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# UTILIZATION OF SELECTIVE MICROBIAL AGENTS IN THE STUDY OF BIOLOGICAL PROBLEMS

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ON THE third of August, 1857, Pasteur presented before the Scientific Society of Lille the first of his studies on the microbial theory of fermentation, "Mémoire sur la fermentation appelée lactique." On this occasion, he expressed his belief that, for each type of fermentation, one would find a specific ferment, characterized not only by its morphology and resistance to inhibitory substances, but also by its specific behavior as a chemical agent.[1]

On December 21st of the same year, Pasteur announced, at the end of his memoir on alcoholic fermentation, that he had observed a new "mode of fermentation" which attacked the *d*-form of tartaric acid, but which was inactive against the *l*-form;[2] experimental details concerning this observation were presented on March 29, 1858;[3] again in 1860 Pasteur described that a mold, *Penicillium glaucum*, exhibited the same specific behavior toward tartaric acid, attacking the *d*-form and not the *l*-form.[4] He pointed out that this selective fermentation afforded an

easy technique for the separation of the *l*-tartaric acid from the racemic mixture, and that the method would probably be applicable to the separation of other isomers. He also emphasized that since "the character of dissymmetry of organic compounds can modify the chemical reactions of physiological order," the phenomenon of specificity which he had observed was probably of great biological significance.

The very beginnings of experimental microbiology thus demonstrated the specific character of the biochemical reactions induced by microörganisms, and suggested to biochemists and physiologists new techniques and new problems. It is hardly necessary to state that bacterial physiology has repeatedly confirmed Pasteur's views on the part played by microbial life in the economy of natural processes. If organic matter does not accumulate in nature, it is because countless species of microörganisms hydrolyze it, oxidize it and eventually break it down to carbon dioxide, ammonia, water, and mineral salts. We know furthermore that, under natural conditions, each one of these microbial species is adapted to the performance of a limited, well-defined biochemical task. One may illustrate this statement by recalling the discovery of bacteria whose sole source of energy is the oxidation of ammonia to nitrites, of others which oxidize nitrites to nitrates, of still others which convert elementary sulfur to sulfuric acid, etc. Several species of bacteria which readily decompose cellulose fail to attack cellobiose or glucose; there are microörganisms which oxidize hydrogen, methane, petroleum, phenol, formol, etc. In fact, it can be stated that one can find in nature, in soil or water for instance, microörganisms capable of performing almost every possible type of biochemical reaction, many of which are not known to take place in the animal or the plant kingdoms. In many cases, the catalysts responsible for these reactions have been extracted from the microbial cells, and have been found to exhibit a remarkable specificity. Because of their cellular origin, these catalysts are able to operate under physiological conditions (pH, temperature, etc.) and this property, together with their specificity, renders them ideal reagents for the analysis of biological problems.

It is apparent, therefore, that given enough time, patience, and skill, the bacteriologist can discover in nature microbial reagents adapted to the study of a great variety of biological problems. The few examples which will be considered in the following discussion have been investigated at the Hospital of the Rockefeller Institute; they have been se-

lected because, in each case, new bacterial species were isolated from soil and active catalysts prepared from cultures of these organisms, in an attempt to discover reagents useful in the study of clinical problems under investigation in our hospital. It is perhaps justifiable, therefore, to emphasize that the facts here reported are not chance findings, but are illustrations of a method which has a distinguished past in bacteriological chemistry and which deserves the consideration of physiologists and biochemists.

*The decomposition of creatinine by bacterial enzymes.* In the course of studies on renal function, it became necessary to develop a method for the quantitative estimation of the very small amounts of creatinine present in blood. The identification and analysis of creatinine in tissues and biological fluids have chiefly depended on colorimetric methods which are so unspecific that many authors have denied the very presence of creatinine in the circulating blood.

It was in an attempt to develop analytical methods specific for creatinine that several species of bacteria capable of attacking this compound were isolated from soil.[5] Two of these bacterial species have been studied with some care; when grown in the proper medium, they have yielded enzyme systems in the form of resting cells which are capable of decomposing creatinine in the absence of bacterial growth. These enzyme systems exhibit a remarkable specificity.

Table I illustrates the effect of the enzyme prepared from the "NC" culture upon a number of substances which give the Jaffe reaction (one of the color tests most commonly used to detect the presence of creatinine). It is clear that, of the Jaffe reactive compounds which were tested, only creatinine was rapidly decomposed. The addition of one methyl group to the creatinine molecule completely inhibited enzymatic activity, and the mere shift of the methyl group from position 3 (in creatinine) to position 5 (in 5-methylglycocyamidine) diminished the rate of decomposition very greatly. Acetyl creatinine and glycocyamidine were also slightly decomposed.

The "NC" enzyme preparation was also tested against a number of substances very closely related to creatinine but which do not give the Jaffe reaction with alkaline picrate. In these cases, the activity of the enzyme was tested by the determination of a characteristic product of the reaction. Urea was selected since it was found to be the most constant product of the action of the NC enzyme upon creatinine.

TABLE I

## DECOMPOSITION BY NC ENZYME OF JAFFE-REACTIVE SUBSTANCES RELATED TO OR ASSOCIATED WITH CREATININE

(Reprinted from the *Journal of Biological Chemistry*)

| COMPOUND* | PERCENTAGE DECOMPOSITION MEASURED BY CHANGE IN JAFFE REACTION |
|---|---|
| | *per cent* |
| Creatinine | 100 |
| 5-Methylcreatinine | 0 |
| Dimethylcreatinine | 0 |
| † Acetylcreatinine | 10† |
| 4-(or 5-) Benzoylcreatinine | 0 |
| 5-Benzylcreatinine | 0 |
| 2-Benzylcreatinine | 0 |
| Glycocyamidine | 10 |
| 5-Methylglycocyamidine | 10 |
| Hunter's chromogenic substance in human erythrocytes | 0 |

* The nomenclature is that used by Greenwald (1925).

† Acetylcreatinine does not undergo further destruction when the incubation is prolonged. This is different from the action with glycocyamine and 5-methylglycocyamidine, since both these compounds may be completely decomposed if the incubation is continued for some hours.

TABLE II

## PRODUCTION OF UREA BY ACTION OF NC ENZYME UPON NON-JAFFE-COMPOUNDS RELATED TO CREATININE

(Reprinted from the *Journal of Biological Chemistry*)

| COMPOUND | PRODUCTION OF UREA* |
|---|---|
| Creatinine | + |
| Creatine | + |
| Methylhydantoin | 0 |
| Methylhydantoic acid | 0 |
| Hydantoin | 0 |
| Guanidineacetic acid | + |
| Methylguanidine | + |
| Arginine | + |
| Sarcosine | 0 |
| Guanidine | Trace |

* + indicates the production of approximately equivalent amounts of urea.

TABLE III

ACTION OF "HR" ENZYME PREPARATION ON COMPOUNDS RELATED TO CREATININE

(Reprinted from the *Journal of Biological Chemistry*)

| COMPOUND | PERCENTAGE DECOMPOSITION MEASURED BY JAFFE REACTION |
|---|---|
| | *per cent* |
| Creatinine | 100 |
| 5-Methylcreatinine | 0 |
| 4- (or 5-) Benzoylcreatinine | 0 |
| 5-Benzylcreatinine | 0 |
| 2-Benzylcreatinine | 0 |
| Diamethylcreatinine | 0 |
| 5-Methylglycocyamidine | 0 |
| Glycocyamidine | 15 |

The results presented in Table II indicate that creatine, guanidine-acetic acid, methylguanidine, and arginine are decomposed at about the same rate as creatinine. It would appear, therefore, that the ring structure of creatinine is but a small factor in determining the specificity of the enzymatic action. On the contrary, the presence of a guanidine-like unit in the molecule appears to be an important determinant of the specificity, since the replacement of one "NH" group by the CO linkage (as in the change from creatinine and creatine to methylhydantoin and methylhydantoic acid) prevents any reaction with the enzyme. It is also interesting that the absence of the methyl group in guanidine (as compared with methylguanidine) markedly reduces the production of urea, as does the shift of the methyl group in creatinine from the 3 to the 5 position.

Cultures of another soil bacillus "HR" have also yielded an enzyme system which attacks creatinine and which is even more specific than the one previously studied. It is shown in Table III that, of the compounds giving the Jaffe reaction which were tested, glycocyamidine is the only one besides creatinine which is attacked by the enzyme, and the rate of its decomposition is far slower than in the case of creatinine; in consideration of the excess of enzyme employed, the inability of the "HR" enzyme to decompose 5-methylglycocyamidine is especially striking.

To determine the effect of the "HR" preparation upon compounds

TABLE IV

ACTION OF "HR" ENZYME ON NON-JAFFE-REACTIVE COMPOUNDS RELATED TO CREATININE 0.200 MG. OF COMPOUND WAS USED IN EACH INSTANCE

(Reprinted from the *Journal of Biological Chemistry*)

| COMPOUND | UREA + $NH_3$ NITROGEN OBTAINED BY HYPOBROMITE METHOD | PERCENTAGE DECOMPOSITION |
|---|---|---|
| | *mg.* | *per cent* |
| Creatinine | 0.0740 | 100 |
| Creatine | 0.0654 | 100 |
| Glycocyamidine | 0 | 0 |
| Glycocyamine | 0 | 0 |
| Methylhydantoin | 0 | 0 |
| Methylguanidine | 0 | 0 |
| Guanidine acetate | 0 | 0 |
| Arginine | 0 | 0 |

related to creatinine, but which do not give the Jaffe reaction, advantage was taken of the fact that, when creatinine is acted upon by the enzyme, all the nitrogen is recovered as ammonia plus urea. It is shown in Table IV that the enzyme does not attack glycocyamine, methylguanidine, or arginine, which are decomposed by the "NC" enzyme. Glycocyamidine does not yield any demonstrable amount of urea or ammonia; it will be remembered, however, that this substance is slowly attacked as determined by disappearance of the Jaffe reaction (Table III). It appears, therefore, that the "HR" preparation slowly opens the ring structure of glycocyamidine, but does not decompose the compound further.

With the help of these bacterial enzymes, it has been possible to develop analytical techniques which are highly specific for creatinine and which have been used by several workers for the study of the metabolism of this substance.[6]

Let us mention in passing that it is possible to extract in solution from the cells of one of the creatinine decomposing cultures an enzyme—an anhydrase—which converts creatine into its anhydride creatinine.[7] This reaction offers an opportunity for the study *in vitro* of (a) the enzymatic production of a biologically important cyclic compound from an aliphatic one, and (b) the enzymatic combination of an amino and a carboxyl group to form the CO–NH linkage. Preliminary experi-

ments indicate that, like the creatinine oxidase, the creatine anhydrase exhibits a great specificity with reference to the substrates which it affects.

*The decomposition of the capsular polysaccharides of pneumococcus by bacterial enzymes.* Virulent pneumococci differ from the avirulent variants of the same bacterial species by the presence of a capsule surrounding the cell. Encapsulated pneumococci can be divided into a number of different serological types, and the type specificity is associated with differences in the chemical composition of the capsular material. The capsular substances of several types of pneumococci have been obtained in a reasonable state of purity and all of them belong to the class of polysaccharides.[8, 9, 10]

On the basis of immunological evidence, it appears therefore that the capsular polysaccharides of the different types of pneumococcus are of paramount importance in determining the serological specificity and conditioning the virulence of these organisms. It was felt that the evidence for this view would become even more convincing if one could obtain specific reagents, enzymes for instance, which, by decomposing the capsular polysaccharides, would render the encapsulated pneumococci inagglutinable in the homologous antisera, and at the same time alter their virulence.

As far as is known, the capsular polysaccharides of pneumococci are not decomposed by enzymes of animal or plant origin, nor are they attacked by common species of bacteria, actinomycetes or molds. It was possible, however, to isolate from soil a new bacterial species, a sporulating bacillus, which hydrolyzes the specific polysaccharide of Type III pneumococcus. A soluble enzyme, capable of catalyzing the same reaction, was separated from cultures of this soil bacillus grown under well defined experimental conditions.[11, 12, 13, 14]

The enzyme depolymerizes the Type III capsular polysaccharide to the aldobionic acid stage. As a result of enzymatic hydrolysis, the capsular substance loses the ability to react *in vitro* with the specific antiserum obtained by immunizing experimental animals with the Type III capsular antigen.

It can be demonstrated by staining reactions that the addition of active enzyme to a suspension of living encapsulated Type III pneumococci causes the disappearance of the capsule; the specific agglutinability of the bacterial cells in the Type III antiserum is at the same time greatly

TABLE V

SPECIFICITY OF THE PROTECTIVE ACTION OF TYPE III ENZYME

(Reprinted from the *Journal of Experimental Medicine*)

| INFECTING DOSE OF PNEUMOCOCCUS | ENZYME (LOT 4-$\alpha$) J.E CC. | | | NO ENZYME | | |
|---|---|---|---|---|---|---|
| | | | | Virulence-controls | | |
| | Pneumococcus Type I | Pneumococcus Type II | Pneumococcus Type III | Type I | Type II | Type III |
| *cc.* | | | | | | |
| 0.1 | .... | .... | S | .... | .... | .... |
| 0.01 | .... | .... | S | .... | .... | .... |
| 0.001 | .... | .... | S | .... | .... | .... |
| 0.0001 | D20 | D34 | S | .... | .... | .... |
| 0.00001 | D34 | D34 | S | D22 | D36 | D34 |
| 0.000001 | D34 | D34 | S | D34 | D36 | D34 |
| 0.0000001 | .... | .... | .. | D34 | D20 | D72 |

S = survived.
D = Death of animal; the numeral indicates the number of hours before death.
— = not done.

impaired. It is important to mention, however, that the enzyme does not kill the bacterial cells; in fact Type III pneumococci grow readily in media containing the enzyme, but they are deprived of their capsules; when the decapsulated cells are now transferred to a new medium not containing the enzyme, the capsule again reappears and restores to the pneumococci their full virulence and their agglutinability in Type III antiserum. It is clear therefore that the action of the enzyme is directed against the preformed capsular polysaccharide, but does not affect the metabolism of the bacterial cell.

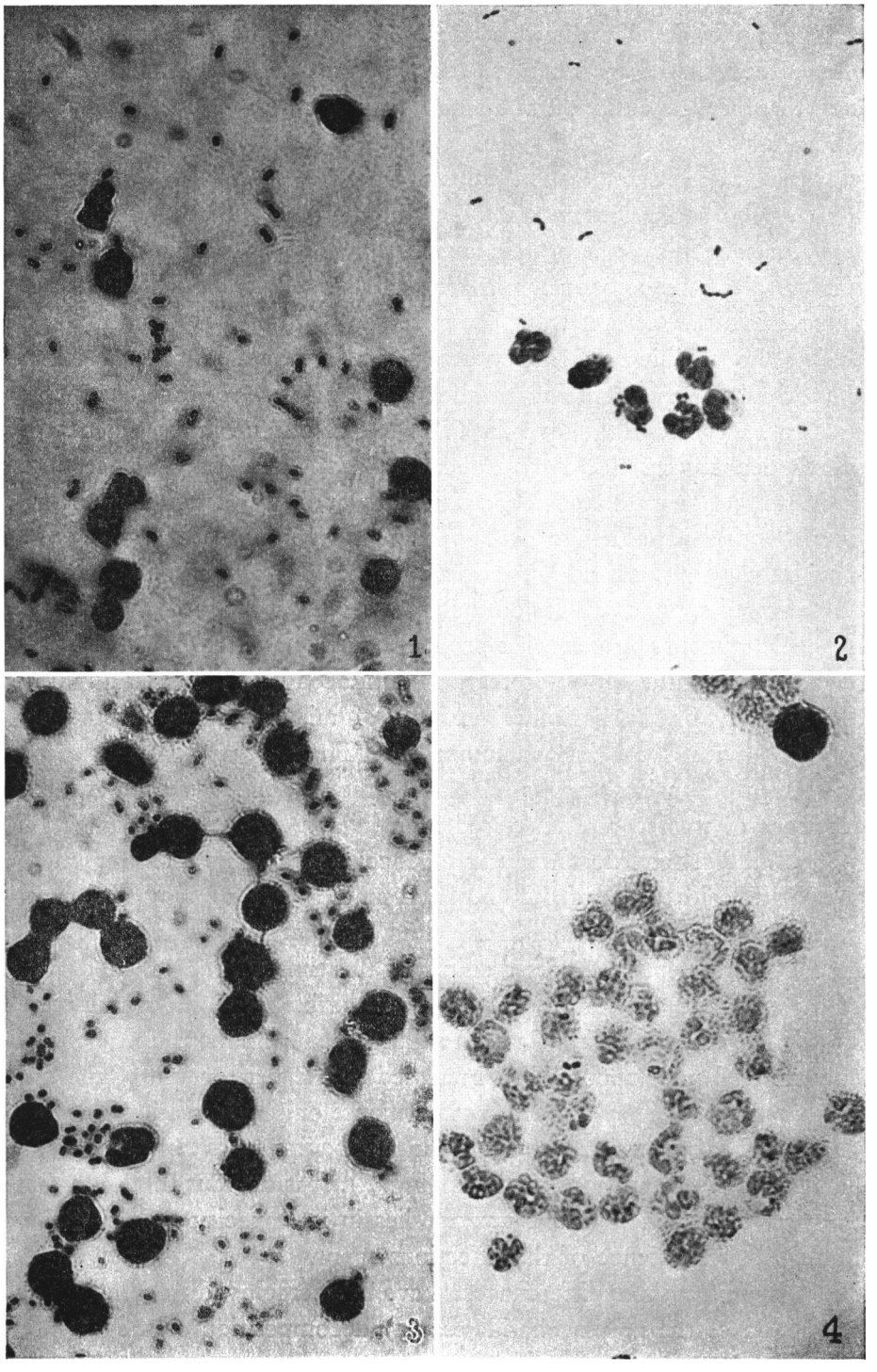
Enzymes capable of attacking the capsular polysaccharides of other pneumococcus types have now been obtained from different strains of soil bacteria.[15, 16, 17, 18, 19] Several of these enzymes exhibit a remarkable specificity, and can differentiate between polysaccharides which give cross reactions in immune antisera; for instance, the polysaccharide of gum acacia which reacts in Type III pneumococcus antiserum is not affected by the enzyme which hydrolyzes the Type III polysaccharide.[13] Even more striking is the difference between the enzymes attacking the polysaccharides of Type III and Type VIII pneumococcus. Both these substances are composed of glucose and glucuronic acid in different ratios, and because of this chemical relationship, they exhibit a certain

amount of cross reaction in immune sera.[20, 21] On the contrary, the bacterial enzymes developed against each one of the polysaccharides fail to attack the other;[19] in other words, the enzymes are even more specific than are the antibodies obtained by immunization of experimental animals.

Not only are the bacterial polysaccharidases capable of hydrolyzing the capsular substances *in vitro*, but they exhibit the same activity *in vivo*. In fact, they can protect experimental animals against infection with virulent pneumococci.[22] In view of the specificity which the enzymes exhibit *in vitro*, it was to be expected that the protection induced would also exhibit a specificity determined by the chemical nature of the capsular polysaccharide of the particular type of pneumococcus used for infection. It is shown in Table V, for instance, that the enzyme which decomposes the Type III capsular substance can protect mice against infection with 1,000,000 fatal doses of pneumococci of this type, but is entirely ineffective against pneumococci of other types. The same polysaccharidase exhibits also a curative effect on the dermal infection of rabbits,[23, 24] as well as on the experimental pneumonia in monkeys of the *M. cynomologos* species, produced with Type III pneumococci.[25]

The mechanism of the protection so induced is revealed by a microscopic study of the peritoneal exudate of mice during the course of infection with Type III pneumococci.[22] The progress of events can be seen in photomicrographs which illustrate the differences in cellular reactions of treated and untreated mice, 2 and 4 hours after injection of one million fatal doses of pneumococci. Two hours after infection, the peritoneal exudate of the untreated mouse (Fig. 1) showed numerous encapsulated cocci free in the fluid. In contrast to this, the pneumococci in the enzyme treated animal at this time (Fig. 2) were devoid of capsules, and only naked bacteria were visible, many of which were already engulfed by leukocytes. At the end of 4 hours, the number of encapsulated pneumococci had increased in the peritoneum of the untreated mouse (Fig. 3); in the treated mouse only an occasional decapsulated organism was seen outside the leukocytes whereas many could be seen within the phagocytic cells (Fig. 4). It is obvious therefore that the protective action of the enzyme lies in its capacity to decompose the capsular substance of the infectious agent.

In summary three different tests have been employed to demonstrate the action of the polysaccharidases: (a) decomposition of the purified


1
2
3
4

capsular polysaccharides, with attendant loss of their specific precipitability in homologous antiserum, (b) destruction of the pneumococcus capsule, both *in vitro* and *in vivo*, (c) protection of experimental animals against infection with virulent pneumococci. All these reactions are type specific. They confirm beyond doubt that the pneumococcus capsules consist of the specific polysaccharides and the latter substances determine the serological specificity of pneumococci and condition their virulence. The polysaccharidases are neither bacteriolytic, nor bactericidal; it is by destroying the protective capsules of the virulent pneumococci that they render the bacteria susceptible to the phagocytic action of the cells of the host, and determine the recovery of the animal.

It is clear that two properties of the enzymes have made possible their application to the study of pneumococcus infections, (a) their specificity, (b) the fact that they can function under physiological conditions. Microbial enzymes have also been used with advantage in studying the chemical nature of bacterial antigens; and there are many other biological problems the analysis of which would be greatly facilitated if enzymes specific for certain substrates were available. The addition of the test substrates to soil or sewage, for instance, will reveal in all cases the existence of microörganisms capable of decomposing them. By isolating these microörganisms in pure culture from the natural

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*LEGENDS FOR PHOTOMICROGRAPHS*

FIG. 1. Photomicrograph of a stained preparation of the peritoneal exudate of a mouse 2 hours after the intraperitoneal injection of 0.01 cc. of a virulent culture of Type III pneumococcus. The bacteria show well-defined capsules and no evidence of phagocytosis is seen. Many polymorphonuclear and a moderate number of mononuclear leukocytes are present. Gram stain. × 1000.

FIG. 2. Photomicrograph of a corresponding preparation of the exudate of a mouse 2 hours after receiving the same amount of culture together with 0.5 cc. of a preparation of the specific enzyme. The bacteria are devoid of capsules. Polymorphonuclear leukocytes predominate and phagocytosis is evident. Gram stain. × 1000.

FIG. 3. Photomicrograph of a stained film of the peritoneal exudate of a mouse 4 hours after injection with 0.01 cc. of culture alone. The bacteria are increased in number, encapsulated, and extracellular. The cellular elements are polymorphonuclear and mononuclear leukocytes in about equal numbers. Gram stain. × 1000.

FIG. 4. Photomicrograph of a corresponding preparation of the exudate of a mouse 4 hours after receiving the same amount of culture together with 0.5 cc. of a preparation of the specific enzyme. Marked phagocytosis has occurred and only an occasional organism is seen outside the accumulated leukocytes, nearly all of which are of the polymorphonuclear type. Gram stain. × 1000.

[Differences in the density of the backgrounds of these four figures are due to the use of color screens in the photographic reproductions. This technique however, alters none of the essential details observed in the original microscopic preparations. (Reproduced by the courtesy of the *Journal of Experimental Medicine.*)]

sources, and growing them under appropriate conditions, it should often be possible to prepare enzymes adapted for use as specific physiological reagents.

*A selective bactericidal principle extracted from cultures of a sporulating bacillus.* The preceding discussion has considered the isolation from natural sources of microörganisms capable of decomposing well-defined organic compounds (creatinine, polysaccharides, etc.). It appeared possible that there also exist microörganisms capable of attacking not only soluble, isolated substances, but also the intact living cells of other unrelated microbial species. Specifically, an attempt was made to recover from soil, microörganisms that could attack the living cells of the pathogenic Gram-positive cocci.[26] To achieve this end, suspensions of living pneumococci, streptococci, and staphylococci were added to a soil mixture which was maintained at neutral reaction under aerobic conditions, in the hope that there would develop in the soil preparation a microbial flora antagonistic to the Gram-positive cocci. In fact, it was possible to isolate from the soil preparation an aerobic sporulating bacillus which can multiply at the expense of the living cells of Gram-positive bacteria. Cultures of this soil bacillus have yielded a soluble principle which kills the susceptible bacterial species;[27] the following discussion deals with the nature, properties and activity of this bactericidal principle.

The bactericidal principle of the soil bacillus can be obtained in a protein-free form which is soluble in alcohol and acetone, but insoluble in water and ether.[28] From the alcohol soluble fraction there have been obtained as crystalline compounds three well-defined chemical entities all of which exhibit bactericidal action *in vitro;* they have been called graminic acid, gramidinic acid, and gramicidin with respective molecular weights of 900, 1000, and 1400.[29, 30] Although the complete structure of these substances is as yet unknown, it can be stated at this time that all of them consist largely of amino acids probably combined as polypeptides. Gramicidin which has been most carefully studied contains 2-3 tryptophane residues per molecule; a large percentage of the other amino acids appear to be present in the *d* (so-called unnatural) form; gramicidin also contains an aliphatic fatty acid but contains neither free acid nor basic group. As stated above, the three crystalline substances exhibit a marked bactericidal effect *in vitro*. For instance, 0.005 mg. of gramicidin is sufficient to kill $10^9$ pneumococci or virulent streptococci

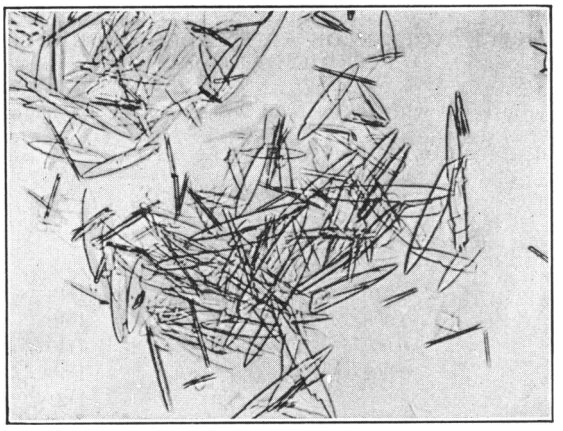

FIG. 5. Photomicrograph of crystals of gramicidin. × 225. (Reproduced by courtesy of Dr. R. D. Hotchkiss.)

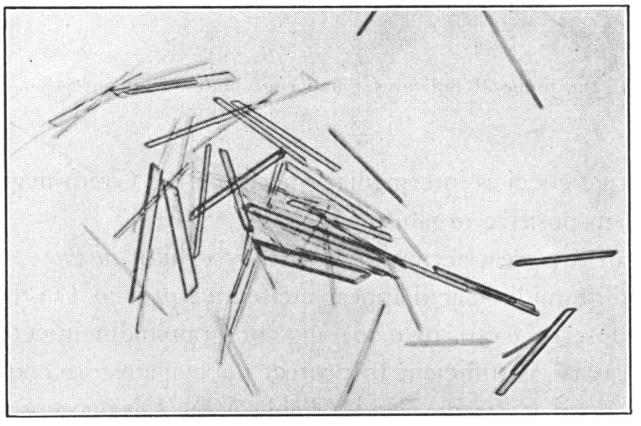

FIG. 6. Photomicrograph of crystals of graminic acid. × 320. (Reproduced by courtesy of Dr. R. D. Hotchkiss.)

within 2 hours at 37° C. Staphylococci, diphtheria bacilli, aerobic sporulating bacilli, in fact all Gram-positive organisms so far tested, are also readily killed under the same conditions although the amount of bacterial substance required varies from one bacterial strain to the other. On the contrary, none of the Gram-negative bacilli have been found to be susceptible, even to much larger amounts of the substance. Meningococci and gonococci are much more susceptible than the Gram-negative bacilli but more resistant than pneumococci or streptococci; this fact may be of some interest, since bacteriologists have often considered the

TABLE VI

PROTECTIVE ACTION OF CRYSTALLINE FRACTION OF BACTERICIDAL AGENT

(All mice infected with 10,000 fatal doses of Type I pneumococcus)

| MATERIAL | AMOUNT | NUMBER OF MICE | RESULT | | |
|---|---|---|---|---|---|
| | *mg.* | | | | |
| Graminic acid ..... | 0.016 | 3 | D40 | D44 | D68 |
| " ..... | 0.008 | 3 | D40 | D40 | D48 |
| " ..... | 0.004 | 3 | D40 | D40 | D40 |
| " ..... | 0.002 | 3 | D40 | D96 | D96 |
| " ..... | 0 | 3 | D24 | D40 | D40 |
| Gramicidin ..... | 0.010 | 3 | S | S | S |
| " ..... | 0.005 | 3 | S | S | S |
| " ..... | 0.002 | 3 | D61 | D114 | S |
| " ..... | 0.001 | 3 | D45 | D46 | S |
| " ..... | 0 | 3 | D27 | D27 | D32 |

S = Survival.
D = Death; the numeral indicates number of hours after infection.

Gram-negative cocci as intermediary between the Gram-negative bacilli and the Gram-positive organisms.

In spite of the great activity which they exhibit *in vitro*, both graminic acid and gramidinic acid appear ineffective *in vivo*. On the contrary, one single dose of 0.001 to 0.002 mg. of gramicidin injected into the abdominal cavity, is sufficient to protect mice against 10,000 fatal doses of pneumococci or streptococci (Table VI). Larger amounts of the material, injected on 3 consecutive days will also protect mice against larger infective doses, or cure them of a well-established infection. The bactericidal substance has proven equally effective against infection with the 5 different types of pneumococci and the 14 different types of hemolytic streptococci (groups A and C) which have been tested; it is permissible to hope, therefore, that it will also prove effective against all virulent strains of these bacterial species irrespective of type specificity; in fact preliminary experiments have recently demonstrated that it does also protect mice against certain strains of staphylococci. On the contrary as could be expected from the *in vitro* experiments, no protection could be obtained against infection of mice with *Klebsiella pneumoniae* (Type B), a Gram-negative bacillus.

Gramicidin is very insoluble in aqueous media; this insolubility may account for the fact that the substance is ineffective against pneumococcus peritonitis in mice when administered by any route (intravenous, intramuscular, subcutaneous) other than the intra-abdominal. Very recently, it has been possible to obtain from autolyzed cultures of the sporulating soil bacillus, a form of the bactericidal substance which is readily soluble in water at neutral reaction; not only does the new preparation cure mice of pneumococcus and streptococcus peritonitis when administered intra-abdominally, but it is also effective by the subcutaneous and intravenous route. Although much remains to be learned about this soluble fraction, it is evident that in some respects it is more effective *in vivo* than the crystalline substance which has been described under the name of gramicidin.

The findings just reported have revealed the existence and to some extent the chemical nature of a new type of bactericidal agent, which, although extremely active against many different species of Gram-positive microörganisms, fails to attack the Gram-negative bacilli. It can be said, therefore, that this new bactericidal principle exhibits a specificity of a peculiar order, one which is correlated with the staining characteristics of the bacterial cells. Since the staining properties are necessarily conditioned by chemical and physical characters of cellular structure, it is perhaps permissible to state that the specificity of the bactericidal agent is related to some structural difference between the Gram-positive and the Gram-negative cells. An analysis of the mechanism of the bactericidal action[31] may therefore reveal important facts concerning cellular structure; this knowledge in turn may indicate what type of chemical structure can be expected to exhibit affinity for the cellular structure of the different bacterial species and may suggest new avenues of approach to the problem of antisepsis. It is also of obvious importance to establish the chemical differences between graminic acid and gramicidin which determine that only the latter is active *in vivo*, whereas both are equally active *in vitro*. This knowledge will give us a clue as to the factors which allow an antiseptic to remain active in the presence of animal tissues, and which thus render it a therapeutic agent.

Finally it is permissible to hope that one will also discover in nature microörganisms antagonistic to other types of pathogens and that the active substances by means of which they exert their antagonistic effect will be isolated. These agents may not themselves be effective in the

animal body. An understanding of their chemical structure and of the mechanism of their action should, however, give the bacteriologist and the chemist useful information and new compounds for the development of chemotherapy on a rational basis.

*The adaptive production of enzymes by bacteria.* It is apparent that the biologist will discover in the microbial world a great variety of useful reagents. On the other hand it is also true that microbial life has revealed a number of physiological processes of general biological significance. For instance, cultural conditions greatly affect the enzymatic constitution of the microbial cell. In some cases in particular, the production of a given enzyme is stimulated when the substrate which it attacks is a component of the culture medium. The bacillus which hydrolyzes the capsular polysaccharide of Type III pneumococcus does not form the specific enzyme when cultivated in ordinary peptone media (in which growth is very abundant), whereas the polysaccharidase is readily produced when the same organism is compelled to use the specific polysaccharide in the course of its growth.[11, 12, 13] Similarly, the "NC" culture which attacks creatinine grows abundantly in peptone solutions, but forms the creatinine oxidase only when creatine or creatinine is a constituent of the culture medium.[5, 7] Karström designated as "adaptive" those enzymes which are produced as a specific response to the presence of the homologous substrate in the culture medium; he differentiated them from the "constitutive" enzymes which are always formed by the cells of a given species, irrespective of the cultural conditions.[33]

Adaptive enzymes exhibit a great specificity with reference to the substrates which they attack, a property which suggests their use in the analysis of biological problems; it is of practical importance therefore, to develop satisfactory techniques for their production. One may wonder also whether the readiness with which microörganisms selectively change their enzymatic constitution in response to changes in the environment may not be of importance in determining the pathology of infectious diseases. Is it not possible that a pathogenic agent growing in living animal tissues may differ in important respects from the same agent grown in laboratory media? In other words, the pathogenic agent may produce during the infectious process, a number of substances which do not appear during growth in the standard laboratory media, and which are the result of the reaction between the para-

site and the tissues of the infected host. These products might account for some of the obscure reactions of infection.

In any case, the very mechanism of production of adaptive enzyme by microörganisms challenges the bacterial physiologist; nothing is known of this mechanism.[32, 33, 34, 35] It seems established that the change in enzymatic construction which results in "adaptation" does not necessarily require the production of new cells. Although production of adaptive enzymes has been described to occur in the absence of cellular division, all evidence available indicates that this formation always involves the synthesis of new protoplasm. It is possible that the synthetic process is, so to speak, oriented or guided by the chemical structure of the substrate, which thus determines the specificity of the enzyme evoked. And it is a common fact, as already pointed out, that adaptive enzymes exhibit a remarkable specificity toward the substrates which stimulate their production.

The phenomenon of adaptive production of enzymes offers great practical possibilities to the bacteriologist. Even more important perhaps, it brings him back into the main channels of biological thought, to the biological problem "par excellence," the problem of adaptation. The study of the mechanism whereby microörganisms produce those enzymes which appear as an adaptive response to the presence of the homologous substrates in the culture medium, bids fair to throw light on some of the reactions involved in specific adaptation.

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