concluded that this extraction method will remove all but traces of histamine from bacterial cultures in 24 hours.

II. THE REMOVAL OF VOLATILE BASES AND AMYL ALCOHOL

As stated before, the acid extract from bacterial cultures will contain considerable amounts of ammonia and volatile amines, which react with the diazo reagent. They can be readily removed by boiling at a slightly alkaline reaction. This process at the same time removes amyl alcohol, which also interferes. Our procedure was as follows:

The acid extract was removed with a capillary pipette and the bulb rinsed with a few cubic centimeters of distilled water. To this, we added a determined amount of 2 per cent Na_2CO_3 solution, that would, after boiling, exactly neutralize to phenolphthalein the 10 cc. of 0.5 per cent sulfuric acid used in the extraction. Finally we added 0.5 cc. of 2 per cent borax solution. (This amount of borax does not interfere with the diazo reaction, though a larger amount will do so.) The fluid was then transferred to a special boiling tube (fig. 2) in which it was boiled vigorously until volatile bases no longer appeared in the vapors. To determine this point, we used as indicator a solution of Brom-Cresol-Green, which was adjusted with minimal acid until its color was a yellowish green. A platinum loopful of this indicator, held in the escaping vapors, will turn blue as long as

volatile bases are coming off. Five to 8 minutes of vigorous boiling usually suffices. The pH of the fluid should be near 9.2. The volume was then made up to 10 cc.; the fluid was now ready for the colorimetric determination.

The boiling tube shown in figure 2 was devised to prevent bumping, which is otherwise very troublesome. It is based upon the principle of creating a "hot spot" and preventing general superheating of the entire bottom. This principle has been

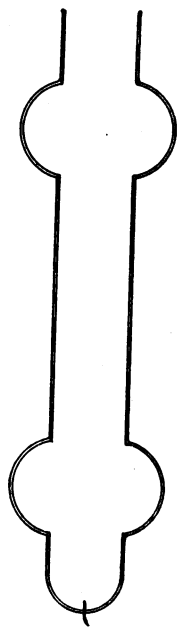

FIG. 2. BOILING TUBE WITH PLATINUM WIRE THROUGH BOTTOM

described by Moroney (1934) in connection with a quite different form of apparatus. The boiling tube was made from a thick walled Pyrex test tube, 200 by 25 mm. in size; a pin hole was blown through the bottom and a short piece of platinum wire passed through it. The hot glass was pinched down on the wire with heavy forceps, and the bottom worked in the flame until a smooth closure was obtained. The wire within the tube must be bare of glass. We have had no trouble with breakage

or leakage of these tubes. A small flame, passing through an 8 mm. hole in an asbestos gauze, is directed on the wire. Fluid can be boiled violently in this tube without any bumping. As some of our extracts frothed a good deal on boiling, the two expansions shown in the figure were blown in the tube.

Histamine solutions can be boiled for a long time in slightly alkaline reactions (pH 9.0 to 10.0) without loss of the amine. With greater alkalinity (pH 10.5 to 11.0 or more) some histamine is destroyed. We found that the removal of volatile bases by the evaporation of a neutral or alkaline solution was unsatisfactory, as there was always considerable loss of histamine. Only acid solutions of histamine can be evaporated to dryness without some loss.

III. THE COLORIMETRIC DETERMINATION OF HISTAMINE

We have followed exactly the method of Koessler and Hanke (1919a) for the diazo reaction and the colorimetric determination of histamine, which we have found very satisfactory. Our chief difficulty, at first, was that small quantities of histamine gave color values that were too low; after re-crystallizing the sulfanilic acid twice from hot water, this difficulty disappeared. For complete details, we refer the reader to Koessler and Hanke's article (1919a). Briefly, their technic is as follows:

Reagents for the test:

0.9 per cent sulfanilic acid in 10 per cent HCl.

5.0 per cent $NaNO_2$ solution.

1.1 per cent Na_2CO_3 solution.

The diazo reagent is prepared by measuring 1.5 cc. of the sulfanilic acid solution into a flask in an ice bath; then 1.5 cc. of the 5 per cent $NaNO_2$ solution are added. After 5 minutes, another 6.0 cc. of the $NaNO_2$ solution are added. In 5 more minutes, the reagent is made up to 50 cc. with cold distilled water. It is ready for use in 15 minutes.

The diazo reaction:

Five cubic centimeters of the 1.1 per cent Na_2CO_3 solution and $(1 - x)$ cubic centimeters of water are measured into a cylinder of the colorimeter (x is the volume of extract to be tested). Two cubic centimeters of the diazo reagent are added, within the space of 5 seconds, to the

alkali in the cylinder and mixed, noting the time to the second. (A stopwatch should be used.) In exactly 1 minute, x cc. of the test fluid are added and mixed. With histamine, the color is first yellow, then red; it is at its maximum in 4 to 5 minutes, after which it fades. The reading is made at the end of 5 minutes after the addition of the test fluid.

The volume x of test fluid should be chosen so that, if possible, the colorimetric reading falls between 5 and 20 mm. This can be determined by a rapid preliminary test: in quick succession, add 1 drop of the sulfanilic acid solution, 5 drops of the $NaNO_2$ solution, and 5 cc. of the Na_2CO_3 solution; then the test fluid is added drop by drop until the desired depth of color is obtained.

For standard solution we used the Congo-Red Methyl-Orange standard of Koessler and Hanke (1919a), which we also found very satisfactory. Two solutions are prepared: a Congo Red solution (2.500 grams of Grubler's Congo Red dissolved in 50 cc. of absolute alcohol, then water added to 500 cc.), and a Methyl Orange solution (0.500 grams of Methyl Orange in 500 cc. of water). The standard is made by adding 1.0 cc. of the Congo Red solution and 1.1 cc. of the Methyl Orange solution to about 250 cc. of water and diluting to 500 cc. This color standard deteriorates slowly and should be checked daily. For this purpose, a 1:10,000 solution of histamine di-hydrochloride, layered with toluene, is kept on hand; 0.25 cc. of this should give a reading of 18.7 mm.

To make the reading, the cup with the test solution is set at 20 mm.; the cup with the standard is moved until a match is found. A correction of 0.3 mm. is subtracted from the reading of the standard to allow for the color of the reagent. Tables given by Koessler and Hanke (1919a) enable one to calculate the concentration of histamine (as the di-hydrochloride) in the extract. Or the concentration may be calculated by the following formula:

$$\frac{\text{Reading of standard cup in mm.}}{\text{Volume of fluid tested in cc.}} \times 1\tfrac{1}{3} = \text{gammas of histamine di-hydrochloride per cc. of test solution.}$$

IV. THE PREPARATION OF HISTAMINE DI-PICRATE FROM THE ACID EXTRACT

As the identification of histamine is not complete unless a crystalline salt is prepared and identified by its melting point and mixed melting point with crystals of pure histamine salt, we prepared the histamine di-picrate from the acid extracts of cultures of all organisms that produced histamine in good yield. As the acid extracts are free from interfering substances, the di-picrates could be prepared and purified with ease.

It was our custom to grow each organism on several different kinds of media, and at several different temperatures. Consequently, when an organism was found positive for histamine, there were always ten or more tubes of extract available for preparing the di-picrate. After the colorimetric determinations were made, the remainders of such positive extracts were combined, adjusted to pH 6.0, and evaporated until the concentration of histamine was approximately 1 part in 1000. To every 9 cc. of this solution, 1 cc. of 10 per cent picric acid in methyl alcohol was added, and the mixture cooled over night. The di-picrates were re-crystallized from hot water 2 to 5 times until their melting points and mixed melting points were 239 to 241°C.

The dry histamine di-picrate can also be crystallized from absolute methyl alcohol. As the quantities were usually small, all crystallizations were done in small test tubes; the crystals were packed on the centrifuge and the supernatant fluids poured off. When the correct melting points were obtained, the di-picrates were dissolved in water and tested with the diazo reagent; in every case the characteristic red color was obtained.

DISCUSSION

When bacteria act upon histidine, it is quite possible that imidazols other than histamine may be produced. Inasmuch as these, if present in the acid extract, would produce a color like that of histamine, we felt it necessary to prepare and study such other derivatives of histidine, as follows:

Imidazol propionic acid. Found by Bertrand and Berthelot (1913) in cultures of their *Bacillus aminophilus.* It was prepared from imidazol lactic acid by the method of Knoop and Windaus (1905).

Imidazol lactic acid. Found in bacterial cultures by Hirai (1933). It was prepared by the method of Fränkel (1903).

Urocanic acid. Found in bacterial cultures by Raistrick (1917). It was prepared by his method from histidine by the action of *Salmonella paratyphi.*

Imidazol. Imidazol and methyl imidazol have not been described as occurring in bacterial cultures; but as they are the analogs of indol and scatol, they are possible bacterial metabolites. Imidazol was prepared from glyoxal sulfate by the method of Ruggli and Henzi (1929); the oxalate was prepared and crystallized from dilute acetone and dilute alcohol; m.p. 234°C.

4-Methyl imidazol. This was prepared by the method of Bernhauer (1929).

(4-Ethyl imidazol and 4-vinyl imidazol are also possible metabolites of histidine. We did not prepare these two substances. They should resemble 4-methyl imidazol in their properties.)

Imidazol ethyl alcohol. According to Ehrlich (1911) this substance is formed by the action of yeast upon histidine. It was prepared by the method of Windaus and Opitz (1911).

Solutions of these 6 imidazols, as well as of histidine and histamine, were studied and compared in the following ways:

1. When made alkaline and extracted in our apparatus in the usual way, only histamine, imidazol, methyl imidazol, and imidazol ethyl alcohol appear in the acid extract. Not the slightest trace of histidine, imidazol propionic acid, imidazol lactic acid, or urocanic acid will appear in the acid extract.

2. When coupled with sulfanilic acid, histamine gives an orange red color that exactly matches the Congo-Red Methyl-Orange standard of Koessler and Hanke (1919a). The color with histidine, urocanic acid, and imidazol ethyl alcohol is only slightly more orange. Imidazol lactic acid, imidazol propionic acid, and methyl imidazol yield a redder color that matches the Congo Red alone. Imidazol, on the other hand, gives a deep orange color, suggesting that, with the 4-position of the nucleus not occupied by a side chain, the coupling may take place at a different point than with the other imidazols.

3. When 1:1000 solutions of these 8 imidazols were treated with phosphotungstic and sulfuric acids, immediate precipitates

appeared with all but the imidazol lactic acid; with the latter, a small precipitate appeared on cooling. When these precipitates were removed by centrifugation, and the supernatant fluids neutralized and tested with the diazo reagent, strong colors developed in every case except that of histamine. *Of these bases, only histamine is completely precipitated by phosphotungstic acid.*

When 1:5000 solutions of these imidazols were tested with phosphotungstic and sulfuric acids, an immediate precipitate appeared only with histamine and imidazol ethyl alcohol. On standing in the refrigerator, small precipitates formed with histidine, urocanic acid, imidazol, and methyl imidazol. In dilutions of 1:10,000, a precipitate formed with histamine only.

Histamine, as Koessler and Hanke (1920) have shown, is completely precipitated by phosphotungstic acid. None of the other 7 imidazols here studied are completely removed. The presence of any of them in an extract can, therefore, be readily demonstrated by testing the supernatant fluid after phosphotungstic acid precipitation (it is necessary only to neutralize the acid). Conversely, if the supernatant fluid does not give the characteristic color with the diazo reagent, these other imidazols are absent. *We made it a practice to test every fluid that gave a histamine-like color in this way.*

As will be explained in the followed paper, in no case did the phosphotungstic acid supernatants of the acid extract contain any imidazols when the culture medium contained an organic compound of nitrogen (such as asparagine or peptone) in addition to histidine itself. But in cultures on a medium in which ammonium salts and nitrates were the only other source of nitrogen, such imidazols were frequently found (though in small quantities) in the supernatant fluids following phosphotungstic acid precipitation.

4. With Nessler's reagent, no precipitate is formed with histidine, imidazol propionic acid, imidazol lactic acid, or urocanic acid in a concentration of 1:1000. White precipitates are formed with histamine, imidazol, methyl imidazol, and imidazol ethyl alcohol. Nessler's reagent will produce a precipitate in a

1:700,000 solution of histamine di-hydrochloride. A modified Nessler's solution, made up with 16 per cent of Na_2CO_3 instead of the usual 10 per cent NaOH, will still precipitate histamine, though it will not precipitate ammonia.

5. Imidazol, methyl imidazol, and imidazol ethyl alcohol are soluble in ether; histamine and the imidazol acids are not.

6. Only histamine and urocanic acid yield picrates that are highly insoluble in water.

In addition to these imidazols, we tested the behavior of certain other substances that are likely to be present in the acid extracts, as follows:

1. Koessler and Hanke (1919c) encountered a base derived from histidine to which they ascribed the formula $HCNH_2$: $CNH_2CH_2CH_2NH_2$, though they did not isolate it. We encountered what is probably the same base; it is found most abundantly in cultures of the Friedlander-aerogenes group of organisms; it is also formed by other bacteria when histidine is the only source of both nitrogen and carbon. This base appears in the acid extracts. With Nessler's reagent, it gives a heavy white precipitate which soon turns yellow. It is incompletely precipitated with phosphotungstic and sulfuric acids. The color formed with the diazo reagent is pale yellow. The presence of this base can usually be neglected, as the color formed by it is so pale.

2. Tyramine might be formed in certain media and appear in the acid extract. As dilute solutions of tyramine are not precipitated by phosphotungstic acid, a test on the supernatant fluid would reveal it. We have never found, with the media used by us, any indications of tyramine.

3. If phenols or cresols are present in the culture, a very small fraction of them will appear in the acid extract. They would not be precipitated by phosphotungstic acid. We have never found phenols or cresols in recognizable quantities in the acid extracts of our cultures (which contained glucose and hence were acid in reaction). Hanke and Koessler (1924) also found that phenols and cresols were not formed in media that became acid.

4. Certain media, especially those that contain peptone and meat extract, will yield an acid extract containing a small amount of color-producing substance. Usually this color is pale yellow or

pale orange. When it is small in amount, it will not interfere with the determination of histamine. It is only necessary then to make a correction of the final reading for the medium. Media that require a large correction (more than 5 to 6 mm. per cubic centimeter) should be avoided.

The method described above has been used in over two thousand determinations of histamine, the results of which are reported in a following paper. This method requires a minimum of attention, labor, and reagents. An extract is obtained which contains histamine in a high state of purity; consequently, the color matches with the standard are very satisfactory. Only where the amount of histamine is very low (less than 5 gamma per cubic centimeter of culture) are the color matches likely to be "fair" rather than "excellent." Histidine and other imidazol acids never appear in the acid extract. With one medium only were histamine-like colors obtained that were in part due to some imidazol base other than histamine; that one medium contained, in addition to histidine, only ammonium salts and nitrates as a source of nitrogen. In all other media where other organic compounds of nitrogen were supplied, the characteristic color was due to histamine alone.

SUMMARY

1. A method for the quantitative extraction of histamine from small quantities of bacterial cultures is described.

2. A quantitative determination of histamine is made on this extract by the colorimetric method of Koessler and Hanke. Histamine di-picrate can also be readily prepared from this extract for positive identification.

3. Various substances that might interfere with the determination of histamine have been studied, and the means of recognizing and eliminating them indicated.

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