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BIOCHEMICAL TESTS FOR BACTERIAL
CHARACTERIZATION. 1. HIPPURATE TEST

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Bacterial characterization is dependent on observations and tests that are both reliable and reproducible. Recently, a series of micromethods using growing cultures was described by Weaver and his colleagues (Arnold and Weaver, 1948; Hannan and Weaver, 1948; Morse and Weaver, 1950; Fabrizio and Weaver, 1951; Bachmann and Weaver, 1951; Hargrove and Weaver, 1951; for review see Cowan, 1953a) and another series using living suspensions by Clarke and Cowan (1952), Cowan (1953b, d) and by Shaw and Clarke (1955). Experience has shown that some of these microtests can be improved by changes in technique (Clarke, 1953; Cowan, 1953c) and that there is a need for new tests to improve bacterial characterization. The present paper concerns one of the more recently developed microtests.

Hajna and Damon (1934) described a medium, with sodium hippurate as the only carbon source, which could be used to distinguish between Aerobacter aerogenes and A. cloacae, either by observing growth or by the persistence of a precipitate produced by the action of ferric chloride with benzoic acid; hippuric acid gives a precipitate which is soluble in excess of ferric salt. In use the test had two disadvantages: (i) like Koser's citrate test the result could be vitiated by carry-over of the medium, (ii) the uninoculated medium sometimes had a slight precipitate which made scanty growth difficult to recognize. Furthermore the ferric salt test depended on a preliminary titration of each batch of medium to find the optimal amount of ferric salt solution.

In a trial of the hippurate medium, changes in pH value of the medium were observed by adding a few drops of indicator solution to duplicate tubes. When the uninoculated medium had a pH value of 6.4, the medium in a positive test had a pH of 6.8-7.2 after 4 days growth, and 7.4-7.6 after 7 days.

From this preliminary experiment was developed a test in which a colour change indicated a positive result; this was easier to read than the precipitate test or the presence or absence of growth.

Media and Methods

A saline suspension containing about 10^8 organisms per ml. was inoculated with a straight wire into tubes containing about 5 ml. of medium prepared according to the formula of Hajna and Damon (1934). Cultures were incubated at 30°C and growth estimated by observing opacity. The hydrolysis of sodium hippurate was detected by adding half-saturated ferric alum solution; the amount to be added was previously determined as the least amount which completely redissolved the precipitate formed when lesser amounts of ferric alum were added to the uninoculated medium.

For the microtest, suspensions were made as described by Clarke and Cowan (1952). Sodium hippurate solutions were sterilized either by Seitz filtration or by autoclaving at 10 lbs for 10 minutes. Sorensen's phosphate buffer at pH 6.8 was used at 0.025 M concentration.

Experimental

Cultures in Hajna and Damon's medium were inoculated in duplicate, incubated, and the opacity of the cultures observed daily. After 7 days incubation ferric alum was added to one set of tubes and bromthymol blue solution to the other. Table 1 gives examples of typical results; strains of Escherichia coli did not grow in the medium and did not produce any change in reaction; on the other hand Klebsiella cultures reacted in different ways, some growing and changing the reaction of the medium, others not growing in it. Neither the opacity of the medium nor the ferric alum precipitate correlated absolutely with the change in pH value, but it seemed likely that the pH change could be used as an indicator of a positive hippurate test, and that by including bromthymol blue in the medium, daily observations could be made. However, before this was tried out an experimental micro-method was developed and the use of growing cultures was abandoned.

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Table 1. The use of (a) opacity, (b) ferric alum precipitate and (c) pH value to detect hippurate-positive tests.

Strain No.	Genus and species	Opacity	Ferric alum	pH value
-	Uninoculated medium			6.4
2794	<u>Klebsiella pneumoniae</u>	++++	++	7.6
204	<u>Klebsiella pneumoniae</u>	-	-	6.4
5051	<u>Klebsiella ozaenae</u>	-	-	6.4
5055	<u>Klebsiella pneumoniae</u>	++	++	6.8
8819	<u>Escherichia coli</u>	-	-	6.4
9194	<u>Klebsiella</u> serotype	+++	++++	7.2

+ to ++++ = increasing degrees of opacity or bulk of precipitate

For the microtest the optimal concentration of sodium hippurate was found to be 1 per cent. Table 2 shows the pH values produced by suspensions of living organisms in different concentrations of hippurate; the suspension of the positive culture (A) had a pH of 6.4 but in 1 per cent hippurate it produced a value of 7.4. In stronger concentrations of hippurate the reaction became more acid, and in 5 and 10 per cent hippurate a value of 6.0 showed that the products of the suspension-substrate reaction were more acid than the suspension. With concentrations less than 1 per cent, positive cultures produced only a small difference between the

Table 2. Effect of varying the concentration of sodium hippurate in the microtest.

(Hippurate solution, 0.06 ml.; buffer solution, 0.06 ml.; indicator solution, 0.04 ml.; suspension, 0.02 ml.)		
pH values after 24 hours at 37°C.		
Hippurate Concentration	Culture A	Culture B
10 per cent	6.0	6.2
5 per cent	6.0	6.2
2.5 per cent	6.4	6.2
1 per cent	7.4	6.4
none	6.4	6.6

Culture A gave a positive reaction in the Hajna and Damon medium; culture B was negative.

test and the control tubes. Suspension of the negative culture (B) had an initial pH value of 6.6 and lower values after incubation with hippurate for 24 hours at 37°C. With 1 per cent sodium hippurate the optimal starting pH value for the test was found to be between 6.4 and 6.8. Unlike most of the other microtests, the results were easier to read when the density of the suspension was rather less than the standard (10^9 organisms per ml.), possibly because the colour of the indicator was masked by the opacity of the suspension.

The microtest finally developed and used in a routine trial was as follows: 1 per cent sodium hippurate, 0.06 ml.; 0.025 M-phosphate buffer, pH 6.8, 0.06 ml.; bromthymol blue indicator solution (British Drug Houses Ltd., London), 0.04 ml.; suspension 0.02 ml. Each test was controlled by a tube containing 0.06 ml. distilled water in place of hippurate solution. Exact volumes were not critical and the test

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Table 3. Examples of positive and negative microtests.

Organism	Test	Control
	Na hippurate 0.06 ml.	none
	Water none	0.06 ml.
	Buffer 0.06 ml.	0.06 ml.
	Indicator 0.04 ml.	0.04 ml.
	Suspension 0.02 ml.	0.02 ml.
pH value after 24 hours at 37°C.		
<u>Escherichia coli</u> (9001)	6.8	7.0
<u>Klebsiella aerogenes</u> (243)	7.4	7.0
<u>Klebsiella pneumoniae</u> (5055)	7.4	7.0
<u>Klebsiella pneumoniae</u> (7242)	7.2	6.8
<u>Klebsiella ozaenae</u> (5050)	6.8	6.8
<u>Cloaca cloacae</u> (5920)	6.8	6.8

could be made with uncalibrated dropping pipettes. Tubes were incubated at 37°C for 24 hours; the pH value of both the test and the control tubes was then estimated by comparison with standard capillary tubes (B.D.H. Capillator). In a negative test there might be no difference in pH value between test and control tubes or the test might be more acid than the control; in a positive result the test would be more alkaline than the control. An arbitrary value of +0.4 pH unit for the difference between the test and the control was taken as the minimum for a positive test. Examples are shown in Table 3.

Table 4. Frequency of positive and negative hippurate microtests.

	+	-
<u>Escherichia coli</u>	0	14
<u>Escherichia freundii</u>	0	4
<u>Klebsiella aerogenes</u>	5	1
<u>Klebsiella pneumoniae</u>	8	0
<u>Klebsiella ozaenae</u>	2	4
<u>Klebsiella rhinoscleromatis</u>	1	3
<u>Klebsiella serotype</u>	7	0
<u>Cloaca cloacae</u>	3	10
<u>Proteus sp.</u>	0	3

The microtest was carried out in routine tests and the results (Table 4) showed that the test might have some value in the classification of members of the family Enterobacteriaceae. All strains tested of Escherichia coli and E. freundii were hippurate-negative, as were three strains of Proteus and one each of Salmonella and Shigella. Trials with these genera were not pursued as the test was primarily intended to help in distinguishing Aerobacter aerogenes from A. cloacae and from Klebsiella pneumoniae (these names being used without prejudice). Apart from motility and gelatin-liquefaction, the distinguishing features between A. aerogenes and A. cloacae are not well established, and Edmunds (1954) classifies the only hippurate-negative strain of A. aerogenes (NCTC 8348) as A. cloacae. I find it difficult, and generally impossible, to distinguish between Aerobacter aerogenes and Klebsiella pneumoniae, and in the tables I have included organisms sent to this Collection as Bacterium aerogenes or Aerobacter aerogenes in the genus Klebsiella. It was hoped that the hippurate test might help to distinguish A. aerogenes from K. pneumoniae, but this it fails to do.

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Discussion

The hippurate microtest is easier to read than the corresponding test using cultures growing in Hajna and Damon's medium. In the two tests the criteria for positivity are different and it is even possible that the end-products tested for are different; thus the tests may not be strictly comparable. Be that as it may, when using the microtest most strains labelled Aerobacter cloacae were negative, whereas those labelled A. aerogenes or Klebsiella pneumoniae were positive. Our strains of A. cloacae form a heterogeneous group so that it was not surprising that the hippurate test was not uniformly negative. At the present time each worker has his own criteria for making the diagnosis of Aerobacter (or Cloaca) cloacae, and the hippurate test may yet find a place in making the distinction between this species and A. aerogenes. In the other problem of distinguishing A. aerogenes from K. pneumoniae, the evidence obtained in the limited trial reported here does not suggest that the test will be of much value.

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