CLXII. HISTAMINASE.

I. THE HISTAMINE-HISTAMINASE REACTION. II. PURIFICATION. III. SPECIES DISTRIBUTION.

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HISTAMINASE is the name which has been applied [Best and McHenry, 1930] to a histamine-inactivating enzyme. Originally found by Best [1929] to be present in ox and horse lung, investigations on the histaminase content of dog tissues showed it to be most plentifully contained in the kidney and in the intestinal mucosa. These studies, utilising fresh tissue *in vitro*, led to perfusion experiments in which it was found that isolated dog kidneys could inactivate large amounts of histamine. The obvious suggestion has been made that this enzymic mechanism, functioning principally in the kidney and in the wall of the small intestine, may constitute one of the body's protections against harmful amounts of histamine.

Some of the properties of histaminase have been previously described. It is heat-labile, being irreversibly inactivated at 60°. For its activity the optimal temperature is 38°, and the optimal p_H range is from 6.9 to 8.0. The enzyme is inhibited slightly by ammonium and magnesium ions, and markedly by calcium, while it is not affected by sodium and potassium. Of the anions, chloride is without effect, sulphate and citrate are inhibitory, and phosphate definitely augments the reaction. As it was found that the histamine-histaminase reaction does not occur in presence of $M/2000$ potassium cyanide, proceeds very slowly in an atmosphere of nitrogen, and is accelerated by oxygen, it has been thought to be oxidative. Under certain conditions, if not always, the enzyme causes disruption of the iminazole ring in the histamine molecule.

Employing the Barcroft-Warburg technique, Gebauer-Fuelnegg and Alt [1932] have measured oxygen consumption during the histamine-histaminase reaction. The enzyme was prepared from dog kidney. No simple molar relation was demonstrated between the amount of oxygen consumed and the amount of histamine inactivated. Three possible discrepancies were noted by these authors between their conclusions, based on oxygen-uptake measurements during the histamine-histaminase reaction, and results obtained from other methods of investigation, which had been reported from this laboratory:

(1) in their experiments more histamine was inactivated at p_H 10 than at 7.4; (2) more histamine was inactivated at 50° than at 37°; (3) potassium cyanide did not completely inhibit the reaction. Since the publication of these results we have repeated the measurements of inactivation at varying temperatures and at varying hydrogen ion concentrations and have found no disagreement with our earlier results.

Several observations by Gebauer-Fuelnegg and Alt are of interest. The enzyme seems to be specific for histamine, as indicated by the lack of oxygen-uptake when active kidney-powder was incubated with several other iminazole derivatives. We had previously found that histaminase does not affect tyramine, an amine possessing the same side-chain as is found in histamine. The results of the brief study by Gebauer-Fuelnegg and Alt of species distribution of histaminase are not in agreement in the case of several species with the data presented in the present paper.

This communication gives results of further studies on the histamine-histaminase reaction, descriptions of some purification procedures and data on the variation in the histaminase contents of kidneys of several species of animals.

As a source of histaminase, a dry powder prepared from ox kidney by extraction of the fats with acetone, removal of acetone with ether and subsequent drying of the tissue was employed in earlier experiments. One powder prepared in this manner showed no loss of activity during storage for one year at laboratory temperature. To secure a stable powder it is essential that the acetone extraction be thorough and that the powder be rapidly dried to remove ether. More recent work has shown that hog kidney is a better source and histaminase powder is now prepared from that tissue by the method described above. The enzyme is removed from the powder by extraction at 38° with phosphate buffer of p_H 7.0 to 7.2. The amount of enzymic action is determined by measuring the amount of histamine remaining after incubation of a mixture containing a known amount of histamine and the tissue or enzyme preparation. All determinations of histamine have been made by the blood-pressure method in etherised cats. For the assays solutions were prepared from the mixtures after incubation by the hydrochloric acid procedure [Best and McHenry, 1930].

I. THE HISTAMINE-HISTAMINASE REACTION.

The results of preliminary work on the rate of the reaction indicated that (1) after mixture of certain proportions of histamine and enzyme, there was, previous to actual inactivation, a phase during which no change in histamine, as shown by the depressor activity, occurred; (2) when the quantity of histamine used was constant, the total amount of amine destroyed depended on the concentration of enzyme; (3) the amount of histamine inactivated by a constant amount of enzyme was not markedly affected by the histamine

concentration. The second and third observations have been confirmed by Gebauer-Fuelnegg and Alt.

More accurate measurements of the rate of inactivation have been made in an attempt to elucidate the nature of the changes occurring. The general method was as follows: a solution of histaminase prepared by extracting kidney powder with phosphate buffer was added to a solution of histamine acid phosphate in phosphate buffer at p_H 7.2. Toluene was used as a preservative and the mixture was kept, with continuous agitation by means of a stirrer or a current of gas, at 38° in a water-bath. Samples for histamine determinations were removed at intervals through a siphon dipping beneath the toluene.

In the first series the proportion of enzyme to histamine was such that the amine would be inactivated in about 24 hours. A histaminase solution was prepared by extracting 30 g. of ox kidney powder with 270 cc. phosphate buffer, p_H 7.2, for one hour with continuous agitation. After filtration 75 cc. were added to phosphate buffer, p_H 7.2, containing 30 mg. histamine acid phosphate (10 mg. base) the volume was made up to 500 cc. with buffer and 50 cc. of toluene were added. There was a definite preliminary period of 5 hours during which no change in the depressor activity of the solution could be detected. The values secured during the second, inactivating phase lie fairly well on a smooth curve (Fig. 1). On semilogarithmically ruled paper the inactivation phase is represented by a straight line. The values of k in the equation $k = \frac{1}{t} \log \frac{a}{a-x}$, where a = initial amount of histamine and x = amount inactivated, have been calculated. When this is done, using for t values from the initial admixture, k is not a constant. If the values of t be corrected by subtracting the length of the lag phase from the elapsed time, k is constant (Table I). As judged from these values for the velocity constant, the reaction is unimolecular.

Table I. *Velocity constant of histamine-histaminase reaction.*

t	$t_1 = t - 5$	a	$a - x$	$k = \frac{1}{t_1} \log \frac{a}{a-x}$
6 hrs.	1	10 mg.	8.5 mg.	0.071
8 "	3	10 "	6.4 "	0.065
10 "	5	10 "	4.7 "	0.066
12 "	7	10 "	3.5 "	0.065
14 "	9	10 "	2.5 "	0.067
16 "	11	10 "	1.6 "	0.072

Determinations of a similar nature have been carried out with varying proportions of enzyme. These all showed the two phases. The duration of the lag phase was inversely proportional to the concentration of histaminase, and the rate of the inactivation phase was directly proportional to the concentration. In each case the rate of the second phase was logarithmic.

Earlier work had shown that in the absence of oxygen and when nitrogen or hydrogen was present almost exclusively the histamine-histaminase reaction

did not occur. Determinations of the rate, similar to those previously made, were carried out with large amounts of oxygen bubbling through the mixture. In these the length of the lag phase was one-half that found without the stream of oxygen but with the same concentration of enzyme and of histamine. No great change was observed in the second phase. In another experiment three sets of measurements were made on the same day using portions from one solution of enzyme in each set. Through one mixture air was bubbled, through the second oxygen and through the third nitrogen. The results are shown in

Fig. 1. Rate of inactivation of histamine.

Fig. 2. Effect of nitrogen and oxygen upon histaminase.

Fig. 2. The rates for air and oxygen are practically identical but nitrogen definitely slowed the reaction, scarcely any inactivation occurring.

It had previously been found that low concentrations of potassium cyanide prevented the inactivation of histamine. It seemed advisable to determine whether the addition of cyanide to a previously cyanide-free mixture in which inactivation was progressing would stop any further action of the enzyme. For this three flasks were used. Each flask contained 75 cc. histaminase solution (the filtrate secured after extraction for one hour of 60 g. of ox kidney powder with 540 cc. phosphate buffer), 30 mg. histamine acid phosphate (10 mg. base), sufficient buffer of p_H 7.2 to make a volume of 500 cc. and 50 cc. toluene. The results are shown in Fig. 3. In the first case 0.002 *M* potassium cyanide was initially present and no inactivation occurred. In the second case an equal amount of cyanide was added when 25 % destruction had taken

place, and it is evident that further inactivation was inhibited. The third curve demonstrates that this particular enzyme preparation was acting in the customary manner. In view of the results of Gebauer-Fuelnegg and Alt [1932] which suggested incomplete inhibition by cyanide it is interesting that we have repeatedly found cyanide to prevent the initiation of the inactivation and to cause its cessation when added at an intermediate stage.

Since cyanide had an inhibitory effect upon the enzyme it seemed desirable to investigate the action of sodium pyrophosphate. In a concentration of 0.05 *M* at p_H 7.2 this salt has no effect upon the histamine-histaminase reaction. As has been suggested by Gebauer-Fuelnegg and Alt, iron is probably excluded as an active agent, while copper may be a factor.

Fig. 3. Effect of cyanide upon histaminase.

Fig. 4. Effect of concentration of phosphate upon histaminase.

Of the various ions investigated, phosphate alone has a definitely accelerating effect upon the action of histaminase. This has been studied quantitatively and the results are given in Fig. 4. Phosphate is not essential for the reaction, which proceeds in the absence of this ion, but the amount of histamine inactivated by a constant amount of enzyme in a constant time is increased when phosphate is present. There is no definite concentration of phosphate which may be regarded as optimal, rather there is an optimal range. The amount of phosphate present in our routine measurements of histaminase action lies within this range.

The changes produced in histamine by histaminase are of considerable interest. In the case of the amine, tyramine, Ewins and Laidlaw [1910] found that, after removal of the amino-group from the side-chain, the remnant was

oxidised to the alcohol and then to the acid. Guggenheim and Loeffler [1916] showed that similar reactions occurred in the cases of phenylethylamine, indolethylamine, and *iso*amylethylamine. They were unable to isolate iminazoyl acetic acid after inactivation of histamine. In view of the destruction of the iminazole ring previously reported [Best and McHenry, 1930], the failure of Guggenheim and Loeffler in the case of histamine is explicable. In experiments with tyramine and tyramine oxidase conducted *in vitro*, Hare [1928] found that deamination occurred but that only half the nitrogen present in the tyramine molecule could be accounted for as ammonia. In an extension of this work [Bernheim, 1931] the same author showed that the course of oxidation of tyramine varied under different conditions. Under certain conditions, *p*-hydroxyphenylacetic acid was produced, and in this case there was complete deamination of tyramine. A quantitative study of ammonia production was not completed. To gain some information on the changes produced in histamine, measurements have been made of ammonia produced during the reaction.

To determine the amount of ammonia various methods have been tried. The procedure found most useful was, after the customary incubation of a mixture of kidney powder and histamine in phosphate buffer, to filter this mixture through Whatman No. 41 paper. The ammonia content of a 10 cc. aliquot portion of the filtrate was measured, using the Van Slyke and Cullen aeration apparatus. In nearly every case duplicates were aerated and afterwards the ammonia from one was determined by titration and from the other by nesslerisation.

In all the ammonia determinations the source of histaminase was a potent hog kidney powder, of which 50 mg. would inactivate 2 mg. histamine in 24 hours at 38°. For each determination 1 g. of this powder and 120 mg. histamine acid phosphate in 100 cc. phosphate buffer were employed. Initially the incubation was carried out in buffer solutions at p_H 7.2 but later two other hydrogen ion concentrations were used. The amount of residual histamine in each case was measured in the customary manner. In place of toluene, "merthiolate"¹ in 0.01 % concentration was used as preservative.

The first series was designed to measure the ammonia produced during the histamine-histaminase reaction under the conditions normally used by us: p_H 7.2, 24 hours' incubation at 38°. At first controls were flasks in which the enzyme had been inactivated by boiling and in these some ammonia was found. The difference between the ammonia content of the active enzyme flask and the boiled control was not of a simple molar nature and it was found that this type of control was not satisfactory. Although there is no histamine in the kidney powder, incubation of the powder in buffer without added histamine gives a demonstrable amount of ammonia, produced from the powder during the incubation, and appreciably greater than the amount found in a boiled control. Consequently similar mixtures of unheated powder and buffer with preservative were used in all subsequent work as controls. Table II, A gives results secured at p_H 7.2.

¹ The Eli Lilly Co.

Table II. *Ammonia formation.*

Histamine inacti- vated mg.	Total possible ammonia from histamine mg.	Ammonia found (mg.)			Ammonia produced from histamine mg.	Ammonia found in % of theoretical
		Titration	Nessler	Average		
A. At p_H 7.2:						
2	0.90	0.61	0.67	0.64	0.29	32
0	0	0.32	0.37	0.35		
2	0.90	0.59	0.61	0.60	0.29	32
0	0	0.29	0.33	0.31		
3	1.35	0.79	0.78	0.79	0.46	34
0	0	0.31	0.35	0.33		
2	0.90	0.57	0.65	0.61	0.32	35
0	0	0.27	0.31	0.29		
4.0	1.80	0.90			0.56	31
0	0	0.34				
1.5	0.68		0.44		0.19	28
0	0		0.25			
					Average = 32	
B. At p_H 5.9:						
1.6	0.72	0.39	0.37	0.38	0.15	21
0	0	0.23	0.22	0.23		
1.6	0.72	0.33	0.29	0.31	0.14	19
0	0	0.17	0.16	0.17		
1.8	0.81	0.55	0.47	0.51	0.15	19
0	0	0.36	0.35	0.36		
1.4	0.63	0.29	0.27	0.28	0.12	19
0	0	0.17	0.15	0.16		
2.0	0.90	0.53	0.44	0.49	0.15	17
0	0	0.35	0.33	0.34		
1.6	0.72	0.34	0.31	0.33	0.14	19
0	0	0.19	0.18	0.19		
2.0	0.90	0.67	0.54	0.61	0.22	24
0	0	0.40	0.38	0.39		
1.8	0.81	0.35	0.35	0.35	0.15	19
0	0	0.19	0.21	0.20		
2.0	0.90	0.64	0.59	0.62	0.20	22
0	0	0.42	0.42	0.42		
					Average = 20	
C. At p_H 8.8:						
3.6	1.62	0.70	0.65	0.68	0.47	29
0	0	0.21	0.21	0.21		
4.0	1.8	0.91			0.49	27
0	0	0.42				
4.0	1.8	0.74			0.53	29
0	0	0.21				
4.0	1.8	0.91			0.49	27
0	0	0.42				
2.0	0.90	0.40	0.41	0.41	0.23	26
0	0	0.17	0.19	0.18		
2.0	0.90	0.48	0.43	0.46	0.27	30
0	0	0.20	0.18	0.19		
3.5	1.61	0.65	0.66	0.66	0.46	29
0	0	0.19	0.21	0.20		
3.1	1.40	0.62	0.55	0.59	0.41	29
0	0	0.19	0.17	0.18		
					Average = 28	

The average amount of ammonia found was 32 % of the quantity which could be produced if all three nitrogen atoms in the histamine molecule had been liberated as ammonia. Under these conditions, then, only one nitrogen, probably from the side-chain, is liberated as ammonia.

It was imperative to make sure that the procedures used were giving reliable results. Aeration of ammonium sulphate solution and of kidney solution containing added ammonium sulphate showed that the analytical method was accurate. Moreover recovery of ammonium sulphate which had been added to digests before incubation indicated that ammonia was not lost during incubation. It is reasonable to conclude that the amount of ammonia measured is the total amount produced during the histamine-histaminase reaction under the experimental conditions.

Determinations were made of ammonia production at two other hydrogen ion concentrations, all other conditions being as similar as possible to those used at p_H 7.2. The results are given in Table II, B and C. As before, control experiments showed that ammonia was quantitatively recovered. While the ratio of ammonia produced to histamine inactivated at p_H 8.8 was slightly lower than at p_H 7.2 the difference is too small to be significant and again one molecule of ammonia was formed per molecule of histamine inactivated. At the other p_H , 5.9, this is obviously not true, the amount of ammonia being much less than at 7.2 and at 8.8. The explanation of this is not obvious at present. Since the amount of ammonia found is less than would be expected if all the inactivated histamine had been deaminised it is reasonable to conclude that a mechanism other than deamination is operative. Previous work in this laboratory had shown that under certain conditions there was a rupture of the iminazole ring. In the histamine-histaminase reaction both deamination and a change in the nucleus may take place and the relative intensities of these two processes may not be the same at different hydrogen ion concentrations.

The rate of ammonia formation in comparison with the rate of loss of physiological activity at p_H 7.2 was measured using a procedure similar to that previously described for velocity measurements. At definite time intervals portions were removed from the incubated mixture and measurements made of histamine and of ammonia present. The control again consisted of a kidney extract without added histamine and the ammonia from histamine was calculated as the difference between that of the histamine mixture and of the control. The results are given in Fig. 5. Calculations were made of the ammonia theoretically possible at each interval, assuming that one molecule had been produced for each molecule of histamine inactivated. Between the curve for these values and that for experimental results there is good agreement. At this p_H , then, the rate of ammonia production is in accordance with the velocity of histamine inactivation.

It seemed possible that urea might be formed during the reaction by a cleavage of the iminazole nucleus. Urea determinations in inactivated mixtures were made by the urease method and it was found that no compound which

could be decomposed by urease to give ammonia had been formed. Urea added to the solution was quantitatively determined by the method, indicating that the urease had not been inhibited by a constituent of the mixture. It may be that a urea derivative which could not be split by urease is formed but certainly urea itself is not produced during the histamine-histaminase reaction.

Fig. 5. Rate of ammonia formation.

II. ATTEMPTS TO PURIFY HISTAMINASE.

Since it is essential to prepare from kidney powder a solution containing the enzyme, conditions for increasing the yield on extraction have been studied. Water gives a potent extract, but the yield is greater when phosphate buffer at p_H 7.0-7.2 is used. Solutions thus secured are unstable but may be stabilised by the addition of glycerol, sufficient to give 15 % concentration. Extracts prepared at 38° are more potent than those at room temperature and these in turn are more potent than those secured by extraction in the cold. Solutions prepared by extracting at $p_H < 5$ are inactive and are not activated by neutralisation after extraction. A slightly increased yield is secured by extraction at p_H 8 but the alkaline solution filters very slowly. Larger yields are given by fairly dilute extraction, and we have found a suitable proportion to be 18 parts of liquid to one part of kidney powder. One such extraction at 38° gives about 60 % of the total potency of the powder, and a further 20 % results from a second extraction. The combined extracts may be concentrated *in vacuo* to one third the original volume without loss in activity, provided that the temperature of the bath surrounding the distillation flask be kept below 50° .

Three methods for the precipitation of histaminase have been investigated. If the concentrated, aqueous solution be poured slowly, with stirring, into a large volume of acetone at room temperature, a precipitate is secured which contains about 70 % of the total activity. This precipitate, after decantation of the acetone, dries quickly in air, and is relatively stable. If alcohol, ether, or mixtures of alcohol and ether are used in place of acetone the precipitate

produced is inactive. Precipitates which settle out from the original aqueous solution after adjustment to p_H 5 are also inactive. Four salts have been tried as precipitating agents. Sodium chloride in 25 % concentration at room temperature and sodium sulphate in various concentrations up to saturation at room temperature do not precipitate any active histaminase, despite the fact that the latter salt gives a heavy precipitate. When a solution is saturated with ammonium magnesium sulphate, histaminase is completely precipitated. This precipitate may be dried in a vacuum oven at temperatures below 50° without decrease in potency, and the dry powder is fairly stable, retaining its activity for at least a month.

The ammonium sulphate precipitate contains much sulphate, but a precipitate with low sulphate content is secured by using 80 to 90 % saturation. At this concentration of ammonium sulphate histaminase is completely precipitated. The conventional method for removing salt by dialysis results in a loss of about 70 % of the enzymic activity. A procedure developed by Brieger and Cohn [1893], and suggested to us by Dr P. J. Moloney, for the separation of tetanus antitoxin from ammonium sulphate has proved useful. This is based on differences in specific gravity. The salt-protein mixture is rubbed up with chloroform, more chloroform added and the whole centrifuged. Two solid layers separate: at the top proteins, and at the bottom the salt. This procedure gives good results with the histaminase precipitate. The top layer is poured on a hard filter-paper and allowed to dry in air. The resulting fine, tan-coloured powder contains over 70 % of the potency. Its activity is about ten times that of the original kidney powder, and it is relatively stable. While this procedure is an improvement on previous methods, it has not yet been possible to prepare histaminase in highly concentrated form.

III. DISTRIBUTION OF HISTAMINASE IN THE KIDNEYS OF VARIOUS SPECIES.

It had previously been shown that, in the dog, histaminase was present in largest amounts in the kidney and in the intestinal mucosa. It seemed desirable to determine the variation in kidney histaminase among several species of animals. For this, where possible, normal animals were placed under ether anaesthesia and the kidneys removed. In some cases tissues were secured from abattoirs as soon as possible after the death of the animal. The human kidneys were autopsy specimens. In larger animals each kidney was tested separately while in smaller animals it was necessary to combine the kidneys from several animals to secure adequate amounts. After finely mincing the tissue, portions were weighed and transferred to 500 cc. Erlenmeyer flasks. In every case 5 g. of tissue were used for each determination. To each flask were added 100 cc. phosphate buffer, p_H 7.2, the required amount of histamine acid phosphate, and 15 cc. toluene. The mixture was incubated for 24 hours at 38° and the residual histamine determined by physiological assay. With each set controls were run, in which the enzyme had been inactivated by heating to boiling.

These controls acted as checks upon the total amount of histamine present, both added and contained in the tissue originally. The amount of histamine inactivated by a given amount of enzyme varies somewhat with the amount of histamine initially present. In each case determinations with varying amounts of histamine were made. The maximum inactivation was secured when 150 mg. of histamine acid phosphate (50 mg. histamine) were used with 5 g. kidney. The results in Table III are based on this proportion, and are averages of determinations made on four or more members of each species.

Table III. *Histamine inactivated by kidneys from various species of animals.*

Animal	Mg. histamine inactivated by 1 g. kidney
Rat	0
Guinea-pig	0
Rabbit	1.6
Dog	1.9
Cat	2.0
Ox	1.6
Hog	3.6
Horse	2.7
Sheep	3.2
Monkey	2.4
Human	0-1.6
Chicken	0

There is no apparent correlation between these results and available figures for histamine distribution in various species (Raxlen, unpublished data), or for sensitivity to histamine [Schmidt and Stähelin, 1929]. Among the individuals in any one species there is considerable variation, as shown in Table IV.

Table IV. *Kidney histaminase variation in members of a species*

Animal	Mg. histamine inactivated by 1 g. kidney	Average
Sheep	4.0, 2.8, 3.2, 4.0, 2.0, 3.2	3.2
Ox	1.2, 2.0, 1.2, 2.0, 1.2, 2.0	1.6
Hog	4.0, 2.8, 3.2, 3.2, 5.2, 3.2	3.6

As far as could be ascertained, the kidneys used in these distribution studies were normal. It would be interesting to investigate the histaminase content of kidneys in various pathological conditions. Preliminary experiments, using cats and dogs, were made in an attempt to alter the histaminase content of the kidney by extended administration of histamine subcutaneously, or by holding the animal in histamine shock. The results have been inconclusive. There has been no marked depletion in kidney histaminase after shock nor has it been found possible to increase kidney histaminase in dogs by giving histamine in small daily doses over a long period.

SUMMARY.

Previous to the actual inactivation of histamine by histaminase there is a lag period, the length of which varies inversely with the enzyme concentration. The rate of the inactivation is logarithmic and, as judged from values secured for the velocity constant, the reaction is unimolecular. The inactivation

is accelerated in an atmosphere containing oxygen and is inhibited in an atmosphere deficient in oxygen. Small amounts of potassium cyanide not only prevent the initiation of the reaction but cause its cessation when added at an intermediate point. Sodium pyrophosphate has no inhibitory effect and the phosphate ion augments the reaction. Measurements of the ammonia production from histamine show that at p_H 7.2 and 8.8 histaminase causes the liberation of one nitrogen atom, presumably that of the side-chain, as ammonia. At p_H 5.9 less ammonia is produced. While attempts at purification have not as yet yielded a very concentrated preparation, information on a number of the properties of histaminase has been obtained. An investigation of kidney-histaminase in various species has shown that it is not demonstrable in the rat, guinea-pig and chicken, and is present in varying amounts in several mammals. Of the tissues so far investigated, hog kidney is the best source of histaminase and a method has been described for the preparation from this tissue of a stable powder which may be used in the study of histaminase.

This investigation has been carried out under the helpful direction of Prof. C. H. Best, whose advice has been greatly appreciated. It is a pleasure to acknowledge the technical assistance of Miss Neta Thompson. The preparation of kidney powder was made possible by the use of large-scale equipment in the Connaught Laboratories, kindly placed at our disposal by the Director, Dr J. G. FitzGerald.

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