CARBOHYDRATE METABOLISM OF ORGANISMS OF THE GENUS MYCOBACTERIUM¹

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I. INTRODUCTION

The present article deals with various aspects of carbohydrate utilization by the organisms of the genus *Mycobacterium* when meat infusion broth constitutes the basis of the media in which the organisms are grown. A correlated study in which a synthetic media was used will be reported in a subsequent article.

There is a scarcity of reported research dealing directly with the problem of carbohydrate utilization by organisms of the genus *Mycobacterium*. Most of the reported work, wherein plain broth has been used as the basis of the media, provides no satisfactory quantitative information as to the extent of carbohydrate utilization. The reason for this will become apparent from subsequent considerations.

Until quite recently, direct quantitative estimations have not been extensively applied to the determination of carbohydrate utilization by bacteria. In the case of the vast majority of bacteria that have been studied, there is associated with the utilization of carbohydrates the production of various organic acids as cleavage products. The accumulation of these acids in the media results in an increase in hydrogen ion concentration. By the use of appropriate indicators this acid production has been readily

¹ The major part of this investigation comprised a thesis presented to the Committee on Graduate Studies of the St. Louis University School of Medicine in partial fulfillment of the requirements for the degree of Master of Science.

demonstrable. This has constituted the most commonly used criterion for carbohydrate utilization.

There has been encountered no increase in acidity accompanying the growth of organisms of the genus *Mycobacterium* when grown in carbohydrate broth (Merrill, 1928, 1929). Using pH changes as an index it is then not clear whether any of the organisms of this group do, or do not, utilize any carbohydrates.

Theobald Smith (1905) long since demonstrated clearly that the addition of glycerol to broth increased the growth of *Myco. tuberculosis (hominis)*. He also demonstrated a terminal acidity when this organism was grown in 3 per cent glycerol broth. The first reaction change, however, was distinctly to the alkaline side. The usual criterion of early acid production thus does not hold even with glycerol. That the presence of glycerol increases the growth of the human type of *Myco. tuberculosis*, however, cannot be doubted. This suggests that glycerol is utilized. In the case of *Myco. tuberculosis (bovis)* the reaction was shown by Smith to become progressively alkaline in glycerol broth. The evidence of utilization is thus inconclusive.

Kendall, Day, and Walker (1914) in working with several species of this genus, including some saprophytic strains, did not in a single instance obtain a primary acidity. They stated that evidently there was an acid production from the glycerol but that it was a "differential acidity," alkaline products being formed in excess. They were, nevertheless, convinced that the glycerol was utilized, but were of the opinion that glucose and mannitol were not utilized. Similar results were later reported by Long and Major (1922). Gamble and Herrick (1922) report utilization of glucose by the tubercle bacillus.

Work done by Frouin and Guillaume (1923) would indicate that some carbohydrates are utilized by *Myco. tuberculosis (hominis)* and their later work (1926) leaves little doubt that some of the carbohydrates are utilized.

The work of Braun et al. (1924, 1924a, 1924b, 1925), which will be considered more fully in a subsequent report, gives quite conclusive evidence that carbohydrates and their derivatives can be utilized by many of the organisms belonging to the genus *Myco-*

bacterium, at least when these organisms are grown in synthetic media.

There are still some workers, however, who believe that aside from glycerol there is no utilization of carbohydrates or their derivatives by these organisms. Recently Weinzirl and Knapton (1927) from their results of an extensive study of the reaction curves of fifteen species of bacteria of the genus *Mycobacterium* on synthetic media stated that glucose, mannitol, and lactose are apparently not utilized. Their methods, however, were not designed to determine whether the carbohydrates were utilized since reaction curves constituted their only criterion for utilization. As will be shown below, results based upon such methods are indefinite.

A problem quite fundamental in character thus presents itself. The *Mycobacteria* apparently utilize carbohydrates without the production of acids. How, then, does the carbohydrate metabolism of these organisms differ from that of most bacteria? What is the extent of carbohydrate utilization by the *Myco-bacteria*? What are the end products? How does the presence of carbohydrates in a medium affect protein metabolism?

II. ORGANISMS USED IN THIS STUDY

The organisms studied comprise a number of the so-called "acid-fast" microorganisms classified in the genus *Mycobacterium*. In all, nineteen organisms were used. The species names and sources from which these were obtained were as follows:

1. *Mycobacterium tuberculosis (hominis)*. Three strains of this organism were used:

a. H-37 strain obtained from the American Type Culture Collection of Chicago.

b. M-1 strain which was isolated by the author from the sputum of a tuberculosis patient at the Veterans Bureau Hospital located at Jefferson Barracks, Mo. This strain was definitely pathogenic for guinea pigs.

c. A saprophytic strain which has been carried in stock in the laboratory for some years. This strain was originally isolated from human sputum. It grows rapidly and is now non-pathogenic for guinea pigs.

2. *Mycobacterium tuberculosis (bovis)* No. 112 of the American Type Culture Collection of Chicago.

3. *Mycobacterium leprae*. Six strains of this organism were used. All were obtained from the American Type Culture Collection of Chicago. Their serial numbers are as follows: 282, 65, 66, 67, 68 and 69.

4. *Mycobacterium avium*, No. 118 of American Type Culture Collection.

5. *Mycobacterium ranae*, No. 110 of American Type Culture Collection.

6. *Mycobacterium chelonae*, No. 114 of American Type Culture Collection.

7. *Mycobacterium smegmatis*, a laboratory culture the exact origin of which is unknown.

8. *Mycobacterium butyricum*, No. 357 of American Type Culture Collection.

9. *Mycobacterium berolinensis*, No. 356 of American Type Culture Collection.

10. *Mycobacterium friburgensis*, obtained some years ago from the University of Pennsylvania.

11. *Mycobacterium stercusis*, No. 77 of American Type Culture Collection.

12. *Mycobacterium phlei*, a laboratory culture, the exact origin of which is unknown.

13. *Mycobacterium piscium*, No. 111 of American Type Culture Collection.

III. REACTION CHANGE DETERMINATIONS

The first undertaking was to determine if there occurred a change in reaction towards the acid side as a result of growth of each of these organisms in glucose broth. In every instance it was observed that the reaction of the media became progressively more alkaline. This was found to be the case in carbohydrate broth as well as in plain broth, i.e., there was no evidence of accumulation of acid in the media. These results are in agreement with those of previous workers (Smith, Kendall et al.). This eliminated any possibility of direct determination of carbohydrate utilization by the generally used indicator method.

Kendall et al. (1914) and Long (1922) determined the reaction

curves of several organisms of this group and observed that these curves differed with the different carbohydrates employed. They were not concerned with the demonstration of the utilization of carbohydrates but it seemed possible that in this study variations in the reaction curves induced by the addition of various carbohydrates might be employed as a method of determining whether the respective carbohydrates were or were not utilized.

To this end the following experiments were carried out. Various factors which might be concerned in reaction curve determinations are first considered; typical reaction changes as observed with various carbohydrates are then presented.

Methods

Standard meat infusion broth, to which 200 mgm. secondary potassium phosphate per liter was added, was used throughout as the basic medium for the reaction curve determinations. The method of determining the reaction changes in the cultures was essentially the same as that used by Long (1922); phenol sulphonphthalein (phenol red) in a concentration of 0.001 per cent being used as the indicator, Clark (1920) buffer systems being used for the standards, and the Dernby and Avery (1918) comparator block method being used in making comparison. The standards gave a definite color change from pH 6.8 to pH 8.0 and approximations of reasonable accuracy could be made down to pH 6.0 and up to pH 8.6, thus giving a satisfactory range for pH determinations.

Tubes of uniform size (18 by 150 mm.) were used throughout the entire work. In the preliminary experiments the media were adjusted to pH 7.2 to 7.4, sufficient carbohydrate was added to make a 1 per cent solution, the media tubed accurately with 10 cc. per tube, and sterilized at 15 pounds for fifteen minutes. One sterilization was adopted, since this yields less hydrolysis (Mudge, 1917).

Most of the organisms of this group are especially adapted for this type of study due to their obligate aerobic requirement which causes them to grow in the form of a pellicle at the surface, leaving the underlying liquid clear. In inoculating the tubes due recog-

nition was given this fact. The inoculum was made to float if possible and, where not possible, it was smeared on the sides of the tube at the surface of the media, care being taken in every case to make certain that some of the inoculum was in contact with the surface of the liquid medium. This insured an early and more or less uniform growth. In some of the later determinations a thin slice of cork was placed in each tube, prior to sterilization, and the inoculum placed on the cork, making it easier to keep the growth floating throughout the duration of incubation.

Some of the crude preliminary tests are considered worthy of mention because of the general principles concerning the metabolism of this group of organisms which are therein illustrated, and also because they throw new light on previous work in which reaction changes in the cultures were studied, especially that of Long and Major (1922).

The variations in reaction changes in the media induced by varying the method of incubation of the cultures were determined. Three organisms, *Myco. leprae*, *Myco. berolinensis*, and *Myco. phlei* were each inoculated into three tubes of plain broth. The three parallel cultures of each organism were incubated under the following conditions: Series I was incubated freely exposed to the relatively dry air of the incubator. In Series II the tubes were incubated in a closed jar, the bottom of which was covered with water. In series III the tubes were capped with celloidin caps. The reaction curves shown in figure 1, A, illustrate the pH changes observed.

A distinct growth with the formation of a pellicle took place in every tube, but the growth was much less in the capped tubes (series III) than in either of the other two, the most luxuriant growth being obtained in the tubes incubated under aerobic conditions (series I).

To better illustrate the effect of free access of air, a capped tube of *Myco. smegmatis* which had been incubated for nine days with little reaction change was opened to the air by puncturing a small hole in the cap. In the next five days the reaction changed from pH 7.4 to pH 8.6 and a heavy pellicle replaced the frail pellicle which had been present at the time of the puncture. These

results indicate that when the tubes are capped there is, in addition to an inhibition of growth, an accumulation of some substance which prevents a change in the reaction of the media towards the

FIG. 1

alkaline side. That this substance is carbon dioxide and the effect which it exerts upon the reaction changes was shown by the following experiment:

Three tubes of plain broth to which indicator (phenol red) had been added, were inoculated with each organism. One of these inoculated tubes was connected by means of a "U" shaped glass tube bearing a rubber stopper at each end to an uninoculated tube of plain broth. A second inoculated tube was similarly connected to a tube containing barium hydroxide, while a third was connected to one containing distilled water. This permitted a direct gas interchange between the tubes thus connected but excluded interchange with the atmosphere. To insure a seal that would be air tight, prior to being used the rubber stoppers were cleaned and soaked in glycerol as directed by Novy and Soule (1925). The reaction curves shown in figure 1, *B* (1), (2), and (3) illustrate the results obtained.

In the first set, a plain broth culture connected with plain broth, it will be noted that the reaction in the inoculated tube changed to pH 7.6 while the reaction in the uninoculated tube changed to pH 6.8. This would indicate that carbon dioxide is produced by the culture, but that decrease in pH is prevented by simultaneous production of alkaline products, since there is a decrease in the pH in the connected tube which is subjected to the same carbon dioxide tension.

In the second set, a plain broth culture connected with barium hydroxide solution, the barium hydroxide solution contained in the side tube absorbed the carbon dioxide as fast as it was formed. A white precipitate of barium carbonate appeared in the barium hydroxide tube. The reaction change in the inoculated tube was more pronounced than in the first set.

In the third set, where distilled water, which has little capacity for absorbing carbon dioxide, was used the reaction change in the inoculated tube was slight.

It will be noted from these results that, although the carbon dioxide was taken out by the barium hydroxide as fast as formed, the culture still did not obtain the degree of alkalinity reached by free exposure to air (see fig. 1, *A*). The growth was also much less abundant. As other tests which were run showed that the reaction change was still less under more anaerobic conditions, it seemed reasonable to assume that the factor concerned in the

lesser amount of growth in this set was the inadequacy of oxygen required for growth.

When pyrogalllic acid and sodium hydroxide were used to absorb the oxygen from the culture tubes, applying the reagents to the cotton stopper and sealing the tube with rubber stoppers and paraffine (technique of Rockwell, 1924) there was obtained no appreciable change in reaction and a very scanty growth of the saprophytic forms, *Myco. phlei* and *Myco. berolinensis*, after twenty days of incubation. These reaction changes are illustrated in figure 1, B (4). From a comparison of these results with those obtained when the barium hydroxide was present in a side tube (fig. 1, B (2)) it is evident that the pH change was distinctly less marked in the tube deprived of oxygen. The carbon dioxide was absorbed by the alkali in both cases, the difference being purely one of oxygen present, a diminution of which caused a corresponding diminution of growth. Evidently, then, it is a general characteristic throughout this group of organisms that, other factors being favorable, the growth is proportional to the oxygen supply. This was conclusively shown to be the case with the tubercle bacillus by the work of Novy and Soule (1925). Any method involving a study of the reaction changes of media which fails to take this factor into consideration would thus of necessity be faulty. Certainly in the light of this consideration the method of determining the reaction curves in tubes capped with tin foil as used by Long and Major (1922) is at best approximate and open to error. Any method of sealing that does not permit of a free gas interchange with the atmosphere, or a constant oxygen and carbon dioxide tension, would be certain to introduce variation in the reaction changes from tube to tube.

The facts presented above suggested the necessity of refraining from interfering with free oxygen supply during the incubation, and for that reason the incubator atmosphere was maintained at a high relative humidity and the tubes were stoppered only with cotton plugs.

In a preliminary series of determinations plain broth, glucose, lactose, sucrose, mannitol, and dextrin broths were used. By the method of sterilization mentioned above it was impossible to

obtain media of the same constant pH. Although all the broth to which the various carbohydrates were added was taken from the same container the pH of the media following sterilization were respectively: plain broth, 7.4; sucrose broth 7.3; dextrin broth, 7.1; glucose broth, 7.0; lactose broth, 7.1; and mannitol broth, 7.3. This eliminated the possibility of making an absolute comparison but the tests were run through to determine whether the difference in the reaction curves would be pronounced enough to indicate a utilization of any of the carbohydrates. With few exceptions, the results were too indefinite to be of any value. Invariably, the change of reaction was towards the alkaline side and the terminal reactions in different media by a given organism were practically the same.

An attempt was made to substitute sterilization by filtration subsequent to the addition of the carbohydrates to the media. This insured an identity of the various media, save for the presence of the different carbohydrates, at the beginning of the determinations. To the broth, which had previously been sterilized in the autoclave, the indicator was added and the reaction of the broth was adjusted to pH 7.2. The carbohydrates were weighed out and added in a 1 per cent concentration. The medium was filtered immediately, using Grade "N" Berkefeld filters. It was then tubed, approximately ten cubic centimeters per tube. Subsequent to filling, the tubes were incubated for at least three days as a check on their sterility.

In the tests reported plain broth, 1 per cent mannitol broth, 1 per cent galactose broth, and 1 per cent glucose broth were used. Each was run in duplicate and the mean pH of the two tubes in the few cases in which they differed was recorded in plotting the reaction curves. In the series reported in figure 2 the incubation was for a duration of seventeen days. It should be remembered in reading the results from the graphs that beyond pH 8.2 the determinations are more or less approximate.

There is no observable change in the color of the indicator used beyond pH 8.6. Consequently none of the curves indicate an alkalinity exceeding this figure, but in the light of later work there is little doubt that in many cases the media became somewhat more alkaline than shown in the graph.

Results

The reaction curves obtained from the plain broth control cultures (first column of fig. 2) present several interesting aspects. The most striking feature is the general similarity of the curves. A close metabolic relationship between the various members of the group is thereby indicated. The typical curves, such as that of *Myco. tuberculosis (hominis)*, present a short period of lag (or absence of reaction change) followed by a rapid increase in alkalinity with subsequent flattening out of the curve. During the lag period a pellicle was formed on the surface of the media and the rapidity of the rise in the pH may be roughly stated to be proportional to the rapidity of growth.

There are, among the curves from the plain broth cultures (first column), a few that vary more or less from the so-called typical curves. In the culture of *Myco. leprae* development of alkalinity is shown to have been somewhat less rapid. This was associated with a less rapid growth but, as will be pointed out later, this is not the only factor concerned in the case of this particular organism, since later quantitative tests have demonstrated that *Myco. leprae* produces less ammonia than the other organisms of the group which were studied.

Again, the period of lag was longer in the culture of *Myco. chelonae*. The optimum temperature for the growth of this organism is about 20°C. but growth even at this temperature is less rapid than that observed for the other organisms at their respective optimal temperatures. The lessened rapidity of growth may thus account for the different type of reaction change. The slight dipping of the curve after fifteen days may be considered to be due to difficulties in the determination of the pH, since an error of pH 0.2 may readily be made beyond pH 8.2 with the indicator used. No such tendency to late diminished alkalinity has been noticed in other tests employing this organism.

The variation of the curve for *Myco. phlei* from the typical is well within the error of the experimental method.

The curves shown in the second column, which represent the reaction changes in mannitol broth cultures of the respective

FIG. 2. REACTION CHANGES INDUCED IN MEDIA BY ORGANISMS OF THE GENUS *MYCOBACTERIUM*

organisms, show more variation. In general, it will be noted that the reaction here also changes towards progressive increased alkalinity, but with less rapidity than in the plain broth controls. This characteristic is likewise observable in the third and fourth columns (galactose and glucose broth cultures). As will be shown below, this tendency is apparently due to the protein-sparing action exerted by the carbohydrates, as a result of which less alkaline protein cleavage products, especially ammonia, accumulate in the media. It must be emphasized, however, that two opposing variable factors are involved. The above factor tends to inhibit protein cleavage and hence reaction change of the media, while the presence of utilizable carbohydrates tends to increase the rapidity of growth of the organisms. Although diminished, there is nevertheless some protein cleavage associated with growth in carbohydrate-containing media. If the rate of growth is sufficiently increased by the presence of the carbohydrate, the protein sparing affect may be counterbalanced and the reaction change may be as rapid as in the plain broth control culture. Such would appear to be the explanation of the curves with *Myco. friburgensis* in mannitol broth, which shows no variation from the curve of the plain broth culture, since this organism has been shown to utilize mannitol (results to be reported in later paper). Utilization of mannitol by *Myco. chelonae* has not been demonstrated. This curve likewise presents no variation from that of the plain broth control.

At the other extreme, protein cleavage may be so retarded by the presence of utilizable carbohydrates that, despite a more luxuriant growth than that present in the plain broth control, there is only slight increase in alkalinity. This is exemplified by the curve for *Myco. phlei* in mannitol broth. A still greater extreme is the curve shown in the same column for *Myco. leprae*. Subsequent tests have shown that the secondary slight decrease in pH in the case of the latter organism is variable but the alkalinity has in no instances been noted to exceed pH 7.4. It is significant that in the quantitative determinations an actual decrease in the ammonia content of the media has been observed in some carbohydrate broth cultures of *Myco. leprae*. This

decrease in ammonia has not been encountered with any other of the *Mycobacteria* studied.

In the mannitol broth column, then, there is one curve at each of the extremes, that is one showing no variation from the control (*Myco. friburgensis*), and a second curve (*Myco. leprae*) exhibiting definite variation from the control. The remaining curves occupy intermediate positions. The curves for *Myco. leprae* and *Myco. phlei* may be of value as a means of distinguishing these organisms from the other species studied. However, the large number of variable factors tending to alter the curves, such as amount of inoculum, amount of inoculum in contact with the surface of the medium, variability in the rapidity of growth from culture to culture etc., definitely limit the value of this distinguishing characteristic.

In the remaining two columns of curves, variations from the plain broth controls similar to those discussed in the mannitol broth cultures may be noted, that is in the presence of the carbohydrate there is in general a retarded reaction change. In the third column the curve for *Myco. friburgensis* in galactose broth is the only one closely simulating the curve of the plain broth control. Varying degrees of the diminished rate of pH change are to be noted in the remaining curves. The curve given for *Myco. chelonei* was obtained at a later date employing a different batch of media, there being very scant growth in the cultures used for the first determination. This fact possibly accounts for the variation from the other types of curves here encountered.

In the fourth column (glucose broth cultures) the curves of several of the organisms resemble closely the curves of their respective plain broth controls; for example, *Myco. tuberculosis*, *Myco. phlei*, and *Myco. friburgensis*. Other curves show merely a longer lag period before the alkalinity developed, e.g., *Myco. smegmatis*, *Myco. butyricum*, and *Myco. berolinensis*. The curve for *Myco. chelonei* constitutes a more definite variation. The growth was scanty and the results indefinite in the first cultures of this organism. The curve given in this case represents the results obtained at a later determination. The growth was more abundant in the latter cultures but even in this case there was

less growth than was obtained in the cultures of the other organisms given. This lesser growth probably accounts for the difference in the type of the reaction curve.

A secondary slightly diminished alkalinity is observable in the glucose broth culture of *Myco. leprae* which, like that appearing with this organism in mannitol broth, is irregular in occurrence. In this same column the curve for *Myco. stercusis* also presents a definite variation from the plain broth control.

The original object of these determinations was to ascertain whether or not mannitol, galactose, and glucose are utilized by the organisms studied. The reaction curves convey indefinite and conflicting evidence. Employing more accurate methods, which will be given below and in a subsequent report, all three of these carbon compounds have been shown to be utilized by all the organisms here studied, with the two exceptions that mannitol has not been shown to be utilized by *Myco. chelonai*, nor galactose by *Myco. leprae*. It must be remembered that the only evidence that carbohydrates are utilized obtainable from the curves is the decreased rate of production of alkalinity observable in some carbohydrate broth cultures. Many of the curves would suggest utilization of the respective carbon compounds; for example *Myco. tuberculosis*, *Myco. leprae* and *Myco. phlei* in mannitol broth; *Myco. tuberculosis*, *Myco. chelonai*, *Myco. smegmatis*, etc., in galactose broth; and *Myco. chelonai* and *Myco. stercusis* in glucose broth. On the other hand, there are not a few cases in which the curves present little or no evidence that utilization has occurred; e.g., all the curves for *Myco. friburgensis*, and several of the curves shown in the fourth column representing the results in glucose broth cultures. Glucose is especially actively utilized by these organisms, yet the curves in most cases indicate no such utilization.

It therefore must be concluded that the determination of reaction change in the media constitutes an indefinite and misleading method of determining carbohydrate utilization by these organisms. Such a method may, however, under carefully controlled conditions, be applicable for differentiation of some of the organisms here studied. The reaction curves obtained when *Myco.*

leprae and *Myco. phlei* were grown in mannitol broth and when *Myco. chelonae* was grown in glucose broth vary sufficiently from the remaining curves in the determinations here given to be of possible differential value.

The significance of these reaction curve determinations undoubtedly rests more upon its clear cut demonstration of the great similarity in the metabolism of the organisms of this group than upon their carbohydrate utilization. These organisms have already been shown to be nearly related (Long, 1923) first, in staining reaction; second, in the possession of a large amount of lipin; and third, serological reactions indicate that their antigenic activity is qualitatively the same. This work offers further evidence that they are very nearly related in their metabolic activities.

IV. DIRECT QUANTITATIVE DETERMINATION OF CARBOHYDRATE UTILIZATION

In order to obtain more exact information concerning the extent to which carbohydrates are utilized by organisms belonging to the genus under consideration direct quantitative determinations of the sugars were made.

Method

Standard broth, prepared as in the preceding work, was used as the base of the media. It was adjusted to pH 7.5 and the various sugars were weighed out and added in amounts to make approximately 1 per cent solutions. The carbohydrate-containing media were then immediately tubed measuring 10 cc. to each tube from burettes. Sterilization was by heat, at 5 pounds pressure for thirty minutes.

The inoculations were made as in the preceding work. The duration of incubation varied as shown in the accompanying table while the temperatures of incubation were as follows: *Myco. avium* 41°C.; *Myco. piscium*, *Myco. ranae*, *Myco. chelonae*, and *Myco. butyricum*, 22° to 25°C.; while the remaining organisms were incubated at 37.5°C.

The Shaffer-Hartman blood sugar quantitative method as recommended for bacteriological procedures by Stiles, Peterson and Fred (1926) was used for the carbohydrate determinations at the termination of incubation of the cultures. Before carrying out the tests for carbohydrates the pathogenic organisms were killed by immersing the cultures in a water bath at 100° C. for ten minutes. Each culture was then diluted to 100 cc. by pouring the entire contents of the tube into a 100 cc. volumetric flask, the culture tube being rinsed three times with distilled water to insure that all the sugar should be transferred into the flask. Following the addition of the lead acetate and potassium phosphate the flask was filled to the mark with distilled water, thoroughly shaken, a portion transferred to a test tube and then, after allowing fifteen to twenty minutes for the precipitate to settle, an aliquot portion of 1 cc. was withdrawn for each determination. Cultures were run in duplicate and two determinations were made with material from each culture.

The carbohydrates used were glucose, fructose, galactose, arabinose, lactose, and sucrose. The latter sugar was hydrolyzed prior to running the determinations by adding 1 cc. of concentrated hydrochloric acid to each tube, incubating at 70°C. for fifteen minutes, and subsequently neutralizing with concentrated sodium hydroxide using phenolphthalein as the indicator.

Results

The results of the main series of determinations are tabulated in table 1. In the first column under each sugar is given the duration of the incubation in days. In each of the next two columns is given the mean value of the two determinations from each culture tube. In the last column is given the average value obtained from the two culture tubes. The results are reported as per cent sugar in the culture media. The values given for the sugars, other than glucose, were arrived at by multiplying the amount of reducing sugar, in terms of glucose, as read from the tables given by Stiles, Peterson and Fred (1926), by the factors given in table 1.

TABLE I
Determination of carbohydrate utilization by quantitative analysis

CULTURE	GLUCOSE* (FACTOR 1.0)			FRUCTOSE* (FACTOR 1.0)			ARABINOSE* (FACTOR 1.14)			GALACTOSE* (FACTOR 1.14)			SUCROSE* (FACTOR 1.0)			LACTOSE* (FACTOR 1.65)		
	Incubation	Culture I	Average	Incubation	Culture I	Average	Incubation	Culture I	Average	Incubation	Culture I	Average	Incubation	Culture I	Average	Incubation	Culture I	Average
	days			days			days			days			days			days		
Controls:																		
Highest.....	16		0.88	18		0.82	19		0.74	20		0.76	30		0.94	28		0.82
Lowest.....	16		0.75	18		0.74	19		0.64	20		0.65	30		0.78	28		0.62
Average of five.....	16-38		0.81	18-38		0.78	19-38		0.70	20-38		0.72	30-38		0.88	28-38		0.78
<i>Myc. tuberculosis</i> (hominus).....	16	0.130.190.16		18	0.070.090.08		19	0.070.070.07	0.07	20	0.120.100.11		30	0.800.860.84		28	0.700.780.74	
<i>Myc. avium</i>	39	0.660.800.73		39	0.140.660.40		39	0.340.290.31	0.31	39	0.650.670.66		30	0.060.080.07		30	0.630.690.66	
<i>Myc. leprae</i> 282.....	16	0.280.260.27		18	0.070.090.08		19	0.620.620.62	0.62	20	0.650.630.64		30	Trace		28	0.760.780.77	
<i>Myc. leprae</i> 65.....	16	0.510.570.54		18	0.140.140.14		19	0.620.620.62	0.62	20	0.680.670.67		30	Trace		28	0.790.720.76	
<i>Myc. leprae</i> 66.....	16	0.230.270.25		18	0.140.100.12		19	0.070.070.07	0.07	20	Trace		30	0.720.860.79		28	0.720.720.72	
<i>Myc. leprae</i> 67.....	16	0.590.570.58		18	0.400.420.41		19	0.550.510.53	0.53	20	0.700.660.68†		30	0.850.730.79		28	0.720.860.79	
<i>Myc. leprae</i> 68.....	16	0.360.400.38		18	0.070.090.08		19	0.070.070.07	0.07	20	0.410.370.39		30	0.080.260.17		28	0.790.730.76	
<i>Myc. leprae</i> 69.....	16	0.160.16		18	0.040.040.04		19	0.000.000.00	0.00	20	0.000.000.00		30	0.800.860.83		28	0.720.740.73	
<i>Myc. piscium</i>	38	0.560.420.49		38	0.490.210.35		38	0.670.620.64	0.64	38	0.780.750.77		38	0.890.890.89		38	0.740.660.70	
<i>Myc. chelonae</i>	38	0.070.070.07		38	0.660.680.67		38	0.560.640.60	0.60	38	0.690.710.70		38	0.700.680.69		38	0.740.760.75	
<i>Myc. ranae</i>	38	0.650.590.62		38	0.300.260.28		38	0.600.580.59	0.59	38	0.830.810.86		38	0.920.920.92		38	0.760.740.75	
<i>Myc. butyricum</i>	38	0.680.780.73		38	0.590.570.58		38	0.380.400.39	0.39	38	0.190.170.18		38	0.820.780.80		38	0.760.760.76	
<i>Myc. smegmatis</i>	16	0.080.080.08		18	0.050.050.05		19	0.000.000.00	0.00	20	Trace		30	0.860.880.87		28	0.790.710.75	
<i>Myc. berolinensis</i>	16	0.200.190.20		18	0.080.080.08		19	Trace		20	0.070.140.10		30	0.760.820.79		28	0.700.720.71	
<i>Myc. phlei</i>	16	0.280.280.28		18	0.070.070.07		19	0.000.000.00	0.00	20	0.120.120.12		30	0.910.890.90		28	0.650.650.65	
<i>Myc. stercois</i>	16	0.370.620.50		18	0.060.060.06		19	0.000.000.00	0.00	20	0.140.160.15		30	0.760.820.79		28	0.650.650.65	
<i>Myc. friburgensis</i>	16	0.130.130.13		18	0.130.070.10		19	0.210.150.18	0.18	20	0.260.420.39		30	Trace		28	0.700.700.70	

* All values represent the per cent of the respective sugars present in the media at the termination of incubation, determined as given in the report.

† More utilization demonstrated in subsequent tests.

The results demonstrate that glucose is utilized by all organisms studied. Utilization by *Myco. avium* is seen to be slight in the results listed. It should be noted, however, that the growth in these cultures was also slight. In subsequent tests greater utilization by this organism was demonstrable.

Fructose was also utilized by all organisms studied. The slight utilization shown by *Myco. chelonae*, again, may be attributed to the slight growth. It is worthy of note that there was, with few exceptions, a more rapid utilization of fructose than of glucose. The growth was also more abundant. This relationship has been repeatedly observed.

Arabinose was definitely utilized by most of the organisms. There was, however, but slight utilization by *Myco. leprae* 282 and *Myco. leprae* 65. This has been rechecked several times with similar results. Greater utilization by *Myco. leprae* 67 was demonstrated in subsequent tests. Some, though slow, utilization by *Myco. piscium* has been shown by subsequent tests. More definite utilization by *Myco. chelonae* was demonstrated in later tests than appears from the table.

There was less tendency towards utilization of galactose. *Myco. leprae* 282 utilizes galactose very slowly. There is apparently no utilization by *Myco. leprae* 65. In later tests *Myco. leprae* 67 was found definitely to utilize galactose. Upon repeated tests, no utilization of galactose by *Myco. chelonae* and *Myco. piscium* could be demonstrated.

Utilization of sucrose is of especial interest. *Myco. avium*, the strains of *Myco. leprae*, 282, 65, and 68 and *Myco. friburgensis* definitely utilize sucrose. There is apparently slow utilization by *Myco. chelonae*. It is possibly significant that the strains of *Myco. leprae* that utilize sucrose are rat strains whereas the strains of human origin do not utilize this carbohydrate.

Lactose is apparently not utilized by any of the organisms.

The summarized conclusions from these experiments as to definite, slight, and no utilization of the carbohydrates studied is given in table 2.

Some determinations have been made to ascertain whether maltose is utilized by any of these organisms. While the results

thus far are not entirely conclusive, definite utilization has been demonstrated only for *Myco. berolinensis*. These results clearly demonstrate a rather wide utilization of carbohydrates by organisms of the genus *Mycobacterium*. There is a distinct variation in the rapidity with which the various carbohydrates are utilized. In the course of the incubation periods adopted for this work, the carbohydrate contained in the media when utilized almost completely disappeared in many cases; in other cases only

TABLE 2

Summary. Carbohydrates utilized by organisms of the genus *Mycobacterium*

ORGANISMS	GLUCOSE	FRUCTOSE	ARABINOSE	GALACTOSE	SUCROSE	LACTOSE
<i>Myco. tuberculosis (hominus)</i>	+	+	+	+	0	0
<i>Myco. avium</i>	±	+	+	0	+	0
<i>Myco. leprae</i> 282.....	+	+	±	±	+	0
<i>Myco. leprae</i> 65.....	+	+	±	0	+	0
<i>Myco. leprae</i> 66.....	+	+	+	+	0	0
<i>Myco. leprae</i> 67.....	+	+	+	+	0	0
<i>Myco. leprae</i> 68.....	+	+	+	+	+	0
<i>Myco. leprae</i> 69.....	+	+	+	+	0	0
<i>Myco. piscium</i>	+	+	0	0	0	0
<i>Myco. chelonae</i>	+	+	+	0	±	0
<i>Myco. ranae</i>	+	+	+	0	0	0
<i>Myco. butyricum</i>	+	+	+	+	0	0
<i>Myco. smegmatis</i>	+	+	+	+	0	0
<i>Myco. berolinensis</i>	+	+	+	+	0	0
<i>Myco. phlei</i>	+	+	+	+	0	0
<i>Myco. stercusis</i>	+	+	+	+	0	0
<i>Myco. friburgensis</i>	+	+	+	+	+	0

The symbols have the following meaning: + indicates definite utilization; ± indicates slight utilization; 0 indicates no evidence of utilization.

a slight decrease was noted. In general it appears that fructose is utilized more rapidly than any other carbohydrate studied.

These results also appear to demonstrate that carbohydrate utilization determinations are of limited value as a means of differentiation of the various species of this genus, since the variations observed between individual strains of the same species are in some cases greater than variations between different species of the same genus.

V. QUANTITATIVE RELATIONSHIP OF CARBOHYDRATE UTILIZED TO
CARBON DIOXIDE AND AMMONIA PRODUCED, AND
REACTION CHANGES IN THE MEDIA

Kendall, Day and Walker (1914) have demonstrated an increase of ammonia in cultures of some Mycobacteria. They stated that this increase was less in the presence of glycerol. No effort was made to correlate the ammonia production with carbohydrate utilization.

Novy and Soule (1925) have carried out extensive studies upon the respiratory relationships of various bacteria. The first study reported dealt with the human type tubercle bacillus. They found that the respiratory quotient, $\frac{\text{CO}_2 \text{ liberated}}{\text{O}_2 \text{ consumed}}$, of a culture of tubercle bacillus varied with the media upon which the organisms were grown. In glycerol media the R.G. value was 0.836. The theoretical value, were the glycerol alone broken down to carbon dioxide and water, is 0.857. When glucose agar was used the respiratory quotient as determined and corrected was 0.992. The theoretical value for glucose is 1.000. This is illustrated by the following equation:

These workers showed that, even in the presence of carbohydrates, the tubercle bacillus will not grow without oxygen. They also demonstrated that the growth is proportional to the oxygen supplied. It would thus appear that molecular oxygen is necessary before the tubercle bacillus can obtain energy from the carbohydrate. In other words, there is apparently no anaerobic cleavage of carbohydrates by this organism.

Novy and Soule did not attempt to correlate the amount of carbohydrate disappearing from the media with the carbon dioxide produced and the oxygen consumed.

Numerous quantitative determinations of end products of carbohydrate and protein bacterial metabolism have been made in connection with organisms other than the Mycobacteria, especially in recent years. In every case, so far as the author has

found, when the end products of carbohydrate metabolism have been studied some organic acids or alcohols have been found. Thus there has been encountered no report of complete utilization of a carbohydrate by a single species of bacteria, that is, complete cleavage of the carbohydrate to carbon dioxide and water. It is significant that in all such determinations facultative anaerobic organisms have been employed.

It is not clear what the end products of carbohydrate utilization are in the case of organisms of the genus *Mycobacterium*. In an effort to obtain some information concerning the nature of these end products, as well as to find an explanation for the characteristic reaction changes induced in the media by these organisms the following experiments were carried out:

First: Determinations were made of reaction change of the media, the change in ammonia content, the titratable alkalinity, the decrease in glucose, or fructose, and the carbon dioxide produced at the termination of a given period of incubation.

Second: These changes were followed, employing a continuous aeration method, at intervals of a few days on a single culture in order to determine whether the relationships found in the above work were maintained throughout the duration of incubation and under unchanging oxygen tension.

Only glucose and fructose were used for these determinations as previous work has shown that the metabolism is apparently similar regardless of the sugar employed.

Method

Meat infusion broth, prepared as stated above, again constituted the basis of the media. When glucose or fructose were used, sufficient was added to make a 1 per cent concentration. The medium was tubed accurately, 10 cc. per tube, employing a 10 cc. volumetric pipette. Sterilization of the media was by heat as in the previous experiment. Inoculation of the tubes was also as previously given.

Immediately after inoculation the tubes were placed in one liter Florence flasks, to which 50 cc. of approximately 0.2 normal barium hydroxide solution had just previously been added. The

flasks were immediately tightly stoppered using specially prepared rubber stoppers. Two or more flasks containing uninoculated controls were similarly prepared for each series. After three to four days incubation the majority of cultures had developed a firm pellicle. From this time on, the flasks were gently rotated at daily intervals to break the crust of barium carbonate which had formed on the surface of the hydroxide, in order to permit more complete absorption of the carbon dioxide formed. At the termination of incubation the flasks were shaken and placed at room temperature for twenty-four hours during which time they were again shaken several times, thus permitting complete absorption of the contained gaseous carbon dioxide.

Each flask was then opened and the amount of hydroxide that had been neutralized by the absorbed carbon dioxide was determined by titration to the disappearance of the blue color of thymolphthalein, employing 0.2 normal oxalic acid. The color imparted by thymolphthalein is intense and clearly visible against the white of the barium carbonate and barium oxalate. Thymolphthalein has the added advantage over phenolphthalein that the change of color is at a somewhat higher pH and as pointed out by Schollenberger (1928) this fact permits a more accurate titration of the carbon dioxide absorbed. The difference between the number of cubic centimeters of standard oxalic acid solution required to neutralize the hydroxide, in the culture flasks on one hand and in the control flasks on the other hand, represented the hydroxide neutralized by carbon dioxide. The number of cubic centimeters difference multiplied by 4.4 gave the milligrams of carbon dioxide absorbed by the hydroxide.

The broth culture was then diluted to 100 cc. in a volumetric flask. Duplicate carbon dioxide determinations employing the Van Slyke apparatus were then run in order to determine the carbon dioxide absorbed or retained by the media. Five cubic-centimeters of the diluted culture were used for each of these determinations. The diluted uninoculated culture was used as control; thus, the carbon dioxide contained in the distilled water used for dilution need not be taken into account since the difference between the control and culture determinations again repre-

sented the cubic centimeters of carbon dioxide contained in 0.5 cc. of undiluted media, or one-twentieth of the total carbon dioxide in the culture. Temperature and barometric corrections were made for each determination. The carbon dioxide thus determined plus that absorbed by the barium hydroxide represented the total carbon dioxide produced by the culture.

Other 10 cc. portions of the diluted media were used for pH determinations. Phenol red and thymol blue (alkaline range) were used as indicators and the pH determined colorimetrically. The cultures when diluted 1-10, as in this work, give a fairly accurate index of the pH as recently shown by Fawcett and Acree (1929).

Two 25-cc. portions of the diluted media were used, first, for the determination of the titratable alkalinity and, second, for the determination of the ammonia content. To each of the 25-cc. portions in N.P.N. tubes was added 0.25 cc. of 0.1 per cent phenol red. Employing $N/40$ sulphuric acid the number of cubic centimeters of acid required to bring the pH of the diluted culture to the same pH as the diluted uninoculated control was determined. This represented the increase in the titratable alkalinity of the culture.

The same 25 cc. portions were then employed for the ammonia determinations. Folin's aeration method was used, the air used being washed by passing it through sodium hydroxide and sulphuric acid solutions. One gram of sodium carbonate and 5 grams of sodium chloride were added to the diluted culture. The ammonia was collected in $N/40$ sulphuric acid. The sulphuric acid neutralized was subsequently determined by back titration with $N/40$ sodium hydroxide using a solution of methyl red, to which was added a small amount of methylene blue, as indicator.

The sugar determinations were run by the Shaffer-Hartman method as previously outlined, except that a protein free filtrate was not prepared. In several control tests it was found that more constant results were obtainable when no effort was made to remove the protein from the media by precipitation. This agrees with the results recently reported by Magee and Smith (1930).

In the interpretation of the results it must be borne in mind

that the values given are in most cases determined from comparison with the uninoculated controls. Since there were several of these for the work here reported it was deemed unnecessary to enlarge the table by their inclusion. The carbon dioxide determinations were controlled as given above. The milligrams of carbohydrate as determined in the uninoculated control tubes, minus the milligrams found in the cultures, gave the amount of carbohydrate that was utilized. The carbon dioxide possible to liberate from the carbohydrate was calculated by multiplying the milligrams of carbohydrate utilized by the factor 1.47, since 1.47 mgm. of carbon dioxide might be obtained from every milligram of a hexose which is quantitatively converted to carbon dioxide and water. Thus:

in which the figures represent one gram molecule of the various substances. Therefore, for every 180 grams of the hexose converted to water and carbon dioxide, 264 grams of the latter would be produced, or $\frac{264}{180}$, or 1.466 grams of carbon dioxide per gram of

hexose. Then, in order to have a direct basis of comparison on a percentage basis between the carbon dioxide actually recovered and that which might possibly be produced from the hexose utilized, the per cent of the possible carbon dioxide which was recovered was determined by dividing the actual carbon dioxide by the possible carbon dioxide. Stated in equation form this relationship would be expressed as follows:

$$\frac{\text{Total CO}_2 \text{ recovered} \times 100}{\text{CO}_2 \text{ possible from carbohydrate utilized}} = \text{per cent of possible CO}_2 \text{ recovered}$$

Theoretically, if the hexose constituted the sole source of carbon, 100 per cent would be the maximum value possible if all the hexose were completely oxidized, but, since other carbon containing compounds are present, one might expect to obtain values greater than 100 per cent, provided the carbohydrate is converted quantitatively into carbon dioxide and water.

TABLE 3
Relationships existing between carbon dioxide production, carbohydrate utilization, pH changes, ammonia production, and changes in the titratable alkalinity of cultures studied

ORGANISM	MEDIA	CO ₂ DETERMINATION			CARBOHYDRATE DETERMINATION			pH	NH ₃ PER 10 CC.	NH ₃ INCREASE PER 10 CC.	NH ₃ INCREASE PER MILLI-GRAM CO ₂ PRODUCED	TITRATABLE ALKALINITY IN CC. N/40 H ₂ SO ₄	TITRATABLE ALKALINITY IN TERMS OF MILLIGRAMS NH ₃	LATTER MINUS NH ₃ INCREASE	DURATION OF INCUBATION
		Absorbed	In media	Total	Utilized	CO ₂ possible	Per cent of possible CO ₂ recovered								

Section A																
<i>Myc. tuberculosis (hominis)</i>	G	71	8	79	52	76	104	7.6	2.8	mgm.	mgm.	1.30.016	3.2	1.4	0.1	7
	G	68	5	73	42	62	118	8.0	2.4	1.20.016	1.20.016	2.8	1.2	0.0	10	
	F	106	9	115	60	88	130	8.0	3.8	2.90.025	2.90.025	3.6	1.5	-1.4	12	
	P	26	7	33				8.4	3.0	1.50.045	1.50.045	4.0	1.7	0.2	7	
	P	44	5	49				8.4	3.2	1.90.041	1.90.041	5.6	2.4	0.5	13	
<i>Myc. smegmatis</i>	G	45	6	51	39	57	90	7.6	1.8	0.30.006	0.30.006	2.4	1.0	0.7	7	
	G	66	6	72	44	65	111	8.0	2.4	1.20.016	1.20.016	3.0	1.3	0.1	10	
	F	91	8	99	56	82	121	8.0	2.8	1.90.019	1.90.019	4.4	1.9	0.0	12	
	P	23	8	31				8.4	2.8	1.30.042	1.30.042	4.0	1.7	0.4	7	
	P	49	6	55				8.7	3.7	2.40.044	2.40.044	6.8	2.9	0.5	13	
<i>Myc. stercusis</i>	G	40	3	43	21	31	139	8.0	2.5	1.50.035	1.50.035	3.0	1.3	-0.2	18	
	F*	53	4	57	45	66	86	7.6	2.3	1.30.023	1.30.023	3.0	1.3	0.0	22	
	P	26	4	30				8.2	3.0	2.00.066	2.00.066	5.2	2.2	0.2	21	

<i>Myc. berolinensis</i>	G	45	4	49	33	49	100	8.0	2.1	1.00.02	3.2	1.4	0.4	10
	F	82	5	87	48	71	122	8.0	2.4	1.50.017	4.0	1.7	0.2	12
	P	46	7	53				8.6	3.7	2.40.045	7.6	3.2	0.8	13
<i>Myc. phlei</i>	G	48	3	51	21	31	164	8.4	2.4	1.40.027	3.8	1.6	0.2	18
	F*	87	4	91	71	104	87	7.6	1.8	0.80.009	2.8	1.2	0.6	22
	P	40	4	44				8.4	3.4	2.40.055	6.0	2.6	0.2	21
<i>Myc. friburgensis</i>	G	69	2	71	45	66	108	7.6	1.0	0.00.0	2.6	1.1	1.1	18
	F	133	3	136	80	118	115	8.4	1.4	0.40.003	4.0	1.7	1.3	22
	P	32	3	35				8.2	1.9	0.90.026	5.2	2.2	1.3	21
<i>Myc. chelonae</i>	G	50	2	52	43	63	83	7.3	1.9	0.90.017	0.8	0.3	-0.6	18
	F	80	4	84	50	74	113	7.6	5.4	4.40.052	2.6	1.1	-3.3	22
	P	22	1	23				7.6	4.2	3.20.14	3.2	1.4	-1.8	21
<i>Myc. butyricum</i>	G	11	1	12	6	9	133	7.1	1.7	0.70.058	0.0	0.0	-0.7	18
	F	97	3	100	67	98	102	7.7	1.4	0.40.004	2.6	1.1	0.7	22
	P	22	1	23				7.6	2.0	1.00.044	3.2	1.4	0.4	21
<i>Myc. leprae</i> 282.....	G	43	1	44	35	51	86	7.3	0.9	-0.10.0	0.6	0.3	0.4	10
	F*	66	3	69	55	81	85	7.2	0.6	-0.40.0	1.4	0.6	1.0	12
	P	15	1	16				7.6	1.7	0.70.044	2.4	1.0	0.3	13
<i>Myc. leprae</i> 67.....	G	26	2	28	13	19	147	7.8	1.5	0.50.018	2.4	1.0	0.5	18
	F*	90	2	92	74	108	86	7.2	1.0	0.00.0	1.2	0.5	0.5	22
	P	41	4	45				8.4	2.7	1.70.038	6.4	2.7	1.0	21

TABLE 3—Concluded

ORGANISM	MEDIA	CO ₂ DETERMINATION			CARBOHYDRATE DETERMINATION			pH	NH ₃ PER 10 CC.	NH ₃ INCREASE PER 10 CC.	NH ₃ INCREASE PER MILLI-GRAM CO ₂ PRODUCED	TITRATABLE ALKALINITY IN CC. N/40 H ₂ SO ₄	TITRATABLE ALKALINITY IN TERMS OF MILLIGRAMS NH ₃	LATTER MINUS NH ₃ INCREASE	DURATION OF INCUBATION	
		Absorbed	In media	Total	Utilized	CO ₂ possible	Per cent of possible CO ₂ recovered									
Section B																
<i>B. typhosus</i>	G	4	0	4	26	38	10	<6.0	1.7	0.20	0.05	-7.6	-3.2	-3.4	7	days
	P	21	3	24				8.4	2.7	1.20	0.05	3.6	1.5	0.3	7	
<i>Staph. aureus</i>	G	11	0	11	39	57	20	<6.0	1.9	0.40	0.036	-10.8	-4.6	-5.0	7	
	F	6	0	6	36	53	11	<6.0	1.7	0.20	0.033	-12.4	-5.2	-5.4	7	
	P	6	1	7				8.0	2.1	0.60	0.086	1.4	0.6	0.0	7	
<i>B. coli</i>	G	10	0	10	40	59	17	<6.0	1.2	0.00	0	-10.4	-4.4	-4.4	7	
	F	9	0	9	36	53	11	<6.0	0.9	-0.30	0	-11.6	-4.9	-4.6	7	
	P	13	2	15				8.2	2.7	1.50	0.10	1.6	0.7	-0.8	7	
<i>B. subtilis</i>	G	27	0	27	50	74	36	<6.0	1.5	0.30	0.011	-6.0	-2.6	-2.9	10	
	F	29	0	29	29	43	67	<6.0	2.9	1.90	0.065	-4.8	-2.0	-3.9	12	
	P	9	1	10				6.8	2.7	1.40	0.14	-1.2	-0.5	-1.9	13	

Section C

<i>Myc. tuberculosis</i> (<i>hominis</i>)	{ 1 per cent.	G	173	11	184	88	129	142	8.6	5.1	4.10.022	6.4	2.7	-1.4	23
	{ 2.5 per cent.	G	308	9	317	212	310	102	8.0	3.2	2.20.007	4.4	1.9	-0.3	23
	{ 3.5 per cent.	G	284	9	293	221	324	90	7.6	1.4	0.40.001	3.2	1.4	1.0	23
<i>Myc. smegmatis</i>	{ 1 per cent.	G	165	12	177	85	125	141	8.6	5.1	4.10.023	6.4	2.7	-1.4	23
	{ 2.5 per cent.	G	277	9	286	211	311	92	8.0	2.5	1.50.005	4.2	1.8	0.3	23
	{ 3.5 per cent.	G	273	7	280	216	315	89	7.7	1.5	0.50.002	2.8	1.2	0.6	23
<i>Myc. tuberculosis</i> (<i>hominis</i>)	{ 1 per cent.	Gl	176	12	188				8.4	5.0	4.00.021	4.2	1.8	-2.2	24
	{ 3 per cent.	Gl	188	9	197				8.0	3.6	2.60.013	2.6	1.1	-1.5	24
	{ 5 per cent.	Gl	157	7	164				8.0	3.4	2.40.014	2.4	1.0	-1.4	24
<i>Myc. smegmatis</i>	{ 1 per cent.	Gl	176	14	190				8.4	4.0	3.00.016	4.6	2.0	-1.0	24
	{ 3 per cent.	Gl	127	1	128				7.4	3.3	2.30.018	1.2	0.5	-1.8	24
	{ 5 per cent.	Gl	134	1	135				7.4	3.3	2.30.017	0.4	0.2	-2.1	24

G = Glucose broth; F = fructose broth; P = plain broth; Gl = glycerol broth.

* These determinations were made immediately after removing the cultures from the incubator without allowing the usual 24-hour period to insure complete carbon dioxide absorption.

The expression "total ammonia per 10 cc." used in the table refers to the total ammonia found in the cultures, and the expression "ammonia increase per 10 cc." is this total, minus the ammonia found present in the uninoculated control. The ammonia present in the uninoculated controls varied in the different batches of media used for the reported determinations between 1.02 and 1.53 mgm. The expression "mgm. ammonia produced per mgm. carbon dioxide recovered" is self explanatory.

The titratable alkalinity is expressed in cubic centimeters of $N/40$ H_2SO_4 required to bring the pH of the culture to the pH of the control; this was determined colorimetrically. In order that a direct comparison may be made between the ammonia increase and the increase in the titratable alkalinity of the media the latter is also expressed in terms of milligrams of ammonia. This value, minus the determined ammonia increase, is given in the next column of the table.

In the control experiments in which non-acid-fast organisms were employed (*B. coli*, etc.) it was necessary to add alkali to bring the pH of the culture to that of the control. This is represented in the column giving titratable alkalinity by a minus sign before the number.

In analyzing the results the limits of the accuracy of the methods employed must be kept in mind. The following control experiments were designed to establish the degree of variation that might be expected.

Accurately weighed amounts of anhydrous Na_2CO_3 were added to 125-cc. Erlenmeyer flasks. These were placed in Novy jars to which a known amount of barium hydroxide had just previously been added. The cover was immediately placed on each jar and the jars partially evacuated by means of a water pump. By means of a tube, one end of which was attached to one outlet of the jar the other end being placed in the flask containing the sodium carbonate, an excess of sulphuric acid was allowed to run into the flask. The stop cocks were then closed and the flasks thus sealed from the atmosphere. After twenty-four hours incubation at $37.5^\circ C.$ approximately 85 per cent of the possible carbon dioxide was demonstrable from titration of the barium

hydroxide. After three days incubation 95 to 98 per cent of the carbon dioxide was recoverable. Similarly the Van Slyke gas analysis method was shown to be correct within 4 per cent by control determinations. Thus, the carbon dioxide determinations are, it may be assumed, correct within 5 per cent.

The sugar determinations are undoubtedly subject to a greater error. Upon control tests in which determinations were made on several uninoculated control tubes of glucose broth a maximum difference of 5 per cent between the lowest and highest values was found. This may thus be taken as the maximum limits of variation between parallel determinations; but, since the average value obtained by the Shaffer Hartman method is subject to at least 5 per cent error, a further possible variation must be taken into consideration. This last is demonstrated by the variations found in the controls throughout this work. Despite the fact that in making the media the glucose was accurately weighed and added in proportion of one gram made up to 100 cc. in volumetric flasks, values ranging from 0.95 to 1.06 per cent glucose were obtained by the Shaffer Hartman method. It should also be noted that the same reagents were used throughout these determinations, a sufficient quantity being prepared in the beginning for all tests. Thus, the maximum variation between the controls was approximately 10 per cent. It seems justifiable to consider this the normal limit of variation of the carbohydrate determination.

Colormetric methods were employed for the determinations of pH and of titratable alkalinity. Absolute accuracy was thus not attained in these determinations. The titratable alkalinity was determined upon but one fourth of the culture, so a variation of at least 0.4 to 0.8 cc. of N/40 acid might be expected to be encountered here. This represents 0.17 to 0.34 mgm. of ammonia.

Finally, since such a minute quantity of ammonia was present, the values obtained here may be stated to be correct only within 0.5 mgm. The widest variation found between all the controls on any one batch of media was 0.51 mgm. per 10 cc., or 0.14 mgm. for the 2.5 cc. of culture media actually used for the determination. This latter represents approximately 0.3 cc. of N/40 NaOH

variation in the actual titrations, since 0.1 cc. of N/40 NaOH is equivalent to 0.0425 mgm. of ammonia. Thus, the maximum error in any one direction of ammonia increase plus titratable alkalinity in terms of ammonia may be stated to approximate 0.85 mgm. Control determinations demonstrated the sodium carbonate, the sodium chloride, and the caprylic alcohol used as antifoam to be free of ammonia.

Results

Results typical of those obtained in a considerable number of determinations are given in table 3. In section A of the table the results obtained from 1 per cent glucose broth, 1 per cent fructose broth, and plain broth cultures of the Mycobacteria are given. A corresponding study in which non-acid-fast bacteria were employed is tabulated in section B. The results obtained when varying concentrations of glucose and of glycerol were added to the broth are reported in section C. The values given represent the average of the determinations upon two parallel cultures. Two such series are reported for the first two organisms listed in the table, when grown in glucose broth and in plain broth, for the purpose of demonstrating the variation normally encountered when the determinations are not made at the same time and when media from the same batch are not employed. In the other cases average results from one series of determinations are given. At least two series of determinations have been made for all the Mycobacteria reported except *Myco. chelonae* and *Myco. butyricum*. In some cases, e.g., *Myco. tuberculosis* and *Myco. smegmatis*, the experiments have been repeated three times.

Despite the limitations cited above to the accuracy of the methods employed, several significant relationships are clearly demonstrated. The following are the more important relationships apparent from data presented in Section A of the table:

1. In the majority of cases more carbon dioxide was produced in the carbohydrate broth cultures than in the control plain broth cultures incubated for approximately the same length of time. In the glucose broth culture of *Myco. tuberculosis*, 79 mgm. of carbon dioxide were produced in seven days; in the control plain broth culture, only 33 mgm. were produced in the same time.

2. The quantity of growth obtained may be stated to be roughly proportional to the carbon dioxide produced. No direct quantitative estimations of the amount of growth were made but it was observed that the bulk of the growth increased with increasing carbon dioxide production. Consequently a more luxuriant growth was obtained in the carbohydrate broth cultures of the *Mycobacteria* than in the plain broth cultures.

3. A significant relationship exists between the carbon dioxide recovered and that which could be produced from the carbohydrate utilized, were it quantitatively converted to carbon dioxide and water. It will be noted that, in the majority of instances, more carbon dioxide was recovered than could have been derived from the carbohydrate disappearing. For example in the glucose broth culture of *Myco. tuberculosis* 79 mgm. of carbon dioxide were recovered while 76 mgm. is all that could have been derived from the 52 mgm. of glucose utilized. In some cases, however, (e.g., the glucose broth culture of *Myco. smegmatis*, the fructose broth culture of *Myco. stercusis*, etc.) less carbon dioxide was recovered than that theoretically possible from the utilized carbohydrate. Seven such low values are given, but as noted in table 3 some of these low values are possibly attributable to the fact that sufficient time was not allowed after removal from the incubator to effect complete absorption of carbon dioxide. Most of these low values are within the limits of experimental error as noted above, while some values lie a little outside such limits. It is noteworthy, however, that in all determinations employing the two strains of *Myco. leprae* the per cent of possible carbon dioxide recovered has averaged less than for the other *Mycobacteria* studied; a single exception is in the results given with *Myco. leprae* 67 when grown in glucose broth. It should be mentioned that, wherever there is slight utilization, the possible error in the per cent of possible carbon dioxide recovered becomes magnified, since an error of as much as 10 mgm. in the glucose determination might possibly be encountered by the methods employed.

Since there is no definite evidence as to just how much of the carbon dioxide was derived from the protein constituents of the media it cannot be concluded from the above relationship alone

that the carbohydrates are completely oxidized to carbon dioxide and water by the Mycobacteria. These results, however, constitute important suggestive evidence that such complete oxidation of the carbohydrate does occur, especially when these results are compared with those reported in section B of the table.

4. The fructose appears to have been utilized more rapidly than the glucose by all the Mycobacteria studied. For example 42 mgm. of glucose were utilized in ten days by *Myco. tuberculosis* while 60 mgm. of fructose were utilized in twelve days. More striking is the utilization of 21 mgm. of glycose in eighteen days and 71 mgm. of fructose in twenty-two days by *Myco. phlei*. The slightly longer incubation period would not account for the differences observed.

5. There was an increase in the alkalinity of all cultures, which was less marked in the carbohydrate broth than in the plain broth. Thus, despite the fact that there was more growth and more carbon dioxide production in the carbohydrate broth cultures the degree of alkalinity was not as great as that found in the plain broth controls. In the seven-day glucose broth culture of *Myco. tuberculosis*, 79 mgm. carbon dioxide was produced incident to a change from pH 7.2 to 7.6 while in the plain broth control culture only 33 mgm. of carbon dioxide was produced incident to a change from pH 7.2 to 8.4. This retardation in the reaction change in the presence of utilizable carbohydrates was suggested in the reaction curves considered above; here it is definitely shown to exist.

6. Intimately related to the reaction changes, were the changes in the ammonia content of the culture media. The ammonia increase in the media was roughly proportional to the reaction change. No such absolute relationship, however, can be shown to exist. For example, when the various cultures had reached a pH of 7.6, values were obtained for ammonia increase ranging from 0.0 mgm. in the case of the glucose broth culture of *Myco. friburgensis* to 1.3 mgm. in the glucose broth culture of *Myco. tuberculosis*. However, the relationships existing between the ammonia increase and the increase in pH varied with the organisms tested, more ammonia being produced in all media tested

incident to the production of a given change in pH by certain organisms than by others. *Myco. friburgensis* produced little ammonia incident to a rather definite reaction change in the plain broth (reaction change pH 7.2 to 8.4; ammonia increase 0.9 mg.) as well as in the carbohydrate broth; conversely *Myco. chelonei* produced large amounts of ammonia with little associated reaction change (in plain broth pH change 7.2 to 7.6; ammonia increase 3.2 mgm.) and finally, in the glucose broth and fructose broth cultures of *Myco. leprae* 282, there was an actual decrease in the ammonia content of the media yet a slight increase in alkalinity was observed, at least in the glucose broth culture.

It is significant that, in every case, far more ammonia was produced in the plain broth culture per mg. of carbon dioxide recovered than in the carbohydrate broth cultures. In the glucose broth culture of *Myco. tuberculosis*, 1.3 mgm. of ammonia was produced incident to the production of 79 mgm. of carbon dioxide, or 0.016 mgm. ammonia per milligram CO_2 , while 1.5 mgm. of ammonia was produced incident to the production of 33 mgm. of carbon dioxide in the plain broth control or 0.0440 mgm. ammonia per mgm. of CO_2 . Thus, if we compare the ratio of milligrams NH_3 increase to milligrams CO_2 recovered we find almost 3 times as much NH_3 produced incident to the production of a milligram CO_2 in the plain broth cultures as in the carbohydrate broth cultures. A study of the column in table 3 giving the milligram ammonia increase per milligram carbon dioxide produced will demonstrate that a similar relationship exists throughout the Mycobacteria excepting in the case of the glucose broth culture of *Myco. butyricum*.

If the ammonia increase in the media may be taken as an index of the protein cleavage, a very distinct protein sparing action is here clearly demonstrated as a result of the presence of the carbohydrate.

7. Another interesting relationship is that existing between the titratable alkalinity and the increase in the ammonia content of the media. In most cultures the increase in the ammonia content of the media alone could account for the determined titratable alkalinity. However, this does not hold true in all cases.

For example there was no increase in the ammonia demonstrated in the glucose broth culture of *Myco. friburgensis*, yet there was a reaction change from pH 7.2 to 7.6 with a titratable alkalinity of the equivalent of 1.1 mgm. of ammonia. Likewise, there was an unmistakable decrease in the ammonia content of some cultures of *Myco. leprae*; yet the reaction changed toward increased alkalinity with a corresponding increase in the titratable alkalinity. In some tests this decrease in ammonia resulting from growth of *Myco. leprae* in the media was observed in plain broth cultures of this organism.

In several cases, e.g., the fructose broth culture of *Myco. tuberculosis*, all cultures of *Myco. chelonae*, and the glucose broth culture of *Myco. butyricum*, it will be noted that the titratable alkalinity (in terms of milligrams of ammonia) is distinctly less than the actual ammonia increase in the media. This indicates the production of compensatory acid products tending to neutralize the ammonia. This relationship has been observed in plain broth cultures as well as carbohydrate broth cultures and is constantly found after long incubation periods in either type of media, becoming progressively greater as incubation is prolonged, or in other words as protein cleavage increases. It is clearly shown in the plain broth culture of *Myco. chelonae* given in table 3, and will again be referred to in connection with other cultures. Thus, the failure of the titratable alkalinity to be equivalent to the ammonia increase is in no way dependent upon the presence of carbohydrates. From the evidence at hand the conclusion therefore seems warranted that the changes in the ammonia content of the media account equally well for such reaction changes and changes in titratable alkalinity in carbohydrate-containing, as in carbohydrate-free, media.

8. From the above considerations it is apparent that there is no evidence that any cleavage products were formed from the carbohydrates that influenced the reaction change or change in titratable alkalinity. It would thus appear that the presence of the utilizable carbohydrate exerts an effect upon the reaction change of the media only to the extent to which it alters the protein metabolism, no cleavage products being formed from the carbohydrates themselves that tend to alter the reaction of the media.

By way of summary, then, it may be stated that the increase in the ammonia content of the cultures would account for all changes in pH and for the determined titratable alkalinity in the majority of cultures, whether in carbohydrate broth or in plain broth. In those case in which such is not the case it would appear that other cleavage products, which may be either acid or alkaline in nature, are produced which play some part in the production of changes in pH and in titratable alkalinity. These latter substances, however, appear to be derived solely from sources other than the carbohydrates since they are produced in plain broth cultures as well as in carbohydrate broth cultures; and furthermore the evidence at hand points to a protein origin since their production by a given organism approximately parallels the protein cleavage produced by that organism.

While the results obtained in this investigation point to a definite similarity in the carbohydrate metabolism of the Mycobacteria it will be noted that there are apparently certain differences in the protein metabolism, as exemplified by differences in ammonia production, relation of ammonia production to change in pH, etc.

9. When we compare the results obtained from cultures of the Mycobacteria with those obtained from cultures of the four non-acid-fast organisms given in section B of the table, marked differences are noted. More carbon dioxide was recovered from the plain broth cultures than from the carbohydrate broth cultures of *B. typhosus* and *B. coli*. This contrasts strikingly with the results obtained with the Mycobacteria. Of special significance is the relationship of the carbon dioxide recovered to that possible of liberation from the carbohydrate utilized. Values ranging from 11 to 67 per cent recovery of the possible carbon dioxide with an average of 24 per cent emphasize the wide difference between the carbohydrate metabolism of these organisms and that of the Mycobacteria in which an average of 111 per cent of the possible carbon dioxide was recovered.

Also, the reaction of the carbohydrate media in the case of the non-acid-fast bacteria became definitely acid, a reaction more acid than pH 6.0 being produced in all cases. The titratable alkalinity, such as was observed in the cultures of the Mycobac-

teria, is here converted into a correspondingly much higher titratable acidity, for example, as much as 12.4 cc. of N/40 NaOH being necessary in the fructose broth culture of *Staph. aureus*, to bring the pH of the culture to that of the uninoculated control. As in the case of the Mycobacteria there is an increase in the ammonia content of the cultures of the non-acid-fast organisms. This increase is likewise greater in the plain broth than in the carbohydrate broth cultures.

The outstanding difference in the metabolism of the two general groups thus appears to be in the mode of utilization of the carbohydrates. In the case of the non-acid-fast organisms studied, there is obviously incomplete utilization of the carbohydrates with the accumulation of incompletely oxidized cleavage products in the media. This is the type of carbohydrate utilization that is demonstrable by the appearance of acid in the media. It is this type of utilization that has been most extensively studied and it is the only type that has been clearly defined in the literature the author has reviewed. It seems remarkable that the possibility of bacteria completely oxidizing carbohydrates to carbon dioxide and water has received such slight attention. The results given above indicate rather definitely that it is by such a process of complete oxidation, unaccompanied by the accumulation of any partially oxidized products in the media at any time, that the Mycobacteria utilize carbohydrates.

10. In order to determine whether the type of metabolism of the Mycobacteria is essentially the same when higher concentration of the glucose is present in the media, the experiments reported in the first half of section C of table 3 were carried out. Essentially the same relationship is here shown to exist between the carbon dioxide recovered to the amount that could be produced from the carbohydrate utilized as outlined for the Mycobacteria above. The high values for per cent of possible carbon dioxide recovered in the case of the 1 per cent glucose broth cultures might be attributable to the fact that practically all the carbohydrate had been utilized since, as will be considered below, the protein metabolism apparently increases as the carbohydrate concentration diminishes. Consequently in the low concentration

of carbohydrate present towards the end of the incubation the amount of carbon dioxide derived from the proteins would increase.

It might thus be suggested that the degree of protein-sparing action exerted by the carbohydrate is definitely dependent upon the number of molecules of that carbohydrate available for potential contact with the organism. It would appear not to be a case merely of the presence of the carbohydrate, (a given protein sparing effect being exerted so long as any of the carbohydrate molecules remain) but rather that the protein sparing effect progressively diminishes as the concentration of the carbohydrate diminishes. Striking evidence that such is actually the case is provided by a study of the effect of higher concentrations of carbohydrate on reaction changes, ammonia production, and titratable alkalinity as contained in the results given in section C of the table.

In the 1 per cent glucose broth cultures of *Myco. tuberculosis* and *Myco. smegmatis*, the terminal reaction was pH. 8.6 in both cases; a terminal reaction of pH 8.0 was found in the 2.5 per cent glucose broth cultures even though considerably more carbon dioxide was produced in these cultures than in the 1 per cent glucose broth cultures; similarly there was still less reaction change in the 3.5 per cent glucose broth cultures. There was a corresponding difference in the ammonia produced, progressively less being formed as the concentration of the carbohydrate was increased.

Thus, in spite of the greater amount of growth in the higher concentration of the carbohydrate as shown by greater carbon dioxide production, there was actually less ammonia produced, 4.1 mgm. increase in ammonia being noted in the 1 per cent glucose broth culture of *Myco. tuberculosis* incident to the production of 184 mgm. of carbon dioxide, as compared to 0.4 mgm. ammonia increase incident to the production of 293 mgm. of carbon dioxide in the 3 per cent glucose broth. This is especially emphasized by the values showing milligrams ammonia increase per milligram carbon dioxide recovered; for example in the cultures of *Myco. tuberculosis* these values are respectively 0.022, 0.007, and 0.001 for the 1, 2.5 and 3.5 per cent glucose broth cultures. A.

pronounced increased protein sparing action with increasing carbohydrate concentration is here clearly shown.

The titratable alkalinity in terms of ammonia is less than the ammonia increase in the 1 per cent glucose broth cultures. This further emphasizes the fact mentioned above that with increasing protein cleavage, whether in plain broth or carbohydrate broth cultures, the ammonia increase becomes progressively greater than the titratable alkalinity as expressed in milligrams ammonia. The relationship here shown to exist between titratable alkalinity and ammonia increase in the 3.5 per cent glucose broth (1.0 and 0.6 with the *Myco. tuberculosis* and *Myco. smegmatis* respectively) further emphasizes the fact that, regardless of how much carbohydrate is utilized, there is no evidence that accumulation of products of the carbohydrate cleavage in any way alters the reaction or titratable alkalinity of the media.

In the second half of Section C are reported results obtained when glycerol replaced the sugars as an extra carbon source. The most significant finding here is that, in any of the concentrations of glycerol employed, much less carbon dioxide was recovered than from the 2.5 and 3.5 per cent glucose broth cultures. It would appear from these results that the addition of glucose in the latter concentration provides a better medium for the growth of these organisms than the addition of glycerol. This is contrary to the usual conception and is worthy of further investigation.

There is evidence of decreasing reaction change and ammonia production upon increasing the glycerol content of the media from 1 to 3 per cent as in glucose but not upon further increasing the concentration to 5 per cent. An increasing protein sparing action with increasing concentration of glycerol, as shown by the relationship of milligrams ammonia increase per milligram carbon dioxide produced, is not definitely shown from these results. Likewise, the ammonia increase is greater in relation to the titratable alkalinity than in the glucose broth cultures. Since the amount of glycerol utilized was not determined, the significance of this variation is not clear. From the amount of ammonia increase it would appear that this is a further manifestation of the production of compensatory neutralizing substances from the

protein constituents of the media. Summing up the results in the glycerol broth, it would appear that the same basic relationships are present as found in the carbohydrate media studied, the glycerol being completely oxidized to carbon dioxide and water.

VI. PROGRESSIVE CHANGES OCCURRING IN THE MEDIA WHEN THE CULTURE IS GROWN UNDER AEROBIC CONDITIONS

The results just reported were obtained from cultures which were grown under conditions which permitted of only a limited supply of atmospheric oxygen. The question arises as to what effect this gradually diminishing oxygen supply has upon the metabolism of the organisms. Novy and Soule have shown that the degree of growth of the human type tubercle bacillus is dependent not upon the oxygen tension but upon the total amount of oxygen available. Their work demonstrated that the oxygen could be used "to the last molecule" and that, thereafter, there were no further changes in the carbon dioxide relationships. There was no demonstrable carbon dioxide production after all the oxygen available had been used. Higher oxygen tensions permitted a more rapid growth but had no effect upon the respiratory quotient. It would appear from their work, therefore, that the gradually diminishing oxygen tension would have no effect upon the metabolic products formed.

What determinations the author has made thus far indicate that gradually diminishing oxygen supply in no way alters the relationships outlined in the preceding section. This work is being continued and will be reported in detail at a later date.

In order that the entire problem may be presented at this time, the results obtained from one of the experiments will be briefly summarized. Employing *Myco. smegmatis* five consecutive determinations were made both upon plain broth and glucose broth cultures; these were made just prior to inoculation of the media and after six, nine, fourteen and twenty days incubation.

A continuous aeration method was devised for these determinations. The method used was an adaptation, with certain modifications, of the methods reported by Krishna (1928) and by

Greaves, Zobell and Greaves (1929). A similar method has been just reported by Walker (1930). A water pump was employed for obtaining negative pressure. The incoming air was drawn successively through sodium hydroxide solution, sulphuric acid; sterile distilled water, the culture flask, N/40 sulphuric acid, and finally a carbon dioxide absorption tower. The latter was similar to that employed by Krishna while the arrangement of the culture flask with provisions for withdrawing specimens for analysis was similar to that used by Greaves *et al.*

By this method, it was possible to carry out all determinations upon the media reported in the previous section and, also, to determine the ammonia and carbon dioxide liberated from the culture.

During the entire incubation period 2023 mgm. of carbon dioxide were recovered from the glucose broth culture. At the termination of the third aeration period there remained no residual $\text{Ba}(\text{OH})_2$ in the absorption tower, the available amount having been completely neutralized by the carbon dioxide. Some carbon dioxide was thus undoubtedly lost. Fifteen hundred milligrams of glucose as determined were utilized, from which 2190 mgm. of carbon dioxide could have been produced. Thus, despite the fact that there was some loss of the evolved carbon dioxide, 93 per cent of the carbon dioxide that could possibly be produced from the glucose utilized was recovered. Thus, the same relationships as were found under gradually diminishing oxygen tension apparently here also obtain, as concerns carbon dioxide output and carbohydrate utilized. Only 1635 mgm. of carbon dioxide were recovered from the plain broth culture.

The reaction of the media became progressively more alkaline in each case, more rapidly in the case of the plain broth culture. Both cultures were adjusted to pH 7.0 in the beginning. After 20 days incubation the reaction of the plain broth culture was pH 9.0 while that of the glucose broth culture was pH 8.6. At pH 8.4, which was reached on the sixth day in the plain broth and on the fourteenth day in the glucose broth, the ammonia content of the two cultures was the same, 1.7 mgm. per 10 cc. At pH 8.6, the ammonia content of the plain broth culture was 2.89 mgm.

per 10 cc., that of the glucose broth culture was 2.6 mgm. per 10 cc. This constitutes further striking evidence that the reaction change induced in the media by any given organism varies directly with the ammonia content, regardless of whether utilizable carbohydrate is present. It is also further evidence that it is presumably the derivatives of the nitrogenous elements of the media that are responsible for all reaction changes, the carbohydrate playing a part only to the degree that its presence alters protein metabolism.

A similar close comparison was noted in the titratable alkalinities of the two cultures. At pH 8.4, the titratable alkalinity of the plain broth culture was 2.1 mgm. NH_3 per 10 cc., while that of the glucose broth culture was 1.6 mgm. per 10 cc.; at pH 8.6, the values were 2.5 and 2.1 mgm., respectively.

In addition it may be mentioned that ammonia is given off by the culture. Recognizable quantities were not obtained until the cultures had reached pH 8.0. As the reaction became more alkaline, progressively more ammonia was liberated. In the first aeration period, during which the reaction of the plain broth culture changed from pH 7.0 to 8.4, 5.6 mgm. of ammonia were recovered; in the next aeration period of 3 days, during which the reaction changed from pH 8.4 to 8.6, 7.4 mgm. of ammonia were recovered; during the change from pH 8.6 to 8.8, 8.2 mgm. of ammonia were recovered; while 15 mg. were recovered in the final aeration period. It must be remembered that these values represent the total ammonia evolved from the 250 cc. of culture media under continuous aeration. The amount evolved from the 10 cc. cultures in the closed flasks reported in the previous section of this paper would, thus, be too slight to introduce appreciable error in those determinations.

VII. GROWTH OF ORGANISMS UNDER ANAEROBIC CONDITIONS

From the work of Novy and Soule and many previous investigators, the stringent oxygen requirements of the organisms belonging to the genus *Mycobacterium* are generally recognized. In order to determine whether this is a general characteristic of

all organisms used in this study attempts were made to grow the organisms under anaerobic conditions.

Glucose agar and plain agar tubes were inoculated, as well as glucose broth and plain broth tubes; these were incubated for twenty-one days in a phosphorous jar without the appearance of growth in any tube. Cultures of *Cl. tetani* in the same jar developed an abundant growth. After removal from the jar the same tubes, without further inoculation, were incubated under aerobic conditions with a resulting abundant growth by the end of one week.

In other experiments the oxygen supply was definitely limited. Test tube cultures of the saprophytic *Mycobacteria* placed in N.P.N. tubes and sealed from the atmosphere with rubber stoppers developed a moderate growth within two weeks. Some of the cultures were tested at this time for pH and for glucose utilization. Other parallel cultures were incubated for eight weeks more and then similarly tested. These latter cultures were found to contain approximately the same per cent of glucose and had the same pH as the cultures tested after 2 weeks incubation. Thus, despite the fact that these cultures had been subjected to 8 weeks further incubation, there had been no further carbohydrate utilization or reaction change. Many tests have been run in which the oxygen supply has been thus limited with the same results. Even if a good growth of the organisms has developed while the free oxygen is gradually decreasing there is no further utilization of carbohydrates or further growth after the oxygen supply has apparently all been exhausted. The organisms do, however, remain viable under anaerobic conditions as the first experiment given above illustrates.

Thus, it would appear that the organisms of this genus possess no ability to obtain energy from carbohydrates under anaerobic conditions. Most bacteria, e.g., *B. coli*, *B. subtilis*, etc., can obtain some energy from carbohydrates by anaerobic cleavage to lactic, acetic, and other acids or to alcohol and carbon dioxide. Organisms of the genus *Mycobacterium* are thus apparently placed in a group by themselves.

VIII. ATTEMPTS AT DEMONSTRATION OF ORGANIC ACID PRODUCTION
BY THE MYCOBACTERIA

In an effort to demonstrate the production of organic acids *Myco. smegmatis* and *Myco. tuberculosis (hominis)* were inoculated into flasks of 1, 2.5, 5, 7.5, and 10 per cent glucose broth and into plain broth. After five days incubation of the 1, 2.5 and 5 per cent glucose broth cultures an effort was made to determine whether any organic acids were present. No evidence of such could be found by distillation. No test could be elicited for lactic acid in the ether extract using ferric chloride. After three weeks incubation, the 5 and 7.5 per cent glucose broth cultures were similarly tested, with no evidence of organic acids present.

Growth did not appear in the 10 per cent glucose broth culture until about the tenth day. Growth was then slow for the subsequent week, after which time the pellicle rapidly became heavier until by the end of one month a very heavy growth was present. No evidence of organic acids could be found from the tests upon these cultures. All cultures were more alkaline when tested than when inoculated.

The same tests were run on cultures which were subjected to a limited oxygen supply in an effort to force utilization. Twenty-five cubic centimeters of the respective media were placed in N.P.N. tubes. After sterilization, inoculations were made and the cotton stoppers pushed down a short distance into the tube and then the tubes were tightly sealed by inserting rubber stoppers. Upon incubation, a pellicle of approximately the same thickness formed on the surface of the media in every tube. The medium in all tubes was more alkaline at the termination of incubation than when inoculated. Again, no organic acids could be demonstrated.

Thus, if organic acids of any type are formed as intermediary products in the carbohydrate metabolism of these organisms they do not accumulate in the media. Also, there is no evidence of any ability on the part of these organisms to derive any energy from carbohydrates in the absence of molecular oxygen. In other words there can be no partial cleavage of carbohydrates by these organisms; apparently they oxidize the carbohydrate molecule

completely or not at all. The end result of carbohydrate utilization in animal organisms is carbon dioxide and water but if animal tissues are subjected to anaerobic conditions there is an accumulation of lactic acid. In the latter case molecular oxygen is, thus, not necessary for the primary cleavage of the carbohydrate. The author knows of no other example in animal or plant physiology where energy is derived from carbohydrates only in the presence of molecular oxygen.

IX. DISCUSSION

That the organisms belonging to the genus *Mycobacterium* do utilize some carbohydrates is clearly demonstrated by the present work. The demonstration of this fact, however, which was the object in view at the beginning of this investigation, is undoubtedly not the only significant aspect of this investigation. New light is thrown on many aspects of the metabolism of this group of organisms and possibly on the metabolism of other bacteria. Combinations of old methods have been used and some new methods have been introduced which are valuable in establishing general relationships in bacterial cultures. These perhaps may be of value if extended to a study of other groups of organisms.

The organisms of the genus *Mycobacterium* apparently have a singularly characteristic type of metabolism. It is due to this fact that some of the fundamental principles governing the carbohydrate metabolism of these organisms have been entirely overlooked. We have probably become too accustomed to considering acid production from carbohydrates as the criterion of fermentation or utilization. The more readily demonstrable type of utilization is of course such a type of incomplete cleavage of the carbohydrate. Here, the reaction progresses rapidly and comes to completion in the course of a few hours with inhibition of further growth of the bacteria, apparently because of accumulation of the cleavage products. In this type of utilization a large amount of carbohydrate is acted upon in a short time with the liberation of a relatively small amount of energy. For example, in the conversion of one gram molecule of glucose to lac-

tic acid approximately 19 calories of heat would be liberated (calculation from the heats of combustion given in Handbook of Chemistry and Physics, Ninth Edition, Hodgman and Lange). On the other hand, if the same amount of glucose were oxidized completely to carbon dioxide and water, approximately 670 calories of heat would be liberated. This is illustrated in the following equations:

Approximately 35 times as much glucose would have to be attacked by the former method of utilization as by the latter method for the obtaining of the same amount of energy. Considered from this standpoint the explanation of the greater rapidity with which the former type progresses becomes clear. It is logical to expect that in the case of those organisms that oxidize the carbohydrate completely, as the Mycobacteria apparently do, there might be a relatively slow, though progressive decrease in the carbohydrate content of the media with a considerable production of energy. This is what has actually been found to occur.

Another significant factor is the rôle played by the per cent of the substrate present in the media. It was clearly pointed out by Kendall (1921) several years ago that carbohydrates exert a protein sparing action. The fact that this action is increased by increasing the concentration of the carbohydrate in the media does not seem to have been previously demonstrated. Possibly this phenomenon is encountered only in the case of the Mycobacteria but it seems possible that, if it occurs in this case, it may occur in other instances. The question of the concentration of the substrate is unquestionably of importance in certain industrial processes dependent upon bacterial action, as for example the manufacture of vinegar (Allen, 1926). If, in this process, the alcohol be allowed to fall below 2 per cent concentration the acetic acid bacteria begin oxidizing the acetic acid which they had previously been producing. The rôle played by the concentration of the substrate, whether it be the original substrate or a cleavage product which under proper conditions the organ-

isms can further oxidize, is a field that has been little investigated and that is rich in possibilities.

In view of the fact that the organisms of this genus have been found to be so closely related in their metabolic activities through the present study and previous studies reported by other workers it seems reasonable to expect that the respiratory relationships found for the tubercle bacillus by Novy and Soule are typical for the entire group. The present investigation, then, aside from demonstrating actual utilization of carbohydrates by the Mycobacteria has gone further in an attempt to correlate the respiratory relationships as established by Novy and Soule with various quantitative biochemical changes occurring within the media.

It is interesting that no inhibition to the growth of the Mycobacteria was noted in this work when the organisms were grown over barium hydroxide. According to the results of Rockwell (1921) such inhibition might be expected to be encountered due to the diminished carbon dioxide tension. The author's observations coincide more nearly with those of Novy and Soule (1928) who found no apparent inhibition to growth upon removal of carbon dioxide as completely as possible.

X. SUMMARY

1. The method of determining carbohydrate utilization by noting the appearance of acid in the media, which is the method generally used for such bacteriological determinations, is not applicable to the determination of utilization of carbohydrates by organisms of the genus *Mycobacterium*.

2. The determination of the reaction curves, using a modification of the method of Long and Major, is an indefinite and impracticable method of determining carbohydrate utilization by organisms of this genus.

3. The reaction curves produced when *Myco. leprae* is grown in mannitol broth, when *Myco. chelonae* is grown in glucose broth, and when *Myco. phlei* is grown in mannitol broth were possibly characteristic enough to be of value as methods of differentiating the strains of these organisms used in this work from each other and from the remaining members of the genus. The reaction

changes are, however, influenced to such a profound degree by slight environmental changes that even these atypical curves are considered too indefinite to be of absolute differential value.

4. The reaction changes in plain broth and carbohydrate broth that typify the organisms of this genus lead toward progressive increase in alkalinity. The reaction change is less rapid in the presence of utilizable carbohydrates.

5. There is a rather wide utilization of carbohydrates by the organisms studied, as demonstrated by direct quantitative carbohydrate determinations. The conclusions as to the carbohydrates utilized are given in table 2.

6. The increase in alkalinity, whether in plain broth or carbohydrate broth cultures, is associated with an increase in the ammonia content of the media. This increase in ammonia in most cases is approximately equivalent to the increase in the titratable alkalinity. In other cases, other substances, either acid or alkaline in character, which are apparently derived from protein cleavage, are involved to some extent in the causation of the reaction changes and the changes in titratable alkalinity. Such changes are accounted for by the ammonia increase, equally as well in the carbohydrate broth cultures as in the plain broth cultures.

7. The ammonia production was distinctly less for the production of a given amount of growth in the presence of utilizable carbohydrates. This diminished ammonia production was associated with a decrease in the degree of reaction change of the media. Also, the higher the carbohydrate concentration, so long as it is within the limits that will permit growth, the less the ammonia increase and the less the reaction change of the media during the production of a constant amount of growth. Thus, not only the presence but also the concentration of a carbohydrate is a factor determining the degree of protein sparing action it exerts.

8. The ammonia content of the media varied directly as the pH, the ammonia contained in the media being approximately the same at any given pH for the same organism in carbohydrate-containing as in carbohydrate-free media. However, there was

no constancy found in the ammonia content of the media at any given pH when different organisms were grown in the same media.

9. The presence of utilizable carbohydrate does not have any direct affect upon the reaction change of the media. Utilizable carbohydrates apparently affect the reaction changes only to the extent that they alter protein cleavage by the organism. Thus, there is no accumulation of cleavage products from the carbohydrate that in any way alter the pH of the medium. This contrasts strikingly with the carbohydrate utilization by most bacteria belonging to other genera.

10. The utilization of carbohydrates by the organisms of the group studied is characterized by a gradual decrease in the carbohydrate contained in the media until it completely disappears. There is, thus, no inhibition to the growth of the organisms at any time by products of the carbohydrate cleavage such as is the case in the majority of organisms the carbohydrate utilization of which have been studied.

11. The carbon dioxide produced has been shown to vary directly with the growth of the organisms, in carbohydrate-containing as well as carbohydrate-free media. The carbon dioxide produced also varied directly with the carbohydrate utilized in the carbohydrate-containing media, and in most cases more carbon dioxide was recovered than could have been derived from the carbohydrate disappearing. This may be compared to a 10 to 18 per cent yield of possible carbon dioxide recovered from cultures of acid-producing organisms each as *B. coli* and *Staphylococcus aureus*. Far more carbon dioxide was found to be liberated per milligram of ammonia produced in the presence, than in the absence, of utilizable carbohydrates.

12. No inhibition to the growth of these organisms was noted when the cultures were placed in jars containing barium hydroxide. Thus, if removal of carbon dioxide does exert inhibition to growth such removal was by the present methods not complete enough to exert any effect.

13. The organisms could not be grown under anaerobic conditions. Cultures grown in limited oxygen supply grew until all the oxygen was used and, thereafter, no further growth or carbo-

hydrate utilization or reaction changes in the media were demonstrable, even after two months' additional incubation. Carbohydrate utilization, thus, takes place only in the presence of molecular oxygen.

14. No acid cleavage products of the carbohydrate utilization could be demonstrated either in media of varying concentrations of carbohydrate or in the presence of varying quantities of available oxygen.

15. The explanation suggested in the preliminary report, namely that if the carbohydrate molecule is attacked at all it is oxidized completely to carbon dioxide and water, without the accumulation of demonstrable amounts of any intermediate products in the media, explains all the phenomena observed relative to carbohydrate utilization by these organisms.

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