Journal of Food Protection, Vol. 44, No. 11, Pages 815-820 (November 1981)

Production of Histidine Decarboxylase and Histamine by *Proteus morganii*

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(Received for publication December 4, 1980)

ABSTRACT

Examination of 22 *Proteus morganii* strains revealed that each possessed histidine decarboxylase activity. Strain GRMO 6 produced maximal activity (nanomoles of CO_2 released /mg cells/h) when grown at ambient temperature (24 C) and at acid pH levels that tended to inhibit growth. Minimal activity was present when the culture was grown at pH 8.5. Histidine decarboxylase activity decreased as the age of the culture increased. Maximal reaction rates occurred at 37 C and pH 6.5. Rapid enzyme and histamine formation occurred in tuna fillets inoculated with *P. morganii* and stored at 24 and 30 C. Histamine levels reached 520 mg/100 g and 608 mg/100 g at 24 and 30 C, respectively. Little enzyme was produced in the inoculated fillets stored at 15 C and in the uninoculated control fillets.

Scombroid fish characteristically contain large amounts of free L-histidine in the muscle, which can give rise to toxic levels of histamine under conditions leading to histidine decarboxylation. Although scombroid food poisoning (histamine intoxication) is usually manifested as a discomfort for a period of hours and very rarely proves fatal, it is not to be taken lightly. Worldwide incidences of scombroid food poisoning indicate the potential for a variety of foodstuffs to induce the intoxication. Although most scombroid poisoning outbreaks are small and isolated, one well-documented outbreak in the United States involved 232 persons in four states who were poisoned from consumption of commercially canned tuna. Two lots of tuna were recalled constituting some 170,000 cans (*13*).

Ferencik (*2*) cited several authors who have reported cases of scombroid food poisoning involving tuna in the United States, Japan, Indonesia and in European countries such as Czechoslovakia. Occurrence of this type of food poisoning is significant in the United States but is much lower on a per capita basis than in those countries which depend more on fish in their diet yet rely less on refrigeration. The consumption of certain dried fish products popular in Japan resulted in many outbreaks. Kawabata et al. (*6*) described 14 outbreaks involving 1,215 people during a 4-year period.

Only a limited number of histidine-decarboxylating bacteria have been isolated and identified from fishery products incriminated in scombroid poisoning. *Proteus morganii* has been most frequently mentioned in relation to histamine formation in fish muscle (*5,7,14,18*). Other bacteria isolated from tuna containing large levels of histamine include *Hafnia alvei* (*5*) and *Klebsiella pneumoniae* (*10,19*).

Recent studies have shown that *P. morganii* can be a contaminant of fish tissue during spoilage and that few other bacteria have as great a capacity to form histamine. Omura et al. (*14*) examined and identified 44 bacterial isolates from spoiled skipjack tuna and broth. Of these, 21 isolates produced large amounts of histamine and were identified as *P. morganii*, 13 weak histamine formers were identified as *Hafnia alvei*, three other species of *Proteus*, one of *Kelbsiella*, and six unidentified bacteria were included in the group of histamine-forming bacteria.

Taylor et al. (*18*) studied 112 bacterial strains from a variety of sources. Twenty-three strains produced greater than 50 nanomoles of histamine/ml in trypticase soy-histidine broth. Thirteen of 15 *P. morganii* strains and three of three *Enterobacter aerogenes* strains produced histamine at this level. In tuna fish infusion broth, the *P. morganii* and *E. aerogenes* cultures produced histamine at much higher levels than did any of the other cultures, including 12 strains of *H. alvei*. The authors indicated that the *H. alvei* cultures would have limited ability to precipitate scombroid poisoning in comparison to the histamine-forming capacity of *P. morganii* and *E. aerogenes*. The incidence of *E. aerogenes* as a contaminant of fishery products is unknown. The *E. aerogenes* cultures used by Taylor et al. (*18*) were from non-marine sources.

The above studies demonstrate that *P. morganii* is a powerful histidine-decarboxylating bacterium. Further-

more, the relationship of *P. morganii* to products incriminated in scombroid poisoning episodes and the fact that the organism has been isolated by several investigators from spoiled tuna clearly indicate that it plays a role in scombroid fish poisoning. Further studies need to be completed that will identify the frequency of occurrence and sources of *P. morganii* on marine fish. The present study considers factors influencing the production of histidine decarboxylase (HD) (4.1.1.22) and histamine by *P. morganii*.

MATERIALS AND METHODS

Source of Proteus morganii *cultures*

The 22 *P. morganii* cultures used in this study were obtained from Dr. Dorthey Jones, Department of Microbiology, University of Leicester, LE17RH, England. The cultures were used by McKell and Jones (*12*) in a taxonomic study of the *Proteus-Providence* bacteria.

Growth conditions and media

P. morganii strains were maintained on tryptic soy agar slants by transfer at 3-week intervals. Preparation of the cultures for the various experiments involved a minimum of two transfers in tryptone-yeast extract broth. This broth consisted of 1% tryptone and 1% yeast extract in distilled water (w/v). One percent inocula were obtained from the broth culture after 24 h of growth. HD activity and histamine were determined in the tryptone-yeast extract broth and in tuna fish infusion broth prepared by the method of Omura et al. (*14*). Fresh Yellowfin tuna was obtained at a seafood market in San Francisco, frozen and shipped with dry ice to the University of Georgia (courtesy of Ms. Ellen Lieber, FDA, San Francisco). The tuna was thawed and homogenized in two volumes of water. The homogenate was steamed for 1 h at 100 C and then filtered. The medium was supplemented with 1% glucose and autoclaved for 15 min at 121 C. The final pH was 5.7.

This tuna was also used to determine effects of storage time and temperature on decarboxylase development and histamine formation in tuna muscle. Fillets were stored at 15, 24 and 30 C. Storage trials consisted of uninoculated fillets and fillets inoculated by dipping them in a suspension of *P. morganii* (Strain GRMO 6) containing approximately 10^4 cells/ml. The fillets were then placed in sterilized containers and stored at the various temperatures. At various intervals during the incubation, both inoculated and uninoculated samples from each of the three temperature trials were removed by excising a 10-g sample. To each sample was added 40 ml of sterile distilled water. Using sterile blades and containers, the samples were homogenized in a Virtis mixer for 3 min at a speed setting of 40. Portions were aseptically removed and analyzed for HD activity, bacterial count and histidine and histamine content.

Isotopic histidine decarboxylase assay

The isotopic enzyme assay was performed as described by Levine and Watts (*11*). The reaction vessel consisted of a polyethylene scintillation vial sealed with a rubber stopper. A 1 × 3-cm piece of rolled filter paper (3-mm Whatman) dipped in phenethylamine and suspended from the stopper by a bent wire clip acted as a CO_2 trap. The stock substrate solution consisted of 0.1 ml of ^{14}C-carboxyl labelled L-(+)-histidine (50 μCi in 25 ml, New England Nuclear, Boston, Massachusetts) in 4.0 ml of a non-radioactive carrier solution prepared by dissolving 30.7 mg of L-histidine in 40 ml of 10^{-4} M HCl.

To each polyethylene reaction vial, 2.4 ml of 1 M sodium acetate at pH 6.5 and containing 100 μg of pyridoxal-5-phosphate/ml was added and equilibrated at 37 C in a shaking water bath. Five-tenths of a g of tissue homogenate or 0.5 ml of a washed bacterial cell suspension was added. The cell suspension was prepared by centrifuging 10 ml of culture broth at 12,100 × *g* for 10 min. The bacterial cell pellet was resuspended in 10 ml of 1.0 M sodium acetate buffer, pH 6.5. The reaction was initiated by addition of 0.1 ml of the ^{14}C-L-histidine substrate to each vial. The vials were incubated for 1 h at 37 C in a Dubnoff Metabolic Shaker.

After incubation, 2.0 ml of 2.0 N HCl were injected (18-gauge needle) through the side of each vial to terminate the reaction and to aid in carbon dioxide liberation. The puncture holes were sealed with tape and the samples were allowed to shake at ambient temperature for 30 min to allow complete liberation and absorption of carbon dioxide onto the trap. To measure non-specific $^{14}CO_2$ release, controls were made by the addition of 2 ml of 2.0 N HCl to the reaction mixture before the reaction was initiated.

Upon complete termination of the reaction, the carbon dioxide-trap paper clips were transferred to polyethylene scintillation vials containing 0.4% Omnifluor (New England Nuclear) in 10 ml of toluene. Each sample was then monitored for ^{14}C for 5 min, using the universal channel of a liquid scintillation counter (Beckman Model LS 7000, Fullerton, California). Activity was expressed as nanomoles of CO_2 released/mg of cells/h or nanomoles CO_2 released/g of tuna muscle/h.

Histidine and histamine analysis

Free histidine levels in fresh and stored tuna muscle were determined, using a Durrum D500 amino acid analyzer (Durrum Instruments, Sunnyvale, CA). Free amino acid extracts were prepared by homogenizing 10 g of tuna muscle in distilled H_2O and centrifuging at 12,000 × *g* for 5 min to remove insoluble muscle components. To 1.0 ml of the clear supernatant fluid was added 4.0 ml of 4.5% (w/v) sulfosalicylic acid solution to facilitate protein precipitation. The mixture was centrifuged at 12,100 × *g* for 10 min and the supernatant fluid was decanted and frozen until amino acid analysis was done. Histamine was determined by the AOAC method (*15*).

RESULTS AND DISCUSSION

Factors influencing histidine decarboxylase production by P. morganii

Examination of the 22 *P. morganii* strains revealed that each possessed HD activity, although at variable levels. Enzyme activity of the cultures and amount of histamine formed during a 12-h growth in tuna fish infusion broth are shown in Table 1. HD activity of the 12-h-old cultures ranged from 14 nanomoles of CO_2/mg of cells/h to 1250 nanomoles of CO_2/mg of cells/h. Histamine content was somewhat greater in the medium with the higher decarboxylase-producing strains than in the medium of the bacteria showing lower levels of activity. However, histamine levels did not vary as widely as HD activity among the cultures. This would be expected if histamine acts as an endproduct inhibitor, thereby slowing down decarboxylation as the amine accumulates in the medium. In this study, culture decarboxylase activity was determined on washed cell suspensions to prevent histamine present in the medium from influencing rate measurements.

After initial studies with the 22 strains, strain GRMO 6 of *P. morganii* was chosen to determine the effects of incubation temperature, medium pH and culture age on histidine decarboxylase activity in tryptone-yeast extract broth. This strain demonstrated vigorous growth and intermediate HD activity when compared to other *P. morganii* strains. Figure 1 shows the effect of incubation for 24 h at 24, 30, 37 and 42 C. Maximal HD activity was obtained when strain GRMO 6 was grown at ambient temperature. The activities decreased from 98 to 12 nanomoles of CO_2/mg of cells/h at 24 and 42 C,

TABLE 1. *Histidine decarboxylase and histamine production by 22 strains of* Proteus morganii *in tuna infusion broth (12-h growth).*

Culture[a]	Enzyme[b]	Histamine[c]
GRMO 1	902[d]	133.4
GRMO 2	638	116.7
GRMO 3	708	177.8
GRMO 4	629	166.7
GRMO 5	870	235.6
GRMO 6	723	266.8
GRMO 7	408	146.7
GRMO 8	682	95.6
GRMO 9	1106	164.5
GRMO 10	689	157.8
GRMO 11	802	37.8
PHL 253	611	88.9
PHL 378	606	80.0
HG 114	14	61.1
NCTC 232	799	115.6
NCTC 1707	1250	111.1
NCTC 2815	1164	163.4
NCTC 2818	406	126.7
NCTC 5845	19	25.6
NCTC 7381	1291	143.4
NCTC 10041	870	132.3
NCTC 10375	844	136.7

[a]Culture designations--used by McKell and Jones, 1976.
[b]Histidine decarboxylase activity--nanomoles of CO_2 released/mg of cells/h.
[c]Histamine--mg/100 ml.
[d]Values are averages of duplicate trials.

respectively. As first observed by Bellamy and Gunsalus (*1*), it appears that conditions maximizing enzyme synthesis, decarboxylation and cell growth are different. The culture grown at ambient temperature had the lowest optical density. The temperature for optimizing growth was 37 C. The drop in enzyme activity, especially evident at 42 C, is probably the result of histidine decarboxylase thermolability.

The effect of initial medium pH levels on HD production is shown in Fig. 2. Eight levels of pH at increments of 0.5 were used, ranging from 5.0 to 8.5. The pH of the medium was adjusted with sterile 1.0 N NaOH or 1.0 N HCl (Fig. 2). Maximal enzyme activity was obtained at pH levels that tended to inhibit growth, suggesting the protective action theory postulated by Koessler et al. (*9*). This theory deals with the induction of certain enzymes whose end products remedy the low pH microenvironmental condition which threatens the microorganism. Activity was highest at pH 5.0 but growth of the culture was very poor. At all other pH levels examined, growth was good. Ienistea (*5*) found that the optimal pH for HD induction in most bacterial species was between 5.0 and 5.5. Very little histidine decarboxylase activity was noted when the culture was grown at pH 8.5. The protective action theory has been demonstrated with other decarboxylases. Gale (*3*) observed that *Streptococcus faecalis* possessed the most active tyrosine decarboxylase system in trypsin-digested casein glucose medium when cells were grown at pH 5.0.

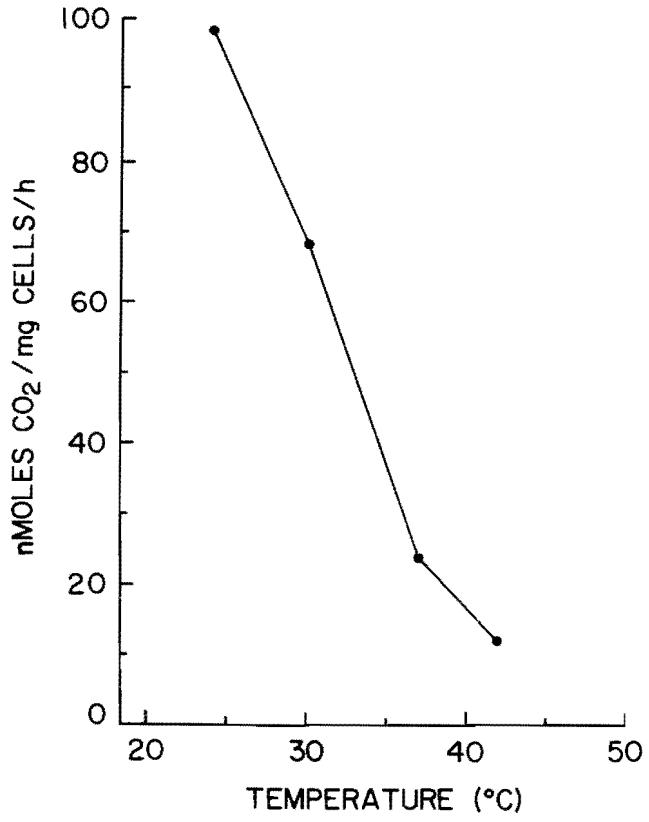

Figure 1. *Effect of incubation temperature on histidine decarboxylase formation by* P. morganii *(24-h growth).*

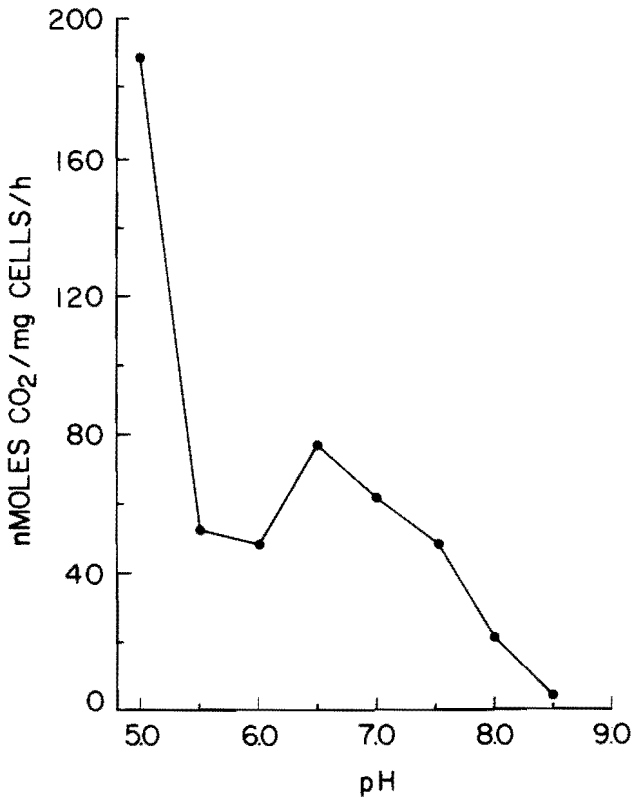

Figure 2. *Effect of initial pH of the media on histidine decarboxylase production by* P. morganii *(24-h growth).*

The influence of incubation time of the culture on histidine decarboxylase activity at 30 C and pH 6.5 was examined for 10, 14, 17 and 21 h. HD activity decreased as the age of the culture increased (Fig. 3). The rapid decrease from 388 to 104 nanomoles of CO_2/mg of cells/h between 10 and 21 h of growth, respectively, was approximately linear. A rise in pH with growth may be responsible for the decrease in activity. Gale (*4*) reached a similar conclusion for other decarboxylases and went so far as to plot activity not against the age of the culture but against the pH of the medium during growth. In most instances, the activity decreased as the pH increased above 6.5.

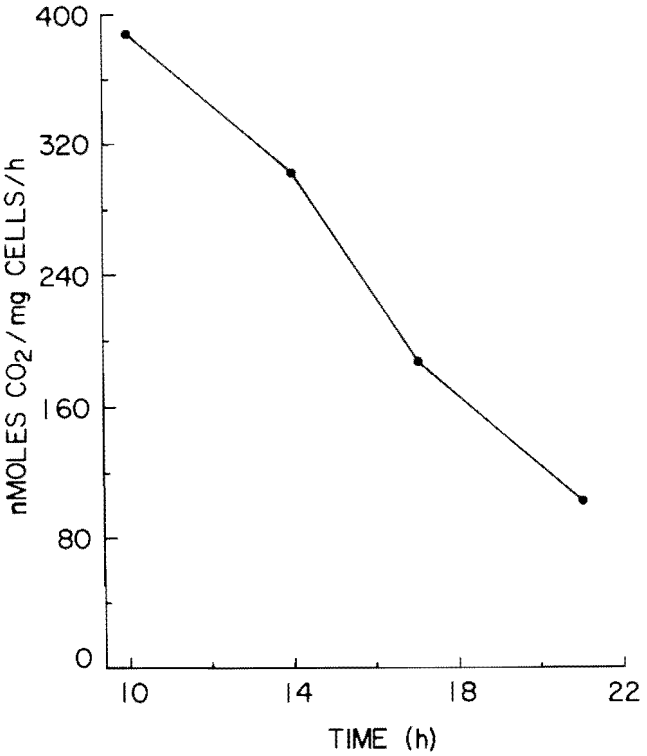

Figure 3. *Effect of incubation time on histidine decarboxylase activity of* P. morganii.

Effect of reaction temperature and pH on histidine decarboxylation

Reaction mixtures were incubated at 25, 30, 37, 45 and 55 C. The measurement HD activity indicated an incubation temperature optimum of 37 C (Fig. 4). Gale (*4*) demonstrated a general decline in activity for most bacterial decarboxylases at incubation temperatures above 40 C. The rate of decarboxylation increased gradually with temperature and started to fall when the higher temperature began to adversely affect the tertiary structure of the enzyme. In the study of the effect of incubation temperature on enzyme production, the level of activity at 37 C was approximately 25% of that found at 24 C. Since both enzyme induction and activity are significant to production of histamine, the optimum temperature for histamine production in tuna must be a compromise between the two parameters.

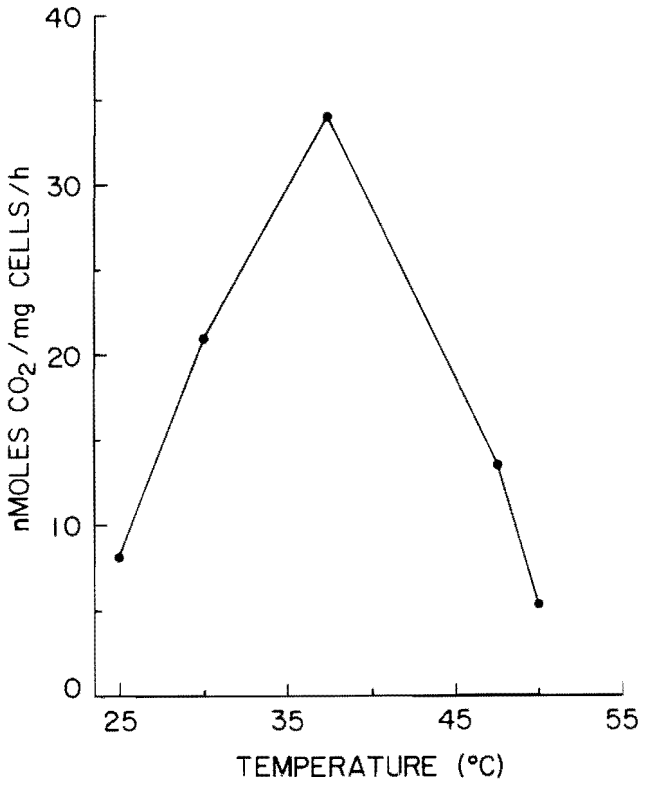

Figure 4. *Effect of reaction temperature on histidine decarboxylase activity.*

To determine the effect of pH on decarboxylase activity, the reaction medium was adjusted from pH 5.0 to 7.5 in increments of 0.5. Optimal activity of the enzyme was obtained at pH 6.5. Histidine decarboxylase activity decreased rapidly on both sides of the optimum with little activity at pH 5.0 (Fig. 5). As previously mentioned, pH 5.0 provided maximum induction of HD synthesis during culture growth. However, the minimal enzyme activity at this pH would greatly inhibit histamine accumulation in the medium. The affinity of the active site for histidine is low at a pH of 7.0 and above (*16*). Kimata (*8*) reported at pH optimum of 6.0 for histidine decarboxylase of *P. morganii.* HD isolated from gastric tissue had a pH optimum of 6.8 (*11*).

Formation of histamine and histidine decarboxylase in tuna fillets

Data on the effects of storage temperature on formation of HD and histamine in tuna fillets are presented in Table 2 and Table 3, respectively. Storage at 15 C resulted in only a slight increase in measurable HD activity in the control fillets containing the normal microflora (Table 2). Fillets inoculated with *P. morganii* and stored at 15 C developed higher levels of HD after 24 h of storage (61.6 nanomoles of CO_2/g/h). The HD activity decreased slightly to 54.2 nanomoles of CO_2/g/h after 48 h of storage. At 15 C, histamine increased from 12.1 mg/100 g in the control to 21.4 mg/100 g and 43.7 mg/100 g in the uninoculated and inoculated tuna, respectively, after 48 h of storage (Table 3). During the same storage period, free histidine decreased from an

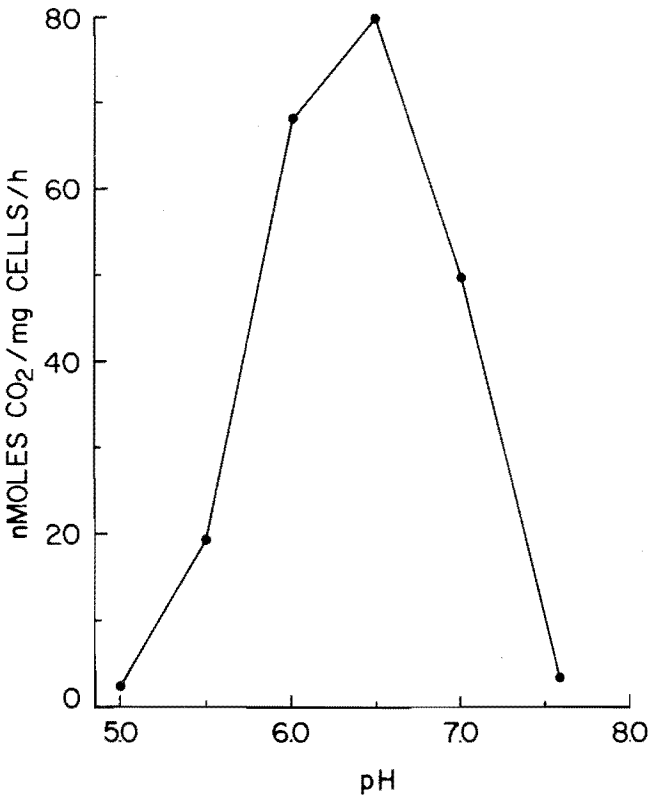

Figure 5. *Effect of reaction pH on histidine decarboxylase activity.*

initial level of 1236 mg/100 g to 989 mg/100 g in the uninoculated fillets and 1030 mg/100 g in the inoculated fillets. The slight decreases in free histidine in the control are most likely due to microbial utilization and loss of the amino acid in small amounts of drip that occurred unavoidably during storage. The increase in histamine concentration at 15 C was not large enough to account for the lowering of free histidine in the fillets. However, the observation that the histamine concentration reached 43.7 mg/100 g in the fillets inoculated with *P. morganii* and stored at 15 C indicates that histamine could be formed to appreciable and even toxic levels at storage temperatures not optimal for bacterial growth or HD activity.

Rapid enzyme and histamine formation occurred in the inoculated fillets stored at 24 and 30 C. (Table 2 and Table 3). Maximal HD activity occurred after 12 h of storage at both temperatures and decreased thereafter. Histamine levels reached 520 mg/100 g and 608 mg/100 g at 24 and 30 C after 24 h of storage. At 24 C, the free histidine content decreased to 279 mg/100 g and at 30 C the free histidine content reached 127 mg/100 g. In the uninoculated fillets, HD activity was somewhat higher than noted in fillets stored at 15 C; however, histamine content reached only 29.4 mg/100 g and 37.1 mg/100 g after 24 h at 24 and 30 C, respectively.

The data demonstrate that storage temperature is a critical factor influencing formation of histamine in tuna. As discussed by Lerke et al. (*10*), tuna or other scombroid fish that are caught in tropical water are particularly vulnerable to build-up of histamine. Likewise, Salguero and Mackie (*17*) showed that little histamine was produced in mackerel muscle at 0 C during extended storage but that appreciable histamine formation occurred at 10 C after 5 days of storage. The requirement for rapid and uninterrupted refrigeration after catch cannot be over-emphasized. Normal muscle pH conditions correspond closely to pH levels required for optimal activity of the enzyme (pH 6.5) and are low enough to permit rapid enzyme synthesis. The ready

TABLE 2. *Histidine decarboxylase activity in tuna fish fillets stored at 15, 24 and 30 C*[a].

Time (h)	15 C		24 C		30 C	
	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
0	0.70[b]	3.6	-	-	-	-
6	1.8	18.1	3.6	30.7	10.6	102.1
12	2.2	46.4	11.5	170.2	20.1	250.8
18	-	-	13.3	109.8	24.0	214.9
24	2.8	61.6	16.6	76.8	31.3	176.5
48	3.5	54.3	-	-	-	-

[a]Values represent averages of duplicate storage trials.
[b]Nanomoles of CO_2/g of muscle/h.

TABLE 3. *Histamine (mg/100 g) in tuna fish fillets stored at 15, 24 and 30 C*[a].

Time of incubation (h)	15 C		24 C		30 C	
	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
0	12.1 (1236)[b]		-		-	
6	13.0	13.3	13.1	13.3	13.0	13.5
12	14.7	19.6	17.2	37.5	19.8	52.8
18	-	-	28.5	164.1	27.5	280.0
24	14.9	31.0	29.4 (1076)[b]	520.0 (279)	37.1 (1169)	608.3 (127)
48	21.4 (989)	43.7 (1030)	-	-	-	-

[a]Values represent averages of duplicate storage trials.
[b]Histidine (mg/100g).

availability of free histidine in the muscle to act both as an inducer and substrate makes scombroid fish muscle an ideal environment for histamine formation. As shown with tuna muscle, *P. morganii*, if given the opportunity to grow, can rapidly synthesize histidine decarboxylase and cause histamine accumulation to levels that can be toxic. Although the literature does not give a completely clear understanding of the frequency of occurrence of *P. morganii* on marine fish, its implication in several outbreaks of scombroid food poisoning suggests that it is a fairly common contaminant.

ACKNOWLEDGMENTS

The authors thank Dr. Dorthey Jones, University of Leicester, for providing the *Proteus morganii* culture and Ms. Ellen Lieber, FDA, San Francisco, for providing the fresh tuna fish used in the study.

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