A SIMPLIFIED METHOD FOR DETERMINING THE APPROXIMATE BACTERIOSTATIC POTENCY OF CHEMICALS*

By ARTHUR R. CADE, Ph.D.

Research Laboratories, Givaudan-Delawanna, Inc., Delawanna, N. J.

THE PURPOSE OF this presentation is threefold: (a) to present a general outline of the phenomena of bacteriostasis as it is understood and employed at the present time; (b) to show where and how bacteriostasis is applied in a practical way; and (c) to show how its relative effectiveness, as exhibited by different chemicals, is determined.

DEFINITIONS

In order that it may be more clear as to what we are talking about, and especially because the terminology as used in the literature is somewhat confusing, we are presenting here definitions of some of the terms as we shall employ them. First, we will endeavor to make a clear distinction between the terms *germicidal action*, *antisepsis*, and *bacteriostasis*. These words have become used to designate situations which overlap considerably. For example, many so-called bacteriostatic conditions are not truly "static" ones, but rather situations in which the functions assumed to be static are merely retarded in their speed of activity such that the end result is the same as though they were truly static. Likewise, the word antiseptic has become so badly misused as to mean almost anything, covering the whole range from truly bacteriostatic action down to truly germicidal; efforts should be made to discontinue its use for defining purposes other than for its original meaning. Literally it means "against sepsis" and, therefore, describes a situation wherein putrefaction (or other forms of decomposition) of the substrate has been prevented. It will be noted that antisepsis refers to a condition in the substrate (i.e., outside of the bacterial cell) brought about by certain activities of the cell; whereas, germicidal and bacteriostatic conditions are situations within the cell itself. It is thus possible to have bacteria present and growing in a substrate (e.g., a cosmetic cream), yet at the same time to have an antiseptic situation exist,

* Presented at the May 20, 1949, Meeting, New York City.

if the proper chemical is present, in the correct amount, to stop the occurrence of the untoward reactions from being brought about. In other words, it is possible to have an "antiseptic cream" which, at the same time, might not be "germ-free."

According to its derivation, a germicide *kills* the bacterial cell, thereby putting it in a condition of no activity, with no recovery possible. A bacteriostatic substance *inhibits* certain cell functions so that there is little or no activity, with recovery possible under proper conditions. Usually these qualifying terms refer to "growth" (either in size or reproduction), but as used today they also include other metabolic processes, i.e., a product which stops the enzyme formation of a certain cell so that no harmful effects are produced in the substrate is considered a bacteriostatic one, whether or not the cell can reproduce.

Location of the Activity	
The Cell	The Substrate
A *germicide* kills the cell.	An *antiseptic* prevents sepsis.
Stops all metabolism. No recovery possible.	May consist of any one or combinations of these others.
A "pseudobacteriostatic" substance inhibits (slows up) cell growth, as well as other metabolic processes.	
A bacteriostatic substance inhibits (stops) growth. Stops most all cell metabolism. Recovery possible.	

Actually one should consider bacteriostasis to include only that condition in which the bacterial cell is entirely "static." Such a situation occurs when a cell goes into a spore formation, wherein a thick membrane is formed around the cell or its nucleus, and, although it metabolizes to a small extent within the spore (just as an animal would do when it hibernates), it is truly "static" as far as "growth" is concerned, as well as to its effects upon the substrate.

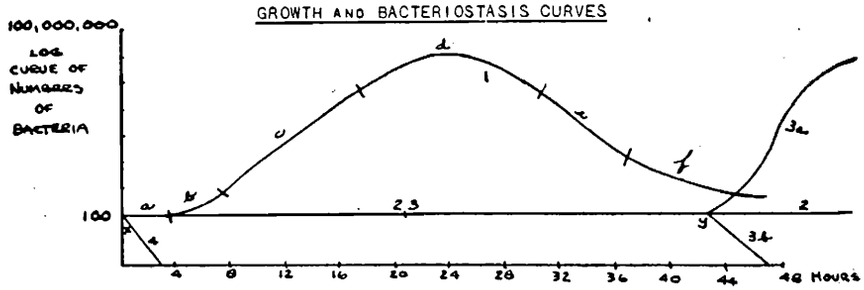
As usually employed, the term bacteriostasis refers, for the most part, to conditions that are not truly "static," but rather which are merely retarded in their speed of action such that the net result, as far as producing harmful or undesirable effects upon the substrate, or on a host, are the same as though the cell was truly "static." However, in these latter situations which should really be called "pseudo-bacteriostasis," there exists a state of equilibrium which can be disturbed by a neutralizing process. Thus, the bacteria in question can "recover from death" and start growing normally again. This is not possible for a germicidal situation.

As a result a bacteriostatic condition, in its broad sense, may be brought about by (a) producing a truly static set-up, as in a spore formation (curve 2); (b) increasing the killing time of the organism (curve 3b); (c) increasing the lag-phase of the bacteria so that the time that it requires to adjust itself to the chemical is prolonged (curve 3a). In each of the three cases the

condition, as far as the net is concerned, remains the same up to some certain point (time), after which in case (a) it continues the same, in case (b) the cell then dies off, while in case (c) the cell, having overcome its interfering influences, starts to grow again. All three are considered as bacteriostatic up to the point where these changes occur. Then (a) continues as bacteriostatic, (b) becomes germicidal, and (c) returns to normal. A standard growth curve is presented also so as to show the comparison with how the same bacteria would grow if no toxic substances were present to interfere with their normal growth processes.

CHART 2.

GROWTH AND BACTERIOSTASIS CURVES



CURVE 1 = NORMAL "GROWTH CURVE", SHOWING (A) THE LAG PHASE, (B) POSITIVE GROWTH PHASE, (C) LOG PHASE OF ACCELERATED GROWTH, (D) RESTING PHASE, (E) LOG DECLINING PHASE OF ACCELERATED DEATH, AND (F) THE DEATH PHASE.

CURVE 2 = TRUE BACTERIOSTASIS.
CURVE 3 = "PSEUDO-BACTERIOSTASIS" ("A" = DELAYED LAG PHASE) AND ("B" = INCREASED KILLING TIME).
CURVE 4 = GERMICIDAL CURVE.

Bacteria and Their Life Processes

Thus far we have seen that bacteriostasis is a phenomenon which involves a chemical substance interfering with the metabolism of a bacterial cell so as to prevent the latter from functioning normally. Besides interfering with the reproductory processes, the prevention of the formation of chemical by-products which may produce undesirable conditions within the medium (e.g., the cosmetic product) is also an end product of this phenomenon.

For our purpose here we will consider a bacterium as being composed of a mass of colloidal material called protoplasm contained within a thin differentially permeable membrane, which substance contains the living material of the cell. It is comprised of carbohydrates, lipoids, proteins, hormones, vitamins, enzymes, water, and a variety of inorganic constituents. To maintain their existence these bacteria utilize food in a manner quite similar to that as used by humans. Chemicals are brought into their environment which they

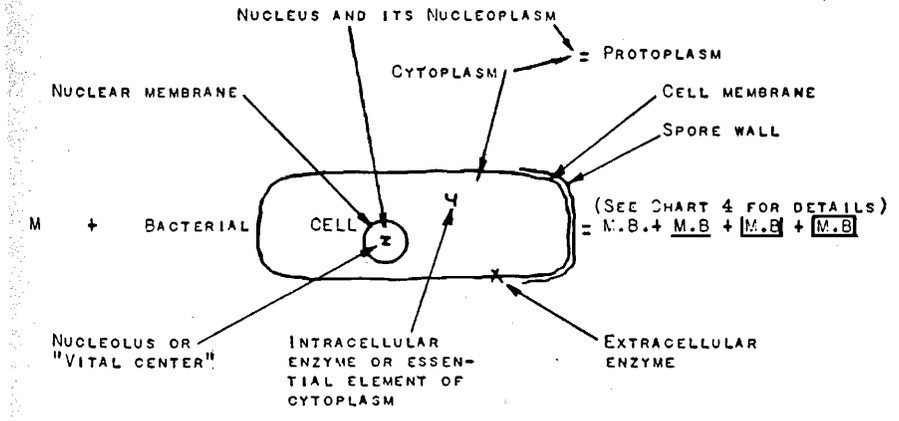
digest, first through enzyme attachment followed by penetration through the cell membrane into the interior, where it is made use of, as in man, to furnish the necessary energy requirements of the cell to do its work and for the building material needed to add new cellular structure or to repair damaged or worn-out ones. These cells manufacture their own enzymes within their structure and apparently bring some of them to specialized points on the cell surface. Within the cell there is also a specialized structure, called the nucleus, in which the vital processes of reproduction are originally carried out. Around this nucleus there exists a membrane to protect it and to separate it from the rest of the cell. The protoplasm of these two parts, which make up the complete cell, are called, respectively, nucleoplasm and cytoplasm.

This picture has been drawn because it is, basically, action at one or more of these three points—namely (X) the extra-cellular enzyme at the cell membrane surface, (Y) the intra-cellular enzyme or "a semivital constituent" in the cytoplasm, and (Z) the "vital essence" or "gene-equivalent" in the nucleus that determines if we have, respectively, bacteriostatic, pseudo-bacteriostatic, or germicidal action involved.

Bacteria grow (i.e., reproduce) by the process of simple fission. One cell goes through the necessary systematic internal changes so that the original one nucleus becomes two. Then, the cell having swelled, elongates, synthesizes the necessary component parts, pinches off at the center, and two daughter cells are thus formed. Interfering with this process is the basic bacteriostatic phenomenon.

CHART 3.

BACTERIOSTASIS AND THE BACTERIAL CELL



Bacteriostasis

We now see how bacteria are brought into being, how they maintain their existence, and how they can be removed from man's environment, at will, by means of the proper use of the proper chemicals. Whether the action so produced is a germicidal one, an antiseptic one, or a bacteriostatic one, depends upon a variety of factors. The same chemical can be used to produce all three of these conditions, as well as the pseudobacteriostatic one, by varying the concentration employed, the time and temperature of contact, etc. In addition, some chemicals are of such a structure that they can attack those parts of the living cell which produce the mild bacteriostatic action, while others combine more or less immediately to produce germicidal actions, with all types of possible variations in between. One thus is able to regulate the procedure or situation desired by making the proper choice of chemical, its concentration, etc.

To express this in a simple graphic form we present the following theoretical reaction. It should be borne in mind that this represents only a simplified situation, so selected as to make the basic problem of bacteriostatic activity clearer. We do not infer that this is an actual, or the only, reaction, that may be going on. Many combinations of these end products are naturally possible. That is, a chemical as used might cause a direct conversion of all of the B to the $\boxed{\underline{M.B}}$; or there may be a situation in which all of the B becomes only $\underline{M.B}$, in which condition it can bring about, through indirect methods, a starvation of the B so attacked. Thus, the end reaction would still be all as $\boxed{M.B}$.

Chart 4.—Equation of Bacteriostasis

(1)	M + B + Ⓑ = (Medication) + (Bacteria) + (Protected cells) =	M.B + *True bacteriostasis*, as in spore formation	$\underline{M.B}$ + *Pseudobacteriostasis* A loose reversible combination of chemical and surface enzyme	$\lvert\underline{M.B}\rvert$ + A reversible combination with the intracellular enzyme, or other component part of the cytoplasm	$\boxed{M.B}$ + Ⓑ *Germicidal action.* Irreversible combination denaturing the "essential life-unit"

(2) $\underline{M.B} + \lvert\underline{M.B}\rvert + 2A = 2M.A\ \ 2B$

(3) $\underline{M.B}$ + dilution or washing = M + B

(4) B + removal of "binding material" O = B

In offering this equation as a graphic exhibition of how bacteriostasis may be brought about, and what effects it may eventually produce, both on or in the cell as well as in the substrate, we make use of the following assumptions. First, that the reaction between the bacteria (B) and the chemical or medication (M) is a simple stoichiometric one of the monomolecular reaction type, wherein one molecule of the chemical reacts with one molecule of the cell constituents. We also assume that M is always in excess, as would be the case if we have complete bacteriostatic or germicidal action. Furthermore we assume, as is actually present in use, that there exists in the medication-bacteria complex some organisms which do not get in contact with the medication because of such physicochemical factors as film formation, agglutination, adsorption, clumping, etc., and thus do not react the same as far as killing or bacteriostasis is concerned, at least during the same time interval. Such organisms are represented as Ⓑ and thus appear on both sides of the equation.

The sum of the amounts of B on the right side of this equation must, naturally, equal 100 per cent of that which entered the reaction at the start (represented on the left). In just what proportion the total B is divided varies as described above. For the purpose of our discussion here we assume that little or none of the organisms exist in the spore form (M.B or true bacteriostasis), but that in the presence of high dilutions of certain chemicals the great majority of the cells will be as represented by $\underline{M.B}$. These eventually with time may become $|\underline{M.B}|$ and finally $\boxed{M.B}$ when the cell dies (Chart 2, curve 3b). Or, eventually the cell by its own repair mechanism may neutralize the M and repair the damage done by the $\underline{M.B} + |\underline{M.B}|$ combination, acting over the extended period of time. Thus, M.A is formed and B is set free, ready to start reproducing again. (Chart 2, curve 3a).

This explains the misnomered "Recovery from death" phenomenon and shows how it is brought about. That is, if during the time interval between where $\underline{M.B}$ and $|\underline{M.B}|$ are present, and before $\boxed{M.B}$ is reached there is added an antidotal substance A to the mixture, it will bring about the reaction

$$\underline{M.B} + |\underline{M.B}| + 2A = 2B + 2M.A$$

(eq. 2, Chart 4). Thus the cell will eventually become free to grow once more. Practical conditions as represented here have occurred in the recent past in connection with the use of mercury compounds. These were formerly thought to be highly germicidal, but now have been found actually to be highly bacteriostatic and not germicidal in anywhere near the previously reported concentrations.

In this same connection, so as to make this picture more complete, we wish to make a brief mention of the "Shippen technique," used for many years as a means for recog-

nizing both the existence and the extent of any bacteriostatic influence on germicidal test data as determined by the standard phenol-coefficient procedure. In general, the method comprises making a subtransfer (4 loopfuls) from the original subtransfer tube. By this sub-subtransfer it is assumed that the toxic material M will be affected through dilution so as to make it no longer capable of acting bacteriostatically. That is

$$\underline{M.B} = M + B$$

Time does not permit going further here to show certain fallacies in this procedure. Suffice it for our current purposes merely to mention the fact.

Another phase of this bacteriostatic influence on micro-organisms, especially as to how it may cause false interpretations of germicidal test data, was shown by the speaker several years ago. Experimental data were presented then (1) which show how, even though a "−, 0" result is obtained by the standard F.D.A. phenol-coefficient and the Cade-Halvorson plate count modification tests, actually at the end-point time of the test (e.g., at the 10-minute contact time period) many viable organisms are present. These organisms may be considered to be, for the most part, in the $|\underline{M.B}|$ condition and therefore die off during the incubation period. However, some of them at least are capable of being neutralized under the influence of a proper antidote and the $|\underline{M.B}| = M + B$, setting free the bacteria to grow again. In other words, from the test findings it has been assumed that all of the organisms were dead—in reality they are not—but are actually in a condition of growing under suitable influences, i.e., they are still potential pathogens.

Mention only can be made here, for lack of time, of one more set of conditions which exist in connection with the practical use of bacteriostatic substances, as well as an interfering influence producing false germicidal test data. This includes the effects, especially, of such substances as quaternary ammonium compounds, wherein some 99+ per cent of the test organisms apparently are truly killed in a short interval of time (even less than 1 minute), but a few bacteria remain viable (even for 30 minutes or more). By the standard F.D.A. phenol-coefficient procedure a "−" result is always obtained by, say a 1–5000 level, whereas by using such special techniques as the Klarmann-Wright semimicro method, (2), the Stuart ring or carrier method (3), the Cade swab technique (4), or others, it can be shown, beyond any doubt, that some viable bacteria are still present. The reason for this situation has been attributed by some to the fact that the quaternary compounds possess high bacteriostatic properties and, therefore, produce these results in a manner similar to that described above. That is: $(2M + 2B = \underline{M.B} + |\underline{M.B}|$ and $(\underline{M.B} + |\underline{M.B}| + 2A = 2M.A + 2B)$.

This, no doubt, is a partial answer to the phenomenon in question, but in addition—and quite likely to a greater degree—there exists in these situations another set of conditions, or influences. These are physico-chemical in nature, and act to prevent contact between the medication and the bacteria by forming a film over the latter; by some form of agglutination of the former; by adsorption of either M or B onto the glass, metal, or rubber, etc., used as the vessel to contain the mixture, or by the normal clumping of the bacteria themselves as they have grown in the culture, etc. By shaking, swabbing, neutralizing, etc. these uncontacted, or partially contacted, organisms are later set free to be found as viable when the vast majority of the others have been killed. These, no doubt, account for many of the "wild plusses" which show up in our test data and cause so much difficulty in evaluating germicidal potencies of the quaternaries. It should also be brought out here that this phenomenon is not one exclusive for quaternary ammonium compounds. It occurs for many other types, if not all, of germicidal substances, but in most cases to a lesser degree. A description of how this fact fits into our general formula will be found above where Chart 4 is discussed.

Practical Aspects of Bacteriostasis

Having shown what bacteriostasis is and how it works on bacteria, we will now present a brief outline as to how the cosmetic and allied industries can make use of these bacteriostatic properties of chemicals. This includes two opposite approaches, namely:

(a) For preservative purposes, wherein the inhibition of some of the normal metabolic processes of the bacterial cell is the goal rather than inhibiting the growth or reproduction abilities of the cell. Thus, the product (cosmetic cream) is "preserved" and kept from deteriorating from either a chemical or a psychological standpoint. Here the effect is outside the cell, i.e., in the substrate.

(b) For therapeutic purposes, wherein growth prevention is the basic aim.

In the former case it is the product itself that requires the bacteriostatic influences applied to it; whereas in the latter case it is the host on which the product is to be applied, that becomes the main object of interest as far as the effects of the bacteriostatic action is concerned.

Test Methods for Bacteriostatic Activity

In general, the following five procedures are used for the purpose of determining if a product possesses bacteriostatic properties, if it is exerting such powers under the specific conditions, or if it is capable of doing the same, and in what limiting concentrations. Each method, however, may produce a different answer as to the end-point concentration, due to the specific nature of

the procedure, to the effects of the component parts of the material used in the test media (agar or broth), to the nature of the test organism (unaffected or partially damaged), as well as to the variations in the diffusion rates as brought about by the nature of the vehicle used for the testing (i.e., the solvent used for the medication). These tests include:

1. *Dilution Methods*
   (a) Using serial dilutions of the chemical in nutrient broth, inoculating the same with a heavy inoculum of a healthy growing culture of the test organism, incubating for 48 hours at 37°C., and observing the end point, which will be where growth starts. That concentration used just prior to the one which permits growth would be considered to be the bacteriostatic strength.
   (b) A similar procedure wherein the serial dilutions and inoculations are made in nutrient agar.
   (c) A method similar to (b) except that the agar is not seeded, but, rather, is streaked across the surface with fresh viable organisms. Growth of organisms on the agar surface furnishes the end-point data in a similar manner to the above.
2. *Zone-of-Inhibition Methods*
   (a) This is the so-called Standard F.D.A. agar-cup or filter-paper procedure, as outlined in Circular 198 of the U. S. Department of Agriculture.
   (b) The writer's "Zone-Reduction" method (details of test to follow).

In previously published papers (5) the writer has shown that there may exist a fallacy in the standard zone test (2a) as far as the interpretations of its results are concerned. That is, namely, that the relative efficiency of a compound from a bacteriostatic standpoint is not in proportion to zone size. In fact, it may even be inversely proportional. This we explain on the basis that it is due to the fact that the size of the clear area or zone produced by this test is determined, not by the factors which bring about the bacteriostatic efficiency of the product but rather, for the most part at least, by the solution potential or diffusion power of the vehicle in which the medication has been incorporated. Thus, the same dilution of a specific chemical can be shown to have all the way from a trace to several millimeter zone size produced under identical conditions of test. For example:

TABLE 1

Chemical	Vehicle Base	Zone Size, Mm.
Hexachlorophene (0.1%)	Petrolatum-lanolin	1
Hexachlorophene "	Glyceryl-monostearate	3
Hexachlorophene "	Carbowax blend	6

Suggested Simplified Method—the "Zone-Reduction" Method

On the basis of the above assumptions we have developed a test which we believe serves to give a reliably accurate answer as to the bacteriostatic potency of a chemical, and one more simple to perform than any of the others mentioned above. It merely involves using the standard F.D.A. zone technique (filter-paper modification) in reverse. Basically, in this new method, instead of measuring the zone size as produced by the chemical we determine the limiting concentration of the chemical at the point where no clear area is produced.

As discussed above, various physical and physico-chemical factors may enter in to influence the diffusion rate or solution pressure, as well as mutual solubility relationships, which factors will determine how far the chemical "M" will diffuse into the agar in the time "T," which is that time such as is required by the bacteria being tested to overcome its lag-phase influences and start growing again. Thus, the point where these two opposing forces meet will set the size of the clear zone area. However, contrary to what some others claim, it is the writer's opinion that the zone size is not a criterion of the relative bacteriostatic efficiency of the product.

Following through on this same basis it seems to us that as long as there is any "M" available in the agar it can and will contact a "B." So long as such a contact is possible, or does actually occur, we have bacteriostasis available or in action. Therefore, in order to determine the real efficiency of the product, all that we need to do is to apply the material absorbed into the filter paper in successive serial dilutions onto the surface of seeded agar and then incubate it in the normal manner. Then, at the end of the selected time period (24 hours, 48 hours, or 72 hours, etc.) remove the filter papers from the agar surfaces and examine under a microscope the area where the papers previously rested. About 25 magnifications is sufficient.

Where no bacteria exist in this area, which will always be the case if any "clear zone" is produced, one has no difficulty to so observe it. When some organisms are growing in this area, say up to 50 per cent of those present in the agar outside of the filter-paper area, it is still quite easy to make such an observation without question. This situation continues to apply up to about where a 25 per cent reduction in colony count is present. From there on, to determine the exact end-point where a zero reduction occurs (thus representing no action between M and B) may be difficult. However, the difference in dilutions or concentrations of M as required to produce a 25 per cent reduction in colony count compared to that as required to show zero reduction does not appear to be materially significant, or enough to make it a serious factor. Equally great errors, on the percentage basis, occur

when endeavoring to duplicate zone sizes or find end-points of bacteriostasis by the various dilution procedures.

It is recognized that this simplified procedure does not determine directly the exact concentration of "M" which is the final limiting bacteriostatic concentration, although this figure can be obtained by indirect methods if it is ever deemed necessary to have it. The amount of medication in the solution into which the filter paper is dipped is not necessarily the same as that present in the seeded agar in the area where bacteriostasis is going on. Variations in the pick-up value of the paper, its retentive power for the chemical, the diffusion power of the material when placed on the agar, the lag phase of the organism, etc., all enter in to affect the end result. If one so wishes, however, these factors can be determined, with a good degree of accuracy, by weighing the filter paper before and after dipping into the solution, for the pick-up value, then by analyzing the paper for its chemical content before and after it has been on the agar for the incubation period, followed by calculating the volume of the agar in which the reduction of count occurs. From such calculations one can obtain very close to what is the actual bacteriostatic concentration of the chemical in question. However, experimental evidence, as shown in the table of data to follow, shows quite conclusively that the differences between the concentration as present in the solution into which the paper is dipped before placing on the agar and that producing the bacteriostatic effect in the agar are not sufficiently great to make a material difference for the purpose at hand. This assumption is based upon the fact that these dilutions so found parallel so closely those as found by the commonly used dilution method, and the closeness with which one can duplicate a result is approximately the same for either method.

Furthermore, as mentioned above, the lag-phase phenomena of bacterial growth plays an important role in determining the actual zone size one obtains when using the standard zone-of-inhibition test. The extent of this lag phase is influenced by, and thus varies with, factors that are not related to the bacteriostatic phenomena. Thus, the zone size is not a direct criterion or a relative variable of the bacteriostatic power of the substance being tested by the zone method.

However, in our Zone-Reduction test method this variable is eliminated. The end point read is that which represents the concentration of the chemical (i.e., molecules, M,) that just neutralizes a definite number of molecules of bacteria (B). The lag-phase phenomenon does not interfere with this reaction, as it is immaterial as to how long it takes for the bacteria to overcome local resistance effects and get started growing, or how far the chemical may diffuse due to solubility influences in the vehicle (including both the agar and the

Chart 5.—Bacteriostatic Dilutions

Chemical	"Zone-reduction" Method	"Dilution" Method
Phenylmercuric benzoate	1–5,000,000 to 1–10,000,000	1–10,000,000
Gentian Violet	1–2,000,000 to 1–5,000,000	1–5,000,000
Mercuric Chloride	1–500,000 to 1–1,000,000	1–1,000,000
Quaternary ammonium cpd.	1–500,000 to 1–1,000,000	1–1,000,000
Hexachlorophene	1–500,000 to 1–1,000,000	1–1,000,000
Acriflavine	1–300,000 to 1–600,000	1–500,000
8-Hydroxyquinolin sulfate	1–50,000 to 1–100,000	1–100,000
Hexylresorcinol	1–20,000 to 1–50,000	1–20,000
Chlorothymol	1–5,000 to 1–10,000	1–10,000
Phenol	1–20 to 1–50	1–200

cream, or whatever you are testing). The regulating factor, which determines the end-point (i.e., the bacteriostatic dilution) is the relative concentrations of the two component parts of the chemical reaction involved (M+B) and not the point in a special artificial medium (agar) where the "M" and the "B" (chemical and bacteria) meet to react.

Summary and Conclusions

It has been the endeavor in preparing this paper to present first a brief over-all description of the phenomena of bacteriostasis, how it acts, how it may be made use of in the field of cosmetics, and how its relative efficiencies and specific values can be determined. Finally we have presented a new method, simple to perform, usable for determining the correlative, as well as actual, bacteriostatic potencies of chemicals.

Bibliography

(1) Cade, A. R., *Soap and Sanitary Chemicals*, 111 (February, 1944); *Ibid.*, 138 (April, 1947); *J. Bact.*, 53, 504 (1947).
(2) Klarmann, E. G., and Wright, E. S., *Soap and Sanitary Chemicals*, **21**, No. 1, 113, (1945); **22**, No. 1, 125, (1946); and **22**, No. 8, 139, (1946).
(3) Stuart, L. S. *Ibid.*, 135 (September, 1947); *J. Assoc. Off. Agr. Chem.*, 401, (1949).
(4) Cade, A. R., *Soap and Sanitary Chemicals* (April, 1947).
(5) Cade, A. R., *Proc. Sci. Section Toilet-Goods Assoc.*, 16 (May, 1947); *Ibid.*, 31 (May, 1948).