UTILIZATION OF CARBOHYDRATES BY PSEUDOMONAS AERUGINOSA

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Pseudomonas aeruginosa is easily identified because it grows well on ordinary laboratory media and produces a pigment. The biochemical activity of the organism, however, has been a subject of controversy.

Moltke (1927) failed to recognize acid production from any of the usual sugars. Wilson and Miles (1948) considered "acid is generally formed from glucose, but from no other sugars". Sandiford (1937), in a comparative study of *P. aeruginosa* and *P. fluorescens*, using only four sugars, glucose, mannitol, sucrose, and lactose, stated that acid is formed from glucose by *P. fluorescens* but not by *P. aeruginosa*. *Bergey's Manual* states "glucose, fructose, galactose, arabinose, maltose, lactose, sucrose, dextrin, inulin, glycerol, mannitol, and dulcitol are not attacked". However, it cites the report of Sandiford that acid is produced from glucose.

In 1942, Elrod and Braun reported a comparative study of *P. aeruginosa* and *Phytomonas polycolor* and stated that acid is produced from glucose, xylose, and arabinose but not from sucrose, mannitol, glycerol, salicin, raffinose, and maltose. The organisms were considered identical by these authors. The introduction by these authors of the use of a synthetic medium for fermentation studies by species in the genus *Pseudomonas* was important. Unfortunately, however, they failed to mention the length of time during which the reaction was observed and apparently did not recognize the usefulness of this synthetic medium.

Clara (1934), who isolated the strains of *Phytomonas polycolor* studied by Elrod and Braun, was cited by the latter as reporting acid production from glucose, galactose, fructose, salicin, mannose, arabinose, xylose, mannitol, and glycerol. In 1946, Gaby reported variable results in which the most active strains produced acid without gas from glucose, glycerol, xylose, and galactose. Although he failed to mention the technique employed, it probably was the conventional peptone carbohydrate broth. Finally, Salvin and Lewis (1946), using the synthetic medium employed by Elrod and Braun, reported the acid production from glycerol, arabinose, glucose, galactose, mannose, and xylose but not from adonitol, dextrin, dulcitol, fructose, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, sorbitol, starch, sucrose, and trehalose by the pyocyanin forming *Pseudomonas* species. Unfortunately these authors again did not specify the length of time during which the reaction was observed.

In the article published by Elrod and Braun (1942) the nitrogen source of the synthetic medium was not given due to an oversight, as the late Dr. Elrod told the author personally, and the oversight was repeated by Salvin and Lewis (1946). In view of the existing confusion, an attempt was made to establish a definite biochemical pattern for *P. aeruginosa*.

MATERIALS AND METHODS

Forty-five strains of *P. aeruginosa* were used in this study. All except two strains were isolated from human sources as follows: ear, 19 strains; leg lesion (ulcer, gangrene, etc.), 7 strains; feces, 7 strains; sputum, 3 strains; eye, 2 strains; spinal fluid, 2 strains; urine, 1 strain; urethra, 1 strain; and cervix, 1 strain.

Two standard strains from the U. S. Army Medical School, 140-A-3 and M-4-C-1, were obtained through the courtesy of Lt. Col. A. C. Sanders, MSC, U. S. Army, director of the bacteriology department, 406th Medical General Laboratory, Tokyo.

All strains of *P. aeruginosa* used were tested for their biochemical pattern with conventional methods. Broth containing 1 per cent peptone and 1 per cent of the test carbohydrate using bromthymol blue as an indicator was employed. Some strains produced slight acidity with glucose, arabinose, and xylose. All strains were motile, indol negative, citrate positive, and liquefied gelatin rapidly; 26 strains were nitrate positive and the remainder were negative.

The formation of pyocyanin is the most important differential criterium for this species. Some strains produced strong pigmentation on plain tryptose phosphate agar (Difco) while others were only weakly active producing a yellow color. Several media such as Loeffler's medium, 5 per cent glycerol agar are known to enhance the production of pyocyanin. Loeffler's medium is not suitable since it is rapidly liquefied. In this study, tryptose phosphate agar containing 5 per cent glycerol and the same agar containing 10 per cent human serum were found most suitable.

Some strains produced pigment more readily in glycerol agar while others favored serum agar. As a result both types of agar plates were used to determine the production of pyocyanin. Solubility of the pigment in water is demonstrated by the fact that it diffuses into the agar, and chloroform solubility was confirmed by pouring the solvent on 48 hour cultures on both media. The bacterial cells usually do not form a suspension with the chloroform, and a greenish blue coloration of the solvent can be observed readily. Strain 140-A-3 is a strong pyocyanin producer, even on a plain agar, and strain M-4-C-1 produces a reddish brown color, probably the pyorubin described by some workers. However, this strain also produced pyocyanin on glycerol agar.

Since the accuracy of this experiment is dependent entirely on the purity of the products employed, a list of material sources of the carbohydrates used is given: glucose, (Merck); fructose (D-levulose), (Pfanstiehl Chemical Company); galactose, (Pfanstiehl Chemical Company); mannitol (D-mannite), (City Chemical Company, New York); trehalose, (Difco); glycerol (The Bender Corporation); arabinose (L-arabinose), (Pfanstiehl Chemical Company); xylose, (Eastman Kodak); mannose, (Difco); and rhamnose, (Difco).

EXPERIMENTAL RESULTS

Alkali production from peptone. As mentioned by Elrod and Braun (1942), *P. aeruginosa* is proteolytic and the alkali produced from peptone may mask the production of acid. If acid is actually produced but an acid reaction does not

develop in a peptone carbohydrate broth, it must be due either to the production of a large amount of alkali from peptone or to the small amount of acid produced from each carbohydrate. Therefore, a study was made to determine the amount of alkali produced from peptone by the organism.

Several types of peptones, such as peptone, tryptose, tryptone, and proteose peptone no. 3 (all Difco), trypticase (B.B.L.), poly peptone (Japanese), were available. In a preliminary trial all the peptones gave essentially the same results, and peptone (Bacto) was used throughout the entire experiment. The medium used was as follows: peptone (Bacto), 10 g; NaCl, 5 g; phenol red (0.2 per cent), 8 ml; and distilled water, 1,000 ml; pH 7.2.

The broth was Seitz-filtered and dispensed aseptically in 5 ml amounts into test tubes which were incubated overnight for a sterility test.

TABLE 1

The amount of alkali produced by various types of enteric bacilli from 0.05 g of peptone (expressed in ml of 0.1 N HCl used to neutralize it)*

ORGANISMS	DAYS							
	1	2	3	4	5	6	7	Total
Pseudomonas aeruginosa	0.20	0.30	0.20	0.30	0.30	0.25	0.05	1.60
Aerobacter aerogenes	0.20	0.20	0.20	0.20	0.20	0.10	—	1.10
Escherichia coli	0.10	0.20	0.20	0.20	0.20	0.10	—	1.00
Salmonella paratyphi	0.10	0.10	0.10	0.05	0.05	0.10	—	0.50
Salmonella schottmülleri	0.25	0.20	0.20	0.20	0.15	0.10	—	1.10
Salmonella hirschfeldii	0.15	0.15	0.10	0.05	0.05	0.10	—	0.60
Salmonella typhosa	0.10	0.15	0.10	0.05	0.10	0.15	—	0.65
Proteus morganii	0.05	0.20	0.20	0.15	0.20	0.10	—	0.90
Proteus vulgaris	0.05	0.20	0.20	0.10	0.10	0.15	—	0.80
Shigella dysenteriae	—	0.05	0.08	0.03	0.05	0.10	—	0.31
Shigella sonnei	—	0.08	0.07	0.03	0.05	0.10	—	0.32

* 5 ml of peptone broth using phenol red as indicator.

Various enteric bacteria such as *Aerobacter aerogenes*, *Escherichia coli*, *Proteus vulgaris*, *Proteus morganii*, *Salmonella paratyphi*, *S. schottmülleri*, *S. hirschfeldii*, *S. typhosa*, *Shigella dysenteriae*, and *S. sonnei* were used for comparative study. All were maintained in the same broth and the inoculum was taken from 18 hour cultures.

The amount of alkali produced was determined by the volume of 0.1 N HCl used to maintain the pH at 7.2. The addition of HCl was continued every 24 hours until the pH remained stable. Five strains of *P. aeruginosa* and two strains each of the other types of organism were used. Average values are listed in table 1. No significant differences were observed within individual strains and the listed values can be taken as those of a single strain. As seen in table 1, the total volume of alkali produced by this organism is only a little more than that produced by *A. aerogenes*, *E. coli*, or the *Proteus* group. It is very likely that the failure of *P. aeruginosa* to show acid production is due to the small amount of acid produced from each carbohydrate.

Acid production from glucose. Stokes and Campbell (1951) demonstrated that glucose is oxidized to gluconic acid and 2-ketogluconic acid by dried cells of *P. aeruginosa* and that no further breakdown occurs. The volume of acid produced by the conversion of 0.05 g of glucose (5 ml of 1 per cent broth) to acid can be calculated as follows: $0.05/180 \times 10.000 = 2.777$ (ml of 0.1 N NaOH). In other words, the maximum amount of the gluconic acids which can be produced from 0.05 g of glucose can be neutralized by 2.777 ml of 0.1 N NaOH. This amount minus alkali produced from the peptone is the total amount available to give an acid reaction in carbohydrate broth (if the reaction goes to completion). Therefore, the less alkaligenic the medium is, the more sensitive will be the reaction. The following synthetic medium was used for the quantitative study of acid production: $(NH_4)_2SO_4$, 2.0 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.1 g; NaCl, 0.2 g;

TABLE 2

The amount of acid produced by various types of enteric bacilli from 0.05 g of glucose (expressed in ml of 0.1 N NaOH used to neutralize it)*

ORGANISMS	DAYS												
	1	2	3	4	5	6	7	8	9	10	11	12	Total
Pseudomonas aeruginosa.....	0.10	0.10	0.10	0.20	0.20	0.17	0.15	0.15	0.15	0.15	0.20	0.15	1.82
Pseudomonas aeruginosa.....	0.05	0.10	0.10	0.10	0.13	0.15	0.15	0.15	0.20	0.20	0.15	—	1.48
Aerobacter aerogenes.....	0.30	2.10	—	—	—	—	—	—	—	—	—	—	2.40
Escherichia coli.....	0.40	2.00	1.80	—	—	—	—	—	—	—	—	—	4.20
Salmonella hirschfeldii.....	0.15	0.35	0.35	0.40	0.30	0.40	0.35	0.30	0.40	0.40	0.40	0.40	†
Shigella sonnei.....	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	†

* 5 ml of synthetic broth containing 1 per cent glucose (phenol red indicator).

† These are not final values since acid production from these organisms continues longer than this. Slow production of acid by these organisms is probably due, in part, to their slow growth.

Two extreme values obtained with *Pseudomonas aeruginosa* are listed in this table.

K_2HPO_4, 0.2 g; phenol red (0.2 per cent), 8.0 ml; and distilled water, 1,000 ml; pH 7.2.

A large quantity of this medium was made up, Seitz-filtered, and dispensed aseptically in 100 ml quantities into screw capped bottles. Carbohydrate to give a concentration of 1 per cent was added to the basic medium whenever required, the pH readjusted, and the medium filtered again. As seen in the formula, the basic medium contains no source of carbon, and, therefore, if the organism fails to utilize the carbohydrate added, it will fail not only to produce acid but to grow.

Titration of total acidity produced from glucose in synthetic medium. Five strains of the organism were inoculated into 5 ml amounts of this synthetic medium containing 1 per cent glucose, and 2 strains each of the above mentioned enteric bacteria were used for comparison. The acid produced was constantly neutralized by 0.1 N NaOH until the pH stabilized. As seen in table 2, acid is produced by *P. aeruginosa* very slowly and the addition of 0.1 ml of 0.1 N NaOH daily was

enough to maintain the pH at 7.2. The pH stabilized after 12 days and the total amounts of alkali used were 1.48, 1.50, 1.64, 1.73, and 1.82 ml by the five strains. The average value was about 1.63. Acid production by organisms such as *A. aerogenes* and *E. coli* was so rapid that it was necessary to add alkali several times daily.

As demonstrated in table 1, the maximum alkalinity produced by the organism from 0.05 g of peptone is equivalent to about 1.60 ml of 0.1 N HCl. From the same amount of glucose the organism is able to produce 2.777 ml of 0.1 N acid if the fermentation goes to gluconic acid completely. The difference between these values is more than 1.0 ml and is more than enough to produce an acid reaction in 5.0 ml of broth containing 1 per cent peptone and 1 per cent glucose. The actual amount of 0.1 N HCl necessary to elicit an acid reaction in this broth is only about 0.20 ml if phenol red is used as the indicator. Six-tenths ml is needed if brom thymol blue is used. The only possible explanation is the incompleteness of the oxidation of glucose in the presence of peptone.

It is known that bacteria do not utilize all available energy sources with the same efficiency. Some substances are more readily utilized than others and in the presence of these substances the less easily utilizable substances are left unattacked or are less completely utilized. The best example for this is the suppression of indol formation from tryptophan in the presence of utilizable carbohydrates as seen in cultures of some enteric bacteria. An unsuccessful attempt was made to determine quantitatively the amount of glucose left over after the growth of this organism. The broth filtrate, either from peptone broth or synthetic medium cultures, contains some unknown reducing substances which react with copper sulfate used in ordinary quantitative tests for glucose (such as Folin and Wu's, or Benedict's methods). These substances cannot be removed by ordinary treatments for removing protein, and positive reactions still persist with Folin and Wu's method, even in a dilution as high as 1 to 10,000. However, the following indirect evidence suggests that the organism utilized peptone in preference to glucose, and only slight acid production occurs in peptone glucose broth.

1. Alkali production in the peptone glucose broth occurs as rapidly as with peptone alone in the first 24 hours.

2. The concentration of peptone can be reduced to as low as 0.1 per cent, and acid production still occurs more slowly than in the synthetic medium in spite of the fact that the organism grows more rapidly in the peptone broth.

Inhibition of fermentation by simple carbon compounds. Acid production by this organism from glucose in synthetic medium is completely inhibited by the addition of sodium citrate or sodium succinate. Although the quantity of glucose remaining after growth could not be demonstrated because of the reason mentioned previously, it is very likely that the organism utilizes these substances in preference to glucose since alkali production in the synthetic medium containing 1 per cent of one of these substances and glucose occurs as rapidly as with them alone. The same type of inhibition can be demonstrated with all of the other fermentable carbohydrates which will be mentioned later.

No investigation was conducted along this line, but it is quite possible that

other readily utilizable simple carbon compounds will also inhibit the fermentation of carbohydrates in the same manner.

Acid production from other carbohydrates. One per cent of the carbohydrate was added to the basic synthetic medium, the pH readjusted to 7.2, the medium Seitz-filtered and dispensed in 2 ml amounts in Kahn tubes. The organisms were maintained in Difco peptone broth (sugar free), and transfers to sugar broth were made with a very small loop in order to minimize the amounts of nutrients carried over in the inoculum. The basic medium itself, with or without carbohydrate added, will turn acid upon standing in the incubator 3 to 5 days. Therefore, observations of the acid reaction after this period are meaningless unless a definite turbidity develops as the result of growth. Two controls were found to be necessary. One is the sugar free basic medium inoculated with organism: the other sterile carbohydrate broth. Both controls remained clear during the period of incubation which lasted 7 days.

TABLE 3

Total acidity produced by various enteric bacilli from 5 ml of 1 per cent carbohydrate broth (expressed in ml of 0.1 N NaOH used to neutralize it)

ORGANISMS	CARBOHYDRATE					
	Glucose	Galactose	Fructose	Mannitol	Glycerol	Trehalose
Pseudomonas aeruginosa	1.63	1.40	0.70	0.74	0.46	0.89
Aerobacter aerogenes	2.40	2.45	3.50	2.50	1.15	3.50
Escherichia coli	4.20	4.10	4.20	2.55	0.40	2.55
Salmonella hirschfeldii	4.35	4.05	5.05	3.50		
Shigella sonnei	2.15	2.05	1.20	1.85		

Blank spaces in this table indicate tests which were not done.

Acid reaction is noted within 24 hours with glucose, galactose, mannitol, glycerol, arabinose, xylose, and mannose, and within 48 hours with fructose. Acid production from trehalose was variable, some strains showing definite acid and growth within 24 hours while others did not. Development of turbidity took as long as 5 days with a few strains. The slow production of acid from fructose and trehalose is probably responsible for the statement of Salvin and Lewis (1946) who stated that fructose and trehalose were not attacked.

The observations were continued until the seventh day when all the control media were also acid. The tubes containing glucose, galactose, fructose, mannitol, glycerol, and trehalose were clearly differentiated from the control tubes by their turbidity as the result of growth, while the tubes with arabinose, xylose, and mannose remained clear. Apparently the organism is unable to use these substances as the sole source of carbon. Positive reactions were noted only by rapid appearance of acid while the control tubes were still alkaline. The experiments were repeated several times and the same results obtained. The acidities produced from these carbohydrates are listed in table 3.

Neither acid production nor growth was noted with adonitol, dextrin, dulcitol, inositol, inulin, lactose, maltose, raffinose, rhamnose, salicin, sorbitol, starch, or

sucrose. Therefore, these substances would have been considered "not attacked" if the results obtained had not been compared with those from a preliminary trial.

Acid production from rhamnose. In a preliminary trial, an agar slant containing 10 per cent carbohydrate and 1 per cent peptone was used. The results obtained with this technique generally agree with those observed in the synthetic medium. However, the slant technique was less sensitive than tests in the synthetic medium (some strains being negative with trehalose). Since large amounts of carbohydrates are required, the use of this technique was quite impractical and was discontinued. However, the results with rhamnose by this technique were definitely positive and an attempt was made to solve the discrepancy. It is already known that the organism is unable to utilize pentoses such as arabinose and xylose as the sole source of carbon for growth, and acid production is slow with trehalose. It was suspected that the organism might be able to produce acid very slowly from the carbohydrates it cannot utilize as the sole source of carbon. The least alkaligenic synthetic medium which will support the growth of the organism would be the ideal one. Several media were tested including the previously mentioned synthetic medium with sodium citrate added. The following non-synthetic medium was found to give satisfactory results as far as detection of acid production is concerned: Peptone, 1.0 g; carbohydrate, 20.0 g; NaCl, 5.0 g; phenol red (0.2 per cent), 8.0 ml; and distilled water, 1,000 ml; pH 7.2. The ratio of carbohydrate to peptone is twice as large as in the agar slant technique, but the amount of carbohydrate required is greatly reduced. The medium is Seitz-filtered and dispensed in 2 ml amounts into Kahn tubes.

All carbohydrates which gave negative results in the synthetic medium were tried. Growth was visible within 24 hours in the sugar free basic medium used as control, but no acid production in any tube was noted even after 48 hours. The tube containing rhamnose became slightly acidic after 3 days, and an acid reaction was definite in 4 days. No acid production was noted after 10 days with any of the carbohydrates mentioned above. An unsuccessful attempt was made to speed up the fermentation of rhamnose by successive transfers to the same broth as soon as the acid reaction appeared. The organism apparently will not attack this substance until all other available carbon sources are exhausted since it is unable to utilize this substance as the sole source of carbon for growth. All strains of *P. aeruginosa* employed behaved exactly the same way.

DISCUSSION

Salvin and Lewis (1946) mentioned many *Pseudomonas* species in their discussion, but there is no reason to consider these *Pseudomonas* strains as being other than *P. aeruginosa*. The differences revealed in the foregoing experiments are entirely due to the techniques employed. Two exceptions noted by Salvin and Lewis (1946) in the fermentation of glucose and arabinose are caused probably by late fermenters of carbohydrates. This is noted with a few of the strains used in this experiment, but delayed fermentation can be reduced by successive transfers to the same carbohydrate medium.

Since the organism is unable to break down the carbon chain in glucose, it is

quite possible that it is also unable to break down the carbon chains of the pentose, and this structure is probably unsuitable in the synthesis of body substances.

P. aeruginosa is not particularly more alkaligenic than enteric bacteria such as *Aerobacter* or *Escherichia* species, as mentioned, but not proved by Elrod and Braun (1942) and Salvin and Lewis (1946). The failure of an acid reaction to develop in peptone carbohydrate broth is due to the preference of the organism to utilize peptone as the carbon as well as the nitrogen source. The relatively large amount of alkali and the small amount of acid produced from peptone and carbohydrate, respectively, are the accessory factors which make the acid reaction less readily demonstrable.

Fermentation tests with some substances such as rhamnose will always be a problem in the future. The organism does not grow when this substance is the sole source of carbon, and if a utilizable carbon source is added, it will utilize this in preference. It becomes mandatory to wait until the utilizable carbon source is exhausted to detect fermentation of the less easily utilizable carbohydrate.

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SUMMARY

Pseudomonas aeruginosa has a definite biochemical pattern. The organism is able to utilize glucose, galactose, fructose, mannitol, trehalose, and glycerol as the sole source of carbon for growth and to produce acid from these substances. Although acid is produced rapidly from arabinose, xylose, and mannose, the organism is unable to utilize these substances as the sole source of carbon for growth. Slow acid production can be demonstrated from rhamnose if the basic medium is able to support growth. The organism cannot utilize this substance as the sole source of carbon for growth and will not attack it until all the other available carbon sources are exhausted. *P. aeruginosa* is unable to attack adonitol, dextrin, dulcitol, inositol, inulin, lactose, maltose, raffinose, salicin, sorbitol, starch, or sucrose.

P. aeruginosa is more alkaligenic than members of the family *Enterobacteriaceae* such as *Aerobacter* and *Escherichia* species. The failure of the organism to produce an acid reaction in peptone carbohydrate broth is due to the preference of the organism to utilize peptone as a source of carbon as well as nitrogen. The relatively large amount of alkali produced from peptone and the small amount of acid produced from each carbohydrate are the accessory factors which make acid production less readily demonstrable. In the synthetic medium described here, however, one carbohydrate is the sole source of available carbon and the organism must attack this substance from the beginning if growth occurs. Carbo-

hydrate utilization can be inhibited by simple carbon compounds such as sodium citrate and sodium succinate.

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