THE MEASUREMENT OF THE OPACITY OF BACTERIAL CULTURES WITH A PHOTO-ELECTRIC CELL.

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(With 6 Figures in the Text.)

Introduction.

In the standardising of bacterial antigens some kind of opacity standard is convenient. The artificial suspensions commonly used are physically unstable and are unreliable guides. G. S. Wilson (1926) pointed out that the use of a bacterial suspension as a standard was open to error, as the opacity of a culture depends on its age as well as on the number of organisms present.

It seemed probable that examination of the opacity of cultures by photometrical methods might give useful information. An attempt to use the Lummer-Brodhun photometer proved unsuccessful, as the instrument was not sensitive enough. A photo-electric cell used as a photometer was found to be sufficiently accurate, and obviated the personal error to which other methods of opacity measurement are liable.

Apparatus.

(Fig. 1.)

The photo-electric cell was used in conjunction with a Dolezalek electrometer, one pair of whose quadrants was earthed, and the other pair connected with the anode of a photo-electric cell. It was convenient to measure the intensity of light falling on the cell by means of a steady deflection of the electrometer needle. The anode of the cell was therefore earthed through a high resistance, of the order of 10^{11} ohms; thus when the anode became charged up a steady potential difference between it and earth was set up, and was measured by the deflection of the electrometer needle. The sensitivity of the apparatus depended on the resistance, which was fixed; and on the potential of both electrometer needle and cell cathode. For the purposes of the experiment about 200 volts on the electrometer needle, and about 80 on the photo-electric cell were found satisfactory.

In the earlier part of the work the light illuminating the cell was supplied by a direct current 40-watt globe. Later it was found more satisfactory to use a standard motor-car lamp. A rheostat and sensitive ammeter were introduced into the lighting circuit, since a variation of 3 per cent. in the current

through the lamp was found to affect the scale reading by 25 per cent. The current was kept constant within 0·1 per cent.

The photo-electric cell was fitted up in a light-tight box in one side of which was an aperture about an inch in diameter, covered by a movable brass shutter. An arrangement was made whereby a culture tube could be placed in an upright position between the light source and the aperture and directly in front of the latter. The shutter was raised for each reading, for a period, about 40 sec., long enough for the electrometer needle to attain a steady position.

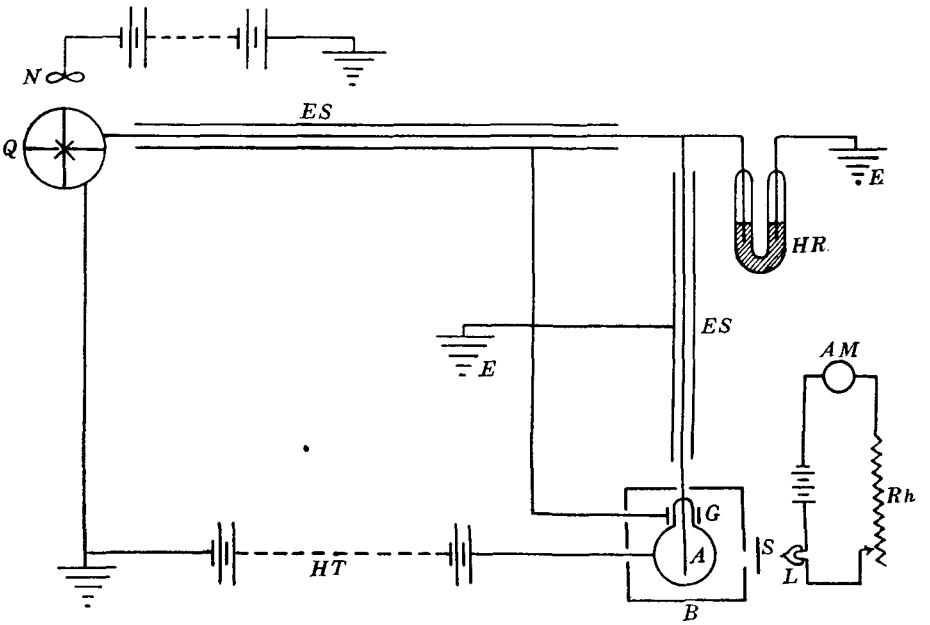

Fig. 1.

A. Anode of Photo-electric Cell; AM. Ammeter in Lighting Circuit; B. Light-tight box; E. Earth; ES. Electrostatic Shield; G. Guard Ring; HT. High Tension Battery; HR. High Resistance; L. Lamp; N. Electrometer needle; Q. Quadrant Electrometer; Rh. Rheostat; S. Brass Shutter.

Technique.

Experiments were carried out to examine the variation in opacity of cultures growing in liquid media. The cultures were interfered with as little as possible apart from the vigorous shaking necessary to distribute the organisms evenly. In nephelometric work, vessels with plane glass sides are generally used; but it was found impossible, in this country, to get such vessels which would also be suitable for use as bacterial culture tubes. It was found that there was no objection to the use of round test-tubes, provided that the instrument was calibrated with the test-tube used in the actual experiment. The fact that the round test-tube acted as a lens actually increased the sensitivity of the instrument in the region of small opacities.

Fig. 2 shows two curves recording the relationship between concentration and deflection. Curve *A* was made with the use of a square tube (from a "Hellige comparator" apparatus), curve *B* with a round test-tube of the same cross-section. It will be seen that over the same range of concentrations curve *B* is the sharper. A suspension of very fine clay particles was used.

It was essential to choose an organism that did not form chains or other aggregations, and that was without tendency to auto-agglutinate in liquid media. A strain of *Salmonella gallinarum* proved very successful, as the individual organisms rarely formed chains or aggregations, and the cultures themselves formed stable homogeneous suspensions. The same strain of *S. gallinarum*

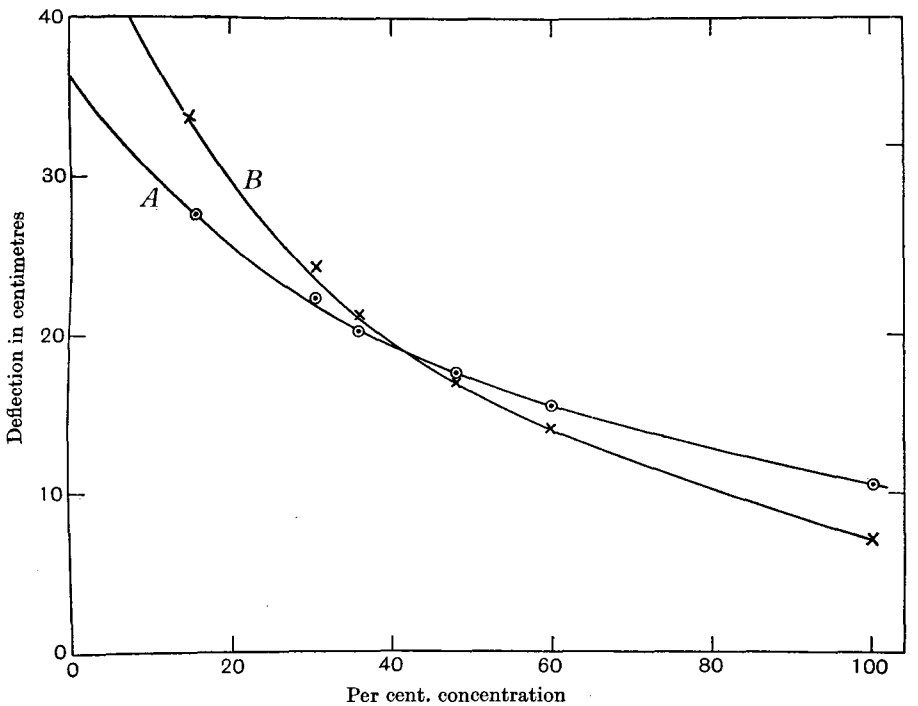

Fig. 2.

A ⊙—⊙ Square tube. B ×—× Round tube.

was used throughout the experiment. At first an attempt was made to cultivate the bacteria on Dolloff's medium; but the growth was slow and ordinary beef bouillon with a *p*H 7·2–7·8 was used.

The procedure for several series of measurements over 24 hours was as follows:

Two test-tubes containing the same amount of bouillon (30 c.c.) received equal inocula from a 24-hour culture of *S. gallinarum*. One of these tubes (*A*) was retained for a series of counts; the other (*B*) being used for the opacity measurement. A third tube (*C*), of the same diameter as *B*, contained an amount of

bouillon equal to the amount in each of the other tubes after inoculation. *C* was left sterile, and served as a control in case of any change in the colour or opacity of the bouillon not caused by the bacteria. Tubes *A*, *B*, and *C* in each series received bouillon from the same batch, and were handled identically as regards storage and sterilisation. This applied also to a quantity of bouillon which was retained for calibrating the opacity apparatus at the end of the run. The opacity measurements and the bacterial counts were of necessity carried out in two different places. Thus after inoculation tubes *B* and *C* had to be transported to the physical laboratory, which meant that they were out of the incubator for about half an hour. A time was prearranged at which they would be put back in the incubator, and tube *A* was left out for the same period of time, an initial count being made during the wait.

The two incubators were adjusted to the same temperature. It is improbable that slight discrepancies between the two could have affected the results. M. W. Jennison and J. W. M. Bunker (1931) found that the generation time of *B. coli* was practically the same at 37° and 42° C. for cultures not recently isolated. It may here be mentioned that in the first experiment only one tube was used, samples being taken for counting from the opacity tube. It was impossible, however, to continue with this procedure.

Total counts were made with a haemocytometer. Owing to lack of time and assistance it was found impossible to perform parallel viable counts. The opacity measurements as well as the counts were made at approximately three-quarter hour intervals, tube *B* being shaken the same number of times before a reading as tube *A* before a count. Each run was completed at 24 hours after inoculation.

The deflection of the electrometer needle in such an arrangement as has been described is not a linear function of the opacity of the suspension. It was therefore necessary to calibrate the instrument with various dilutions of some standard suspension. It would have been possible to make one calibration suffice for all the runs, but the chance that the bouillon from different batches might vary in colour, or that some variation in sensitivity of the apparatus might occur over a period of several weeks, suggested the advisability of calibrating separately for each experiment. The 24-hour culture was used as a standard in each case, and dilutions were made with bouillon set aside for the purpose. The relationship between scale reading and concentration of the 24-hour culture was then graphically established by a curve such as curve *B* of Fig. 2. The concentration corresponding to any scale reading could then be read off from the curve, the given concentration in each case representing a certain number of 24-hour-old bacteria. The total number at 24 hours was taken from the count at that time. Thus the opacity of the culture at any age was defined by the number of 24-hour-old organisms necessary to give the same scale reading.

Now if the opacity of the culture depended only on the number of bacteria present, or, in other words, if the bacteria of all ages had similar optical pro-

perties, then it was to be expected that the "opacity curve" and the growth curve would be identical. This, however, was not the case. The divergence in the two curves lay outside the bounds of experimental error. The following were possible explanations of the divergence:

(1) That the bacteria might in some way affect the bouillon and change its colour or opacity. The difference between growth curve and "opacity curve" would then include changes in the bouillon itself.

(2) That the bouillon itself, apart from any effects of the bacteria, might change in colour or opacity on standing.

An experiment was devised to control the first possibility. Eight similar tubes of bouillon received equal inocula (about 2 million per c.c.), a ninth tube being left sterile, and the cultures were left to grow for varying times. Each was in turn passed through a Seitz filter, and the opacity of the bacteria-free filtrate examined. In order not to have to depend on one reading for each tube, a series of readings was made in every case, each series consisting of the readings taken with several different currents in the lighting circuit. The eight growing tubes were examined at times varying from 1 to 26 hours after inoculation. In no case did the opacity vary appreciably from that of the filtered bouillon of the sterile tube. This experiment was also a check on the second possibility mentioned above. An additional control was, however, provided by the use of tube *C*, whose opacity reading remained constant within 5 per cent. throughout each run.

In addition to the 24-hour runs an experiment was performed to compare directly the number of bacteria of different ages which would give some standard opacity. A number of similar tubes of bouillon which had received equal inocula at the same time were incubated. They were taken one by one, each at a different stage of growth, and successive dilutions were made with bouillon, the scale reading corresponding to each dilution being noted. Thus for each stage of growth a curve similar to curve *B* of Fig. 2 was obtained. Some scale reading was selected which appeared on every curve, and the opacity represented by it taken as the standard. The number of bacteria producing it at each stage in the growth of the culture could then be established.

EXPERIMENTAL RESULTS.

Exp. 1. An opacity curve was made, with no parallel counts. The form showed a close similarity to the usual type of bacterial growth curve. The opacity of the culture is defined by the number of 24-hour-old bacteria that give the same scale reading. The term "*opacity curve*" will have this meaning throughout the paper.

Exp. 2. In this experiment bouillon of *p*H 7·6 was used, and parallel counts were made from the tube used in the opacity determination, the original inoculum being about 10 million per c.c. Unfortunately it was not possible to incubate the culture until 2 hours after inoculation. The opacity curve and

count curve show the greatest divergence at 3 hours, where the number of organisms is 35 million and 13 million respectively.

At 7·3 hours the curves meet and continue together, the number at the meeting point being 398 million per c.c.

Exp. 3. Bouillon of *p*H 7·8 was used. The original inoculum was 10 million per c.c., and the counts were made from a duplicate tube. The opacity and count curves show a point of greatest divergence at 1·75 hours; the number of 24-hour bacteria was 29 million and the number of growing bacteria 10 million per c.c. The curves cross at 3·4 hours, the number of organisms being 159 million per c.c. The opacity curve then proceeds below the count curve, with a point of greatest divergence at about 7 hours, where the number of 24-hour bacteria is 347 million per c.c. The curves meet again at 10 hours and proceed together.

Exp. 4. Bouillon *p*H 7·8 was used. The original inoculum was 130 million per c.c., and the two curves were practically coincident all the way (see Fig. 6).

Exp. 5. Bouillon *p*H 7·5. The original inoculum was about 15 million per c.c. In this case no early readings were taken, but there seems to be a point of greatest divergence at about 2 hours, where the opacity count was about 100 million per c.c., and the actual count 44·7 million per c.c. The curves meet at 9 hours, where the count is 400 million per c.c.

Exp. 6. The filtration experiment mentioned above (see Technique, p. 498).

Exp. 7. Bouillon *p*H 7·6 was used. The original inoculum was about 2·0 million per c.c. The greatest divergence between the curves occurs at 3 hours, where the number of growing organisms was 6·3 million per c.c. The number of 24-hour organisms giving the same opacity was 44 millions per c.c. The curves meet at approximately 8 hours after inoculation, the number of organisms being 400 million per c.c. at that time. In this experiment the two curves are very nearly parallel in the logarithmic stage.

Exp. 8. Bouillon *p*H 7·6. The original inoculum was 3·2 million per c.c. The greatest divergence between the curves occurs at 3 hours, where the number of 24-hour-old bacteria is 45 million, and of growing bacteria 5·6 million per c.c. The curves cross at 7 hours, the number of organisms at that time being 280 million. These curves are again very nearly parallel in the logarithmic stage (see Fig. 3).

Exp. 9. This experiment was made to determine the numbers of bacteria in cultures of various ages which would give some standard opacity. The method has already been described (see Technique, p. 498). The results are best presented by means of a figure (Fig. 4).

Each curve was made with a culture of different age, and each shows the deflection of the electrometer needle corresponding to different numbers of

bacteria from the culture in question. The numbers were estimated from the growth curve constructed from counts made on a parallel culture. The count at the actual time of each reading was read from the growth curve, and according to the dilution used the actual number of organisms was calculated. It will be seen that the order of the curves is as follows: 4·7, 5·8, 7·2 and 14·1 (coincident), 26, 8·7 hours. Although cultures younger than 4·7 hours were examined, their small turbidity made it impossible for the dilution curves to be of value in this experiment. For example, the maximum number of bacteria in the 3·6-hour curve was 19 million per c.c. This was too near the end-point of all the curves to be of value.

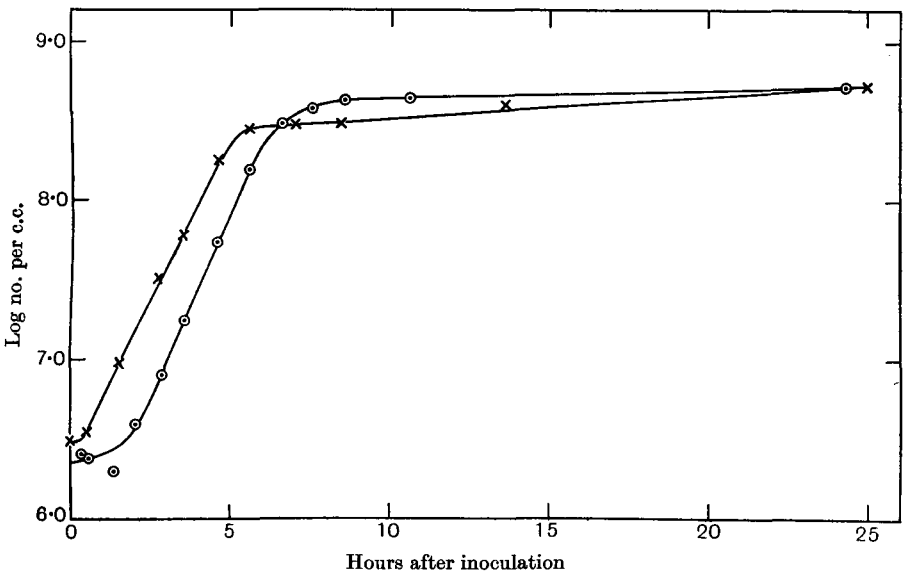

Fig. 3.

Actual count ⊙—⊙. Opacity count ×—×.

If, now, some deflection included in all the curves be selected, it is possible to determine the number of organisms producing it in each culture. As an arbitrary standard, take the deflection produced by 200 million per c.c. of the 26-hour-old bacteria. The reading is 20·8 cm., and the corresponding numbers of organisms in the other cultures are recorded in Table I.

Table I.

Time after inoc. (hours)	Number per c.c. (millions)
4·7	60
5·8	111
7·2	178
8·7	238
14·1	178
26·0	200

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G. S. Wilson (1926) made a series of estimations of the numbers of *B. aertrycke* (*mutton*) which at different stages of growth would correspond to an opacity standard consisting of 500 million per c.c. of *B. coli*. It is of interest to take the same number as standard in this experiment in order to compare

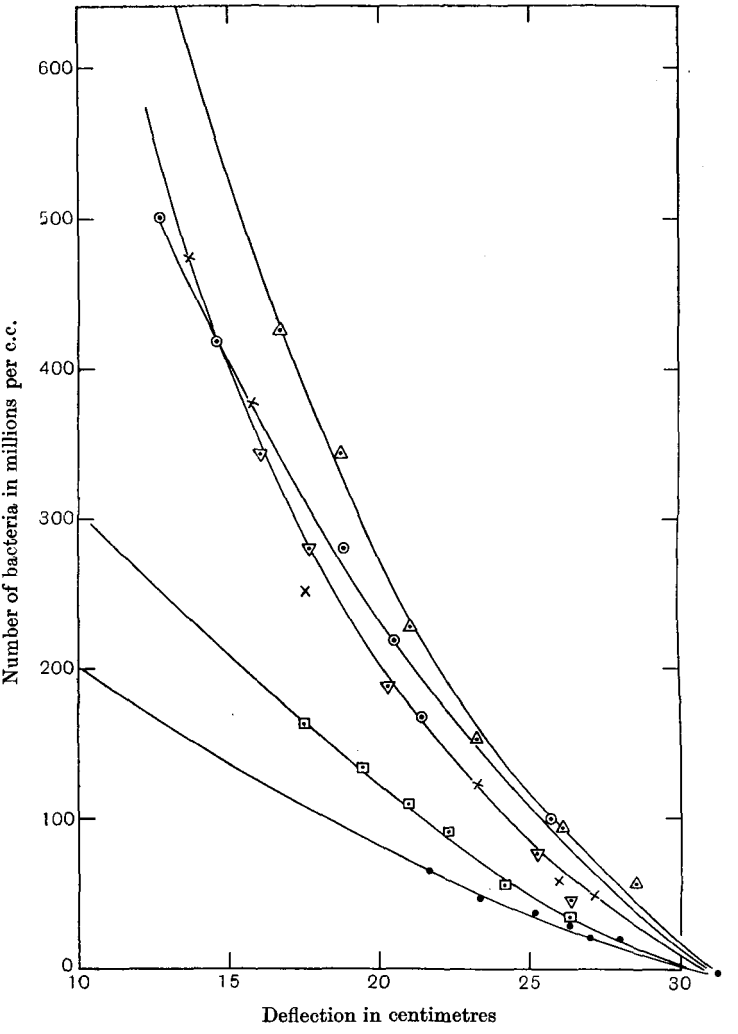

Fig. 4.

⊙ 26 hours. × 14·1 hours. △ 8·7 hours. ▽ 7·2 hours. ⊡ 5·8 hours. ● 4·7 hours.

the results. This can only be done by extrapolating all the curves except the one made with the 26-hour culture, and the figures can therefore not be relied upon as accurate quantitative results. Table II shows, nevertheless, that the agreement between the two sets of results for cultures up to 7 hours old is very striking:

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Table II. *Standard of deflection* 13 *cm.*

Time after inoc. (hours)	Number per c.c. (Alper & Sterne) (millions)	Number per c.c. (Wilson) (millions)
4·7	178	200
5·8	246	260
7·2	528	500
8·7	646	550
14·1	528	610
26·0	500	700

DISCUSSION.

The results obtained confirm Prof. Wilson's finding for *B. aertrycke* that the opacity of a bacterial suspension is not in general dependent only on the number

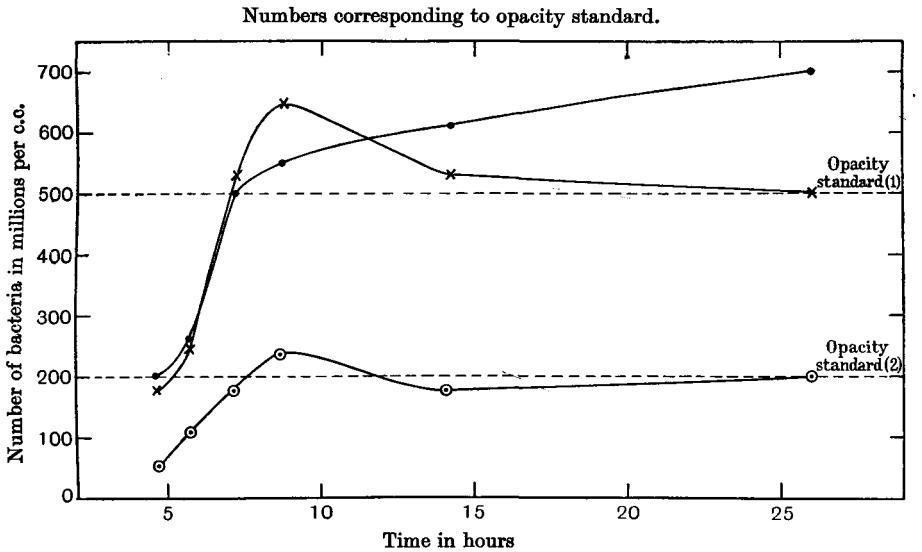

Fig. 5.

●—● G. S. Wilson. Standard = 500×10^6/c.c.
×—× Alper and Sterne. Standard = 500×10^6/c.c. (1).
⊙—⊙ Alper and Sterne. Standard = 200×10^6/c.c. (2).

of organisms present, but also on the age of the culture. Fig. 5 shows the number of bacteria corresponding to a standard opacity as found in the present experiments. It will be seen that the opacity of the 24-hour organism is attained at about 7 hours; the points at which the opacity and growth curves cross, in the series of results exemplified by Fig. 3, are the points where the opacity of the 24-hour-old bacteria is first attained. In those experiments where comparatively small inocula were used, the times of crossing were as follows: 7·3, 4·3, 7·0, 9·0, 8·0, 7·2 hours. The mean of all these times is 7·1 hours. In *Exp.* 4 the curves were coincident from inoculation. This experiment will be referred to later.

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It seems, therefore, that whatever the changes in opacity may be due to, a culture about 7 hours old behaves optically like one 24 hours old. At 8·7 hours the number of bacteria required to give some standard opacity is a maximum. This is represented, in Fig. 3, by the point *after* crossing where the difference between the curves is greatest. It is possible that the presence of this maximum in many of the experiments is due to a combination of experimental errors in counting and opacity measurement. There seems little room for doubt, however, that the 7-hour cultures have the same opacity as at 24 hours; and it seems significant that Wilson's curve, made with a similar type of organism, crosses the opacity standard ordinate at about 7 hours after inoculation, his standard having been made with a 24-hour-old culture of *B. coli*.

The general shape of the opacity curve (Fig. 3) bears a resemblance to the ordinary growth curve in that it possesses lag, logarithmic, and stationary stages. The slope of the curve in the logarithmic stage is less steep in the opacity curve, and the lag stage is not as long as in the growth curve. Before the opacity and growth curves cross, the opacity curve lies above the count curve, a point of maximum divergence occurring at 3·0 hours. The times of maximum divergence in the different experiments are as follows: 3·0, 1·75, 2·0, 3·0, 3·0 hours (mean 2·6 hours). Again *Exp.* 4 was an exception, since the curves were coincident.

Now if a comparison be made between the number of 24-hour-old bacteria required to give a certain opacity, and the number of growing bacteria producing that opacity, the ratio between them will be given by the difference in the ordinates of the opacity and count curves, since they are on a logarithmic scale. The ratio is greatest at the point of maximum divergence, and varies with the size of the original inoculum. Table III gives the different ratios.

Table III.

No. of Exp.	Original inoculum Millions per c.c.	Ratio at approx. 3 hours
4	130	1
5	15	2·2
2	10	2·7
3	10	2·9
8	3·2	7·0
7	2·0	8·0

Thus it seems clear that the number of 24-hour bacteria required to absorb the same amount of light as, roughly, 3-hour bacteria is much greater when the original inoculum is small. The probable meaning of this fact will be suggested later. The mean greatest ratio between "opacity" and actual number is 4·6, if we omit that experiment where the original inoculum was 130 million per c.c. Up to 7 hours after inoculation the ratio is always greater than one (except in *Exp.* 4, where it is one), *i.e.* the number of 24-hour-old bacteria absorbing a certain amount of light is always greater than the number of 7-hour-old or younger bacteria absorbing that amount of light.

The fraction of light absorbed when a beam passes through a cloudy medium

is a complicated function of the size and number of the particles, their refractivity and reflectivity, the wave-length of the light and the thickness of the medium. It is therefore not possible in our present state of knowledge of the optical properties of bacteria to ascribe the variation in opacity definitely to one or more factors. As Wilson suggests, however, the opacity is probably to a large extent a measure of the total protoplasmic mass, and the variation in opacity is due to a variation in the size of the organism during growth.

Clark and Ruehl (1919) demonstrated a change in the morphology of bacteria during growth, and Henrici (1926) investigated the morphological variation of *Bacillus megatherium* throughout the lag and logarithmic stages. His curves for cell size showed a shorter lag than his curves for numbers of cells, due to an increase in size of the cells prior to division commencing. As seen above, the opacity curves also showed a shorter lag phase than the growth curves, so that the interdependence of opacity and cell size would seem likely and would well account for the observed changes in the opacity of a bacterial culture.

Interpreted in this way the present series of curves would show that *Salmonella gallinarum* increases in size up to about 3 hours, and then decreases until 7 hours when it attains the size of a 24-hour *S. gallinarum*. After 7 hours there is possibly a decrease in size, or at any rate in opacity. This can, however, be only tentatively suggested. It is interesting to compare the times of maximum size noticed by other authors. Henrici found a progressive change in the form of *B. megatherium*, reaching a maximum at $5\frac{1}{2}$ hours, followed by a gradual change to the original form which was complete at 10 hours. Stanhope Bayne-Jones and E. F. Adolph (1931) found that the mean volumes of the adult individuals in a culture of *B. megatherium* increased during the first 4 hours, after which they decreased.

If, as is suggested, the variations in opacity and in size of the bacteria are closely related, the maximum size occurring at about 3 hours should be represented by a corresponding minimum in the curves of Fig. 5, which represent the numbers required at all stages to give a standard opacity. That the minimum is absent is explained by the fact that the first useful quantitative results were obtained only after 4·5 hours' growth. An arrangement is under contemplation whereby the earlier part of the curve may be investigated.

A further result of Henrici is confirmed by these experiments. It has been shown that with very small inocula the variation in size is greater than with large inocula, the ratio between the actual number of bacteria at 3 hours and the number of 24-hour bacteria absorbing the same amount of light being 7·0, with an inoculum of 2 million per c.c., and the corresponding ratio being one, when the original inoculum was 130 million per c.c. Now Henrici finds that the change in size is much more marked with light than with heavy seedings, the number of gradations in growth rate between the maximum and the minimum being greater according as the initial seeding is greater. The coincidence of the curves of *Exp.* 4 (Fig. 6) is explained in this way. If the opacity is indeed

largely dependent on the size of the organism, it would seem that with a large inoculum there is very little change in the mean bacterial size either before growth commences, or while it is taking place. It may also be pointed out that there was practically no lag phase.

Henrici points out that "since the cells change in size while growing, the number of cells is not a true measure of growth, which could only be obtained by computing the volume of the protoplasm." If it be accepted that the opacity can afford a measure of the protoplasmic mass, then the "opacity curves" should be a truer measure of growth than the usual count curve. In any event,

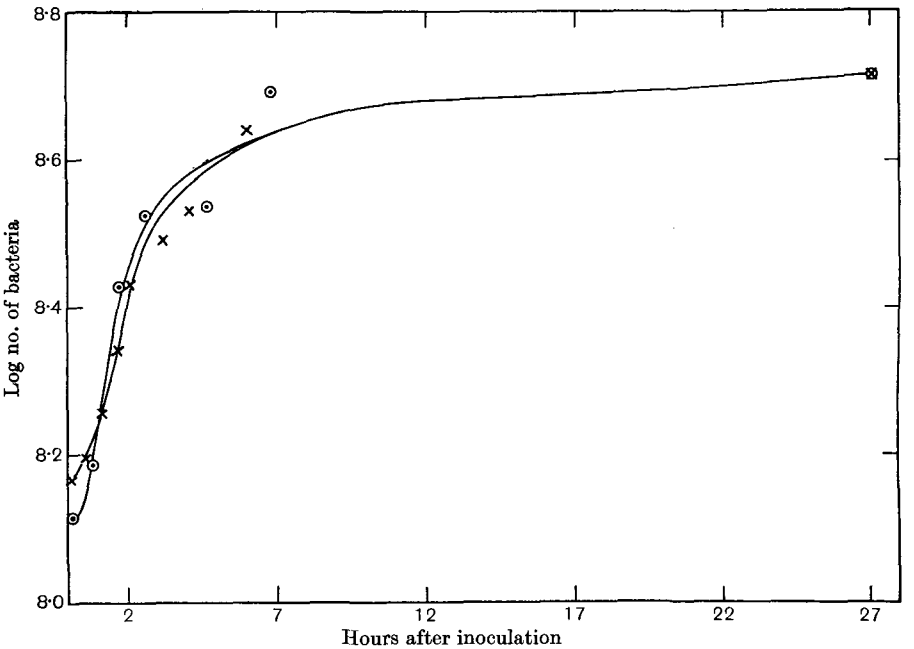

Fig. 6.

⊙—⊙ Actual count. ×—× Opacity count.

such curves can be used to define the growth of a culture in terms of organisms of a standard size, the standard throughout the experiments here described being the 24-hour-old organism.

Summary.

1. A photo-electric cell was used to measure photometrically the opacity of cultures of *Salmonella gallinarum* growing in bouillon.

2. The opacity of a culture was defined as the number of 24-hour-old bacteria which when suspended in the same type of bouillon as those under investigation would absorb the same amount of light. Seven experiments were made to compare the opacities of growing cultures with their actual numbers throughout the first 24 hours of growth. An additional experiment

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was made to find directly the numbers of bacteria of different ages which would absorb the same amount of light.

3. The ratio between opacity and number was greatest at about 3 hours, decreasing to unity at about 7 hours; the optical behaviour of cultures 7 hours old was thus the same as of cultures 24 hours old. It is possible from the results that the ratio decreased to a minimum at about 9 hours, thereafter again rising to unity. The value of the greatest ratio was greater as the inoculum was less. The mean greatest ratio was 4·6, the mean time being 3 hours. This means that it would take about 4·6 times as many 24-hour-old bacteria as 3-hour bacteria to absorb the same amount of light.

4. It was shown that the variation in the opacity-number ratio was in all likelihood due to changes in the size of the organism during growth.

5. The results suggest that the opacity curves give a truer picture of actual growth than the ordinary growth curves, since the cells change in size during growth.

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