XCIX. THE INFLUENCE OF SURFACE TENSION ON THE GROWTH OF BACTERIA.

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ONE of the factors which may be anticipated to affect the growth and metabolism of bacteria is surface tension.

The relation of this physical factor to other biological problems such as muscle action or the deposition of salts in plant cells [Macallum, 1911] has been the subject of much study; the relation to bacterial growth has received little attention.

If, however, one considers the results obtained with surface films by J. J. Thomson [1888] and others it is evident that the increased concentration of substances, such as peptone, at the air/liquid interface ought to have a pronounced effect on those organisms growing at the surface. This should especially be the case with micro-organisms like *C. diphtheriae* where experience has shown that efficient toxin production is associated with good pellicle formation.

In a study of certain factors affecting toxin production my attention was drawn to this aspect of the subject by the statements of practically every bacteriologist who has worked in this field as to the necessity of removing all but inevitable traces of fat from the medium before it is used. The fact that pellicle is also essential would appear to be connected with the particular conditions of the air/liquid interface.

In a paper published in 1919 Larson, Cantwell and Hartzell [1919] made some very interesting statements regarding the effect of lowering the surface tension of media on the growth and habit of bacteria. The protocols of their experiments are unfortunately lacking in details of the methods used for determining surface tension. On lowering the surface tension of their media they observed that anaerobic bacteria grew in the presence of air, that organisms, usually forming pellicles, grew throughout the liquid and did not form a film and that spore-bearing organisms no longer produced spores. Evidently the unusual environment of the bacteria completely changed their habit of growth.

As my interest was in the behaviour of organisms whose pellicle forming properties are important, I devoted some attention to a study of the effects of lowered surface tension on the growth of *C. diphtheriae* and extended these observations to other organisms.

Certain general observations may be made here regarding surface tension, although the subject is discussed among others by Freundlich [1922] and Bayliss [1923].

In the first place no standard method has as yet been arrived at for the determination of its value. In most instances in biological research the stalagmometer has been used and has proved to be a satisfactory instrument if employed in a strictly comparative way. It is in the comparison of the results of one worker with those of another that difficulties arise. This was seen in the meiostagmin reaction of Ascoli and Izar [1910], which is possibly of real biological significance and yet has been found so difficult to repeat by other workers that it has not had general acceptance.

The stalagmometer represents the transition from a purely dynamic method such as the jet method of Lord Rayleigh and the static method of either the capillary tube or the measurement of pull of a solid being torn from the surface of a liquid. Both static and dynamic methods give practically concordant results when pure non-associating liquids are dealt with. It is only when we come to deal with liquids containing substances depressing or elevating surface tension that we find difficulties.

With the drop weight method, the longer the time taken to form the drop, the lower or higher the surface tension value obtained, until a number is reached which is close to that obtained by the static method. That this is so will be seen in some of the present results. The earlier values for bacteriological media obtained with the stalagmometer and only moderately slow dropping are very close to those given by Larson and his co-workers. When the number of drops was decreased and the time taken for each individual drop to fall very much prolonged, values were obtained which were about 10 dynes per cm. lower than in the first method, and these were very close to those obtained by the static ring method to be described later.

Larson, Cantwell and Hartzell [1919] discuss some of the possible causes of the change in the behaviour of bacteria through lowering the surface tension. They point out that the conditions for bacterial nutrition are very different at the surface from those in the interior owing to the great increase in the concentration of nutrient substances such as peptone in the surface layer. This must be correct, for Metcalf [1905] in investigating the behaviour of thin films of peptone solution showed that these consisted of a layer of almost undiluted peptone. We also know that other thin films of protein solutions such as the froth of egg-albumin consist almost entirely of the solid component.

The conclusions of Larson cannot altogether be accepted without reserve. The bacteria/water interface must also present an enrichment atmosphere and before it can be assumed that the air/water interface is a situation of

increased concentration of nutritive substances for the cells we ought to be informed regarding the system chitin/water or hemicellulose/water concentration differentiated with the air/water system before we can generalise respecting situation of increased or decreased nutritional environment.

For lack of more precise information the terms chitin and hemicellulose are here used to designate the composition of the bacterial envelope. It seems more than probable that the composition varies, and it may be this very reason which determines that some bacteria in their search for the most suitable surroundings seek the surface of the liquid while others are attracted to the interior of the medium.

There is also an important effect which is not mentioned by Larson and his colleagues. It is known that in the contest for the surface layer those substances which are more powerful in decreasing the surface tension tend to remove those which are less efficient. This may be the double cause of the deleterious action of small quantities of fats when these are not removed from the medium. They not only lower the surface tension but in so doing expel the nutrient substances of less surface tension-depressing power from the surface of the liquid. Thus the surface layer is no longer as rich in nutrient substances but has a high concentration of substances such as soaps which may be directly toxic to those bacteria existing near the interface. In speaking of the interface not only is the air/water junction implied, but also the bacterial/aqueous interface.

EXPERIMENTAL.

The present experiments were designed to enlarge the observations of Larson, Cantwell and Hartzell, and if possible throw some light on the relation between changes in surface tension and the pellicle formation of *C. diphtheriae*. As Larson and his colleagues had recommended the use of sodium ricinoleate for changing the surface tension of the medium it was decided to employ it. The sample used was made from castor oil by following their directions. A 5 % solution of the soap in water was used as a stock solution.

In the first experiments a stalagmometer of the Traube [1887] type was used, dropping at the rate of 15 drops per minute. The drops fell directly into free air and were counted. As a preliminary, certain combinations of nutrient broth made from English and imported beef combined with 2.0 % of Parke Davis, Witte and Morson's peptones were examined; referring the surface tension to dynes per cm. the following results were obtained:

Table I.

Beef	Peptone	Surface tension in dynes per cm.
Imported	Witte	53.4
"	Parke Davis	58.2
"	Morson	59.6
English	Witte	53.3
"	Parke Davis	58.0
"	Morson	59.6

These results agree fairly closely with those of Larson, Cantwell and Hartzell who found the surface tension of their media to be between 58 and 60 dynes per cm. The principal factor determining the surface tension of the medium appears to be the peptones, and of those used in the present experiments Witte's appears to have the greatest effect. In order to test this, 1.0 % solutions of the three peptones were made up, the specific gravity and surface tension were determined and the effect of equalising the hydrogen ion concentration ascertained. The results were as follows:

Peptone	S.G.	Surface tension dynes per cm.	p_H	Surface tension dynes per cm. at p_H 7.6
Witte	1.006	54.6	6.6	54.2
Parke Davis	1.005	61.5	6.3	62.1
Morson	1.006	60.5	5.6	60.6

It will be seen that the change produced by reducing the hydrogen ion concentration of the peptone solutions to a common number was slight.

In order to examine the effect of sodium ricinoleate on a nutrient broth, the 5.0 % solution of this salt was progressively diluted and the surface tension of the dilutions determined. They were as follows:

Table II.

Dilution	Sodium ricinoleate %	Surface tension dynes per cm.
0	5.0	32.8
4/10	2.0	33.6
1/10	0.5	39.7
1/25	0.2	47.0
1/50	0.1	52.0
1/100	0.05	57.0

As the surface tension of the peptone broth was already between 55 and 60 dynes per cm., it was decided to use much higher dilutions of sodium ricinoleate. The concentration of the soap in the medium is given in the following table and the surface tension before and after sterilisation:

Table III.

Na ricinoleate %	Surface tension dynes per cm.	
	Before sterilisation	After sterilisation
0.125	40.6	41.2
0.062	43.0	43.4
0.031	48.1	48.4
0.015	51.5	51.6
0.0	59.5	59.2

It will be observed that any hydrolysis of the soap which may have occurred as the result of heating to a high temperature did not measurably affect the surface tension of the media.

The media given in the above table were taken in lots of 10 cc., sterilised

and inoculated with the following rapidly growing organisms: *B. coli*, *C. diphtheriae*, *B. pyocyaneus*, *B. proteus*, *Vibrio septique*, *B. welchii* and *B. subtilis*, and examined after 48 hours' incubation at 37°.

Table IV.

Organism	Concentration of Na ricinoleate %				
	0	0.015	0.031	0.062	0.125
<i>B. coli</i>	+	+	+	+	+
<i>C. diphtheriae</i>	x x +	++	+	?	?
<i>B. pyocyaneus</i>	x x x +	x x x +	x x x +	x x +	x x +
<i>B. proteus</i>	x x x +	x x +	x x +	x x +	x x +
<i>V. septique</i>	x x +	+	?	?	?
<i>B. welchii</i>	+	+	?	?	?
<i>B. subtilis</i>	+	+	?	?	?

Note. x represents pellicle formation; + represents growth.

As will be seen, *B. coli* appears to be relatively indifferent to a concentration of soap which is definitely inhibitory to the anaerobes. Among the more affected is *C. diphtheriae*. This is in agreement with the statements arrived at empirically regarding the necessity of removing all fats from the medium if the growth of this organism is to be satisfactory.

By incubating the cultures for a further 24 hours the differences were accentuated.

Table V.

Organism	Concentration of Na ricinoleate %				
	0	0.015	0.031	0.062	0.125
<i>B. coli</i>	++	++	++	++	++
<i>C. diphtheriae</i>	x x x +	x x x x +	++	+	?
<i>B. pyocyaneus</i>	x x x x +	x x x +	x x x +	x x x +	x x x +
<i>B. proteus</i>	x x +	x x x +	x x x +	x x +	x x +
<i>V. septique</i>	x +	x x x +	+	+	x +
<i>B. welchii</i>	x +	x x +	+	?	?
<i>B. subtilis</i>	x x x +	x x x x +	+	+	?

The only additional point of interest in this table is the apparently slight stimulation to growth seen with the lowest concentration of soap used.

This experiment was repeated except that *B. typhosus* was added to the series. *B. sporogenes* showed itself extremely sensitive to the action of soap. *B. typhosus* grew, but with lessened vigour, even in the more concentrated tubes of soap broth.

The next set of experiments was made to test the effect of soaps on glucose fermentation.

For this purpose peptone broth containing varying quantities of sodium ricinoleate was tubed in portions of 10 cc. and sterilised. Each tube contained a Durham tube. To each tube after autoclaving was added 0.2 cc. of a sterile 10 % glucose solution with a sterile pipette. The tubes were placed in the incubator for 24 hours to control their sterility. The tubes were each inoculated with a loopful of actively growing culture and placed in the incubator at 37°.

The results at the end of 24 hours are given in the following tables:

Table VI.

	Concentration of sodium ricinoleate %				
	0	0.035	0.052	0.104	0.156
Time—24 hours.					
<i>B. paratyphosus</i> B	++0ggg	++0g	++0gg	++0gg	++0ggg
<i>B. subtilis</i>	+++pp0	000	000	000	000
<i>B. welchii</i>	++0ggg	000	000	000	000
<i>C. diphtheriae</i>	000	000	000	000	000
<i>B. murisepticus</i>	++pp0	000	000	000	000
<i>B. paratyphosus</i> A	++0ggg	++0gg	++0gg	++0g	++0gg
<i>B. sporogenes</i>	++0gg	000	000	000	000
<i>B. coli</i>	+++p(?)g	++p(?)g	++p(?)g	++p(?)g	++p(?)g
<i>B. pyocyaneus</i>	++pp0	++pp0	++pp0	++pp0	++pp0
<i>B. typhosus</i>	++00	++00	++00	++00	++00
<i>B. proteus</i>	++0gg	++0g	++0gg	++0g	++0g
Time—48 hours.					
<i>B. paratyphosus</i> B	+++pggg	++0g	++0gg	++0gg	++0gg
<i>B. subtilis</i>	+++pp0	+p0	+00	000	000
<i>B. welchii</i>	+++0ggg	000	+0g	000	000
<i>C. diphtheriae</i>	+p0	000	000	000	000
<i>B. murisepticus</i>	+++p0	++p0	000	000	000
<i>B. paratyphosus</i> A	++0gg	++0gg	++0gg	++0gg	++0gg
<i>B. sporogenes</i>	+++0g	++0g	+0g	+0g	000
<i>B. coli</i>	++0gg	++pgg	++0gg	++0gg	++0gg
<i>B. pyocyaneus</i>	++p0	++p0	++p0	++p0	++p0
<i>B. typhosus</i>	++00	++00	++00	++00	++00
<i>B. proteus</i>	++0gg	++0gg	++0gg	++0gg	++0gg
Time—96 hours.					
<i>B. paratyphosus</i> B	++0gg	++0g	++0gg	++0gg	++0gg
<i>B. subtilis</i>	+++pp0	++pp0	++pp0	000	000
<i>B. welchii</i>	++0ggg	000	+0g	000	+?00
<i>C. diphtheriae</i>	++pp0	000	000	000	000
<i>B. murisepticus</i>	+++pp0	++pp0	+00	000	000
<i>B. paratyphosus</i> A	++0gg	++0gg	++0gg	++0gg	++0gg
<i>B. sporogenes</i>	++0gg	++0gg	++0gg	++0gg	+00
<i>B. coli</i>	+++0gg	+++ppgg	+++ppgg	+++0gg	+++pgg
<i>B. pyocyaneus</i>	++pp0	++pp0	++pp0	++pp0	++pp0
<i>B. typhosus</i>	++00	++00	++00	++00	++00
<i>B. proteus</i>	++0gg	++0g	++0gg	++0gg	++0g

Note. The signs used are +=growth, p=pellicle, g=gas. Where 0 occurs the respective characteristic is absent.

It will be noted that among those bacteria which are most susceptible to the action of the ricinoleate are *B. welchii* and *B. subtilis*. Organisms flourishing in the intestine do not seem to be at all susceptible even to large quantities of soap, confirming similar observations of Larson [1922].

In view of the susceptibility of some of these organisms to quite small quantities of soap it was decided to repeat the above experiment, using smaller concentrations, the amount of glucose employed being the same. Only the 24 hours' and 96 hours' results are recorded here.

Table VII.

	Concentration of sodium ricinoleate %				
	0	0.012	0.025	0.050	0.075
Time—24 hours.					
<i>B. paratyphosus</i> B	× × 0gg	× × 0gg	× × 0gg	× × 0gg	× × 0gg
<i>B. subtilis</i>	× × pp0	× × pp0	× × pp0	× × pp0	000
<i>C. diphtheriae</i>	× × pp0	000	000	000	000
<i>B. murisepticus</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>B. paratyphosus</i> A	× × 0gg	× × 0gg	× × 0gg	× × 0gg	× × 0g
<i>B. coli</i>	× × ppg	× × ppg	× × pg	× × ppg	× × ppg
<i>B. pyocyaneus</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>B. typhosus</i>	× × 00	× × 00	× × 00	× × 00	× × 00
<i>B. proteus</i>	× × 0gg	× × 0g	× × 0g	× × 0gg	× × 0g
<i>B. enteritidis</i>	× × ppgg	× × 0gg	× × 0g	× × 0g	× × 0gg
Time—96 hours.					
<i>B. paratyphosus</i> B	× × 0gg	× × 0gg	× × 0gg	× × 0gg	× × 0gg
<i>B. subtilis</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × ppp0
<i>C. diphtheriae</i>	× × pp0	× pp0	000	000	000
<i>B. murisepticus</i>	× × pp0	× × × pp0	× × × pp0	× × pp0	× × pp0
<i>B. paratyphosus</i> A	× × 0gg	× × 0gg	× × 0gg	× × 0gg	× × 0gg
<i>B. coli</i>	× × 0gg	× × 0gg	× × ppg	× × 0g	× × 0g
<i>B. pyocyaneus</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>B. typhosus</i>	× × 00	× × 00	× × 00	× × 00	× × 00
<i>B. proteus</i>	× × 0gg	× × 0g	× × 0g	× × 0g	× × 0g
<i>B. enteritidis</i>	× × 0gg	× × 0gg	× × 0gg	× × 0g	× × 0g

It will be noted that here again *C. diphtheriae* shows itself unusually susceptible to small concentrations of soaps.

The next series of experiments was directed towards ascertaining whether changes in the surface tension of the medium took place during growth of *C. diphtheriae*. The reason for this was partly the interesting statements of Warden and his co-workers [1921] on the constitution of toxins in general. If toxin production be contingent on the dispersion of fatty acids on certain colloid aggregates, then one would anticipate that changes would occur in the surface tension of the medium during the transfer. Furthermore, it might be expected that during the early growth of the bacteria and the consequent introduction of large colloidal particles, the surface tension-lowering substances already present in the medium would be adsorbed by the bacteria with a rise in their surface tension.

In order to get better results than had been obtained with the original form of Traube stalagmometer it was decided to use a drop weight method with a much slower rate of drop and to weigh the drops as formed. In the first set of experiments 30 drops were weighed and these were collected in a small weighing bottle which enclosed the tip of the stalagmometer. In this way disturbances from currents of air and from evaporation of the liquid were avoided. The total time for dropping was 3½ minutes. The average weight of a drop of water from this tip was 0.105 g.

The results with this method were very satisfactory from a comparative point of view, quadruplicate weighings of 30 drops of water did not differ by more than 0.2 %. With peptone solutions the results were not so concordant, a difference of 1.0 % being obtained.

A series of very careful experiments was then made in which five drops of the medium were weighed, the time taken for a single drop of water to fall being about 45 seconds. Excellent results were given by the slow method of drop, and as was to be expected the values for surface tension approximated to those given by the static method when peptone solutions were tested. The differences obtained did not appear to justify the employment of the slower method of drop.

A series of tubes of bouillon peptone was set up and inoculated with the pellicle of a rapidly growing culture of *C. diphtheriae*. At stated times a tube of the culture was taken from the incubator, the culture centrifuged and the clear liquid examined. The results were as follows:

Hours	Surface tension dynes per cm.
control	57.8
24	58.2
72	58.9
120	55.8
168	58.2

It will thus be seen that during the early period of growth the surface tension rose owing to the adsorption by the bacteria of surface tension-lowering substances in the original medium. Following this there is a discharge of these materials into the medium, due, probably, to death and autolysis of bacteria, and the surface tension falls. The last determination gives a high value, but whether this is associated with other changes in the medium cannot be stated.

A second experiment was made in which not only the surface tension was determined, but at the same time the reaction and the production of carbonates were estimated. In this experiment quantities of bouillon of 100 cc. were inoculated. These were contained in flat medicine bottles which were incubated lying on their larger sides. The surface of medium exposed to the air was about 115 cm. The results were as follows:

Table VIII.

Hours	p _H	CO ₂ from 1 cc. medium by acid	Surface tension dynes per cm.
0	8.0	0.09	60.06
72	8.4	0.27	62.20
120	8.4	0.29	62.00
168	8.5	0.30	61.37
240	8.5	0.34	62.70
336	8.6	0.34	62.99
432	8.5	0.32	62.41
768	8.5	0.30	62.90

The results of this experiment run quite differently from the preceding one. In the former it was noted that the surface tension of the medium rose slowly after inoculation to fall later. In the present series the rise occurs but the surface tension of the medium does not fall. The results in both series are the average of very closely agreeing duplicate determinations. In the second series, the surface exposed and the amount of pellicle formed is much greater

than in the test-tube series. This would tend to give a much larger amount of pellicle and leave a greater effective surface for the adsorption of surface tension-lowering substances.

In order to obviate some of the difficulties of the dynamic method it was decided to devise an apparatus which would give static readings. It will not be necessary to mention the various types which are already described in the literature some of which are known to give a high degree of accuracy when properly used.

It seemed that the pull given by a platinum ring would, on account of the ease of cleaning, be the most convenient for the purpose. This method has quite recently been used by du Nouy [1919] who obtained an increasing pull by utilising a torsion wire as had been done by Searle. The apparatus of du Nouy is a very expensive one and it seemed possible that by utilising the device of a dividing chain and ordinary hand scales one could produce the same effect with rather more gradual increase in weight than is afforded by the torsion wire. The essential features of the apparatus (Fig. 1)¹ are a small hand balance (*A*) with arms 8 cm. in length, turning with a load of 2 mg., and a platinum ring (*G*) 0.75 mm. thick, 3.1 cm. in outside diameter, supported from a small platinum ring by three very thin platinum wires. The ends of the rings were soldered together with silver. Care was taken to have the larger ring perfectly flat and in subsequent handling all tendency to deform it was avoided. This was done by always removing the ring with a stout hooked wire fastened to a handle. When not in use the ring was kept in a wide mouthed bottle containing strong sulphuric acid and potassium dichromate. The ring was washed in tap water before use and gently dried over a flame. The rest of the apparatus was made up of "Meccano" parts.

The arrangement of the apparatus was as follows:

The balance was suspended from a framework (*B*) 42 cm. high and 30 cm. wide at the base. The frame was slightly sprung inwards at the top and suitably braced to increase its rigidity. Holes were drilled in the horn pans (*C* and *D*) and from the right hand pan a small gold coated eyeglass chain (*F*) fell which ended in a hook supporting the platinum ring (*G*).

From the other fell a much longer (*E*) chain which was doubled upwards and was fastened to a hook which could be made to travel up and down by means of a rack and pinion (*H*). The position of the hook was determined by a fixed millimeter scale (*L*). By lowering the hook, an increase in weight, due to increase in the pan portion of the chain, was put upon the pan. By adding weights to the right hand pan (*C*) it was easy to construct a curve giving the increments of weight on the millimeter scale. In the present apparatus one millimeter roughly corresponded to 2 mg.

An improvement to the apparatus as drawn consisted in gearing down the rate of rotation of the wheel by means of a second smaller wheel fixed near

¹ I am indebted to Mr Colbrand of the Engineering School for the isometric scale drawing of the apparatus.

the bottom of the pillar and connected with the former by a small catgut driving cord. The handle of this spindle was made from the largest sized meccano wheel. This slight alteration is a considerable improvement in the smoothness of movement of the ratchet.

The vessel used for holding the liquid to be examined was a large watch glass (*J*). This was supported on a platform which could also be raised and lowered by a rack and pinion (*K*) so that when the platinum ring was immersed in the fluid, after being balanced without contact, the balance could be returned to the zero point. The movable platform allowed the liquid to be raised gently until contact with the platinum ring was secured.

All determinations were made at room temperature which was 16–18°. For more precise work the container or the whole apparatus could be enclosed in a simple thermostat. Using fresh tap water to determine the water constant of the apparatus the weight required to tear the ring from the surface was 1.698 g.

The method of making a determination was as follows.

The scales were balanced by shot so that the pointer for the millimeter scale was at zero. The watch glass was provided with a few cc. of the solution to be examined, and was slowly moved up until the platinum ring was just in contact with the surface of the fluid. Weights were then added until the ring was being pulled away from the liquid without breaking contact. The weights were gently placed on the scale pan with as little disturbance as possible. With a little experience one easily recognises when it is time to have recourse to the chain for finally increasing the weight. At this point, by means of the rack and pinion on the left, the chain weight is *gradually* lowered. This should be done with as even and slow a motion as possible. At a certain point the ring is torn from the liquid and readings of the weights in the pan and on the millimeter scale are noted, and the scale reading converted into milligrams from the plotted curve. This is added to the pan weights and from the total weight the surface tension of the liquid is deduced. The experiment is then repeated to ensure concordance. With a total weight of over 1 g., individual experiments do not vary much more than 5–7 mg., so that the apparatus acts with an accuracy well under 1 %. As was to be expected the values obtained for the surface tension of solutions of peptones were considerably below those obtained by the ordinary drop methods. On an average the values were about 7–10 dynes per cm. lower. This difference was almost entirely due to a function of the dropping rate of the stalagmometer, for the experiments with the drop weight method in which the drops were produced at a very slow rate gave results very close to those obtained with the static method.

An experiment with *C. diphtheriae* will indicate to what extent growth of the organism in a peptone broth medium affects the surface tension. The strain used was P.W. 8 growing actively in the same medium. The culture used for inoculation was two days old. These experiments were performed in test-tubes.

The initial surface tension of the medium was 50.2 dynes per cm.; the p_H 7.8.

The following table gives the curve of the surface tension as measured by the static method:

Table IX.

Hours	p_H	Surface tension in dynes per cm.
0	7.8	50.2
24	—	52.2
48	6.8	47.1
72	7.5	45.7
120	8.2	47.6
144	8.5	46.9
192	8.5	45.6
240	8.5	45.2
288	8.5	45.6

This table also gives the interesting result that during the very early stage of growth some of the substances which produce a fall in surface tension are apparently adsorbed on the bodies of the young bacteria and therefore the surface tension rises. With death and autolysis, which occur later, fatty substances are liberated and with this the tension falls. It is noteworthy that this is not coincident with an alkaline reaction of the medium, for at the 48th hour, when the nutrient broth had a reaction of p_H 6.8, the tension dropped to 47.1 dynes per cm. It thus agrees with the first experiment in which the surface tension was determined by the drop weight method. This experiment was also performed with test-tube cultures.

The differences due to the media being contained in deep layers in test-tubes or in shallow layers with a larger surface exposed to air were so striking, that it was decided to repeat them in shallow layers on a larger scale and to use for comparison strains which were known to be toxigenic and those which were non-toxigenic. There were also drawn into the investigation three media which experience has shown to be the most suitable for the production of toxin.

Two strains of non-toxigenic *C. diphtheriae* were used, which were obtained from the National Type Collection at the Lister Institute. The toxigenic strain was P. W. No. 8. Two cultures of this strain were used, one of which had been subcultured in this laboratory for some years, the other being a fresh culture from the Lister Institute. No difference in behaviour between the two cultures was noticed. The three media employed were (1) the ordinary peptone broth, (2) Hartley's [1922] modification of Douglas's medium and (3) Martin's medium made up in accordance with the direction of Abt and Loiseau [1922].

The media were sterilised in 1300 cc. Fernbach flasks containing 500 cc. of medium. The necks of the flask were plugged with cotton wool. Through the neck passed a glass tube the upper part of which was bent at a right angle. This was provided with a rubber tube and screw clip. By attaching the rubber tube to a small wash bottle and gently sucking, a small amount of the

contents of the Fernbach flask could be removed without disturbing the flask in the incubator. The samples drawn off were usually so clear that it was not necessary to centrifuge them. The device is similar to that employed by Wolf and Harris [1917] in their study of anaerobic fermentation except that the cultures are maintained aerobically.

Only sufficient of the medium was withdrawn to determine the hydrogen ion concentration and the surface tension. At the end of the experiment the culture was examined for contamination. None such was ever found. Only one of the three experiments will be given although the qualitative results were very similar.

Table X.

		Medium											
		Martin				Douglas				Peptone broth			
		P.W. No. 8		Gentry		P.W. No. 8		Gentry		P.W. No. 8		Gentry	
Hours		p_H	S.T.	p_H	S.T.	p_H	S.T.	p_H	S.T.	p_H	S.T.	p_H	S.T.
0		7.5	44.8	7.5	44.9	7.5	46.6	7.5	45.7	7.6	44.9	7.6	45.7
24		7.2	45.6	7.1	48.6	7.0	48.0	7.0	48.1	6.8	47.8	6.6	48.3
48		7.4	47.0	7.2	49.1	7.2	48.0	7.3	49.6	6.6	48.4	6.8	50.3
72		8.0	48.3	7.6	52.8	7.7	49.0	7.8	53.0	7.2	48.6	7.5	54.1
96		8.3	49.2	8.2	54.0	8.0	50.0	8.0	53.1	7.7	49.0	8.0	54.7
120		8.4	50.2	8.4	55.1	8.2	51.6	8.2	52.6	8.0	51.3	8.2	55.4
144		8.4	51.1	8.5	55.4	8.2	52.0	8.4	54.6	8.2	51.6	8.4	57.8
168		8.4	51.6	8.5	54.9	8.2	51.9	8.4	54.1	8.1	51.2	8.5	57.4
264		8.5	52.6	8.6	57.8	8.4	54.0	8.6	55.7	8.4	53.7	8.7	58.6
384		8.5	52.8	8.7	57.5	8.6	52.8	8.6	53.8	8.6	51.6	8.7	58.2
576		8.8	54.8	8.8	55.3	8.7	53.3	8.8	54.3	8.8	51.6	8.8	57.9

The main point to be observed is the continuous rise in the surface tension of the medium during the growth of the culture and what is more important during a time when it may be assumed that growth has practically ceased.

Speaking generally it may be assumed that the toxin production under the conditions of the experiment had reached its maximum at the fifth day. Curves were drawn of the rise in surface tension and it was found that this rise was, on the whole, steepest during the first 120 hours of the experiment. What is more striking is the divergence of the curves for a given medium produced by a toxigenic and a non-toxigenic strain. In each pair of experiments the curve of rise in surface tension given by a non-toxigenic strain is much steeper, and the ultimate rise in surface tension much higher than with a toxigenic bacillus. This would lead one to believe that the adsorption of surface tension-lowering substances by the bodies of the bacteria is more complete when little toxin is formed.

It may be that the envelope of the bacteria, by reason of its content in fat plays an important part in determining toxin production. Those strains of bacteria whose outermost coating has the greater amount of fat will not adsorb the metabolic products of surface tension-lowering capacity on which toxin production depends. That such substances are elaborated is indicated, not only by the experiments of Warden to which allusion has already been made, but also by the more recent investigations of Walbum [1923] and Doyon

[1922]. Walbum, in particular, believes that a non-toxic "protoxin" is elaborated by the bacillus, and this in contact with peptone or peptone broth produces the active toxin. One may take it that the "protoxin" is of a surface active character, and if adsorbed on the bodies of the bacteria would be removed from a sphere where toxin can be formed. This would give a partial explanation for the fact that non-toxic strains of bacteria remove the surface tension-lowering substances from the medium.

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