From: The Laboratory for Special Bacteriology. Head: G. Frostell, Royal School of Dentistry, Stockholm, Sweden.

A TITRATION METHOD FOR THE DETERMINATION OF ACID AND ALKALI PRODUCTION BY MICRO-ORGANISMS

by

GÖRAN FROSTELL

Many important biological processes in nature involve a change in the hydronium ion concentration of the environment due to the metabolic activity of micro-organisms. Therefore, it is often of great interest to be able to determine quantitatively the acid or the alkali production capacity of various organisms or mixtures of organisms. Many methods are available for such a determination.

Numerous authors have studied the production of acid or alkali in cultures of growing organisms. Some have considered that under such conditions many metabolic processes may be involved simultaneously and the results will therefore often be difficult to interpret. In some experiments the incubation period has been kept short in order to avoid an appreciable multiplication of the organisms.

More clearcut results will often be obtained by the study of "washed" suspensions of organisms. The original substrate is eliminated and the organisms are suspended in a substrate mixture of known composition, rendering it possible to determine quantitatively the ability of an organism to carry out certain chemical reactions. Great numbers of cells may be used in a single experiment and subsequently the experimental period may be kept short.

Determination of the substances produced may be performed

by quantitative chemical analysis. Many authors, however, have measured the pH before and after the experiment whereafter the acid or alkali production has been determined by titration back to the original pH.

Stephan & Hemmens (1947), *Strålfors* (1950), *Singer* (1951) and others have studied the acid or alkali production of suspensions of oral micro-organisms in relation to dental caries. They followed the changes of the hydronium ion concentration occurring in the suspensions when the cells acted on a sugar or on urea. *Strålfors* used a medium, the buffering capacity of which was known, and he was able to calculate the acid production rate of a number of strains of oral micro-organisms.

Acid production by micro-organisms may also be determined by manometric methods (*Warburg* and others). If the organisms are suspended in a bicarbonate buffer under carbon-dioxide atmosphere, the acid produced by the organisms will drive off carbon dioxide from the suspension to the gas phase, the amount of gas being equivalent to the production of such acids. (See *Umbreit et al.* 1957).

Often, however, when studying fluctuations of the hydronium ion concentration in a biological system, the investigator is more interested in the capacity of the cells or the material in question to produce hydronium ions from the substrate than to determine the actual amount and caracter of the acids or alkalis produced.

Willstätter et al. (1927) and *Stedman et al.* (1932) used a titration method when determining choline esterase activity. *David Glick* (1937) worked out a modification of their indicator method. When determining the choline esterase activity of human serum he titrated with 0.02 or 0.01N NaOH, keeping the hydronium ion concentration constant and controlled by electrometrical pH-determination using the glass electrode. The present author worked out a titration method (*Frostell* 1957), which makes it possible to determine the "acid production activity" or the "alkali production activity", i.e. the production of hydronium or hydroxyl ions per time unit by suspensions of micro-organisms at constant or nearly constant pH. Since considerable experience has now been obtained with this method (*Frostell*, 1959 a, 1959 b, 1960; *Frostell & Strålfors*, in print, *Takazoe & Frostell*, in print) a more detailed description of the me-

thods and apparatus used by the author will be given in the present paper.

THEORY OF THE METHOD

By the titration method the production of hydronium or hydroxyl ions is measured in a system consisting of an active principle (a suspension of bacteria or other cells, an enzyme solution), a substrate and a buffer. The changes of acidity occurring as a result of the acid or alkali production activity and read on a pH-meter are compensated by subsequent addition of base or acid. The amount of base or acid required per unit of time to keep the pH constant is a measure of the activity of the system.

If the experiment is performed with an unbuffered system or with a system with a low buffering capacity there will be extensive and rapid changes of the pH, on the one hand owing to the metabolic activity, on the other as a result of the addition of acid or base. If the buffer capacity of the system is high and the metabolic activity is low there will be minute indications on the instrument, the titration will be performed at long intervals, and great amounts of acid or base will be required in order to get back to the original pH. If the buffer capacity is low the titration must be performed with small quantities of acid or base at short intervals. The amount of acid or base per time unit required to keep the pH constant will, however, be the same in both instances, provided the same amount of active principle is used in the two systems. However, even if the metabolic activity as measured by the titration method is independent of the buffer capacity of the system, it is important to choose a suitable buffer concentration.

If a completely protolysed non-volatile acid or base is produced, and this substance does not enter into a chemical reaction with the substrate, an amount of acid or base will be required to keep the pH constant which is equivalent to the alkali or acid produced.

If the acid or base produced is only partially protolysed, the conditions are more complicated. The production of hydronium ions or hydroxyl ions will be less than the total production of the acid or the base in question, but will, provided the pH is constant, be proportional to that total production. The proportionality depends on the degree of ionization.

If, as a result of the metabolic activity, a partially protolysed acid is produced, the concentration of this acid (HA) will increase during the experiment. The acid will immediately protolyse partially to H_3O^+ and A^-. During a titration with NaOH, H_3O^+ will form water with OH^-, and Na^+ and A^- will accumulate. Since the sodium salt of a weak acid is a strong electrolyte which is completely dissociated,

there will be no formation of NaA. Thus, when the concentration of A^- increases and the H_3O^+ is kept constant, an accumulation of HA will occur. Consequently, the buffer capacity of the system will increase during the experiment.

In the neighbourhood of neutrality and at alkaline pH, the protolysis of lactic acid and many other weak acids is almost complete, and the amount of alkali required for the titration is equivalent to the total acid production. At low pH, however, the molar amount of NaOH required to keep the pH constant is lower than the molar production of acid. With the titration method the production of hydronium ions is measured directly.

Even more complicated conditions arise if the acid or base produced is volatile (formic acid, acetic acid, carbonic acid, ammonia). If the experiments are performed in an apparatus open to the air under constant stirring, or if a gas is bubbled through the suspension, it may be postulated that the partial pressure of the volatile substance over the surface of the suspension is zero or close to zero. Consequently, some of the acid or base produced will vanish and will not be measured during the experiment. If volatile acids or bases are produced, the titration method will be expected to give values of metabolic reaction activity which are lower than those which would have been obtained if the system had been closed. The difference from the total activity will be proportional to the amounts of volatile substance produced.

Strålfors (1956) made an investigation of the anaerobic fermentation of oral micro-organisms, using the *Warburg* technique. He found a close coincidence between the results obtained with the *Warburg* technique and the technique with an open system which he used in 1950.

In any case, if the stirring, the temperature, the hydronium ion concentration and the gas stream through the suspension are kept constant, an equilibrium between a possible production and evaporation of volatile acids or bases will rapidly establish itself, and the acid or alkali production activity as determined by the titration method will be constant and proportional to the total acid or alkali production. When this occurs, the activity curve will be a straight line.

If, as a result of the metabolic activity, a volatile acid and a volatile base are produced simultaneously (as in experiments on urease activity) the conditions are complex. Ammonia produced is distributed partly as NH_3 and partly as ammonium ion. If the pH is less than 7 the risk that NH_3 will disappear during the experiment is small, but at a pH over 7 the loss of ammonia may be considerable. Consequent on the hydrolysis of urea, carbon dioxide is also formed. The latter reacts with water and forms carbonic acid, which protolyses into H_3O^+, HCO_3^- and CO_3^{--}. Since the hydratization rate of carbon dioxide is low and the titration experiments are performed with a

system open to the air and the carbon dioxide pressure of the gas bubbled through the suspension is close to zero, it may be assumed that the carbon dioxide formed from urea will pass over into the gas phase quite rapidly. An equilibrium will establish itself between the production of carbon dioxide, the formation of carbonate

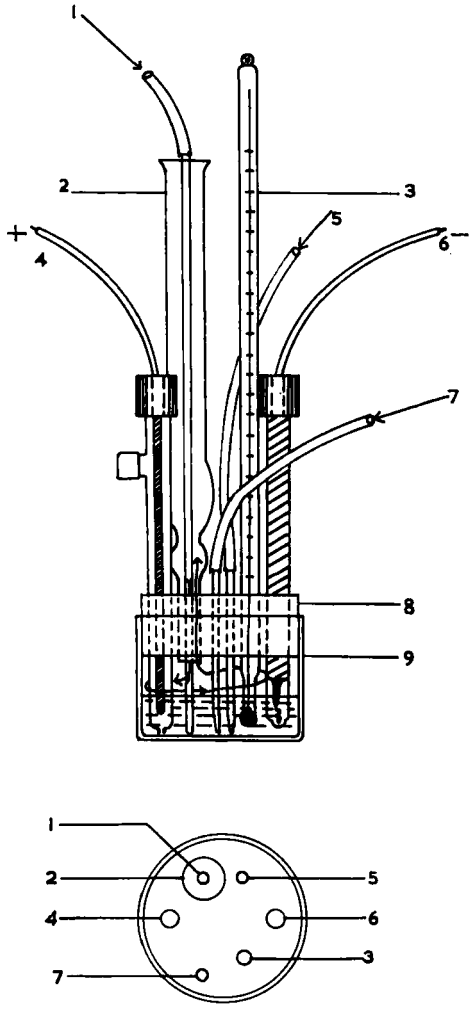

Fig. 1. Schematic drawing of the reaction bottle showing the experimental arrangement.

1. Tube for the introduction of gas. 2. Gas outlet. 3. Thermometer. 4. and 6. Glass and calomel electrodes. 5. and 7. Tubes for the introduction of acid and alkali.

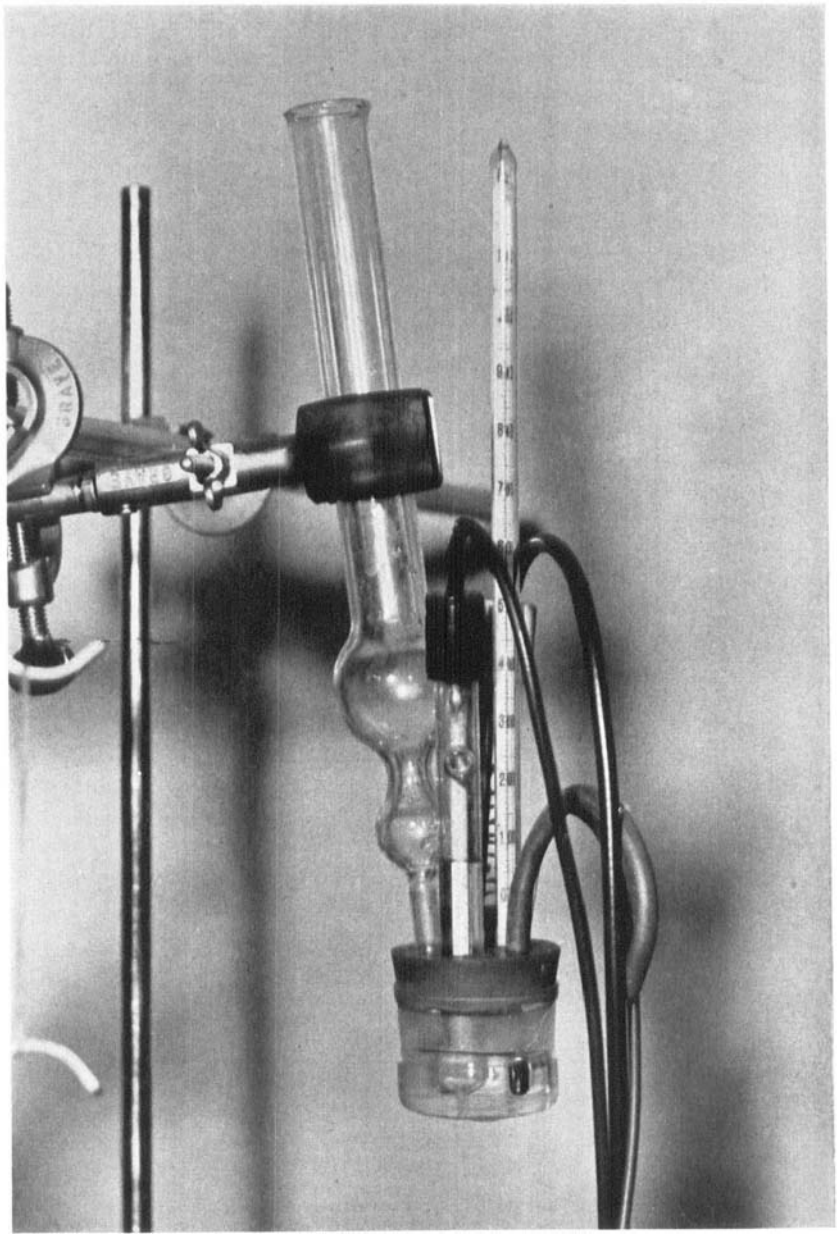

Fig. 2. The reaction bottle. The gas tube is not in position.

and bicarbonate ions and the loss of carbon dioxide to the gas. Since carbonic acid is a weak acid and ammonia is a weak base it may be expected that the salt $(NH_4)_2CO_3$ will accumulate to some extent during the experiment. The expected result will be that the activity determined as production of alkali is lower than the activity determined as production of ammonia but proportional to it. Such a system is not capable of producing hydroxyl ions to the same extent as NH_4^+ ions, a fact which thus may be illustrated by the titration method. If, on the contrary, the experiments are performed with a closed system, the carbon dioxide pressure of the system will increase. With increasing concentration an increased hydratization will take place with the formation of carbonic acid, carbonate and bicarbonate ions. Consequently, the alkali production activity as measured by the method will decrease when thus more ammonia will be neutralized.

Further, the possibility exists that acids or bases produced will react with some constituent of the system forming neutral substances. In such a case the titration method will make it possible to perform a determination of the ability of the system to produce hydronium ions, which is closer to actual conditions than the result of a chemical determination of the amount of acid produced.

Thus it is obvious that many factors influence the capacity of a biological system to produce hydronium or hydroxyl ions. Frequently, many of these factors will be imperfectly known at the evaluation of an experiment. Consequently, it is hazardous to calculate the hydronium or hydroxyl ion production activity from the results of a chemical determination of the substances formed. The titration method makes it possible to measure this production in a very direct way.

APPARATUS

The reaction bottle is a glass container the volume of which is about 30 ml (Figs. 1 and 2). The flask is closed by a rubber stopper through which holes are made for the introduction of the two electrodes of a pH-meter (4, 6), two fine glass tubes with pointed ends (one for admission of acid and one for base, 5, 7), a thermometer (3) and a tube (2) for the admission and release of gas.

The reaction bottle is mounted in a water-bath on a horizontal axis (Figs. 3 and 3 a). This axis may be moved to and fro by a motor within a range of 15 cm, keeping the solution in the bottle in motion.

Two burettes graded in hundredths of a ml are mounted beneath the apparatus. From the burettes, fine tubes made of rubber or acrylic lead the solutions to the reaction bottle (5, 7). Thus it is possible to titrate the solution directly even when the reaction bottle is in motion.

For admission and release of gas a tube (2) of glass is used. The gas is admitted through a fine glass tube (1) inserted through the tube (2), which has an opening 2 cm from the lower end. The gas is

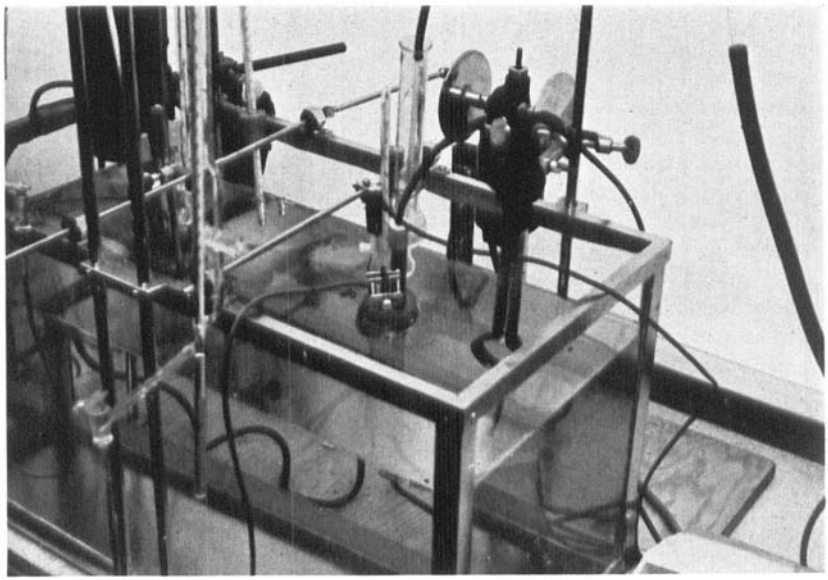

Fig. 3. The reaction bottle mounted in the waterbath.

thus admitted over the surface of the solution and will leave the reaction bottle through tube 2.

PROCEDURE

The pH-meter of the apparatus is calibrated to a buffer solution of known pH. The author has used a Beckman Zeromatic pH-meter, which has proven to be extremely stable.

The reaction bottle containing 5—10 ml of the buffersuspension mixture is mounted in the apparatus after the electrodes have been thoroughly rinsed with distilled water and dried by a stream of air. The pH of the solution — the calibration of the pH-meter — is checked and the bottle is lowered into the water and left for ten minutes to allow temperature equilibration to occur.

If the experiment is to be performed anaerobically or, for example, under an atmosphere of oxygen, the gas in question is admitted through the tube (1) and allowed to flow constantly when temperature equilibration occurs.

To create anaerobic conditions the author used nitrogen gas taken from a pressure bottle. The gas passed through two bottles containing an alkaline pyrogallol solution to a height of 60 cm. It was found, however, that the gas contained small amounts of oxygen after the passage. Later, the author used nitrogen which passed through a tube containing pure heated copper and two tubes containing calcium

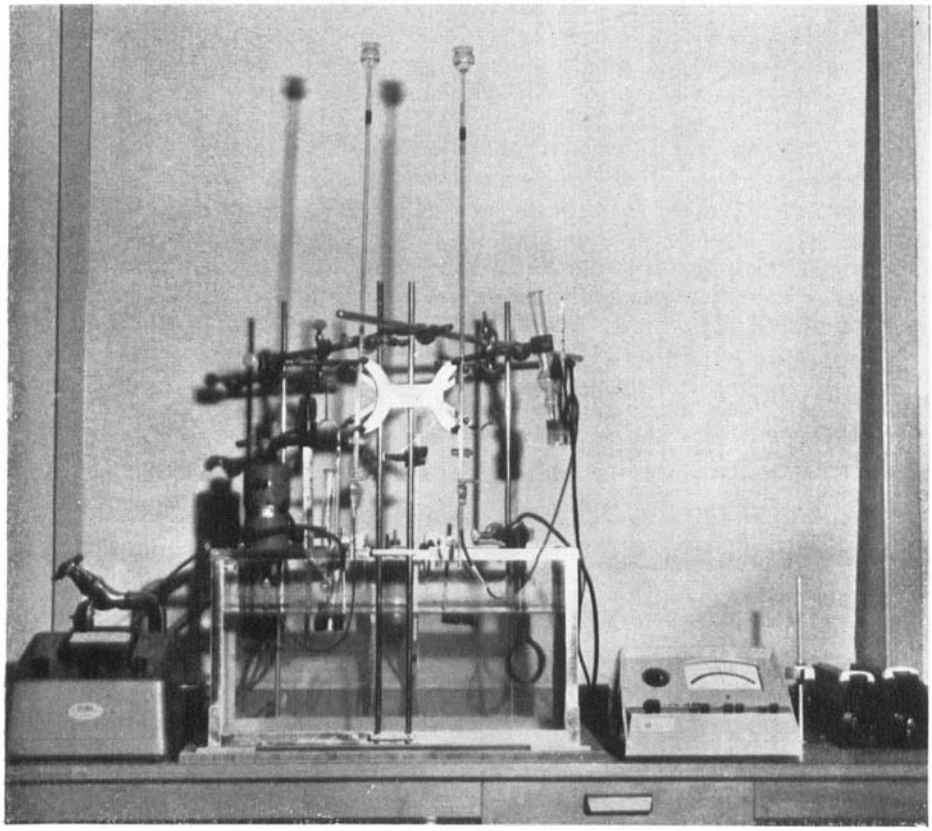

Fig. 3 a. View of the apparatus permitting the simultaneous performance of two experiments.

chloride. No oxygen was found in the gas so treated before or after its passage through the reaction bottle as determined by the *Haldane* method.

If a chemical determination is to be performed on the test solution before the beginning of the experiment a sample of known volume may be taken by a pipette through the tube 2.

At the start of the experiment proper, an amount of a solution containing the substance on which the micro-organisms or cells are going to act is pipetted through tube 2. This solution is warmed beforehand. At the same time a stop watch is set going.

The pH of the mixture in the reaction bottle will change after some time as a result of the enzymatic activity. When the pH has shifted about one-tenth of a unit an amount of acid or base is added from one of the burettes and the pH is read and noted. The concentrations

of buffer, acid or base may be chosen in such a way that one tenth of a ml of acid or base corresponds to a change of about one tenth of a pH-unit. A change of 0.1—0.3 pH-units per minute is ideal.

At the end of the experiment the stop-watch is stopped at exactly the pH-value obtained immediately after addition of the substrate and the time is read to the nearest second. If a chemical analysis is to be performed on the contents of the bottle, new samples may be taken at the end of the experiment through tube 2, or preferably from the bottle after it has been taken out of the apparatus. The metabolic activity may also be stopped by addition of excess acid or base. An inhibitor must not be added to the solution in the apparatus, because the rubber stopper and the electrodes may be contaminated and this may influence a following experiment.

EXAMPLES OF EXPERIMENTS AND RESULTS

1. Alkali production from urea by a staphylococcus strain

A study on the urease activity of oral staphylococci is described in a separate paper (*Frostell,* 1959 a).

A suspension of staphylococci (*Staphylococcus albus* (1), *Frostell,* 1957, 1959) was used. The cells were suspended in a 10 mM phosphate buffer (pH 6.9). Of this suspension 1.0 ml was transferred to the reaction bottle and 5.0 ml of a 10 mM phosphate buffer were added. After 10 minutes, when the temperature of the mixture was 37.5° C and the pH was 6.5, an amount of 1.0 ml of a urea solution (6.25 g/50 ml) was added. The temperature of the added solution was about 37° C.

The reaction bottle was constantly kept in motion (about 50 strokes per minute in each direction). Small amounts of 0.10N HCl were added from the burette when required, or about twice a minute. After about 10 minutes the experiment was stopped by the addition of 5 ml of the 0.10N HCl solution giving a final pH of about 2.2. When the bottle was taken out of the apparatus, 0.2 ml of a 20 per cent $CuSO_4$ solution was added. 0.44 ml of acid was required to keep the pH constant at 6.5 during the experiment. When the amounts of acid added were plotted against time a straight line was obtained, indicating that the alkali production activity was constant (Fig. 4).

Ammonia determination was performed on 1.0 ml of bacterial suspension + 5.0 ml of phosphate buffer in order to determine the ammonia content of the solution at the start of the experi-

ment. After the experiment the ammonia content of the contents of the reaction bottle *in toto* was determined (the electrodes were thoroughly rinsed with distilled water, this water being added to the contents of the bottle). The ammonia determination was performed according to the method of *Ljungdahl* (1922), using a *Parnas-Wagner* apparatus.

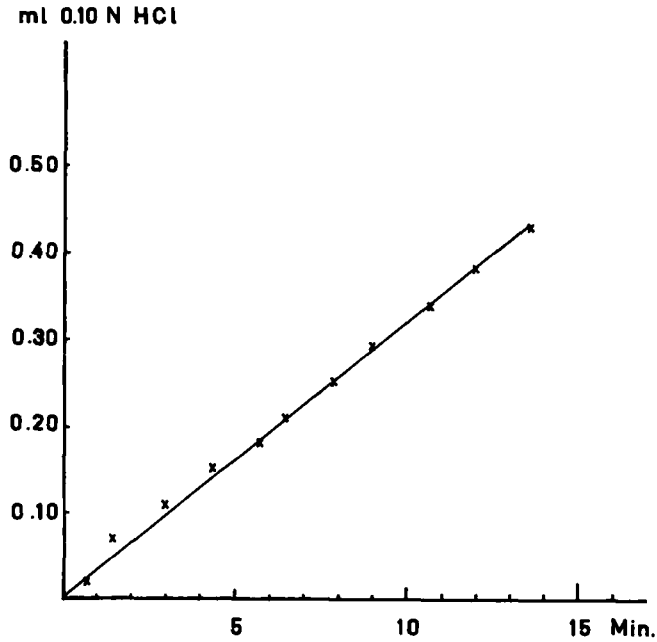

Fig. 4. Alkali production from urea by a suspension of *Staphylococcus albus*. As the activity is constant, the graph shows a straight line.

It was found that an amount of ammonia equal to 0.82 ml 0.10N HCl was formed during the experiment. A correction was made for the production of ammonia from urea during distillation (0.08 ml 0.10N HCl), *Frostell* 1959 a.

The dry weight of the micro-organisms was obtained after 3.0 ml of the suspension were dried at 105°C for 18 hours on a watch-glass. A correction was made for the salts of the buffer. This correction was made with respect to the volume of the organisms which was calculated from the wet weight. The specific gravity of the organisms was considered to be 1.10.

When thus the wet weight, the dry weight, the amount of alkali and the ammonia formed as well as the length of the experimental period were known, the alkali production activity per minute per mg wet or dry weight could be expressed in moles of NaOH or moles of ammonia (*Strålfors*, 1950).

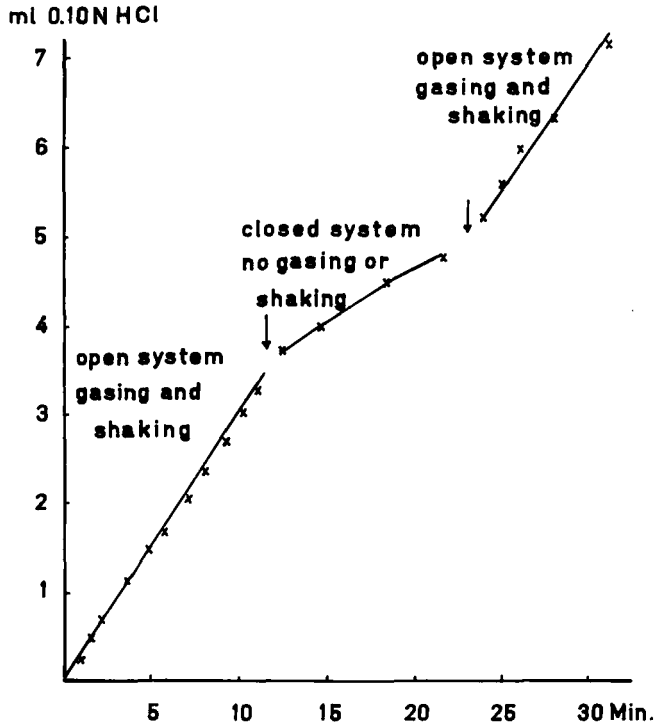

Fig. 5. Influence of gasing and shaking on the alkali production from urea by a staphylococcus strain.

This experiment was repeated 12 times with the same suspension. The error of the determination of the alkali production activity was ± 4.9 per cent (variation coefficient). The error of the determination of ammonia production activity was ± 3.6 per cent (variation coefficient). The number of experiments was small but the performance allows an appreciation of the magnitude of the experimental error.

In some experiments of this type, air was led through the sus-

pension and then through 0.01N HCl, which was then titrated. There was no detectable escape of ammonia.

In order to study the effect of airation and stirring on the alkali production from urea by a staphylococcus strain, the following experiment was performed:

Nitrogen gas was led through the suspension for approximately 10 minutes when the cells acted on urea. The test bottle was shaken vigorously in the apparatus. Then the gas stream was stopped and the bottle was kept motionless. After about 10 minutes the gas stream was again led through the solution and the test bottle was once more kept in motion. (Fig. 5). The experiment shows that the urease activity determined as alkali production activity by the titration method increases when the carbon dioxide is aerated out of the system.

2. Acid production from glucose by a lactobacillus strain

These experiments were performed anaerobically at pH 6.5. The procedure was the same as described under (1) except that the concentration of buffer was lower and that nitrogen gas was admitted through tube 1 before and during the experiment. Lactate production was determined according to the method of *Barker & Sumnerson* as reported by *Umbreit et al.* (1957). Three samples were taken before and after every experiment. The error of the method was calculated from 20 double and tripple experiments.

The error of the determination of acid production activity was ± 6.9 per cent (variation coefficient).

The error of the determination of the lactate concentration was ± 5.0 per cent (variation coefficient).

In order to determine the risk of error due to evaporation of volatile acids, the following experiment was performed:

0.1 ml of analytically pure formic acid and 0.1 ml of acetic acid were pipetted into 9.8 ml of a 0.2M phosphate buffer, the pH of which was 6.5. The same amounts of these acids were pipetted into 9.8 ml of a 0.2M phosphate buffer, the pH of which was 5.0. The tubes were placed in a waterbath, the temperature of which was 37°C and air was bubbled through the solutions for 16 minutes. The air was led into 15.0 ml of a 0.01N NaOH solution.

This solution was then titrated against 0.01N HCl. It was found that an amount of volatile acids corresponding to 0.10 ml 0.01N HCl had passed over from the tube in which the pH was 6.5 and 0.11 ml from the other tube.

It was calculated that if during an experiment 25 per cent of the acids produced were present in the system as formic acid and 25 per cent as acetic acid, a total loss of activity of less than 0.1 per cent would occur.

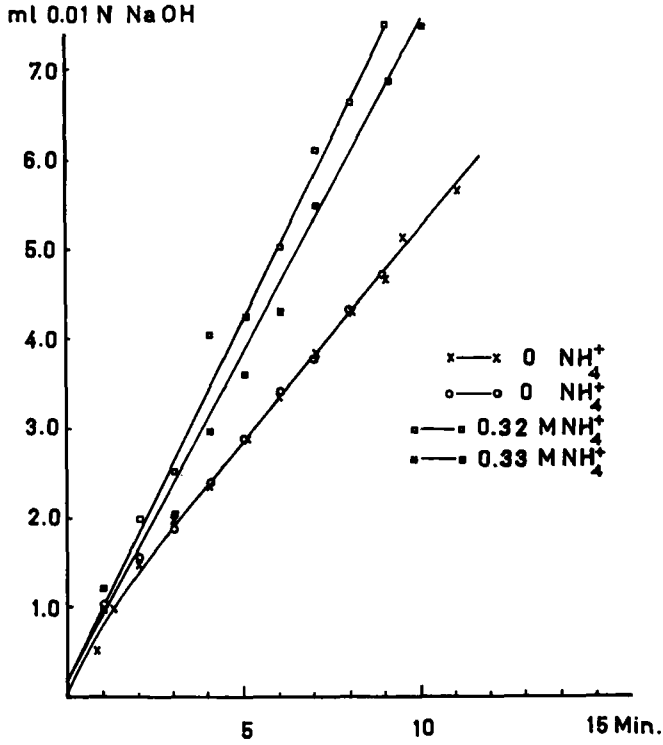

Fig. 6. Influence of ammonium ion on the acid production of lactobacilli.

3. Influence of ammonium chloride on the acid production of lactobacilli

The results of an investigation on the effect of the ammonium ion on the glycolysis of lactobacilli are reported in a separate paper (*Frostell* 1959 b).

The experimental conditions were as described under (2).

Fig. 6 shows the results of two experiments performed at pH 6.5 where the ammonium ion concentration was 0.32 and 0.33 at the beginning of the experiment and two control experiments where no ammonia was added. Fig. 6 shows that the ammonium salt exerted a stimulatory effect on the acid production. The lactate determination revealed a stimulation of lactate production corresponding to the stimulation of acid production measured titrimetrically.

4. Urease activity in the presence and in the absence of glucose

Soy bean meal was suspended in a weak phosphate buffer (10 mM, pH 6.9). 10.0 ml of this solution was added to the reaction bottle. After warming for 10 minutes until the temperature was 37.5 °C 1.0 ml of an urea solution (6.25 g/50 ml) was added. Titration was performed with a 0.10N HCl solution. The pH was kept at 6.5. A similar experiment was made with the same urease buffer suspension, the only difference being that the solution contained 50 mM of glucose as well. (Fig. 7).

It was found that the alkali production activity of the soy bean meal decreased on standing. Since the glucose experiments were carried out after the control experiments, the activity of the glucose containing suspension was somewhat lower than that of the controls in all the three double experiments performed. It was judged that the effect of glucose on the urease activity of soy bean meal, if any, was insignificant. No effort was made to study the question in greater detail, and the experiments are mentioned here only to give an idea of the possible use of the method.

5. Ureolytic activity of dental plaque material

The ureolytic activity of dental plaque material has been determined in different patient groups (*Frostell,* 1960). Plaque material was scraped from between the teeth of a patient and taken to a little stainless steel spoon (*Krasse,* 1954). The wet weight of the material was determined and a suspension of the material was prepared. From the plaque suspension so obtained 6.0 ml

were transferred to each of two reaction bottles and the latter were mounted in the apparatus. The pH was found to be 6.76. After 10 minutes, when temperature equilibration was reached, the pH of one of the bottles was adjusted to 6.5, and 1.0 ml of a urea solution (2 g/50 ml) was added to that bottle. Two stop-watches were set at work.

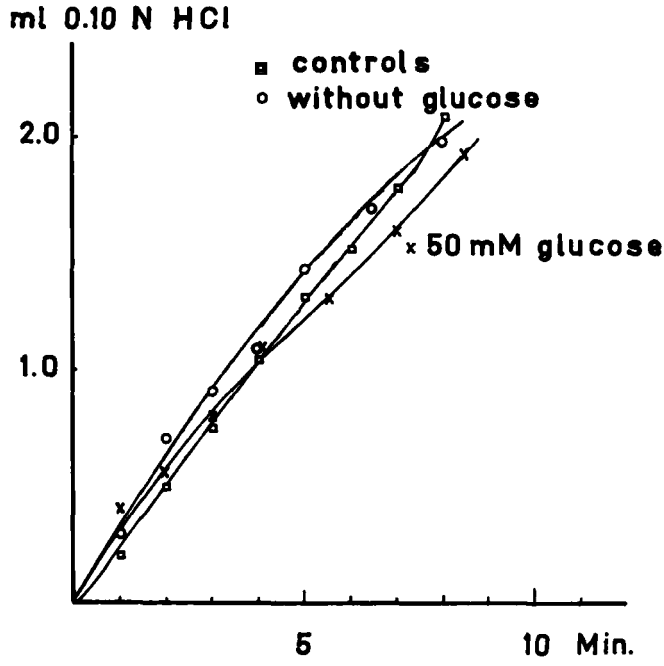

Fig. 7. Alkali production from urea of a soy bean meal suspension in the presence and in the absence of glucose.

Titration was performed with a 0.01N HCl solution against alkali production so that the pH was kept at 6.5. After 40 minutes, one of the stop-watches was stopped when the pH was exactly the same as immediately after the addition of urea at the beginning of the experiment. The bottle was taken out of the apparatus and 0.2 ml of a 20 per cent $CuSO_4$ solution was added. At that time the other stop-watch was stopped. As quickly as possible 0.2 ml of the same copper solution was added to the other bottle.

Ammonia determination was performed on the whole contents of the two beakers (*Frostell*, 1959 a).

Alkali production or ammonia production per minute per mg wet weight could then be determined as described earlier.

Since it was difficult to obtain great masses of dental plaque material, an artificial plaque material was created. Suspensions of different kinds of micro-organisms used in the laboratory were mixed and some ml of a highly active staphylococcus suspension were added. The suspensions were mixed thoroughly. The cells were centrifuged down and were — after drying with blotting paper — used as plaque material.

In each experiment two samples were weighed separately and tested for urease activity separately as described. Ten such double experiments were performed. From the values obtained the errors of the methods were determined.

The error of the determinaion of the alkali production activity was calculated at ± 8.2 per cent (variation coefficient).

The error of the determination of ammonia production activity was ± 9.8 per cent (variation coefficient).

These errors include the error of the determination of the wet weight. The number of experiments is small but gives an idea of the magnitude of the errors.

DISCUSSION

The titration method makes it possible to determine with some degree of accuracy the production of hydronium or hydroxyl ions in systems of reacting organisms or enzymes. This method is considered to be of special interest in dental research when it is the question of the capacity of isolated micro-organisms or mixtures of organisms or of tooth debris to change the pH of the environment. This capacity may be of fundamental importance for the occurrence of dental caries or for the formation of salivary calculus.

As already mentioned, titration methods for the determination of acid or alkali production are not new in bacteriology. The present method, however, differs from earlier procedures in that the titration is performed with very short intervals during the experiment, thus providing shifts of the pH only within a narrow

pH-range. The experiments are performed at constant — or nearly constant — pH under continuous control. A curve showing the production of acid or base during every phase of the experiment can be obtained. This is of fundamental importance, since changes of metabolic activity may occur during an experiment not only as a result of a changed pH but also owing to many other factors, such as to exhaustion of the organisms, gradual poisoning, changes of permeability of the cell wall etc. and technical mishaps such as changes in temperature or in the stirring.

Compared to the procedure elaborated by *Strålfors* (1950) — from which in fact the present method has developed — it is clearly an advantage that the pH may be kept within a narrow range. On the other hand, an experiment *ad modum Strålfors* will give much valuable information of the activity in different pH-ranges. However, if the influence of various substances on the activity is to be evaluated it will be very difficult to analyse the curves obtained if the effect of the substance is influenced by the pH, as shown for Na-N-lauroylsarcosinate *(Frostell,* 1957).

There are, however, some definite drawbacks connected with the present method as hitherto used by the author: (1) The system is not closed and therefore the formation or presence of volatile acids or bases may influence the results; (2) As a result of the continuous titration the concentrations of the substances in the substrate mixture will change; (3) It is difficult to perform several experiments simultaneously.

Since the system is not closed, volatile acids or bases will disappear, as already pointed out. However, when it has been shown that the activity curve is a straight line, the investigator is justified in drawing the conclusion that the activity as determined by the titration method is proportional to the total activity. It would be extremely difficult to reproduce the exact diffusion conditions and gas pressures existing as for example in the dental plaques and the tissue coatings in the oral cavity in experiments *in vitro*. Thus, the results obtained with the present method — as with other methods — will be the results under the conditions of the experiment and e.g. not an exact reproduction of the capacity of the organisms in the oral cavity.

It will be possible, perhaps, to make the method more accurate by using a closed system, which it is quite possible to do in

aerobic as well as in anaerobic experiments. This, however, will not make the experiment more similar to natural conditions, but it will partly eliminate the risk of loss of volatile acids or bases.

The continuous titration will cause changes in the concentrations of substances in the solution during the experiment. The dilution of the bacterial suspension will not cause changes of the metabolic activity of the cells (*Passmore & Yudkin*, 1937, *Strålfors*, 1950); e.g. the acid or alkali production activity is proportional to the number of organisms irrespective of the distance between the cells. Changes in ionic composition or ionic strength occurring during the experiment may, however, cause changes of metabolic activity. No significant changes in acid or alkali production activity occurred as a result of changes of ionic composition or ionic strength of the magnitude occurring during the titration, as shown by many control experiments. When the influences of various substances on the alkali or the acid production activity are studied, however, a significant change in the concentration of the substance in question may take place (*Frostell* 1959 b).

There are two ways to avoid the influence of this drawback of the method. The solution used for titration (acid or base) may be of the same composition as the substrate except with regard to the base or the acid. Very precisely working microburettes may be used so that the acid or base titrated may be more concentrated and the changes of the volume kept small.

If a large series of similar experiments are to be performed the first method may be tried, but if the composition of the substrate mixture is changed between every two experiments the method is not practical.

With the present apparatus, the experimenter and one assistent can perform two experiments at the same time. It would be possible to perform several experiments simultaneously if automatically controlled titration units were used. Provided the acting bacterial suspension or the enzyme system is not stable such an arrangement is of fundamental value. When working with stable preparations, however, the benefit of the possibility of performing several experiments simultaneously is perhaps illusory, since the control of the single experiment will often suffer.

The technical procedure of the method which is described in

this paper may be developed further. Experiments in the directions here outlined are in progress.

As stated earlier, manometric techniques are widely used for the determination of acid production of micro-organisms. With these methods the total amount of acids produced, which are stronger than the carbonic acid, can be measured with great accuracy. If the chemical nature, the dissociation constants, the amounts of the acids produced, the buffer capacity, and the pH of the medium are known, the production of hydronium ions can be calculated. This is, however, practically never the case. Different types of organisms produce different acids in varying amounts and proportions. The present method makes a direct determination of the "acid production activity" possible.

A further advantage of the present method as compared with manometric techniques would seem to be that concentrated suspensions of micro-organisms may be used in the experiments and yet the buffer capacity of the system may be kept low. This is doubtless of great value in certain types of experiments. *Pearlman* (1951), when using the *Warburg* manometric technique for the evaluation of the influence of ammonium ion concentration on the glycolysis of lactobacilli used concentrated phosphate buffers in order to balance the ammonium ion and thus the inhibiting action of the phosphate ion overshadowed the stimulating effect of the ammonium ion (*Frostell,* 1959 b). *Strålfors* (1950) pointed out that rapid changes in the ionic composition of the substrate surrounding the cells in concentrated suspensions of micro-organisms may occur as a result of the activity of the cells and may be of importance in the biochemical processes in the mouth. It is suggested that such phenomena may be studied by the present method. It has, for example, been advocated that rapid changes in the phosphate concentration may occur in the dental plaque as a result of phosphate uptake by the micro-organisms during the rapid acid production on the administration of sugar. If such a phenomenon occurs, it may enhance the solution of enamel apatite during the acid attack. The author believes that the titration method may be used in order to answer this question.

Urease activity may be determined by manometric techniques (*Umbreit et al.,* 1957). These methods, however, will give little

information about the capacity of the system to produce hydroxyl ions. The titration method gives an indication of the capacity of an organism in this respect (*Frostell,* 1959 b). Further, the titration method made it possible to study the alkali production activity and the acid production activity in one and the same experiment.

Though the author has little experience of manometric techniques he believes that the titration method presents some advantages over these methods for the study of certain problems, especially in connection with oral biochemical phenomena. It must be kept in mind, however, that the method is not fully developed, that it is not as accurate as manometric methods and that it has not the same general applicability.

When performing experiments with the titration method it is of the utmost importance to consider what is actually happening in the system and what is measured. If, for example, the urease activity is studied at alkaline pH, considerable losses of ammonia may occur if the system is not closed. At pH 6.5, however, there is little risk. If the pH is low, carbon dioxide will be driven out of the solution and there will be little or no formation of carbonate. On the contrary, if a closed system had been used carbonate would have accumulated and the alkali production activity would subsequently have been less.

What then is actually measured? The author would explain it in the following way: The alkali production activity of the organism under the conditions of the experiments is determined. The values obtained are relative values differing more or less from those under natural conditions. However, it must be of great value for the understanding of biochemical properties of staphylococci to be able to determine that the capacity of producing alkali from urea is about 20 times as great as the acid production capacity from glucose under similar conditions. In order to be able to make a more precise evaluation of the relative importance of different micro-organisms for the urease activity of dental plaque material, the author has determined the ammonia production as well as the alkali production of plaque material and different micro-organisms. Thus, through the combination of the determinations of alkali or acid production with a chemical determination of one or some of the components formed during

the experiment, it will be possible to draw more definite conclusions concerning the relative importance of different organisms for the changes of the hydronium ion concentration occurring in biological systems.

SUMMARY

A method for the determination of the acid or the alkali production of suspensions of micro-organisms is described. In a system consisting of micro-organisms, a buffer and a substrate from which hydronium or hydroxyl ions are produced by the organisms, the hydronium ion concentration is kept constant by subsequent titration of acid or base. The amount of alkali or acid required per unit of time to keep the pH constant is a measure of the acid or alkali production activity of the system. The method has also been used for the determination of the acid or alkali production of enzyme-substrate mixtures.

The theory of the method, the apparatus and the experimental procedure are described and examples of experiments and results are given. Experiments may be performed aerobically or anaerobically.

The method may be used in the study of various bacteriologico-biochemical problems, such as the acid production activity of lactobacilli and other micro-organisms or mixtures of organisms, the influence of various substances on the acid production of micro-organisms, the urease activity of micrococci and other organisms, and the ureolytic activity of dental plaque material.

It is pointed out that the method can be further developed and that it has some drawbacks. Suggestions are given as to how to avoid their effects.

RÉSUMÉ

UNE MÉTHODE DE DOSAGE POUR LA DÉTERMINATION DE LA PRODUCTION ACIDE OU ALCALINE DES MICRO-ORGANISMES

L'auteur décrit une méthode pour la détermination de la production acide ou alcaline d'une suspension de micro-organismes. Dans un système composé de micro-organismes, d'une solution tampon et d'un substrat à partir duquel les micro-organismes

produisent des ions hydrogènes ou hydroxyles, la concentration de l'ion hydrogène est ensuite maintenue constante par titrage d'acide ou de base. La quantité d'acide ou de base nécessaire par unité de temps pour garder le pH constant est la mesure de l'activité de production acide ou alcaline du système. Cette méthode a été aussi employée pour la détermination de l'activité des enzymes.

L'auteur décrit les aspects théoriques et techniques de la méthode et les appareils et donne des exemples d'essais et de résultats. Les expériences peuvent être faites en milieu aérobie ou en milieu anaérobie.

Cette méthode peut être utilisée pour l'étude de divers problèmes de biochimie microbienne tels que l'activité de production acide des bacilles lactiques et autres micro-organismes ou combinaisons d'organismes, l'influence de diverses substances sur la production acide des micro-organismes, l'activité uréase des microcoques et autres organismes et l'activité uréolytique des plaques dentaires.

L'auteur fait remarquer que la méthode peut être ultérieurement developpée et qu'elle a quelques inconvénients. Des suggestions pour en éviter les conséquences sont présentées.

ZUSAMMENFASSUNG

EINE TITRATIONSMETHODE ZUR BESTIMMUNG DER SÄURE- ODER ALKALIPRODUKTION VON MIKROORGANISMEN

Eine Methode zur Bestimmung der Säure- oder Alkaliproduktion in Suspensionen von Mikroorganismen wird beschrieben. In einem System bestehend aus Mikroorganismen, einem Puffer und einem Substrat, aus dem die Mikroorganismen Hydrogenium- oder Hydroxyljonen produzieren, wird die Hydrogeniumjonenkonzentration durch eine abwechselnde Titration von Säure oder Alkali konstant gehalten. Die titrierte Menge von Säure oder Alkali pro Zeiteinheit ist ein Mass für das Alkali- oder Säureproduktionsvermögen des Systemes. Diese Methode ist auch zur Bestimmung der Säure- oder Alkaliproduktionsaktivität von Enzymen verwendet worden.

Die Theorie der Methode, der Apparat und das experimentelle Verfahren werden beschrieben und Beispiele von Experimenten

und Resultaten werden mitgeteilt. Die Experimente können unter aeroben oder anaeroben Bedingungen ausgeführt werden.

Die Methode kan zum Studium verschiedener bakteriologisch-biochemischer Probleme verwendet werden, zum Beispiel zur Bestimmung der Säureproduktionsaktivität der Laktobazillen und anderer Mikroorganismen, der Einwirkung von verschiedenen Substanzen auf die Säureproduktionsaktivität von Mikroorganismen, der Ureaseaktivität von Mikrokokken und anderer Organismen und der Ureolyseaktivität der Zahnbeläge.

Es wird darauf hingewiesen, dass diese Methode noch verbessert werden kann, und dass die Methode einige Fehlerquellen hat. Es wird erklärt, wie man diese in gewissen Fällen ausschalten kann.

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Address: *G. Frostell*
Royal School of Dentistry
Stockholm, Sweden