The Influence of pH and Histidine Dipeptides on the Production of Staphylococcal α -Toxin

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SUMMARY

The histidine-induced production of α -toxin in *Staphylococcus aureus* strain Wood 46 declined rapidly when the pH was raised above 8.0 in the medium. This was related to the degradation of histidine by a histidase. The staphylococcal histidase was thermostable, had a K_m of 1.2×10^{-2} M and pH optimum near 9.0. Both histidine and urocanic acid raised the intracellular level of histidase when added to the medium. The enzyme was inhibited by cysteine, but not by EDTA. It was slowly oxidized to an inactive form, activity being restored by glutathione or mercaptoethanol.

Histidine dipeptides had a similar or more potent stimulating effect than histidine on the production of staphylococcal α -toxin in a synthetic medium. Glycyl-L-histidine and L-alanyl-L-histidine entered the organisms more readily than free histidine. The dipeptides were hydrolysed intracellularly, and a considerable portion of the released histidine was further degraded to urocanic acid.

L-Homocarnosine (γ -aminobutyryl-L-histidine) did not stimulate α -toxin production. The uptake and intracellular hydrolysis of this dipeptide were significantly slower than those of dipeptides with the free amino group in the α -position. Accumulation of intracellular histidine could not be demonstrated without an exogenous source of histidine. This was also examined under conditions when α -toxin was produced. The results indicated that stimulation of α -toxin production was not directly correlated with the intracellular level of free histidine. Accordingly, the mediator of the stimulating effect is still unknown.

INTRODUCTION

A factor from yeast autolysate stimulating α -toxin production in *Staphylococcus aureus* was identified as histidine (Dalen, 1973*a*) and, with a synthetic amino acid medium, histidine induced α -toxin production (Dalen, 1973*b*).

Hendricks & Altenbern (1968) found that α -toxin synthesis declined rapidly when cultures became more alkaline than pH 7.4, even though growth proceeded at an unaltered rate. Arvidson & Holme (1971) reported an optimal pH of 7.0 for α -toxin formation, while no toxin was produced at pH 9.0.

The effect of histidine dipeptides on the production of staphylococcal α -toxin would be of interest since they are known to stimulate the production of tetanus toxin (Mueller & Miller, 1956). Furthermore, some dipeptides are more readily transported into staphylococci than free amino acids (Rowlands, Gale, Folkes & Marrian, 1957).

The present work relates the pH effect on α -toxin production to the breakdown of histidine by a staphylococcal histidase. The uptake of certain histidine dipeptides and their effect on the formation of staphylococcal α -toxin are reported.

METHODS

Organism and growth conditions. *Staphylococcus aureus* strain Wood 46 was propagated as described earlier (Dalen, 1973*a*). Two different media were used for fluid cultures, one based on Casamino acids (Difco) and the other a synthetic medium composed of 11 amino acids, glucose, vitamins and salts (Dalen, 1973*a*).

Incubation conditions and measurements of bacterial concentrations and haemolytic activity were as indicated by Dalen (1973*a*). In testing the various histidine dipeptides, bacteria were pre-incubated in the synthetic amino acid medium (6 to 8 mg dry wt bacteria/ml) for 2 to 3 h. The bacteria were then diluted in fresh amino acid medium to 1 mg dry wt bacteria/ml. The compounds to be tested were 1 mM, and the cultures (5 ml in 50 ml Erlenmeyer flasks) were incubated at 37 °C for 2 h with shaking (30 to 40 strokes/min).

Preparation of bacterial extracts and partial purification of histidase. Bacteria were grown for 14 to 16 h in Casamino acid medium at 37 °C on a rotary shaker. One volume of fresh medium with tris-chloride buffer (final concentration 0.05 M, pH 8.5) was then added. The culture was supplemented with histidine (final concentration 1 mM) and further incubated for 3 to 4 h. Bacteria were collected by centrifuging and washed once with 0.05 M-tris-chloride buffer, containing 0.145 M-sodium chloride (pH 7.2). Lysostaphin (a gift from Dr P. A. Tavormina, Mead-Johnson Research Center, Evansville, Indiana, U.S.A.) in 50 µg/ml of the same buffer was added (100 µg enzyme/g wet wt bacteria) and incubated for 30 min at 37 °C to lyse the bacteria. RNase and DNase were then added (each at 50 µg/g wet wt bacteria) and incubation was continued for 30 min. The material was then centrifuged for 60 min at 12000 g. The supernatant was heated at 70 °C for 4 min, and precipitated protein was removed by centrifuging (30 min at 12000 g). Samples (5 ml) from the supernatant were applied to a 40 × 5 cm column of Sephadex G-100 (Pharmacia AB, Uppsala, Sweden), equilibrated with 0.05 M-tris-chloride buffer (pH 7.5). The column was eluted with the same buffer and 5 ml fractions were collected and assayed for histidase activity.

Enzyme assays. Histidine ammonia lyase (EC. 4.3.1.3, histidase) was assayed by measuring the formation of urocanate from histidine (Lessie & Neidhardt, 1967) by following the increase in extinction at 277 nm using a Unicam SP 800 spectrophotometer (Unicam Instruments Ltd, Cambridge). ΔE of 1.0 was assumed to be equivalent to 55 nmol of urocanate formed/ml of reaction mixture. The reaction mixtures (3 ml) contained 0.05 M-tris-chloride buffer (pH 9.2), 0.02 M-reduced glutathione, between 40 and 90 µg of protein and 0.01 M-K-histidine, which was adjusted to pH 9.2. The reactions were started by the addition of histidine.

Urocanase was assayed by measuring the decrease in extinction at 277 nm, associated with urocanate degradation. The reactions were performed in 0.05 M-tris-chloride buffer (pH 7.5) and 10⁻⁴ M-urocanate (3 ml). Between 40 and 90 µg of protein were added.

Protein determination. This was done by the Folin phenol method (Lowry, Rosebrough, Farr & Randall, 1951) using crystalline serum albumin as standard.

Polyacrylamide gel electrophoresis. The electrophoresis was carried out as indicated earlier (Dalen, 1973*b*), except for the staining of proteins, which was done with Coomassie Brilliant Blue, 0.05 % in 10 % trichloroacetic acid (Chrambach, Reisfeld, Wyckoff & Zaccari, 1967).

The earlier method (Dalen, 1973*b*) for analysis for extracellular proteins was slightly modified. Culture fluids were freed of bacteria by centrifuging and filtering through Millipore filters (pore size 0.45 µm). To fluids with a low protein content, 5 mg cellulose was added as a slurry to 10 ml fluid, which was the usual volume employed. Trichloroacetic acid (TCA) was added to a concentration of 5 % (w/v), and precipitation took place for 2 h at 4 °C.

The precipitate was washed twice with ethanol:0.05 M-tris-chloride (pH 7.5), 7:3. The precipitate was dried by evaporation and redissolved in 0.2 ml of 0.05 M-tris-chloride buffer (pH 7.5), with 8 M-urea. The cellulose was usually removed before gel electrophoresis. In contrast to earlier experiments, no urea was used in the polyacrylamide gels.

Detection of histidase activity on polyacrylamide gels. The gels were incubated immediately after the electrophoretic runs for 10 min in 0.05 M-tris-chloride buffer (pH 9.2) containing 2 mM-glutathione and 0.1 % eosin (v/v). They were then brought into a second solution containing 0.05 M-tris-chloride buffer (pH 9.2), glutathione (2 mM) and L-histidine (20 mM). The gels were observed at 5 min intervals in a Chromatovue cabinet (Ultraviolet Products Inc., San Gabriel, California, U.S.A.) with the 254 nm light source. Histidase activity was revealed as a dark band appearing against a bright yellow background, due to the absorption of urocanate (Roth & Hug, 1971).

Degradation of histidine by intact staphylococci. Bacteria (2 mg dry wt bacteria/ml) were incubated with 3 mM-histidine for 45 min and samples (5 ml) were removed at 15 min intervals. The incubation conditions were: phosphate buffer 0.07 M, pH 7.0; pyrophosphate buffer 0.07 M, pH 9.2; amino acid medium with phosphate buffer 0.07 M, pH 7.0; and amino acid medium with pyrophosphate buffer 0.07 M, pH 9.2 (vol. 20 ml). The bacteria were collected by centrifuging, and washed once in phosphate buffer. The bacteria were boiled for 10 min in distilled water (1 ml), and the fluids were freeze-dried after centrifuging. Supernatants from the cultures were also freeze-dried. Thin-layer chromatography was done on pre-coated plastic sheets (Polygram Cel 300, Macherey-Nagel & Co., Düren, Germany) with *n*-propanol:acetic acid:water (75:1.5:23.5). The Pauly reagent was employed to locate histidine and urocanic acid.

Intracellular uptake of histidine and histidine peptides. Bacteria were collected by centrifuging after pre-incubation in the amino acid medium, and washed once in a buffer containing sodium chloride, 0.082 M, magnesium chloride 0.5 mM, glucose 10 mM and Sørensen's phosphate buffer 0.03 M, pH 7.0. The washed bacteria were then suspended in the same buffer to a concentration of 1 mg dry wt bacteria/ml. Histidine and the histidine dipeptides to be tested were added at 1 mM (vol. 15 ml) and incubated with shaking at 37 °C. Samples (5 ml) were taken after 15, 30 and 60 min incubation and immediately filtered through Millipore filters (pore size 0.65 μ m). The collected bacteria were washed twice in 20 ml ice-cold distilled water and extracted with 5 ml of 60 % ethanol at 4 °C for 24 h. The extracts were freeze-dried and redissolved in 50 μ l volumes of distilled water for examination by thin-layer chromatography.

Thin-layer chromatography was done on plastic sheets covered with cellulose (MN Polygram cel 300, Macherey-Nagel & Co.) with iso-propanol:formic acid:water (40:2:10) as solvent. Pauly reagent was used for staining, and a Photovolt Densicord (Photovolt, New York, N.Y., U.S.A.) for quantitation of the spots.

Chemicals. Histidine and the various histidine dipeptides were obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A. Other chemicals were analytical grade reagents from various sources.

RESULTS

The yield of α -toxin, after induction with histidine in the amino acid medium, declined rapidly with increasing pH obtained either by including sodium bicarbonate in the medium (Fig. 1) or with tris-chloride buffer.

Since degradation of histidine at an alkaline pH was an explanation of the findings, this possibility was explored with intact bacteria at pH 7.0 and pH 9.2. Thin-layer chromato-

Fig. 1. Effect of pH on α -toxin production in a synthetic medium containing sodium bicarbonate. Inocula, 1 mg dry wt bacteria/ml. α -Toxin activity and the pH of cultural fluids were measured after 90 min incubation. □, pH; ○, haemolytic units.

graphy of extracts from bacteria and concentrates of supernatants, prepared after 15, 30 and 45 min incubation with 3 mM-histidine, showed that urocanic acid appeared in increasing amounts. Most urocanic acid was formed, both extra- and intracellularly, at pH 9.2. At pH 7.0 urocanic acid appeared in smaller quantities and only intracellularly.

Histidase activity was always present in the bacteria, but increased when urocanic acid or histidine was added to the medium. Histidine was more efficient than urocanic acid in inducing histidase when equimolar concentrations were compared at pH 7.0. A more detailed study on the induction of staphylococcal histidase remains to be done.

Histidase activity was found mainly intracellularly. Trace activity could be demonstrated in extracellular fluids concentrated 50-fold by ultrafiltration of overnight cultures in the Casamino acid medium.

Urocanase activity, as with histidase, was mainly found intracellularly and was thermolabile and completely inactivated by heating at 70 °C for 4 min. Its optimum pH was near 7.0; other properties were not investigated.

Properties of staphylococcal histidase. The preparative procedure gave an approximately sixfold purification and a recovery of 68 %. Histidase was eluted from columns of Sephadex G-100 in a single fraction with a K_a value of 0.06. Electrophoretic analysis on polyacrylamide gel of the active fraction from gel filtration revealed four weak bands in addition to histidase. With activity-staining for histidase, only one active band was found (R_f 0.60), both in crude extracts and in the active fraction from gel filtration.

Histidase which had been passed on a Sephadex G-100 column was active without the addition of reducing agent but glutathione or mercaptoethanol increased activity by about 10 %. There was, however, a gradual loss of activity during storage. Activity was restored by adding glutathione or mercaptoethanol, which were equally effective. Dithiothreitol and cysteine were inactive. Iodoacetamide (10^{-3} M) added to the reaction mixture did not inhibit enzyme activity.

Histidase was resistant to heat (Fig. 2), and its pH optimum was close to 9.0 (Fig. 3). The enzyme showed 25 % of maximum activity at pH 7.0.

The K_m of histidase was determined by a Lineweaver & Burk (1934) plot and was 1.2×10^{-2} M (Fig. 4).

Fig. 2

Fig. 3

Fig. 2. Effect of heating on histidase activity. Samples were heated for 4 min. Activity is expressed as a percentage of the maximum activity, which was 1 nmol/min/ml.

Fig. 3. Influence of pH on histidase activity. Activity is expressed as a percentage of maximum activity, which was 1 nmol/min/ml.

Fig. 4. Double-reciprocal plot showing the dependence on histidine concentration of the rate of formation of urocanic acid by histidase. Velocities are expressed as nmol urocanic acid formed/min/ml, and histidine concentrations in mM.

Pseudomonas histidase is very sensitive to EDTA, which is inhibitory at a concentration of 10^{-5} M (Lessie & Neidhardt, 1967). Staphylococcal histidase did not share this property. EDTA (10^{-2} M) gave 40 % inhibition, while a concentration of 10^{-3} M did not affect enzyme activity. Inhibition of *pseudomonas* histidase by pyrophosphate was ascribed to its metal-chelating properties (Lessie & Neidhardt, 1967). Pyrophosphate (pH 9.2), tested up to 15 mM, was without inhibitory effect on the staphylococcal enzyme. Mg, Ca, Mn and Zn did not influence histidase activity up to a concentration of 1.2 mM. Cysteine, which inhibits mammalian and *pseudomonas* histidases (Peterkofsky & Mehler, 1963), also inhibited staphylococcal histidase (Fig. 5).

Inhibitory activity of various imidazoles was tested up to a concentration of 8 mM. Imidazole acetic acid, histidinol, histamine, acetyl-histidine, glycyl-L-histidine, L-carnosine and L-homocarnosine were all inactive.

Fig. 5. Double-reciprocal plots showing the inhibition exerted by various concentrations of cysteine on the action of histidase. $\circ$, No addition; $\square$, 0.4 mM cysteine added; $\bullet$, 5 mM cysteine added. Velocities are expressed as nmol urocanic acid formed/min/ml, and histidine concentrations in mM.

Table 1. *Comparative activities of histidine peptides in α -toxin production by Staphylococcus aureus*

The various compounds were added to the amino acid medium at a concentration of 1 mM. Inoculum, 1 mg dry wt bacteria/ml; incubation time, 120 min.

Compound	Relative amount of α -toxin (%)	Haemolytic units/ml
Glycyl-L-histidine	100	22
L-Histidyl-glycine	88	18
L-Alanyl-L-histidine	76	16
L-Histidyl-L-alanine	64	14
Acetyl histidine	39	9
L-Carnosine	30	8
L-Homocarnosine	20	5
L-Histidine	68	15
Control	21	5

The effect of histidine and various histidine peptides on α -toxin production. The relative amounts of α -toxin formed in the amino acid medium after addition of various histidine dipeptides are shown in Table 1. L-Homocarnosine (γ -aminobutyryl-L-histidine) and L-carnosine (β -alanyl-L-histidine) did not stimulate α -toxin production, whilst the other peptides tested had a similar or more potent stimulating effect than free histidine.

Incubation at pH 6.3, at which histidase is inactive, gave less production of α -toxin. The differences in stimulating effect between the various compounds tended to be smaller, but the extracellular protein patterns revealed by polyacrylamide gel electrophoresis were the same at pH 6.3 and 7.0.

Analysis of extracellular fluids by polyacrylamide gel electrophoresis. Bacteria grown in the amino acid medium produced one major extracellular protein when incubated for 2 h (Fig. 6a). Inclusion of histidine or one of the active histidine dipeptides in the medium

Fig. 6. Polyacrylamide gel electrophoresis of extracellular proteins from *Staphylococcus aureus*. Concentrates from 10 ml of the amino acid and 4 ml from the Casamino acid fluids were used for each gel. Inoculum, 1 mg dry wt bacteria/ml; incubation time, 120 min. Arrows indicate the zones with haemolytic activity. (a) Amino acid medium; (b) amino acid medium with 1 mM-L-histidine; (c) amino acid medium with 1 mM-glycyl-L-histidine; (d) Casamino acid medium.

induced the additional formation of several other proteins (Fig. 6*b* and *c*). The free amino acid and the dipeptides induced a similar extracellular protein pattern. α -Haemolytic activity was located 3 mm below the top of the gels.

Bacteria grown under identical conditions in the Casamino acid medium produced a different extracellular protein pattern (Fig. 6*d*). The dominating protein band (R_f 0.53) was found with this medium irrespective of the inoculum size and length of incubation.

Uptake of peptides. The intracellular uptake of glycylhistidine and alanylhistidine was higher than of histidine (Fig. 7*a*). The dipeptides were rapidly split to the free amino acids, and only traces of dipeptides were found intracellularly. Histidine was broken down further to urocanic acid, which was found in considerable amounts intracellularly (Fig. 7*b*).

Uptake of L-homocarnosine was clearly different from the two other dipeptides. This was investigated using 2 mg dry wt bacteria/ml. When compared with histidine the uptake was 30% after 60 min incubation. Intracellularly, 40% was found as free histidine, and 60% as homocarnosine. Homocarnosine was therefore not only transported more slowly than the other dipeptides but also less readily hydrolysed.

Fig. 7. (a) Uptake of histidine and histidine dipeptides by *Staphylococcus aureus*, measured as intracellular free histidine/mg dry wt bacteria, ●, Histidine (1 mM); ■, glycyl-L-histidine (1 mM); ▲, L-alanyl-L-histidine (1 mM). (b) Uptake of histidine and histidine dipeptides in *Staphylococcus aureus*, measured as urocanic acid accumulated intracellularly. ●, Histidine (1 mM); ■, glycyl-L-histidine (1 mM); ▲, L-alanyl-L-histidine (1 mM).

Endogenous formation of histidine and urocanic acid during growth. Serine stimulates the production of α -toxin in stagnant cultures with low inocula in the amino acid medium without histidine (Dalen, 1973a). To see whether this was the consequence of intracellular accumulation of histidine, the intracellular levels of histidine and urocanic acid were measured after 2, 9 and 23 h incubation in shake cultures with an inoculum of 1 mg/ml and in stagnant cultures with an inoculum of 25 μ g/ml. The experimental conditions would allow the detection of a minimum of 10^{-10} nmol of histidine and urocanic acid/mg dry wt bacteria. There was no evidence of accumulation of histidine or urocanic acid above this limit.

DISCUSSION

The decline in production of α -toxin with increasing pH of the medium was well correlated with the action of histidase. The degradation of the inducer, even at pH 7.0, might explain the weak stimulatory properties of histidine on α -toxin formation at this pH when small inocula and prolonged incubation were used (Dalen, 1973a).

Histidine-degrading enzymes are induced by urocanic acid in *Aerobacter aerogenes* and *Pseudomonas* (Newell & Lessie, 1970), while histidine is the physiological inducer in *Bacillus subtilis* (Chasin & Magasanik, 1968). These findings are based on genetic studies, and similar studies should be done with *Staphylococcus aureus* before definite conclusions can be drawn about the inducer in this organism. Both urocanic acid and histidine gave a rise in histidase activity, suggesting that urocanic acid was the inducer. The fact that histidine was superior to urocanate does not contradict this. A similar finding in *A. aerogenes* has been related to a slower rate of entry of urocanate as compared with histidine into the bacterial cell (S. Schlesinger, P. Scotto and B. Magasanik).

A pH optimum near 9.0 of staphylococcal histidase is similar to that reported for other bacterial and mammalian enzymes. The K_m value of 1.2×10^{-2} M is also similar to that of *Bacillus subtilis*, *Pseudomonas fluorescens* and *Aerobacter aerogenes* enzymes, which vary between 0.3×10^{-2} and 2×10^{-2} M (Schlesinger *et al.* 1965). The enzymes from Gram-negative organisms are easily oxidized, which seems not to be the case with the enzyme from *B.*

subtilis as it is insensitive to thiol reagents (Hartwell & Magasanik, 1964). Staphylococcal enzyme resembled *B. subtilis* enzyme in this respect. There is no evidence of multiple active forms of histidase in staphylococci although these have been found in *Pseudomonas* (Soutar & Hassall, 1969).

In contrast to histidase from Gram-negative bacteria (Lessie & Neidhardt, 1967), EDTA did not inhibit the staphylococcal enzyme. The effect of EDTA on the *Bacillus subtilis* enzyme seems not to have been investigated. Cysteine, however, inhibited the enzyme in staphylococci as in *Pseudomonas*. The effect on the latter enzyme has been related to the metal-chelating properties of cysteine (Peterkofsky & Mehler, 1963), an explanation which is unlikely in the case of staphylococcal enzyme.

The transport of histidine has been extensively studied in *Salmonella typhimurium* (Ames & Roth, 1968). There are at least two systems for histidine transport in this organism. One is a general aromatic system with a low affinity for histidine, whilst the other is a specific high affinity system. Transport of histidine in *Staphylococcus aureus* seems not to have been investigated. Transport of dipeptides and oligopeptides is mediated by two distinct systems in *Escherichia coli* and, as far as it has been investigated, the same seems to be true for *Staph. aureus* (Payne, 1968). Oligopeptides use a common transport system which is also capable of transporting dipeptides. Several types of dipeptide permease systems seem to exist. They are specific for dipeptides, requiring a free α -amino group and a free C-terminal carboxyl group for their action (Payne & Gilvarg, 1968). This work shows that histidine dipeptides are more readily transported into staphylococci than the free amino acids, indicating an efficient dipeptide permease system. The findings also make the presence of a high-affinity specific histidine permease improbable in staphylococci. With glutamic acid and dipeptides containing glutamic acid similar findings were made (Rowlands *et al.* 1957). However, in their system there was a smaller difference between the rates of transport of amino acid and dipeptide. The possible existence of an additional oligopeptide permease system thus makes staphylococci equipped to utilize a variety of exogenous sources of histidine.

The peptides were readily cleaved intracellularly. Several attempts to show dipeptidase activity in extracellular fluids were unsuccessful. This indicates the presence of intracellular peptidases in staphylococci. Studies of such peptidases in *Escherichia coli* have revealed several types with varying specificities and requirements for activity (Sussman & Gilvarg, 1971). Homocarnosine, having the free amino group in the γ -position, accumulated slowly intracellularly and was also less readily cleaved than the dipeptides with free α -amino groups.

Our findings emphasize the resemblance between the stimulation of the production of staphylococcal α -toxin and of tetanus toxin (Miller, Gray & Eaton, 1960). Free histidine did not stimulate tetanus toxin production. This could be caused by a slow transport of free amino acid or a rapid breakdown intracellularly, or both. The effect of histidine dipeptides on tetanus toxin synthesis was related to their rate of hydrolysis intracellularly, which would indicate a correlation between intracellular free histidine and toxin synthesis. From the effect of histidine and the various histidine dipeptides on staphylococci, a correlation between the intracellular level of free histidine and the stimulating effect on α -toxin synthesis might be adduced. However, this was contradicted by the fact that α -toxin was formed in the absence of exogenous histidine. Histidine is then synthesized, and the intracellular level of free histidine as shown here must be very low. How the stimulating effect of histidine is mediated is therefore still unknown.

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