COLICINE K

I. The Production of Colicine K in Media Maintained at Constant pH

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The amassing of bacteria for chemical study is a problem of some magnitude, as those who are engaged in this type of investigative work can attest. Our early studies on the type-specific polysaccharides of Pneumococci (1) and Friedländer bacilli (2) were accompanied by numerous difficulties, both in the handling of large volumes of liquid and in the separation of non-diffusible broth components from the bacterial polysaccharides themselves. In 1946 a culture medium was described (3) which contains only dialyzable constituents and which supports the growth of many fastidious microorganisms. This medium has been of great assistance in circumventing these earlier difficulties, for it yields bacterial cells which are essentially free of adsorbed broth components. Furthermore, should one wish to obtain soluble and non-diffusible bacterial constituents from the medium, their separation and subsequent purification is enormously facilitated because the components of the medium itself can be removed by dialysis.

Many culture media fail to yield maximum bacterial populations not because the nutrients become exhausted, but because it has not been possible to maintain the pH of the environment at an optimal value throughout the growth cycle. There are many ways of controlling the pH of bacterial cultures,—the use of buffers, the addition of calcium carbonate, or control by the manual addition of alkali are means which are familiar to us all (4, 5). No procedure, however, has surpassed that devised by Longsworth and MacInnes (6) for investigative purposes. This will be described further on.

During the past several years our laboratory has been concerned with the isolation and identification of certain antibacterial agents termed colicines (7). These are substances of unknown nature, elaborated by certain strains of enteric microorganisms, which have the remarkable property of killing specifically and selectively still other strains of enteric bacilli. The colicines, of which there are now known some seventeen specific types, are believed to be related to the bacteriophages. Unlike the latter, however, the colicines are incapable of reduplication. It is the purpose of this series of investigations to determine the nature of one of these agents, colicine K, and to ascertain whether this sub-

stance does indeed bear any relationship to the coli dysentery phage T_6, the virus to which it is presumably related. Before undertaking such a program it is of course necessary to establish the conditions for the optimum production of colicine K. From that which follows it will be seen that this has in part been achieved.

In our experience, colicines are elaborated in the culture medium only when conditions for growth of the host cell are controlled (8), both in respect to the pH of the medium and to the nutrients which the cells receive. In order to maintain the pH of the medium constant, an apparatus has been devised similar to that of Longsworth and MacInnes (6). In the equipment which will be described, an electronic device has been substituted for the Compton electrometer used by these investigators, and a 5 liter pyrex bottle was used for the growth vessel. With this apparatus many hundreds of liters of a colicine K–producing strain of *Escherichia coli* have been grown under conditions whereby the antibacterial agent is produced in quantities sufficiently large to render its isolation and purification feasible.

Materials and Methods

Strains of Microorganisms.—In the experiments to be described, a strain of *E. coli* known as K235 (7, 9) was employed. This microorganism produces the antibacterial agent, colicine K. It was kindly supplied by Dr. Pierre Fredericq of the University of Liége to whom we are indeed grateful. When streaked on neopeptone agar, the culture yielded translucent smooth colonies about 4 mm. in diameter. This microorganism produces a specific thermostable O antigen, as well as a thermolabile surface L antigen. The strain was therefore characterized by the symbol L_+ according to the nomenclature of Kaufmann (10). Since the colonies of this bacillus are translucent, the strain was finally characterized by the symbol K235 L_+T. Several months after receiving the culture from Dr. Fredericq, a variant was isolated which differed from the parent both in colonial form and in the fact that it elaborated far more colicine. The opaque mucoid colonies of the variant appeared to be identical in antigenic structure with the parent. This variant was termed *E. coli* K235 L_+O. Both the O and T variants of *E. coli* K235 L_+ lose their labile surface L antigen when repeatedly transferred on solid nutrient agar. Two new variants, termed *E. coli* K235 L_-O and L_-T have thus been obtained. Colonies of these are morphologically very similar to their parents, and they can be readily differentiated by serological means. The experiments described in this communication were conducted with the L_+O and the L_-T variants of *E. coli* K235.

Culture Media.—Two different culture media were employed in these studies. The basic medium consisted of 1 per cent Difco casamino acids (technical) dissolved in 0.03 M phosphate buffer at pH 7.0 and sterilized by autoclaving. To 15 liters of this was added the dialyzed extract of 1.5 pounds of meat infusion (3) which had been sterilized by filtration (medium I). 300 ml. of sterile 50 per cent glucose was likewise added. Medium II was prepared by adding the sterile dialysate of 15.0 gm. of Difco yeast extract and 450 ml. of sterile 50 per cent glucose solution to the basic casamino acid medium. In all instances the pH of the sterile media was adjusted to the desired value by adding sterile acid or alkali.

Colony Counts and Turbidity Measurements.—The growth of the culture was followed by turbidity measurements and colony counts. Samples were removed at specified time intervals and diluted in the medium in which they were grown. The dilutions were such that the turbidity readings were always of approximately the same value. Measurements were made in a

phototurbidometer. When colony counts were made, the sample was diluted so that 1 ml. of the final dilution contained approximately 10^3 organisms. 0.1 ml. of this was added to 2.5 ml. of 0.5 per cent nutrient agar maintained at 46°C. The contents of the tube was then poured on 1.5 per cent nutrient agar contained in a Petri dish. The colonies which developed were counted after 18 hours of incubation at 37°C. Duplicate determinations were made in all instances.

Titration of Colicine Activity.—The colicine K activity of a given culture medium was determined by measuring the ability of the medium to inhibit the growth of the test organism *E. coli* B. This bacillus is exceedingly susceptible to the action of colicine K and it serves as an ideal test organism. Two procedures were employed. Method A, the first and more pre-

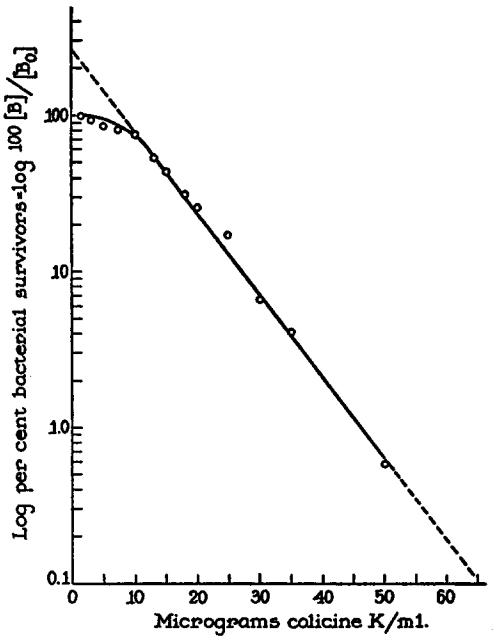

FIG. 1. Effect of varying concentrations of colicine K on survival of *E. coli* B.

cise, was used for following colicine production in cultures of *E. coli* K235 L_T grown in medium I. Method B, the second and more convenient, was employed for studying the growth and colicine production of K235 L_4O in medium II. The procedures were as follows:—

Method A.—0.1 ml. of the colicine solution to be assayed was added to 2.5 ml. of nutrient agar (0.5 per cent) maintained at 46°C. 0.2 ml. of a suspension of *E. coli* B containing 250 to 500 cells was now added, and the mixture was poured on a nutrient agar (1.5 per cent) plate. As a rule 3 to 4 different dilutions of the sample were made and tested, and duplicate determinations were carried out. The plates were incubated at 37°C. for 18 hours, and the number of surviving bacteria were scored and their percentage calculated. The concentration of colicine in a particular dilution was determined from the graph given in Fig. 1 or by calculation from Equation 1. The total concentration of colicine in 1 ml. of the original sample was then determined by multiplying by the dilution factor.

Method B.—Petri dishes marked in 8 equal segments and containing 18 ml. of nutrient agar (2 per cent) were overlayered with 2.5 ml. of soft nutrient agar (0.5 per cent) containing

5×10^7 cells of *E. coli* B. Twofold serial dilutions of the colicine sample to be tested were made in nutrient broth and 0.02 ml. of each dilution was placed on the nutrient agar surface, using one segment for each dilution. The solutions were added with a standardized nichrome wire loop about 5 mm. in diameter. After standing at room temperature for an hour the drops had dried and the dishes were then incubated for 6 hours at 37°C. The antibacterial activity of the colicine was manifested by the fact that no growth occurred in those areas which had been covered by a sufficient concentration of colicine. The last dilution which still gave a clear area was defined as containing 1 unit of colicine K per ml. Thus, if a drop of supernate from the culture medium which had been diluted 1:800 still gave an area in which no growth of the test organism occurred, the dilution contained 1 unit of colicine K per ml. The original undiluted supernate contained, therefore, 800 units per ml. The method of assay is remarkably reproduceable provided the number of test organisms per plate is kept reasonably constant and the incubation time is always the same.

Apparatus.—An apparatus for controlling automatically the pH of a liquid medium for the growth of bacteria was described by Longsworth and MacInnes (6). A glass electrode and potassium chloride bridge were immersed in the culture medium, and a calomel electrode completed the electrochemical cell whose electromotive force reflected the pH of the bacterial environment. The E.M.F. of this cell was balanced by a potentiometer which could be preset to a value corresponding to the desired pH for the experiment at which the liquid culture medium was initially adjusted. A Compton electrometer served to indicate any imbalance between the glass electrode cell and the potentiometer which would result from a lowering of the pH as acid was elaborated during the growth of the bacteria. In order to maintain the pH at a constant value any deflection of the electrometer resulting from a pH lowering caused the addition of alkali from a solenoid operated buret, activated by a photoelectric device which observed such electrometer deflections by means of suitable optical system.

For our work we have retained the type of solenoid buret used by Longsworth and MacInnes, but have replaced the electrometer-photoelectric cell assembly by an electronic amplifier. It has the advantage of being portable and relatively fool proof in the hands of biological technicians. Since it is extremely difficult to obtain a direct current amplifier with sufficient time stability in electrical circuits having as high a resistance as is the case with glass electrodes, use was made of a stable pulse amplifier. A condenser was alternately charged from the E.M.F. of the glass electrode cell and from that of the potentiometer by a continually rotating cam. This provided a cyclic input into the amplifier which, in turn, operated the solenoid buret for the addition of sodium carbonate as acid developed in the system.[1]

The schematic arrangements for this apparatus are shown in Figs. 2 and 3 and are briefly described in the accompanying legends. The position of the equipment in the assembly of the apparatus for the growth of bacteria under controlled conditions is shown in *E* of Fig. 4. The glass electrode and calomel electrode—saturated KCl bridge were connected to the electronic apparatus and immersed into the culture medium of known pH. The temperature and pH dials were set at the appropriate values and the potentiometer was balanced with the adjustments shown in Fig. 2. Balance was indicated by the milliammeter *M* in Fig. 3. From then on the apparatus functioned automatically. The pH of the medium was maintained at a constant value by the cyclical alternate inputs from the glass electrode cell and from the potentiometer as described above. When these two inputs of E.M.F. departed from equality (because of the formation of acid in the culture medium) the imbalance, amplified by the

[1] The electrical pH control unit was constructed for us by Process and Instruments Company, Brooklyn. The electronic details (of a more technical nature) will soon be published in an appropriate electrical journal by Mr. George Katz, to whom we are indebted for the electronic design.

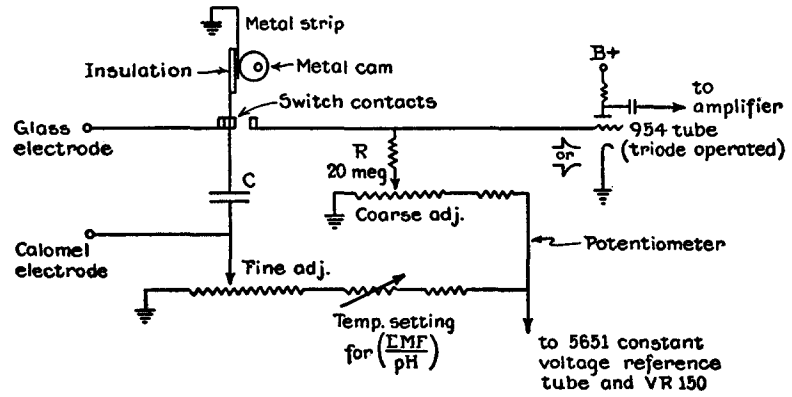

FIG. 2. Diagrammatic electrical input circuit of pH control unit.

Metal strip in contact with cam is grounded to eliminate friction potentials against the polystyrene insulation. Capacitor C is limited in size to minimize the time of charging. Capacitor C and switch have low leakage to ground. The larger the value of R the larger is the pulse and the better is the discrimination against 60 cycle contact "bounce" and other transients. Balancing against the E.M.F. of the glass electrode cell the potentiometer which contains a coarse and a fine adjustment is also provided with a variable resistance to set the appropriate value for the ratio E.M.F./pH at the temperature of the experiment. Reference tube 5651 is operated from a VR 150. Switch contacts are platinum-iridium (on steel backing) soldered to the switch springs. Input pulse to tube 954 is positive Λ or negative V depending on whether the capacitor voltage is larger or smaller than the voltage opposing the pH cell E.M.F.

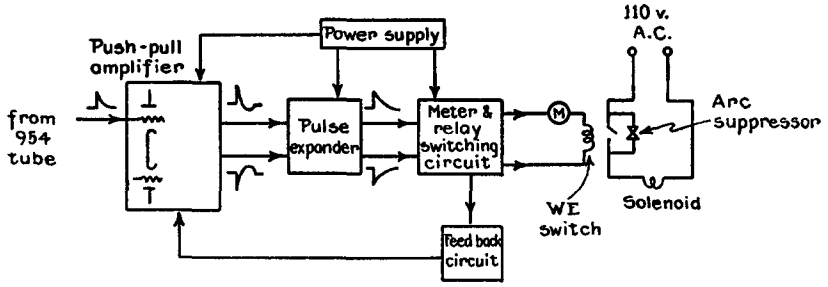

FIG. 3. Diagrammatic electrical amplifier circuit of pH control unit.

Push-pull amplifier controls switching for addition of alkali (or acid). Pulse expander permits sufficient time for operation of meter, M, and relay. Feedback circuit develops voltage proportional to pulse amplitude and is fed back to decrease gain (thereby decreasing amplitude of overshoot and increasing the dynamic range). Switch WE is a Western Electric mercury relay type 276B. The mercury wipes the contacts and prevents pitting. Meter, M, has a zero centered 1-0-1 milliampere sensitivity scale. Arc suppressor is a Federal telephone and radio type 15A5PS5 which further protects contacts and minimizes electrostatic transient disturbances from the arc. Solenoid is a guardian type AZC intermittent duty unit. Power supply is of an appropriate conventional type.

apparatus, activated the solenoid buret which in turn released sodium carbonate into the medium until balance was restored.

For the mass cultivation of bacteria, a 5 gallon pyrex bottle A (Fig. 4) containing 15

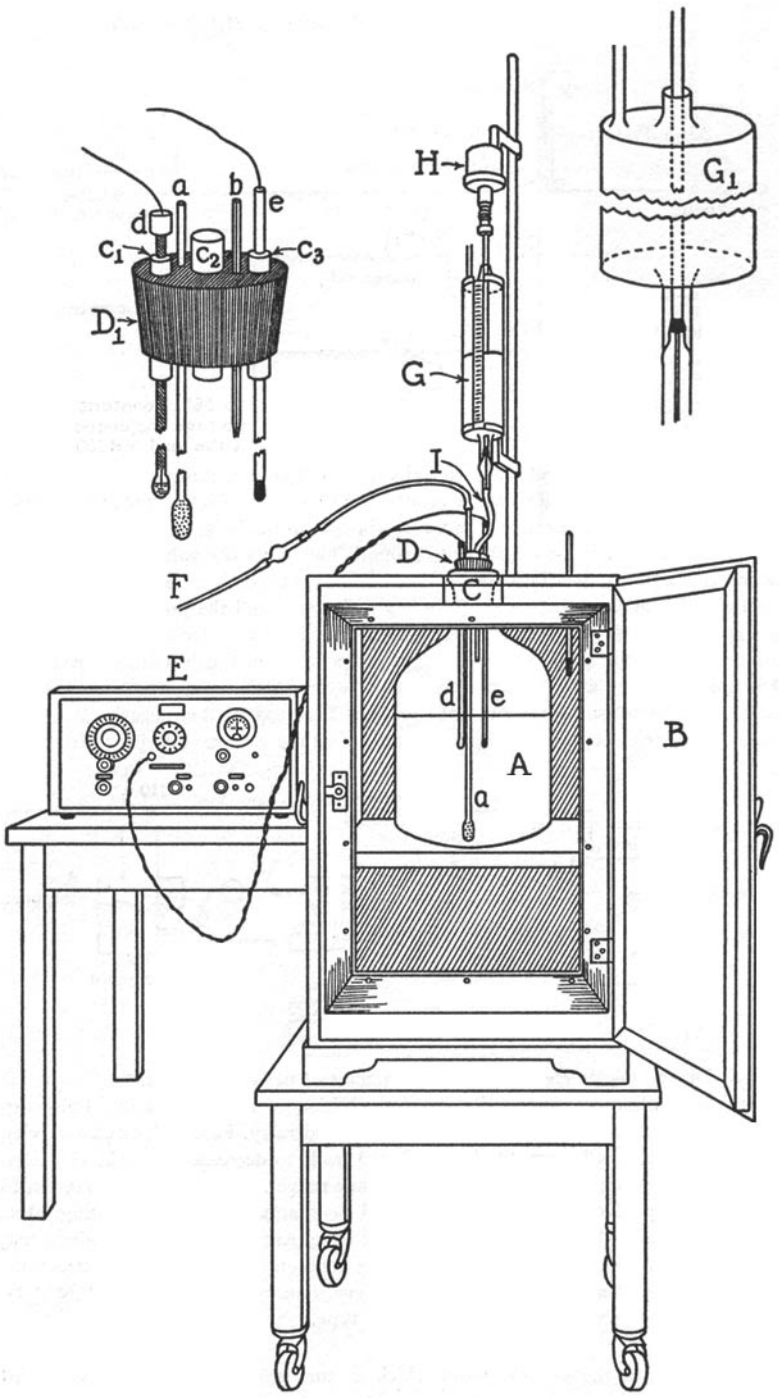

FIG. 4. Apparatus for growing bacteria at constant pH.

liters of culture medium was placed in a specially constructed incubator *B*, maintained at 37°C., 24 hours prior to its use. The incubator bore a hole in its top through which the neck of the bottle protruded. A slot, closed by the removable piece *C*, readily permitted placing the bottle in position. An assembly D_1 (*D* in large fig.) consisting of a No. 12 rubber stopper bearing a sintered glass aeration tube (*a*), a capillary tube (*b*) (150 mm. in length, through which sodium carbonate was added), and 3 glass tubes c_1, c_2, and c_3, (15 × 100 mm.) was placed in the flask. Tubes c_1 and c_3 served as receptacles for the glass electrode (*d*) and for the calomel electrode and saturated KCl bridge (*e*).[2] Both were fitted with a No. 2 rubber stopper to the receptacles c_1 and c_3. The opening c_2, which served as a port both for the escape of air and for the removal of samples, was covered with a sterile aluminum cap. This entire assembly was heat-sterilized in a paper wrapping prior to its use.

The glass electrodes (*d*) and the calomel half-cell (*e*) were sterilized by immersion in 2 per cent sodium hypochlorite for 15 minutes prior to their use, washed with 70 per cent alcohol, and then placed in position (Tubes c_1 and c_3). After the electrodes were connected to the meter *E* which was set at the desired pH, a compressed air line *F* was attached to the aeration tube (*a*). The latter bore a sterile calcium chloride tube filled with non-absorbent cotton which served as a filter for sterilizing the air. The latex rubber tubing *I*, sterilized in 70 per cent ethyl alcohol, connected the solenoid operated buret to the capillary tube (*b*). The buret, which is shown in detail, (*G*), bore a movable rubber tipped rod and was connected at its upper end through a chuck to the solenoid *H*. The buret itself had a capacity of 1 liter and was filled with 1.5 M sodium carbonate. A few drops of tributyl phosphate were added to the medium to prevent foaming, and a stream of air (3 liters per minute) was blown through the liquid. Assembling the apparatus required about 15 minutes.

EXPERIMENTAL

Colicine K Assay Curve.—The concentration of colicine K in the culture media can be ascertained by a procedure involving the use of a standard reference curve constructed by plotting the percentage survival of a given number of test organisms (*E. coli* B) against varying quantities of colicine K to which the cells had been subjected. In order to prepare such a curve it was necessary to procure colicine K itself. The latter was obtained in the following manner:—

15 liters of medium II was seeded with 1.5×10^8 cells of *E. coli* K235 L_4O at 5:00 p.m. Growth of the organisms in the automatic pH control apparatus shown in Fig. 4 attained a maximum value at 37°C. with aeration and at pH 7.0, some 17 hours after seeding. At this time the population reached a level of about 1.2×10^{10} B/ml. and the consumption of 1.5 M sodium carbonate abruptly ceased. To the culture was now added 100 ml. of chloroform and after stirring for 15 minutes the dead bacteria were removed by centrifugation. The supernate was rapidly concentrated *in vacuo* (4 hours) to 1 liter and dialyzed against distilled water at 5°C. for 24 hours. The contents of the dialysis membranes was again concentrated and dialyzed until free of salts and nutrients; the material remaining in solution was isolated by freeze drying. Approximately 5 gm. of substance was obtained. This material, a pale yellow amorphous powder, possessed very potent colicine K activity.

[2] The glass electrode (40 cm. in length) was supplied by the Beckman Instrument Company, Pasadena. The calomel electrode–saturated KCl bridge was of the same length. It was constructed from a pyrex glass tube sealed at the bottom and containing a small quantity of mercury and calomel. An asbestos fibre had previously been sealed into the wall of the tube 1 inch above the surface of the mercury-calomel mixture to permit contact with the culture medium. Contact with the mercury was made through an inner glass tube bearing a sealed-in platinum wire.

An assay procedure, designed to measure the *relative* concentration of colicine K in any given sample, was devised, employing this material as a reference substance and using *E. coli* B as the test organism.

A series of tubes, each containing 2.5 ml. of nutrient agar (0.5 per cent) was seeded with 250 to 500 bacilli and a known concentration of crude colicine K was added to each. The mixture was poured on nutrient agar plates and the latter were incubated for 18 hours at 37°C. The number of surviving bacteria was then determined by colony count and their percentages were calculated.

A plot of the per cent survivors against the concentration of added colicine K is presented in Fig. 1. This curve was employed as a reference standard and from it the relative colicine K content of any sample could be ascertained. One unit of colicine K was arbitrarily chosen to be equivalent to one microgram of the standard preparation and hence 1 mg. would contain 1000 units. It should be pointed out that the curve presented in Fig. 1 was constructed using impure colicine K. When the pure substance becomes available these values can of course be corrected.

The colicine assay curve is shown in Fig. 1 and represents a plot of log 100 $[B]/[B]_0$ against D. Here $[B_0]$ represents the number of bacteria per milliliter present in the original untreated suspension and [B] the corresponding number of cells which survived exposure to a concentration D of colicine. Within the concentration range 10 to 50 μg. of colicine per ml. the curve is essentially linear and can be expressed by the equation:—

$$\log \frac{100[B]}{[B_0]} = 2.423 - 0.0523D \qquad (1)$$

In this equation the value—0.0523 represents the slope of the linear portion of the curve and 2.423 is the value of the extrapolated intercept. In order to determine the concentration of colicine in an unknown sample it is merely necessary to determine the per cent survivors in a given population of test microorganisms which had been subjected to an appropriate dilution of the sample and then to make use either of the graph or Equation 1.

Colicine Production by E. coli K235 L_T in Medium I at Different pH Values. —In order to ascertain the optimum condition for the production of colicine K, the growth of two variants of *E. coli* K235, L_+O, and L_T, was studied. These organisms were grown in two different media and at various values of pH.

One liter of medium I contained in a two liter wide mouth Erlenmeyer flask was seeded with a culture of K235 L_T. The flask was fitted with the assembly shown in Fig. 4, and used in the production of mass quantities of colicine K. The pH of the medium was maintained at the desired value by the automatic addition of 1.0 M sodium carbonate, and the flask was held at 37°C. by immersion in a constant temperature water bath.

The flask was seeded with approximately 5×10^6 B/ml. and placed in the incubator at 3 a.m. Samples were withdrawn for assay beginning at 9 a.m., and at hourly intervals there-

after, when aeration was commenced and the bacterial population had reached approximately 1 × 10^8 B/ml. The turbidity, viable bacterial concentration, and colicine K content were determined. In each sample the colicine K concentration in the whole chloroform-killed culture was determined, as well as that in the cell-free supernate.

Fig. 5 represents a plot of the amount of colicine K elaborated by *E. coli* K235 L_T at various intervals during its growth in medium I and maintained at different values of pH. From the figure it can be seen that at pH 6.0 little colicine K was liberated into the medium by the rapidly multiplying microorganisms. It will also be observed that as the cell concentration increased, the amount of colicine in the whole culture diminished. Thus, there appears to be an inverse relationship between the bacterial population and the colicine K content of the cells themselves. Attempts were made to obtain cultures con-

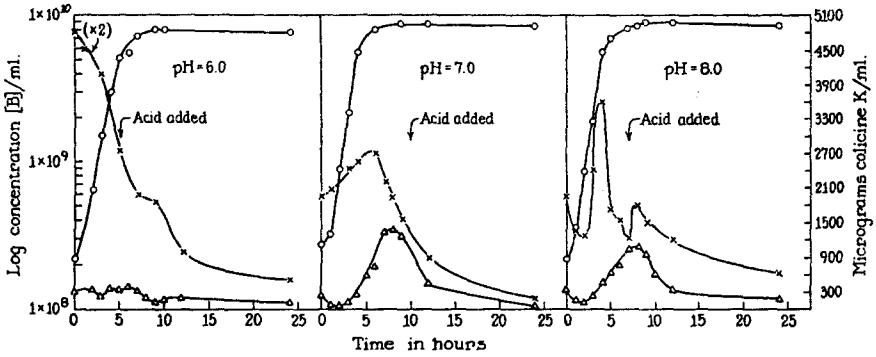

FIG. 5. Colicine K production by *E. coli* K235 L_T at various pH values. O—O—, bacterial count; ×—×, colicine content, whole culture; △—△, colicine content, supernate.

taining larger amounts of intracellular colicine by permitting them to grow without aeration, but without success. It was also observed that as the bacteria grew to maturity, the concentration of intracellular colicine did not increase in proportion to the number of bacteria. When the growth experiment was conducted at pH 7.0, the concentration of colicine K in the whole culture reached a maximum 5 hours after aeration was initiated. It was not until some 3 hours later, however, that the colicine content of the cell-free supernate attained its maximum concentration. At this time the bacterial population had reached a maximum and the culture stopped consuming sodium carbonate. Even though aeration was continued at this juncture and the pH of the medium was maintained at 7.0 by the automatic addition of 1.N HCl, rapid inactivation of the colicine in the supernate occurred. When growth was permitted to take place at pH 8.0 the results were essentially the same as those obtained at pH 7.0, although the total amount of colicine liberated was somewhat less.

It should be stated that colicine K is stable over wide ranges of pH. If solu-

tions are aerated at 37°C. between pH 2.0–9.5 there is but little diminution in activity. The reason for the rapid disappearance of colicine from the growing cultures will be discussed later.

In sum, it can be concluded that the maximum release of colicine K from growing cultures of *E. coli* K235 L_T occurred when the pH of the medium was maintained at approximately 7.0 and at that point at which the culture ceased to consume sodium carbonate. Incubation beyond this interval invariably resulted in a great diminution in the colicine content of the whole culture.

Colicine K Production by E. coli K235 L_+O in Medium II at Different pH Values.—From the foregoing it is apparent that the elaboration of colicine K by *E. coli* K235 L_T is determined in large measure by maintaining the pH of the medium at an optimum value. In order to learn whether still higher yields

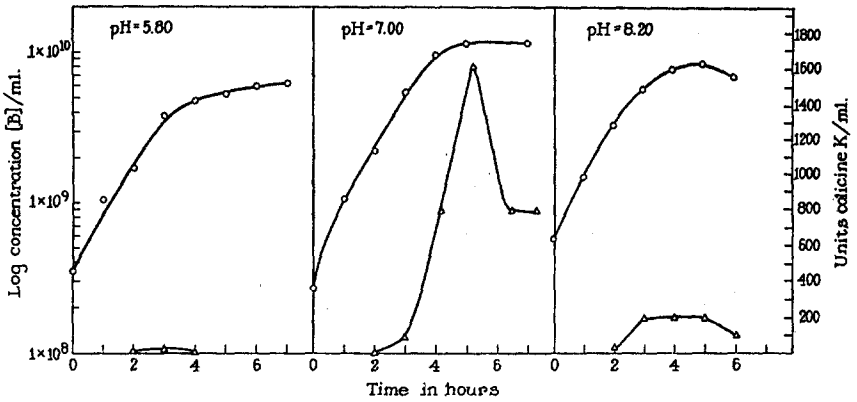

FIG. 6. Colicine K production by *E. coli* K235 L_+O at various pH values. O—O—, bacterial count; △—△—, colicine content.

of the antibacterial agent could be obtained than in the previous experiments, growth of the variant L_+O was studied at three different values of pH, using the same basic casamino acid medium to which a dialyzed extract of yeast, instead of meat, had been added and in which the glucose concentration was increased to 1.5 per cent.

The results, presented in Fig. 6, reveal clearly that in this medium the L_+O strain of *E. coli* K235 produce a maximum amounts of colicine K only when growth was permitted to take place near neutrality. Here the bacterial population reached a peak slightly greater than 1×10^{10} B/ml. at which point some 1600 units per ml. of colicine K were present in the culture medium. At pH 7.0 the liberation of colicine K paralleled the multiplication of bacteria until growth ceased; at this juncture the culture suddenly stopped consuming sodium carbonate and the colicine content of the supernate began to diminish. Just why this sudden drop occurred is not clear, but it is possible that the cells released an enzyme which destroys the antibacterial agent (11).

When the growth experiment was conducted at pH 5.8 the bacterial concentration reached half that obtained at pH 7.0, but the release of colicine into the surrounding medium was negligible. At pH 8.2, on the other hand, the growth of the bacteria and release of colicine did not parallel one another, for at no time were more than 200 units per ml. present in the medium despite the fact that the concentration of bacilli reached 8.0×10^9 cells per ml. Although no graphic presentation is made, it should be stated that when the L_T variant was grown in medium II at pH 7.0 and colicine assays were made by method B, its growth rate was identical with that of the L_+O variant. The concentration of colicine K in the culture medium, however, never exceeded 400 units per ml.

Neither the parent strain, K235 L_+T sent us originally by Doctor Fredericq, nor its other variants produced as much colicine as did the L_+O variant. The latter yielded from 4 to 8 times as much colicine K as did any of the others. It should also be mentioned that colicine K was not produced in significant quantities by any of the strains when grown in synthetic medium. Significantly enough, if to the latter was added 0.1 per cent of dialyzed yeast or meat extract, the production of colicine K was greatly enhanced, though it was never as great as when the organisms were grown in the casamino acid media. From the foregoing it is apparent that the production and release of colicine K from the various strains of *E. coli* K235 is dependent upon a number of factors and that among these the precise control of the pH of the environment and nutrients are of utmost importance.

DISCUSSION

From the experimental work which has been presented, it is seen that the strain of *E. coli* designated as K235 yields colicine K in maximal quantities only when the organisms are grown under special environmental conditions. Those procedures which induce colicine production in other strains of *E. coli* have been found to be without effect upon strain K235 (12). Thus, irradiation of the bacilli with ultraviolet light or subjecting them to the action of inducing agents such as hydrogen peroxide, sulfhydryl compounds, or the nitrogen mustards has each failed to bring about liberation of colicine K in rapidly growing cultures of the two variants employed in this study.

It was found, however, that an increased output of colicine K could be achieved if the pH of the culture was maintained near neutrality. A still further enhancement in the yield was obtained when dialyzed yeast extract was added to the medium. This nutritional factor was not one of the B complex vitamins, for when the latter were tested individually or in combination they were found to be without effect.

In attempting to understand why the release of colicine K from those strains of *E. coli* which have here been under study is so dependent upon the pH of the environment, it should be borne in mind that not all the cells in the growing culture need necessarily elaborate the antibacterial agent. Indeed, colicine

production has been likened by others to the elaboration of a lytic phage by a lysogenic bacillus. In a culture of colicinogenic bacilli it is possible that some nutritional factor is required, and that the latter functions most effectively near neutrality to bring about release of the colicine. It is also conceivable that some intracellular enzyme system is responsible for the synthesis, and that the latter operates effectively only within a narrow pH range. Whatever the factors, it is apparent that a rigid control of the pH of an enriched nutrient medium is essential for the production and release of colicine from *E. coli* K235.

SUMMARY

An apparatus for maintaining the pH of an actively growing bacterial culture at constant level has been described. Using this apparatus it has been shown that the production of maximal amounts of colicine K from a strain of *E. coli* known as K235 is dependent upon an enriched nutrient medium which is maintained at pH 7.0.

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