J. Physiol. (1951) 113, 218–227

DECARBOXYLATION OF HISTIDINE AS A SOURCE OF ERROR IN EXTRACTING HISTAMINE FROM TISSUES

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(*Received* 7 *June* 1950)

Although the acid hydrolysis method described by Barsoum & Gaddum (1935) and later modified by Code (1937) has been used by many workers for the extraction of histamine from tissues including blood, there have been a number of objections to it. Chief of these is that because of the relatively drastic treatment necessary to destroy other active substances such as adenylic acid present in the tissues, some of the histamine estimated to be present in the final extract may have been formed or released as a result of the acid hydrolysis. As expressed by Dale (1948) it is doubtful whether 'the histamine found in the product (of acid hydrolysis) will be only that which was preformed in the original blood or other material'. In particular, it is possible that histamine might result from acid hydrolysis of amino-acids and other 'incoagulable protein fractions' (Dale), including histidine and carnosine. Rocha e Silva (1944*a*) has found that histamine can be quantitatively recovered by extraction according to Code's method of pharmacologically inactive preparations containing combined histamine.

Earlier evidence on the hydrolytic formation of histamine had been reassuring. Abel & Kubota (1919) had detected no histamine formation when L-histidine hydrochloride was boiled for 2 hr. with concentrated hydrochloric acid. But the chemical methods then available for estimating histamine were not very sensitive and it is possible that small quantities of histamine were in fact formed but could not be detected. However, the acid hydrolysis mixture did not include trichloroacetic acid. In 1933, Gavin, McHenry & Wilson found that no histamine was formed from egg albumin, casein or L-histidine HCl when preparations of the substances were heated at 95° C., for 60 min. with concentrated HCl. Again, no trichloroacetic acid was present.

In 1941, Åkerblom published results showing that histidine can be readily decarboxylated to histamine by the reagents used in Code's extraction process. He examined biologically the histamine content of extracts of aqueous solutions

of histidine and of horse blood to which known amounts of histidine were added. He concluded that probably because of the trichloroacetic acid present, decarboxylation of histidine to histamine can occur 'strikingly easily' and from 10 mg. histidine in 10 ml. water extracted according to Code's method, Åkerblom reported the formation of at least 10 μg. histamine. Furthermore, the amount of histamine extracted from 10 ml. of histamine-free horse blood to which 10 mg. histidine had been added was greater than that obtained from 10 mg. histidine in water. Emmelin, Kahlson & Wicksell (1941), on the other hand, could find no histamine in extracts prepared by Code's method from aqueous solutions containing 1–10 mg. histidine/10 ml.

The problem seemed worth investigating further and this account records some observations on the occurrence of histamine in extracts of solutions of histidine in water and when added to various tissues. While these experiments were being made, Schmiterlöw (1949) was confirming and extending the results found by Åkerblom (1941).

METHODS

Extraction procedure. This has been as far as possible that given by Code (1937). He made no definite statement about the nature of the heated bath on which the acid hydrolysis was performed. Some workers originally used a sand-bath for this purpose, but it has probably been generally replaced by the water-bath. This point may be important, since it is possible that the 'bumping' and splashing which might occur when the extraction flasks were heated on a sand-bath could lead to small amounts of solution drying off on the side of the flask and being exposed to a temperature in excess of that of the boiling solution. Charring might even occur and at a sufficiently high temperature breakdown of histidine is more probable. However, Code and others were careful to note that water was added to the mixture to prevent desiccation. This is very unlikely to occur if a water-bath is used to heat the flasks, and this has been used in most of the experiments reported here.

To 10 ml. of a solution of histidine, 15 ml. of 10 % (w/v) trichloroacetic acid was added as described by Code for blood or plasma. Since filtration was unnecessary, after standing the mixture for 30 min. a further 20 ml. of trichloroacetic acid solution was added in place of the usual four washings of the filtrate.

When tissue extracts were used these were prepared in accordance with Code's directions, trichloroacetic acid being added and the tissue ground up in this with sand, in a mortar. After standing, the clear fluid was obtained by filtration with mild suction and the filter washed with trichloroacetic acid.

10 ml. of concentrated hydrochloric acid was then added to the extract or histidine-trichloroacetic acid mixture in a flask and the flasks heated under a reflux air condenser on a boiling water-bath for 90 min. Acid was removed by distillation under reduced pressure on a water-bath at 70–80°. 10 ml. of re-distilled ethyl alcohol was then added and distilled off, this being done four times in all to obtain a dry or nearly dry residue, which was taken up, as described by Code, in distilled water, neutralized to pH 7 (thymol blue) with 0·2 N-NaOH and made up to a convenient volume, usually 10 ml.

In a few experiments, some modifications were made in the usual extraction procedure to determine whether these affected the results obtained. Thus, for concentrated hydrochloric acid, normal acid was substituted in the hydrolysis, traces of metallic ions were added and in some experiments either the HCl or the trichloroacetic acid was omitted or the quantities of each varied. The effect of the presence of varying amounts of protein and of other tissue constituents on histidine decarboxylation, was studied by addition of reconstituted dried human plasma, tissue

extracts, blood or serum, before extraction of the histamine. Three samples of L-histidine HCl from three different sources were used as well as a sample of racemic histidine.

A few experiments were done to determine whether removal of the excess trichloroacetic acid from the extracts before hydrolysis affected the amount of histamine found in samples of human blood and serum.

Assays were done on duplicate samples of human blood and serum treated with trichloroacetic acid in the usual way. From one of each pair of samples excess trichloroacetic acid was removed before hydrolysis by shaking with trioctylamine, a procedure which Hughes & Williamson (1951) have shown to be effective in removing practically all the acid. Hydrolysis and final solution of the extracts were then performed in the usual way.

A blank extraction of 10 ml. distilled water was always included with each series of experiments.

Recovery of histamine from aqueous solution. Assays were also made of extracts obtained by treating aqueous solutions of histamine, to ensure that recovery was quantitative when the extraction procedure was applied to aqueous solutions instead of tissues.

Assay of histamine in extracts. The assays of the histamine activity of the extracts were made in the usual way on isolated strips of guinea-pig terminal ileum suspended in oxygenated Tyrode's solution at 37° C., to which atropine was sometimes added to give a concentration of $0{\cdot}5 \times 10^{-6}$ or 10^{-7}. As this reduced the sensitivity of the preparation considerably it was only done when spontaneous contractions of the ileum made assay difficult. The bath had a capacity of about 2 ml. and the intestine usually responded, in the absence of atropine, to the addition of 0·2 ml. or less of a solution of histamine, $0{\cdot}5 \times 10^{-7}$. At this level of sensitivity one could detect the presence of 0·02 μg. histamine (base)/ml. Sometimes the sensitivity of the preparation was sufficiently high to allow estimations of 0·01 μg./ml. The activity of the test solution has been matched against that of standard histamine solutions, test and unknown solutions being added to the bath alternately (Gaddum, 1936). We have used histamine acid phosphate in these experiments. All the quantitative references to histamine in this paper are to the base, using the conventional approximation which considers histamine acid phosphate to contain one-third of its weight of histamine base. This means that the figures for estimated 'histamine' are 8·5% less than the absolute value in terms of the base. Additional evidence that the active principle of the extracts was histamine was obtained by noting as suggested by Reuse (1948) that a specific antihistamine such as mepyramine ('neoantergan') in suitable amounts, inhibited equally the response to equi-effective doses of histamine standard and of the extract. The ileum used was usually fresh but it was confirmed that intestine which had been stored in the refrigerator for up to 3 days, and occasionally up to 5 days, could often be used for the assay after suspension in oxygenated Tyrode's solution for 1–2 hr.

Histidine determination. Histidine was determined microbiologically by a modification of the method of Barton-Wright (1946). The response of the *Leuconostoc mesenteroides* P 60 was measured by turbidimetric estimation of growth after incubation for 18 hr. The responses in the presence of test samples containing unknown amounts of histidine were compared with those obtained in the presence of known amounts of histidine. The detailed procedure is described by Hughes & Williamson (1951).

RESULTS

Histamine content of stock histidine. Repeated tests were made to confirm that the histidine samples used in these experiments did not contain histamine. Solutions of 0·1% (w/v) L-histidine hydrochloride were found to have no stimulant effect on the isolated ileum. Histidine is known to inhibit the action of histamine and it is possible that if histamine were present in the stock histidine its effect might not be apparent owing to this antihistamine action of histidine. However, in a concentration of 0·1% (w/v) (the highest used here) the antihistamine effect of histidine is negligible. Previous workers have described such an effect with 0·5 M. histidine (approx. 10% (w/v) solution)

(Halpern, 1939; Rocha e Silva, 1944*b*; MacKay, 1938; Edlbacher, Jucker & Baur, 1937).

In the figure are seen the contractions produced by small quantities of histamine made up either in Tyrode's solution alone or in Tyrode's solution containing 0·1% L-histidine hydrochloride. The presence of this concentration of histidine does not interfere with the effect of this small concentration of histamine. This is also likely to obtain when higher concentrations of histamine are present.

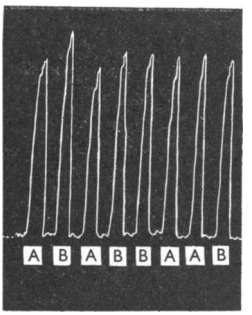

Fig. 1. The effect of $0{\cdot}5 \times 10^{-7}$ histamine is not inhibited in the presence of 0·1% histidine HCl. Contractions of guinea-pig ileum due to 0·4 ml. of solution containing (A) histamine only, (B) histamine and histidine.

Recovery of histamine from aqueous solutions. When solutions containing 10 μg. of histamine in 10 ml. of distilled water were extracted, recovery of histamine was practically complete. Twelve such extracts were examined and found to contain 10·0, 9·4, 10·2, 10·1, 9·5, 9·2, 9·7, 10·0, 10·0, 9·2, 8·0 and 9·7 μg. histamine in 10 ml. (Mean $\pm$ S.E., $9{\cdot}6 \pm 0{\cdot}15$ μg.) This confirms results which Code (1937) obtained under the same conditions.

Formation of histamine by hydrolysis of histidine solutions. Several solutions of histidine were used, containing from 0·1 to 10 mg. L-histidine hydrochloride in 10 ml. of distilled water.

From the figures given in Table 1*a* it is clear that significant quantities of histamine were formed during the extraction, according to Code's method, of aqueous solutions containing 5–10 mg. L-histidine hydrochloride. At the lower concentrations, there was rarely any detectable quantity of histamine in the extract. As can be seen, there is a considerable variation in the amount of histamine extracted in different experiments from samples of histidine solution treated exactly similarly. No satisfactory explanation can be given for this. Histamine was formed whether the hydrolysis took place on a sand-bath or water-bath. The amount appeared to be greater if the sand-bath was used (Table 1*b*).

TABLE 1*a*. The formation of histamine from aqueous solutions containing 5 or 10 mg. L-histidine HCl/10 ml. extracted according to Code's method (1937). Mixture hydrolysed on water-bath

mg. L-histidine HCl/10 ml.	Histamine content of extract (μg./10 ml.) ml. 10% trichloroacetic acid present			
	0	10	20	30 or 35
0	<0·10, <0·20, <0·10, <0·13, <0·10, <0·05, <0·10, <0·13	<0·13 <0·05 <0·13 —	<0·10 <0·10 <0·25 —	<0·07, <0·10, <0·30, <0·10, <0·20, <0·20, <0·13, <0·13, <0·25
0·1	<0·07, <0·10, <0·10	<0·05	—	<0·07 <0·30
1·0	<0·20, <0·07	<0·05, <0·13	<0·10, <0·10 <0·25, <0·25	0·10, <0·20, <0·13, <0·20 <0·20
5	— <0·07, <0·10, <0·10, <0·10,	1·76, 0·23 1·2, <0·17 <0·19, 1·18,	— 0·16, 0·16, <0·10, <0·10,	<0·30, 1·82, 4·54, 0·86, 3·4, 3·95,
10	<0·10, <0·10, 0·33, 0·10, 0·37, <0·10, <0·10, <0·10, <0·10	0·18	0·34, 0·53, 1·02, 0·30, 0·63, 0·68, 0·67, 0·25, 0·67	3·33, 2·28, 0·28, 0·30, 0·52, 1·25, 0·13.

TABLE 1*b*. Comparison of amount of histamine formed from 10 mg. L-histidine in the presence of 35 ml. 10% trichloroacetic acid, hydrolysed for 90 min. (*a*) on water-bath, (*b*) on sand-bath

	μg. histamine formed
(*a*) Hydrolysis on water-bath	2·28, 3·33, 4·54, 3·95, 0·86, 3·4, 0·13, 0·3, 0·52, 1·25
(*b*) Hydrolysis on sand-bath	8·2, <0·25, 9·48, 5·5, 4·6, 10·0, 2·27, 1·25, 1·31, 0·28, 1·54, 5·68, <0·25, 3·3, 2·9, 11·5, 2·6, 9·5

Mean values and standard error

(*a*) water-bath $2{\cdot}06 \pm 0{\cdot}52$ μg. histamine (10)
(*b*) sand-bath $4{\cdot}25 \pm 1{\cdot}22$ μg. histamine (17)

The difference between the means is not significant (*t* test) but the difference between the variations is highly significant ($F = 9{\cdot}35$).

Treatment of blanks containing 10 ml. of distilled water never resulted in an extract which had any effect on the intestine.

Factors responsible for decarboxylation. The probability that the trichloroacetic acid is responsible for the decarboxylation of histidine is supported by some of the results in Table 1*a*, whence it is seen that when 10 mg. of histidine is heated with HCl in the absence of trichloroacetic acid no histamine was found in nine out of twelve tests. However, the amount of trichloroacetic acid present does not appear to be very critical as the following typical experimental result shows. All the manipulations were done at the same time and under identical conditions. (T.C.A. signifies trichloroacetic acid.)

	Histamine (μg.)
10 mg. histidine + 35 ml. 10% T.C.A. + 10 ml. HCl	0·13
10 mg. histidine + 10 ml. 10% T.C.A. + 10 ml. HCl	0·13
1 mg. histidine + 35 ml. 10% T.C.A. + 10 ml. HCl	<0·1
1 mg. histidine + 10 ml. 10% T.C.A. + 10 ml. HCl	<0·1
10 ml. distilled water + 35 ml. 10% T.C.A. + 10 ml. HCl	<0·1
10 ml. distilled water + 10 ml. 10% T.C.A. + 10 ml. HCl	<0·1

However, the results given in Table 1*a* show that there is usually more histamine found in extracts in which a large excess of trichloroacetic acid was present.

Some further experiments on the same problem were performed by adding histidine to various amounts of reconstituted dried human plasma and then extracting these mixtures, each made up to 10 ml. with distilled water, in the usual way. The reduction of the amount of free trichloroacetic acid by the quantity required to precipitate the plasma proteins did not affect consistently the formation of histamine from the added histadine. Histamine present in the final solution was always greater in samples to which histidine had been added.

	Histamine found (μg./10 ml. mixture)	
Plasma vol. ml.	Plasma alone	Plasma + 10 mg. histidine
0	—	1·25
1	0·1	0·55
2	0·1	1·1
2	<0·1	0·4
4	0·1	0·24
4	<0·1	0·41

Other factors. The results of experiments in which normal instead of concentrated hydrochloric acid was used and others where traces of metallic salts were added to the hydrolysis mixture did not suggest that these were factors concerned in the formation of histamine from histidine.

Removal of trichloroacetic acid before hydrolysis of extracts of blood and serum. The histamine activities of the resulting samples treated in the two different ways did not differ significantly. Removal of excess trichloroacetic acid by trioctylamine, before hydrolysis, did not affect the result (Table 2). These results are in accord with those of Code who obtained figures for the histamine content of blood and plasma which agreed well with those found by Barsoum & Gaddum who removed excess trichloroacetic acid with ether, before hydrolysis.

TABLE 2. Histamine content of human blood and serum. Effect of removal by trioctylamine of excess trichloroacetic acid present in extracts, before acid hydrolysis

	Histamine activity (μg./5 ml.)	
	With T.C.A.	Without T.C.A.
5 ml. blood	0·12	0·125
5 ml. serum	<0·02	0·02

DISCUSSION

In view of the present results and those recently reported by Schmiterlöw (1949), it appears to be established that under the conditions of the extraction procedure of Code (1937), for extraction of histamine from blood and other tissues, pure L-histidine hydrochloride can be decarboxylated to yield histamine in detectable quantities, provided that the histidine is present in a concentration

of more than 0·1% (w/v). It is profitable to examine the importance of this finding in view of the concentrations of histidine likely to be found in animal tissue extracts. Free histidine in rabbit and human serum has been estimated by Schwartz, Reigert & Bricka (1938) to be 1–2 mg./100 ml. By a microbiological method Hughes & Williamson (1951) have obtained similar values for the concentration of histidine present in blood and serum from man and rabbit. These indicate that if a 10 ml. sample of whole blood from man, rabbit or cat or of serum from man or rabbit, is taken for histamine determination, it is likely to contain 0·1–0·2 mg. of histidine. Further, when human, rabbit or cat blood was hydrolysed by boiling with hydrochloric acid after precipitation of the proteins with trichloroacetic acid as described above, there was no significant increase in the amount of histidine determined microbiologically (Hughes & Williamson, 1951). On the basis of these results it is unlikely that a detectable quantity of histamine would be formed from the histidine present when blood is extracted by Code's method. The same is true for serum and presumably for plasma also.

Emmelin (1945*a*, *b*) has shown that estimates of the histamine content of extracts of plasma from the guinea-pig, rat, rabbit and cat, obtained chemically or by ultrafiltration do not differ significantly whichever method is used. Chemical extraction involving acid hydrolysis did not give samples with higher histamine content. Schmiterlöw (1949) has reported that extracts of horse blood using an alcoholic extraction procedure contain about one-half of the amount of histamine found in extracts made by Code's method. Schmiterlöw finds that the difference (0·062 μg. histamine dihydrochloride $\equiv$ 0·038 μg. histamine base/ml. of blood) is within the range of the amounts of histamine formed when he treats solutions of horse haemoglobin by Code's method. These haemoglobin solutions (0·125 g./ml.) contained about the same amount of haemoglobin as is found in horse blood, an amount which is estimated to contain 14 mg. (11% w/w) of histidine, and on extraction yielded solutions containing an average of 0·092 μg. histamine dihydrochloride ($\equiv$0·077 μg. histamine base)/ml. Schmiterlöw (1949) suggests that the differences between the estimations of histamine made by the two methods when applied to horse blood, might be due to a decarboxylation of the histidine present in haemoglobin during the extraction procedure according to Code.

However, although we, like Schmiterlöw, have been able to show a significant formation of histamine from histidine using the chemical extraction method when the histidine concentration was 1 mg./ml. (Table 1*a*), extracted solutions containing less than 0·5 mg. histidine/ml. had no histamine activity.

If it requires the formation (from haemoglobin in the instance under consideration) of 0·5–1 mg. histidine/ml. to supply enough material to give a detectable quantity of histamine by decarboxylation of part of it, then this new formation of histidine by breakdown of haemoglobin should be shown in the

estimations of histidine present in blood before and after hydrolysis. Unfortunately, this information is not available for horse blood but only for blood from the cat, rabbit and man. The increase required according to the above analysis is of the order of 0·5–1 mg./ml., or 50–100 mg./100 ml. In human blood the normal histidine content is about 1·5 mg./100 ml. and this is unchanged after acid hydrolysis. It is impossible to apply Schmiterlöw's argument to the blood which we have tested as no check of its histamine content was made. The discrepancy between his conclusions based on results with horse blood and ours on blood from other species remains unexplained. It may be due to some difference in the haemoglobin of these species. One other point of contrast between Schmiterlöw's method and those used here is that he heated his hydrolysis mixtures over an open flame and we have used a water-bath for the greater part of this work, including the mixtures in which changes in histidine content were followed.

Code's results and those of our few experiments suggest that the quantity of histamine found in blood, plasma or serum is unaffected by the presence of trichloroacetic acid during the acid hydrolysis.

It would appear then that none of the histamine estimated to be present in samples of blood and serum from man, rabbit or cat is likely to have been formed during the extraction process described by Code.

The situation is rather different from some tissues other than blood. In striated muscle there may be a very considerable increase in the amount of histidine present in the extract after hydrolysis. Some of this histidine may be decarboxylated and contribute to the apparent histamine content of the extract. For example, 5 g. of cat's skeletal muscle contains, before hydrolysis, about 0·3 mg. of histidine, but after hydrolysis the amount is increased to 6·5 mg. (Hughes & Williamson, 1951), an amount from which a detectable quantity of histamine may be formed during the hydrolysis, according to the results presented above.

Hughes & Williamson have also shown that this increase in the amount of histidine present after hydrolysis can be explained by the breakdown of carnosine present in muscle. This action can occur in the presence of hydrochloric acid alone, in the absence of trichloroacetic acid. The amounts of histidine formed are considered unlikely to be sufficient to provide a concentration which might inhibit the effect on guinea-pig ileum of any histamine present in the extract. If this were not so it would mean that the actual amounts of histamine in the final extract would be even higher than those found.

There was no increase in the histidine content of extract of cat's gastric mucosa after hydrolysis. The histidine content of gastric mucosa is low, of the same order as that found in blood (Hughes & Williamson, 1951), so that the histamine estimated to be present in mucosal extracts is unlikely to have been derived from histidine during the chemical extraction.

In conclusion, it may be said that although formation of histamine by decarboxylation of histidine in aqueous solution can be demonstrated if the histidine concentration exceeds 0·05–0·1%, this finding does not indicate any significant error from this cause when histamine is estimated in whole blood rather than plasma from man, cat or rabbit. However, especially in the rabbit, in which most of the blood histamine has been shown to be present in the platelets, there may be other reasons against the use of whole blood when estimating histamine after a chemical extraction procedure. Schmiterlöw's evidence indicates that estimates on horse blood are also erroneous.

It seems clear that chemical extraction of skeletal muscle involves formation of some histamine and the histamine content of the extract probably does not represent the amount originally present.

SUMMARY

1. Acid hydrolysis by Code's method (1937) of solutions containing 5–10 mg. of histidine hydrochloride in 10 ml., may result in the formation of significant, but variable, quantities of histamine.

2. The decarboxylation of histidine during the extraction is probably due to the trichloroacetic acid present.

3. Since whole blood, serum and plasma from man, cat and rabbit contain only 0·1–0·2 mg. of histidine in 10 ml., and since there is no evidence of any new formation of histidine during the extraction, decarboxylation of histidine is not a source of error in the extraction and estimation of histamine in these fluids.

4. The extraction method is unsuitable for the estimation of histamine in skeletal muscle, owing to the formation of histamine from carnosine during the hydrolysis.

5. Decarboxylation of histidine is unlikely to be a source of error in the determination of histamine in chemical extracts of cat's gastric mucosa.

We wish to thank Professor E. J. Wayne for helpful criticism and discussion.

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