ACID PRODUCTION GRAPHICALLY REGISTERED AS AN INDICATOR OF THE VITAL PROCESSES IN THE CULTIVATION OF BACTERIA.

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PLATE 54.

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The investigations described below are a continuation of earlier publications[1] concerning acid production by bacteria of the *coli* group. In the previous investigations the typical acid curve for *Bacillus coli* was determined by titrating at regular intervals the acid produced in the course of *coli* cultivation. But this method was unsatisfactory for several reasons. In the first place, as many *coli* cultures were needed as points were required on the curve. Secondly, there could be no absolute assurance even with the most painstaking care that all the cultures were produced or maintained under constant experimental conditions. And thirdly, when the experiment occupied a longer time, the presence of the observer was required at all hours of the day. A method by which all points necessary for plotting the curve could be obtained from a single culture without depriving the latter of any of its contents or in any other way disturbing the experiment would be more advantageous. Conductivity measurements were attempted as a possible solution of this problem, but, as was to be expected, the presence of strongly dissociated salts such as sodium chloride masked the small changes in conductivity caused by the slightly dissociated acids which were produced.

[1] Fischer, A., and Andersen, E. B., *Centr. Bakteriol., 2te Abt.*, 1912, xxxiii, 289. Fischer, A., *ibid., Ite Abt., Orig.*, 1913, lxix, 474.

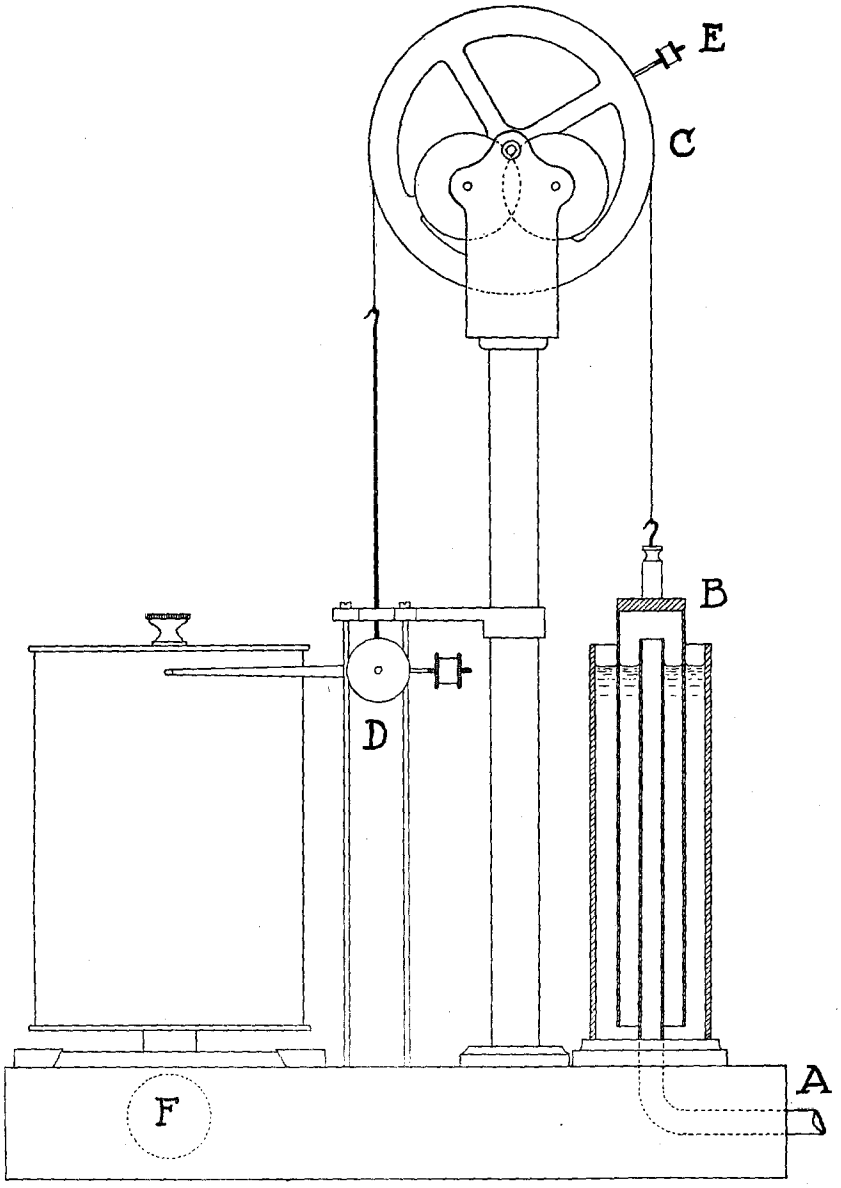
Method.

The method finally adopted was as follows: By the addition of calcium carbonate to the culture, the production of acid was easily followed by the measurement of the carbon dioxide liberated. This in turn was accomplished by a small spirometer, the movements of which were recorded on a rotating cylinder. This method had the following advantages: (1) the acid curve was obtained from the same culture; (2) the process was written in full, whereas formerly only single points were given; (3) constant attention was unnecessary. The procuring of a sensitive self-registering spirometer was a problem in itself. Such an apparatus was formerly utilized for bacteriological experiments and described by Weissenberg.[2]

Great care was taken to make the apparatus used in the present work as sensitive as possible.[3] It is mounted in a heavy mahogany box with glass sides and double doors (Text-fig. 1 and Fig. 1). The apparatus rests on three legs, of which two are separate screws. The carbon dioxide developed from the culture is passed through a glass tube conductor to a tube protruding outside (Text-fig. 1, *A*). This tube is furnished with a three-way stop-cock, not shown on the drawing, which permits communication between the spirometer cylinder and either the culture or the atmosphere. As will be seen from the drawing, the tube protrudes from under a brass cylinder, which is suspended in a glass vessel, containing a 25 per cent glycerol solution. The average diameter of the cylinder is 10 cm. Therefore each 10 cc. of gas at atmospheric pressure will raise the bell 1 cm. By means of a silk thread which is placed over a pulley *C*, the bell is connected with and at the same time partly balanced by the counterweight *D*, which bears the indicator with the writing pen. The pulley is borne upon an upright brass stand, and to reduce friction to a minimum the bearings consist of two pairs of smaller wheels, as shown in the drawing. Since the motion of the pulley wheel is very slow, the inertia of the wheel causes no difficulty. To permit the horizontal adjustment of the axis of the pulley its pointed ends rest in the conically hollowed ends of two steel screws.

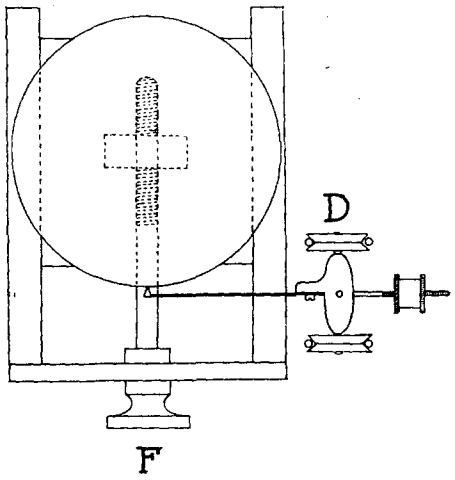
[2] Weissenberg, H., *Centr. Bakteriol., 2te Abt.*, 1902, viii, 370.

[3] The apparatus was made by a mechanical engineer, J. Olsen, Hallinsgade, Copenhagen.



TEXT-FIG. 1. Diagram of the self-registering spirometer used.

As the weight of the cylinder depends upon the extent of immersion in the fluid, this is corrected by a moveable weight *E*, which is placed on the upper part of the pulley in such a position that when the bell is half way out of the fluid it is perpendicularly above the axis of the pulley. If the bell should ascend higher, its weight will increase in proportion to the ascent, or, what amounts to the same thing, in proportion to the angle described by a point on the wheel. Simultaneously the weight moves to the other side of the axis, and by its weight almost always counterbalances the bell, since its moment with regard to the axis of the wheel is proportional to the sine of the angle



TEXT-FIG. 2. Detailed drawing of the rotating cylinder and writing pen.

described. If the diameter of the wheel is large enough so that a given shifting of the bell causes only a small revolution, one may approximately assume the sine of the angle as the angle itself. In this way the varying movements of the weight will almost compensate the varying weight of the bell. If the bell is near its lowest position and the weight on the same side of the axis, the situation is exactly the same. As shown in Text-fig. 2, the counterweight *D* bears two brass wheels which can run against four vertical steel rails. Enough space is allowed between the wheels and the rails so that the wheels do not touch them unless the cylinder presses the pen. The revolving

cylinder has a height corresponding to the bells and a circumference of 48 cm. and revolves by means of a clockwork once in 24 hours. On the roller is placed ordinary millimeter paper. Each hour is represented by 2 cm., while the vertical movement of the indicator of 1 cm. represents a gas evolution of 10 cc. The clock is wound up every 6th day. The whole roller (Text-fig. 2) is placed on a stage which, by means of a screw and bolt, can be moved towards or away from the pen. This is controlled from the outside by means of a pinion wheel *F*. This roller is so adjusted that the wheels on *D* are placed exactly against two of the controlling rails. In order to steady the hands during the motion up and down, the silk thread is not fastened directly to *D* but to a steel hook of considerable length.

For the culture the ordinary tuberculin bulbs were found most suited for the purpose, partly because an ample surface was obtained. Again it was easy to mix by shaking the contents so as to neutralize the acid as quickly as it was formed. Finally, the wide opening was convenient for the introduction of a delivery tube, filling funnel, etc.

To control the conditions of the experiments as much as possible, the culture medium was always prepared in quantities sufficient for a whole series of experiments. Where the experiments did not demand certain changes in the composition of the media, the latter consisted of 2 per cent peptone (Witte's), 0.5 per cent sodium chloride, and 0.5 per cent sugar, generally glucose or lactose. The volume used in each bulb was 100 cc., and to this were always added 100 gm. of calcium carbonate (Kahlbaum). This amount was more than enough to neutralize the largest amount of acid which could be produced by the bacteria in the culture. The bulbs of culture media and calcium carbonate were closed with cotton plugs and autoclaved for half an hour at 120°C., after which the cotton plugs were paraffined and the bulbs placed in a cool dark place ready for use.

For inoculating the cultures precultures were used in order to render the number of bacteria as constant as possible. 24 hours before the beginning of the experiment over 10 cc. of the nutrient medium from a 24 hour agar culture were inoculated, consisting of the following composition: 1 per cent peptone, 0.5 per cent sodium chloride, and 0.5 per cent sugar. In this preliminary culture with a comparatively small amount of nutrient material, the bacteria will have reached

a fairly constant number after 24 hours, even if more or less bacteria had been introduced by means of the platinum needle. The whole of this preculture is added to the tuberculin bulb at the beginning of the experiment. Afterwards the cotton plug is replaced by a sterilized rubber cork, through which passes a glass tube. The rubber tube which leads to the spirograph should be fastened to the glass tube. The bulb is then placed in the incubator and by means of a special shaking apparatus is kept in slow motion so that the culture fluid slowly flows over the layer of calcium carbonate which quickly settles at the bottom.

RESULTS.

There is a marked rise in the curve during the first 2 hours, a result corresponding to the evolution of 20 cc. of gas. This is due to the fact that the air in the tuberculin bulb expands until it reaches the temperature of the incubator. After this the curve becomes horizontal but will rise again, indicating that acid production has begun. In order to insure that the temperature in the incubator had remained constant during the day, a thermograph was placed in the incubator so that a temperature curve was obtained for each curve from the spirograph.

The acid curve produced in this way corresponded exactly to the one found by titration methods on a former occasion. In the first experiments it was important to adjust the composition of the nutrient medium so that the entire process could be made to occur within 24 hours. It was quickly found that the amount of carbohydrate present was the deciding factor. As already stated, in order to adjust the process 0.5 per cent concentration of the specified sugar, as a rule, was used. The process stops as soon as the carbohydrate is consumed. This is shown on the curve as a peculiar sharp break. If more than 0.5 per cent is added, for instance 2 per cent, the production of acid will continue for several days, and the process gradually becomes weaker, finally stopping as a result of the accumulated acids, calcium salts, and cleavage products of the peptone. That this is so may be seen by the fact that the addition of more nourishment does not cause noticeable continuation of the process.

Of the 150 experiments with the spirograph, many were made to test how well the apparatus answered the purpose for which it was designed, and how much could be deduced from the experiments; *i.e.*, how well the experiments could be controlled for obtaining comparative results. It was shown that the changes in pressure and temperature which may take place during a series of experiments are less than the percentage deviation between experiments which are repeated, the deviation amounting at most to about 5 per cent. The temperature in the thermostat can be kept constant, and a changed reading of about 1 per cent will correspond to an alteration in the temperature of the room of 2°C., and 2 per cent to a change in the barometer of 5 mm. That the change in temperature and pressure during 24 hours does not materially influence the curve was shown by making a control test with an uninoculated tuberculin bulb and with the spirometer so arranged that the bell stood half way up from the fluid. In this way almost a horizontal line was obtained.

Before proceeding to the results it should be mentioned that a point on the curve does not conform to the amount of acid produced at that particular moment. This was shown by killing the bacteria when they were vigorously producing acid. It was found that the neutralization of the acid takes place with a certain lag, so that the carbon dioxide evolution takes place for some time after all the bacteria have been killed. For this experiment a strong solution of sublimate (10 cc., 6 per cent) was added to the culture. In this instance and in others where additions to the contents of the bulbs were made during an experiment, the procedure was carried out in such a manner that the curve was not altered by the displacement of air during the filling. A filling funnel is placed in the rubber cork of the tuberculin bulb, where the delivery tube is inserted. When the tap is opened, the contents drop into the bulb and the expelled air will then return to the filling funnel, and consequently not change the pressure in the bulb or spirometer.

These experiments were intended to produce a picture of the growth of the bacteria in a culture; in other words, to make the process visible in a way which can always be reproduced under identical experimental conditions. In a future communication we shall endeavor to give a theoretical explanation of the curves produced. Un-

der constant experimental conditions it has been possible to obtain definite curves. By altering these conditions and by studying the curves obtained, various influences on the life and physiology of the bacteria can be studied.

The investigations which we shall now discuss, but which are as yet not completed, relate especially to the influence of bacteria belonging to the *coli* group on the typical acid curve produced by *Bacillus coli communis*. The reason for employing the *coli* curve was not so much on account of its biology as because it is easy to obtain, and its relation to the carbohydrates presents a special interest, as it can destroy a great number of these with the formation of acid, and is at the same time aggressive towards proteins. Each member of the *coli* group behaves peculiarly towards the different carbohydrates and proteins. Lastly, it is important that the bacteria should be easy to cultivate with a small amount of nutrient material.

Before describing the investigations with mixed infections I shall briefly state the influence of changes in the composition of the nutrient media on the curve.

In order to compare the experiments quantitatively one can derive several numerical relationships from the curves. First, there is the reaction balance, which is ascertained by the combined production of carbonic acid (vital capacity). The process is considered to be completed if at the end of an hour no more than 1 cc. of carbon dioxide is produced. Secondly, there is the speed of reaction, which may be measured by the time consumed for the total carbon dioxide production. This is supplemented by information concerning the speed of the process after the lapse of a certain number of hours (vitality momentum) with the greatest speed attained by the process (optimum vitality). From the numerical data so obtained other curves can be derived, which, for instance, show what influence ascending concentrations of carbohydrates have upon the process. We may, for example, using the amount of sugar as abscissæ and the carbon dioxide production as ordinates, show how lactose, glucose, and peptone in ascending concentration affect the production of carbon dioxide (Table I and Text-figs. 3 to 6). Text-fig. 3 shows the original lactose and glucose curves as registered by the spirograph, reduced about one-ninth.

TABLE I.

Influence of Ascending Concentrations of Carbohydrates on the Acid Curve of B. coli communis.

Glucose.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
per cent	*hrs.*	*cc.*	*hrs.*	*cc.*	*cc.*	
0.3	5.0	35	13.5	5		Process stops abruptly.
0.5	5.0	60	13.5	12		
1.0	4.5	170	29.5	16	13	
2.0	3.5	360	55.5	18	15	
3.0	3.0	540	71.5	19	17	
4.0	3.0	510	98.0	19	16	
5.0	3.5	535	99.0	20	17	

Lactose.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide in 24 hrs.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
per cent	*hrs.*	*cc.*	*hrs.*	*cc.*	*cc.*	
0.3	7.0	35	17.0	9		Process stops abruptly.
0.5	8.0	72		7	6	
1.0	5.5	45		6	5	
2.0	7.5	60		4	4	
3.0	6.0	90		11	10	
4.0	5.0	110		12	10	
5.0	4.0	120		14	12	

Peptone.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
per cent	*hrs.*	*cc.*	*hrs.*	*cc.*	*cc.*	
0.2		1		0	0	
0.5	14	5		0	1	
1.0	6	26		4	3	
1.5	8	28		4	4	

Results with Glucose.

0.5 Per Cent Glucose.—After a lapse of 5 hours the formation of acid begins to be visible on the curve which continues to rise steadily for about 11 hours with a total production of 60 cc. of carbon dioxide. After $13\frac{1}{2}$ hours from the beginning of the experiment a sharp break in the curve is obtained without any previous change in its ascent. From here the curve continues in a horizontal line. This sudden break in the curve, which can take place in less than 3 minutes, can be explained only by the fact that this point marks the disappearance of sugar. This was shown by the fact that sugar could be detected before the break but not afterwards.

1 Per Cent Glucose.—A far greater production of acid is obtained with this sugar concentration. After a lapse of $4\frac{1}{2}$ hours the rise begins and continues for 25 hours, reaching a total production of 170 cc. of carbon dioxide.

2 Per Cent Glucose.—The rise in the curve begins after $3\frac{1}{2}$ hours and continues quite rapidly for 42 hours, reaching a total production of 360 cc. of carbon dioxide. It should be noted that the higher the initial concentration of sugar, the less sharp is the break in the curve. In these instances there is sugar enough and the process does not stop suddenly for lack of nourishment but is gradually retarded because of the inhibiting action of the end-products in the culture.

3 Per Cent Glucose.—After 3 hours the rise begins and continues sharply for $68\frac{1}{2}$ hours with a total production of 540 cc. of carbon dioxide.

4 Per Cent Glucose.—The ascent begins after $3\frac{1}{2}$ hours and continues sharply for about 98 hours, after which the curve breaks, the end being reached after the production of 510 cc. of carbon dioxide.

5 Per Cent Glucose.—After $3\frac{1}{2}$ hours the curve rises sharply for 99 hours and then becomes horizontal. The total production is 535 cc. of carbon dioxide.

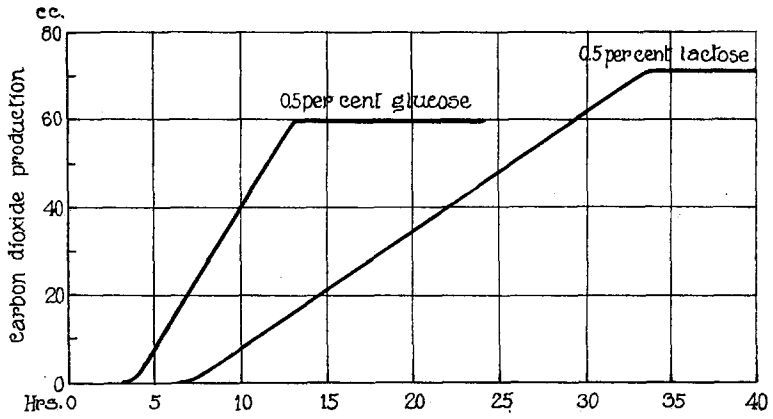
Results with Lactose.

The lactose experiments give only the first part of the curve, as the experiments were interrupted. The curve deviates greatly from the glucose curves. First, it takes somewhat longer for the rise to

begin; secondly, the rise is not so sharp; and thirdly, from the experiments which were completed the duration of carbon dioxide production is greater than in the glucose experiments.

0.3 Per Cent Lactose.—The ascent begins after 7 hours, and lasts about 11 hours, terminating with as sudden a break in the curve as in the case of the curve obtained with the lower concentrations of glucose. The total production was about 35 cc. of carbon dioxide.

0.5 Per Cent Lactose.—The rise begins after about 8 hours and lasts about 27 hours (compare the glucose curve, Text-fig. 3). 72 cc. of carbon dioxide were produced.



TEXT-FIG. 3. The original lactose and glucose curves as registered by the spirograph, reduced about one-ninth.

1 Per Cent Lactose.—After about $5\frac{1}{2}$ hours the ascent proceeded with an average production of 3 cc. per hour and reached about 50 cc. in 24 hours, when the experiment was interrupted.

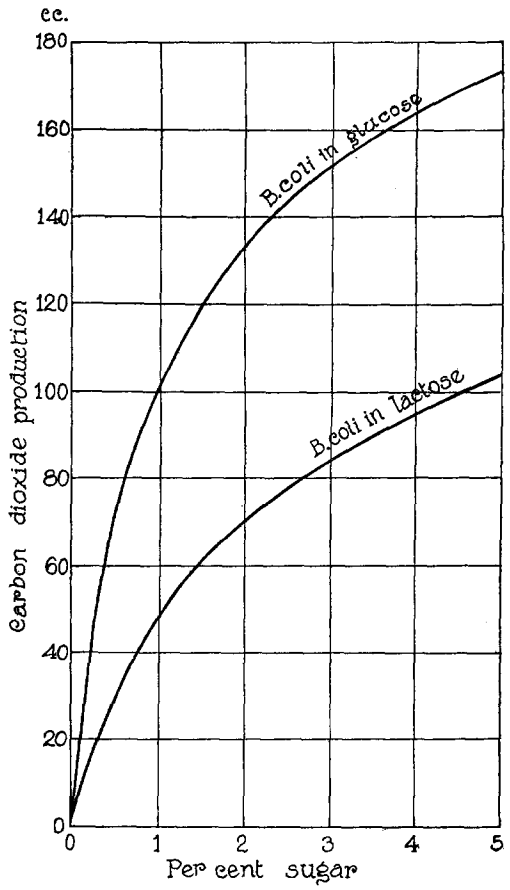
2 Per Cent Lactose.—After about $7\frac{1}{2}$ hours the rise began and gave a total production of 60 cc. in 24 hours.

3 Per Cent Lactose.—After 6 hours the rise began and reached about 90 cc. in 24 hours.

4 Per Cent Lactose.—After about 5 hours the rise began and yielded 110 cc. in 24 hours.

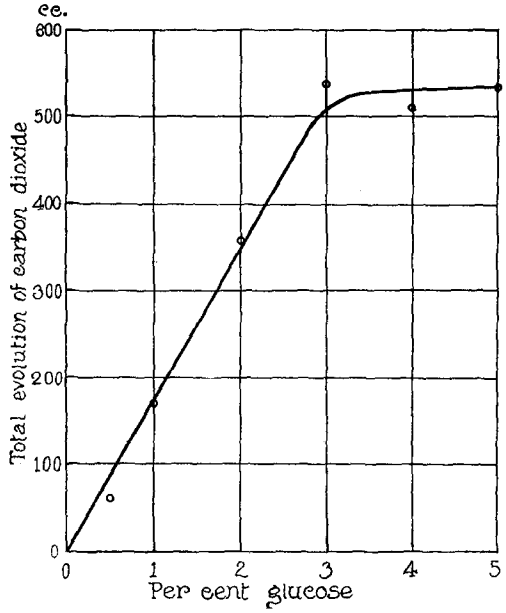
5 Per Cent Lactose.—After 4 hours the rise began and yielded 120 cc. in 24 hours.

It is seen from Table I and the curve (Text-fig. 5) that an ascending concentration of glucose increases the total volume of carbon dioxide produced until about 3 per cent is reached. Greater concentrations of glucose produce no further addition to the total amount of carbon

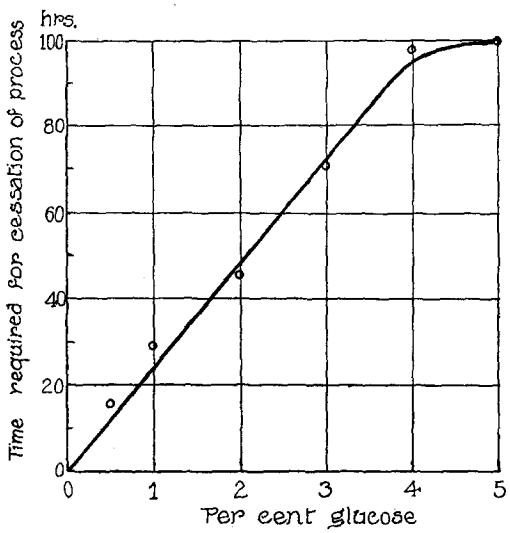


TEXT-FIG. 4. Comparison of the lactose and glucose curves during the first 24 hours.

dioxide formed, whereas the time consumed by the process steadily increases (Text-fig. 6). This indicates that the sugar inhibits the growth of the bacteria. The lactose curve was observed only during the first 24 hours, and when compared with the corresponding period

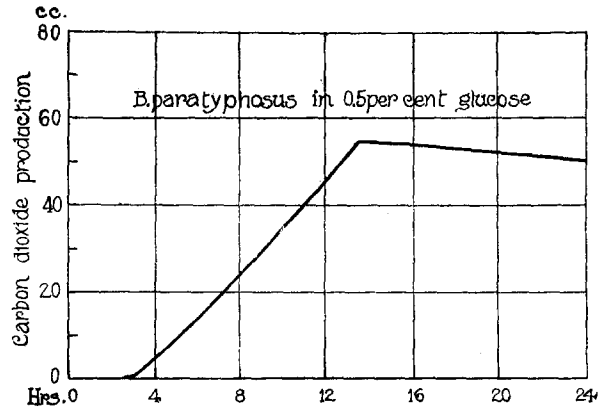


TEXT-FIG. 5. Curve showing the effect of an ascending concentration of glucose upon the total volume of carbon dioxide.



TEXT-FIG. 6. Curve showing the length of time required for the cessation of carbon dioxide production with ascending concentrations of glucose.

of the glucose curves (Text-fig. 4) we perceive, as already mentioned, the longer latent period in the former, the more gradual rise, and, as would appear from the experiments which were completed, a larger total carbon dioxide production. With an increase in the amount of peptone up to 3 per cent the latent period (the time before the rise begins) becomes shorter. When we compare the curve for *Bacillus coli* in 0.5 per cent glucose with the curve for *Bacillus paratyphosus* B also in 0.5 per cent glucose, both curves are seen to be similar, until the point is reached where the process stops. Then while the *coli* curve, as we have seen, continues as a horizontal line, the *para-*



TEXT-FIG. 7. Curve showing the amount of carbon dioxide produced by *B. paratyphosus* B in 0.5 per cent glucose.

typhosus curve (Text-fig. 7) begins to drop (Table II). In the *paratyphosus* curve the ascent begins after 3 hours and after about 12 hours 55 cc. of carbon dioxide have been produced. It then stops suddenly like the *coli* curve, is horizontal for 2 hours, and then drops 5 cc. during a period of 12 hours more. We have not yet been able to explain this. The same curve was found previously by the titration method. By this method it was easier to explain the drop as due to the possible sudden production of ammonia by the bacteria. But here where we are dealing with a method which should show results only in one direction, the case becomes more difficult. At first we naturally thought of the possibility of a leakage of carbonic acid

formed through the connecting tubes. All rubber tubes were therefore replaced with glass tubes, but the curve did not change. That it is a phenomenon connected with the life of the bacteria we have ascertained by killing all the bacteria with sublimate. If ammonia is produced by the bacteria this would be retained in the culture, so that the latter would be able to bind more carbon dioxide than normally.

TABLE II.

B. coli communis and B. paratyphosus B in Mixture.

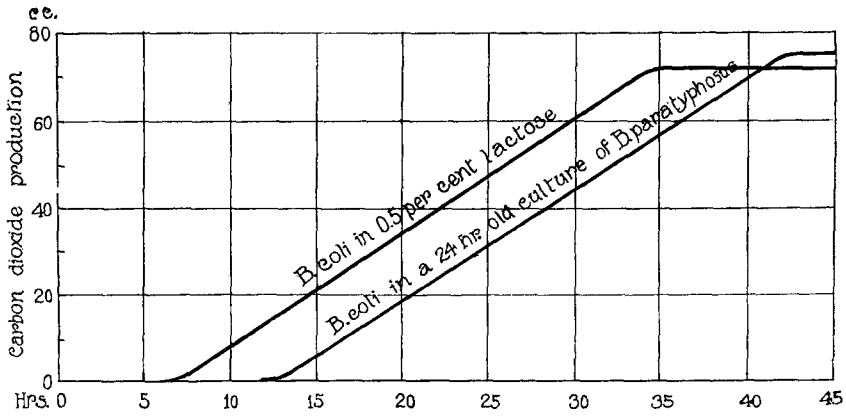
Bacteria.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.	
				After 12 hrs.	After 24 hrs.
Glucose 0.5 per cent.					
	hrs.	*cc.*	*hrs.*	*cc.*	*cc.*
B. coli	3.5	57	13.0	14	0
B. paratyphosus B	3.0	55	13.5	13	0
B. coli added to 24 hr. culture of *paratyphosus*		−16	24.0	−2	−2
B. paratyphosus added to 24 hr. culture of *coli*		−3	24.0	−0.5	0
B. coli + *B. paratyphosus* B	6.0	53	14.5	13.5	−2
Lactose 0.5 per cent.					
B. coli	4.5	76	20.0	11	0
B. paratyphosus B	4.0	−18	24.0	−3	0
B. coli added to 24 hr. culture of *paratyphosus*	12.0	74	44.0	0	3
B. paratyphosus added to 24 hr. culture of *coli*		−14	24.0	−2	−3
B. coli + *B. paratyphosus* B	7.0	48	24.0	6	6

When *Bacillus paratyphosus* is added to lactose media the result is different, since here no acid is produced. But after about 3 hours the curve falls about 20 cc. in the course of 24 hours.

If *Bacillus coli* (Text-fig. 8) is added to a 24 hour *paratyphosus* experiment in lactose, the curve will cease to fall and become a horizontal line in about 14 hours. After this we obtain a steady rise, which, as a rule, lasts a little longer than with the *coli* experiment. In other

words, the latent period for the formation of acid by *coli* is long, which can be explained by the fact that *coli* must overcome the profuse *paratyphosus* growth with its metabolic products. Lack of peptone is partly responsible for this as we have seen that a similarly long latent period was obtained with smaller amounts of peptone. If in like manner we add *coli* to a 24 hour *paratyphosus* growth in glucose, the curve continues to fall. This demonstrates that the decline in the curve is independent of the sugar present, whether glucose or lactose.

We have also proceeded in the opposite direction in order to ascer-



TEXT-FIG. 8. Comparison of the results obtained in lactose with *B. coli* and with a 24 hour *paratyphosus* culture to which *B. coli* has been added.

tain the result when *paratyphosus* is added to a 24 hour *coli* culture. In glucose a fall was noted after the *coli* had stopped producing acid. The same happened in lactose, but the fall was somewhat more abrupt. We finally undertook to determine the effect of adding an equal mixture of *paratyphosus* and *coli* at the beginning of the experiment. In glucose we obtained a curve similar to the *paratyphosus* curve. In lactose we obtained a somewhat longer latent period (7 hours) than with *coli* alone; after that a rise of somewhat longer duration (18 hours) was noted, next a horizontal line (2 to 3 hours), and then the usual fall.

SUMMARY.

We may formulate the results as follows:

1. *Bacillus paratyphosus* in lactose-peptone-water causes a fall in the curve. The explanation of this must be left to later investigations.

2. *Bacillus paratyphosus* in glucose-peptone-water shows at the beginning a rise similar to that produced by *coli*, but a fall in the curve follows.

3. If *Bacillus coli* is added to a 24 hour *paratyphosus* lactose culture a very long latent period is obtained before the formation of acid by *coli* becomes apparent, presumably due to accumulated basic metabolic products.

4. If *Bacillus coli* is added to a 24 hour *paratyphosus* glucose culture the curve is not changed from the normal to any perceptible degree.

5. *Bacillus coli communis* and *Bacillus paratyphosus* in lactose in mixed cultures give a longer latent period than *coli* alone. The first ascending part of the curve is due to acid formation by the *coli* which in the beginning is dominant. Later *paratyphosus* dominates and causes an ultimate decline in the curve.

6. *Bacillus coli communis* and *Bacillus paratyphosus* in glucose in mixed culture behave like *Bacillus paratyphosus* in pure culture.

The purpose of these investigations has been for the present to show the usefulness of the method. It is hoped that by these investigations material of particular interest relating to the biochemical and physiological processes within the bacterial culture will be obtained.

EXPLANATION OF PLATE 54.

FIG. 1. Photograph of the self-registering spirometer used to measure acid production of bacteria.

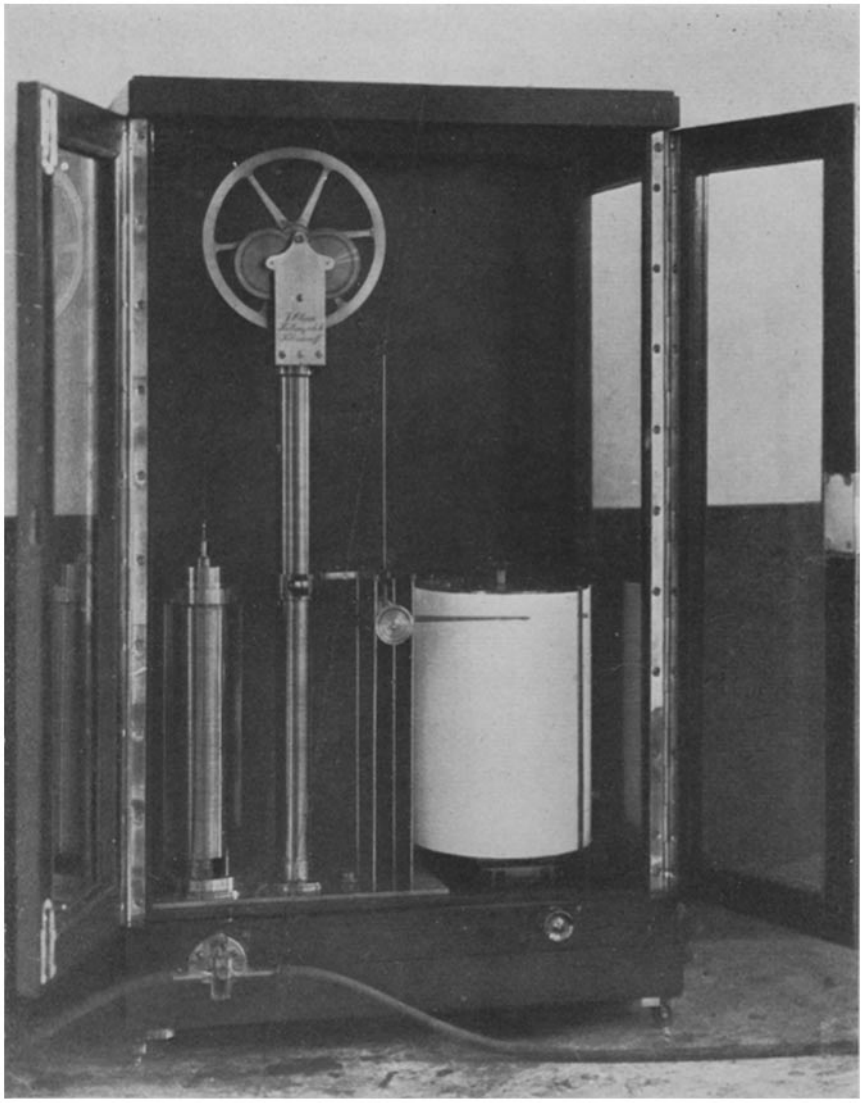


FIG. 1.

(Fischer: Acid production in cultivation of bacteria.)