THE PRODUCTION OF DIPHTHERIA TOXIN.

By W. H. PARK, M. D.,
AND
A. W. WILLIAMS, M. D.

(*From the Bacteriological Laboratory of the Health Department of the City of New York.*)

The production of diphtheria antitoxin was undertaken by the New York City Health Department in September, 1894. The number of horses, at first limited to a few, was gradually increased until in January, 1895, forty were under treatment. In order to supply sufficient toxin for the injection of these horses it had to be produced in large quantities. It was also desirable to obtain the toxin in as concentrated a form as possible, so that the horses, which required ever-increasing amounts, should not receive subcutaneously too large quantities of fluid. The statements of Roux and Behring were so explicit as to the methods of producing toxin that their advice was followed for a time without questioning. At considerable expense and trouble virulent diphtheria bacilli were either grown for several weeks in broth contained in especially constructed flasks and under a constant current of fresh air, as advised by Roux, or the bouillon cultures contained in Erlenmeyer flasks were allowed to stand in the incubator at 35° for from four to six weeks, as recommended by Behring.

The results were not satisfactory. The toxin produced by these methods had generally a strength of which 0·1 cubic centimetre would kill a 250-gramme guinea-pig in four to five days (Behring's normal toxin). Occasionally 0·05 cubic centimetre would kill, while, on the other hand, not infrequently 0·2 cubic centimetre or even more was required.

The moderate strength of the toxin obtained with abundant growth of bacilli, and the fact that the toxin was never found to increase at later periods in cultures after they had developed for three weeks at

35° C., but rather was frequently found to slowly disappear when left longer in the incubator, made us resolve to thoroughly investigate in a practical way the length of time needed for the production of toxin and the best conditions, both as to culture and media, under which to obtain it. The rapid production of very poisonous toxin in human and experimental diphtheria suggested to us that under favourable conditions it would probably be as quickly produced by very virulent bacilli in bouillon.

The experimental work described in this paper was done in the Bacteriological Laboratory of the Health Department of the City of New York, of which Dr. Hermann M. Biggs is the director, and comprises two series of investigations. The first, begun in June, was terminated in the latter part of July; the second, begun in October, is still unfinished.

Technique.

The bouillon in each set of experiments, after being prepared from meat in the usual way, was first poured into a single vessel and made neutral to litmus. From this common stock portions were removed, and, after having added the amounts of normal soda solution and peptone desired, were poured into 600-cubic-centimetre Erlenmeyer flasks. All the flasks contained the same amount of bouillon—250 cubic centimetres—and were similarly stoppered with cotton. In the second series of experiments each flask was covered by a paper cap as an additional precaution to prevent contamination. All the flasks were placed in the same incubator and kept at a temperature of 35° C. By these means all the conditions for the growth of the different cultures were exactly similar except those which we purposely made different, such as the amounts of peptone and of alkali, and the degrees of virulence of the different bacilli used. The inoculation of the bouillon in all the flasks of each series with the diphtheria bacilli was made from a single bouillon culture of forty-eight hours' growth. Each flask was inoculated with an equal amount—2·5 cubic centimetres—of this thoroughly shaken culture.

In order to test the influence of the reaction, of the percentage of peptone, and of the degree of virulence possessed by the bacillus upon the production of toxin, the following scheme was carried out. The details of the second experiment, as being the most extensive, will be first described, and then later the points in which the first one differed from it. The bouillon used contained only faint traces of glucose derived from the meat. After being mixed thoroughly it was divided into three portions. To one half of the bouillon one per cent peptone, to one third two per cent peptone, and to one sixth four per cent peptone was added. Each one of the three portions of neutral bouillon was then further subdivided into four equal parts. The first part was left neutral, to the second five cubic centimetres, to the third ten cubic centimetres, and to the fourth fifteen cubic centimetres of normal soda solution (forty grammes of sodium hydrate to the litre) were added. With these different varieties of bouillon seventy-two 600-cubic-centimetre Erlenmeyer flasks were partly filled, each flask receiving two hundred and fifty cubic centimetres. These flasks, after being both plugged with cotton and covered with loosely fitting paper caps, were sterilized on three successive days for one hour each in Arnold steam sterlizers.

The flasks were now finally divided into three groups, each of which contained an equal number of all the different varieties of bouillon. For the inoculation three cultures were employed which differed somewhat in virulence. The one named 8, which was obtained two months previously from a very mild case of diphtheria, killed a 500-gramme guinea-pig in a dose of 0·002 cubic centimetre. Culture named R came from a moderately severe diphtheria, while J was from a rapidly fatal case. These two were equally virulent, killing the same-sized guinea-pigs in doses of about 0·01 cubic centimetre. None of these cultures had been passed through the bodies of guinea-pigs, being merely transplanted from the pellicle into fresh bouillon every two days. The flasks of each division were inoculated on October 27th with 2·5 cubic centimetres of a forty-eight hours' growth from a single flask of one of the three cultures. The

flasks were then all placed in the same incubator and kept at a temperature of 35° C.

After twenty-four hours' growth in the thermostat a measured quantity was withdrawn from each flask, after its contents had been shaken, by means of sterilized pipettes and placed in sterile glass bottles; after the addition of 0·5 per cent of carbolic acid, these bottles were stored in the ice chest. From a certain number of the flasks larger quantities were withdrawn and immediately filtered. To the filtered culture fluid, after testing its sterility, 0·5 per cent carbolic acid was also added.

At the time that the fluid was removed from each flask agar plates were made from each culture in order to test its purity. From eight cultures the agar plates were made with a tube of fluid agar to which a loop full of the well-shaken culture had been added. The colonies which developed were then counted after forty hours' growth at 35° C.

Similar drawings were made from all of the seventy-two flasks upon each of the first days, and then at longer intervals up to the seventy-fifth day.

If from time to time one or more flasks became contaminated they were withdrawn. We were, however, very fortunate in this respect in this second experiment. We believe this to be partly due to the protection of the cotton plugs from dust by the paper caps.

The first series of experiments differed only so far as the bouillon was concerned, in that we added greater amounts of alkali to some of the flasks and did not use as great amounts of peptone in others. The two cultures were H and P, each killing 500-gramme guinea-pigs in doses of 0·025 cubic centimetre. In this experiment we commenced to withdraw the culture fluid on the tenth day, and did not immediately filter any of the test samples. As the details are somewhat intricate, they are summarized in the following table:

TABLE I.

Showing the Number of Flasks employed, the Amount of Alkali added, and the Cultures used in the Experiments.

First experiment, 30 flasks.		Second experiment, 72 flasks.	
One per cent peptone.	Neutral, 3 flasks.	One per cent peptone.	Neutral, 9 flasks.
Two thirds of flasks	5 c. c., " "	36 flasks divided equal-	5 c. c., " "
inoculated with culture	10 " " "	ly between the 3 cul-	10 " " "
P and one third with	20 " " "	tures, 8, R, and J.	15 " " "
culture H.	30 " " "	Two per cent peptone.	Neutral, 6 "
Two per cent peptone.	Neutral. " "	24 flasks divided equal-	5 c. c., " "
One flask of each lot	5 c. c. " "	ly between the 3 cul-	10 " " "
inoculated with H, the	10 " " "	tures.	15 " " "
others with P.	15 " " "	Four per cent peptone.	Neutral, 3 "
	25 " " "	12 flasks divided equal-	5 c. c., " "
		ly between the 3 cul-	10 " " "
		tures.	15 " " "

Neutral = neutral to litmus.

5, 10, 15 c. c. = 5, 10 and 15 cubic centimetres of normal sodium-hydrate solution added to each litre of neutral bouillon.

The Bacillus 8 killed 500-grm. guinea-pigs in 0·002 c. c. dose in 7 days.

"	R	"	"	"	"	0·01	"	"	"	2	"	0·005 c. c. not fatal.
"	J	"	"	"	"	0·01	"	"	"	2	"	0·005 " "
"	P	"	"	"	"	0·025	"	"	"	2	"	0·01 " "
"	H	"	"	"	"	0·025	"	"	"	2	"	0·01 " "

THE PERIOD AT WHICH THE DIPHTHERIA BACILLI BEGIN TO PRODUCE TOXIN IN APPRECIABLE AMOUNT, AND AT WHICH THE TOXIN HAS ACCUMULATED TO THE GREATEST EXTENT.

The work of Roux and Yersin * upon this subject is not only the earliest, but also the most extensive. With but minor additions it comprises practically all that has been published upon the subject. Their results as contained in four reports may be summarized as follows:

If a flask of slightly alkaline bouillon is inoculated with a quantity of virulent bacilli and kept at a temperature of about 37° C., the bacilli rapidly proliferate and produce certain changes in the broth. The reaction of the broth becomes acid in a few days, and then again in a variable length of time alkaline. These changes require under the ordinary methods of growth three and four weeks, but when the bacilli grow in broth to which a very free access of air is permitted

* Roux. *Annales de l'Institut Pasteur*, 1888, 1889, 1890, 1894.

they may take place within two weeks. Roux and Yersin state positively that strong toxin is not produced by the bacilli in less than three weeks in broth cultures grown in the usual manner, and in those to which a free access of oxygen is supplied by the constant air current in not less than two weeks. They also assert that strong toxin is never produced while the reaction is still acid.

So far as we can determine, the essential portion of their statement that strong toxin is produced only after the lapse of a number of days and after the alkaline reaction has been established has been accepted by all German and other investigators. Some, indeed, have discovered that toxin is produced at times somewhat earlier than Roux mentions. Thus Spronck and van Furenhout,* as shown in their report, have recently obtained strong toxin from a very virulent bacillus within thirteen days in a culture grown under the ordinary conditions, and Aronson † states that by a special method —that of inoculating the bouillon from the pellicle of a forty-eight-hour culture of virulent bacilli—he obtained very strong toxin after eight days.

The table on page 170 gives the results of our tests of the amount of toxin present in samples of broth withdrawn from different cultures at different periods of growth.

The results tabulated there show that the most virulent bacillus used by us (No. 8) produced appreciable toxin within four hours and strong toxin within twenty-four hours, 0·1 cubic centimetre killing a 324-gramme guinea-pig in three days. Moreover, this toxin was produced while the bouillon was of an acid reaction (see table VII).

By the fifth day the amount of toxin in this sample of bouillon reached its maximum, 0·005 cubic centimetres killing a 430-gramme guinea-pig in two and a half days, and then remained practically stationary, having diminished but little as late as the fifty-sixth day. The other two cultures produced fairly strong toxin in four days, and here also it reached its maximum between the fifth and seventh

* Spronck. *Annales de l'Institut Pasteur*, October 25, 1895.

† Hans Aronson. *Berichte der Pharmeutischen Gesellschaft*, May 2, 1895.

TABLE II.

Time of Production of Toxin.

Length of time of the growth of the bacilli in the culture before the withdrawal of the sample tested.	CULTURES.														
	No. 8. 2 per cent.* 5 c. c.†			No. 8². Filtrate. 1 per cent.* 5 c. c.†			R. 1 per cent.* 0 c. c.†			P. 2 per cent.* 10 c. c.†			H. 1 per cent.* 0 c. c.†		
	Weight of guinea-pigs in gms.	Amount inoculated, c. c.	Result of inoculation.	Weight of guinea-pigs in gms.	Amount inoculated, c. c.	Result of inoculation.	Weight of guinea-pigs in gms.	Am't inoculated, c. c.	Result of inoculation.	Weight of guinea-pigs in gms.	Am't inoculated, c. c.	Result of inoculation.	Weight of guinea-pigs in gms.	Am't inoculated, c. c.	Result of inoculation.
4 hours.‡	...	.....	.....	300	2	D. in 2½ d.									
1 day.	324	0·1	D. in 3 d'ys.	403	0·025	" 4 "	415	1·00	No react'n.						
2 days.	505	0·01	" 24 "												
" "	548	0·1	" 40 h.	470	0·025	" 40 hrs.	480	1·00	"						
3 "	492	0·025	" 2 d'ys.	545	0·025	" 2 d'ys.	396	0·25	D. in 2½ d.						
4 "	495	0·01	" 2½ "	...	.....	.....	483	0·25	" 3 d'ys.						
5 "	430	0·005	" 2½ "	560	0·0125	D. in 2 d'ys.	390	0·10	" 2½ "						
7 "	530	0·01	" 40 h.	451	0·005	" 3 "	438	0·10	" 4 "						
9 "	529	0·005	" 8½ d.	...	.....	.....	335	0·10	" 4 "						
10 "	...	.....	.....	...	.....	.....	...	....	.....	409	0·25	D. in 40 h.	322	0·25	D. in 40 h.
11 "	440	0·01	D. in 2½ d.	421	0·01	No react'n.									
" "	...	.....	.....	312	0·025	Slight infiltration.									
17 "	520	0·005	Induration.												
18 "	...	.....	.....	...	.....	.....	...	....	.....	395	0·25	" 24 "	395	0·25	" 60 "
25 "	490	0·01	D. in 2½ d.												
26 "	...	.....	.....	...	.....	.....	...	....	.....	421	0·25	" 40 "	306	0·25	No react'n.
35 "	380	0·01	D. in 5½ d.	367	0·05	No react'n.	385	0·10	Considerable infiltration.						
40 "	...	.....	.....	...	.....	.....	...	....	.....	477	0·25	Induration.			
56 "	340	0·01	D. in 2½ d.	...	.....	.....	290	0·10	No react.#	458	0·25	No react'n.			

* Percentage of peptone. † Amount of normal soda solution added to each litre of neutral bouillon.
‡ This culture was grown in a different bouillon, but inoculation was made with same bacillus by means of a platinum loopful of the pellicle.
# This culture was slightly contaminated.

days. These same results have been repeated whenever the bouillon was equally suitable for a vigorous growth of the bacillus and for the quick production of toxin.

During the past three months bouillon made from fourteen different lots of beef has been used. In all but three the development of the toxin has been about as rapid as that in the experiments given in the table. The growth of the bacilli and the production of toxin in one lot were extremely interesting, being similar to the classical description of Roux and Yersin. During the first day the bacilli grew vigorously and a fairly thick pellicle formed. At the end of twenty-four hours the original alkaline reaction changed to slightly acid, the growth of the bacilli lessened, and the pellicle partially sank to the bottom of the flask and did not immediately reform. These conditions remained up to the twelfth day, when the acidity began to diminish. On the sixteenth day the pellicle had reformed; the bouillon was turbid and alkaline. No toxin was present on the twelfth day; strong toxin on the eighteenth. In the two other samples part of the cultures remained acid, and no toxin was produced. In one the growth was very scanty. But two of fourteen samples of non-putrid meat, therefore, were unfit for the purpose of producing strong toxin.

In view of the facts brought out in the preceding experiments, the positive statements of Roux, Behring, and many others that strong diphtheria toxin is never produced until after the culture has grown for several weeks are certainly very surprising.

The explanation seems to us to be that Roux and Yersin must have had in their first experiments bouillon similar to the three lots last mentioned. Getting uniformly no toxin until the third or fourth week in this variety of bouillon, they came to the conclusion that the bacilli under no conditions produced it earlier. All bacteriologists who have been interested in the production of diphtheria toxin since then seem to have accepted to a very great degree their opinions without themselves thoroughly investigating whether they were in fact correct.

The gradual disappearance of toxin in some of the older cultures

is well shown in the table in cultures P and H, where fairly strong toxin present in the cultures on the sixteenth day had almost disappeared upon the fifty-sixth and twenty-sixth day respectively, while in cultures 8^2 and J the toxin present on the fourth day had appreciably diminished in some flasks on the eleventh and eighteenth days. We had even a more striking experience with some cultures grown under a constant current of air; strong toxin was present on the seventh day and entirely absent on the fourteenth day.

The Relation between the Activity of the Growth of the Bacilli and the Amount of Toxin produced.

In all of the cultures in which strong toxin developed quickly there was a rapid and abundant growth of the bacillus, with the formation of a more or less thick pellicle within the first thirty-six hours. An abundant growth of the bacilli did not, however, necessarily indicate the production of strong toxin. In the broth to which fifteen cubic centimetres of normal NaOH solution was added per litre, and which generally produced very little toxin, we have noticed the same abundant and rapid growth.

In the broth to which twenty cubic centimetres and thirty cubic centimetres of normal NaOH solution was added per litre, and in which practically no toxin was produced, the growth was slow at first, but later nearly equalled that in the broth to which less alkali was added. In all of the broths which have remained acid for some time and which have produced toxin of some strength only after the reaction has become alkaline, the growth during the stage of acid reaction has been somewhat scanty, the pellicle, if formed at all, being slight.

The Relation between the Number of Living Bacilli in a Culture and the Production of Toxin.

This was sought to be determined in the following way: Eight of the flasks containing the cultures (four with one per cent peptone and four with two per cent, each lot comprising all four degrees of alkalinity), from which samples were being daily drawn as described

earlier in this paper, were selected for examination as to the number of living bacilli in each cubic centimetre of broth at different periods of growth. The different alkalinities and proportions of peptone gave nearly similar results. The method employed was as follows: The culture fluid in a flask in which the living bacilli were to be counted was first thoroughly shaken and then $\frac{1}{450}$ cubic centimetre was removed by means of a platinum loop. This was inoculated into a tube of sterilized fluid agar cooled down to 40° C., and the whole thoroughly shaken and poured into a Petri dish. The colonies were counted at the end of forty hours' growth in the incubator. The results are shown in the following table:

TABLE III.

Showing Average Number of Colonies of Bacilli in One Field of Microscope at Different Days.

PEPTONE AND ALKALINITY.	24 hours.	48 hours.	72 hours.	4 days.	5 days.	7 days.	9 days.	11 days.
R—2 per cent, neutral.....	18	30	43	25	42	16	$1\frac{1}{8}$	$4\frac{1}{8}$
1 " 5 c. c.....	32	47	45	27	18	10	1	8
1 " 10 "	60	42	25	22	31	3	$1\frac{1}{8}$	$1\frac{1}{8}$
1 " 15 "	47	57	40	56	60	13	$1\frac{3}{8}$	$9\frac{3}{8}$

In this trial, therefore, the number of living bacilli reached its highest total at the end of forty-eight hours. From the third to the fifth day the number of living bacilli, as shown by the plates, remained nearly the same. Thereafter it rapidly diminished, until at the ninth day it was but one thirtieth of its former number. Then for a time a moderate increase occurred, the number finally falling again two weeks later. The toxin increased rapidly while there were numerous living bacilli present in the culture, and then when they decreased the toxin ceased to accumulate rapidly, and even in some cases gradually diminished. Here, if new toxin was still being produced, that already present must have been even more rapidly destroyed.

In two cultures in which the acid reaction occurred early and persisted, the number of bacilli never rose above one third of the number in the above test or in other tests where similar broth was used.

THE AMOUNT OF TOXIN CONTAINED WITHIN THE BODIES OF THE BACILLI AS COMPARED WITH THAT HELD IN SOLUTION IN THE BOUILLON AT DIFFERENT PERIODS IN THE GROWTH OF THE CULTURE.

In endeavouring to solve this problem the following experiments were made: First, from two of the cultures—one very virulent (8), the other somewhat less so (R)—a portion was removed each day after they had been thoroughly shaken and immediately filtered. To the filtrate, after its sterility had been proved, was added 0·5 per cent carbolic acid. The filtrate was found to contain as much toxin as the unfiltered portion of the cultures removed on the same day (see Table IV).

Second, from the quantities removed at different periods in the growth of the culture the bacilli left on the filter, after the passage of the culture fluid and of considerable quantities of sterile water, were scraped off and allowed to soak in a 0·5-per-cent alkaline carbolic solution for one week. The quantity of water was equal to the amount of culture filtered. The watery extract alone and the watery extract together with the dead bacilli were then injected into guinea-pigs. Whether the filtrate contained or did not contain very strong toxin, neither the water in which the bacilli had remained nor the water plus the dead bacilli was sufficiently toxic to produce any marked reaction in 500-gramme

TABLE IV.

Showing Relative Strength of Filtrate and Carbolized Culture.

Culture used.	One day's growth.			Two days' growth.			Three days' growth.			Seven days' growth.		
	Weight of guinea-pig in gms.	Am't injected in c. c.	Result.	Weight of guinea-pig in gms.	Am't injected in c. c.	Result.	Weight of guinea-pig in gms.	Am't injected in c. c.	Result.	Weight of guinea-pig in gms.	Am't injected in c. c.	Result.
R—1 per cent peptone, neutral reaction.												
Carbolized Culture.....	415	1·00	No reaction.	480	1·00	No reaction.	483	0·25	Died in 3 days.	459	0·25	Died in 40 hours.
Filtrate.....	435	1·00	No reaction.	490	1·00	No reaction.	413	0·25	Died in 3 days.	472	0·25	Died in 40 hours.

TABLE V.

Showing Influence of Percentage of Peptone and of Degree of Alkalinity upon the Production of Toxin.

Peptone.	C. c. of normal solution of NaOH to litre of neutral broth.	CULTURES. No. 8—First day. Weight of guinea-pig in gms.	Am't inoculated, c. c.	Result of inoculation.	No. 8—Fifth day. Weight of guinea-pig in gms.	Am't inoculated, c. c.	Result of inoculation.	R—Ninth day. Weight of guinea-pig in gms.	Am't inoculated, c. c.	Result of inoculation.	J—Seventeenth day. Weight of guinea-pig in gms.	Am't inoculated, c. c.	Result of inoculation.
Per ct.													
1	0	325	0·10	Slight induration.	253	0·10	D. in 26 h.	335	0·10	Died in 40 hours.	595	0·10	Died in 3 days.
1	5	445	0·10	" "	453	0·10	" 40 "	425	0·10	" 3 days.	503	0·10	" 4 "
1	10	440	0·10	Died in 4½ days.	435	0·10	" 40 "	412	0·10	Slight infiltration.	480	0·10	Considerable infiltration.
1	15	...	....	.....	...	...	.....	...	....	.....	307	1·00	Died in 40 hours.
1	"	340	1·00	Slight induration.	425	1·00	" 2½ d.	470	1·00	Died in 27 days.	536	0·10	No reaction.
2	0	407	0·10	No local reaction.	435	0·10	" 40 h.	370	0·10	" 2½ "	645	0·10	Died in 2½ days.
2	5	324	0·10	Died in 3 days.	430	0·10	" 26 "	351	0·10	" 40 hours.	533	0·10	Died in 40 hours.
2	10	468	0·10	No reaction.	490	0·10	" 40 "	325	0·10	" "	557	0·10	" "
2	15	...	....	.....	...	....	.....	...	....	.....	345	1·00	" 45 hours.
2	"	432	1·00	Slight induration.	590	1·00	" 3½ d.	480	1·00	No reaction.	450	0·10	No reaction.
4	0	575	0·10	" "	410	0·01	" 45 h.	465	0·10	Died in 40 hours.	485	0·10	Died in 45 hours.
		Second day.											
4	5	500	0·10	Died in 24 hours.	520	0·01	" 40 "	448	0·10	" "	555	0·10	Died in 2½ days.
		First day.											
4	10	496	0·10	Slight induration.	395	0·01	" 40 "	560	0·10	" "	498	0·10	" "
4	15	...	....	.....	484	0·01	No local reaction.						
4	"	421	1·00	Much infiltration.	298	1·00	D. in 17 h.	495	0·10	" "	550	0·10	" 3 days.

guinea-pigs in one-cubic-centimetre doses. These tests would seem to show that when under certain conditions toxin is not found in the first days in a culture, it is not because it is locked up in the bodies of the bacilli, but that the conditions are such that the bacilli, although growing perhaps vigorously and possessing full virulence, do not produce toxin.

Influence of Percentage of Peptone and Degree of Alkalinity upon the Production of Toxin.

We notice in the experiments of Table V that culture 8, which gave the strongest toxin, produced it most quickly with the ten-cubic-centimetre alkalinity in one per cent peptone and with the five-cubic-centimetre alkalinity in the two-per-cent solution. After the third day the toxin was equally strong in the neutral, five- and ten-cubic-centimetre alkalinities for both percentages of peptone. The four-per cent peptone gave nearly the same results, except that the toxin was somewhat stronger.

With all three amounts of peptone fifteen cubic centimetres of normal soda solution to the litre greatly lessened the formation of toxin. In the earlier experiments still larger amounts of alkali—twenty to thirty cubic centimetres to the litre—almost completely prevented the development of toxin, though not materially inhibiting the growth of the bacilli after the first few days.

In culture R the results were similar for one- and two-per-cent cent peptone bouillon, but with four per cent peptone about equal amounts of toxin were produced in all four degrees of alkalinity. With culture J toxin was produced under the same conditions as in R, except that the first toxin produced in the broth with one per cent peptone was in that containing fifteen cubic centimetres of alkali to the litre.

These experiments indicate, therefore, that the diphtheria bacilli will produce strong toxin when the quantities of alkali and of peptone vary within considerable limits. With neutral broth toxin is usually produced, but not so surely, and, as a rule, not so quickly nor to such an extent as in that to which five to seven cubic centimetres

of alkali have been added per litre. The added alkali is probably necessary in bouillon containing considerable traces of glucose. With ten cubic centimetres to the litre the production of toxin is a little less reliable, and with fifteen cubic centimetres much less so. With four per cent peptone we have found the toxin to be more constantly produced in the decidedly alkaline bouillon, with all the degrees of alkalinity employed.

For the production of toxin on a large scale we have usually adopted the custom of dividing the bouillon into several portions. To one of these two per cent peptone is added, and to the others one per cent and four per cent. To the whole after neutralizing to litmus we add five to ten cubic centimetres of normal soda solution to the litre. The larger amount of alkali is added to the bouillon containing four per cent peptone. From repeated trials we have found the two-per-cent and four-per-cent peptone bouillon to have averaged a stronger toxin than the one-per-cent, but not infrequently in single trials the one per cent peptone has given the strongest toxin. Larger amounts of peptone (six per cent to ten per cent) have been tried once experimentally. In this single trial the toxin in the cultures on the seventh day was somewhat stronger in the bouillon containing the larger amounts of peptone, as is shown in the following table:

TABLE VI.

Showing Relation of Toxicity to Percentage of Peptone.

Amount of Peptone added.	Toxicity.		
	Weight of guinea-pig in gms.	Amount inoculated in c. c.	Result of inoculation.
Per cent.			
2	298	0·01	Died in 3 days.
4	355	0·01	" 40 hours.
6	562	0·01	" 3 days.
8	745	0·01	" 3½ "
10	675	0·01	" 40 hours.

The neutralization of the bouillon in these experiments was done by Mr. James A. Miller, assistant chemist to the laboratory. In doing this it developed that the amount of peptone added to broth influences greatly its reaction to phenolphthalein; also with different

peptones, even from the same house, the reactions to this indicator vary considerably. The peptone which we use requires in a two-per cent solution ten cubic centimetres of normal NaOH to the litre to neutralize to phenolphthalein. In a four-per-cent solution twenty cubic centimetres to the litre are required. As peptone is decidedly alkaline to litmus, it will be seen that the greater the amount added, the greater the alkalinity of the broth to this indicator, while, on the other hand, to phenolphthalein the acidity is increased at the rate of five cubic centimetres normal solution to the litre for every one per cent added. We have found for diphtheria cultures the litmus to be the best indicator if different percentages of peptones are employed. It is perfectly possible, however, after having decided the proper degree of alkalinity for each percentage of each variety of peptone, to use phenolphthalein for future testing.

The Relation between the Reaction of the Broth Cultures and the Amount of Toxin.

Litmus paper was the indicator used in determining the reaction. The guinea-pigs into which the inoculations were made weighed from four to five hundred grammes. The amount inoculated in each instance, except those indicated, was 0·1 cubic centimetre.

The results from these experiments are given in Table VII, on the following page. The broth, after becoming alkaline in any culture, continued so as long as tested. These results show that strong toxin is produced under certain conditions while the broth is still acid. This occurred in those cases where the period of acid reaction was brief and where the amount of acid was not very great. In those cases where the period of acid reaction is longer little if any toxin seems to be produced until just before the change to alkalinity. The amount of acid present in the broth, therefore, must be small to allow the development of toxin.

TABLE VII.

Showing the Relation between the Reaction of the Broth Cultures and the Amount of Toxin.

CULTURES.	1ST DAY.		2D DAY.		3D DAY.		4TH DAY.		5TH DAY.		12TH DAY.		18TH DAY.		35TH DAY.	
	Reaction.	Toxicity.	Reaction.	Toxicity.	Reaction.	Toxicity.	Reaction.	Toxicity.	Reaction.	Toxicity.	Reaction.	Toxicity.	Reaction.	Toxicity.	Reaction.	Toxicity.
8—1 per cent peptone, 0 c. c. alkali.	Acid.	Slight infiltration.	Acid.	Death in 4½ days.	Neutral.	Death in 40 hrs.	Alk.	.....	Alk.	Death in 26 hrs.	Alk.	.....	Alk.	.....	Alk.	
8—1 per cent peptone, 5 c. c. alkali.	Acid.	Slight infiltration.	Acid.	Death in 24 hrs.	Alk.	Death in 23 hrs.	Alk.	.....	Alk.	Death in 40 hrs.	Alk.	.....	Alk.	.....	Alk.	
8—2 per cent peptone, 0 c. c. alkali.	Acid.	(1·0 c. c.) slight infiltration.	Acid.	(1·0 c. c.) death in 5½ days.	Acid.	(1·0 c. c.) death in 40 hrs; no local reaction.	Acid.	Death in 2½ days.	Alk.	Death in 40 hrs.	Alk.	.....	Alk.	.....	Alk.	
8—2 per cent peptone, 5 c. c. alkali.	Acid.	Death in 3 days.	Acid.	Death in 40 hrs.	Alk.	Death in 40 hrs.	Alk.	.....	Alk.	Death in 40 hrs.	Alk.	.....	Alk.	.....	Alk.	
R—1 per cent peptone, 0 c. c. alkali.	Acid.	(1·0 c. c.) no local reaction.	Acid.	(1·0 c. c.) no local reaction.	Neutral.	(¼ c. c.) death in 2½ days.	Alk.	(¼ c. c.) death in 3 days.	Alk.	Death in 2½ days.	Alk.	.....	Alk.	.....	Alk.	
8[3]—2 per cent peptone, 5 c. c. alkali.	Acid.	.....	Acid.	.....	Acid.	.....	Acid.	.....	Acid.	.....	Acid.	(½ c. c.) no local reaction.	Alk.	Death in 18 hrs.	Alk.	
8[4]—1 per cent peptone, 5 c. c. alkali.	Acid.	.....	Acid.	.....	Acid.	.....	Acid.	.....	Acid.	.....	Acid.	(1 c. c.) no local reaction.	Acid.	.....	Acid.	(1 c. c.) no local reaction.

The Influence of Glucose upon the Growth of the Diphtheria Bacilli and upon the Production of Toxin.

Buchner * and Behring † mention that bacteria produce acid in media containing sugar. Theobald Smith ‡ made a more thorough investigation of this subject. He examined bouillon made from forty-four samples of meat. Of these, eleven contained no glucose, so far as indicated by the fermentation test. The remaining seventy-five per cent contained from faint traces up to as much as 0·3 per cent of glucose. In bouillon in which no sugar, or at most only a trace of sugar, was present no appreciable acid was produced by the growth of bacteria. He found that as much as 0·3 per cent of glucose favoured the growth of many bacteria, while over five per cent was, as a rule, harmful. He noted that when the growth of the bacteria continued abundant the acid reaction changed to the alkaline within a short time, but that where the amount of acid produced was sufficient to inhibit the growth of the bacteria, alkali was no longer produced, and the culture bouillon remained permanently acid. He considers that small amounts of glucose may be a valuable nutrient to the bacteria, or, on the other hand, that the small amount of acid produced may be of advantage in preventing a too intense alkalinity.

The especial influence of the presence of glucose in the broth upon the growth of the diphtheria bacillus and upon the production of toxin has been lately investigated by Spronck # and van Furenhout. They found that if to a bouillon which contained no glucose and in which the growth of the diphtheria bacilli caused no acid there was added 0·15 per cent of glucose, the character of the growth changed, as shown in the following summary:

1. Bouillon containing no glucose. The bacilli develop rapidly; the broth is turbid and remains alkaline. Type B of Spronck.

2. Bouillon type B to which 0·15 per cent glucose is added.

* Buchner. *Arch. f. Hygiene*, Bd. iii, p. 361, 1885.

† Behring. *Zeitschr. f. Hygiene*, Bd. vii, p. 178, 1889.

‡ Theobald Smith. *Centralblatt für Bakteriologie und Parasitenk.*, Bd. xviii, No. 1, 1895.

Spronck. *Annales de l'Institut Pasteur*, October 25, 1895.

Growth at first active. Reaction of bouillon changes to acid, and growth of bacilli decreases. After ten days to two weeks the reaction becomes alkaline and the bouillon turbid, the growth of the bacilli increasing. Type C.

3. Bouillon type B to which 0·2 per cent glucose is added. The bouillon soon becomes acid, and this reaction is usually permanent. The growth of the bacilli is scanty. Type A.

4. Bouillon type B to which glucose is added in the ratio of 1 to 1,000. The bouillon remains alkaline, the alkalinity being at first slightly reduced and, after the fifth day, greater than at first.

Type B gives the greatest amount of toxin, type C the next, and type A the least. In type A there may be none whatever produced. Spronck states that meat, as a rule, contains sufficient glucose to give a growth of type A. That if such meat is kept for a number of days until it becomes slightly putrid, the glucose will be destroyed by fermentation, and that the growth of the bacilli in the bouillon from such meat will be of the type B.

Original investigations. It is probable that the conditions under which cattle live and the different laws affecting their slaughter and the sale of their meat for food may have an influence upon the amount of glucose present in the beef of different countries.

In New York it is practically impossible to obtain the absolutely fresh meat spoken of by Spronck, since the law prevents the sale of meat for food until forty-eight hours after the slaughter. Indeed, meat as ordinarily obtained at the retail butchers has been kept from five to seven days. Bouillon from such meat has proved, almost without exception, suitable for the growth of the diphtheria bacilli and for the production of strong toxin. As tested chemically, these lots of meat have contained but small traces of glucose.

The usual type of growth has been one active from the start, the broth having an acid reaction only upon the second and perhaps third day.

The development of the toxin has been similar to that in the two experiments previously described. In cultures from only two out of

fourteen lots of bouillon was the acid stage protracted. In one the bouillon remained acid until the fourteenth day, and then became decidedly alkaline (Spronck's type C). In the other the culture remained permanently acid in the one-per-cent peptone bouillon without the production of toxin (Spronck's type A). In two-per-cent peptone bouillon, however, the growth in the latter was of type C.

In order to carry out the plan suggested by Spronck we sent directly to the slaughterhouse and obtained a large quantity of chopped beef from a recently killed animal. A portion of this meat was immediately boiled in the usual amount of water, another portion was soaked over night, another for five and a half days, and the remainder left soaking for two days longer until it became slightly putrid. The bouillon made from the fresh meat was less acid than that from the kept meat. That soaked five days required the addition of three cubic centimetres, and that seven and a half days nine cubic centimetres of normal soda solution to each litre to make them of the same reaction as the bouillon from the fresh meat. The bouillon in all four lots after being neutralized to litmus had seven cubic centimetres of normal soda solution added to each litre. In both the four-per-cent peptone and the two-per-cent peptone bouillon made from all four lots the diphtheria bacillus grew equally well. The period of acid reaction was very brief in all, but was most marked in the bouillon from the meat kept five days. To a portion of each of the four lots an extra twelve cubic centimetres of alkali was added. In these there was no stage of acid reaction whatever.

We have not found, therefore, that the amount of glucose present in the meat obtained by us in the usual way is apt to be of any serious hindrance to the abundant and rapid production of toxin. By this we do not mean to indicate that in meat obtained from other sources it might not be in sufficient amounts to be deleterious.

To test the effects of different amounts of glucose in broth we added from 0·05 to six per cent to different lots. To one lot of neutral bouillon in which the growth of the bacilli produced no acid the

addition of 0·1 per cent of glucose produced no appreciable effect; 0·2 per cent of glucose, however, entirely changed the character of the growth. The bouillon became acid on the first day and remained so. The growth of the bacilli was stopped and no toxin was produced. In another sample of bouillon, however, which contained four per cent peptone and was strongly alkaline to litmus (thirteen cubic centimetres to the litre), and gave to phenolphthalein a reaction of 0·25, an amount of glucose up to 0·5 per cent had no inhibiting effect on the growth of the bacillus, nor on the production of toxin, 0·01 cubic centimetre of a five days' filtrate killing a four hundred gramme guinea-pig in three days. The acid reaction developed at the end of twenty-four hours. At the end of forty-eight hours the bouillon was again alkaline. One per cent glucose added to this bouillon was sufficient to cause such a production of acid that the further growth of the bacilli was prevented. Indeed, on the seventh day the culture was sterile.

A curious result appeared when the acidity of the cultures to which different amounts of glucose had been added was tested with phenolphthalein. All the cultures in the same bouillon to which different amounts of glucose had been added and in which the acid reaction remained permanent were found not only intensely acid, but also equally acid. This seemed to us to indicate that the bacilli had grown in all the samples of bouillon containing the different amounts of glucose until the acidity had become so excessive that their further growth was prevented. Those cultures in which the glucose was entirely consumed before the growth of the bacilli was stopped became alkaline as the development of the bacilli continued. We found that cultures in which the acid produced was great enough to stop the growth of the bacilli again developed if sufficient alkali was added. In the bouillon containing excessive amounts of glucose, with each new addition of alkali there would be a new growth of bacilli and a new production of acid, until finally, all the glucose having been used up, the alkaline reaction became permanent and the culture became that of type C. These incomplete experiments indicate that the composi-

tion of the bouillon, and especially its degree of alkalinity, influence greatly the effect of whatever amounts of glucose may be present in the meat.

Summary.

Toxin of sufficient strength to kill a 400-gramme guinea-pig in three days and a half in a dose of 0·025 cubic centimetre developed in suitable bouillon, contained in ordinary Erlenmeyer flasks, within a period of twenty-four hours. In such bouillon the toxin reached its greatest strength in from four to seven days (0·005 cubic centimetre killing a 500-gramme guinea-pig in three days). This period of time covered that of the greatest growth of the bacilli, as shown both by the appearance of the culture and by the number of colonies developing on agar plates.

The bodies of the diphtheria bacilli did not at any time contain toxin in considerable amounts.

The type of growth of the bacilli and the rapidity and extent of the production of toxin depended more on the reaction of the bouillon than upon any other single factor.

The best results were obtained in bouillon which, after being neutralized to litmus, had about seven cubic centimetres of normal soda solution added to each litre. An excessive amount of either acid or alkali prevented the development of toxin.

Strong toxin was produced in bouillon containing peptone ranging from one to ten per cent. The strength of toxin averaged greater in the two- and four-per-cent peptone solutions than in the one-per-cent.

When the stage of acid reaction was brief and the degree of acidity probably slight, strong toxin developed while the culture bouillon was still acid; but when the stage of acid reaction was prolonged, little if any toxin was produced until just before the fluid became alkaline.

Glucose is deleterious to the growth of the diphtheria bacillus and to the production of toxin when it is present in sufficient amounts to cause by its disintegration too great a degree of acidity

in the fluid culture. When the acid resulting from decomposition of glucose is neutralized by the addition of alkali the diphtheria bacillus again grows abundantly. Glucose is not present, at least as a rule, in sufficient amounts in the meat as obtained from the New York butchers to prevent the rapid production of strong toxin if the bouillon is made sufficiently alkaline.

In our experiments, when other conditions were similar, the strength of the toxin was in proportion to the virulence and vigour of growth of the bacillus employed.