LEAFLET X

INOCULATIONS WITH BACTERIA CAUSING PLANT DISEASE

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Introduction

The methods for studying the pathogenicity of bacteria in plants, and for making a few selected cognate investigations are briefly treated. The procedures for handling certain organisms and for studying the diseases they induce, vary so widely that no given directions apply to the group as a whole. The selected representative methods included are given primarily as guides to the beginner and need modification according to circumstances. Two excellent reviews have appeared recently by Allen and Allen (1950) and Hildebrandt (1950).

Difficulty in interpretation frequently is encountered from variations in results that depend on the methods used. A given bacterial characteristic may sometimes be positive when measured by one method and be negative when measured by a slightly different technique. Students should employ a known positive and a known negative as controls when making critical determinations. The method used should always be given or cited when a characteristic is listed, so that its validity can be estimated by the reader.

A number of topics discussed in Leaflet VII regarding bacteria pathogenic on animals are applicable to bacteria pathogenic on plants. These include particularly: (1) identification of the active agent as the bacterial cell or its products; (2) distinction between invasion and the power to cause disease after entry; (3) variability in virulence of the pathogen, which requires single-cell cultures, and in susceptibility of the host, which frequently calls for plants with known genetic constitution, when critical studies are involved; and (4) relations between reactions induced in the test tube and in the host.

The pathogenicity of a microörganism may be proved by fulfilling Koch's postulates, which have been stated and modified in various ways, and which are so important that they are repeated here. One summarized statement follows: (1) The causal agent must be associated in every case with the disease as it occurs naturally, and conversely the disease must not appear without this agent. (2) The causal agent must be isolated in pure culture and its specific characteristics determined. (3) When the host is inoculated under favorable conditions with suitable controls, the characteristic symptoms of the disease must develop. (4) The causal agent must be reisolated, usually by means of the technique employed for the first isolation, and identified as that first isolated. Obviously, the demonstration of pathogenicity is made only after repeated trials, preferably with a number of different isolates which are of unquestioned purity. While the technique for cultivating causal agents on artificial media has not yet been worked out, their pathogenicity is established in other ways (e.g., Rivers, 1937). When

causal relations are being worked out, one may well differentiate between predisposing, inciting, and continuing causes. Various factors that influence the physiology of the plant may also affect pathogenicity.

The ability to induce disease often has been considered a single characteristic. In reality, pathogenicity is a combination of many characteristics, of which we can consider only a few. The microörganism must have a susceptible host that is growing in a suitable environment (especially temperature, moisture, light, and mineral nutrition). It must enter the host plant. It must establish itself inside by finding suitable nutrients and by overcoming any antagonistic factors encountered. It must come out of the plant again; it must find a means of transfer by one way or another to a new host; it must withstand the winters. Variations may occur independently in any one of these and comparable items. Without a suitable combination of such factors, no epidemic[1] will develop.

Unusual succulence of the host plant and certain other abnormalities sometimes may enable saprophytes to invade the tissue and to give the appearance of pathogens. Such conditions have led to erroneous conclusions. To insure against such conclusions, one should hold the environmental conditions for experimental inoculations as nearly as possible like those occurring in nature at the time of natural infection. When difficulty occurs with artificial inoculations, careful continued observation of the host plant at the time of natural infection may reveal the cause of the trouble.

The simpler methods for preparing and using both ordinary and differential media, for making isolations, and for studying the morphology and physiology of such bacteria have been adequately described in Leaflets II, IV, V, VI, and elsewhere. This Leaflet, therefore, is concerned primarily with methods of inoculation.

In advanced research, investigators working with pathogens, whether against plants, animals, or men, have many common interests. These include, for example, (1) life cycles, referring to changes in the morphology of individual cells and the relation of these different forms to virulence; (2) changes in colony characters and physiology, including particularly changes in pathogenicity; (3) factors attending changes, such as the time, frequency, and conditions of origin, as well as the influence of environment, and relations to earlier and succeeding generations; (4) statistical analyses to classify the origin and frequency of the variations observed; and (5) life histories of the pathogens in relation to entrance into the host, location, exit, and transmission to a new host.

Certain characteristics of plants not possessed by animals facilitate basic research on pathogenicity. These include: (1) Large numbers of hosts are easily available. The number used, whether 10 or 10,000,

[1]"Epidemic," in the original Greek meaning "on the people," was early applied to plant diseases, together with many other medical terms. It is an old and common word in plant pathology, although on etymological grounds its use for human disease alone is preferred by some authorities. This Leaflet follows the broad definition from Gould's Medical Dictionary, "Epidemic: of a disease affecting large numbers or spreading over a wide area."

is selected on the basis of experimental needs. (2) The initial costs and expenses of maintaining plants are relatively low. (3) The species of plants studied frequently contain varieties or selections possessing several degrees of resistance and susceptibility. (4) Plants are suited to a wide range of experimental procedures, such as regulation of internal temperature and moisture, that are not feasible with animals. (5) Epidemics are induced with relative ease and without concern for the health of the technician or the public. (6) The genetic purity of the host can be assured. Seeds from long lines of successively self-fertilized parents often are available. When this is not sufficient, one can commonly find or develop experimental units all genetically identical through vegetative propagation on their own roots. With such material, any variations secured can be studied without concern that the genetics of the host may have been obscuring pathogenicity. (7) Certain plant materials are being cultivated *in vitro* on media containing only nutrients for which the chemical formulae are known. This technique offers many interesting possibilities for investigations on the interactions of hosts and pathogens.

Simple Representative Inoculation Methods

The actual procedures for making inoculations vary with circumstances. Some simpler methods are considered briefly by way of illustration. Ways for testing the relative efficiency of several techniques are considered in a later section.

SOIL "INOCULATION"

The introduction of large numbers of pathogenic bacteria into the soil depends upon growing sufficient quantities in cultures, either on agar or in liquid media. Special flasks, bottles, and other containers having adequate flat surfaces are useful. Most plant pathogens are aerobic and need incubation under pronounced aerobic conditions for the best growth. When agar is used, the surface growth is washed or scraped off after sufficient growth has appeared, and a suspension is made. When a liquid medium is employed, a satisfactory bacterial count per cubic milliliter develops, with an organism like *Agrobacterium tumefaciens* (Smith and Town.) Conn., with a medium less than 2 cm. deep or with one well aerated by shaking or by some other means. Satisfactory aeration may be secured in deep liquid cultures by bubbling sterile air through a sintered glass or other aerator placed in the medium. In large containers aeration can be improved by a few pounds of pressure. This forces more air to dissolve in the liquid. Maintaining such pressure also reduces contamination from leaky valves. Chemicals that poise the oxidation-reduction potential may be helpful. The highest count of active bacterial cells may occur somewhat before the maximum turbidity is attained. Bacterial gum may cause considerable turbidity. Usually the whole culture is employed for soil treatment; but one should avoid adding too much extraneous matter with the inoculum. Such aerated liquid cultures also work well with many fungi.

Soil may be "inoculated" by pouring liquid suspensions on relatively

dry soil, by allowing the water to be absorbed long enough to avoid puddling, and by mixing. The quantity of culture used for each plant varies. One might begin with 1 part of culture to 10 parts of soil and use a handful of this mixture about the roots of each plant.

Inoculations through the soil are considerably more difficult than those with various other methods.

SEED INOCULATION

Perhaps the easiest way to infect a large population is through treatment of the seed. Legume root nodule bacteria from a fresh, active culture grown on agar are shaken into a water suspension and are commonly spread on the seed just before planting. Many commercial inocula are prepared by mixing the culture with some moisture absorbing powder, such as autoclaved ground peat. Wood flour is also quite absorbent, and contains almost no bacteria. If the seed is drill-sown, it is made only moist enough to "fix" the bacteria on the seed, and then dried sufficiently so as not to clog the drill. To secure uniform results it is best to use plenty of bacterial culture. Fred, Baldwin, and McCoy (1932) have reviewed this general subject.

SPRAY INOCULATION

Spraying is the method most commonly used to inoculate growing plants. It is particularly useful with bacteria that enter the host plants through natural openings such as stomata, water pores, and nectaries. For many simple tests, suspensions of bacteria are merely sprayed on the surfaces of susceptible leaves, stems, flowers, fruits, etc. For more exact tests, as for comparative virulence, one suspends the growth from an agar culture in water, saline solution (0.9% NaCl), or a selected buffer (such as suitable mixtures of dilute K_2HPO_4 and KH_2PO_4), and standardizes the concentration according to a selected and measured turbidity. If the bacteria have been grown in liquid culture, the entire culture may be used. This procedure, however, is often unsatisfactory because, after spraying, secondary organisms may grow in the nutrient medium. It is frequently better to separate the bacteria from the medium by means of a centrifuge and to resuspend the cells as with the growth from agar media.

The number of bacteria in a suspension may be determined, for example, (1) by a Breed count or by direct examination in a Petroff-Hausser counting chamber; or (2) by mixing a known volume of the bacteria with previously counted suspensions of yeast or red blood cells, by making smears, and by determining the relative number of bacteria and cells. Bacterial suspensions often are duplicated by comparing their turbidity with that of a graded series of barium sulfate standards (described by Riker and Riker, 1936). A common density for a bacterial suspension has the turbidity of a solution obtained by mixing 1 ml. of a 1% solution of barium chloride with 99 ml. of dilute sulfuric acid. Turbidity can be measured accurately and rapidly with a suitable instrument.

The prepared bacterial suspension is filtered through cheesecloth, to remove small pieces of agar or other materials which might clog the

spray nozzle, and is placed in the spraying device. The plants are sprayed so that good coverage is given especially to the lower sides of leaves which commonly have more stomata. A strong spray may force the bacteria through the stomata into the leaves. The plants are placed in an environment where they will not dry off for a number of hours.

Certain additional precautions may be necessary for best results. For example: (1) The relative humidity of the air surrounding the host plant is maintained at saturation before as well as after inculation. This and suitable light help to provide wide open stomata. The length of time necessary varies with the host plant and the parasite. A saturated atmosphere for 6 to 18 hours in both instances favors infection with many leaf parasites. Various kinds of moist chambers, e.g., that described by Keitt *et al.* (1937), can be used in the greenhouse. Small outdoor plantings can be covered for a short time with a cloth tent (Keitt, 1918) and water sprayed over the exterior. The amount of moisture in the air apparently influences the intercellular humidity and, correspondingly, the susceptibility of the host. (2) If the plant parts are difficult to wet because of a waxy covering, the surface can be gently rubbed with a moist cloth. For work on a large scale, the suspension of the organism can be made in a solution of a spreader (e.g., castile soap, 1 : 1000) to reduce surface tension. The concentration varies according to requirements. Some spreaders, however, are toxic. (3) A reduced oxygen supply may be important if the pathogen is a facultative anaerobe. For example, the protective wound-cork formation in potato tubers requires abundant oxygen, while certain bacterial pathogens, such as *Erwinia carotovora* (Jones) Holland, grow well with little oxygen. (4) Water pressure, suction, prolonged spraying, and other means can be used to saturate the intercellular spaces below the stomata and thus to improve the penetration of bacterial suspensions into these regions. This is particularly important with a pathogen, like that causing black fire of tobacco (Johnson, 1937), which is often not aggressive. With this method it is possible to induce necrotic areas on plants not ordinarily considered hosts of the microörganism used. Since bacteria that are usually considered saprophytes have caused damage under these circumstances, care is necessary while interpreting such results. For example, such saprophytes would hardly fulfill the first of Koch's postulates, as given earlier.

WOUND INOCULATION

Suspensions of bacteria, small portions of culture, or of diseased tissue can be introduced into healthy plants through wounds when they do not readily gain entrance through natural openings or when heavier or more rapid infection is desired. The simplest procedure is to smear the point of a dissecting needle with the bacterial mass and to insert the needle into the plant tissue. If large numbers of inoculations are to be made, various instruments are useful. For example, Ivanoff (1934) has described an inoculator in detail. It consists of a hypodermic needle (size varied according to needs) with end closed and smooth-walled opening made above the point, of a suitable chamber to hold a bacterial suspension, and of a valve to regulate the flow. This

needle with a side opening may be used with an ordinary syringe. The common hypodermic needle when pushed into a plant clogs too easily to be practical.

Known small numbers of bacteria may be introduced into micro-wounds by means of a micromanipulator. Such wounds may resemble those made by insects (Hildebrand, 1942).

INSECT INOCULATION

The translocation of microörganisms causing plant disease and their introduction into susceptible plants by insects is a large and relatively undeveloped field. The simplest technique with active insects like cucumber beetles or leafhoppers is merely to place the plant to be inoculated in the same insect cage with an infested diseased plant (Leach, 1940).

For virus diseases, inoculation with slow-moving insects, like aphids, is accomplished by placing a paper on a caged plant to be inoculated, and by laying on this paper a portion of a diseased leaf which carries aphids. As the new leaf tissue dries, the insects crawl over the paper to the fresh leaf below. When insects are involved, a variety of special cages (Leach, 1940) may be necessary.

All stages in the life cycle of the insect employed must be considered because inoculation capabilities often vary in this respect. The insect should be identified by a competent authority, and if significant results are obtained, a specimen should be deposited in a permanent reference collection.

A detailed discussion of methods for studying insect transmission has appeared (Leach, 1940). Some knowledge of the mouth parts of insects and of their feeding and breeding habits is necessary if insects are to be used successfully for inoculating bacterial plant pathogens. Sometimes they merely open infection courts for the microörganisms to enter. Aphids and leafhoppers are particularly important as carriers of virus diseases.

Before claims are made about insect transmission of a plant disease, demonstrations of the following (Leach, 1940) seem a minimum for proof: (1) close, but not necessarily constant association of the insect with diseased plants; (2) regular visits by the insect to healthy plants under conditions suitable for the transmission of disease; (3) presence of the pathogen or virus in or on the insect in nature or after visiting a diseased plant; (4) experimental production of the disease by insect visitation under controlled conditions and with adequate checks.

FUNGUS INOCULATION

In general, inoculations with the spores or mycelia of fungi differ only in detail from those made with bacteria. For pathogenic fungi, variations in the mode of entrance and in other important characters require modified procedures. Some of the more common methods are discussed by Riker and Riker (1936).

VIRUS INOCULATION

Brief mention is given to inoculations with viruses without implication that they are microörganisms. Experimental inoculations are

more commonly accomplished by mechanical processes, insects (see Insect Inoculations), and grafting.

Mechanical inoculation of a virus frequently is made by grinding diseased tissue in a mortar with a little water and by rubbing the juice lightly over leaves of the host plant. With some viruses, the following modifications may be helpful. A favorable reaction between pH 7.0 and 8.5 may be obtained by placing a little M/10 K_2HPO_4 in the mortar before the leaves are triturated. Sometimes viruses have to be protected from rapid oxidation by means of 0.5% anhydrous Na_2SO_3. Just enough friction by a finger, cheesecloth, or similar agent is employed to injure the leaf hairs. With viruses difficult to transmit, better infection may be induced if a fine abrasive material (e.g., carborundum powder, 600 mesh) is lightly dusted on the leaf before it is rubbed. Some plant viruses are highly infectious. (Usually washing with soap and water is sufficient to remove infectious material from the technician's hands.) When the mechanical methods and insect vectors fail, two possibilities are left.

Budding or another form of grafting may be employed and is sometimes the only successful means of virus transmission. When grafts are made, special precautions are necessary to prevent desiccation of the grafted parts before union has been accomplished. This may be achieved by providing high air humidity, by suitable wrappers, or by spraying the scions with one of the commercial wax emulsions.

Some investigators use dodder to carry certain viruses not otherwise transmitted from one plant to another.

TREATMENT WITH BACTERIAL PRODUCTS

The metabolic products found in bacterial cultures are prepared and employed in a variety of ways which are not yet well worked out. Perhaps the least change occurs in the bacterial cells if they are centrifuged from a liquid culture and dried while frozen. The culture filtrate may be concentrated under reduced pressure at a little above room temperature and then "lyophilized" if desired.

A fermented culture or an aqueous extract may be sterilized and placed in a small container. If leaves with petioles or growing tops are removed from the host plant and are placed with the cut surfaces in such liquids, they commonly show injury within one day or two if much toxic material is present. Bacterial contamination may be reduced if the pH is approximately 4.0. A rigid control of temperature and relative humidity is essential to repeat the results. Care is necessary while interpreting such injury because many constituents of media may be toxic, e.g., ammonia in alkaline material or mineral salts. Likewise, many nonparasitic as well as parasitic fungi produce toxic substances in culture that are not necessarily the reason for pathogenicity.

The metabolites are sometimes applied either in liquid form or in a paste made with inert material, like lanolin, a polymer of ethylene glycol, or flour. The paste has the advantage of furnishing a continuous supply of material over a longer period with relatively less desiccation. It is commonly applied to a wound. The liquid can be introduced into the vascular system of a potted plant by placing cut roots extending

from the base of the pot, or a cut petiole, into a container of the material. Likewise, a cup can be made from a rubber stopper and sealed on a plant stem with vaseline. The cup is filled with liquid, under which a cut is made into the vascular system, so that the liquid is taken by the plant directly into the transpiration stream. The stem can be opened to form a small cavity which is kept filled by means of a capillary tube and funnel. If an enzyme like pectinase is being tested, thin sections of tissue need merely be immersed in a few drops of the liquid.

So many substances appearing in cultures influence plants in one way or another that rigid controls are necessary in searching for the products responsible for pathogenicity. Whenever feasible, an attenuated culture of the same organism or a closely related nonpathogenic culture is carried in a parallel series of trials.

The methods of testing for plant "hormones" and "vitamines" are being improved so rapidly that one should consult an active investigator for the latest procedure.

ANTIBODY PRODUCTION

Questions on the development of antibodies in plants following inoculation or natural infection are discussed in a considerable literature reviewed by Chester (1933; 1935). A number of controversial points are involved.

The injection of plant bacteria into an experimental animal commonly results in the production of antibodies useful for various investigations. Suitable methods appear in Leaflet VIII.

Cognate Considerations

STRAIN VARIATIONS

When studies involving strain variations are made, it is well to consider Frobisher's (1933) comment, "Plating and fishing of colonies, while generally useful, is not a sufficiently reliable method of purifying cultures in work involving bacterial variations. It is sometimes extremely difficult, if not impossible, to separate bacterial species by this means. Single-cell methods are much more reliable and, it would seem, furnish the only satisfactory means of solving our problems, but even such procedures as are at our disposal require very expert manipulation and may lead to error." The relative unreliability of the poured-plate technique for such studies has been discussed by Riker and Baldwin (1939). The need for cultures with a known origin from a single cell has stimulated much work on methods for securing them. Literature on this work has been reviewed by several writers, e.g., Hildebrand (1950). Unfortunately, some reports on bacterial variations have appeared in which the cultures were purified merely by several successive dilution plates, and such purified cultures were called "single-cell cultures." This misleading use of a well-established phrase provides both the investigator and the reader with a false sense of security.

Variations may be induced among plant pathogens by procedures very similar to those employed on other bacteria. Some of the considerations involved in such studies are discussed by Riker (1940).

The pathogenicity of crown gall bacteria can be destroyed (Van Lanen, Baldwin, and Riker, 1940) with certain amino acids and related compounds added to common media. Attenuation was commonly secured in 20 to 30 successive transfers. The rate of attenuation was increased if bacterial growth was retarded by the strength of the compound (e.g., 0.1 to 0.3% glycine) and by an alkaline reaction (e.g., pH 8.0).

The virulence of partly attenuated cultures was restored by long cultivation on suitable media and by ultra violet irradiation (Duggar and Riker, 1940). Likewise, when a virulent culture was inoculated into a tomato stem above an inoculation with an attenuated culture, the gall about the attenuated culture was approximately as large as that about the virulent culture. A gall induced by a plant hormone served as well as that from a virulent culture (Riker, 1942).

PATHOGENS ACTING TOGETHER

Combinations of microörganisms sometimes induce symptoms different from those caused by any one alone. So long as the pathogens can be cultivated on artificial media, the principles in Koch's postulates can be applied with two or more causal agents. For example, a simple inoculation with one organism may involve a series of susceptible plants growing in a suitable environment with the living causal agent, and a parallel control series. With two causal agents, however, there should be four series of plants as follows: (1) with both living pathogens, (2) with only one living pathogen, (3) with only the other living pathogen, and (4) with neither living pathogen. Correspondingly, three causal agents would require eight series of plants.

One should not overlook the fact that in nature pure cultures seldom exist except in the most advanced margin of the lesion.

CULTURES FROM ANOTHER LOCALITY

The use of a culture of a pathogen not already present on local plants requires critical consideration. The progress of bacteriology calls for reasonable freedom in the movement of cultures. This science, however, has a duty in the protection of local plant populations and requires that cultures or strains brought into a new locality should be handled with proper consideration of all the factors involved. It must be insisted that cultures be secured and studied only after both the investigators and their administrators have fully considered and accepted the responsibilities involved. Younger research workers and particularly graduate students are advised to employ such cultures only after detailed plans have been made in conference with their advisors.

Various laws apply to the shipment of infected material.

RELATIVE EFFICIENCY IN TECHNIQUE

The best methods of procedure for making inoculations and for recording results have not always been worked out and are not obvious from inspection. If the question is of sufficient importance, the answer may be secured statistically. A doubt may appear, with a leaf-spot organism, for example, whether to spray or to make needle punctures.

Likewise, when infection develops, the question may occur whether the results should be recorded in terms of total number of lesions, of total tissue involved, the effect of the disease on yield, or of some other criterion. Such possibilities may be tested by means of the frequently described "analysis of variance." Thus the best method for making the trials and for recording the results may be determined. In general, the method that gives the greatest value for the variance ratio, "F", is the most desirable. This value indicates a greater uniformity in readings from different trials with the same technique, or a greater differentiation of the varieties used or treatments employed without a proportional increase in error.

ANTIBIOTICS

Numerous recent reports have shown that various higher plants and saprophytic microorganisms produce substances that adversely affect certain bacteria. Many instances occur in which various higher and lower forms of plant life make chemicals that inhibit successful plant inoculations.

To secure the latest methods for plant diseases, one should consult an active investigator in this rapidly developing field.

RECORDS

Taking notes on plant inoculations presents various problems depending upon the experiment in hand. To assist with such records, a tentative protocol appears in Table 1. For some lines of work it is obviously too complex while for others it is clearly too simple.

TABLE 1. TENTATIVE PROTOCOL FOR PLANT INOCULATIONS

- Host:
  - Variety .....
  - History .....
  - Age .....
  - Morphological condition .....
  - Stomata open .....
  - Physiological condition .....
  - Susceptibility .....
  - Environment .....
  - Treatment before .....
  - Treatment after .....
- Pathogen:
  - Strain .....
  - History .....
  - Culture on .....
    - at .....°C.
    - for .....days
- Inoculum used:
  - Diseased tissue .....
  - Entire culture .....
  - Bacteria:
    - Turbidity .....
    - Number per ml. .....
  - Filtrate .....
  - Products .....
  - Amount used per plant.....
- Manner of inoculation:
  - Through soil .....
  - Through wounds.....
  - By sprays .....
    - Spreader used .....
  - By insects (name) .....
    - Stage in life cycle .....
  - Other means .....
- Incubation:
  - Time .....
  - Environment:
    - Temperature .....
    - Moisture .....
    - Light .....
    - Intensity .....
    - Length of day .....
    - Soil nutrients .....
- Symptoms:
  - Location .....
  - Age of parts affected .....
  - Severity .....
  - Description:
    - Early .....
    - Medium .....
    - Final .....
- Effect on yield:
  - Quantity .....
  - Quality .....

A number of the items listed for records may be critical factors for the success or failure of an experiment. Since each one cannot be discussed, several examples are mentioned. (1) Infection may fail if the incubation temperature is either too low or too high. Many plant pathogens operate best between 18° and 30° C. (2) Plenty of moisture is usually important for disease development, a deficiency of water often being responsible for negative results. (3) The age of the plant or of the part inoculated may influence the result. The relatively young leaves are frequently more susceptible than old leaves to bacterial leaf spots. (4) Some varieties of plants are highly resistant to pathogens which readily attack other varieties. Similarly, different strains of bacteria often vary in pathogenicity.

INTERPRETATION OF RESULTS

The results of research are valid only in accord with the reliability of the methods employed and the accuracy of their interpretation. After an experiment has been performed it is insisted that a report of such work must not be published for the use of others until repeated determinations have been made and the results have been satisfactorily analyzed. The simpler experiments are commonly performed with suitable controls at least in duplicate or triplicate, and carried through three separate times. A good investigator does not become so enthusiastic about an experiment that he fails to view it impartially and to accept sound evidence against it. On the contrary, he makes every reasonable effort before publishing to find an error in the experiment itself or in the conclusions drawn from it.

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INDEX

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INDEX[1]

Acetyl-methyl-carbinol, determination of ..... V_{42} 29
Acid dyes ..... IV_{54} 2
Acid-fast staining ..... IV_{54}, 9-10; V_{54} 10
Acid fuchsin ..... II_{50} 17
Acid production in milk ..... V_{54} 25
Acids, fatty, determination of ..... VI_{52} 8
Acidity, determination of ..... IX_{42} 15-17
Acidity, titratable ..... IX_{42} 15-16
Aerobes, media for ..... II_{50} 2
Aerobes to absorb oxygen ..... III_{42} 6
Agar, ammonium phosphate ..... II_{50} 17
  beef-extract (nutrient) ..... II_{50} 7
  blood ..... II_{50} 13
  egg yolk ..... II_{50} 14
  meat infusion ..... II_{50} 7
  nitrate ..... II_{50} 18
  semi-solid ..... II_{50} 8
  sodium caseinate ..... II_{50} 7
  specifications of ..... II_{50} 6
  starch ..... II_{50} 18
  sugar ..... II_{50} 16-17
Agglutination ..... $VIII_{47}$ 3, 5-9
  as test for obligate anaerobes ..... III_{42} 13
Agglutination test, macroscopic,
    preparation of antigen for ..... $VIII_{47}$ 6
    procedure for ..... $VIII_{47}$ 7-9
    tabulation of ..... $VIII_{47}$ 7
  microscopic, procedure for ..... $VIII_{47}$ 6-7
Agglutinin absorption ..... $VIII_{47}$ 9-10
Albert's diphtheria stain (including Laybourne modification) ..... IV_{54} 13
Alcohols, cleavage of ..... V_{54} 18-22
Alkaline egg medium ..... II_{50} 18
Alkaline gentian violet (Kopeloff & Beerman's) ..... IV_{54} 7
Amboceptor, anti-sheep cell ..... $VIII_{47}$ 14
Amino acids, estimation of ..... VI_{52} 12
Ammonia, determination of ..... VI_{52} 22
Ammonia tests ..... VI_{52} 22
Ammonium oxalate crystal violet ..... IV_{54} 7
Ammonium phosphate media (liquid and agar) ..... II_{50} 17
Ammonium salts, action of bacteria on ..... VI_{52} 21
Anaerobic bacteria ..... III_{42} 3-23
  enrichment of ..... III_{42} 16
  fermentation reactions of ..... III_{42} 12
  inoculation technics ..... III_{42} 9-20
  isolation methods for ..... III_{42} 18-19
  media for ..... II_{50} 3, 12-13
  microscopic examination of ..... III_{42} 14-16
  morphology of ..... III_{42} 14
  motility of ..... III_{42} 14
  pathogenicity tests for ..... III_{42} 12
  preliminary culture of ..... III_{42} 3-5
  presumptive tests for ..... III_{42} 5
  putrefactive changes of ..... III_{42} 11
  separation from aerobes ..... III_{42} 13, 18-19
  serological reactions ..... III_{42} 13
  spore formation of ..... III_{42} 15
  storage of ..... III_{42} 20
  toxin production by ..... III_{42} 21

[1] In this index Roman numerals refer to the leaflet number, inferior Arabic numerals to the year of the edition cited, and large Arabic numerals to the page of the leaflet

Andrade's indicator ..... II_{50} 17
Anilin gentian violet (Ehrlich) ..... IV_{54} 6
Animals, laboratory, autopsy of ..... VII_{48} 9-10
  use of ..... VII_{48} 5
Anthony's capsule stain ..... IV_{54} 18
Antibiotics, production of, media for ..... II_{50} 20
Antibody, definition of ..... $VIII_{47}$ 4
Antigen(s), definition of ..... $VIII_{47}$ 4
  for complement fixation test, preparation of ..... $VIII_{47}$ 14
    titration of ..... $VIII_{47}$ 16-17
  for macroscopic agglutination test, preparation of ..... $VIII_{47}$ 6
Antigenic variations ..... VII_{48} 11
Antitoxins, titration of ..... $VIII_{47}$ 19-20
Autopsy of laboratory animals ..... VII_{48} 9-10

Bacteremia ..... VII_{48} 8
Bacterial genetics ..... VI_{52} 16
Bacterial smears, preparation of ..... IV_{54} 2
Bacterial stains, general ..... IV_{54} 4-6
Bacteriostasis ..... II_{50} 18
Bailey's flagella stain (Fisher and Conn modification) ..... IV_{54} 17
Bartholomew and Mittwer's "cold" spore stain ..... IV_{54} 12
Basic dyes ..... IV_{54} 2
Basic fuchsin ..... IV_{54} 4, 5, 9
Beef-extract agar ..... II_{50} 7
  broth ..... II_{50} 6
  gelatin ..... II_{50} 18
Beef heart infusion medium ..... II_{50} 12
Beef liver infusion medium ..... II_{50} 8
Bioassays, synthetic media for ..... II_{50} 21
Biochemical methods ..... V_{54} 12-26; VI_{52} 3-23
  in study of pathogenic properties ..... VII_{48} 13
Blood agar ..... II_{50} 13
  corpuscles, red, sheep's, preparation of ..... $VIII_{47}$ 14
  culture ..... VII_{48} 8-9
  serum, coagulated ..... II_{50} 18
Böhme test for indole ..... V_{54} 15
Brain medium ..... II_{50} 8
Brewer anaerobic jar ..... III_{43} 10
Brewer culture dish ..... III_{43} 12
Brom cresol green ..... II_{50} 4; V_{54} 21; IX_{48} 7, 8
Brom cresol purple ..... II_{50} 4; V_{54} 21; IX_{48} 7, 8, 13
  in milk ..... V_{54} 25
Brom phenol blue ..... II_{50} 4; V_{54} 21; IX_{48} 7, 8, 13
Brom phenol red ..... II_{50} 4
Brom thymol blue ..... II_{50} 4; IX_{48} 7, 8, 13, 17
Broth, beef extract (nutrient) ..... II_{50} 6
  carbohydrate ..... II_{50} 16
  glucose ..... III_{37} 4
  meat infusion ..... II_{50} 7
  nitrate ..... II_{50} 18
  thioglycollate ..... II_{50} 7
  yeast extract ..... II_{50} 8
Brucella, media for ..... II_{50} 15
Buffer action ..... IX_{48} 16
Buffer standards ..... IX_{48} 9-11
Burdon's stain for fat droplets ..... IV_{54} 19
Burke's modification of Gram stain ..... IV_{54} 7-8
2,3-Butylene glycol, determination of ..... VI_{52} 20

Calomel electrode ..... IX_{48} 2-4
Calomel half-cell ..... IX_{48} 19
Capsule stains ..... III_{43} 15; IV_{54} 17-19; V_{54} 10
Carbohydrates, cleavage of ..... V_{54} 18-20
Carbohydrate media ..... II_{50} 16-17
Carbol-fuchsin (Kinyoun's or Ziehl's) ..... IV_{54} 4, 5, 9
Carbol gentian violet (Nicollé) ..... IV_{54} 5
Carbon and oxidation-reduction balances ..... VI_{52} 8