ELECTROPHORESIS OF DIPHTHERIA BACILLI

III. THE DEVELOPMENT OF A SIMPLIFIED TECHNIQUE FOR ELECTROPHORETIC MEASUREMENT OF THE VIRULENCE OF DIPHTHERIA BACILLI

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Received for publication December 24, 1927

Because of the somewhat complex form of the apparatus used in the electrophoresis measurements reported in the two preceding papers of this series, and the time and number of operations involved in growing and preparing cultures for the test, we undertook the task of attempting to develop reliable, simplified methods that might be utilizable in the public health laboratory.

Several types of depression slide cells have been devised. The first attempt with point platinum electrodes did not provide a suitable method because of polarization and electrolysis at the electrodes when broth media were used as suspending menstrua. Even though platinum black was deposited upon the electrodes, gas bubbles soon appeared and caused equivocal mobilities.

Another type of cell with non-polarizing electrodes was devised resembling the cell described by Taylor.[1] Although it is not practical for routine laboratory tests, uses for it may be found elsewhere.

The type of cell finally adopted is made as follows: Glass slides are cut into desired sizes and cemented to a thick glass slide forming a rectangular cell with grooves providing places for platinum electrodes. In figures 1 and 2 are shown the top and side views of the cell with dimensions and arrangement of apparatus.

[1] Taylor, C. V., Proc. Soc. Exper. Biol. and Med., 1925, **23**, 147. Cf. also Gelfan, S., ibid., 1926, **23**, 308.

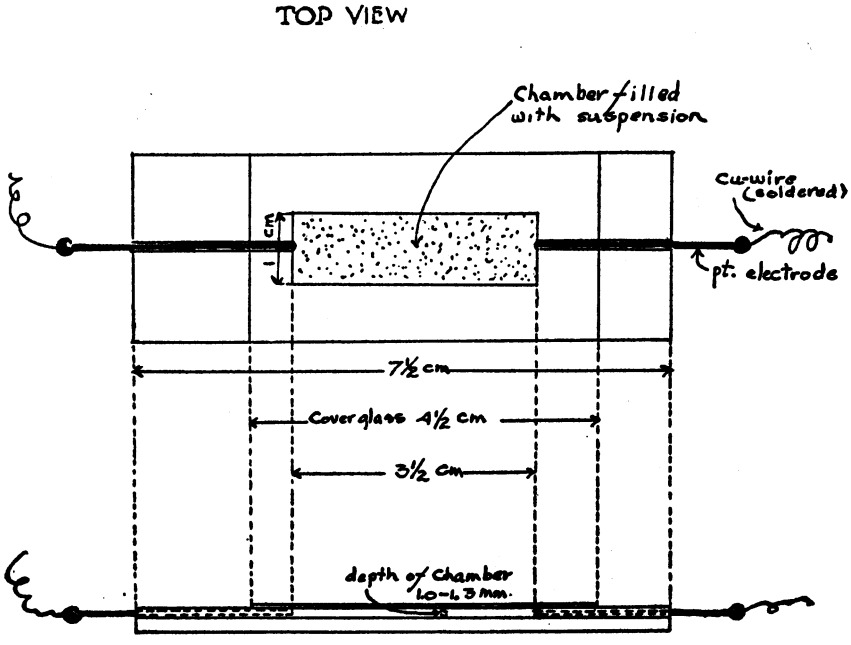

FIG. 1

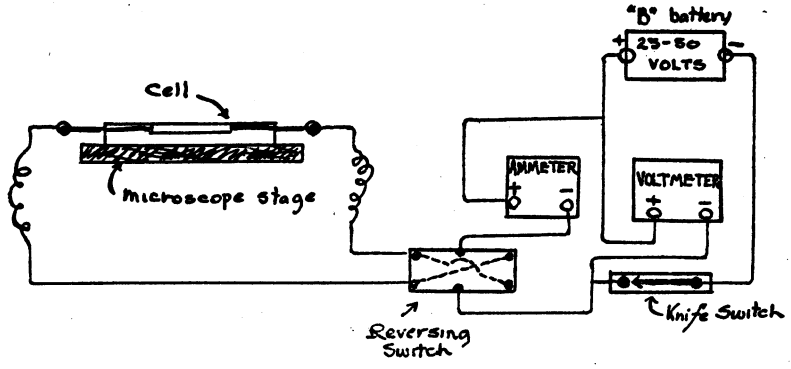

FIG. 2. WIRING DIAGRAM FOR THE SIMPLIFIED ELECTROPHORESIS CELL

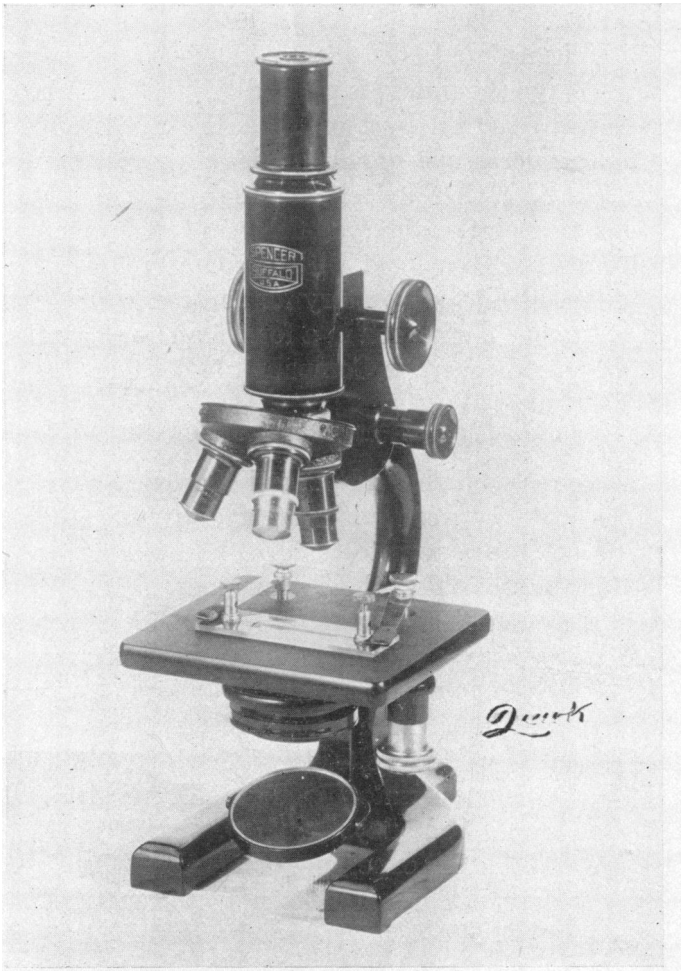

FIG. 3. IMPROVED FORM OF THE "SLIDE CELL" FOR SUSPENSIONS IN DISTILLED WATER

The platinum electrodes are cemented part way in the grooves with deKhotinsky cement, leaving 0.5 cm. or less of the end of the wire uncovered.

The cell is cleaned and filled with distilled water suspensions of bacteria. A large coverglass is pressed tightly[2] over the cell and excess water is removed by blotting. The whole slide is now mounted on a microscope provided with a compensating ocular (25×) with a Sedgwick–Rafter micrometer (each large square being 34 μ square on the observed field) and an 8 mm. objective. The electrodes are connected with a source of direct current. (We use a 45 volt dry cell battery for routine work.) In figure 3 we show the simple slide cell in a form improved by Dr. Klopsteg of the Central Scientific Company of Chicago.

PROOF OF TRUE ELECTROPHORESIS

Preliminary tests on this simple cell showed the usual differences in velocity between virulent and avirulent diphtheria bacilli observed in previous tests with the large apparatus. It was necessary to prove that these preliminary results were not a matter of chance and that the simplified cell showed true electrophoresis. This was tested by observing whether the velocity was proportional to the impressed voltage. Using a three-times-washed forty-eight-hour broth culture of *C. hoffmanni* with the source of potential at 114 volts, the following results were obtained. The culture (in water) was an old one that had been held in the ice chest several days (table 1).

It is apparent from this experiment and others that follow that the cell shows true cataphoresis as tested by this method.

After placing the cell in water overnight, washing in 50 per cent HCl and rinsing in distilled water, a similar test was conducted, but the forty-eight hour broth culture of *C. hoffmani* was used after one washing with water. As will be seen in table 2 remarkably true electrophoresis was again obtained.

[2] It was found that observed velocities varied greatly unless the coverglass was fitted tightly on the slide. This effect is due to the resulting change in the position of the V_s (stationary layer) from the calculated one, if the depth of the cell is increased or decreased.

TABLE 1

RESISTANCE	VOLTS	MILLIAMPERES	MOBILITY	MOBILITY
ohms			*μ/second*	*μ/second/volt*
0	114	8.0	77.3	0.678
6,200	80	5.8	49.3	0.616
18,900	50	3.5	32.4	0.648
42,400	30	2.0	20.2	0.674
Average				0.667

Ten measurements of velocity (5 with one and 5 with reversed orientation of the electrical field) were made at V_s 1262.

TABLE 2

IMPRESSED VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
30	12.1	0.247
50	18.9	0.264
80	29.5	0.271
100	37.8	0.264
114	44.7	0.255
Average		0.260

TABLE 3

Culture no. 111

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
114	48.2	0.422
100	40.0	0.400
80	29.9	0.374
60	22.2	0.370
50	17.1	0.342
40	13.6	0.340
30	9.0	0.300
20	6.23	0.312
13	4.8	0.369
Average		0.359

That these experiments were conducted with cultures that had been washed only once should be especially noted.

TABLE 4

Culture no. 85

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
114	72.2	0.633
100	64.7	0.647
80	54.7	0.683
60	38.2	0.637
40	26.3	0.665
30	20.4	0.680
20	12.9	0.644
13	8.35	0.643
Average		0.654

TABLE 5

Culture no. 86

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
100	82.4	0.824
80	63.8	0.798
50	37.0	0.739
40	27.9	0.697
30	24.4	0.813
20	11.7	0.585
13	7.9	0.607
Average		0.723

TABLE 6

Culture no. 110

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
114	66.3	0.582
100	64.2	0.642
80	48.6	0.608
50	28.7	0.574
30	17.0	0.567
13.5	7.9	0.585
Average		0.593

Once washed forty-eight-hour broth cultures of Nos. 111, 85, 86, 110, Park 8 and *C. hoffmanni* were each tested at various voltages. Tables 3, 4, 5, 6, 7 and 8 show the observed velocities in μ per second and μ per second per volt for these cultures of virulent and avirulent corynebacteria.

TABLE 7

Culture C. hoffmanni

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
114	69.3	0.607
100	68.7	0.687
80	54.4	0.680
60	39.8	0.663
40	24.3	0.607
30	18.4	0.613
13	8.1	0.623
Average		0.640

TABLE 8

Culture Park no. 8

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
100	43.5	0.435
60	22.0	0.367
40	12.8	0.322
30	8.2	0.366
20	6.0	0.334
13±	3.1	0.420
Average		0.374

It may be recalled that[3]

$$\text{P.D.} = \frac{4\pi\eta v}{KX} \qquad (1)$$

[3] This is the abbreviated form of the Helmholtz-Lamb equation (cf. Winslow, Falk and Caulfield: Jour. Gen. Physiol., 1923, **6**, 177). According to Debye and Hückel (Physik. Zeitschr., 1924, **25**, 49) instead of 4π, for spherical particles the formula should include 6π. With this correction, P.D. as a ζ (zeta) potential becomes equal to P.D. as the θ potential deduced from the Stoke's law (cf. Harkins: Atoms, Ions, Salts and Surfaces, in The Newer Knowledge of Bacteriology and Immunology, edited by Jordan and Falk, Chicago, 1928).

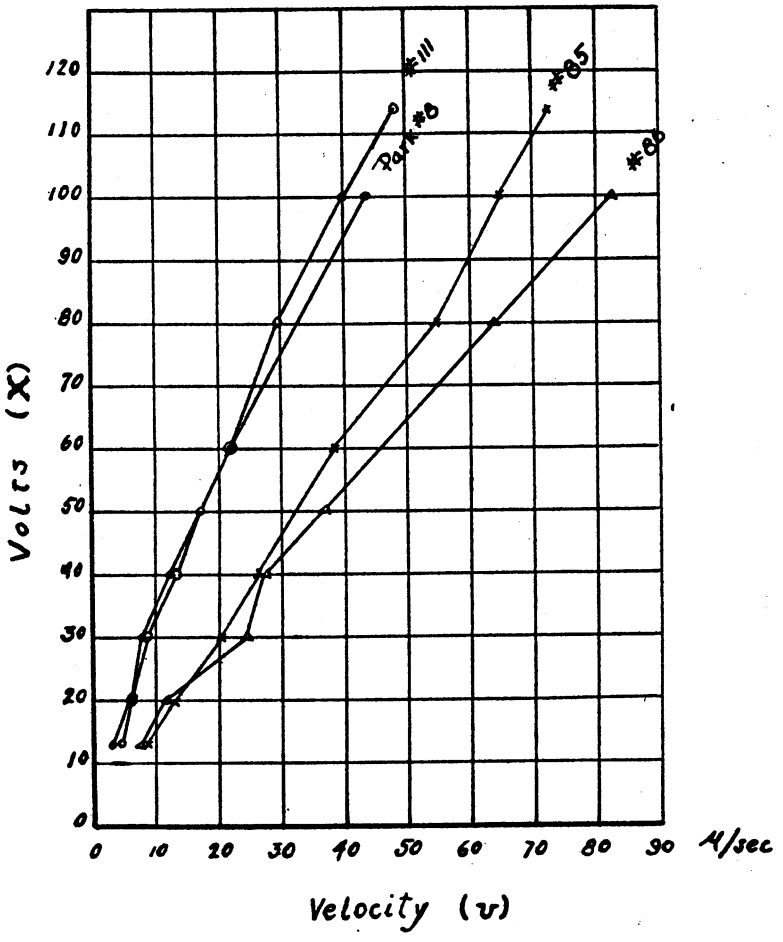

FIG. 4

For suspensions of a given nature in a particular menstruum

$$\text{P.D.} = k \cdot \frac{v}{X} \tag{2}$$

where k is a constant $\left(=\frac{4\pi\eta}{K}\right)$, v is electrophoretic velocity in centimeters per second, X is the impressed voltage in c. g. s. units.

It is obvious from equation (2) for any given suspension (P.D. and k being independent of v and of X and being constant) that:

$$v \propto X \tag{3}$$

Hence, plotting mobility against voltage for a given washed culture a straight line should be obtained if true electrophoresis is observed. Figure 4 shows graphically the results of some of the experiments listed in tables 3 to 8.

TABLE 9

110 volts—4 ocular units (circa 140μ)

CULTURE	MEAN MOBILITIES			VIRULENCE OR TOXIGENICITY (OLD TESTS)
	Seconds/140μ	μ/second/110 volts	μ/second/volt	
Park no. 8	3.4	41.2	0.375	+
No. 85	2.8	50.0	0.455	+
Hoffmanni	2.2	63.6	0.578	−
No. 111	1.9	73.7	0.670	−

TABLE 10

Three-times-washed forty-eight-hour broth cultures

CULTURE	MOBILITY		VIRULENCE
	μ/second/25 volts	μ/second/volt	
Park no. 8	2.6	0.104	+
No. 85	4.1	0.164	+
Hoffmanni	5.0	0.200	−
No. 89	6.6	0.264	−
No. 103	7.2	0.288	−

Measurements were made on once-washed suspensions from four-day Witte's peptone veal broth. The whole broth cultures had been kept in the ice chest for forty-eight-hours. The results of these readings are shown in tables 9 and 10.

It is apparent from tables 9 and 10 that electrophoresis in this simplified cell affords a method for determination of virulence or toxigenicity of diphtheria bacilli.

A larger series of cultures was tested after forty-eight hour

growth in 2 per cent Witte's peptone veal broth to which 0.1 per cent glucose had been added. The bacilli were thrown out of the broth and washed three times in distilled water. It will be observed in this experiment (table 11) that cultures nos. 110 and 109 occupy anomalous positions. Subsequent tests revealed that culture no. 109 shows toxigenic powers only after seven days' growth in broth. We believe, however, that most of the anomalous velocities which we observed in these and other preliminary tests were due in part to the inaccurate position of the V_s (stationary layer) caused by not pressing the coverglass tightly on the cell. Subsequent experiments were performed (*a*) with the cover-

TABLE 11

STRAIN	MOBILITIES				AVERAGE	VIRULENCE (OLD TESTS)
	25 volts	50 volts	25 volts	50 volts		
	μ/second	*μ/second*	*μ/second/volt*	*μ/second/volt*		
No. 102	10.5	22.2	0.420	0.444	0.432	+
Park no. 8	10.7	23.3	0.427	0.466	0.447	+
No. 110	11.5	23.1	0.460	0.462	0.461	−
No. 85	11.7	23.3	0.468	0.466	0.467	+
No. 93	13.8	29.1	0.552	0.582	0.568	−
No. 109	19.1	42.2	0.764	0.844	0.804	+ (?)
Hoffmanni	20.4	43.6	0.816	0.872	0.844	−
No. 111	42.4	71.6	1.300	1.430	1.370	−

glass resting on a heavy cushion of water, (b) with the coverglass too tightly pressed so that the glass was slightly warped, (*c*) pressed gently with four points of pressure and surplus water blotted away; all showed wide differences in velocity at the same impressed voltage with the same culture. The true mobility is observed under condition (*c*).

An experiment with a large series of strains is recorded in table 12 to show anomalous potentials observed before we were aware of errors resulting from not properly adjusting the coverglass on the cell. The three-times-washed suspensions were obtained from forty-eight-hour 2 per cent Witte's peptone veal broth cultures and from eighteen hour growths on glycerol agar slants.

Following the P.D. determinations, toxigenicity tests with the clear supernatant broth were made in guinea pigs, using 3.0,

TABLE 12

CULTURE	P.D.		TOXIGENICITY TESTS				VIRULENCE	P.D. AGAR CULTURE
			3 cc.	1 cc.	0.5 cc.	0.1 cc.		
			Death in days					
	μ/second/25 volts	*μ/second/volt*	*days*	*days*	*days*	*days*		*μ/second/25 volts*
No. 109	7.9	0.32	½	1	1	3	+	15.9
No. 86	8.0	0.32	1½	1½	1½		+	10.7
Park no. 8	11.6	0.46	3	3			+	7.3
No. 93	13.2	0.53					−	17.3
No. 89	14.2	0.57					−	17.0
No. 110	16.2	0.65					−	10.5
No. 88	17.0	0.68					−	15.4
No. 85	17.6	0.70	3				+	11.1
No. 105	19.0	0.76					−	
No. 111	19.0	0.76					−	11.3
No. 95	20.5	0.82					−	13.7
No. 104	21.3	0.85	1½	3		3	+	
Hoffmanni	22.5	0.90					−	
No. 103	23.0	0.92					−	11.8
No. 102	23.3	0.93	1	½	3		+	9.3
No. 92	23.6	0.94					−	15.7

TABLE 13

Retests of virulence of agar slant cultures (eighteen to twenty-four hours) were made with cultures nos. 85, 102 and 104 using antitoxin controls

CULTURES	SUBCUTANEOUS INJECTIONS			
	3 cc.	1 cc.	0.5 cc.	0.1 cc.
	Death in days			
	days	*days*		
No. 85	6	8		
No. 102	3	Sick		
No. 104	1¼	1¼		

1.0, 0.5 and 0.1 cc. quantities inoculated subcutaneously. The results are recorded in table 13.

It is apparent that these three cultures were virulent, but killed only those guinea pigs that received 1.0 or 3.0 cc.

A number of cultures obtained from the Chicago Department of Health were tested after two and seven days' incubation. These strains were grown in 2 per cent Witte's peptone veal broth and washed three times in distilled water before each test. The pH of the two-day suspensions was determined colorimetrically. The two-day suspensions had been held in the ice-chest two days after washing. Table 14 shows the results of these tests.

TABLE 14

CULTURE	2-DAY CULTURE		7-DAY CULTURE		2 CC. OF 48 HOURS BROTH SUBCUTANEOUSLY, VIRULENCE
	μ/second/25 volts	pH	μ/second/25 volts	μ/second/ volt	
No. 102	4.6	6.2	6.2	0.249	+
No. 95	5.0	6.2			+*
No. 85	6.5	6.2	6.4	0.256	+
No. 104	6.8	6.4	4.5	0.179	+
Park no. 8	7.1	6.6	5.9	0.236	+
No. 109	7.8	6.0	6.2	0.270	+
No. 86	7.9	6.6	8.4	0.337	+
No. 110	8.2	6.2	9.7	0.387	−
No. 88	8.5	6.4	8.7	0.347	−
No. 103	8.9	5.7	12.7	0.509	− (±)†
No. 93	9.7	6.4	9.1	0.365	−
No. 105	9.9	6.4	8.7	0.349	−
No. 89	10.8	6.0			−
Hoffmanni	14.2	6.6	11.8	0.470	−
No. 92	16.5	6.8	14.3	0.572	−

* Negative on first test. Positive on retest. In some experiments this culture is found to give a high velocity and is avirulent.

† This culture is sometimes slightly toxigenic.

This experiment shows that the acidity of the suspensions does not account for the velocities observed. The inverse relationship between electrophoretic mobility and virulence, i.e., high mobility and avirulence or non-toxigenic bacilli, and low mobility and toxigenicity or virulence is demonstrated in these tests. The dividing line between virulent and avirulent strains in this test is 7.9 to 8.2 for forty-eight-hour cultures and 8.4 to 8.7 for seven-day cultures. The strains giving the intermediate measurements

had been isolated from middle ear infections, scarlet fever and other unusual sources and conditions. They were purposely chosen to show the limitations or values of this method of testing virulence. Guinea pig tests for virulence and toxigenicity have not been more regular than electrophoresis with these intermediate strains: Cultures Nos. 88, 110 and 103 have been found to be slightly toxigenic in some tests when 3 cc. of seven-day broth cultures were injected into guinea pigs and at other times have been non-toxigenic under conditions supposedly similar.

TABLE 15

48-HOUR BROTH CULTURE	TIME FOR COMPLETE PRECIPITATION	VIRULENCE	P.D. (μ/SECOND/25 VOLTS)
	minutes		
No. 102	2	+	4.6
No. 95	2	+	5.0
No. 85	2	+	6.5
No. 104	20	+	6.8
No. 109	2	+	7.8
Park no. 8	2	+	7.1
No. 86	20	+	7.9
No. 110	20	−	8.2
No. 88	20	−	8.5
No. 103	20	±	8.9
No. 95	20	±	5.0
No. 92	20	−	16.5
Hoffmanni	More than 20	−	14.2

However, the electrophoretic mobility has varied in a correlated manner with these variations.

An experiment was designed to show the stability of suspension in the second change of distilled water and correlation with virulence and electrophoretic mobility. Table 15 indicates that there is a relationship between the time to precipitate the cells at 1500 r.p.m. and virulence with the concomitant mobility, although other factors undoubtedly enter.

Table 16 shows the results of testing seven-day, three-times-washed bacilli from broth cultures, making the P.D. measurements with the small apparatus.

Seven-day-old cultures of our collection are readily identified

by the electrophoresis method of determining the toxigenicity of corynebacteria. In a preceding paper it was shown that certain toxigenic strains did not produce demonstrable toxin (by guinea pig method) until three, four, and five or more days. Conversely, certain strains (culture No. 19 described as a type) produce their maximum toxigenicity after a week's growth. It was shown that

TABLE 16

CULTURE	μ/SECOND/25 VOLTS	TOXIGENICITY TESTS, 2 CC. SUBCUTANEOUSLY	VIRULENCE
No. 102	Motionless	Died 40 hours	+
Park no. 8	0.5	Died 18 hours	+
No. 109	1.8	Died 4 days	+
No. 85	Motionless	Died 72 hours	+
No. 105	2.2	Sick 18 hours, died 7 days	±
No. 95	4.6	Died 40 hours	−
No. 103	5.5	Survived*	±
No. 88	19.5	Survived	−
Hoffmanni	20.0	Survived	−

* Seven-day culture killed test animal in twenty-four hours in other tests.

TABLE 17

CULTURE	SMALL APPARATUS SUSPENSIONS	TOXIGENICITY TESTS, 2 CC. INJECTED SUBCUTANEOUSLY
	μ/second/25 volts	
No. 85	1.6	Died 1 day
No. 102	2.2	Died 1 day
Park no. 8	3.2	Died 18 hours
No. 95	3.3	Died 2 days
No. 109	(4.7) (6.8)	Died 4 days
No. 88	7.9	Survived
No. 105	11.0	Sick 18 hours—survived
Hoffmanni	19.0	
No. 103	21.2	

the mobility curve varied inversely with that of toxigenicity. Hence, it is possible that a few strains may escape identification by this method of measuring the mobility of three-times-washed bacilli suspended in distilled water. However, these equivocal results are less apt to be obtained when mobilities of bacilli in whole broth cultures are measured in the large apparatus. *It*

must be repeated that standard guinea pig tests for toxigenicity and virulence of borderline strains are no more accurate than these physical measurements.

Table 17 represents another experiment on three-times-washed bacilli as well as measurements on whole broth cultures. The bacilli were grown seven days in 2 per cent Witte's peptone veal broth to which 0.1 per cent glucose had been added. Toxigenicity tests were conducted with the clear supernatant broth obtained by centrifugating the whole broth cultures.

From numerous similar experiments it was plain that true electrophoresis measurements are obtainable by use of this simplified cell if the coverglass is fitted properly and three-times-washed cultures are used as test suspensions.

The data show that toxigenic diphtheria bacilli may be identified by the method.

IDENTIFICATION OF UNKNOWN DIPHTHERIA CULTURES FOR THE CHICAGO DEPARTMENT OF HEALTH BY USE OF THE SIMPLE ELECTROPHORESIS CELL (P.D. OF WASHED SUSPENSIONS)

It has been shown that there were significant differences in electrophoretic mobilities between toxigenic and non-toxigenic corynebacteria. These potential differences were found when measurements were made on diphtheria bacilli in the menstrua in which they were grown or on three-times-washed bacilli suspended in distilled water. It was found that cultures tested in this way must be incubated at least forty-eight hours before unequivocal results may be obtained. This is also the case when measurements are made on distilled water suspensions of bacilli. In some instances a culture did not show low mobility until after a seven-day incubation period.

The work reported in this section is an actual demonstration of the reliability of this method for identifying diphtheria bacilli in the Department of Health laboratory. One hundred and fifty-three cultures of corynebacteria were obtained from forty-nine sources during an outbreak of diphtheria in a Chicago Public School. The virulence of each of these strains was determined

by the Bureau of Laboratories and Research of the Chicago Department of Health, using the guinea pig test for virulence. At the same time transplants of these cultures were given to us. The city laboratory findings were unknown to us until after the completion of the electrophoresis experiments.

Our cultures were grown in veal infusion, 2 per cent Witte peptone broth, pH 7.2, to which 0.1 per cent glucose had been added. Approximately 50 cc. of broth were placed in 250 cc. flat bottom flasks and sterilized. After two- and seven-day incubation periods at 37°C. about 10 cc. of the whole broth culture were centrifugated at 2000 r.p.m. for several minutes and the sediment of bacilli resuspended in distilled water and washed three times with distilled water. Mobilities were then determined in the simplified cell, using 48 volts D.C.

Table 18 shows P.D. observed after two- and seven-day incubation periods and the reports from the Chicago Department of Health. It will be observed that our physical measurements detected 100 per cent of the "*C. hoffmanni*" strains, and 100 per cent of the "pleomorphic granular short bacilli." Two cultures of "multigranular long bacilli" were called virulent by the electrophoresis method but were found to be avirulent by the Chicago Department of Health guinea pig tests. We were 94.4 per cent accurate in detecting "typical *C. diphtheriae*." We called culture 7118B avirulent. Upon retesting by the animal virulence test this strain proved to be virulent. With this one exception, our errors were on the side of safety because we called those strains virulent which were found to be avirulent on retests by the Department of Health.

At this point the characteristics of the slide cell method may be recapitulated:

1. The apparatus is simple.
2. The electrophoretic measurement is made quickly and easily.
3. It demands the use of a small quantity of three-times-washed suspensions from broth cultures at least forty-eight hours old.
4. Unless older cultures are used, strains which in culture pro-

TABLE 18

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O. P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7537–2	–	–	20.0	21.2
–4	–	–	13.6	22.6
–5	–	–	19.3	20.0
7494–1	+	+	14.8	4.8
–2	+	+	19.3	7.6
–4	+	+	6.1	10.6
–5	+	+	11.3	11.3
7593	–	–	19.3	13.6
7579–2	–	–	18.7	19.3
–5	–	–	28.3	12.3
7470–4	–	–	22.6	17.0
7578	+	+	(4.2) 6.8	8.5
7118–1	+	+	4.0	13.8
–2 A	+	+	7.4	9.7
–3	+	+	6.8	17.0
–4	+	–	19.3	18.8
–5 B	+	–	19.3	17.0
7156–1	+	+	10.0	9.7
–2	+	+	16.0	9.1
–3	+	+	11.3	5.6
–4	+	+	13.6	6.6
–5	+	+	13.6	7.6
7180–5	–	–	20.0	17.0
7110–1	+	+	6.8	9.1
–3	+	+	10.9	10.9
–4	+	+	6.8	11.3
–5	+	+	19.3	8.5

* If a mobility measurement as low as (or lower than) 11.3 appeared, the culture was called "virulent."

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O. P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7264–1	+	+	5.7	8.5
–2	+	+	4.3	12.6
–3	+	+	6.8	7.9
–4	+	+	4.6	6.2
–5	+	+	4.3	10.9
7278–4	+	+	4.6	11.3
7245–1	+	+	3.6	9.1
–2	+	+	9.7	13.6
–4†	+	+	11.3	11.3
–5	+	+	13.6	9.7
7508–5	–	–	20.0	20.0
–6	–	–	26.0	13.6
–7	–	–	26.0	26.0
7476–1	+	+	8.5	11.3
–2	+	+	8.5	7.6
–3	+	+	12.6	7.6
–4	+	+	5.8	11.3
–5	+	+	9.7	7.6
7295–5	–	–	19.3	13.6
7108–1	+	+	5.2	6.1
–2†	+	+		
–3	+	+	8.5	7.6
–4	+	+	26.0	6.2
–5	+	+	11.3	7.6
7216–1	–	–	13.5	17.0
–2	–	–	26.0	13.6
7194–1	–	–	34.0	34.0
–2	–	–	34.0	17.0
–3	–	–	34.0	20.0
–4	–	–	18.7	28.3
–5	–	+	11.3	8.3

† Contamination.

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O. P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7533-3	+	+	8.5	11.3
-4	+	±	19.3	11.3
7169-2	+	+	6.2	9.1
7468-4	−	−	19.3	17.0
7137-2	+	+	4.2	9.7
7329-1	−	±	20.2	8.5
-3	−	+	11.3	6.8
-4	−	±	15.4	13.6
7514-2	−	−	11.3	17.0
-5	−	+	9.7	6.5
8381-1	+	+	6.2	6.8
-2	+	+	17.0	11.3
-3	+	+	6.2	6.8
7740-2	+	+	7.5	8.5
-3	+	+	16.1	4.2
7772-1	+	+	10.9	7.7
-4	+	+	6.8	7.7
7666-3	+	+	9.7	11.3
-4	+	+	4.9	3.7
7597-3	+	+	7.5	6.8
-4	+	+	9.7	6.8
7744-1	+	+	8.1	11.3
-2	+	+	8.1	11.3
-3	+	+	6.8	11.3
-4	+	+	10.9	4.2
-5	+	+	11.3	4.2
7806-2	+	+	11.3	9.4
-3‡	+	−	22.6	34.0
-4	+	+	8.5	4.2

‡ Yellow colony.

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O. P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7825–2	–	–	15.6	34.0
–4	–	–	17.0	64.0
–5	–	–	18.5	11.3
7666–4	–	–	17.0	34.0
–5	–	–	17.0	34.0
7812–1	+	+	7.5	9.6
–2	+	+	20.0	10.0
–4	+	+	8.5	9.6
7658–1	–	–	18.5	34.0
–2	–	–	15.6	34.0
8394–A1	–	+	6.5	11.3
–A4	–	+	3.7	4.1
–A5	–	+	10.6	9.7
–B2	–	+	7.7	6.5
7677–1	+	+	8.5	7.6
–4	+	+	11.3	4.8
–5	+	+	7.2	7.6
7717–1	+	+	2.2	3.7
–2	+	+	4.2	3.7
–3	+	+	9.7	4.2
–4	+	+	17.0	12.5
–5	+	+	10.3	3.5
8350–1	+	+	8.5	8.1
–2	+	+	17.0	5.0
7780–1	–	+	14.0	9.7
–2	–	+	6.8	7.5
–3	–	+	2.2	11.3
–5	–	+	4.2	4.2
8939–1	–	+	10.0	10.0
–2	–	+	3.7	6.8
–3	–	+	3.7	6.8
–4	–	+	8.5	6.8
–5	–	+	48.0	15.6

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O. P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7244–1	–	+	6.8	6.4
–2	–	–	10.3	8.5
–3	–	–	22.6	8.5
–4	–	+	6.8	6.2
–5	–	–	17.0	6.2
7279–1	+	+	8.1	7.6
–2	+	+	7.5	5.6
–3	+	+	11.3	12.6
–4	+	+	12.5	10.9
–5	+	+	6.8	5.6
7196–1	+	+	11.3	6.8
–2	+	+	17.0	6.8
–3	+	+	8.7	4.2
7332–1	+	+	6.8	7.6
–2	+	+	6.8	7.6
–3	+	+	8.5	9.7
–4	+	+	6.8	7.6
–5	+	+	6.8	7.6
7208–1	+	+	4.8	6.8
–2§	+	+	13.6	34.0
–3	+	+	9.7	34.0
–4	+	+	4.9	6.8
–5	+	+	6.2	6.8
7374–1	+	+	2.2	2.0
–2	+	+	6.2	6.8
–3	+	+	6.2	6.8
–4	+	+	7.5	6.8
–5	+	+	6.8	11.3
7371–1	+	+	12.5	4.4
–2	+	+	6.8	6.8
–3	+	+	7.5	10.9
–4	+	+	6.8	6.5
–5	+	+	6.8	6.5

§ Mixed yellow colony.

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O. P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
Park no. 8	+	+	2.9	7.6
	+	+	6.5	8.0
	+	+	2.9	9.1
	+	+	4.2	3.5
	+	+	2.9	7.6
Hoffmanni	−	−	19.3	34.0
	−	−	19.3	64.0
	−	−	14.0	34.0
	−	−	34.0	34.0

duce toxin slowly (i.e. seven days) will frequently be reported as "nonvirulent."

5. In general, it gives results which when checked against guinea pig tests are reliable.

The details of the technique are given below. It will be noted that a further manipulative disadvantage of the method is the necessity for making the mobility measurements with the microscope focussed on the "stationary level."

To meet certain obvious objections to these points in technique, we undertook a series of experiments to develop an apparatus which would (*a*) permit the use of growth taken directly from the original Loeffler medium; (*b*) eliminate the necessity for even forty-eight-hour subculturing, and the washing of the cultures, and (*c*) eliminate the necessity for focussing in the "stationary level." To these ends, we have developed the "capillary tube" method.

It may be recalled that when electrophoresis is conducted in a closed system, the electrical double layers between the fluid and the walls (glass) are located in the positions indicated in the schematic diagram of figure 5. Under the influence of the applied electrical field, the (positive) electrical layers in the water are given a positive velocity to the cathode and drag the successively

contiguous layers of water along at lesser and lesser velocities. The return streaming of the water occurs in the middle of the cell (if it is symmetrical). Hence the electrophoretic velocities of suspended particles vary at different levels in the cell and the "true" velocity is observed only at the position of the "stationary level" of the fluid. For this reason the mobility measurements are made with the microscope focussed at the "stationary level." Mooney[4] has pointed out that these effects of the endosmotic streaming of the fluid upon the electrophoretic mobility may be eliminated by using a cell of capillary dimensions. The principle

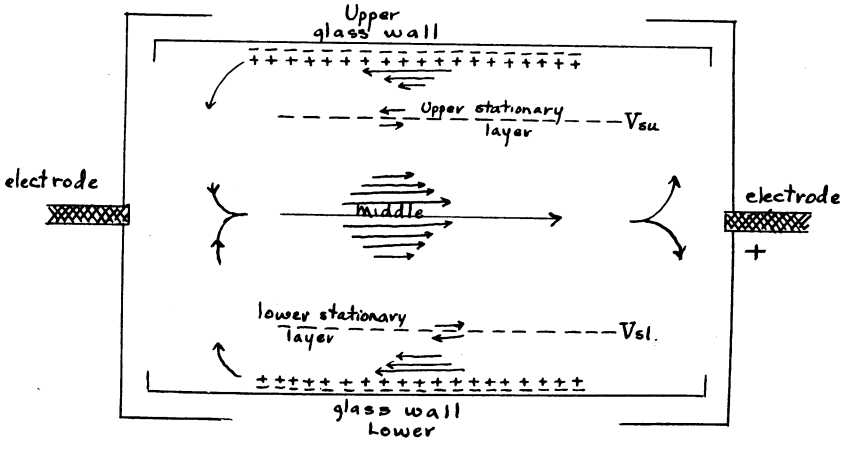

FIG. 5

of the method is that the electrical double layers at the fluid-wall interphase are brought so close together that, by the viscous resistance of the fluid, they drag all of the fluid along at approximately the same velocity. Applied to the electrophoresis of bacteria, the situation is this: Under the influence of the external electrical field, all of the fluid (water) in a glass capillary tube moves in one direction (to the cathode) at a velocity which is practically without a gradient across the diameter of the tube. Bacteria suspended in the water, being electronegatively charged, tend to move in the opposite direction to the anode. Hence, under any

[4] Phys. Rev., 1927, **29**, 218.

given set of conditions *the observed velocity of the bacteria is the difference between the velocity of the endosmotic streaming of the water* (*End.*$_w$) *and the velocity of electrophoresis of the bacterium* (*Elect.*$_b$). Thus:

$$\text{Observed mobility} = \text{End.}_w - \text{Elect.}_b$$

In a series of measurements, if the necessary precautions are taken, End.$_w$ is sensibly constant. Hence, *the observed mobility of the particle* (*bacterium*) *varies inversely as its true mobility*, and is

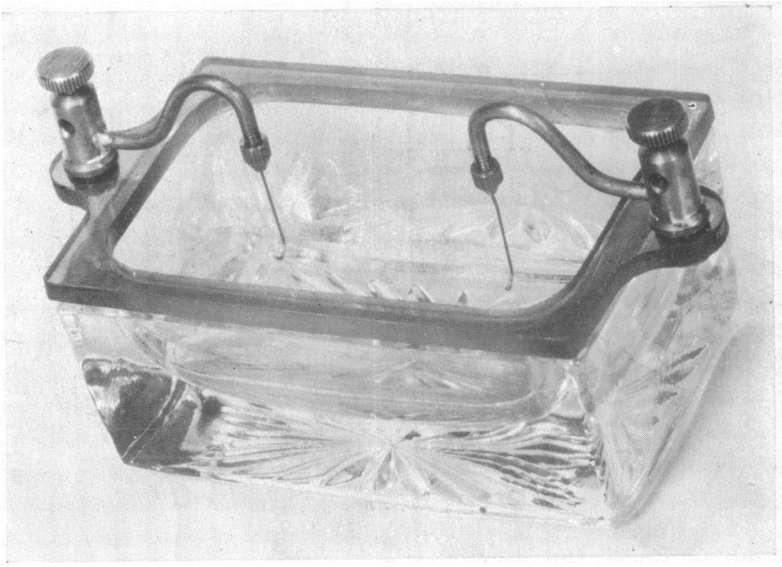

FIG. 6. THE CELL USED IN THE CAPILLARY TUBE METHOD

approximately independent of its position in the capillary tube. Furthermore, by the simple arrangement of apparatus shown in figure 6, polarization at the electrodes is practically eliminated by the interposition of distilled water between the electrodes and the open ends of the capillary tube.

With the capillary tube apparatus, the technique is as follows:

1. An isolated colony with the characteristics of the diphtheria bacillus is picked from the original Loeffler's medium growth with a *clean* loop.

2. It is emulsified in a small quantity of water in a clean crystal.

3. A loopful of this suspension is used for the routine microscopical examination to detect bacilli with the morphology (shape, size, granular, barred, solid, etc. characteristics) of diphtheria bacilli.

4. If the microscopical examination is "positive," a clean capillary tube is filled with the suspension (by capillarity) and is used for the electrophoretic measurement.

The details of the apparatus and technique are given below.

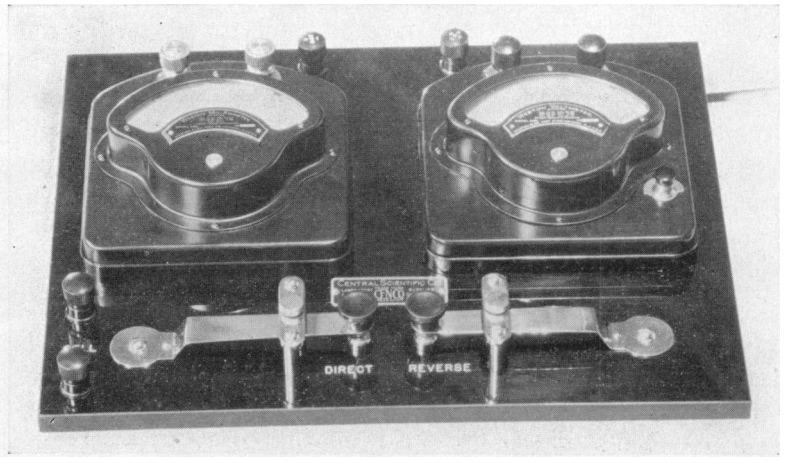

FIG. 7. A CONVENIENT ARRANGEMENT OF THE ELECTRICAL APPARATUS FOR ELECTROPHORESIS

DETAILS OF TECHNIQUE

A. The slide cell method

1. Cultures. If pure cultures are already available they should be grown in veal infusion, 2 per cent Witte peptone broth, initial pH = 7.2 to 7.4, at 37°C., preferably for at least forty-eight hours. If original cultures from throat, nose, etc., on Loeffler's medium are to be used, three to six separate colonies should be transferred to broth and used as for pure cultures. Sediment the bacteria by centrifugation; resuspend in distilled water and repeat twice more. Use these three-times-washed organisms in

distilled water in a suspension that is definitely cloudy to the naked eye. The distilled water suspension should have a pH near 7.0.

2. Cell.[5] Clean by brushing with soap and water and rinsing under running tap, and then, distilled water. Fill with suspension nearly to overflowing with a fine tipped pipette. Place coverglass over cell without trapping air bubbles. Press down coverglass so that it rests on the glass and not merely on the fluid in the cell. Wipe up excess fluid with filter paper. Connect electrodes to source of direct current voltage and place the cell on the stage of the microscope so that the objective is over the central portion of the cell.

3. Source of voltage. Any direct current source may be used. If the lighting circuit is D.C. this is satisfactory. A rheostat may be used to reduce the voltage applied to the cell electrodes. It is preferable to use ordinary radio "B" batteries, from which 25 or 45 to 50 volts can be taken. An impressed voltage of 25 to 50 volts (preferably the higher voltage) is satisfactory.

4. Microscope and equipment. Any microscope with a calibrated head on the fine adjustment screw will serve. We have found the following optical system satisfactory: objective—Bausch and Lomb, 8 mm., 0.50 n.a., 21× (long focal working distance); ocular—Bausch and Lomb, 25×, compensating; ocular micrometer—Sedgwick-Rafter ruling, placed over the diaphragm of the ocular. (Any similar equipment will serve.) Calibrate the ocular micrometer with a stage micrometer so that the diameter of each large square in the ocular micrometer is known in micra. Have available a good stop-watch reading to a fifth of a second.

5. Calibration of the cell. Fill the cell with water, cover with the coverglass, bring the calibrated head of the fine adjustment screw to zero and focus the microscope on the bottom of the chamber in the cell with the coarse adjustment. By turning the fine adjustment head until the microscope is focussed on the bottom of the coverglass, determined the depth of the cell chamber

[5] The cells and accessory equipment are made according to our specifications by the Central Scientific Company of Chicago.

in units on the head of the fine adjustment screw. Using the formula given below, focus the microscope on the upper or lower "stationary level." Do not change this focus.

In a cell of the kind described here, the stationary layers are located at a distance of $1/2\ D \div \sqrt{3}$ above and below the middle of the cell. Thus, if a cell is 100 units deep on the micrometer screw head, the middle is 50 units; $1/2\ D = 1/2$ depth $= 50$; and $50 \div \sqrt{3}$ is 28.9; the lower stationary level (*Vsl*) is located at 21.1 units from the bottom of the cell and the upper one (*Vsu*) at 78.9 units from the bottom. These levels, determined for each cell, are the ones at which the electrophoresis measurements are made.

6. Illumination. Natural or artificial illumination should be adjusted as for hanging drop examinations.

7. Electrical connections. The cell should be wired to the source of voltage through a reversing switch so that the polarity of the electrodes can be reversed quickly, easily and without jarring or moving the cell. It is desirable to have a small ammeter and a small voltmeter in the circuit to provide direct checks on the circuit. The voltmeter should show that the impressed potential is constant and the ammeter that practically no current is flowing (i.e., less than 10 milliamperes) and hence that the suspension in the cell is a very poor conductor. An extra main switch in the circuit, as indicated in our diagram, (fig. 2) is desirable. (Note: All that is *essential* is the cell, a good "B" battery and a reversing switch).[6]

8. Measurements. After the eye has become adjusted to the illumination of the field of vision in the microscope, the bacteria in clumps or singly should be plainly visible. With one hand on the reversing switch or keys and the other controlling a stopwatch throw on the voltage. As bacteria come into focus, moving across the field of vision, determine the time it takes for them to pass the diameter of one, two or more squares on the ocular micrometer. Make at least five measurements on different bacteria or clumps. Reverse the orientation of the electrical

[6] The Central Scientific Company of Chicago has prepared the electrical circuit in a compact and convenient arrangement. (See fig. 7.)

field by reversing the switch or keys and repeat the measurements with the bacteria moving in the opposite direction. Average the measurements for equal numbers of observations in the two directions. This average measurement is a velocity in "seconds per[7] micra." Calculate velocity in "micra per second." Dismount the cell and clean as before.

B. The capillary tube method

1. Cultures. Pick from the Loeffler's medium which has been incubated twelve to twenty-four hours at 37°C. three to six separate colonies that resemble colonies of diphtheria bacilli. Transfer each colony to a few drops of sterile distilled water on a clean slide or watch glass. Use a loopful of this suspension for the routine microscopical examination. If this is "positive," proceed with the electrophoretic measurement. A satisfactory suspension has a turbidity of about 1 billion organisms per cubic centimeter.

2. Capillary tube. This should be a little longer than the distance between the electrodes and should have an internal diameter of approximately 0.2 to 0.3 mm. (With a little practice capillaries of these internal dimensions can be drawn from heavy wall tubing. If a number of measurements are to be made, it is desirable to have on hand a set of capillaries of the same diameter. With the optical system recommended, the diameter of the capillary is somewhat less than the diameter of the field of vision).[8] The capillary tube is cleaned first with dichromate-sulfuric acid cleaning solution and then with running tap and distilled water. Water may be run through by inserting the end of the capillary in a piece of soft rubber tubing attached to the hub of a 5 cc. hypodermic syringe.

Holding the capillary between clean forceps, let it fill with the test suspension in the watch glass by capillarity. The cell should be filled with distilled water to a definite level (marked), constant in all measurements, and the capillary laid across the

[7] The value here is determined by the stage micrometer equivalents of the ocular micrometer units and by the number of ocular micrometer unit squares across which the bacteria moved in each measurement.

[8] The cell and capillary tubes are supplied by the Central Scientific Company of Chicago.

electrodes with the ends projecting the same distance beyond the two electrodes.

3. The source of voltage, the electrical circuits, connections, etc., and the optical system are the same as for the slide cell method described above.[9]

4. Measurements. Focus the microscope on the middle of the interior of the capillary. Apply the external voltage and record the velocity of movement of a bacterium. *Immediately*, reverse the polarity and repeat a measurement. Repeat this process until 6 to 10 measurements have been obtained. Convert the measurements to velocities in micra per second (μ/sec.).

Caution. It is recommended that these methods should be used by Schick-negative individuals only.

STANDARDIZATION OF ELECTROPHORESIS MEASUREMENTS

Instead of presenting specific recommendations on the standardization of electrophoretic measurements against guinea pig tests, we prefer to list the following which—though they recommend departures in principle from certain accepted procedures—we believe are theoretically as well as technically correct.

1. When standardizing the electrophoretic methods, first make measurements with pure cultures (prepared as indicated above) known by quantitative guinea pig tests to be (*a*) highly toxigenic; (*b*) moderately toxigenic, and (*c*) non-toxigenic.

2. It should be found that for such a series of cultures the electrophoretic velocities fall in a sequence which (*a*) with the slide cell method is an increasing velocity sequence, and (*b*) with the capillary tube method is a decreasing velocity sequence.

3. In routine practice, with each series of "unknown" cultures examined, there should be used as controls at least three cultures known to be highly-, moderately- and non-toxigenic, respectively. (We have found that with a little practice, some 12 to 20 slide cell and some 10 to 15 capillary tube measurements can be made in an hour, particularly if a helper is available to clean apparatus between measurements, or if several clean cells or capillary tubes of the same dimensions are prepared in advance.)

[9] A water immersion lens is perhaps preferable to the one already specified (i.e., Bausch and Lomb 7 mm. "water," 0.50 n.a.).

4. In reporting virulence (toxigenicity) results from electrophoresis measurements, cultures or suspensions with extremely high or extremely low velocities should be reported as a "negative" or "positive" (according to which method of measurement is used); and cultures with intermediate velocities should be reported as + (−) or − (+) according to which velocity extreme they approach, and subject to reexamination by an electrophoretic method or by a suitable quantitative guinea pig test.

When multiple measurements of several colonies (or cultures from several colonies) taken from a throat, nose or other streak culture show significantly different results, the diagnosis should be determined by that set of measurements which gives velocities most nearly approaching the velocity of cultures known to be highly virulent (toxigenic).

5. The last preceding statement applies as well to measurements made on suspensions or cultures which are apparently or certainly mixed or contaminated.

In conclusion, we would emphasize that we have presented here a series of presumptive methods. It is hoped that workers in other laboratories will experiment with them. They may find, as we have found, that they are useful and reliable in providing rapid, tentative "virulence diagnoses" without using animals, without waiting days for the results of animal tests and where animal tests are inconvenient or impossible. Further experimentation may bring to light shortcomings of the techniques that may be avoidable. The values of the methods can be determined only as the results of wider experience become available.

Finally, we would add that the studies summarized in this series of papers may be useful in research as well as in routine observations, particularly because they provide significant evidences on apparent quantitative gradations in toxigenicity. It may not be gratuitous to remark that, in our opinion, the older qualitative notion has dictated practices which have resulted in missing many "virulent" strains of diphtheria bacilli and in complicating diphtheria epidemiology to an extent that may have been significant in the failure of epidemiological methods in the past to effect control of diphtheria incidence and morbidity.

The simplified electrophoresis methods described here may also be useful in other fields of study.