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Antibiotic selection : antibiosis, antimetabolites, assay methods and bacterial sensitivity tests

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ANTIBIOTIC SELECTION

Antibiosis, Antimetabolites, Assay Methods and Bacterial Sensitivity Tests.

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INTRODUCTION

Serving an externship in a local hospital, I first learned of the methods used for testing bacterial sensitivity to the antibiotics. The thought occurred as to whether or not this laboratory procedure could be adapted to use in the office of the practicing physician, who does not have the readily available facilities of a hospital laboratory.

In order to learn more about the tests themselves, the literature was consulted. To gain some background in the principles involved led to a survey of assay methods, from which the bacterial sensitivity tests were derived. This in turn led to a review of the investigations which resulted in the phenomenon of antibiosis and the concept of essential metabolites.

Because of the voluminous amount of available literature on these subjects, no attempt is made to give a complete review. Rather, this thesis is a brief review of the investigations and discoveries which have been instrumental in the development of methods for testing for bacterial sensitivity to the antibiotics, and the question of whether the bacterial sensitivity tests can be an office orocedure or not is answered.

ANTIBIOSIS

One needs only peruse any of various history books to find related the scourge of epidemics and the havoc they created, but it was not until in the 1880's that medical bacteriologists raised the question of what became of all the bacteria causing such diseases as typhoid, bubonic plague, cholera, dysentery and tuberculosis. Would not the soil and water be filled with such bacteria? The soil and water were searched for the answer and the results of various experiments established that pathogens do not survive long in soil or water.

To relate a few of the many experiments we find that DeBary (10) in 1879 was the first to emphasize the significance of antagonistic relations among microorganisms.

In 1893 Frankland (34) observed that the typhoid bacteria die in 9 to 13 days in unsterile surface water but survived 20 to 25 days in sterilized polluted water or in pure deep well water. Similar results were obtained by Jordon and associates (41) who found that Eberthella typhosa survived 15 to 25 days in sterilized water but died within 4 days in raw river or canal water.

In 1902 Russell and Fuller (54) showed that saprophytic bacteria were directly responsible for the destruction of the pathogens.

Thus it was found that in a natural milieu as soil or water in which a mixed microbacterial population exists there occur numerous examples of antagonism. While this relationship was first visualized as due primarily to competition for nutrients as aptly expressed by Pfeffer (49) who wrote " the entire world and all the friendly and antagonistic relationships of different organisms are primarily regulated by the necessity of obtaining food", it soon became clear that antagonism among microorganisms embraced phenomena other than mere competition for or exhaustion of nutrients. For example Smith (67) in 1905 demonstrated that when two or more organisms live in close proximity they may exert mutually antagonistic, indifferent or favorable effects.

Lasseur (44) regarded antagonism as a very complex phenomenon, stating that it is the result of numerous and often little known activities, then proposed that it influences the morphology of the organism. Indeed Hettche(40) in 1934 showed that the morphology of the diptheria organism may be changed and this is

often accompanied by a reduction in virulence. Also Emmerich and Saida(19) found that pyocyanase brings about morphological changes in Bacillus anthracis. In 1940 Gardner(35) found that penicillin by inhibiting fission of bacteria led to abnormal growth of cells followed by autolysis.

To go back several years, in other fields of investigation, we find that as early as 1877 Pasteur (48) had noted the phenomenon of antagonism when he observed that by simultaneous inoculation of Bacillus anthracis and various other bacteria the development of anthrax in sensitive animals could be repressed. In 1885 Cantani (7) applied the above phenomenon when he treated a patient suffering with tuberculosis with a culture of saprophytic bacteria designated Bactium termo, and the results were highly favorable.

The great amount of investigative work done in regard to antagonism among microorganisms is too voluminous to record here, be it sufficient to list the various types of antagonism which have been recognized:" 1. Antagonism in vivo vs antagonism in vitro; the former being often designated as antibiosis(44). 2. Repressed, bactericidal and lytic forms of antagonism, as well as antagonism of function vs antagonism of

growth. 3. Direct, indirect and true antagonism. 4. One-sided and two-sided antagonism; antagonism between strains of the same species and among different species; or iso- and hetero-antagonism." (70)

Among the various types of antagonism the most definite and best understood is that which results in the formation of antagonistic substances. The nature of these substances or toxins produced by different bacteria or fungi is not always the same as some can be destroyed by heat, exposure to light or by filtration; others are resistent to heat and ultra-violet rays, while still others are readily adsorbed by filters from which they can be removed by special solvents.

Likewise the mechanism of action of the antagonist varies greatly, appearing to be dependent on the specific nature of the active substances. The antagonistic action may be either bacteriostatic or bactericidal or both. They also vary in effectiveness when injected into the animal body, some being highly toxic others having low toxicity.

Although Lasseur (43) confined the term antibiosis to antagonism in vivo, antibiosis is now defined as " bacterial antagonism; an association between two or more organisms which is detrimental to

one of them." (12)

ANTIMETABOLITES

Let us turn again to the latter part of the 19th Century for a brief review of investigations in other fields of microbiology, particularily as regards metabolism. In 1870 Kuhne (43) introduced the term enzyme to mean a biological catalyst. Buckner (5) in 1897 proposed that the microorganisms produce their effects through the agency of intracellular enzymes. However it was not until 1926 that the first enzyme, urease (66), was crystallized.

Within the next eleven years nine other enzymes were crystallized and much was written about the mechanism of enzyme action. From this work has emerged two main theories:

> "1. Bayliss' hypothesis. This suggests that the substrate, or substance acted upon, is adsorbed upon the surface of the enzyme (assumed to be a colloid). This brings about a closer orientation of the reacting molecules, permitting the substrate to be acted upon. 2. Michaelis' hypothesis. This is a chemical view as contrasted with Bayliss'

physical approach. Michaelis believes that the catalyst reacts with the substrate, forming an intermediate compound. The latter then decomposes into the new product plus the original enzyme." (42)

But even in 1938 it was not known that there were proteolytic enzymes in streptococci thus Lockwood (46), working on action of sulfanilamide, denounced his own hypothesis that the proteolytic enzymes of streptococci were inhibitied by sulfanilamide. Mc Intosh and Whitby(47), in 1939, also working on the mechanism of sulfanilamide action proposed that the drug acted by blocking the vital food supply of bacteria by inactivating an enzyme. Stamp (64) was able to extract a substance from streptococci which was antagonistic to the action of sulfanilamide and proposed that this substance was a necessary nutritive factor.

Green (38) in 1940 obtained a substance from Brucella abortus and various other bacteria which he termed "P" factor. This " P " factor counteracted the action of sulfanilamide and he concluded that sulfanilamide inhibited an enzyme reaction fundamental in metabolism of bacteria.

Woods (74) obtained an extract from yeast which counteracted the action of sulfanilamide. He believed

this substance identical with the Stamp factor and Green's " P" substance. Woods identified this active material as p-aminobenzoic acid and looked on it as an "essential metabolite" (Fildes) which is acted upon by a special enzyme system, and that the sulfonamides inhibited growth of microorganisms by competing with it for the enzyme.

Fildes (22, 23) established the " essential metabolite" concept when he wrote " an essential metabolite is a substance or chemical group which takes an essential part in a chain of synthesis necessary for bacterial growth. A growth factor which must be supplied in the nutrients is an essential metabolite which the cell cannot synthesize."

This hypothysis provides a rational basis for the mode of action of chemotheraputic agents since it would explain inhibitions of growth by any drug which interferred with the normal metabolism of an essential metabolite. The ways in which any drug would interfere with metabolism of an essential metabolite are: " 1) by oxidizing a substance required in the reduced form, 2) by molecular combination to yield an inactive product, or 3) by competition for an enzyme associated with an essential metabolite."(22)

CHEMOTHERAPY AND CHEMOTHERAPUTIC AGENTS

With the preceding brief review of antibiosis and antimetabolites as a background we now turn to the subject of chemotherapy and development of chemotheraputic agents.

Although the phenomenon of antibiosis had been suggested as a possible mode of treatment of certain infections in the latter part of the 19th Century (7, 19, 20, 48) it was not until the period between 1908 and 1913 that the fundamentals of chemotherapy were established. To Paul Ehrlich (16) goes the credit for stating the principles of chemotherapy, for it was he who used the term " chemotherapy" to mean treatment of parasitic disease by " direct attack upon invading organisms - viruses, fungi, bacteria, spirochetes, protozoa, or helminths." Ehrlich (16) also early realized that although a great many drugs were effective at some concentration in vitro, toxicity for the host might prevent their systemic utilization. As the specificity of protein interactions was little understood during this period, it was his view that drugs combined with tissue cells through receptor side chains(17).

To-day Ehrlich's (16, 17) theories could be

reformulated in terms of enzyme-mediated steps in metabolic processes, some playing a role in both parasite and host and others being unique to organism or host tissue.

The discovery of the chemotheraputic value of prontosil by Domagk (11) in 1935 and subsequent use of the sulfonamides was remarkable, not that these drugs were more potent than the well known antiseptics in inhibiting bacterial growth, but because effective sulfonamide concentrations could be tolerated in body fluids without prohibitive toxicity. Thus the era of systemic antibacterial chemotherapy was introduced.

In 1928 Fleming (25) was studying variations of staphylococci when a culture was accidentally contaminated by a fungus. This resulted in lysis of the bacterial colonies. He carefully saved a culture of this mold and later identified it as Penicillium notatum. Even though Fleming (26) suggested that penicillin could be used as a dressing for septic wounds, a major war was necessary to bring the miracle drug to the physician. To Florey and Chain and coworkers (1,8,27) goes credit for working out certain of the basic chemical and biologic properties as well as production methods of penicillin. They also studied

the effects of penicillin on animals.

This gave impetus to the search for other antibiotics which have the combination of effective antibacterial activity and low toxicity for man. As result of this search many antibiotics were found, however only a few notable ones possessed the above combination. Those which can be used systemically and are now available to the physician are: Streptomycin (55), Polymyxin (65), Chloromycetin (6, 14, 15), Aureomycin (13), Neomycin (71) and Terramycin (24).

ASSAY METHODS

Before the antibiotics are available for use by the physician there is first production of the antibiotic and then assay of the antibiotic in order to determine its potency and to correlate dosage. As assay methods for most of the antibiotics are similar to those for penicillin only the latter will be discussed.

Up until 1943 aside from the report of the Oxford group (1) little information had appeared pertaining to the microbiological aspects of penicillin, yet the matter of establishing with some degree of certainty and accuracy the potency of penicillin solutions was

an important issue confronting workers engaged in penicillin studies. Because of the wide divergencies in penicillin assay from one laboratory to another it was difficult to interpret findings and correlate dosage.

However all penicillin assay methods have in common the determination of the amount of penicillin which will cause an arbitrarily established degree of inhibition of the growth of a susceptible test bacterium. In essence, the methods differ in the mechanics used to measure the inhibition.

The most widely used assay method for penicillin is that of the Oxford group (1), the Oxford cup method, although numerous modifications of this original method are employed. To eliminate the day to day variations the Oxford group introduced the concept of the penicillin unit, later known as the Oxford unit, which is the amount of penicillin which under conditions of the cup assay gives an inhibition zone 24mm. in diameter, or that amount of penicillin dissolved in 50 ml. of meat extract broth which just inhibits com letely the growth of the test strain of Staphylococcus aureus. Thus the potency of any penicillin sample is obtained by direct comparison with the primary standard or indirectly against secondary

standards whose potencies have previously been established by the primary Oxford standard.

The advantage of the cup assay while it is not the most accurate, is that a large number of unknown samples of penicillin can be run with commensurate accuracy and speed yet a minimum of labor.

The Oxford Cup method and the Cylinder Plate method, described by Abraham and associates (1), are alike except that in the latter method glass or porcelian cylinders are used to hold the penicillin solutions. Essentially the assay consists of placing five cylinders on an agar plate previously seeded with the test organism. Into three of the cylinders are placed the unknown penicillin samples and in the other two a standard penicillin solution is used. The plates are then incubated and after 15 to 16 hours the zones of inhibition are carefully measured and by compariaon with standard the potencies of unknowns are determined. Technique of the Cylinder Plate method is given below.

The Cylinder Plate Method (1)

The modification is that the Oxford Cup is replaced by glass or porcelian cylinders one centimeter long with an outside diameter of 7.9 _ 0.1 mm. which have been ground perfectly smooth at one end with

the outside edge beveled. The cylinder may be made from pyrex glass tubing or preferably from porcelain tubing. The nutrient agar medium for the assay plates must be clear, support good growth, and easily duplicated from batch to batch thus the following was used as it gave good results. Referred to as medium I.

Bactopeptone	5.0 gms.
Bacto yeast extract	3.0 gms.
Commercial hydrated glucose	1.0 gms.
Bacto agar	15.0 gms.
Distilled water to make	1000 ml.

Another medium designated medium II is used for inoculation of the assay plates as it does not contain stringy or granular growth.

Medium II.

Bacto pystone	5.0 gms.
Bacto yeast extract	1.5 gms.
Commercial hydrated glucose (Cerelose)	1.0 gms.
Sodium chloride	3.5 gms.
1% phosphate buffer pH 7.0	500 ml.
Distilled water to make	1000 ml.

Flood petri plates containing 22 ml. of agar with 3 ml. of medium I which has been melted and cooled to 48-50 C and then inoculated with 1% inoculation (1 ml. of a 24 hour broth culture per 100 ml. of agar). After hardening of the agar, cylinders are dropped from a height of 1/8 inch onto the agar

surface. On each plate 5 cylinders are placed equidistant from the center. Glass covers are now replaced with unglazed porcelain tops and the dishes allowed to stand at room temperature for 45 to 60 minutes. If room temperature is quite high the inoculum may be reduced or the standing time reduced, or if the zones are quite large the inoculum may be increased or plates incubated.

Two cylinders on each plate are filled with a standard penicillin solution containing 1 unit per ml., and the other three cylinders are filled with the test sample. After the cylinders are filled, the plates are incubated at 37 C for 15 to 16 hours.

The diameter of each circle of inhibition is measured to the nearest 0.25 mm. Readings for the standards and the test samples are averaged, unless it is obvious that a cylinder has been jarred or that it leaks badly. A standard curve is made and the unit value of each sample is calculated in terms of its respective standard.

Factors affecting this method are: (1) depth of agar medium, (2) pH of the agar medium, (3)

concentration of the inoculum, (4) the stock culture, (5) the relationship between the rate of diffusion of penicillin solution and growth of the test organism, (6) condition of the ground glass surface of the cylinder (crack or chip causes leaks).

Tube Dilution Method.

Another method of assay is the tube dilution or serial dilution method, and like the cup assay it is widely used. Because of its accuracy, many laboratories use this method as a check on the cylinder plate method. It is relatively sim le and fast to run.

Many workers consider the tube dilution method a modification of the serial dilution used by Fleming (25) in 1929. Foster and Woodruff (31) described the broth dilution method devised by Squibb Biological laboratories, which is essentially the same as the method of Schmidt and Moyer (56).

This procedure consists of adding, by suitable dilution procedures, different amounts of penicillin to either liquid or solid media which have been inoculated with a test organism. On comparison with the standard, the potency of the unknown sample can de determined.

Technique of Tube Dilution Method (Schmidt & Moyer)

Medium II as described in Cup assay method is made

up and 200 ml. portions are placed in 500 ml. flasks. These are sterilized and refrigerated. As each flask is used it is inoculated with 1 ml. of a 20 hour broth culture of Staphylococcus aureus. The broth and inoculum are thoroughly mixed and the broth is then immediately pipetted in various amounts to sterile test tubes. The sample of penicillin which is being diluted must be substantially free from bacteria which are insensitive to penicillin. Dilutions of 1/10, 1/100, 1/500, 1/600, 1/700, 1/800, 1/900, 1/1000, 1/1100, and 1/1200 are made and added to tubes of broth, the tubes are shaken to thoroughly mix the broth and penicillin. Incubate at 37 C for 18 hours, or better, for 40 hours. The calculation for converting dilution to Oxford units is based on the highest dilution showing no growth.

For example: (18 hour reading) Takes approximately 0.045 Oxford units per ml. to inhibit growth, then to convert

1/900- 900X0.045= 40 units/ of sample
1/1000+

(40 hour reading) Say 0.1 unit per ml. is required to inhibit growth, then

1/400- 400X0.1 = 40 units/ of sample.
1/500+

Turbidimetric Method.

Another valuable method of assay is the turbidimetric method. The outstanding feature of this method is that its sensitivity permits measurement of relatively small differences in penicillin activity with a reasonable accuracy.

Essentially this method consists of adding to series of flasks containing broth inoculated with a test organism, varying amounts of a penicillin standard solution and to another series like amounts of unknown penicillin sample. The flasks are incubated for 3 to 5 hours on a shaking machine, the growth stopped and turbidities read with an Evelyn photoelectric coloroimeter.

Technique of this method as described by Foster and Wilker (30) is given below.

Turbidimetric Technique. A standard curve is made: five points in duplicate (0, 0.1, 0.2, 0.3, and 0.4 Oxford units/ 10 ml.) define the curve.

Using 2.0 ml. of an overnight aerated broth culture of Bacillus adhaerans (or Staphylococcus aureus) inoculate 200 ml. of sterile nutrient broth, then place 10 ml. amounts on 50 ml. Erlenmeyer flasks. The standard penicillin solution contains 1.0 Oxford units / ml. Quantities of 0, 0.1, 0.2,

0.3, and 0.4 ml. of this are added to the respective flasks in duplicate.

Then unknown samples are diluted to contain 1.0 Oxford unit/ml. on the basis of the expected potency and 0.1, 0.2, and 0.3 ml. added in duplicate flasks as above. All dilutions are made in advance and the penicillin added to the medium all at one time.

The flasks then are placed on shaking machine at 37 C for 3 to 5 hours after which the growth is stopped by cooling in ice water or adding a drop of disinfectant.

Using an Evelyn photoelectric colorimeter the turbidities are read and the standard curve is obtained by plotting the percent transmissible light against penicillin concentration. Then potencies of the unknowns are computed from the standard curve.

Rammelkamp Method.

Rammelkamp (51) developed an assay method based on hemolysis of red blood cells by hemolytic streptococci. In this procedure a culture of hemolytic streptococci is diluted with veal infusion broth containing erythrocytes. A measured amount of this is added to each tube of two

series, one series containing varying dilutions of a standard penicillin solution the other series containing like dilutions of unknown penicillin solution. Then incubation for 18 hours at which time the tubes are examined for hemolysis. By comparison with the control, the potency of the unknown is determined. This method can be used to determine penicillin levels of blood, spinal fluid, joint fluid, and urine.

Technique of Rammelkamp method. To the first two tubes of a series of small culture tubes, 0.2 cc of unknown sample of penicillin is added. The rest of the tubes contain 0.2 cc of veal infusion broth. Then serial dilutions are made. A control run with each determination is made up from a standard of penicillin in a solution of 0.85% sodium chloride in a concentration of 20 Oxford units per cc. Serial dilutions of this are then made.

A group A strain of hemolytic streptococcus obtained from the blood stream of a patient with erysipilas was used as test organism. Dilution of 12 hour broth culture is made in veal infusion broth containing 1% erythrocytes so that the final number of organisms varies between 1,000 and

10,000 cc. Then 0.5 cc of this dilution is added to each tube as well as to the control series containing known amounts of penicillin. The cultures are then incubated for 18 hours and the tubes then examined for hemolysis. By comparison with the control the unit per cc can be determined.

Modifications.

Numerous modifications of the above methods have been reported. One of the more important modifications is that devised by Vincent and Vincent (69). Their modification was the use of a thick filter paper disc (no. 470½ in. diameter Schelcher and Schuell Co. Inc. N. Y.) saturated with the penicillin sample and substituted for the sample containing small cylinder used in the Cylinder plate method. The inhibition by unknown sample disc then compared to inhibition by disc saturated with a standard penicillin solution.

Epstein et. al. (21) described a similar method, using filter paper discs but using flat pyrex baking dishes as culture dishes. Thus a larger number of unknown samples could be run on one culture.

Rake and Jones (50) in 1943 described a modification of the Rammelkamp method which has advantage of time. They used as a test organism a streptococcus

pyogenes and by preparing the various components of the test ahead, the test is run at optimum conditions thus a reading could be obtained in 60-90 minutes.

Both Thomas and associates (68) and Sherwood and associates (60) employed a wire loop to deliver unknown penicillin solutions to a seeded plate. Thomas et. al. used a 4 mm (22-23 gauge) platinum loop to deliver one loopfull from each solution of penicillin to an agar plate previously seeded with a susceptible organism. Sherwood et. al. used a 4 mm 24 guage nichrome wire loop to place antibiotic substances upon sterile absorbent paper discs which lay on the surface of inoculated nutrient agar.

Various principles have been tested for possible use in measuring penicillin, particularily for assays requiring only a short time. For the most part, unpromising results have been obtained. In the case of some, the special technique and preparation are not conveniently adaptable. A few of these are: (1) Methylene blue reduction by washed cells of a susceptible organism. (2) Microscopic observation of cessation of mobility of bacteria. (3) Inhibition of luminescence in cultures of luminescent bacteria. (4) Microscopic observation of appearance of enlarged and involution cell shapes.

(5) Titration of acid formed by lactic acid bacteria, Staphococcus aureus, etc. (31)

TEST OF BACTERIAL SENSITIVITY TO ANTIBIOTICS

While each of the antibiotics available today have a relatively wide spectrum of antimicrobial activity, there is considerable overlapping of the spectra. Thus the clinician is often faced with the problem of selecting the correct antibiotic. Among the factors influencing this choice, one of the most important is the relative susceptibility of the microorganism or microorganisms responsibile for the infection.

While it is well recognized that microorganisms vary considerably in their susceptibility to the antibiotics, not only do individual species vary in their susceptibility, but the different species very markedly in their sensitivity to the same antibiotic. More-over different strains of the same species may exhibit wide variations in their sensitivity to the same antibiotic (33).

Thus it can be said that in general the sensitivity of any species or strain of microorganisms to any one antiobiotic is quite independent of its susceptibility to any other antimicrobial agent.

To aid the physician in selection of the correct antibiotic and especially in the case where the identity of the etiologic agent is a question, several methods to test bacterial sensitivity to antibiotics have been devised.

The Bondi Disc Method.

Bondi and associates (4) realizing the value of a routine method for rapid determination of bacterial sensitivity to antibiotics described such a method in 1947. Since rapidity of such a determination is more valuable than great accuracy, the latter is sacrificed to some extent. This method is essentially a modification of the penicillin assay method of Vincent and Vincent (69). It consists of placing filter paper discs saturated with antibiotic solution on the surface of a blood agar plate immediately after inoculation of the plate with the clinical speciman. The plates are incubated overnight and the zones of inhibition are measured for determination of the most effective antibiotic. Up to three antibiotics can be tested on each plate.

Technique of Bondi Disc Method. The medium used is a veal infusion broth prepared according to the method of Wright (75) to which is added 2% try tose and 10% defibrinated horse blood.

For each clinical speciman two plates are made, one for anaerobic and one for aerobic incubation.

A swab or loopfull of the clinical speciman is drawn once across one side of the agar surface. This primary inoculum is restreaked with a sterile inoculating loop and is spread with parallel streaking over 1/3 of the surface of the plate. After flaming the loop, the plate is successively turned at right angles and streaked in the same manner, care being taken to touch previous inoculum with each streak until the entire surface has been covered.

The discs are cut with a cork borer or paper punch 6.5 mm. in diameter out of Whatman #2 filter paper. These are then sterilized in a petri dish in hot air oven. With sterile forceps one of the dishes is dipped into a solution of commercial penicillin carrying 15 units 1 ml. The disk is then placed on the surface of each primary inoculum on left side of the plate, and a disc prepared with streptomycin carrying 500 micrograms 1 ml. is placed on the right side of the plate. The discs should be 20 mm. apart and on a line parallel to the line of streak so that the inoculum around

each disc is comparable. Two plates are made for each clinical speciman, one for anaerobic and one for aerobic incubation.

The plates are incubated overnight and the next morning the zones of inhibition are measured and compared to determine the more effective antibiotic.

In 1950 Scott (57) reports the testing sensitivity to aureomycin by Bondi Disc technique. To prepare aureomycin for the test, 15 milligrams of recrystallized aureomycin hydrochloride is accurately weighed, and dissolved in 10 ml. of sterile water. After mixing well by inversion, it is furtner diluted to a 1:10 solution by addition of 9 parts of sterile water, thus giving a final concentration of 150 micrograms per ml. The technique for testing is same as given above.

Ditch-plate method.

In 1950 Rice and Lonergan (53) described the ditch-plate method for testing bacterial sensitivity to antibiotics. This method originally was used in assessing bacterial resistance to sulfonamides. The method consists of removing three parallel 1 cm. wide strips of agar from an agar plate. Each ditch is filled with a different antibiotic solution. The

at right angles to the ditches. The plate is incubated for 12 to 18 hours following which those with zones of inhibition compared to each other.

Technique of Ditch-plate Method. Bacto-blood agar base with 5% citrated human blood is used. Melted agar is poured into a 14 cm. petri dish and after solidifying, three parallel strips of agar 1 cm. in width are removed using sterile scapel and forceps. Blood agars are prepared to contain, respectively, buffered crystalline Penicillin G sodium, 10 units / ml., Streptomycin, calcium chloride complex, 500 units ml., and soluble Aureomycin hydrochloride, 10 micrograms/ml. These are carefully poured to fill the previously prepared ditches. After solidifying the plates should be used at once. Organisms to be tested are inoculated in parallel streaks, 1.5 to 2 cm. apart, at right angles to the ditches containing the antibiotic. For comparison, each plate should be controlled with a standard stock culture of a gram positive coccus and a gram negative "coliform" bacillus. With two controls, 3 to 4 unknown organisms may be tested on a 14 cm. petri dish.

The plates are then incubated at 37 C for

12-18 hours and the zones of inhibition are measured.

The width of the inhibition zone is not an index of the comparative antibacterial power of different antibiotics since other factors enter, particularily the rate of diffusion through the gel. It is however an index of antibacterial quality when compared with the control.

Dried Disc Method. (67)

The main difference between this and the Bondi disc method is that in this method the discs are prepared by dipping filter paper disc in antibiotic to be tested, then dried and stored. These discs remain stable for 6 months. The discs are placed on surface of an inoculated petri plate, such as in Bondi Disc method. The zones of inhibition obtained with these discs has been found to guide to the sensitivity of the organism and to the adequacy of the antibiotic dosage.

Dried Disc Preparation Technique. (67) Using a 9 mm. cork borer, discs are cut out of No. 633 Hoyle Mills blotting paper. The discs are separated in a large petri dish and sterilized in a hot air oven.

1. Dried Penicillin Discs. Using a 50 drop pipette each disc is impregnated with one drop (0.02ml.) of a solution of penicillin containing 1000 units/ml. The discs are then placed in a petri dish, the lid left slightly open and then dried in an incubator for 2 hours. Any moisture forming in the lid of the petri dish is removed with a sterile blotter and the dried discs then stored in a refrigerator. They remain stable for 6 to 8 months.

Sensitivity interpretation: Measurements are made from edge of the disc to the line of growth:

15 mm. = 0.075 Units/ml. tube sensitivity. (Sensitive Organism)
8 mm. = 0.25 Unit/ml. tube sensitivity. (Moderately sensitive organism.)
3 mm. = 0.3 Unit/ml. tube sensitivity. (Slightly sensitive organism.)
nil = 0.5 Unit/ ml. tube sensitivity. (Resistant organism.)

2. Dried Streptomycin Discs. Method of preparation is same as described for penicillin discs. A 10,000 microgram/ml. solution of streptomycin is used. The dried streptomycin discs are stable for 6 months. Sensitivity interpretation same as for penicillin discs.

3. Dried Aureomycin Discs. Discs are prepared in the same way as penicillin discs, impregnation

being made with a 2,500 microgram/ml. solution of aureomycin. This solution is prepared by dissolving the hydrochloride salt in sterile distilled water, and buffered with acetate of pH5. Discs are stable for 6 months.

Because of the instability of aureomycin in solution, reliable methods for tube sensitivity testing were not found, however the values given below are at least relative and correspond respectively with the zoning.

12 mm. = 0.25 microgram/ml.
(Sensitive organism.)
5 mm. = 2.5 microgram/ml.
(Moderately sensitive organism.)
nil. = 20 microgram/ml.
(Resistant organism.)

4. Dried Chloromycetin Discs. Impregnate discs with a solution of 2,500 micrograms/ml. of chloromycetin and dry, as described for penicillin discs. These discs are stable for at least 2 months.

Sensitivity interpretation - same as given under penicillin discs.

Agar plates are inoculated with the clinical speciman and dried disc of each antibiotic is laid on the surface of the inoculated plate at least 15 mm. from the edge of the plate and from each other. Incubate for 12-18 hours and measure zones of inhibition.

Interpret as given in the preceding method.

The Boyle Tablet Method.

A search of the literature failed to reveal a description of this method. However, this method of testing is used in numerous laboratories, one of which is the University of Nebraska Bacteriology Laboratory. The following description was obtained from the instructions which accompany the tablets. (The tablets are produced by Boyle & Company, Los Angles 33, California.)

The various antibacterial drugs are incorporated into compressed tablets. These tablets are made in two strengths for each antibiotic, and are applied to the surface of inoculated petri plates. The plates are incubated for 12 to 18 hours and the readings then made. At the present time tablets are made for testing the following: penicillin, streptomycin, aureomycin, chloromycetin, and terramycin.

Technique of Boyle Tablet Method. As stated above, five antibiotics are incorporated into compressed tablets. Drug concentrations in these tablets have been selected which will indicate the relative sensitivity of bacteria in the range of the levels which are found in the body fluids of patients

receiving treatment.

The tablets are placed on surface on an inoculated petri plate. Any desired solid medium, such as nutrient agar, blood agar, chocolate agar, or Eosin-Methylene-blue agar, may be used and inoculation may be either the streak or pour method. The plates are then incubated and may be read as soon as sufficient growth has taken place which is usually 12 to 18 hours.

The tablets are made in two strengths as follows:

Peni-Test Tablets:

Strength A produces inhibition zones comparable to those produced by a buffer solution containing 10 units/ml.

Strength B corresponds to a solution containing 1 unit/ml. when tested against a standard organism.

Strepto-Test Tablets:

Strength A tablets produce zones of inhibition comparable to a buffer solution containing 0.1 mg. of streptomycin/ml.

Strength B tablets produce zones of

inhibition comparable to a buffer solution containing 0.01 mg/ml. of streptomycin.

Aureo-Test Tablets:

Strength A tablets produce inhibition zones comparable to a solution containing 0.025mg/ml. This value approximates the lower urinary levels of 32 to 256 micrograms/ml.(9)

Strength B tablets produce zones comparable to a solution of 0.0025 mg/ml. This corresponds to blood levels found in aureomycin therapy which was found by Herrell and associates (39) to be 2 to 4 micrograms/ml.

Chloro-Test Tablets:

Strength A tablets produce zones comparable to a solution containing 0.25 mg/ml. This corresponds to urine levels found by Ley and associates (45) to be 100 to 400 micrograms/ml.

Strength B tablets produce zones comparable to a solution containing 0.025 mg./ml. which corresponds to clinical blood

levels. The blood levels of chloromycetin in patients under therapy were found to be from 10 to 30 micrograms/ml. by both Ley and coworkers (45) and Smadel and coworkers(61).

Terra-Test Tablets:

Have been produced and are now available. At this writing the amounts of Terramycin used in the tablets is not available.

Interpretation of results. If an organism is inhibited by both Strength A and Strength B the organism is relatively sensitive, however if inhibited by Strength A but not by Strength B it is considered as relatively resistant to the antibiotic tested.

Dessicated Disc Method of Severens (59).

This method is essentially the same as that of the Dried Disc method of Thomson(67). The report has not yet appeared in the literature although the discs have just been placed on the market under the name of Bio-Test.

The discs are impregnated with five different antiobiotics and contain the following amounts of antibiotic:

1. Penicillin - 2 units, 2. Aureomycin - 2 micrograms, 3. Streptomycin - 2 micrograms, 4. Terramycin - 2 micrograms, and 5, Chloromycetin - 2 micrograms. Those concentrations were chosen because it was found that a barely preceptible inhibition of bacteria by a disc of this concentration would result in a good clinical response when treated with the generally accepted dose.

The discs are placed on inoculated blood agar plates and zones of inhibition by various antibiotics compared to determine the agent of choice. Blood agar plates are used as standard since a good correlation exists between results obtained on blood agar and the clinical results observed.

Adaptable Methods.

Although not described in the literature, any of the assay methods described in this paper can be adapted to the testing of bacterial sensitivity to antibiotics.

Where accuracy of a high degree is desirable the tube dilution method is used. This method has been used in determining the amount of antibiotic to be used in the disc or tablet of the antibiotic methods in which the disc or tablet are used. Should an organism be resistant to any of the antibiotics tested by disc or tablet

method, the tube dilution method is used to determine to which antibiotic and in what concentration is necessary to inhibit growth of the resistant organism. From the result it may be determined what dosage would be necessary to maintain to overcome the infection.

Thus the tube dilution method, while not used for routine testing, is a valuable supplementary technique.

EVALUATION OF THE BACTERIAL SENSITIVITY TESTS

Bondi Disc Method.

When Bondi (4) described his technique for determining sensitivity of bacteria to penicillin and streptomycin there were several methods available for determining susceptibility. However, these procedures required first the isolation of the organism in pure culture, before running the determination for susceptibility. Furthermore in the case of a mixed infection separate determinations had to be carried out for each organism. Thus the technique described by Bondi saved at least one day in time, and because the test for sensitivity is made at the same time the primary culture is made, the necessity for separate determinations in case of a mixed infection is eliminated.

Although a considerable number of discs can be cut and sterilized beforehand, the procedure of dipping each disc in the antibiotic solution just before placing the disc on an inoculated plate requires time. Also slight differences in concentration of the antibiotic from disc to disc may occur.

Unless a large number of tests are run daily, the fact that aqueous antibiotic solutions begin to deteriate in strength within a week is a disadvantage of the Bondi method.

Ditch-plate Method.

The advantage of this method is that a number of organisms may be tested on a single plate for sensitivity to three separate antibiotics. Also, direct comparison of the effects of the antibiotics on each organism may be made under identical conditions.

Like the Bondi Disc method measured inoculums of an 18 to 24 hour broth culture are not required thus a gain in time of at least 18 hours.

The preparation of the "ditches" requires time and they should be identical which is rather difficult to accomplish.

Thus like the Bondi method the Ditch-plate method would not be too practical as an office procedure.

Dried Disc Method (Thompson)

In this method the discs are cut, sterilized, impregnated with the different antibiotics, dried, and then can be stored in the refrigerator. They will remain stable for at least 6 months. Thus any number of discs can be prepared in a relatively short time and are ready for use whe ever it is desired to test an organism for susceptibility to various antibiotics.

By using a pipette to deliver a measured amount of antibiotic solution to each disc gives uniformity of concentration of the antibiotic from disc to disc.

Since the individual discs are very light in weight and stick to the solid medium surface well enough, the plates may be inverted for incubation thus the moisture collects on the petri dish cover rather than on the inoculated medium surface, favoring growth of colonies.

The objection to this method as an office procedure, lies mainly in preparation of the discs.

Boyle Tablet Method.

The objection of preparation of discs are eliminated by this method. They are stable and do not require refrigeration for storage. They give good results on any desired solid medium. By having two strengths

of each antibiotic, the sensitivity of an organism to any one antibiotic is readily determined, as well as comparison with sensitivity to the various antibiotics.

Inhibition of the organism by the lower strength (strength B) tablets means that a good clincial response may be expected when treated with the generally accepted dose for that antibiotic. The greater strength (strength A) tablets are of such concentration, that this dose, while higher than the generally accepted dose, is deliverable.

A disadvantage is that it is best to incubate without inverting the plates, since the tablets occasionally slip when the plates are inverted, or due to weight of tablet they may fall from the medium surface.

As an office routine for the clinician this method is quite acceptable. It is inexpensive, requires only preparation of solid medium for inoculation of the clinical speciman, and may be read as soon as growth has taken place.

Dessicated Disc Method (Bio-Test)

The discs are less expensive than the Boyle tablet. They are stable and do not require refrigeration.

The concentration of antibiotic in each disc is such that if the disc effects a barely preceptible

inhibition of bacteria a good clinical response will result with the generally accepted dosage for that antibiotic.

Another advantage is that the plates may be inverted for incubation, thus moisture does not form on the inoculated surface.

Blood agar is used as a standard medium in this method as it was found that a good correlation exists between results obtained on this medium and the clinical results obtained. Thus for best results only blood agar should be used with this method.

As an office procedure this method is inexpensive and requires only the preparation of blood agar, and an incubator to run the test.

SUMMARY

1. The phenomenon of antibiosis was reviewed.

2. The concept of essential metabolites was given, with a review of the investigation leading to the establishment of this concept.

3. A brief history of chemotherapy and chemotheraputic agents was given.

4. A few of the assay methods and modifications thereof were reviewed. The technique of the more widely used assay methods was given in detail.

5. Tests for testing bacterial sensitivity to antibiotics were described.

6. The advantages and disadvantages of the various tests of bacterial sensitivity to antibiotics were given.

CONCLUSIONS

With the increasing number of available antibiotics, it behoves the clinician to make a more careful diagnosis of infectious diseases. To aid his choice of the most suitable antibiotic for use in a given case, the bacterial sensitivity tests are of great value.

In conclusion, either the Boyle tablet method or the Bio-test method can be efficiently used by any physician, whether he has the facilities of a hospital laboratory or not, as either of the two above methods are readily adaptable to use in the office of the practicing physician.

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