Antibiotics

That Come

From Plants

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An antibiotic is an organic chemical substance that is produced by a plant, an animal, or a micro-organism. It selectively checks the growth of bacteria, viruses, fungi, and other disease-producing organisms, or completely destroys them. The term is new, but the idea that such substances can cure diseases is as old as the hills.

Investigations since 1943 have disclosed that many plants, plant parts, and plant extracts have therapeutic values (a point that primitive man knew) and contain antibiotic agents (a point that primitive man might have suspected). Among them are the lotus, olive, laurel, myrtle, asphodel, and garlic, which were used as medicines in ancient Assyria; dates, figs, onions, lettuce, crocus, and opium, used as remedies by the Egyptians; the juices of celery, parsley, asparagus, peppers, and cabbage, favored as a medicine by Greeks and Romans; and amber, musk, manna, cloves, peppers, rhubarb, nutmeg, camphor, croton oil, and nux vomica, which the Arabians introduced and told their neighbors to take for aches and pains.

Only a few antibiotics so far have been isolated from the higher plants in pure form. The detection, isolation, characterization, and evaluation of antibiotics require the coordinated efforts and experiences of plant physiologists, bacteriologists, chemists, and medical men.

The differences in the types of plants investigated, the available quantities of particular varieties, and the kinds of equipment used for processing mean that the methods of extracting plant antibiotics are almost as numerous as the groups of scientists here and abroad who are investigating them. The organisms against which the extracts are tested are varied and many, too.

As to procedure, the investigators disagree. One group believes that only fresh green plants should be used, because, they say, plants should be harvested quickly and handled immediately before enzymatic processes can destroy the antibiotic substances or produce toxic byproducts. In theory, that method is preferred; it can be conducted successfully on a small scale.

Another group works almost entirely with dried material; the commercial preparation of an antibiotic would almost always require the drying or longer handling of the plant material.

In our laboratory, we use some features of both methods. We first assay the extracts of freshly cut green plants and, having detected antibiotic activity, we then dry another portion in a forced-draft oven, usually at 85° C., for extraction. If the antibiotic material is not destroyed by the drying process, we dry a quantity sufficient for work during the winter, when the fresh plants may not be available. We have found it desirable, if possible, to separate the plants into their various parts—roots, leaves, stems, and flowers—since the antibiotic may be present in one or more but not in all parts of the plant. Plants may be extracted with water, saline solution, dilute acid or alkali, or organic solvents, such as alcohol, acetone, and ether. The use of lower-boiling solvents is preferred in order to avoid excessive heating when concentrating the extracts.

The detection and measurement of antibiotic activity can be accomplished by a number of methods.

In the cylinder-cup and paper-disc methods, a Petri dish containing a solid nutrient agar is inoculated evenly with the micro-organism. In the first, cylinders, made of porcelain, glass, or stainless steel, are then placed on the inoculated agar, and the plant extract is pipetted into the cylinders. In the other method, paper discs are immersed in the plant extract long enough to become saturated with the solution, after which they are placed on the inoculated agar. The agar plate is incubated for a fixed period of time at a temperature that is optimum for the growth of the organism. Any zones of inhibition around the cylinders or discs are measured to obtain a relative indication of the amount of antibiotic agent in the extract.

The serial dilution and streak (or transplant) methods differ from the first two mainly in that graded amounts of the plant extract to be tested are incorporated in the nutrient medium, and the micro-organism is then added.

We use all of these methods in our survey work and in purification of antibiotic agents present in plant extracts. We find it advantageous to use routinely several typical test organisms— the gram-positive bacterium *Staphylococcus aureus,* the gram-negative bacterium *Escherichia coli,* the fungus *Fusarium oxysporum* f. *lycopersici,* and the acid-fast bacterium *Mycobacterium phlei.* Plant extracts may inhibit the growth of one or more or all of the organisms, but usually when a crude plant extract inhibits the growth of all four the presence of more than one antibiotic in the plant is indicated.

The widespread antibiotic activity in plants is attested by the published reports of various investigators. At Oxford University, E. M. Osborn in 1943 tested the antibiotic activity of water extracts from 2,300 species of plants that belong to 166 families; she used *Staphylococcus aureus* and *Bacterium* (*Escherichia*) *coli* as test organisms, and found that 63 genera contained substances that inhibited the growth of one or both test organisms.

In the botanical and bacteriological laboratories at Indiana University, D. W. Sanders and his associates tested the juices from 120 plant species, collected mostly in Indiana, using *Escherichia coli* and *Bacillus subtilis* as test organisms. Twenty-two of the plants showed varying degrees of bacterial inhibition.

H. J. Carlson and his associates at Western Reserve University extracted and tested more than 200 plants, collected in Oregon, in an attempt to separate substances inhibitory to malaria parasites, bacteria, and viruses. Many of them were found to contain substances that were bacteriostatic or bactericidal to micro-organisms *in vitro,* i. e., in laboratory test. Extracts prepared from five of the plants— buttercup, sagebrush, the mountain pasque, dwarf waterleaf, and juniper— were evaluated by many comprehensive tests *in vivo* (i. e., in experimental animals) and by *in vitro* tests. Salt extracts of all five plants were found to have antibacterial and antimalarial activity *in vitro.* Two of the plants, sagebrush and dwarf waterleaf, contained substances that protected chickens during the blood phase of malaria, and an ether-insoluble, water-soluble fraction of a steam distillate of mountain pasque protected mice heavily infected with pneumococcus organisms (*Diplococcus pneumoniae* type 19). Extracts obtained from a species of sumac, *Rhus hirta,* showed marked bacteriostatic activity against gram-negative bacteria but were less effective in inhibiting the growth of gram-positive bacteria and fungi.

L. E. Hayes studied 231 plant species, collected in Ohio, as possible sources of antibiotics. Extracts from 46 inhibited the growth of one or more species of the test organisms—*Staphylococcus aureus, Escherichia coli, Erwinia carotovora,* and *Phytomonas tumefaciens.*

About 100 plants listed in the Indian Pharmacopoeia for the treatment of diseases that are definitely of bacterial origin have been found by Mariam George and associates of the Indian Institute of Science to contain antibiotics. R. R. Rao and associates reported that garlic extract in low concentration was bacteriostatic *in vitro* against the *Mycobacterium tuberculosis* organism.

At the University of Vermont, Thomas Sproston, Jr., and associates tested 73 plant extracts, directing particular attention toward those that show fungicidal or fungistatic properties. The most active antifungal extracts were obtained from *Impatiens* (wild touch-me-not), *Cucumis melo* L. (muskmelon), and *Tropaeolum majus* L. (nasturtium). The crystalline antifungal agent isolated from *Impatiens* was 2-methoxy-1,4-naphthoquinone.

At Columbia University, B. C. Seegal and M. Holden found that the pressed juice or the steam distillate from the pressed juice of buttercup is a strong antibiotic with a wide range of activity. The extracts were effective *in vitro* against selected gram-positive and gram-negative pathogenic cocci and bacilli, against *Mycobacterium tuberculosis*, and against three yeasts, two of which are potential human pathogens. The toxicity of the active principle, protoanemonin, however, is sufficient to preclude its use as an effective therapeutic agent.

At the New York State Agricultural Experiment Station, C. S. Pederson and Paul Fisher noted that the undesirable gram-negative aerobic bacteria on the surface of cabbage leaves ordinarily disappear shortly after the cabbage is cut. They attributed this change in the number of micro-organisms to the presence of bactericidal substances in the cabbage tissue, which caused marked reduction in the number of gram-negative bacteria in 6 to 24 hours. The extract was less active toward gram-positive bacteria and was inactivated by heat.

We have tested some 300 plant ex-

tracts, and of these approximately 50 percent showed inhibition against one or more of the representative gram-positive, gram-negative, and acid-fast bacteria or fungi.

In the preliminary survey of plants for antibiotic activity, we use methanol as a solvent in the preparation of extracts. The methanol extract is evaporated to dryness on a water bath under reduced pressure, and water is added to the residue until 1 milliliter of water extract represents 1 gram of dry plant material. Any water-insoluble material is removed by centrifuging, and the supernatant solution is poured off and tested for antibiotic activity. If any is found, the stability of the antibiotic principle or principles to heat is determined by autoclaving a portion of the aqueous extract for 15 minutes at 15 pounds steam pressure.

THE MANY UNIDENTIFIED antibiotic principles of the higher plants vary greatly, not only in their potency and distribution within the plant but also with the species and variety of plant. For instance, E. H. Lucas and R. W. Lewis at Michigan State College reported that the active principles found in the scarlet berries of *Lonicera tatarica,* one of the honeysuckles, were not present in species and varieties of honeysuckles having dark-red, orange, yellow, or purple fruits. C. S. Pederson and Paul Fisher, in their investigation of the bactericidal action of cabbage juice, found that four of the varieties tested showed very marked bactericidal action, five showed intermediate effect, and four showed still less effect. E. M. Osborn concluded from her survey of 2,300 plants that specificity and activity of plant antibiotics were similar among members of the same family. Our own tests on unpurified plant extracts did not indicate any great difference among species and varieties of plants in the same family. There may be differences in antibiotic activity due to climatic conditions, but, so far as we know, none of the investigators in the field has studied this phase intensively.

The ultimate goal of any scientist working on the production of antibiotic substances from plant materials is the preparation of the active principle in a pure and crystalline form. The structure and biological properties of the crystalline antibiotic can then be determined, and its synthesis in the laboratory may possibly be accomplished.

The method of isolation varies with the nature of the antibiotic. Some of the substances are acidic, some neutral, some basic, and some proteinaceous.

The compounds in the acidic group can be extracted at low pH by organic solvents immiscible with water. The basic compounds can be precipitated by base precipitants. Some antibiotics are purified by extracting the plant material with one solvent and then distributing the crude extract between two immiscible solvents. The plant material may also be extracted with a suitable solvent, after which the extract is concentrated and run through a chromatographic column containing an adsorbent capable of removing the active substance. The antibiotic may then be removed from the adsorbent with a suitable solvent, purified, and crystallized. The pure crystalline compound may be identified by determining its chemical constituents and ascertaining its physical and chemical properties. Determination of the spectrum, color reactions, with various reagents, sulfhydryl inactivation, and other special methods are useful in characterizing an antibiotic.

THE FOREGOING description of the procedure for isolating a pure antibiotic has necessarily been very general, as the methods vary with the compound isolated. A few examples will be cited briefly.

In England, N. G. Heatley and associates isolated a crystalline antibiotic from *Crepis taraxacifolia* by adsorption of the active principle from an aqueous extract on charcoal and further purified it by adsorption on alumina. The suggested chemical formula for the antibiotic, named crepin, is $C_{14}H_{16}O_4$, and it is designated as a beta, gamma unsaturated lactone. The crystals are polymorphic, soluble in alcohol and ether, very slightly soluble in water, heat stable, acid stable, and alkali unstable. Crepin is most active against gram-positive bacteria, but its activity is diminished by serum. It destroys the motility of leucocytes at a concentration of 1:450,000 and is lethal at higher concentrations.

Another antibiotic principle was isolated from *Spiraea aruncus* L. (goat's beard). This crystalline compound is thought to be an alpha, beta unsaturated lactone with a suggested formula $C_{10}H_{14}O_4$. It has very low activity against gram-positive and gram-negative bacteria.

C. J. Cavallito and J. H. Bailey of the Sterling-Winthrop Research Institute have isolated antibiotic agents from the following plants: *Arctium minus* (common burdock), *Asarum canadense* (wild ginger), *Allium sativum* (garlic), and *Centaurea maculosa* (spotted knapweed). A crystalline antibiotic from *Arctium minus* was obtained by fractionation of a water extract and subsequent crystallization from ethyl acetate. The suggested formula for this compound is $C_{15}H_{20}O_5$, and it probably is an unsaturated lactone. The crystals are colorless prisms soluble in alcohols, chloroform, ethyl acetate, dioxane, and acetone; slightly soluble in water; insoluble in petroleum ether; optically active; melting at 115° to 117° C. and resolidifying to lose antibiotic activity. The antibiotic is irreversibly inactivated by cysteine. It is active only against gram-positive bacteria. The LD_{50}—that is, the amount that will kill 50 percent of the animals—for mice is 90 milligrams per kilogram intravenously. The substance is not lethal but is toxic at 500 mg./kg. when administered orally.

Two antibiotic principles were isolated from *Asarum canadense* (wild ginger). They are designated as compounds *A* and *B*, with the suggested formulas $C_{12}H_{20}O_8N_2S$ and $C_{16}H_{11}O_7N$, respectively. Both compounds are al-

most insoluble in water, benzene, and petroleum ether, but are soluble in alcohol, acetone, chloroform, ethyl acetate, and dioxane. Both are active only against gram-positive bacteria. Preliminary toxicity tests indicate A to be lethal in about 3 days after intraperitoneal injection of 5 mg./kg. in sesame oil.

Allium sativum (garlic) yielded a sulfur-containing antibiotic, which was equally active toward gram-positive and gram-negative bacteria. The compound was isolated as a colorless liquid, stable to acid but unstable to heat and alkali, soluble in petroleum ether, and irreversibly inactivated by cysteine. The exact formula for this compound is not known, but two are suggested:

$$CH_2{=}CH - CH_2 - S - S - CH_2 - CH{=}CH_2$$
$$\overset{\|}{\underset{O}{}}$$

or

$$CH_2{=}CH - CH_2 - S - O - S - CH_2 - CH{=}CH_2$$
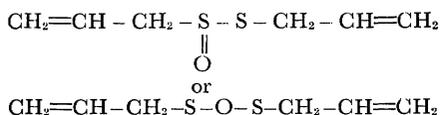
In toxicity tests with mice, the LD_{50} was 60 mg./kg. intravenously and 120 mg./kg. subcutaneously.

The dried leaves of the spotted knapweed, *Centaurea maculosa,* yielded about 1.5 percent of an antibiotic which is active against gram-positive and gram-negative bacteria. The suggested formula for this unsaturated lactone is $C_{20}H_{26}O_7$. Its activity is rapidly destroyed by cysteine and thioglycolate.

Two compounds, lupulon and humulon, isolated from the resins of mature hops, were recently found to have antibiotic activity toward gram-positive and acid-fast bacteria (*Mycobacterium tuberculosis*) in research on hops utilization at the Western Regional Research Laboratory.

We have investigated the antibiotic principles in the tomato plant. The total substance (or substances) in crude tomato plant extracts that exhibited antibiotic activity was named tomatin. Tomatin inhibits both gram-positive and gram-negative bacteria, but most noteworthy is its ability to inhibit certain fungi that are pathogenic to plants or animals. Included among these are three plant-wilt organisms—*Fusarium oxysporum* f. *lycopersici* (tomato wilt), *Fusarium oxysporum* f. *pisi* (pea wilt), and *Fusarium oxysporum* f. *conglutinans* (cabbage yellows)—and six fungi that are pathogenic to human beings—*Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum, Epidermophyton floccosum, Trichophyton mentagrophytes,* and *Candida albicans.*

Fractionation of the tomatin extract has resulted in the separation of an individual crystalline antibiotic, tomatine, which has antifungal activity but little or no antibacterial activity. Rutin, a flavone glycoside, also has been isolated from the extract. Rutin does not have antibiotic activity, but its degradation product, quercetin, does have antibacterial properties. Therefore, we assume that at least part of the antibacterial activity exhibited by tomatin is due to the presence of a small amount of quercetin.

Tomatine was isolated from a crude tomatin concentrate by repeated precipitation from an alkaline solution, dissolving the alkaline precipitate in hot 70-percent ethanol, and cooling, thereby precipitating an amorphous substance, which was dried and then dissolved in hot 80-percent dioxane. Crystalline tomatine was obtained on cooling the dioxane solution.

Tomatine has been characterized as a glycosidal alkaloid. It is soluble in ethanol, methanol, dioxane, and propylene glycol; it is almost insoluble in water, in ether, and in petroleum ether. It appears to be stable in strong alkali, but is readily hydrolyzed in boiling hydrochloric acid solution to yield an insoluble crystalline product, tomatidine hydrochloride, and a clear supernatant solution rich in reducing sugars. Tomatine melts at 263° to 267° C. with decomposition.

Tomatidine hydrochloride is easily converted to tomatidine, which is now considered to be a steroid secondary amine. In cooperation with the National Institutes of Health, tomatidine has been chemically degraded to a sterol,

Δ16−*allo*−pregnen−3(β)−ol−20−one. This compound may prove to be valuable starting material in the synthesis of biologically active sterols.

Crystalline tomatine and its aglycone, tomatidine, are more effective against the pathogenic fungi associated with human diseases than against the fungus, *Fusarium oxysporum* f. *lycopersici,* that causes the tomato wilt. They are almost completely without effect on the gram-negative bacterium *Escherichia coli* and are very slightly effective against the gram-positive bacterium *Staphylococcus aureus.* The oral toxicity of these compounds has been determined. Essentially no toxic effects have been observed when albino rats were fed diets containing up to 0.04 percent of these two compounds for 200 days; likewise, subacute and acute oral toxicity results were favorable. However, they are very toxic if administered by intravenous injection.

We have found that sweetpotato vines, which are sometimes used as silage, contain highly active antifungal and antibacterial substances. It is perhaps significant that the edible tuber also contains these substances. From an active water-soluble resinous fraction, a buff-colored crystalline-appearing solid and a clear red-brown liquid, with a distinctively characteristic odor, have been obtained. The solid material exhibits selective activity toward the gram-negative bacterium (*E. coli*), and the liquid toward gram-positive bacteria (especially *Mycobacteria*) and toward fungi.

The banana skin has been referred to as "nature's bacteria-proof wrapper." In our investigations we found that the green banana skin and pulp contain antifungal substances, but ripe banana skin and pulp (naturally and ethylene-ripened) contain both antifungal and antibacterial substances. Our results indicate that antibiotics in the banana skin and pulp appear during the ripening process. Of particular significance is the fact that an antibacterial factor active toward acid-fast bacteria (*Mycobacteria*) does not appear until the banana is well-ripened. The antifungal substance, which inhibits the growth of disease-causing fungi, has been separated from the antibacterial fractions.

AN ANTIBIOTIC must undergo extensive laboratory and clinical evaluation before it can be put into general use as a therapeutic agent. If tests on experimental animals show that the material is either nontoxic or of very low toxicity and that it is capable of protecting or curing infected animals, the compound may be tried on humans with their knowledge and consent. Care must be taken to prove that the antibiotic will kill or suppress the growth of a variety of pathogenic micro-organisms, that it will be readily absorbed into the body, that it has no damaging action on body cells, and that it is stable and effective in the presence of body fluids, cells, and tissue enzymes. If a compound passes those tests, it may be classified as a satisfactory chemotherapeutic agent. None of the few antibiotics isolated thus far from plants have passed all the tests, although some show promise.

The search for antibiotics in higher plants is relatively new. Thousands of plants have been tested for antibiotic activity by numerous investigators, but the search, of necessity, has been narrow. An investigator does not have the time to consider each plant individually when he is trying to survey a larger part of the plant kingdom. He first makes a preliminary survey of a number of plants as efficiently as he can by assaying the extracts against a sufficient number of representative micro-organisms. From such a survey he can select the species and varieties that warrant his further attention. The problem then is to isolate pure substances from the varieties of plants that showed promise in the preliminary survey and to prove the therapeutic possibilities of these pure substances.

P. S. SCHAFFER, *as biochemist in charge of the antibiotic section,*

biologically active compounds division, Bureau of Agricultural and Industrial Chemistry, is engaged in research on the detection, isolation, and characterization of antibiotics from agricultural sources. He entered the Department of Agriculture in 1930 and before his present assignment worked on insecticides with the Bureau of Entomology and Plant Quarantine and on milk proteins, fats, and other milk products with the Bureau of Dairy Industry.

WILLIAM E. SCOTT, an associate chemist in the biologically active compounds division, Bureau of Agricultural and Industrial Chemistry, is engaged in research on antibiotics from plants. Before joining the Bureau staff he conducted research on food products in the Production and Mar-keting Administration, concentrating mainly on vitamins, proteins, and amino acids. He has also conducted investigations on equipment and apparatus designed by industrial concerns for research on cereal products.

THOMAS D. FONTAINE, head of the biological active compounds division, joined the staff of the Bureau of Agricultural and Industrial Chemistry as a chemist in the oil, fat, and protein division of the Southern Regional Research Laboratory in 1941. Before entering the Department of Agriculture, he worked at Mellon Institute on the proteins, amino acids, and enzymes of cottonseed. His fields of research include plant disease, antibiotics from plants, and natural and synthetic plant growth regulators.

INTEREST IN the use of drugs to treat rheumatoid arthritis and related diseases is keen. The most pressing problem in 1950 was to produce cortisone, pregnenolone, artisone, and desoxycorticosterone in quantities large enough for the needs of arthritics. The drugs were first prepared from animal sources, but those sources, it quickly became apparent, yielded too little to meet the demand.

Turning to the plant kingdom, scientists began an intensive search for plants that contain suitable antiarthritic precursors. They have found that the sapogenins, a little-known group of compounds, are excellent precursors for antiarthritic drugs. In plants, the sapogenins are combined with sugars. The combination, known as saponins, is highly poisonous. An acid treatment removes the sugars from the saponins, after which the nonpoisonous sapogenins can be recovered. Some excellent sources of sapogenins have been found in the yucca, agave, and yam, which are native to Mexico and our Southwestern States.

Department of Agriculture botanists are collecting and identifying plants belonging to species that contain sapogenin. Department chemists are investigating the plants to determine whether they contain suitable antiarthritic precursors. When the best plant sources are known, agronomists will determine the best growing conditions, geneticists will find strains with higher sapogenin content, and chemists will develop methods for large-scale isolation of the sapogenins and their conversion to antiarthritics.—*Monroe E. Wall, Eastern Regional Research Laboratory.*