MODIFIED BENZIDINE TEST FOR THE DETECTION OF CYTOCHROME-CONTAINING RESPIRATORY SYSTEMS IN MICROORGANISMS[1]

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Received for publication July 27, 1959

Most aerobic and facultative bacteria contain a cytochrome system that serves as a terminal electron carrier during aerobic respiration. The majority of these bacteria also produce catalase that can decompose any hydrogen peroxide that might be produced by flavoprotein oxidases. These two iron-porphyrin systems generally are not present in anaerobic bacteria or in lactic acid bacteria. This correlation has led to employment of the simple test for catalase as a general test for iron-porphyrin systems, and a negative catalase test is a common criterion for differentiating lactic acid bacteria from morphologically similar organisms. However, catalase-positive lactic acid bacteria have been described (Felton *et al.*, 1953; Dacre and Sharpe, 1956; Vankova, 1957; Deibel and Niven, 1960) as have catalase-negative staphylococci (Lucas and Seeley, 1955; Everall and Stacey, 1956), tubercle bacilli (Coleman and Middlebrook, 1956; Knox *et al.*, 1956), and pseudomonads (Silliker, *personal communication*).

A benzidine test has been employed in clinical chemistry to detect the iron porphyrins of blood in urine and feces. In the presence of iron-porphyrin compounds and hydrogen peroxide, benzidine forms quinoidic bonds that impart a blue color to the reaction mixture (Malowan, 1952). This reaction has not been applied to the detection of iron porphyrins in bacteria although it has been used to detect hydrogen peroxide production in bacteriological media (McClung *et al.*, 1946; Kraus *et al.*, 1957).

The benzidine test is subject to a number of interfering conditions such as excessive fat in the specimen or the presence of copper, bromides, iodides, nitric acid, or formalin (Hepler, 1952) To avoid these difficulties Ham (1953) proposed the use of benzidine dihydrochloride in place of the more sensitive benzidine base reagent.

[1] Journal paper no. 187, American Meat Institute Foundation.

The present study has shown that the benzidine test using the dihydrochloride is quite satisfactory for detecting the presence of cytochrome systems in bacteria and is not confused by the presence of trace levels of catalase. Thus, this test when performed as described would seem to be a superior criterion for differentiating lactic acid bacteria from morphologically related organisms.

MATERIALS AND METHODS

Organisms. The lactic acid bacteria, staphylococci, micrococci, and bacilli were from the stock culture collection of the American Meat Institute Foundation. The *Salmonella*, *Paracolobactrum* and *Desulfovibrio* strains were obtained from Dr. J. H. Silliker, Swift and Company, Chicago, Illinois. Representative strains of *Microbacterium flavum* and *Microbacterium lacticum* were obtained from Dr. M. J. Pelczar, University of Maryland, College Park, Maryland. The *Pneumococcus*, *Corynebacterium*, and *Shigella dysenteriae* strains were obtained through the courtesy of Dr. B. A. Freeman, The University of Chicago, Chicago, Illinois. The *Clostridium*, *Erysipelothrix*, and *Propionibacterium* strains were from the American Type Culture Collection. The *Escherichia*, *Proteus*, *Aerobacter*, and other *Shigella* strains were isolated from clinical sources at St. James Hospital, Chicago Heights, Illinois.

A catalase-negative strain of *Staphylococcus aureus* was obtained from Dr. H. W. Seeley, Cornell University, Ithaca, New York. Two strains of catalase-negative pseudomonads were received from Dr. J. H. Silliker, Swift and Company, Chicago, Illinois. Three catalase-positive strains of *Lactobacillus plantarum* were obtained from Dr. M. E. Sharp, The University of Reading, England.

Reagents. The benzidine solutions were prepared by the method of Bing and Baker (1931; also see Ham, 1953). One gram of benzidine-

2 HCl (Fisher) or benzidine base (Merck) was partially dissolved in 20 ml of glacial acetic acid, 30 ml of distilled water were added and the solution heated gently, cooled, and 50 ml of ethyl alcohol (95 per cent) were added. The development of a slight yellow color in the dihydrochloride upon storage does not alter the sensitivity of the reagent (Ham, 1953). At refrigerator temperatures the reagent is stable for at least 1 month.

Fresh hydrogen peroxide solutions were prepared each week by diluting 30 per cent reagent grade hydrogen peroxide (Merck).

Methods of performing the benzidine test. After good growth of the respective organisms was obtained (24 to 48 hr) the plate was flooded with the benzidine dihydrochloride solution followed by the addition of an approximately equal volume of 5.0 per cent hydrogen peroxide. The benzidine dihydrochloride reagent must come in contact with all of the microbial growth before the introduction of the hydrogen peroxide. If the culture in question possessed iron-porphyrin compounds, a blue-green to deep blue coloration of the microbial growth promptly developed. Some variation in the intensity of the color was noted depending on the species and strain examined. Only the microbial growth (either individual colonies or confluent growth) evidenced a positive test. The medium, itself, never gave a positive reaction unless high concentrations of iron were added.

The benzidine test, as described by Bing and Baker (1931), for the occult blood determination employed 0.6 per cent concentration of hydrogen peroxide. However, preliminary studies showed that 4 to 6 per cent peroxide gave much more rapid and intense color development. Consequently, a 5.0 per cent solution was employed throughout this study.

All attempts to perform the benzidine test directly on broth cultures failed. This may have been due to an actual dilution of the iron-porphyrin compounds in the culture, a dilution of the reagents, or a masking effect due to the color of the medium. However, if cultures of benzidine-positive organisms were first centrifuged and the bacterial sediment resuspended in 0.5 ml of the reagent followed by the addition of 0.5 ml of 5.0 per cent hydrogen peroxide, a positive test could be obtained. In the later phases of this study this method of testing was applied to a large number of cultures and the results were in good agreement with the plate method of testing. The benzidine-positive cultures turned the entire 1-ml content of the tube a blue-green to blue color. Occasionally a benzidine-negative strain (agar plate test) when tested in this manner evidenced a very small speck of blue coloration; however, the tube contents remained uncolored.

RESULTS AND DISCUSSION

Sensitivity and specificity of the benzidine reagents. It has been established that under certain conditions a large number of compounds produce colored substances in the presence of the benzidine base reagent but not the benzidine dihydrochloride reagent (Ham, 1953). Preliminary experiments were conducted to compare the relative sensitivity and specificity of benzidine dihydrochloride and benzidine base for the detection of bacterial iron-porphyrin systems.

A comparison of the sensitivity of the two benzidine reagents was obtained by preparing duplicate, decimal dilutions of crystalline hemin (Armour) in an ethyl alcohol-glacial acetic acid-water mixture. Tubes containing varying concentrations of hemin in 1 ml of the diluting mixture were developed by the addition of 0.5 ml of benzidine reagent and 0.5 ml of 5.0 per cent peroxide. The lowest concentration of hemin detectable with the benzidine dihydrochloride reagent was 1 μg per ml. At this level a faint green color was produced. However, at the 1 μg per ml level the benzidine base reagent gave a strong positive test. At a concentration of 0.1 μg per ml, neither reagent produced a positive test.

To detect possible interfering substances, a large number of the more commonly employed bacteriological plating media were prepared, poured into plates, and streak inoculated with a benzidine-negative (*Streptococcus faecalis*) and a benzidine-positive organism (*Escherichia coli*). After incubation, inoculated and uninoculated plates were developed with the two benzidine reagents. None of the uninoculated media gave a positive reaction with benzidine dihydrochloride, although in some instances a diffuse blue reaction was noted when serum was added to the medium. Of course, the employment of blood agar is prohibited. As demonstrated in table 1, media containing moderate levels of ferrous or ferric ion often produced a faint color reaction. The *E. coli*

TABLE 1

Effect of iron on the benzidine test

Conc. of Iron Salt Added*	Plates Developed with Benzidine Dihydrochloride			Plates Developed with Benzidine
	Escherichia coli†	*Streptococcus faecalis*†	Uninoculated	Uninoculated
None	+	−	−	−
+0.001% $FeCl_3$	+	−	−	−
+0.01% $FeCl_3$	+	−	−	(±)
+0.1% $FeCl_3$	+	−	Wk +	Wk +
+0.001% $FeCl_2$	+	−	−	−
+0.01% $FeCl_2$	+	−	(±)	Wk +
+0.1% $FeCl_2$	+	−	+	+

* The basal medium was tryptone glucose yeast agar (Difco).

† These results refer to the reaction given by the bacterial growth and are independent of the color given by the media that have a high iron content.

growth gave a satisfactory positive test and the *S. faecalis* growth gave a negative test with either reagent on all media. The positive reaction develops within approximately 15 sec and may fade after 2 to 5 min.

Despite its lesser sensitivity (or, possibly because of it) the benzidine dihydrochloride reagent is considered to be more suitable for this test because of its relative freedom from interfering reactions. Furthermore, the benzidine base is reported to be a highly carcinogenic compound in contrast to the benzidine dihydrochloride.

Benzidine testing of various microbial genera. Representative strains of a large number of microbial genera were screened for the presence of iron porphyrins. These organisms were streaked on appropriate agar media and after incubation sufficient to produce good growth they were tested with the benzidine dihydrochloride. The results of this screening are summarized in table 2. It is noteworthy that none of the various media employed offered any interference with the test. Furthermore, the microbial growth always gave either a definite positive or a totally negative reaction. Either the characteristic green to deep blue-green color developed promptly or the microbial growth was completely devoid of color.

From the data presented in table 2 it further appears that this test clearly delineates the so-called lactic acid group of bacteria from other facultative and aerobic microorganisms. Although some of the pediococci and lactobacilli have been shown to synthesize modest amounts of catalase, these strains failed to give a positive benzidine test. Thus, the benzidine test would appear to be superior to the catalase test for differentiating lactic acid bacteria.

The results obtained with the genus *Microbacterium* presented an interesting deviation in that all of these organisms were benzidine-positive with the exception of the species *Microbacterium thermosphactum*. Three strains of *M. flavum* and 5 strains of *M. lacticum* gave positive tests, but the 6 strains of *M. thermosphactum* gave negative tests. These results would suggest that *M. flavum* and *M. lacticum* are physiologically close to the corynebacteria and the *M. thermosphactum* is more closely related to the lactic acid bacteria.

The negative benzidine reaction of the *Erysipelothrix* strains, as well as the absence of the catalase activity in these bacteria (Breed *et al.*, 1957), suggests that further study and re-evaluation of the taxonomic position of this genus might be fruitful. With the exception of patho-

TABLE 2

Response of various microorganisms to the benzidine (dihydrochloride) test

Benzidine-positive		Benzidine-negative	
Genus	No. of strains	Genus	No. of strains
Shigella	6	*Leuconostoc*	6
Salmonella	7	*Lactobacillus*	21
Paracolobactrum	4	*Pediococcus*	28
Proteus	20	*Streptococcus*	56
Pseudomonas	14	*Diplococcus*	1
Escherichia	23	*Clostridium*	6
Aerobacter	6	*Erysipelothrix*	2
Staphylococcus	18	*Desulfovibrio*	3
Micrococcus	21		
Neisseria	2		
Bacillus	4		
Corynebacterium	2		
Propionibacterium	2		
Debaryomyces	6		
Torulopsis	2		
Candida	4		
Other fungi	6		

TABLE 3

Comparison of catalase activity and the benzidine test

Species	No. of Strains	Catalase Activity		Benzidine Dihydrochloride Test*
		TYG agar	APT agar	
Lactobacillus plantarum	2	+	−	−
Pediococcus cerevisiae	14	6+; 8−	−	−
Pediococcus homari	14	7+; 7−	−	−
Microbacterium thermosphactum	6	5+; 1−	4+; 2−	−
Staphylococcus aureus, N55	1	−	−	+
Shigella dysenteriae	1	−	−	+
Pseudomonas sp.	2	−	−	+

* Identical results were obtained with APT (Evans and Niven, 1951) agar and TYG (Felton *et al.*, 1953) agar.

TABLE 4

Comparison of the sensitivity of the benzidine test and the test for catalase activity

Test Reagent	Catalase Conc* (μg/ml)					
	10,000	1,000	100	10	1	0.1
Benzidine dihydrochloride	+	+	+	−	−	−
Benzidine base	+	+	+	+	−	−
Hydrogen peroxide	+	+	+	+	+	−

* Crystaline catalase (Armour).

genicity, which is a poor criterion for classification purposes, these organisms are physiologically similar to the lactobacilli. Additional physiological information such as the type of acid produced from glucose would be helpful in establishing an improved classification of this genus.

The reaction of only a few strictly anaerobic bacteria to the benzidine test was determined. The small number of *Clostridium* and *Desulfovibrio* strains tested in this study were found to be benzidine-negative. However, from the reports of Postgate (1954) and Kamen and Vernon (1954) in which cytochrome pigments were detected in various anaerobic bacteria, it is possible that such organisms and some other strict anaerobes might be benzidine-positive.

The results of the benzidine test and the known occurrence of cytochrome systems in the various genera presented in table 2 are in good agreement. Cytochrome components have been detected in the majority of benzidine-positive genera listed in table 2, whereas cytochrome pigments generally are absent in the benzidine-negative genera (Smith, 1954; Clark *et al.*, 1955).

Relation of catalase activity and the benzidine test. As shown in the preceding section, the lactic acid bacteria that produce catalase do not give a positive benzidine test. Media having a low level of glucose are required to demonstrate catalase in most of these organisms and even then the reaction generally is quite weak.

A collection of pediococci with weak catalase activity, as well as 2 catalase-positive strains of *Lactobacillus plantarum*, were cultured in duplicate on a medium with a low glucose content (TYG agar; Felton *et al.*, 1953) and on APT agar (Evans and Niven, 1951). One set of each was then tested with the benzidine dihydrochloride reagent and the other developed with hydrogen peroxide for the detection of catalase activity. As shown in table 3, regardless of the medium employed, the catalase-positive strains of pediococci, lactobacilli, and *M. thermosphactum*, as well as the catalase-negative strains of these species, gave a negative benzidine test. On the other hand, catalase-negative strains of *S. aureus*, *S. dysenteriae*, and *Pseudomonas* sp. gave a positive benzidine test.

A direct test was made of the relative sensitivity of the catalase and benzidine tests by preparing decimal dilutions of crystalline catalase (Armour) in triplicate and developing one series with hydrogen peroxide, another with the benzidine dihydrochloride reagent, and the third series with the benzidine base reagent. As shown in table 4, the catalase test was much more sensitive than the benzidine test, particularly when

the benzidine dihydrochloride reagent was used for the latter test.

Several methods were employed in an attempt to increase the amount of iron-porphyrin compounds produced in cells of *M. thermosphactum* and pediococci that produce small amounts of catalase but give a negative benzidine test. Cultures were grown in a wide variety of liquid media under highly aerobic conditions, but none of the cell crops gave a positive benzidine test. Solid media were supplemented with varying amounts of iron salts, glycine, and succinate, or low levels of hemin, and similar negative results were obtained. Thus, it would appear that the cleavage between benzidine-positive and benzidine-negative strains is both sharp and stable. It is presumed to correlate directly with the presence or absence of a functional cytochrome system of terminal respiration.

ACKNOWLEDGMENTS

The authors are indebted to the numerous contributors who generously provided many of the cultures employed in this study. Appreciation also is extended to Dr. P. T. Fagan, Clinical Laboratories, St. James Hospital, Chicago Heights, Illinois, in whose laboratories a portion of this study was conducted.

SUMMARY

The benzidine test for iron-porphyrin compounds has been modified and adapted so that it is suitable for the routine detection of cytochrome-containing respiratory systems in microorganisms. It has been shown to be much more suitable than the catalase test for the delineation of the lactic acid group of bacteria and their differentiation from morphologically similar bacteria. Also, it would appear to be of value in studies on the physiological relationship of other groups or genera of microorganisms.

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