

A STUDY OF ACID-PRODUCTION BY DIPHTHERIA BACILLI *

EMILY L. MOSHAGE AND JOHN A. KOLMER

From the Laboratory of the Philadelphia Hospital for Contagious Diseases, Philadelphia

While investigators are in general accord regarding the power of virulent diphtheria bacilli to split dextrose with the formation of organic acids, there is considerable difference of opinion regarding the action of these bacilli on other carbohydrates. It is generally agreed that avirulent bacilli are less able than virulent bacilli to produce acids from carbohydrates or even lack this power entirely, and various observers have therefore advocated fermentation tests for the differentiation of various diphtheria-like bacilli from one another and from the true diphtheria bacillus.

HISTORICAL REVIEW

The earliest work on this subject consisted in the titration of cultures for acidity, the cultures having been grown in broth of known titer for varying periods of time. In this manner Biggs, Park, and Beebe¹ early observed and drew attention to the varying powers of diphtheria bacilli in the production of acid in dextrose broth, and differentiated between true and pseudodiphtheria bacilli according to their virulence for the guinea-pig and their power of acid-production in this medium. This power of true diphtheria bacilli to produce acids from dextrose was likewise observed by Theobald Smith,² who drew attention to the absence of acid-production in broths containing the disaccharids lactose and saccharose. L. Martin³ reported that true diphtheria bacilli were able to split dextrose, galactose, levulose, and to a lesser degree saccharose and glycerin; he observed negative results with lactose, maltose, raffinose, arabinose, dulcitol, and mannitol.

Shortly thereafter several investigators undertook to differentiate between virulent and nonvirulent diphtheria bacilli and the pseudobacillus of Hofmann-Wellenhof by means of tests for acidity in broth containing dextrose and other carbohydrates. While, as previously stated, practically all investigators have found that the true diphtheria bacillus produces relatively large amounts of acids from dextrose, there is not by any means a uniformity of opinion regarding the value of this test in differentiating among the various kinds of diphtheria bacilli. The investigations of Peters,⁴ Kurth,⁵ Schmitz,⁶ and others

* Received for publication December 10, 1915.

¹ Centralbl. f. Bakteriologie, 1895, 17, p. 765.

² Tr. Assn. Am. Phys., 1896, 11, p. 37.

³ Ann. de l'Inst. Pasteur, 1898, 12, p. 26.

⁴ Deutsch. med. Wchnschr., 1897, 23, p. 133.

⁵ Ztschr. f. Hyg. u. Infektionskrankh., 1898, 28, p. 409.

⁶ Ibid., 1913, 75, p. 513.

have led them to minimize the value of these acid-production tests in differentiating among the members of the group of diphtheria bacilli, while Bronstein and Grünblatt,⁷ Riemsdijk,⁸ and others regard acid-production from glucose an absolute criterion of differentiation among diphtheria and pseudodiphtheria bacilli.

With the advent of the serum-water medium of Hiss containing 1% of carbohydrate with litmus as an indicator, a means was afforded for applying these acid-production tests with a number of carbohydrates on a large scale and in a manner much simpler than that of the titration method. Using this medium, Knapp⁹ studied 27 cultures of diphtheria-like bacilli and reported very favorably upon it as a means of differentiating *Bacillus hofmanni* and *Bacillus xerosis* from the true diphtheria bacillus. Similar results were reported by Zinsser.¹⁰ Hamilton and Horton¹¹ were unable to confirm Knapp's results, but found that the true diphtheria bacillus always produced acid in dextrin broth.

Graham-Smith,¹² using the Hiss medium and a number of different carbohydrates, studied 39 races of diphtheria bacilli, and found marked acid-production with glucose, levulose, galactose, maltose, and dextrin; variable results with lactose and saccharose; and no acid at all with mannite. Differences were found between the series grown on plain broth plus the carbohydrate and that grown on the same carbohydrate in Hiss' medium, probably due to the fact that some of the bacilli grew poorly on the former but well on the latter.

Kolmer,¹³ in a study of acid-production with 72 cultures that had been tested for virulence by guinea-pig inoculation, found that virulent bacilli produced acid most frequently with dextrose, and next most frequently with dextrin. With the remaining sugars the results varied. Cultures proving nonvirulent that produced acid with some of the sugars, especially dextrose and dextrin, were regarded as nonvirulent diphtheria bacilli. The sugar tests were found of most practical value in dealing with the solid types so frequently found in normal noses and throats.

More recently Hine¹⁴ has advocated these acid-production tests as a means of differentiation, and on this basis he divides diphtheria bacilli into 3 groups. The 1st group, comprising the true Klebs-Loeffler bacilli, produces acid with dextrose, maltose, and dextrin, and generally with lactose, but not with saccharose; the 2nd group, comprising bacilli morphologically indistinguishable from the true bacillus and usually observed in cultures from the skin and adjacent mucous membranes, produces acid with glucose and saccharose, and frequently with maltose and lactose, but not with dextrin; the bacilli of the 3rd group produce acid with dextrose, saccharose, maltose, and dextrin, but not with lactose. He reports that he has never found any but true virulent diphtheria bacilli to produce acid with dextrose and dextrin but not with saccharose, and he advocates these tests as a practical means of differentiation superior to the guinea-pig-inoculation test. *Bacillus hofmanni* in all these investigations was found unable to produce acids with any of the sugars mentioned.

⁷ Centralbl. f. Bakteriöl., I, O., 1902, 32, p. 425.

⁸ Ibid., 1914, 75, p. 229.

⁹ Jour. Med. Research, 1904, 7, p. 475.

¹⁰ Ibid., 1907-1908, 17, p. 227.

¹¹ Jour. Infect. Dis., 1906, 3, p. 128.

¹² The Bacteriology of Diphtheria, 1908, p. 157.

¹³ Jour. Infect. Dis., 1912, 11, p. 56. Arch. Pediat., 1912, 29, p. 94.

¹⁴ Jour. Path. and Bacteriol., 1913-1914, 18, p. 75.

During the past 15 months, in connection with the routine animal-inoculation tests for virulence, we have studied a number of cultures from various sources, the object being (1) to note the acid-production with various carbohydrates by cultures of diphtheria bacilli from various sources, of varying morphology, and of known virulence as determined by animal-inoculation tests; and (2) to ascertain the practical value of these tests in aiding differentiation among the members of the diphtheria group.

MATERIALS AND METHODS OF STUDY

Cultures.—In all, 392 cultures of diphtheria bacilli have been studied. Of these, 233 were isolated from cultures of the throat; 83 from the nose; 58 from the ear in cases of suppurative otitis media; 12 from the skin; and 6 from the eye. All the cultures were from patients in the wards of the Philadelphia hospital for contagious diseases, most of them from persons suffering with diphtheria. All cultures had been tested for virulence by the guinea-pig-inoculation test. The technic employed and the results observed are given in separate papers dealing with that subject.¹⁵ General toxemia and local edema at the site of inoculation were accepted as evidences of virulence; absence of toxemia and local reaction over a period of 4 days were regarded as indicating absence of virulence. However, if the guinea-pig showed definite edema at the site of inoculation, even tho without the well-marked general toxemia and without succumbing by the 4th day, the result was interpreted as positive, that is, as indicating virulence of the bacilli.

The morphology of the bacilli was recorded according to Wesbrook's classification. While cultures of granular or solid types of the bacilli tend to preserve their group characteristics, we have found so many variables in a culture of a granular type of bacillus, that no attempt has been made to classify the sugar reactions, according to the subvarieties, as bacilli of the A, B, C, D, etc., types. Instead, all granular types are listed under the one heading "granular," as we have included only the common varieties. The solid varieties were divided into 2 main types; namely, the long solid bacilli corresponding to the C₂ type of Wesbrook, and the short solid bacilli corresponding to the D₂ types.

Technic.—Hiss's serum-water medium, containing 1% of sugar and colored with litmus or azolitmin, was employed in most of this work.

Cultures of bacilli were grown in plain or glucose broth for 3 days before transplantation to the Hiss medium. In this manner all cultures were trained to grow in a fluid medium before subculturing—a matter of importance especially in relation to the rapidity, and to some extent the degree, of acid-production and coagulation in the Hiss medium.

A number of sugars were used with each culture. All cultures were grown for 4 days at 37 C. and the results recorded according to acid-production and coagulation. With many of the cultures the results were recorded each day for 4 days. When changes occurred in the medium, the culture was examined for purity; this was found to be absolutely necessary in guarding against occasional contamination.

¹⁵ Kolmer and Moshage: Jour. Infect. Dis., 1916, 19, p. 1. Kolmer, Woody, and Moshage: Am. Jour. Dis. Child., 1916, 11, p. 257.

The following carbohydrates were used:

Monosaccharids	{	Dextrose	Disaccharids	{	Maltose
		Levulose			Lactose
		Galactose			Saccharose
Trisaccharids	{	Raffinose	Polysaccharids	{	Dextrin
		Arabinose			Alcohol mannite

RESULTS

Acid-Production in Hiss Serum-Water Media.—The results obtained are summarized in Tables 1 and 2 according to the type of bacillus in the culture and the results of guinea-pig-inoculation tests for virulence.

TABLE 1
ACID-PRODUCTION IN HISS SERUM-WATER MEDIA

Sugars	Virulent Cultures				Nonvirulent Cultures			
	Total	Number Producing Acid	No Change	Percentage Producing Acid	Total	Number Producing Acid	No Change	Percentage Producing Acid
ACID-PRODUCTION BY GRANULAR TYPES OF DIPHTHERIA BACILLI (MOSTLY TYPE C)								
Dextrose.....	70	68	2	97	31	29	2	94
Levulose.....	43	38	5	90	7	6	1	86
Galactose.....	64	58	6	91	20	18	2	85
Maltose.....	55	28	27	51	18	8	10	42
Lactose.....	64	20	44	31	14	4	10	29
Saccharose.....	73	28	45	40	43	14	29	32
Raffinose.....	40	8	32	20	19	2	17	10
Arabinose.....	24	2	22	4	18	1	17	5
Dextrin.....	81	73	8	90	22	19	3	86
Mannite.....	77	0	77	0	47	0	47	0
ACID-PRODUCTION BY LONG SOLID TYPES OF DIPHTHERIA BACILLI								
Dextrose.....	48	47	1	98	87	83	4	95
Levulose.....	11	10	1	90	29	26	3	90
Galactose.....	60	49	11	82	115	93	22	81
Maltose.....	34	14	20	41	29	9	20	31
Lactose.....	62	30	32	48	118	42	76	35
Saccharose.....	54	4	50	7	112	7	105	6
Raffinose.....	18	2	16	11	78	6	72	7
Arabinose.....	18	1	17	5	15	0	15	0
Dextrin.....	75	37	38	49	138	54	86	40
Mannite.....	74	0	74	0	140	0	140	0
ACID-PRODUCTION BY SHORT SOLID TYPES OF DIPHTHERIA BACILLI (MOSTLY D ₂)								
Dextrose.....	30				2			
Levulose.....	30				0			
Galactose.....	31				1			
Maltose.....	30				1			
Lactose.....	30				0			
Saccharose.....	27				0			
Raffinose.....	30				0			
Arabinose.....	12				0			
Dextrin.....	27				0			
Mannite.....	30				0			

TABLE 2

SUMMARY OF THE PERCENTAGES OF CULTURES OF THE VARIOUS TYPES OF DIPHTHERIA BACILLI WHICH PRODUCED ACID

Sugars	Granular Types		Long Solid Types		Short Solid Types
	Virulent	Nonvirulent	Virulent	Nonvirulent	Nonvirulent
Dextrose.....	97	94	98	95	6
Levulose.....	90	86	90	90	0
Galactose.....	91	85	82	81	3
Maltose.....	51	42	41	31	3
Lactose.....	31	29	48	35	0
Saccharose.....	40	32	7	6	0
Raffinose.....	20	10	11	7	0
Arabinose.....	4	5	5	0	0
Dextrin.....	90	86	49	40	0
Mannite.....	0	0	0	0	0

Virulent diphtheria bacilli are more prone to ferment carbohydrates than are nonvirulent bacilli; this is generally true of all types independently of source and morphologic character. Acid-production by diphtheria bacilli therefore runs somewhat parallel with toxin-production; as shown in the tables, however, it is commonly observed that a culture nonvirulent for guinea-pigs may be capable of splitting carbohydrates. Further study on the relationship of the carbohydrate-splitting ferments to the soluble toxins of diphtheria bacilli is given in another communication.¹⁷

Acid-production generally was found most frequently with dextrose, levulose, galactose, and dextrin.

The granular or beaded types of bacilli generally yielded slightly higher percentages of acid-production than did the long solid types.

The majority of the cultures of the short solid thick D_2 types of bacilli failed to produce acids with any of the carbohydrates used in this study. Bacilli of this type resemble the Hoffmann-Wellenhof bacillus rather closely in morphologic and biologic characters. The typical pseudodiphtheria, or Hofmann, bacillus does not ferment any sugar, and for this reason we have found acid-production tests of most value in identifying this bacillus. A bacillus of this morphology, fermenting dextrose and proving nonvirulent for the guinea-pig, we regard as a true nonvirulent diphtheria bacillus.

Cultures of virulent bacilli of the granular or beaded types show most constant and vigorous acid-production; the short solid nonvirulent races show little or no tendency toward acid-production. Between these extremes is a group of bacilli with valuable fermentative powers.

¹⁷ Kolmer and Moshage: Jour. Infect. Dis., 1916, 19, p. 28.

Considerable interest is attached to the fermentation of saccharose; in our experience about 36% of cultures of granular bacilli and 6% of the long solid types of bacilli produced acids with this sugar. This property is by no means confined to cultures of diphtheria-like bacilli isolated from the conjunctiva (*B. xerosis*), so that, contrary to certain statements, the use of this sugar proved to be of little value in differentiating between true diphtheria bacilli and *B. xerosis* on the basis that the former is unable to cleave saccharose while the latter possesses this property.

In common with other observers we have not encountered cultures of diphtheria bacilli capable of definitely cleaving mannite. Occasionally acid has been produced, but further study has invariably shown the culture to be contaminated. With prolonged incubation a small percentage of cultures turn the culture medium a reddish tint, but evidences of definite acid-production and coagulation in the Hiss medium have not been observed.

TABLE 3
RAPIDITY OF ACID-PRODUCTION BY DIPHTHERIA BACILLI WITH VARIOUS CARBOHYDRATES

Sugars	Total	Percentage of Acid Produced During a Period of 4 Days			
		1st Day	2d Day	3d Day	4th Day
Dextrose.....	131	73	27
Levulose.....	78	63	29	8	..
Galactose.....	150	62	26	10	2
Maltose.....	92	46	40	14	..
Lactose.....	79	35	60	3	2
Saccharose.....	85	62	25	10	3
Raffinose.....	48	80	20
Arabinose.....	10	35	60	5	..
Dextrin.....	131	65	30	5	..
Mannite.....	82	0	0	0	0

Rapidity of Acid-Production.—As shown in Table 4, the majority of cultures produced sufficient acid definitely to affect litmus in the first 24 hours of incubation. Rapidity of acid-production is probably parallel to some extent with rapidity of growth and multiplication of the bacilli; the growth of our cultures in a fluid medium before transference to the Hiss medium influenced the rate of acid-production during the first day or two. While cultivation of the bacilli for periods of time exceeding 4 days may yield higher percentages of positive results, in our experience cultures capable of splitting a carbohydrate in the Hiss medium show this property within 4 days in the majority of instances.

Rapidity of acid-production, as shown in Table 3, was most apparent in media containing dextrose, levulose, galactose, and dextrin, these 4 carbohydrates being those which diphtheria bacilli are most able to cleave. Cultures failing to produce appreciable amounts of acid with these sugars in 24 hours usually do so within 48 hours and more certainly within 4 days.

These facts were further shown in a series of titrations for total acidity of cultures in neutral broths each containing 1% of one of the various carbohydrates. The results of titrations with two cultures are shown in Table 4.

TABLE 4
RAPIDITY OF ACID-PRODUCTION BY DIPHTHERIA BACILLUS IN CARBOHYDRATE BROTHS

	Sugars	Results of Titrations for Acidity During a Period of 14 Days after Inoculation													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
A culture of a granular type isolated from the throat of a convalescent from diphtheria; virulent for guinea-pigs	Dextrose...	8	12	14	16	16	17	16	16	14	16	18	18	20	21
	Levulose...	8	14	20	23	24	25	24	24	26	26	26	26	25	24
	Galactose...	6	15	16	19	20	22	20	20	19	20	20	20	20	20
	Lactose...	4	7	12	13	14	17	14	14	12	12	11	12	10	8
	Saccharose...	0.5	2	2	3	4	7	8	9	11	13	15	17	17	17
	Raffinose...	0.5	3	5	6	6	6	0	0	0	0	0	0	0	0
	Dextrin...	4	10	12	14	15	16	16	16	15	16	16	18	18	18
A culture of a long solid (C ₂) type isolated from the throat of a convalescent; virulent for guinea-pigs	Mannite....	0	0	0	Tr.	Tr.	Tr.	0	0	0	0	0	0	0	0
	Dextrose...	7	9	12	10	13	12	12	14	14	16	15	15	15	16
	Levulose...	7	8	10	11	12	12	12	13	14	16	15	15	15	14
	Galactose...	2	4	3	4	4	4	4	6	9	11	11	11	11	14
	Lactose...	2	3	6	7	6	7	8	8	8	10	10	11	12	12
	Saccharose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Raffinose...	0	Tr.	Tr.	0	0	0	0	0	0	0	0	0	0	0
	Dextrin...	2	6	10	12	11	11	13	14	14	16	15	15	15	14
	Mannite....	0	0	0	0	0	0	0	0	0	0	0	0	0	0

These titrations were made daily over a period of 2 weeks. All titrations were made with $n/20$ sodium hydroxid, with phenolphthalein as the indicator. Duplicate titrations were conducted at incubator temperature with unfiltered cultures without boiling, and again after boiling for 2 minutes. The results shown in the tables were those observed with the latter method, the end reaction being taken as the first definite pink which held after the second boiling for a minute or two. The titrations of unheated cultures (not boiled) showed uniformly a slightly higher acidity, but otherwise the results were closely parallel with the results shown in the table.

The highest degrees of acidity occurred with dextrose, levulose, galactose, and dextrin; acid-production also occurred in the lactose broths but more slowly; a few cultures produced acid with saccharose and raffinose while others did not (as shown in Table 4). While

traces of acid were at times discovered in the mannite broths, the majority of the cultures failed entirely to cleave this carbohydrate.

Acid-Production By Diphtheria Bacilli From Various Sources.—Diphtheria bacilli of the same morphology isolated from different parts of the body vary in their power to produce acids in carbohydrate media. These differences, however, are apparently largely dependent on the question of virulence, as already pointed out. Bacilli from the throat and nose are usually more virulent than those from the ear; those derived from the skin and adjacent mucous membranes, such as the conjunctiva and genitalia, are usually nonvirulent and poor acid-producers. The general rule, however, that granular types of bacilli are likely to be more virulent than the short varieties is likewise observed in acid-production tests, in which they are more likely to produce acid.

Cultures of diphtheria bacilli from the throat generally possess most marked fermentative powers; those from the nose and ear are less active and those from the eye and skin (including the genitalia), least active.

Coagulation of Hiss Serum Water.—Coagulation of the serum in Hiss's medium is frequently observed, the time of its occurrence and the degree of coagulation being dependent on the time of production and the amount of organic acids formed. For example, with the monosaccharids coagulation is frequently observed on the 2nd day and sometimes on the 1st day of incubation; with other less readily fermented sugars coagulation is delayed and may be absent on the 4th day, even tho some acid has been produced.

PRACTICAL VALUE OF ACID-PRODUCTION TESTS

In our experience acid-production tests are of limited value in the classification of diphtheria bacilli. The difficulty of securing pure carbohydrates for these tests constitutes a disturbing factor which may account in part for the irregularities and inconstant results. From the practical standpoint, these tests have generally proved successful in the identification and classification of the Hofmann bacillus on the basis that it does not produce appreciable quantities of acids with carbohydrates. For the purpose of differentiating between virulent and non-virulent bacilli, these tests have failed to prove of any value; the typical virulent diphtheria bacillus produces acids most characteristically with dextrose, dextrin, levulose, and galactose, and the false types, or Hof-

mann's bacilli, produce no acids at all; but between these extremes is a large group of bacilli of varying morphology and from different sources which present varying and inconstant degrees of virulence and fermentative power.

Considerable interest is attached to cultures of diphtheria-like bacilli from the conjunctivae. Such bacilli from the normal eye, which are practically always nonvirulent, are well known under the name of *B. xerosis*. These micro-organisms are capable of fermenting saccharose, and we have found them capable of attacking other sugars as well, including dextrin and dextrose; as previously stated, we have not found that fermentation of saccharose and absence of fermentation of dextrin by these bacilli suffice to differentiate *B. xerosis* from the true diphtheria.

CONCLUSIONS

Acid-production by diphtheria bacilli in Hiss's serum-water-carbohydrate media is most marked with dextrose, dextrin, levulose, and galactose.

A small percentage of cultures from the throat and nose also produce acid with saccharose.

Nonvirulent diphtheria-like bacilli from the conjunctiva are capable of fermenting not only saccharose but also other sugars as well, including dextrin and dextrose.

Granular types of bacilli generally produce acids more frequently than do the solid types, and long solid types more frequently than the short varieties.

In general, the time of appearance and degree of acid-production from carbohydrates are somewhat parallel with the degree of virulence of the diphtheria bacilli.

Acid-production tests on different carbohydrates are not sufficiently regular to be depended on in determining the harmfulness of a given culture.

Acid-production tests are of most value in the study and classification of the solid varieties of the diphtheria-like bacilli; cultures proving nonvirulent for guinea-pigs and producing no acid with sugars are classified as the pseudo, or Hofmann's types; nonvirulent cultures producing acid with one or more sugars are regarded as non-virulent diphtheria bacilli.