

Bacterial Culture with Controlled pH¹

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In view of the generally acknowledged importance of hydrogen-ion concentrations in the culture of bacteria, it is rather surprising that few studies have been done with culture methods in which pH is controlled at fixed levels. The academic and practical values of data thus obtained are obvious and it seems that technical difficulties may have inhibited extensive work of this kind.

Longworth and MacInnes (1935) described a functionally satisfactory apparatus for pH control but their work was done at a time when electrical measurement of hydrogen-ion concentration was in its infancy. It is probable that one not well versed in electronics would be discouraged by the technical aspects of assembling equipment of the kind they employed.

MATERIALS AND METHODS

The basic features of the apparatus described in the present paper are those of Kempe, Halvorson, and Piret (1950); however, these authors did not work with pure cultures and many essential procedures entailed were therefore omitted from their paper.

To aid in further work on the culture of microorganisms under conditions of controlled pH, a detailed description of an apparatus and a technique suitable for experimental work are submitted.

The apparatus consists essentially of a recorder, an amplifier, glass and calomel electrodes, a culture vessel containing a mechanically operated stirring bar, and an alkali reservoir with a solenoid actuated delivery valve. Accessory equipment includes a pressure sterilizer, electric heating unit, and electric incubator. The specifications and sources for the equipment are given in detail to facilitate purchase, construction, and assembly. The assembled apparatus is shown in figure 1.

Recorder. Pyromaster potentiometer (model 531-42, the Bristol Co.²), range 3-10 pH, 115 V, 60 cycles, 0-50 MV. The factory was requested to alter the machine from proportional input to on-off response.

Amplifier. RX pH amplifier with automatic control (model 9001, Beckman Instruments, Inc.³). A resistance

thermometer (Beckman model³ 8950-72) was connected with the amplifier.

Voltage stabilizer. (Not shown in figures.) A rather labile source of alternating current necessitated the interposition of a voltage stabilizer between the recorder and the line. (Raytheon⁴ model VR6112 CP.)

Glass electrode. General purpose, heat resistant, glass electrodes (Beckman model 8990-80)³ and amber glass electrodes (Beckman model 8990-90)³ were equally satisfactory. These electrodes may be sterilized with steam at 10 to 15 pounds pressure. A piece of black rubber tubing 22 mm long and of proper inside diameter to fit over the electrode (variable diameter) is forced upward to the base. The outer surface of the tubing is fitted to the inside of a Pyrex test tube (16 mm inside diameter). The base of the electrode is packed into the electrode adapter (87566) by means of the electrode clamping nut (87571). A 10-foot lead is furnished.

The resistance of electrodes to the heat of steam sterilization is quite variable. Some electrodes were ruined by one sterilization while others withstood as many as 15. We used 8 electrodes in performing 82 experiments.

It was found that the sterilization process described was not only more convenient, but less destructive than sterilization in an autoclave. The electrodes were scrubbed with detergent before sterilization and the pressure was carefully limited to 10 pounds and maintained for 15 minutes. These precautions which were most rigorously adhered to in later experiments were thought to increase the "life" of the electrodes. We now feel that an electrode can be used in from 10 to 15 experiments.

Calomel electrode. The calomel electrode (Beckman model 8970-92)³ was furnished with a 10-foot lead. An agar bridge for use with this electrode was constructed by bending 8-mm OD glass tubing into the shape of three sides of a square. Each of the arms was cut to a length of 165 mm, and the ends were fire polished. A bell-shaped skirt was made for one arm by cutting a short length of rubber tubing which was slipped up the arm to a distance of 130 mm. A longer piece of rubber tubing of larger diameter was placed over the shorter

⁴ Raytheon Manufacturing Company, Waltham, Massachusetts.

¹ This paper is part of a thesis submitted to the Graduate School of Washington State College in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology and Public Health.

² Bristol Company, Waterbury, Connecticut.

³ Beckman Instruments, Inc., Fullerton, California.

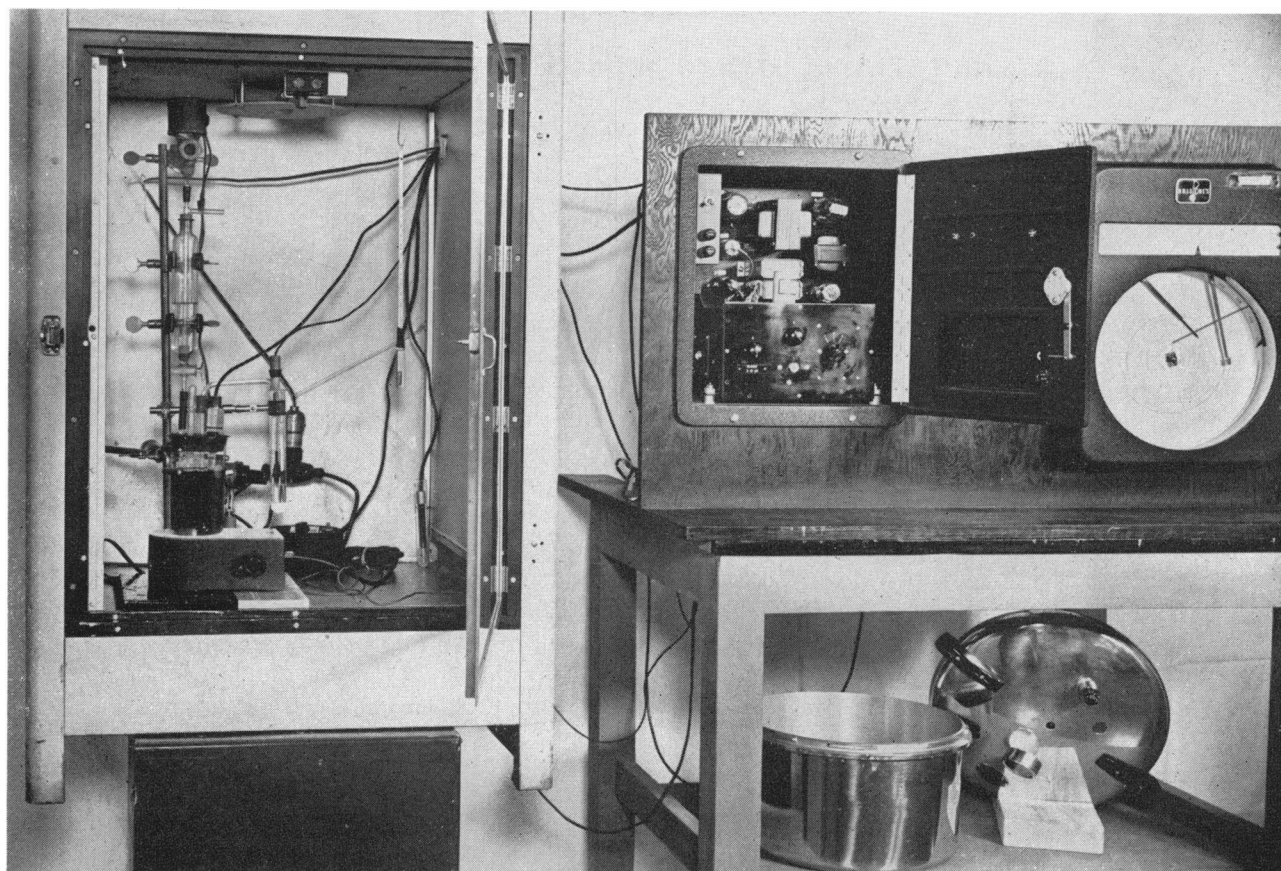


FIG. 1. Complete assembly for culture with controlled pH. A resistance thermometer (not depicted elsewhere) is shown in the far right corner of the incubator. A lamp for transillumination of the culture vessel is partially visible behind the vessel.

and smaller piece to form the bell skirt. This tubing was selected to make a close fit over 17-mm Pyrex tubing 46 mm long.

Cultural vessel. A 600-ml Berzelius beaker (Corning Glass Co.,⁵ No. 1040, tall form without spout) was fitted with a No. 14 rubber stopper cut to a thickness of 24 mm. The top of the stopper was thus nearly flush with the rim of the vessel and permitted insertion of the electrodes to a depth of 4 cm into the medium. The stopper was drilled to accommodate the following: (1) An inoculation tube of Pyrex tubing, 11 mm OD by 102 mm long (figure 4, no. 1). (2) An agar bridge support cut from Pyrex glass tubing of 12 mm OD by 70 mm long (figure 4, no. 2). (3) A glass electrode sleeve (figure 4, no. 3) to accommodate the glass electrode made by taking the upper 27 mm of a Pyrex culture tube 18 mm OD. (4) An alkali addition tube (figure 4, no. 4) cut from capillary tubing, 7 mm OD, with a bore of 1.75 mm. The length of this tube was 80 mm. The bore was tapered by drawing the tubing in a flame to an inside diameter of 0.75 mm. The tube was fitted at the top with a 60-mm section of 1/4-inch rubber tubing.

All of the tubes except the alkali addition tube were

⁵ Corning Glass Works, Corning, New York.

inserted into the holes in the rubber stoppers so that the lower ends were nearly flush with the bottom of the stopper. The alkali addition tube protruded approximately 1/2 inch. Glass caps were cut from test tubes of suitable diameter to fit over the tops of all tubulations except the glass electrode sleeve which was plugged with wrapped cotton for sterilization of the unit.

Magnetic stirrer. A 1 x 1/4 inch Alnico⁶ stirring bar sealed in "Teflon"⁷ was placed in the beaker with the medium prior to sterilization. The magnetic stirrer used most frequently was a "Mag-Mix" made by the Precision Scientific Company.⁸

Sterilizing unit. The lid of a 16-qt. Presto cooker, manufactured by the National Pressure Cooker Company,⁹ was drilled and tapped for a 1/2-inch male pipe fitting. The hole was drilled about 2 1/2 inches from the center of the lid. The tapped hole accommodates the electrode adapter of the glass electrode which is tightened by means of a wrench. It was found advisable to prepare a lid holder to facilitate placement of the

⁶ General Magnetic Corp., Detroit, Michigan.

⁷ E. I. duPont de Nemours & Co., Wilmington, Delaware.

⁸ Precision Scientific Company, Chicago, Illinois.

⁹ National Pressure Cooker Company, Eau Claire, Wisconsin.

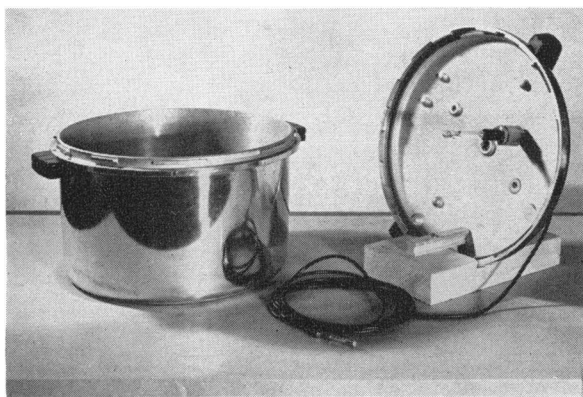


FIG. 2. Glass electrode sterilization unit

lid on a bench with the electrode in place. Construction of the holder can best be understood by referring to the photograph (figure 2). An electric hot plate was used as a source of heat.

Alkali buret and automatic solenoid valve. The buret is a modification of one used by Longworth and MacInnes (1935). The one used by us is obsolete and unlikely to be generally available, but the principle of construction may be applied to other cylindrical separatory funnels. A funnel of 125-ml capacity graduated in 1-ml units (Kimble,¹⁰ catalog No. 29040) was used. The inside bottom shoulder of the funnel was ground with valve grinding compound to form a seat for a gray rubber stopper. The stopper was tapered to fit the seat by grinding it on a high-speed grinding tool. A hole was cut in the stopper to accommodate a glass rod, 5 mm by 240 mm, which passed through a short length of 8-mm glass tubing set in a No. 3 rubber stopper at the top of the funnel. The upper end of the glass rod was inserted into a short length of rubber tubing and an aluminum rod was inserted into the other end of the tubing. The aluminum rod was coupled with a cotter key to a solenoid plunger made from a metal rod and substituted for the adjusting screw of the solenoid. The solenoid used by us is a "Sporlan"¹¹ type 73 P (11 watts, 115 V, 60 cycles). Spacers were inserted to obtain the desired travel ($\frac{5}{16}$ "') of the rod.

The side arm tubulation of the sleeve in the upper stopper is shown in the figures. The purpose of the tubulation was to admit air and thus permit free flow of the alkali, but it was not necessary for the purpose. A stopcock on the stem of the buret permits adjustment of the rate of flow. This reservoir and valve apparatus has a minor disadvantage in that the glass rod valve stem displaces the liquid (2.5 ml at 100 ml) to an amount proportional to the volume of alkali in the buret and thus causes slight errors in reading.

Incubator. It is evident that any incubator with

¹⁰ Kimble Glass Company, Toledo, Ohio.

¹¹ Sporlan Valve Company, Palisades Park, New Jersey.

forced circulation and of adequate size may be used. It is only necessary to cut a hole in the wall to admit the various wire leads. The Cenco¹² incubator, No. 46023, was found suitable for the assembled apparatus as depicted in the figures. The total height of the apparatus was 30 inches.

Grounding of machine. The instruction manuals emphasize that troubles and complaints are often traceable to improper or insufficient grounding of equipment. Inasmuch as circumstances of installations will vary, no specific instructions for wiring will be given here.

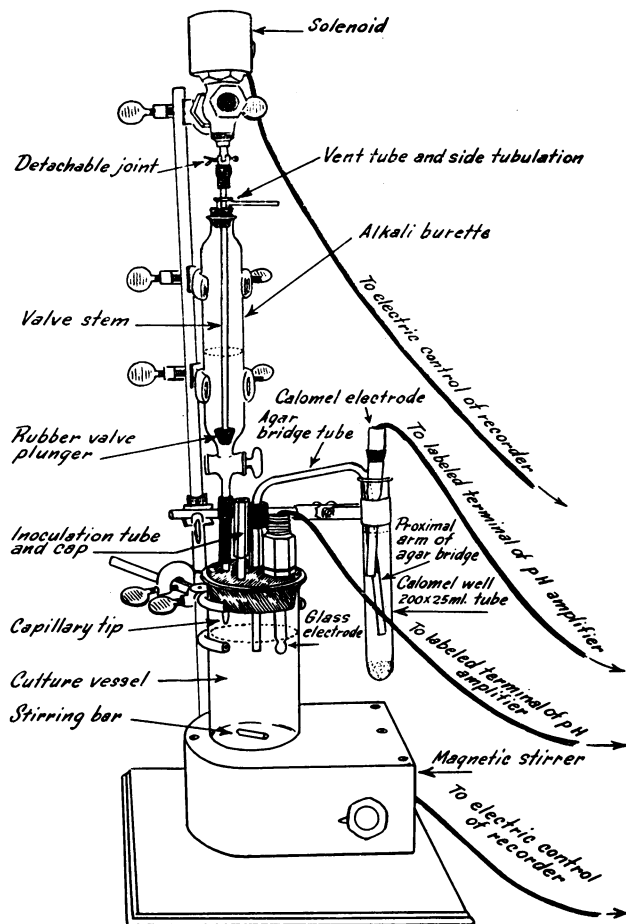


FIG. 3. Culture unit assembly

Three groundings must be made: (1) the measuring circuit ground; (2) the case ground; (3) incubator ground.

The measuring circuit and the measuring circuit ground can not be put on the same conduit because of the possibility of induced voltage. Insulation must be dry to avoid electrical leakage and the grounding system must be free of applied potential. Tight connections are necessary to eliminate contact resistance.

Preparation of apparatus: calibration. Prior to each

¹² Central Scientific Company, Chicago, Illinois.

use, the recorder and amplifier are systematically checked for calibration following the instructions in the manual furnished by the manufacturer. Ordinarily no difficulties or delay are anticipated or encountered, and, therefore, adjustments are made as time permits on the day of use. Two commercial buffer standards with different pH values are used in standardization.

Sterilization of glass electrode. The stored electrode is removed from distilled water and the lead wire is passed upward through the adapter aperture of the pressure cooker lid. The electrode adapter is inserted into the threaded aperture and tightened with a wrench. A small wire basket containing an open test tube (16 mm inside diameter) is placed in the cooker and 500 ml of water are poured into the cooker. The lid is then firmly sealed and placed on a hot plate until steam emerges from the open petcock, which is then closed. When 10 pounds of pressure are indicated on the dial, the petcock is opened very slightly and the temperature of the plate adjusted to maintain that pressure (not over 15 pounds). After the pressure has been maintained for 15 minutes, the heat is turned off and the entire apparatus allowed to cool until the electrode is needed.

Medium. A medium of the kind desired is prepared as usual and adjusted to a pH near the desired level. Four hundred and twenty-five milliliters are put into the culture vessel and a Teflon coated magnetic bar is dropped in. The vessel is sealed as shown in figure 4

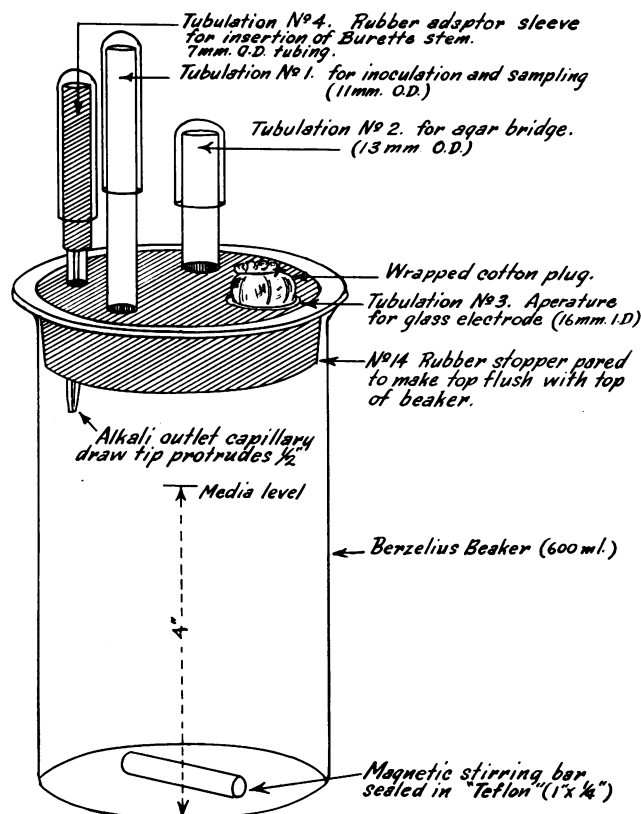


FIG. 4. Culture vessel assembled for sterilization

and autoclaved. Final fine adjustment of pH of the media may be done with the entire apparatus fully assembled by manual operation of the switches of the magnetic stirrer and the solenoid valve of the buret. Alternatively, and especially if acid is needed to obtain a lower pH, pipetting may be done through one of the tubulations of the vessel for both addition of reagent and pH determination on a sample. The latter technique using another potentiometer has the advantage that standardization of the unit electrodes may be done by comparison of readings on the two potentiometers.

We have not found it necessary to sterilize the 1 N solutions of sodium hydroxide or hydrochloric acid.

Agar bridge. A gel containing 3 per cent agar and 1 per cent sodium chloride is prepared and 30-ml volumes are distributed while hot into 50-ml Erlenmeyer flasks. These flasks are capped and autoclaved and may be stored until used. When needed, the two ends of the agar bridge tube are placed on the gel in two such flasks and the distal end, that is, the end with the skirt, is wrapped with paper. The assembly is autoclaved with the bottoms of the flasks on a horizontal plane. In the process, air contained in the tube is displaced by melted agar. An occasional large bubble entrained in the tubing can be removed by tilting the assembly. Small bubbles may be left in place. The bridge and the culture vessel are cooled to about 37 C before joining.

Assembling the apparatus. When the pressure cooker is cooled, and all pressure has been released, the lid is removed and, immediately upon opening the cooker, the test tube sterilized with the glass electrode is placed over it to maintain sterility. The electrode is removed with a wrench. The test tube is then removed and the electrode inserted into tubulation No. 3 (figure 4).

The sterile wrapping is removed from the agar bridge assembly which is then inserted into tubulation No. 2 of the culture vessel.

The apparatus assembled thus far is placed on a magnetic stirrer in the incubator. The stem of the alkali reservoir buret is flamed thoroughly and inserted into the rubber tubulation (figure 4, no. 4) of the culture vessel.

The proximal end of the agar bridge is placed in a 200 x 25 mm tube and submerged to a depth of about 8 cm in saturated KCl solution. An excess of KCl crystals is placed in the tube. The calomel electrode is then placed in the tube to a submerged depth of about 5 cm. No support is needed as the base of the electrode is wedged between the bridge and the tube.

All electric wires are passed through a hole in the wall of the incubator and are connected to appropriate jacks and current outlets as follows: (1) The incubator power wire is connected to an A.C. 110 V line outlet; (2) the magnetic stirrer and the solenoid valve of the buret are connected to the "input" electric control of

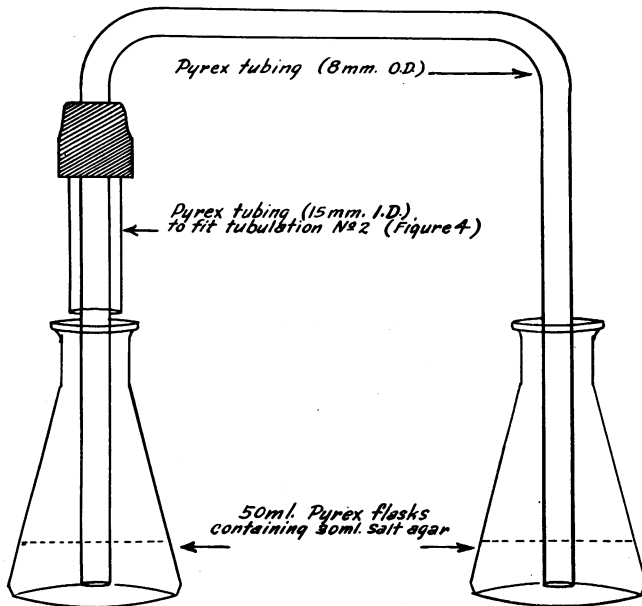


FIG. 5. Agar bridge tube assembled for sterilization

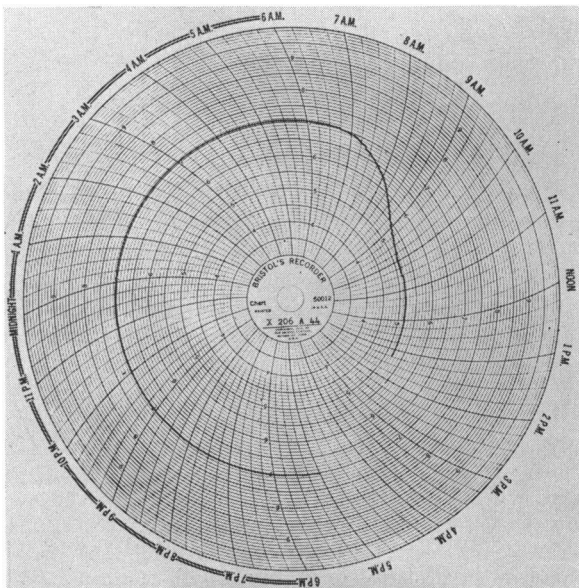


FIG. 6. A typical chart. Initial pH 7.0. No control

the recorder; (3) the plugs of the calomel and glass electrodes are inserted into the appropriate jacks of the amplifier; (4) the resistance thermometer bulb wires are inserted into the appropriate jacks of the amplifier.

Jacks for insertion of the above leads are labelled on the machine. Wires of the calomel electrode and the resistance thermometer are usually left in place between runs. The lead of the glass electrode is the only one which must be passed through the hole in the incubator for each operation of the apparatus.

Inoculation. Inoculation is done with a pipette by introducing it through tubulation No. 1 of the stopper of the culture vessel. We used an inoculum of washed

and detoxified spores deposited below the surface of the medium.

Operation. A Bristol Recorder Chart²(No. 50012) is used for graphic record of pH. The instruction manual gives adequate directions for placing of the pen, inking, and so forth. The set point at which it is desired to maintain pH is determined by adjustment of a pointer, the "set point index" on the face of the dial, to the desired point. In most of the experiments, the pH of control was set only 0.1 or 0.2 units below the initial pH.

Thereafter, operation of the apparatus is completely automatic, requiring only the changing of charts once

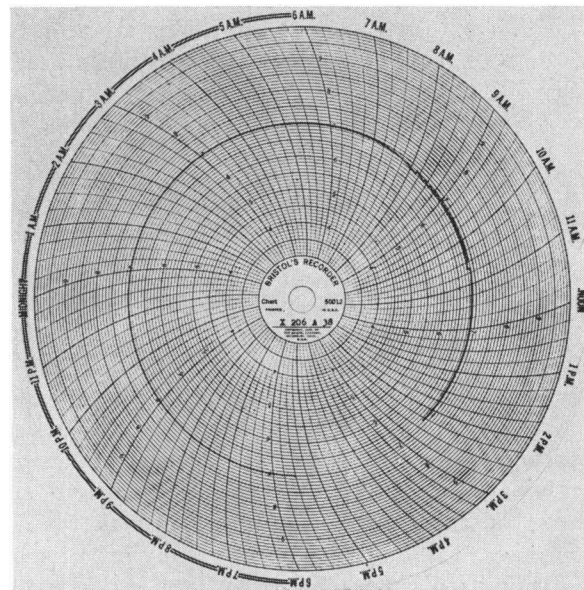


FIG. 7. A typical chart. Initial pH 7.0. Set point for control at pH 6.8.

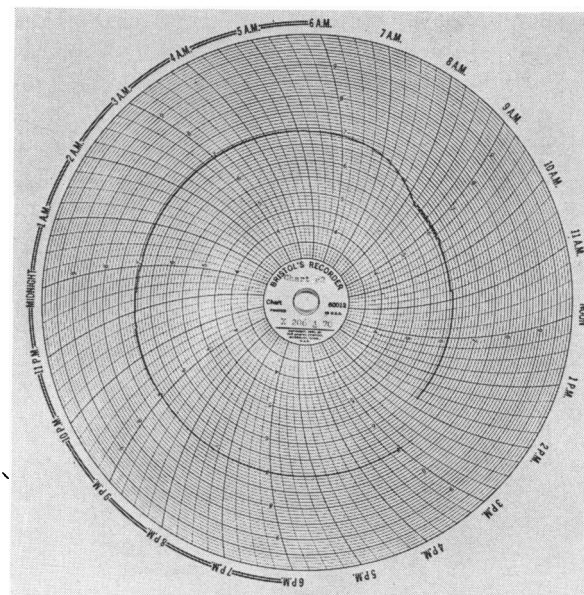


FIG. 8. A typical chart. Initial pH 7.0. Set point for control at pH 6.1.

every 24 hours and standardization of the recorder once a day (operational check). Directions for standardization are given in the manual.

Termination of operations. As a rule the apparatus was kept in operation for an arbitrary period of about 3 hours after the last addition of alkali or in uncontrolled experiments for the same period after a base line of pH was reached. The pen is then lifted from the face of the chart and switches of both instruments are turned to the off position. The glass electrode plug is removed from its jack in the amplifier and the wire is drawn through the hole in the incubator. The electrode is left in place. The rubber adapter sleeve of tubulation No. 4 is clamped off and the stem of the buret is pulled from it. The calomel electrode and the bridge are removed from the well but left in place in the culture vessel. The vessel is then free and can be taken to a "sterility room" for sampling. When samples are to be taken, the vessel is placed on a magnetic stirrer to insure homogeneity of the contents.

RESULTS

Some typical charts obtained in experiments on the culture of *Clostridium botulinum* type C in yeast autolysate-lactalysate are shown in figures 6, 7, and 8. The inoculum consisted of about 60,000 spores in each case. When active vegetative cells are inoculated, acid production occurs earlier. It may be seen that control of pH is practical within from 0.1 to 0.2 pH units and we have considered this range sufficiently narrow for our purposes. The data obtained in many experiments will be submitted separately.

DISCUSSION

In our studies of cultures with controlled pH, we have obtained information which cannot be duplicated by ordinary culture techniques. The use of buffers does not afford comparable control. Furthermore, the range of action of a single buffer is not so wide as that of a machine control, and the use of different buffers introduces variables which may confuse the results obtained.

Machine control of pH eliminates many of the undesirable features encountered with buffers and the availability of commercial apparatus simplifies the technical aspects of the problem. Too, the present availability of heat-resistant electrodes has made pure culture studies practical. A yeast autolysate-lactalysate medium suggested by Wayne I. Jensen (Personal communication, 1952) has further broadened the usefulness of the apparatus inasmuch as strict anaerobes may be cultured in it from spore inocula without special precautions for anaerobiosis. At the time our apparatus was devised we had not encountered the paper of Kempe *et al.* and we used the solenoid valve and gravity flow of alkali for adjustment of pH instead of the pump described by them. The alkali addition apparatus was the one item which caused most trouble in operation, and it is probable that a motor-driven pump would be much more satisfactory. A peristaltic-action pump (American Instrument Co.,¹³ Bulletin 2250-N) should prove adaptable to the requirements of the work.

The method of culture described here, while not so simple as ordinary techniques, is sufficiently simple for practical use on a laboratory scale.

SUMMARY

A laboratory unit has been devised for the continuous control of pH in bacterial cultures grown under anaerobic conditions. Typical data are given for growth of *Clostridium botulinum* type C in the apparatus.

Since this manuscript was submitted for publication we have used the peristaltic pump and have found it more satisfactory than the solenoid valve.

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¹³ American Instrument Company, Silver Spring, Maryland.