THE PREPARATION OF SILICIC ACID JELLIES FOR BACTERIOLOGICAL MEDIA

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Although silicic acid jellies are inferior to agar, gelatin, blood serum or egg white in the preparation of solid media for the majority of bacteriological requirements, there are certain specialized purposes, such as the isolation of autotrophic bacteria, for which an inorganic jelly is necessary. Theoretical advantages from the utilization of silica gel as a specialized substitute for organic gels have not been realized in practice, largely as a result of the erroneous assumption that inorganic gels are inert in the medium. The present study deals with the influence of time, temperature, dialysis and autoclaving on the pH and the rigidity of silica jellies prepared for bacteriological purposes.

Many modifications of two classical methods for the preparation of silicic acid jellies have been described by bacteriologists. The "sol" method, first applied in bacteriology by Kuhne (1890) and later used by Winogradsky (1891) and by Omeliansky (1899), was adequately reviewed by Bojanovsky (1925) and recently employed by Boltjes (1935) in obtaining pure cultures of nitrifying bacteria. This procedure has a distinct advantage in that the dialyzed and sterilized sol is gelled after addition of the bacterial suspensions to the plates. However, it was not considered of general utility because of the special apparatus required for the preparation of the dialyzed sol and because the liquid of syneresis exuded on the surface of the plates permits a film of contaminants to cover the pure colonies which may develop in the depths of the gel. Boltjes overcame this difficulty with the aid of a micromanipulator for colony picking.

Because of its apparently greater simplicity, the "gel-in-plate" method recommended by Winogradsky (1925) has been less carefully developed and more widely used. In this procedure, the mixture of silicate and acid, at an undetermined pH, is allowed to harden in the Petri dishes. The gel is dialyzed in tap water and distilled water to remove the excess sodium chloride, after which the nutrient salts are added. No provision is made for final sterilization. Various modifications such as those by Stevens and Temple (1908) and by Doryland (1916) have provided for sterility, but disposal of liquid of syneresis and of excess sodium chloride has been neglected. Therefore, in the case of this "gel-in-plate" method, one has been forced to choose between a good nutrient medium which could not be sterilized and a sterile medium with an unfavorable salt concentration. The production of gels which fulfill the strict requirements of uniform nutrient content, suitable pH, optimal salt concentration, dry surface, and autoclave sterility has not been accomplished by the methods hitherto available.

For several years previous to this study silica gels were prepared by the authors at pH 7.4, using bromthymol blue indicator (see methods), on the assumption that the gel should be made at the pH desired in the medium. Although the color of the medium was observed to become a deeper blue on standing, this change was attributed to an increased opacity of the gel rather than to a decreased H-ion concentration.

These gels were prepared from sterile reagents and were dialyzed aseptically in a solution of inorganic salts to impregnate the plate with nutrients and to remove the excess sodium chloride. Although such plates permitted the isolation of pure cultures of autotrophic bacteria (*Nitrosomonas*),[1] they occasionally failed to provide favorable conditions for growth. It was finally learned that these failures were due to the anomalous fact that the pH of the dialyzed gels was often higher than that of the original jelly or of the dialyzing fluid. Furthermore, the inconvenience attending aseptic dialysis was so great that this procedure was

[1] A practical method for this purpose is being published separately (Hanks and Weintraub, 1936b).

considered impracticable. The gels were next dialyzed in open baths of the nutrient solution and attempts were made to sterilize them in the autoclave. Autoclaving further increased the alkalinity of the medium and usually resulted in its complete peptization.

Therefore, it became necessary to undertake an investigation of the H-ion changes in gels made in various ways and to study the influence of dialysis and autoclaving on the rigidity and pH of the medium.

METHODS OF STUDY

The method for the preparation of the gels differed from the classical procedure. Previous titration of the reagents was made unnecessary by the addition of 5 cc. of 0.04 per cent aqueous bromthymol blue or bromcresol purple per liter of the stock solutions of sodium silicate and of hydrochloric acid. The gels were prepared at the desired pH by pouring the sodium silicate into a chosen volume of the acid in a large flask until the proper color of the indicator appeared. The pH of a small sample of the mixture was then quickly determined electrometrically and further additions of acid or silicate were made as required. In order to delay gel formation in rapidly hardening mixtures until the pH could be adjusted and the mixture could be distributed among 20 or 30 Petri dishes, the reagents were previously chilled to 4°C. All batches of gel were made in such volume that duplicate or triplicate plates (containing approximately 30 cc.) could be used for each variable studied. This method permitted the preparation of any desired number of plates in which the nutrients, the pH, the hardening time, and the SiO_2 content of the gels were standardized automatically. Furthermore, the gels were easily duplicated in successive batches.

Although firm gels were formed by mixtures of 3.5 per cent sodium silicate (Baker's 40 per cent solution, diluted to 3.5 per cent Na_2SiO_3) and 3 per cent of concentrated hydrochloric acid by volume (approximately 0.3 N), the changes in the gels during dialysis and autoclaving made it desirable to employ 6 and 5 per cent, or 7 and 6 per cent, concentrations of silicate and acid,

respectively. Since approximately equal volumes of these reagents were required, various salts were incorporated in the jellies by adding a double amount of these salts to the stock solution (acid or silicate) in which they were most stable.

All pH measurements were made at 25°C., with a calomel half-cell and quinhydrone electrode. Rapid determinations on chilled acid-silicate mixtures were made possible by keeping a number of tubes containing 5 cc. of distilled water and excess quinhydrone in a bath at 32°C. Shaking 3 cc. of the chilled mixture in one of these tubes quickly brought the temperature to 25°C. The pH of the gels was determined by macerating approximately 2 cc. of the gel with a glass stirring rod, in a short tube containing solid quinhydrone, until the gel was reduced to a smooth paste. The gel paste was then diluted with distilled water at 25°C. to a final volume of approximately 8 cc. and the suspension was shaken. Gels which had been covered with water of syneresis or with dialyzing fluid were rinsed with distilled water before samples were removed.

The gels were dialyzed at 40°C. in ten times their volume of the various solutions which were employed. Dialysis was hastened by arranging the plates, without lids, on edge in the dialyzing fluid with the exposed surface of the gels facing slightly downward.

Properly prepared gels withstood autoclaving at ten pounds of steam pressure for 15 minutes. Occasional cracks at the periphery of the medium were caused by expansion of air in this rapidly heated portion of the gel before the autoclave was sealed, but these slight imperfections did not impair the usefulness of the plates. Since bubbles and cracks throughout the medium resulted from rapid decreases in the pressure during autoclaving, the steam chamber was sealed absolutely tight after displacement of the air and the pressure was carefully controlled. Good results were also obtained by the more laborious method of raising the pressure in the tightly sealed autoclave to the desired point and then exchanging the air for steam *without permitting fluctuations in pressure.*

EXPERIMENTAL RESULTS

pH changes in non-dialyzed gels

The early observations suggesting a pH drift in gels prepared at a known pH were confirmed. Figure 1 shows the course of the alkaline shift in a batch of gel made at pH 6.3. One lot of plates was autoclaved immediately and then stored at 30°C.; a second lot was maintained at 30°C. while a third lot was kept at 4°C. Autoclaving promptly alkalinized the gel to pH 7.4. The pH

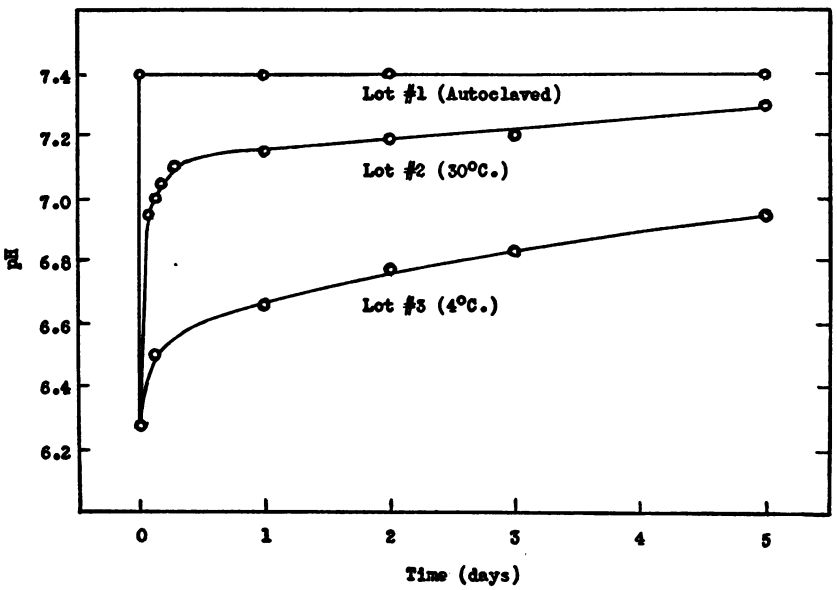

FIG. 1. THE pH CHANGES IN SILICA GELS AS INFLUENCED BY TEMPERATURE

of the gels stored at 30°C. rose rapidly at first and then more slowly; at this temperature the gels attain the stable pH of autoclaved gels after 14 to 21 days. The process of H-ion change in the jellies stored at 4°C. was much slower and more prolonged.

All batches of gel prepared between pH 6.0 and 7.5 ultimately showed a rise of 1.1 to 1.2 pH units, regardless of differences in initial H-ion concentration or in the storage temperature. These results demonstrate that the final pH of non-dialyzed jellies is much higher than the pH at which the jellies are prepared. This

phenomenon doubtless explains one of the unknown faults of the gels of Stevens and Temple, of Doryland and of those prepared earlier by the authors.

Nutrient salts can be incorporated in silica jellies at the time of preparation and the initial pH of the acid-silicate mixtures can be adjusted to produce the desired pH after autoclaving. The rise will be about 0.8 pH in non-dialyzed media which contain 0.1 per cent of phosphates. However, such gels are not generally useful for the cultivation of bacteria because of the high concentration of sodium chloride. The excess salt must be removed by dialysis.

Changes in pH and firmness of the gels during dialysis in various solutions

Dialysis induces marked changes in the pH and the rigidity of the gels and results in their peptization in the autoclave unless special attention is given to the ions incorporated in the gel or added to the dialyzing solutions. The information to be presented will make it clear that for some purposes the "stabilizing" salts can be added only to the gels and in other cases may be added to both the gels and the dialyzing fluids.

As a general principle, the dialyzing fluid should always have the composition of the nutrient solution required by the organisms to be cultivated. This is important because the addition of materials to individual plates will not provide a uniform content of nutrients in all the plates and also because the addition of substances to a finished medium may cause further changes in pH. Since nutrient solutions to be used for various purposes will differ in composition, it seemed advisable to attempt the stabilization of gels in solutions containing a simple assortment of ions. Phosphate mixture (M/150) was chosen as an ingredient which would be involved universally.

Figure 2 shows the pH of the gels and the solutions at the indicated intervals during dialysis of silica gels in various solutions, and also the pH and the condition of the gels after autoclave sterilization. It may be seen that the gels were made unsuitable for autoclaving by dialysis in distilled water of pH 5.7, in phos-

phate buffer of pH 7.4 and in phosphate buffer +0.1 per cent NaCl (pH 7.4). On the other hand, the gels were stabilized in the slightly acid phosphate buffer (pH 6.2), in the ammonium sulphate medium[2] (pH 7.4) and in the phosphate buffer +0.1 per cent NH_4Cl (pH 7.4). Comparison of the results obtained with these different solutions is instructive.

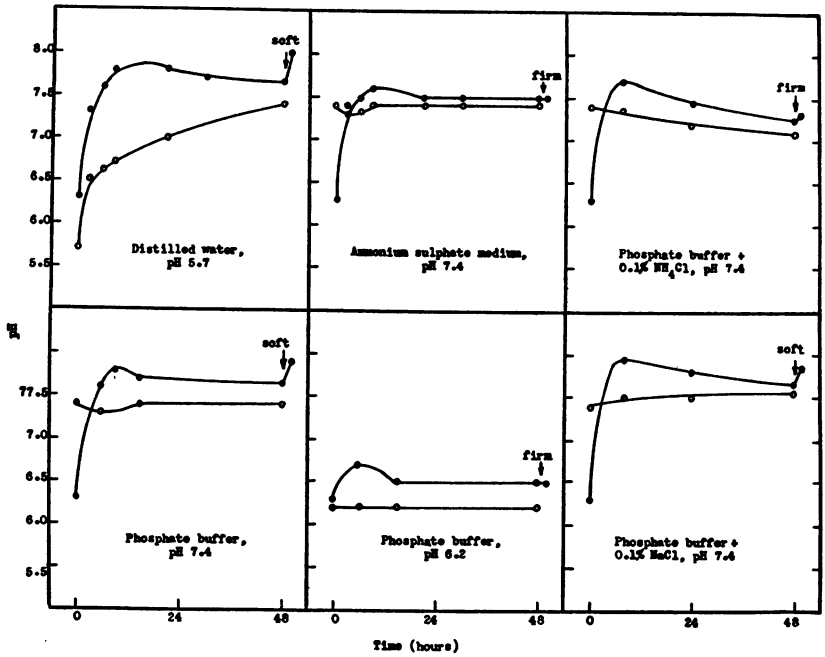

FIG. 2. THE RESULTS OF DIALYZING SILICA JELLIES IN VARIOUS SOLUTIONS
● — Gels
○ — Dialyzing fluids
↓ Arrow indicates autoclaving

In the first place it is obvious that dialysis in distilled water accomplished nothing more than the removal of excess sodium chloride and a weakening of the gel; the nutrient salts had not been introduced and the gel could not be sterilized. In contrast, dialysis in the ammonium sulphate medium produced a stable

[2] Ammonium sulphate medium: 1 gram $(NH_4)_2SO_4$, 0.2 gram KH_2PO_4, 0.8 gram K_2HPO_4, traces of $FeSO_4$ and $MnSO_4$, in 1 liter of tap water.

nutrient gel[3] which was autoclaved without appreciable change in pH or firmness. That the ammonium ion, rather than increased concentration of electrolyte, was the important factor in the stabilization of the gel at pH 7.4 is demonstrated by the fact that phosphate buffer containing 1 gram of ammonium chloride per liter also stabilized the gel, while at the same pH phosphate buffer containing an equivalent amount of sodium chloride was unsatisfactory. The stabilizing influence of small amounts of ammonium solves the problem of preparing dialyzed gels at a neutral or slightly alkaline pH. However, under certain circumstances the ammonium must be excluded from a medium and other means of stabilizing the gels must be sought.

From a comparison of the results of dialysis in the phosphate buffer solutions at pH 7.4 and 6.2 it is clear that a slight increase in the concentration of hydrogen ions had a remarkable effect on the stability of silica gels. Much less hydrogen than ammonium ion was required to produce stabilization of the gels. However, the biological activity of the hydrogen ion limits its usefulness. Recognition of the stabilizing effect of this ion is important because it indicates that the pH of gels lacking ammonium should always be as low as is consistent with the purpose for which they are prepared.

The time required for dialysis is indicated by the character of the curves in figure 2. The curves for the pH of the gels and the salt solutions always diverge during the early hours of dialysis. They then approach each other and tend to establish a constant relationship within 0.1 to 0.3 pH units. Dialysis may be regarded as sufficiently complete whenever this relationship has been attained. If dialysis is terminated before its completion, the jellies are considerably more alkaline than the dialyzing fluid and may be unstable.

Inasmuch as the non-dialyzed jellies were alkalinized during storage, it was also important to determine whether this cause of variations in the pH of stored gels would influence the final pH and firmness of the gels at the end of dialysis. Because of the

[3] Without further treatment, except drying, such gels are complete and satisfactory media for the isolation of pure cultures of ammonia-oxidizing bacteria.

temperature conditions to which they had been subjected, each lot of the jelly shown in figure 1 had a different pH at the end of 5 days of storage. The individual lots of this jelly (pH 7.4, 7.3 and 6.9, respectively) were dialyzed separately in the ammonium sulphate medium at pH 7.4. All of these gels reached identical H-ion concentrations by the end of 9 hours of dialysis. The results already described (see fig. 2) for the dialysis of freshly prepared gels in the ammonium sulphate solutions were duplicated throughout the remainder of the dialyzing period and on autoclaving. Therefore, non-dialyzed gels may be stored in any convenient way until one wishes to start the dialysis.

Since the final pH of the gels dialyzed in the buffer solution or in solutions containing ammonium is controlled by that of the dialyzing solution, some latitude in the initial pH of the gels is allowable. The gels may be reproduced with sufficient accuracy for bacteriological purposes by merely combining the acid and silicate solutions until a conveniently recognized color of the chosen indicator is observed, e.g., grass green to bromthymol blue or wine red to bromcresol purple.

The effect of various salts incorporated in the gels

On theoretical grounds, it might be expected that stabilization would be effected by the use of salts of calcium, aluminum or iron. Unfortunately, the precipitation of these ions by phosphate, which is always present, precludes their addition to the dialyzing fluids. Since it had been learned that the incorporation of the ammonium ion in the acid-silicate mixtures would protect the gel during dialysis in the plain phosphate buffer of pH 7.4, the effects of incorporating the di- and tri-valent ions in the gel were studied.

By the addition of calcium chloride, ferrous sulphate or aluminum chloride to the acid solution, jellies were made which contained the same concentration of each of the cations as was given by 1 gram of ammonium sulphate per liter. The stabilizing effects of the four cations were compared by autoclaving the gels following dialysis in the buffer solution of pH 7.4. As previously observed, the ammonium gels were stabilized and no permanent change in the pH of the dialyzing solution was induced. On the

other hand, in the calcium, iron and aluminum gels the final pH was too high for satisfactory stabilization, because of a slight alkaline drift which occurred in the dialyzing solutions. When allowance was made for this drift, by employing dialyzing solutions of pH 7.0, all the gels were of satisfactory firmness and had final hydrogen ion concentrations ranging from pH 7.2 to 7.4 after autoclaving. Of the three gels lacking ammonium, the aluminum and the iron gels were clear whereas the calcium gel became opaque during dialysis (precipitation of calcium phosphate) and did not permit microscopic examination through the bottom of the closed plate.

The gel containing ferrous sulphate, when made with bromcresol purple at an initial wine red color, quickly developed a yellow appearance and determinations with the quinhydrone electrode indicated considerable acidification. The factors involved in this apparent pH change could not be worked out because of a disagreement between the pH values obtained colorimetrically and electrometrically. However, following dialysis in the buffer solution of pH 7.0, determination with the two H-ion methods were in agreement and stable gels were produced with pH 7.2 after autoclaving. More experience with the iron gels is needed before the course of their pH changes can be adequately understood.

PREPARATION OF THE MEDIUM

The procedure finally evolved for the preparation of silicic acid media consisted of three steps:

(a) Preparation of gels in which were incorporated the salts for nutritional purposes, or for stabilizing the firmness and pH of the medium.

(b) Dialysis of the gels in solutions which removed the excess sodium chloride, provided the nutrients required in the medium, and stabilized the firmness and pH of the gel.

(c) Sterilization of the medium in the autoclave.

The precautions to be observed in autoclaving the medium have been mentioned.

For the pure-culture isolation of bacteria it is necessary that the surface of the sterilized medium be absolutely dry. The plates are covered with a towel during autoclaving. After removal of the plates from the *cooled* autoclave, each is held on edge to allow the excess fluid to drain out. The sterile towel is then touched to the crack between the plate and the lid, to act as a wick for absorbing the residual fluid, so that no capillary film remains between the halves of the dish to permit contamination. The plates are then inverted and placed at 37°C. for 48 hours or at 28°C. for 96 hours in order to dry the surface completely.

Silicic acid jellies cannot be inoculated by streaking. The inoculum can be spread evenly over the surface by holding the plate in a nearly horizontal plane and causing it to oscillate through a short arc by a rapid side to side movement of the wrist. The fluid should be taken up quickly by the gel.

DISCUSSION

The foregoing data illustrate the most obvious and, perhaps, the more important changes which take place in silica gels during the preparation of a bacteriological medium. Since the phenomenon of pH rise in non-dialyzed gels and the stabilizing influence of various ions on the pH and firmness of these gels during dialysis will be discussed elsewhere (Hanks and Weintraub, 1936a) from a more theoretical point of view, it is desirable in this paper to state only briefly the factors which are believed to be involved.

The initial pH of the acid-silicate mixtures probably represents a titration equilibrium between the hydrogen and hydroxyl ions of the acid and the silicate. The changes in pH which accompany the hardening of the jelly are explained on the basis of an ionic exchange between sodium and hydrogen ions which are competing for positions on the gel framework. The factors controlled the success with which various cations compete for positions on soils, clays, permutits and other hydrous colloids have been admirably summarized by Jenny (1932) and are known to depend on the size, charge and degree of hydration of these ions. Among the monovalent ions, the firmness of adsorption is indicated by the lyotropic series: $Na^+ < K^+ < NH_4^+ < H^+$ and the ease with

which each ion may be displaced is expressed by arranging the list in the reverse order. This means that hydrogen ions will release or displace sodium ions even though the latter are present in much greater concentration. A progressive adsorption of hydrogen ions by the gel framework and a release of sodium ions into the capillary water of the non-dialyzed gels would appear to explain the alkalinization described in this paper.

Since the pH shift in non-dialyzed jellies represents merely a redistribution of the ions, it is understood why the pH rise during the storage of plates did not influence the pH of the plates at the end of dialysis. The pH of the gel at the time it is placed in the bath is altered by further ionic exchange during dialysis. A correlation between the position of the ions in the lyotropic series and their influence on the stability of the gels was demonstrated in this study. Gels dialyzed at pH 7.4 in solutions containing only sodium or potassium ions were alkalinized and were softened or peptized on autoclaving, while equivalent concentrations of ammonium ion produced stabilization. In the absence of the ammonium ion, stabilization was secured with relatively slight increases in the concentration of hydrogen ions. These results are in agreement with the demonstration by Wiegner (1931) and Baver (1929) that the viscosity, degree of hydration and the general stability of clays and other soil colloids are markedly affected by the nature and concentration of the adsorbed ions.

The incorporation of certain salts in the gels was based on the hypothesis that properly selected ions might be adsorbed with sufficient firmness to remain in the gel during dialysis in plain buffer solution and thus promote stabilization at a slightly higher pH than was otherwise possible. From the results obtained it would appear that calcium, aluminum or iron were slightly less effective than equivalent amounts of ammonium. The disadvantages in the use of calcium and the peculiarities observed in the case of iron have been mentioned. The incorporation of more than one salt in the same gel might be advantageous.

SUMMARY AND CONCLUSIONS

1. A method, which does not require previous titration of the reagents, is given for the preparation of silica gels. This method

facilitates the preparation of large batches of jelly in which the nutrients, the hardening time, and SiO_2 content and the pH are readily duplicated.

2. The universally disappointing attempts to employ silica jelly as a solidifying agent in bacteriological media are shown to result from the fact that each step in the preparation of the medium is attended by hitherto unsuspected changes.

3. The changes which occur in the pH of non-dialyzed jellies, and in the pH and firmness of jellies during dialysis and autoclaving, are described.

4. Stabilization of the jellies during dialysis and autoclaving depends on the ions incorporated in the jelly and on the ions furnished by the dialyzing solutions.

5. Stabilization is secured by maintaining the pH below 7.0, by the incorporation of ammonium, aluminum, calcium or iron salts in the gels, or by dialysis in solutions containing the ammonium ion.

6. This study shows that it is practical to make stable, sterilizable jellies for bacteriological purposes by adherence to the following principles:

(a) Ions which tend to stabilize the gel structure during dialysis should be incorporated at the time of gel preparation.

(b) In certain instances, the ions in the nutrient dialyzing solution will afford stabilization; in such cases the incorporation of the stabilizing ion in the jelly is not mandatory but is desirable.

(c) Dialysis to remove excess sodium chloride should always be carried out in a nutrient solution of the final composition desired in the medium. The pH of the dialyzing fluid should be as low as is consistent with the purpose for which the medium is intended.

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