THE RELATION OF DEXTROSE TO THE PRODUCTION OF TOXIN IN BOUILLON CULTURES OF THE DIPHTHERIA BACILLUS.

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The publication of certain investigations by Spronck and van Furenhout * in 1895 concerning the inhibitory action of muscle sugar upon the production or accumulation of toxin in peptone bouillon cultures of the diphtheria bacillus was the starting point of a series of investigations into this practically very important subject. Before the publication of this paper I had pursued a similar line of observations. The method employed was to test the amount of muscle sugar present in beef bouillon in the fermentation tube and compare it with the relative toxin production. It was found that the least amount of sugar was associated with the largest accumulation of toxin. Owing to the scarcity of beef containing but traces of sugar the work progressed slowly and did not appear until 1896.† In the meantime Park and Williams ‡ had found that so far as the beef used by them was concerned, the inhibitory action of the muscle sugar could be neutralized by making the bouillon sufficiently alkaline. Cobbett § in a later paper confirms the relations between muscle sugar and toxin production. Blumenthal|| reports upon the use of large quantities of sugar (grape and milk sugar) in cultures of the diphtheria bacillus. His results are wholly unintelligible to me. Among other things he states that lactose is acted upon by diphtheria bacilli, whereas I find that the addition of lactose does not influence the culture whatever. He also

**Annal. de l'Inst. Pasteur*, 1895, ix, p. 758.

† *Trans. Assoc. American Physicians*, 1896, xi, p. 37.

‡ *Journal of Experimental Medicine*, 1896, i, p. 164.

§ *Annal. de l'Inst. Pasteur*, 1897, xi, p. 251.

|| *Deutsche med. Wochenschr.*, 1897, p. 382.

states that sugar bouillon inoculated with diphtheria bacilli becomes "very frequently" acid. This depends upon the bouillon, whether free from muscle sugar or not, and the kinds of sugar added. The chemical changes under like conditions are absolutely constant. Blumenthal goes so far as to vindicate a therapeutic action for sugar because of its supposed inhibitory action on toxin production, a very premature inference, as this paper will show.

Madsen * in an otherwise interesting paper presents nothing new concerning the factors favoring or opposing toxin production.

More recently Martin and Spronck have published methods by which they claim to have produced unusually toxic culture fluids. Martin† prepares his peptones by the self-digestion of the stomachs of swine. The resulting fluid is added to an equal quantity of fermented bouillon. The mixture is heated to 70° C. and then passed through a Pasteur filter. If Martin's method should prove to yield all that is claimed for it, it would be superior to any now in use. A recent careful trial has convinced me that it is liable to fail in producing the looked-for result. I obtained from bouillon prepared in this way a filtrate having from $\frac{1}{5}$ to $\frac{1}{8}$ the toxic power of the filtrate obtained according to the method given below. The fermentation tube revealed the presence of a considerable amount of sugar. Whether this was the cause of the failure I am not prepared to state. That the method may under certain circumstances accomplish all that is claimed for it I will not gainsay, but it does not appear to act uniformly and the results cannot be predicted as with the method to be described. Spronck's ‡ new method utilizes, in place of beef juice, the boiled and filtered extract of the yeast of commerce to which he adds salt and 2 per cent peptone. This fluid yields a toxin 20 times stronger than does the bouillon from decomposed beef, the minimum fatal dose for a 500-gramme guinea-pig being now .005 cc.

The process which I wish to describe is a slow evolution of the past three years. Owing to the necessity of keeping on hand large quantities of diphtheria toxin for practical purposes, the investigations could

* *Zeitschr. f. Hygiene u. Infectionskrankheiten*, 1897, xxvi, p. 157.

† *Annal. de l'Inst. Pasteur*, 1898, xii, p. 26.

‡ *Annales de l'Inst. Pasteur*, 1898, xii, p. 700.

not be pushed rapidly and I contented myself with gradually introducing modifications and carefully noting results. The process cannot be considered essentially new excepting in so far as the minor details here added are absolutely necessary to its success. In the course of the work it was found, contrary to all the views hitherto expressed, *that dextrose is not in itself inimical to toxin production, that a certain quantity is in fact essential to an abundant accumulation of toxin.* It was found that the muscle sugar naturally present in beef and the ordinary chemically pure dextrose, added after the former had been removed by fermentation, act in a quite different manner and that we must assume either that the muscle sugar undergoes a decomposition under the influence of the diphtheria bacillus different from that which ordinary dextrose undergoes, or else that there are other still unknown inhibitory substances in the beef which are removed with the muscle sugar during the preliminary fermentation. Leaving a discussion of the experiments which demonstrated this peculiar behavior of unfermented bouillon aside for the present I will give a description of the process as at present in use.

PREPARATION OF PEPTONE BOUILLON FOR TOXIN PRODUCTION.

It should be stated at the outset that now and then bouillon containing a little muscle sugar will yield a toxin as strong as bouillon specially prepared. This outcome cannot be predicted however. Following the suggestions of Park and Williams that the difficulty can be overcome by the increased alkalinity of the bouillon I have returned again and again to unfermented bouillon without obtaining so good results as with the new method. Until the beef used in different localities has been compared, these discrepancies cannot be explained satisfactorily.

1. The beef infusion is prepared in the usual way and kept in the cold for 12 to 24 hours. The beef juice is then expressed, and its reaction, which will in general be found to vary from 3 to 4 per cent acidity,* must be reduced to 1.5 to 2 per cent by the addition of normal sodium carbonate solution. The fluid is then heated to 40° C., inoculated with

* See below for a definition of these terms.

30-50 cc. of a 12- to 24-hour bouillon culture of *B. coli* and placed in the incubator for 16 hours or over night. Next morning the acidity will have risen again to 3-3.5 per cent and a scum may have formed on the surface. The odor varies and may be distinctly sour or slightly putrefactive.

2. The fermented infusion is next mixed with the white of egg in the proportion of one egg to a litre of infusion, and boiled in a water-bath or an Arnold sterilizer for 45 to 60 minutes.

3. The boiled infusion, previously cooled off to favor any precipitation, is filtered and then receives 2 per cent Witte peptone, 0.5 per cent common salt, and after these have been dissolved by gentle heat, enough normal sodium carbonate solution to bring the acidity down to about 0.8 per cent. The fluid is boiled or steamed again for 20 or 30 minutes and then filtered. The reaction may become slightly more acid than the calculation allows but no further addition of alkali is necessary.

4. The filtered fluid is distributed into Fernbach flasks in shallow layers 2.5 ctm. deep and autoclaved (at 110° to 115° C. for about 30 minutes).* Each flask should have 2 or 3 cotton-plugged openings to facilitate ventilation.

5. Before inoculation with the diphtheria bacillus 5 cc. of a sterile 20 per cent solution of dextrose or about 0.1 per cent (autoclaved and kept on hand in small tubes) is added per litre.†

6. The culture employed should form membranes promptly and leave the fluid clear. This property can be induced in freshly isolated cultures by 5 to 10 transfers in bouillon of the kind here described. These can be made in large test tubes kept in an inclined position to increase the surface area.

7. The culture fluid becomes distinctly alkaline to phenolphthalein in from 6 to 8 days and may then be regarded at its maximum toxicity.

Before proceeding to a discussion of the more essential points—the relation of dextrose and peptone to the accumulation of toxin—a brief explanation of the minor details of this method will be in order.

* The necessity for autoclave sterilization was pointed out by me in *Journal of Experimental Medicine*, 1898, iii, p. 647.

† Several trials have shown that the efficiency of the bouillon is not impaired by adding the dextrose before the final autoclaving. This would materially simplify the work and reduce the chances of contamination. The amount of sugar has been increased by me to .15 and even to .2 per cent without interfering with rapid alkali production. In some instances the toxin was markedly increased, in no instance reduced in amount. For different bacilli the most favorable quantity should be determined by trials.

The process of preparing dextrose-free bouillon was first described by me in 1897.* Subsequently Martin† used the same process but substituted yeast, or left the fermentation to be accomplished by the miscellaneous bacteria already in the infusion.‡ The first method suggested by Spronck I found inadequate. The bouillon prepared from the old beef frequently contained large quantities of fermentable substance and was never entirely free from it. The process here suggested produces a bouillon which permits no growth whatever in the closed branch of the fermentation tube. It is in fact almost wholly free from reducing substances. While the ordinary bouillon, even when gas is not produced in it, still contains enough reducible substances to decolorize methylene-blue in the fermentation tube over night, the bouillon thus prepared does so only after 3 or 4 days in the incubator.§ In order to obtain this result, however, it is necessary to reduce the initial acidity of the raw infusion as directed, otherwise the additional acid formed during the fermentation may become inhibitory. It is also necessary to warm the infusion if large quantities are prepared, otherwise 3 or 4 hours will be lost and the infusion may still contain acid-forming substances next morning. The fermented infusion even after prolonged boiling forms such a loose clot that the filtration may become exceedingly tedious. To obviate this, egg-albumen should be added. Careful tests showed that the dextrose added in the egg-albumen cannot be recognized in the finished bouillon and is therefore a negligible quantity. The other parts of the process need no special explanation. The initial acidity recommended has been found the best level from which to start. The reason for the final addition of about 0.1 per cent dextrose will be given farther on.

With this method in use there has been no noticeable fluctuation in the toxic strength of the culture fluid after 6 to 8 days' incubation. The end reactions are in all cases absolutely the same excepting in flasks accidentally contaminated. In fact the production of toxin has

* *Journal of Experimental Medicine*, 1897, ii, p. 543, and 24th Annual Report of the State Board of Health of Mass. (a comparative study of the toxin production of diphtheria bacilli) issued October, 1897.

† Loc. cit.

‡ It might be supposed that the fermentation would lead to the production of various toxins. Repeated injection of 5 cc. of the finished product into the peritoneal cavity of guinea-pigs had no effect whatever.

§ Th. Smith, Reduktionserscheinungen bei Bakterien, etc., *Centralbl. f. Bakt.*, 1896, xix, p. 181.

been brought to the level of a chemical process. Formerly, the peptone was frequently suspected of being at fault, but the uniform results now obtained indicate that this suspicion was unfounded. The culture employed in these investigations, with the exception of certain final tests to be described farther on, is the one used by Park and Williams in their investigations and denominated by them "No. 8." The efficiency of the method is most convincingly shown by a record of the toxic strength of the culture filtrate. Park and Williams in their article state that .005 cc. of their strongest toxin proved fatal to a 500-gramme guinea-pig in 3 days. Martin states that the m. f. d.* for a 500-gramme guinea-pig of the strongest toxin he obtained with the same bacillus was .002 cc. In another place he mentions the dose of .005 cc. for a 500-gramme pig. The following consecutive record of diphtheria toxins prepared according to the procedure described shows the unvarying results obtainable. The test was made in all cases from mixtures of about 4 litres each of filtered culture fluid, either immediately after filtration or some months later:

Lot	1.	.01 cc.	fatal to	318-gramme	pig	in	28	hours.	
"	2.	.01 cc.	"	362	"	"	36	"	±
		.0035 cc.	"	257	"	"	"	"	±
		.0025	"	253	"	"	60	"	±
		.0023	"	256	"	"	5½	days.	
"	3.	.01 cc.	"	322	"	"	36	hours	±
"	4.	"	"	276	"	"	36	"	+
"	5.	.01 cc.	"	300	"	"	28	"	
		"	"	"	"	"	36	"	±
		.005	"	309	"	"	40	"	
"	6.	.01	"	376	"	"	"	"	±
		.005	"	432	"	"	48	"	—
"	7.	.01	"	356	"	"	24	"	
		.005	"	312	"	"	36	"	±
"	8.	.01	"	360	"	"	"	"	±
		.005	"	378	"	"	54	"	
"	9.	.005 cc.	"	371	"	"	36	"	±

* Abbreviation for minimum fatal dose.

Lot 1.	0	.01 cc.	fatal to	405-gramme	pig	in	57	hours.
"	11.	"	"	390	"	"	$5\frac{1}{4}$	days.
"	12.	"	"	250	"	"	36	hours ±
"	13.	"	"	378	"	"	30	" ±
		"	"	383	"	"	"	" ±
"	14.	"	"	365	"	"	54	" ±
		"	"	365	"	"	56	" ±
"	15.	"	"	442	"	"	60	" ±

In Lots 1 and 2 the 2 per cent peptone was added from a sterile solution just before inoculation. In lot 13 there was still some muscle sugar. In lot 11 the dextrose was added *before* the final autoclaving.

In estimating the absolute toxicity of culture filtrates the relative susceptibility of the guinea-pigs used must be taken into consideration. Animals from some sources seem to be much more susceptible to diphtheria toxin than those from others. For several years the writer had been experimenting only upon guinea-pigs reared under his supervision. During this time all animals used exhibited a remarkably uniform susceptibility. Latterly guinea-pigs purchased from a dealer had to be used and it was soon evident that for them the m. f. d. was about $\frac{1}{2}$ to $\frac{2}{3}$ of that to which the home-bred pigs succumbed. Similar differences were noticed when toxin-antitoxin mixtures in which there was a slight excess of toxin were injected. Many of the animals used by the writer for breeding purposes had passed through a single inoculation with toxin or toxin plus antitoxin at least 3 or 4 months previously. Recently Behring * states that he uses such guinea-pigs in the same way but has not noticed any increased resistance in the progeny. He states furthermore that Ehrlich obtained from a breeder a race of diphtheria-immune guinea-pigs and that in England these animals present a considerable degree of resistance to diphtheria toxin. The more susceptible animals used by the writer were as a rule thin and had a thin skin, while those raised for the laboratory had a thicker skin and were in excellent condition. This possible variation in the resistance to the diphtheria toxin must first be taken into account before we can positively decide which method may give the strongest

* *Deutsche med. Wochenschr.*, 1898, p. 621.

toxin. However, with the figures quoted above and those to follow as a basis there seems little to choose between Martin's complicated and not certain method and the simple one I have described.

THE RELATION OF DEXTROSE TO THE REACTION CURVE OF PEPTONE BOUILLON AND TO TOXIN PRODUCTION.

The daily changes in the reaction of the culture fluid are perhaps the best available indications of the activity of the bacilli and of the toxin production. In order to follow this change closely without disturbing the surface membrane which forms within 24 hours the following procedure was adopted:

A large Fernbach flask* (Figure 1, reduced to $\frac{1}{3}$ size) within which a litre of bouillon occupies a layer 2.5 ctm. deep, is modified so as to have 3 cotton-plugged openings. Through one lateral opening a siphon (*A*) passes which has joined by means of rubber tubing to its lower free end a protected mouth-piece according to Maassen (*B*). The other lateral opening may have in it a small funnel (*C*) through which alkalies, acids or other fluids may be added without disturbing the larger plug or breaking the surface membrane. The three openings are also very favorable to free ventilation. The flask after inoculation is placed on a shelf in the thermostat so that the longer arm of the siphon may pass through a hole in the shelf. From day to day or oftener if desired fluid may be removed for various tests without disturbing the flask or imperiling the purity of the contents. Care should be taken to reject the fluid in the siphon as it has been under anaërobic conditions since the former withdrawal of fluid. The samples thus obtained were titrated with phenolphthalein as an indicator according to the method suggested by Fuller† and the values obtained are expressed throughout this article in per cent of a normal solution of acid or alkali. The sign minus (—) whenever used signifies acid, the sign plus (+) alkaline reaction toward phenolphthalein.‡

* Made for the writer by Whitall, Tatum & Co., N. Y.

† Procedures recommended for the study of bacteria, Concord, N. H., 1898, p. 19. See also *Journ. Amer. Public Health Assoc.*, 1895, p. 386.

‡ This use of the signs is contrary to the notation adopted by the committee which edited the "Procedures," etc., and of which the writer was a member. My reason for the use of + for alkalinity is that all aërobic bacteria, both obligatory and facultative, tend normally towards an alkaline reaction. The tendency towards an acid reaction is in a sense abnormal and, if continued, destroys the organisms producing it. This notation was adopted after careful deliberation as being more logical.

The presence or absence of sugar in the finished bouillon was determined with the aid of the fermentation tube and *B. coli*. The rise in acidity of the fluid in the closed branch, whether gas appears or not, is an indication of the presence of dextrose or allied substances (excluding glycogen). The amount of acid produced is proportional to the amount

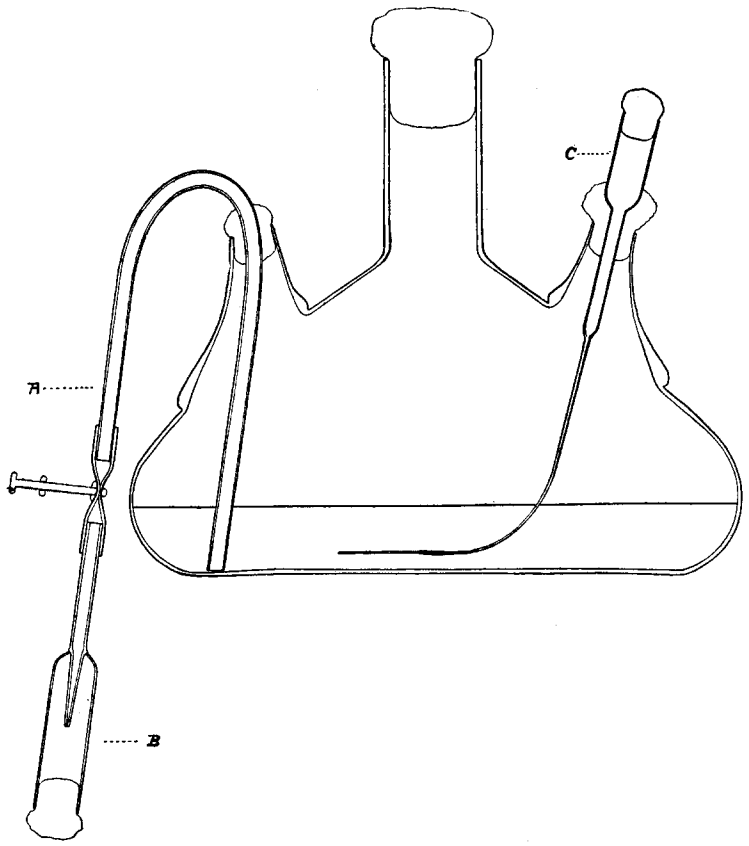

Fig. 1.

Fernbach Flask, modified by the writer. Figure reduced to ⅓ size.

of sugar present up to a certain limit varying for different bacteria and probably never exceeded by the sugar in beef broth. A total absence of sugar is indicated by an absence of growth in the closed branch, *B. coli* (as well as other facultative anaërobes) becoming obligatory aërobes when sugar is absent.

As stated in the introduction, it has been generally assumed that the amount of acid formed by diphtheria bacilli in presence of muscle sugar is responsible for the feeble toxin production. In the course of these studies I was early convinced that these bacilli in their multiplication can without trouble produce and neutralize much larger quantities of acid derived from dextrose, artificially added, than are as a rule formed in unfermented bouillon. In order to attempt an explanation of this paradox, it became necessary to learn what strength of acid is injurious to the toxin. As a preliminary test dextrose was added to a full-grown alkaline culture to see what effect the acids, produced by the diphtheria bacillus itself, had on its toxin.

I. February 5, 1897. To a 10-day litre culture with reaction + 0.2 (*i. e.* nearly as strong an alkaline reaction as such cultures can attain) of which .01 cc. is fatal to a 340-gramme guinea-pig in 2½ days, enough sterile dextrose solution is added to make a one per cent solution.

February 7. A good membrane has formed in place of the former one shaken down February 5.

February 10, reaction—4.77.

" 13, 0.03 cc. produces no longer any local effect.

" 17, 0.5 cc. " " " " " "

" 18, reaction as on February 10, no change.

" 23, one cc. of this culture, which had been filtered and stored in the cold carefully neutralized with NaHO has no effect on a guinea-pig.

II. February 17, 1897. To a 22-day culture of another diphtheria bacillus of which the m. f. d. is now 0.04 cc. one per cent dextrose is added.

February 18. Renewed multiplication.

" 19. Complete membrane.

" 23. Reaction, —4.55; 0.5 cc. has no effect on a guinea-pig.

" 27. Subculture remains sterile.*

These experiments show that with the two cultures tested the maximum acidity does not exceed 4.5 to 5 per cent. 5 or 6 days after the beginning of renewed growth and acid formation the toxin present at the start is completely destroyed. Later the bacilli themselves are killed.

* Cobbett (l. c.) states that diphtheria bacilli are *not* killed by the acids they produce, while colon and other bacilli are so destroyed.

It now became necessary to determine the effect of different degrees of acidity within the maximum. This could be most expeditiously done by adding to the finished and filtered toxin certain acids in known quantities. For this purpose lactic acid and chlorhydric acid were chosen. The toxin employed contained about 0.2 per cent carbolic acid. The acidified toxin was kept in partly-filled, cork-stoppered bottles in the thermostat to imitate as nearly as possible the usual conditions of the culture.

I. M. f. d. of toxin about 0.04 cc. Lactic acid added to an acidity of 2 per cent. After 1, 6 and 13 days no appreciable loss in toxicity.

II. M. f. d. of toxin 0.036 cc. Lactic acid added to — 4.4 per cent. After 24 hours .08 cc. produced only local necrosis. After 3 days 0.3 cc. no longer fatal. After 6 days 1 cc. produces only transitory œdema.

III. The same toxin (m. f. d. = .036 cc.) receives chlorhydric acid to — 4.47 per cent. After 6 days 1 cc. produced severe necrosis locally.

IV. This and the following test were made a year later. M. f. d. of toxin .032 cc. Lactic acid was added to produce reactions of — 3.5, — 4, — 4.5 and — 5 per cent. Actual acidity found to be — 3.66, — 4.15, — 4.65, — 5.12 per cent.

After 5 days none of the 4 acidified toxins produced any local lesion in doses of 0.064 cc. After 11 days 0.5 cc. of lowest acid toxin (— 3.66 per cent) had no effect.

V. The same toxin brought to an acidity of 2.8 and 3.2 per cent with lactic acid was tested after 5 and 9 days. The m. f. d. of the first toxin after 5 days was about .045 cc., after 9 days 0.1 cc. The second toxin produced only a slight local effect in a dose of 0.06 cc. after 5 days. After 9 days 1 cc. still produced local necrosis.

The m. f. d. of the control toxin rose in 5 days from .032 cc. to .045 cc.; in 9 days to .06 cc.*

These tests though incomplete in many respects indicate that an acid reaction of 2.5 to 3 per cent destroys the toxin only very slowly, while above 3 per cent the destruction is more rapid. A reaction of

* The destruction of toxin in cultures provided with protecting bacillar membranes does not proceed so rapidly as this in the thermostat. From an earlier experiment the following figures may be quoted:

After	8	days of growth	0.02	cc.	fatal to a	410	gramme	guinea-pig	in 2	days.
"	34	"	0.02	"	"	435	"	"	3	"
"	56	"	0.04	"	"	305	"	"	3½	"

— 3.5 per cent or above is quite rapidly destructive. The maximum amount of acid produced by most diphtheria bacilli (4.5 to 5 per cent),

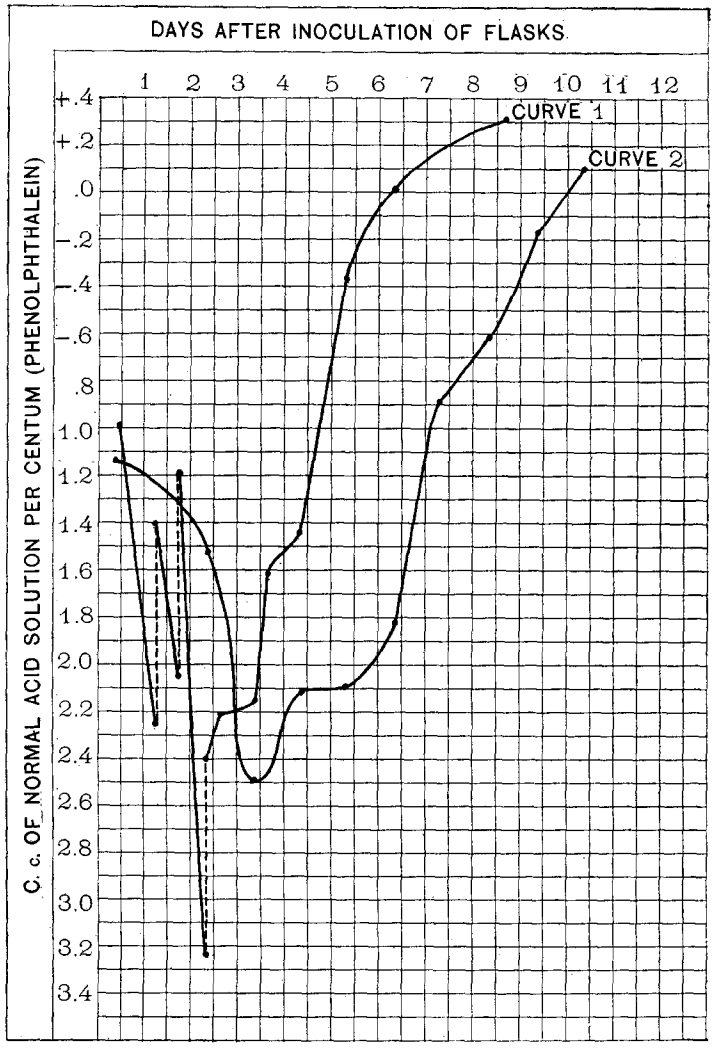

FIG. 2.

which is destructive to both toxin and bacilli, is equivalent to .164 to .182 per cent pure chlorhydric acid.

The quantitative production of acids in ordinary, unfermented

bouillon varies considerably, but it rarely rises above 3 per cent if the initial acid reaction is fairly low (0.8 to 1 per cent). It is difficult,

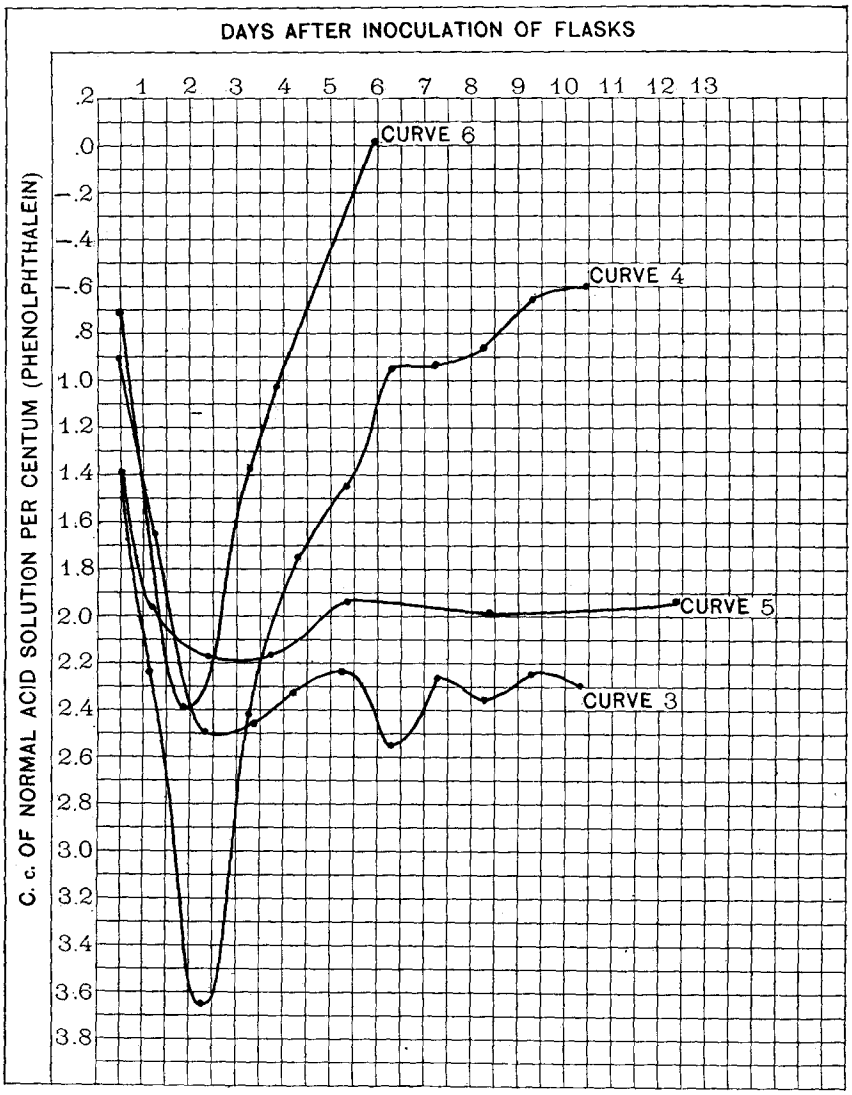

FIG. 3.

therefore, to harmonize the inhibitory power of these acids, if it really exists, with the figures quoted above—that an acidity of 2.5 to 3 per

cent is only slowly destructive. On the other hand a higher temporary degree of acidity is compatible with an abundant accumulation of toxin when the acids are derived from dextrose added. From among the many experiments made the following are selected as illustrating the rapid acid production in presence of both muscle sugar and dextrose, the rapid alkali production in presence of large quantities of the latter, and the marked difference in the final accumulation of toxin in bouillon containing muscle sugar and in that containing only ordinary dextrose:

June 27, 1897. 1200 cc. of peptone bouillon containing still a very small quantity of muscle sugar (about .02 per cent) receives 18 cc. of a sterile 20 per cent solution of dextrose, or about 0.3 per cent.

June 28. Initial acidity 1 per cent. Inoculated with diphtheria bacilli.

" 29. 8.30 A. M. Membrane present; acidity 2.23; 10 cc. normal NaHO added.

" 29. 5.30 P. M. Acidity 2.16; 10 cc. NaHO again added.

" 30. 8.30 A. M. Acidity 3.23; 10 cc. NaHO again added.

" 30. 4.30 P. M. Acidity 2.22.

July 1. 9 A. M. " 2.16.

" 1. 5.30 P. M. " 1.62.

" 2. 9.40 A. M. " 1.46.

" 3. 9 A. M. " 0.38.

" 4. 9 A. M. " 0.00.

" 6. 3 P. M. Alkalinity 0.3. Culture pure; 0.04 cc. fatal to a 300-gramme guinea-pig in 36 hours.

" 14. 0.04 cc. fatal to a 313-gramme guinea-pig in 36 hours.*

At this time the best toxin obtained from this bacillus, *i. e.* before the present method had been perfected was about .015 cc. for the m. f. d. for a 300-gramme guinea-pig. The above inoculations indicate a m. f. d. of .02 cc. The curve of this culture is platted as Curve 1, Fig. 2, in which the dotted line indicates the reduction in acidity

*In this experiment the alkali was added to prevent the acidity from ascending to the inhibitory and destructive limit. The experiment was primarily conceived to determine whether the bacterial metabolism in the presence of large quantities of dextrose would be inimical to toxin production.

brought about by the added alkali. The most favorable condition encountered with ordinary unfermented bouillon is shown by Curve 2, Fig. 2. The amount of muscle sugar was about 0.15 per cent. The reaction returned quite promptly to the neutral point and the toxicity of the bouillon was about .02 cc. But this course is not to be anticipated and may in fact be exceptional. Curve 3, Fig. 3, has been a more common type with beef used in this laboratory. The bouillon contained 1 per cent peptone and about 0.15 per cent muscle sugar. The initial acidity was reduced with alkali to 0.8. After inoculation the acidity rose to 2.45 where it remained for 14 days. On the 17th day the toxicity was about 0.09 cc.

Curve 4, Fig. 3, represents the course of a culture in dextrose-free bouillon containing 2 per cent peptone and about 0.18 per cent dextrose added before inoculation. On the 3d day the acidity had risen to 3.6. On the 5th day, when the acidity was still 1.44, the toxicity was 0.015 cc. On the 10th day it was 0.01 cc.

The following parallel tests with bouillon from the same beef, one lot fermented with *B. coli*, the other not, are still more demonstrative.

I. Dextrose-free bouillon receives in sterile solutions 1 per cent peptone and 0.6 per cent dextrose. Initial reaction — 1.4. After 7 days, reaction feebly alkaline to phenolphthalein, m. f. d. about .008 cc.

II. The unfermented bouillon containing about 0.1 per cent muscle sugar receives 1 per cent peptone. The initial reaction is — 1.4. After inoculation the acidity rises to — 2.13 and remains there for 20 days (Curve 5, Fig. 3). Toxicity at this time about 0.06 cc.* It should be noted that the bouillon used contained only one per cent peptone.

From these few illustrations among many it is evident that the amount of acid formed in ordinary peptone bouillon is not sufficient to account for the marked interference with growth, for when dextrose is added to fermented bouillon the acidity may be much greater during the first 2 days as illustrated by Curve 6, Fig. 3. There is, how-

* The inoculations were as follows:

I. 0.01 cc. fatal to 289-gramme guinea-pig in 44 hours.

II. 0.05 cc. produces local necrosis in a 289-gramme guinea-pig. After 17 days guinea-pig weighs 355 grammes.

ever, a prompt production of alkali which makes the curve of these cultures quite acute in outline. The cultures in unfermented bouillon usually languish at a comparatively low degree of acidity and the accumulation of toxin is correspondingly light.

In searching for the cause of this peculiar inhibition of the growth of the diphtheria bacillus in ordinary bouillon it occurred to me that possibly the glycogen of the muscular tissue might be responsible. This factor however was eliminated by a single experiment. The presence of glycogen had no effect upon the reaction curve of diphtheria cultures. The cultures proceeded as in dextrose-free fermented bouillon. In other words, diphtheria bacilli do not attack glycogen as they do dextrose.

Very many experiments have been made during the past two years to determine the influence of adding peptone and alkali before and after the first boiling of the beef infusion, also the behavior of peptone added before the final autoclaving and after it in sterile solution. It was thought that possibly the interaction of the different substances in presence of carbohydrates or a slight excess of either acid or alkali might produce modifications of the peptones or other substances sufficient to influence the production of toxins favorably or unfavorably. These experiments were made with the same beef infusion, each set of flasks having their contents modified in some way. Without going into detail concerning these tedious trials, it may be stated that if a bouillon gives rise to much toxin when prepared according to one method, it will yield equally goods results whatever be the order of neutralizing or adding the various ingredients. With reference to the addition of dextrose I may state that it largely disappears when added to the raw infusion. When added to the boiled and filtered infusion it is not lost.

Bouillon may even remain markedly acid during the preparation without losing its toxin-producing capacity, provided the acidity be properly reduced before use. In one instance the final acidity through some oversight was left at 3 per cent. After a reduction to 0.7 per cent with 23 cc. normal soda solution per litre and the addition of 0.12 per cent dextrose in sterile solution the fluid was inoculated. After 6 days the toxicity

was .007 cc. The course of the reaction is shown by Curve 6, Fig. 3. A duplicate flask yielded the same toxin.*

The unusually good results obtained with the dextrose-free peptone bouillon might reasonably raise the query whether the miscellaneous bacteria, including *B. coli* in the beef infusion, may not produce some substance from which toxin is easily obtained by diphtheria bacilli. It has already been stated that the infusion is considerably altered during the fermentation since the coagulation formed by boiling fails to cohere as in fresh infusion and renders filtration very difficult. Bouillon prepared in this way without peptone and tested parallel with that to which 2 per cent peptone had been added produced barely 1 per cent of the toxin found in the peptonized fluid, *i. e.* while .01 cc. or less of the peptonized bouillon proved fatal to guinea-pigs, 1 cc. of the peptone-free bouillon contained only a trace of toxin in spite of good growth, membrane formation, and final alkalinity. Nor does such bouillon give rise to any indol when indol-producing bacteria have multiplied in it. We are, therefore, justified in concluding that the fermentation does not yield any substance available for toxin production, but simply eliminates some inhibiting substance from the bouillon while the true source of the toxin is the peptone added to it.

OTHER FACTORS MODIFYING TOXIN PRODUCTION.

Besides the presence or absence of muscle sugar as a factor in the production of diphtheria toxin, there are several others which have a

* In one experiment made recently the fermented bouillon was simply left acid and without dextrose. The object was to obtain thereby the same amplitude or range of reaction otherwise secured by making the bouillon more alkaline and adding dextose which furnishes the acids. The result was as good as when the latter method is employed. Thus a flask of 2 per cent peptone bouillon with an initial reaction of — 2.2 yielded on the 9th day an alkaline fluid whose m. f. d. was about 0.004 cc. (compare with Table I, p. 391, with which this test was made). Whether this procedure would be always successful and capable of taking the place of the alkali plus dextrose can only be decided after repeated trials. Another modification of the process consisted in the fermentation of the boiled and filtered broth instead of the raw infusion. The peptone was added after the bacteria (B. coli and others) had been eliminated by boiling and filtration. This modification, tried but once, also yielded a strong toxin.

distinct influence and which, differently employed by different observers, prevent any very accurate comparison of published results. Among these the most important are:

1. The amount of peptone used.
2. The manner in which the stock cultures have been kept.
3. The oxygen supply (character of the culture flask and the depth of the layer of fluid).

Other possible modifying influences, such as the method of preparing and sterilizing the bouillon and the initial reaction do not in my experience have any marked influence so long as the reaction attained by the culture does not reach the inhibitory limit above — 3.5 per cent.

The amount of peptone.—It is a well-known fact that of the peptones added to culture media only a small amount is utilized by bacteria. For a number of years the writer used only ¼ per cent peptone for culture media. The return to one per cent was simply to conform to current methods. In the production of diphtheria toxin 2 per cent has been used by some, 1 per cent by others. A number of special trials have been made in combination with the present method of preparing the bouillon, to determine the relative efficiency of different amounts of peptone.

The same dextrose-free bouillon, placed in thin layers, 2-2.5 cm. deep, in Erlenmeyer flasks, receives (*a*) 0.5, (*b*) 1, and (*c*) 1.5 per cent peptone. The initial reaction is — 0.87, — 0.91, and —0.8 respectively. Each flask receives 0.1 per cent dextrose and is then autoclaved. On the ninth day after inoculation the fluid is alkaline in all flasks.

(a).	.01 cc.	fatal to	240-gramme	guinea-pig	in	1½	days	±,	m. f. d. .005 cc.
	.005 cc.	"	254	"	"	" 3¾	"		
(b).	.008 cc.	"	235	"	"	" 1½	"	±	" .0025 cc.
	.003 cc.	"	300	"	"	" 1¾	"		
(c).	.005 cc.	"	268	"	"	" 1½	"	±	" .0025 cc.
	.003 cc.	produces induration only.							

Leaving aside the result of the last test as quite irregular, we notice the large amount of toxin produced in bouillon containing but 0.5 per cent peptone. The difference between 1 and 1.5 per cent peptone may be regarded as trifling. A second experiment was made subse-

quently in which not only the peptone but also the dextrose and the stock culture were varied. The other conditions remained the same. The cultures, which were derived from the same original stock (Park and Williams), had the following history:

α. Grown in bouillon for a number of years.

β. Grown on Löffler's (horse) serum for 9 months, before that in bouillon.

γ. Grown on serum for 18 months before that time in bouillon. β and γ were passed through two tubes of bouillon before use. The bouillon was fermented.

TABLE I.

Culture.	Peptone in per cent.	Dextrose in per cent.	Initial reaction.	Reaction (after 9 days).	Toxicity (after 9 days).	Estimated m. f. d. for 250-280-gramme guinea-pig.
a	1	0.1	−1.	+.2	.008 cc. fatal to 273-gramme guinea-pig in $1\frac{3}{4}$ days ±	.005 cc.
					.008 cc. " 255 " " " " " ±	"
β	1	0.1	−1.	+.2	.008 cc. " 273 " " " 3 "	.007
a	2	0.2	−.95	+.05	.008 cc. " 284 " " " 31 hours−	.002 +
β	2	0.2	−.95	+.15	.008 cc. " 284 " " " 31 " +	.003 −
a	2	0.1	−1.	+.05	.005 cc. " 301 " " " $3\frac{3}{4}$ days	.005 −*
					.008 cc. " 280 " " " $1\frac{3}{4}$ " ±	.005
γ	2	0.1	−1.	+.15	.008 cc. " 280 " " " $1\frac{3}{4}$ "	.005

*Test on 7th day.

Table I shows that with a suitably prepared bouillon the accumulation of toxin in the presence of 1 per cent peptone may be nearly, if not quite as great, as in the presence of 2 per cent. Other tests not here described taken together with these have convinced the writer that probably 1.5 per cent peptone is as efficient as 2 per cent in bouillon prepared as herein detailed. The large amount of dextrose used up by this bacillus in presence of 2 per cent peptone and the conse-

quent increase in the toxicity of the culture fluid is well shown in the 3rd and 4th lines of the table.

The stock culture.—The favorable influence of the continued growth in bouillon is evident from the preceding experiment and deserves careful attention. Bouillon cultures of diphtheria bacilli undergo certain changes when, as first suggested by Park and Williams, the bacilli are transferred at short intervals from tube to tube. Those which I have treated in this way grow at first diffusely through the bouillon and only a very faint pellicle appears on the surface after some days. If the cultivation be continued, the membrane becomes heavier and the tendency to a diffuse clouding becomes more or less checked. If such culture be shaken up after a growth of 4 or more days and compared with one inoculated directly from serum, it will be found full of flakes and of a decidedly yellowish tinge as compared with the uniformly turbid original. The color approaches that of the bouillon because the flakes disperse the light less than the fine powdery suspension in the original culture. A tendency to cohere is developed in the bouillon, which tendency favors surface growth. This condition is favorable to toxin production, especially in bouillon made from unfermented beef. In fermented bouillon the difference is less marked. The great advantage of the latter bouillon appears when cultures are made of bacilli grown on Löffler serum and those freshly isolated from the throat whose capacity to grow on the surface is restricted. This is the only explanation that can be found at present for the many failures to obtain strong toxin some years ago when the work of preparing antitoxin was started with ordinary bouillon. Martin obtained strong toxin with his new method from fresh cultures. The two additional cultures I have tested show equally satisfactory results. Both were isolated in 1896. One of them, No. 14, of a series of 42 cultures * was the best toxin producer of the series at that time when Spronck's method of using old beef was still used. Early in 1897 the m. f. d. of a one per cent peptone-bouillon culture for a 300-gramme guinea-pig was 0.036 to 0.04 cc. In February, 1898, the test of a fresh culture yielded a m. f. d. of 0.036 cc. In

* *Twenty-fourth Ann. Rep. Mass. State Board of Health*, p. 543.

June another culture yielded a m. f. d. of 0.03 cc. From that time until October this bacillus was passed through bouillon every 4 days to improve the growth in membrane which had always been rather feeble. The toxin then produced in a bouillon containing 2 per cent peptone, filtered and stored but not tested until 4 months later, had the surprisingly low fatal dose of about 0.007 cc. The culture was then returned to Löffler's serum until March, 1899, when, after a passage through 3 tubes of bouillon it yielded a m. f. d. of 0.012 cc.

The second bacillus, No. 12 of the same series, yielded in 1896 and 1897 with Spronck's method a m. f. d. of 0.04 cc. to 0.45 cc. It has been grown continuously on Löffler's (horse) serum. In March, 1899, after a passage through 4 tubes of bouillon its toxicity was tested together with bacillus No. 14. It yielded a toxin having a m. f. d. of .02 cc. for a 260-gramme guinea-pig. More recently this figure was brought down to .015 cc. as shown in Table II below.

The influence of continuous cultivation in a favorable bouillon is illustrated in some recent tests with bacillus No. 14. Three cultures were used in the comparative test:

a. Grown as described above (about 4 months on bouillon, then about 6 months on serum, then for about 15 days on bouillon).

b. Grown for about 15 days on bouillon, before that on serum.

c. Grown for about 24 hours on bouillon, before that on serum.

The three cultures were then inoculated into fermented bouillon containing 2 per cent peptone and 0.1 per cent dextrose and the fluid tested after 10 days' growth:

a. 0.02 cc. fatal to 255-gramme guinea-pig in $1\frac{5}{6}$ days—m. f. d. .012.
b. 0.02 cc. " 263 " " " $3\frac{4}{5}$ " " " .02.
c. 0.02 cc. " 267 " " " $2\frac{3}{4}$ " " " .018.

The influence of the prolonged culture in bouillon had not been wiped out by the succeeding cultivation on serum in (*a*), for it produced a toxin about 50 per cent stronger than the bacillus grown on serum alone.

The marked increase in the toxicity of the bouillon cultures of these two bacilli (No. 12 and No. 14) since their isolation in 1896, is attributable in part to a doubling of the quantity of peptone, in part to the acquired power of surface growth, and in part to the thorough

removal of the muscle sugar and the addition of dextrose. The last factor evidently puts the bouillon into the most favorable condition for toxin production while the others aid in hastening it. This is of no small importance when we consider that in the thermostat there appears to be a continuous destruction of toxin going on side by side with its production. By intensifying and, therefore, shortening the life of the culture, the flasks can be removed after 6 to 10 days, according to the bacillus used, and much toxin thereby saved from destruction.

In Table II a final illustration of the relative influence of these several factors is given.

The culture used is No. 12, which had never been grown in bouillon, and which was, therefore, better adapted for this experiment than the culture of Park and Williams, whose membrane-forming power had been developed by them some years ago. The letter α stands for the continuous cultivation in bouillon through 17 transfers at intervals of 4 or 5 days, β for the same bacillus grown on Löffler's serum since its isolation until 24 hours before use when a bouillon culture was prepared. The bouillon used was deprived of its muscle-sugar by fermentation and some dextrose was added. The cultures were in Erlenmeyer flasks as for the experiment in Table I. In the α flasks after inoculation the membranes formed promptly and became heavy. The fluid remained clear. In the β flasks, on the other hand, the membranes were quite feeble and the bouillon well clouded throughout.

As a result, alkali production was much more rapid in the membrane-forming cultures than in the others, since it took the latter 15 days to reach the point probably attained by the former in 6 or 7 days. Nevertheless the accumulation of toxin did not go parallel to alkali production in the flasks containing one per cent peptone, for the toxicity was the same for the alkaline and the still acid culture on the 9th day. The relatively large amount of toxin in the 4th flask was probably due to the fact that on the 7th day a fairly good membrane had formed. After the 9th day a better membrane appeared on the 3d flask and that on the 4th partly subsided. As a result, the 4th flask lost while the 3d gained in toxin as shown by the second test made on the 15th day. The uncertain yield of bacilli without de-

cided membrane-forming capacity as well as the disadvantages of prolonged stay in the thermostat are here unexpectedly demonstrated.

TABLE II.

Culture used.	Peptone in per cent.	Dextrose in per cent	Initial reaction.	Reaction (after 9 days).	Reaction (after 15 days).	Bacillus No. 12. (Toxicity after 9 days, with * after 15 days.)	Estimated m. f. d. for 250-280-gramme guinea-pig.
α	2	.11	−.75	+.5	—	.02 cc. fatal to 411-gramme guinea-pig in 3½ days	.015 cc.
α	1	.11	−.8	+.5	—	.25 cc. nearly fatal to 383-gramme guinea-pig. Large slough	.025
β	2	.11	−.75	−.65	+.1	.05 cc. fatal to 268-gramme guinea-pig in 6½ days	.05
						.03 cc. produces moderate necrosis only	
						*.04 cc. fatal to 278-gramme guinea-pig in 5 days	.04
β	1	.11	−.8	−.7	+.1	.03 cc. " 353 " " 4½ "	.025
						*.04 cc. " 267 " " 2½ "	.036

It must not be inferred from these experiments that the membrane-forming bacillus is capable of overcoming the obstacles inherent in unfermented bouillon. The most favorable conditions under which the bacillus of Park and Williams, grown continuously in bouillon, may multiply in unfermented bouillon do not bring the toxicity up to the concentration obtainable in fermented bouillon to which dextrose has been added. This is clearly shown by the following tests of 5 different lots of unfermented bouillon recently made under the same conditions as those governing the experiment in Table I. The acid reaction was in all cases reduced by adding normal Na_2CO_3. The flasks were inoculated with the culture kept growing in bouillon only. The column headed " Muscle-sugar test " indicates the acidity of the closed branch of the fermentation-tube culture of *B. coli* over and above the acidity of the sterile bouillon. Allowing an increase of

acidity of 1 per cent for 0.1 per cent of sugar* it will be seen that the amount is between 0.09 and .17 per cent—not more than the amount of dextrose that is easily managed and utilized by diphtheria bacilli.

TABLE III.

Designation of bouillon.	Muscle-sugar test.	Initial reaction.	Final reaction after 11 days.	Result.
A	1.3	−.85	+0.	.02 cc. fatal to 244-gramme guinea-pig in 1¾ days ± m. f. d.=.012 cc.
B	1.1	−8.5	−2.	.02 cc. fatal to 243-gramme guinea-pig in 1½ days ± m. f. d.=.01 cc.
C	1.4	−.8	−1.5	.02 cc. fatal to 245-gramme guinea-pig in 30 hours. .008 cc. " 253 " " " 2½ days ± m. f. d.=.007 cc.
D	0.9	−.8	−1.8	.01 cc. fatal to 251-gramme guinea-pig in 2⅔ days. m. f. d.=.009 cc.
E	1.6	−0.7	+.15	.01 cc. produces large slough in 265-gramme guinea-pig. m. f. d.=.012 cc.

Tables I and III show that ½ to ⅕ of the amount of toxin obtainable in fermented bouillon plus dextrose is produced in ordinary bouillon under identical conditions. For bacilli without the training for surface growth the result would have been far worse, as all experimenters have amply testified.† Table III also shows that the bacillus employed, in spite of its surface growth, was unable to make more than 2 out of 5 lots of bouillon alkaline within 11 days. Nevertheless, a considerable amount of toxin had accumulated. The toxin production goes on in spite of unfavorable conditions, probably in virtue of the persistent membrane growth and the large amount of available peptone. The table furnishes further illustration of the fact that a strongly alkaline end reaction does not necessarily imply the greatest toxic accumulation. A comparison of this with preceding tables

* See *Journ. Boston Soc. Med. Sciences*, June, 1898, for this estimate.

† Dr. W. H. Park informs me that he obtains no better results than these with unfermented bouillon at the present time.

shows that 1 per cent peptone or even less in fermented bouillon may accomplish more than 2 per cent in ordinary bouillon.

The oxygen supply.—Concerning the need of abundant oxygen in cultures of diphtheria bacilli there is general agreement and but little need be said. Ventilation is readily secured in the modified Fernbach flask shown in Fig. 1 (p. 381), and this flask I have invariably found superior to the other forms of the Fernbach and to Erlenmeyer flasks when the layer of culture fluid was about 2.5 cm. deep. By reducing the depth of the layer these flasks become more efficient.

CONCLUSIONS.

1. Dextrose is not in itself injurious but rather favorable to toxin production. When added in quantities not exceeding 0.2 per cent to peptone bouillon freed from fermentable acid-producing substances (muscle sugar) it leads to a maximum accumulation of toxin by utilizing the available peptone to the best advantage.

2. The different courses taken by cultures of diphtheria bacilli in ordinary unfermented peptone bouillon containing muscle sugar and in peptone bouillon made from fermented infusion to which 0.1 to 0.2 per cent dextrose has been added are manifested by an increased production of toxin in the latter as well as by a rapid return from an acid to an alkaline reaction. In the former an acid reaction may prevail even under most favorable conditions.

3. These differences may be explained by assuming either that the acid products of the muscle sugar are different from those of dextrose and non-utilizable, or else that the bouillon contains certain other unknown inhibitory substances removed during fermentation. The use of synthesized media and an analysis of the acid products in fermented bouillon plus dextrose and in unfermented bouillon would aid in explaining the differences.

4. Among the accessory conditions which favor the toxin production in unfermented bouillon, as pointed out by Park and Williams, are increased quantities of peptone, well developed surface growth of the diphtheria bacilli, and a low initial acid reaction (phenolphthalein). In fermented bouillon these accessory conditions are also favoring, though of less importance.