THE EFFECT OF INORGANIC SALTS ON THE PRODUCTION OF SMALL COLONY VARIANTS BY STAPHYLOCOCCUS AUREUS

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Received for publication July 31, 1941

Small colony variants, or G. forms, have been reported for a number of bacterial species (see Koser and Dienst (1934), Swingle (1935), and Haddow (1938), for reviews of the literature) but because of the infrequency of their occurrence as compared with many other bacterial variants they have not received an equivalent amount of attention. The characteristics of the small colony variants of several bacterial species have been investigated, particularly *Shigella dysenteriae* and related species by Hadley, Delves and Klimek (1931), and Haddow (1938), and *Staphylococcus aureus* by Hoffstadt and Youmans (1932–1934a, b), Swingle (1935), and Chinn (1936). The biological significance of these forms has been interpreted differently by the above investigators. Hadley, Delves and Klimek, and Hoffstadt and Youmans ascribe to them a position in the life cycle of the organism, whereas Koser and Dienst, Swingle, and Chinn all support the theory that the small colony variants are merely forms possessing a temporarily lowered rate of metabolism.

Two general types of small colony variants of *Staphylococcus aureus* may be recognized on the basis of the stability of the small colony form and these may be conveniently designated as "Unstable" and "Stable." Unstable small colony variants are those which after 24 to 48 hours exhibit colorless, translucent colonies which vary from microscopic size to those about 0.5 mm. in diameter, but which upon subculture to plain meat-infusion agar revert within 24 hours to normal-appearing *Staphylococcus aureus* colonies. Stable small colony variants, on the other hand, retain the typical small colony size and appearance for a period varying from several days to several months following the initial transfer.

In a preliminary paper one of us (Youmans, 1937) reported the rapidity with which these forms appeared when grown in media containing inhibiting concentrations of barium chloride and a possible mechanism for the action of this salt was suggested. The present report deals with an extension of this work using several barium salts and other inorganic salts added to a variety of media. The results obtained with a few organisms other than staphylococci have also been included.

METHODS

Seventeen strains of *Staphylococcus aureus* were employed. All were isolated in the laboratories of the Michael Reese Hospital and the Cook County Hospital, Chicago, from a variety of human *Staphylococcus* infections.

Four basic media were employed: 1) meat-infusion broth prepared from fresh beef hearts; 2) a solution of 1.0 per cent peptone in distilled water; 3) 1.0 per cent peptone solution to which was added meat-extract activator solution; 4) 1.0 per cent peptone solution to which was added vitamin B_1 and nicotinic acid. The activator solution was prepared according to the method of Hughes (1932) by extracting 50 gm. of commercial meat extract dissolved in 100 ml. of distilled water with 6 to 8 volumes of acetone. The acetone was decanted and removed *in vacuo* at 50° to 60°C. The residue was taken up in 50 ml. of distilled water and re-extracted with 6 to 8 volumes of acetone which was decanted from the precipitate and removed *in vacuo* as before. The final residue was dissolved in 50 ml. of distilled water and, after adjusting the pH to 7.0, was sterilized by filtration through a Berkefeld N filter. Four ml. of this solution were added to 100 ml. of 1.0 per cent peptone solution.

The inorganic salts tested were barium chloride, barium nitrate, barium nitrite, sodium chloride, lithium chloride, magnesium chloride and calcium chloride. The desired concentrations of these salts were made up accurately in one or more of the above basic media and the pH adjusted to 7.0. The solutions were sterilized either by filtration through Berkefeld N filters or by autoclaving, and were tubed in 5 ml. amounts.

Each tube of the salt-containing medium was inoculated with 2 to 3 drops of an 18- to 24-hour culture of the desired organism grown in the same basic medium used in preparing the salt concentrations. The tubes were incubated at 37°C., observed daily for evidence of growth and plated every two days by spreading evenly over the surface of thick meat-infusion agar plates a small amount of the culture from each tube. This procedure was continued for a period of 24 to 30 days. The plates were examined daily for a period of at least 4 days since the appearance of the small colony variant is frequently slow.

All strains of bacteria were purified by repeated single-colony isolations. The media were controlled for sterility both by incubation before use and by incubation and plating a set of uninoculated salt dilutions along with the inoculated tubes. One or more tubes of the medium containing no salt were also inoculated and plated at the same time as the salt dilutions as a control on any change brought about by the basic medium alone.

RESULTS

Meat-infusion broth: When barium chloride was added to meat-infusion broth a voluminous precipitate usually appeared which varied in amount with the quantity of barium chloride added. This could be removed by filtration, which resulted in loss of some of the barium, or could be prevented by increasing the hydrogen ion concentration. The results obtained with this medium, however, were very irregular. Many strains of staphylococci grew even in 5 per cent barium chloride when loss due to precipitation was prevented, and the appearance of small colony variants was seldom observed. When these variants did appear, however, they were always in the highest concentrations of barium chloride and were usually of the stable variety.

One per cent peptone: When the pH of a solution of 1.0 per cent peptone was adjusted to 7.0, no precipitate was formed on adding barium chloride in concentrations up to 5.0 per cent. However, growth of the staphylococci upon this medium was very poor, seldom occurring in concentrations of barium chloride greater than 0.75 per cent. Growth in the 1.0 per cent peptone solution alone was never heavy, although it always occurred within 24 hours. Small colony variants seldom appeared upon this medium. Many strains failed to show any change, even after several weeks' incubation. Several strains gave rise at times to small colony variants in the highest concentrations of barium chloride in which growth was present. These small colony variants were usually of the unstable variety.

Peptone activator medium: Although the growth of the different strains of staphylococci in this medium varied considerably, all grew in higher concentrations of barium chloride when the activator was added than when this ingredient was omitted. One strain grew only in 0.5, 16 in 1.0, 6 in 2.0, 4 in 3.0 and 1 in 4.0 per cent barium chloride. Growth, especially in the higher concentrations of barium chloride, was usually slow to appear, the first indication being a faint turbidity within 24 to 48 hours which gradually increased but never equalled that of the control, in which marked turbidity was visible within 24 hours. With increase in concentration of barium chloride there was a decrease in the amount of growth.

All seventeen strains of *Staphylococcus aureus* gave rise to small colony variants in this medium. The appearance of the variants was as a rule very rapid, the majority of the strains producing them within 4 days, and many in 2 days. The appearance of the variants was often coincident with the appearance of growth, though frequently only normal forms appeared at first, to be later either partially or completely replaced by small colony variants.

The different strains of staphylococci varied considerably in regard to the number of small colony variants produced. In many cases after only 2 to 4 days' incubation the entire culture would be transformed into the small colony variant form. Other strains never showed complete conversion; the plates would constantly show a varying number of normal *Staphylococcus* colonies surrounded by hundreds of small colony variant colonies.

Quite frequently all of the small colony variants upon a plate proved to be of the stable variety. Certain strains of staphylococci seemed to produce the stable type more frequently than others, but with any strain the number of stable forms might vary from none to 100 per cent of the colonies. Usually the number of stable colonies was large (over 50 per cent). That this is an enormous increase in the number of stable forms over those hitherto reported is well illustrated by the fact that Swingle (1935) obtained only 9 stable small colony variants out of several hundred examined.

The results obtained with the peptone activator medium plus barium chloride were far more regular and striking than with any other medium employed; nevertheless they were not entirely consistent. Failures were sometimes encountered in attempts to repeat results previously obtained with a given strain

of *S. aureus*. This may have been primarily due to variation in the potency of the activator solution since different preparations varied in their growth-stimulating properties. Only those preparations which promoted growth of the staphylococci in the higher concentrations of barium chloride were suitable. However, it is also possible that variation in the *S. aureus* cultures at different times may have played a rôle since occasionally even with potent activator solutions no small colony variants were obtained.

Nicotinic acid and vitamin B_1: In an attempt further to standardize the procedure a synthetic medium was tried. This proved to be entirely impractical because of the precipitating action of the barium. Nicotinic acid and vitamin B_1 were also added to 1.0 per cent peptone solution on the chance that the peptone might be deficient in these substances. The results, however, were identical

TABLE 1

The highest concentrations of salts which permitted growth of staphylococci in peptone activator media, and their specific potencies

STRAIN	NaCl	LiCl	$MgCl_2$	$CaCl_2$	$BaCl_2$	$BaNO_2$	$BaNO_3$
			Per cent concentration				
M-2	15.0	4.0	11.0	9.0	1.0	3.0	4.0
M-6	17.0	4.0	11.0	9.0	1.0	3.0	4.0
C-6	15.0	4.0	11.0	9.0	0.5	3.0	4.0
M-16	15.0	4.0	11.0	9.0	1.0	3.0	4.0
M-18	17.0	4.0	11.0	9.0	3.0	3.0	4.0
M-19	17.0	4.0	11.0	9.0	2.0	3.0	4.0
			Specific potencies				
M-2	1.0	2.6	2.1	3.0	53.1	20.8	16.6
M-6	1.0	3.0	2.5	3.5	61.7	24.1	19.3
C-6	1.0	2.6	2.1	3.0	104.1	20.8	16.6
M-16	1.0	2.6	2.1	3.0	53.1	20.8	16.6
M-18	1.0	3.0	2.5	3.5	20.7	24.1	19.3
M-19	1.0	3.0	2.5	3.5	30.8	24.1	19.3

with those obtained with 1.0 per cent peptone solution alone. This indicates that probably the main function of the meat-extract activator solution is to furnish added nutrient material.

OTHER SALTS

Six strains of *S. aureus* were grown in the peptone activator medium to which had been added various concentrations of other salts. As an additional control, the same medium, containing the same concentrations of barium chloride previously used, was inoculated at the same time from the same culture used to inoculate the other salt tubes. Only those experiments in which small colony variants were obtained in the control barium chloride tubes were considered valid. The highest concentrations of these salts in which growth was obtained with the six strains of *Staphylococcus aureus* are shown in table 1.

Barium nitrate: The amount of growth, as indicated by turbidity and plating, was far greater than in similar concentrations of barium chloride, and was greater even in 4 per cent barium nitrate than in the control medium which contained no salt. This could be accounted for on the basis of the energy obtained from the reduction of the nitrate to nitrite, which was found to have taken place in all the tubes in which growth was evident. No small colony variants were obtained in the presence of this salt.

Barium nitrite: The production of small colony variants occurred in the presence of this salt in much the same manner as with barium chloride. It seemed to be slightly less effective since 2 strains which gave rise to small colony variants in the presence of barium chloride failed to do so in barium nitrite, and higher percentages of unstable variants were found.

Lithium chloride: Only one of the six strains gave rise to any small colony variants, which were first observed upon the 12th day of incubation in only the 3 and 4 per cent concentrations. These small colony variants, while few in number, were all of the stable variety.

Sodium chloride: Calcium chloride and magnesium chloride: No small colony variants were ever obtained in the presence of these salts.

No other changes, except the occasional occurrence of white *albus* variants, were observed in the above experiments. All controls were consistently negative.

OTHER ORGANISMS

Peptone activator medium containing barium chloride was inoculated with stock laboratory strains of the following organisms: *Sarcina lutea, Escherichia coli, Shigella dysenteriae, Shigella paradysenteriae* (Flexner), *Eberthella typhosa, Salmonella schottmuelleri, Pseudomonas aeruginosa* and *Bacillus subtilis.* None of the above organisms except *S. lutea* formed small colony variants at any time. The *S. lutea* culture produced stable and unstable small colony variants after 14 days' incubation in the barium chloride medium.

OTHER EXPERIMENTS

Since the *Staphylococcus* cultures were carefully purified by single colony selection and since it has previously been shown by Hoffstadt and Youmans (1938) that small colony variants of *S. aureus* may be obtained from cultures started from a single cell, it appears unlikely that the presence of the barium chloride merely provides a suitable medium for the growth of small colony variants present in the original culture while inhibiting the growth of the normal form. However, a few experiments were done to test this possibility. Inoculations were made directly into barium chloride activator medium from 8-month-old agar slant cultures of six strains of staphylococci. Similar inoculations were made from cultures of the same organisms that had been purified by repeated single colony isolations. No difference was noted in the number or stability of the small colony variants obtained from either, nor were any small colony variants found on the control plates of the new or old cultures. In addition,

pure cultures of stable small colony variants were inoculated into various concentrations of barium chloride media to determine whether they would grow in higher concentrations of this salt than the normal form of the organisms. In all cases the inhibiting concentrations were the same for both forms. Stable small colony variants, as well as the normal forms, also grow less readily in the presence of barium chloride than in its absence, and normal staphylococci and these variants may grow side by side in the same concentrations of the salt.

Numerous attempts were made to produce small colony forms from reverts obtained from small colony variants with the thought that having once passed through this stage they might more easily be changed into the small colony form. However, no difference was ever noted between the reverts and the original culture in this respect.

The cultural characteristics and fermentation reactions were determined for all the original *S. aureus* strains, a large number of stable small colony variant strains and reverted forms obtained from these. The stable small colony variants without exception fermented only glucose, and all reverted forms had the same cultural characteristics and fermentation reactions as the original *S. aureus* cultures. Microscopically the small colony variants showed the typical staphylococcus morphology.

A number of stable small colony variants were tested with *Staphylococcus* bacteriophage to detect any difference in resistance to this agent from the normal form of *S. aureus*. In all cases the small colony variants and the original culture were found to be equally susceptible. This is at variance with the results of Hoffstadt and Almaden (1934) who found the G form to be bacteriophage-resistant.

REVERSION

In our experience the reversion of the stable small colony variant has always been sudden and spontaneous. Some stable strains may be carried for days or weeks without evidence of reversion and then suddenly show normal *S. aureus* colonies after only 24 hours' incubation. Sometimes a whole culture will revert in this manner. Other strains either continually show a few normal *S. aureus* colonies upon subculture, or produce them sporadically. Eventually, however, all stable small colony variants revert. The most striking feature of these reverted colonies is the intensity of the pigmentation, a far more pronounced orange than that of the original *S. aureus* culture. Upon subculture however, the pigmentation always reverts to that of the original culture. This increase in pigmentation, while common, is not always observed.

Reversion also occurs in broth, since subculture from broth cultures often shows normal *S. aureus* colonies. Either a portion or all of a culture may change under these conditions. The presence of glucose in the medium markedly favors reversion.

The unstable forms always revert upon the first subculture though they may resemble the stable form upon the original isolation plate. When allowed to remain upon the original isolation plate the colonies gradually become larger and

assume the pigmentation of the normal form. This may occur within 24 to 48 hours or it may take several days longer. The pigmentation may progress to a far more brilliant orange than is observed with the normal form, in a manner similar to the reverts of the stable small colony variants. There may be a considerable variation in the size of these unstable colonies upon first isolation, from colonies almost microscopic in size to those about 0.5 mm. in diameter. The larger colonies upon close observation may show faint traces of pigmentation. These larger colonies regain normal size and pigmentation more rapidly than the smaller.

DISCUSSION

Winslow and his collaborators (1934) have demonstrated that all inorganic salts stimulate bacterial growth in low concentration and inhibit growth in high concentration. These workers have also demonstrated that each cation has a specific potency in regard to its growth-stimulating or inhibiting action and if the specific potency of sodium chloride is taken as 1.0 the potencies of the others bear a constant relationship to this. For example, with *E. coli* as the test organism the specific potencies of several chlorides were as follows: sodium chloride, 1.0; potassium chloride, 1.2; lithium chloride, 3.0; barium chloride, 5.0; magnesium chloride, 9.0; calcium chloride, 12.0; manganese chloride, 400; zinc chloride, 700; and cadmium chloride, 3000.

It is of interest to compare the results obtained with the various salts used in the present study in the same manner as was done by Winslow (1934) with *E. coli*. Table 1 shows the highest per cent concentration of the inorganic salts in which growth was obtained with six strains of staphylococci and also specific potencies as calculated from the molar concentrations. Although the specific potencies differ from those given for *E. coli* by Winslow, due possibly to the different organisms, media and methods employed, it can be seen that barium chloride and barium nitrite had from 7 to 104 times the inhibiting action of any of the other salts used, except barium nitrate, and these two salts were the only ones that consistently stimulated the production of small colony variants. These results suggest that there may be a relationship between the growth inhibiting capacity of a cation and its ability to cause this type of bacterial variation. Furthermore, they indicate that there is a qualitative difference between cations in this respect, since equivalent inhibiting concentrations of magnesium chloride, calcium chloride and sodium chloride failed to bring about the production of small colony variants. The lack of effect of barium nitrate, as previously mentioned, was probably due to the energy obtained from the reduction of the nitrate to nitrite, which actually resulted in a stimulation of growth.

In a previous report one of us (Youmans, 1937) suggested the possibility that the action of barium chloride might be due to its enzyme-inhibiting or destroying power since Quastel and Woolridge (1927) have shown that this salt was the most effective of many in inhibiting the metabolism of washed suspensions of *E. coli*. Isaacs has pointed out (1932) the close similarity between the action of many germicides on bacteria and their action on enzymes and concludes that

the primary germicidal action in many cases is one of enzyme destruction. This same author (1930a, b) has shown that after exposing suspensions of *B. coli*, streptococci and a saprophytic tubercle bacillus to heat the enzymatic functions of some of the bacterial cells were partially destroyed without killing the organisms or preventing growth. This loss in enzymatic activity was manifested by a marked retardation in the rate of growth, which resulted in a lag phase prolonged for a period of days. The description by Isaacs of some of these colonies bear a striking resemblance to the unstable small colony variants of *Staphylococcus aureus*. Chinn (1936) has shown that the stable small colony variants of *Shigella paradysenteriae* Sonne and *Staphylococcus aureus* are actually metabolically less active than the normal forms, in that they have a reduced generation time, reduced fermentative powers, lessened ability to reduce methylene blue and a slower cataphoretic velocity.

The suggestion comes readily that the small colony variants of *Staphylococcus aureus* are merely composed of cells unable to metabolize as well as the parent form due to the temporary loss or inactivity of certain enzymatic functions. It is impossible to say whether this loss of enzymatic activity is due to a decrease in cell permeability which Winslow (1934) gives as the reason for the growth-inhibiting action of cations, or to destruction or inhibition of certain enzymes. However, the results of the present work show this may be brought about under suitable conditions more or less specifically by the barium ion, which McCalla (1940) has shown is actually adsorbed to the surface of the bacterial cell. It is possible, of course, that other cations than those tried might prove more effective than barium chloride, or, that under suitable conditions magnesium, calcium, sodium or lithium might be effective. The failure to obtain similar results with the other organisms used, except *S. lutea*, is not surprising since it is not to be expected that all species of bacteria would be affected identically by a given agent.

We do not believe that our results furnish any evidence in support of the theory previously suggested by Hoffstadt and Youmans (1932–1934a) that the small colony variants occupy a position in the life cycle of *Staphylococcus aureus* similar to that postulated for the G forms of *Shigella dysenteriae* by Hadley, Delves and Klimek (1931). These variants of *Staphylococcus aureus* are not filterable [Swingle (1935) and Hoffstadt and Youmans (1934a)], reversion is invariably rapid and spontaneous instead of slow and continuous, and, insofar as determined, they differ from the normal form only in their decreased metabolic activity. Reversion takes place most readily when an easily available source of energy is present, such as glucose, and the reverts are identical in all respects with the original culture. Furthermore these variants appear most readily under conditions distinctly unfavorable for growth. What is probably even more important, when a specific inhibiting agent such as barium chloride is used, the number of small colony variants and the stability of these forms is proportional, in most cases, to the concentration of the barium ion. However, even in the presence of the specific agent, when sufficient energy is available for growth, as with barium nitrate, no small colony variants are obtained. The

same effect can be observed to a certain extent when too rich a basic medium, such as meat-infusion broth, is used. On the other hand when a poor basic medium is used growth is apparently completely inhibited in the higher concentrations of barium chloride before variation can occur. This supports the contention of Rettger and Gillespie (1935), at least insofar as this type of variation is concerned, that "variation is possible only when favorable and unfavorable influences are so balanced as to permit slow growth in the face of untoward circumstances". Furthermore, there is no evidence of a regular cyclic series of changes, the sequence apparently being S $\rightleftarrows$ G, not S $\rightleftarrows$ R $\rightleftarrows$ G as previously suggested by Hoffstadt and Youmans (1932).

SUMMARY

Seventeen strains of *Staphylococcus aureus* were grown in various concentrations of barium chloride made up in four different types of media: 1) meat-infusion broth, 2) 1 per cent peptone, 3) 1 per cent peptone activator medium, 4) 1 per cent peptone to which was added nicotinic acid and vitamin B_1. The maximum concentration of barium chloride in which growth was obtained varied with each medium, being greatest with meat-infusion broth and least with 1 per cent peptone alone.

All strains produced numerous stable and unstable small colony variants within 2 to 6 days when grown in the barium chloride activator medium. The number and stability of these variants was, in most cases, directly proportional to the concentration of barium chloride in which growth occurred. The other basic media gave less consistent results.

Staphylococci were also grown in peptone activator medium to which was added barium nitrate, barium nitrite, magnesium chloride, calcium chloride, lithium chloride and sodium chloride. Barium nitrite gave results similar to barium chloride. Barium nitrate stimulated growth, probably due to the energy obtained from the reduction of the nitrate to nitrite, and no small colony variants were obtained. Similarly, no small colony variants were obtained with any of the other salts except lithium chloride which produced them on one occasion from only one strain.

The growth-inhibiting activity of the various inorganic salts in the order of their effectiveness was found to be: barium chloride, barium nitrite, barium nitrate, calcium chloride, lithium chloride, magnesium chloride and sodium chloride.

Laboratory strains of *Sarcina lutea*, *Escherichia coli*, *Shigella dysenteriae*, *Shigella paradysenteriae* Flexner, *Eberthella typhosa*, *Salmonella schottmuelleri*, *Pseudomonas aeruginosa* and *Bacillus subtilis* were also tested for the production of small colony variants when grown in barium chloride activator medium. *Sarcina lutea* was the only organism from which small colony variants were obtained.

The morphological and cultural characteristics of the small colony variants of *Staphylococcus aureus* and the methods of reversion were studied.

The mechanism of the action of barium chloride in producing small colony variants of *Staphylococcus aureus* and the significance of these forms is discussed.

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