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Chlorpromazine as Permeabilizer and Reagent for Detection of Microbial Peroxidase and Peroxidaselike Activities

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Chlorpromazine was used to perform a test for the detection of microbial peroxidase activities. The compound acts as both a cell permeabilizer and a reagent in the procedure developed which allows the detection of peroxidase and peroxidaselike reactions both semiquantitatively in whole cell determinations and quantitatively in cell-free supernatants.

The antipsychotic drugs belong to the class of phenothiazines that possess multiple effects on eucaryotic and procaryotic cells. These effects include membrane permeabilization (6, 9), alteration of cyclic nucleotide metabolism (7), intercalation into DNA (5), and antimicrobial activity (4; L. Galeazzi, thesis, corso di laurea, Università di Camerino, Camerino, Italy, 1984).

Interestingly, phenothiazines, and particularly chlorpromazine, have also been used as reagents for developing tests for the demonstration of the peroxidaselike activity of hemoglobin (1, 3).

Peroxidase tests are also utilized in microbiology for the differentiation and characterization of bacterial strains. Generally, the bacterial peroxidase tests are used to perform semiquantitative whole cell enzymatic determinations. However, currently used semiquantitative assays for microbial peroxidases present some drawbacks, particularly the need for preliminary permeabilization of bacterial cells (8). For example, often the peroxidase test with *o*-dianisidine does not show any color formation with whole cells that have not been previously permeabilized. On the other hand, some procedures of permeabilization may interfere with enzyme detection (8).

Taking into account that chlorpromazine has been well established to cause alterations in the permeability of bacterial cell walls and membranes (6) and moreover to constitute an optimal reagent for detecting peroxidase activity (1, 3), we decided to develop a microbiological application of the chlorpromazine-peroxidase reaction. In this paper we describe an assay which demonstrates that chlorpromazine may contemporaneously act both as a cell permeabilizer and as a reagent for the detection and quantitative estimation of bacterial peroxidase activities.

The microbial strains tested were from the American Type Culture Collection (ATCC), Rockville, Md., and from specimens submitted to our clinical laboratory (Table 1). Bacterial suspensions for whole cell peroxidase determinations were prepared from colonies harvested from Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.). Although cells could be added directly to the reaction mixture with an inoculating loop, cell suspensions in water were used because they provide standardized inocula which may be turbidimetrically controlled. Inocula which gave an absorbance of about 1.5 to 2 at 540 nm in the final reaction mixture were used to avoid false-negative reactions due to weak color formation at lower inocula.

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Chlorpromazine hydrochloride of drug pure grade (Farmitalia-Carlo Erba, Milan, Italy), 25 g/liter in double-distilled water, was utilized. H_2O_2 (40%; Farmitalia-Carlo Erba, Milan, Italy) was used. Benzidine for the reference method (2) was from E. Merck AG, Darmstadt, Federal Republic of Germany.

To 2 ml of chlorpromazine solution 0.1 ml of the bacterial suspension was added. To obtain cell permeabilization the mixture was allowed to stand at room temperature. Then the reaction was started by the addition of 0.01 ml of the H_2O_2 solution. Visual readings were performed immediately after hydrogen peroxide addition and monitored for 10 min. This procedure gives an easy and clear semiquantitative evaluation of whole cell peroxidase and peroxidaselike activities. Upon treatment with chlorpromazine, in some bacteria an enzymelike activity can be detected also extracellularly, allowing the spectrophotometric monitoring of the reaction with the supernatants obtained after cell permeabilization. After H_2O_2 addition we performed time-dependent measurements at room temperature, monitoring the absorbance at 527 nm in cuvettes with a 10-mm light path. The wavelength of 527 nm was chosen after examination of the absorption spectra of the reaction product(s). The specificity of the reaction was confirmed by testing the activities of pure enzymes: peroxidase from horseradish and catalase from bovine liver, both obtained from Sigma Chemical Co., St. Louis, Mo.; catalase served as a negative control. All the spectrophotometric determinations reported were performed with a Uvikon 860 spectrophotometer (Kontron, Milan, Italy).

The peroxidase activity, i.e., color formation, is detected within the first 2 min after the addition of hydrogen peroxide. A pink to red color develops which gradually may turn to red-purple, and successively a slight or rapid decrease in the color intensity occurs. The color never develops in reactions not containing hydrogen peroxide; on the other hand hydrogen peroxide alone fails to give any color when added to the chlorpromazine solution in the absence of peroxidase. When catalase activity is present a stream of small bubbles gradually evolves, forming a temporary foam on the surface. However, it is important to emphasize that catalase does not carry out the same reaction as peroxidase (see Fig. 2) and that chlorpromazine did not inhibit the detection of catalase activity (bubbles) when this activity was present. Thus the assay can also function well in combined peroxidase-catalase detection. Table 1 shows the results of semiquantitative evaluation of whole cell peroxidase tests performed on 10 different microorganisms. The chlorpromazine method

TABLE 1. Whole cell peroxidase tests[a]

Strain	Peroxidase test results	
	Conventional benzidine method	Chlorpromazine test
Streptococcus species group A	−	−
Streptococcus pyogenes ATCC 19615	−	−
Streptococcus faecalis ATCC 19433	−	−
Staphylococcus aureus, catalase negative	+2	+2
Staphylococcus aureus	+2	+3
Staphylococcus epidermidis ATCC 12228	+3	+3
Proteus mirabilis	+3	+3
Escherichia coli ATCC 25922	+2	+2
Pseudomonas aeruginosa ATCC 27853	+5	+5
Saccaromyces cerevisiae	+4	+4

[a] Reactions were semiquantitatively rated − to +5 (white to dark green for benzidine and white to red-purple for chlorpromazine). As reported in the text the standardized bacterial suspensions for whole cell peroxidase determinations were prepared from colonies harvested from Mueller-Hinton agar.

was compared with a conventional (benzidine) method; as is usually done in whole cell peroxidase tests (8) the results were read by the naked eye semiquantitatively with the color formation expressed as a degree of reaction, with clear positivity equal to +5 and a negative reaction equal to −. Figure 1 shows the absorption spectrum and peak detection of the color developed in the chlorpromazine-peroxidase reaction; the following spectrophotometric measurements were done at the wavelength of 527 nm. Figure 2 clearly and quantitatively shows that catalase does not carry out the same reaction as peroxidase. In fact, purified catalase (500 U/ml) fails to give any color formation in the chlorpromazine test, whereas purified peroxidase (0.05 U/ml) gives a clear-cut positive reaction. Figure 3 shows the quantitative time-dependent spectrophotometric monitoring of the peroxidase activity (of various microbial strains) released extracellularly after treatment with chlorpromazine and then assayed as described above. Boiling of the peroxidase-positive control (pure enzyme) results in a negative activity in the chlorpromazine test. However, when very high concentrations of peroxidase are tested (450 U/ml) also after heat treatment some color formation takes place. Also, some bacteria highly positive in the peroxidase test, when autoclaved, still show some color formation, probably due to a peroxidase-like activity which is detectable also in the benzidine test. Thus, the assay reveals the overall peroxidase reactions of a bacterial suspension. The assay easily detects the peroxidase activity both of strains with high catalase activity and of a catalase-negative, peroxidase-positive mutant of *Staphylococcus aureus*; moreover, it never gave false-positive reactions with members of the family *Streptococcaceae*, which are known to be benzidine negative (2). Microbial cells showed their greatest activity in the chlorpromazine test when grown on media containing agar, whereas broth cultures gave low or negative results. Also, the agar medium used is of importance. In conclusion, the procedure de-

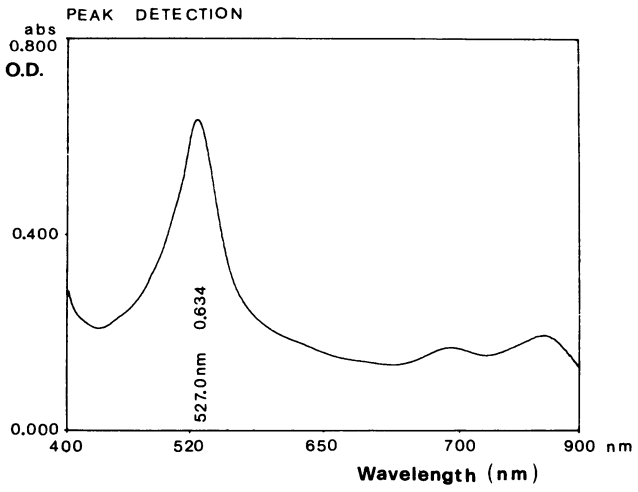

FIG. 1. Absorption spectrum and peak detection of the colored product of chlorpromazine oxidation in the peroxidase reaction. O.D., Optical density.

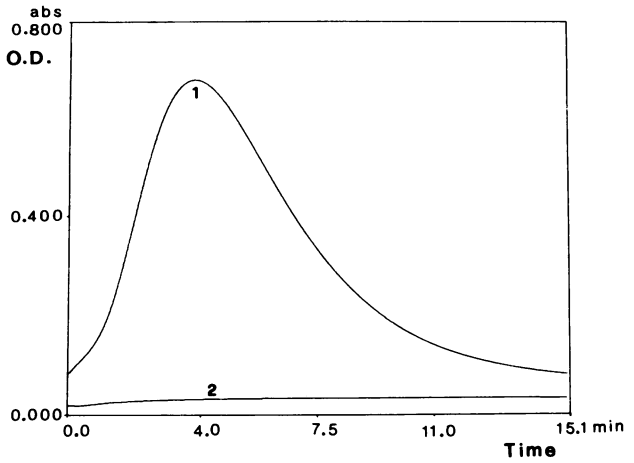

FIG. 2. Reaction of purified peroxidase and catalase in the chlorpromazine assay. The curves represent time-dependent spectrophotometric measurements of the absorbances at 527 nm and quantitatively demonstrate that catalase (curve 2) does not carry out the same reaction as peroxidase (curve 1). O.D., Optical density.

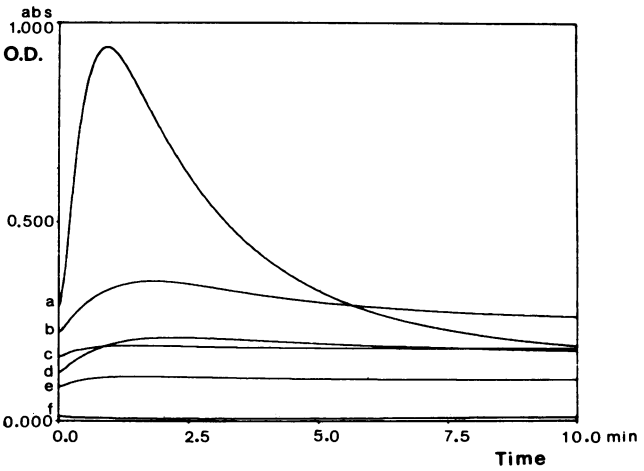

FIG. 3. Time-dependent spectrophotometric measurements (527 nm) of the peroxidaselike activities found in the supernatants obtained after cell permeabilization with chlorpromazine, tested as described in the text. Curves: a, *S. cerevisiae*; b, *P. mirabilis*; c, *S. aureus*; d, *E. coli*; e, *S. aureus* (catalase-negative mutant); f, reactive blank (chlorpromazine solution plus H_2O_2). O.D., Optical density.

scribed is simple and rapid to perform, and its development and optimization may allow a low-cost and safe additional peroxidase test in microbiology.

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