Metabolism of Pyrithiamine by the Pyrithiamine-requiring Mutant of *Staphylococcus aureus*

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(Received 13 November 1967)

1. A mutant strain of *Staphylococcus aureus* that requires pyrithiamine for its optimum growth was found to utilize pyrithiamine during the exponential phase of growth. 2. Pyrithiamine was deaminated by the organism to form oxypyrithiamine, the reaction being enzymic with no cofactor requirement. 3. On prolonged incubation of *S. aureus* A cultures, the concentration of deaminating enzyme increased in the culture broth, from which pyrithiamine-deaminating enzyme could be isolated by solvent fractionation. 4. Oxypyrithiamine is not a competitive analogue of thiamine although it inhibited the growth of the parent strain of *S. aureus*; the inhibition index of this compound, however, was lower than that of pyrithiamine.

In earlier publications (Das & Chatterjee, 1962*a,b*, 1963; Sinha & Chatterjee, 1967*a,b*) some of the biochemical characteristics of a pyrithiamine-requiring mutant of *Staphylococcus aureus* were described. While the fate of pyrithiamine [1-(4-amino-2-methylpyrimid-5-ylmethyl)-3-(β -hydroxyethyl)-2-methylpyridinium bromide] in the pyrithiamine-requiring mutant strain has been studied, it was found that the micro-organism elaborates a new enzyme that can deaminate the antimetabolite. The deaminated product was shown to be oxypyrithiamine [3-(β -hydroxyethyl)-1-(4-hydroxy-2-methylpyrimid-5-ylmethyl)-2-methylpyridinium bromide]. Oxypyrithiamine has been found to possess growth-inhibitory activity against the parent-strain *S. aureus* although this substance is less potent as an inhibitor than pyrithiamine.

In this paper, studies on the enzymic deamination of pyrithiamine by the pyrithiamine-requiring mutant of *S. aureus*, some of the properties of the crude enzyme isolated from the mutant strain and the effect of oxypyrithiamine on the growth of the parent strain are presented.

MATERIALS AND METHODS

Growth conditions. The pyrithiamine-requiring mutant of *S. aureus* (*S. aureus* A) was isolated and maintained by the methods of Das & Chatterjee (1962*a*). The organism was grown in liquid medium of the following composition: Difco vitamin-free casein hydrolysate (4.0g.), glucose (3.0g.), cysteine (0.02g.), nicotinic acid (0.02g.) and K_2HPO_4 (5.0g.), pH 7.0, in a final volume of 1 l. Pyrithiamine solution was sterilized separately by passage through a bacterial filter (Morton type) and added to give a con-

centration of 1 μ g. of pyrithiamine/ml. of medium. The organism was grown at 37° for 20 hr. in a rotary shaker (120 rev./min.) unless otherwise stated. Inhibitory doses of pyrithiamine and oxypyrithiamine were determined by inoculating a loopful of the organism (viable count about 10^6) from 48 hr.-old culture in the medium containing different amounts of either pyrithiamine or oxypyrithiamine. Growth was measured turbidimetrically with a Klett-Summerson photoelectric colorimeter (filter no. 54) after 24 hr. incubation at 37°.

Detection of the enzymic reaction products of pyrithiamine. Reaction products and residual pyrithiamine were isolated from culture filtrates as described in the Results section and examined by one-dimensional ascending paper chromatography on Whatman no. 4 chromatography paper. Solvent systems used were butan-1-ol-acetic acid-water (4:1:2, by vol.) and hexan-1-ol-acetic acid-water (4:2:1, by vol.).

Pyrithiamine-deaminating activity of cell fractions of the mutant organism and culture filtrate. Cell walls were prepared by the method of Cummins & Harris (1956), but treatment of the cell-wall preparation with either pepsin or trypsin was omitted. Cell-free extract was prepared by disintegrating the cells with Ballotini beads (grade 12) in 0.2 M-tris-HCl buffer, pH 7.7, in a Mickle disintegrator and centrifugation of the disintegrated suspension, as described by Das & Chatterjee (1963). Pyrithiamine-deaminating activity of the broth after incubation of the cells for various times was tested after centrifuging off the cells at 0°, followed by dialysis at 0° against water overnight.

Substrates. Pyrithiamine was purchased from Calbiochem, Los Angeles, Calif., U.S.A., and oxythiamine from the California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A. Oxypyrithiamine was prepared from pyrithiamine essentially as described by Raydon (1951) for the preparation of oxythiamine from thiamine with some minor modifications. The melting point (193°, decomp.) of the compound and its characteristic colour

reactions (positive diazosulphanilic acid reaction, negative thiochrome test) conjointly with analytical data coincided with those of the preparation of Cerecedo & Eich (1955).

Analytical methods. Pyrithiamine was determined by the method of Rindi & Perri (1961). Ultraviolet spectrophotometric studies were made on a Beckman spectrophotometer (model DU). Protein of the cell-free extract and crude enzyme was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The NH_3 produced by the enzymic deamination of pyrithiamine was determined by the method of Conway (1957). Bacterial dry weight was determined by a gravimetric method (Das & Chatterjee, 1962a).

Heat-killed bacteria. A suspension of *S. aureus* A in 0.85% NaCl solution was heated at 60° for 90 min.

RESULTS

Utilization of pyrithiamine by *S. aureus* A during the growth. *S. aureus* A was grown in 50 ml. of liquid medium with 1 μg . of pyrithiamine/ml. for 24 hr. at 37° in 250 ml. Erlenmeyer flasks in a rotary shaker. After growth the cells were centrifuged out at 0° and the supernatant was collected. The cells (21 mg. dry wt.) were then washed consecutively with 10 ml. of water and 10% (v/v) propan-1-ol solution; the washings obtained by centrifugation at 0° were mixed with the original culture supernatant. The combined supernatants were dried under vacuum. The solid residue thus obtained was dissolved in water and passed through a column (5.0 g.) of active charcoal (G.R.; E. Merck A.-G., Darmstadt, Germany). After the column had been washed with water, the adsorbed materials were eluted with 10% (v/v) propan-1-ol. The eluate was evaporated under vacuum and the solid residue tested for the presence of pyrithiamine by the method of Rindi & Perri (1961). The residue obtained from the supernatants under the above experimental condition contained only a trace amount of pyrithiamine. This indicated utilization of pyrithiamine by *S. aureus* A during its growth. When the utilization of pyrithiamine by *S. aureus* A during various periods of incubation was being studied it was further noted that pyrithiamine was utilized actively by the mutant strain during its exponential phase of growth (Fig. 1).

To investigate the fate of pyrithiamine during cell growth, dense suspensions of exponential-phase *S. aureus* A were incubated with pyrithiamine. Cells in the exponential growth phase were collected after 18 hr. of growth by centrifuging and, without being washed, suspended in 0.1 M-tris-hydrochloric acid buffer, pH 7.7. To 1 ml. of suspension (12 mg. bacterial dry wt.) 2 ml. of 0.1 M-tris-hydrochloric acid buffer, pH 7.7, and 80 μg . of pyrithiamine in solution were added and the volume was made up to 3.5 ml. with water. The suspension was incubated at 37° for 2 hr. with occasional shaking. After

Fig. 1. Utilization of pyrithiamine by *S. aureus* A during growth. Concentration of pyrithiamine (●) at different periods was measured by the method of Rindi & Perri (1961). Growth of the organism (O) (for details see the text) was measured turbidimetrically.

incubation, the cells were centrifuged off at 0° and the supernatant was passed through a column of active charcoal (2 g.) that was subsequently thoroughly washed with water, and the adsorbed material was eluted with 10% (v/v) propan-1-ol as above. The eluted material was next passed through Amberlite IRC-50 (H^+ form) and eluted with 0.1 N-hydrochloric acid, as described by Rindi & Perri (1961). After the removal of hydrochloric acid under vacuum, a portion of the eluate was examined by paper chromatography for the presence of pyrithiamine and its reaction products. After development, the paper was dried in air and the reaction products were located under ultraviolet light. Pyrithiamine and another spot with an R_F value greater than that of pyrithiamine were present. The latter spot was cut out and the unknown compound eluted with water. The ultraviolet spectrum of the unknown compound differed from that of pyrithiamine (two absorption maxima at 235 m μ and 271 m μ) in having only a single absorption maximum at 268 m μ . Further, the unknown spot did not give any fluorescent reaction product when the chromatogram was sprayed with 0.25% (w/v) potassium ferricyanide in 5 N-sodium hydroxide. On the basis of these properties and R_F values (Table 1) the compound appeared to be oxypyrithiamine. The experiment was repeated with heat-killed bacteria, which served as control.

Effect of oxypyrithiamine on the growth of *S. aureus* and *S. aureus* A. Since oxypyrithiamine is not an inhibitor of thiamine pyrophosphokinase (Cerecedo & Eich, 1955) and oxypyrithiamine is formed by *S. aureus* A from pyrithiamine, the effect of oxypyrithiamine on the growth of *S. aureus* and *S. aureus* A was investigated. Oxypyrithiamine at a

Table 1. *Comparison of properties of oxypyrrithiamine and the reaction product formed from pyrithiamine by a suspension of S. aureus A*

The reaction mixture contained 80 μ g. of pyrithiamine, 2 ml. of cell suspension (12 mg. dry wt.), 2 ml. of 0.1 M-tris-HCl buffer, pH 7.7, and water to a final volume of 3.5 ml. The reaction mixture was incubated at 37° for 2 hr. For details of the analytical procedures see the text.

Compound	Absorption maximum (m μ)	R_F		Formation of fluorescent product with alkaline $K_3Fe(CN)_6$ reagent
		Butan-1-ol-acetic acid-water (4:1:1, by vol.)	Hexan-1-ol-acetic acid-water (4:2:1, by vol.)	
Pyrithiamine	235, 271	0.32	0.28	Positive
Oxypyrrithiamine	268	0.54	0.44	Negative
Reaction product	268	0.54	0.44	Negative

concentration of 10.2 μ g./ml. inhibited the growth of the parent *S. aureus* whereas a much lower concentration (0.24 μ g./ml. of medium) of pyrithiamine inhibited the growth of the organism. The mutant organism failed to grow in the absence of pyrithiamine in the medium. Whereas oxypyrrithiamine (1 μ g./ml.) could replace pyrithiamine, thiamine (1 μ g./ml.) or oxythiamine (1 μ g./ml.) could not replace either pyrithiamine or oxypyrrithiamine.

Localization of the enzyme. Formation of oxypyrrithiamine by the cell suspension of *S. aureus* A suggested the presence of an enzyme in the organism that could deaminate pyrithiamine, and this possibility was investigated as follows. Cells collected after 18 hr. growth were broken in a Mickle disintegrator and the disrupted cells were fractionated to give cell-wall and soluble fractions as described in the Materials and Methods section. These fractions and also the cell-free broth were tested for enzymic activity. The deaminating activity of the cell-free supernatant was tested by incubating dialysed broth (pH adjusted to 7.7) with pyrithiamine. With cell-wall preparation and cell-free extract, pyrithiamine was added and the pH adjusted to 7.7 with 0.1 M-tris-hydrochloric acid buffer. Deaminating activity of intact cells was determined by suspending the cells in 0.1 M-tris-hydrochloric acid buffer, pH 7.7. The heat-killed bacteria were similarly suspended in 0.1 M-tris-hydrochloric acid buffer, pH 7.7, with pyrithiamine and were used as the control in the measurement of the deaminating activity of the intact bacteria. All the reaction mixtures were incubated at 37° for 90 min. with occasional shaking.

Owing to the lack of an amino group at the 4'-position oxypyrrithiamine does not give a fluorescence product (pyrithiochrome), and this allowed the amount of pyrithiamine deaminated to be determined by measuring the disappearance of pyrithiamine, which was determined fluorimetrically by the method of Rindi & Perri (1961). One unit of enzyme is defined as the amount of enzyme that deaminates 1 μ mole of pyrithiamine/min. at

pH 7.7 in 0.1 M-tris-hydrochloric acid buffer at 37°. Cell-free supernatant obtained from an 18 hr. culture of *S. aureus* A contained negligible activity. The intact organism contained 2.6 units of enzyme/mg. dry wt. of cells. On washing the cells once with 2% (w/v) sodium chloride solution about 10% of the total activity of the unwashed intact cells was lost, and on repeated washings the activity could be brought down to as low as 50% of the total activity normally retained by the cells. In another experiment treatment of intact cells with toluene did not increase the deaminating activity. The cell-free extract and the cell-wall fraction contained 5 units/mg. of protein and 10.2 units/mg. dry wt. of cell wall respectively. On prolonged incubation of *S. aureus* A in the liquid medium, the deaminating activity of the cell-free supernatant gradually increased up to 72 hr., after which the activity decreased again (Table 2). The deaminating activity of the intact cells during prolonged incubation decreased gradually with a concomitant decrease in the viable count.

Isolation of the crude enzyme. *S. aureus* A was grown in a flat-bottomed flask (4 l.) containing 2 l. of medium inoculated with 250 ml. of a shaken culture grown for 24 hr. The culture was incubated at 37° for 72 hr. with constant shaking. Cell-free supernatant was collected by centrifugation at 0°. To each 100 ml. of the cell-free broth 100 ml. of cold acetone (cooled to -16°) was added slowly with constant stirring. The precipitated protein with other materials from 2 l. of culture were centrifuged off at 0° and suspended in 5 ml. of water, and the suspension was dialysed against water at 0° overnight. After the dialysis the precipitate was removed by centrifugation at 0°. The clear supernatant was used as the enzyme source. This crude enzyme contained an appreciable amount of pyrithiamine-deaminating activity.

Spectral changes during the deamination of pyrithiamine by the crude enzyme preparation. The crude enzyme preparation was tested for pyrithiamine-deaminating activity both by spectral

Table 2. *Appearance of pyrithiamine-deaminating activity in the cell-free culture supernatant*

Reaction mixtures contained 10ml. of dialysed cell-free broth, 0.35 μ mole of pyrithiamine, 300 μ moles of 0.1 M-tris-HCl buffer, pH 7.7, incubated at 37° for 90 min. Details of the determination of deaminating activity of whole cells are described in the text.

Period of growth (hr.)	Units of enzyme/ mg. dry wt. of the cells	Approx. viable count (cells/ml.)	Units of enzyme/ 10ml. of cell-free supernatant
18	2.6	10 ⁶	5
36	2.0	10 ^{7.5}	28
54	1.6	10 ⁷	40
72	1.4	10 ⁵	71
84	0.8	10 ^{4.2}	51

Fig. 2. Ultraviolet spectra of pyrithiamine before (—) and after (---) incubation with pyrithiamine deaminase for 90 min. Reaction mixture contained 0.5 μ mole of pyrithiamine, 1.0 ml. of crude enzyme (equivalent to 1.5 mg. of protein) and 0.1 M-tris-HCl buffer, pH 7.7, to a final volume of 3.0 ml. After incubation at 37° for 90 min. the reaction was stopped by heating the mixture in a boiling-water bath. Precipitated protein was centrifuged off. A portion of the supernatant was appropriately diluted and ultraviolet spectra were taken. The spectrum of the pyrithiamine was measured under similar conditions, except that the enzyme preparation was heated in a boiling-water bath for 2 min. before the addition to the assay mixture.

changes and release of ammonia during the reaction. Spectral changes were investigated by incubating pyrithiamine (0.1 μ mole) with 0.3 ml. of diluted enzyme preparation (equivalent to 0.3 mg. of protein) and 0.1 M-tris-hydrochloric acid buffer, pH 7.7, to 1 ml. in a Beckman spectrophotometric

Fig. 3. Liberation of NH_3 from pyrithiamine by crude pyrithiamine deaminase from *S. aureus* A. Assay mixtures in Conway micro-diffusion cells contained 1.0 μ mole of pyrithiamine and 2 ml. of enzyme preparation (equivalent to 3 mg. of protein), and the volume was made up to 5 ml. with 0.1 M-tris-HCl buffer, pH 7.7. After incubation at 37° NH_3 was determined by the method of Conway (1957) by the direct nesslerization of trapped NH_3 .

cuvette at 37°. The reaction was started by the addition of the enzyme and extinctions were measured at 235 $\text{m}\mu$ and 271 $\text{m}\mu$ at 10 min. intervals. The extinction at 235 $\text{m}\mu$ fell sharply to a low value within 90 min., but that at 271 $\text{m}\mu$ did not show much change. The reaction was sensitive to 6% (w/v) trichloroacetic acid solution or heat. In another experiment a reaction mixture containing 0.5 μ mole of pyrithiamine, 1.0 ml. of enzyme preparation (equivalent to 1.5 mg. of protein) and 0.1 M-tris-hydrochloric acid buffer, pH 7.7, to 3 ml., was incubated for 90 min. The reaction was stopped by heating in a boiling-water bath for 2 min. and the precipitated protein was removed by centrifugation. The pH of the clear supernatant was

brought to pH 7.0, the solution appropriately diluted and its ultraviolet spectrum determined. A zero-time ultraviolet spectrum of the supernatant was made on a similar reaction mixture containing boiled enzyme preparation (Fig. 2).

Release of ammonia from pyrithiamine in the presence of crude enzyme was tested as follows. Reaction mixtures containing 1.0 μ mole of pyrithiamine, 2 ml. of enzyme preparation (equivalent to 3.0 mg. of protein) and 0.1 M-tris-hydrochloric acid buffer, pH 7.7, to 5 ml., were placed in Conway micro-diffusion cells. The reaction was started by the addition of the enzyme preparation and the reaction mixtures were incubated at 37° for various periods. Production of ammonia by deamination of pyrithiamine was determined as described by Conway (1957) (Fig. 3).

Optimum pH of the enzyme. Study of the deamination of pyrithiamine at 37° by the crude enzyme at various pH values showed that activity decreased with decreasing pH and was strongly inhibited at about pH 5.0. On increasing the pH above 8.5 the activity fell sharply. The optimum pH value of the enzyme was 7.5–7.8.

DISCUSSION

The pyrithiamine-requiring mutant of *S. aureus* A shows various biochemical and morphological differences when compared with the parent strain. The mutant organism needs pyrithiamine in the medium for its optimum growth; utilization of glucose is much depressed and the tricarboxylic acid cycle and amino acid utilization are stimulated in the mutant strain (Das & Chatterjee, 1962*a,b*, 1963; Sinha & Chatterjee, 1967*b*). It has further been found that there was deletion of thiamine pyrophosphokinase in *S. aureus* A (Sinha & Chatterjee, 1967*a*).

The present study of the metabolism of pyrithiamine by the pyrithiamine-requiring mutant strain has shown that pyrithiamine is primarily deaminated by an enzymic reaction and that there is no cofactor requirement for the process. An appreciable amount of the enzyme was found to be loosely bound to the cell wall. With the increase of the age of the culture the enzyme concentration was increased in the broth medium. This finding indicates that the enzyme was not readily released into the surrounding medium, and the increase in the concentration of the enzyme in the broth after prolonged incubation was probably due to autolysis of the cells. Treatment of cells with toluene did not increase the enzyme activity and this

might indicate the deaminating enzyme to be an exoenzyme. It appears that the first step in the utilization of pyrithiamine by the mutant strain is enzymic deamination with the formation of oxypyrithiamine, since oxypyrithiamine could replace pyrithiamine for the growth of the mutant organism. The failure of thiamine to replace pyrithiamine or oxypyrithiamine suggests that the role of pyrithiamine in the life process of the mutant strain is not to replace thiamine functionally. If a hydroxylated pyrimidine fragment of pyrithiamine were essential for the metabolic function(s) of the mutant, oxythiamine would have replaced pyrithiamine or oxypyrithiamine. The results also indicate the requirement for both pyrimidine and pyridine fragments of pyrithiamine. The pyrithiamine mutant of *S. aureus* seems to bypass the thiamine pyrophosphate-dependent metabolic pathways, perhaps through the conversion of pyrithiamine into some other compound that might play an essential role in the altered activity of the mutant strain. Oxypyrithiamine, however, inhibits the growth of the parent strain of *S. aureus* and, since oxypyrithiamine is not a competitive analogue of thiamine (Cerecedo & Eich, 1955), this indicates that in this organism pyrithiamine inhibits metabolic process(es) other than thiamine pyrophosphokinase activity (Sinha & Chatterjee, 1967*a*).

The authors thank Dr J. A. DeMoss of the University of California, U.S.A., for his helpful suggestions. Thanks are also due to Professor S. C. Roy, Professor J. J. Ghosh, Dr T. K. Roy, Dr D. Chakraborty and Dr K. Das for their helpful discussion. The co-operation of Mr Amit B. Kundu is also gratefully acknowledged. The investigation was supported by a grant-in-aid of the Council of Scientific and Industrial Research, India.

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