AN IMPROVED FERRIC CHLORIDE TEST FOR DIFFERENTIATING PROTEUS-PROVIDENCE GROUP FROM OTHER ENTEROBACTERIACEAE

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It has been found by a number of investigators that cell suspensions and sonic extracts of one species (*Proteus vulgaris*) of the family Enterobacteriaceae contain an enzyme which catalyzes the oxidative deamination of most of the L-amino acids to their corresponding α-keto acids (Bernheim *et al.*, 1935; Uemura, 1942*a*, *b*; Uemura, 1944; Stumpf and Green, 1944).

Henriksen (1950) utilized this faculty of *P. vulgaris* to develop a test for differentiating the Proteus group from other enteric organisms. When cell suspensions are incubated with phenylalanine, ferric ions react with the formed phenylpyruvic acid to yield a green color which, however, fades quickly.

This study reports a simplification of the above test and its trial with 22 amino acids giving, in some cases, different colors specific to the acid; tryptophan proved most suitable for the detection of the Proteus-Providence group. Preliminary results were reported earlier (Singer, 1953).

MATERIALS AND METHODS

Cultures. The cultures tested included strains of the following groups of Enterobacteriaceae mostly obtained from local isolates: Escherichia (120 strains), Salmonella (60), Arizona (6), Bethesda (60), Ballerup (4), Shigella (50), Alkalescens (15), Dispar (15), Klebsiella (120), *Proteus rettgeri* (25), *P. morganii* (60), *P. mirabilis* (110), *P. vulgaris* (15), *Proteus* sp. urease negative and Providence (100). All stock cultures were maintained on nutrient agar slants.

Amino acids. The various amino acids used were obtained from the Nutritional Biochemical Corporation.

α-Keto acids. The following keto acids, as sodium salts, were kindly supplied by Dr. A. Meister: α-ketoisocaproic acids, α-ketoisovaleric acid, α-keto-γ-methylthiobutyric acid, phenylpyruvic acid, and *p*-hydroxyphenylpyruvic acid. Indole-3-pyruvic acid was obtained from Bios Laboratories, Inc.

Ferric chloride tests. The following procedure was finally adopted: An 18 to 24 hour old nutrient agar slant culture was suspended in 1 ml saline and transferred to a 5 ml test tube. Equal volume of 0.2 per cent of the L- or DL-amino acid dissolved in saline was added, and the tubes were shaken in a Kahn shaker for 15 to 30 minutes at room temperature. Upon the addition of one or two drops of 10 per cent ferric chloride solution, various colors developed, specific to the amino acid tested. With tryptophan, one additional drop of 50 per cent HCl was added in order to destroy the red-orange color formed from tryptophan alone with ferric chloride, and thus to intensify the cherry-red color produced with the indolepyruvic acid formed from tryptophan by the organisms.

RESULTS

Ferric chloride reaction with incubated amino acids. The amino acids L-histidine, L-leucine, DL-isoleucine, DL-norleucine, DL-methionine, DL-norvaline, L-phenylalanine, and L-tryptophan, incubated with cell suspensions of the various organisms obtained from nutrient agar slant cultures as described above, gave different colors with ferric chloride as shown in table 1. All colors except that derived from tryptophan faded gradually. With DL-alanine, DL-arginine, L-aspartic acid, L-cysteine, L-cystine, L-glutamic acid, glycine, DL-lysine, DL-ornithine, L-proline, L-hydroxyproline, DL-serine, DL-threonine, DL-valine, and L-tyrosine the color was almost indistinguishable from that of ferric chloride alone.

Hydrazones. In order to correlate the ferric chloride test with the presence of the corresponding keto acids formed from the amino acids by oxidative deamination, the incubation mixture was tested for hydrazone formation.

TABLE 1

Correlation between the formation of hydrazone and the formation of color upon the addition of ferric chloride from various amino acids incubated with various groups and subgroups of Enterobacteriaceae

Groups and Subgroups	Hydrazone	Amino Acids							
		Histidine	Leucine	Isoleucine	Norleucine	Methionine	Norvaline	Phenylalanine	Tryptophan
Escherichia	−	Y	Y	Y	Y	Y	Y	Y	Y
Salmonella	−	Y	Y	Y	Y	Y	Y	Y	Y
Arizona	−	Y	Y	Y	Y	Y	Y	Y	Y
Bethesda	−	Y	Y	Y	Y	Y	Y	Y	Y
Ballerup	−	Y	Y	Y	Y	Y	Y	Y	Y
Shigella	−	Y	Y	Y	Y	Y	Y	Y	Y
Alkalescens	−	Y	Y	Y	Y	Y	Y	Y	Y
Dispar	−	Y	Y	Y	Y	Y	Y	Y	Y
Klebsiella	−	Y	Y	Y	Y	Y	Y	Y	Y
Proteus rettgeri	+	G	GV	O	O	V	O	G	CR
Proteus morganii	+	G	GV	O	O	V	O	G	CR
Proteus mirabilis	+	G	GV	O	O	V	O	G	CR
Proteus vulgaris	+	G	GV	O	O	V	O	G	CR
Proteus sp. urease negative	+	G	GV	O	O	V	O	G	CR
Providence	+	G	GV	O	O	V	O	G	CR

Y = Yellow color indistinguishable from that of ferric chloride alone; G = green; GV = grayish-violet; O = olive; V = violet; CR = cherry-red.

Two 18 hour old agar slants were suspended in 10 ml saline and transferred to 50 ml Erlenmeyer flasks. Ten ml of a 0.2 per cent solution of the various amino acids were added, and the mixture was shaken in a Kahn shaker for 30 minutes at room temperature. The suspension was adjusted with acetic acid to pH 3.8 and heated in boiling water for 10 minutes; it was then centrifuged. The supernatant was treated dropwise with saturated solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid until a precipitate was formed. The results are summarized in table 1. It was found that in all cases where the ferric chloride test was positive, hydrazone formation occurred.

Ferric chloride reaction with authentic samples of α-keto acids. Further evidence to prove that the color produced with ferric chloride in the oxidation of the amino acids is due to the formation of keto acids is given in the following experiments. One milligram of the keto acid was dissolved in distilled water, and a drop of 10 per cent ferric chloride solution was added. The colors obtained with the various keto acids are given in table 2. Comparing table 1 with 2, it can be seen that in all cases the colors are identical.

Routine screening test for Proteus-Providence group. In the course of this study it was found that among the Enterobacteriaceae only cell suspensions of Proteus and Providence, when incubated with tryptophan, produced a very intensive and stable cherry-red color. This suggested the use of tryptophan as a substrate for the ferric chloride test, thus replacing the urea medium described by Rustigian and Stuart (1941), Ferguson and Hook (1943), and Singer (1950) for the differentiation of the Proteus-Providence group from other enteric bacteria.

TABLE 2

Color formation from α-keto acids with ferric chloride

Keto Acid	Corresponding Amino Acid	Color with FeCl₃
α-Ketoisocaproic acid	leucine	gray-violet
α-Keto-γ-methylthiobutyric acid	methionine	violet
Phenylpyruvic acid	phenylalanine	green
Indole-3-pyruvic acid	tryptophan	cherry-red
p-Hydroxyphenylpyruvic acid	tyrosine	yellow*
α-Ketoisovaleric acid	valine	yellow*

* Indistinguishable from the color of ferric chloride alone.

For routine screening of this group, the following procedure is recommended:

Colonies isolated from primary plates of Difco SS agar or MacConkey agar are inoculated on Difco triple sugar iron agar slants and incubated for 24 hours at 37 C. The growth is washed off with 0.5 ml of 0.2 per cent aqueous tryptophan solution, then transferred to a 5 ml test tube, and shaken for one hour. One drop of 50 per cent HCl and a drop of a 10 per cent ferric chloride are added. A very intensive cherry-red color develops instantly with strains of Proteus-Providence group. Reliability and accuracy of this procedure have been proved in hundreds of cases in the laboratory of the senior author.

DISCUSSION

It is well known (Henecka, 1950; Houben-Weyl, 1953) that certain α and β-keto acids give a positive color reaction with ferric chloride in aqueous or alcoholic solution. The ability of the carbonyl group to enolyze determines whether a positive or a negative ferric chloride reaction is obtained.

This reaction was used by Henriksen (1950) to differentiate the Proteus group, which can oxidize amino acids to their corresponding α-keto acids, from other enteric bacteria lacking this faculty. Phenylalanine is used as a substrate, and a green color is formed with the ferric ions and the phenylpyruvic acid produced in the oxidation of phenylalanine. It has, however, the disadvantage that the color is rather unstable and fades quickly.

In the present study, a simplified ferric chloride reaction yielded various colors with the amino acids histidine, leucine, isoleucine, norleucine, methionine, norvaline, phenylalanine, and tryptophan, previously incubated with cell suspensions of Proteus and Providence. With other amino acids, the color was almost indistinguishable from that of ferric chloride alone. None of the other groups of the enteric bacteria oxidized the amino acids.

The ferric chloride test which gives a sensitive, very intensive, and stable cherry-red color with indole-3-pyruvic acid formed from tryptophan could be used in routine tests in place of phenylalanine. The results were identical with those obtained by Henriksen (1950). They also confirm the recent suggestion of Singer and Bar-Chay (1954) that the Providence group is closely related to Proteus.

It was found further that the positive ferric chloride reaction, in all cases, was correlated with the formation of hydrazones. Moreover, available authentic samples of a number of the α-keto acids with ferric chloride gave, in all cases, a specific color similar to that obtained from the corresponding incubated amino acids. It is suggested, therefore, that this ferric chloride test can be used as an auxiliary method for detecting the formation of α-keto acids from a number of amino acids.

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SUMMARY

Among the Enterobacteriaceae, only the Proteus-Providence group can oxidize amino acids to their α-keto acids. A number of the derived keto acids react with ferric chloride to form a specific color, thus offering a method for the detection of such keto acids.

Using tryptophan as a substrate, a simplification of the method described by Henriksen for differentiating the Proteus-Providence group from other enteric bacteria is recommended for routine tests.

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