RAPID METHODS FOR THE DETECTION OF CARBOHYDRATE FERMENTATION

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This report deals with a comparative study of typical methods for determining the fermentative abilities of microorganisms. The aim of the first studies was to select the best method for use with members of the Enterobacteriaceae and other easily cultivable species, using as criteria, rapidity, dependability, and simplicity. Later studies have involved adapting the method to some less easily cultivable organisms.

METHODS FOR USE WITH ENTEROBACTERIACEAE AND OTHER EASILY CULTIVABLE ORGANISMS

Most of the rapid methods that have been used by other workers fall into four categories:

(1) Methods using drops of a medium in the bottom of a petri dish (Bronfenbrenner and Schlesinger, 1918; Seiffert, 1942; Turner-Graff, 1932):—In preliminary studies we tried the Turner-Graff procedure, together with modifications of it, but the results did not warrant the inclusion of a method of this type in the more extensive studies. Acid production could be determined reliably but the minimum times required, e.g., 1 hr for glucose, 4 hr for lactose, were longer than with other methods, and gas production could not be detected.

(2) Methods using filter paper strips or discs containing carbohydrates, with or without nutrient medium (Knox, 1949; Spaur and Wynne, 1951; Snyder et al., 1951; Snyder, 1954), or discs of formalin-denatured gelatin containing carbohydrates (Kohn, 1953):—Most workers have found these methods reliable for the detection of acid production but deficient for the detection of gas production. In studies with a limited number of cultures, we found Kohn's method to be reliable, but at least 1 hr was required for acid production from glucose and at least 18 hr for gas production. On the basis of simplicity and results of preliminary studies, we chose Snyder's method for more extensive study.

(3) Methods using dense suspensions of bacteria as inocula for small amounts of substrate in small tubes (Hannan and Weaver, 1948; Clarke and Cowan, 1952; Cowan, 1953; Pickett, 1955; Pickett et al., 1955):—The Hannan and Weaver method and the method developed by Pickett and his coworkers depend upon the use of a nutrient substrate. We tried the Pickett method using media in tablet form, consisting of glucose, lactose, mannitol, sucrose, and salicin as prepared by the Medical Research Specialties Co. Results were obtained rapidly and reliably except that fermentation of lactose by two strains of the Arizona group (Paraclostridium arizonae) could not be demonstrated in 24 hr although it could by some of the other methods. Studies of variations in indicators, media, and test conditions with the various methods in this group, led us to the development of modifications of the Hannan and Weaver, and Pickett methods which appeared to give improved results. On the basis of the results obtained, these modified methods were selected for more extensive study.

The Clarke and Cowan method (1952) (or methods, since they used three different procedures) is based upon the use of "washed" suspensions of cells and a non-nutrient substrate. These procedures are assumed to depend more upon preformed enzymes. We found the procedure in which cell suspension, carbohydrate solution and buffer-indicator solution are mixed in a small tube and incubated in a water bath to be the best of the three. However, we discarded it for the more extensive studies because it did not show acid production quite as rapidly as the modified

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Hannan and Weaver, and Pickett procedures and because it did not show gas formation.

(4) Methods using capillary tubes (Clarke and Cowan, 1952; Morris, 1955)—Using the Clarke and Cowan procedure, we found that the observation of results was difficult and that the results were not obtained as rapidly as with some of the methods of other types. For these reasons no method of this type was included in the more extensive studies.

Modified Pickett method. The medium used was the same as that which Pickett (1955) recommended for use in tablet form except that the binding agent (polyvinylpyrrolidone) was omitted; the crystal violet was omitted as unnecessary when completely sterile precautions are to be used; and the K₂HPO₄ was reduced to 0.45 g per L. The reduction in phosphate content was on the basis of results of preliminary studies which showed the reduction to produce a more rapid test. Further reduction resulted in false reactions. The basal medium was adjusted to pH 7.5 to 7.6 and autoclaved. Seitz-filtered, 20 per cent solutions of carbohydrates were added aseptically to the basal medium to give a concentration of 2 per cent and the medium was dispensed in 1.0-ml amounts into 10 by 75 mm tubes. The media were preheated in a 37 C water bath and inoculated by adding 0.2-ml amounts of a cell suspension. Suspensions were prepared by harvesting growths from the surfaces of 4.0 to 4.5 hr tryptose-beef extract agar slant cultures in 1.0-ml amounts of saline. After inoculation, each tube was gently shaken to disperse the inoculum into the surface-layers of the medium. An inoculated basal medium tube (carbohydrate-free) was included for each test culture.

Snyder plate method. This method was used as described by Snyder (1954) using 1 per cent agar in the plates and the concentrated nutrient medium with neutral red indicator plus carbohydrates in the discs. The plates were inoculated with 1-ml quantities of the saline suspensions of organisms prepared as for the modified Pickett method. Four impregnated discs were placed on each plate.

Snyder tube method. This method was used as described by Snyder (1954) using discs containing the concentrated medium with phenol red indicator plus carbohydrates, except that the discs were cut in half and added to 1.0-ml amounts of sterile 0.2 per cent agar in 10 by 75 mm tubes. The tubes were preheated in a 37 C water bath and then inoculated with 0.1-ml amounts of the saline suspension of cells.

Modified Hannan-Weaver method. The procedure of the test was as described by Hannan and Weaver (1948) except for changes in the medium. The medium was used as double strength medium since the inocula to be used were the saline suspensions of the organisms. The KH₂PO₄ content was reduced to 0.8 g per L and 0.072 g of phenol red per L was used as the indicator. Phenol red was also used in the cap agar. The pH of the media was adjusted to 7.5 to 7.6 instead of 7.0 as in the original Hannan and Weaver procedure. As in the modified Pickett method, an inoculated basal medium tube (carbohydrate-free) for each culture was used as a control.

Results with Enterobacteriaceae

The fermentation of glucose, mannitol, maltose, and lactose by one strain each of Salmonella enteritidis, Salmonella typhosa, Shigella flexneri, Escherichia coli, Aerobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa, and an Alcaligenes sp., and two strains of the Arizona group (Pseudomonas arizonae) was tried by the usual Durham fermentation tube procedure using nutrient broth as a substrate and by the modified Pickett, Snyder plate, Snyder tube, and modified Hannan-Weaver procedures.

Results with the Durham fermentation tube macroprocedure were according to the published descriptions of the species except that acid production from lactose by the Arizona group (P. arizonae) strains could not be demonstrated in 72 hr. Failure of the strain of P. aeruginosa to produce acid from glucose is in accord with the results that are usually obtained although this species is known to attack glucose.

Except for minor variations in times, the modified Pickett and Hannan-Weaver techniques gave identical results. Both gave the same results as the Durham fermentation tube macroprocedure except that they were more sensitive, so as to demonstrate acid production from lactose by both the Arizona strains and from glucose by P. aeruginosa. Except for these strains, acid production from glucose was evident in from 5 to 10 min; that from mannitol in from 30 to 60 min; and that from maltose and lactose in from 35 min to 4 hr. The demonstration of gas production usually required longer periods of time, varying from 50 min to 12 hr with different strains of organisms and different carbohydrates.
The Snyder tube method usually gave the most rapid demonstration of gas production but was slightly slower in the demonstration of acid production and failed in the demonstration of acid production from lactose within 24 hr by the Arizona strains. The Snyder plate method was unsatisfactory in that it required from 3 to 6 hr for demonstration of acid production, and failed in the demonstration of acid production from lactose by the Arizona strains and gas production from glucose by E. coli and both Arizona strains.

On the basis of the results that have been reported above, the modified Pickett and Hannan-Weaver methods were chosen for further testing against the Durham fermentation tube macroprocedure. This testing was done with 11 strains of salmonellae: S. typhosa (2), S. paratyphi (2), S. schottmuelleri (3), S. cholerasuis (2), S. ballerup, S. typhimurium; and 10 strains of shigellae: S. sonnei (4), S. flexneri, S. dispar, S. alkalescens (4).

The results with the Durham fermentation tube macroprocedure were according to the published descriptions of the species except that acid production from maltose by the strain of S. flexneri was not demonstrated within the 24 hr of the test. The two rapid methods were reliable with respect to acid production. The modified Hannan-Weaver method showed acid production by S. flexneri in 5 hr, and the modified Pickett method in 24 hr. Acid production by the other strains was evident in from 6 to 75 min by both methods. Gas production was shown by the modified Hannan-Weaver method in from 1 hr to 6 hr and by the modified Pickett method in from 2.5 to 12 hr. The modified Pickett method failed in the demonstration of gas production by the two strains of S. paratyphi from glucose and mannitol and by one of the strains from maltose. The modified Hannan-Weaver method failed in the demonstration of gas production by one of the strains of S. paratyphi and one of the strains of S. schottmuelleri from maltose.

It may be concluded that the modified Hannan-Weaver method is the most useful of the methods that have been tried for the rapid demonstration of the fermentation of carbohydrates by members of the Enterobacteriaceae and other easily cultivable organisms. It is even more reliable than the usual Durham fermentation tube macroprocedure for the demonstration of acid production. It fails, very rarely, in the demonstration of gas production.

**METHOD FOR USE WITH STREPTOCOCCI**

Except for the use of a different medium for preparing the inoculum, the method employed was that of McCollum (1949). This method was developed as a modification of the Hannan-Weaver method.

The test medium contains: tryptose, 10 g; proteose peptone no. 3, 10 g; NaCl, 5 g; KH2PO4, 1 g; and distilled water, 1000 ml. To prepare the medium, the ingredients are dissolved by heating, the pH is adjusted to 8.0, and 36 mg of phenol red per L of medium are added. This basal medium is boiled for 2 min, dispensed in 100-ml amounts in rubber-stoppered dilution bottles, and sterilized in the autoclave. To each bottle, 5 ml of a 20 per cent solution of the desired test carbohydrate that has been sterilized by filtration are added aseptically. McCollum found it necessary to add 20 ml of sterile fresh horse serum aseptically to 50 ml of sterile basal medium for testing some delicate strains. This has not been necessary with any of the strains that we have used.

The medium is dispensed into sterile 5 by 50 mm tubes in 0.15-ml amounts by means of sterile capillary pipettes and the tubes are placed in a 37 C water bath for preheating prior to inoculation. They are inoculated with 3-mm loopfuls of packed cells and are incubated in the water bath.

To prepare the inocula, the cultures are grown in brain heart infusion broth (Difco) or trypticase soy broth (BBL) which has been adjusted to pH 8.0, dispensed into tubes, and autoclaved at 10 pounds pressure for 10 min. When the cultures have reached the vigorous growth phase (10 to 18 hr) they are centrifuged and the packed cells are used as inocula as soon as possible.

Tubes of inoculated carbohydrate-free basal medium and of uninoculated media are necessary as controls.

The procedure, as described, was tested by comparison with the usual macrotest procedure for detection of fermentation of glucose, maltose, lactose, trehalose, and sorbitol by stock strains of *Streptococcus pyogenes*, *Streptococcus faecalis*, and *Streptococcus agalactiae* and 5 unidentified strains of streptococci from various sources. Acid production was detected reliably in 1 to 4 hr by the rapid microtest as compared to 24 to 96 hr by the macrotest procedure.

McCollum (1949) recommended that when fermentation of a carbohydrate that is attacked by adaptive enzymes is to be tested, the medium
used to grow the inoculum should contain 0.5 per cent of that carbohydrate. Our tests have shown that this procedure does not shorten significantly the time required to obtain fermentation of maltose, trehalose, or sorbitol. With 5 of 8 strains, the time required for fermentation of lactose was one or two hr shorter when the strains had been grown on a lactose-containing medium. The time saved on these strains is not sufficient to warrant the extra labor and expense required to produce an inoculum of adapted cells.

METHOD FOR USE WITH NEISSERIAE

Based on preliminary studies of methods for detecting fermentation by members of the genus *Neisseria* described by Cameron and Castles (1946), Vera (1948), and Mullaney (1956), a method has been developed which is essentially another modification of the Hannan-Weaver method for demonstrating fermentation.

Any of three basal media may be used: (1) phenol red broth base (Difco); (2) cystine tryptase broth (BBL); (3) a modification of the medium which has been described for use in detecting the fermentations of streptococci, in which the KH2PO4 is omitted, the pH is dropped to 7.3, and cresol red is substituted for phenol red. The last medium gives slightly more rapid results. Sufficient 20 per cent Seitz-filtered carbohydrate to produce a concentration of 1 per cent is added to the sterile basal medium.

The medium is tubed in 0.2 ml amounts in 10 by 75 mm tubes and preheated in a 35 to 36 C water bath. The tubes are inoculated with 0.2-ml amounts of cell suspension. Tubes of carbohydrate-free basal medium should be inoculated as controls. Incubation is in the water bath.

The inocula are prepared from heavily inoculated slants of gonococcus medium base (Difco) (with the agar concentration increased to 1.5 per cent) enriched with hemoglobin (Difco) (2 per cent) and supplement A (Difco) (1 per cent), that have been incubated in a candle jar for 18 hr at 37 C. The growths from the surfaces of the slants are harvested in 1.0-ml amounts of the basal medium being used in the test.

The results with this method have been entirely reliable, strains of *Neisseria gonorrhoeae* fermenting glucose in 4.0 to 4.5 hr, those of *Neisseria meningitidis* fermenting glucose and maltose in 18 to 24 hr, and those of *Neisseria catarrhalis* failing to ferment glucose or maltose. Recently this method has been used successfully by Mr. John Doucoutes in a Lexington clinic with suspensions of cells from primary colonies as the inocula. With these less dense suspensions the time was only slightly lengthened.

SUMMARY

A comparative study of a number of rapid methods for detecting carbohydrate fermentation by members of the Enterobacteriaceae and other easily cultivable species of bacteria has led to the selection of a modification of the method developed by Hannan and Weaver (1948) as the most reliable. The original method of Hannan and Weaver is modified by reducing the KH2PO4 content of the medium to 0.04 per cent, using phenol red as the indicator, and adjusting its pH to 7.5 to 7.6. The modified Hannan-Weaver method has been found to be more sensitive than a macromethod with Durham fermentation tubes using a nutrient broth base for the demonstration of acid production, showing fermentation by slow acid formers in 5 to 24 hr when the macromethod failed in 72 hr. Except for slow fermenters, acid production from glucose can be shown in 5 to 45 min. Somewhat longer periods are required for disaccharides. Gas production is demonstrated in from 1 to 6 hr. During extensive series of tests, the rapid method has failed in two instances to show gas production.

With a modification of the Hannan-Weaver method, based on the work of McCollum (1949), it has been possible to obtain reliable fermentation reactions with streptococci within 1 to 4 hr.

With a further slight modification of the method, it has been possible to obtain fermentations of glucose by *Neisseria gonorrhoeae* within 4.0 to 4.5 hr and of glucose and maltose by *Neisseria meningitidis* within 18 to 24 hr.

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