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TRANSGLUTAMINASE AND HISTAMINE INCORPORATION *IN VIVO**

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Abstract—Incorporation of histamine into liver protein of mice was investigated; the activity of transglutaminase, the enzyme that catalyzes the incorporation *in vitro* of amines into protein was determined simultaneously. Although the amount of histamine bound to liver protein is negligible in untreated control mice, after the administration of *Salmonella typhosa* endotoxin and *Haemophilus pertussis* vaccine, agents which increase transglutaminase activity in mouse liver, a definite fixation of histamine in liver proteins was found. The increase in enzyme activity and the amount of protein-bound histamine showed a significant correlation. When the incorporation of the amine was studied with ^{14}C, the relatively low radioactivity of the incorporated histamine suggested an extensive dilution of the labeled amine by unlabeled histamine. Methods for the determination of small amounts of protein-bound unlabeled histamine and of labeled histamine from ^{14}C-labeled proteins are described.

AS DISCUSSED in the preceding report,[3] difficulties were encountered in demonstrating transglutaminase activity *in vivo* despite its unequivocal action *in vitro*. After the injection of unlabeled histamine into normal mice, and also after that of ^{14}C-histamine of a specific activity deemed sufficient to indicate its fixation on protein, no significant increase of labeled or unlabeled protein-bound histamine was found in the liver protein. Since we are dealing with a Ca^{2+}-dependent enzymatic reaction, it was thought that the distribution of enzyme, Ca^{2+}, and substrate in the cell may not permit interaction of the partners of the enzymatic system with the administered amine; but even when histamine was administered and the Ca^{2+} metabolism was disturbed by the administration of parathyroid hormone and thioacetamide, no increased fixation was found. In another series of experiments, histamine, labeled or unlabeled, was administered in large doses with negative results. The possibility had to be considered that the administered amine did not enter the amine pool in close proximity to the enzyme system.

It was therefore decided to search for conditions under which the activity of transglutaminase may be increased, in the hope that then a fixation of amine in proteins might be demonstrated. For reasons presented in the preceding paper, the effect of pertussis vaccine and various bacterial endotoxins was studied and a considerable

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increase in enzyme activity found after the administration of these agents. As described in the present report, in mice so treated, the administration of histamine led to a fixation of significant amounts of amine in liver proteins.

In the first experiments, histamine fixation in female mice treated with *H. pertussis* vaccine was demonstrated. Since a larger increase of the enzyme after administration of the endotoxin fraction from *S. typhosa* occurred within 24 hr after injection, and male mice showed in various aspects a more reproducible response than female mice, experiments were continued with males and endotoxin from *S. typhosa*.

METHODS

The methods applied were designed to determine by bioassay or isotope techniques minute amounts of histamine bound in covalent linkage to a large excess of protein (ratio approximately 1 to 10^6). It was therefore necessary to scrutinize each step of the procedure from that point of view. The use of ^{14}C-histamine added some further complications inherent in the work with isotopic metabolites *in vivo*. The methods of isolation of histamine from protein for the purposes of bioassay, which are also applicable to experiments with ^{14}C-histamine *in vivo*, are described.

Collection and purification of liver protein. The livers had to be secured within a time period and in a manner that eliminated postmortem enzymatic activities and, particularly, fixation of the amines on the proteins.

The mice were killed by a blow on the head, decapitated, and the livers ground in a mortar precooled with solid carbon dioxide. The usual time elapsed between the death of the animal and the freezing of the liver did not exceed 30 sec. To the frozen layer of liver tissue 10% TCA (trichloracetic acid) was added, and the tissue sample was brought into a suspension by stirring and, finally, homogenization in a Potter-Elvehjem homogenizer.

The liver suspension in 10% TCA was centrifuged and the precipitate washed according to Siekevitz.[4] First the precipitate was washed by centrifugation three times with 5% TCA. The supernatant fluids of the 10% TCA suspension and of the first 5% TCA washing were combined and stored in the cold for a determination of free histamine in the liver. The precipitate was then suspended in 5% TCA at 90° for 15 min in order to solubilize and remove nucleic acids. After centrifugation the sedimented protein was washed once with ethanol, once with a mixture of ether : ethanol : chloroform (2 : 2 : 1) and finally with ether. The powder was dried in a desiccator over potassium hydroxide to constant weight. If ^{14}C-histamine was employed, the radioactivity of the protein sample was determined.

Although the Siekevitz procedure has proved satisfactory in experiments in many laboratories on incorporation of labeled amino acids into proteins, it appeared advisable to extend the purification of the protein samples in our experiments to exclude the possibility that the ultimately determined histamine may have been absorbed and not bound to protein. The dried protein samples were therefore dissolved by stirring in 66% (v/v) thioglycolic acid and precipitated by adjusting the pH to 6·5. The protein was separated by centrifugation and the procedure repeated. After the two treatments with thioglycolic acid, the precipitate was washed three times with 5% TCA, twice with the ether : ethanol : chloroform mixture and once with ether and dried to constant weight as described above and its radioactivity counted if so

required. The efficiency of the purification procedure is demonstrated in Table 1 in which the radioactivity of liver protein samples of mice which received ^{14}C-histamine is given at different steps of purification.

On the basis of the results shown in Table 1, it appeared advisable to process all protein samples through at least one treatment with thioglycolic acid which appeared sufficient to bring the protein close to a constant radioactivity. In some cases (experiments 4, 5, and 6) this was accomplished by the application of the Siekevitz procedure alone.

TABLE 1. PURIFICATION OF ^{14}C-HISTAMINE PROTEIN*

Exp. no.	Siekevitz procedure	Thioglycolic acid treatment First	Second
1	64	47	46
2	71	63	55
3	59	51	47
4	28	28	
5	68	68	
6	95	75	

* In counts per min/mg protein; histamine · 2HCl, specific activity 7·25 mc/mMmole, was used. Mice received 0·75 mg of histamine base (50 μc) per animal. Protei n sample in each experiment was obtained from 6 to 10 livers.

The increase in weight of the protein samples, owing to a reaction of thioglycolic acid with protein disulfide, may in part account for the decrease in counts. An attempt to measure the extent of changes in the protein molecule, after thioglycolic acid treatment, was made by the determination of the nitrogen to sulfur ratios in (1) protein washed by the Siekevitz procedure alone, (2) after one thioglycolic acid treatment, and (3) after two thioglycolic acid treatments. The averaged N to S ratios found in two experiments were (1) 6·1, (2) 5·4, and (3) 6·0 respectively. It seems, therefore, that the treatment with thioglycolate does not significantly influence the N to S ratios.

In all incorporation experiments the histamine dosage was kept constant, at 37·8 mg/kg, which is close to the LD_{50} for mice sensitized to histamine after administration of *H. pertussis* vaccine.[5] The purification procedure of the liver protein was adequate as shown by the results of specially designed experiments in which high doses of unlabeled and ^{14}C-histamine were administered to normal mice.

The LD_{50} for normal mice is known to be considerably higher, 620 to 915 mg/kg;[6] high doses in this range were tested in normal mice, and in these experiments a modification of the washing procedure was adopted. When the dose of histamine exceeded 100 mg/kg, it was found necessary to carry out the precipitation from thioglycolic acid from a large volume of fluid; 200 ml of distilled water was added to solutions of about 0·6 to 0·8 g of protein in 15 to 20 ml of 66% (v/v) thioglycolic acid, and the protein was precipitated by adjusting the pH to 6·5 (Tables 2 and 3).

Hydrolysis of protein. The liver protein powder was hydrolyzed in 6 N HCl at 100° for 18 hr—samples up to 200 mg in sealed tubes and larger samples under reflux (3 ml HCl/100 mg protein). The hydrolysates were filtered, brought to dryness in a rotary evaporator, and the excess of acid removed by repeated addition and removal of water.

Removal of ammonia from the hydrolysate. Since the presence of free ammonia interferes with the bioassay of histamine, its removal was essential. The residue was dissolved in a few milliliters of water, the pH of the solution brought to 11 to 12 with N NaOH, and the solution brought to dryness *in vacuo*. The residue was redissolved in water and, if necessary, the pH readjusted to 11 to 12, and the solution concentrated

TABLE 2. HISTAMINE IN LIVER PROTEIN OF NORMAL MICE AFTER THE ADMINISTRATION OF LARGE DOSES OF THE AMINE

Dose (mg/kg)	No. of animals	Thioglycolic acid treatment (μg histamine/g protein precipitated from volume)		
		Standard First	Large	
			First	Second
Normal	6	<0·1*	0·1	
50	6	0·2	0·08, 0·08	
100	6	0·2	0·1	
150	6	3·3		
200	8	4·2		
400	10	3·9	0·12, 0·13, 0·1	0·1
600	8	4·5		

* <: Indicates inability of chlorprophenpyridamine to inhibit the effects of the histamine-like action of the sample on the guinea pig ileum. In the standard experiments the protein was processed through the Siekevitz procedure, dissolved in thioglycolic acid and reprecipitated from a small volume of fluid. In the experiments labeled "large" the protein was reprecipitated from a large volume of fluid (200 to 250 ml per 0·6 to 0·8 mg of protein powder).

TABLE 3. INCORPORATION OF ^{14}C-HISTAMINE INTO LIVER PROTEIN OF MICE TREATED WITH *S. typhosa* ENDOTOXIN*

Histamine (mg/kg)	Protein precipitated from volume (μg histamine/g protein)		Protein precipitated from volume (counts per min/mg)			
			Standard		Large	
	Standard	Large	Protein	Histamine DBS	Protein	Histamine DBS
37·5	0·5	0·5	54·0	6·4	56·0	6·2
37·5	1·1	1·25	44·0	6·5	45·0	6·6

* In each of the experiments dry protein powder obtained after application of the Siekevitz procedure was counted and divided into two equal parts; both samples were dissolved in thioglycolic acid; one of the samples was submitted to the standard procedure while the other was reprecipitated from a large volume of fluid. Subsequently, each sample was hydrolyzed and an aliquot was taken after addition of carrier histamine for isolation and recrystallization of histamine as dichlorobenzene sulfonate (histamine DBS). The protein powder was counted for radioactivity on steel planchets, the histamine DBS was counted in the scintillation counter. Each sample was corrected for background and for self-absorption.

to 3 to 4 ml. By this procedure, the ammonia derived mainly from the hydrolysis of the protein amide groups was reduced to 0·1 % of the ammonia concentration of the original hydrolysate without any loss in histamine content.

Presence of substances that may interfere with the bioassay. Conventional determinations of histamine in tissue are concerned with the amine that occurs in protein-free

filtrates. The butanol extraction procedure therefore appears sufficient to remove any possibly interfering substances. In protein hydrolysates containing minute amounts of histamine there is present a large variety of amino acids that might interfere with the bioassay. Such interference has been reported for arginine.[7] In preliminary experiments the basic amino acids, including arginine, were removed by resin chromatography without retention of added histamine. It was found later that butanol extraction under controlled conditions was sufficient to separate any inhibitory substances from the protein hydrolysate and to remove successfully any substance interfering with the bioassay. The only interfering substance that follows the amine in the extraction procedure is ammonia which had been eliminated previously by evaporation of the hydrolysates at alkaline pH. When the outlined procedure was followed, the recovery of histamine (1 to 5 μg) added to the hydrolysate of 1 g of protein amounted to 95 to 100%.

Extraction of histamine. The method for the extraction of histamine from the alkaline solution is a modification of the procedure by Shore *et al.*[8] To 4 to 5 ml of the hydrolysate (pH 12) 1·5 g of sodium chloride and 12 ml of *n*-butanol were added and the two phases of fluid vigorously shaken for 5 min in centrifuge tubes with Teflon-lined caps. The phases were separated by centrifugation; the aqueous layer was removed and the organic layer shaken with 3 ml of sodium chloride saturated 0·1 N NaOH followed by centrifugation. A 10-ml aliquot of the butanol solution (12·5 ml) was extracted with 3 ml of 0·1 N HCl after the addition of 15 ml of *n*-heptane. After vigorous shaking the emulsion was separated by centrifugation, and the aqueous phase was brought to dryness in the rotary evaporator. The residue was taken up in oxygen-saturated Tyrode's solution and the pH adjusted to 8·4 to 8·6. This solution was used in various dilutions for the bioassay or, if desired, for determination of radioactivity.

Determination of histamine in protein-free tissue extracts. The combined supernatant fluid of 10% TCA and the first 5% TCA extracts of liver were freed from TCA by passing the solution through a Dowex-2 (Cl^- form) column (0·75 ml resin/ml 7·5% TCA). The columns were washed with distilled water until neutrality, and the TCA solution was passed through followed by distilled water. The eluates were brought to dryness in a rotary evaporator, the residue was taken up in 3 to 4 ml of distilled water, and the solution was adjusted to pH 12 and extracted with butanol as described above.

The bioassay. In the first series of experiments histamine was determined on the guinea-pig ileum immersed in a 15-ml organ bath. Since a more sensitive method was desirable in the later experiments, the superfused guinea-pig ileum was used.[9] The superfusing fluid was oxygenated Tyrode's solution (pH 8·5 to 8·6) which flowed over the suspended ileum at 3 to 4 ml/min. The identity of the agonist in the extract, with histamine, was tested by the effect of an antihistamine (chlorprophenpyridamine) upon the contractions. Dilutions of the extracts and standard histamine solutions were applied to the ileum, giving assays of the standard (2 and 2) dose design.[10] In a series of twelve assays of "known" amounts of histamine, the mean deviation of the estimated from the true value was $\pm 5\%$.

Accuracy and reproducibility of the isolation procedure. Aqueous solutions of histamine in quantitites of 2 and 5 μg were processed through the butanol procedure, the percentage recoveries as measured by bioassay were 66·4 $\pm$ 4·3 (SE) in eight experiments

and 69·8 ± 4 in four experiments respectively. In another series of experiments ^{14}C-histamine dihydrochloride (13 μg, specific activity 1·1 μc/mmole) was added to 1 g of purified mouse liver protein prior to hydrolysis, and the extraction procedure was applied. Radioactivity was measured in the hydrolysates before butanol extraction and also in final acid extracts of the same samples. In three such experiments the percentage recovery based on radioactivity was 90 ± 3 in the hydrolysates and 60 ± 6 in the final acid extracts.

Histamine is lost during the extraction procedure owing to the distribution of the base between the aqueous and butanol phases. An approximate loss of 10% of histamine occurs during hydrolysis. The recovery as measured by radioactivity and by bioassay shows a satisfactory agreement, the average over-all recovery of histamine being 62% without consideration of the loss during hydrolysis. In the tables the values of histamine determined by the butanol procedure are the raw data not corrected for the two-thirds recovery but corrected for the aliquots taken during the procedure (10 ml of butanol extract taken from a total of 12·5 ml).

Histamine formation during hydrolysis. The minute amounts of histamine finally determined may have arisen during the purification procedure of the liver protein and particularly during its acid hydrolysis. It has been reported recently that considerable amounts of histamine were formed during the digestion of histidine with acid (20 μg/100 mg).[11] Since the histamine in hydrolysates from livers of untreated mice (Tables 4 and 5) was below or just at the limit of the sensitivity of the bioassay, a significant formation of histamine during purification of the protein and its hydrolysis appeared unlikely. Nevertheless, the yield of substances with histamine-like properties from histidine was studied under various conditions. Histidine HCl . H_2O (California Corp. for Biochemical Research) was recrystallized five times from water, and the crystals were washed twice with alcohol and twice with ether. It was found that such preparation, not exposed to conditions of hydrolysis but extracted with butanol, yielded a histamine-like substance in amounts up to 0·7 μg/100 mg (bioassay). This amount was decreased under conditions of hydrolysis. Assuming an average histidine content of proteins of 3%, the maximal yield of "histamine" (0·25 μg from 30 mg histidine/g protein) would be above that found in the proteins of the livers of control as well as treated animals.

Isolation of histamine after administration of ^{14}C-histamine in vivo. Histamine administered to animals is rapidly metabolized and, when the ^{14}C-labeled amine is employed, the label in the purified protein will be found not only in the incorporated histamine but also in various amino acids which, during their synthesis, have incorporated radioactive carbon derived from the labeled carbon atom no. 2 of histamine. It is therefore necessary to isolate the histamine in order to have a valid measure of the incorporation into protein of ^{14}C-histamine *in vivo*. The radioactivity of the isolated histamine when compared with that of the original protein provides data as to the distribution of counts among the various fractions. Preliminary experiments were carried out with the silver fractionation procedure.[12] Although this procedure provides fractions of amino acids, a simplified method employing butanol extraction was finally adopted and is described here. Unlabeled carrier histamine dihydrochloride (6 to 10 mg) was added to the protein hydrolysate and by repeated concentration *in vacuo* after the addition of water, the bulk of the hydrochloric acid was

TABLE 4. PROTEIN-BOUND HISTAMINE, FREE HISTAMINE, AND TRANSGLUTAMINASE LEVELS IN LIVERS OF FEMALE MICE*

Exp. no.	Treatment	No. of animals†	Protein-bound histamine (μg/g protein)	Free histamine (μg/liver)	Transglutaminase (units/mg protein)
1	Control	26	<0·06	0·65	24·2
2	*H. pertussis* vaccine	54	0·09	0·36	61·8
3	Histamine (37·5 mg/kg)	24	0·14	178·2	36·5
4	*H. pertussis* + histamine	28	0·54	62·7	73·5
5	Histamine (75·0 mg/kg)	40	0·30	435·0	35·0
6	*H. pertussis* + histamine	17	0·58	386·0	60·0

* Mice were killed 16 to 17 hr after the second administration of vaccine (6×10^9 organisms/20 g) which followed the first by 10 days. Histamine was injected 30 min before the animals were killed; < indicates that histamine-like effects on the ileum were not completely counteracted by antihistaminic drug.

† Number of animals used: experiment no. (1) $2 \times 10 + 1 \times 6$; (2) $8 \times 5 + 1 \times 6 + 1 \times 8$; (3) 4×6; (4) $2 \times 6 + 2 \times 8$; (5) 4×10; (6) $2 \times 6 + 1 \times 5$.

TABLE 5. PROTEIN-BOUND HISTAMINE, FREE HISTAMINE, AND TRANSGLUTAMINASE LEVELS IN LIVERS OF MALE MICE*

Exp. no.	Treatment	Protein-bound histamine (μg/g protein)	*mean*	Free histamine (μg/liver)	*mean*	Transglutaminase (units/mg protein)	*mean*
1	Control	<0·06 <0·07 <0·10 <0·08	<0·08	0·23 0·19 0·33 0·09	0·21	18·8 13·4 22·3 21·0	18·8
2	*S. typhosa*	<0·03 <0·06 <0·08 <0·10	<0·07	0·35 0·24 0·24 0·12	0·24	81·0 80·4 53·9 55·9	68·0
3	Histamine	<0·07 <0·10 <0·16 <0·1	<0·11	11 8 5·9 3·9 8·0	7·9	41·0 27·9 27·8 24·9	30·2
4	*S. typhosa* + histamine	1·1 0·88 0·44 0·34	0·68	20·9 5·1 13·6 6·0	11·4	68·2 63·8 52·6 51·5	58·3

* Lipopolysaccharide from *Salmonella typhosa* was given intraperitoneally in doses of 5 mg/kg. Histamine base = 37·5 mg/kg. Each value represents a mean of four experiments. Each experiment was performed on pooled material from 8 to 10 mice. Animals were killed 16 to 17 hr after endotoxin administration and 30 min after histamine injections; < indicates that histamine-like activity, assayed on guinea pig ileum, was not eliminated by antihistaminic drug.

removed. The residue was dissolved in dilute sodium hydroxide solution to bring the pH to 12. The alkaline solution was brought to dryness. The residue was dissolved in water, the pH checked (11 to 12) and, after bringing the solution to dryness, the residue was extracted three times with 30 ml of hot *n*-butanol (60 to 70°). The butanol solution (approximately 90 ml) was filtered and re-extracted twice with about 5 ml of N HCl for each extraction. After the addition of the first hydrochloric acid solution, *n*-heptane was added in a volume twice that of the butanol solution. The acid extracts were combined, brought to dryness, and the residue extracted with methanol in which histamine dihydrochloride is soluble. The alcoholic solution was brought to dryness and the residue dissolved in a solution of 20% dichlorobenzene sulfonate. The histamine derivative crystallized in the cold and was twice recrystallized from a 10% solution of dichlorobenzene sulfonate (mp 225 to 227°). The final yield was between 60 and 70% of the theory. The radioactivity of the histamine dichlorobenzene sulfonate was determined on stainless steel planchets in a thin-window gas-flow counter or in a scintillation counter. The values were corrected back to the amount of carrier histamine added.

Animal experiments. The animals used, and the various bacterial preparations and their administration, have been described in the previous report.[3] Details of the experiments will be found in the tables and in Figs. 1 and 2. In each experiment six to ten

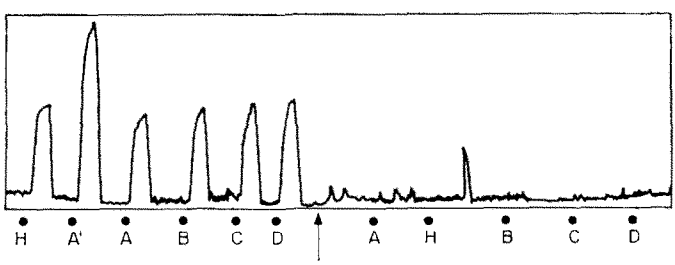

FIG. 1. Effect of extracts of liver protein hydrolysates from normal female mice injected with histamine (75 μg/kg). H = histamine, 6 mg/ml in Tyrode's solution. A, B, C, D, = extracts of hydrolysates from 25 mg protein/ml. A′ = same extract as A but from 50 mg protein/ml.
At arrow: 2 mg chlorprophenpyridamine.

mice were used and their livers pooled. When enzyme and histamine determinations were to be carried out on the same livers, a small piece was removed from each liver; these pieces were combined and homogenized, as previously described. The remainder, corresponding to the major part of the liver, was frozen. One liver provided approximately 180 mg (based on nitrogen determination) of protein after the second thioglycolic acid treatment; this amount corresponds to about 70% of the original protein content of the liver.

In some experiments fixation of histamine in protein was studied in the perfused rat liver. The livers were prepared according to the method described by Burton *et al.*[13] Thirty min after the perfusion had been established,[14]C-histamine was added to the perfusing blood; 45 min later the liver was removed and the liver protein prepared as described previously. Albumin and globulin fractions from the perfusate plasma were

prepared by half-saturation with ammonium sulfate and purified by the Siekevitz procedure in order to remove free histamine.

Material. ^{14}C-histamine (Nuclear-Chicago), with a radioactivity of 7·25 mc/mmole, was used. The purity of each sample was checked by paper chromatography in butanol : pyridine : water.[14]

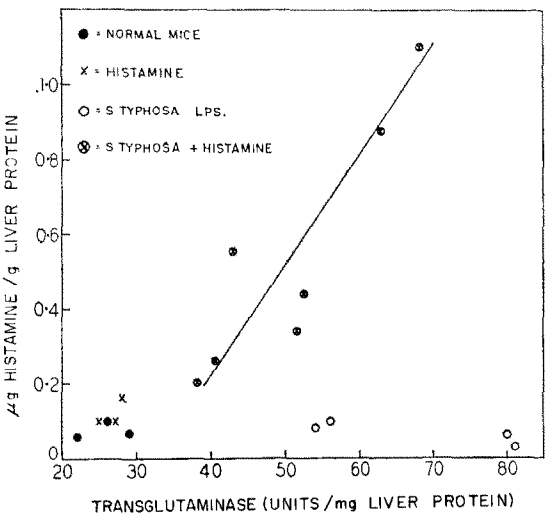

FIG. 2. Transglutaminase activity and protein-bound histamine in liver of endotoxin-treated male mice.

RESULTS

Protein-bound histamine in normal and pertussis-treated female mice

In the first extensive series of experiments, protein-bound and free histamine were determined in livers of female mice with and without the administration of histamine and of pertussis vaccine. In addition, transglutaminase was determined in the livers of the animals (Table 4).

Histamine could not be demonstrated in the hydrolysates of liver protein from normal mice. The concentrated extracts of the hydrolysates did cause contraction of the guinea pig ileum, but this effect was not blocked by an antihistamine (chlorprophenpyridamine). The histamine content of these extracts was thus clearly less than the apparent estimate from the assay (see Table 4).

In mice treated with pertussis vaccine, 9 of 10 of the liver protein hydrolysates tested contained measurable amounts of histamine; the extracts had to be diluted before addition to the ileum, and the contractions they produced were blocked by the antihistamine. Nevertheless, in most cases the amounts of histamine found were very small, and these results cannot be regarded as an unequivocal demonstration of the occurrence of protein-bound histamine in the livers of vaccine-treated mice which did not also receive exogenous histamine.

In experiments in which histamine was administered to mice, the doses of the amine used were 1·5 and 0·75 mg/20 g given intraperitoneally. The latter dose corresponds to the LD_{50} for histamine in mice treated with pertussis vaccine.[5] Although

normal mice tolerated both doses, the pertussis mice showed evidence of toxicity (respiratory embarrassment and convulsions), and many died with the larger dose. Animals which did not survive or were clearly moribund 30 min after the injection were rejected when the remaining mice were sacrificed. Even after administration of the lower dose to normal mice, significant amounts of protein-bound histamine were found (Table 4), and it is of interest that in these experiments in which histamine was given to normal mice, the liver transglutaminase was higher than that usually found in untreated animals. The administration of histamine to vaccine-treated animals leads to an increase of protein-bound histamine in the liver. Simultaneously, in the same animals, transglutaminase activity was increased significantly (Table 4).

The administration of 37·5 mg histamine/kg to pertussis-treated mice increased the liver protein-bound histamine 3 to 15 times while the free histamine increased by 60 to 500 times.

Protein-bound histamine in endotoxin-treated mice

Although the experimental results obtained with pertussis-treated female mice suggest that increased transglutaminase activity is accompanied by an increase of protein-bound histamine, it seemed desirable to repeat the experiments on male mice in which hormonal influences do not introduce variations in histamine metabolism; and furthermore, to test the effect of endotoxin from *S. typhosa*, the administration of which resulted in a more rapid increase of enzyme activity (Table 5).

No protein-bound histamine could be demonstrated in male control mice and animals which had received endotoxin or histamine (experiments 1, 2, 3). On the other hand, after endotoxin treatment and histamine administration, protein-bound histamine was increased considerably, and the effects of the extracts on the ileum were completely blocked by the antihistamine. The results show clearly the effect of endotoxin treatment on the level of enzyme activity and protein-bound histamine. A number of additional experiments was carried out in which ^{14}C-histamine was administered to endotoxin-treated mice. The experiments were exact repetitions of experiment 4 in Table 5. The results obtained in these, and also the data from the four experiments averaged in Table 5, were plotted against enzyme activity (Fig. 2). Quite unexpectedly, the plot revealed a correlation of the amount of protein-bound histamine and transglutaminase activity, with a correlation coefficient of 0·9 ($P < 0{\cdot}001$).

Experiments with ^{14}C-histamine

Before discussing the results above, those experiments in which ^{14}C-histamine was employed will be described. The data on the increase of protein-bound histamine, after the administration of ^{14}C-histamine, have been recorded in Fig. 2. In order to correlate the histamine content of the protein with its radioactivity, it was necessary to ascertain which fraction of the counts found in the protein was actually in the amine and which was incorporated into amino acids owing to the metabolism of the amine. Table 6 gives the results of experiments 2 and 3 in which the silver fractionation procedure was used, and of experiment 5 in which the butanol procedure was used. The data do not give a clear answer to the question of whether or not a larger number of counts from administered ^{14}C-histamine is incorporated into the liver proteins of endotoxin-treated than into those of normal mice. Of the total counts, a varying percentage is found in histamine attached to the protein; this varies from 4 to 45% in the liver

protein of endotoxin-treated mice. The amount of protein-bound histamine as given by the bioassay is considerably greater than that calculated on the basis of the counts recovered in histamine.* The administered labeled histamine is apparently diluted by endogenous, unlabeled histamine prior to incorporation. It is difficult to see where a pool of free, unlabeled, endogenous histamine to effect a dilution of this magnitude could arise, but it must be stressed that we do not know at which stage of the fate of the administered histamine such a dilution could occur. It might be considered that an accumulation of large amounts of extracellular histamine could lead to an increase of intracellular unlabeled histamine by a delay in the egress of the amine from the cell, with a resulting dilution of the labeled amine.

TABLE 6. INCORPORATION OF ^{14}C-HISTAMINE INTO LIVER PROTEINS OF MICE*

Exp. no.	Animal preparation	Counts per min/mg protein	Counts per min in isolated histamine/mg protein	Percent of histamine counts of total†	Histamine/g (protein) calculated from		Ratio
					Count	Bioassay	
1	N	40	2·4	6·0	0·05	<0·03	0·6
2	T	46	1·7	3·7	0·04	0·3	8·0
3	T	75	2·6	3·5	0·05	0·4	8·0
4	N†	39	13·7	35·0	0·23	1·3	6·0
5	T†	96	43·0	45·0	0·7	2·3	3·0
6	T	55	5·9	10·0	0·1	0·5	5·0
7	T	44	6·6	15·0	0·11	1·2	11·0

* Each animal received 37·5 mg/kg of ^{14}C-histamine (7·2 μc/μmole). N, normal mice; T, endotoxin (*S. typhosa*)-treated mice. One μg of histamine = 46,000 counts at infinite thinness in the thin-window counter and 60,000 counts in the scintillation counter. In experiments 1 to 3 the isolated histamine dichlorobenzene sulfonate was counted in the thin-window counter, and in experiments 4 to 7 in the liquid scintillation counter. For direct comparison with the other value the radioactivity measured in the scintillation counter should be multiplied by a factor of 0·6; the bioassay values are corrected for the 60% yield during the procedure to make the value comparable with the radioactivity which refers to the total in 1 g protein.

Each experiment was performed on pooled material from 8 to 10 mice.

† These experiments were done during the hot season; the normal mice were unusually histamine-sensitive.

In experiments 4 and 5 (Table 6) the amounts of histamine incorporated into liver proteins in the normal and endotoxin-treated animals exceeded the values obtained in all other experiments. It may be significant that these experiments were carried out during the first hot spell in June 1961, and the normal mice of this group of animals exhibited a histamine sensitivity otherwise encountered only in pertussis vaccine-treated animals. Transglutaminase levels were 19 units/mg protein in experiment 4, and 50·2 units/mg protein in experiment 5.

* In order to exclude the presence of 1-methylhistamine in the histamine fraction this compound was added in some experiments as carrier in place of unlabeled histamine. No significant counts were recovered in the isolated methylhistamine derivative.

Perfusion experiments

The experiments with perfused rat livers were carried out with the expectation that a more striking histamine incorporation would be found in protein than was achieved in the mice *in vivo*; this was not the case. Hydrolysates of protein from rat livers perfused without added histamine contained significant amounts of histamine (0·13 and 0·14 μg/g liver protein). When perfused for 45 min after the addition of 0·75 mg of histamine to the perfusing blood, the liver protein-bound histamine increased (0·48 and 0·57 μg/g). Some protein-bound histamine was also found in the globulin fraction of the perfusate, 0·13 and 0·17 μg/g without histamine administration, increasing to 0·61 and 1·4 μg/g when histamine was added to the perfusate; no protein-bound histamine was found in the albumin fraction even after perfusion in the presence of exogenous histamine. This observation is of interest since serum α- and β-globulins are acceptors of amines in the transglutaminase reactions *in vitro* whereas albumin is not.[15, 16]

The role of transglutaminase in the fixation of histamine in protein

Although a correlation between transglutaminase activity and the concentration of protein-bound histamine was found in endotoxin-treated animals, these results do not establish a causal relationship between the increased enzyme activity and the level of protein-bound histamine. Even the isolation of a γ-glutamyl histamine peptide from the proteins would be only suggestive of such a causal relationship and would present a rather cumbersome approach to this question. Since the action of transglutaminase was shown to be reversible and is mainly hydrolytic at pH 7,[17] the removal of ^{14}C-histamine incorporated *in vivo* by action *in vitro* of a purified preparation of transglutaminase was attempted. In preliminary experiments carried out in collaboration with Dr. M. J. Mycek, the cleavage of histamine from β-lactoglobulin and from a protein suspension of the mouse liver into which the amine had been incorporated by transglutaminase action *in vitro* was compared with the cleavage of histamine from liver protein of normal and endotoxin-treated mice which had received ^{14}C-histamine. Before the data could be evaluated, it was necessary to ascertain the fraction of counts still residing in histamine, by the procedures described above, the for histamine protein obtained *in vivo*. The data suggest that the liberation of histamine, by the action of transglutaminase, from histamine lactoglobulin and histamine liver protein obtained *in vitro* was of the order of magnitude of that obtained from histamine liver protein obtained *in vivo* (6 to 10%). These data provide suggestive evidence that transglutaminase was responsible for the incoporation of histamine *in vivo* as *in vitro*, but they will be conclusive only when a more extensive cleavage of histamine from protein by transglutaminase action has been achieved.

DISCUSSION

In normal male mice significant fixation on protein of histamine could be demonstrated only after the administration of large amounts of the amine and pretreatment of the animals with endotoxin, which leads to an increase of transglutaminase activity in the liver. The increases in protein-fixed histamine and in transglutaminase activity appear to be correlated. Although similar results were obtained in female mice, a slight but significant fixation of histamine was also found without pretreatment with

endotoxin on some of the animals. Such female mice, although considered control animals, showed an exceptionally high transglutaminase activity in the livers.

The fixation on proteins of an amine occuring normally in the mammalian body, such as histamine, has not been demonstrated unequivocally previously. Block and associates[18] have furnished evidence that mescaline, but not β-phenylethylamine, may be fixed in liver protein in mice *in vivo*. A repetition of the mescaline experiment by Clouet[19] led to equivocal results. In one of the experiments, the counts found in liver proteins after mescaline administration to mice equaled those of proteins purified from a liver homogenate to which mescaline had been added. In other experiments, a slight increase of radioactivity in the liver proteins over those obtained in the control experiments was found. If it is assumed, for the purpose of this discussion, that the work of Block and associates demonstrated the incorporation *in vivo* of mescaline into liver proteins, the question arises of whether the fixation of mescaline is also mediated *in vivo* by transglutaminase and not by the enzyme system assumed by these authors on the basis of their experiments *in vitro*.

It should be noted that an increased fixation of histamine in proteins in mice was found only after the administration of massive amounts of histamine. This finding might suggest that the physiological substrate of transglutaminase is an amine other than histamine and that the fixation of histamine under our experimental conditions may be taken as an indication of a reaction in which histamine is not normally involved. It may be recalled in this connection that various amines and the ε-amino group of protein-bound lysine may act as replacing agents in the transglutaminase reaction *in vitro*, and recent experiments have shown that various proteins may act in a similar manner.[20] The possibility can therefore not be excluded that, *in vivo*, transglutaminase catalyzes the attachment of proteins to each other and that this process is particularly active in endotoxin-treated animals.

In the interpretation of the quantitative aspects of the histamine incorporation into proteins, it is noteworthy that the amount of histamine incorporated does not correspond to, but exceeds, the values calculated from the specific radioactivity of the ^{14}C-histamine fixed. This finding suggests that intracellular histamine is in close contact with the enzymatic incorporation system and is mobilized by the injection of the large amounts of histamine and that it is the former histamine mainly which in varying amounts is fixed on the proteins.

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